Irisin Is Expressed and Produced by Human Muscle and Adipose Tissue in Association With Obesity and Insulin Resistance

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Context: Recently irisin (encoded by *Fndc5* gene) has been reported to stimulate browning and uncoupling protein 1 expression in sc adipose tissue of mice.

Objective: The objective of the study was to investigate *FNDC5* gene expression in human muscle and adipose tissue and circulating irisin according to obesity, insulin sensitivity, and type 2 diabetes.

Design, Patients, and Main Outcome Measure: Adipose tissue *FNDC5* gene expression and circulating irisin (ELISA) were analyzed in 2 different cohorts (n = 125 and n = 76); muscle *FNDC5* expression was also evaluated in a subcohort of 34 subjects. In vitro studies in human preadipocytes and adipocytes and in induced browning of 3T3-L1 cells (by means of retinoblastoma 1 silencing) were also performed.

Results: In both sc and visceral adipose tissue, *FNDC5* gene expression decreased significantly in association with obesity and was positively associated with brown adipose tissue markers, lipogenic, insulin pathway-related, mitochondrial, and alternative macrophage gene markers and negatively associated with *LEP*, *TNF*α, and *FSP27* (a known repressor of brown genes). Circulating irisin and irisin levels in adipose tissue were significantly associated with *FNDC5* gene expression in adipose tissue. In muscle, the *FNDC5* gene was 200-fold more expressed than in adipose tissue, and its expression was associated with body mass index, *PGC1*α, and other mitochondrial genes. In obese participants, *FNDC5* gene expression in muscle was significantly decreased in association with type 2 diabetes. Interestingly, muscle *FNDC5* gene expression was significantly associated with *FNDC5* and *UCP1* gene expression in visceral adipose tissue. In men, circulating irisin levels were negatively associated with obesity and insulin resistance. Irisin was secreted from human adipocytes into the media, and the induction of browning in 3T3-L1 cells led to increased secreted irisin levels.

Conclusions: Decreased circulating irisin concentration and *FNDC5* gene expression in adipose tissue and muscle from obese and type 2 diabetic subjects suggests a loss of brown-like characteristics and a potential target for therapy. **(*J Clin Endocrinol Metab* 98: E769–E778, 2013)**

In recent years, the interaction between adipose and muscle tissues has been increasingly recognized to play an important role in body weight regulation. Both adipose and muscle tissues secrete cytokines and other peptides, named adipokines and myokines, which contribute to tissue communication that is essential to maintain metabolic homeostasis. For instance, adiponectin is a hormone produced specifically by adipose tissue, which increases

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Abbreviations: AT, adipose tissue; BMI, body mass index; Ct, threshold cycle; FNDC5, fibronectin type III domain containing 5; HbA1c, glycosylated hemoglobin; HDL, high-density lipoprotein; KD, knockdown; PGC1, PPAR-γ coactivator-1; PPIA, cyclophilin A; PPAR, peroxisome proliferator-activated receptor; Rb1, retinoblastoma 1; SAT, sc AT; SVF, stroma vascular cell fraction; UCP1, uncoupling protein 1; VAT, visceral AT.

AMP-activated protein kinase-induced oxidative metabolism and glucose uptake by the muscle (1, 2). Similarly, leptin plays a role as a muscle insulin sensitizer (3, 4). Otherwise, some myokines such as IL-6 and IL-15, mainly produced after physical exercise, display beneficial effects on metabolism interacting with the adipose tissue (5, 6). Exercise-induced muscle IL-6 production increases insulin-stimulated glucose metabolism in muscle and adipose tissue, enhancing insulin-stimulated glucose disposal and fatty acid via AMP-activated protein kinase activation (7). IL-15 leads to reduced adipogenesis and increased fatty acid mobilization from adipose tissue depots (8, 9).

Irisin is a novel myokine, proteolytically processed from the product of the *FNDC5* gene prior to being released into the circulation (10). Irisin is regulated by peroxisome proliferator-activated receptor-γ coactivator-1 (PGC1)-α, and it has been proposed to mediate the beneficial effects of exercise on metabolism, inducing the browning of sc adipocytes and thermogenesis by increasing uncoupling protein 1 (UCP1) levels (10). In mice, irisin causes a significant increase in total body energy expenditure and resistance to obesity-associated insulin resistance. In humans, contradictory effects of physical exercise on irisin production have been reported (11–13). Timmons et al (11), using gene expression arrays, detected an exercise-induced increase of *FNDC5* mRNA in human muscle biopsies from old but not from young subjects. On the other hand, 2 recent studies found an association of *FNDC5* gene expression and irisin levels with physical exercise and *PGC1-α* mRNA level (12, 13). In human tissues, the distribution of FNDC5 expression was strongly increased in muscle in comparison with adipose tissue, similar to the findings described in mice (12). In fact, age-related muscle loss correlated to decreased circulating irisin concentration, muscle mass being the main predictor of this in humans (12). In this latter study, circulating irisin concentration levels were inversely correlated with adiponectin and positively correlated with body mass index (BMI), fasting glucose, and total cholesterol. Furthermore, after bariatric surgery-induced weight loss, circulating irisin levels as well as muscle *FNDC5* gene expression were significantly down-regulated. According to these authors, these correlations may suggest a compensatory role for irisin in response to deterioration of insulin sensitivity and glucose/lipid metabolism (12).

The stimulation of beige adipocytes in human adipose tissue has been postulated as a possible therapeutic way to improve obesity-associated metabolic disturbances (14). Because irisin is a circulating factor that activates beige fat cells in rodents (10), it could represent one way to perform this action in humans. In the current study, we aimed to investigate *FNDC5* gene expression in skeletal muscle, human adipose tissue, and circulating irisin in association with the expression of browning and adipogenic genes in both sc and visceral adipose tissue, according to obesity and type 2 diabetes. The possible relationship among obesity-associated metabolic disturbances, insulin sensitivity, and circulating irisin was also investigated. In vitro, we studied the potential irisin secretion from human preadipocytes and after their differentiation into adipocytes. Finally, we explored irisin secretion after inducing browning by retinoblastoma 1 (Rb1) silencing of mouse 3T3-L1 cells.

