Lipidomic approach in young adult triathletes: effect of supplementation with a polyphenols-rich juice on neuroprostane and F_2-dihomo-isoprostane markers

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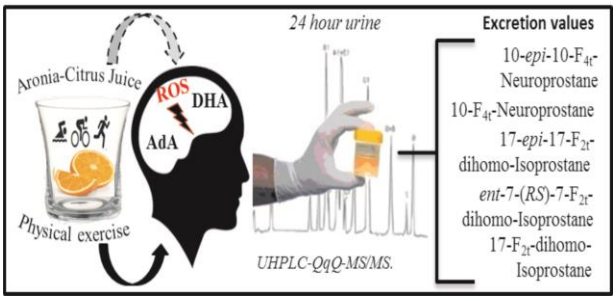

Our juice rich in polyphenolic compounds with an adequate training has been able to influence the excretion values of oxidative stress biomarkers relation to central neuronal system.

Abstract

The aim of the this study was to determine the effect of a polyphenols-rich juice (aronia-citrus juice, ACJ) on F_4-neuroprostanes and F_2-dihomo-isoprostanes -markers of oxidative stress associated with the central nervous system (CNS) – in 16 elite triathletes under a controlled diet for triathlon training (145 days). In the triathletes, a decrease of the lipid peroxidation markers after ACJ intake, associated with neuronal membrane degradation (10-*epi*-10-F_{4t}-neuroprostane and, 10-F_{4t}-neuroprostane) was observed when we compared with placebo stage values. Regarding the F_2-dihomo-isoprostanes, a significant decrease of the neuromotor system damage biomarkers (17-F_{2t}-dihomo-isoprostane) with an increase of training load during the study was observed although the decrease of the load training at the last stage showed a significant increase of the values of *ent*-7-(*RS*)-7-F_{2t}-dihomo-IsoP suggesting a possible role in adaptation post-training. On the other hand, the changes in the excretion of 17-*epi*-17-F_{2t}-dihomo-IsoP provided a positive connection between physical exercise and ACJ intake. Thus, the results showed in this clinical study in young triathletes will help to elucidate novel interactions and mechanisms between the excretion of lipid peroxidation metabolites from CNS, supplementation of polyphenols-rich juice in the diet and physical exercise during a training season.

Running head: Urinary biomarkers of oxidative stress from central nervous system

Suplementary Keywords: Polyphenols, Oxidative stress, F_4-neuroprostanes; F_2-dihomo-isoprostanes, Aronia-Citrus Juice; Athletes, Biomarkers.

1. Introduction

Exercise-induced reactive oxygen species (ROS) production could be an important signaling pathway to induce biological adaptations to training [1, 2]. In addition, regarding the effect of exercise on the brain, regular and moderate aerobic exercise appears to promote the antioxidant capacity, but anaerobic or high-intensity exercise, aerobic-exhausted exercise, or the combination of both types of training could worsen the antioxidant response[3]. The literature shows that polyphenols (abundant in plants and derived foods such as fruits and vegetables) can provide protection against exercise-induced muscle damage and oxidative stress (OS) thanks to their antioxidant and anti-inflammatory properties [4, 5]. There has also been growing recognition of the possible beneficial influence of polyphenols on the development and health of brain structure and function [6, 7], as well as their positive effects that involve a decrease in oxidative/inflammation damage in the nervous system [8, 9].

The use of antioxidant supplementation is common in athletes, primarily to prevent overproduction of ROS and its deleterious impact on cells and tissues through lipid and protein protection. There is evidence that beverages, such as fruit juice, containing a diversity of polyphenol compounds can have a favorable impact on human health [5, 9]. It has been mentioned that fruit juices can provide a blend of polyphenols in a single serving of the drink that cannot be obtained from a portion of fruit [10, 11]. For example, combination of aronia (*Aronia melanocarpa*) with citrus juices has provided synergistic effects of flavanones plus anthocyanins, among other bioactive compounds [12]. Black chokeberry (*Aronia melanocarpa*) contains high amounts of polyphenol compounds which are bioavailable and show health-promoting properties for the human by different mechanisms[13]. Among them, the intake of this berry may be beneficial against OS, in both human and animals [14]. Also, citrus flavonoids

have antioxidant and anti-inflammatory bioactivities. Previous *in vitro* and *in vivo* studies showed that these flavonoids exert neuroprotection at high and low doses [15]. Supplementation with the polyphenols-rich juice used in this study- aronia-citrus juice (ACJ)- may provide health protection to triathletes (200 mL/day), according to previously published results [13]. In fact, the bioavailability of flavanones (eriodictyol and hesperetin) in the triathletes was augmented after the ACJ intake (during 2 weeks) by the physical exercise compared to sedentary volunteers. Besided, the intake of this ACJ, in conjunction with adequate training, was able to influence the plasmatic and urinary values of OS biomarkers (15-F_{2t}-IsoP; also termed 8-iso-prostaglandin-$F_{2\alpha}$ urinary biomarker, as well as the biomarkers guanosine-3',5'-cyclic monophosphate and 8-hydroxyguanine analyzed in plasma samples) [16]. Llorach *et al.* published recently a metabolomic study in healthy volunteers after regular ACJ intake (250 mL/day) during 16 weeks and found the association with markers of intake of the component of juice: proline betaine, ferulic acid, and two unknown mercapturate derivatives [17].

Regarding lipid oxidation markers, F_2-dihomo-isoprostanes (F_2-dihomo-IsoPs) and F_4-neuroprostanes (F_4-NeuroPs) are formed by a free radical, non-enzymatic mechanism from adrenic acid (AdA, C22:4 n-6) [18, 19] and docosahexaenoic acid (DHA, C22:6 n-3) [20], respectively. F_4-NeuroPs originate from DHA, an essential constituent of nervous tissue, highly enriched in neurons and highly prone to oxidation [21]. F_2-dihomo-IsoPs are specific markers generate from AdA and are potential markers of free radical damage to myelin in human brain [18]. Currently, the researchers tend to focus more on the assessment of these biomarkers in disease conditions and their increase in different biological fluids [19, 22-24]. Besides, no attention has been paid to the investigation of these central nervous system (CNS)

degradation markers and their relationship with physical exercise, to the ability of nutrition with functional foods enriched in polyphenols to attenuate to this type of OS generation, or to the elucidation of potential pathways of the OS biomarkers with exercise adaptation and/or the effect of functional foods on the CNS.

