

Salivary and serum irisin in healthy adults before and after exercise

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Abstract

Irisin is an exercise-induced cytokine mainly secreted by myocytes. Circulating level of irisin can increase in response to acute exercise, promoting pleiotropic effects on health. Generally, irisin is evaluated in blood, however, its collection is invasive. Saliva sample would not have any risk associated with blood collection and would represent a less invasive method for irisin detection. Until now, there are only a few studies that have analyzed irisin levels in saliva. In the present research, five healthy male adults performed an incremental exercise until exhaustion on cycle ergometer. Serum and saliva samples were collected before exercise and 15min, 24h and 48h after reaching the exhaustion. Irisin was detected by ELISA assay. Serum and salivary irisin levels increased from baseline to 24h post exercise and reverted to basal levels after 48h of rest. A significant rise of both serum and salivary irisin level at 24h ($p \leq 0.05$) compared to baseline levels was found. Furthermore, a significant correlation between irisin percentage change in serum and saliva from baseline to 24h post exercise was detected ($r=0.92$, $p < 0.05$). Despite the relatively limited sample, this research suggests that collecting saliva samples might represent a valid and less invasive method to detect irisin level changes induced by exercise.

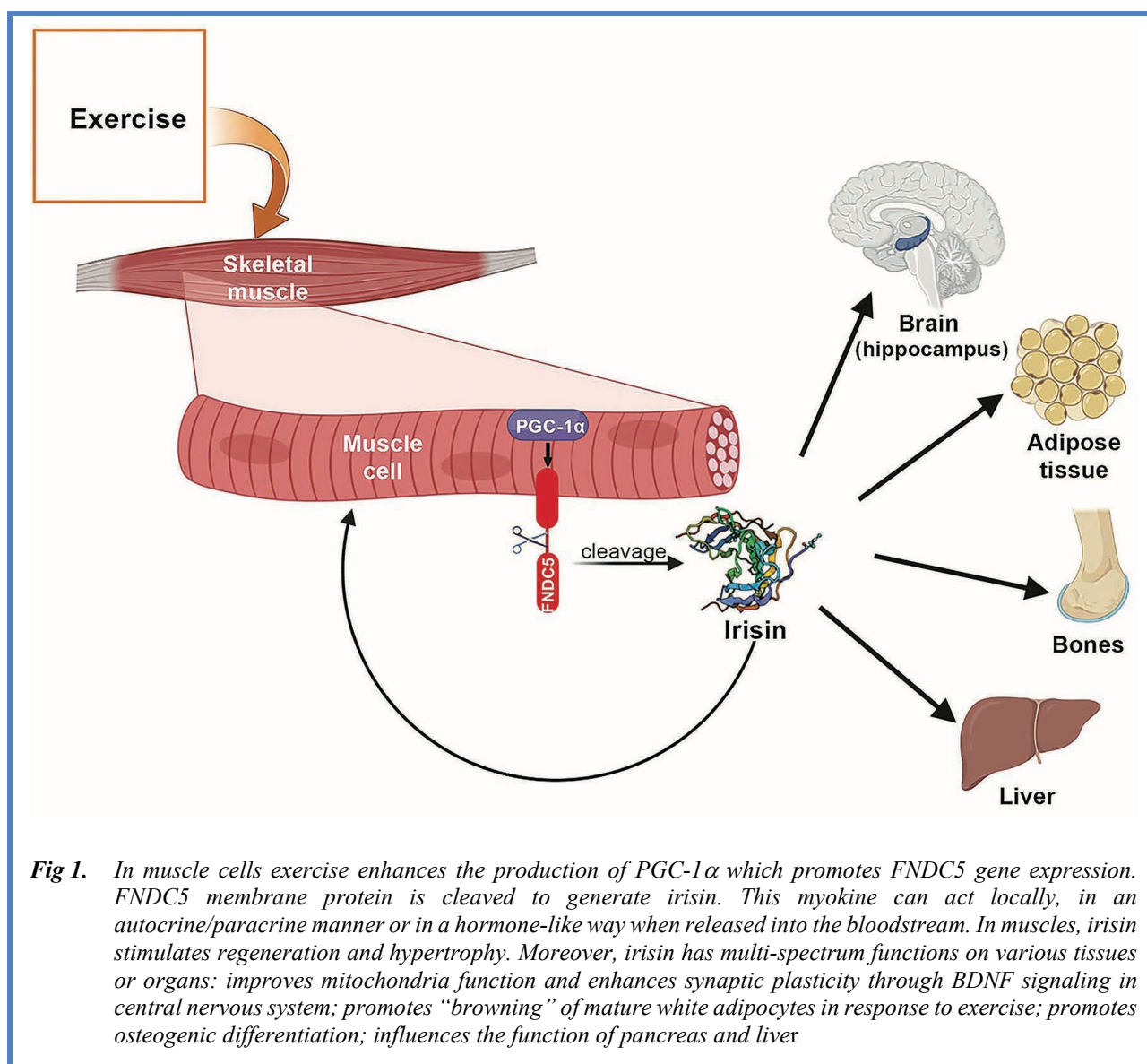
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Ten years ago, a novel myokine called irisin, usually secreted by exercising muscles, was discovered.¹ Physical exercise induces an increase of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) that determines Fibronectin type III domain-containing protein 5 (FNDC5) expression. Irisin is obtained through a proteolytic cleavage of FNDC5 protein and it is released into the bloodstream.¹ Primary roles of irisin include browning white adipose tissue, improving liver and systemic glucose metabolism, keeping bone and musculoskeletal homeostasis, and playing a neuroprotective function (Figure 1).² This myokine modulates muscle cells activity in an autocrine or paracrine manner.³ *In vitro* studies demonstrated the ability of irisin to stimulate the expression of specific mitochondrial transcription factors, resulting in mitochondrial content and oxygen consumption increase. Moreover, irisin can play a regulatory role in muscle growth and differentiation. Indeed, it stimulates muscle

