TITLE:

Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind randomized controlled trial

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KEY POINTS SUMMARY

- Recent studies have indicated that antioxidant supplementation may blunt adaptations to exercise, e.g., mitochondrial biogenesis induced by endurance training. Studies on humans are, however, sparse and results are conflicting.
- Isolated vitamin C and E supplements are widely used, and unravelling the interference of these vitamins in cellular and physiological adaptations to exercise is of interest to those who exercise for health purposes and to athletes.
- Our results show that vitamin C and E supplements blunted the endurance training-induced increase of mitochondrial proteins (COX4), which is needed for improving muscular endurance.
- The training-induced increases in VO_{2max} and running performance were not detectably affected by the supplementation.
- The present study contributes to the understanding of how antioxidants interfere with adaptations to exercise in humans, and the results indicate that high dosages of vitamin C and E should be used with caution.

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ABSTRACT

In this double-blind, randomized, controlled trial we investigated the effects of vitamin C and E supplementation on endurance training adaptations in humans.

Fifty-four young men and women were randomly allocated to receive either 1000 mg vitamin C and 235 mg vitamin E daily or a placebo for 11 weeks. During supplementation, the participants completed an endurance training programme consisting of 3-4 sessions per week (primarily running), divided into high intensity interval sessions (4-6x4-6 minutes; >90% of maximal heart rate (HR_{max})) and steady state continuous sessions (30-60 minutes; 70-90% of HR_{max}). Maximal oxygen uptake (VO_{2max}), submaximal running, and a 20 m shuttle run test were assessed and blood samples and muscle biopsies were collected, before and after the intervention.

The vitamin C and E group increased their VO_{2max} (8±5%) and performance in the 20 m shuttle test (10±11%) to the same degree as the placebo group (8±5% and 14±17%, respectively). However, the mitochondrial marker cytochrome c oxidase subunit IV (COX4; +59±97%) and cytosolic peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1alpha; +19±51%) increased in m. vastus lateralis in the placebo group, but not in the vitamin C and E group (COX4: -13±54%, PGC-1alpha: -13±29%; $p \leq 0.03$, between groups). Furthermore, mRNA levels of CDC42 and mitogen-activated protein kinase 1 (MAPK1) in the trained muscle were lower in the vitamin C and E group ($p \leq 0.05$, compared to the placebo group).

Daily vitamin C and E supplementation attenuated increases in markers of mitochondrial biogenesis following endurance training. However, no clear interactions were detected for

improvements in VO_{2max} and running performance. Consequently, vitamin C and E supplementation hampered cellular adaptions in the exercised muscles, and although this was not translated to the performance tests applied in this study, we advocate caution when considering antioxidant supplementation combined with endurance exercise.

INTRODUCTION

Paragraph 1: Aerobic endurance exercise is highly recommended by health authorities for its health rewarding effects (Garber *et al.*, 2011), and in many sports, a high muscular aerobic energy capacity and VO_{2max} are prerequisites for elite performance (Saltin & Astrand, 1967). Strategies for obtaining optimal endurance training effects include not only certain training methods – e.g. interval training (Gibala, 2007), but also nutritional measures (Hawley *et al.*, 2011). Supplements containing antioxidants and vitamins are widely used for the purpose of improving health and athletic achievements (Petroczi *et al.*, 2007;Kennedy *et al.*, 2013). Isolated vitamin C and E supplements are among the most commonly used, despite tentative evidence for the purported effects of these vitamins on health, sport performance and recovery from muscle damage (Padayatty *et al.*, 2003;Nikolaidis *et al.*, 2012).

Paragraph 2: Contrary to common beliefs, studies have recently demonstrated that antioxidant supplementation may interfere with exercise-induced cell-signalling in skeletal muscle fibres (Ristow & Zarse, 2010;Hawley *et al.*, 2011). In turn, such changes in cell-signalling could potentially blunt or block adaptations to training (Peternelj & Coombes, 2011;Gliemann *et al.*, 2013;Morales-Alamo & Calbet, 2013). For example, Gomez-Cabrera et al (2008) investigated whether high dosages of vitamin C affected adaptation to endurance exercise training in both an animal and a human model (1000 mg/d in the human study; male participants). Interestingly, endurance performance increased to a greater extent in animals treated with the placebo compared with animals treated with vitamin C. Furthermore, markers for mitochondrial biogenesis (i.e., peroxisome proliferator-activated receptor gamma co-activator 1 alpha (PGC-1alpha)) increased only in animals treated with the placebo. In the human experiment, changes in VO_{2max} were not significantly different between the supplement and placebo groups. Unfortunately, these authors did not test endurance capacity

111 or collect muscle biopsies from the participants to verify the results of the animal study. In
112 another study with untrained and trained male participants, Ristow et al (2009) demonstrated
113 that four weeks of vitamin C (1000 mg/d) and E (400 IU/d) supplementation blunted training-
114 induced increases in the mRNA expression of genes associated with mitochondrial biogenesis
115 and endogenous antioxidant systems in skeletal muscle (e.g., PGC-1alpha and glutathione
116 peroxidise). Furthermore, Braakhuis et al (2013) observed that supplementation with 1000 mg
117 per day of vitamin C for three weeks slowed female runners during training, although no
118 differences were found in a 5 km time trial or in an incremental treadmill test after the
119 intervention period.
120
121 ***Paragraph 3:*** Contrary to these studies, Yfanti et al (2010;2011;2012) found no negative
122 effects of vitamin C (500 mg/d) and E (400 IU/d) supplementation in male participants who
123 trained five times a week for 12 weeks on a cycle ergometer. The antioxidant supplementation
124 did not influence changes in VO_{2max} and maximal power output (cycling), or activity of the
125 enzymes citrate synthase (CS) and beta-hydroxyacyl-CoA dehydrogenase (HAD) in skeletal
126 muscle. Similarly, Roberts et al (2011) reported no effects of vitamin C (1000 mg/d)
127 supplementation on adaptations to high-intensity running training in male participants.
128 VO_{2max} and endurance performance (10 km time trial and YoYo tests) improved equally in
129 supplemented and placebo groups. The conflicting results from these human studies are
130 reflected in recent animal studies (Gomez-Cabrera *et al.*, 2012;Nikolaidis *et al.*,
131 2012;Braakhuis, 2012).
132
133 ***Paragraph 4:*** Accordingly, it seems clear that antioxidant supplementation potentially
134 inhibits favourable cellular responses to endurance training. On the other hand, the
135 discrepancy between studies invites further investigation. Therefore, we studied the influence

of vitamin C and E supplementation on adaptations to aerobic endurance training, hypothesising that high dosages of vitamin C and E, ingested shortly before and after exercise, would blunt physiological adaptations to 11 weeks of endurance training. The hypothesis was tested in a study with a double-blind, randomized, controlled trial design, in which both training and nutrition were tightly controlled. We combined performance tests with physiological measurements (VO_{2max}) and biochemical/molecular analyses of blood and muscle.

METHODS

Participants

Paragraph 5: Fifty-four young, healthy men and women participated in the experiment (Table 1 and Figure 1). Forty of the volunteers were defined as recreationally endurance-trained individuals, because they had been endurance training 1-4 times per week for 6 months prior to the study. The endurance training was mainly running and cycling. Fourteen volunteers were defined as untrained, because they had not trained regularly (≥ 1 session per week) during the previous 6 months. Sixty-eight volunteers were recruited to the study, but 14 participants (7 from each group) dropped out of the study during the training intervention. Five participants were injured during training (ankle sprains, and achilles pains), while nine dropped out for reasons unrelated to the study.

Paragraph 6: The volunteers were instructed not to take any form of supplements or medication (except contraceptives). Individuals who did use multi-vitamin supplements, etc., were asked to stop taking them at least two weeks before the beginning of the study.

Paragraph 7: The study was approved by the Regional Ethics Committee for Medical and Health Research of South-East Norway and performed in accordance with the Helsinki Declaration. All participants signed a written consent form.

Experimental design

Paragraph 8: After pre-tests and assessments (e.g., VO_{2max} and muscle biopsies), the participants were randomly allocated to a vitamin C and E supplemented group or a placebo group. The randomization was stratified by gender and VO_{2max}. All participants started to take supplements or placebo as they started on the endurance training programme. All tests were

replicated after 11 weeks of training. The experiment was a double-blind, randomized, controlled trial.

Paragraph 9: Blood samples and muscle biopsies collected before the intervention period were preceded with three days of rest, and scheduled again three days after the last exercise session. However, due to practical reasons, a few participants provided samples two and four days after the last exercise session. There was no group bias in the sampling time points.

