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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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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 the urine. The quantification was carried out using solid-phase extraction, liquid chromatography coupled with triple quadrupole mass spectrometry. The physical activity decreased the excretion of some PG, IsoP, TX, and LT metabolites from arachidonic acid, γ-dihomo-linolenic acid, and eicosapentaenoic acid. The ACJ also reduced the excretion of 2,3-dinor-11β-$PGF_{2\alpha}$ and 11-dehydro-TXB_2, although the levels of other metabolites increased after juice supplementation (PGE_2, 15-keto-15-F_{2t}-IsoP, 20-OH-PGE_2, LTE_4, and 15-*epi*-15-E_{2t}-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, 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.

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Introduction

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

In the field of sports science and elite sports environment, biomarkers are used to make inferences about the athlete's underlying physiology and health, particularly in the context of adaptation to training and the impact of environmental stressors.[11] Metabolomics and lipidomics data indicate that intensive and prolonged exercise is associated with extensive

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lipid mobilization and oxidation, including many components in the pathway of linoleic acid conversion and related oxidized derivatives or oxylipins.[12] The lipid metabolism constitutes a network of pathways that are related at multiple biosynthetic hubs.[13] Oxygenated lipids are collectively known as oxylipins.[14] Eicosanoids, a subset of oxylipins, are signaling molecules that have been used as biomarkers for a global picture of changes in lipid peroxidation and vascular events as a consequence of chronic exercise and the supplementation of polyphenols.[5–8,12–15] Eicosanoids are a family that includes prostaglandins (PGs), leukotrienes (LTs), thromboxanes (TXs), and isoprostanes (IsoPs), which are lipid mediators involved in the physiopathology of all organs, tissues, and cells.[16,17] PGs and TXs, collectively termed prostanoids, are formed when arachidonic acid (AA), a 20-carbon unsaturated fatty acid, is released from the plasma membrane by phospholipases and metabolized by the sequential actions of prostaglandin G/H synthase, or cyclooxygenase (COX). TXA_2 is synthesized from prostaglandin H_2 (PGH_2) by thromboxane synthase, and it is non-enzymatically degraded into biologically inactive thromboxane B_2 (TXB_2).[18] On the other hand, there are four primary bioactive PGs generated *in vivo*: prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).[18] Besides AA, another polyunsaturated fatty acid (PUFA) is dihomo-γ-linolenic acid (DGLA), a 20-carbon n-6 (C20:3 n-6) derived *in vivo* from α-linolenic acid (c18:3 n-6). Through a series of free radical reactions, COX metabolizes DGLA and AA to form various bioactive metabolites: namely, the 1 and the 2 serie of PGs (PG1 and PG2), respectively.[19] The LTs also contain 20 carbons, but lack the 5-carbon ring structure.[20] They are AA metabolites derived from the action of 5-LOX (5-lipoxygenase). The immediate product of 5-LOX is LTA_4 (leukotriene A_4), which is enzymatically converted into either LTB_4 (leukotriene B4), by LTA_4 hydrolase, or LTC_4 (leukotriene C_4), by LTC_4 synthase.[20] The glutathione conjugate forms are termed *cys*-LTs (cysteinyl leukotrienes) and include leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), and leukotriene E_4 (LTE_4). The *cys*-LTs are potent bronchoconstrictors and vasoconstrictors.[13] The biosynthesis of eoxins (EX), structural isomers of *cys*-LTs, is initiated *via* the 15-lipoxygenase (15-LOX) pathway. Also, there is another pathway that occurs *in vivo* through a free radical-mediated mechanism to yield a series of PG-like compounds termed IsoPs, independent of the catalytic activity of COX.[21,22] The F_2-isoprostanes (F_2-IsoPs) are an *in vivo* index of OS.[16] Furthermore, F_1-phytoprostanes (F_1-PhytoPs) and F_3-IsoPs are also generated from α-linolenic acid (ALA) and eicosapentaenoic acid (EPA).[23,24] Finally, the 3-serie prostanoids, derived from the COX oxidation of EPA, may mediate the anti-inflammatory effects of this fatty acid.[25]

Based on the preceding discussion, the primary goal of this randomized, double-blind, placebo-controlled, and crossover study was to ascertain the effects of a serving (200 mL) of *Aronia–citrus* juice (ACJ) on the generation and metabolism of oxylipins, using a lipidomic approach. Also, the study design allowed the assessment of the changes produced by elite training sessions. We screened biomarkers from AA *via* LOX (LTs, cysLTs, and EXs), as well as other IsoPs, PGs, and TXs that complement our schematic of oxylipins (52 lipid mediators (Fig. 1)).

Materials and methods

Physical characteristics of participants

The anthropometric measurements were made according to the methods of the International Society of Advancement of Kinanthropometry (ISAK),[26] and all tests were performed by the same, internationally certified anthropometrist (Level 2 ISAK) with the objective of decreasing the technical errors of measurement. The body composition was determined by GREC Kinanthropometry consensus,[27] using a model consisting of the determination of total fat by Withers' formula,[28] lean weight by using the procedure described by Lee *et al.*,[29] and residual mass by the difference in the weight (Table 1).

Dietary intake

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

Aronia–citrus juice and placebo beverage

The polyphenol-rich juice composition was based on a mixture of *citrus* juice (95%) with added *Aronia melanocarpa* juice (5%). This juice was developed on an industrial pilot scale (HERO Spain S.A., Alcantarilla, Murcia) with organoleptically-acceptable criteria to mimic the composition of the flavonoids of the original beverage developed by Gonzalez-Molina *et al.*[32] The nutrient and caloric content of the ACJ that the triathletes consumed is summarized in Table 2, detailing the percentage contribution of the juice to the total diet.

The placebo beverage composition was based on a mixture of water, authorized red dye, flavoring, and sweetener, possessing sensory characteristics close to those of ACJ (see Garcia-Flores *et al.*[33,34] for further information about ACJ composition and nutritional planning).

