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Abstract

This study investigated the presence and the role of oxylipins and their precursors in small urinary extracellular vesicles (uEVs) collected from ultramarathon runners during the Tor des Géants® to explore the metabolic adaptation to an endurance exercise.

Oxylipins are potent signalling compounds originated from polyunsaturated fatty acids (PUFAs) and involved in the regulation of the immune system response. During intense exercise, EVs are strongly released to mediate systemic immune adaptation by promoting cell-cell interaction and organ crosstalk through a plethora of signalling chemicals.

A mass spectrometry-based method was fully developed and validated for the targeted profiling of fifty-two oxylipins (i.e., isoprostanoids, prostaglandins, epoxy- and hydroxy-fatty acids, specialized pro-resolving mediators) and four PUFAs in small uEVs.

Both F_2- and E_2-isoprostanes were detected in small uEVs of the ultramarathon runners, suggesting the onset of an oxidant insult. 5-F_{2t}-IsoP exhibited significant pre- to post-race variations, thus potentially representing a non-invasive marker of *in-vivo* lipid peroxidation. The presence of specialized pro-resolving mediators suggests the activation of pro-resolution signalling cascades resolving inflammation. These outcomes may help managing post-exercise recovery and improve training.

Keywords (6 max)

Oxylipins; exosomes; F_2-isoprostanes; oxidative stress; metabolic adaptation; ultramarathon

Introduction

Urine analysis is common in the clinical practice, but the recent identification of extracellular vesicles (EVs) in this specimen has opened new perspectives for biomarker discovery as well as physiological and pathological studies [1] [2]. EVs are small (30 to 10,000 nm in diameter) double membrane bound vesicles secreted by various cell types and present in biofluids such as urine, blood, cerebrospinal fluid, and saliva [3,4]. They play a pivotal role in communication among cells, which dynamically use their cargo to respond to physiological or pathological changes [5,6]. This makes EVs excellent candidates as non-invasive diagnostic biomarkers in clinical settings [7,8], but the absence of standardized isolation and purification protocols hampers research progress [9,10].

EVs contain proteins, nucleic acids, mRNAs, and lipids [11], however much less is known about the composition and functional significance of the lipidic fraction [12]. Recent mass spectrometry-based studies have identified numerous lipid species in urinary EVs (uEVs), including cholesterol and phosphatidyl serine 18:0/18:1 [13]. Notably, approximately 280 distinct molecular lipids have been identified in EVs derived from patients with prostate cancer, suggesting a disease-specific lipid composition [14]. The role of lipids in EVs has been also evidenced in cardiovascular and neurodegenerative diseases, including schizophrenia [15,16]. EVs not only carry bioactive lipids but also enzymes associated with the lipid metabolism [17–20] . Oxylipins, i.e. a class of compounds deriving from the enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids (PUFAs), are

of particular interest [21]. Oxylipins include isoprostanoids (IsoPs) and isofuranoids (IsoFs), prostaglandins (PGs), leukotrienes (LTs), epoxy- and hydroxy-PUFAs, and specialized pro-resolving mediators (SPMs e.g., lipoxins, resolvins, protectins, maresins) [22]. Studies have demonstrated that EVs transport PGs to target cells, inducing a prostaglandin-dependent biological response [23]. For instance, PGE_2 has been detected in murine mammary adenocarcinoma-derived EVs, suggesting its potential involvement in tumor growth [24]. Additionally, elevated levels of $PGF_{2\alpha}$ have been measured in patients with paroxysmal nocturnal hemoglobinuria, demonstrating a differential metabolite cargo between plasma EVs of patients and controls [25]. EVs also transport enzymes responsible for LT synthesis and granulocyte recruitment, thereby regulating the inflammatory response [26].

Oxylipins contribute to a variety of biological functions, including immune regulation and cellular metabolic reprogramming [27]. The body undergoes multisystemic metabolic adjustments in response to various stimuli, including extreme physical exercise. While intense exercise can induce muscle damage and inflammation, the body typically adapts by improving its ability for recovery [28]. When coupled with altitude hypoxia, strenuous physical activity may exacerbate the inflammatory response [29]. Acute exercise bouts trigger an increase in circulating EVs that are hypothesized to mediate organ crosstalk, thereby promoting systemic adaptation. Exploring the signalling networks that underlie these benefits is of particular interest because of their potential therapeutic implication in metabolic diseases [30].

The unique biophysical properties make urine a valuable biological matrix for isolating EVs, facilitating the non-invasive physiology and pathology studies. However, harmonized analytical protocols for isolating pure pellets of uEVs, particularly exosomes within the 30-150 nm size range, remain elusive. Size-based isolation methods, without further separation procedures, allow to obtain pellets containing small uEVs, with exosomes likely representing the larger fraction.

Considering the critical role of intercellular communication and physical activity in human health, we propose a novel MS-based profiling method for the determination of fifty-two oxylipins and their precursors extracted from small uEVs collected from urine samples provided from mountain ultramarathon runners. This approach aims to investigate the regulation of inflammation and the metabolic adaptation to endurance exercise in a new and non-invasive way. Our procedure will be a valuable tool for quantifying a wide panel of key inflammatory mediators and markers of oxidative stress in future EVs studies.

Materials and methods

Chemicals and materials

Acetonitrile (ACN) and methanol (MeOH) hypergrade for LC-MS LiChrosolv® (purity ≥ 99.9%), and water (H_2O) for chromatography (LC-MS grade) LiChrosolv® (purity ≥ 99.9%) used for sample treatment and UHPLC-ESI-MS/MS analysis were from EDM Millipore (Milan, Italy). Commercially available oxylipins (purity ≥99%), 15-F_{2t}-isoprostane, 15-F_{2t}-isoprostane-d_4, 15-E_{2t}-isoprostane, 15-E_{2t}-isoprostane-d_4, prostaglandin E_2, prostaglandin E_2-d_4, prostaglandin D_2, thromboxane B_2, leukotriene B_4, lipoxin A_4, lipoxin A_4-d_5, lipoxin B_4, resolvin E_1, resolvin D_1, resolvin D_1-d_5, resolvin D_2, resolvin

D_3, resolvin D_4, resolvin D_5, 17(*R*)-resolvin D_1, 17(*R*)-resolvin D_1-d_5, 17(*R*)-resolvin D_3, 17(*R*)-resolvin D_4, neuroprotectin D_1, protectin DX, maresin-1, maresin-1-d_5, 7-*epi*-maresin-1, maresin-2, 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, 15-HETE, 20-HETE, 20-HETE-d_6, 8,9-DiHETE, 11,12-DiHETE, 14,15-DiHETE, 13-hydroxy-9Z,11E-octadecadienoic acid (HODE), 8,9-epoxyeicosatrienoic acid (EET), 11,12-EET, 14,15-EET, 14,15-EET-d_{11}, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA), n-3 docosapentaenoic acid (DPA_{n-3}), linoleic acid (LA) and LA-d_4 were from Cayman Chemical (Michigan, USA). Not commercially available oxylipins, i.e. 5-F_{2t}-isoprostane, 5-*epi*-5-F_{2t}-isoprostane, 8-F_{3t}-isoprostane, 8-*epi*-8-F_{3t}-isoprostane, 18-F_{3t}-isoprostane, 20-F_{4t}-neuroprostane, 20-*epi*-20-F_{4t}-neuroprostane, 10-F_{4t}-neuroprostane-d_4, 10-*epi*-10-F_{4t}-neuroprostane-d_4, 14(*R,S*)-14-F_{4t}-neuroprostane, 14(*R,S*)-14-F_{3t}-neuroprostane, 4(*R,S*)-4-F_{4t}-neuroprostane, C21-15-F_{2t}-isoprostane, tetranor-NPD_1, dinor-NPD_1, 7(*R,S*)-ST-Δ^8-11-dihomo-isofuran, ent-7(*R,S*)-7-F_{2t}-dihomo-isoprostane, 17-F_{2t}-dihomo-isoprostane, 16(*S*),9(*R*)-linotrin, diH-DPA_{n-3}, diH-DPA_{n-6} and diH-AdA were synthesized at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France), according to procedures reported elsewhere [31–35]. Sterile polypropylene containers were from Eppendorf (Milan, Italy)**,** whereas Phenex™-RC syringe filters (0.2 μm regenerate cellulose, 4 mm of diameter) were from Phenomenex (California, USA). A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and a Hermle Z-326 K Centrifuge, (Wehingen, Germany) were used for sample vortex-mixing and centrifugation, respectively. The removable needle micro-extraction by packed sorbent (MEPS) 250 μL syringe for HTA 300APlus (Thermo Scientific & Varian 8400 systems) and MEPS silica-C18 Barrel Insert and Needles (BINs) were purchased from SGE Analytical Science (Melbourne, Australia). The automated HT4000 Series Sample Prep workstation was purchased from HTA S.R.L. (Brescia, Italy).

Preparation of standard solutions and quality control samples

Stock solutions of commercially available oxylipins (100-1000 μg/mL in organic solvent [refer to product insert from Cayman Chemicals]), as well as those synthetized at IBMM (Montpellier, France) (1000 μg/mL in MeOH), were stored at -80 °C and used for the preparation of intermediate standard mixtures containing all the fifty-two oxylipins and four PUFAs in MeOH:ACN (1:1, *v/v*) mixed solvent.

