



RESEARCH ARTICLE

Impact of 10-day bed rest on serum levels of irisin and markers of musculoskeletal metabolism

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Abstract

The bed rest (BR) is a ground-based model to simulate microgravity mimicking skeletal-muscle alterations as in spaceflight. Molecular coupling between bone and muscle might be involved in physiological and pathological conditions. Thus, the new myokine irisin and bone-muscle turnover markers have been studied during and after 10 days of BR. Ten young male individuals were subjected to 10 days of horizontal BR. Serum concentrations of irisin, myostatin, sclerostin, and haptoglobin were assessed, and muscle tissue gene expression on vastus lateralis biopsies was determined. During 10-days BR, we observed no significant fluctuation levels of irisin, myostatin, and sclerostin. Two days after BR (R+2), irisin serum levels significantly decreased while myostatin, sclerostin, and haptoglobin were significantly increased compared with BR0. Gene expression of myokines, inflammatory molecules, transcription factors, and markers of muscle atrophy and senescence on muscle biopsies were not altered, suggesting that muscle metabolism of young, healthy subjects is able to adapt to the hypomobility condition during 10-day BR. However, when subjects were divided according to irisin serum levels at BR9, muscle ring finger-1 mRNA expression was significantly lower in subjects with higher irisin serum levels, suggesting that this myokine may prevent the triggering of muscle atrophy. Moreover, the negative correlation between *p21* mRNA and irisin at BR9 indicated a possible inhibitory effect of the myokine on the senescence marker. In conclusion, irisin could be a prognostic marker of hypomobility-induced muscle atrophy, and its serum levels could protect against muscle deterioration by preventing and/or delaying the expression of atrophy and senescence cellular markers.

Abbreviations: ASI, Space Agency; BDC1 and BDC2, 1 and 2 days pre-BR day; BMD, bone mineral density; BMI, body mass index; BR, bed rest; CSA, cross-sectional area; ERK, extracellular signal-regulated kinase; *FNDC5*, fibronectin type III domain-containing protein 5; HDT, head-down tilt; IGF-1, insulin-like growth factor-1; IL-6, interleukin-6; ISS, International Space Station; *MuRF1*, muscle ring finger-1; OP, osteopenia/osteoporosis; R+1 and R+2, 1 and 2 days after the end of BR; SST, serum separator tubes; *Tfam*, mitochondrial transcription factor A; TGF β , transforming growth factor beta; TNF- α , tumor necrosis factor-alpha; TVP, deep thrombosis; VL, vastus lateralis.

Angela Oranger and Giuseppina Storlino contributed equally to this work.

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KEYWORDS

bed rest, cell senescence, haptoglobin, irisin, muscle atrophy, myostatin, sclerostin

1 | INTRODUCTION

Exposure to space environment induces many human body adaptations thereby altering the physiology of different organs and systems such as musculoskeletal one.

During space flight, changes occur over a period of weeks to months, first in the muscle and then in the bone. It has been shown that after the first week of space mission, astronauts showed a 4%–8% drop of muscle volume.¹ Other authors showed that 5–11 days in space were sufficient to observe a size decrease of all muscle-fiber types from the vastus lateralis (VL) muscle.^{2,3} Moreover, it has been well established that space flight determines the loss of 1%–2% bone mass per month compared to pre-flight and ground controls.^{4,5} Microgravity condition reduces the loading on weight-bearing bones, resulting in adaptive changes that induce loss of bone integrity, calcium and phosphate homeostasis alteration and calcium absorption decrease.⁶

Due to both the infrequent mission launches and the difficulties of studying changes in human physiology on astronauts during and after space flight missions, ground models that partially simulate micro- and hypo-gravity have been used in biomedical research. One of these, which has been widely validated, is the bed rest (BR) in humans^{7,8} which determines an important minimization of weight-bearing activity on all postural body tissues.⁹ However, this model is not appropriate for the study of cardiovascular adaptations, given that failed to simulate fluid redistribution towards the head, as really occur in in-flight astronauts. Thus, when cardiovascular evaluations have been necessary, head-down tilt (HDT) angles range from 4 to 15 have been adopted in numerous human BR study.¹⁰ Both BR and HDT were considered microgravity simulation techniques suitable also for long-term studies.¹¹ However, compared with spaceflight, gravitational force on bodyweight is not lost, and in parallel there is an extensive compression of the skin surface against the bed, which is absent in astronauts.¹¹ In any case, it appears well established that the BR model is appropriate to study multisystem effects of reduced physical activity and unweighting.⁷ Indeed, the observations on the musculoskeletal system in studies on human model of simulated microgravity on ground, as bedridden condition, are largely congruent to data obtained from spaceflight experiments. There is evidence that in this ground model, VL and soleus atrophy characterized by reduced cross-sectional area (CSA) and fascicle length are observed.^{9,12}

Some studies demonstrated a decrease in CSA of about 20% in older subjects as early as after 5 days of BR,^{13–15} while in two other studies the CSA decrease was slightly lower (15%) and occurred later (14 days BR) in both middle-aged¹⁶ and older adults.^{17,18} Skeletal muscle atrophy correlated to CSA decrease occurs when the balance between protein synthesis and degradation is altered in favor of the degradation rate,¹⁹ and atrophy related-genes such as F-box only protein 32 Atrogin-1 and muscle ring finger-1 (*MuRF1*) are involved in muscular ubiquitination and protein degradation.²⁰ Interestingly, literature data suggest a link between muscle atrophy and haptoglobin, the major serum hemoglobin-binding protein which is highly produced in response to the acute phase of inflammation. Indeed, a study performed on haptoglobin knockout young mice suggested that degradation systems contribute to muscle atrophy in haptoglobin KO mice by increasing the expression of *MuRF1*.¹⁹ Importantly, the negative regulator of muscle growth myostatin, a member of the transforming growth factor beta (TGF β) family expressed and secreted by skeletal muscle, if up-regulated, it is responsible for muscle atrophy.²¹