Based on the foregoing statements, the aim of this work was to evaluate urinary biomarkers of OS associated with the CNS, namely four F_4-NeuroPs and four F_2-dihomo-IsoPs, and whether the supplementation of the diet with one serving (200 mL/day) of ACJ during 45 days could produce changes in these OS biomarkers. In this study, the identification was carried out by UHPLC-QqQ-MS/MS thanks to its superior advantages to others used in other studies to distinguish the regioisomers and diastereomers of the metabolites in samples [20]. This is the first study to investigate these CNS degradation markers in relation to physical exercise, as well as the influence of nutrition with functional foods enriched in polyphenols.

2. Materials and methods

2.1 Physical characteristics of participants

The anthropometric measurements were performed according to the International Society for the Advancement of Kinanthropometry (ISAK: http://www.isakonline.com), in all cases by the same internationally certified anthropometrist (level 2 ISAK) to minimize the technical error of measurement. The body composition was determined by GREC Kineanthropometric consensus, using a model which consists of: total fat by Withers' formula [25], lean weight by a previous procedure [26], and residual mass by the difference in weight (Table 1).

2.2 Dietary intake of participants

The diet was kept constant to avoid any interference with urinary analysis (Table 2). The calculation of the dietary parameters and caloric intake was accurately designed and overviewed during the experimental intervention by nutritionists and specific software was used for the calculation. The data were calculated using the software available on the website (http://www.easydiet.es), with the additional assistance of the Spanish and USDA databases (http://www.bedca.net/ and http://www.nal.usda.gov/fnic/foodcomp/search/). The dietary assessment and planning for our volunteers were estimated based on their energy needs [27], on their energy expenditure [28], and on different recommendations for triathletes [29], as well as sports men/women [30]. The dietary fullfilment was individually conducted for each elite triathlete by the University of Alicante nutritionists (Chief responsible of the dietary control: Dr. José Miguel Martínez-Sanz). Dietary information was obtained via 24-h recall [31], in which they described in detail all foods and drinks consumed 24 hours prior to each provision of urine.

2.2.1 Aronia-Citrus juice and placebo beverage

The juice composition was based on a mixture of citrus juice (95%) with 5% *Aronia melanocarpa* juice, based on a drink model developed before [32]. The composition was developed in the industry at pilot scale with organoleptically-acceptable criteria, to mimic the flavonoids composition of the original beverage. The supplementation with this natural fruit juice has been used in others studies as aforesaid in the introduction, the daily dose being around 200 mL [13, 17] in healthy subjects. The nutrients content and caloric supply of the ACJ are summarized in Table 3, as well as the contents of fruit flavanones, flavones, and

anthocyanins. The results were expressed as milligrams per serving of juice. One serving of juice corresponds to 240 mL according to the FDA (U.S. Food and Drug Administration), but in this study it was adjusted to 200 mL, to adapt to the caloric requirements of the triathletes. The placebo beverage was a mixture of water, authorized red dye, flavoring, and sweetener, with sensory characteristics very similar to those described for the ACJ . This placebo drink has been used in two other previous research [17, 33].

2.3 Training load

Triathlon is a sport where three exercises (swimming, cycling, and running) are performed in a continuous way, these three are being the most common exercises among human forms of locomotion [34]. The quantification of training programs was addressed to evaluate their effects on physiological adaptation and subsequent performance [35]. The training load quantification was performed using the objective load scale (ECOs), to learn more about this scale, refer to the papers below [34, 36] .The training loads developed by triathletes in the present trial were similar to those found in other studies [13, 37, 38]. The values of daily and weekly trainings have been summarized to assess the ECOs of each volunteer, depending on their physical characteristics and the intensity of the training program (the ECOs data presented are the average of the individual ECOs of the triathletes; Figure 1). Briefly, and from a general point of view, the intensity was exponentially –not linearly– considered, with the aim of leveling off the total training stress for a given performance level. The volume was quantified by time and this allowed better comparison of different performance levels and terrain conditions (pavement, uneven laps) [34] .

2.4 Study design

Sixteen Caucasian triathletes (6 training women and 10 training men), aged 19-21 years from the University of Alicante (Spain) agreed to participate in the project. The recruitment started on 28th-29th October 2010 and was completed on 24th-25th March 2011. The volunteers were non-smokers, had stable food habits, and did not receive any medication (the specific absence of the acute administration of anti-inflammatory drugs) during the experimental procedure. The study was approved by the Bioethics Committee of the University Hospital of Murcia, in accordance with the principles of the Declaration of Helsinki, and all participants signed written informed consent.

This was a randomized, double-blind, placebo-controlled, and crossover study (Figure 1). Before the supplementation with ACJ, two urine-sampling periods (as controls) were used: the first was a control baseline (C-B) with loads training minimal (ECOs) and the second control (Control-Training: C-T) started with an increase in ECOs; both lasted 15 days. Both groups consumed ACJ or placebo during 45 days (200 mL beverage). Ten days were utilized as the washout period without drink intake, while maintaining the training and the control diet. Subsequently, the intervention protocol was repeated, swapping the two groups according to the corresponding drink intake and maintaining their ECOs. The drink intake was 15 minutes after their training finished, to improve the bioavailability of ACJ [13]. After the crossover period, the control post-treatment (CP-T) was started for the last 15 days of study without supplementation and with decreases of ECOs (active recovery phase) with the objective of analyzing the post-training adaptations. Twenty-four-hour urine samples were collected at the end of each period (as shown in Figure 1). To learn more about study design, refer to the paper previously published [16].

2.5 Sample collection and preparation

Twenty-four-hour urine samples were collected on the last day of each stage. They were collected in sterile and clear polystyrene pots with screw caps and were protected from light. One milliliter of the urine excreted over 24-hours was analyzed and used for the absolute calculation of the amounts of F_4-NeuroPs and F_2-dihomo-IsoPs excreted by all volunteers. All F_4-NeuroPs and F_2-dihomo-IsoPs were assayed using the method previously described [22].

2.6 Chemicals and Standards

Four F_4-NeuroPs (4(*RS*)-4-F_{4t}-NeuroP, 4-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, and, 10-F_{4t}-NeuroP) as well as four F_2-dihomo-IsoPs (*ent*-7(*R*)-7-F_{2t}-dihomo-IsoP, *ent*-7*(S)*-7-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, and 17-*epi*-17-F_{2t}-dihomo-IsoP) were utilized in this experiment. Three deuterated internal standards (d_4-4(*RS*)-F_{4t}-NeuroP, d_4-10-*epi*-10-F_{4t}-NeuroP, and d_4-10-F_{4t}-NeuroP) were used for the quality control of the analyses (Figure 2). All standards were synthesized using our published strategies [39-41]. The β-glucuronidase, type H2, from *Helix pomatia* and BIS-TRIS (Bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane) used was purchased from Sigma-Aldrich (St. Louis, MO, USA). All LC-MS grade solvents were from J.T. Baker (Phillipsburg, NJ, USA). The Strata X-AW SPE cartridges (100 mg 3 mL^{-1}) were obtained from Phenomenex (Torrance, CA, USA).

