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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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Running Head: Nutrient cocktail protects against physical inactivity

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ABSTRACT

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

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

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

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

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

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New & Noteworthy:

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

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

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

Abbreviations:

Akt: Protein kinase B

AUC: Area under the curve

BSA: Bovine serum albumine

CSA: Cross sectional area

CPT1: Carnitine palmitoyl transferase 1

DHA: Docosahexaenoic acid

DRI: Dietary references intake

DXA: Dual energy X-ray

EPA: Eicosapentaenoic acid

FABPpm: Fatty acid binding protein plasma membrane

FAT/CD 36: Fatty acid transport CD36

FATP1: Fatty acid transport protein 1

FM: Fat mass

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

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GSH: Glutathione

GSK3: Glycogen synthase kinase 3

GSSG: Glutathione disulfide

HDL: High density lipoprotein

HMW: High molecular weight

HPLC: High performance liquid chromatography

IRMS: Isotope ratio mass spectrometry

LBM: Lean body mass

LCcoA: Long chain acyl co-A

LPL: Lipoprotein lipase

MDA: Malondialdehyde

MET: Metabolic equivalent

MPE: Molar percent enrichment

mtGPAT: mitochondrial glycerol-3phosphate acyltransferase

NEFA: Non-esterified fatty acids

NFkB: Nuclear factor kappa beta

NPRQ: non-protein respiratory quotient

OGTT: Oral glucose tolerance test

PBS: Phosphate buffered saline

PGC1α: Peroxysome proliferator-activated receptor gamma co-activator 1 alpha

ROS: Reactive oxygen species

TG: Triglycerides

UL: Upper limit

VL: Vastus lateralis

VLDL: Very low-density lipoprotein

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122 ω-3: Omega-3 fatty acids

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INTRODUCTION

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

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

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

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

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

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

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

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

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

While these cocktails have the potential to reduce the risk of metabolic disease there are no studies in the literature examining their impact on physical inactivity-induced metabolic alterations in humans. The purpose of this study was to determine the preventive effect of a nutrient cocktail composed of polyphenols, ω-3, vitamin E and selenium on the metabolic and physiological changes that occur during 20 days of reduced daily steps and exercise in trained men. To further trigger metabolic deterioration, dietary fructose supplementation was provided during the last 10 days of the trial. We hypothesized that the cocktail supplementation would reduce and/or prevent the deterioration in glucose and lipid metabolism, insulin sensitivity and muscle atrophy that are associated with reduced physical activity and fructose overfeeding.

METHODS

Subjects

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

Experimental design

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

Cocktail composition and doses

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

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

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Body composition

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

Physical Activity Patterns

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

Glucose tolerance and substrate use

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

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

Exogenous fructose oxidation

Exhaled breath samples were collected at the same time as blood samples. Breath $^{13}C/^{12}C$ isotopic ratio was measured in triplicate on a GasBench system (Thermo Scientific, Germany) connected to a continuous-flow isotope ratio mass spectrometer (IRMS, DELTA V, Thermo Scientific, Germany). U-^{13}C-fructose oxidation was calculated as the cumulated percentage dose recovery of ^{13}C in expired CO_2 per hour over the OGTT, as previously described (42).

Liver *de novo* lipogenesis

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

Blood sample analyses

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

Muscle biopsies and parameters

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

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

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

Protein expression: Muscle samples were homogenized in 10 volumes of lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 100 mM NaF, 5 mM Na3VO4, 1% Triton X-100, 1% SDS, 40 mM β-glycerophosphate and protease inhibitor mixture [P8340; Sigma-Aldrich]) and centrifuged at 10,000 g for 10 min (4°C). Sixty micrograms of protein extract were loaded into Stain-Free 4-20% precast gels (4568095; Bio-Rad) before electrophoretic transfer onto nitrocellulose membranes (Bio-Rad; Trans-Blot Turbo Blotting System). After transfer, the membranes were blocked with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20 (TBS-T) containing 5% skimmed milk or bovine serum albumin (BSA) and incubated overnight at 4°C with primary antibodies. The membranes were then incubated for 1 h with a peroxidase-conjugated secondary antibody. The immunoblots were revealed using a Pierce ECL kit (32106; Thermo Scientific), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc Touch Imaging System and quantified with Image Lab™ Touch Software (version 5.2.1). Stain-Free technology or red Ponceau or GAPDH were used as loading control.