The role of skeletal muscle as a secretory organ and the effects of its myokines exerted in an autocrine/paracrine and endocrine fashion on various tissues have emerged over the last decade. Irisin is one of the recent myokines released into circulation during skeletal muscle contraction. Its precursor undergoes proteolytic cleavage of the extracellular domain releasing a small peptide, irisin, into the bloodstream. This myokine acts as an exercise-mimetic hormone playing key functions in the whole-body metabolism.²² In vitro studies on C2C12 myoblast cell lines showed that treatment with rec-Irisin resulted in a significant increase in mitochondrial biogenesis and content, and oxygen consumption²³; in human myocytes irisin reduced *myostatin* gene expression.²⁴ Recent in vitro study, performed under microgravity conditions, demonstrated the anabolic effect of irisin on osteoblasts, thus providing a first step that might suggest a way in preventing bone loss in astronauts.²⁵ Moreover, in vivo data on hindlimb unloaded mice, a recognized animal model of simulated microgravity on ground, revealed that irisin promotes osteogenesis, increases osteocyte viability, prevents disuse-induced loss of bone and muscle mass, and accelerates fracture healing.^{26,27} On humans it has been proved a positive correlation between irisin and bone mineral density (BMD) in healthy young adult athletes,²⁸ while in adults with prediabetes, irisin inversely correlated

with serum sclerostin levels,²⁹ a well-known inhibitor of bone formation. Irisin was also negatively associated with vertebral fragility fractures and with sarcopenia^{30,31} in postmenopausal women. A few years ago, we showed that in humans irisin serum levels negatively correlated with age and its levels are lower in patients with osteopenia/osteoporosis (OP) compared to healthy controls. Moreover, analyzing the senescence marker *p21*, it has been found a significant increase in its mRNA expression in the bone biopsies of OP patients compared to control ones.³²

In accordance with literature data, we hypothesized that the myokine irisin might be involved in preserving the integrity of the musculoskeletal system under unloading conditions.

Therefore, we agreed with the Capodistra Research Centre on a 10-day BR study on 10 volunteer subjects on whom we studied during and after the BR period first the serum levels of irisin and known markers of musculoskeletal atrophy and inflammation, then the possible correlations of irisin with markers of atrophy and senescence.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was part of the Italian Space Agency (ASI) project 'MARS-PRE Bed Rest SBI 2019'. The study was performed following the criteria established during the last revision of the Declaration of Helsinki. The study was approved by the National Ethical Committee of the Slovenian Ministry of Health (ref. number: 0120-304/2019/9). Subjects participating in the study were made aware of all the procedures involved in the study and underwent a complete medical evaluation. The volunteers were administered a questionnaire and interview by appropriate committee. The subjects were informed of the possibility to interrupt the study at any time. Following the signature of informed consent, subjects were enrolled and submitted to the study.

2.2 | Subjects

This study was conducted in the General Hospital of Izola (Izola, Slovenia). Ninety-three requests for participation from young males were received for this study. Of this first ninety-three, only forty-six were subsequently interviewed, and sixteen passed all the inclusion/exclusion criteria and the medical examination. The inclusion/exclusion criteria are listed below. Ten homogenous subjects were enrolled in the study in order to obtain a statistical power >0.85. An additional two subjects were considered

as a reserve for the study. The sample size was determined based on statistical power analysis using the G-power3 calculator, imposing power values (1- β), α levels and effect size to reach a probability of (1- β) obtained from previous studies.

In the study, these 10 male individuals (20–30 years, mean 23 ± 5 years; body mass: 77.5 ± 10.0 kg; body height: 1.81 ± 0.04 m; body mass index (BMI): 23.5 ± 2.5 kg·m⁻²) were recruited and subjected to 10 days of horizontal BR.

Healthy individuals were recruited following approval by the local ethics committee. All patients were subjected to horizontal BR for 10 days in standard air-conditioned hospital rooms of the General Hospital of Izola and were provided constant surveillance and 24-h medical care. Each subject was evaluated from two days before to two days after 10 days of BR. Two days before the BR, the subjects adapted to the environment and diet but remained in an orthostatic position. During BR no changes from lying position, muscle stretching, or static contractions were allowed while during the two days post-BR, subjects remained in bed or wheelchair. Each volunteer was screened and those who had any of the following conditions were excluded: chronic diseases requiring clinical treatment, habitual intake of drugs and/or medicines, regular smoking, blood coagulation defects, previous history of deep thrombosis (TVP) with D-dimer values >500 μ g/L, acute and chronic bone, neuromuscular, cardiovascular and metabolic conditions; previous history of embolism, inflammatory diseases, psychiatric disorders, epilepsy, participation in agonistic sports and ferromagnetic installation.

2.3 | Bed rest

Groups of five subjects each were placed in air-conditioned hospital rooms. Also, participants were constantly controlled and monitored during the 24 h. All participating subjects were maintained on a controlled eucaloric diet receiving three meals a day. Diet energy requirement was established for each individual subject taking into account their BR condition.³³ The macronutrient composition was 60% carbohydrate, 25% fat, and 15% protein. During the entire duration of the experiment, the volunteers performed all daily routine activities in bed, without being able to remove themselves from the supine position of the BR. No muscle contraction tests, or any form of physical exercise were permitted during the BR. The BR subjects slept from 10:00 pm to 7:00 am.

Our protocol included 2 days of adaptation to the environment and diet, Pre-BR Day (BDC2) for collection of all control parameters, 10 days of BR and 2 days of recovery (R+1 and R+2). During the entire course of the

experiment, serum was collected from each subject and muscle biopsies were taken at times BR0 and BR9.

2.4 | Biochemical measurements

Blood samples were always collected fasting, at 7.00 a.m., on the day of interest. Since a previous study reported a possible day-night fluctuation of irisin synthesis in humans, with its lowest levels early in the morning and its peak levels around 9:00 pm,³⁴ we opted for a morning blood draw ruling out possible physiological spikes later in the day, unrelated to BR status. Blood was collected into serum separator tubes (SST) and allowed to clot for 30 min at room temperature before centrifugation for 15 min at 1000 g. After centrifuging, the samples were aliquoted and stored at -80°C until analysis.

Irisin serum concentrations were detected using a competitive ELISA kit (Adipogen, Liestal, Switzerland) with an inter-assay coefficient of variation $\leq 6.9\%$. The ELISA kit allows the largest range of measurement (0.001–5 $\mu\text{g}/\text{ml}$) and is the most sensitive (0.001 $\mu\text{g}/\text{ml}$). The ELISA kit includes a polyclonal antibody recognizing naïve Irisin and recombinant Irisin under competition in Irisin-coated plates.

Sclerostin serum levels were detected using the enzyme immunoassay ELISA kit (Biomedica, Wien, Austria). The ELISA kit allows a range of measurement (0–240 pmol/L) and the sensitivity is 3.2 pmol/L. Regarding cross-reactivity, the assay does not detect SOSTDC1/Nogging and does not cross-react with rat or mouse sclerostin.

