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# Saliva as a non-invasive tool for monitoring oxidative stress in swimmers athletes performing a $VO_{2max}$ cycle ergometer test

Denise Biagini<sup>a</sup>, Tommaso Lomonaco<sup>a,\*</sup>, Silvia Ghimenti<sup>a</sup>, Jonathan Fusi<sup>b</sup>, Eugenio Cerri<sup>b</sup>, Francesca De Angelis<sup>a</sup>, Francesca Giuseppa Bellagambi<sup>c</sup>, Camille Oger<sup>d</sup>, Jean Marie Galano<sup>d</sup>, Emilia Bramanti<sup>e</sup>, Ferdinando Franzoni<sup>b</sup>, Roger Fuoco<sup>a</sup>, Fabio Di Francesco<sup>a</sup>

<sup>a</sup> Department of Chemistry and Industrial Chemistry, University of Pisa, Via Giuseppe Moruzzi 13, 56124, Pisa, Italy

<sup>b</sup> Department of Clinical and Experimental Medicine, University of Pisa, Via Roma 67, 56124, Pisa, Italy

<sup>c</sup> Univ Lyon, CNRS, Université Claude Bernard Lyon 1, Institut de Sciences Analytiques, UMR 5280, 5 rue de la Doua, F-69100, Villeurbanne, France

<sup>d</sup> Institut des Biomolécules Max Mousseron, IBMM, Université de Montpellier, CNRS, ENSCMFaculté de Pharmacie, 15 avenue Charles Flahault, BP 14491, F-34093,

e Institute of Chemistry of Organometallic Compounds, CNR, Via Giuseppe Moruzzi 3, 56124, Pisa, Italy

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Keywords: Saliva Prostanoids Isoprostanes Carbonyl compounds Oxidative stress VO<sub>2max</sub> cycle ergometer test ABSTRACT

Biomarkers of oxidative stress are generally measured in blood and its derivatives. However, the invasiveness of blood collection makes the monitoring of such chemicals during exercise not feasible. Saliva analysis is an interesting approach in sport medicine because the collection procedure is easy-to-use and does not require specially-trained personnel. These features guarantee the collection of multiple samples from the same subject in a short span of time, thus allowing the monitoring of the subject before, during and after physical tests, training or competitions. The aim of this work was to evaluate the possibility of following the changes in the concentration of some oxidative stress markers in saliva samples taken over time by athletes under exercise. To this purpose, ketones (i.e. acetone, 2-butanone and 2-pentanone), aldehydes (i.e. propanal, butanal, and hexanal), α,β-unsaturated aldehydes (i.e. acrolein and methacrolein) and di-carbonyls (i.e. glyoxal and methylglyoxal) were derivatized with 2,4-dinitrophenylhydrazine, and determined by ultra-high performance liquid chromatography coupled to diode array detector. Prostaglandin  $E_2$ ,  $F_2/E_2$ -isoprostanes,  $F_2$ -dihomo-isoprostanes,  $F_4$ neuroprostanes, and F2-dihomo-isofuranes were also determined by a reliable analytical procedure that combines micro-extraction by packed sorbent and ultra-high performance liquid chromatography-electrospray ionization tandem mass spectrometry. Overall the validation process showed that the methods have limits of detection in the range of units of ppb for carbonyls and tens to hundreds of ppt for isoprostanes and prostanoids, very good quantitative recoveries (90-110%) and intra- and inter-day precision lower than 15%. The proof of applicability of the proposed analytical approach was investigated by monitoring the selected markers of oxidative stress in ten swimmers performing a VO<sub>2max</sub> cycle ergo meter test. The results highlighted a clear increase of salivary by-products of oxidative stress during exercise, whereas a sharp decrease, approaching baseline values, of these compounds was observed in the recovery phase. This study opens up a new approach in the evaluation of oxidative stress and its relation to aerobic activity.

#### 1. Introduction

Free radicals are usually produced as part of normal metabolic processes to modulate cell signaling and vital physiological functions [1,2]. The cellular redox homeostasis is guaranteed by the presence of scavenging defense systems that minimize the excess of free radicals [3]. However, an imbalance between antioxidant defenses and production of free radicals causes a condition called oxidative stress (OS)

that induces cell and tissue damages [4]. Oxidative stress has different pathophysiological impacts on biological processes, exacerbating several diseases such as diabetes [5] and cardiovascular disease [6]. The most important free radicals in living cells are reactive oxygen species (ROS) and reactive nitrogen species (NOS), both of them are involved in biological reactions with lipids and proteins [7]. The in vivo detection of these free radicals is challenging due to their very short half-life (e.g.  $\cdot OH \sim 10^{-9}$  s) [8,9]. Thus, the monitoring of OS normally involves the

\* Corresponding author.

Montpellier Cedex 05, France

E-mail address: tommaso.lomonaco@unipi.it (T. Lomonaco).

determination of sub-products of lipid peroxidation in blood, such as aldehydes (e.g. methylglyoxal, diacetyl, acrolein, crotonaldehyde, and hexanal [10,11]) and prostaglandin  $F_2$ -like compounds that are produced in vivo by non-enzymatic free radical-catalyzed peroxidation of arachidonic acid [12].

In order to counteract the effects of oxidative stress, the scientific community has commonly accepted the concept that performing a regular physical activity of moderate intensity brings significant health benefits, including prevention and treatment of chronic diseases [13–15]. Among the molecular mechanisms related to these beneficial effects, an enhancement of antioxidant defenses and a drop of lipid peroxidation have been evidenced both in adult and in aged individuals [16]. Nevertheless, prolonged and intense exercise can result in free radicals (over)production leading to an impairment of the antioxidant defenses of humans and a consequent modification of the cellular components. In this regard, ROS and NOS may oxidize proteins provoking a change of their tridimensional structure that may limit in turn the functional activities, including gene transcription [17]. The oxidation of polyunsaturated free fatty acids and lipoproteins induces changes in fluidity and permeability of the cell membrane [18]. It impairs physical performance by altering contractile function and/or accelerating muscle damage/fatigue [19], and may lead to a vicious cycle of ROS generation and DNA damage [20]. This scenario is further exacerbated by the cytotoxic effect of endogenous aldehydes, e.g. acrolein, methylglyoxal, and crotonaldehyde [21]. Recent studies have been focused on isoprostanes as markers of oxidative stress after exercise [22]. F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs) were found to increase in athletes during extreme endurance exercise and in ultra-marathon runners [23,24]. Moreover, specific markers of oxidative damage to the brain belonging to new families of isoprostanes, such as F2-dihomo-Isoprostanes (F<sub>2</sub>-dihomo-IsoPs) and F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs) have been recently reported in literature. They originate from the free radical peroxidation of adrenic and docosahexanoic acid, respectively, and showed a significant change of the urine level as a results of increasing or decreasing training loads. A decrease of these markers associated to neuronal membrane degradation was observed in urine using polyphenols-rich juice as antioxidant supplementation to triathletes [25,26].