Working standard solutions of the fifty-six analytes were prepared by diluting intermediate stock solutions with LC-MS H_2O and then stored at 4 °C up to 1 month. A mixture of deuterated internal standards (ISs) composed of 15-F_{2t}-isoprostane-d_4, 15-E_{2t}-isoprostane-d_4, 10-F_{4t}-neuroprostane-d_4, 10-*epi*-10-F_{4t}-neuroprostane-d_4, C21-15-F_{2t}-isoprostane, prostaglandin E_2-d_4, lipoxin A_4-d_5, resolvin D_1-d_5, 17(*R*)-resolvin D_1-d_5, maresin-1-d_5, 20-HETE-d_6, 14,15-EET-d_{11} and LA-d_4 was prepared at 20 ng/mL through sequential dilutions of the stock solutions (100 μg/mL in organic solvent [refer to product insert from Cayman Chemicals]) with MeOH:ACN (1:1, *v/v*). Working standard solutions were used to obtain five-point calibration curves (see concentration levels in Table 1). These solutions, added with 5 μL of the ISs mixture (20 ng/mL), were subjected to the MEPS procedure and analysed by UHPLC-ESI-MS/MS.

A pooled sample of small urinary EVs (uEVs) was obtained by mixing known aliquots of samples collected from nominally healthy subjects (n=10). This pooled sample was spiked with known amount of the intermediate stock solutions to obtain quality control samples (QCs) at low, medium, high levels (i.e., level 1^{st}, 3^{rd}, 5^{th} of the calibration curve) and then used for method validation. The volume of the spiked standard solution never exceeded 10% of the total small uEV sample volume.

Small uEV isolation

Small uEVs were isolated from urine samples in a polypropylene tube through ultracentrifugation (UC), the most used technique for isolating urinary EVs among immunoprecipitation/affinity capture, size exclusion chromatography, filtration, precipitation, and density-gradient centrifugation [2]. Despite longer duration, lower yield, and purity when compared to other techniques, UC is still well-suited for studies where large volumes of low viscosity are available (e.g., urine samples) [1]. We followed a previously reported procedure for pelleting small urinary EVs from endurance athletes [36]. Briefly, frozen urine samples stored at –80 °C were thawed at 37 °C and centrifuged multiple times at 4°C to remove cells, debris and large EVs (10 min at 300 × g, 20 min at 2000 × g, and 30 min at 10,000 × g). These UC cycles were followed by a further cycle at 70 min at 100,000 × g (4 °C) to collect the pellet and by a final ultracentrifugation for 60 min at 100,000 × g to wash the sample. Serial UC cycles at 100,000 × g or more allow to pellet small EVs [37]. After resuspending the pellet in 100 µL of PBS 1X (i.e., 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), an immediate quality control of isolation was carried out by checking the nucleic acid-to-protein ratio (NPr) by measuring UV absorbance at 260 nm and 280 nm (NanoDrop® 1000 Spectrophotometer, Thermo Scientific, Waltham, MA). The NPr was reported as a means of evaluating the purity of EVs samples [38].

Oxylipin and PUFA extraction from uEVs

The optimization of the extraction of oxylipins and PUFAs from small uEVs was performed by testing three solvent mixtures i.e., 50:50 v/v MeOH:H_2O, 50:50 v/v ACN:H_2O, and 50:50 v/v ACN: aqueous saline solution (50:50 *v*/*v* mixture of $CuSO_4 \cdot 5\ H_2O$ 10% *w*/*v* and $Na_2WO_4 \cdot 2\ H_2O$ 12% *w*/*v* [39]). For each experiment, the urinary exosome sample (50 µL), was added with 5 µL of the IS mixture (20 ng/mL), 5 µL of a methanolic solution of the antioxidant butylhydroxytoluene (BHT, 1.5 mg/mL) [40], and 200 µL of each iced solvent mixture. After vortex-mixing (2000 rpm for 1 min), the sample was placed in a Branson ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT) in ice bath for 10 min (freq.: 40 kHz, high power) for ultrasonication (US). After centrifugation (25 °C, 10000 rpm for 5 min), and filtration at 0.2 µm, the supernatant was diluted with water up to 3 mL and subjected to a clean-up procedure by means of MEPS technique. Each extraction solvent was tested in triplicate. The extraction efficiency was evaluated by spiking the uEVs sample at a mid-concentration level of the analytes both pre- and post-extraction, and it was calculated as the ratio between the peak area of the post- and pre-extraction spiked sample. Upon selection of the optimal extraction mixture, we tested different percentages of organic solvent (i.e., 30, 50, 70%) in triplicate to obtain the best extraction performances.

uEV lysis method

EVs are generally disrupted to facilitate the analyte extraction by a proper solvent [41]. Here, we tested two approaches i.e., ultrasonication (US) and freeze−thaw cycle (FT), which we compared to control (CT, no lysis step). Each procedure was tested in triplicate. For each experiment, 50 µL of the uEVs sample were added with 5 µL of the IS mixture (20 ng/mL), 5 µL of a BHT solution in methanol (1.5 mg/mL), and 200 µL of the iced 50:50 v/v MeOH:H_2O mixture. Then the sample was vortex-mixed (2000 rpm) for 1 min. In case of the US lysis, the sample was placed in a Branson ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury, CT) in ice bath for 10 min (freq.: 40 kHz, high power). For the FT lysis, the sample was dipped into dry ice (-78 °C) for 1 min and then thawed at 37 °C for 1 min. The procedure was repeated three times. The resulting suspensions were centrifuged (25 °C, 10000 rpm for 5 min), filtrated at 0.2 µm, and diluted with water up to 3 mL before the MEPS extraction.

MEPS-UHPLC-MS/MS analysis

The analysis of small uEVs was performed in accordance with our in-house analytical procedure that couples the Micro-Extraction by Packed Sorbent (MEPS) technique to liquid chromatography tandem mass spectrometry for the simultaneous quantification of fifty-two oxylipins and four PUFAs in biofluids [39,42,43].

The MEPS C18 cartridge was activated by drawing and discharging 3 times 100 µL of MeOH (3 x 100 µL), and then conditioned with 3 x 100 µL of H_2O at 0.6 mL/min. The diluted supernatant (3000 µL) was loaded up and down twelve times at 0.3 mL/min by discarding it. The cartridge was then washed with 100 µL of a H_2O:MeOH (95:5 *v/v*) mixture at 0.6 mL/min to remove potential interferences. Analytes were eluted with 30 µL of MeOH at 0.3 mL/min and then injected into the UHPLC-MS/MS instrument.

The instrument consisted of an Agilent 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer, which was equipped with a Jet Stream electrospray (ESI) ionization source (Agilent Technologies, USA). The chromatographic separation was achieved at 0.7 mL/min using a Polaris 3 C18-A column (50 × 4.6 mm, 3 µm, Agilent Technologies, USA) and a gradient elution with a mobile phase consisting of 0.1% aqueous formic acid (A) and 50:50 *v/v* MeOH:ACN (B). The multisampler and the column compartment were set at a 4 and 25 °C, respectively. The injection volume was 20 µL. The Agilent 6495 Triple Quadrupole mass spectrometer detector operated in ESI negative ionization mode and performed multiple reaction monitoring with unit mass resolution. Mass spectrometer control, data acquisition and data analysis were performed with the MassHunter Workstation software (B.07.00). Each analyte was detected using two specific MRM transitions, the most abundant one was used for quantification (Q) of the target compound whereas the other for its identification (q). A deviation ≤ ± 0.10 min of the expected retention time compared to working standard solutions and a qualifier/quantifier (q/Q) ratio within 20% of the ratio measured in working standard mixtures were required for the identification of the analyte in urinary exosomes. Details of the operating UHPLC-MS/MS conditions are shown elsewhere [39].

Method validation

The analytical method was validated according to the IUPAC guidelines [44], and included the evaluation of limits of detection (LODs), calibration curves, matrix effect, recovery, and intra-day and inter-day precision. LODs were calculated as three times the standard deviation (s.d.) of the "low level spiked blank". Five-point external and internal calibration curves were obtained by analysing triplicate working standard solutions and pooled uEV samples spiked with known amounts of standards, respectively. These solutions, as well as spiked uEV samples, were added with 5 µL of the ISs solution (20 ng/mL), then subjected to the MEPS procedure and analysed by UHPLC-ESI-MS/MS. The analyte (a) to internal standard (IS) peak area ratios (Y, A_a/A_{IS}) were plotted versus the corresponding concentration ratios (X, C_a/C_{IS}). Calibration curves (Y=mX) for all the analytes were fitted by the Deming regression, which considers measurement errors for both dependent and independent variable. The matrix effect was evaluated by comparing, at a confidence level of 95%, the slopes of the calibration curves obtained with working solutions (external) and spiked uEV samples (internal). In case of a matrix effect, internal calibration curves were used for analyte quantitation. Recovery was assessed by analysing a pooled uEV sample spiked at three concentration levels (low, mid, and high) in triplicate (n = 3 at each concentration level) within the same day and on three consecutive days. The analyte recovery was calculated as the percentage ratio of the difference between the average analyte concentration measured in the spiked and not spiked samples to the expected concentration. Intra- and inter-day precision was expressed as relative standard deviation (RSD) of measurements performed on the spiked samples in a single day and on three consecutive days, respectively.

Subjects and snapshot of the race

Eighteen experienced ultra-marathon runners (only Caucasian males, age 38 ± 13 yr, weight 75 ± 8 Kg, height 1.77 ± 0.05 m) voluntarily participated to the study. This was performed during the 10^{th} edition of the extreme mountain ultra-marathon (MUM) of the world Tor des Géants® (TDG), which is a 330 km race with an altitude difference of 24,000 m taking place on September 8-15th, 2019.

During basal evaluation (at pre-race), a questionnaire was administered to collect clinical and training experience data on the subjects.