Supplementation and nutrition

Paragraph 10: The C and E vitamin and placebo pills were produced under Good Manufacturing Practice (GMP) requirements at Petefa AB (Västra Frölunda, Sweden). Each vitamin pill contained 250 mg of ascorbic acid and 58.5 mg DL-alpha-tocopherol acetate. The placebo pills had the same shape and appearance as the vitamin pills.

Paragraph 11: The pills were analysed by a commercial company, Vitas (Oslo, Norway), two years after production, with no sign of degradation of the vitamins (per pill: vitamin C: 255±7 mg, vitamin E: 62±2 mg). The experiments were conducted within this time period. No traces of the vitamins were found in the placebo pills.

Paragraph 12: The participants consumed two pills (500 mg of vitamin C and 117 mg vitamin E) 1-3 hours before every training session and two pills in the hour after training. On non-training days the participants ingested two pills in the morning and two pills in the evening. Thus, the daily dosage was 1000 mg of vitamin C and 235 mg vitamin E. The supplement intake was confirmed in a training diary.

Paragraph 13: The participants were asked to drink no more than two glasses of juice and four cups of coffee or tea per day. Juices especially rich in antioxidants, such as grape juice, were to be avoided.

Paragraph 14: We aimed to keep the participants in energy balance, and encouraged the participants to continue their normal diets. The participants completed a weighed food registration dietary assessment over four days (Black *et al.*, 1991) at the start and end of the intervention period. The participants used a digital food weighing scale (Vera 67002; Soehnle-Waagen GmbH & Co, Murrhardt, Germany; precision 1 g). The dietary registrations were analysed with a nutrient analysis programme (Mat på data 4.1; LKH, Oslo, Norway).

Body composition

Paragraph 15: Inbody 720 (a bioimpedance apparatus) was used to assess body composition before and after the training intervention (Biospace Co., Ltd., Seoul, Korea). The apparatus has been validated (compared with Dual-energy X-ray absorptiometry, DXA) for estimating fat mass and lean mass in men and women (Anderson *et al.*, 2012).

Endurance training

Paragraph 16: The training programme was divided into three periods (Table 2). In period 1 the participants exercised three times per week, two continuous sessions (30 and 60 min) and one interval session (4x4 min). In period 2 one extra interval session was added (4 sessions per week). In periods 2 and 3 the number of runs per interval session was increased, while the exercise intensity was similar throughout the training period. The exception was that the less experienced runners (untrained participants) used 3-6 sessions to gradually increase the intensity. The intensity was high in every session, except during the 60 min run (moderate

intensity). Running was the main exercise form, but one running session per week could be substituted by cycling, cross-country skiing or similar whole body activity.

Paragraph 17: Training intensity was controlled using the Borgs scale (rating of perceived exertion) and heart rate monitors (Polar RS400/RS800CX, Kempele, Finland). The heart rate monitor was worn in every session and the training data were collected and controlled by the investigators. Moreover, each participant was instructed to fill out a training diary, in which they logged mean heart rate, running distance and perceived effort (not reported).

VO_{2max} and submaximal workloads

Paragraph 18: All participants underwent a familiarization session for VO_{2max} measurements (mixing chamber; Jaeger Oxycon Pro, Hoechberg, Germany) on a treadmill (Woodway ELG 90/200 Sport, Weil am Rhein, Germany). The pre-test for VO_{2max} started with 7 minutes at two submaximal running speeds (5.3% inclination), corresponding to 60 and 85% of the VO_{2peak} reached during the familiarization session. VO_2, respiratory exchange ratio (RER), heart rate (Polar RS400, Kempele, Finland) and rating of perceived exertion (Borgs scale) were measured during the last 2 minutes at each velocity. Capillary blood from a finger-stick was sampled within 1 minute after each workload and blood lactate concentration was measured (YSI 1500 Sport Lactate Analyzer, YSI INC, Yellow Springs, Ohio, USA). The same submaximal running velocities were used for both the pre- and post-tests.

Paragraph 19: After a 10 minute rest, the participants performed the VO_{2max} test. The running velocity (5.3 % inclination) was increased by 1 km/h in three 1 minute stages, before 0.5 km/h increases per minute until exhaustion (total duration: 4-8 minutes). Lactate was measured as detailed above.

20 m shuttle run test (Beep test)

Paragraph 20: The 20 m shuttle run test is a multistage shuttle run test that measures aerobic fitness; the test has shown good reliability (Leger *et al.*, 1988). The participants ran a distance of 20 m between two lines and placed one foot on the line each time a beep sounded (from a CD player); the interval between beeps decreased over time. The test had 21 levels and started at a speed of 8 km/h and increased with 0.5 km/h per minute. The participants ran until exhaustion, which was defined as not completing the distance within the time-limit after one warning. The untrained participants completed a familiarization session before this test.

Muscle tissue sampling and pre-analytic handling

Paragraph 21: Muscle biopsies from the mid-portion of the right m. vastus lateralis were collected before and after the training intervention. The post-training insertion was located proximally to the pre-training site (approximately 3 cm). The procedure was conducted under local anaesthesia (Xylocain adrenalin, 10 mg/ml + 5 µg/ml, AstraZeneca, UK). Approximately 200 mg (2-3 x 50-150 mg) of muscle tissue was obtained with a modified Bergström-technique. Tissue intended for homogenization and protein measurements was quickly washed in physiological saline, and fat, connective tissue, and blood were removed before the sample was weighed and quickly frozen in isopentane cooled on dry ice. Tissue intended for mRNA analyses was placed in RNAlater (Ambion, Life Technologies, Carlsbad, CA). Samples for immunohistochemistry were mounted in Tissue-Tek (Cat#4583, Sakura Finetek, CA, USA) and quickly frozen in isopentane cooled on liquid nitrogen. All muscle samples were stored at -80 ^{0}C for later analyses.

Protein immunoblot

Paragraph 22: About 50 mg of muscle tissue was homogenized and fractionated into cytosol, membrane, nuclear, and cytoskeletal fractions, using a commercial fractionation kit according to the manufacturer's procedures (ProteoExtract Subcellular Proteo Extraction Kit, Cat#539790, Calbiochem, EMD Biosciences, Germany). Protein concentrations were assessed with a commercial kit (BioRad DC protein micro plate assay, Cat#0113, Cat#0114, Cat#0115, Bio-Rad, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK), and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich).

Paragraph 23: Cytosol, membrane, and nuclear fractions were analysed by the western blotting technique. Equal amounts of protein were loaded per well (9-30 µg) and separated on 4-12% SDS-PAGE gels under denaturized conditions for 35-45 min at 200 volts in cold MES running buffer (NuPAGE MES SDS running buffer, Invitrogen, CA, USA). Proteins were thereafter transferred onto a PDVF-membrane (Immuno-blot, Cat#162-0177, Bio-Rad, CA, USA), at 30 volts for 90 min in cold transfer buffer (NuPAGE transfer buffer, Cat#NP0006-1, Life Technologies, CA, USA). Membranes were blocked at room temperature for 2 hours in a 5% fat free skimmed milk and 0.05% TBS-T solution (TBS, Cat#170-6435, Bio-Rad, CA, USA; Tween 20, Cat#437082Q, VWR International, PA, USA; Skim milk, Cat#1.15363, Merck, Germany). Blocked membranes were incubated with antibodies against HSP60 (mouse-anti HSP60, Cat#ADI-SPA-807, Enzo Life Sciences, NY USA; diluted 1:4000), HSP70 (mouse-anti HSP70, Cat#ADI-SPA-810, Enzo Life Sciences, NY USA; diluted 1:4000), and COX 4 (mouse-anti-COX4, Cat#Ab14744, Abcam, Cambridge, UK; diluted 1:1000) overnight at 4 °C, followed by incubation with secondary antibody (goat anti-mouse, Cat#31430, Thermo Scientific, IL, USA; diluted 1:30000) at room temperature for 1 hour. All antibodies were diluted in a 1% fat free skimmed milk and 0.05% TBS-T solution.

