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## **Aronia – citrus juice (polyphenol-rich juice) intake and elite triathlon training: a lipidomic approach using representative oxylipins in urine**

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1 ***Aronia-citrus* juice intake (polyphenol rich juice) and elite triathlon training: A lipidomic**  
2 **approach using representative oxylipins in urine**

3  
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**Abstract**

In the present study, we examined whether particular urinary oxylipins (isoprostanes (IsoPs), leukotrienes (LTs), prostaglandins (PGs), and thromboxanes (TXs)) in 16 elite triathletes could alter during 145 days of training. Within this time span, 45 days were dedicated to examining the effects of the intake of a beverage rich in polyphenols (one serving: 200 mL per day) supplemented in their diet. The beverage was a mixture of *citrus* juice (95%) and *Aronia melanocarpa* juice (5%) (ACJ). Fifty-two oxylipins were analyzed in urine. The quantification was carried out using solid-phase extraction, liquid chromatography coupled to triple quadrupole mass spectrometry. The physical activity decreased the excretion of some PGs, IsoPs, TXs, LTs metabolites from arachidonic acid,  $\gamma$ -dihomo-linolenic acid, and eicosapentaenoic acid. The ACJ also reduced the excretion of 2,3-dinor- $11\beta$ -PGF<sub>2 $\alpha$</sub>  and 11-dh-TXB<sub>2</sub>, although the levels of other metabolites increased after juice supplementation (PGE<sub>2</sub>, 15-keto-15-F<sub>2t</sub>-IsoP, 20-OH-PGE<sub>2</sub>, LTE<sub>4</sub>, and 15-*epi*-15-E<sub>2t</sub>-IsoP), compared to the placebo. The metabolites that increased in abundance have been related to vascular homeostasis and smooth muscle function, suggesting a positive effect on the cardiovascular system. In conclusion, the exercise influences mainly the decrease in oxidative stress and the inflammation status in elite triathletes, while ACJ supplementation has a potential benefit regarding the cardiovascular system that is connected in a synergistic manner with elite physical activity.

**Key Word:** Urinary oxylipins; Polyphenols; Juice; Athletes; Training.

## 64 **Introduction**

65 Currently, it is not clear whether polyphenol supplementation exerts beneficial effects on oxidative  
66 stress (OS) and/or the inflammation status in the area of sport.<sup>1,2</sup> Many studies analyzing the effects  
67 of dietary polyphenols on human health have been performed in the last decade, with increasing  
68 numbers of reports studying flavonoids and polyphenols in general.<sup>3,4</sup> Polyphenol supplementation  
69 in exercise studies includes mainly extracts, juices, infusions, or increased intake of polyphenol-rich  
70 foods (including functional foods).<sup>1</sup> In athletes of different disciplines, polyphenols have shown an  
71 antioxidant potential that can be beneficial for the reduction of the effects of oxidative damage  
72 during intense exercise, apparently without an anti-inflammatory effect.<sup>4</sup> Furthermore, it is also  
73 necessary to take into account the effect of the physical exercise, since this external factor has  
74 shown a positive effect on lipid peroxidation and/or OS as a consequence of its chronic practice.<sup>5-8</sup>  
75 In 2005, Petersen<sup>9</sup> mentioned that regular exercise induces an anti-inflammatory response rather  
76 than a pro-inflammatory response. Regular exercise training promotes increases in enzymatic and  
77 non-enzymatic antioxidants in muscle fibers, resulting in improved endogenous protection against  
78 exercise-mediated oxidative damage.<sup>10</sup>

79 In the field of sports science and elite sports environment, biomarkers are used to make  
80 inferences about the athlete's underlying physiology and health, particularly in the context of  
81 adaptation to training and the impact of environmental stressors.<sup>11</sup> Metabolomics and lipidomics  
82 data indicate that intensive and prolonged exercise is associated with extensive lipid mobilization  
83 and oxidation, including many components in the pathway of linoleic acid conversion and related  
84 oxidized derivatives or oxylipins.<sup>12</sup> The lipid metabolism constitutes a network of pathways that are  
85 related at multiple biosynthetic hubs.<sup>13</sup> Oxygenated lipids are known collectively as oxylipins.<sup>14</sup>  
86 Eicosanoids, a subset of oxylipins, are signaling molecules that have been used as biomarkers for a  
87 global picture of changes in lipid peroxidation and vascular events as a consequence of chronic  
88 exercise and the supplementation of polyphenols.<sup>5-8, 12-14</sup> Eicosanoids are a family that includes  
89 prostaglandins (PGs), leukotrienes (LTs), thromboxanes (TXs), and isoprostane (IsoPs), which are

90 lipid mediators involved in the physiopathology of all organs, tissues, and cells.<sup>17</sup> The PGs and  
91 TXs, collectively termed prostanoids, are formed when arachidonic acid (AA), a 20-carbon  
92 unsaturated fatty acid, is released from the plasma membrane by phospholipases and metabolized  
93 by the sequential actions of prostaglandin G/H synthase, or cyclooxygenase (COX). TXA<sub>2</sub> is  
94 synthesized from prostaglandin H<sub>2</sub> (PGH<sub>2</sub>) by thromboxane synthase, and it is non-enzymatically  
95 degraded into biologically inactive thromboxane B<sub>2</sub> (TXB<sub>2</sub>).<sup>18</sup> On the other hand, there are four  
96 primary bioactive PGs generated *in vivo*: prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), prostacyclin (PGI<sub>2</sub>),  
97 prostaglandin D<sub>2</sub> (PGD<sub>2</sub>), and prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>).<sup>18</sup> Besides AA, another polyunsaturated  
98 fatty acid (PUFA) is dihomo- $\gamma$ -linolenic acid (DGLA), a 20-carbon n-6 (C20:3 n-6) derived *in vivo*  
99 from  $\alpha$ -linolenic acid (c18:3 n-6). Through a series of free radical reactions, COX metabolizes  
100 DGLA and AA to form various bioactive metabolites: namely, the 1 and the 2 series of PGs (PG1  
101 and PG2), respectively.<sup>19</sup> The LTs also contain 20 carbons, but lack the 5-carbon ring structure.<sup>20</sup>  
102 They are AA metabolites derived from the action of 5-LOX (5-lipoxygenase). The immediate  
103 product of 5-LOX is LTA<sub>4</sub> (leukotriene A<sub>4</sub>), which is enzymatically converted into either LTB<sub>4</sub>  
104 (leukotriene B<sub>4</sub>), by LTA<sub>4</sub> hydrolase, or LTC<sub>4</sub> (leukotriene C<sub>4</sub>), by LTC<sub>4</sub> synthase.<sup>20</sup> The  
105 glutathione conjugate forms are termed *cys*-LTs (cysteinyl leukotrienes) and include leukotriene C<sub>4</sub>  
106 (LTC<sub>4</sub>), leukotriene D<sub>4</sub> (LTD<sub>4</sub>), and leukotriene E<sub>4</sub> (LTE<sub>4</sub>). The *Cys*-LTs are potent  
107 bronchoconstrictors and vasoconstrictors.<sup>13</sup> The biosynthesis of eoxins (EX), structural isomers of  
108 *cys*-LTs, is initiated via the 15-lipoxygenase (15-LOX) pathway. Also, there is another pathway that  
109 occurs *in vivo* through a free radical-mediated mechanism to yield a series of PG-like compounds  
110 termed IsoPs, independent of the catalytic activity of COX.<sup>21, 22</sup> The F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs) are  
111 an *in vivo* index of OS.<sup>16</sup> Further, F<sub>1</sub>-phytoprostanes (F<sub>1</sub>-PhytoPs) and F<sub>3</sub>-IsoPs are also generated  
112 from  $\alpha$ -linolenic acid (ALA) and eicosapentaenoic acid (EPA).<sup>23, 24</sup> Finally, 3-series prostanoids,  
113 derived from COX oxidation of EPA, may mediate the anti-inflammatory effects of this fatty acid.

114 <sup>25</sup>

115 Based on the preceding, the primary goal of this randomized, double-blind, placebo  
116 controlled, and crossover study was to ascertain the effects of a serving (200 mL) of *Aronia-citrus*  
117 Juice (ACJ) on the generation and metabolism of oxylipins, using a lipidomic approach. Also, the  
118 study design allowed the assessment of the changes produced by elite training sessions. We  
119 screened biomarkers from AA via LOX (LTs, cysLTs, and EXs), as well as other IsoPs, PGs, and  
120 TXs that complement our schematic of oxylipins (52 lipid mediators (Figure1)).

## 121 **Materials and methods**

### 122 *Physical characteristics of participants*

123 The anthropometric measurements were made according to the International Society of  
124 Advancement of Kinanthropometry (ISAK),<sup>26</sup> and all tests were performed by the same,  
125 internationally certified anthropometrist (Level 2 ISAK) with the objective of decreasing technical  
126 errors of measurement. The body composition was determined by GREC Kinanthropometry  
127 consensus,<sup>27</sup> using a model consisting of total fat by Withers' formula,<sup>28</sup> lean weight by the  
128 procedure described by Leet et al.,<sup>29</sup> and residual mass by the difference in the weight (Table 1)

### 129 *Dietary intake*

130 The calculation of the dietary parameters and caloric intake was accurately designed and  
131 overviewed during the experimental intervention by nutritionists, using specific software for the  
132 calculation (website: <http://www.easydiet.es> and with the additional assistance of the Spanish and  
133 USDA databases (<http://www.bedca.net/> and <http://www.nal.usda.gov/fnic/foodcomp/search/>). The  
134 dietary assessment and planning were based on the sport nutrition guidelines.<sup>30, 31</sup>

### 135 *Aronia-citrus juice and Placebo beverage*

136 The polyphenol rich juice composition was based on a mixture of citrus juice (95%) with  
137 added *Aronia melanocarpa* juice (5%). This juice was developed on a industrial pilot scale (HERO  
138 Spain S.A., Alcantarilla, Murcia) with organoleptically-acceptable criteria to mimic the flavonoids

139 composition of original beverage developed by Gonzales-Molina et al.<sup>32</sup> The nutrients content and  
140 caloric supply of the ACJ that the triathletes consumed are summarized in Table 2, detailing the  
141 percentage contribution of the juice to the total diet.