All athletes were in good health, 4 subjects were smokers (22%), 1 suffered from hypertension (5%), 2 (11%) subjects suffered from hypercholesterolemia. On average, the subjects had 8 ± 9 years of ultra-endurance experience. No specific limitations were imposed to the use of antioxidants, minerals/vitamin supplements, medications and/or herbs during the race.

Urine samples were collected pre- and post-race by voluntary voiding in a sterile container provided to the subjects.

All subjects were fully informed of the procedure and the risks involved and, before data collection, signed a written informed consent outlining study requirements. The procedure was conducted according to the Declaration of Helsinki and approval was obtained from the institutional Ethics Committee of the Azienda USL, Valle d'Aosta, Aosta Hospital, Italy (USL VdA N°895-September 2019).

Data analysis

GraphPad Prism (GraphPad Prism 9.0, GraphPad Software Inc., San Diego, CA, USA) was used for the statistical data analyses. Univariate paired parametric t-test and multivariate exploratory methods were performed on the dataset. The original data were pre-processed (data integrity and missing value check) and normalized (i.e., data transformation and data scaling) before the statistical analyses. Continuous variables with a normal distribution were reported as mean ± standard deviation, whereas variables with skewed distribution were described by median with lower (25th percentile) and upper (75th percentile) quartiles.

Results and discussion

Oxylipin and PUFA extraction from small uEVs

Both MeOH and ACN are recognized as optimal extraction solvents for intracellular metabolomics across different cell types [45,46]. In this study, we tested these two organic solvents in combination with H2O (in a 1:1 ratio) to find the most effective extraction mixture for isolating oxylipins and PUFAs encapsulated within small uEVs. Additionally, we tested ACN in combination with an aqueous saline solution (composed of 50:50 *v/v* mixture of $CuSO_4 \cdot 5\ H_2O$ 10% *w/v* and $Na_2WO_4 \cdot 2\ H_2O$ 12% *w/v*)), a combination previously optimized for simultaneous oxylipin extraction and fine protein precipitation from plasma samples [39,47].

The oxylipin profile extracted using the MeOH:H_2O mixture exhibited slightly higher average concentration than that obtained with the ACN:H_2O mixture ($p = 0.027$). Both the mixtures yielded significantly higher chromatographic peaks than the ACN:saline solution mixture ($p<0.0001$). Results are shown in the heatmap in Figure 1, where the compounds are listed in order of chromatographic elution.

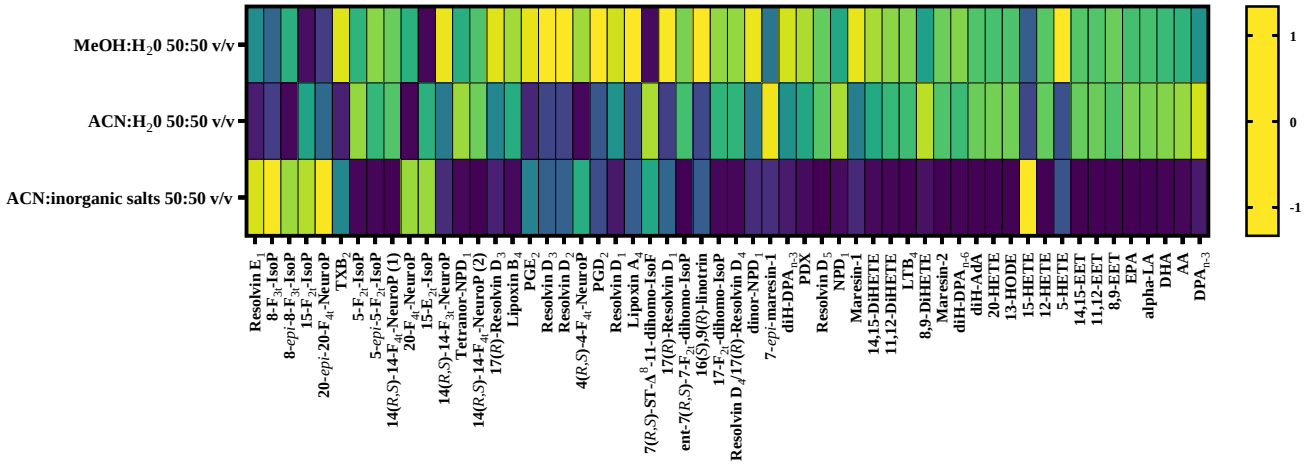

Figure 1. Heatmap of autoscaled peak area of oxylipins and PUFAs measured in small uEVs upon extraction with 50:50 *v/v* MeOH:H_2O, 50:50 *v/v* ACN:H_2O, and 50:50 *v/v* ACN: aqueous saline solution.

Reinicke *et al*. used a saline mixture (MeOH*BHT/H_2O*$ZnSO_4$ (80:20 *v/v*) for lipid extraction and protein precipitation from macrophage-derived vesicles [48]. Contrary to their findings, the organic

solvent:saline solution mixture did not generally yield superior results compared to the use of water combined with pure organic solvents alone. The ACN:saline solution mixture proved more effective only for extracting more polar compounds (logP ≈ 2-3), such as resolvin E_1, 8-F_{3t}-IsoP, 15-F_{2t}-IsoP, and 20-*epi*-20-F_{4t}-NeuroP. In contrast, both MeOH and ACN in combination with H_2O were better suited for extracting more apolar compounds (logP ≈ 5-6), including EETs, HETEs, and PUFAs. MeOH consistently exhibited good extraction efficiency (70-115 %) across all compounds, including SPMs and PGs with intermediate polarity (logP ≈ 3-4), as confirmed by the extraction efficiency percentage values reported in Table S1. Based on these findings, the MeOH:H_2O mixture was chosen for all subsequent experiments. A 50% of MeOH composition provided the best compromise for simultaneous extraction of compounds with a logP ranging from 3 to 6 (Figure S1). Our results align with existing literature where the use of 50:50 *v/v* organic phase:water mixtures is widely described in metabolomics. Luo *et al*. proposed it as the best mixture for in-depth yeast metabolome profiling [49], and it has been suggested as the most efficient reagent for cellular (e.g., HeLa and MCF-7 cells) and exosome metabolomics [41,50]. Therefore, the 50:50 v/v MeOH:H_2O mixture was selected as the extracting solvent for oxylipins and PUFAs from small uEVs. The resulting supernatant underwent MEPS extraction for further clean-up and pre-concentration. Handling the sample on ice, using a fast extraction procedure, and adding BHT as an antioxidant likely aided in preserving PUFAs from *ex-vivo* lipid peroxidation prior to UHPLC-MS/MS analysis.

Small uEVs lysis method

In cellular metabolomics, the use of an effective disruption method to lyse cells and vesicles is crucial for promoting metabolite extraction into the solvent medium. Previous studies have reported various methods for cell disruption, including glass-bead-assisted, freeze-thaw-cycle, and ultrasonication lysis techniques [49,51]. In this study, we investigated freeze-thaw (FT) and ultrasonication (US) methods for vesicles disruption and compared them to a control sample (no disruption).

Thirty-six out of fifty-six compounds were detected in samples from at least one of the three procedures and, thus, considered for data analysis. The autoscaled peak areas are shown in the heatmap in Figure 2.

The levels of oxylipins and PUFAs did not significantly increase upon vesicle disruption by both FT and US compared to the control sample. However, these two lysis methods allowed the detection of early eluting peaks including compounds as isoprostanoids and prostanoids that were below the limits of detection in the control sample. Specifically, the FT method facilitated the detection of chromatographic signals for 15-E_{2t}-IsoP and 14(*R,S*)-14-F_{3t}-NeuroP, while the US method allowed the detection of 14(*R,S*)-14-F_{4t}-NeuroP. These compounds, mainly iso- and neuroprostanes, are crucial indexes of oxidative stress and expressed at very low concentrations (ranging from tens of ppt to units of ppb) in their free form.

Both FT and US significantly decreased ($p < 0.05$) the levels of more apolar compounds, such as HETEs, and PUFAs, which generally exhibit higher peak signals and concentration levels (ppb level). Compared to FT, the US method increased the number of overexpressed compounds (e.g., 2.5-fold for resolvin D_2) and reduced the number of chemicals with no variation ($p > 0.05$) upon the disruption treatment. As suggested by Wu *et al*., ultrasonication resulted the best option to lyse cells and vesicles

[51]. In our case, it represented the best compromise for the simultaneous detection of the fifty-six analytes while only losing 20-30% of the peak signal for compounds with the highest concentration levels, as HETEs (e.g., 12-HETE, 15-HETE, and 20-HETE) and PUFAs (e.g., EPA, DHA, AA). For this reason, it was chosen as the lysis method in the following experiments.

