ORIGINAL ARTICLE

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Irisin Correlates Positively With BMD in a Cohort of Older Adult Patients and Downregulates the Senescent Marker p21 in Osteoblasts

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ABSTRACT

Irisin is a myokine produced by skeletal muscle during exercise in both mice and humans. We previously showed that irisin treatment ameliorates immobility-induced osteoporosis and muscular atrophy in mice. Data in humans showed a positive association between irisin and bone mineral density (BMD) in athletes and a population of healthy children. However, the role of this myokine regarding the state of muscle and bone in the same population remained to be determined. For this purpose, 62 patients (age 68.71 ± 12.31 years) undergoing total hip or knee replacement were recruited. Our results showed that irisin serum levels negatively correlated with age ($R = -0.515$; $p = .000018$) and positively correlated with femoral BMD ($R = 0.619$; $p = .001$) and vertebral BMD ($R = 0.201$; $p = .0001$). Irisin was also positively associated with *Fndc5* mRNA in muscle biopsies ($R = 0.248$; $p = .016$), as well as with Osteocalcin (*Ocn*) mRNA in bone biopsies ($R = 0.708$; $p = .006$). In skeletal muscle, FNDC5 positive fibers positively correlate with BMD of total femur ($R = 0.765$; $p = .0014$) and BMD of femoral neck ($R = 0.575$; $p = .031$), Interestingly, by analyzing patients divided by their *T*-score, we found lower irisin levels ($p = .0011$) in patients with osteopenia/osteoporosis (OP) compared to healthy controls matched for age and sex. By analyzing the senescence marker *p21*, we found a significant increase of its mRNA expression in the bone biopsies of OP patients compared to control ones. Therefore, we investigated in vitro whether rec-irisin had a direct effect on this senescence marker, showing that *p21* mRNA expression was significantly downregulated in osteoblasts by the treatment with irisin. Overall, these results indicate that higher irisin levels are associated with a lower rate of age-related osteoporosis and that irisin could be effective in delaying the osteoblast aging process, suggesting a potential senolytic action of this myokine. © 2020 American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: BONE-MUSCLE INTERACTIONS; IRISIN; OSTEOPOROSIS; SARCOPENIA; SENESCENCE

Introduction

Although increasing life expectancy is regarded as one of the main achievements of modern science, at the same time the rise of overall median age also leads to an increased incidence of chronic diseases in the older adults.[1] The pathologies linked to bone, such as osteoporosis, may therefore strongly increase in the coming years.[2] Throughout life, the skeleton is constantly renewed by the process of bone remodeling through which bone is reabsorbed by osteoclasts to be replaced by new bone produced by osteoblasts. With aging, however, increased bone resorption overcomes bone formation, resulting in the negative bone balance that leads to the onset of osteoporosis.[2–5] Nevertheless, the older adult population suffering from osteoporosis is often affected by a concurrent pathology, sarcopenia, which leads to a progressive loss of muscle mass and strength,[6] amplifying the risk of fractures during the aging process.[7,8]

Extensive prospective studies have shown that activating skeletal muscles during exercise might be a key aspect to improve quality of life by reducing mortality by 20% to 40% and mitigating the aging process.[9,10] This positive outcome is determined not only by the reinforcement of the muscular apparatus that increases mechanical load on the skeleton, but also by biochemical signals, the myokines, secreted during contraction, which act on various body parts. Among these myokines, irisin is a

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hormone-peptide synthesized from skeletal muscle performing key actions in bone metabolism and energy homeostasis.[11,12] We found that irisin treatment prevented muscle atrophy and bone loss in hindlimb-suspended mice, a murine model to simulate weightlessness.[13] In humans, we and other authors showed a positive correlation between irisin and bone mineral density (BMD) in young adult athletes.[14,15] Of note, in football players, we found stronger linear association at the right arm, lumbar vertebrae, and head. This result suggested that the effect of irisin on bone mass could be systemic, rather than specific to the bone sites where the load is applied, given that arms, lumbar vertebrae, and head receive a lower impact from mechanical loading in this type of sport.[15] Furthermore, our group also identified a positive association between circulating levels of irisin and bone during adolescence both in healthy children[16] and in a population of children affected by type 1 diabetes mellitus under insulin replacement therapy.[17] We also observed a significant reduction of irisin concentration in osteoporotic women affected by hyperparathyroidism (PHPT) compared with healthy matched controls, further supported by in vitro data showing that parathyroid hormone (PTH) treatment in C2C12 myotubes downregulated the expression of the irisin precursor, FNDC5.[18] Irisin inversely correlated with serum sclerostin levels in adults with prediabetes[19] and it was negatively associated with vertebral fragility fractures[20] and with sarcopenia[21] in postmenopausal women.

To our knowledge, no studies have been conducted so far to investigate existing links between circulating irisin, FNDC5 expression in skeletal muscle, and bone status in the same population of human subjects. Therefore, we evaluated possible correlations of irisin with parameters obtained from bone and muscle biopsies in a population of older adult subjects. Aiming to understand if irisin could be effective in delaying the cellular aging process, we also evaluated its possible senolytic action on osteoblast cell culture in vitro.

Patients and Methods

Population

Sixty-two patients (16 male and 46 female) undergoing total hip or knee replacement were recruited from the Orthopaedics and Traumatology Clinic, Policlinico of Bari, and the Department of Orthopaedics and Traumatology, Policlinico Tor Vergata Foundation, Rome. The average age of the patients was 68.71 $\pm$ 12.31 years. The patients were subsequently divided in osteopenic/osteoporotic patients (n = 18) and healthy subjects (n = 16) based on their femur T-score. The exclusion criteria were anti-osteoporosis therapy and physical impairments affecting the performance of daily activities.