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

Data and statistical analysis

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

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

RESULTS

Subjects' characteristics and changes in time spent physically active and sitting

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

Glucose tolerance and insulin sensitivity

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

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

Lipid metabolism

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

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

Anti-oxidant capacity

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

Skeletal muscle analysis

Muscle parameters are presented in **Table 5**. While type I fibers CSA was lower in both groups at the end of the intervention, the decrease in type IIa CSA observed in the control group, was prevented by the cocktail supplementation. In line with this observation, ubiquitination content, a protein marker of proteolysis processes, increased only in the control group. At the mitochondrial level both Mitofusin-2 and PGC1α contents dropped in response to the intervention. We failed to see a protective effect of the cocktail. FATP1, involved in fatty acid transportation at the mitochondrial level, tended to rise in the cocktail group only ($p=0.07$), supporting the maintenance of lipid oxidation during the intervention in the supplemented but not in the control group. No changes in citrate synthase activity, skeletal muscle intracellular signaling protein contents and oxidative stress markers were observed.

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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Clinical trial registration: NCT03313869

Disclosure summary

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

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Figure 1: Design of the study

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

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

Figure 3: Carbohydrate metabolism

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

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

Figure 4: Lipid metabolism and free radicals defense

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

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

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Table 1: Anthropometry and physical activity data

Means are ± SEM. [1]Mixed models are adjusted on baseline.

Table 2: Fasting metabolites and hormones

Means are ± SEM. [1]Mixed models are adjusted on baseline.

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

HOMA index: Homeostasis model assessment of insulin resistance; TG: Triglycerides; HDL: High Density Lipoprotein; LDL: Low Density Lipoprotein; HMW: High Molecular Weight

Table 3: Fasting oxidative stress and damages

Means are ± SEM. [1]Mixed models are adjusted on baseline.

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

KRL: Total antiradical defense potential (in French, Kit Radicaux Libres); Reseda (in French, RESErves Défenses Antioxydantes) 1, 2 and 3: antiradical defense reserves unmasked by treatment of serum with respectively glucosides, sulphates or glucuronides. GSH: Reduced Glutathione; GSSG: Oxidized Glutathione.

Table 4: Fasting energy & substrate oxidation

[1]Mixed models are adjusted on baseline, fat mass and fat-free mass. Data are presented as LSMeans ± SEM.

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

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

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Table 5: Vastus lateralis western blots and enzyme activity

Means are ± SEM. [1]Mixed models are adjusted on baseline.