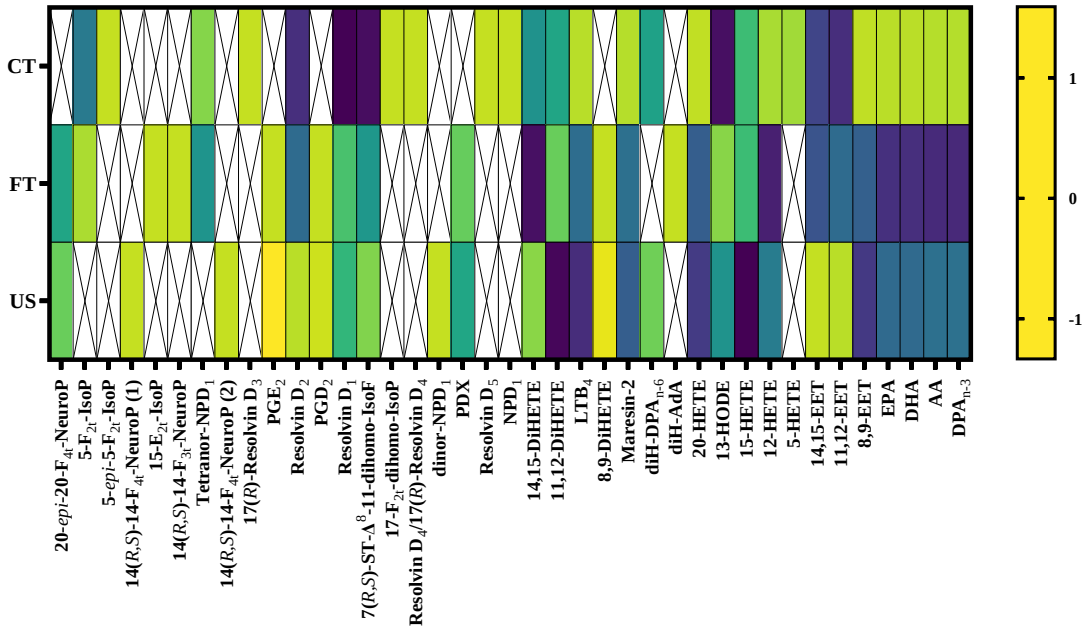

Figure 2. Heatmap of autoscaled peak area of oxylipins and PUFAs measured in small uEVs upon lysis by ultrasonication (US) and freeze−thaw cycle (FT) *versus* control (CT, no lysis step). The cross in the box refers to a concentration value below LOD.

Analytical figures of merit

LOD values ranged between 10-400 pg/mL for oxylipins and 0.10-3 ng/mL for PUFAs. All analytes showed satisfactory linearity, with R^2 values ranging from 0.957-1 within the tested concentration range. Recoveries were not significantly different ($p > 0.05$) across the tested concentration levels and were generally satisfactory for most analytes during both intra- (overall range: 84-116%) and inter-day (overall range: 85-116%) experiments. Intra- and inter-day precisions consistently remained at or below 15%. The slopes of the calibration curves obtained from working solutions (external curve) and spiked samples (internal curve) were not significantly different at a 95% confidence level for thirty-four out of fifty-six compounds (see Table 1), suggesting the absence of any measurable matrix effect. However, a slight significant difference ($p<0.05$) was observed for twenty-two out of fifty-six analytes (see Table 1). In such cases, internal calibration curves were used for analyte quantitation.

Compound	Calibration levels	Slope ± s.d (R^2)	LOD	Intra-assay	Inter-assay

Table 1 reports the mean slopes and standard deviations (n= 3) of the five-point calibration curves, LOD values, and overall intra- and inter-day recovery, along with the corresponding RSD.

Table 1. Full list of quantified oxylipins and PUFAs and relevant analytical parameters i.e., calibration curve, limit of detection, intra-assay and inter-assay recovery, intra-assay and inter-assay precision.

Real case scenario: Tor des Géants® mountain ultramarathon runners

	(ng/mL)		(pg/mL, *ng/mL)	recovery % (RSD)[a]	recovery % (RSD)[b]
PUFAs					
AA	1; 5; 10; 25; 50	19 ± 1 (0.999)	1.7*	91(5)	101(9)
DHA		59 ± 5 (0.999)	2.7*	90(12)	100(7)
DPA_{n-3}		10.7 ± 0.4 (0.991)	0.10*	102(12)	100(14)
EPA		29.1 ± 0.3 (0.999)	1.3*	93(7)	100(11)
Isoprostanoids (IsoPs, NeuroPs, dihomo-isoPs), isofurans (IsoFs) and prostanoids (prostaglandins (PGs), leukotrienes (LEU), thromboxanes (TX))					
5-F_{2t}-IsoP	0.02; 0.10; 0.25; 0.50; 1	0.7 ± 0.1 (0.999)	30	96(9)	114(7)
5-*epi*-5-F_{2t}-IsoP		0.62 ± 0.05 (0.999)	10	116(3)	96(10)
15-F_{2t}-IsoP		2.0 ± 0.2 (0.995)	10	105(6)	103(11)
8-F_{3t}-IsoP		0.79 ± 0.07 (0.997)	20	98(4)	113(14)
8-*epi*-8-F_{3t}-IsoP		0.7 ± 0.1 (0.999)	20	99(6)	105(12)
18-F_{3t}-IsoP		0.14 ± 0.01 (0.990)	30	104(8)	101(10)
15-E_{2t}-IsoP		3.82 ± 0.02 (0.986)	15	97(7)	86(15)
4(*R*,*S*)-4-F_{4t}-NeuroP		0.69 ± 0.06 (0.998)◊	30	99(4)	100(11)
20-F_{4t}-NeuroP		0.07 ± 0.01 (0.971)◊	30	84(13)	116(13)
20-*epi*-20-F_{4t}-NeuroP		0.075 ± 0.003 (0.998)	40	97(15)	112(13)
14(*R*,*S*)-14-F_{4t}-NeuroP		0.07 ± 0.01 (0.992)◊	25	98(11)	100(8)
14(*R*,*S*)-14-F_{3t}-NeuroP		0.6 ± 0.05 (0.997)◊	25	92(14)	98(10)
ent-7(*R*,*S*)-7-F_{2t}-dihomo-isoP		0.84 ± 0.07 (0.997)	100	104(7)	110(10)
17-F_{2t}-dihomo-isoP		0.71 ± 0.02 (0.995)	120	105(8)	103(5)
7(*R*,*S*)-ST-Δ^8-11-dihomo-isoF		0.29 ± 0.03 (0.995)	60	101(2)	107(5)
PGE_2	0.1; 0.5; 1; 2.5; 5	5.2 ± 0.5 (1)	50	106(9)	101(5)
PGD_2		0.79 ± 0.03 (0.996)	30	99(4)	102(6)
LTB_4	0.5; 5; 10; 25; 50	4.4 ± 0.5 (0.995)◊	30	95(6)	103(12)
TXB_2		0.51 ± 0.07 (1)◊	10	106(9)	106(8)
Hydroxy/dihydroxy-PUFAs					
5-HETE	0.5; 5; 10; 25; 50	27 ± 1 (0.986)	120	88(3)	115(10)
12-HETE		22 ± 1 (0.992)◊	90	96(5)	104(6)
15-HETE		63 ± 8 (0.977)◊	260	116(7)	107(8)
20-HETE		5.4 ± 0.1 (0.991)◊	60	97(12)	115(11)
13-HODE	2.5; 25; 50; 125; 250	31 ± 4 (0.992)	1.1*	91(9)	100(14)
8,9-DiHETE	0.1; 0.5; 5; 10; 25	0.271 ± 0.005 (0.983)	140	98(5)	113(9)
11,12-DiHETE		24.6 ± 2.2 (0.999)	80	95(4)	100(8)
14,15-DiHETE		18.6 ± 0.5 (0.997)	30	95(12)	100(14)
Epoxy-PUFAs					
8,9-EET	0.5; 5; 10; 25; 50	16.7 ± 0.8 (0.993)◊	100	107(14)	97(17)
11,12-EET		3.2 ± 0.4 (0.957)◊	400	90(11)	110(5)
14,15-EET		14 ± 1 (0.986)◊	250	91(10)	91(4)
Pro-resolving (lipoxins, resolvins, maresins, protectins)					
Lipoxin A_4	0.05; 0.5; 2.5; 5; 20	1.61 ± 0.06 (0.969)◊	10	100(12)	87(16)
Lipoxin B_4		0.62 ± 0.07 (0.991)◊	30	100(8)	102(14)
Resolvin D_1		0.83 ± 0.09 (0.997)◊	10	100(7)	110(12)
Resolvin D_2		0.18 ± 0.01 (0.999)◊	30	99(2)	100(8)
Resolvin D_3		0.36 ± 0.02 (1)	25	100(11)	89(17)
Resolvin D_4		0.96 ± 0.07 (0.997)	60	100(1)	100(7)
Resolvin D_5		4.1 ± 0.4 (0.996)	50	109(4)	99(1)
17(*R*)-Resolvin D_1		1.30 ± 0.08 (0.997)	40	100(3)	114(17)
17(*R*)-Resolvin D_3		1.5 ± 0.1 (0.982)	20	97(1)	90(16)
17(*R*)-Resolvin D_4		0.96 ± 0.07 (0.997)	60	100(1)	100(7)
Resolvin E_1		1.0 ± 0.1 (0.988)	10	97(13)	101(9)
Maresin-1		0.72 ± 0.04 (1)◊	60	99(10)	100(13)
7-*epi*-maresin-1		0.9 ± 0.1 (0.998)	20	100(10)	102(12)
Maresin-2		3.7 ± 0.2 (0.996)	20	99(8)	100(4)
Neuroprotectin D_1		3.2 ± 0.1 (0.982)◊	10	99(9)	101(7)
dinor-Neuroprotectin D_1		5.6 ± 0.4 (0.994)◊	130	100(6)	103(10)
Tetranor-Neuroprotectin D_1		0.18 ± 0.01 (0.984)◊	150	111(5)	103(7)
Protectin DX		4.9 ± 0.3 (0.987)◊	50	98(6)	85(11)
diH-AdA		3.9 ± 0.2 (1)	80	100(10)	101(12)
diH-DPA_{n-3}		2.6 ± 0.3 (0.995)	20	99(7)	101(9)
diH-DPA_{n-6}		10.8 ± 0.8 (0.998)◊	40	100(3)	102(7)
16(*S*),9(*R*)-linotrin		3.55 ± 0.09 (0.994)	10	98(3)	100(9)

Oxylipins and their precursors were quantified in small urinary uEVs from experienced ultramarathon runners participating in the Tor des Géants® ultramarathon. Pre- and post-race samples were collected from eighteen athletes. Eight oxylipins (i.e., 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 15-E_{2t}-IsoP, 13-HODE, 16(*S*),9(*R*)-linotrin, lipoxin B4, resolvin D_1 and

resolvin D_2) and four PUFAs (i.e., AA, EPA, DPA_{n-3}, DHA) were detected in more than 50% of the samples and thus considered for statistical analysis. The number of lipid mediators detected is consistent with that reported by Reinicke *et al*. studying the expression of bioactive lipids in macrophage-derived extracellular vesicles [48]. Seven out of twelve compounds were detected in both the pre- and post-race samples from all participants, while four out of twelve were detected in more than half of the population. The median concentration levels approached the LODs for 15-E_{2t}-IsoP, resolvin D_1 and resolvin D_2, they resulted around LLOQs (SLLOQ= 5 x s.d. of the spiked blank) for 5-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, 16(*S*),9(*R*)-linotrin, lipoxin B4 and DPA_{n-3}, and higher than LOQs values for 13-HODE and the other PUFAs (AA, EPA, DHA). The median (lower and upper quartiles) concentration levels of the compounds detected in pre- and post-race samples are reported in Table S2.