2.7 UHPLC-QqQ-MS/MS analyses

The separation of F_4-NeuroPs and F_2-dihomo-IsoPs in the urine samples was performed by Ultra High Pressure Liquid Chromatography-triple Quadrupole-Tandem Mass Spectrometry (UHPLC-QqQ-MS/MS), Agilent Technologies, Waldbronn, Germany), using

the set-up described by [22]. Data acquisition and processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies, Waldbronn, Germany). The identification and quantification of F_4-NeuroPs and F_2-dihomo-IsoPs were carried out using the authentic markers previously described [22].

2.8 Statistical analyses

Specific differences between the amounts of F_4-NeuroPs and F_2-dihomo-IsoPs excreted (ng 24 h^{-1}) in the different stages were analyzed by Friedman´s non-parametric repeated measures analysis of variance (ANOVA), since the normality and/or equal variance tests failed. When a significant difference was found in the ANOVA, a pair-wise comparison was performed using the Wilcoxon signed rank test with Bonferroni correction. *A posteriori*, sample size was calculated using the value r, calculated by $r=Z/\sqrt{N}$, in which Z is the Z-score that SPSS produce, and N is the size of the study on which Z is based. A r value of 0.1, 0.3, or 0.5 was considered to show a small, moderate, or large effect, respectively [42]**.** The data are shown as mean ± SD, as well as the quartiles (upper values 75%, median 50%, and lower values 25%), of the F_4-NeuroPs and F_2-dihomo-IsoPs excreted throughout the study. Because the crossover period data, of the two phases did not differ, data from both groups were pooled into one placebo or ACJ treatment. The statistical analyses were carried out using the SPSS 23.0 software package (LEAD Technologies Inc. Chicago, USA). The graphs were carried out using the Sigma Plot 12.0 software package (Systat Software, Inc. SigmaPlot for Windows).

3. Results y discussion

In a previous study realized in our group, we observed that urinary levels of the F_4-NeuroPs and F_2-dihomo-IsoPs remained constant during a short triathlon training (2-weeks) at sea level [43]. This study analyzed the same eight biomarkers in the urine, but the present trial had a longer period (145 days), furthermore analyzing the effect of the supplementation of our rich-polyphenols juice (200 mL) in the diet. The identification was confirmed according to their molecular mass, the characteristic MS/MS fragmentation product ions, and the retention time relative to the corresponding standard. Only six biomarkers were quantified (Table 4). Our volunteers did not show representative differences through of the experimental study, according working Group of Kinanthropometrics procedure (Table 1). The majority of our triathletes ranged from 19 to 21 years old (Table 1), belonging to the young adult period in accordance to the human life-stages. According to our current knowledge [44], this life-stage is ideal for quantification of these specific markers for DHA and AdA peroxidation (F_4-NeuroPs and F_2-dihomo-IsoPs), since in sedentary and healthy young adults we detected low amounts of oxidative damage biomarkers. Thereby, the evaluation in this group indicated a behavior more real of the effects due to triathlon training and supplementation of our ACJ in the diet on lipid peroxidation from CNS.

3.1 F_2-dihomo-Isoprostanes

The F_2-dihomo-IsoPs are specific markers for free radical-induced AdA peroxidation, being potential markers of free radical damage to myelin in the human brain [18]. For example, in cerebrospinal fluid, the F_2-dihomo-IsoPs levels were associated with some neuropsychological symptoms of Alzheimer's disease [45]**.** De Felice *et al* published[23] that the

plasma F_2-dihomo-IsoPs were involved in the pathogenesis of Rett syndrome. In this assay , the urinary biomarkers derived from AdA were detected in all samples during the whole period of the study, and ranged from ~1787 to ~4813 ng 24 h^{-1} (Table 4). The two F_2-dihomo-IsoP metabolites of the 17-series showed significant changes (Table 4); the values decreased with the increase of ECOs training and continued to decline during the ACJ intake. Particularly, 17-*epi*-17-F_{2t}-dihomo-IsoP differed significantly among the C-B values compared to C-T (Z=-2.783, *P*=0.005, r= 0.695), placebo (Z=-3.124, *P*=0.002, r= 0.781), and ACJ stages (Z=-3.408, *P*=0.001, r= 0.852), respectively. The excretion of 17-F_{2t}-dihomo-IsoP reached its highest value in C-B. The Bonferroni correction of the results from the Wilcoxon test gave $P < 0.005$, showing that the C-B value was statistically higher than those from placebo (Z = -3.124, *P* = 0.002, r = 0.781), ACJ (Z = -3.067, *P* = 0.002, r = 0.766), and CP-T (Z = -3.181, *P* = 0.001, r = 0.795) (Figure 3). Therefore, our results demonstrated that the F_2-dihomo-IsoPs values had significant changes due to increase or decrease of the training loads, as well as, the influence depending on the time (acute or chronic). The OS elicits different responses depending on the type of the organ tissue and its endogenous antioxidant levels, upon acute and chronic exercise [3]. In fact, regular aerobic, moderate training or physical activity programs could increase the resistance against OS to promote antioxidant capacity in the brain[3]. Highlighting also that our athletes have no influence according their range age, since a research found that *ent*-7(*R*)-7-F_{2t}-dihomo-IsoP, *ent*-7-*epi*-7-F_{2t}-dihomo-IsoP, 17-F_{2t}-dihomo-IsoP, and 17-*epi*-17-F_{2t}-dihomo-IsoP in sedentary and healthy volunteers between the ages of 13 and 35 years did not have significant differences [44].