regeneration and hypertrophy through the activation of satellite cells, and promotes the improvement of muscle function, mass and healing.^{2,4-6}

Irisin is generally analyzed in blood,⁷ the best body fluid for evaluation of different biomarkers.⁸ However, blood collection exposes to potential risks the individuals, including discomfort of needle, infection at the venipuncture site, and bruising. Moreover, it is also less preferred in research involving children, elderly, as well as patients for whom venous access is not easy. As a result, an alternative method for irisin detection appears desirable. Collection of saliva holds several advantages, such as being a noninvasive, easy, safe, and cost-effective procedure that can be applied to large populations compared to conventional venipuncture procedure and requires no special qualified personnel. In addition, participants' acceptance of saliva sample collection might be much better when compared to obtaining serum or biopsy samples.



Saliva is the product of secretion from the salivary glands.⁹ Moreover, it is known that some molecules enter the saliva from the blood by passing through the intercellular spaces using a passive diffusion process, filtration, or active transport.¹⁰ These molecules, such as miRNAs, can be used as diagnostic biomarkers of tumors, autism, Parkinson disease and sleep disorder.¹¹ Only a few studies have investigated the irisin level in saliva.¹²⁻¹⁷ Findings have shown that this myokine is present in a different concentration over blood. Despite this, saliva sample seems to represent a valid biological specimen for irisin detection. The limited number of studies, measuring irisin in saliva, present many limitations regarding the collection and analysis of samples. Indeed, there are some variables that can significantly influence the results, like the method of collection as well as the degree of stimulation.¹⁸ Moreover, as the consistency of saliva may vary within a

person and between people,¹⁹ this aspect needs to be considered when analyzing irisin and validating a possible method for exercise-induced cytokine detection. Finally, several commercial kits for irisin detection are available, nevertheless, not every kit is extremely sensitive and capable of detecting irisin with high specificity.¹³

In this study we analyzed the irisin levels in saliva and serum samples obtained from healthy adults before and after an exercise protocol. Moreover, we verified whether there was a correlation between the level changes of salivary and serum irisin in these subjects.