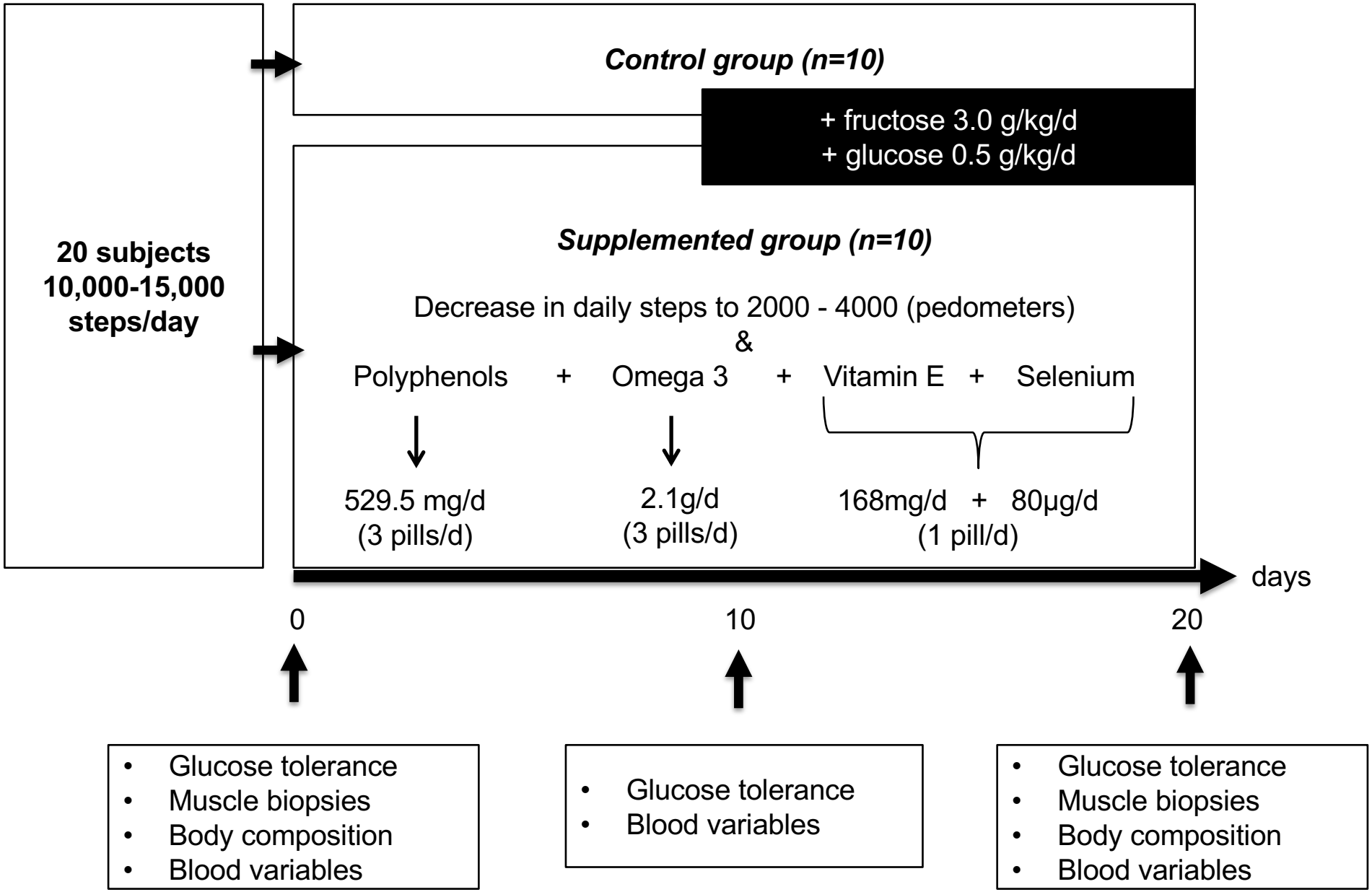
Post-hoc tests: *p<0.05, **p<0.01, ***p<0.001 vs control. #p<0.05, ##p<0.01, ###p<0.001 vs baseline.

Muscle fiber type : CSA: Cross Sectional Area; MHC: Myosin Heavy Chain; Western blots: pAKT: phosphorylated Protein Kinase B; pGSK3: phosphorylated Glycogen Synthase Kinase 3; MURF-1: Muscle-Ring-finger protein 1; HSP90: Heat Shock Protein 90; NRF2: Nuclear Factor erythroid-derived 2 like 2; CBR1: Carbonyl Reductase 1; AKR7A2: Aldo-Keto Reductase Family 7 Member A2; TNFα: Tumor Necrosis Factor α; PGC1α: Peroxisome proliferator-activated receptor gamma coactivator 1α; FATP1: Fatty Acid Transport Protein 1. Enzyme activity: citrate synthase.