Otherwise, the Friedman test showed a significant difference in the *ent*-7-(*R*)-7-F_{2t}-dihomo-IsoP values (Table 4), and also a significant increase in CP-T compared with C-T

stage. In CP-T, the training load was decreased around 50 % after 115 days with high load training (1008 ± 105 ECOs). Post hoc analysis with the Wilcoxon signed-rank test showed that values were higher in the CP-T stage (Figure 3), although only the C-T stage (Z=-3.389, P=0.001, r= 0.847) differed significantly with the Bonferroni correction ($P < 0.005$). This result indicates that an acute decrease of training loads after chronic exercise programme may stimulate the adaptation response where this oxidative product deriving from radical attack on AdA (ent-7(*RS*)-7-F_{2t}-dihomo-IsoP), could play a role in this adaptation post-training, although typically the F_2-dihomo-IsoPs provide a relatively-selective insight into oxidative damage to myelin since they are the oxidative products deriving from radical attack on AdA. These markers are also considered to reflect cerebral white matter injury[46]; however, we should also remember that AdA is present in other organs, like kidney and adrenal glands[18, 47]. Thereby, physical exercise effects on OS from kidney and adrenal glands could also reflect similar results. Besides, a previous study reflected that the urinary levels of F_2-IsoP decreased with chronic exercise in most of the cases and chronic exercise may rarely result in increased urine F_2-IsoP levels [48], while some studies have supported no changes. Our results are consistent with the three changes that were mentioned by Nikolaidis, M. G *et al* [48] in their review**,** since any change in the e*nt*-7-*epi*-7-F_{2t}-dihomo-IsoP values was also observed [18] remaining at constant levels throughout the study with no statistical differences.

Regarding to the possible role of the compounds from our juice on the lipid peroxidation from AdA (whatever the current physiological origin: brain white matter, adrenal gland or kidney), the 17-*epi*-17-F_{2t}-dihomo-IsoP in ACJ stage was significantly lower that CP-T values (Z=-3.013, P=0.003, r= 0.753) (Figure 3). From our point of view, this significant difference perhaps is due to over-activation of the steroid biosynthesis pathway

314 in the particular case of citrus juices [49], since this pathway is mainly located in the adrenal
315 glands and gonads as well as within nervous system. There is evidence of neurotrophic and
316 neuroprotective effects on the CNS involving steroid mechanism, for example the
317 progesterone has been linked with a decreased of the amount of LPP [50]. A steroid conjugate
318 from progesterone (17-hydroxyprogesterone) was identified as metabolite significantly after
319 the citrus juice intake [49], suggesting a possible role on OS status. Another explanation is that
320 due to food biomarkers discovered after the ingestion of ACJ in healthy volunteers: proline
321 betaine, ferulic acid, and two mercapturate derivatives [17], they may be related with the
322 decrease of 17-*epi*-17-F_{2t}-dihomo-IsoP levels in combination with the training sessions. For
323 example, the proline betaine (specific and sensitive markers of citrus fruit intake) had a
324 lowering effect on plasma homocysteine concentration in a healthy volunteers [51] Lowering
325 plasma homocysteine levels has been related with lowered OS, conversely if this amino acid
326 increases its levels can lead to prooxidative activity, age-related cognitive impairment,
327 neurodegenerative and cerebrovascular disease [52]. In addition, ferulic acid provides
328 protection also against lipid peroxidation and prevents the attacks to the membrane. Acting
329 as an antioxidant potential due to its structural characteristics, the presence of electron
330 donating groups on the benzene ring and to its carboxylic acid group [53]. In biological models,
331 the ferulic acid showed a role as inhibitor or disaggregating agent of amyloid structure
332 suggesting a positive effect in the first steps to trigger Alzheimer's disease [54]. Alzheimer's
333 disease has been related with the increase of F_2-dihomo-IsoPs levels [18]. On the other hand,
334 it is noteworthy that ACJ, besides their phytochemicals, provides other compounds such as
335 vitamins and minerals, that appear to have or help antioxidative activities providing health
336 benefits. The vitamin C from the mixture (from citrus to aronia) is a representative compound
337 [32]. Ascorbic acid (vitamin C) is an electron donor and reducing agent, so it prevents the

oxidation of the biomolecules [55]. Ascorbic acid is accumulated in adrenal glands and central nervous system, indicative the importance of ascorbate function in CNS , even with plasmatic levels low [56]. Besides its function as a reactive oxygen species scavenger also helps to restore other substances with antioxidant properties, such as alpha-tocopherol (vitamin E) or glutathione(antioxidant in plants)[55]. Anti-oxidative effects related to mineral intake from aronia and/or citrus did not find conclusive data, although, orange juice consumption exhibited to enhance the absorption of minerals (iron, aluminum, calcium, zinc, and selenium) from the diet [57] . And besides, we found that in animal models the hesperidin intake (a monomethylated flavanone found abundantly oranges) due to its antioxidant and antiinflamatory properties showed protective effects on the bone mineral density [58]. The minerals in vivo are involved in the production of free radical, since can accelerate or delay the oxidative stress and neurodegeneration occurring in the CNS [56]. Therefore, minerals and vitamins from our ACJ, maybe have involved in the lipid peroxidation pathways for this result.

Nonetheless, further research is needed on the correlation of potential beneficial effects of polyphenols-rich dietary supplements and their particular mechanisms of action of each compound lonely or in conjunction with others on the markers of central nervous system degradation in athletes**,** although some experimental studies have indicated positive biological effects of polyphenols-rich dietary supplements in athletes[5, 9, 13, 59, 60]. Thus, we are developing further research to clarify the positive influence that the intake of functional fruit juices and polyphenols could have in athletes [16].

3.2 F_4-neuroprostanes

The F_4-NeuroPs originate from the free radical-catalyzed peroxidation of DHA - an essential constituent of nervous tissue- highly enriched in neurons and highly susceptible to oxidation [21]. Looking our findings, we note a possible effect of ACJ at the neuronal level, since 10-*epi*-10-F_{4t}-NeuroP and 10-F_{4t}-NeuroP were not detected during the intake period compared to placebo stage. In C-T, two F_4-NeuroPs (10-*epi*-10-F_{4t}-NeuroP (Z = -2.845, $P = 0.004$, r = 0.711 and 10-F_{4t}-NeuroP (Z = -2.499, $P = 0.012$, r = 0.624)) showed a decrease before the crossover intake of the beverages (placebo or ACJ) (Figure 3).The 10-F_{4t}-NeuroP values continued to decline significantly in the placebo stage (Z =-3.130, $P = 0.002$, r = 0.782) (Figure 3). During the ACJ stage and CP-T, these F_4-NeuroPs were not detected (Table 4). The decline of the excretion of the NeuroPs in our study could partially be attributed to the ingestion of bioactive compounds found in our polyphenols-rich juice. There is evidence showing that citrus fruits intake could alter the OS of the CNS [7] and particularly, polyphenols may alter brain function at three locations: outside the CNS (for instance, by improving cerebral blood flow or by modulating signaling pathways from peripheral organs to the brain), at the blood–brain barrier (*e.g.*, by altering multi-drug-resistant protein-dependent influx and efflux mechanisms of various biomolecules), and inside the CNS (*e.g.*, by directly modifying the activity of neurons and glial cells). In addition, citrus fruits, which are rich in and abundant sources of hesperidin and other polyphenols, are promising for the development of general food-based neuroprotection and "brain foods" [15]. A recent review gathered evidence about the neuroprotective actions of the flavonoids mentioned that may influence the survival cascade and transcription factors by modulating the redox potential of neurons and glia. I*n vivo* activities of flavonoids in the brain remain to be elucidated, but have shown potential functions against oxidative damage [61], as has been shown in this study.