293 Membranes with the PGC-1alpha molecular weight were blocked at room temperature for 2
294 hours in a 1% BSA solution (BSA 10% in PBS; deionized H_2O; Cat#37525, Thermo
295 Scientific, IL USA). Blocked membranes were incubated with primary antibodies against
296 PGC-1alpha (rabbit-anti-PGC-1alpha, C-Terminal (777-7979), Cat#516557, Calbiochem,
297 MA, USA; diluted 1:2000) overnight at 4 °C, followed by incubation with secondary antibody
298 (goat anti-rabbit IgG, Cat#7074, Cell Signaling Technology, MA, USA; diluted 1:1000) at
299 room temperature for 1 hour. Both primary and secondary antibodies were diluted in 1% BSA
300 and deionized H_2O solution. Between stages, membranes were washed in 0.05% TBS-T
301 solution. Bands were visualized using an HRP-detection system (Super Signal West Dura
302 Extended Duration Substrate, Cat#34076, Thermo Scientific, IL, USA). Chemiluminescence
303 was measured using a CCD image sensor (Image Station 2000R or Image Station 4000R,
304 Kodak, NY, USA), and band intensities were calculated with the Carestream molecular
305 imaging software (Carestream Health, NY, USA). All samples were run as duplicates and
306 mean values were used for statistical analyses.
307
308 ***Immunohistochemistry***
309 ***Paragraph 24:*** Cross sections 8 µm thick were cut using a microtome at -20 ^{0}C (CM3050,
310 Leica, Germany) and mounted on microscope slides (Superfrost Plus, Thermo Scientific, MA,
311 USA). The sections were then air-dried and stored at -80 °C. The muscle sections were
312 blocked for 30 min with 1% BSA (bovine serum albumin; Cat#A4503, Sigma Life Science,
313 MO, USA) and 0.05% PBS-T solution (Cat#524650, Calbiochem, EMD Biosciences, CA,
314 USA). They were then incubated with antibodies against myosin heavy chain type 2 (1:1000;
315 SC71, gift from Prof. S. Schiaffino), CD31 (capillaries; 1:200; Dako, clone JC70A, M0823)
316 and dystrophin (1:1000; Cat#ab15277, Abcam, Cambridge, UK) overnight at 4°C followed by
317 incubation with appropriate secondary antibodies (Alexa Fluor, Cat#A11005 or Cat#A11001,

Invitrogen, CA, USA). Between stages the sections were washed 3x5 min in 0.05% PBS-T solution. Muscle sections were finally covered with a coverslip and glued with ProLong Gold Antifade Reagent with DAPI (Cat#P36935, Invitrogen Molecular Probes, OR, USA) and left to dry overnight at room temperature. Muscle sections were visualized using a high resolution camera (DP72, Olympus, Japan) mounted on a microscope (BX61, Olympus, Japan) with a fluorescence light source (X-Cite 120PCQ, EXFO, Canada). Fibre type distribution, fibre cross-sectional area, and capillaries were identified by TEMA software (CheckVision, Hadsund, Denmark). All staining counts were manually approved/corrected independently by two investigators. Capillarisation was expressed as capillaries around each fibre (CAF) and CAF related to fibre area (CAFA), for type 1 and type 2 (2a and 2x) fibres.

Gene expression analyses

Paragraph 25: Total RNA was isolated using a "RNeasy Fibrous Tissue Mini Kit" (Qiagen, CA, USA, Cat#74704) according to the manufacturer`s instructions. RNA quantity and quality were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Agilent Bioanalyser combined with "Agilent RNA 6000 Nano Kit" (Agilent Technologies, Palo Alto, CA, USA). A "High-Capacity cDNA reverse transcription kit" (Applied Biosystems, Foster City, CA, USA, Cat# 4368814) was used for cDNA synthesis. Q-RT-PCR was performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems) using 140 ng cDNA in a custom-made Taq-Man Low Density Array (Applied Biosystems). Primers for the following genes were included in the array (abbreviated name; Applied Biosystems Assay ID): CRYAB (Hs00157107_m1), CAT (Hs00156308_m1), CDC42 (Hs00741586_mH), CS (Hs00830726_sH), COL4A1 (Hs01007469_m1), COX4I1 (Hs00971639_m1), CYCS (Hs01588973_m1), ESRRA (Hs00607062_gH), FOXO1 (Hs01054576_m1), SLC2A4 (Hs00168966_m1), GPX1

(Hs00829989_gH), HIF1A (Hs00936368_m1), HMOX1 (Hs00157965_m1), HSPB2 (Hs00155436_m1), HSPD1 (Hs01036747_m1), HSPA1A:HSPA1B (Hs00359147_s1), HSF1 (Hs00232134_m1), IGF2 (Hs00171254_m1), IL6 (Hs99999032_m1), LAMA4 (Hs00158588_m1), MAPK1 (Hs01046830_m1), MAPK3 (Hs00385075_m1), NFKB1 (Hs00231653_m1), NFKB2 (Hs00174517_m1), NID2 (Hs00201233_m1), NOX1 (Hs00246589_m1), CYBB (Hs00166163_m1), NOX3 (Hs00210462_m1), NOX4 (Hs01558199_m1), NOX5 (Hs00225846_m1), NQO1 (Hs00168547_m1), NFE2L1 (Hs00231457_m1), NFE2L2 (Hs00232352_m1), NRF1 (Hs00602161_m1), PPARGC1B (Hs00991676_m1), PPARGC1A (Hs01016724_m1), PPARA (Hs00947539_m1), PPARG (Hs01115512_m1), RELA (Hs00153294_m1), SOD1 (Hs00916176_m1), SOD2 (Hs00167309_m1), TXN (Hs00828652_m1), VEGFA (Hs00900055_m1). Endogenous controls included in the assay were: 18S, GAPDH (Hs99999905_m1), GUSB (Hs99999908_m1), HPRT1 (Hs99999909_m1), TBP (Hs99999910_m1). RQ Manager version 1.2 (Applied Biosystems) and Microsoft Excel 2010 were used for the data analysis. The expression levels were quantified using the cycle threshold (Ct) normalized against the average of the endogenous controls GUSB and HPRT1. ΔCt represents the Ct value of the target gene minus (average) Ct value of the endogenous control, and is used to calculate $2^{-\Delta Ct}$. A target gene was determined as "not expressed" when the average Ct was ≥ 35.

Blood sampling and handling

Paragraph 26: Venous blood was collected in the morning after 12 hours of fasting. Heparin and EDTA coated tubes were immediately centrifuged at 1500 g for 10 min at 4°C. Care was taken to keep the collected plasma cooled (on ice) between steps, and to freeze the treated samples rapidly in dry ice. Heparin plasma destined for vitamin C analysis was immediately mixed in equal volumes with metaphosphoric acid before freezing; the further analysis

procedure is described by Karlsen et al (2005). Vitamin E was analysed in EDTA plasma, as described by Bastani et al (2012). Plasma (heparin) 8-iso PGF 2a analyses have previously been described by Bastani et al (2009). All samples were stored at -80°C until analysis.

Statistics

Paragraph 27: The numbers of participants included in the different tests and analyses are given in Figure 1. All data were tested for Gaussian distribution with the D'Agostino & Pearson omnibus normality test. A two-way ANOVA was used to evaluate the effect of training (time) and vitamin C and E supplementation (absolute values, pre and post). A Holm-Sidak multiple comparisons test was applied for post hoc analyses. Between groups differences in relative changes (%) from before to after the intervention period (pre-post changes) were assessed with an unpaired Student's t-test or the Mann Whitney test (dependent on distribution). Relative changes within each group were assessed with a paired Student's t-test or Wilcoxon signed rank test (dependent on distribution). For mRNA data, Mann Whitney U tests were used to compare changes between groups, and Wilcoxon signed rank tests were used for within-group analyses. Data are given as mean and standard deviation (SD) in text and tables. The figures display max-min values, 25^{th} and 75^{th} quartiles and the medians (boxplot), as some of the biochemical variables were not normally distributed. Outliers were defined by Tukey's rule. Effect size was calculated as the differences between the group means divided by the combined SD. Graphpad Prism(R) (version 6.00, La Jolla California USA, www.graphpad.com) was used for statistical analyses.

RESULTS

Paragraph 28: The participants reported 97±5% adherence to the supplements. A survey conducted after the training period confirmed that the group affiliation was indeed concealed for the participants. The vitamin C and E supplementation raised plasma levels of both vitamin C (before: 81±24 µM, after: 114±30 µM; $p<0.001$) and vitamin E (alpha-tocopherol; before: 27±7 µM, after: 35±11 µM; $p=0.009$; Figure 2). No changes were found in the placebo group (vitamin C: before: 80.9±17.2 µM, after: 81.1±19.9 µM; $p=0.70$; vitamin E: before 25.9±6.6 µM, after: 26.6±4.2 µM; $p=0.66$).

Paragraph 29: In contrast to the C+E vitamin group (before: 87.1±49 $pg \cdot ml^{-1}$, after: 85.5±43 $pg \cdot ml^{-1}$), 8-iso PGF 2a increased in the placebo group (before: 74±33 $pg \cdot ml^{-1}$, after: 88.2±29 $pg \cdot ml^{-1}$, $p=0.03$), the difference between the groups being statistically significant ($p=0.03$; Figure 3).