Training load

Training load quantification was performed using the Objective Load Scale (ECOs) developed by Cejuela-Anta and Esteve-Lanao.[35] The training loads designed for the triathletes in the present work were similar to those found in other studies.[5,30,33,36] The method used in the current work allowed the quantification of the training loads in triathlon (swim, bike, run, and transitions).[37] The values of daily and weekly training were determined and summarized to assess the ECOs

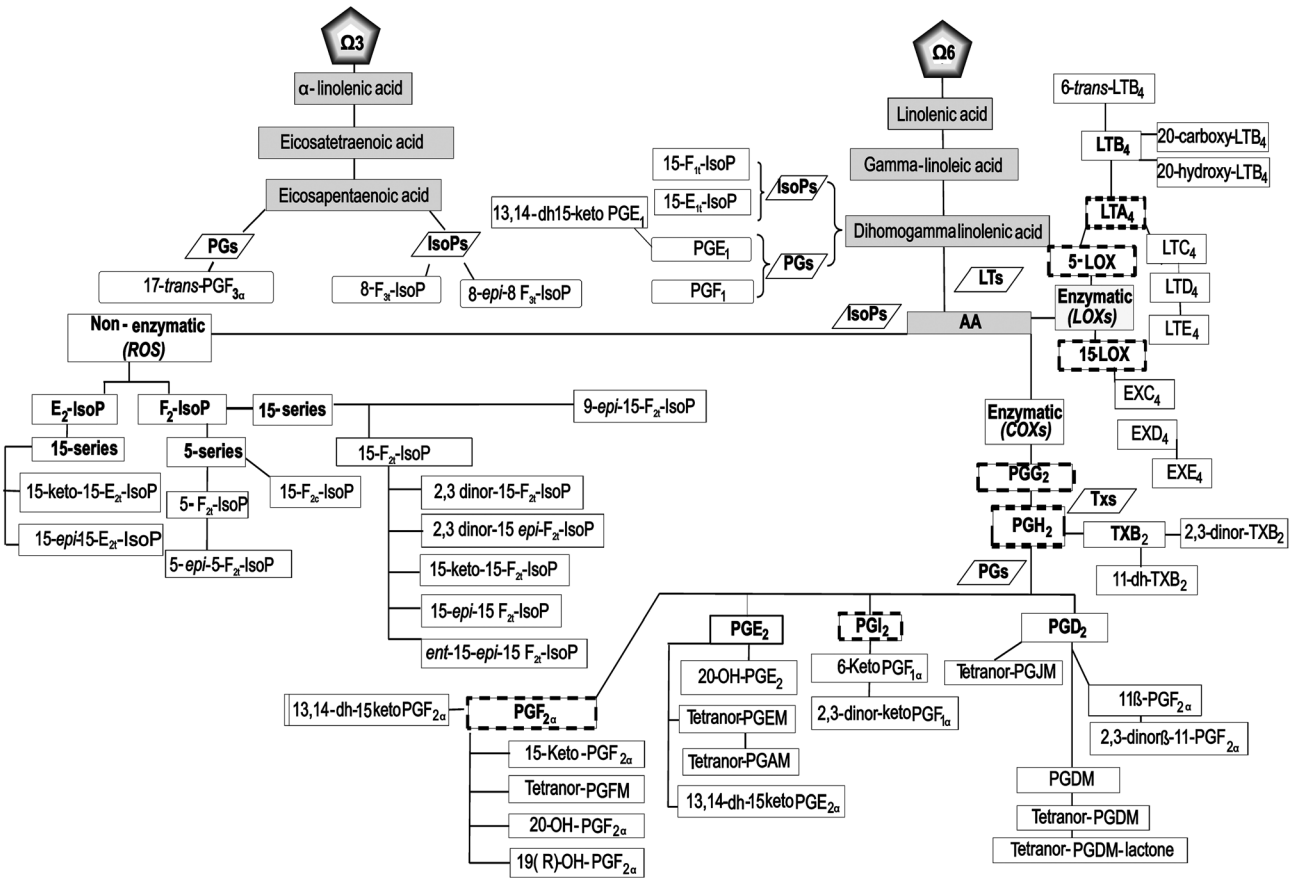

Fig. 1 Flow chart: pathway of the oxylipins analyzed in this study. The nomenclature of the metabolites is described in the text.

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

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

[a] Body mass index. Abbreviations: 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; MCS, medial calf skinfold; SES, supraespinale skinfold; SS, subscapular skinfold; TS, triceps skinfold.

of each volunteer, depending on their physical characteristics and the intensity of the training program (the ECOs data presented in this work are the average of the individual ECOs of the triathletes). The variations of the ECOs are shown in Fig. 2 for better orientation.

Study design

Sixteen triathletes (6 training women and 10 training men) from the University of Alicante (Spain) agreed to participate in the project. An elite athlete in the context of sports medicine is

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^{-1})	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
Sugar (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
Water ingestion (mL d^{-1})	1500[d]	1500[d]

(B) ACJ	200 mL	%
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
Flavanones (mg)		
Eriocitrin	22.9 ± 0.16	
Hesperidin	27.08 ± 0.28	
Flavones (mg)		
Vicenin-2	1.18 ± 0.04	
Diosmetin-6,8-di-*O*-glucoside	15.5 ± 0.38	
Diosmin	<0.5	
Anthocyanins (mg)		
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	
Hydroxycinnamic acids (mg)		
Neochlorogenic acid	39.44 ± 0.34	
Chlorogenic acid	29.38 ± 0.26	
∑*Quercetin derivatives* (mg)	8.62 ± 0.26	

(A) Dietary parameters and caloric intake of the triathletes during the study. [a] Saturated fatty acids. [b] Monounsaturated fatty acids. [c] Polyunsaturated fatty acids. [d] This was the daily water intake required; furthermore, the athletes drank extra liquids during the nutritional intervention (200 mL day^{-1} of ACJ or placebo), as well as during their sessions of training (400 mL to 600 mL $hour^{-1}$ 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 per 200 mL, and the phytochemical study of the juice was performed according to the procedure of Gonzalez-Molina (2008).[32]

an athlete with potential for competing in the Olympics or as a professional athlete.[38] The volunteers were non-smokers, had stable food habits, and did not receive any medication (the specific absence of acute administration of anti-inflammatory drugs) during the experimental procedure. The study was approved by the Bioethics Committee of the University Hospital of Murcia and was in accordance with the Declaration of Helsinki. All participants provided written informed consent to a protocol approved by the institution.[39] The recruitment started on 28th–29th October 2010 and was completed on 24th–25th March 2011. This study had a randomized, double-blind, placebo-controlled, and crossover design (Fig. 2) and was previously approved by nutritional experts. We assumed an equal allocation of volunteers to each beverage using computer-generated simple randomization with consecutive codes linked to the preparation of the placebo or ACJ. An impartial outsider, without the knowledge of the study, helped us to select the randomization code and indicated the assignment order. The volunteers remained blinded throughout the study as well as the researchers responsible for the outcome measurements and the data analysis (see Garcia-Flores *et al.*[33,34] for further information).