The ethics committee of "Policlinico Tor Vergata" (approval reference number prot. 13107/2017), University of Rome, Rome, Italy approved all experiments described in the present study, and informed consent was obtained from all participants.

Determination of serum irisin

Serum concentration of irisin was measured by a competitive ELISA kit (AdipoGen, Liestal, Switzerland; Cat. No. AG-45A-0046YEK-KI01), following the manufacturer's instructions. This kit has detection limit 0.001 μg/mL, intraassay coefficient of variation (CV) 6.9% and interassay CV 9.07%. In this assay, a polyclonal antibody recognizes under competition the native irisin and recombinant irisin in irisin-coated plate, measuring the colorimetric reaction by spectrophotometer (Eon; BioTek Instruments, Inc., Winooski, VT, USA). Serum samples were obtained after centrifugation (1,000xg at 4°C for 20 min) and then stored at −80°C.

Bone mass measurements

The BMD was calculated by means of dual-energy X-ray absorptiometry (DXA) analysis (EXPLORER QDR series-Mo 010-157; Hologic, Inc., Bedford, MA, USA), calibrated using a lumbar spine phantom and following the Hologic guidelines. All scanning and analyses were performed by the same operator (FR) to guarantee uniformity. The percent coefficient of variation with repositioning (precision error) of whole-body DXA measurements was 1.1%, 0.9%, 2.3%, 0.5%, and 2.8% for BMC, BMD, fat mass (FM), fat-free soft tissue mass (FFSTM), and per cent FM, respectively. Participants were scanned in supine position, with their body and limbs fully extended and inside the limits set by the scan lines. The BMD (g/cm^2) was measured in the lumbar vertebrae, total femur, and femoral neck. The BMD was used to calculate the T-score, a parameter indicating BMD compared with that normally expected in a healthy young adult of the same sex (normal is between +1 and −1; osteopenia is between −1 and −2.5; osteoporosis is ≤ −2.5).

Bone and muscle biopsies

Orthopedic surgery was performed in an operating theater equipped with a turbulent ventilation system. We used the direct lateral approach to expose the hip joint. We adopted traditional total knee replacement involving a 7-cm to 8-cm incision over the knee for knee replacement. The same surgeon and the same anesthetist performed all the surgical procedures and anaesthesiology. We took bone and muscle biopsies during hip and knee replacement surgery. Bone and muscle biopsies analyzed were taken via the joint parts that were removed in the operational theater. We analyzed bone biopsies from the head of the femur removed during hip replacement, femoral condyle from both medial and lateral compartments during knee replacement, and vastus muscle near to the join part. After complete removal of soft tissues from bone biopsies, bone and muscle samples were immediately stored at −80°C until the required procedures were performed.

Immunohistochemistry

Skeletal muscle biopsies were performed during surgery in patients undergoing hip or knee replacement. The muscle samples were halved for biochemical and morphological analysis, for the latter, the samples were fixed by immersion in 4% paraformaldehyde in PBS at 4°C for 2 hours, then embedded in paraffin wax. Five-micrometer (5-μm) sections were cut, collected on Vectabond-treated slide (Vector Laboratories, Burlingame, CA, USA) and submitted to immunostaining. The following protocol was performed: protease treatment (Proteinase K; Roche, Indianapolis, IN, USA) 0.1 mg/mL in PBS for 2 min at 37°C; blocking of endogenous alkaline phosphatase in Dual Endogenous Enzyme-Blocking target (Agilent Dako, Santa Clara, CA, USA) at room temperature (RT) for 10 min; incubation with rabbit-FNDC5 (diluted 1:200; Abcam, Cambridge, UK) primary antibody at RT for 30 min; revealing with Biotinylated Secondary Antibody (AB2; Agilent Dako REAL™ Detection System), at RT for 15 min, Streptavidin Alkaline Phosphatase (Agilent Dako REAL™

Detection System) at RT for 15 min, Chromogen (RED) (Agilent Dako) at RT for 20 min; counterstaining with Mayer's hematoxylin (Sigma-Aldrich, St. Louis, MO, USA); mounting in glycergel (Agilent Dako). Specific pre-immune serum replacing the primaries antibodies served as a negative control.

Cell cultures

Mouse preosteoblast-like cells MC3T3-E1 were plated at 10×10^3 cells/cm^2 and cultured in α-MEM (Gibco, Thermo-Fisher, Waltham, MA, USA) with 10% of fetal bovine serum (FBS) (Gibco, Thermo-Fisher) until they reached the confluence in a humidified atmosphere (37°C, 5% CO_2) (Hera cell 150; Thermo-Fisher, Waltham, MA, USA). Upon confluence, to induce differentiation of preosteoblastic MC3T3-E1, we cultured them with α-MEM + 10% FBS supplemented with 5 μg/mL ascorbic acid and 1×10^{-2}M β-Glycerophosphate for 10 days, changing the medium every 2 days. Human osteoblasts were obtained from long bone fragments. The bones were processed and cut in small pieces. Bone pieces were put in α-MEM + 10% collagenase (0.1 mg/mL) (Sigma-Aldrich) and stirred for 30 min at 37°C. The digestion with fresh alpha-MEM + 10% collagenase was repeated for 30 min at 37°C. Then, supernatant was thrown away, and digested bone fragments were cultured in 12.5-cm^2 flask in α-MEM + 10% FBS + 0.2 ng/mL FGF until confluence. After confluence, cells were amplified 1:2 until no longer than the fourth passage. To induce differentiation, human osteoblasts were cultured with α-MEM supplemented with 5 μg/mL ascorbic acid and 1×10^{-2}M β-Glycerophosphate for 21 days, changing the medium every 2 days.