PUFAs are essential components of cell membranes with an influence on membrane flexibility and various cellular processes. They have a key role in modulating signalling pathways, especially in the context of inflammation. During inflammation, immune cells release PUFAs in free form, bound to proteins, or within extracellular vesicles [52]. The omega-3 DHA has been identified as a main component in extracellular vesicles derived from EBV-positive lymphoma cells, contributing to settle an immunosuppressive phenotype in tumour microenvironments [53]. Here, we proved that small uEVs not only transport omega-3 and omega-6 PUFAs but also their oxidation products, such as AA-derived IsoPs (F_2/E_2-IsoPs) and SPMs, including lipoxin B4 and some D-series resolvins.

Isoprostanes are prostaglandin-like compounds generated by the non-enzymatic oxidation of arachidonic acid. Their biosynthesis is primarily driven by reactive oxygen species, which makes them valuable markers for oxidative stress and lipid peroxidation. F_2-IsoPs exhibit potent biological activities and may act as mediators in vasoconstriction, platelet aggregation, angiogenesis inhibition, and atherosclerosis by activating prostanoid receptors [54]. E-ring IsoPs, such as 15-E_{2t}-IsoP, are even more potent than 15-F_{2t}-IsoP in activating of TP and EP3 receptors. 15-E_{2t}-IsoP has been reported to stimulate monocytes binding to endothelial cells and exert contractile activity on gastrointestinal smooth muscle [55,56].

PUFAs can also undergo enzymatic oxidation mediated from COX, LOX, and CYP450 enzymes. At the very beginning of the inflammatory insult, COX-2 and 5-LOX generate pro-inflammatory mediators such as PGs, TXs, and LTs [57]. PGE_2 triggers a lipid mediator switch that leads to the production of anti-inflammatory and pro-resolving mediators, such as CYP450-derived epoxy-PUFAs and 12/15-LOX-derived SPMs, which facilitate the inflammation resolution and restore homeostasis. Biosynthetic enzymes such as COX, LTC4 synthase, and LTA4 hydrolase have been identified in exosomes [58]. Interestingly unlike macrophage-, neutrophil- and plasma-derived exosomes reported in literature [59,60], we did not detect any pro-inflammatory COX- and LOX-derived mediators in the small uEVs we collected. Instead, the small uEVs of these athletes contained various resolution mediators, including lipoxin B_4, resolvin D_1, and resolvin D_2, which is consistent with results from Calderin *et al*. who compared 4 weeks-trained and sedentary mice [61]. Exercise training has been shown to enhance SPM production and stimulate the catabolism of pro-inflammatory mediators (e.g., leukotrienes and prostaglandins), highlighting the multisystemic benefits of long-term endurance exercise [30]. Recent studies have recognized physical activity as an agonist of inflammation resolution, with maximal physical exertion leading to a rapid increase in the urinary excretion of lipoxin A_4 post-exercise [62] as well as specific temporal response of serum lipoxins, resolvins, and

protectins to resistance exercise [63]. Our results proved the presence of SPMs in small uEVs of ultramarathon runners, potentially transported and released throughout the body to regulate the inflammation resolution and the trained immunity.

Although uEVs are mainly derived from various cell types of the urogenital tract, resident immune cells, and microorganisms, non-vesicular molecules can be encapsulated into EVs and released into the urinary tract after endocytosis by renal tubular cells. Additionally, a subset of circulating EVs may enter the urine thanks to perturbations of the membrane pore integrity, transcytosis by podocytes, or endothelial fenestrae of glomerular filtration [1]. The potential contribution of stressed skeletal muscle to SPM biosynthesis, either directly or via cell-cell interactions, remains unexplored. Skeletal muscle and other tissues are recognised to have an endocrine function and to release exerkines (e.g., peptides, nucleic acids, and metabolites) into the circulation in response to acute endurance exercise for multisystemic adaptations [64]. Exerkines, are often found in EVs modulating cell-cell interactions and organ crosstalk. Oxylipins and their precursors may be reasonably classified as exerkines mediating the response between the muscular system and other tissues.

None of the investigated oxylipins and PUFAs showed significantly different concentration levels between pre- and post-race, except for 5-F_{2t}-IsoP, which increased after the race [median (lower and upper quartiles) PRE: 0.018 (0.014-0.023) ng/mL *vs.* POST: 0.034 (0.025-0.043) ng/mL, p=0.0374] (Figure 3). The 5-series F_2-IsoPs are the most abundant positional isomers of the well-known 15-series IsoPs produced *in-vivo*. The increase of 5-F_{2t}-IsoP demonstrates the occurrence of a cellular oxidant injury. Recently, considerable attention has been focused on this molecule to assess oxidative stress due to its higher concentrations in biological fluids compared to 15-F_{2t}-IsoP [65]. Unlike 15-F_{2t}-IsoP, no vasoactive role has not been assigned to this isomer [66,67].

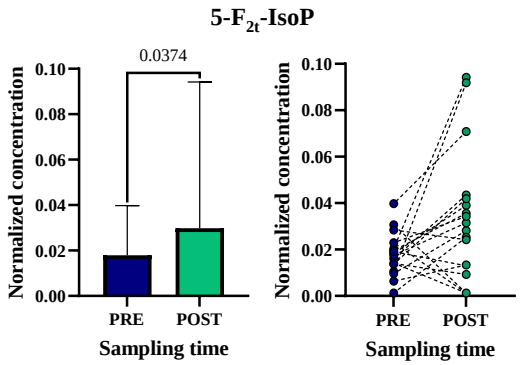

Figure 3. Normalized (log-transformation and autoscaling) concentration levels of 5-F_{2t}-IsoP measured pre- and post-TDG race in ultramarathon runners (n=18). The univariate parametric t-test was performed upon data normalization.

Several studies on strenuous exercise and ultramarathon runs have consistently demonstrated a systemic increase in lipid peroxidation and isoprostanes [68–73], which depend on the type, intensity, and duration of exercise [70]. The TDG is considered one of the most challenging mountain ultramarathons globally, covering approximately 330 km with an altitude ranging from 322 to 3,300 m. Lipid peroxidation, resulting from an imbalance between ROS and antioxidants, has been extensively documented in athletes performing the TDG race. Mrakic-Sposta *et al*. observed a decrease in antioxidant activity after race, coupled with a pronounced generation of reactive oxygen species (ROS)

and biomarkers of oxidative stress and inflammation [74]. F_2-IsoPs considerably increased in response to the physical exertion demanded by this mountain ultramarathon race [75]. The transport of lipid oxidation products has been observed to accelerate with the increase of oxidized HDL following strenuous exercise [76]. During prolonged endurance activities, the body increasingly relies on fat metabolism for energy, making circulating fatty acids more susceptible to peroxidation by ROS [74]. The oxygen demand of muscle and ROS production increase with the exercise intensity [77]. Principal sources of ROS include mitochondrial electron transport, catecholamine metabolism, xanthine oxidase reaction, haemoglobin auto-oxidation, and neutrophil activation [78]. Without adequate compensation from the antioxidant system, activation of these pathways may lead to harmful oxidant insult and muscle damage. Mastadoulis *et al*. reported a 2-fold increase in F_2-IsoPs after a 50 km ultramarathon, along with the immediate disappearance of labelled α-tocopherol ingested by athletes the previous night [79]. In this context, antioxidant supplementation (1000 mg vitamin C and 300 mg RRR-α-tocopheryl acetate) was found to reduce oxidative damage without affecting inflammation regulation, which is crucial to preserve the metabolic adaptation of the body to exercise [80].

Limitations

Limitations are mainly related to the absence of harmonized analytical protocols to isolate uEVs and the exosomal fraction, as well as for extracting lipid oxidation products from these vesicles. Alongside the few studies proposed so far in literature, our work represents a starting point to address this goal for a straightforward detection and quantification of exosomal oxylipins and PUFAs. In addition, the low concentration levels of oxylipins point out the need of more sensitive methods that may extend the range of detectable metabolites.