Urine sample collection and preparation

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

Chemicals and analytes

Seven IsoPs derived from AA (15-F_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 2,3-dinor-15-F_{2t}-IsoP, *ent*-15-*epi*-15-F_{2t}-IsoP, 9-*epi*-15-F_{2t}-IsoP, and 15-keto-15-E_{2t}-IsoP); 31 enzymatic metabolites of AA (PGD_2, PGDM (PGD metabolite), tetranor-PGDM lactone (tetranor-PGD metabolite lactone), 11-β-$PGF_{2\alpha}$, 2,3-dinor-11-β-$PGF_{2\alpha}$, tetranor-PGJM (tetranor-PGJ metabolite), tetranor-PGDM (tetranor-PGD metabolite), 6-keto-$PGF_{1\alpha}$, PGE_2, 20-OH-PGE_2, tetranor-PGEM (tetranor-PGE metabolite), tetranor-PGAM (tetranor-PGA metabolite), 13,14-dihydro-15-keto-PGE_1, 13,14-dihydro-15-keto-PGE_2, 13,14-dihydro-15-keto-$PGF_{2\alpha}$, $PGF_{2\alpha}$, tetranor-PGFM (tetranor-PGF metabolite), 20-OH-$PGF_{2\alpha}$, 19(R)-OH-$PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, thromboxane B_2 (TXB_2), 2,3-dinor-TXB_2, 11-dehydro-thromboxane B_2 (11-dh-TXB_2), leukotriene (LT) B_4, 20-carboxy-LTB_4, 20-hydroxy-LTB_4, 6-*trans*-LTB_4, LTC_4, LTE_4, EXC_4, and EXE_4); four metabolites of DGLA (PGE_1, $PGF_{1\alpha}$, 15-F_{1t}-IsoP, and 15-E_{1t}-IsoP); and one metabolite of EPA (17-*trans*-$PGF_{3\alpha}$) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The authentic markers [$^2H_4$]-13,14-dihydro-15-keto-PGE_1, [$^2H_4$]-13,14-dihydro-15-keto-PGE_2, [$^2H_4$]-13,14-dihydro-15-keto-$PGF_{2\alpha}$, [$^2H_4$]-6-keto-$PGF_{1\alpha}$, [$^2H_4$]-TXB_2, [$^2H_4$]-20-carboxy-LTB_4, [$^2H_4$]-LTB_4, and [$^2H_4$]-8,12-iso-$iPF_{2\alpha}$-VI were also purchased from Cayman Chemicals.

Four IsoPs derived from AA (15-*epi*-15-E_{2t}-IsoP, 2,3-dinor-15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP, and 5-*epi*-5-F_{2t}-IsoP) and two metabolites of EPA (8-F_{3t}-IsoPs and 8-*epi*-8-F_{3t}-IsoPs) were synthesized according to our published procedures,[40–44] while 2,3-dinor-6-keto-$PGF_{1\alpha}$, [$^2H_3$]-2,3-dinor-6-keto-$PGF_{1\alpha}$, EXD_4, 15-F_{2c}-IsoPs, and [$^2H_4$]-15-F_{2c}-IsoPs were provided as described by Balgoma *et al.* (2013).[45] The enzyme β-glucuronidase, type H2 from *Helix pomatia*, and BIS–TRIS (bis-(2-hydroxyethyl)-amino-

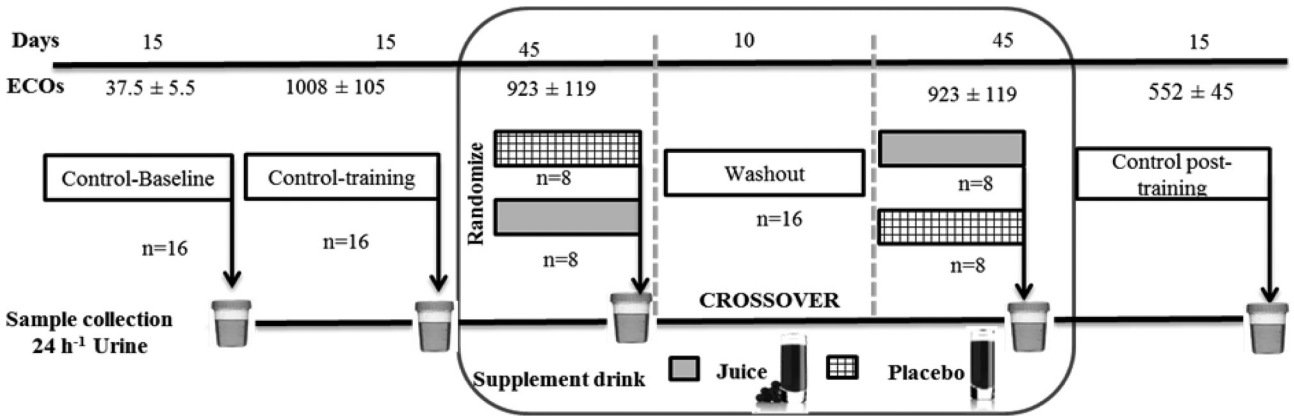

Fig. 2 Study design: this crossover study was randomized, double-blind, and placebo-controlled. Sixteen athletes, randomly divided into two groups, were assigned to supplementation of either 200 mL of ACJ or 200 mL of placebo. After 45 days of supplementation and a 10-day washout period, the beverages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

tris(hydroxymethyl)-methane) were obtained from Sigma-Aldrich (St Louis, MO, USA). All LC-MS grade solvents were obtained from J.T. Baker (Phillipsburg, NJ, USA). The Strata X-AW, 100 mg per 3 mL SPE cartridges were purchased from Phenomenex (Torrance, CA, USA). Ammonium acetate, methoxyamine hydrochloride, and isopropanol were purchased from Sigma-Aldrich. Milli-Q ultrapure deionized water was used (Millipore Corporation, Billerica, MA). Methanol and acetonitrile were obtained from Rathburn (Walkerburn, Scotland, UK). Acetone, acetic acid, and formic acid were purchased from Fisher. Aqueous ammonia (25%, w/v) was obtained from Merck (Darmstadt, Germany).

UHPLC-MS/MS analyses

The samples were analyzed according to two methods described previously by Medina *et al.*[46] and Balgoma *et al.*[45] for the purpose of a deeper analysis of the generation and metabolism of oxylipins by our volunteers.