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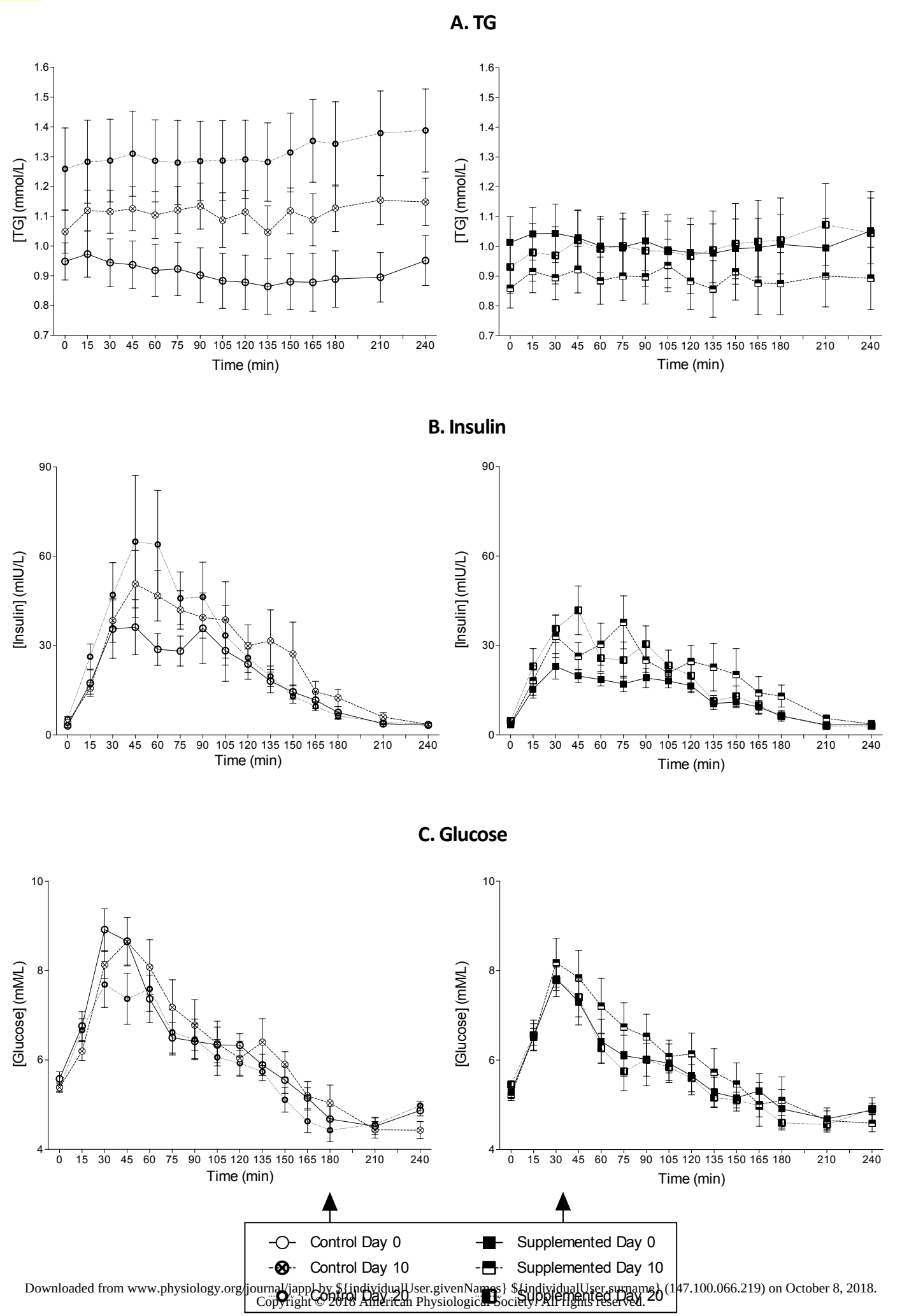
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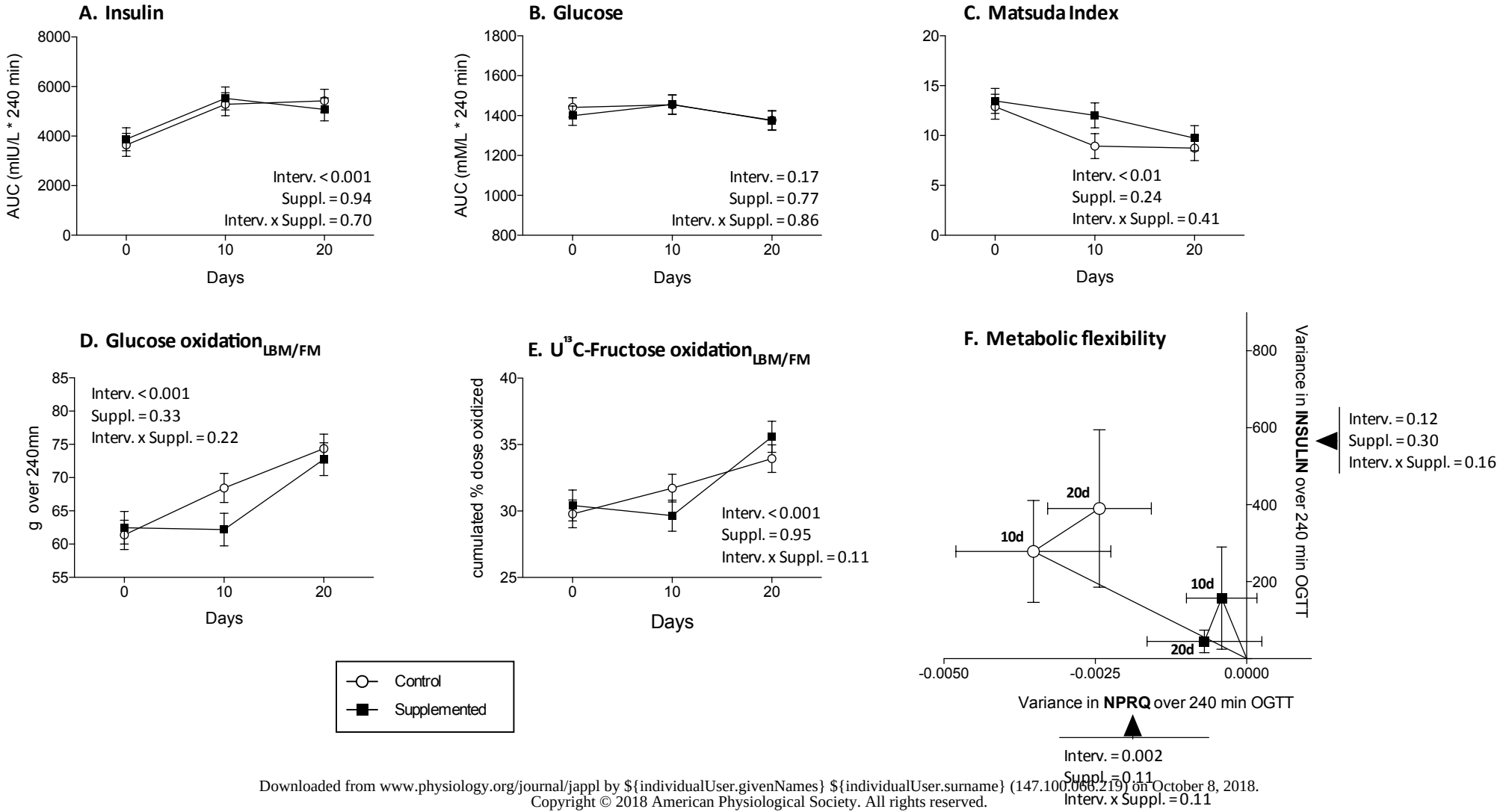