Subjects and Methods

Subjects' recruitments for adipose tissue and muscle study

Two hundred forty-three adipose tissue samples (125 visceral and 118 sc abdominal fat depots) from a group of Caucasian participants, with different degree of obesity (BMI between 20 and 58 kg/m^2) were analyzed. In a subcohort of the last, consecutive 29 morbidly obese subjects, we also investigated circulating irisin in association with sc and visceral *FNDC5* gene expression. Finally, in a subgroup of 34 participants, muscle tissues (rectus abdominis muscle) were also obtained. The cohorts were recruited at the Endocrinology Service of the Hospital Universitari Dr Josep Trueta (Girona, Spain). All subjects reviewed that their body weight had been stable for at least 3 months before the study, and all subjects gave written informed consent after the purpose, nature, and potential risks for the study were explained to them.

Adipose tissue (visceral and sc) and muscle samples were obtained during elective surgical procedures (cholecystectomy, surgery of abdominal hernia, and gastric bypass surgery), washed, fragmented, and immediately flash frozen in liquid nitrogen before be stored at −80°C. The isolation of adipocyte and stroma vascular cell fraction (SVF) is reported as supplementary information.

Subjects' recruitments for the insulin sensitivity study

Seventy-six Caucasian men were recruited and studied in an ongoing study dealing with nonclassical cardiovascular risk factors in northern Spain. Subjects were randomly localized from a census and they were invited to participate. The participation rate was 71%. A 75-g oral glucose tolerance test according to the American Diabetes Association Criteria was performed in all subjects. Subject characteristics and inclusion criteria were detailed in the Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. All subjects gave written informed consent after the purpose of the study was explained to them. The Ethical Committee of the Hospital Universitari Dr Josep Trueta approved the protocol.

Subjects were studied in the postabsorptive state. BMI was calculated as weight (in kilograms) divided by height (in meters) squared. Subjects' waists were measured with a soft tape midway between the lowest rib and the iliac crest; the hip circumference

was measured at the widest part of the gluteal region; and the waist to hip ratio was accordingly calculated.

Insulin sensitivity was measured using the frequently sampled iv glucose tolerance test as previously reported (Ref 15 and Supplementary Data).

Analytical methods

Serum glucose, glycosylated hemoglobin (HbA1c), serum insulin, total serum triglycerides, and high-density lipoprotein (HDL) cholesterol measurements are detailed in Supplemental Data. Plasma irisin concentrations were measured using an ELISA (SK00170-01; Aviscera Bioscience Inc, Santa Clara, California). The intra- and interassay coefficients of variation for these determinations were between 4% and 6% and between 8% and 10%, respectively.

Human adipocyte differentiation

Subcutaneous human preadipocytes (Zen-Bio Inc, Research Triangle Park, North Carolina) were cultured and differentiated as previously described (16). The release of irisin by adipocytes was corrected by the number of cells, the time, and the volume of the medium and expressed as femtomoles per (thousand cells × hours).

Induction of browning of 3T3-L1 adipocytes

The embryonic fibroblast mouse cell line 3T3-L1 (American Type Culture Collection, Manassas, Virginia) was maintained in DMEM containing 20 mM glucose, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Two days after confluence, an insulin (5 μg/mL), dexamethasone (0.5 μM), and isobutylmethylxanthine (0.5 mM) mixture was added for 2 days, followed by 5 days with insulin (5 μg/mL) alone. Differentiation was verified by morphological assessment and adipogenic gene expression.

Permanent silencing was performed using Rb1-targeted and control short hairpin RNA lentiviral particles (sc-29468-V and sc-108080l; Santa Cruz Biotechnology, Santa Cruz, California) and following the manufacturer instructions. Positive 3T3-L1 preadipocytes harboring short hairpin RNA cassette for Rb1 were selected by puromycin (3 μg/mL) selection 60 hours after infection. In 3T3-L1 Rb1 knockdown cells, white adipogenesis was significantly decreased in parallel with the increase of brown adipocyte differentiation (data not shown, J. M. Moreno-Navarrete, unpublished data). The release of irisin was corrected by the number of cells, the time, and the volume of the medium and expressed as femtomoles per (thousand cells × hours).

Gene expression

RNA was prepared from all these samples using RNeasy lipid tissue mini kit (QIAGEN, Izasa SA, Barcelona, Spain). The integrity of each RNA sample was checked by an Agilent bioanalyzer (Agilent Technologies, Palo Alto, California). Total RNA was quantified by means of a spectrophotometer (GeneQuant; GE Health Care, Piscataway, New Jersey) and reverse transcribed to cDNA using a high-capacity cDNA archive kit (Applied Biosystems Inc, Madrid, Spain) according to the manufacturer's protocol.

Real-time quantitative PCR was conducted using a Light Cycler 480 Probes Master (Roche Diagnostics, Barcelona, Spain) using Taqman or SyBr Green technology suitable for relative genetic expression quantification and running the same amount of tissue total mRNA (10 ng) to compare gene expression levels between tissues.

The commercially available and prevalidated TaqMan primer/probes sets used were as follows: endogenous control cyclophilin A (*PPIA*; 4333763) and target genes such as fibronectin type III domain containing 5 (*FNDC5*; Hs00401006_m1); peroxisome proliferator-activated receptor-γ (*PPARγ*; Hs00234592_m1); solute carrier family member 4 (*SLC2A4* or *GLUT4*; Hs00168966_m1); insulin receptor substrate 1 (*IRS-1*; Hs00178563_m1); fatty acid synthase (*FASN*; Hs00188012_m1); acetyl-CoA carboxylase (*ACACA*; Hs00167385_m1); and PR domain containing 16 (*PRDM16*; Hs00922674_m1); leptin (LEP, Hs00174877_m1); uncoupling protein 1 (mitochondrial, proton carrier) (UCP1, Hs00222453_m1); interleukin 10 (IL10, Hs00961622_m1); tumor necrosis factor (TNF, Hs01113624_g1); CD68 (*CD68*, Hs00154355_m1); mannose receptor, C type 1 (*MRC1* or *CD206*, Hs00267207_m1).