384 The health effects of polyphenols depend on the amount consumed and their
385 bioavailability. The bioavailability is a key aspect to exert antioxidant activity in human,
386 since many polyphenols have a scarce bioavailability and are extensively metabolized [62].
387 According to our previous study, the bioavailability of flavanones from ACJ intake increased
388 in the triathletes, suggesting that over-activation of the microbiota and intestinal motility
389 were caused by physical exercise - helping to increase the bioavailability of the compounds
390 in the ACJ [13]. The results obtained in this study with the ACJ supplementation (one serving,
391 200 mL), which was adjusted to the normal diet of our athletes (the intake always being
392 around 15 minutes after training for 45 days) suggest an effect of the ACJ due to the
393 combination with the physical exercise. Based on the physiological changes that may re-
394 establish colonic motility after exercise, when blood flow is restored, allowing maximum
395 exposure and absorption of nutrients including polyphenols and thus, the increase the
396 flavonoids bioavailability [62]. In support of the above affirmation, Gomez, Pinilla [8] mentioned
397 that the combination of polyphenols intake and physical activity can deliver more beneficial
398 effects than intervention alone or the mixed effects of exercise. For example, a study in
399 athletes showed that the increase of the intake of anthocyanins can limit the exercise-induced
400 oxidative damage to red blood cells, most probably by enhancing the endogenous antioxidant
401 defense system. These athletes daily consumed 150 mL of chokeberry juice - providing 23
402 mg/100 mL anthocyanin - during a period of one month [60]. Other nutritional intervention in
403 athletes also showed the protective effect against OS induced by the consumption of
404 polyphenols from grape extract (400 mg/day) [59]. Furthermore, berry extracts could have
405 effects associated with their ability to maintain metabolic homeostasis, thus protecting
406 membranes from lipid peroxidation and affecting synaptic plasticity [63]. *In vitro* and animal
407 models has been proved the beneficial effects of polyphenols on exercise-induced OS, muscle

damage and exercise performance, but in human studies further research is required for the better assessment of their benefits [4]. Currently, the mechanisms by which the physical exercise exerts its effects in the brain remain largely unknown although the researchers have provided promising evidences about physical exercise-induced outcomes for several prevalent neurological and psychiatric conditions (CNS) [64]. The reductions of the oxidative stress have been a possible evidence to suggest positive effects on the CNS health[3, 64]. Thus, our study provides evidence of the effect of the intake of ACJ (rich in polyphenols) during a training period with regard to decrease of the NeuroPs values, suggesting a potential positive effect on the nervous system during training.

Another interesting point besides the apparent absence of 10-*epi*-10-F_{4t}-NeuroP and 10-F_{4t}-NeuroP in the ACJ stage, was the significant changes in the values of these NeuroPs during the stages in which they were detected (C-B, C-T, and placebo stage) (Table 4). The excretion of these metabolites tended to decrease, as we could observe for 10-F_{4t}-NeuroP during the study, but, in the placebo stage, 10-*epi*-10-F_{4t}-NeuroP exhibited a significant increase ($Z = -2.543$, $P = 0.011$, $r = 0.635$) in the placebo period, compared with C-T, but returned to previous values in C-B. This behavior of the stereoisomers can depend on different mechanisms, but the precise roles of these isomers *in vivo* have not been elucidated yet. In the urine analysis of the systemic neuroprostane-like compounds (isoprostane, IsoPs) formed *in vivo* via the non–enzymatic, free radical-initiated peroxidation of polyunsaturated fatty acids, it is important to consider that these molecules are not only excreted as the original form since they are extensively metabolized in the liver, producing a biotransformation of the metabolites [65]. For example, in a study of smokers mentioned, all IsoPs are equally increased by any source of OS (e.g., smoking), but some are more

efficiently metabolized, so that their determined concentrations appear less affected by variations at oxidant levels [66]. This would make that highly-metabolized IsoPs appear less correlated with smoking than less-metabolized IsoPs. Another possibility was that exposure to different types of oxidants may affect the mechanisms that create IsoPs, thereby affecting their distribution**.** In our study, the closest relationship was between chronic physical exercise and the metabolite 10-*epi*-10-F_{4t}-NeuroP.

Finally, two F_{4t}-NeuroPs (4-(*RS*)-4-F_{4t}-NeuroP and 4-F_{4t}-NeuroP) were analyzed in this study, but they were below the limit of detection/quantification. Therefore, these data are not shown. In previous work, 4-(*RS*)-F_{4t}-NeuroP and 4F_{4t}-NeuroP were also not detected [22] In addition, other mediator of oxidative stress from omega-3 fatty acid, but this from docosapentaenoic acid (4-F_{3t} NeuroP), was only detected in the 22.22% of the 45 young adults volunteers [44] .Thus, the latest data continue to support the idea that the NeuroPs do not appear to be specific biomarkers in healthy and sedentaries or healthy volunteers.

4. Conclusions

The F_4-NeuroPs, 10-*epi*-10-F_{4t}-NeuroP and 10-F_{4t}-NeuroP, were not detected after the consumption of ACJ. These changes in the excretion values suggest health benefits which could be attributed to the ingestion of bioactive coumpounds that include partial co-responsibility of flavonoids and others phenolics found in ACJ on the oxidative status neuronal membrane. The changes in the excretion of 17-*epi*-17-F_{2t}-dihomo-IsoP show the positive connection between physical exercise and ACJ intake, suggesting that combination of polyphenols intake and physical activity can deliver beneficial effects on neuromotor

system .The physical exercise by itself was also able to exert different responses depending the increases (17-F_{2t}-dihomo-IsoP) or the decreases (*ent*-7-(*RS*)-7-F_{2t}-dihomo-IsoP) of the traininig loads. Thus, the chronic intake of one serving of ACJ rich in polyphenols (200 mL, adjusted to the diet) and an adequate training influenced the OS of the CNS in young adults triathletes will help to elucidate novel interactions and mechanisms among excretion of lipid peroxidation metabolites, supplementation of polyphenols-rich juice in the diet and physical exercise during a training season.

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Figure captions

Figure 1. Study design. This crossover study was randomized, double-blind, and placebo-controlled. Sixteen athletes (n=16), randomly divided into two groups (n=8), were assigned supplementation with either 200 mL of ACJ (Aronia citrus juice) or 200 mL of placebo. After 45 days of supplementation and a 10-days washing-out period, the beverages were reversed. Urine samples were collected on the last day at the end of each stage. The training load was quantified by the Objective Load Scale (ECOs).