Paragraph 30: We found no significant difference in energy intake between the C+E vitamin group and placebo group (~10500±3500 kJ in both groups), or for macro- or micro-nutrients (data not shown). Through their regular diet, the C+E vitamin group consumed 104±72 mg of vitamin C and 11±4 mg of vitamin E per day, while the placebo group consumed 102±50 mg and 11±4 mg, respectively ($p>0.7$ between groups).

Paragraph 31: The C+E vitamin group reduced body mass by 1.0±2.0% ($p=0.02$), due to a 5.3±8.6% ($p=0.005$) loss of fat mass, but these changes were not different from those in the placebo group (Table 3). The estimated muscle mass was stable in both groups.

Paragraph 32: All participants performed 38-45 exercise sessions during the 11 week intervention. The training diary and heart rate data showed no differences in training intensity and perceived exertion between the groups (data not shown).

Paragraph 33: VO_{2max} improved to the same degree in both groups (C+E vitamin: 52.9±7.6 to 57.2±9.6 $ml \cdot min^{-1} \cdot kg^{-1}$, placebo: 52.9±8.6 to 57.1±7.4 $ml \cdot min^{-1} \cdot kg^{-1}$), as did the performance in the 20 m shuttle run test (C+E vitamin: 1660±570 to 1800±540 meters, placebo: 1670±550 to 1870±550 meters; Figure 4).

Paragraph 34: The subgroup of previously untrained participants increased their VO_{2max} more than the trained participants (12.6±6.2%; $p<0.001$, untrained vs. trained), but there were no differences between the untrained participants in the C+E vitamin group vs. the placebo group ($p=0.98$).

Paragraph 35: During submaximal velocity running, corresponding to 58±7 and 80±7% of pre VO_{2max}, the putative training effects were slightly larger in the placebo than in the C+E vitamin group, specifically for heart rate and RER values (Table 4). However the group differences only reached a statistical tendency ($p=0.08$-0.09; effect size = 0.5 for both variables).

Paragraph 36: The COX4 protein content in membrane fractions (including the mitochondrial components) of samples from m. vastus lateralis increased with training only in the placebo group ($p=0.01$). A similar trend was seen for the COX4 mRNA levels from the muscle biopsies (Figure 5).

Paragraph 37: The PGC-1alpha mRNA levels increased during training only in the C+E vitamin group (Figure 6), but no significant changes were found for PGC-1alpha protein content either in the cytosol or in the nuclear fraction in either group. However, a small, but significant group difference was found for the change of PGC-1alpha protein levels in the cytosolic fraction ($p=0.03$).

Paragraph 38: The heat shock proteins 60 and 70 (HSP60 and HSP70) did not change during training, either at the mRNA level (some of the data given in Figure 5) or the protein level in the cytosolic and nuclear fractions (Figure 7).

Paragraph 39: The mRNA levels of CDC42 and MAPK1 decreased in the C+E vitamin group, and the changes were statistically different from those in the placebo group ($p \leq 0.05$; Figure 8).

Paragraph 40: With no group differences in the mRNA levels, VEGF mRNA ($p=0.018$) and CRYAB mRNA (alphaB-crystallin; $p=0.018$) decreased in the placebo group (supplementary table display results for all analysed genes).

Paragraph 41: No changes or group differences were found for fibre cross-sectional area or capillarisation (Table 5). When the groups were combined, there was a trend towards an increased proportion of type 2 fibres ($p=0.08$).

DISCUSSION

Paragraph 42: In the present study we investigated the effects of vitamin C and E supplementation on adaptations to endurance exercise during an 11-week double-blind, randomized, controlled trial (n=54). The main findings were that the supplementation blunted the training-induced up-regulation of cytosolic PGC-1alpha and the mitochondrial COX4 protein in m. vastus lateralis, without altering the training-induced improvements in VO_{2max} and running performance. The supplementation decreased the gene expression of the signalling proteins CDC42 and MAPK1, but did not alter stress proteins or capillarisation

Cellular effects

Paragraph 43: Although conflicting results exist, animal models have demonstrated that high dosages of antioxidant supplements can shut down specific (redox sensitive) cell signalling pathways, and thereby, decrease synthesis of new muscle mitochondria and endogenous antioxidant production (Kang *et al.*, 2009;Hawley *et al.*, 2011;Strobel *et al.*, 2011;Villanueva & Kross, 2012;Feng *et al.*, 2013). Importantly, both health benefits and improved athletic performance in response to endurance training seem dependent on such cellular adaptations (Coffey & Hawley, 2007;Ristow & Zarse, 2010). With human participants, we herein provide novel evidence that high dosages of vitamin C and E reduce the endurance training-induced increase of COX4 (in vastus lateralis), which suggests a blunted mitochondrial biogenesis. The exact mechanism behind this effect is not possible to decipher. However, as suggested by Ristow et al (2009;2010), we assume that the antioxidants attenuated the generation of reactive oxygen and/or nitrogen species (RONS), and thereby inhibited redox-sensitive signalling and blunted the induction of genes such as PGC-1alpha (as discussed further below).

Paragraph 44: Our observations are in conflict with findings in a recent human study by Yfanti et al (2010), who reported that supplementation with vitamins C and E did not alter training adaptations, as assessed by changes in citrate synthase (CS) and beta-hydroxyacyl-CoA dehydrogenase (HAD) activity in m. vastus lateralis. A plausible explanation of this discrepancy could be that Yfanti et al supplemented with 500 mg vitamin C per day, rather than 1000 mg per day as used in the present study. Furthermore, our participants were instructed to take the supplements in two doses (half dosage: 500 mg vitamin C and 117.5 mg vitamin E), 1-3 hours before and within one hour after each exercise session. By contrast, participants in the study by Yfanti et al consumed their vitamin supplement only at breakfast. Considering the pharmacokinetics of vitamin C in plasma (which decrease within a few hours; (Padayatty *et al.*, 2004)), this might have caused a different cellular response to the supplementation.

Paragraph 45: We and others (Morton *et al.*, 2009a;Feng *et al.*, 2013) have used COX4 as a marker of mitochondrial content, and COX4 and total mitochondrial contents are found to correlate significantly (Larsen *et al.*, 2012). Nevertheless, as a surrogate marker for mitochondrial content, we should keep in mind that the COX4 content is not directly comparable with changes in enzyme activity, such as citrate synthase as measured by Yfanti et al (2010).

Paragraph 46: Mitochondrial biogenesis seems primarily regulated by PGC-1alpha, which controls the expression of both nuclear and mitochondrial gene transcription, through proteins such as NFR1/2 and TFAM (Lanza & Sreekumaran, 2010). The up-stream activators of PGC-1alpha comprise MAPK (p38 and ERK1/2) and AMPK (Lanza & Sreekumaran, 2010;Hawley *et al.*, 2011). In our study we observed that vitamin C and E supplementation blunted any rise

511 of the muscle cytosolic PGC-1alpha levels and lowered the gene expression of CDC42 and
512 MAPK1 (ERK2). These responses are consistent with the changes that we observed for
513 COX4. By contrast, PGC-1alpha mRNA was increased only in the vitamin C and E
514 supplemented group, and the nuclear PGC-1alpha protein levels were unchanged in both
515 groups. Further complicating the issue, others have recently reported that PGC-1alpha is
516 dispensable for exercise-induced mitochondrial biogenesis in mice (Rowe *et al.*, 2012).
517
518 ***Paragraph 47:*** Notably, our biopsies were collected 2-4 days after the last training session,
519 meaning that they do not reflect any immediate activation, subcellular movement of proteins
520 (e.g. nuclear translocation of PGC-1alpha), or gene expression during exercise.
521
522 ***Paragraph 48:*** CDC42 is a member of the Rho family of small GTPases (Jaffe & Hall, 2005).
523 Among various functions, CDC42 exerts certain effects via MAPKs (Maillet *et al.*, 2009), and
524 has been shown to be ROS-sensitive (Li *et al.*, 2009). Nielsen et al (2010) reported no
525 changes in the protein levels of CDC42 in response to 12 weeks of endurance training, but a
526 decrease with cessation of training. Cessation of training is certainly strongly associated with
527 a decrease in muscular fitness, including mitochondrial capacity (Henriksson, 1992).
528 Accordingly, the lower CDC42 gene expression may reflect an adverse effect of the vitamin C
529 and E supplementation, further supporting the negative effect observed on COX4 levels, and
530 sheds light on possible mechanisms for antioxidant interactions.
531
532 ***Paragraph 49:*** There were no significant changes in the HSP60 and HSP70 levels (mRNA or
533 cytosolic and nucleic protein). This suggests no accumulated cellular stress during the
534 endurance training, with or without C and E vitamin supplementation (Morton *et al.*, 2009b).
535 Stable HSP levels contrast with the observations of previous studies (Liu *et al.*, 2006;Morton

et al., 2009b). This difference may reflect the fact that our participants (from whom we collected muscle biopsies) were recreationally endurance trained as they entered the study (Morton *et al.*, 2009b). Similarly, the training status of the participants was probably the reason for the stable capillary density conditions.