142 The placebo beverage composition was based on a mixture of water, authorized red dye,  
143 flavoring, and sweetener, giving sensory characteristics close to those of ACJ (see Garcia-Flores et  
144 al.,<sup>33,34</sup> for further information about ACJ composition and nutritional planning).

#### 145 *Training load*

146 The training load quantification was performed using the Objective Load Scale (ECOs)  
147 developed by Cejuela-Anta and Esteve-Lanao.<sup>35</sup> The training loads designed by the triathletes in  
148 the present work were similar to those found in other studies.<sup>5,30,33</sup> This method used in the current  
149 work allowed the quantification of the training loads in triathlon (swim, bike, run, and transitions).  
150<sup>37</sup> The values of daily and weekly training were determined and summarized to assess the ECOs of  
151 each volunteer, depending on their physical characteristics and the intensity of the training program  
152 (the ECOs data presented in this work are the average of the individual ECOs of the triathletes). The  
153 variations of the ECOs are displayed in Figure 2 for better orientation.

#### 154 *Study design*

155 Sixteen triathletes (6 training women and 10 training men) from the University of Alicante  
156 (Spain) agreed to participate in the project. An elite athlete in the context of sports medicine is an  
157 athlete with potential for competing in the Olympics or as a professional athlete.<sup>38</sup> The volunteers  
158 were non-smokers, had stable food habits, and did not receive any medication (the specific absence  
159 of acute administration of anti-inflammatory drugs) during the experimental procedure. The study  
160 was approved by the Bioethics Committee of the University Hospital of Murcia and was in  
161 accordance with the Declaration of Helsinki. All participants provided written informed consent to a  
162 protocol approved by the institution.<sup>39</sup> The recruitment started on 28<sup>th</sup>-29<sup>th</sup> October 2010 and was



163 completed on 24<sup>th</sup>-25<sup>th</sup> March 2011. This study was a randomized, double-blind, placebo controlled,  
164 and crossover design (Figure 2) and was previously approved by nutritional experts. We assumed  
165 an equal allocation of volunteers to each beverage using computer-generated simple randomization  
166 with consecutive codes linked to the preparation of the placebo or ACJ. An impartial outsider,  
167 without the knowledge of the study, helped us to select the randomization code and indicated the  
168 assignment order. The volunteers remained blinded throughout study as well as the researchers  
169 responsible for the outcome measurements and the data analysis (see Garcia-Flores *et al.*,<sup>33, 34</sup> for  
170 further information).

#### 171 *Urine sample collection and preparation*

172 Twenty-four-hour urine samples were collected at the end of each stage (C-B, control  
173 baseline, C-T, control training, placebo intake stage, ACJ: *Aronia-citrus* juice intake stage, and CP-  
174 T, control post-training). All samples collected were immediately frozen (-80 °C) to preserve the  
175 sample integrity until the time of analysis.

#### 176 *Chemicals and analytes*

177 Seven IsoPs derived from AA: 15-F<sub>2t</sub>-IsoP; 15-keto-15-F<sub>2t</sub>-IsoP; 15-*epi*-15-F<sub>2t</sub>-IsoP; 2,3-  
178 dinor-15-F<sub>2t</sub>-IsoP; *ent*-15-*epi*-15-F<sub>2t</sub>-IsoP; 9-*epi*-15-F<sub>2t</sub>-IsoP; 15-keto-15-E<sub>2t</sub>-IsoP, 31 enzymatic  
179 metabolites of AA: PGD<sub>2</sub>; PGDM (PGD metabolite); tetranor-PGDM lactone (tetranor-PGD  
180 metabolite lactone); 11-β-PGF<sub>2α</sub>; 2,3-dinor-11-β-PGF<sub>2α</sub>; tetranor-PGJM (tetranor-PGJ metabolite);  
181 tetranor-PGDM (tetranor-PGD metabolite); 6-*keto*-PGF<sub>1α</sub>; PGE<sub>2</sub>; 20-OH-PGE<sub>2</sub>; tetranor-PGEM  
182 (tetranor-PGE metabolite); tetranor-PGAM (tetranor-PGA metabolite); 13,14-dihydro-15-keto-  
183 PGE<sub>1</sub>; 13,14-dihydro-15-keto-PGE<sub>2</sub>; 13,14-dihydro-15-keto-PGF<sub>2α</sub>; PGF<sub>2α</sub>, tetranor-PGFM  
184 (tetranor-PGF metabolite); 20-OH-PGF<sub>2α</sub>; 19(R)-OH-PGF<sub>2α</sub>; 15-*keto*-PGF<sub>2α</sub>, thromboxane  
185 B<sub>2</sub> (TXB<sub>2</sub>); 2,3-dinor-TXB<sub>2</sub>; 11-dehydro-thromboxane B<sub>2</sub> (11-dh-TXB<sub>2</sub>); leukotriene (LT) B<sub>4</sub>, 20-  
186 carboxy-LTB<sub>4</sub>, 20-hydroxy-LTB<sub>4</sub>, 6-*trans*-LTB<sub>4</sub>; LTC<sub>4</sub>; LTE<sub>4</sub>; EXC<sub>4</sub>; and EXE<sub>4</sub>, four metabolites

187 of DGLA (PGE<sub>1</sub>; PGF<sub>1 $\alpha$</sub> ; 15-F<sub>1t</sub>-IsoP; 15-E<sub>1t</sub>-IsoP), and one metabolite of EPA (17-*trans*-PGF<sub>3 $\alpha$</sub> )  
188 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The authentic markers [<sup>2</sup>H<sub>4</sub>]-  
189 13,14-dihydro-15-keto-PGE<sub>1</sub>, [<sup>2</sup>H<sub>4</sub>]-13,14-dihydro-15-keto-PGE<sub>2</sub>, [<sup>2</sup>H<sub>4</sub>]-13,14-dihydro-15-keto-  
190 PGF<sub>2 $\alpha$</sub> , [<sup>2</sup>H<sub>4</sub>]-6-keto PGF<sub>1 $\alpha$</sub> , [<sup>2</sup>H<sub>4</sub>]-TXB<sub>2</sub>, [<sup>2</sup>H<sub>4</sub>]-20-carboxy-LTB<sub>4</sub>, [<sup>2</sup>H<sub>4</sub>]-LTB<sub>4</sub>, and [<sup>2</sup>H<sub>4</sub>]-8,12-iso-  
191 iPF<sub>2 $\alpha$</sub> -VI were also purchased from Cayman Chemicals.

192 Four IsoPs derived from AA (15-*epi*-15-E<sub>2t</sub>-IsoP; 2, 3-dinor-15-*epi*-15-F<sub>2t</sub>-IsoP; 5-F<sub>2t</sub>-IsoP;  
193 5-*epi*-5-F<sub>2t</sub>-IsoP) and two metabolites of EPA (8-F<sub>3t</sub>-IsoPs and 8-*epi*-8-F<sub>3t</sub>-IsoPs) were synthesized  
194 according to our published procedures,<sup>40-44</sup> while 2, 3-dinor-6-keto-PGF<sub>1 $\alpha$</sub> , [<sup>2</sup>H<sub>3</sub>]-2, 3-dinor-6-keto-  
195 PGF<sub>1 $\alpha$</sub> , EXD<sub>4</sub>, 15-F<sub>2c</sub>-IsoPs, and [<sup>2</sup>H<sub>4</sub>]-15-F<sub>2c</sub>-IsoPs were provided as described by Balgoma, *et al.*,  
196 2013<sup>45</sup>. The enzyme  $\beta$ -glucuronidase, type H2 from *Helix pomatia*, and BIS-TRIS (Bis-(2-  
197 hydroxyethyl)-amino-tris(hydroxymethyl)-methane) were from Sigma-Aldrich (St. Louis, MO,  
198 USA). All LC-MS grade solvents were from J.T. Baker (Phillipsburg, NJ, USA). The Strata X-AW,  
199 100 mg 3 mL<sup>-1</sup> SPE cartridges were purchased from Phenomenex (Torrance, CA, USA).  
200 Ammonium acetate, methoxyamine hydrochloride, and isopropanol were purchased from Sigma-  
201 Aldrich. Milli-Q ultrapure deionized water was used (Millipore Corporation, Billerica, MA).  
202 Methanol and acetonitrile were from Rathburn (Walkerburn, Scotland, UK). Acetone, acetic acid,  
203 and formic acid were from Fisher. Aqueous ammonia (25%, w/v) was from Merck (Darmstadt,  
204 Germany).

#### 205 UHPLC-MS/MS analyses

206 The samples were analyzed according to two methods described previously by Medina, *et al.*<sup>46</sup> and  
207 Balgoma, *et al.*<sup>45</sup>, for the purpose of a deeper analysis of the generation and metabolism of  
208 oxylipins by our volunteers.