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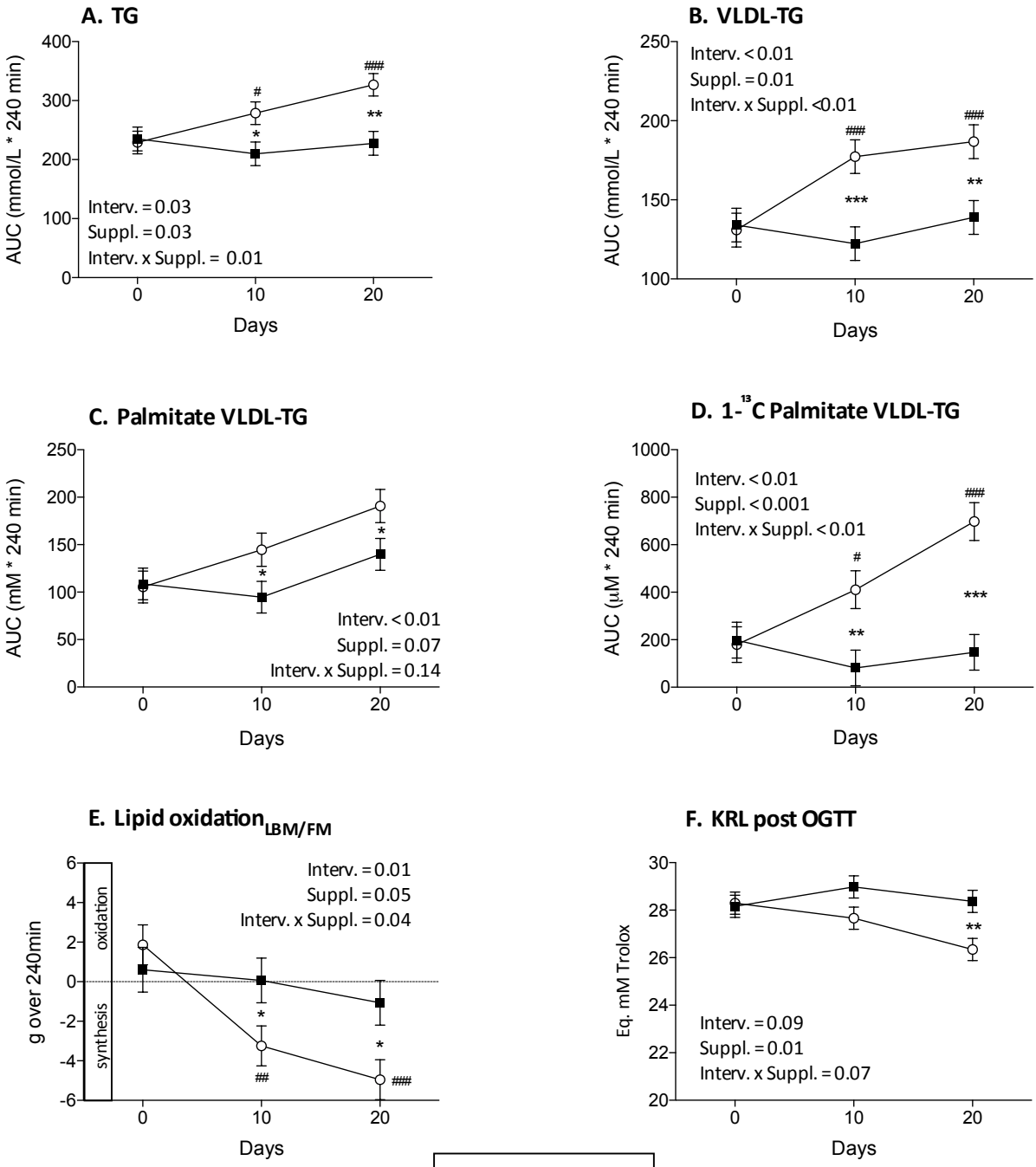
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Table 1. Anthropometry and physical activity data