Materials and Methods

Five healthy males ages 20-55 years, mean body mass index (BMI) 22.9 \pm 3.7 kg/m², peak of oxygen consumption 54.0 \pm 16.5 mL/kg/min were recruited.

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Procedures of present study was approved by the Ethics Commission of the Department of Psychology (CERPS) of the Catholic University of the Sacred Heart of Milan and written informed consent for study participation, permission for personal data treatment and biochemical analysis was obtained from all participants upon enrolment. This study was conducted in accordance with the ethical standards of the responsible committee on human experimentation and the ethical principles established in the Declaration of Helsinki of 1975, as revised in 2013.

Anthropometric parameters included weight and height measurement, following the procedures described by Gordon et al.,²⁰ and the calculation of BMI.

Participants were asked to arrive in the laboratory after 2 h fast and 48 h of rest. All the participants performed an incremental exercise until exhaustion on a cycle ergometer. The exercise took place in a room with a relative humidity less than 60% and a temperature ranging from 18°C to 22°C.

To avoid the influence of circadian variation, assessments took place at the same time of the day, from 9:00 a.m. to 1:00 p.m. Four samples of peripheral venous blood (3 mL each one) and saliva (1.5 mL each one) were drawn simultaneously at each sampling time. First samples were collected at baseline, second samples were obtained 15 min after reaching the exhaustion during the maximal exercise test, third samples were taken approximately 24 h after and the last samples were