#### 209 UHPLC-QqQ-MS/MS for thirty-seven metabolites

210 The separation of the metabolites present in the urine was performed using a UHPLC  
211 coupled with a 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), using the set-up  
212 described by Medina, *et al.*<sup>46</sup>. The main changes are as follows: after being clarified with  
213 MeOH/HCl (200 mM), the urine samples were centrifuged at 10000 rpm for 5 min. The solid phase  
214 extraction was as follows: 1) preconditioning of cartridge with MeOH (2 mL) and then MilliQ  
215 water (2 mL); 2) loading of urine sample; 3) washing of cartridge with MilliQ water (4 mL); 4)  
216 elution of cartridge with MeOH (1 mL). Subsequently, the MeOH was evaporated from the extract  
217 by speed Vac concentrator and the extract was reconstituted in 200  $\mu$ L of mobile phase (A:B)  
218 (90:10). The changes in the identification and quantification of metabolites were as follows:  
219 chromatographic separation was carried out on an ACQUITY UPLC BEH C<sub>18</sub> column (2.1  $\times$  150  
220 mm, 1.7  $\mu$ m; Waters), the column temperatures being 6  $^{\circ}$ C (left) and 6  $^{\circ}$ C (right). The flow rate was  
221 0.15 mL min<sup>-1</sup>, using the linear gradient scheme (t, %B): (0.00; 60), (7.00; 60), (7.01; 73), (10.00;  
222 73), (10.01; 80), (18.00; 100), (19.00; 100), and (19.01; 60). The operating conditions for the MS  
223 parameters were as follows: gas flow: 8 L min<sup>-1</sup>, nebulizer: 30 psi, capillary voltage: 4000 V, nozzle  
224 voltage: 2750 V, gas temperature: 325  $^{\circ}$ C, and jet stream gas flow: 8 L min<sup>-1</sup>. The MS parameters  
225 were in the range of 50 to 160 V and the collision energy was in the range of 0 to 24 V. The  
226 acquisition time was 19.01 min for each sample, with a post-run of 3.0 min for the column  
227 equilibration. The quantification of the oxylipins was carried out by daily preparation of calibration  
228 curves (concentration range 3.9 nM to 1  $\mu$ M) using standard solutions. The matrix effect, recovery  
229 of extraction, and overall process efficiency for each analyte were assessed using post-extraction  
230 addition, established by Matuszewski, *et al.*<sup>47</sup>. The values were within the requested range for all  
231 the metabolites.

232 The sensitivity, precision, and accuracy were established with the same parameters by the  
233 Guidance for Industry-Bioanalytic Method Validation (the intraday and interday values were in the  
234 range of 80-120% for all the metabolites).<sup>48</sup> By this method, the metabolites determined were:

235 PGDM, PGD<sub>2</sub>, tetranor-PGDM lactone, 11- $\beta$ -PGF<sub>2 $\alpha$</sub> , 2,3-dinor-11 $\beta$ -PGF<sub>2 $\alpha$</sub> , tetranor-PGDM, tetranor-  
236 PGJM, PGE<sub>2</sub>, 20-OH-PGE<sub>2</sub>, tetranor-PGEM, tetranor-PGFM, 15-keto- PGF<sub>2 $\alpha$</sub> , 20-OH-PGF<sub>2 $\alpha$</sub> , 19  
237 (R)-OH-PGF<sub>2 $\alpha$</sub> , 2,3-dinor-6-keto PGF<sub>1 $\alpha$</sub> , 6-keto PGF<sub>1 $\alpha$</sub> , 15-F<sub>2t</sub>-IsoP, 15-keto-15-F<sub>2t</sub>-IsoP, 15-*epi*-  
238 15F<sub>2t</sub>-IsoP, 2, 3-dinor-15-F<sub>2t</sub>-IsoP, *ent*-15-*epi*-15F<sub>2t</sub>-IsoP, 9-*epi*-15-F<sub>2t</sub>-IsoP, 2, 3-dinor-15-*epi*-15F<sub>2t</sub>,  
239 5-F<sub>2t</sub>-IsoP, 5-*epi*-5F<sub>2t</sub>-IsoP, 15-keto-15E<sub>2t</sub>-IsoP, 15-*epi*-15E<sub>2t</sub>-IsoP, 11-dh-TXB<sub>2</sub>, 17-*trans*-PGF<sub>3 $\alpha$</sub> , 8-  
240 F<sub>3t</sub>-IsoP, 8-*epi*-8-F<sub>3t</sub>-IsoP, PGE<sub>1</sub>, PGF<sub>1 $\alpha$</sub> , 15-E<sub>1t</sub>-IsoP, and 15-F<sub>1t</sub>-IsoP. The quantification of the  
241 IsoPs, PGs, and TXs detected was performed using authentic markers. Data acquisition and  
242 processing were performed using Mass Hunter software version B.04.00 (Agilent Technologies).

#### 243 *UHPLC-TQ-MS/MS for sixteen metabolites*

244 For the remaining 16 lipid metabolites (LTs, PGs, TXs, and IsoPs), two different analytical  
245 methods based on Balgoma *et al.*<sup>45</sup>, using the same analytical platform: UPLC Acquity- coupled to  
246 a Xevo TQS mass spectrometry system (Waters, Milford, MA) (LC-MS/MS).

#### 247 *Statistical analysis*

248 The metabolites were analyzed individually as well as by series or family, using the  
249 excretion values ( $\mu\text{g } 24 \text{ h}^{-1}$ ) obtained throughout the study (C-B, C-T, placebo stage, ACJ stage, and  
250 CP-T). The 24-h urine was used for the absolute calculation of the amount of the LTs, EXs, IsoPs,  
251 PGs, and TXs excreted; the volume of urine excreted by the volunteers was  $1212.42 \pm 716.50 \text{ mL}$   
252  $24 \text{ h}^{-1}$ , on average, over the assay. The data shown are the mean  $\pm$  SD (Table 3), as well as the  
253 quartiles (upper values 75%, median 50%, and lower values 25%) (Figure 3). We employed non-  
254 parametric statistical tests since the data did not satisfy the assumption of normality. The Friedman  
255 test was used; if the *P*-value was significant, the *post hoc* Wilcoxon signed-rank test was used to  
256 decide which groups were significantly different from each other. The Bonferroni correction was  
257 applied, this correction was calculated by dividing the *P*-value (*P*=0.05) by the number of tests,  
258 namely 10 (if the metabolite was detected in all the stages). Thus, our results were adjusted to

259  $P \leq 0.005$ . The statistical analyses were made using the SPSS 23.0 software package (LEAD  
260 Technologies, Inc. Chicago, USA). The graphs were plotted using the Sigma Plot 12.0 software  
261 package (Systat Software, Inc., SigmaPlot for Windows).

## 262 **Results and Discussion**

263 Currently, the evidence is insufficient to make recommendations for the use of polyphenol  
264 supplementation by elite athletes.<sup>1, 4, 49, 50</sup> So, we wanted to make an in-depth examination of the  
265 primary lipid peroxidation biomarkers using a study design which allows observation of the effects  
266 of physical exercise and polyphenolic-rich beverage intake. A total of 52 oxylipins were screened in  
267 the triathletes' urine (Table 3). The mass spectral information of the oxylipins identified was based  
268 on Medina *et al.*<sup>46</sup> and Balgoma *et al.*<sup>45</sup> In total, 37 metabolites - 17 PGs, 14 IsoPs, two LTs, one  
269 EX, and three TXs - were detected in the urine samples of the triathletes. Therefore, 15 metabolites  
270 (PGD<sub>2</sub>, tetranor-PGJM, 6-keto-PGF<sub>1 $\alpha$</sub> , 20-OH-PGF<sub>2 $\alpha$</sub> , 19(R)-OH-PGF<sub>2 $\alpha$</sub> , 15-*keto*-PGF<sub>2 $\alpha$</sub> , 15-F<sub>1t</sub>-  
271 IsoP, 8-*epi*-8-F<sub>3t</sub>-Isop, LTC<sub>4</sub>, EXC<sub>4</sub>, EXE<sub>4</sub>, 6-*trans*-LTB<sub>4</sub>, 20-carboxy-LTB<sub>4</sub>, 20-hydroxy-LTB<sub>4</sub>,  
272 and 13, 14-dihydro-15-*keto* PGE<sub>1</sub>) were not detected.

273

### 274 *Prostaglandin and thromboxane metabolites derived from Arachidonic acid*

275

276 Recent publications have demonstrated changes in lipid peroxidation as a consequence of  
277 chronic exercise.<sup>5-8</sup> A prior study by our group showed a decrease in the values of urinary PGs  
278 (tetranor-PGEM and 11- $\beta$ -PGF<sub>2 $\alpha$</sub> ) after a chronic training program.<sup>5</sup> Our current results are similar,  
279 showing a decline in these biomarkers due to the elite training program. In our urine samples, 17  
280 PGs from different families were quantified. Our data show means in the range from  $0.04 \pm 0.08 \mu\text{g}$   
281  $24 \text{ h}^{-1}$  (PGE<sub>2</sub>) to  $41.2 \pm 24.4 \mu\text{g} 24 \text{ h}^{-1}$  (PGDM). The PGs are potent oxylipins involved in numerous  
282 homeostatic biological functions and inflammation.<sup>18</sup> The literature mentions that regular exercise  
283 induces an anti-inflammatory response rather than a pro-inflammatory response.<sup>4, 9</sup> In this context,