The prevalidated SybrGreen primers were purchased from Sigma S.A. (Barcelona, Spain), and the primers sequences used were as follows: endogenous control human cyclophilin A (*PPIA*) forward, 5′-CAAATGCTGGACCCAACACAA-3′, and reverse 5′-CCTCCACAATATTCATGCCTTCTT-3′; peroxisome proliferator-activated receptor-γ, coactivator 1α (*PGC1α*) forward, 5′-GCAATTGAAGAGCGCCGTGTGA-3′, and reverse, 5′-CTGTCTCCATCATCCCGCAGAT-3′; peroxisome proliferator-activated receptor-γ, coactivator 1β (*PGC1β*) forward, 5′-GCTGACAAGAAATAGGAGAGGC-3′, and reverse, 5′-TGAATTGGAATCGTAGTCAGTG-3′; transcription factor A, mitochondrial (*TFAM*) forward, 5′-AAGATTCCAAGAAGC TAAGGGTGA-3′, and reverse, 5′-CAGAGTCAGACAGATT TTTCCAGTTT-3′, cytochrome c oxidase III (*MTCO3*) forward, 5′-GCCCCCAACAGGCATCA-3′, and reverse, 5′-GGA TGTGTTTAGGAGTGGGACTTC-3′; fat-specific protein 27 (*FSP27*) forward: 5′-gaggtccaacgcagtccagctg-3′, and reverse: 5′-gtacgcactgacacatgcctggag-3′.

The reaction was performed in a final volume of 7μL. The cycle program consisted of an initial denaturing of 10 minutes at 95°C and then 40 cycles of a 15-second denaturizing phase at 95°C and a 1-minute annealing and extension phase at 60°C. A threshold cycle (Ct value) was obtained for each amplification curve, and a ΔCt value was first calculated by subtracting the Ct value for human PPIA RNA from the Ct value for each sample. Fold changes compared with the endogenous control were then determined by calculating 2-ΔCt, so gene expression results are expressed as an expression ratio relative to *PPIA* gene expression according to the manufacturers' guidelines.

Protein preparation

Proteins were extracted from adipose tissue by using a Polytron PT-1200C homogenizer (Kinematica AG, Lucerne, Switzerland) directly in radioimmnunoprecipitation assay buffer (0.1% sodium dodecyl sulfate; 0.5% sodium deoxycholate; 1% Nonidet P-40; 150 mM NaCl; and 50 mM Tris-HCl, pH 8.0), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride). Cellular debris and lipids were eliminated by centrifugation of the solubilized samples at 13 000 rpm for 60 minutes at 4°C, recovering the soluble fraction below the fat supernatant and avoiding the nonhomogenized material at the bottom of the centrifuge tube. Protein concentration was determined using the RC/DC protein assay (Bio-Rad Laboratories, Hercules, California).

Statistics

Statistical analyses were performed using SPSS version 12.0 software for Windows (SPSS, Chicago, Illinois). All assays were performed at least in duplicate and reported as mean ± SD. The comparison between groups was performed using a 2-way ANOVA followed by post hoc analysis (using DMS and Bonferroni post hoc tests). Parameters that did not fulfill normal distribution were mathematically transformed (log10) to improve symmetry for subsequent analyses. The relation between variables was analyzed by bivariate correlation (Pearson's or Spearman's test) and multiple linear regression models (using a stepwise method). To analyze in vitro experiments, a Mann Whitney *U* test (nonparametric test) was used.

Results

Human adipose tissue

Anthropometric and clinical characteristics of all participants are detailed in Table 1. In this cohort of subjects, *LEP* and *CD68* gene expression in visceral adipose tissue (VAT) were significantly associated with BMI (r = 0.47, $P < .0001$, and r = 0.30, $P < .0001$) and fasting glucose (r = 0.38, $P < .0001$ and r = 0.20, $P = .01$). *TNFα* gene expression was significantly associated with BMI (r = 0.27, $P = .004$). In sc adipose tissue (SAT), *LEP* gene expression was associated with BMI (r = 0.25, $P = .008$).