Athletes may take vitamin-based supplements after a race to limit oxidative damage, even if it is not clear if the body's natural antioxidant defense system is sufficient to balance the increase of free radicals [27]. In this case, a personalized and targeted antioxidant supplementation, tuned with respect to the resistance of each athlete to the attack of free radicals, should guarantee the correct counterbalance between anti-/ pro-oxidants, and improving athlete recovery to reduce cellular damage and maintaining peak athletic performance.

Few articles have reported saliva analysis as an alternative source of useful information in physiological research related to the performance of sports teams [28–31]. In the last decades, the monitoring of human health conditions using non-invasive fluids analysis (e.g. breath, saliva, and sweat) in alternative to traditional blood and urine assays has attracted increasing attention in the field of medical diagnosis [32-35] and therapy monitoring [36,37]. In particular, saliva analysis may provide a comprehensive characterization of exercise-related oxidative stress markers thanks to the rapid equilibrium between blood and saliva across salivary membranes [38]. Moreover, the use of saliva to monitor oxidative stress during the physical exercise is an attractive approach because it shows several advantages compared to blood and its derivatives: i) non-invasive collection procedures, ii) no need for trained personnel to collect the sample, iii) low risk of infections, and iv) simpler chemical composition compared to blood and its derivatives [39,40]. Most of these features make the collection of several saliva samples in a pre-determined time span from one subject very easy, particularly appealing in the field of physical tests, training or competitions. In this regard, the concomitant monitoring of a comprehensive panel of by-products related to the oxidative stress might provide a

more informative picture of the process under investigation, enabling a better understanding of the physiopathology and its implication in physical exercise.

The aim of this work was to evaluate saliva analysis as a non-conventional innovative analytical approach to monitor oxidative stress during physical exercise. For this purpose, reliable analytical procedures for the determination of selected markers of the oxidative damage, i.e. carbonyls, isoprostanes, and prostanoids, in stimulated saliva samples were developed and validated. Finally, the procedures were applied to saliva samples collected from 10 swimmers athletes performing a cycle ergometer test for the determination of the maximal aerobic capacity (VO<sub>2max</sub>).

## 2. Materials and methods

### 2.1. Chemicals and materials

2,4-dinitrophenilhydrazine (2,4-DNPH, purity of 97%), TO11/IP-6A Aldehyde/Ketone-DNPH Mix (15 µg/mL of acetaldehyde-2,4-DNPH, acetone-2,4-DNPH, acrolein-2,4-DNPH, benzaldehyde-2,4-DNPH, butyraldehyde-2,4-DNPH, crotonaldehyde-2,4-DNPH, 2,5-dimethylbenzaldehyde-2,4-DNPH, formaldehyde-2,4-DNPH, hexaldehyde-2,4-DNPH, isovaleraldehyde-2,4-DNPH, propionaldehyde-2,4-DNPH, o-tolualdehyde-2,4-DNPH, m-tolualdehyde-2,4-DNPH, ptolualdehyde-2,4-DNPH, valeraldehyde-2,4-DNPH), acetone (purity  $\geq$  99.9%), 2-butanone (purity  $\geq$  99.9%), 2-pentanone (purity  $\geq$  99.9%), propanal (purity  $\geq$  99.9%), butanal (purity  $\geq$  99.9%), hexanal (purity  $\geq$  99.9%), acrolein (purity  $\geq$  99.9%), methacrolein (purity  $\geq$  99.9%), glyoxal (purity of 40%), methylglyoxal (purity of 40%), magnesium sulfate (purity  $\geq$  99.5%), formic acid (purity of 98%), ammonium acetate (purity  $\geq$  99.9%), and ammonium formate (purity  $\geq$  99.9%) where purchased from Sigma Aldrich (Milan, Italy). Water, acetonitrile, and methanol where purchased from Sigma Aldrich (Milan, Italy) at LC/MS grade.

Commercially available prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), F<sub>2</sub>- and E<sub>2</sub>-iso-prostanes (IsoPs), 15-F<sub>2t</sub>-IsoP and 15-E<sub>2t</sub>-IsoP (purity  $\geq$  99%) were from Cayman Chemical (Michigan, USA).

Not commercially available F<sub>4</sub>-NeuroPs (20-F<sub>4t</sub>-NeuroP, 20-*epi*-F<sub>4t</sub>-NeuroP), F<sub>2</sub>-dihomo-IsoPs (*ent*-7(R,S)-7-F<sub>2t</sub>-dihomo-IsoP) and F<sub>2</sub>-dihomo-IsoF (7(R,S)-ST- $\Delta^8$ -11-dihomo-IsoF) were synthesized at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France) according to procedures reported elsewhere [41–44].

All the solutions and saliva samples were stored in sterile polypropylene containers from Eppendorf (Milan, Italy).

Phenex<sup>M</sup>-RC syringe filters (0.2 µm regenerate cellulose, 4 and 15 mm of diameter) were from Phenomenex (California, USA).

The eVol<sup> $\circ$ </sup> XCHANGE analytical syringe (20–500 µL) and microextraction by packed sorbent silica-C18 Barrel Insert and Needles (BINs) were purchased from SGE Analytical Science (Melbourne, Australia).

Salivette roll-shaped polyester swabs were purchased from Sarstedt (Nümbrecht, Germany).

A Macherey Nagel Pehanon narrow range (5.2 < pH < 8.1) pH paper strips (Düren, Germany) with a resolution of 0.3 pH units was used to estimate the salivary pH.

An Amicon Ultra-0.5 mL Centrifugal Filters (Darmstadt, Germany) with a cut-off of 3 kDa was used to purify saliva samples.

## 2.2. Equipment

A VELP Scientifica ZX4 Advanced Vortex Mixer (Usmate, Italy) and an Eppendorf 5804 R Centrifuge, equipped with an A-4-44 swinging bucket rotor (Milan, Italy), were used for sample vortex-mixing and centrifugation, respectively. The derivatization reaction was carried out using a Julabo SW22 thermostatic water bath (Milan, Italy), which guarantees an accurate control of the temperature (resolution of 0.1 °C). A Radwag AS220/X balance (Milan, Italy) was used to weight Salivette swabs before and after sampling.