284 the results for the concentrations of metabolites from the PGD<sub>2</sub> pathway are notable since they have  
285 been implicated in both the development and resolution of inflammation. For the PGD<sub>2</sub> pathway,  
286 the Friedman test revealed statistically significant differences ( $\chi^2(4)=42.143, P<0.001$ ). The CP-T  
287 value was significantly lower, compared to all other stages (Figure 3, A). Moreover, without the  
288 Bonferroni correction, the ACJ stage was different from C-T ( $Z=-2.155, P=0.031$ ). Individually and  
289 concerning the PGD<sub>2</sub> metabolites, PGDM was the metabolite that showed the highest excretion  
290 levels. Prostaglandin D<sub>2</sub> is a COX product of AA that activates D prostanoid receptors to modulate  
291 vascular, platelet, and leukocyte function *in vitro*.<sup>51</sup> The Friedman test revealed statistically  
292 significant changes (Table 3) in this metabolite; the Wilcoxon test showed that the CP-T value was  
293 lower than for C-T ( $Z=-3.237, P<0.001$ ). The 11- $\beta$ -PGF<sub>2 $\alpha$</sub>  content in the CP-T stage was  
294 significantly lower than in all other stages (CB,  $Z=-3.124, P=0.002$ ; C-T,  $Z=-3.124, P<0.001$ ;  
295 placebo,  $Z=-3.237, P=0.001$ ; and ACJ,  $Z=-3.067, P=0.002$ ). The 2, 3-dinor-11- $\beta$ -PGF<sub>2 $\alpha$</sub>  excretion in  
296 the ACJ stage was lower than for C-B ( $Z=-2.953, P=0.003$ ) and C-T ( $Z=-3.124, P=0.002$ ). The ACJ  
297 stage also showed a lower value of this compound compared to the placebo stage, though this was  
298 not statistically significant when applying the correction ( $P=0.009$ ). In the last control stage, the  
299 excretion of tetranor-PGDM was decreased when compared to C-T ( $Z=-3.010, P=0.003$ ), placebo  
300 ( $Z=-3.233, P=0.001$ ), and ACJ ( $Z=-2.856, P=0.004$ ) (Table 3). According to research carried out by  
301 Morrow *et al.*,<sup>52</sup> PGDM is a major urinary metabolite of PGD<sub>2</sub> with a unique lower side-chain that  
302 readily undergoes reversible cyclization. In our study, the urinary excretion of PGDM was highest  
303 under basal conditions, but showed a decreased about 70% by the end of the experiment. This  
304 suggests that in our triathletes there was a reduction in the inflammation status since the hallmark of  
305 inflammation is the enhanced secretion of pro-inflammatory immune mediators such as PGs.<sup>49, 53</sup> A  
306 study in humans using liquid chromatography-tandem mass spectrometry mentioned that tetranor-  
307 PGDM was much more abundant than the PGD<sub>2</sub> metabolites 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  and 2, 3-dinor-11 $\beta$ -PGF<sub>2 $\alpha$</sub>   
308 in the urine of healthy volunteers.<sup>51</sup> In our elite triathletes, 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  and 2, 3-dinor-11 $\beta$ -PGF<sub>2 $\alpha$</sub>  (F-  
309 ring metabolites) were much more abundant than tetranor-PGDM (D-ring metabolite). This leads us

310 to believe that physical exercise affects quantitatively the excretion of metabolites of this PGD  
311 pathway, when compared to non-athletes volunteers. Concerning the effect of ACJ intake on the  
312 excretion of PGD<sub>2</sub> metabolites, we observed a positive influence, since 2, 3-dinor-11 $\beta$ -PGF<sub>2 $\alpha$</sub>   
313 showed a significant decrease when compared to the first controls; also, the excretion of PGDM  
314 showed a significant reduction (in the placebo stage it remained constant). Previous studies, both *in*  
315 *vivo* and *in vitro*, have also reported some influence on the cardiovascular system due to  
316 supplementation in the diet of polyphenols.<sup>1,13,47</sup> In addition, a study by our group analyzed the  
317 biomarker implicated in iron metabolism, hepcidin, and revealed that long-term training using  
318 ECOs reduces inflammation and, hence, could be responsible for the decrease in hepcidin in  
319 triathletes found in this study.<sup>54</sup>

320 Metabolites from the PGE pathway showed a significant decrease after increased training,  
321 suggesting that physical exercise also played a role in the decline in excretion of these metabolites.  
322 The metabolites of the PGE<sub>2</sub> pathway in C-B and C-T was higher, but subsequently fell ( $\chi^2$   
323 (4)=21.962,  $P=0.001$ ) (Figure 3, A). As well, we cannot rule out an effect of ACJ intake on  
324 inflammation since the excretion of PGE<sub>2</sub> (detected in all periods) increased in comparison to the  
325 placebo stage ( $0.04 \pm 0.08$  vs.  $0.19 \pm 0.30$ ). The placebo period showed lower values than C-B ( $Z=-$   
326  $2.98$ ,  $P=0.003$ ) and C-T ( $Z=-3.180$ ,  $P=0.001$ ), although the excretion values did not decrease  
327 significantly between C-B and C-T ( $Z=-2.669$ ,  $P=0.008$ ). The other three metabolites of the E  
328 pathway (20-OH-PGE<sub>2</sub>, tetranor-PGEM, and tetranor-PGAM) were mainly detected in the two  
329 control periods (C-B and C-T), but in the beverage intake stages and the CP-T stage the number of  
330 volunteers that excreted these biomarkers decreased. The 20-OH-PGE<sub>2</sub> was excreted by the  
331 majority of the volunteers after the juice intake, compared to the placebo. PGE<sub>2</sub> is involved in all  
332 processes leading to the classic signs of inflammation (redness, swelling, and pain), but also shows  
333 anti-inflammatory properties.<sup>18</sup> For example, according to recent *in vivo* studies, this lipid mediator  
334 is related to numerous physiological and pathophysiological processes in the kidney,<sup>55</sup> involving a



335 significant role in modulating the effect of vasopressin on the osmotic water reabsorption in the  
336 renal collecting duct cells - where it attenuates antidiuretic action.<sup>56</sup> In addition, it has been  
337 mentioned that the induction of prostanoids during exercise alters clotting factors, increases  
338 vascular tone, and helps adapt muscle cells to contractile activity.<sup>57</sup> Based on the above, our results  
339 suggest a potential effect of ACJ intake on the inflammatory process and vascular system.

340       Regarding the F and I pathways, the metabolites were scarcely detected in the urine  
341 samples or did not differ significantly during the study. Concerning the TXs, the primary enzymatic  
342 metabolite of TXA<sub>2</sub> is 11-dh-TXB<sub>2</sub>, which has been validated as a reliable and noninvasive  
343 biomarker-integrated index of *in vivo* platelet activation<sup>58</sup>. A previous report observed that 22  
344 sedentary subjects subjected to standardized, aerobic, high-amount-high-intensity training for eight weeks  
345 showed significant decreases in the urinary excretion of 11-dh-TXB<sub>2</sub>.<sup>59</sup> The authors related this  
346 result to platelet activation and hence it may be relevant to explain why long-term physical exercise  
347 is beneficial for the cardiovascular system. According to our results, the excretion of 11-dh-TxB<sub>2</sub>  
348 showed a significant decrease in the ACJ ( $Z=-2.953$ ,  $P=0.003$ ) and CP-T ( $Z=-3.069$ ,  $P=0.002$ )  
349 stages, compared to C-T (Table 3). The 11-dh-TXB<sub>2</sub> decreased significantly in the last period when  
350 the training load was lower; ACJ also had a considerable influence, reducing the values, suggesting  
351 a cardiovascular benefit.

352

### 353 *Leukotrienes*

354       Two metabolites (LTB<sub>4</sub> and LTE<sub>4</sub>) were detected in all stages and in the majority of the  
355 volunteers. The Friedman test showed significant changes in LTB<sub>4</sub> and the subsequent Wilcoxon  
356 signed-rank test revealed higher values in the ACJ stage compared with the placebo ( $Z=-2.166$ ,  
357  $P=0.03$ ), C-T ( $Z=-2.668$ ,  $P=0.008$ ), and CP-T ( $Z=-2.166$ ,  $P=0.03$ ) stages. However, no  $P$ -value was  
358 below 0.005. Contrarily, LTE<sub>4</sub> showed a significant decrease in the placebo stage, relative to the  
359 baseline values ( $Z=-2.784$ ,  $P=0.005$ ). Also, the placebo stage differed from the ACJ stage ( $Z=-$



360 1.960,  $P=0.05$ ), but not significantly so after Bonferroni correction. The excretion values of the CP-  
361 T stage were lower than for C-B ( $Z=-2.668$ ,  $P=0.008$ ) and C-T ( $Z=-1.931$ ,  $P=0.053$ ), but not  
362 statistically so (Table 3). In summary, the urinary metabolites  $LTB_4$  and  $LTE_4$  showed significant  
363 changes; in particular, the ACJ stage presented higher values than the placebo phase. These findings  
364 are the opposite of those mentioned in the current literature, since most polyphenols-intake studies  
365 have shown decreased excretion in healthy people.<sup>50, 60</sup> It has been demonstrated that flavonoids  
366 can modulate the activity of enzymes that are involved in the metabolism of AA in macrophages -  
367 such as phospholipase  $A_2$ , COXs, and LOXs; inhibition of these enzymes by flavonoids lowers the  
368 production of the mediators of inflammatory reactions.<sup>60</sup> Yoon and Baek, 2005<sup>61</sup> mentioned also  
369 that polyphenols are inhibitors of both COX and LOX and that a general rule is "more COX  
370 inhibitions and less LOX inhibitions with polyphenols that contain few hydroxyl substituents (with  
371 none in ring B)". This suggests that polyphenols, including those in our juice rich in polyphenols,  
372 have more effect on an inflammatory cascade of COX-2, which allows the LOX branch to  
373 accelerate the formation of LTs. This explanation seems to describe to a certain extent the change  
374 produced in the excretion values in our study. On the other hand, due to the decline in the ECOs  
375 load, a decrease in the excretion of  $LTE_4$  was detected. Other reports have mentioned that elite  
376 athletes show an increased risk of respiratory symptoms related to asthma, especially those that  
377 participate in endurance sports - such as swimming, running, and cycling - and in winter sports.  
378 This risk to the respiratory system arises because, during physical activity, the elite athletes increase  
379 their water and heat loss through respiration.<sup>62</sup> This has strong ties with the LTs results since they  
380 play a key role in perpetuating airway inflammation - leading directly to airflow obstruction through  
381 the effects on vascular permeability, mucus production, and smooth muscle constriction.<sup>63</sup> A  
382 training program can result in a depletion of LTs and/or a slow *cys*-LTs response to exercise, which  
383 may be responsible for the protective effect of training programs on respiratory symptoms.<sup>64</sup> Our  
384 study shows that post-training could change the excretion of *cys*-LTs, and therefore might have an  
385 effect on the airway pathway.