Conclusions

This work suggests that small uEVs are not only lipid carriers, but also transport oxidation products regulating oxidative stress and inflammation. The detection of both F_2- and E_2-IsoPs in small uEVs as well as the significant variation observed between 5-F_{2t}-IsoP pre- and post-ultramarathon race levels mark a significant advancement. This latter finding suggests the potential use of urinary 5-F_{2t}-IsoP as a non-invasive marker of *in-vivo* lipid peroxidation. Moreover, the presence of specialized pro-resolving mediators indicates the onset of inflammation following intense physical activity and the subsequent activation of anti-inflammatory and pro-resolution signalling cascades. These outcomes hold significant implications in the field of exercise physiology and show an innovative approach to manage exercise recovery and musculoskeletal damage.

Author contribution

Conceptualization: [Danilo Bondi, Simona Mrakic-Sposta]; Methodology: [Denise Biagini, Silvia Ghimenti, Alessio Lenzi]; Formal analysis and investigation: [Tommaso Lomonaco, Danilo Bondi, Simona Mrakic-Sposta, Carmen Santangelo, Guido Giardini, Gennaro D'Angelo]; Writing - original draft preparation: [Denise Biagini]; Writing - review and editing: [Tommaso Lomonaco, Fabio Di Francesco, Federico Vivaldi, Danilo Bondi, Simona Mrakic-Sposta, Carmen Santangelo, Vittore Verratti, Alessandra Vezzoli]; Funding acquisition: [Fabio Di Francesco]; Resources: [Thierry Durand,

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References

[1] U. Erdbrügger, C.J. Blijdorp, I. V. Bijnsdorp, F.E. Borràs, D. Burger, B. Bussolati, J.B. Byrd, A. Clayton, J.W. Dear, J.M. Falcón-Pérez, C. Grange, A.F. Hill, H. Holthöfer, E.J. Hoorn, G. Jenster, C.R. Jimenez, K. Junker, J. Klein, M.A. Knepper, E.H. Koritzinsky, J.M. Luther, M. Lenassi, J. Leivo, I. Mertens, L. Musante, E. Oeyen, M. Puhka, M.E. van Royen, C. Sánchez, C. Soekmadji, V. Thongboonkerd, V. van Steijn, G. Verhaegh, J.P. Webber, K. Witwer, P.S.T. Yuen, L. Zheng, A. Llorente, E.S. Martens-Uzunova, Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles, J Extracell Vesicles 10 (2021). https://doi.org/10.1002/jev2.12093.

[2] C. Grange, A. Dalmasso, J. John Cortez, B. Spokeviciute, B. Bussolati, EXPLORING THE ROLE OF URINARY EXTRACELLULAR VESICLES IN KIDNEY PHYSIOLOGY, AGEING, AND DISEASE PROGRESSION 2 3, n.d.

[3] D. Armstrong, D.E. Wildman, Extracellular vesicles and the promise of continuous liquid biopsies, J Pathol Transl Med 52 (2018) 1–8. https://doi.org/10.4132/jptm.2017.05.21.

[4] Y. Zhang, Y. Liu, H. Liu, W.H. Tang, Exosomes: Biogenesis, biologic function and clinical potential, Cell Biosci 9 (2019). https://doi.org/10.1186/s13578-019-0282-2.

[5] X. Wang, L. Tian, J. Lu, I.O.L. Ng, Exosomes and cancer - Diagnostic and prognostic biomarkers and therapeutic vehicle, Oncogenesis 11 (2022). https://doi.org/10.1038/s41389-022-00431-5.

[6] D. Gonçalves, S.N. Pinto, F. Fernandes, Extracellular Vesicles and Infection: From Hijacked Machinery to Therapeutic Tools, Pharmaceutics 15 (2023). https://doi.org/10.3390/pharmaceutics15061738.

[7] X. Wang, J. Xia, L. Yang, J. Dai, L. He, Recent progress in exosome research: isolation, characterization and clinical applications, Cancer Gene Ther 30 (2023) 1051–1065. https://doi.org/10.1038/s41417-023-00617-y.

[8] M.N. Huda, M. Nafiujjaman, I.G. Deaguero, J. Okonkwo, M.L. Hill, T. Kim, M. Nurunnabi, Potential Use of Exosomes as Diagnostic Biomarkers and in Targeted Drug Delivery: Progress in Clinical and Preclinical Applications, ACS Biomater Sci Eng 7 (2021) 2106–2149. https://doi.org/10.1021/acsbiomaterials.1c00217.

[9] J. Chen, P. Li, T. Zhang, Z. Xu, X. Huang, R. Wang, L. Du, Review on Strategies and Technologies for Exosome Isolation and Purification, Front Bioeng Biotechnol 9 (2022). https://doi.org/10.3389/fbioe.2021.811971.

[10] G.K. Patel, M.A. Khan, H. Zubair, S.K. Srivastava, M. Khushman, S. Singh, A.P. Singh, Comparative analysis of exosome isolation methods using culture supernatant for optimum yield, purity and downstream applications, Sci Rep 9 (2019). https://doi.org/10.1038/s41598-019-41800-2.

[11] T. Altadill, I. Campoy, L. Lanau, K. Gill, M. Rigau, A. Gil-Moreno, J. Reventos, S. Byers, E. Colas, A.K. Cheema, Enabling metabolomics based biomarker discovery studies using molecular phenotyping of exosome-like vesicles, PLoS One 11 (2016). https://doi.org/10.1371/journal.pone.0151339.

[12] T. Skotland, K. Sandvig, A. Llorente, Lipids in exosomes: Current knowledge and the way forward, Prog Lipid Res 66 (2017) 30–41. https://doi.org/10.1016/j.plipres.2017.03.001.

[13] T. Skotland, N.P. Hessvik, K. Sandvig, A. Llorente, Exosomal lipid composition and the role of ether lipids and phosphoinositides in exosome biology, J Lipid Res 60 (2019) 9–18. https://doi.org/10.1194/jlr.R084343.

[14] A. Llorente, T. Skotland, T. Sylvänne, D. Kauhanen, T. Róg, A. Orłowski, I. Vattulainen, K. Ekroos, K. Sandvig, Molecular lipidomics of exosomes released by PC-3 prostate cancer cells,

Biochim Biophys Acta Mol Cell Biol Lipids 1831 (2013) 1302–1309. https://doi.org/10.1016/j.bbalip.2013.04.011.
[15] M. Agudiez, P.J. Martinez, M. Martin-Lorenzo, A. Heredero, A. Santiago-Hernandez, D. Molero, J.M. Garcia-Segura, G. Aldamiz-Echevarria, G. Alvarez-Llamas, Analysis of urinary exosomal metabolites identifies cardiovascular risk signatures with added value to urine analysis, BMC Biol 18 (2020). https://doi.org/10.1186/s12915-020-00924-y.
[16] Y. Du, L. Chen, X.S. Li, X.L. Li, X.D. Xu, S. Bin Tai, G.L. Yang, Q. Tang, H. Liu, S.H. Liu, S.Y. Zhang, Y. Cheng, Metabolomic Identification of Exosome-Derived Biomarkers for Schizophrenia: A Large Multicenter Study, Schizophr Bull 47 (2021) 615–623. https://doi.org/10.1093/schbul/sbaa166.
[17] W. Wang, N. Zhu, T. Yan, Y.N. Shi, J. Chen, C.J. Zhang, X.J. Xie, D.F. Liao, L. Qin, The crosstalk: Exosomes and lipid metabolism, Cell Communication and Signaling 18 (2020). https://doi.org/10.1186/s12964-020-00581-2.
[18] A. Conigliaro, S. Fontana, S. Raimondo, R. Alessandro, Exosomes: Nanocarriers of biological messages, in: Adv Exp Med Biol, Springer New York LLC, 2017: pp. 23–43. https://doi.org/10.1007/978-981-10-4397-0_2.
[19] K. Sagini, E. Costanzi, C. Emiliani, S. Buratta, L. Urbanelli, Extracellular vesicles as conveyors of membrane-derived bioactive lipids in immune system, Int J Mol Sci 19 (2018). https://doi.org/10.3390/ijms19041227.
[20] G.M. Kalinec, L. Gao, W. Cohn, J.P. Whitelegge, K.F. Faull, F. Kalinec, Extracellular Vesicles From Auditory Cells as Nanocarriers for Anti-inflammatory Drugs and Pro-resolving Mediators, Front Cell Neurosci 13 (2019). https://doi.org/10.3389/fncel.2019.00530.
[21] S.C. Dyall, L. Balas, N.G. Bazan, J.T. Brenna, N. Chiang, F. da Costa Souza, J. Dalli, T. Durand, J.-M. Galano, P.J. Lein, Polyunsaturated fatty acids and fatty acid-derived lipid mediators: Recent advances in the understanding of their biosynthesis, structures, and functions, Prog Lipid Res (2022) 101165.
[22] W.W. Christi, J.L. Harwoo, Oxidation of polyunsaturated fatty acids to produce lipid mediators, Essays Biochem 64 (2020) 401–421. https://doi.org/10.1042/EBC20190082.
[23] C. Subra, K. Laulagnier, B. Perret, M. Record, Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies, Biochimie 89 (2007) 205–212. https://doi.org/10.1016/j.biochi.2006.10.014.
[24] X. Xiang, A. Poliakov, C. Liu, Y. Liu, Z. Bin Deng, J. Wang, Z. Cheng, S. V. Shah, G.J. Wang, L. Zhang, W.E. Grizzle, J. Mobley, H.G. Zhang, Induction of myeloid-derived suppressor cells by tumor exosomes, Int J Cancer 124 (2009) 2621–2633. https://doi.org/10.1002/ijc.24249.
[25] F. Vallejo, J. Enrique Yuste, R. Teruel-Montoya, G. Luengo-Gil, N. Bohdan, S. Espín, N. García-Barberá, C. Martínez, V. Vicente, J.C. Espín, I. Martínez-Martínez, First exploratory study on the metabolome from plasma exosomes in patients with paroxysmal nocturnal hemoglobinuria, n.d.
[26] J. Esser, U. Gehrmann, F.L. D'Alexandri, A.M. Hidalgo-Estévez, C.E. Wheelock, A. Scheynius, S. Gabrielsson, O. Rdmark, Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration, Journal of Allergy and Clinical Immunology 126 (2010). https://doi.org/10.1016/j.jaci.2010.06.039.
[27] E. Boilard, Extracellular vesicles and their content in bioactive lipid mediators: More than a sack of microRNA, J Lipid Res 59 (2018) 2037–2046. https://doi.org/10.1194/jlr.R084640.
[28] Y. Li, C. Han, J. Wang, J. Zhou, C. Liang, K. Ranganna, Y. hua Song, Exosomes mediate the beneficial effects of exercise, in: Adv Exp Med Biol, Springer New York LLC, 2017: pp. 333–353. https://doi.org/10.1007/978-981-10-4304-8_18.