An Agilent 1290 Infinity II LC system coupled to an Agilent 1290 diode array detector (DAD) was used for the determination of 2,4-DNPH adducts. The chromatographic separation was carried out using a Zorbax Eclipse plus reversed-phase column ( $50 \times 2.1 \text{ mm}$ ,  $1.8 \mu\text{m}$ ) coupled to a guard column ( $5 \times 2.1 \text{ mm}$ ,  $1.8 \mu\text{m}$ ), both from Agilent Technologies (Santa Clara, USA). The UHPLC-DAD instrument was controlled by OpenLAB software (v. A.01.05) from Agilent Technologies (Santa Clara, USA).

An Agilent 1290 Infinity II LC system coupled to an Agilent 6495 triple quadrupole mass spectrometer detector was used for the determination of isoprostanes and prostanoids. The chromatographic separation was carried out using a Polaris 3 C-18 reverse-phase column ( $50 \times 4.6 \text{ mm}$ ,  $3 \mu \text{m}$ ) coupled to a guard column ( $5 \times 4.6 \text{ mm}$ ,  $3 \mu \text{m}$ ), both from Agilent Technologies (Santa Clara, USA). The UHPLC-MS/MS instrument was controlled by MassHunter software (v. B.07.01) from Agilent Technologies (Santa Clara, USA).

#### 2.3. Standard solutions and quality control samples

Working solutions of TO11/1P-6A mixture were prepared gravimetrically by diluting 100-, 20-, 10- and 2-fold the stock solution (15  $\mu$ g/mL) with a mixture of acetonitrile and water (70:30 %v/v) and then stored in amber vials at 4 °C for one week.

Stock solution of ketones (i.e. acetone, 2-butanone and 2-pentanone), aldehydes (i.e. propanal, butanal, and hexanal),  $\alpha$ , $\beta$ -unsaturated aldehydes (i.e. acrolein and methacrolein) and di-carbonyls (i.e. glyoxal and methylglyoxal) were prepared gravimetrically by dissolving 5 µL of each pure compound in 10 mL of acetonitrile. All stock solutions were prepared every week and then stored in amber vials at 4 °C. Working mixtures were prepared daily by diluting 8000-, 3000-, 1600-fold the stock solutions with water to obtain different concentration levels of target compounds.

Stock solutions of 2,4-DNPH (12 mM) were prepared gravimetrically by dissolving 25 mg of the pure compound in 10 mL of acetonitrile and stored in amber vials at 4  $^{\circ}$ C for one week. The solution was acidified by adding 0.2 mL of formic acid.

Stock solutions of 15-F<sub>2t</sub>-IsoP, PGE<sub>2</sub> and 15-E<sub>2t</sub>-IsoP (1000 µg/mL) were prepared by dissolving 1 mg of pure compound in 1 mL of a mixture of acetonitrile and methanol (50:50 v/v). These solutions were protected from light and stored at -20 °C. 1000 µg/mL stock solutions of the remaining isoprostanes were prepared in methanol at Institut des Biomolécules Max Mousseron (Montpellier, France) and shipped in dry ice to our lab. Once arrived, they were immediately stored at -20 °C, and used to prepare monthly intermediate stock solutions of the analytes at 10 µg/mL in a 50:50 v/v acetonitrile:methanol mixture. A standard solution containing all the analytes at 10 ng/mL concentration level (MIX) was prepared weekly through sequential dilutions of each stock solution with LC-MS water and then stored at 4 °C. This solution was used to prepare working standard solutions and quality control samples in the range 0.05–1 ng/mL.

Stock solutions of internal standards (i.e. 8-isoPGF<sub>2</sub> $_{\alpha}$ -d4, 8-isoPGE<sub>2</sub>-d4 and PGE<sub>2</sub>-d4) were supplied in methyl acetate at 100 µg/mL concentration level and stored at -20 °C. A working standard mixture at 20 ng/mL (MIX ISs) was prepared weekly through sequential dilutions with LC-MS water from the stock solutions and stored at 4 °C.

Pooled saliva samples were obtained by mixing known aliquots of stimulated saliva samples collected from 10 nominally healthy control volunteers that did not performed the exercise test. This pooled sample was daily spiked with known amounts of analytes to obtain standard saliva samples at different quality control levels.

#### 2.4. Exercise protocol and sample collection

Ten swimmers (6 males and 4 females) performed an incremental

#### Table 1

Age, height, weight, body mass index (BMI), maximal oxygen consumption  $(VO_{2max})$ , and maximal working capacity (MWC) of the enrolled swimmers athletes (n = 10).

Characteristics	Value (mean $\pm$ standard deviation)
Age (y)	$23 \pm 5$
Height (cm)	$178 \pm 8$
Weight (Kg)	71 ± 7
BMI (Kg/m <sup>2</sup> )	$22 \pm 2$
VO <sub>2max</sub> (mL/Kg min)	49 ± 7
MWC (W)	$270 \pm 40$

cycle ergometer exercise test of maximal aerobic power in order to estimate the  $VO_{2max}$  [45]. The study was conducted at the "Dipartimento di Medicina Clinica e Sperimentale" of the University of Pisa in accordance with the Declaration of Helsinki. All subjects provided written informed consent before starting the experiments. Eating habits of swimmers were evaluated using a questionnaire. They followed a Mediterranean diet characterized by 50% carbohydrates, 30% proteins, and 20% fats. All subjects were non-smokers and did not take any supplements.

Table 1 reports the anthropometric characteristics of the enrolled swimmers athletes.

The exercise test was performed by an electrically braked cycle ergometer (Quark PFT ergo, COSMED, Italy). The workload was 25 W for the first 5 min followed by an increase of 25 W every minute until impossibility to maintain the pace due to fatigue. The evaluation of fatigue was done according to the Borg Scale [46]. During exercise, pedaling cadence was constant at 70 rpm. Stimulated saliva samples were collected by moving the swab in the mouth for 1 min at the following times: 5 min before the test  $(t_0)$ , at the maximal exercise peak  $(t_1)$ , 2.5  $(t_2)$ , 5 min  $(t_3)$  and 10  $(t_4)$  minutes after the VO<sub>2max</sub>. The timing of the experiment (3:00 p.m. to 5:00 p.m.) was chosen to minimize the influence of circadian variation in salivary composition [47]. Respiratory quotient is calculated as the ratio between the volume of carbon dioxide released over the volume of oxygen absorbed during respiration [48]. Samples were recovered by centrifuging the swabs at 5000 rpm for 5 min at 4  $^{\circ}$ C and stored at -80  $^{\circ}$ C until the analysis. Immediately after sampling, two independent measurements of the salivary pH were performed by using pH paper strips. The salivary flow rate (grams per minute) was calculated from the ratio of sample weight and collection time, assuming a unit density for saliva [49].