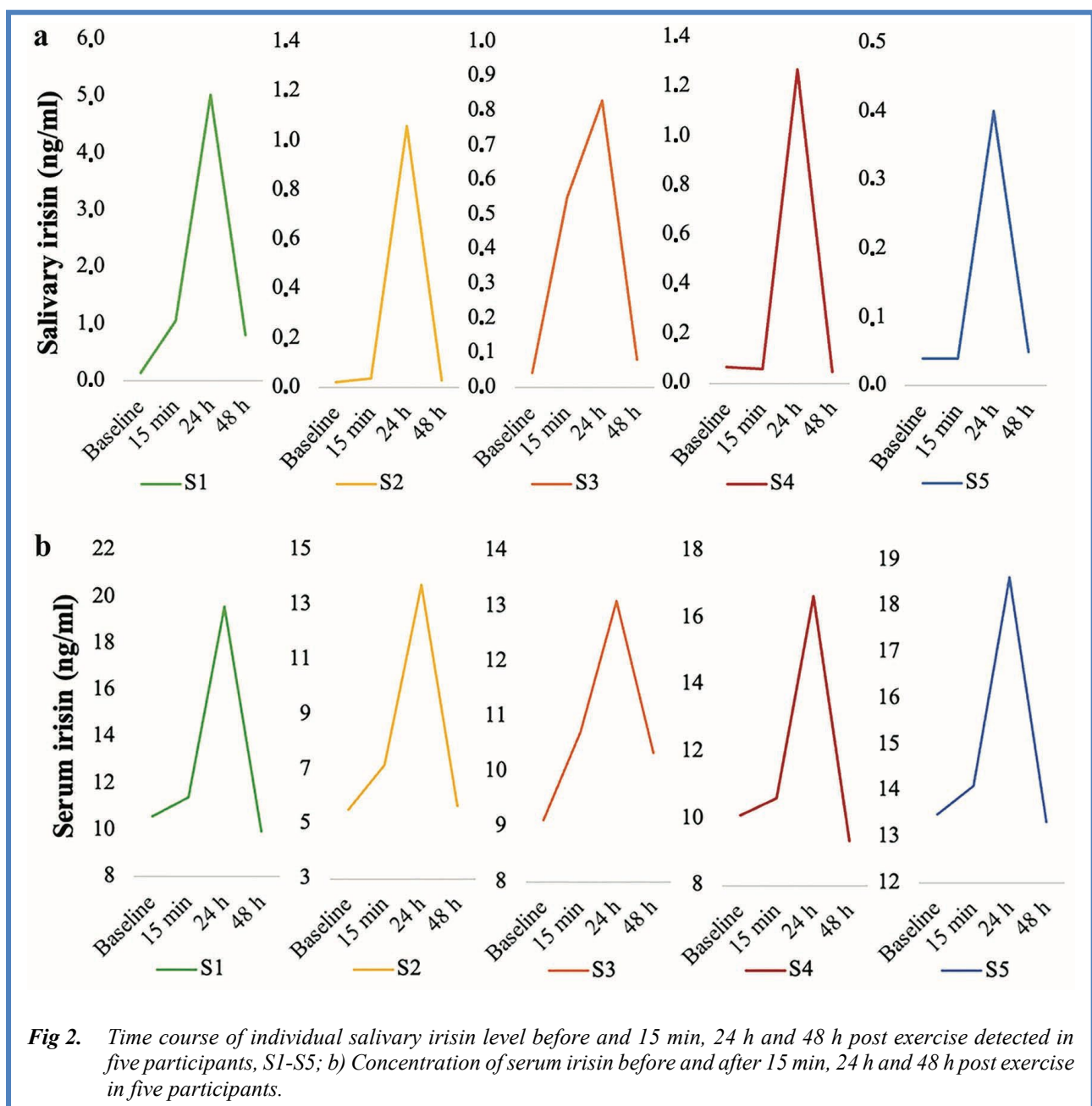


Fig 2. Time course of individual salivary irisin level before and 15 min, 24 h and 48 h post exercise detected in five participants, S1-S5; b) Concentration of serum irisin before and after 15 min, 24 h and 48 h post exercise in five participants.

collected after 48 h of rest. Vacutainers were drawn using a vacutainer system and were immediately centrifuged at 10,000 rpm for 10 min. Soon after, serum was separated and stored at -80°C for subsequent analysis. After the oral rinse with water, participants were asked to drool into a sterile Eppendorf tube. Saliva samples were quickly centrifuged at 10,000 rpm for 2 min and kept at -80°C . In saliva samples, protein concentration was quantified using Bradford Assay (Bio-Rad). Total protein amount was used to normalize the irisin concentration in saliva samples. Serum and saliva irisin levels were detected by ELISA (Irisin kit: Cat. EK-067-29, Phoenix Pharmaceuticals), according to the manufacturer instructions, using the Victor Nivo multimode plate reader (PerkinElmer). Inter-assay variation for irisin was $<10\%$. SPSS software (Version 27) was used for statistical analyses. The effect of exercise on irisin level was analyzed by non parametric test (Wilcoxon signed-rank test). Spearman's correlation analysis was used to assess the relationships between serum and saliva irisin

percentage change. The limit for statistical significance was always considered $p < 0.05$.

Results and Discussion

Primary purpose of the current research was to evaluate the effect of an exercise protocol on irisin levels in saliva and serum of healthy adults. We assessed serum and salivary irisin in 5 males before and 15 min, 24 h and 48 h post exercise. Figure 2a and b shows salivary and serum irisin profile of each participant before and after exercise, evidencing that physical activity causes a consistent increase of this myokine. Salivary irisin, normalized for total protein content, (baseline: 0.06 ± 0.05 ng/ml; 15 min: 0.35 ± 0.45 ng/mL; 24 h: 1.71 ± 1.87 ng/mL) and serum (baseline: 9.77 ± 2.87 ng/mL; 15 min: 10.80 ± 2.48 ng/mL; 24 h: 15.93 ± 2.42 ng/mL) increased from baseline to 24 h post exercise, and then the irisin concentration decreased up to 48 h post exercise (saliva 48 h: 0.20 ± 0.33 ng/mL; serum 48 h: 9.72 ± 2.75 ng/mL). A significant difference of serum irisin levels at 15 min ($p \leq 0.05$) and 24 h ($p \leq 0.05$), and of salivary irisin levels at 24 h ($p \leq 0.05$)