Irisin treatment

To investigate the intracellular signal activated by irisin in differentiated human and murine osteoblasts, cells were starved for 1 hour and subsequently stimulated with rec-irisin (100 ng/mL) for 0-1-5-10-20 minutes. Then, cell cultures were lysed for the analysis of Erk phosphorylation by Western blot. Differentiated murine osteoblasts were treated with vehicle (PBS) or 100 ng/mL of irisin (Adipogen, Liestal, Switzerland) for 8 and 24 hours and then subjected to RNA and protein extraction. For experiments in the presence of IgG neutralizing irisin, the primary anti-irisin antibody (Abcam) was added to the cell cultures 10 min prior to the addition of rec-irisin and used at a concentration of 0.2 μg/mL according to the manufacturer's instructions. To investigate the effect of longer stimulation by rec-irisin, differentiated murine osteoblasts were treated for 6 days with continuous or intermittent pulses of rec-irisin. For the continuous protocol, cells were treated with 100 ng/mL of irisin for 6 days, refreshing the medium every 48 hours. For the intermittent protocol, cells were treated with 100 ng/mL irisin for 3, 8, or 24 hours; after each treatment-pulse the medium was replaced with medium without rec-irisin until 48 hours, repeating the cycle three times. At the end of the three cycles (6 days), cells were lysates and subjected to RNA extraction.

Real-time PCR

We homogenized the skeletal muscle and bone biopsies using an Ultra-Turrax T8 homogenizer (Ika, Staufen im Breisgau, Germany). We used RNeasy Mini Kit (Qiagen, Hilden, Germany) to extract total RNA. To perform reverse transcription in thermal cycler (My cycler; Bio-Rad Laboratories, Hercules, CA, USA) we used iScript Reverse Transcription Supermix (Bio-Rad Laboratories). To perform real-time PCR on CFX96 real-time system (Bio-Rad Laboratories) we used SsoFast EvaGreen Supermix (Bio-Rad Laboratories) for 40 cycles (denaturation 95°C for 5 s; annealing/extension 60°C for 10 s) after an initial 30-s step for enzyme activation at 95°C. Primers were designed by using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). We chose beta 2 microglobulin (B2M) as housekeeping gene for human tissues and Gapdh as housekeeping for murine osteoblasts because they are stably expressed in these samples. All primers and sequences are reported in Table 1. All primers span an exon-exon junction. Each transcript was assayed in triplicate and quantitative measures were obtained using the delta-delta threshold cycle (ΔΔCT) method and expressed as a fold change compared to control.

Western blot

A total of 20 μg of protein from human and murine cell cultures were solubilized with lysis buffer (50mM Tris (Tris(hydroxymethyl)aminomethane)-HCl (pH 8.0), 150mM HCl, 5mM ethylenediaminetetraacetic acid, 1% NP40, and 1mM phenylmethyl sulfonyl fluoride). The protein concentration was measured by DC™ Protein Assay (Bio-Rad Laboratories). Cell proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The blots were incubated overnight at 4°C using primary antibody anti-pERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Erk (Santa Cruz Biotechnology), and anti-p21(Abcam). Subsequently, membranes were incubated for 2 hours at room temperature with appropriate IRDye-labeled secondary antibodies (680/800 CW) (LI-COR Biosciences, Lincoln, NE, USA). For immunodetection, the Odyssey infrared imaging system was utilized (LI-COR Biosciences). All data were normalized to background and loading controls.

Table 1. Sequence, Accession Number (NM_), and Product Length for Each Primer

Primer	Accession number	Forward primer	Reverse primer	Product length (bases)
β2M Human	NM_004048.4	AGATGAGTATGCCTGCCGTG	TTCAAACCTCCATGATGCTGC	97
Fndc5 Human	NM_001171940.2	TCAATGATGTCATACTGGCGGC	TCATCGTCGTCGTGGTCCTGTTC	73
p21 Human	NM_001291549.1	GGCTTCCTCTTGGAGAAGATCA	GGCAGACCAGCATGACAGATT	64
Ocn Human	NM_199173.6	GCTTGGACACAAAGGCTGCAC	CTCACACTCCTCGCCCTATTG	109
Gapdh Murine	NM_001289726.1	ACACCAGTAGACTCCACGACA	ACGGCAAATTCAACGGCACAG	145
p21 Murine	NM_001111099.2	GCAGAATAAAAGGTGCCACAGG	AAAGTTCCACCGTTCTCGGG	176
p16^{Ink4a} Murine	NM_001040654.1	GAACTCTTTCGGTCGTACCC	AGTTCGAATCTGCACCGTAGT	92

Statistical analysis

IBM SPSS statistics version 22 (IBM Corp., Armonk, NY, USA) was used to analyze data. In this study we reported comparisons between serum concentration of irisin and bone parameters. All variables were checked for normality (Shapiro-Wilk normality test) to see the population distribution. Unpaired two-tailed *t* test for mean values and Pearson's correlation coefficient for linear regression analysis were used to compare parameters with normal distribution. Parameters with non-normal distribution were instead evaluated with the Mann-Whitney test for the mean values and with the Spearman's coefficient for linear regression analysis. The results were considered statistically significant for p values <.05. Investigators were blinded for the analysis of difference in bone and muscle parameters between non-osteoporotic and osteoporotic patients.

Results

Irisin is positively associated with BMD

Table 2 shows the participant characteristics. Parametric variables are reported as mean and standard deviation (SD), and nonparametric variables as median and interquartile range (IQR). No value differed between genders.