#### 2.5. Sample analysis

Method 1: an aliquot of saliva sample (0.2 mL) was transferred into an Amicon tube and centrifuged at 12500 rpm for 25 min at 4 °C. The derivatization reaction was carried out using a modified version of Andreoli's procedure [50]. Briefly, an aliquot (0.1 mL) of ultrafiltrate was added to the Eppendorf tube together with 0.1 mL of 2,4-DNPH (12 mM) and incubated at 30.0  $\pm$  0.1 °C for 60 min. This solution was then filtered with a RC syringe filter (4 mm diameter, 0.2 µm pore size) and injected (20 µL) into the UHPLC-DAD system. Chromatographic separation was carried out at 0.5 mL/min using a water (A)/acetonitrile (B) mobile phase. The following gradient was used: isocratic for 2 min (A/B = 70/30), followed by an increase of acetonitrile up to 65% in 17 min. The column was rinsed with a mixture of A (10%) and B (90%) for 4 min followed by a re-equilibration step (30% of B) of 7 min. The UHPLC run time was 30 min. The Agilent 1290 high performance wellplate autosampler and the 1290 thermostatted column compartment was set at 4 °C and 30 °C, respectively. Before every injection, needle was washed for 60 s with a water-acetonitrile mixture (80:20, v/v). Acetone, 2-butanone, 2-pentanone, propanal, butanal, hexanal, acrolein, methacrolein and benzaldehyde were monitored acquiring the

signal at 370 nm, crotonaldehyde, cyclohexanone and pentanal at 380 nm and glyoxal and methylglyoxal at 420 nm. For each signal, bandwidth and reference wavelength were set at  $\pm$  5 nm and at 550  $\pm$  20 nm, respectively. The DAD spectrum was acquired from 200 to 600 nm, with a scan rate of 20 scans/s.

Method 2: saliva sample extraction was performed by a modified version of a method previously described [51]. Briefly, an aliquot of saliva (0.5 mL) was added with 0.01 mL of MIX ISs (20 ng/mL) and then diluted to 3 mL with LC-MS water. The diluted saliva sample was vortex mixed for 30 s, filtered with a RC syringe filter (15 mm diameter, 0.2 µm pore size) and then subjected to microextraction by packed sorbent (MEPS) procedure prior to UHPLC-MS/MS analysis. The MEPS procedure was performed through an off-line semi-automated system. assembled in our lab, combining the eVol® XCHANGE analytical syringe (20-500 µL, SGE Analytical Science, UK) and the AL-4000 Programmable Syringe Pump (World Precision Instrument, USA). The cartridge was activated and then conditioned by drawing and discharging three times an aliquot (100 µL) of methanol and water, respectively. After that, 500 µL of the diluted sample were loaded up and discharged six times in order to retain isoprostanes and prostanoids. The cartridge was then washed one time with an aliquot (100 µL) of a water:methanol mixture (95:5 v/v) to remove potential interferences and to minimize the matrix effect. Finally, the analytes were eluted from the cartridge with 50 µL of pure methanol. Sampling and elution were performed at 0.3 mL/min, whereas all the other steps at 0.6 mL/min. An aliquot (20  $\mu$ L) of the extract was injected into the UHPLC-MS/MS instrument for the analysis. Chromatographic separation was carried out at 0.7 mL/ min using a 0.1% aqueous formic acid (A)/50:50 methanol:acetonitrile (B) mobile phase. The following gradient was used: isocratic for 2 min (A/B = 90/10), then 45% (B) at 2.1 min, isocratic for 3 min, up to 60% (B) in 3.5 min, isocratic for 0.5 min, 80% (B) at 9 min, isocratic for 5 min, re-equilibration to initial conditions up to 18 min. The multisampler and the column compartment were set at a 4 °C and 25 °C. respectively. The mass spectrometer operated in ESI negative ionization mode and performed multiple reaction monitoring (MRM) experiments with unit mass resolution, monitoring at least two transitions for each compound. For all analytes, the ESI operation conditions were: drying gas temperature 240 °C, drying gas flow 18 L/min, nebulizer gas pressure 30 psi, sheath gas temperature 360 °C, sheath gas flow 12 L/ min, capillary voltage 5000 V and nozzle 1500 V. The fragmentor voltage was fixed at 380 V and high and low pressure funnel voltages were set at 160 V for all mass transitions. Each analyte was detected using two specific MRM transitions, the most abundant was used for quantification (Q), whereas the second one for the identification of the target compound (q). A deviation of  $\pm$  0.10 min of the expected retention time compared to working standard solutions and a qualifier/ quantifier (q/Q) ratio within 10% of the ratio measured in working standard mixtures were required for analyte identification [52].

## 2.6. Methods validation and statistical analysis

The analytical method validation was performed in accordance with the IUPAC guidelines [53] and included the evaluation for each analyte of limit of detection (LOD) and quantification (LOQ), calibration curve, matrix effect, recovery, intra-day and inter-day precision.

LOD and LOQ values were calculated as three and ten times the standard deviation (sb) of the "low level spiked blank" with carbonyls (5 ng/mL), isoprostanes and prostanoids (0.05 ng/mL). These samples were analyzed five times.

Five-point calibration curves (n = 3 at each concentration level) were obtained in the range 25–250 ng/mL for carbonyls and 0.05–1 ng/mL for isoprostanes and prostanoids. Optimal equation curves were evaluated by the Deming regression analysis, which considers measurement errors for both dependent and independent variables [54].

The recovery, precision and matrix effect of the analytical procedures were evaluated by analyzing in triplicate (n = 3 at each concentration level) within the same day and on three consecutive days a pooled saliva sample spiked with 50, 100 and 250 ng/mL of carbonyls and 0.05, 0.5 and 1 ng/mL of isoprostanes and prostanoids. The recovery was calculated as the percentage ratio of the difference between the analyte concentrations measured in the spiked and the non-spiked samples to the concentration in spiked samples.

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. The matrix effect was evaluated by comparing the slopes of the calibration curves obtained with working solutions and spiked saliva samples at a confidence level of 95%.