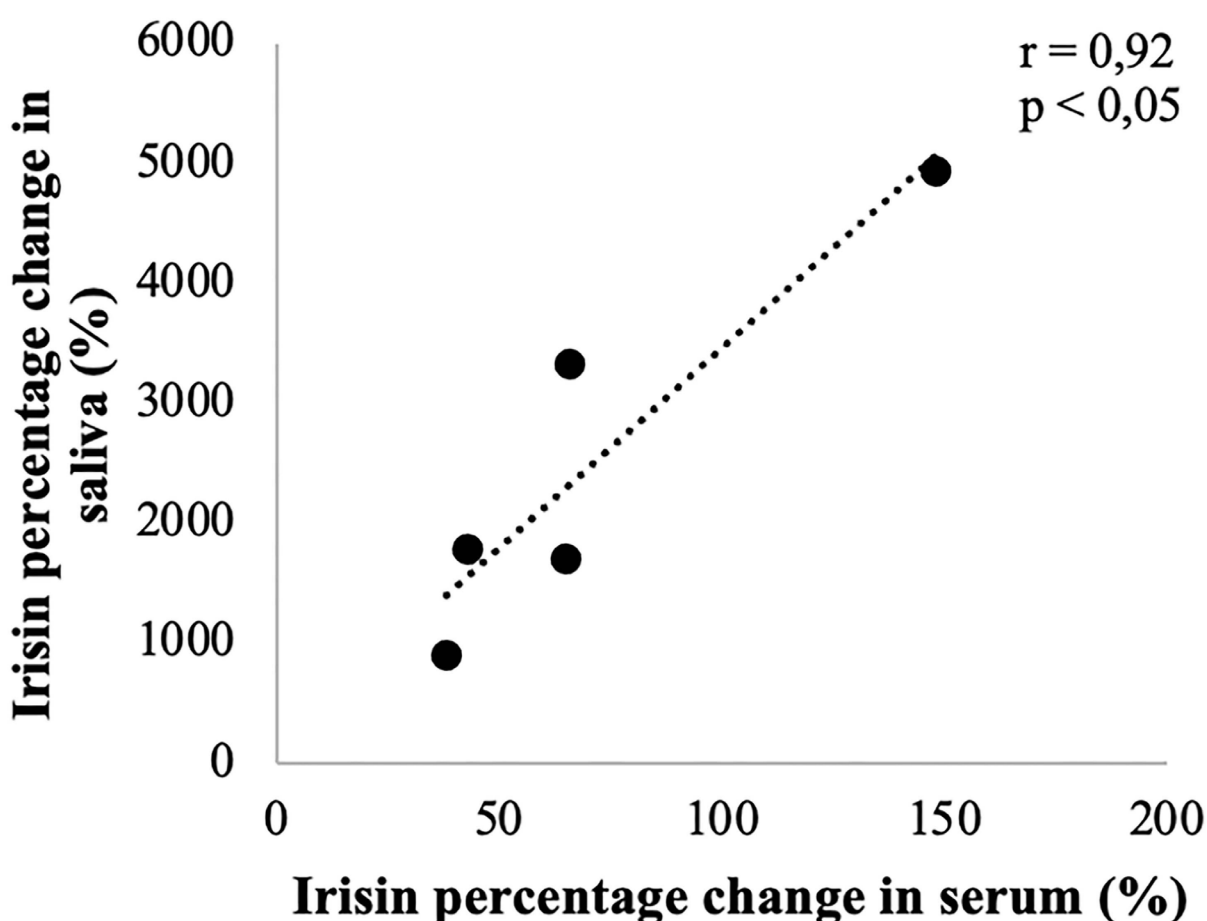


Fig 3. Correlation between irisin percentage change in saliva and serum from baseline to 24 h postexercise in 5 subjects

compared to baseline levels was detected. Data in Figure 2 also indicates that irisin is more concentrated in serum in comparison with saliva.