[29] S. Mrakic-Sposta, D. Biagini, D. Bondi, T. Pietrangelo, A. Vezzoli, T. Lomonaco, F. Di Francesco, V. Verratti, OxInflammation at High Altitudes: A Proof of Concept from the Himalayas, Antioxidants 11 (2022). https://doi.org/10.3390/antiox11020368.
[30] A. Safdar, M.A. Tarnopolsky, Exosomes as mediators of the systemic adaptations to endurance exercise, Cold Spring Harb Perspect Med 8 (2018). https://doi.org/10.1101/cshperspect.a029827.
[31] C. Oger, Y. Brinkmann, S. Bouazzaoui, T. Durand, J.-M. Galano, Stereocontrolled access to isoprostanes via a bicyclo [3.3. 0] octene framework, Org Lett 10 (2008) 5087–5090.
[32] A. Guy, C. Oger, J. Heppekausen, C. Signorini, C. Defelice, A. Fürstner, T. Durand, J.M. Galano, Oxygenated Metabolites of n-3 polyunsaturated fatty acids as potential oxidative stress biomarkers: Total synthesis of 8-F3t-IsoP, 10-F 4t-NeuroP and [D4]-10-F4t-NeuroP, Chemistry - A European Journal 20 (2014) 6374–6380. https://doi.org/10.1002/chem.201400380.
[33] C. Oger, V. Bultel-Poncé, A. Guy, T. Durand, J.M. Galano, Total synthesis of isoprostanes derived from adrenic acid and EPA, European J Org Chem (2012) 2621–2634. https://doi.org/10.1002/ejoc.201200070.
[34] L. Balas, S.K. Dey, S. Béraud-Dufour, D.E. Riechers, O.A. Landau, J. Bertrand-Michel, T. Durand, N. Blondeau, Linotrins: Omega-3 oxylipins featuring an E,Z,E conjugated triene motif are present in the plant kingdom and alleviate inflammation in LPS-challenged microglial cells, Eur J Med Chem 231 (2022). https://doi.org/10.1016/j.ejmech.2022.114157.
[35] P. Fischer, V. Bultel-Poncé, A. Guy, I. Gonzales, P.A. Conde, J.M. Galano, T. Durand, C. Oger, Straightforward Syntheses of Phytoprostanes and Dihomo-phytoprostanes-Non-enzymatic Metabolites of γ-Linolenic, Dihomo-γ-linolenic and Stearidonic Acids, European J Org Chem 2022 (2022). https://doi.org/10.1002/ejoc.202200085.
[36] T. Pietrangelo, C. Santangelo, D. Bondi, P. Cocci, R. Piccinelli, F. Piacenza, E. Rosato, S.N.A. Azman, E. Binetti, M. Farina, M. Locatelli, V. Brunetti, C. Le Donne, L. Marramiero, E.S. Di Filippo, V. Verratti, S. Fulle, V. Scollo, F. Palermo, Endurance-dependent urinary extracellular vesicle signature: shape, metabolic miRNAs, and purine content distinguish triathletes from inactive people, Pflugers Arch 475 (2023) 691–709. https://doi.org/10.1007/s00424-023-02815-x.
[37] I.M. Sall, T.A. Flaviu, Plant and mammalian-derived extracellular vesicles: a new therapeutic approach for the future, Front Bioeng Biotechnol 11 (2023). https://doi.org/10.3389/fbioe.2023.1215650.
[38] D. Sun, Z. Zhao, S. Spiegel, Y. Liu, J. Fan, P. Amrollahi, J. Hu, C.J. Lyon, M. Wan, T.Y. Hu, Dye-free spectrophotometric measurement of nucleic acid-to-protein ratio for cell-selective extracellular vesicle discrimination, Biosens Bioelectron 179 (2021). https://doi.org/10.1016/j.bios.2021.113058.
[39] D. Biagini, M. Franzini, P. Oliveri, T. Lomonaco, S. Ghimenti, A. Bonini, F. Vivaldi, L. Macera, L. Balas, T. Durand, MS-based targeted profiling of oxylipins in COVID-19: A new insight into inflammation regulation, Free Radic Biol Med 180 (2022) 236–243.
[40] W.A. Yehye, N.A. Rahman, A. Ariffin, S.B. Abd Hamid, A.A. Alhadi, F.A. Kadir, M. Yaeghoobi, Understanding the chemistry behind the antioxidant activities of butylated hydroxytoluene (BHT): A review, Eur J Med Chem 101 (2015) 295–312. https://doi.org/10.1016/j.ejmech.2015.06.026.
[41] X. Luo, M. An, K.C. Cuneo, D.M. Lubman, L. Li, High-Performance Chemical Isotope Labeling Liquid Chromatography Mass Spectrometry for Exosome Metabolomics, Anal Chem 90 (2018) 8314–8319. https://doi.org/10.1021/acs.analchem.8b01726.
[42] D. Biagini, S. Antoni, T. Lomonaco, S. Ghimenti, P. Salvo, F.G. Bellagambi, R.T. Scaramuzzo, M. Ciantelli, A. Cuttano, R. Fuoco, Micro-extraction by packed sorbent combined with UHPLC-

ESI-MS/MS for the determination of prostanoids and isoprostanoids in dried blood spots, Talanta 206 (2020) 120236.
[43] D. Biagini, S. Ghimenti, A. Lenzi, A. Bonini, F. Vivaldi, C. Oger, J.-M. Galano, L. Balas, T. Durand, P. Salvo, Salivary lipid mediators: Key indexes of inflammation regulation in heart failure disease, Free Radic Biol Med (2023).
[44] M. Thompson, S.L.R. Ellison, R. Wood, Resulting from the Symposium on Harmonization of Quality Assurance Systems for Analytical Laboratories, 2002.
[45] Z. Ser, X. Liu, N.N. Tang, J.W. Locasale, Extraction parameters for metabolomics from cultured cells, Anal Biochem 475 (2015) 22–28. https://doi.org/10.1016/j.ab.2015.01.003.
[46] S. Dietmair, N.E. Timmins, P.P. Gray, L.K. Nielsen, J.O. Krömer, Towards quantitative metabolomics of mammalian cells: Development of a metabolite extraction protocol, Anal Biochem 404 (2010) 155–164. https://doi.org/10.1016/j.ab.2010.04.031.
[47] D. Biagini, P. Oliveri, A. Baj, D.D. Gasperina, F.D. Ferrante, T. Lomonaco, S. Ghimenti, A. Lenzi, A. Bonini, F. Vivaldi, C. Oger, J.M. Galano, L. Balas, T. Durand, F. Maggi, F. Di Francesco, The effect of SARS-CoV-2 variants on the plasma oxylipins and PUFAs of COVID-19 patients, Prostaglandins Other Lipid Mediat 169 (2023). https://doi.org/10.1016/j.prostaglandins.2023.106770.
[48] M. Reinicke, S. Shamkeeva, M. Hell, B. Isermann, U. Ceglarek, M.L. Heinemann, Targeted Lipidomics for Characterization of PUFAs and Eicosanoids in Extracellular Vesicles, Nutrients 14 (2022). https://doi.org/10.3390/nu14071319.
[49] X. Luo, S. Zhao, T. Huan, D. Sun, R.M.N. Friis, M.C. Schultz, L. Li, High-performance chemical isotope labeling liquid chromatography-mass spectrometry for profiling the metabolomic reprogramming elicited by ammonium limitation in yeast, J Proteome Res 15 (2016) 1602–1612. https://doi.org/10.1021/acs.jproteome.6b00070.
[50] X. Luo, X. Gu, L. Li, Development of a simple and efficient method of harvesting and lysing adherent mammalian cells for chemical isotope labeling LC-MS-based cellular metabolomics, Anal Chim Acta 1037 (2018) 97–106. https://doi.org/10.1016/j.aca.2017.11.054.
[51] Y. Wu, L. Li, Development of isotope labeling liquid chromatography-mass spectrometry for metabolic profiling of bacterial cells and its application for bacterial differentiation, Anal Chem 85 (2013) 5755–5763. https://doi.org/10.1021/ac400330z.
[52] R. Marion-Letellier, G. Savoye, S. Ghosh, Polyunsaturated fatty acids and inflammation, IUBMB Life 67 (2015) 659–667. https://doi.org/10.1002/iub.1428.
[53] C. Aslan, S. Maralbashi, F. Salari, H. Kahroba, F. Sigaroodi, T. Kazemi, P. Kharaziha, Tumor-derived exosomes: Implication in angiogenesis and antiangiogenesis cancer therapy, J Cell Physiol 234 (2019) 16885–16903. https://doi.org/10.1002/jcp.28374.
[54] P. Montuschi, P.J. Barnes, L.J. Roberts, Isoprostanes: markers and mediators of oxidative stress, The FASEB Journal 18 (2004) 1791–1800. https://doi.org/10.1096/fj.04-2330rev.
[55] J.M. Galano, Y.Y. Lee, C. Oger, C. Vigor, J. Vercauteren, T. Durand, M. Giera, J.C.Y. Lee, Isoprostanes, neuroprostanes and phytoprostanes: An overview of 25 years of research in chemistry and biology, Prog Lipid Res 68 (2017) 83–108. https://doi.org/10.1016/j.plipres.2017.09.004.
[56] O.S. Ahmed, J.-M. Galano, T. Pavlickova, J. Revol-Cavalier, C. Vigor, J.C.-Y. Lee, C. Oger, T. Durand, C.-Y. Lee, Moving forward with isoprostanes, neuroprostanes and phytoprostanes: where are we now? Moving forward with isoprostanes, neuroprostanes and phytoprostanes: where are we now? Moving forward with isoprostanes, neuroprostanes and phytoprostanes: where are we now?, Essays Biochem 2020 (2020) 463–484. https://doi.org/10.1042/EBC20190096ï.