VO_{2max} and performance

Paragraph 50: The various cellular effects of the vitamin C and E supplementation are interesting, but performance outcomes are more important for athletes. Thus, in contrast to the cellular observations, the increases in VO_{2max} (~8%) and the improvements in running performance (20 m shuttle run test; ~10-14%) were similar in both groups. This is in line with recent human studies where increased VO_{2max} due to endurance training was unaffected by vitamin C and E supplementation (Aguilo *et al.*, 2007;Yfanti *et al.*, 2010;Roberts *et al.*, 2011). Interestingly, Gomez-Cabrera et al (2008) reported that rats that were supplemented with vitamin C showed the same increases in VO_{2max} as placebo animals. However, the vitamin C supplementation strongly suppressed improvements in endurance performance (running to exhaustion). No group differences were detected in the present study, yet it is intriguing to note that the four participants with the largest improvements in running performance were all in the placebo group (effect size = 0.3 in favour of the placebo group). Although speculative, this could suggest that there are considerable inter-individual differences in the effects of vitamin C and E supplementation. Sub-group analyses showed, however, no effect of initial training status or gender on the gain in VO_{2max} and running performance during the training period (data not shown).

Paragraph 51: In further support of (mild) negative effects of the vitamin C and E supplementation, we observed improved fat oxidation (indicated by reduced RER values) and

reduced heart rates at submaximal workloads in the placebo group, while no significant changes were detected in the vitamin C and E group. The group differences were of moderate effect size, but did not reach statistical differences (p=0.08-0.09). Improved fat oxidation at steady state submaximal workloads could theoretically be due to both a selective up-regulation of enzymes, such as beta-HAD, or a gross increase in the mitochondrial mass, or both (Spina *et al.*, 1996). Unfortunately, we did not measure cellular markers for fat oxidation; however, our observation of a group difference in the COX4 levels, indicating increased levels of mitochondrial proteins, could be related to the RER findings.

Paragraph 52: Although we recruited a high number of participants, compared to similar studies (Nikolaidis *et al.*, 2012), we may have been underpowered to detect small, but potentially true biological effects; e.g. changes in RER-values and running performance. For these variables, we had only 30-45% power to detect statistical group differences of the observed 3-4%.

C and E vitamin in plasma and changes of 8-iso PGF2a

Paragraph 53: Plasma measurements supported the efficiency of the vitamin C and E supplementation – even though the C and E vitamin levels among our young, healthy participants were at the upper range of reference values at baseline (Karlsen *et al.*, 2005;Gomez-Cabrera *et al.*, 2008;Yfanti *et al.*, 2010;Braakhuis *et al.*, 2013).

Paragraph 54: 8-iso PGF2a is an established oxidative stress marker (Basu & Helmersson, 2005), and interestingly, the vitamin C and E supplementation inhibited an elevation of 8-iso PGF2a that occurred in the placebo group. Vitamin C and E supplements (alone) have been found to reduce 8-iso PGF2a levels (Basu & Helmersson, 2005), although intriguingly,

586 vitamin E has been shown to act as a pro-oxidant in certain experiments (Bowry *et al.*,
587 1992;Abudu *et al.*, 2004). Endurance training has been found to lower the 8-iso PGF2a
588 plasma concentration, especially in individuals with initially high levels (Roberts *et al.*,
589 2002;Campbell *et al.*, 2010;Arikawa *et al.*, 2013). Contrary to these training studies, we
590 observed an increase in the placebo group. This increase might be explained by the intensive,
591 high-frequency running programme for participants with normal baseline 8-iso PGF2a levels.
592
593 **Supplement considerations**
594 ***Paragraph 55:*** Our participants were supplemented with DL-alpha-tocopherol acetate, the
595 synthetic form of vitamin E. The bioavailability and biological action of natural (D-alpha-
596 tocopherol/RRR- alpha-tocopherol) may be different (Traber *et al.*, 1994;Burton *et al.*, 1998).
597 Thus, we must be careful when comparing our results with studies that have administered the
598 natural form of vitamin E. Concerning vitamin C, there seem to be no differences in blood
599 and tissue bioavailability of synthetic and natural or flavonoid-rich vitamin C (Carr *et al.*,
600 2013).
601
602

CONCLUSION

Paragraph 56: Vitamin C and E supplementation did not affect the endurance training-induced increase in VO_{2max} and running performance (20 m shuttle test). However, at the muscle cellular level, the supplementation blunted the training-induced increase in mitochondrial COX4 protein content. Group differences in PGC-1alpha (cytosolic protein level), and CDC42 and MAPK1 mRNA levels provide further evidence that antioxidant supplementation may have interfered with exercise-induced cell signalling in skeletal muscle. Moreover, the cellular results appeared to some degree to be reflected in physiological adaptations, as measured under submaximal workloads (heart rate and RER). Thus, supplementation with high dosages of vitamin C and E appears to diminish some of the endurance training-induced adaptations in human skeletal muscles. We suggest that high dosages of isolated antioxidants should be used with caution when simultaneously engaged in endurance training.

COMPETING INTERESTS

None.

AUTHOR CONTRIBUTION

All authors approved the final version for publication.

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TABLES

Table 1. Characteristics of the participants in the vitamin C and E group and the placebo group.

	C+E-vitamin N=27: 14 women, 13 men	Placebo N=27: 14 women, 13 men
Age (years)	25±5	24±6
Height (m)	1.74±0.10	1.76±0.10
Body mass (kg)	74±14	70±12
VO_{2max} ($ml \cdot min^{-1} \cdot kg^{-1}$)	53±9	53±8

806 Table 2. Outline of the endurance training programme.

Weeks	Period	Day#1	Day#2	Day#3	Day#4
1-3	1	Continuous: 30 min: 82-87% of HR_{max}; Borg: 15-17	Interval: 4x4 min: >90% of HR_{max}; Borg: 16-18	Continuous: 45-60 min: 72-82% of HR_{max}; Borg: 13-16	
4-8	2	Continuous: 30 min: 82-87% of HR_{max}; Borg: 15-17(18)	Interval: 5x4 min: >90% of HR_{max}; Borg: 16-18	Continuous: 60 min: 72-82% of HR_{max}; Borg: 13-16	Interval: 4x6 min: >90% of HR_{max}; Borg: 16-18
9-11	3	Continuous: 30 min: 82-87% of HR_{max}; Borg: 15-17(18)	Interval: 6x4 min: >90% of HR_{max}; Borg: 16-18	Continuous: 60 min: 72-82% of HR_{max}; Borg: 13-16	Interval: 5x6 min: >90% of HR_{max}; Borg: 16-18

807 HR_{max}: Maximal heart rate, Borg: Borg scale of perceived exertion (6-20).

808 Table 3. Body composition before and after the 11-week intervention period.

	C+E-vitamin			Placebo			*P-value group diff. (%-change)*
	Pre	*Post*	*%-change*	*Pre*	*Post*	*%-change*	
Body mass (kg)	73.9±14.2	73.1±13.7*	-1.0±2.0**	70.2±11.8	69.5±12.5	-1.1±2.8	0.856
Fat mass (kg)	15.5±7.1	14.6±6.8*	-5.3±8.9**	12.6±5.8	12.2±5.9	-3.3±12.1	0.497
Fat%	20.8±8.2	19.8±7.9*	-4.6±7.7**	18.1±7.1	17.6±7.2	-2.0±11.0	0.324
Muscle mass (kg)	32.9±7.2	33.0±7.1	0.4±2.2	32.4±6.6	32.3±6.8	-0.4±2.6	0.206

809 Within group changes: *: $p<0.05$; **: $p<0.01$. Exact p-values for group comparisons of
810 relative changes between groups are also displayed.
811
812

Table 4. Changes in oxygen uptake (VO_2), heart rate (HR), respiratory exchange rate (RER) and lactate during submaximal workloads at approximately 60% and 80% of VO_{2max} at baseline.