The second purpose of this study was to ascertain a possible correlation between the expression level of serum and salivary irisin induced by physical exercise. As shown in Figure 3, a significant and positive correlation was found between irisin percentage change in serum and saliva from baseline to 24 h post exercise ($r=0.92$, $p<0.05$). Hence, the results reported here show that changes in irisin levels can be detected in both serum and saliva after an incremental exercise. Furthermore, the variations in irisin present the same trend in both biological samples. However, in order to achieve reliable results, we set up an experimental plan that took into account different variables for the quantification of salivary irisin: i) method of collection; ii) hydration; iii) sensitive and specific method for irisin detection.

i) It has been demonstrated that the degree of stimulation (active or passive) and the method of collection influence the relative contribution of the different salivary glands to whole saliva and can modify saliva analytes contents.^{21,22} Ravara et al. pointed out the importance to standardize the methods for collection, processing, and storage of saliva specimens to ameliorate the use of this body fluid in clinical analysis.²² In previous studies researchers indifferently used unstimulated or stimulated methods to obtain whole saliva samples for irisin analysis.^{12-14,16,17} As recently Jasim et al., by comparing different saliva collection approaches, identified stimulated whole saliva as the simplest and with low variability method for analyses of biomarkers,¹⁸ we also collected saliva through a self-stimulated approach.

ii) None of the previous research standardized salivary irisin levels by considering the intra- and interpersonal diversity of total salivary protein amount, like indicator of hydration status. For this reason, contrasting results were reported. Indeed, some studies have revealed that salivary irisin levels were higher than blood levels in healthy adults.^{12,13} Instead, Bakal and colleagues,¹⁵ assessing healthy subjects and patients with acute appendicitis and abdominal pain, have found similar irisin concentration in blood compared to saliva. In another study,¹⁴ the total protein concentration in saliva from five Prader Willi Syndrome patients and five healthy controls was previously quantified. However, the amount of proteins has not been considered for standardizing the saliva concentration, despite patients' saliva was thicker, and total protein concentration was higher compared to that of healthy controls.

iii) Finding from this study revealed that salivary irisin levels in both controls and patients were higher than serum.¹⁴ Considering the interpersonal and intrapersonal differences of the saliva consistency, we

quantified the total protein concentration in saliva samples, and we related the abundance of the target protein (irisin) to the total protein amount in each sample. This approach takes account of the state of hydration of participants.

iv) There is no consensus for the normal concentration of circulating irisin in humans. This could be due to the use of different ELISA kits, determining enormous variation in irisin quantification. The best method to measure irisin levels is still quantitative mass spectrometry. Nevertheless, it has been shown that some commercial ELISA kits allow to obtain results similar to those of mass spectrometry.²³ We have measured irisin levels using an ELISA kit that has been validated by Western blot, which has visualized three bands (15, 20, and 26 kDa), and the amino acid sequence of the molecules isolated from each of the three bands has been verified via mass spectrometry.²⁴

In conclusion, we have focused on the potential role of saliva to detect irisin changes induced by physical exercise. In serum and saliva samples of all assessed participants, increased levels of this myokine 24 h post exercise were observed, which reverted to baseline levels after 48 h of rest. A key strength of the study is the use of a high sensitive and specific kit for irisin detection and the normalization of salivary irisin level with total protein amount within a sample, allowing us to obtain standardized results, not dependent on the different saliva concentration, due to the "personal hydration status". This study is limited by the relatively small number of subjects involved.

Nevertheless, the finding, while preliminary, suggests that collecting saliva samples might represent a valid method for quantify irisin level changes in response to exercise and can be used especially for frail population or when a time course analysis is required. Future studies conducted on a larger sample size should be conducted to confirm these preliminary observations.

List of acronyms

BMI - body mass index

CERPS - Ethics Commission of the Department of Psychology

FNDC5 - Fibronectin type III domain - containing protein 5

PGC1- α - PPAR γ -coactivator-1- α

Contributions of Authors

SM collected blood and saliva samples, performed irisin evaluation and edited the manuscript; ET collected exercise data and wrote the manuscript; PV enrolled the participants; CP conceived physical exercise protocol; PV, ET and CG supervised physical exercise; AS performed protein quantification; AM critically reviewed biological data; DT conceived the study, supervised it and revised the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare they have no financial, personal, or other conflicts of interest.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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