	Baseline		day 10		day 20		Mixed model[1]		
Variables	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 ($min.day^{-1}$)	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

	Baseline		day 10		day 20		[1] Mixed model		
Variables	**Control**	**Supplemented**	**Control**	**Supplemented**	**Control**	**Supplemented**	**Supplementation**	**Intervention**	**Interaction**
	n=10	n=10	n=10	n=10	n=10	n=10			
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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Table 3. Fasting oxidative stress

	Baseline		day 10		day 20		[1] Mixed model		
Variables	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 4. Fasting substrate oxidation

	Baseline		day 10		day 20		[1] Mixed model		
Variables	Control	Supplemented	Control	Supplemented	Control	Supplemented	Supplementation	Intervention	Interaction
	n=10	n=8	n=10	n=8	n=10	n=8			
NPRQ	0.93 ± 0.03	0.94 ± 0.03	1.04 ± 0.03	1.02 ± 0.03	1.07± 0.03	0.97 ± 0.03	0.20	<0.01	0.16
Lipid oxidation (mg/min)	24.48 ± 8.47	15.24 ± 9.54	-8.47 ± 8.47	-6.64 ± 9.51	-19.77 ± 8.48	4.03 ± 9.50	0.53	<0.01	0.18
Glucose oxidation (mg/min)	196.08 ± 15.08	203.71 ± 16.88	232.07 ± 15.05	220.82 ± 16.88	252.86 ± 15.04	254.50 ± 16.90	0.97	<0.01	0.81