In all subjects, irisin serum levels correlated negatively with age ($R = -0.515$; $p = .000018$) (Fig. 1*A*) and positively with the BMD of femurs ($R = 0.619$; $p = .001$; $n = 22$) (Fig. 1*B*) and vertebrae ($R = 0.201$; $p = .0001$; $n = 21$) (Fig. 1*C*). Expectedly, BMD of femur ($R = -0.653$; $p = .0004$; $n = 24$) (Fig. 1*D*) and vertebrae ($R = -0.416$; $p = .035$; $n = 27$) (Fig. 1*E*) correlated negatively with the age of the patients. No statistically significant correlation was observed between serum irisin levels and calcium ($R = 0.283$; $p = .152$; $n = 27$) (Fig. 1*F*) or C-terminal telopeptide of type I collagen (CTX) concentration ($R = 0.053$; $p = .829$; $n = 19$) (Fig. 1*G*).

To assess whether the levels of circulating irisin were consistent with the expression of its precursor in muscle, we measured FNDC5 mRNA levels in skeletal muscle biopsies from these subjects. Figure 2*A* shows that FNDC5 mRNA levels were positively associated with irisin serum levels ($R = 0.248$; $p = .016$; $n = 24$). Furthermore, while seeking a connection between bone and muscle tissues, we found that FNDC5 expression in muscle biopsies positively correlated with osteocalcin mRNA in bone biopsies ($R = 0.708$; $p = .006$; $n = 12$) (Fig. 2*B*). To further demonstrate that the expression of irisin in skeletal muscle was associated with improved bone mass, we evaluated possible correlations between its protein levels and BMD. Skeletal muscle biopsies were immunolabeled for FNDC5 by means of immunohistochemistry. Results showed that the number of FNDC5-positive fibers in muscle biopsies were positively associated with BMD of total femurs ($R = 0.765$; $p = .0014$; $n = 14$) (Fig. 2*C*) and femoral neck ($R = 0.575$; $p = .031$; $n = 14$) (Fig. 2*D*). The cellular immunoreactivity of FNDC5 was detected at the sarcolemma and as a diffuse, punctate signal in the sarcoplasm, recognizable in longitudinally and cross-sectioned muscle fibers (Fig. 2*E*).

Lower irisin levels in osteopenic/osteoporotic subjects

Among the recruited subjects, we selected a subgroup of patients affected by mild/severe bone loss according to their femur *T*-score (*T*-score ≤ −1). Figure 3*A* shows that irisin serum levels were significantly lower in patients with osteopenia/osteoporosis (OP) ($n = 18$) compared to healthy matched controls ($n = 16$) ($p = .0011$). Consequently, we evaluated whether there might be a relationship between reduced levels of circulating irisin observed in OP and the cellular senescence. By analyzing the expression of senescence markers in bone and muscle tissues, we found higher expression of *p21* mRNA in the bone biopsies of OP patients ($n = 7$) compared to control patients ($n = 4$) ($p = .042$) (Fig. 3*B*), whereas the expression of *p21* was not significantly different between the muscle biopsies of the two subgroups of patients (OP $n = 11$; controls $n = 5$) ($p = .41$) (Fig. 3*C*).

Treatment with rec-irisin decreases the expression of *p21* mRNA in murine osteoblasts

We have previously shown that irisin stimulated the phosphorylation of mitogen-activated protein kinases (MAPKs) Erk1 and Erk2 (pErk) in bone marrow preosteoblasts[12] and in osteocytes.[22] Here we found that incubation with rec-irisin (100 ng/mL) activates Erk phosphorylation in differentiated human osteoblasts and MC-3T3 murine osteoblasts (Fig. 4*A*,*B*). Having proven that rec-irisin has a receptor-mediated effect on mature osteoblasts, we consequently assessed whether *p21* expression in osteoblasts was a possible target of irisin action. Gene expression analysis showed that irisin induced a rapid downregulation of *p21* mRNA levels within 8 hours (threefold, $p = .03$) compared to untreated control (Fig. 4*C*). Moreover, by pretreating MC-3T3 osteoblasts with 600mM of H_2O_2 for 2 hours followed by 8 hours of treatment with rec-irisin, we observed that the increase of *p21* expression after H_2O_2 stimulation (4.5-fold, $p = .017$) was 2.5-fold

Table 2. General Characteristics of the Population

Characteristic	All ($n = 62$)	Male ($n = 16$)	Female ($n = 46$)	p
Age (years)	68.71 ± 12.31	69.00 ± 13.10	72.08 ± 10.93	.38
Irisin (mg/mL)	3.47 (2.38; 8.62)	2.980 (2.1; 5.93)	3.656 (2.44 ± 9.08)	.40
BMC vertebrae (g)	54.14 ± 13.11	59.73 ± 10.67	46.950 (44.39; 58.93)	.12
BMD vertebrae (g/cm^2)	0.97 ± 0.22	0.966 (0.83; 1.07)	0.94 ± 0.21	.45
BMC femur (g)	35.42 ± 10.10	38.27 ± 8.80	33.52 ± 10.74	.26
BMD femur (g/cm^2)	0.803 (0.75; 1.01)	0.94 ± 0.22	0.85 ± 0.19	.25
S-Calcium (mg/dL)	8.100 (7.70; 8.50)	8.20 ± 0.65	8.24 ± 0.69	.87
S-Phosphate (mg/dL)	3.24 ± 0.84	3.42 ± 1.14	3.13 ± 0.62	.45
iPTH (pg/mL)	35.60 ± 15.38	36.68 ± 14.69	34.87 ± 16.19	.75
25-OH Vitamin D (ng/mL)	17.00 ± 9.66	14.15 ± 7.95	17.21 ± 10.34	.40
CTX (ng/mL)	0.48 ± 0.31	0.47 ± 0.22	0.48 ± 0.37	.95

Parametric variables are reported as mean ± SD, and nonparametric variables as median (IQR).