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

The separation of the metabolites present in the urine was performed using a UHPLC system coupled with a 6460 QqQ-MS/MS system (Agilent Technologies, Waldbronn, Germany), using the setup described by Medina *et al.*[46] The main changes are as follows: after being clarified with MeOH/HCl (200 mM), the urine samples were centrifuged at 10 000 rpm for 5 min. The solid-phase extraction was as follows: (1) preconditioning of the cartridge with MeOH (2 mL) and then MilliQ water (2 mL); (2) loading of the urine sample; (3) washing of the cartridge with MilliQ water (4 mL); and (4) elution of the cartridge with MeOH (1 mL). Subsequently, the MeOH was evaporated from the extract by using a SpeedVac concentrator and the extract was reconstituted in 200 μL of the mobile phase (A : B) (90 : 10). The changes in the identification and quantification of the metabolites were as follows: chromatographic separation was carried out on an ACQUITY UPLC BEH C_{18} column (2.1 × 150 mm, 1.7 μm; Waters), the column temperatures being 6 °C (left) and 6 °C (right). The flow rate was 0.15 mL min^{-1}, using the linear gradient scheme (t, %B): (0.00; 60), (7.00; 60), (7.01; 73), (10.00; 73), (10.01; 80), (18.00; 100), (19.00; 100), and (19.01; 60). The operating conditions for the MS parameters were as follows: gas flow: 8 L min^{-1}, nebulizer: 30 psi, capillary voltage: 4000 V, nozzle voltage: 2750 V, gas temperature: 325 °C, and jet stream gas flow: 8 L min^{-1}. The MS parameters were in the range of 50 to 160 V and the collision energy was in the range of 0 to 24 V. The acquisition time was 19.01 min for each sample, with a post-run of 3.0 min for the column equilibration. The quantification of the oxylipins was carried out by daily preparation of calibration curves (concentration range 3.9 nM to 1 μM) using standard solutions. The matrix effect, recovery of extraction, and overall process efficiency for each analyte were assessed using post-extraction addition, established by Matuszewski *et al.*[47] The values were within the requested range for all the metabolites.

The sensitivity, precision, and accuracy were established with the same parameters provided by the Guidance for Industry-Bioanalytic Method Validation (the intraday and interday values were in the range of 80–120% for all the metabolites).[48] The metabolites determined by using this method were: PGDM, PGD_2, tetranor-PGDM lactone, 11-β-$PGF_{2\alpha}$, 2,3-dinor-11β-$PGF_{2\alpha}$, tetranor-PGDM, tetranor-PGJM, PGE_2, 20-OH-PGE_2, tetranor-PGEM, tetranor-PGFM, 15-keto-$PGF_{2\alpha}$, 20-OH-$PGF_{2\alpha}$, 19 (R)-OH-$PGF_{2\alpha}$, 2,3-dinor-6-keto-PGF_{1a}, 6-keto-PGF_{1a}, 15-F_{2t}-IsoP, 15-keto-15-F_{2t}-IsoP, 15-*epi*-15F_{2t}-IsoP, 2,3-dinor-15-F_{2t}-IsoP, *ent*-15-*epi*-15F_{2t}-IsoP, 9-*epi*-15-F_{2t}-IsoP, 2,3-dinor-15-*epi*-15F_{2t}, 5-F_{2t}-IsoP, 5-*epi*-5F_{2t}-IsoP, 15-keto-15E_{2t}-IsoP, 15-*epi*-15E_{2t}-IsoP, 11-dh-TXB_2, 17-*trans*-$PGF_{3\alpha}$, 8-F_{3t}-IsoP, 8-*epi*-8-F_{3t}-IsoP, PGE_1, $PGF_{1\alpha}$, 15-E_{1t}-IsoP, and 15-F_{1t}-IsoP. The quantification of the IsoPs, PGs, and TXs detected was performed using authentic markers. Data acquisition and processing were performed using the MassHunter software version B.04.00 (Agilent Technologies).

UHPLC-TQ-MS/MS for sixteen metabolites

For the remaining 16 lipid metabolites (LTs, PGs, TXs, and IsoPs), two different analytical methods based on Balgoma *et al.*[45] were employed, using the same analytical platform: ACQUITY UPLC coupled with a Xevo TQS mass spectrometry system (Waters, Milford, MA) (LC-MS/MS).

Statistical analysis

The metabolites were analyzed individually as well as by serie or family, using the excretion values (μg per 24 h) obtained throughout the study (C-B, C-T, placebo stage, ACJ stage, and CP-T). The 24 h urine was used for the absolute calculation of the amount of the LTs, EXs, IsoPs, PGs, and TXs excreted; the volume of urine excreted by the volunteers was 1212.42 ± 716.50 mL per 24 h, on average, over the assay. The data shown are the mean ± SD (Table 3), as well as the quartiles (upper values 75%, median 50%, and lower values 25%) (Fig. 3). We employed non-parametric statistical tests since the data did not satisfy the assumption of normality. The Friedman test was used; if the *P*-value was significant, the *post hoc* Wilcoxon signed-rank test was used to decide which groups were significantly different from each other. The Bonferroni correction was applied; this correction was calculated by dividing the *P*-value ($P = 0.05$) by the number of tests, namely 10 (if the metabolite was detected in all the stages). Thus, our results were adjusted to $P \leq 0.005$. The statistical analyses were made using the SPSS 23.0 software package (LEAD Technologies, Inc., Chicago, USA). The graphs were plotted using the SigmaPlot 12.0 software package (Systat Software, Inc., SigmaPlot for Windows).

Results and discussion

Currently, the evidence is insufficient to make recommendations for the use of polyphenol supplementation by elite athletes.[1,4,49,50] So, we wanted to make an in-depth examination of the primary lipid peroxidation biomarkers using a study design which allows the observation of the effects of physical exercise and polyphenolic-rich beverage intake. A total of 52 oxylipins were screened in the triathletes' urine (Table 3). The mass spectral information of the oxylipins identified was based on Medina *et al.*[46] and Balgoma *et al.*[45] In total, 37 metabolites – 17 PGs, 14 IsoPs, two LTs, one EX, and three TXs – were detected in the urine samples of the triathletes. Therefore, 15 metabolites (PGD_2, tetranor-PGJM, 6-keto-$PGF_{1\alpha}$, 20-OH-$PGF_{2\alpha}$, 19(R)-OH-$PGF_{2\alpha}$, 15-keto-$PGF_{2\alpha}$, 15-F_{1t}-IsoP, 8-*epi*-8-F_{3t}-Isop, LTC4, EXC_4, EXE_4, 6-*trans*-LTB_4, 20-carboxy-LTB_4, 20-hydroxy-LTB_4, and 13, 14-dihydro-15-keto-PGE_1) were not detected.