Table 1. Anthropometric and Clinical Parameters of Study Subjects From Cohort 1

	Nonobese	Obese	Nonobese T2D	Obese T2D	*P* Value
n	35	56	6	28	
Gender (men/women)	11/24	9/47	2/4	6/22	.5
Age, y	51.1 ± 13.4	45.9 ± 10.9[a]	55.5 ± 13.2	43.9 ± 10.4[a,b]	**.02**
BMI, kg/m^2	25.8 ± 2.7	45.4 ± 8.2[a,b]	28.19 ± 1.38	44.5 ± 5.3[a,b]	**<.0001**
Fasting glucose, mM	5.03 ± 0.63	5.2 ± 0.72[b]	8.91 ± 3.59[a]	8.22 ± 3.64[a]	**<.0001**
Fasting insulin, pM	67.32 ± 54.82	73.22 ± 28.45	56.56 ± 11.10	124.78 ± 62.59[a,c]	**.02**
HOMA-IR	2.36 ± 2.2	2.53 ± 1.36	3.04 ± 0.28	5.1 ± 3.2[a,c]	**.04**
VAT					
PPARγ, R.U.	0.027 ± 0.019	0.0061 ± 0.004[a]	0.016 ± 0.009	0.0040 ± 0.0016[a]	**<.0001**
FSP27, R.U.	0.71 ± 0.21	0.51 ± 0.25[a]	0.65 ± 0.09	0.39 ± 0.15[a]	**.003**
GLUT4, R.U.	0.057 ± 0.029	0.024 ± 0.023[a]	0.039 ± 0.016	0.013 ± 0.005[a]	**<.0001**
IRS1, R.U.	0.015 ± 0.008	0.010 ± 0.004[a]	0.011 ± 0.004	0.008 ± 0.005[a]	**.002**
LEP, R.U.	0.21 ± 0.165	0.34 ± 0.21	0.33 ± 0.13	0.37 ± 0.14	.08
FASN, R.U.	0.20 ± 0.11	0.071 ± 0.06[a]	0.10 ± 0.09	0.047 ± 0.04[a]	**<.0001**
ACC, R.U.	0.039 ± 0.03	0.016 ± 0.009[a]	0.027 ± 0.016	0.014 ± 0.009[a]	**<.0001**
UCP1, R.U.	0.0011 ± 0.0009	0.0012 ± 0.001	0.00082 ± 0.0006	0.0005 ± 0.0003	.2
PRDM16, R.U.	0.0038 ± 0.0013	0.0035 ± 0.002	0.0038 ± 0.0009	0.0032 ± 0.0028	.8
PGC1α, R.U.	0.014 ± 0.009	0.0096 ± 0.007	0.0086 ± 0.006	0.0071 ± 0.004[a]	**.04**
MTCO3, R.U.	39.1 ± 17.9	21.4 ± 8.6[a]	25.6 ± 19.8	20.7 ± 13.8[a]	**<.0001**
IL10, R.U.	0.0051 ± 0.004	0.0031 ± 0.0025	0.0012 ± 0.0005	0.0025 ± 0.002	.5
TNFα, R.U.	0.0041 ± 0.003	0.0064 ± 0.0051	0.0024 ± 0.0014	0.0046 ± 0.002	.2
CD206, R.U.	0.035 ± 0.026	0.021 ± 0.015[a]	0.023 ± 0.015	0.023 ± 0.009[a]	**.01**
CD68, R.U.	0.15 ± 0.09	0.19 ± 0.08	0.21 ± 0.09	0.24 ± 0.1[a]	**.01**
FNDC5, R.U.	0.0065 ± 0.002	0.0041 ± 0.003[a]	0.0048 ± 0.001	0.0039 ± 0.002[a]	**<.0001**
SAT					
PPARγ, R.U.	0.044 ± 0.022	0.009 ± 0.004[a,b]	0.031 ± 0.02	0.008 ± 0.004[a,b]	**<.0001**
FSP27, R.U.	1.29 ± 0.75	1.01 ± 0.49	1.20 ± 0.81	0.94 ± 0.39	.5
GLUT4, R.U.	0.069 ± 0.032	0.047 ± 0.03[a]	0.040 ± 0.008	0.025 ± 0.012[a,c]	**.001**
IRS1, R.U.	0.015 ± 0.008	0.011 ± 0.006[a]	0.013 ± 0.006	0.009 ± 0.003[a]	**.03**
LEP, R.U.	0.64 ± 0.35	0.89 ± 0.32[a]	0.66 ± 0.16	0.82 ± 0.37	**.045**
FASN, R.U.	0.46 ± 0.35	0.078 ± 0.05[a]	0.12 ± 0.10[a]	0.053 ± 0.040[a]	**<.0001**
ACC, R.U.	0.037 ± 0.030	0.021 ± 0.015[a]	0.025 ± 0.010	0.016 ± 0.009[a]	**.008**
UCP1, R.U.	0.00011 ± 0.0001	0.00013 ± 0.0001	0.000086 ± 0.00004	0.00004 ± 0.00007	.1
PRDM16, R.U.	0.0041 ± 0.002	0.0033 ± 0.002	0.0025 ± 0.0003	0.0024 ± 0.001[a]	.06
PGC1α, R.U.	0.011 ± 0.009	0.0057 ± 0.005[a]	0.010 ± 0.003	0.0059 ± 0.0045[a]	**.02**
MTCO3, R.U.	32.5 ± 18.03	23.15 ± 8.5[a]	22.3 ± 2.4	20.7 ± 13.8[a]	**<.0001**
IL10, R.U.	0.0014 ± 0.0011	0.0019 ± 0.0014	0.0013 ± 0.0006	0.0014 ± 0.0009	.6
TNFα, R.U.	0.0050 ± 0.0036	0.0037 ± 0.0024	0.0028 ± 0.0005	0.0041 ± 0.0034	.3
CD206, R.U.	0.021 ± 0.009	0.016 ± 0.011	0.021 ± 0.01	0.0020 ± 0.010	.4
CD68, R.U.	0.22 ± 0.11	0.19 ± 0.10	0.22 ± 0.11	0.23 ± 0.09	.06
FNDC5, R.U.	0.0052 ± 0.003	0.0037 ± 0.0027[a]	0.0034 ± 0.0030	0.0028 ± 0.0016[a]	**.02**

Abbreviations: HOMA-IR, homeostasis model assessment insulin resistance index; R.U., relative units; T2D, type 2 diabetes. Bold values mean that the value is statistically significant, $P < .05$.

[a] $P < 0.05$ compared with nonobese and nondiabetic participants performing DMS and Bonferroni post hoc tests.

[b] $P < 0.05$ compared with non-obese and T2D participants performing DMS and Bonferroni post hoc tests.

[c] $P < 0.05$ compared with obese and non-diabetic participants performing DMS and Bonferroni post hoc tests.

These data were in agreement with the known inflammatory state of VAT vs SAT (17, 18).

In both SAT and VAT, *FNDC5* gene expression was significantly decreased in association with obesity (Table 1 and Figure 1A). In SAT, *FNDC5* gene expression was decreased significantly in type 2 diabetic participants (0.0029 ± 0.002 vs 0.0042 ± 0.003 R.U., $P = .01$), but this association was lost after controlling for BMI. No differences were found in SAT (0.0040 ± 0.002 vs 0.0038 ± 0.003 R.U., $P = .7$) and VAT (0.0051 ± 0.0028 vs 0.0046 ± 0.003 R.U., $P = .5$) *FNDC5* gene expression according to gender. In both SAT and VAT, *FNDC5* gene expression was negatively associated with BMI and positively associated with *GLUT4*, *IRS1*, *FASN*, *ACC*, *PRDM16*, *UCP1*, and *MTCO3* gene expression (Table 2 and Figure 2 and Supplemental Figure 1). In addition, in SAT, *FNDC5* gene expression was negatively associated with *LEP* and *FSP27* and positively associated with *PGC1α*, *IL10*, *CD206*, and *CD68* gene expressions. In VAT, *FNDC5* was also negatively associated with *TNFα* gene expression (Table 2).

In multiple linear regression models, BMI ($\beta = -0.31$, $P = .005$) contributed independently to SAT *FNDC5* gene expression variance after controlling for age and gender. Furthermore, *UCP1* ($\beta = .24$, $P = .01$), *PRDM16* ($\beta = .22$, $P = .04$), *IRS1* ($\beta = .32$, $P = .003$), *FASN* ($\beta = .36$, $P = .001$), *MTCO3* ($\beta = .32$, $P = .003$), *LEP* ($\beta = -.47$, $P < .0001$), *FSP27* ($\beta = -.32$, $P = .003$), and *CD206* ($\beta = .38$, $P = .001$) contributed independently to *FNDC5* gene expression variance after controlling for age, gender, and BMI. Otherwise, BMI ($\beta = -.33$, $P = .001$) contributed independently to VAT *FNDC5* gene expression variance after controlling for age and gender, and only UCP1 ($\beta = .24$, $P = .01$) contributed independently to the *FNDC5* gene expression variance after controlling for age, gender, and BMI. The associations with *IRS1*, *FASN*, *PRDM16*, and *MTCO3* gene expressions were lost after controlling for BMI.

Only a few numbers of participants with type 2 diabetes were under treatment [metformin (n = 4), glitazones (n = 3), and insulin (n = 4)]. No drug effects were found to influence *FNDC5* gene expression in VAT and SAT.