	C+E-vitamin			**Placebo**			***P-value group diff. (%-change)***
60 % of pre VO_{2peak}	*Pre*	*Post*	*%-change*	*Pre*	*Post*	*%-change*	
VO_2 ($ml \cdot min^{-1} \cdot kg^{-1}$)	30.9±5.9	30.4±6.3	-1.4±8.7	30.3±4.5	29.1±5.2	-3.6±11.3	0.430
HR ($beats \cdot min^{-1}$)	140.8±13.2	136.2±12.7	-3.0±6.7	140.9±17.3	131.7±15.9	-6.3±7.2**	0.095
RER (VCO_2:VO_2)	0.89±0.05	0.89±0.05	0.3±5.4	0.91±0.04	0.89±0.04	-1.7±5.1	0.168
Lactate (mmol·l)	1.6±0.9	1.3±0.5	-3.5±33.4	1.5±0.9	1.3±0.7	-6.1±32.5	0.776
80 % of pre VO_{2peak}	*Pre*	*Post*	*%-change*	*Pre*	*Post*	*%-change*	
VO_2 ($ml \cdot min^{-1} \cdot kg^{-1}$)	42.4±7.9	42.5±8.9	-0.1±6.7	41.7±5.2	41.4±6.0	-0.4±9.0	0.919
HR ($beats \cdot min^{-1}$)	170.1±11.1	165.0±13.3	-2.9±5.9	169.8±15.5	161.6±14.6	-4.7±4.4**	0.214
RER (VCO_2:VO_2)	0.93±0.04	0.92±0.05	-1.5±5.3	0.95±0.04	0.91±0.03	-3.9±4.5**	0.083
Lactate (mmol·l)	3.8±2.2	2.5±1.3	-27.4±25.1**	3.3±2.2	2.4±1.3	-18±26.0**	0.270

Within group changes: *: $p<0.05$; **: $p<0.01$. Exact p-values for group comparisons of relative changes between groups are also displayed.

828 Table 5. Fibre type distribution, fibre area, and capillarisation.

	C+E-vitamin			Placebo			*P-value group diff. (%-change)*
	Pre	*Post*	*%-change*	*Pre*	*Post*	*%-change*	
Fibre type 1 (%)	54±12	51±12	-3.9±22.9	49±13	44±11	-7.4±28.5	0.124
CSA (μm^2) fibre type 1	5070±1614	5202±1409	5.6±22.1	5021±1702	4893±1206	3.7±35.2	0.455
CAF fibre type 1	4.4±0.9	4.4±0.9	-0.6±13.1	4.1±0.8	4.2±0.7	1.3±13.6	0.774
CAFA fibre type 1	0.9±0.2	0.9±0.2	-1.6±26.0	0.9±0.2	0.9±0.3	7.0±35.9	0.746
CSA (μm^2) fibre type 2	4831±1646	5245±2048	11.3±34.3	5845±2207	6019±2368	4.5±34.2	0.234
CAF fibre type 2	3.8±1.0	3.8±1.0	3.1±17.1	4.0±0.7	4.0±0.9	0.7±14.4	0.730
CAFA fibre type 2	0.8±0.2	0.8±0.2	0.0±30.3	0.7±0.2	0.8±0.5	10.4±57.0	0.579

829 CAF: capillaries around each fibre; CAFA: CAF/fibre area.

FIGURE LEGENDS

Figure 1. Outline of the numbers of trained and untrained participants in each group, and the numbers of participants in tests and analyses applied.

Figure 2. Boxplot (max-min values, 25^{th}-75^{th} quartiles, and median) of percentage changes in the plasma levels of vitamin C and vitamin E in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); #: difference between groups; ∗: within group changes.

Figure 3. Boxplot of percentage changes in plasma 8-iso-prostane in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); #: difference between groups; ∗: within group changes.

Figure 4. Boxplot of percentage changes in VO_{2max} and the 20 m shuttle run test in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); #: difference between groups; ∗: within group changes.

Figure 5. Boxplot of percentage changes in COX4 mRNA, COX4 (protein), HSP60 mRNA and HSP60 (protein) in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); ∗: within group changes. Exact p-values denote tendencies for group differences.

Figure 6. Boxplot of percentage changes in PGC1alpha mRNA and PGC1alpha in cytosol and nuclear fractions in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); #: difference between groups; ∗: within group changes.

Figure 7. Boxplot of percentage changes in the HSP60 and HSP70 levels in cytosol and nuclear fractions in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule).

Figure 8. Boxplot of percentage changes in CDC42 mRNA and MAPK1 mRNA in the vitamin C and E group and the placebo group. ●: outliers (Tukey's rule); #: difference between groups; ∗: within group changes.

Figure 1

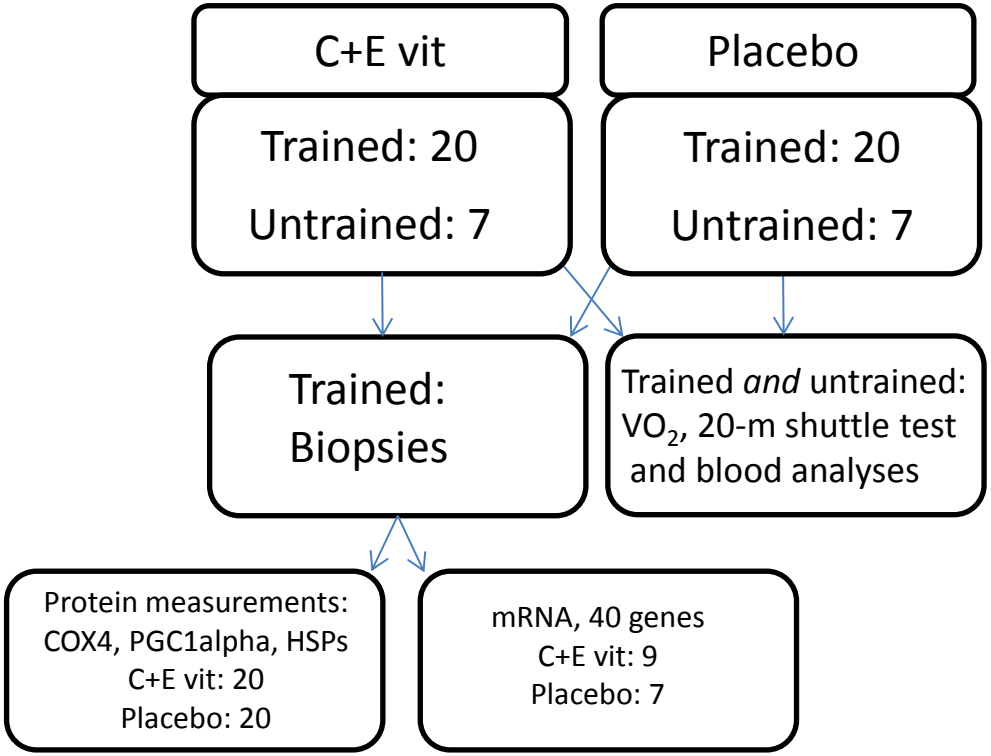
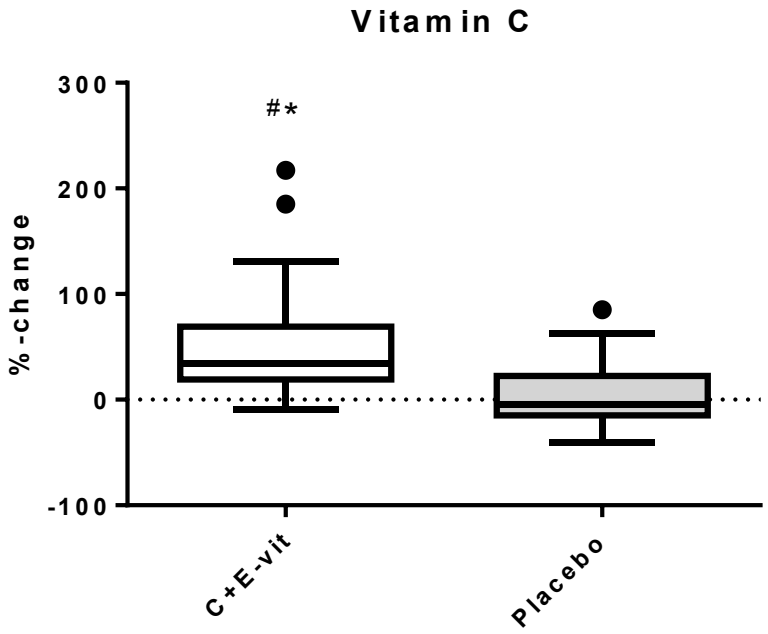