The Myostatin assay employs the quantitative enzyme immunoassay technique. Antibody specific for myostatin has been pre-coated into a microplate (Cusabio, Wuhan, Hubei, China). The detection range is 0.625–20 ng/ml. For the sensitivity of this kit, the minimum detectable dose of human myostatin typically $<0.312\text{ ng}/\text{ml}$. This assay has high sensitivity and excellent specificity for detection of the total form of human myostatin. No significant cross-reactivity or interference between human myostatin and analogues was observed.

Haptoglobin serum levels were detected with quantitative sandwich enzyme immunoassay technique. Monoclonal antibody specific for human Haptoglobin has been pre-coated into a microplate (R&D systems, Minneapolis, US). The ELISA kit allows a minimum detectable range of measurement (0.031–529 ng/ml) and the mean of minimum detectable dose was 0.192 ng/ml. This assay recognizes human Haptoglobin, especially human Haptoglobin 1–1 and Haptoglobin 2–2, also no significant cross-reactivity or interference was observed in this assay.

For all assays (Irisin, Sclerostin, Myostatin and Haptoglobin), the colorimetric reaction was measured

using a spectrophotometer (Eon, BioTek, Winooski, Vermont) at the end of the assay.

2.5 | Real-time PCR

Muscle biopsies were homogenized with ultra-turrax T8 (Ika, Staufen im Breisgau, Germany). Total RNA was obtained using spin columns (RNeasy, Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase I treatment was used to remove genomic DNA contamination (Qiagen, Hilden, Germany) and RNA integrity was tested on agarose gels. Reverse transcription was obtained using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, California, US). The resulting cDNA (125 ng) was subjected to quantitative PCR (qPCR) using the SsoFast EvaGreen Supermix (Bio-Rad, Hercules, California, US) on a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules, California, US) for 40 cycles (denaturation 95°C for 5 s; annealing/extension 60°C 10 s) after an initial 30 s step for enzyme activation at 95°C . To validate the specificity of amplification products, melting curve was led between 65 and 96°C , with 0.5°C incrementing every 10 s.

Primers were designed by using Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences were provided in Table 1, where it is also specified the accession number (NM_) and the product length for each primer. All primers span an exon-exon junction. A geometric mean of three housekeeping genes (*Gapdh*, *β 2-microglobulin* and *b-actin*) was used to normalize data. Each transcript was tested in triplicate and quantitative measures were calculated using the $\Delta\Delta\text{CT}$ method and expressed as a fold change compared to control.

2.6 | Statistical analysis

To obtain a statistical power of 0.85, power analysis was performed based on a previously published study analyzing irisin serum level variations according to the musculoskeletal status of human subjects.³² The results of the power analysis indicated that, in order to reach a power of 0.85, it was necessary to recruit 10 participants.

Values were expressed as mean \pm SEM. Variation between time-points from BDC1 to R+2 among the mean values of irisin, sclerostin, myostatin and haptoglobin serum levels in all subjects were subjected to Shapiro–Wilk test to evaluate if data are or not normally distributed and subsequently, the significance was calculated by student *t*-test or Mann–Whitney test, respectively. Thus, before performing comparisons, the Shapiro–Wilk test was performed. Unpaired two tailed *t*-test for mean

TABLE 1 Table sequence, accession number (NM_) and product length for each primer

Gene name and ID	Forward (5'–3') and template	Reverse (5'–3') and template	Product length
GAPDH NM_001256799.3	AATGGGCAGCCGTTAGGAAA	GCCCAATACGACCAAATCAGAG	166
β -2-microglobulin NM_004048.4	AGATGAGTATGCCTGCCGTG	TTCAAACCTCCATGATGCTGC	97
B-Actin NM_001101.5	GCCGCCAGCTCACCAT	AATCCTTCTGACCCATGCC	168
FNDC5 NM_001171940.2	TCATCGTCGTGGTCTGTTC	TCAATGATGTCATACTGGCGGC	70
Myostatin NM_005259.3	CAGGCACTGGTATTTGGCAG	AACGGATTCAGCCCATCTTCTC	163
Haptoglobin NM_001126102.3	CGCCACAGAAGGAGATGGAG	TTGGGCTTCCCACATACTGC	108
Murf-1 NM_032588.4	GAGCCACCTTCTCTTGACTG	CTCAGGGCGTCTGCTATGTG	145
p53 NM_001126118.2	CTACCAGGGCAGCTACGGTTT	ACAGACTTGGCTGTCCAGAAT	60
p16 NM_001195132.2	CAGAAATGATCGGAAACC	CTACGCATGCCTGCTTCTA	50
p21 NM_001220777.2	GGCAGACCAGCATGACAGATT	GGCTTCTCTTGGAGAAGATCA	64
Tfam NM_001270782.2	CGGGTCCAGTTGTGATTGC	ACACAAAACCTGAAGGGGGAGC	196

values was used to compare parameters with normal distribution. Parameters with non-normal distribution were instead evaluated with the Mann–Whitney test and, for linear regression analysis, with the Spearman's coefficient. The results were considered statistically significant for p values $\leq .05$. Real-Time-PCR data are statistically analyzed as previously described for serum evaluations and are presented as box-and-whisker plots, from max to min, with all data points shown. For linear regression, r and p values are indicated into the graph. IBM SPSS statistics version 22 and GraphPad Prism 7.0 were used to analyze data.

3 | RESULTS

3.1 | Circulating levels of irisin, haptoglobin and muscle-bone turnover markers before, during and after human BR

Serum samples collected from the peripheral blood of the volunteers recruited for a 10-day BR study were used to assess by ELISA assay the levels of irisin, myostatin and sclerostin, which are markers of muscle and bone turnover respectively, as well as haptoglobin, a protein involved in acute phase response of inflammation.

The detections were performed over time: two days before BR, during BR, and two days after BR. No significant changes in the mean serum values of irisin during the entire BR period were found, although upward and downward fluctuations were observed. Interestingly, we detected significant lower levels of the myokine two days after the BR period (R+2) compared to both the values at BR0 ($*p < .05$) and the two days before the beginning of BR (BDC2) ($^{\#}p < .05$) (Figure 1A).

No significant change was also detected for myostatin and sclerostin, whose serum levels increased significantly only two days after the end of BR (R+2), both compared with serum values at BR0 and BDC2. As shown in Figure 1B,C, overlapping the patterns of serum determinations, we showed that when irisin decreased myostatin and sclerostin increased significantly. A different path was observed for mean serum haptoglobin levels, which increased on day 4 of BR and remained elevated both until the end of BR and 2 days later, (R+2), compared to BDC2 (Figure 1D).