A two-tailed *p*-value of < 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism (v. 8.0) from GraphPad Software Inc. (La Jolla, USA) and MetaboAnalyst 4.0 (https://www.metaboanalyst.ca).

## 3. Results and discussion

# 3.1. Optimization of the extraction procedure for the determination of carbonyls in saliva samples

Aliquots (0.2 mL) of pooled saliva sample spiked with 100 ng/mL of carbonyls were derivatized in triplicate with 0.2 mL of 2,4-DNPH (12 mM) for 60 min at 30 °C. Then, liquid-liquid extraction (LLE), salting-out liquid-liquid extraction (SALLE) and ultra-filtration (UF) with a 3 kDa cut-off were compared in terms of recovery and simplicity of the analytical workflow. An aliquot (0.4 mL) of hexane was added to the derivatized sample and the resulting mixture was vortex-mixed for 30 s at 12500 rpm. After separation and evaporation of the organic phase, the extract was reconstituted with 0.05 mL of a water/acetonitrile mixture (65:35 v/v) and then injected into the UHPLC-DAD instrument. Single and multiple extractions were compared to optimize the LLE method. For a single extraction, analytes recovery ranged between 70 and 80% (RSD 15%) whereas the second extraction did not significantly increase recovery. Salting-out liquid-liquid extraction approach was tested in order to simplify the sample treatment. This approach exploits the principle that the solubility of organic solvent in the water phase can be modulated by modifying the ionic strength of the medium. Magnesium sulfate (5 M) and ammonium formate (5 M), two of the most common inorganic salts used in this field [55], showed a limited capability to separate acetonitrile layer, leading to a poor analytes recovery (40-50%, RSD 13%). Taking into account the low molecular weight and high hydrophilicity of the target analytes, the possibility to ultrafiltrate the sample (12500 rpm for 25 min at 4 °C) with a molecular weight cut-off of 3 kDa was tested. The experiments were conducted using 0.2 mL of a pooled saliva sample spiked with 100 ng/mL of carbonyls. Data highlighted an almost quantitative recovery (90-100%, RSD 5%) for all the analytes, with the exception of 2pentanone and hexanal that showed a slightly lower recovery (80%, RSD 6%), probably due to their affinity with the plastic material of the device [56]. Thus, the ultra-filtration method was selected according to the highest recovery and high-throughput of this approach compared to the classical LLE.

# 3.2. Optimization of MEPS extraction procedure for the determination of isoprostanes and prostanoids in saliva samples

Compared to the standard SPE approach, MEPS has the main advantage to reduce the amount of sample and solvents needed for the analyte extraction without spoiling the pre-concentration factor [57]. The presence of mucins and other proteins does not allow the direct loading of saliva into the MEPS, thus ultra-filtration (3 kDa cut-off), protein precipitation and sample dilution with water as treatments to minimize the saliva viscosity and preserve the lifetime of the MEPS cartridge were comparatively tested. The experiments were carried out

# Table 2

Calibration range, slope and limits of detection (LOD) of the target compounds.

Analyte	Calibration range (ng/mL)	Slope (RSD <sup>a</sup> )	R <sup>2</sup>	LOD (ng/mL)
Acetone	25–250	0.202 (7%)	0.995	6
2-Butanone	25–250	0.238 (7%)	0.997	2
2-Pentanone	25–250	0.310 (8%)	0.998	3
Propanal	25–250	0.289 (6%)	0.997	1
Butanal	25–250	0.136 (10%)	0.994	2
Hexanal	25–250	0.068 (12%)	0.997	2
Acrolein	25–250	0.509 (6%	0.999	1
Methacrolein	25–250	0.306 (8%)	0.997	1
Glyoxal	25–250	0.419 (10%)	0.995	3
Methylglyoxal	25–250	0.979 (13%)	0.996	2
15-F <sub>2t</sub> -IsoP	0.05–1	$4.0 \times 10^5$ (5%)	0.999	0.01
15-E <sub>2t</sub> -IsoP	0.05–1	$1.7 \times 10^5$ (6%)	0.999	0.005
PGE <sub>2</sub>	0.05–1	$1.7 \times 10^{5}$ (8%)	0.999	0.005
20-epi-20-F4t-NeuroP	0.05–1	$2.1 \times 10^4$ (10%)	0.995	0.2
20-F <sub>4t</sub> -NeuroP	0.05–1	$1.3 \times 10^4$ (8%)	0.999	0.08
$7(R,S)$ -ST- $\Delta^8$ -11-dihomo-IsoF	0.05–1	$3.4 \times 10^5$ (9%)	0.996	0.2
ent-7(R,S)-7-F22t-dihomo-IsoP	0.05–1	$6.4 \times 10^4$ (3%)	0.997	0.05
17-F <sub>2t</sub> -dihomo-IsoP	0.05–1	$6.7 \times 10^4$ (9%)	0.997	0.06

<sup>a</sup> Calculated from three calibration curves.

using 0.5 mL of pooled saliva sample spiked with 200 and 50 pg/mL of isoprostanes and prostanoids. Ultra-filtration with a 3 kDa cut-off at 12500 rpm and 4 °C for 60 min gave the worst performances showing a very low recovery of 20-30% (RSD 10%) for all the analytes, probably due to the interaction between the plastic material and target chemicals, as already mentioned [56,58]. Protein precipitation with acetonitrile (acetonitrile/saliva volume ratio of 1) entailed an almost quantitative recovery (95-100%, RSD 10%) for all the compounds and represents an alternative strategy for the pre-treatment of the sample, confirming the results reported elsewhere [59]. Nevertheless, fast and easy-to-use dilution of the sample with water (water/saliva volume ratio of 6) was preferred to protein precipitation, in order to avoid the evaporation step required for the reduction of the organic phase content before MEPS loading [60]. Lower water-to-saliva ratios (i.e. 3 or 1) were soon excluded because of frequent sorbent occlusion just after ten MEPS extractions. Regarding the recovery of analytes from MEPS, no

#### Table 3

Recovery, intra- and inter-day precision relevant for the measurement of carbonyls, isoprostanes and prostanoids compounds in spiked pooled saliva samples.