Figure 2



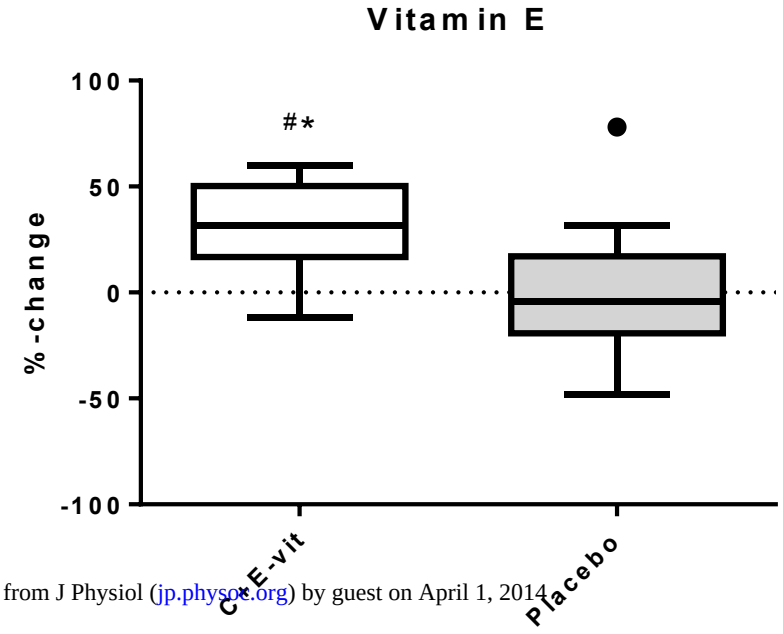


Figure 3

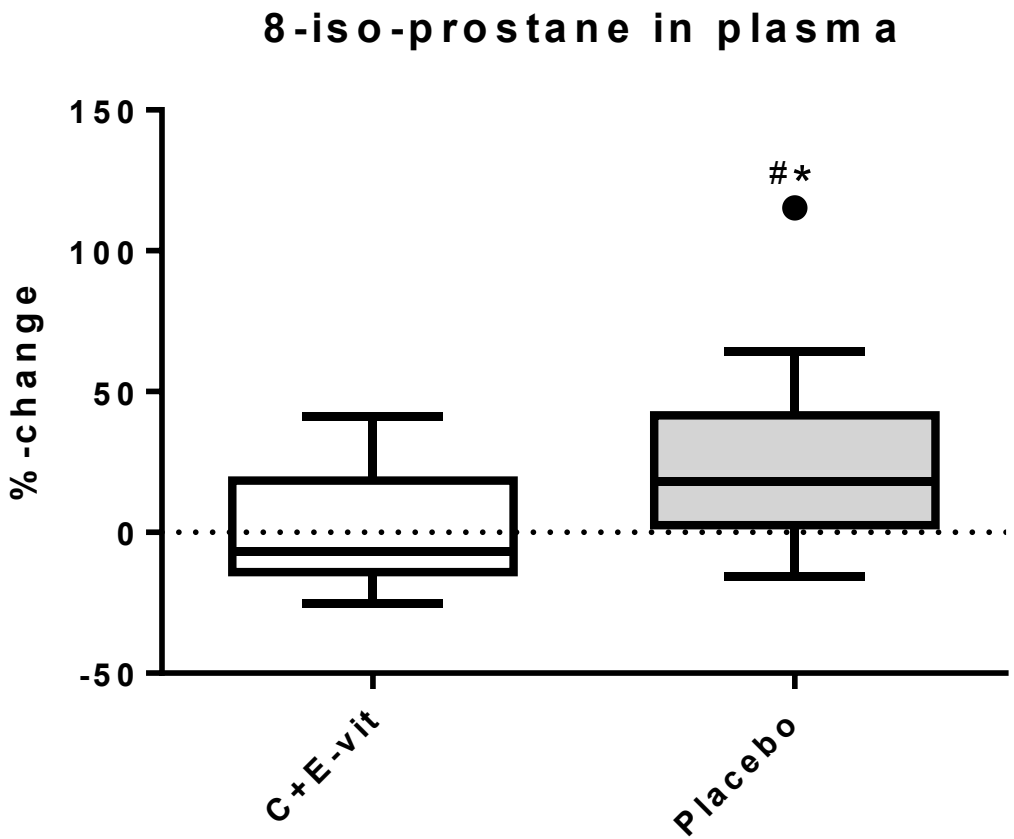


Figure 4

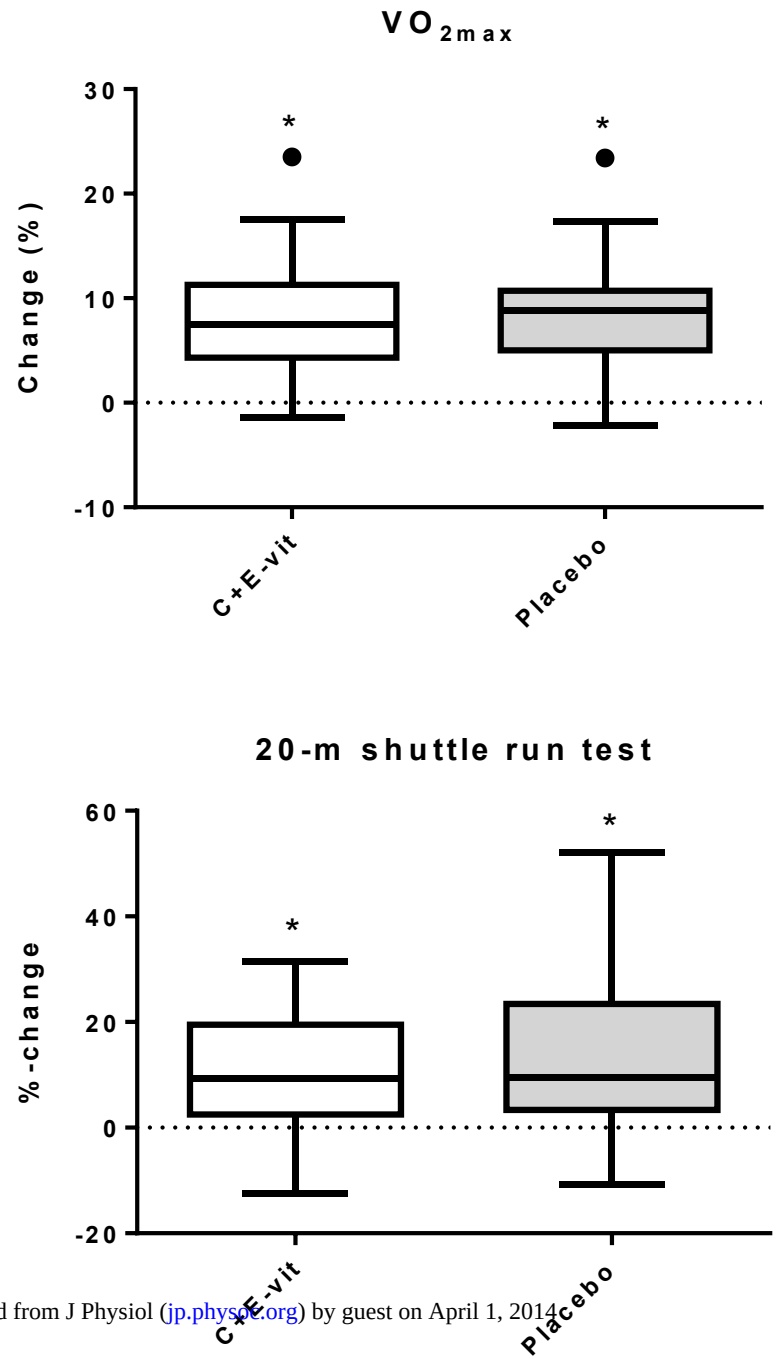