We can therefore describe that at R+2, analysis of the serum levels of the molecules mentioned above showed that irisin levels were significantly reduced by about 34.5% ($p < .011$) compared to the first day of BR (Figure 2A). In contrast, at the same time point, we found a significant increment in serum levels of myostatin by 41.5% ($p < .016$) (Figure 2B), ($p < .013$) sclerostin by 34.8% (Figure 2C) and

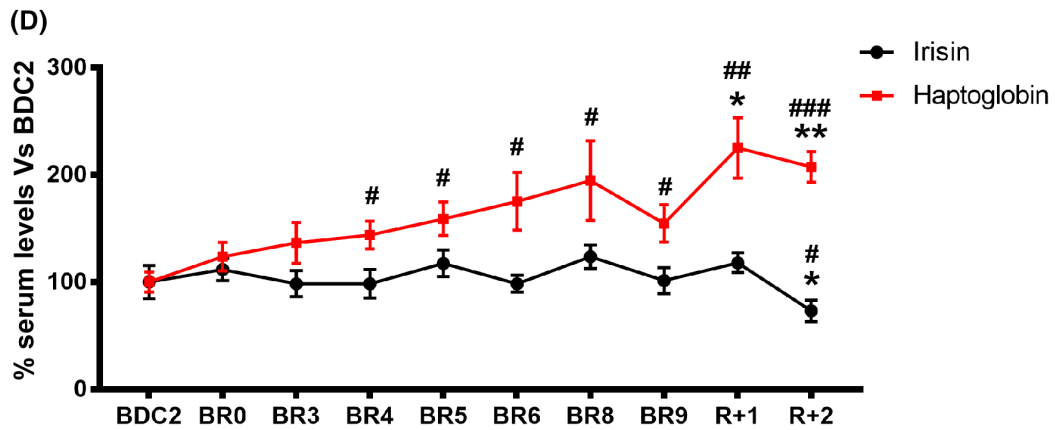
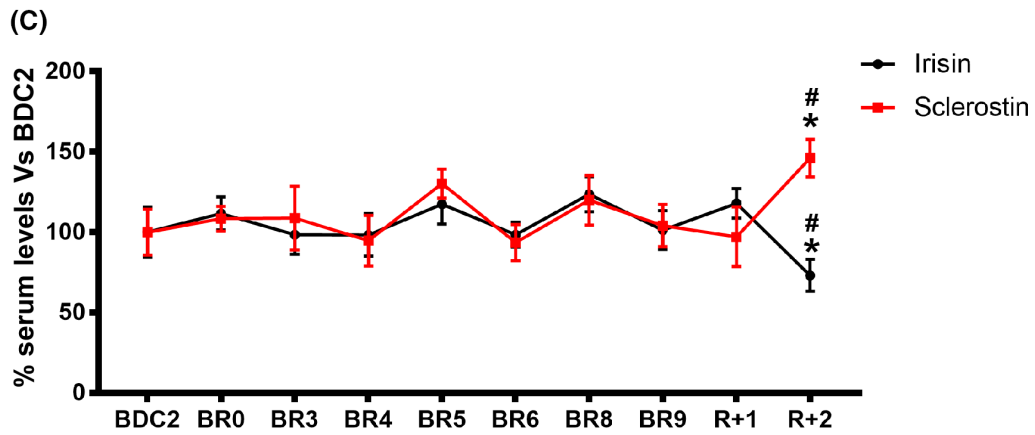
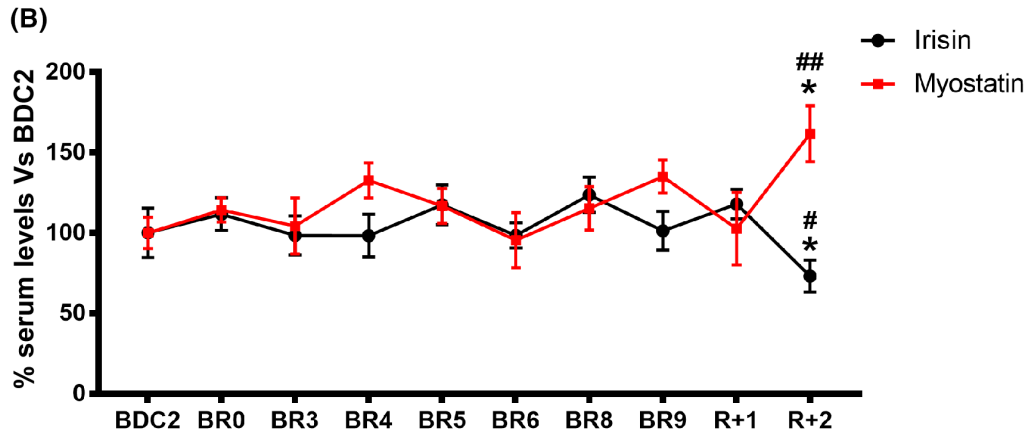
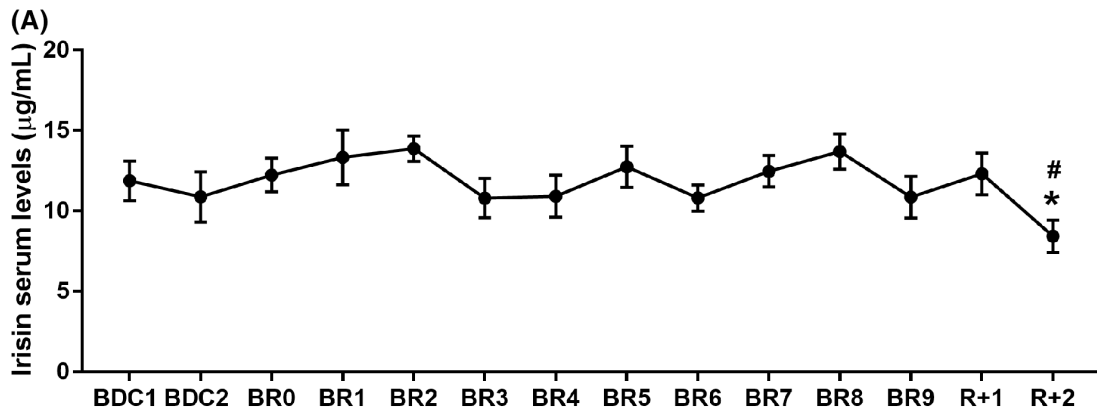