386

387 *Isoprostanes derived from Arachidonic acid*

388

389 The measurement of F<sub>2</sub>-IsoPs is known to be an index of OS *in vivo*.<sup>14</sup> Regarding the level of total  
390 IsoPs derived from AA in urine, a significant reduction was observed; reflecting mainly the OS  
391 decrease in the CP-T stage (Figure 3, C). When the sum of all the IsoPs was submitted to the  
392 Friedman test, a significant *P*-value ( $\chi^2(4)=91.035$ ,  $P\leq 0.001$ ) was obtained. The total IsoPs ranged  
393 from  $6.10 \pm 6.47 \mu\text{g } 24 \text{ h}^{-1}$  (C-B) to  $3.42 \pm 5.9 \mu\text{g } 24 \text{ h}^{-1}$  (CP-T). The Wilcoxon signed-rank test  
394 showed a tendency of the excretion to fall over the study (Figure 3, C). The IsoPs showed  
395 significant variation in their urinary excretion when the values were analyzed by series: 15-F<sub>2t</sub>-IsoPs  
396 ( $\chi^2(4)=33.360$ ,  $P\leq 0.001$ ), 5-F<sub>2t</sub>-IsoPs ( $\chi^2(4)=12.893$ ,  $P=0.012$ ), and 15-E<sub>2</sub>-IsoPs ( $\chi^2(4)=14.484$ ,  
397  $P=0.006$ ) (Figure 3, B).

398 These data suggest that chronic exercise decreased OS levels in our elite athletes.  
399 According to the review by Nikolaidis *et al.*,<sup>65</sup> in most of the cases in which they analyzed this  
400 behavior the levels of urinary F<sub>2</sub>-IsoP were decreased by chronic exercise. In other studies,<sup>5, 62-64</sup>  
401 physical activity also was the primary factor that decreased the urinary OS biomarker (IsoPs). The  
402 literature mentions that regular exercise training increases the levels of enzymatic and non-  
403 enzymatic antioxidants in muscle fibers, resulting in improved endogenous protection against  
404 exercise-mediated oxidative damage.<sup>10</sup> Furthermore, in athletes of different disciplines,  
405 polyphenols have shown an antioxidant potential that can be beneficial in the reduction of oxidative  
406 damage effects during intense exercise.<sup>4</sup> In our study, considering the metabolites individually, we  
407 observed an increase in 15-*epi*-15-E<sub>2t</sub>-IsoP and 15-keto-15-F<sub>2t</sub>-IsoP, but this change was not linked  
408 to physical exercise directly since the increase was in the ACJ stage, when compared to the placebo.  
409 This result suggests a potential role for the compounds from ACJ intake in these IsoP pathways.  
410 Recent reports have shown that the E-type IsoPs are potent vasoconstrictors at low nanomolar  
411 concentrations.<sup>41</sup> 15-E<sub>2t</sub>-IsoP (also referred to as 8-iso-PGE<sub>2</sub> or iPE2-III) was found to be a

412 powerful and efficient constrictor in the ductus arteriosus of chicken, acting through the  
413 thromboxane receptor.<sup>68</sup> Also, other studies with animals have shown both vasoconstrictive and  
414 vasodilatory effects of 15-E<sub>2t</sub>-IsoP, suggesting biological activity of this molecule in the  
415 cardiovascular system.<sup>69</sup> On the other hand, 15-keto-15-F<sub>2t</sub>-IsoP is a metabolite derived from 15-  
416 F<sub>2t</sub>-IsoP. In an animal study, it was demonstrated that this IsoP probably acted as a partial agonist at  
417 the TP-receptor, mediating contraction and inducing a weak endothelium-independent relaxation at  
418 high concentrations.<sup>70</sup> Therefore, the increase in abundance of these metabolites could reflect  
419 participation of the compounds from ACJ - for example, the flavonoids (polyphenols)<sup>71</sup> - or of  
420 proline betaine, ferulic acid, or other metabolic derivatives (nutritional biomarkers)<sup>72</sup> in the  
421 stimulation of some IsoPs related to the effects on vascular smooth muscle. Also, it should not be  
422 forgotten that, as well as phytochemicals, ACJ contains a variety of vitamins, minerals, and fiber  
423 that could have influenced this result.<sup>73, 74</sup>

424

#### 425 *Metabolites derived from Eicosapentaenoic acid and Dihomo- $\gamma$ -linolenic acid*

426

427 Regarding metabolites derived from DGLA, PGE<sub>1 $\alpha$</sub>  was detected and the Friedman test  
428 revealed significant changes among the experimental periods ( $\chi^2$  (3)=29.624,  $P\leq 0.001$ ). The  
429 Wilcoxon test showed that the CP-T value was significantly lower (C-T,  $Z=-3.408$ ; placebo,  
430  $Z=3.294$ ; ACJ,  $Z=-3.324$ ,  $P=0.001$  in all cases) compared to most of the other stages (Table 3).  
431 According to the literature, through a series of free radical reactions, COX metabolizes DGLA and  
432 AA to form various bioactive metabolites - namely, the 1 and the 2 series of prostaglandins (PG1  
433 and PG2), respectively. Unlike the PG2s, which are viewed as pro-inflammatory, the PG1s possess  
434 anti-inflammatory and anticancer activity.<sup>19</sup> During our study, PGE<sub>1</sub> was detected in all stages,  
435 showing statistically significant differences (Table 3). These results suggest a decrease in this  
436 metabolite in urine when there is a decline in ECOs, although the values during C-T were higher  
437 than in C-B, since the acute physical exercise could have stimulated this pathway. PGE<sub>1</sub> has been

438 shown to possess anti-inflammatory properties and to modulate vascular reactivity.<sup>75</sup> On the other  
439 hand, 15-E<sub>1t</sub>-IsoP was mainly detected in C-B ( $0.5 \pm 0.1 \mu\text{g } 24 \text{ h}^{-1}$ ), suggesting that physical exercise  
440 is an external factor that could have influenced the diminution of its values.

441

442 Regarding the metabolites derived from EPA, 8-*epi*-8-F<sub>3t</sub>-IsoP was not detected and 8-F<sub>3t</sub>-  
443 IsoP was detected only during C-B ( $3.4 \pm 2.3 \mu\text{g } 24 \text{ h}^{-1}$ ). The elite training decreased the values of  
444 8-F<sub>3t</sub>-IsoP, suggesting again that physical exercise is an external factor that could influence the  
445 reduction of biomarkers concomitantly with the decline in the training loads of the athletes (CP-T).  
446 These IsoPs are formed by the free radical-induced peroxidation of EPA *in vivo* and *in vitro*. The  
447 F<sub>3</sub>-IsoPs are spontaneously generated in abundance *in situ* in response to OS and both are useful as  
448 biomarkers of OS.<sup>23, 76</sup>

449

## 450 Conclusions

451

452 This study contributes to a better comprehension of the behavior of urinary biomarkers  
453 related to OS and inflammation status (IsoPs, LTs, PGs, and TXs) in athletes after an elite training  
454 period and supplementation of 200 mL of ACJ (a functional beverage rich in polyphenols). The  
455 findings indicate that physical exercise is an external factor that influenced mainly the OS  
456 biomarkers (F<sub>2</sub>-IsoPs) and inflammation biomarkers (11-dh-TxB<sub>2</sub>, PGE<sub>2</sub>, PGDM, tetranor-PGFM,  
457 PGF<sub>1 $\alpha$</sub> , PGE<sub>1</sub>, and LTE<sub>4</sub>) in triathletes. Furthermore, our collective results regarding ACJ intake  
458 show that supplementation stimulated the excretion of some metabolites related to vascular  
459 homeostasis and smooth muscle (15-*epi*-15-E<sub>2t</sub>-IsoPs, 15-keto-F<sub>2t</sub>-IsoP, 20-OH-PGE<sub>2</sub>, PGE<sub>2</sub>, LTE<sub>4</sub>,  
460 and LTB<sub>4</sub>), indicating a potential role in the cardiovascular system. This work could help to increase  
461 our knowledge about the effect of chronic exercise and sports drinks on human lipid metabolism.  
462 Moreover, it could aid the design of new beverages for athletes.

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464

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475 The authors declare that they have no conflict of interest.

**476 Author Contribution**

477 LA García-Flores carried out the analytical processes and wrote and discussed the present paper. S  
478 Medina, C Gómez, and C Wheelock supervised the analytical processes and developed the  
479 discussion of the paper. R Cejuela (coach) monitored the physical exercise training of the  
480 triathletes. J M Martínez-Sanz was nutritionist of the triathletes and monitored the nutritional plan.  
481 C Oger, Jean-Marie Galano, and Thierry Durand provided the markers for the study and helped  
482 with the review of the manuscript. A Hernández-Sáez helped to the analytical processes. Federico  
483 Ferreres helped with the experimental procedures linked to UHPLC-QqQ-MS/MS. Ángel Gil-  
484 Izquierdo and Sonia Medina designed, supervised, and discussed this research work.

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487

488 **Figure captions**

489 **Figure 1** Flow chart: pathway of the oxylipins analyzed in this study. The metabolites nomenclature  
490 is described in the text.

491 **Figure 2** Study design: this crossover study was randomized, double-blind, and placebo-controlled.  
492 Sixteen athletes, randomly divided into two groups, were assigned to supplementation of either 200  
493 mL of ACJ or 200 mL of placebo. After 45 days of supplementation and a 10-day washout period,  
494 the beverages were reversed. Three controls were used: baseline control, control training, and  
495 control post-training with duration of 15 days. Urine samples were collected at the end of each  
496 stage. The training load quantification was by the Objective Load Scale (ECOs).<sup>5, 33, 36</sup>

497 **Figure 3** Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of the  
498 urinary oxylipins throughout the study ( $\mu\text{g } 24 \text{ h}^{-1}$ ). The level of statistical significance was set at  
499  $P < 0.005$  with Bonferroni correction (\*\* =  $P < 0.005$  and \*\*\* =  $P < 0.001$ ). A) Prostaglandins by  
500 family, B) Isoprostanes by serie, and C) Total isoprostanes, both  $\text{F}_2$ -isoprostanes and  $\text{E}_2$ -  
501 isoprostanes.