Figure 2. Chemical structures of F_4-NeuroPs, F_2-dihomo-IsoPs, and deuterated internal standards. A: F_4-NeuroPs, B: F_2-dihomo-IsoPs

Figure 3. Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of the A) F_2-dihomo-IsoPs and B) F_4-NeuroPs in 24 h^{-1} urine throughout the study (ng 24 h^{-1}). • Outliers data are show. *: shows a significant difference compared to the C-B stage, §: shows a significant difference compared to the ACJ and ‡: shows a significant difference compared to C-T stage. Significant *P*-values are shown according to post hoc analysis with Wilcoxon signed-rank tests (with a Bonferroni correction $P<0.005$, for F_2-dihomo-IsoPs and $P<0.016$, for F_4-NeuroPs). Abbreviations: C-B; Control Baseline, C-T; Control Training, ACJ; Aronia-Citrus Juice, CP-T; Control Post-Treatment.

Table 1. Physical and metabolic characteristics and training loads of the elite triathletes.

Physical characteristics	**Stages of study**				
Male (n=10)	**CB**	**CT**	**Placebo**	**ACJ**	**CP-T**
Age (y)	19.0 ±1.7	19.0 ± 1.7	19.0 ± 1.7	19.4 ± 1.3	19.6 ± 1.3
Weight (kg)	69.0 ± 6.2	69.0 ± 6.4	70.7 ± 6.9	71.2 ± 4.6	72.2 ± 6.8
Height (m)	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
BMI[a] (kg m^{-2})	22.2 ± 1.0	22.2 ± 1.0	21.7 ± 1.4	21.6 ± 1.3	21.8 ± 1.7
Total fat (kg)	9.2 ± 2.8	8.8 ± 2.6	8.0 ± 1.7	6.4 ± 2.8	6.8 ± 1.2
Lean weight (kg)	31.4 ± 2.1	30.5 ± 2.7	31.6 ± 3.0	33.8 ± 3.2	32.4 ± 2.4
Subscapular skinfold (mm)	9.6 ± 3.0	9.5 ± 2.1	9.1 ± 1.7	8.6 ±2.0	8.6 ± 1.8
Triceps skinfold (mm)	8.9 ± 3.0	9.7 ± 2.6	8.7 ± 2.1	7.4 ± 2.4	7.3 ± 1.5
Biceps skinfold (mm)	5.4 ± 2.4	4.7 ± 1.5	4.1 ± 0.6	4.5 ± 1.5	3.7 ± 0.4
Iliac crest skinfold (mm)	12.0 ± 2.6	13.1 ± 4.1	12.5 ± 4.2	11.2 ± 3.4	9.6 ± 2.5
Supraspinale skinfold (mm)	9.0 ± 2.6	8.9 ± 2.8	8.7 ± 2.5	7.6 ± 1.9	6.7 ± 1.4
Abdominal skinfold (mm)	16.4 ± 8.0	15.5 ± 6.8	14.5 ± 5.9	11.8 ± 5.2	10.0 ± 3.7
Front thigh skinfold (mm)	14.9 ± 4.4	14.0 ± 4.4	11.5 ± 2.3	10.1 ± 2.9	10.0 ± 2.5
Medial calf skinfold (mm)	9.0 ± 3.0	9.5 ± 3.1	8.2 ± 2.1	7.2 ± 2.3	7.3 ± 1.8
Training loads ECOs	37.5 ± 5.5	1008 ± 105	923 ± 119	923 ± 119	552 ± 45
Female (n=6)	**CB**	**CT**	**Placebo**	**ACJ**	**CP-T**
Age (y)	21.0 ± 3.0	21.0± 3.0	21.08 ± 3.0	21.0 ± 3.0	21.0 ± 3.0
Weight (kg)	54.8 ± 12.2	54.8 ± 11.6	56.2 ± 4.8	54.4 ± 5.0	53.1 ± 2.9
Height (m)	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
BMI[a] (kg m^{-2})	21.2 ± 4.1	21.2 ± 4.1	20.7 ± 1.3	21.6 ± 2.4	20.5 ± 1.6
Total fat (kg)	8.7 ± 4.1	8.9 ± 4.7	9.2 ± 0.9	7.5 ± 1.2	7.3 ± 1.4
Lean weight (kg)	20.8 ± 3.6	20.6 ± 2.7	20.8 ± 2.4	19.4 ± 2.8	20.9 ± 2.0
Subscapular skinfold (mm)	12.7 ± 6.7	13.4 ± 8.2	11.7 ± 2.5	10.7 ± 1.9	9.9 ± 2.8
Triceps skinfold (mm)	16.3 ± 2.3	18.4 ± 3.8	19.3 ± 5.4	16.1 ± 4.6	17.4 ± 4.6
Biceps skinfold (mm)	10.3 ± 2.8	9.8 ± 3.2	7.2 ± 0.4	5.7 ± 1.0	5.7 ± 1.3
Iliac drest skinfold (mm)	19.7 ± 4.5	17.1 ± 6.9	20.9 ± 4.5	17.3 ± 3.7	13.7 ± 4.3
Supraspinale skinfold (mm)	14.3 ± 6.5	14.4 ± 6.9	15.0 ± 1.0	12.8 ± 2.1	11.6 ± 2.5
Abdominal skinfold (mm)	23.1 ± 5.9	23.6 ± 6.9	24.5 ± 4.7	21.3 ± 4.1	17.9 ± 4.6
Front thigh skinfold (mm)	27.2 ± 5.2	26.4 ± 5.0	25.8 ± 3.6	23.8 ± 12.5	26.0 ± 5.4
Medial calf skinfold (mm)	14.8 ± 3.8	13.9 ± 3.0	15.7 ± 2.1	12.5 ± 1.8	14.4 ± 2.9
Training loads ECOs	37.5 ± 5.5	1008 ± 105	923 ± 119	923 ± 119	552 ± 45

[a] Body Mass Index. CB; Control Baseline, CT; Control Training, ACJ; Aronia-Citrus Juice, CP-T; Control Post-Treatment

Table 2. Dietary parameters and caloric intake of the triathletes during the study