Analyte	Recovery (%)	Intra-day <sup>a</sup> RSD	Recovery (%)	Inter-day <sup>b</sup> RSD
Acetone	95	18%	98	25%
2-Butanone	102	9%	98	13%
2-Pentanone	79	11%	82	10%
Propanal	86	5%	89	7%
Butanal	94	11%	95	13%
Hexanal	82	16%	85	17%
Acrolein	107	3%	103	5%
Methacrolein	104	6%	99	9%
Glyoxal	97	12%	98	11%
Methylglyoxal	91	12%	93	14%
15-F <sub>2t</sub> -IsoP	116	9%	117	7%
15-E <sub>2t</sub> -IsoP	117	4%	112	9%
PGE <sub>2</sub>	123	11%	116	13%
20-epi-20-F4t-NeuroP	116	15%	119	11%
20-F4t-NeuroP	110	3%	117	5%
$7(R,S)$ -ST- $\Delta^8$ -11-	225	6%	240	7%
dihomo-IsoF				
ent-7(R,S)-7-F <sub>2t</sub> -	211	4%	216	8%
dihomo-IsoP				
17-F <sub>2t</sub> -dihomo-IsoP	208	10%	198	12%

<sup>a</sup> Calculated from three replicates at each concentration level.

<sup>b</sup> Calculated from three replicates at each concentration level and on three consecutive days.

loss of the analytes during the washing step with 100  $\mu$ L of MeOH:water 95:5 v/v neither carry over effect were observed for all the analytes investigated. A complete analyte elution was achieved by a single withdraw-eject cycle with 50  $\mu$ L of methanol. The elution with a second aliquot of methanol did not produce any detectable chromatographic signal. The fast and easy-to-use MEPS procedure resulted very innovative. The procedure was semi-automated, highly reproducible (RSD < 5%) and accurate (recovery > 90%). Compared to time consuming, tedious and multistage procedures reported in literature [58,61], our approach entails saliva clean-up and analyte pre-concentration in about 30 min by using a reduced amount of organic solvents (i.e. 50-100  $\mu$ L). The eluate was directly injected into the UHPLC system without any additional drying step, ensuring the straightforward 10-fold enrichment of the analyte.

# 3.3. Analytical figures of merit

#### 3.3.1. Limits of detection and calibration curves

Table 2 reports calibration range, slope of the calibration curves and limits of detection for the investigated compounds.

As reported in Table 2, detection limits in the range 1–3 ng/mL were observed for carbonyls, except for acetone (6 ng/mL). As for isoprostanes and prostanoids, the limits of detection were in the range 0.005–0.2 ng/mL and allowed to assess oxidative damage across human health outcomes [62]. The LODs were much lower than those reported in literature [63–65], as in the case of 15- $E_{2t}$ -IsoP and PGE<sub>2</sub>, whose LODs were as low as 0.01 ng/mL. Only Peña-Bautista et al. reported lower limits of detection (0.01–0.02 ng/mL) for  $F_{2t}$ -dihomo-IsoFs [61]. Finally, the five-points calibration curves (n = 3 of each concentration level) of all the investigated analytes showed a coefficient of determination (R<sup>2</sup>) higher than 0.995, indicating a good linearity over the tested concentration range.

## 3.3.2. Recovery and precision

The analytes recovery and the corresponding intra- and inter-day RSD values are reported in Table 3.

Analyte recovery was not significantly influenced (p > 0.05) by concentration level and was satisfactory for most of the analytes when performing both intra- (overall range: 79–123%) and inter-day (overall range: 82–119%) experiments (Table 3). For acetone, 2-butanone, butanal, hexanal, acrolein and methacrolein, the recovery was also confirmed by comparing the peak area of both standard solution and spiked saliva samples with those observed by injecting the TO11/1P-6A mixture, highlighting that the yield of reaction (%) was quantitative, as discussed

#### Table 4

Mean value of pH, flow rate and concentrations of target analytes measured in saliva samples collected from the enrolled swimmers athletes (n = 10) 5 min before the test ( $t_0$ ), at the maximal exercise peak ( $t_1$ ), and 2.5 ( $t_2$ ), 5 min ( $t_3$ ) and 10 ( $t_4$ ) minutes after the VO<sub>2max</sub>. Data reported as mean  $\pm$  standard deviation (range).

Characteristics	Value					
	t <sub>o</sub>	t <sub>1</sub>	t <sub>2</sub>	t <sub>3</sub>	t <sub>4</sub>	
pH	$6.8 \pm 0.3$	$6.8 \pm 0.4$	$6.8 \pm 0.4$	$6.9 \pm 0.4$	$6.9 \pm 0.3$	
	(6.3–7.2)	(6.1–7.6)	(6.1–7.4)	(6.5–7.4)	(6.5–7.4)	
Flow rate (mL/min)	$1.2 \pm 0.5 (0.7 - 2.0)$	$1.1 \pm 0.3$	$1.2 \pm 0.5$	$1.2 \pm 0.5$	$1.2 \pm 0.4$	
		(0.6–1.5)	(0.6–1.9)	(0.5–1.9)	(0.6–1.8)	
Acetone (ng/mL)	$150 \pm 108$	$415 \pm 152$	$355 \pm 138$	$235 \pm 112$	$175 \pm 101$	
	(125–250)	(350–560)	(245–453)	(165–320)	(110-220)	
2-Butanone (ng/mL)	$15 \pm 18$	$60 \pm 40$	$82 \pm 56$	$54 \pm 44$	$57 \pm 24$	
	(2-50)	(22–116)	(11–170)	(23–125)	(29–80)	
2-Pentanone (ng/mL)	99 ± 35	$197 \pm 93$	$234 \pm 113$	$180 \pm 85$	$203 \pm 70$	
	(50–133)	(102–334)	(110-401)	(78–310)	(156–307)	
Propanal (ng/mL)	$5 \pm 1$	$13 \pm 5$	$12 \pm 5$	$8 \pm 3$	6 ± 4	
	(3–6)	(8-21)	(4-20)	(4-11)	(4–9)	
Butanal (ng/mL)	9 ± 3	$14 \pm 5$	$10 \pm 4$	$14 \pm 11$	9 ± 4	
	(4-12)	(10–25)	(6-15)	(6-32)	(6-14)	
Hexanal (ng/mL)	< LOD	< LOD	< LOD	< LOD	< LOD	
Acrolein (ng/mL)	< LOD	< LOD	< LOD	< LOD	< LOD	
Methacrolein (ng/mL)	< LOD	< LOD	< LOD	< LOD	< LOD	
Glyoxal (ng/mL)	57 ± 35	76 ± 24	85 ± 40	$72 \pm 28$	$73 \pm 22$	
	(16–112)	(50-115)	(25–128)	(16-125)	(13–95)	
Methylglyoxal (ng/mL)	$12 \pm 9$	$20 \pm 5$	$25 \pm 16$	$25 \pm 24$	$19 \pm 18$	
	(2-22)	(17-30)	(2-48)	(4-57)	(5-45)	
15-F <sub>2t</sub> -IsoP (pg/mL)	$14 \pm 6$	$17 \pm 7$	$12 \pm 1$	$11 \pm 2$	$14 \pm 2$	
	(9-23)	(10-26)	(11-13)	(10–14)	(11–16)	
15-E <sub>2t</sub> -IsoP (pg/mL)	< LOD	< LOD	< LOD	< LOD	< LOD	
PGE <sub>2</sub> (pg/mL)	48 ± 43	81 ± 89	71 ± 45	$73 \pm 51$	$112 \pm 161$	
	(14–139)	(10-309)	(11-155)	(7–154)	(13-495)	
20-epi-20-F <sub>4t</sub> -NeuroP (pg/mL)	$3198 \pm 1663$	$1052 \pm 570$	666 ± 290	$1630 \pm 757$	$2131 \pm 1456$	
	(970-4804)	(370–1992)	(322-1153)	(822-2321)	(264-4362)	
$7(R,S)$ -ST- $\Delta^8$ -11-dihmo-IsoF <sup>a</sup> (pg/mL)	$360 \pm 166$	$277 \pm 104$	$295 \pm 119$	$296 \pm 10$	197 ± 28	
	(223–684)	(185–403)	(205–429)	(285–305)	(178–217)	