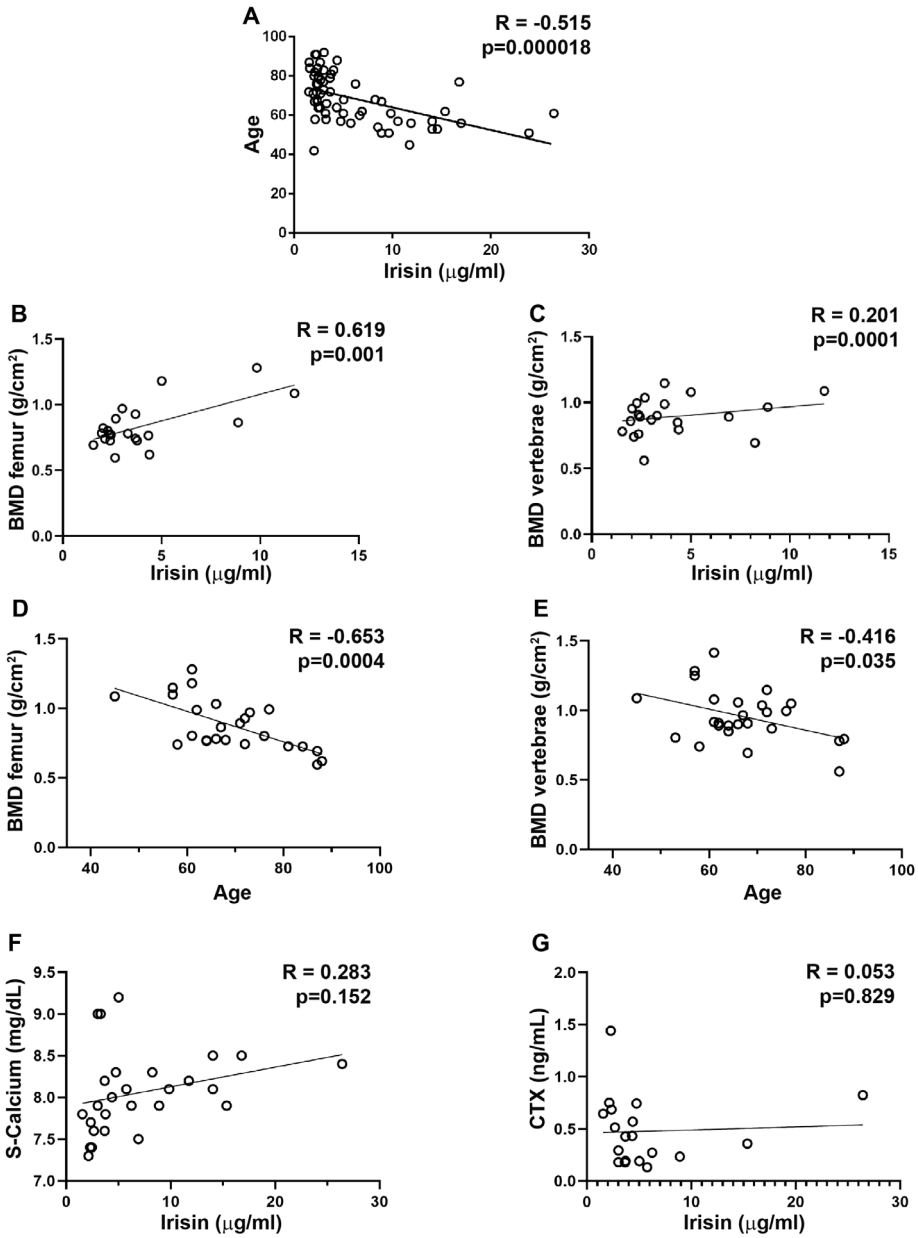

Fig 1. (*A*) Negative correlation between age and irisin serum levels in all subjects ($n = 62$). Irisin serum levels positively correlated with BMD of femurs ($n = 22$) (*B*) and with BMD of vertebrae ($n = 21$) (*C*). BMD of femur ($n = 24$) (*D*) and vertebrae ($n = 27$) (*E*) were negatively associated with age. No statistically significant correlation has been observed between irisin and calcium ($n = 27$) (*F*) or C-terminal telopeptide of type I collagen (CTX) ($n = 19$) (*G*) serum levels. Linear regression, *R* and *p* values as indicated.

lower if cells were then maintained in the presence of irisin ($p = .04$) (Fig. 4*D*). To further validate the inhibitory effect of irisin on p21 expression, we measured its protein levels. Western blot analysis showed that rec-irisin downregulates p21 significantly after 8 hours of treatment ($p = .024$). Although not statistically significant, the inhibitory effect showed a tendency to persist for up to 24 hours (Fig. 4*E*). To rule out nonspecific effects of irisin, we treated murine osteoblasts in the presence of a neutralizing antibody (IgG). As shown in Fig. 4*F* and *G*, irisin-induced reduction of p21 mRNA ($p = .014$) and protein levels ($p = .0003$), respectively, were blunted by the addition of an IgG neutralizing the irisin peptide. Additionally, we also evaluated $p16^{Ink4a}$ mRNA expression in osteoblast cell cultures, but no significant change was observed following irisin treatment (Supplementary Fig. S1).