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**Table 1.** Physical and metabolic parameters and training loads of the triathletes

Physical characteristics	<i>C-B</i>		<i>C-T</i>		<i>Placebo</i>		<i>ACJ</i>		<i>CP-T</i>	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Years	<b>21.08 ± 3.0</b>	19.0 ± 1.7	<b>21.08 ± 3.0</b>	19.0 ± 1.7	<b>21.08 ± 3.0</b>	19.0 ± 1.7	<b>21.08 ± 3.0</b>	19.4 ± 1.3	<b>21.08 ± 3.0</b>	19.6 ± 1.3
Weight (kg)	<b>54.8 ± 12.2</b>	69 ± 6.2	<b>54.8 ± 11.6</b>	69 ± 6.4	<b>56.2 ± 4.8</b>	70.7 ± 6.9	<b>54.4 ± 5.0</b>	71.2 ± 4.6	<b>53.1 ± 2.9</b>	72.2 ± 6.8
Height (m)	<b>1.6 ± 0.1</b>	1.8 ± 0.1	<b>1.6 ± 0.1</b>	1.8 ± 0.1	<b>1.6 ± 0.1</b>	1.8 ± 0.1	<b>1.6 ± 0.1</b>	1.8 ± 0.1	<b>1.6 ± 0.1</b>	1.8 ± 0.1
BMI <sup>a</sup> (kg m <sup>-2</sup> )	<b>21.2 ± 4.1</b>	22.2 ± 1.0	<b>21.2 ± 4.1</b>	22.2 ± 1.0	<b>20.7 ± 1.3</b>	21.7 ± 1.4	<b>21.6 ± 2.4</b>	21.6 ± 1.3	<b>20.5 ± 1.6</b>	21.8 ± 1.7
Total fat (kg)	<b>8.7 ± 4.1</b>	9.2 ± 2.8	<b>8.9 ± 4.7</b>	8.8 ± 2.6	<b>9.2 ± 0.9</b>	8.0 ± 1.7	<b>7.5 ± 1.2</b>	6.4 ± 2.8	<b>7.3 ± 1.4</b>	6.8 ± 1.2
Lean weight (kg)	<b>20.8 ± 3.6</b>	31.4 ± 2.1	<b>20.6 ± 2.7</b>	30.5 ± 2.7	<b>20.8 ± 2.4</b>	31.6 ± 3.0	<b>19.4 ± 2.8</b>	33.8 ± 3.2	<b>20.9 ± 2.0</b>	32.4 ± 2.4
SS (mm)	<b>12.7 ± 6.7</b>	9.6 ± 3.0	<b>13.4 ± 8.2</b>	9.5 ± 2.1	<b>11.7 ± 2.5</b>	9.1 ± 1.7	<b>10.7 ± 1.9</b>	8.6 ± 2.0	<b>9.9 ± 2.8</b>	8.6 ± 1.8
TS (mm)	<b>16.3 ± 2.3</b>	8.9 ± 3.0	<b>18.4 ± 3.8</b>	9.7 ± 2.6	<b>19.3 ± 5.4</b>	8.7 ± 2.1	<b>16.1 ± 4.6</b>	7.4 ± 2.4	<b>17.4 ± 4.6</b>	7.3 ± 1.5
BS (mm)	<b>10.3 ± 2.8</b>	5.4 ± 2.4	<b>9.8 ± 3.2</b>	4.7 ± 1.5	<b>7.2 ± 0.4</b>	4.1 ± 0.6	<b>5.7 ± 1.0</b>	4.5 ± 1.5	<b>5.7 ± 1.3</b>	3.7 ± 0.4
ICS (mm)	<b>19.7 ± 4.5</b>	12.0 ± 2.6	<b>17.1 ± 6.9</b>	13.1 ± 4.1	<b>20.9 ± 4.5</b>	12.5 ± 4.2	<b>17.3 ± 3.7</b>	11.2 ± 3.4	<b>13.7 ± 4.3</b>	9.6 ± 2.5
SES (mm)	<b>14.3 ± 6.5</b>	9.0 ± 2.6	<b>14.4 ± 6.9</b>	8.9 ± 2.8	<b>15.0 ± 1.0</b>	8.7 ± 2.5	<b>12.8 ± 2.1</b>	7.6 ± 1.9	<b>11.6 ± 2.5</b>	6.7 ± 1.4
AS (mm)	<b>23.1 ± 5.9</b>	16.4 ± 8.0	<b>23.6 ± 6.9</b>	15.5 ± 6.8	<b>24.5 ± 4.7</b>	14.5 ± 5.9	<b>21.3 ± 4.1</b>	11.8 ± 5.2	<b>17.9 ± 4.6</b>	10.0 ± 3.7
FTS (mm)	<b>27.2 ± 5.2</b>	14.9 ± 4.4	<b>26.4 ± 5.0</b>	14.0 ± 4.4	<b>25.8 ± 3.6</b>	11.5 ± 2.3	<b>23.8 ± 12.5</b>	10.1 ± 2.9	<b>26.0 ± 5.4</b>	10.0 ± 2.5
MCS (mm)	<b>14.8 ± 3.8</b>	9.0 ± 3.0	<b>13.9 ± 3.0</b>	9.5 ± 3.1	<b>15.7 ± 2.1</b>	8.2 ± 2.1	<b>12.5 ± 1.8</b>	7.2 ± 2.3	<b>14.4 ± 2.9</b>	7.3 ± 1.8

**a** Body Mass Index. Abbreviation: ACJ; *Aronia-citrus* Juice; AS, Abdominal skinfold; BS, Biceps skinfold; CB; Control Baseline; CP-T; Control Post-Treatment; CT; Control Training; FTS, Front Thigh skinfold; ICS, Iliac Crest skinfold; Medial Calf skinfold; SES, Supra espinal skinfold; SS, Subscapular skinfold; TS, Triceps skinfold;

**Table 2.** Dietary parameters: caloric intake of the triathletes during the study and nutritional composition of the *Aronia-citrus* Juice (ACJ)

A)	Male triathletes	Female triathletes
Energy intake (kcal d <sup>-1</sup> )	2820.0 ± 241.2	2072.6 ± 223.4
Carbohydrate (g d <sup>-1</sup> )	326.1 ± 63.5	211.3 ± 43.9
Dietary fiber (g d <sup>-1</sup> )	27.3 ± 7.4	15.5 ± 4.4
Sugar (g d <sup>-1</sup> )	121.3 ± 33.9	80.5 ± 18.3
Proteins (g d <sup>-1</sup> )	133.7 ± 12.9	83.5 ± 9.0
Total lipids (g d <sup>-1</sup> )	113.7 ± 13.3	107.1 ± 14.4
SFA <sup>a</sup> (g d <sup>-1</sup> )	33.5 ± 6.5	29.6 ± 4.4
MUFA <sup>b</sup> (g d <sup>-1</sup> )	56.5 ± 5.5	56.6 ± 7.5
PUFA <sup>c</sup> (g d <sup>-1</sup> )	16.9 ± 2.7	15.9 ± 6.7
Vitamin C (mg d <sup>-1</sup> )	178.9 ± 71.9	135.0 ± 60.4
Vitamin A (µg d <sup>-1</sup> )	2970.0 ± 913.9	1427.4 ± 573.1
Vitamin E (mg d <sup>-1</sup> )	21.0 ± 5.6	13.9 ± 3.4
Vitamin D (mg d <sup>-1</sup> )	988. ± 47.5	751.6 ± 163.0
Iron (mg d <sup>-1</sup> )	20.9 ± 2.4	14.9 ± 2.6
Selenium (mg d <sup>-1</sup> )	149.8 ± 21.5	103.0 ± 17.4
Water ingestion (mL d <sup>-1</sup> )	1500*	1500*
<b>B) ACJ</b>	<b>200 mL</b>	<b>%</b>
Energy intake (kcal)	76.0	2.6
Proteins (g)	0.9	0.6
Carbohydrate (g)	18.0	2.6
Sugar (g)	6.6	5.2
Fat (g)	0.1	0.1
<i>Flavanones (mg)</i>		
Eriocitrin	22.9 ± 0.16	
Hesperidin	27.08 ± 0.28	
<i>Flavones (mg)</i>		
Vicenin-2	1.18 ± 0.04	
Diosmetin-6,8-di-O-glucoside	15.5 ± 0.38	
Diosmin	<0.5	
<i>Anthocyanins (mg)</i>		
Cyanidin 3-O-galactoside	30.16 ± 0.20	
Cyanidin 3-O-glucoside	2.62 ± 0.04	
Cyanidin 3-O-arabinoside	18.36 ± 0.40	
Cyanidin 3-O-xyloside	2.22 ± 0.03	
Total anthocyanins	53.4 ± 0.70	
<i>Hydroxycinnamic acids (mg)</i>		
Neochlorogenic acid	39.44 ± 0.34	
Chlorogenic acid	29.38 ± 0.26	
$\Sigma$ Quercetin derivatives (mg)	8.62 ± 0.26	

A) Dietary parameters and caloric intake of the triathletes during the study. <sup>a</sup> Saturated fatty acids, <sup>b</sup> Monounsaturated fatty acids, <sup>c</sup> Polyunsaturated fatty acids.\* This was the daily water intake required, furthermore, the athletes drank extra liquids during the nutritional intervention (200 mL /day of ACJ or Placebo), as well as during their sessions of training( since 400 mL to 600 mL /hour of water). B) The nutritional composition of ACJ; %, contribution of the juice to the diet. The values of the phenolic content are mean ± standard deviation (n=3), expressed as mg 200 mL<sup>-1</sup> and the phytochemical study of the juice was performed according to Gonzales-Molina, 2008. <sup>32</sup>.