Prostaglandin and thromboxane metabolites derived from arachidonic acid

Recent publications have demonstrated changes in lipid peroxidation as a consequence of chronic exercise.[5–8] A previous study by our group showed a decrease in the values of urinary PGs (tetranor-PGEM and 11-β-$PGF_{2\alpha}$) after a chronic training program.[5] Our current results are similar, showing a decline of these biomarkers due to the elite training program. In our urine samples, 17 PGs from different families were quantified. Our data show means in the range from 0.04 ± 0.08 μg per 24 h (PGE_2) to 41.2 ± 24.4 μg per 24 h (PGDM). The PGs are potent oxylipins involved in numerous homeostatic biological functions and inflammation.[18] The literature mentions that regular exercise induces an anti-inflammatory response rather than a pro-inflammatory response.[4,9] In this context, the results for the concentrations of metabolites from the PGD_2 pathway are notable since they have been implicated in both the development and resolution of inflammation. For the PGD_2 pathway, the Friedman test revealed statistically significant differences (χ^2 (4) = 42.143, $P < 0.001$). The CP-T value was significantly lower, compared to all other stages (Fig. 3A). Moreover, without the Bonferroni correction, the ACJ stage was different from C-T ($Z = -2.155$, $P = 0.031$). Considering the PGD_2 metabolites individually, PGDM was the metabolite that showed the highest excretion levels. Prostaglandin D_2 is a COX product of AA that activates D prostanoid receptors to modulate vascular, platelet, and leukocyte function *in vitro*.[51] The Friedman test revealed statistically significant changes (Table 3) in this metabolite; the Wilcoxon test showed that the CP-T value was lower than that for C-T ($Z = -3.237$, $P < 0.001$). The 11-β-$PGF_{2\alpha}$ content in the CP-T stage was significantly lower than that in all other stages (CB, $Z = -3.124$, $P = 0.002$; C-T, $Z = -3.124$, $P < 0.001$; placebo, $Z = -3.237$, $P = 0.001$; and ACJ, $Z = -3.067$, $P = 0.002$). The 2,3-dinor-11-β-$PGF_{2\alpha}$ excretion in the ACJ stage was lower than that for C-B ($Z = -2.953$, $P = 0.003$) and C-T ($Z = -3.124$, $P = 0.002$). The ACJ stage also showed a lower value of this compound compared to the placebo stage, though this was not statistically significant when applying the correction ($P = 0.009$). In the last control stage, the excretion of tetranor-PGDM was decreased when compared to C-T ($Z = -3.010$, $P = 0.003$), placebo ($Z = -3.233$, $P = 0.001$), and ACJ ($Z = -2.856$, $P = 0.004$) (Table 3). According to research carried out by Morrow *et al.*,[52] PGDM is a major urinary metabolite of PGD_2 with a unique lower side-chain that readily undergoes reversible cyclization. In our study, the urinary excretion of PGDM was highest under basal conditions, but showed a decrease of about 70% by the end of the experiment. This suggests that in our triathletes there was a reduction in the inflammation status since the hallmark of inflammation is the enhanced secretion of pro-inflammatory immune mediators such as PGs.[49,53] A study in humans using liquid chromatography-tandem mass spectrometry mentioned that tetranor-PGDM was much more abundant than the PGD_2 metabolites 11β-$PGF_{2\alpha}$ and 2,3-dinor-11β-$PGF_{2\alpha}$ in the urine of healthy volunteers.[51] In our elite triathletes, 11_{β}-$PGF_{2\alpha}$ and 2,3-dinor-11_{β}-$PGF_{2\alpha}$ (F-ring metabolites) were much more abundant than tetranor-PGDM (D-ring metabolite). This leads us to believe that physical exercise affects quantitatively the excretion of metabolites of this PGD pathway, when compared to non-athlete volunteers. Regarding the effect of ACJ

Table 3 Urinary isoprostanes and prostaglandins (μg per 24 h) from arachidonic acid, dihomo-γ-linoleic acid, and eicosapentaenoic acid detected in the urine samples of triathletes