FIGURE 1 Time courses of irisin, myostatin, sclerostin, and haptoglobin serum levels during bed rest. Irisin serum levels ($\mu\text{g}/\text{mL}$) are significantly lower at time point R+2 ($n = 10$) compared to both time points BDC2 ($n = 10$) and BR0 ($n = 10$) (A). Comparing percentage trends of irisin, myostatin, sclerostin, and haptoglobin serum levels (B–D). Myostatin percentage serum levels increase significantly at time point R+2 ($n = 8$) compared to time points BDC2 ($n = 8$) and BR0 ($n = 8$) (B). Sclerostin percentage serum levels are significantly higher at time point R+2 ($n = 8$) compared to time points BDC2 ($n = 8$) and BR0 ($n = 8$) (C). The serum percentage of haptoglobin is significantly higher from time point BR4 ($n = 8$) than BDC2 ($n = 8$), while percentage levels are significantly higher than BR0 at time points R+1 ($n = 8$) and R+2 ($n = 8$) (D). In all three time-courses, irisin percentage serum levels are reduced at time point R+2 ($n = 10$) compared to BDC2 and BR0 time points (B–D). Shapiro–Wilk test, and Student *t*-test or Mann–Whitney test were performed. Data are presented as mean \pm SEM. $^{\#}p < .05$, $^{\#\#}p < .01$, $^{\#\#\#}p < .001$ vs. BDC2; $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. BR0.

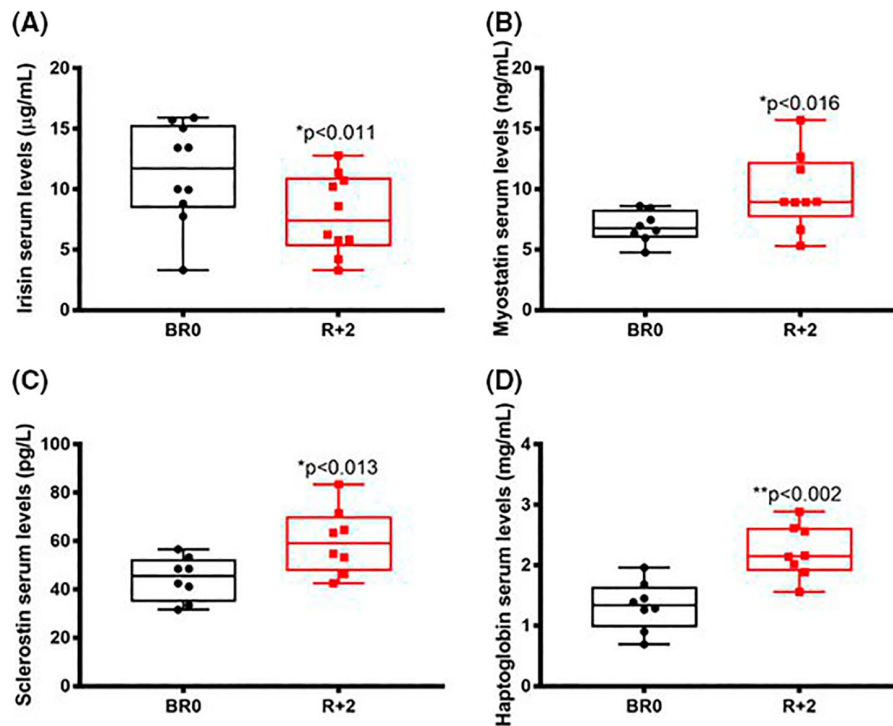


FIGURE 2 Irisin, myostatin, sclerostin, and haptoglobin serum levels at time points BR0 and R+2. Irisin serum levels are significantly lower in subjects at time point R+2 ($n = 10$) compared to the same subjects at time point BR0 ($n = 10$) (A). Myostatin serum levels are significantly higher in subjects at time point R+2 ($n = 8$) compared to the same subjects at time point BR0 ($n = 8$) (B). Sclerostin serum levels are significantly increased in subjects at time point R+2 ($n = 8$) compared to the same subjects at time point BR0 ($n = 8$) (C). Haptoglobin serum levels are significantly enhanced in subjects at time point R+2 ($n = 8$) compared to the same subjects at time point BR0 ($n = 8$) (D). Shapiro–Wilk test, and Student *t*-test or Mann–Whitney test were performed. Data are presented as box-and-whisker mean \pm SEM, from max to min, with all data points shown. $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$.

haptoglobin by 67.7% ($p < .002$) (Figure 2D) compared to BR0 values.

3.2 | Gene analysis in muscle biopsies from subjects during 10-day BR

Real-Time PCR analysis was performed on muscle biopsies at BR0 and BR9 of 8 subjects recruited for this BR study. No change in the gene expression of the irisin precursor, *fibronectin type III domain-containing protein 5* (*FNDC5*), was observed at BR9 compared to BR0

($p = .85$) (Figure 3A). Similarly, no change was detected in the expression of *myostatin* ($p = .52$) (Figure 3B) and *haptoglobin* ($p = .72$) (Figure 3C) mRNA levels. The lack of a modulatory effect on myokines and inflammatory molecules suggested that muscle metabolism of young healthy subjects was not affected by 10 days of BR. This was confirmed by the lack of changes in the gene expression of mitochondrial transcription factor A (*Tfam*) ($p = .77$; Figure 3D) and *MuRF1*, a muscle-specific E3 ubiquitin ligase activated during skeletal muscle atrophy ($p = .72$; Figure 3E) in muscle biopsies at BR9 compared to BR0. Thus, to assess possible correlations between serum

