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A MEPS-UHPLC-MS/MS analytical platform to detect isoprostanoids and specialized pro-resolving mediators in the urinary extracellular vesicles of mountain ultramarathon runners

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The authors declare no competing interests.

Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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., isoprostanooids, prostaglandins, epoxy- and hydroxy-fatty acids, specialized pro-resolving mediators) and four PUFAs in small uEVs.

Both F₂- and E₂-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; F₂-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, PGE₂ has been detected in murine mammary adenocarcinoma-derived EVs, suggesting its potential involvement in tumor growth [24]. Additionally, elevated levels of PGF_{2α} 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 (H₂O) 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 E₂, prostaglandin E₂-*d*₄, prostaglandin D₂, thromboxane B₂, leukotriene B₄, lipoxin A₄, lipoxin A₄-*d*₅, lipoxin B₄, resolvin E₁, resolvin D₁, resolvin D₁-*d*₅, resolvin D₂, resolvin

D₃, resolvin D₄, resolvin D₅, 17(*R*)-resolvin D₁, 17(*R*)-resolvin D₁-*d*₅, 17(*R*)-resolvin D₃, 17(*R*)-resolvin D₄, neuroprotectin D₁, 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-9 Z ,11 E -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-NPD₁, dinor-NPD₁, 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 Biomolécules 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 μ m 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 μ L 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 μ g/mL in organic solvent [refer to product insert from Cayman Chemicals]), as well as those synthesized at IBMM (Montpellier, France) (1000 μ g/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 H₂O 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 E₂-*d*₄, lipoxin A₄-*d*₅, resolvin D₁-*d*₅, 17(*R*)-resolvin D₁-*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 μ g/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 μ L 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 1st, 3rd, 5th 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 $-80\text{ }^{\circ}\text{C}$ were thawed at $37\text{ }^{\circ}\text{C}$ and centrifuged multiple times at $4\text{ }^{\circ}\text{C}$ to remove cells, debris and large EVs (10 min at $300 \times g$, 20 min at $2000 \times g$, and 30 min at $10,000 \times g$). These UC cycles were followed by a further cycle at 70 min at $100,000 \times g$ ($4\text{ }^{\circ}\text{C}$) to collect the pellet and by a final ultracentrifugation for 60 min at $100,000 \times g$ to wash the sample. Serial UC cycles at $100,000 \times g$ 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:H₂O, 50:50 v/v ACN:H₂O, and 50:50 v/v ACN: aqueous saline solution (50:50 v/v mixture of $\text{CuSO}_4 \cdot 5\text{ H}_2\text{O}$ 10% w/v and $\text{Na}_2\text{WO}_4 \cdot 2\text{ H}_2\text{O}$ 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 ($25\text{ }^{\circ}\text{C}$, 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:H₂O 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 H₂O 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 H₂O: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 \times 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 $\leq \pm 0.10$ 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 μ L 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 10th 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 \pm 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 CuSO₄·5 H₂O 10% w/v and Na₂WO₄·2 H₂O 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:H₂O mixture exhibited slightly higher average concentration than that obtained with the ACN:H₂O 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.

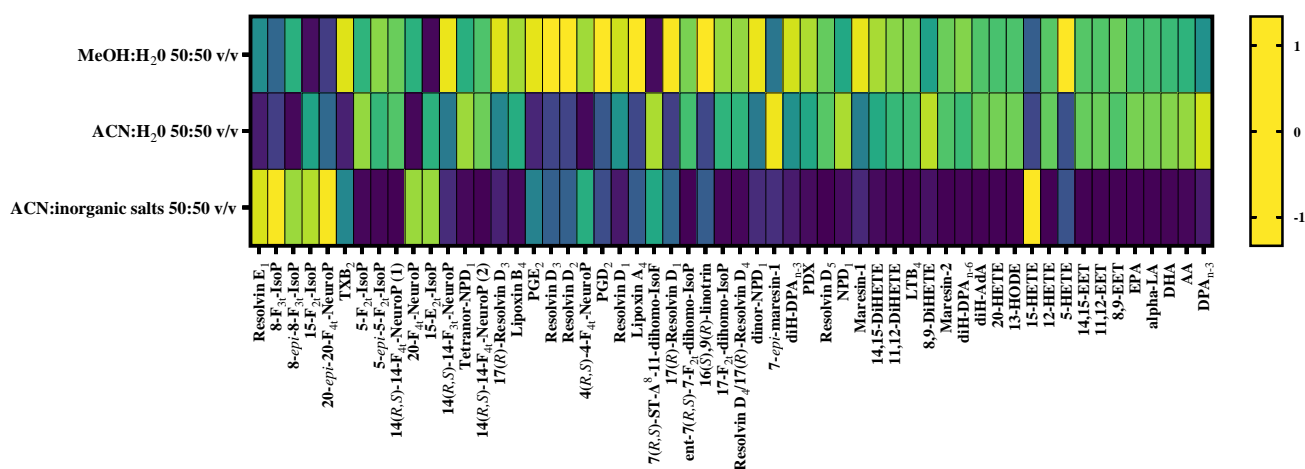


Figure 1. Heatmap of autoscaled peak area of oxylipins and PUFAs measured in small uEVs upon extraction with 50:50 v/v MeOH:H₂O, 50:50 v/v ACN:H₂O, and 50:50 v/v ACN: aqueous saline solution.