	Male triathletes	**Female triathletes**
Energy intake (kcal)	2820.0 ± 241.2	2072.6 ± 223.4
Carbohydrate (g d^{-1})	326.1 ± 63.5	211.3 ± 43.9
Dietary fiber (g d^{-1})	27.3 ± 7.4	15.5 ± 4.4
Sugars (g d^{-1})	121.3 ± 33.9	80.5 ± 18.3
Proteins (g d^{-1})	133.7 ± 12.9	83.5 ± 9.0
Total lipids (g d^{-1})	113.7 ± 13.3	107.1 ± 14.4
SFA[a] (g d^{-1})	33.5 ± 6.5	29.6 ± 4.4
MUFA[b] (g d^{-1})	56.5 ± 5.5	56.6 ± 7.5
PUFA[c] (g d^{-1})	16.9 ± 2.7	15.9 ± 6.7
Vitamin C (mg d^{-1})	178.9 ± 71.9	135.0 ± 60.4
Vitamin A (µg d^{-1})	2970.0 ± 913.9	1427.4 ± 573.1
Vitamin E (mg d^{-1})	21.0 ± 5.6	13.9 ± 3.4
Vitamin D (mg d^{-1})	988. ± 47.5	751.6 ± 163.0
Iron (mg d^{-1})	20.9 ± 2.4	14.9 ± 2.6
Selenium (mg d^{-1})	149.8 ± 21.5	103.0 ± 17.4

Dietary parameters and caloric intake of the triathletes during the study. [a] Saturated fatty acids, [b] Monounsaturated fatty acids, [c] Polyunsaturated fatty acids.

Table 3.

ACJ	**200 mL**
Energy intake (kcal)	76
Proteins (g)	0.9
Carbohydrate (g)	18
Fat (g)	0.06
Phenolics compounds [a]	
Total Flavonoids (mg)	129.31 ± 1.79
Hydroxycinnamic acids (mg)	68.82 ±0.6

The values are means ± standard deviation (n=3, expressed as mg per 200 mL of juice). [a] To find out about more detailed analysis of the phenolics compounds from this juice, see the reference [16]

Table 4. Urinary F_4-neuroprostanes and F_2-dihomo-isoprostane (ng 24 h^{-1}) [Z] determined throughout the assay

From	Analyte (ng 24 h^{-1})[Z]	X^2	*df*	*Sig*	Stages of study C-B (n=16)	C-T (n=16)	Placebo [a] (n=16)	ACJ [a] (n=16)	CP-T (n=16)
Ω3	***Neuronal membrane degradation***								
DHA	10-*epi*-10-F_{4t}-NeuroP	11.37	2	0.003	**4930.3** ±1844.4	**2953.2** ± 1176.3	**4135.4** ± 1005.0	n.d	n.d
	10-F_{4t}-NeuroP	20.93	2	0.000	**2711.6** ± 294.5	**1909.9** ± 116.7	**891.6** ± 372.7	n.d	n.d
Ω6	***Neuromotor system degradation***								
AdA	17-*epi*-17-F_{2t}-dihomo-IsoP	27.14	4	0.000	**2689.4** ± 487.5	**2018.6** ± 507.0	**2016.6** ± 330.4	**1787.0** ± 328.6	**2319.9** ± 444.9
	17-F_{2t}-dihomo-IsoP	24.48	4	0.000	**3604.4** ± 628.4	**2677.7** ± 444.7	**2842.8** ± 316.7	**2559.1** ± 504.4	**2607.1** ± 450.9
	Ent-7(*R*)-7-F_{2t}-dihomo-IsoP	22.56	4	0.000	**4045.3** ± 763.5	**3551.1** ± 534.2	**3914.9** ± 444.2	**4070.2** ± 599.5	**4639.7** ± 612.8
	Ent-7-*epi*-7-F_{2t}-dihomo-IsoP	8.80	4	0.066	**4179.0** ± 815.7	**4020.6** ± 1115.9	**4216.3** ± 629.4	**4813.23** ± 1040.9	**4255.0** ± 834.2

The data are shown as means ± SD. N.d: not detected. [Z] The volume of urine excreted by the volunteers was 1212.42 ± 716.50 ml per 24 h^{-1}, on average, in all the periods. [a] Average of the two urine collections in the crossover period (Placebo/ACJ). C-B; Control Baseline, C-T; Control Training, ACJ; Aronia-Citrus Juice, CP-T; Control Post-Treatment.

Figure 1

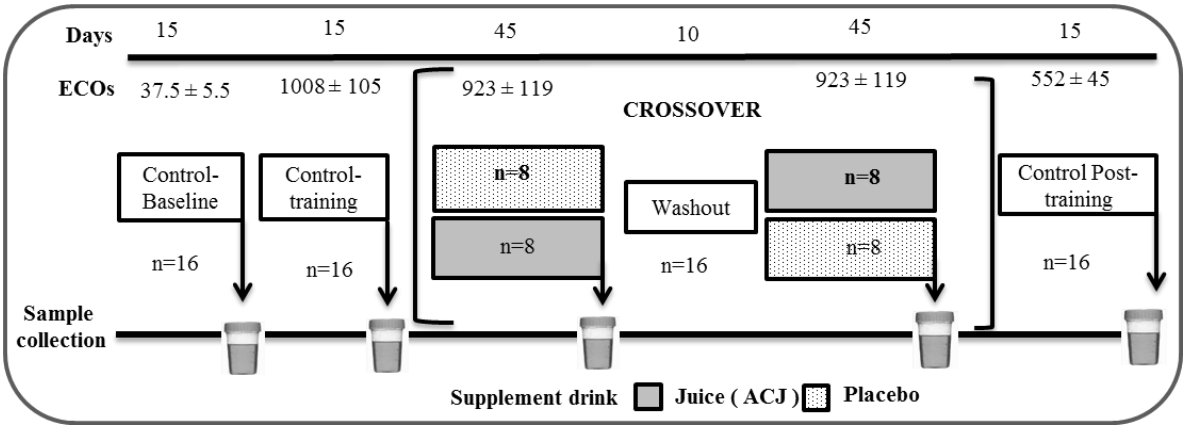

Days
15
15
45
10
45
15
ECOs
37.5 ± 5.5
1008 ± 105
923 ± 119
923 ± 119
552 ± 45
CROSSOVER
Control-Baseline
Control-training
n=8
n=8
Washout
n=8
n=8
Control Post-training
n=16
n=16
n=16
n=16
Sample collection
Supplement drink
Juice (ACJ)
Placebo