Figure 4*H* and *I* summarizes the effect on the expression of *p21* mRNA after continuous or intermittent treatment with rec-irisin (100 ng/mL) on MC3T3 osteoblasts for 6 days (three cycles of 48 hours each). Both types of exposure were effective in downregulating *p21* mRNA expression with a greater effect when

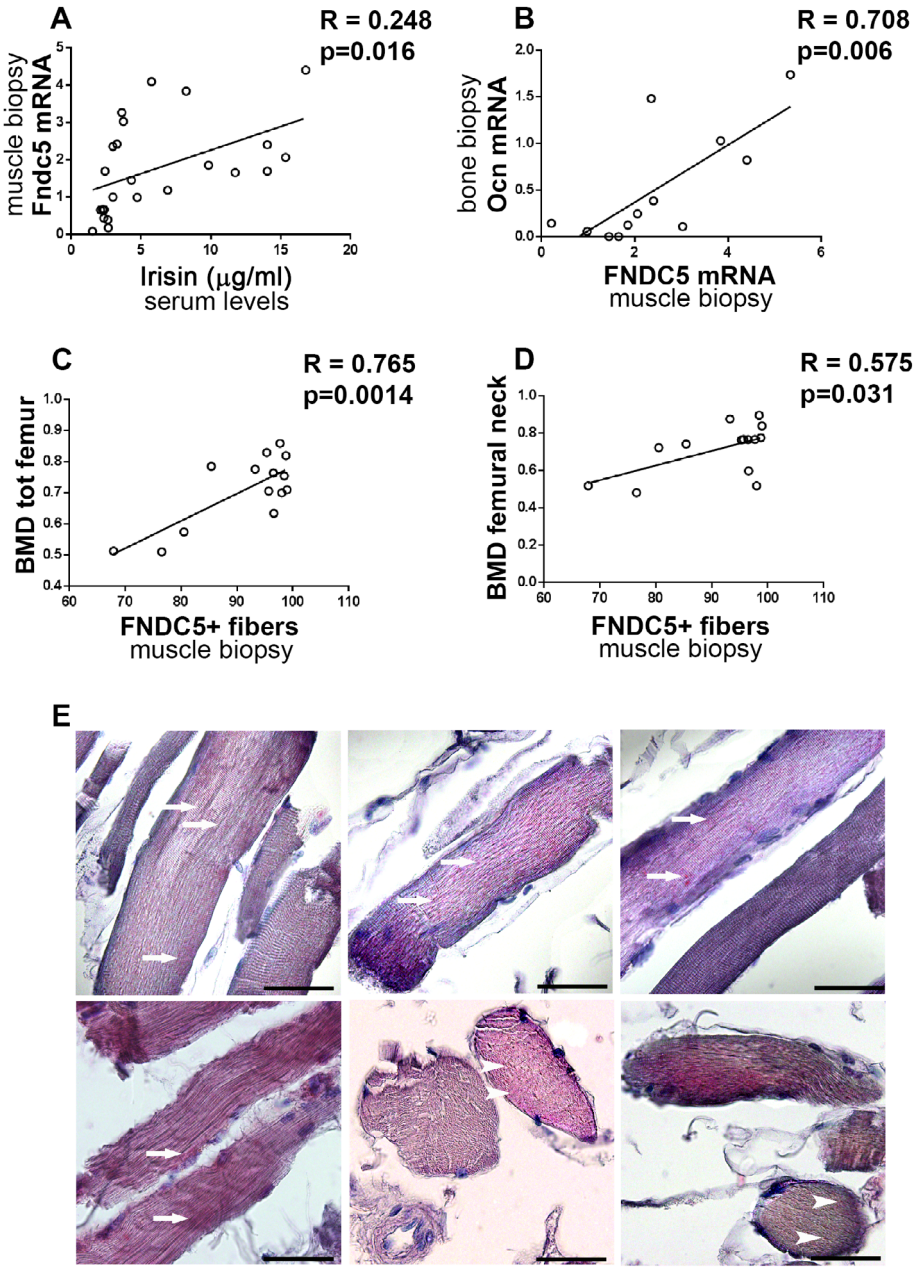

Fig 2. (*A*) Irisin serum levels are positively associated with *FNDC5* mRNA levels in skeletal muscle biopsies ($n = 24$). (*B*) *FNDC5* mRNA positively correlates with *Ocn* mRNA in bone biopsies ($n = 12$). The number of FNDC5-positive (FNDC5+) fibers in skeletal muscle are positively associated with total femur BMD ($n = 14$) (*C*) and femoral neck BMD ($n = 14$) (*D*). Linear regression, *R* and *p* values as indicated. Representative images of skeletal muscle biopsies immunolabeled for FNDC5 (*E*). The cellular immunoreactivity of Fndc5 was detected at the sarcolemma and as a diffuse, punctate signal in the sarcoplasm recognizable in longitudinally and cross-sectioned muscle fibers (arrows). Nuclear counterstaining with Gill's hematoxylin. Scale bars = 50 μm.

MC-3T3 osteoblasts were intermittently treated with rec-irisin, independently of treatment duration for each cycle (Fig. 4*I*).

Discussion

This study highlights a positive correlation between serum irisin levels and femoral and vertebral BMD in a population of older adult subjects. We also showed that the levels of circulating irisin were consistent with the expression of its precursor FNDC5 in the skeletal muscle of these subjects. Interestingly, FNDC5 in muscle was positively associated with osteocalcin mRNA expression in bone biopsies. This result supports our previous observation of an existing positive correlation between irisin and osteocalcin in a population of healthy children[16] and it is in line with in vitro data showing that long-term treatment with rec-irisin increased osteocalcin expression in primary mouse and rat osteoblasts.[23]

We previously showed a positive association between serum irisin and bone quality in healthy children[16] and in children with type 1 diabetes mellitus.[17] Likewise, we and others found a