<sup>a</sup> Analyte concentration was estimated using spiked saliva samples calibration curve.

elsewhere [66]. An overestimated recovery up to about 200% was obtained for compounds belonging to the families of  $F_{2t}$ -dihomo-IsoPs and  $F_{2t}$ -dihomo-IsoFs (Table 3) and eluting at the end of the chromatographic run of *method 2*. These results suggested the presence of matrix effect giving rise to enhancement of the signal, as reported elsewhere [67].

The calculation of the overall intra- and inter-day RSD showed a satisfactory precision that was always lower than or equal to 17%, except for acetone with a value that reached up to 25% (Table 3).

### 3.3.3. Interferences and matrix effect

The shape and position of the analyte peaks were not affected by the presence of the saliva sample, which suggests the absence of any detectable matrix effect. This aspect was definitely confirmed by comparing the slopes of the calibration curves obtained with working solutions and spiked pooled saliva samples. For most of the analytes investigated, the slopes were not significantly different at a confidence level of 95%. The difference became strongly significant for 7(R,S)-ST- $\Delta^8$ -11-dihomo-IsoF, ent-7(R,S)-7-F<sub>2t</sub>-dihomo-IsoP and 17-F<sub>2t</sub>-dihomo-IsoP, with slopes of the "matrix" curves 2-fold higher than those of "solvent" curves due to a broad ion enhancement effect. The presence of ion enhancement, as already described by Sánchez-Illana et al. [63] and Peña Bautista et al. [61], led to an inaccurate quantitation of such analytes, unless of the use of the standard addition approach [68] or the employment of an analogue labelled internal standard [69]. The absence of commercially available deuterated analogues for most of these new isoprostanes and the laborious work required for their stereochemical synthesis [70,71] made their accurate quantitation still an open issue. In our opinion, the use of an internal standard different from the analogue, as widely suggested in literature [63,68,69], would not be sufficient to exactly mimic the behavior of the analyte during extraction and analysis, as reported by Chocholoušková et al. [72]. For all these reasons, a qualitative/semi-quantitative determination of F2tdihomo-IsoPs and  $F_{2t}$ -dihomo-IsoFs was proposed for the preliminary targeted screening analysis of saliva samples. Only after the assessment of the presence of these compounds in saliva samples and their effective role as potential indicators of OS, additional efforts would be evaluated in order to ensure their accurate quantitation, as already guaranteed for the majority of the analytes investigated.

#### 3.3.4. Stability

The stability of standard working solutions was evaluated at room temperature (25  $\pm$  1 °C) and 4 °C, whereas the stability of saliva samples was also studied at -80 °C. The initial concentration of the analytes (t = 0 h) was used as the reference values and the stability was evaluated using ANOVA model at a confidence level of 95%.

The stock solutions of ketones, aldehydes,  $\alpha$ , $\beta$ -unsaturated aldehydes and di-carbonyls were stable at 4 °C up to one week, longer storage times (i.e. 1 month) entailed a significant decrease (-60%) of aldehydes,  $\alpha$ , $\beta$ -unsaturated aldehydes and di-carbonyls peak areas due to the high reactivity of such chemicals in water solutions. A slight decrease (-20%) of ketones peak area was observed after one month. The standard aqueous working solutions of carbonyls were stable at room temperature and 4 °C for one day. Once derivatized, the carbonyl-2,4-DNPH adducts were stable at both room temperature and 4 °C up to one week. Carbonyls in saliva were stable at 4 °C up to one day and one month at -80 °C, and after one freeze-thaw cycle.

Standard stock solutions of isoprostanes and prostanoids were stable up to 1 month at -80 °C, whereas standard working solutions were stable within a working day at room temperature and up to 1 week at 4 °C. The methanol extract of saliva samples was stable throughout the duration of a typical sequence of UHPLC-MS/MS analysis (storage in the autosampler for about 24 h at 4 °C). Isoprostanes and prostanoids in saliva were stable after one week at 4 °C and three months at -80 °C, and after one freeze-thaw cycle.

# 3.4. Application to real samples: monitoring oxidative stress during an incremental cycle ergometer exercise test

The optimized procedures were employed to determine by-products of oxidative stress in saliva samples collected from ten swimmers performing an incremental cycle ergometer exercise test of maximal aerobic power. Table 4 reports the salivary characteristics measured in the volunteers' saliva samples before, at the maximal exercise peak and after the exercise session.