Figure 2

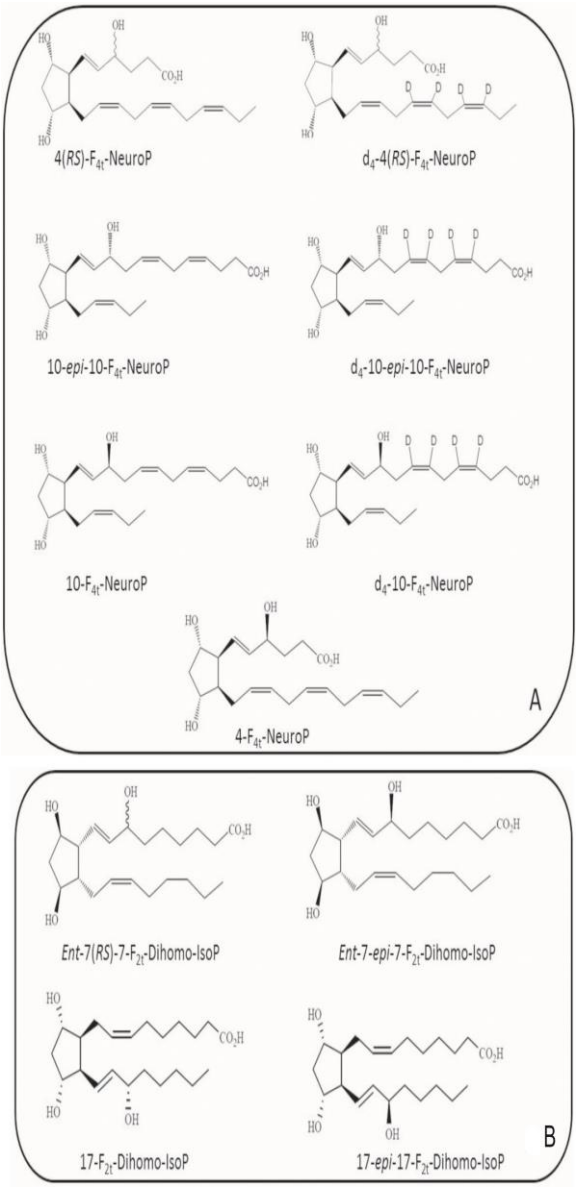
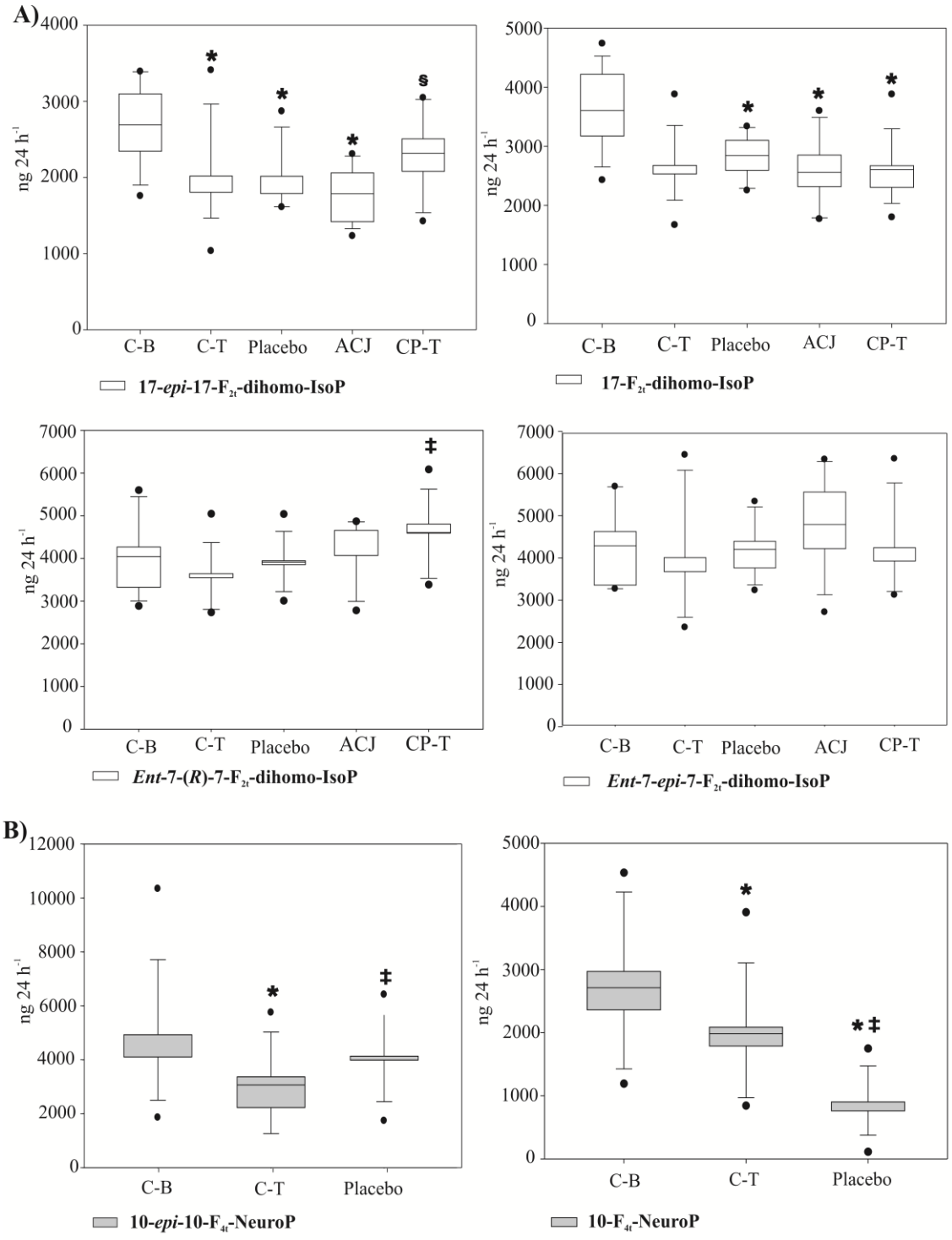

4(RS)-F4t-NeuroP
d4-4(RS)-F4t-NeuroP
10-epi-10-F4t-NeuroP
d4-10-epi-10-F4t-NeuroP
10-F4t-NeuroP
d4-10-F4t-NeuroP
4-F4t-NeuroP
A
Ent-7(RS)-7-F2t-Dihomo-IsoP
Ent-7-epi-7-F2t-Dihomo-IsoP
17-F2t-Dihomo-IsoP
17-epi-17-F2t-Dihomo-IsoP
B

Figure 3


A)
ng 24 h-1
C-B
C-T
Placebo
ACJ
CP-T
17-epi-17-F2t-dihomo-IsoP
17-F2t-dihomo-IsoP
Ent-7-(R)-7-F2t-dihomo-IsoP
Ent-7-epi-7-F2t-dihomo-IsoP
B)
10-epi-10-F4t-NeuroP
10-F4t-NeuroP