		Stage of study												
		C-B		C-T		P		ACJ		CP-T		Friedman test		
	Analyte (μg per 24 h)	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	χ^2	df	Sig
Arachidonic acid														
PGs														
D pathway	PGDM	**31.1**	24.6	**41.2**	24.4	**16.1**	14.0	**19.3**	14.3	**10.0**	13.9	*19.7*	*4*	***0.001***
	Tetranor-PGDM lactone	**2.4**	2.4	**1.1**	1.2	**1.2**	0.6	**0.9**	1.1	**1.4**	1.7	*3.8*	*4*	*0.430*
	11-β-$PGF_{2\alpha}$	**4.3**	2.1	**7.5**	4.1	**7.1**	3.9	**7.4**	5.2	**1.7**	4.1	*18.8*	*4*	***0.001***
	2,3-dinor-11β-$PGF_{2\alpha}$	**8.9**	5.5	**6.2**	2.0	**6.8**	7.3	**2.8**	2.0	**4.3**	5.2	*20.9*	*4*	***<0.001***
	Tetranor-PGDM	**3.2**	2.5	**3.8**	2.7	**2.7**	1.6	**2.0**	1.2	**0.6**	0.7	*21.3*	*4*	***<0.001***
E pathway	PGE_2	**0.51**	0.50	**0.15**	0.14	**0.04**	0.08	**0.19**	0.30	**0.44**	0.08	*13.5*	*4*	***0.009***
	20-OH-PGE_2	**3.8**	4.6	**2.0**	2.1	**2.1**[b]	0.6	**0.9**	1.0	**4.3**[c]	3.1	*4.2*	*2*	*0.122*
	Tetranor-PGEM	**2.6**	2.2	**1.2**	1.7	**0.9**[c]	0.9	**2.4**[d]	1.6	**3.0**[c]	2.6	—	—	—
	Tetranor-PGAM	**2.9**	3.3	**2.4**	4.5	**2.3**[d]	1.9	**1.3**[e]	0.8	**2.1**[d]	1.7	—	—	—
	13,14-dihydro-15-keto-$PGF_{2\alpha}$	—	—	**2.9**	—	**6.1**	—	—	—	—	—	—	—	—
	13,14-dihydro-15-keto-$PGE_{2\alpha}$	—	—	—	—	—	—	—	—	**0.2**[a]	0.2	—	—	—
F pathway	Tetranor-PGFM	**0.9**	0.4	**1.8**	—	**1.6**	—	—	—	—	—	—	—	
	$PGF_{2\alpha}$	**3.5**[b]	1.6	**2.7**	—	**2.7**	—	**5.1**[b]	2.5	**3.7**	—	—	—	—
I pathway	2,3-dinor-6-keto-$PGF_{1\alpha}$	**2.1**	2.7	**2.2**	2.4	**2.0**	1.9	**2.2**	3.0	**1.9**	2.3	*2.3*	*4*	*0.680*
F_2-Isoprostane														
15-serie	15-F_{2t}-IsoP	**3.2**	0.7	**2.7**	0.5	**2.5**	0.5	**2.1**	0.6	**1.6**	0.4	*16.1*	*4*	***0.002***
	15-keto-15-F_{2t}-IsoP	**1.4**	1.4	**0.4**	1.0	**1.0**	—	**0.2**	0.4	**3.02**[d]	1.9	*6.1*	*2*	***0.046***
	15-*epi*-15-F_{2t}-IsoP	**4.3**	4.3	**2.8**	2.7	**1.5**	1.3	**3.1**	6.2	**1.0**	0.8	*4.8*	*4*	*0.298*
	2,3-dinor-15-F_{2t}-IsoP	**16.5**	9.4	**14.8**	6.5	**11.4**	7.4	**9.5**	5.6	**10.2**	12.7	*8.3*	*4*	*0.081*
	ent-15-*epi*-15-F_{2t}-IsoP	**0.7**	1.0	**0.4**	0.5	**0.1**	0.1	**0.3**	0.5	**0.1**	0.1	*4.9*	*4*	*0.297*
	9-*epi*-15-F_{2t}-IsoP	**2.7**	1.6	**1.4**	0.8	**1.0**	0.4	**1.3**	0.9	**1.2**	0.8	*15.1*	*4*	***0.004***
	2,3-dinor-15-*epi*-15-F_{2t}-IsoP	**3.0**	2.2	**1.4**	0.5	**1.3**	1.4	**1.2**	0.5	**1.5**	1.4	*9.1*	*4*	*0.057*
	15-F_{2c}-IsoPs	**8.4**	4.3	**8.2**	4.9	**6.4**	2.9	**7.0**	3.7	**5.3**	3.3	*5.4*	*4*	*0.250*
5-serie	5-F_{2t}-IsoP	**11.2**	5.6	**10.7**	5.8	**9.0**	4.3	**11.9**	6.8	**7.5**	4.7	*4.5*	*4*	*0.332*
	5-*epi*-5F_{2t}-IsoP	**7.2**	4.6	**5.5**	4.5	**2.9**	2.0	**4.7**	3.4	**4.9**	2.5	*13.3*	*4*	***0.010***
E_2-Isoprostane														
15-serie	15-Keto-15-E_{2t}-IsoP	**3.3**	0.5	**2.3**	0.4	**1.7**	0.3	**1.9**	0.2	**2.1**	0.6	*8.5*	*4*	*0.073*
	15-*epi*-15-E_{2t}-IsoP	**2.7**	4.1	**2.1**	3.8	**2.0**[b]	1.6	**1.3**	1.5	**3.5**	6.1	*1.0*	*3*	*0.785*
LT	LTB_4	**0.03**	0.02	**0.02**	0.02	**0.03**	0.02	**0.06**	0.04	**0.03**	0.02	*9.7*	*4*	***0.040***
cys-LT	LTE_4	**0.13**	0.07	**0.11**	0.09	**0.06**	0.03	**0.12**	0.11	**0.05**	0.05	*9.9*	*4*	***0.040***
EX	EXD_4	—	—	**2.1**[b]	2.6	**0.1**	—	**0.2**	—	—	—	—	—	—
TXs	TXB_2	—	—	—	—		—	**0.1**	—	**0.1**	—		—	—
	11-dehydro-TXB_2	**0.3**	0.2	**0.5**	0.2	**0.3**	0.2	**0.2**	0.1	**0.2**	0.1	*21.8*	*4*	***<0.001***
	2,3-dinor-TXB_2	**3.3**[f]	0.7	**3.1**[e]	0.5	**2.9**[f]	0.4	**2.1**[e]	0.5	**2.4**[d]	1.0	—	—	—
Eicosapentaenoic acid														
PG	17-*trans*-$PGF_{3\alpha}$	**1.1**	1.7	**1.2**	1.7	**0.7**	1.0	**0.2**	0.4	**2.9**[b]	2.8	*1.52*	*3*	*0.676*
IsoP	8-F_{3t}-IsoP	**3.2**	2.3	**0.6**[a]	0.1	**1.0**[b]	0.4	**1.6**[d]	1.0	—	—			
Dihomo-γ-linolenic acid														
PGs	PGE_1	**0.3**	0.2	**0.6**	0.3	**0.5**	0.3	**0.4**	0.2	**0.1**	0.1	*29.6*	*4*	***<0.00***
	$PGF_{1\alpha}$	**2.1**[f]	0.4	**0.05**	—	**3.8**[b]	2.6	—	—	**1.1**	—	—	—	—
IsoP	15-E_{1t}-IsoP	**0.5**	0.1	—	—	—	—	**0.3**[a]	0.3	—	—	—	—	—

The data are shown as mean ± standard deviation (SD) in μg per 24 h. The volume of urine excreted by the volunteers was 1212.42 ± 716.50 mL per 24 h, on average, in all the periods. 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, P: placebo, ACJ: *Aronia–citrus* juice, CP-T: control post-treatment.

intake on the excretion of PGD_2 metabolites, we observed a positive influence, since 2,3-dinor-11β-$PGF_{2\alpha}$ showed a significant decrease when compared to the first controls; also, the excretion of PGDM showed a significant reduction (in the placebo stage, it remained constant). Previous studies, both *in vivo* and *in vitro*, have also reported some influence on the cardiovascular system due to polyphenol supplementation in the diet.[1,13,47] In addition, a study by our group analyzed the biomarker implicated in iron metabolism, hepcidin, and revealed that long-term training using ECOs reduces inflammation and, hence, could be responsible for the decrease of hepcidin in triathletes found in this study.[54]

The concentration of the metabolites from the PGE pathway showed a significant decrease after increased training, suggesting that physical exercise also played a role in the decline in the excretion of these metabolites. The levels of the