Irisin levels in adipose tissue (AT) lysates were measured by an ELISA in 12 SAT and 16 VAT samples. AT irisin levels strongly correlated with *FNDC5* gene expression in both SAT (r = 0.65, $P = .02$) and visceral (r = 0.54, $P = .02$) adipose tissues. Interestingly, VAT irisin protein levels were positively associated with insulin pathway-related gene expression [such as *IRS1* (r = 0.63, $P = .015$) and *GLUT4* (r = 0.70, $P = .01$)] and negatively with BMI (r = −0.55, $P = .02$).

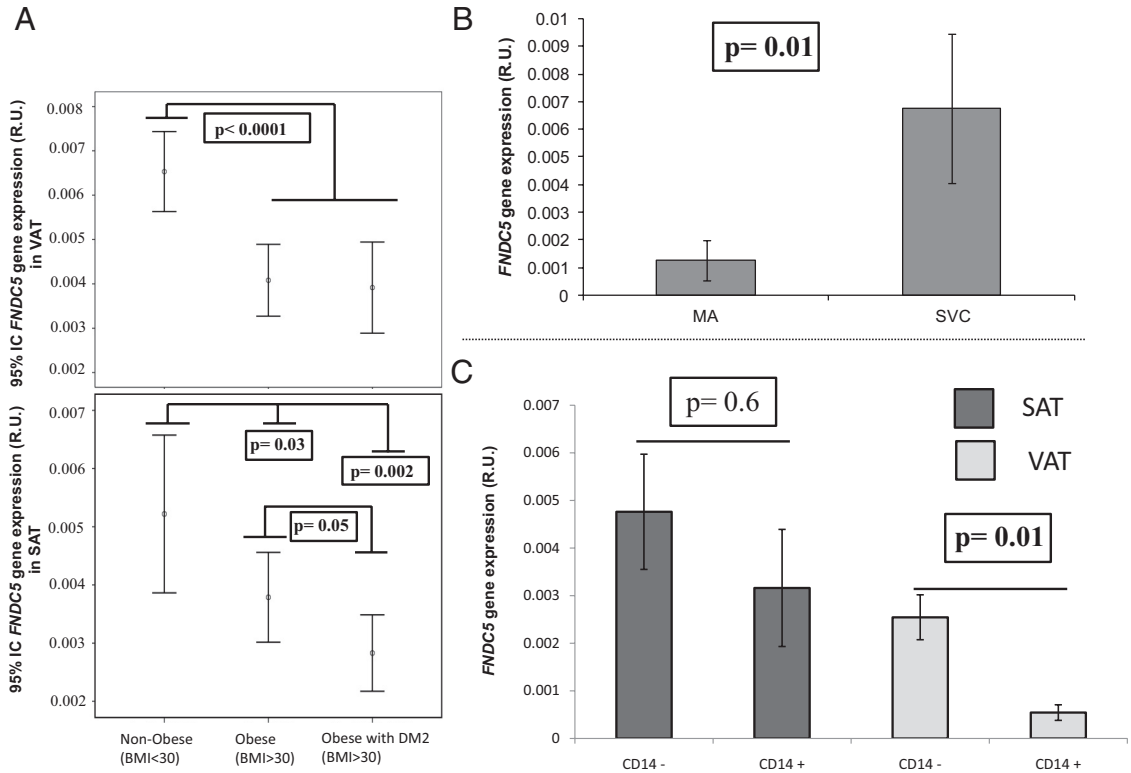

Figure 1. A, *FNDC5* gene expression in SAT and VAT according to obesity and type 2 diabetes. B, *FNDC5* gene expression in adipose tissue fractions [adipocytes (MA) and stromovascular cells (SVCs)]. C, *FNDC5* gene expression in $CD14^+$ and $CD14^-$ cells from SVCs from SAT (n = 5) and VAT (n = 4).

Table 2. Bivariate Correlation Among *Fndc5* Gene Expression and Anthropometric and Clinical Parameters of Study Subjects From Cohort 1

	VAT (n = 125)		SAT (n = 118)	
	r	*P* Value	r	*P* Value
Age, y	0.23	**.04**	0.01	.9
BMI, kg/m^2	−0.45	**<.0001**	−0.26	**.008**
Fasting glucose, mM	0.03	.7	−0.20	.06
Fasting insulin, pM	−0.08	.6	−0.21	.1
HOMA-IR	−0.034	.9	−0.24	.05
PPARγ, R.U.	0.21	.1	0.15	.2
FSP27, R.U.	−0.06	.6	−0.34	**.002**
GLUT4, R.U.	0.49	**<.0001**	0.27	**.008**
IRS1, R.U.	0.39	**<.0001**	0.39	**<.0001**
LEP, R.U.	−0.21	.1	−0.47	**<.0001**
FASN, R.U.	0.25	**.02**	0.24	**.03**
ACC, R.U.	0.31	**.004**	0.21	**.04**
UCP1, R.U.	0.29	**.004**	0.27	**.008**
PRDM16, R.U.	0.39	**<.0001**	0.28	**.008**
PGC1α, R.U.	0.15	.3	0.36	**.001**
MTCO3, R.U.	0.37	**.001**	0.30	**.004**
IL10, R.U.	0.22	.06	0.31	**.005**
TNFα, R.U.	−0.25	**.02**	0.04	.7
CD206, R.U.	0.06	.6	0.37	**.001**
CD68, R.U.	0.07	.5	0.29	**.005**

Abbreviations: HOMA-IR, homeostasis model assessment insulin resistance index; R.U., relative units. Bold values mean that the value is statistically significant, $P < .05$.

FNDC5 gene expression was significantly increased in the SVF compared with adipocytes (Figure 1B). *FNDC5* gene expression was similarly expressed in SVF-isolated $CD14^+$ and $CD14^-$ cells in SAT, whereas in VAT, *FNDC5* gene expression was significantly more expressed in $CD14^-$ than in $CD14^+$ cells (Figure 1C).