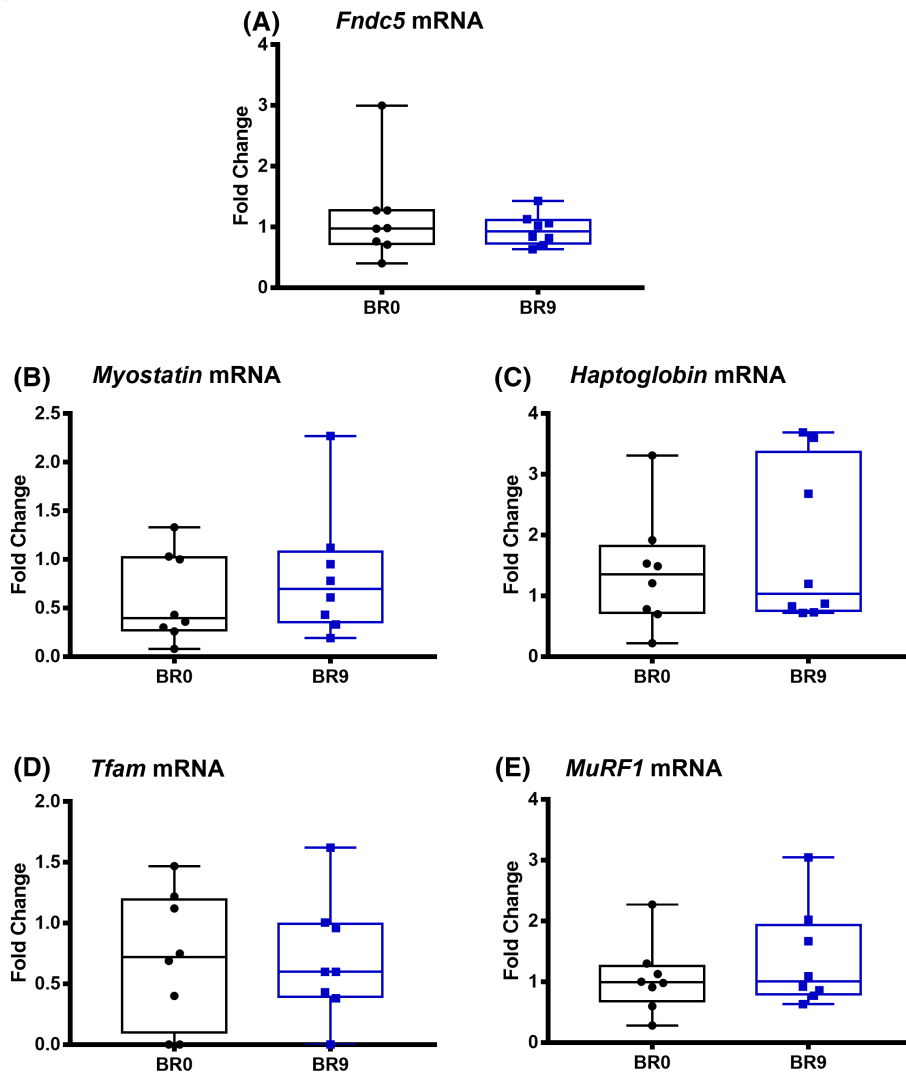


FIGURE 3 Muscle biopsies qPCR. Quantitative PCR (qPCR) showing mRNA expression levels of *FNDC5* (A), *Myostatin* (B), *Haptoglobin* (C), *Tfam* (D), and *MuRF-1* (E) at time points BR0 ($n = 8$) and BR9 ($n = 8$). Gene expression was normalized to a geometric mean of three housekeeping genes (*Gapdh*, $\beta 2$ -microglobulin and *b-Actin*) and plotted as fold increase or decrease from the subjects at time point BR0. Shapiro–Wilk test, and Student *t*-test or Mann–Whitney test were performed. Data are presented as box-and-whisker mean \pm SEM, from max to min, with all data points shown. * $p < .05$, ** $p < .01$, *** $p < .001$ vs. BR0.

irisin levels and skeletal-muscular changes after BR, we divided subjects according to lower and higher than the average of irisin serum levels evaluated at BR0 and BR9 (Figure 4A). Interestingly, we observed that *MuRF1* but not *myostatin*, *haptoglobin* and *Tfam* RNA levels, differ according to serum irisin levels. Specifically, we found that *MuRF1* expression at BR9 was significantly lower (-59.4% ; $p = .029$) in subjects with high irisin than those with low irisin serum levels (Figure 4B), suggesting that higher serum levels of this myokine may be involved in preventing the triggering of muscle atrophy. Finally, we investigated whether changes occurred in the expression of senescence markers in skeletal muscle during BR. Quantitative PCR analysis on skeletal muscle biopsies showed that the mRNAs for *p16* ($p = .58$; Figure 5A), *p53* ($p = .27$; Figure 5B), and *p21* ($p = .87$; Figure 5C) did not

differ significantly between BR9 and BR0. However, at BR9 we found a negative correlation between *p21* mRNA fold change, but not *p16* and *p53*, with irisin serum levels ($r = -0.738$; $p = .04$) (Figure 5D), suggesting a possible inhibitory effect of the myokine on the senescence marker, as already demonstrated in an in vitro study on osteoblasts.³²

4 | DISCUSSION

The present research performed on young male volunteers during 10-day BR allowed us to analyze the circulating levels of molecules contributing to the physiological control of musculoskeletal system during and following adaptation to the change in mechanical load and fluid