**Table 3.** Urinary isoprostanes and prostaglandins ( $\mu\text{g } 24 \text{ h}^{-1}$ ) from arachidonic acid, dihomo- $\gamma$ -linoleic acid, and eicosapentaenoic acid detected in the urine samples of triathletes

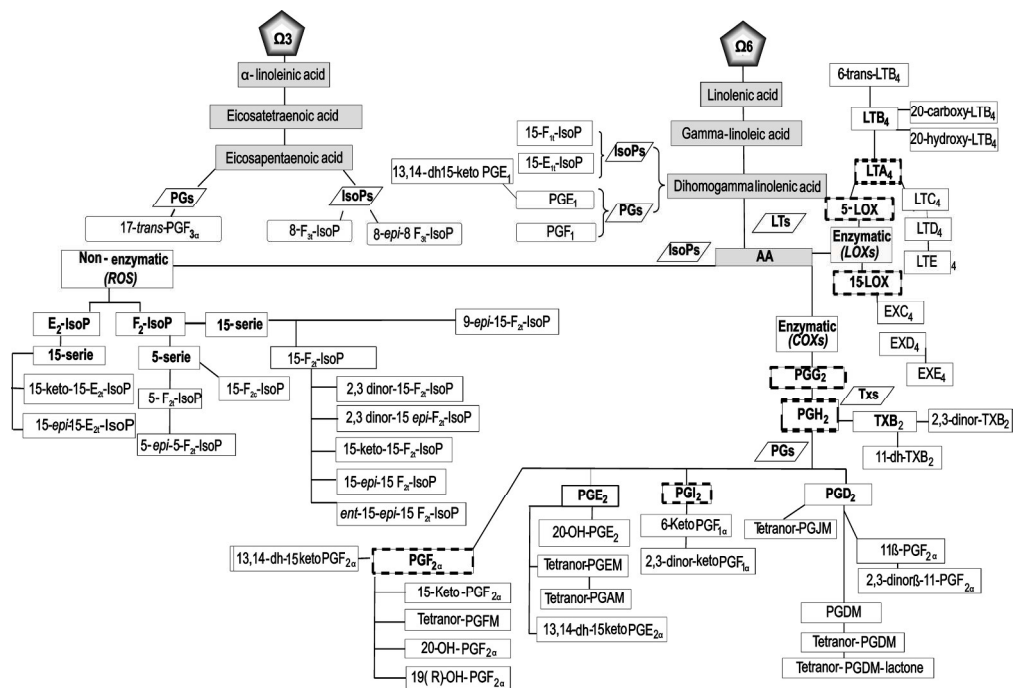
		Stage of study										Friedman Test		
Analyte ( $\mu\text{g } 24 \text{ h}^{-1}$ )		C-B		C-T		P		ACJ		CP-T		$\chi^2$	<i>df</i>	<i>Sig</i>
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Arachidonic Acid PGs	<b>D pathway</b>													
	PGDM	<b>31.1</b>	24.6	<b>41.2</b>	24.4	<b>16.1</b>	14.0	<b>19.3</b>	14.3	<b>10.0</b>	13.9	19.7	4	<b>0.001</b>
	Tetranor-PGDM lactone	<b>2.4</b>	2.4	<b>1.1</b>	1.2	<b>1.2</b>	0.6	<b>0.9</b>	1.1	<b>1.4</b>	1.7	3.8	4	0.430
	11- $\beta$ -PGF <sub>2<math>\alpha</math></sub>	<b>4.3</b>	2.1	<b>7.5</b>	4.1	<b>7.1</b>	3.9	<b>7.4</b>	5.2	<b>1.7</b>	4.1	18.8	4	<b>0.001</b>
	2,3-dinor-11 $\beta$ -PGF <sub>2<math>\alpha</math></sub>	<b>8.9</b>	5.5	<b>6.2</b>	2.0	<b>6.8</b>	7.3	<b>2.8</b>	2.0	<b>4.3</b>	5.2	20.9	4	< <b>0.001</b>
	Tetranor-PGDM	<b>3.2</b>	2.5	<b>3.8</b>	2.7	<b>2.7</b>	1.6	<b>2.0</b>	1.2	<b>0.6</b>	0.7	21.3	4	< <b>0.001</b>
	<b>E pathway</b>													
	PGE <sub>2</sub>	<b>0.51</b>	0.50	<b>0.15</b>	0.14	<b>0.04</b>	0.08	<b>0.19</b>	0.30	<b>0.44</b>	0.08	13.5	4	<b>0.009</b>
	20-OH-PGE <sub>2</sub>	<b>3.8</b>	4.6	<b>2.0</b>	2.1	<b>2.1<sup>b</sup></b>	0.6	<b>0.9</b>	1.0	<b>4.3<sup>c</sup></b>	3.1	4.2	2	0.122
	Tetranor-PGEM	<b>2.6</b>	2.2	<b>1.2</b>	1.7	<b>0.9<sup>c</sup></b>	0.9	<b>2.4<sup>d</sup></b>	1.6	<b>3.0<sup>c</sup></b>	2.6	-	-	-
	Tetranor-PGAM	<b>2.9</b>	3.3	<b>2.4</b>	4.5	<b>2.3<sup>d</sup></b>	1.9	<b>1.3<sup>c</sup></b>	0.8	<b>2.1<sup>d</sup></b>	1.7	-	-	-
	13,14-dihydro-15-keto PGF <sub>2<math>\alpha</math></sub>	-	-	<b>2.9</b>	-	<b>6.1</b>	-	-	-	-	-	-	-	-
	13,14-dihydro-15-keto PGE <sub>2<math>\alpha</math></sub>	-	-	-	-	-	-	-	-	<b>0.2<sup>a</sup></b>	0.2	-	-	-
	<b>F pathway</b>													
	Tetranor-PGFM	<b>0.9</b>	0.4	<b>1.8</b>	-	<b>1.6</b>	-	-	-	-	-	-	-	-
PGF <sub>2<math>\alpha</math></sub>	<b>3.5<sup>b</sup></b>	1.6	<b>2.7</b>	-	<b>2.7</b>	-	<b>5.1<sup>b</sup></b>	2.5	<b>3.7</b>	-	-	-	-	
<b>I pathway</b>														
2,3-dinor-6-keto PGF <sub>1<math>\alpha</math></sub>	<b>2.1</b>	2.7	<b>2.2</b>	2.4	<b>2.0</b>	1.9	<b>2.2</b>	3.0	<b>1.9</b>	2.3	2.3	4	0.680	
<b>F<sub>2</sub>-Isoprostane</b>														
<b>15-series</b>														
15-F <sub>2t</sub> -IsoP	<b>3.2</b>	0.7	<b>2.7</b>	0.5	<b>2.5</b>	0.5	<b>2.1</b>	0.6	<b>1.6</b>	0.4	16.1	4	<b>0.002</b>	
15-keto-15-F <sub>2t</sub> -IsoP	<b>1.4</b>	1.4	<b>0.4</b>	1.0	<b>1.0</b>	-	<b>0.2</b>	0.4	<b>3.02<sup>d</sup></b>	1.9	6.1	2	<b>0.046</b>	
15- <i>epi</i> -15-F <sub>2t</sub> -IsoP	<b>4.3</b>	4.3	<b>2.8</b>	2.7	<b>1.5</b>	1.3	<b>3.1</b>	6.2	<b>1.0</b>	0.8	4.8	4	0.298	
2,3-dinor-15-F <sub>2t</sub> -IsoP	<b>16.5</b>	9.4	<b>14.8</b>	6.5	<b>11.4</b>	7.4	<b>9.5</b>	5.6	<b>10.2</b>	12.7	8.3	4	0.081	
<i>ent</i> -15- <i>epi</i> -15-F <sub>2t</sub> -IsoP	<b>0.7</b>	1.0	<b>0.4</b>	0.5	<b>0.1</b>	0.1	<b>0.3</b>	0.5	<b>0.1</b>	0.1	4.9	4	0.297	
9- <i>epi</i> -15-F <sub>2t</sub> -IsoP	<b>2.7</b>	1.6	<b>1.4</b>	0.8	<b>1.0</b>	0.4	<b>1.3</b>	0.9	<b>1.2</b>	0.8	15.1	4	<b>0.004</b>	
2,3-dinor-15- <i>epi</i> -15-F <sub>2t</sub> -IsoP	<b>3.0</b>	2.2	<b>1.4</b>	0.5	<b>1.3</b>	1.4	<b>1.2</b>	0.5	<b>1.5</b>	1.4	9.1	4	0.057	
15-F <sub>2c</sub> -IsoPs	<b>8.4</b>	4.3	<b>8.2</b>	4.9	<b>6.4</b>	2.9	<b>7.0</b>	3.7	<b>5.3</b>	3.3	5.4	4	0.250	
<b>5-series</b>														
5-F <sub>2t</sub> -IsoP	<b>11.2</b>	5.6	<b>10.7</b>	5.8	<b>9.0</b>	4.3	<b>11.9</b>	6.8	<b>7.5</b>	4.7	4.5	4	0.332	
5- <i>epi</i> -5F <sub>2t</sub> -IsoP	<b>7.2</b>	4.6	<b>5.5</b>	4.5	<b>2.9</b>	2.0	<b>4.7</b>	3.4	<b>4.9</b>	2.5	13.3	4	<b>0.010</b>	
<b>E<sub>2</sub>-Isoprostane</b>														
<b>15-series</b>														
15-keto-15-E <sub>2t</sub> -IsoP	<b>3.3</b>	0.5	<b>2.3</b>	0.4	<b>1.7</b>	0.3	<b>1.9</b>	0.2	<b>2.1</b>	0.6	8.5	4	0.073	
15- <i>epi</i> -15-E <sub>2t</sub> -IsoP	<b>2.7</b>	4.1	<b>2.1</b>	3.8	<b>2.0<sup>b</sup></b>	1.6	<b>1.3</b>	1.5	<b>3.5</b>	6.1	1.0	3	0.785	
<b>LT</b>														
LTB <sub>4</sub>	<b>0.03</b>	0.02	<b>0.02</b>	0.02	<b>0.03</b>	0.02	<b>0.06</b>	0.04	<b>0.03</b>	0.02	9.7	4	<b>0.040</b>	
<b>Cys-LT</b>														
LTE <sub>4</sub>	<b>0.13</b>	0.07	<b>0.11</b>	0.09	<b>0.06</b>	0.03	<b>0.12</b>	0.11	<b>0.05</b>	0.05	9.9	4	<b>0.040</b>	
<b>EX</b>														
EXD <sub>4</sub>	-	-	<b>2.1<sup>b</sup></b>	2.6	<b>0.1</b>	-	<b>0.2</b>	-	-	-	-	-	-	

Continuation of Table 3.