Human muscle

In muscle samples *FNDC5* gene was 200-fold more expressed than in adipose tissue (1.54 ± 0.79 vs 0.0047 ± 0.0029 R.U. in VAT or vs 0.0038 ± 0.0028 R.U. in SAT, $P < .0001$). Muscle *FNDC5* gene expression was positively associated with BMI ($r = 0.42$, $P = .02$), and the mitochondrial markers *PGC1α* ($r = 0.43$, $P = .01$), *PGC1β* ($r = 0.44$, $P = .01$), *TFAM* ($r = 0.50$, $P = .005$), and *MTCO3* ($r = 0.51$, $P = .004$) and negatively associated with age ($r = -0.44$, $P = .01$), but not with *IL15* ($r = 0.09$, $P = .6$). In multiple linear regression analysis, age ($\beta = -0.43$, $P = .02$) contributed independently to the *FNDC5* gene expression variance in muscle after controlling for gender and BMI. In addition, *PGC1β* ($\beta = .44$, $P = .01$), *TFAM* ($\beta = .49$, $P = .003$), and *MTCO3* ($\beta = .46$, $P = .006$) contributed independently to the *FNDC5* gene expression variance in muscle after controlling for age, gender, and BMI.

In obese participants, *FNDC5* gene expression in muscle was significantly decreased in association with type 2 diabetes (1.36 ± 0.64 vs 2.03 ± 0.82 R.U., $P = .045$). These type 2 diabetes patients were not under drug treatment.

Interestingly, muscle *FNDC5* gene expression was significantly linked to *FNDC5* and *UCP1* gene expression in SAT ($r = 0.57$, $P = .01$, and $r = 0.45$, $P = .04$, respectively) but not in VAT ($r = 0.10$, $P = .7$, and $r = 0.19$, $P = .5$, respectively). Furthermore, muscle *FNDC5* gene expression was significantly associated with sc and visceral AT irisin levels in both sc and visceral adipose tissues ($r = 0.98$, $P = .03$, and $r = 0.95$, $P = .04$, respectively, $n = 4$).

Circulating irisin and adipose tissue FNDC5 expression

In a subcohort of consecutive 29 morbidly obese subjects, circulating irisin concentration was significantly associated with both *FNDC5* gene expression in sc ($r = 0.44$, $P = .01$) and visceral ($r = 0.38$, $P = .04$) adipose tissues. These associations were maintained after controlling for age and BMI. No significant differences in circulating irisin according to gender were found (424.4 ± 72.7 ng/mL in men vs 509.1 ± 122.2 ng/mL in women, $P = .2$).

Circulating irisin, BMI, and insulin sensitivity

Anthropometrical and analytical characteristics of this cohort are shown in Tables 3 and 4. Excluding diabetic subjects, circulating irisin decreased in association with obesity (Table 3). In fact, circulating irisin was negatively associated with BMI ($r = -0.37$, $P = .001$), percent fat mass ($r = -0.26$, $P = .03$), waist to hip ratio ($r = -0.31$, $P = .008$) and positively associated with insulin sensitivity ($r = 0.24$, $P = .04$). No correlation was found with fasting glucose, HbA1c, fasting triglycerides, HDL cholesterol, or C-reactive protein. In a multiple linear regression analysis, BMI ($\beta = -.33$, $P = .004$) contributed independently to circulating irisin variance after controlling for age, waist to hip ratio and insulin sensitivity. Circulating irisin concentration was decreased significantly in participants with type 2 diabetes (Table 4).

In vitro studies

Human adipocytes

Irisin secretion into the media was measured before and after the differentiation process (at day 14) of human sc adipocytes. Secreted irisin concentration was 0.071 ± 0.010 fmol/(thousand cells × hour) in media from the preadipocytes and decreased to 0.035 ± 0.003 fmol/(thousand cells × hour) in the adipocyte media after differentiation (Figure 3A).

In 3T3-L1 cells after inducing browning features

The strong association between FNDC5 and brown adipocyte genes is supported by the browning effects of irisin on white adipose tissue (10). Because we found that

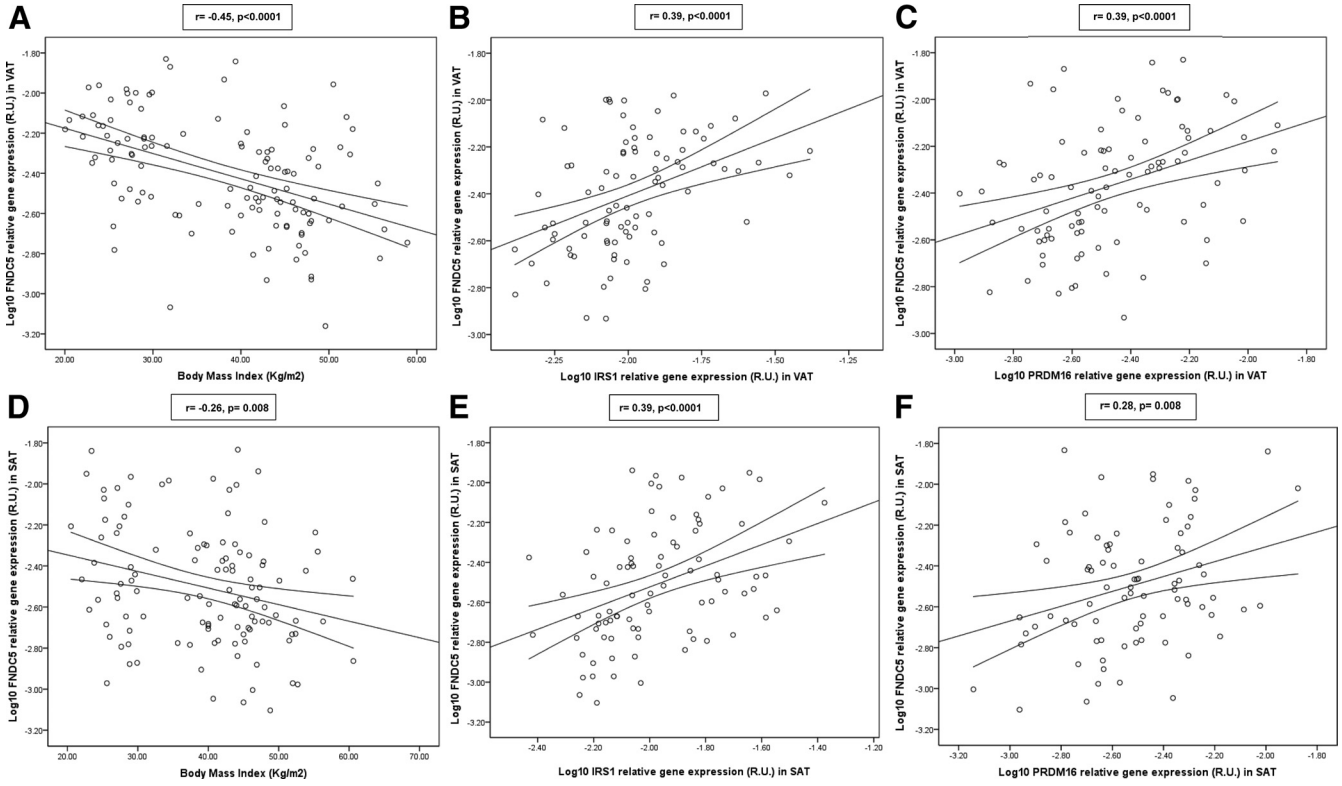

Figure 2. Correlation between *FNDC5* gene expression and BMI, *IRS1*, and *PRDM16* in SAT and VAT.