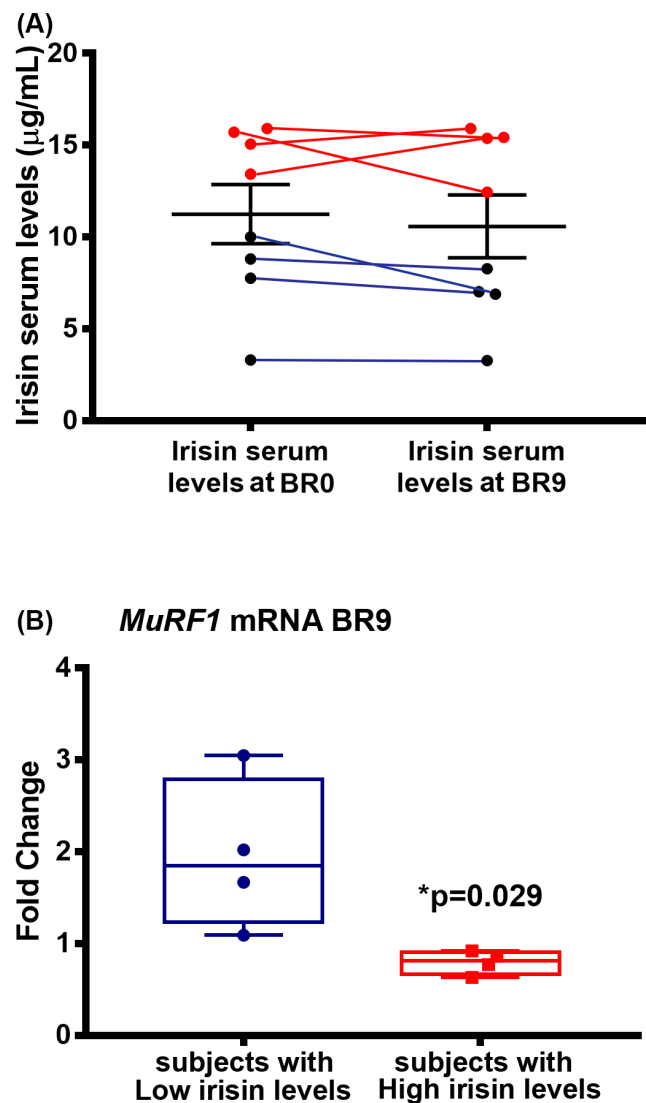


FIGURE 4 Distribution of irisin serum levels at BR0 and BR9 and *MuRF1* mRNA expression levels at BR9 depending on subject irisin levels. Variation of irisin serum levels ($\mu\text{g/ml}$) at time point BR0 and BR9 ($n = 8$) (A). Quantitative PCR (qPCR) showing mRNA expression levels of *MuRF1* at BR9 (B) depending on low (blue) or high irisin (red) serum levels. The distribution is based on the mean of irisin serum levels. Gene expression was normalized to a geometric mean of three housekeeping genes (*Gapdh*, $\beta 2$ -microglobulin and *b-Actin*) and plotted as a fold increase from the subjects with irisin low levels. Shapiro–Wilk test, and Student *t*-test or Mann–Whitney test were performed. Data are presented as box-and-whisker mean \pm SEM, from max to min, with all data points shown. * $p < .05$, ** $p < .01$, *** $p < .001$ vs. Irisin low levels.

redistribution. We studied the circulating levels of the myokine irisin, whose anabolic role on bone and muscle has been recently demonstrated,^{35–37} and of myostatin and sclerostin, well-known molecules controlling muscle and bone mass respectively.^{38,39} Additionally, as an inflammatory state can arise as a result or outcome of lack of mobility,⁴⁰ we also measured the serum haptoglobin levels.

Because unloading-induced muscle atrophy is one of the most relevant problems for astronauts during their stay in space,⁴¹ and 10-day BR has been shown to be sufficient to induce skeletal muscle atrophy and functional decline in the same group of volunteers used in the present study,⁴² we first evaluated the serum levels of irisin, never studied before on human models of simulated microgravity and ever more linked to muscular atrophy.^{26,43,44} Our data demonstrate no significant fluctuation in the levels of this myokine in healthy subjects throughout the BR period, and a significant reduction two days after the end of BR. We speculated that the unexpected up and down fluctuation might be a compensatory effect for young healthy subjects who adapt to the load variations and, maintaining the myokine values at control levels, counteract the possible effects of muscle damage due to disuse. Instead, the significant reduction in serum irisin levels observed at R+2 could be the cause and/or consequence of the subjects' resumption of a mild mobility condition to which they are exposed, remaining in a wheelchair for 2 days, and further load changes before returning to an orthostatic condition. Thus, probably at the end of BR, leaving the supine resting position caused an alteration of the compensatory mechanisms that kept irisin values constant, resulting in a collapse of the myokine levels with a subsequent loss of its musculoskeletal protective role. As for irisin, myostatin levels also fluctuated up and down during BR, however, its levels increased significantly precisely when those of irisin decreased.

Prior to this study, few data in humans demonstrated the existence of an inverse interplay between myostatin and irisin, whereas numerous studies in vitro and in mouse models suggested a negative correlation between the two myokines.⁴⁵ Thus, irisin and myostatin are both synthesized by skeletal muscle, and their secretion is inversely regulated by physical activity.⁴⁶ Previous data showed that irisin and its precursor were highly expressed in the skeletal muscle of myostatin deficient mice,⁴⁵ suggesting that their hyper-muscular phenotype⁴⁷ may also depend on increased irisin synthesis. In addition, irisin-mediated activation of extracellular signal-regulated kinase, along with a concomitant increase in insulin-like growth factor-1 expression, represses myostatin gene expression in muscle cells.²⁴

Similarly to myostatin, serum levels of sclerostin, a glycoprotein that has inhibitory effects on Wnt signaling in osteoblasts,³⁹ were significantly increased 2 days after BR. Consistently, an earlier study performed on a longer BR protocol demonstrated delayed sclerostin modulation. Buehlmeier et al. analyzed 16 older (60-year-old) and 8 younger (23-year-old) men undergoing BR for 14 days. Sclerostin levels increased between days 11 and 14 and remained elevated during recovery in all age groups.⁴⁸ In