[57] F.O. Martinez, S. Gordon, M. Locati, A. Mantovani, Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression, The Journal of Immunology 177 (2006) 7303–7311. https://doi.org/10.4049/jimmunol.177.10.7303.
[58] M. Record, K. Carayon, M. Poirot, S. Silvente-Poirot, Exosomes as new vesicular lipid transporters involved in cell-cell communication and various pathophysiologies, Biochim Biophys Acta Mol Cell Biol Lipids 1841 (2014) 108–120. https://doi.org/10.1016/j.bbalip.2013.10.004.
[59] J. Donoso-Quezada, S. Ayala-Mar, J. González-Valdez, The role of lipids in exosome biology and intercellular communication: Function, analytics and applications, Traffic 22 (2021) 204–220. https://doi.org/10.1111/tra.12803.
[60] M. Record, Exosomal lipids in cell-cell communication, in: Emerging Concepts of Tumor Exosome-Mediated Cell-Cell Communication, Springer New York, 2013: pp. 47–68. https://doi.org/10.1007/978-1-4614-3697-3_3.
[61] E.P. Calderin, J.-J. Zheng, N.L. Boyd, L. Mcnally, T.N. Audam, P. Lorkiewicz, B.G. Hill, J. Hellmann, Exercise-induced specialized proresolving mediators stimulate AMPK phosphorylation to promote mitochondrial respiration in macrophages, n.d. www.molecularmetabolism.com.
[62] S. Gangemi, G. Luciotti, E.D.' Urbano, A. Mallamace, D. Santoro, G. Bellinghieri, G. Davı`,Davı`, M. Romano, M. Romano, 1152/japplphysiol. 01004, J Appl Physiol 94 (2003) 2237–2240. https://doi.org/10.1152/japplphysiol.
[63] Exercise roles of bioactive lipid mediators • 111, n.d.
[64] L.S. Chow, R.E. Gerszten, J.M. Taylor, B.K. Pedersen, H. van Praag, S. Trappe, M.A. Febbraio, Z.S. Galis, Y. Gao, J.M. Haus, I.R. Lanza, C.J. Lavie, C.H. Lee, A. Lucia, C. Moro, A. Pandey, J.M. Robbins, K.I. Stanford, A.E. Thackray, S. Villeda, M.J. Watt, A. Xia, J.R. Zierath, B.H. Goodpaster, M.P. Snyder, Exerkines in health, resilience and disease, Nat Rev Endocrinol 18 (2022) 273–289. https://doi.org/10.1038/s41574-022-00641-2.
[65] L. Iuliano, D. Praticò, C. Greco, E. Mangieri, G. Scibilia, G.A. FitzGerald, F. Violi, Angioplasty Increases Coronary Sinus F 2-Isoprostane Formation: Evidence for In Vivo Oxidative Stress During PTCA, 2001.
[66] X. Hou, L.J. Roberts, F. Gobeil, D.F. Taber, K. Kanai, D. Abran, S. Brault, D. Checchin, F. Sennlaub, P. Lachapelle, D.R. Varma, S. Chemtob, Isomer-specific contractile effects of a series of synthetic F 2-isoprostanes on retinal and cerebral microvasculature, Free Radic Biol Med 36 (2004) 163–172. https://doi.org/10.1016/j.freeradbiomed.2003.10.024.
[67] S. Marlière, J.L. Cracowski, T. Durand, O. Chavanon, J. Bessard, A. Guy, F. Stanke-Labesque, J.C. Rossi, G. Bessard, The 5-series F2-isoprostanes possess no vasomotor effects in the rat thoracic aorta, the human internal mammary artery and the human saphenous vein, Br J Pharmacol 135 (2002) 1276–1280. https://doi.org/10.1038/sj.bjp.0704558.
[68] L. Alejandra García-Flores, S. Medina, R. Cejuela, J. Miguel Martínez-Sanz, C. Oger, J.-M. Galano, T. Durand, T. Casas-Pina, P. Martínez-Hernández, F. Ferreres, Assessment oxidative stress biomarkers -neuroprostanes and dihomo-isoprostanes-in elite triathletes urine after two weeks of moderate altitude training, 50 (2016). https://doi.org/10.3109/10715762.2015.1111514ï.
[69] S. Mrakic-Sposta, M. Gussoni, A. Vezzoli, C. Dellanoce, M. Comassi, G. Giardini, R.M. Bruno, M. Montorsi, A. Corciu, F. Greco, L. Pratali, Acute Effects of Triathlon Race on Oxidative Stress Biomarkers, Oxid Med Cell Longev 2020 (2020). https://doi.org/10.1155/2020/3062807.
[70] A. Vezzoli, C. Dellanoce, S. Mrakic-Sposta, M. Montorsi, S. Moretti, A. Tonini, L. Pratali, R. Accinni, Oxidative Stress Assessment in Response to Ultraendurance Exercise: Thiols Redox

Status and ROS Production according to Duration of a Competitive Race, Oxid Med Cell Longev 2016 (2016). https://doi.org/10.1155/2016/6439037.
[71] K. Arakawa, A. Hosono, K. Shibata, R. Ghadimi, M. Fuku, C. Goto, N. Imaeda, Y. Tokudome, H. Hoshino, M. Marumoto, M. Kobayashi, S. Suzuki, S. Tokudome, Open Access Journal of Sports Medicine ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/djsm20 Changes in blood biochemical markers before, during, and after a 2-day ultramarathon, Open Access J Sports Med (2016) 7–43. https://doi.org/10.2147/OA.
[72] N. Giovanelli, L. Biasutti, D. Salvadego, H.K. Alemayehu, B. Grassi, S. Lazzer, Changes in skeletal muscle oxidative capacity after a trail-running race, Int J Sports Physiol Perform 15 (2020) 278–284. https://doi.org/10.1123/ijspp.2018-0882.
[73] D. Jesús Ramos-Campo, V. Ávila-Gandía, F. Alacid, F. Soto-Méndez, P.E. Alcaraz, F. Javier López-Román, J. Ángel Rubio-Arias, D. Jesús Ramos Campo, Title: Muscle damage, physiological changes and energy balance in ultra-endurance mountain event athletes Affiliation, n.d. www.nrcresearchpress.com.
[74] S. Mrakic-Sposta, M. Gussoni, S. Moretti, L. Pratali, G. Giardini, P. Tacchini, C. Dellanoce, A. Tonacci, F. Mastorci, A. Borghini, M. Montorsi, A. Vezzoli, Effects of mountain ultra-marathon running on ROS production and oxidative damage by micro-invasive analytic techniques, PLoS One 10 (2015). https://doi.org/10.1371/journal.pone.0141780.
[75] G. Vernillo, A. Savoldelli, A. Zignoli, P. Trabucchi, B. Pellegrini, G.P. Millet, F. Schena, Influence of the world's most challenging mountain ultra-marathon on energy cost and running mechanics, Eur J Appl Physiol 114 (2014) 929–939. https://doi.org/10.1007/s00421-014-2824-y.
[76] C. Le Goff, M. Viallon, J.F. Kaux, P. Andonian, K. Moulin, L. Seidel, G. Giardini, L. Gergelé, P. Croisille, E. Cavalier, G.P. Millet, Kinetics of Cardiac Remodeling and Fibrosis Biomarkers During an Extreme Mountain Ultramarathon, Front Cardiovasc Med 9 (2022). https://doi.org/10.3389/fcvm.2022.790551.
[77] F. Wang, X. Wang, Y. Liu, Z. Zhang, Effects of Exercise-Induced ROS on the Pathophysiological Functions of Skeletal Muscle, Oxid Med Cell Longev 2021 (2021). https://doi.org/10.1155/2021/3846122.
[78] V. Penn, J. (Photographer) Higham, Route One food run : a rollicking road trip to the best eateries from Connecticut to Maine, n.d.
[79] A. Mastaloudis, S.W. Leonard, M.G. Traber, Original Contribution OXIDATIVE STRESS IN ATHLETES DURING EXTREME ENDURANCE EXERCISE, 2001.
[80] A. Mastaloudis, J.D. Morrow, D.W. Hopkins, S. Devaraj, M.G. Traber, Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners, Free Radic Biol Med 36 (2004) 1329–1341. https://doi.org/10.1016/j.freeradbiomed.2004.02.069.