irisin was produced at substantial levels in human preadipocytes and adipocytes, we aimed to investigate the effects of induced browning in 3T3-L1 Rb1 knockdown (KD) cells on irisin production. These cells expressed high levels of brown adipocyte genes, such as *UCP1* and *PRDM16* (data not shown, J. M. Moreno-Navarrete, unpublished data). Interestingly, irisin secretion was significantly higher in 3T3-L1 Rb1 KD than 3T3-L1 C cells (Figure 3B).

Discussion

FNDC5 gene expression has been described in skeletal muscle of mice, and *PGC1α* is one of its main regulators (10). After the initial submission of this article, 3 independent papers have confirmed *FNDC5* gene expression in human skeletal muscle (11–13), being the main source of circulating irisin in both animal models and humans. In agreement with these results, we found a 200-fold in-

Table 3. Anthropometric and Clinical Parameters of Study Subjects (Cohort 2) According to Obesity Status (Excluding Subjects With Type 2 Diabetes)

	Lean	Overweight	Obese	*P* Value
n	18	34	17	
Age, y	47.28 ± 10.15	51.62 ± 10.64	54 ± 11.83	.17
BMI, kg/m^2	23.33 ± 1.2	27.23 ± 1.58[a]	32.47 ± 2.6[a,b]	**<.0001**
Waist to hip ratio	0.89 ± 0.034	0.93 ± 0.065	1.01 ± 0.065[a,b]	**<.0001**
Percent fat mass, %	4.9 ± 3.6	9.95 ± 9.01[a]	20.53 ± 12.6[a,b]	**<.0001**
Fasting glucose, mM	5.27 ± 0.66	5.25 ± 0.47	5.49 ± 0.49	.3
HbA_{1c}, %	4.75 ± 0.44	4.78 ± 0.27	4.94 ± 0.37	.2
Insulin sensitivity, $10^{-4} \cdot min^{-1} \cdot pM^{-1}$	1.44 ± 0.21	1.23 ± 0.22[a]	0.96 ± 0.31[a,b]	**<.0001**
Fasting triglycerides, mg/dL	91.66 ± 62.5	87.64 ± 49.1	107.5 ± 43.4	.43
HDL cholesterol, mg/dL	54.01 ± 15.51	54.45 ± 13.28	52.43 ± 11.1	.87
C-reactive protein, mg/L	0.188 ± 0.16	0.22 ± 0.2	0.43 ± 0.3[a,b]	**.01**
Circulating irisin, ng/mL	2157.9 ± 600.7	1783.7 ± 426.9[a]	1652.2 ± 421.8[a]	**.006**

Bold values mean that the value is statistically significant, $P < .05$.

[a] $P < .05$ vs lean participants performing DMS and Bonferroni post hoc tests.

[b] $P < .05$ vs overweight participants performing DMS and Bonferroni post hoc tests.

Table 4. Anthropometric and Clinical Parameters of Study Subjects (Cohort 2) According to Type 2 Diabetes

	Control	Type 2 Diabetes	*P* Value
n	69	7	
Age, y	50.9 ± 10.93	53.57 ± 10.32	.54
BMI, kg/m^2	27.61 ± 3.8	29.58 ± 3.4	.18
Waist to hip ratio	0.93 ± 0.071	0.96 ± 0.056	.32
Fat mass, kg	10.54 ± 10.5	14.45 ± 14.12	.46
Fasting glucose, mM	5.31 ± 0.53	6.45 ± 0.61	**<.0001**
Insulin sensitivity, $10^{-4} \cdot \text{min}^{-1} \cdot \text{pM}^{-1}$	1.21 ± 0.30	0.84 ± 0.52	**.02**
Fasting triglycerides, mg/dL	95.97 ± 54.9	128.85 ± 56.8	.14
HDL cholesterol, mg/dL	53.83 ± 13.15	48.67 ± 10.6	.3
Circulating irisin, ng/mL	1848.9 ± 507.5	1507.7 ± 222.1	**.006**

Bold values mean that the value is statistically significant, $P < .05$.

creased expression of *FNDC5* in muscle relative to adipose tissue and a strong relationship among *FNDC5*, *PGC1α*, and the expression of other mitochondrial genes (*PGC1β*, *TFAM*, and *MTCO3*) but not with *IL15* (a specific myokine).

In line with the browning effects of muscle-produced irisin, observed only in SAT (10), we have found that *FNDC5* in muscle was linked to the expression of *FNDC5* and *UCP1* in SAT but not in VAT.

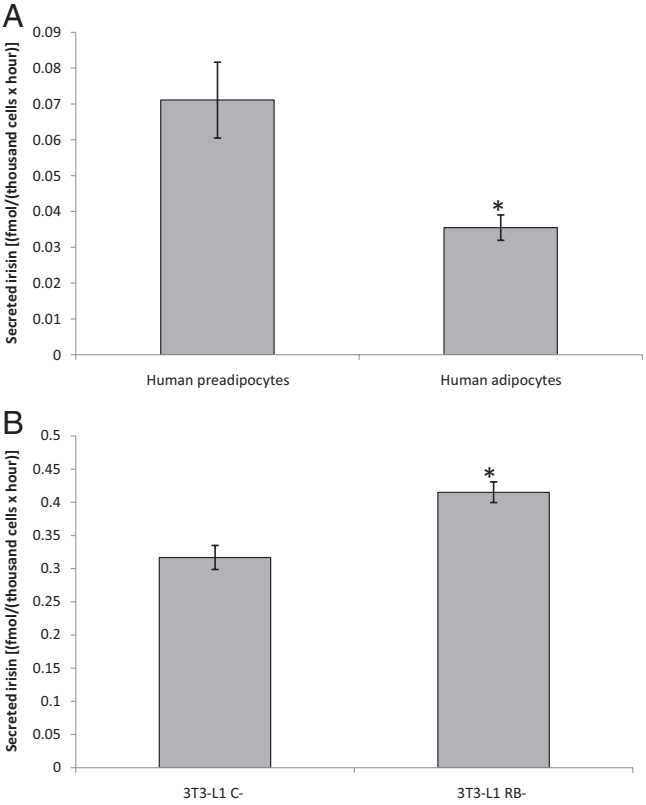

Figure 3. A, Secreted irisin [femtomoles per (thousand cells × hours)] from human sc preadipocytes and adipocytes at the end of adipocyte differentiation (day 14). *$P < .05$, compared with human preadipocytes. B, Secreted irisin [femtomoles per (thousand cells × hours)] in 3T3-L1 Rb1 KD and control cells at the end of adipocyte differentiation (day 7). *$P < .05$ compared with 3T3-L1C cells.