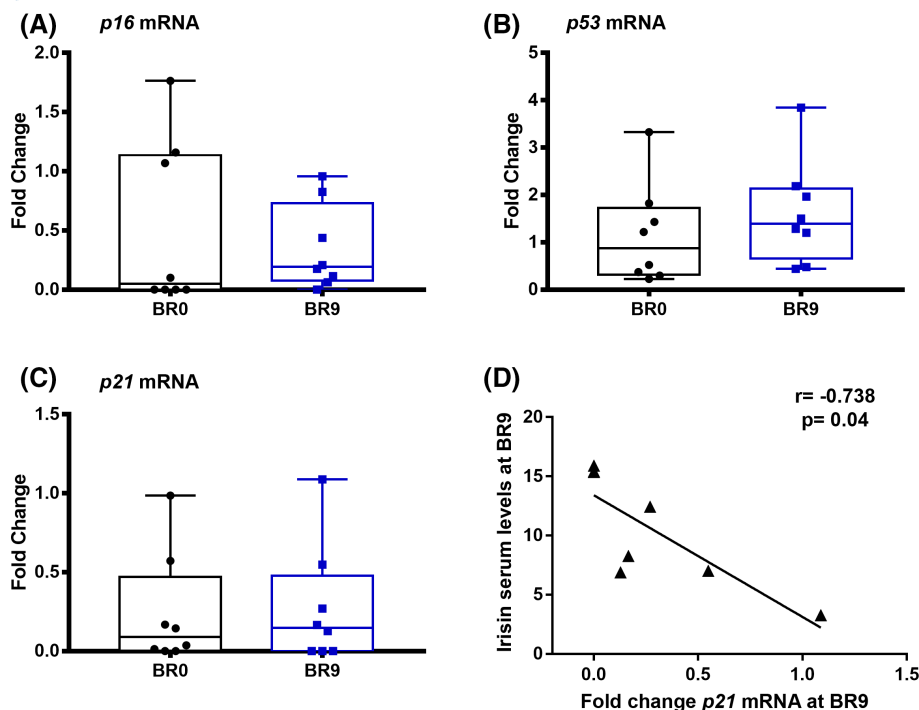


FIGURE 5 Absence of variation of senescence marker gene expression and negative correlation between *p21* mRNA and irislin serum levels at BR9. Quantitative PCR (qPCR) showing mRNA expression levels of *p16* (A), *p53* (B), and *p21* (C), at time points BR0 ($n = 8$) and BR9 ($n = 8$). Negative correlation between *p21* mRNA fold change expression levels ($n = 7$) and irislin serum levels ($n = 7$) at time point BR9 (D). Gene expression was normalized to a geometric mean of three housekeeping genes (*Gapdh*, $\beta 2$ -microglobulin and *b-Actin*) and plotted as fold increase or decrease from the subjects at time point BR0. Shapiro–Wilk test, and Student *t*-test or Mann–Whitney test were performed. Data are presented as box-and-whisker mean \pm SEM, from max to min, with all data points shown. Spearman analysis, linear regression and *r* and *p* values are indicated. * $p < .05$, ** $p < .01$, *** $p < .001$ vs. BR0.

contrast, the study of Frings-Meuthen et al., performed on healthy young males for 14 days of 6° head-down-tilt BR and 21 days of head-down-tilt BR, showed in the first type of protocol an early increase of sclerostin (8 days BR), while in the second protocol its serum levels increased significantly at 10- and 14-days.⁴⁹ Moreover, it is not surprising that in our study the increase in sclerostin coincided with a decrease in irislin, since previous studies showed an inverse correlation between these two molecules in adults with prediabetes.²⁹

As for myostatin and sclerostin, the increase in haptoglobin serum levels coincided with decreased levels of irislin 2 days after the end of BR. However, unlike the other markers, haptoglobin is significantly increased already at BR4 compared to BR0, reaching the highest levels at R+1 and R+2.

Haptoglobin is the major serum hemoglobin-binding protein and a marker of acute phase inflammation response, secreted by the liver in response to tumor necrosis factor alpha and interleukin-6 stimulation.⁵⁰ Numerous studies are emerging to assess haptoglobin levels under microgravity conditions since haptoglobin-hemoglobin binding limits the pro-oxidative action of free hemoglobin. Therefore, it is relevant to understand the contribution

of haptoglobin in preventing oxidative stress and subsequent muscle atrophy during chronic inflammation. Consistent with our findings, a recent study performed by proteomic analysis on plasma samples obtained from 13 cosmonauts before and after long-duration missions to the International Space Station (ISS) and from five healthy humans included in 21-day head-down BR and dry immersion experiments revealed that haptoglobin levels increased after return to Earth, as well as in both simulated microgravity ground experiments.⁵¹ However, results of haptoglobin modulation during BR have been discordant, mainly because of different time-points of protein measurement and for the overall duration of the BR protocol. For example, in a study performed for 60 days of 6° head-down tilt BR, haptoglobin levels remained unchanged during BR measured at BR20, BR49, and 13 and 30 days after BR.⁵² Moreover, it has been shown that the absence of haptoglobin, investigated in haptoglobin knock-out mice, induces muscle atrophy and expression of atrophy-related genes such as *MuRF1*¹⁹ and the mitochondrial transcription factor *Tfam*. However, our results showed that both *MuRF1* and *Tfam* expression in skeletal muscle biopsies were not modulated by 10-day BR. Presumably, increased haptoglobin levels during the BR period could

represent a useful mechanism to early prevent muscle-skeletal atrophy.

Literature data demonstrate that skeletal muscle tissue can rapidly adapt to mechanical load variation.⁵³ However, in skeletal muscle biopsies, we observed no change in the expression of the irisin precursor *FNDC5*, *myostatin*, and *haptoglobin*. These results suggest that muscle metabolism of young and healthy subjects was not affected by 10 days of BR.

However, in order to evaluate possible correlations between irisin serum levels and skeletal-muscular adaptation to unload conditions, when we divided subjects according to irisin serum levels at BR9 as lower and higher than the mean, we observed that *myostatin*, *haptoglobin* and *Tfam* were not significantly different, while the atrophy related gene *MuRF1* was reduced in subjects with higher irisin suggesting a possible mechanism that involves this myokine in modulating muscle response in BR conditions.

In parallel, to evaluate BR impact on skeletal-muscle senescence, we analyze *p16*, *p53*, and *p21* senescence-related genes, which result highly expressed during aging or in such stress conditions.^{54–56} Their expression levels on skeletal muscle biopsies were not significantly modulated during BR, however, we found an inverse correlation between *p21*, but not *p16* and *p53* (data not shown) expression levels on skeletal muscle biopsies and irisin serum levels, in agreement with our previous findings showing that *p21* expression was negatively affected by irisin.³² These results suggested a possible involvement of the myokine irisin in modulating senescence of skeletal muscle, an event that could contribute to unload associated muscle-skeletal atrophy.

In conclusion, although the small number of subjects enrolled in this study and the variability of data, our results suggest a possible involvement of the myokine irisin in muscle and bone adaptation to microgravity-simulated conditions. Moreover, the lower expression of *MuRF1* in subjects with higher irisin serum levels and the inverse correlation between irisin and the senescence marker *p21* indicates that this myokine may contribute to prevent the onset of atrophy and aging of skeletal muscle. Thus, although further studies are needed, we can hypothesize that irisin serum levels could represent an early prognostic marker of muscle atrophy associated with mechanical unload, a condition that usually precedes the bone mass reduction.^{57,58} A limitation of our study is that the enrolled subjects had lower mean age (23 ± 5 years) than the astronauts (46 ± 9 years). However, in view of a possible strategy to counteract skeletal muscle damage due to changes or absence of load, consisting of physical activity on ISS and assumption of anabolic molecules, irisin, the new exercise-mimetic hormone might represent an

additional supplementation for treat and/or prevent muscle and bone loss in astronauts.

AUTHOR CONTRIBUTIONS

Angela Oranger, Giuseppina Storlino, Graziana Colaianni, Silvia Colucci, and Maria Grano: designed the research. Manuela Dicarlo, Roberta Zerlotin, Patrizia Pignataro, Lorenzo Sanesi, Marco Narici, Rado Pišot and Bostjan Simunič: performed the research and acquired the data. Angela Oranger, Giuseppina Storlino, Graziana Colaianni, Silvia Colucci, and Maria Grano: analyzed and interpreted the data. All authors are involved in drafting and revising the manuscript.

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DISCLOSURES

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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