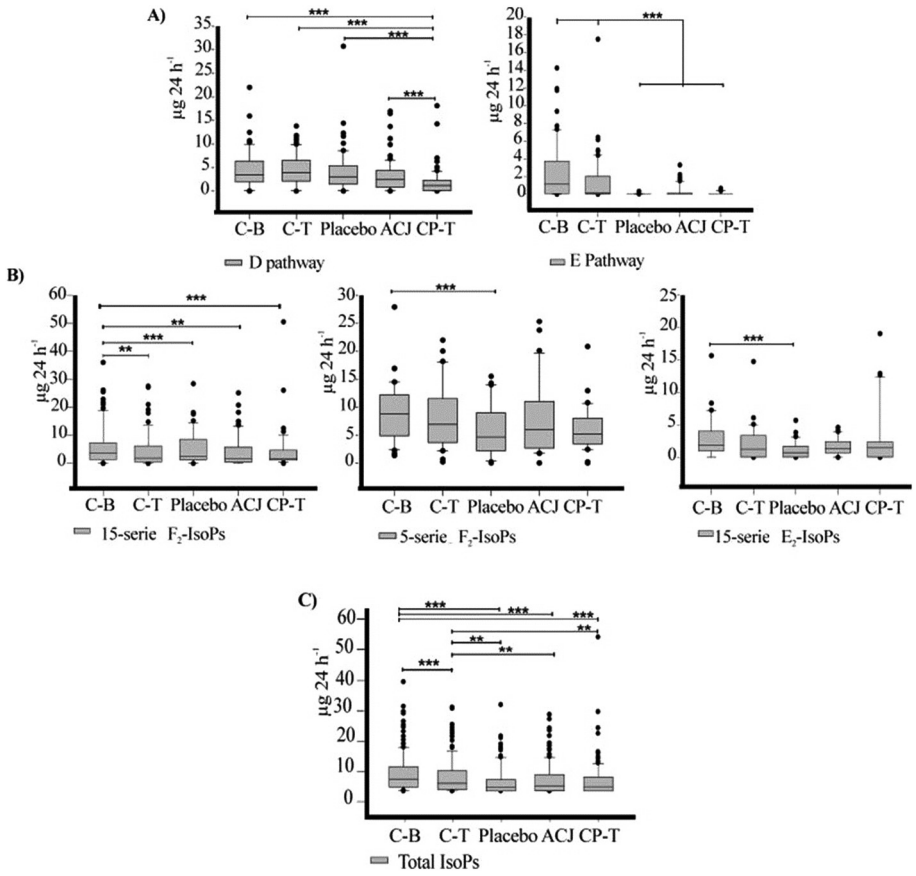

Fig. 3 Box plots with quartiles (upper values 75%, median 50%, and lower values 25%) of the urinary oxylipins throughout the study (μg per 24 h). The level of statistical significance was set at $P < 0.005$ with the Bonferroni correction (** = $P < 0.005$ and *** = $P < 0.001$). (A) Prostaglandins by family, (B) isoprostanes by serie, and (C) total isoprostanes, both F_2-isoprostanes and E_2-isoprostanes.

metabolites of the PGE_2 pathway in C-B and C-T were higher, but subsequently declined (χ^2 (4) = 21.962, P = 0.001) (Fig. 3A). Also, we cannot rule out an effect of ACJ intake on inflammation since the excretion of PGE_2 (detected in all the periods) increased in comparison with the placebo stage (0.04 ± 0.08 *vs.* 0.19 ± 0.30). The placebo period showed lower values than C-B ($Z = -2.98$, $P = 0.003$) and C-T ($Z = -3.180$, $P = 0.001$), although the excretion values did not decrease significantly between C-B and C-T ($Z = -2.669$, $P = 0.008$). The other three metabolites of the E pathway (20-OH-PGE_2, tetranor-PGEM, and tetranor-PGAM) were mainly detected in the two control periods (C-B and C-T), but in the beverage intake stages and the CP-T stage, the number of volunteers that excreted these biomarkers decreased. 20-OH-PGE_2 was excreted by the majority of the volunteers after the juice intake, compared to the placebo. PGE_2 is involved in all processes leading to the classic signs of inflammation (redness, swelling, and pain), but also shows anti-inflammatory properties.[18] For example, according to recent *in vivo* studies, this lipid mediator is related to numerous physiological and pathophysiological processes in the kidney,[55] indicating a significant role in modulating the effect of vasopressin on the osmotic water reabsorption in the renal collecting duct cells – where it attenuates antidiuretic action.[56] In addition, it has been mentioned that the induction of prostanoids during exercise alters clotting factors, increases vascular tone, and helps adapt muscle cells to contractile activity.[57] Based on the above, our results suggest a potential effect of ACJ intake on the inflammatory process and vascular system.

Regarding the F and I pathways, the metabolites were scarcely detected in the urine samples or did not differ significantly during the study. Regarding the TXs, the primary enzymatic metabolite of TXA_2 is 11-dehydro-TXB_2 (11-dh-TXB_2), which has been validated as a reliable and noninvasive biomarker-integrated index of *in vivo* platelet activation.[58] A previous report observed that 22 sedentary volunteers subjected to standardized, aerobic, high-amount–high-intensity training for eight weeks showed significant decreases in the urinary excretion of 11-dh-TXB_2.[59] The authors related this result to platelet activation and hence it may be relevant to explain why long-term physical exercise is beneficial for the cardiovascular system. According to our results, the excretion of 11-dh-TxB_2 showed a significant decrease in the ACJ ($Z = -2.953$, $P = 0.003$) and CP-T ($Z = -3.069$, $P = 0.002$) stages, compared to C-T (Table 3). The 11-dh-TXB_2 decreased significantly in the last period when the training load was lower;

ACJ also had a considerable influence, reducing the values, suggesting a cardiovascular benefit.

Leukotrienes

Two metabolites (LTB_4 and LTE_4) were detected in all the stages and in the majority of the volunteers. The Friedman test showed significant changes in LTB_4 and the subsequent Wilcoxon signed-rank test revealed higher values in the ACJ stage compared with the placebo ($Z = -2.166$, $P = 0.03$), C-T ($Z = -2.668$, $P = 0.008$), and CP-T ($Z = -2.166$, $P = 0.03$) stages. However, no *P*-value was below 0.005. In contrast, LTE_4 showed a significant decrease in the placebo stage, relative to the baseline values ($Z = -2.784$, $P = 0.005$). Also, the placebo stage differed from the ACJ stage ($Z = -1.960$, $P = 0.05$), but not significantly so after the Bonferroni correction. The excretion values of the CP-T stage were lower than those for C-B ($Z = -2.668$, $P = 0.008$) and C-T ($Z = -1.931$, $P = 0.053$), but not statistically so (Table 3). In summary, the urinary metabolites LTB_4 and LTE_4 showed significant changes; in particular, the ACJ stage presented higher values than the placebo phase. These findings are the opposite of those mentioned in the current literature, since most polyphenol intake studies have shown decreased excretion in healthy people.[50,60] It has been demonstrated that flavonoids can modulate the activity of enzymes that are involved in the metabolism of AA in macrophages – such as phospholipase A_2, COXs, and LOXs; the inhibition of these enzymes by flavonoids lowers the production of the mediators of inflammatory reactions.[60] Yoon and Baek (2005)[61] also mentioned that polyphenols are inhibitors of both COX and LOX and that a general rule is "more COX inhibitions and less LOX inhibitions with polyphenols that contain few hydroxyl substituents (with none in ring B)". This suggests that polyphenols, including those in our juice rich in polyphenols, have more effect on an inflammatory cascade of COX-2, which allows the LOX branch to accelerate the formation of LTs. This explanation seems to describe to a certain extent the change produced in the excretion values in our study. On the other hand, due to the decline in the ECOs load, a decrease in the excretion of LTE_4 was detected. Other reports have mentioned that elite athletes show an increased risk of respiratory symptoms related to asthma, especially those that participate in endurance sports – such as swimming, running, and cycling – and in winter sports. This risk to the respiratory system arises because, during physical activity, the elite athletes increase their water and heat loss through respiration.[62] This has strong ties with the results of LTs since they play a key role in perpetuating airway inflammation – leading directly to airflow obstruction through the effects on vascular permeability, mucus production, and smooth muscle constriction.[63] A training program can result in a depletion of LTs and/or a slow *cys*-LTs response to exercise, which may be responsible for the protective effect of training programs on respiratory symptoms.[64] Our study shows that post-training could change the excretion of *cys*-LTs, and therefore might have an effect on the airway pathway.