Figure 5

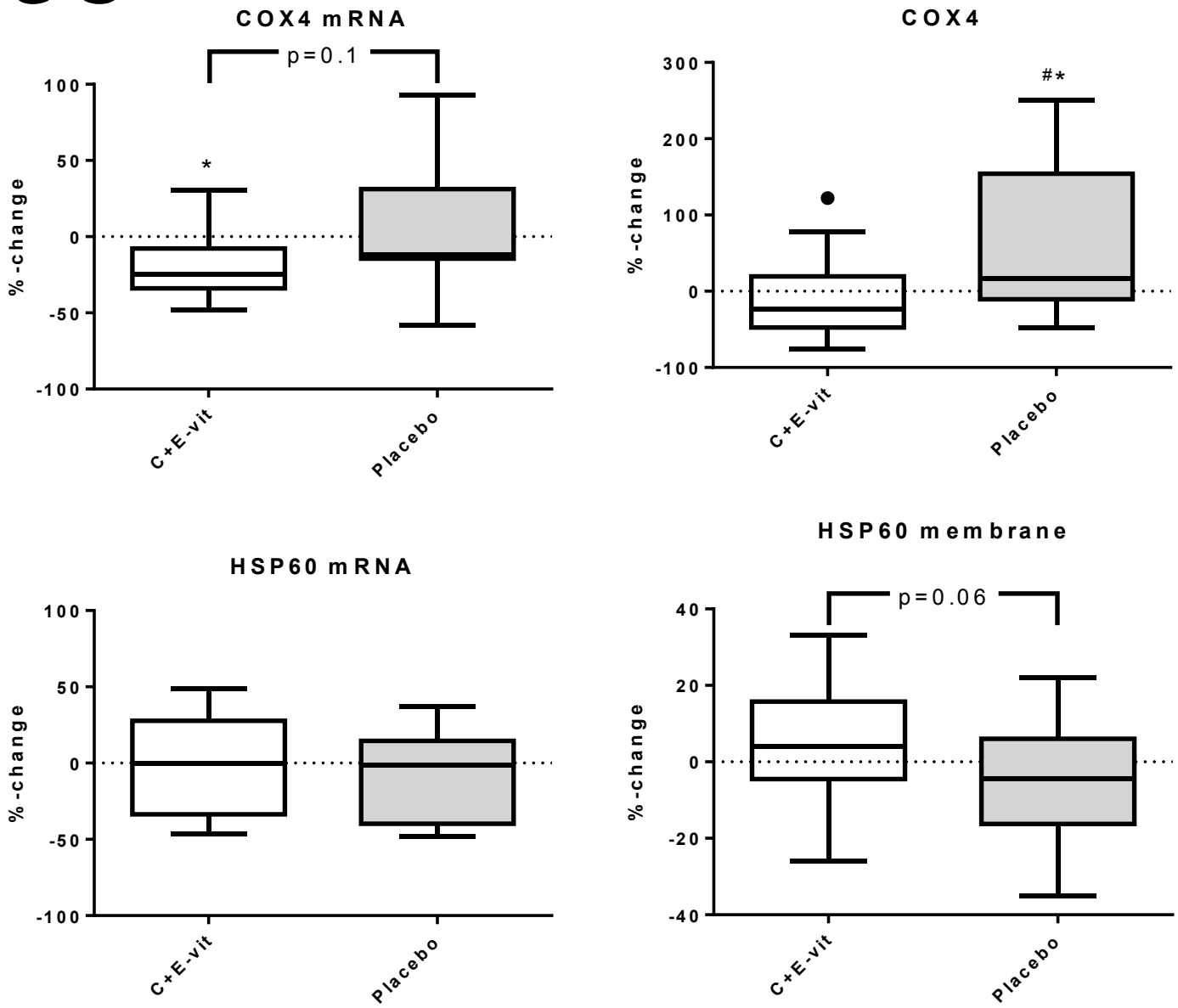


Figure 6

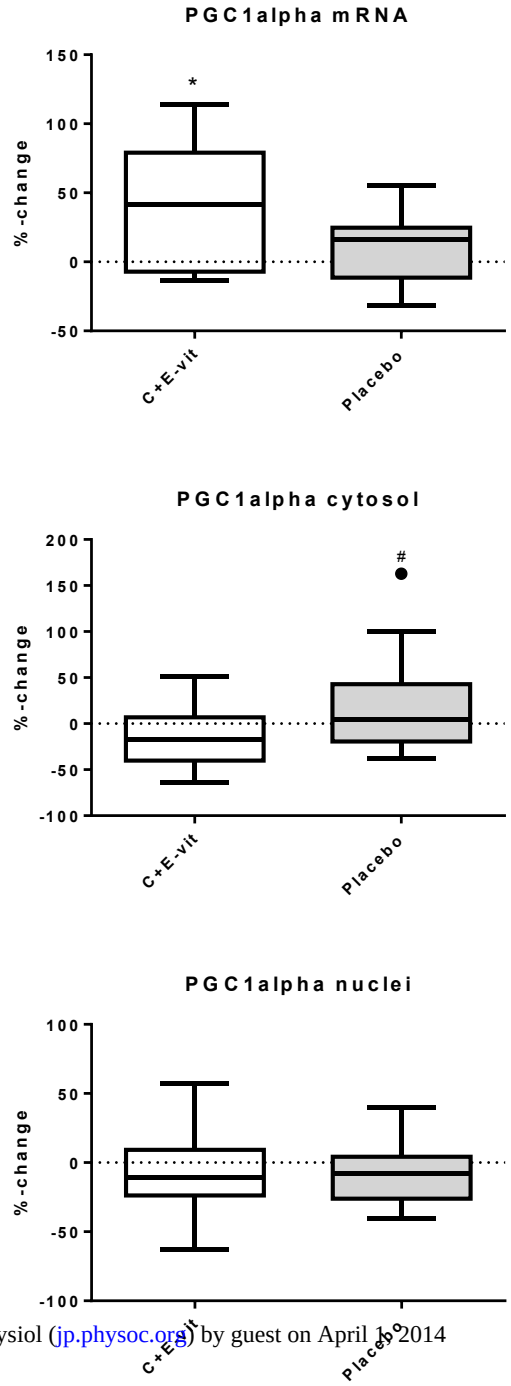


Figure 7

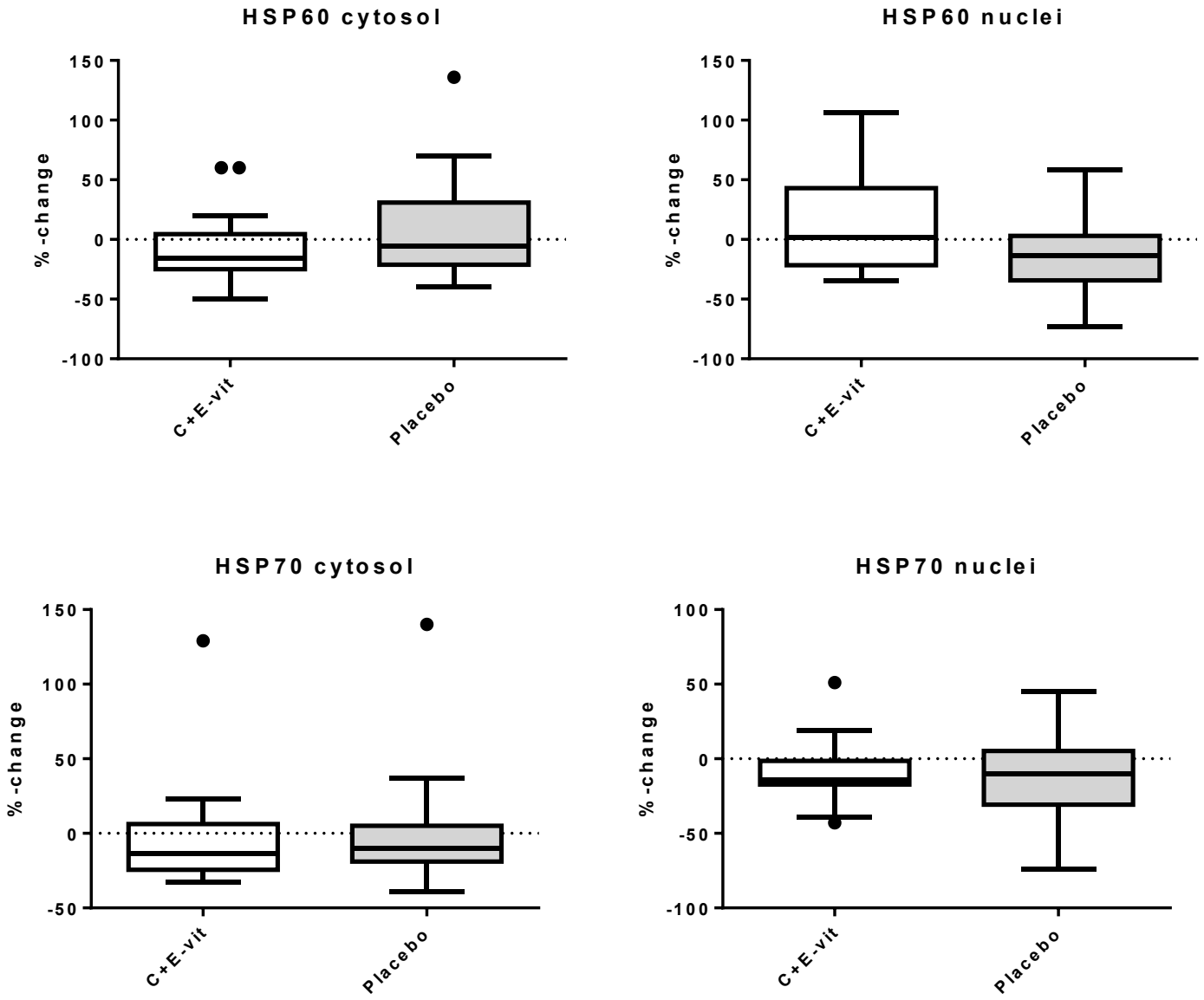


Figure 8