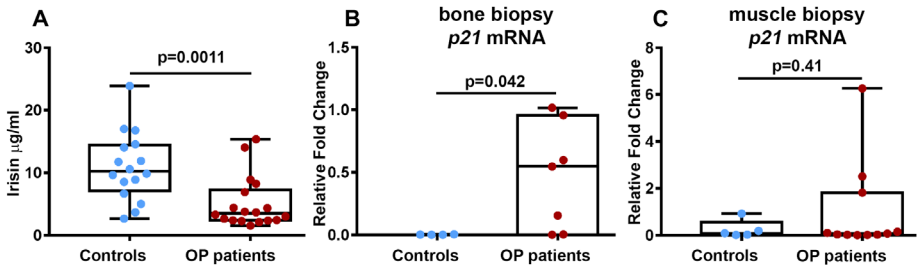

Fig 3. Irisin serum levels are significantly lower in patients with osteopenia/osteoporosis (OP) ($n = 18$) compared to healthy matched controls ($n = 16$). Not normally distributed data. (*A*) Mann-Whitney test was used to compare groups. *p21* mRNA expression is higher in bone biopsies from OP patients ($n = 7$) than controls ($n = 4$). Not normally distributed data. (*B*) Mann-Whitney test was used to compare groups. In skeletal muscle biopsies, *p21* mRNA expression in OP patients ($n = 11$) is not significantly different compared to controls ($n = 5$). Not normally distributed data. (*C*) Mann-Whitney test was used to compare groups. *p21* mRNA expression was normalized to *beta2microglobulin* and plotted as fold-increase from the control samples. Data are presented as box-and-whisker plots with median and interquartile ranges, from max to min, with all data points shown.

positive correlation between irisin and BMD also in young adults playing sports at competitive level.[14,15] In this study we highlight that serum irisin levels and *FNDC5* expression in muscle are associated with higher BMD in the older adult population. This result is further supported by the observation that we detected lower irisin levels in subjects with mild/severe bone loss compared to healthy controls. In parallel, we found that the expression of *p21*, a cell cycle inhibitor that drives senescence, is significantly higher in patients with bone loss.

The senescence process leads to an irreversible arrest of cell maturation in which, although senescent cells develop severe chromatin changes, they remain metabolically active and resistant to apoptosis.[24–27] During aging, senescent cells accumulate in all tissues altering the physiological homeostasis and promoting the progress of age-dependent diseases.[28] It is widely demonstrated that the administration of molecules selectively ablating senescent cells, termed senolytics, delays the onset of age-related diseases in murine models characterized by premature aging (progeroid mice).[29] Mounting evidence shows that the senescence process compromises the function of the osteoprogenitor cells and triggers osteoclastogenesis, thus leading to the onset of osteoporosis, one of the most widespread aging-related diseases.[30–32]

Although a unique marker of senescent cells has not yet been identified, most senescent cells express high levels of $p16^{Ink4a}$,[33] a cell cycle inhibitor, and *p21* and *p53*, the effector molecules of chronic senescence, which are highly expressed during tissue aging and under particular stress conditions.[34,35] In the elegant work by Farr and colleagues,[36] the authors reported that expressions of *p21* and $p16^{Ink4a}$ were significantly higher in the bone biopsies from old versus young women, whereas *p53* expression was unchanged. In line with this result, we found that *p21*, but not *p53* (Supplementary Fig. S2*A*,*B*), was higher in bone biopsies from osteopenic/osteoporotic versus healthy subjects. The upregulation of *p21* expression supports the hypothesis that age-related osteoporosis is at least partly linked to cellular senescence. Interestingly, in muscle biopsies, *p21* expression did not differ in subjects with bone loss compared with controls, suggesting that osteoporosis is not always accompanied by muscle senescence or possibly that bone cells senescence precedes that of muscles.

This study has several limitations. First, the research was limited by a small sample size for most outcomes as well as the cross-sectional human study design. Moreover, another limitation of this study is that we have not determined causality between accelerated senescence and lower concentration of circulating irisin in patients with bone loss. Similarly, the inverse correlation between irisin and age in this group of subjects does not allow us to establish a direct or indirect effect of this myokine on the reduction of *p21* expression, because the process of senescence can be independent of chronological age. Further studies are required to ascertain whether irisin plays a key role in mitigating age-related bone loss in humans. However, we provided in vitro evidence that irisin inhibits *p21* expression in osteoblasts. First, we found that irisin treatment, in both human and murine mature osteoblasts, rapidly activates phosphorylation of Erk1/2, thus suggesting a direct receptor-mediated action of the myokine on these cells. Consequently, we sought to investigate whether *p21* was modulated by short-term treatment with rec-irisin, showing that the myokine significantly inhibited its expression, also when it was added in culture media after acute treatment with hydrogen peroxide, which upregulates the expression of senescence markers.[37] The irisin-mediated downregulation of p21 mRNA and protein levels was specific as shown by adding a neutralizing antibody against irisin, which blunted this effect. However, although no statistically significant variation from vehicle-treated osteoblasts has been observed, treatment with irisin in the presence of the neutralizing antibody still showed a tendency to reduce p21, suggesting that the effect of this myokine was not fully inhibited. Moreover, in order to assess whether a longer treatment with rec-irisin was effective in maintaining *p21* expression at low levels in osteoblasts or whether this effect was merely transient, we cultured these cells for 6 days in the presence of rec-irisin administered continuously or intermittently. Both types of treatment have been effective, with greater effect if irisin was administered with intermittent pulses, as we already showed for downregulation of *Sost* in osteocytes[22] and increase of bone mass in murine models in vivo.[12]

In summary, by evaluating circulating irisin levels and bone parameters in a group of older adult subjects, our study shows