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Table 5. Vastus lateralis western blots and enzyme activity

	Baseline		day 20		[1]Mixed model		
Variables	**Control**	**Supplemented**	**Control**	**Supplemented**	**Supplementation**	**Intervention**	**Interaction**
	n=8	n=10	n=10	n=9			
Muscle fiber type							
CSA MHC1 (µm2)	5330.6 ± 399.0	4968.8 ± 314.3	3951.5 ± 267.6	4453.4 ± 349.2	0.27	<0.01	0.18
CSA MHC2A (µm2)	5477.8 ± 478.3	5184.0 ± 329.4	4303.4 ± 230.3$^{\#\#\#}$	4990.3 ± 261.7 ***	0.001	<0.01	<0.01
CSA MHC hybride (µm2)	0.0 ± 0.0	582.3 ± 582.3	2763.8 ± 759.8	2514.2 ± 756.6	0.35	<0.001	0.25
MHC1 (%)	67.8 ± 5.8	62.3 ± 4.2	52.7 ± 3.4	46.7 ± 4.2	0.38	0.001	0.85
MHC2A (%)	32.2 ± 5.8	37.7 ± 4.2	46.0 ± 2.9	51.4 ± 4.1	0.37	<0.01	0.89
Hybrids (%)	0.0 ± 0.0	0.0 ± 0.0	1.3 ± 0.7	1.9 ± 1.0	0.79	0.02	0.79
Insulin/glucose metabolism							
pAKT serine 473/AKTtotal	15.00± 8.04	15.03 ± 4.56	8.77 ± 2.69	17.71 ± 5.56	0.08	0.61	0.21
pGSK3α[3]	2.91 ± 1.28	2.36 ± 0.92	1.74 ± 0.50	2.88 ± 1.13	0.14	0.61	0.19
pGSK3β	2.00 ± 1.09	1.44 ± 0.79	1.19 ± 0.48	1.13 ± 0.45	0.57	0.22	0.58
Protein synthesis/degradation							
atrogin-1	0.17 ± 0.08	0.20 ± 0.09	0.25 ± 0.10	0.25 ± 0.12	0.55	<0.05	0.62
MURF-1	0.017 ± 0.004	0.016 ± 0.004	0.016 ± 0.003	0.015 ± 0.002	0.94	0.71	0.87
Ubiquitination	0.64 ± 0.06	0.36 ± 0.04	0.83 ± 0.08	0.36 ± 0.05 **	0.01	0.1	0.09
HSP90	4.59 ± 0.26	4.26 ± 0.41	4.26 ± 0.34	4.41 ± 0.70	0.35	0.77	0.40
Oxidative stress							
NRF2	6.52 ± 1.55	4.39 ± 0.62	4.99 ± 0.76	5.28 ± 0.63	0.68	0.50	0.45
Catalase	2.00 ± 0.84	1.20 ± 0.41	1.14 ± 0.28	1.43 ± 0.38	0.55	0.68	0.59
CBR1	4.13 ± 0.60	4.61 ± 0.78	4.55 ± 0.50	4.30 ± 0.79	0.52	0.97	0.41
AKR7A2	8.41 ± 0.59	8.51 ± 0.79	8.92 ± 0.63	8.17 ± 0.83	0.33	0.86	0.33
TNFα	3.55 ± 0.59	3.39 ± 0.61	2.97 ± 0.50	3.60 ± 0.76	0.21	0.52	0.19
Mitochondria markers							
mitofusine	5.98 ± 0.77	6.14 ± 1.51	4.18 ± 0.96	4.36 ± 1.15	0.95	<0.01	0.99
PGC-1α	0.37 ± 0.04	0.25 ± 0.02	0.38 ± 0.09	0.20 ± 0.03	0.21	0.65	0.64
Citrate synthase activity (µmol/min/mg protein)	0.17 ± 0.03	0.14 ± 0.02	0.14 ± 0.03	0.15 ± 0.03	0.44	0.81	0.28
FATP1	3.50 ± 0.33	2.71 ± 0.39	3.34 ± 0.34	3.40 ± 0.70	0.07	0.45	0.07

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