In humans, muscle *FNDC5* gene expression increased with obesity (11, 12). Huh et al (12) speculated that this association might be a compensatory mechanism. Reinforcing this hypothesis, we found that muscle *FNDC5* gene expression was positively associated with BMI ($r = 0.42$, $P = .02$) but negatively associated with age ($r = -0.44$, $P = .01$). In fact, age ($\beta = -.43$, $P = .02$) contributed independently to the *FNDC5* gene expression variance in muscle after controlling for gender and BMI. Timmons et al (11) detected an exercise-induced increase of *FNDC5* mRNA in human muscle biopsies from old (precisely the subjects with the lowest *FNDC5* levels in our study) but not from young subjects. These data are not comparable with our data because *FNDC5* gene expression in our study was measured only in the basal state. In addition, we also found that muscle *FNDC5* mRNA was significantly decreased in subjects with type 2 diabetes. Whether this is pathophysiologically linked to type 2 diabetes should be explored more in depth in future studies.

In the current study, we found an inverse association of *FNDC5* gene expression in human SAT and VAT with obesity. In addition, we found a positive association of FNDC5 gene expression with brown adipose tissue markers (*PRDM16* and *UCP1*), lipogenic (*FASN* and *ACC*), and the expression of insulin-pathway related genes (*GLUT4* and *IRS1*), mitochondrial (*MTCO3* and *PGC1α*), and alternative macrophage markers (*IL-10* and *CD206*).

FNDC5 gene expression was also negatively associated with *LEP*, *TNFα*, and *FSP27*, which is a negative regulator of white transdifferentation to brown adipose tissue (19, 20). Interestingly, FSP27-deficient mice showed improved insulin sensitivity and were resistant to diet-induced obesity, and FSP27-deficient white adipocytes had reduced lipid storage, smaller lipid droplets, increased mitochondrial activity, and a higher expression of several brown adipose tissue-selective genes. Furthermore, in adipose tissue, in FSP27-deficient mice (with browning phenotype), the expression levels of genes involved in oxidative phosphorylation, the TCA cycle, fatty acid synthesis,

and fatty acid oxidation were increased (20). Otherwise, no correlation was found in association with the master adipogenic transcription factor PPARγ (21). Interestingly, we confirmed the presence of irisin in adipose tissue lysates, being significantly correlated with AT *FNDC5* gene expression.

In vitro experiments revealed that irisin was produced in human preadipocytes and adipocytes and in 3T3-L1 adipocytes. Of note, in 3T3-L1 Rb1 KD cells, which showed and increased expression of brown adipocyte gene markers (*UCP-1* and *PRDM16*), secreted irisin into the medium was higher than in 3T3-L1 control cells at the end of adipocyte differentiation, suggesting that the stimulation of browning on adipocytes may enhance irisin production. In agreement with current results, Huh et al (12) reported that muscle *FNDC5* gene expression was 100-fold increased vs AT. Surprisingly, considering the expected relatively poor contribution of adipose tissue to circulating irisin concentration, we found a significant association between AT *FNDC5* mRNA and circulating irisin concentration. Taking into account all these data, we speculate that circulating irisin might induce the browning effects of human adipose tissue, leading to improved function and capacity of adipose tissue (ie, increasing the expression of adipogenic genes) and enhancing adipose tissue *FNDC5* gene expression, irisin being produced in a positive feedback by the adipose tissue itself.

All these associations led us to speculate para- or autocrine production of irisin from adipose tissue, which may act by increasing the adipose tissue capacity to burn fuel (glucose and fatty acids). The cross-sectional associations suggest a scenario in which irisin enhances white adipocyte transdifferentiation (by increasing *UCP1* and *PRDM16*) into beige adipocytes and as a consequence, the capacity of adipose tissue (mainly SAT) to uptake glucose (by increasing *IRS1* and *GLUT4*) or to synthesize fatty acids (by increasing *FASN* and *ACC*), to promote lipid mobilization (by decreasing *FSP27*) or to burn these fuels (by increasing *MTCO3* and *PGC1*α). In parallel to these effects, irisin could contribute to the modulation of obesity-induced inflammatory/antiinflammatory balance (by increasing *CD206* and *IL10* and decreasing *TNF*α and *LEP*). The strong association between *FNDC5* and *CD206* in SAT also suggests that alternative macrophages might contribute to irisin production at the same level of other cells contained within the stroma vascular cell fraction (such as endothelial cells). In fact, *FNDC5* gene expression was similar in $CD14^+$ (containing alternative and classical macrophages) and $CD14^-$ (containing endothelial cells and preadipocytes) in SAT.

Circulating irisin has been recently found to be directly associated with muscle mass and estradiol levels and inversely associated with age in a cohort of 117 middle-aged women (12). Circulating irisin also tended to be associated with BMI, glucose, adiponectin, and cholesterol levels, but these correlations were lost after controlling for estradiol levels and muscle mass. In the current study, BMI was the main contributor to circulating irisin after controlling for age, waist to hip ratio, and insulin sensitivity. These different associations could be interpreted in the context of the well-known inverse associations between muscle mass and fat mass or BMI in men (22).

van Marken Lichtenbelt et al (23) showed that the amount of brown adipose tissue was significantly decreased in association with obesity, with a negative linear relationship between brown adipose tissue, BMI, and percent body fat. Possibly the decreased para- or autocrine production of irisin in muscle/ adipose tissue in obese subjects could be behind the obesity-associated lower amounts of brown or beige adipocytes in human adipose tissue. In agreement with this hypothesis, circulating irisin levels were significantly decreased with obesity and type 2 diabetes.

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