		Stage of study										Friedman Test		
TXs	Analyte ( $\mu\text{g } 24 \text{ h}^{-1}$ )	CB		CT		P		ACJ		CP-T		$\chi^2$	df	Sig
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
	TXB <sub>2</sub>	-	-	-	-	-	-	<b>0.1</b>	-	<b>0.1</b>	-	-	-	-
	11-dh-TXB <sub>2</sub>	<b>0.3</b>	0.2	<b>0.5</b>	0.2	<b>0.3</b>	0.2	<b>0.2</b>	0.1	<b>0.2</b>	0.1	21.8	4	< <b>0.001</b>
	2,3-dinor-TXB <sub>2</sub>	<b>3.3<sup>f</sup></b>	0.7	<b>3.1<sup>e</sup></b>	0.5	<b>2.9<sup>f</sup></b>	0.4	<b>2.1<sup>e</sup></b>	0.5	<b>2.4<sup>d</sup></b>	1.0	-	-	-
<b>Eicosapentaenoic acid</b>														
<b>PG</b>	17- <i>trans</i> -PGF <sub>3<math>\alpha</math></sub>	<b>1.1</b>	1.7	<b>1.2</b>	1.7	<b>0.7</b>	1.0	<b>0.2</b>	0.4	<b>2.9<sup>b</sup></b>	2.8	1.52	3	0.676
<b>IsoP</b>	8-F <sub>3<math>\gamma</math></sub> -IsoP	<b>3.2</b>	2.3	<b>0.6<sup>a</sup></b>	0.1	<b>1.0<sup>b</sup></b>	0.4	<b>1.6<sup>d</sup></b>	1.0	-	-	-	-	-
<b>Dihomo-<math>\gamma</math>linolenic Acid</b>														
<b>PGs</b>	PGE <sub>1</sub>	<b>0.3</b>	0.2	<b>0.6</b>	0.3	<b>0.5</b>	0.3	<b>0.4</b>	0.2	<b>0.1</b>	0.1	29.6	4	< <b>0.00</b>
	PGF <sub>1<math>\alpha</math></sub>	<b>2.1<sup>f</sup></b>	0.4	<b>0.05</b>	-	<b>3.8<sup>b</sup></b>	2.6	-	-	<b>1.1</b>	-	-	-	-
<b>IsoP</b>	15-E <sub>1<math>\gamma</math></sub> -IsoP	<b>0.5</b>	0.1	-	-	-	-	<b>0.3<sup>a</sup></b>	0.3	-	-	-	-	-

The data are shown as means  $\pm$  standard deviations (SD) in  $\mu\text{g } 24 \text{ h}^{-1}$ . The volume of urine excreted by the volunteers was  $1212.42 \pm 716.50 \text{ ml } 24 \text{ h}^{-1}$ , on average, in all the periods. The average of the two plasma samples in the crossover period (placebo/ACJ). The statistical P-value from the Friedman test is indicated in italics and bold letters show the significant P-values. The mean values with letters in superscript were found in a reduced number of volunteers within the experimental groups, thus the number of volunteers was a=2, b=3, c=4, d=5, e=6, and f=7. Abbreviations: C-B: control baseline, C-T: control training, ACJ: *Aronia-citrus* juice, CP-T: control post-treatment.

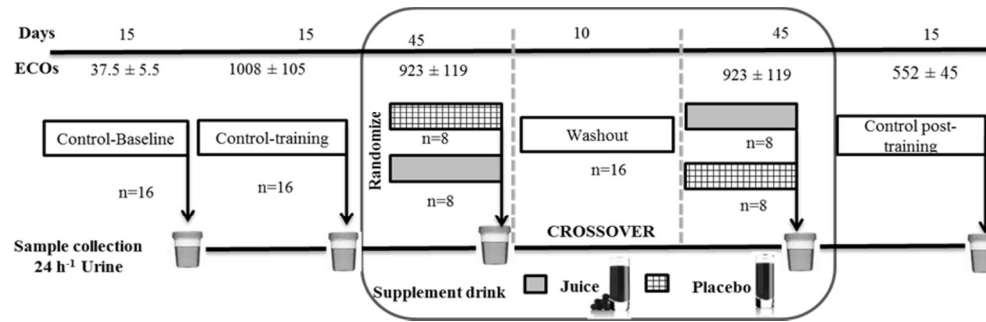




Flow chart: pathway of the oxylipins analyzed in this study. The metabolites nomenclature is described in the text.

107x72mm (600 x 600 DPI)





Study design: this crossover study was randomized, double-blind, and placebo-controlled. Sixteen athletes, randomly divided into two groups, were assigned to supplementation of either 200 mL of ACJ or 200 mL of placebo. After 45 days of supplementation and a 10-day washout period, the beverages were reversed. Three controls were used: baseline control, control training, and control post-training with duration of 15 days. Urine samples were collected at the end of each stage. The training load quantification was by the Objective Load Scale (ECOs). 5, 30, 33

51x16mm (600 x 600 DPI)

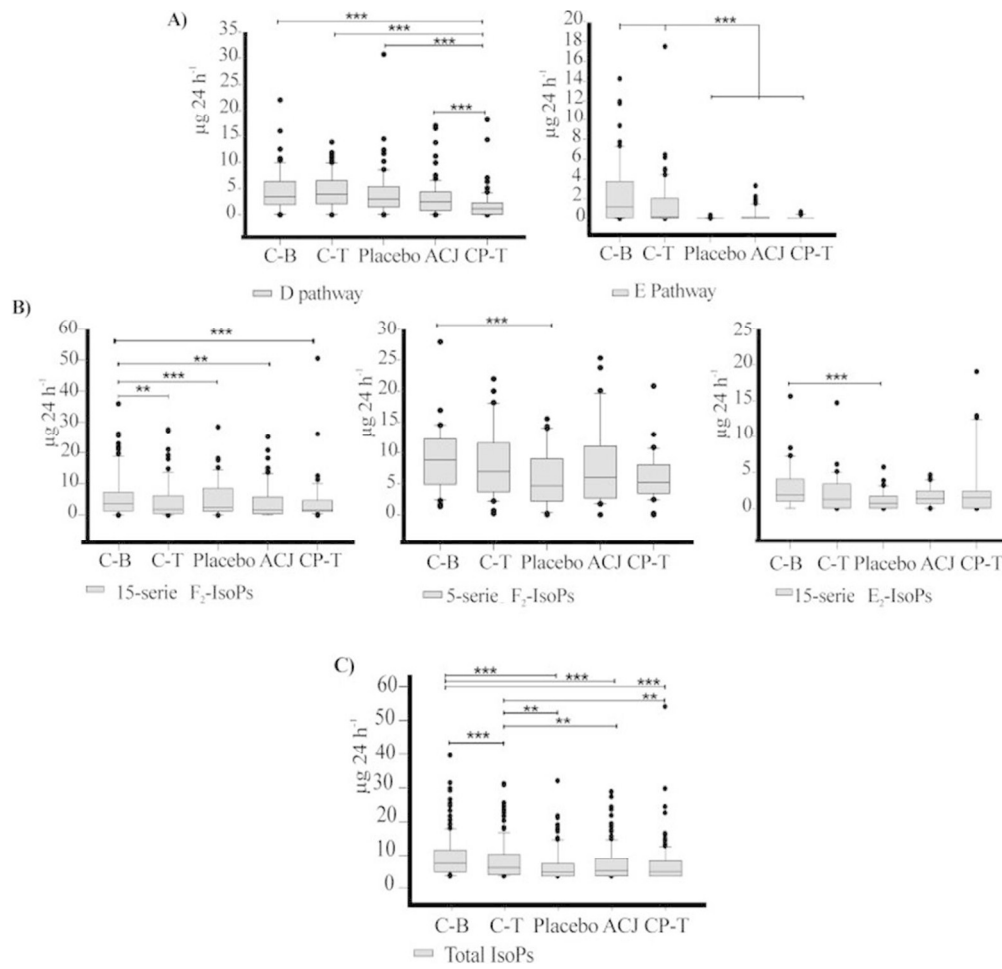
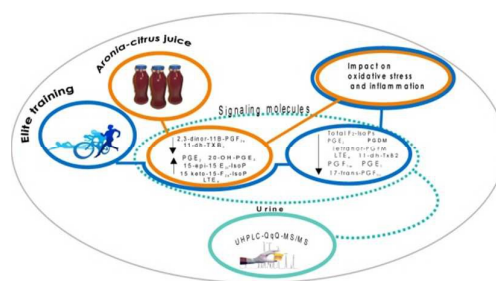


Figure 3 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of the urinary oxylipins throughout the study (µg 24 h<sup>-1</sup>). The level of statistical significance was set at P<0.005 with Bonferroni correction (\*\* = P<0.005 and \*\*\* = P<0.001). A) Prostaglandins by family, B) Isoprostanes by serie, and C) Total isoprostanes, both F<sub>2</sub>-isoprostanes and E<sub>2</sub>-isoprostanes.

190x181mm (150 x 150 DPI)



The ACJ supplementation has a potential benefit regarding the cardiovascular system that is connected in a synergistic manner with elite physical activity.