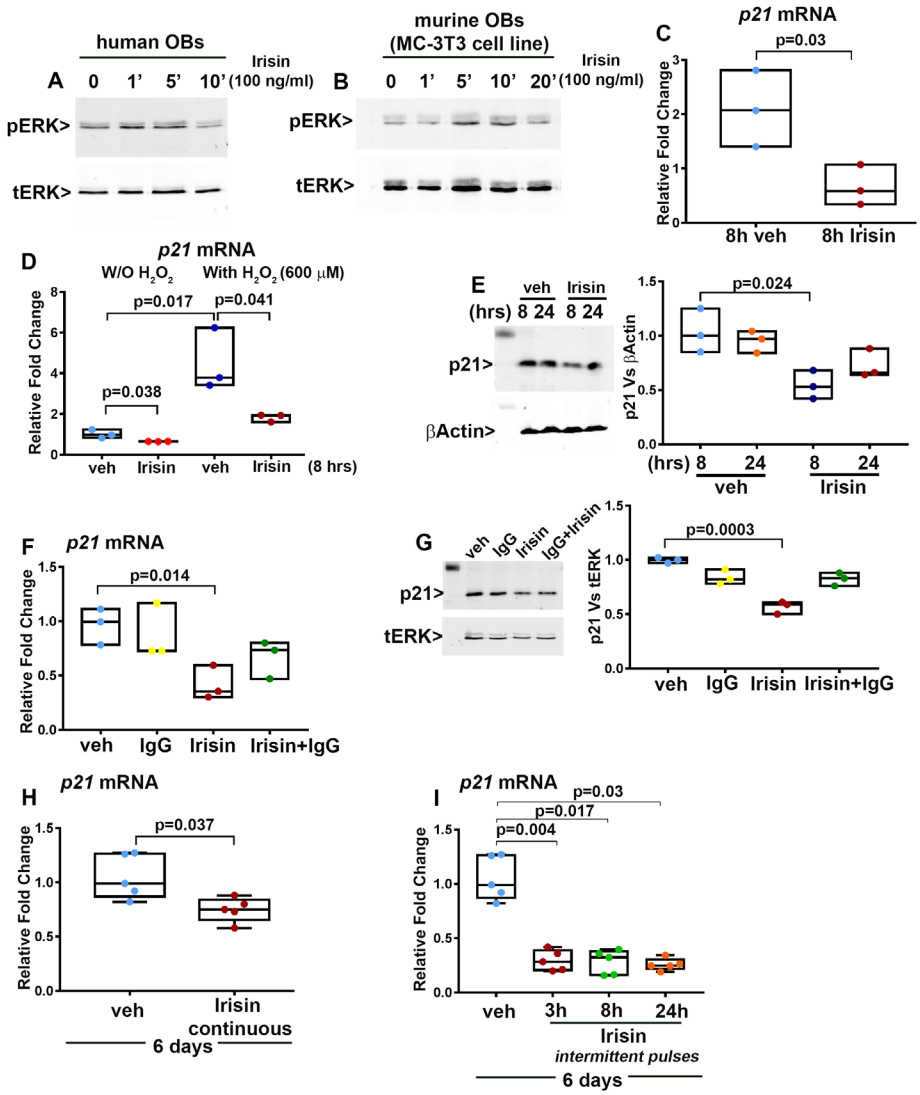

Fig 4 (A,B). Western immunoblotting showing Erk phosphorylation (pErk) triggered by rec-irisin (100 ng/mL) in human OBs and MC-3 T3 cell line (murine OBs) (representative Western blot image of $n = 3$ independent experiments). Quantitative PCR showing downregulation of *p21* mRNA expression after 8 hours of treatment with 100 ng/mL rec-irisin in murine OBs. Normally distributed data. Unpaired Student's *t* test was used to compare groups (*C*) and after pretreatment with 600μM of H_2O_2 for 2 hours followed by treatment with rec-Irisin (100 ng/mL) for 8 hours. (*D*) ANOVA was used to compare groups. Western immunoblotting and densitometric quantitation of p21 expression in murine OBs after 8 to 24 hours of treatment with 100 ng/mL rec-irisin, normalized to control loading (β-actin) (representative Western blot image of $n = 3$ independent experiments). (*E*) ANOVA was used to compare groups. mRNA expression levels of *p21* were assayed (qPCR) after 8 hours of treatment with 100 ng/mL rec-irisin ±0.2 μg/mL neutralizing antibody against irisin (IgG) in murine OBs. (*F*) ANOVA was used to compare groups. Western immunoblotting and densitometric quantitation of p21 expression in murine OBs after 8 hours of treatment with 100 ng/mL rec-irisin ±0.2 μg/mL neutralizing antibody against irisin (IgG), normalized to control loading (tERK) (representative Western blot image of $n = 3$ independent experiments). (*G*) ANOVA was used to compare groups. mRNA expression levels of *p21* were assayed (qPCR) after 6-day culture with rec-irisin refreshed every 48 hours (continuous protocol). Normally distributed data. Unpaired Student's *t* test was used to compare groups (*H*) and after 6-day culture with rec-irisin added for 3-hour, 8-hour, or 24-hour pulses. After each treatment-pulse, the medium was changed, and the procedure repeated every 48 hours for three times (intermittent protocol). (*I*) ANOVA was used to compare groups. Gene expression was normalized to *Gapdh* and plotted as fold-increase from the nontreated control (veh) sample ($n = 3$–5 independent experiments). For figures with three values per group, data are presented as boxplot with median and interquartile ranges. For figures with five values per group, data are presented as box-and-whisker plots with median and interquartile ranges, from max to min. All data points are shown.

that this myokine is positively associated with femoral and vertebral BMD and that, with the onset of bone loss, irisin levels decrease and at the same time *p21* expression in bone tissue increases. Although a proof of a direct action of irisin on *p21* levels in vivo needs further studies, we provide in vitro evidence showing that rec-irisin decreases the expression of this senescence effector in osteoblasts. Therefore, given the emerging role of irisin as an osteoanabolic agent, our results suggest that it

could represent a possible therapeutic option to delay age-related osteoporosis.

Disclosures

The authors declare that there is no conflict of interest regarding the publication of this paper. All authors approved the final version of the submitted manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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PEER REVIEW

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