Reinicke *et al.* used a saline mixture (MeOH*BHT/H₂O*ZnSO₄ (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 ($\log P \approx 2-3$), such as resolvin E₁, 8-F_{3t}-IsoP, 15-F_{2t}-IsoP, and 20-*epi*-20-F_{4t}-NeuroP. In contrast, both MeOH and ACN in combination with H₂O were better suited for extracting more apolar compounds ($\log P \approx 5-6$), including EETs, HETEs, and PUFAs. MeOH consistently exhibited good extraction efficiency (70-115 %) across all compounds, including SPMs and PGs with intermediate polarity ($\log P \approx 3-4$), as confirmed by the extraction efficiency percentage values reported in Table S1. Based on these findings, the MeOH:H₂O mixture was chosen for all subsequent experiments. A 50% of MeOH composition provided the best compromise for simultaneous extraction of compounds with a $\log P$ 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:H₂O 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 D₂) 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.

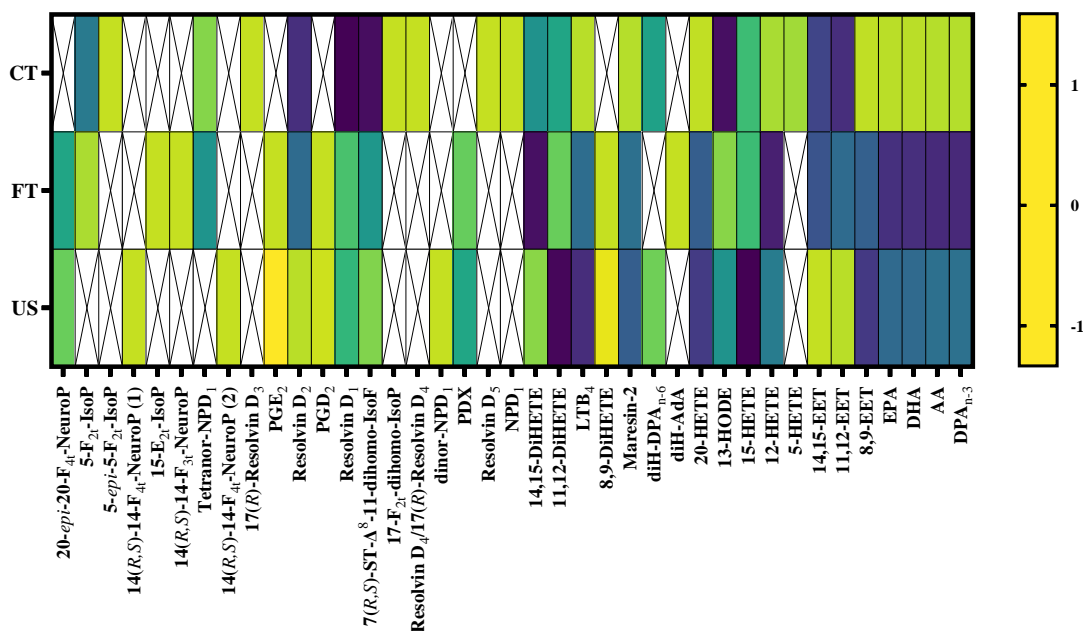


Figure 2. Heatmap of autoscaled peak area of oxylipins and PUFAs measured in small uEVs upon lysis by ultrasonication (US) and freeze–thaw cycle (FT) *versus* control (CT, no lysis step). The cross in the box refers to a concentration value below LOD.

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.

Table 1 reports the mean and slopes and standard deviations (n=3) of the five calibration curves, LOD values, and overall

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

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

ied 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 D₁ and

resolvin D₂) 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 D₁ and resolvin D₂, 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 (F₂/E₂-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. F₂-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]. PGE₂ 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 B₄, resolvin D₁, and resolvin D₂, 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 A₄ 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 exerkinins (e.g., peptides, nucleic acids, and metabolites) into the circulation in response to acute endurance exercise for multisystemic adaptations [64]. Exerkinins, are often found in EVs modulating cell-cell interactions and organ crosstalk. Oxylipins and their precursors may be reasonably classified as exerkinins 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 F₂-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].

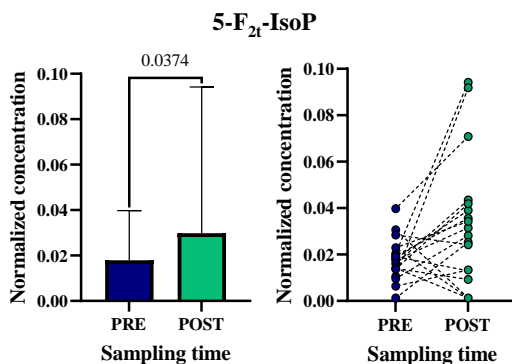


Figure 3. Normalized (log-transformation and autoscaling) concentration levels of 5-F_{2t}-IsoP measured pre- and post-TDG race in ultramarathon runners (n=18). The univariate parametric t-test was performed upon data normalization.

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]. F₂-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 F₂-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 F₂- and E₂-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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