2-butanone, 2-pentanone, propanal, butanal, glyoxal, methylglyoxal, PGE<sub>2</sub>, 20-epi-20-F<sub>4t</sub>-NeuroP and 7(R,S)-ST- $\Delta^8$ -11-dihomo-IsoF were successfully detected in saliva samples of all ten swimmer athletes enrolled, whereas 15-F<sub>2+</sub>-IsoP was detected only in three subjects (i.e. V2, V3 and V9). Swimmers showed significantly higher (p < 0.05) mean value of propanal measured at the maximal exercise peak  $(t_1)$ (13 ng/mL) and at 2.5 min after the VO<sub>2max</sub> (t<sub>2</sub>) (12 ng/mL) compared to the level in the sample collected 5 min before the test (5 ng/mL). Acetone was significantly higher (p < 0.05) only at the maximal exercise peak (t1) (415 ng/mL vs 150 ng/mL at t0). In the same way, salivary levels of 20-epi-20-F4t-NeuroP were significantly decreased (p < 0.05) at t<sub>1</sub> and t<sub>2</sub> compared to the level measured at t<sub>0</sub>. Butanal and PGE<sub>2</sub> showed a slight increase (p > 0.05) of the mean value at t<sub>1</sub>, contrarily 2-butanone, 2-pentanone, glyoxal and methylglyoxal showed the highest values at 2.5 min after the  $VO_{2max}$  (t<sub>2</sub>). No significant trend overtime was observed for 15-F<sub>2t</sub>-IsoP and 7(R,S)-ST- $\Delta^8$ -11-dihomo-IsoF. These results are in accordance with the concept that exercise performed at high intensity causes an imbalance between ROS/RNS generation and antioxidant levels, leading to the production of byproducts of oxidative stress [10-12]. According to our results, it has been observed that exercise affects only some oxidative stress markers, as reported in literature [73]. Increasing and/or decreasing trends could be hidden by confounding factors, such as the intensity of the exercise and the training level of the subjects [74]. Thus, saliva analysis was hypothesized to be more effective when performing a longitudinal study, in which each subject acts as his/her own control [34].

Fig. 1 shows the clustering analysis results in the form of heatmap. For each swimmer the variations over time of salivary concentrations of the target analytes were normalized to values measured at the baseline sample ( $t_0$ ). The purple and red color in the plot represent the highest and lowest value measured, respectively.

All the swimmers showed a remarkable increase (2 to 3-fold) of 2butanone, 2-pentanone and propanal concentration in saliva at  $t_2$ . For all these analytes, a sharp decrease, approaching baseline values, of the salivary levels was observed at 5 min ( $t_3$ ) and 10 min ( $t_4$ ) after the maximal exercise peak ( $t_1$ ). Thus, these findings suggest that 10 min of active recovery were adequate to restore the balance between radical generation and antioxidants levels, confirming the high fitness level of the enrolled volunteers. Thus, one could speculate that exercise plays a beneficial role against oxidative stress because of its ability to boost up the antioxidant defense mechanisms.

Interestingly, the degree of increase for 2-butanone and 2-pentanone was positively correlated (p < 0.05) with the respiratory quotient measured at the peak exercise. Such increase might support the hypothesis that during exercise the human metabolism needs to produce energy from any metabolic source to maintain the physical performance. In addition, the observed correlation points out a direct shift in carbohydrate fatty acid metabolism over the course of performance.

Acetone, glyoxal, methylglyoxal, 20-*epi*-20-F<sub>4t</sub>-NeuroP and 7(*R*,*S*)-ST- $\Delta^{8}$ -11-dihomo-IsoF showed different behavior. As an example, the salivary levels of acetone, glyoxal and methylglyoxal did not show significant variation overtime in all the swimmers, whereas for 20-*epi*-20-F<sub>4t</sub>-NeuroP and 7(*R*,*S*)-ST- $\Delta^{8}$ -11-dihomo-IsoF a decrease between the baseline level and the exercise peak was observed. Also García-Flores et al. found a reduction of F<sub>2</sub>-dihomo-IsoP metabolites (i.e. 17-F<sub>2t</sub>-dihomo-IsoP and 17-*epi*-17-F<sub>2t</sub>-dihomo-IsoP) with the increase of a triathlon training [25]. A possible explanation could be that a regular aerobic moderate training could increase the resistance against oxidative stress to favor antioxidant capacity in the brain [75]. These results confirmed the complexity of the metabolic pathways involved in the production of these oxygenated metabolites of polyunsaturated fatty acids.

Our analytical approach allows both to evaluate the epigenetic response to sport in terms of increasing antioxidant systems and to obtain useful information on the severity and evolution of several pathologies such as heart failure and diabetes [5,6].

#### 4. Conclusions

This paper reports two validated analytical procedures for the determination of carbonyls, isoprostanes and prostanoids in stimulated saliva samples collected from athletes performing an incremental cycle ergometer exercise test of maximal aerobic power. Overall, our methods showed satisfactory limits of detection (i.e. units of ppb for carbonyls and tens to hundreds of ppt for isoprostanes and prostanoids), quantitative recovery (90–110%) and intra- and inter-day precision lower than 15%.

Preliminary results were obtained by monitoring ten swimmers performing a  $VO_{2max}$  cycle ergo meter test. A significant increase (3–15fold) of most of the target analytes during exercise with a sharp decrease up to the baseline levels 10 min later the maximal exercise peak was observed. Nevertheless, some inconsistencies of the results were observed, probably due to the training level of the subject and to different eating habits. Moreover, also the large inter-individual variability in exercise-induced oxidative stress may be considered as a confounding factor. Since this large inter-subject variability is not a new concept, we postulated that saliva analysis could be more successful if longitudinal monitoring was performed for each subject over time more than referring to nominal "reference" values. In this way, each subject



**Fig. 1.** Heatmap of salivary levels measured for all the enrolled swimmers athletes (n = 10) during the cycle ergometer exercise test of maximal aerobic power. Data were normalized with respect to the baseline sample ( $t_0$ ). Each square represents a collection point.

would act as his/her own control.

These results strongly support the hypothesis that saliva analysis can be used as a non-invasive tool to monitor oxidative stress in patients with various pathologies and in subjects during exercise, helping to obtain useful information on the progress of the disease and on the adaptation to the physical exercise. Further investigations are planned, working on a wider population, in order to increase the statistical power of our results and try to confirm the observed trends.

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### CRediT authorship contribution statement

Denise Biagini: Methodology, Validation, Formal analysis, Writing - original draft. Tommaso Lomonaco: Conceptualization, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration. Silvia Ghimenti: Methodology, Validation, Resources. Jonathan Fusi: Formal analysis, Supervision, Project administration. Eugenio Cerri: Formal analysis, Supervision. Francesca De Angelis: Methodology, Writing - original draft. Francesca Giuseppa Bellagambi: Methodology, Writing - original draft. Camille Oger: Formal analysis, Writing - original draft. Jean Marie Galano: Formal analysis, Writing - original draft. Emilia Bramanti: Formal analysis, Writing - review & editing. Ferdinando Franzoni: Formal analysis, Supervision. Roger Fuoco: Formal analysis, Writing - review & editing, Supervision, Funding acquisition. Fabio Di Francesco: Formal analysis, Writing - review & editing, Supervision, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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