Isoprostanes derived from arachidonic acid

The measurement of F_2-IsoPs is known to be an index of OS *in vivo*.[16] Regarding the level of total IsoPs derived from AA in urine, a significant reduction was observed, reflecting mainly the OS decrease in the CP-T stage (Fig. 3C). When the sum of all the IsoPs was submitted to the Friedman test, a significant *P*-value (χ^2 (4) = 91.035, $P \leq 0.001$) was obtained. The total IsoPs ranged from 6.10 ± 6.47 μg per 24 h (C-B) to 3.42 ± 5.9 μg per 24 h (CP-T). The Wilcoxon signed-rank test showed a tendency of the excretion to decline over the course of the study (Fig. 3C). The IsoPs showed significant variation in their urinary excretion when the values were analyzed by series: 15-F_{2t}-IsoPs (χ^2 (4) = 33.360, $P \leq 0.001$), 5-F_{2t}-IsoPs (χ^2 (4) = 12.893, $P = 0.012$), and 15-E_2-IsoPs (χ^2 (4) = 14.484, $P = 0.006$) (Fig. 3B).

These data suggest that chronic exercise decreased the OS levels in our elite athletes. According to the review by Nikolaidis *et al.*,[65] in most of the cases in which they analyzed this behavior, the levels of urinary F_2-IsoP were decreased by chronic exercise. In other studies,[5,66,67] physical activity was also the primary factor that decreased the urinary OS biomarker (IsoPs). The literature mentions that regular exercise training increases the levels of enzymatic and non-enzymatic antioxidants in muscle fibers, resulting in improved endogenous protection against exercise-mediated oxidative damage.[10] Furthermore, in athletes of different disciplines, polyphenols have shown antioxidant potential that can be beneficial in the reduction of the effects of oxidative damage during intense exercise.[4] In our study, considering the metabolites individually, we observed an increase of 15-*epi*-15-E_{2t}-IsoP and 15-keto-15-F_{2t}-IsoP, but this change was not linked to physical exercise directly since the increase was in the ACJ stage, when compared to the placebo. This result suggests a potential role for the compounds from ACJ intake in these IsoP pathways. Recent reports have shown that the E-type IsoPs are potent vasoconstrictors at low nanomolar concentrations.[41] 15-E_{2t}-IsoP (also referred to as 8-iso-PGE_2 or iPE2-III) was found to be a powerful and efficient constrictor in the ductus arteriosus of chicken, acting through the thromboxane receptor.[68] Also, other studies with animals have shown both vasoconstrictive and vasodilatory effects of 15-E_{2t}-IsoP, suggesting a biological activity of this molecule in the cardiovascular system.[69] On the other hand, 15-keto-15-F_{2t}-IsoP is a metabolite derived from 15-F_{2t}-IsoP. In an animal study, it was demonstrated that this IsoP probably acted as a partial agonist at the TP-receptor, mediating contraction and inducing a weak endothelium-independent relaxation at high concentrations.[70] Therefore, the increase in the abundance of these metabolites could reflect the participation of the compounds from ACJ – for example, the flavonoids (polyphenols)[71] – or of proline betaine, ferulic acid, or other metabolic derivatives (nutritional biomarkers)[72] in the stimulation of some IsoPs related to the effects on vascular smooth muscle. Also, it should not be forgotten that, along with phytochemicals, ACJ contains a variety of vitamins, minerals, and fiber that could have influenced this result.[73,74]

Metabolites derived from eicosapentaenoic acid and dihomo-γ-linolenic acid

Regarding metabolites derived from DGLA, $PGE_{1\alpha}$ was detected and the Friedman test revealed significant changes among the experimental periods (χ^2 (3) = 29.624, $P \leq 0.001$). The Wilcoxon test showed that the CP-T value was significantly lower (C-T, $Z = -3.408$; placebo, $Z = 3.294$; ACJ, $Z = -3.324$, $P = 0.001$ in all cases) compared to most of the other stages (Table 3). According to the literature, through a series of free radical reactions, COX metabolizes DGLA and AA to form various bioactive metabolites – namely, the 1 and the 2 series of prostaglandins (PG1 and PG2), respectively. Unlike the PG2s, which are viewed as pro-inflammatory, the PG1s possess anti-inflammatory and anticancer activity.[19] During our study, PGE_1 was detected in all the stages, showing statistically significant differences (Table 3). These results suggest a decrease of this metabolite in urine when there is a decline in ECOs, although the values during C-T were higher than in C-B, since the acute physical exercise could have stimulated this pathway. PGE_1 has been shown to possess anti-inflammatory properties and to modulate vascular reactivity.[75] On the other hand, 15-E_{1t}-IsoP was mainly detected in C-B (0.5 ± 0.1 μg per 24 h), suggesting that physical exercise is an external factor that could have influenced the diminution of its values.

Regarding the metabolites derived from EPA, 8-*epi*-8-F_{3t}-IsoP was not detected and 8-F_{3t}-IsoP was detected only during C-B (3.4 ± 2.3 μg per 24 h). The elite training decreased the values of 8-F_{3t}-IsoP, suggesting again that physical exercise is an external factor that could influence the reduction of biomarkers concomitantly with the decline in the training loads of the athletes (CP-T). These IsoPs are formed by the free radical-induced peroxidation of EPA *in vivo* and *in vitro*. The F_3-IsoPs are spontaneously generated in abundance *in situ* in response to OS and both are useful as biomarkers of OS.[23,76]

Conclusions

This study contributes to a better comprehension of the behavior of urinary biomarkers related to OS and inflammation status (IsoPs, LTs, PGs, and TXs) in athletes after an elite training period and supplementation of 200 mL of ACJ (a functional beverage rich in polyphenols). The findings indicate that physical exercise is an external factor that influenced mainly the OS biomarkers (F_2-IsoPs) and inflammation biomarkers (11-dh-TxB_2, PGE_2, PGDM, tetranor-PGFM, $PGF_{1\alpha}$, PGE_1, and LTE_4) in triathletes. Furthermore, our collective results regarding ACJ intake show that supplementation stimulated the excretion of some metabolites related to vascular homeostasis and smooth muscle (15-*epi*-15-E_{2t}-IsoPs, 15-keto-F_{2t}-IsoP, 20-OH-PGE_2, PGE_2, LTE_4, and LTB_4), indicating a potential role in the cardiovascular system. This work could help to increase our knowledge about the effect of chronic exercise and sports drinks on human lipid metabolism. Moreover, it could aid the design of new beverages for athletes.

Author contribution

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

Conflicts of interest

The authors declare that they have no conflict of interest.

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