**TITLE:** Association between circulating irisin levels and the promotion of insulin resistance during the weight maintenance period after a dietary weight-lowering program in obese patients

**RUNNING TITLE:** Weigh regain, insulin resistance and irisin

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ABSTRACT

*Objective.* Weight regain is associated with the promotion of insulin resistance. The newly discovered myokine irisin, which was proposed to be involved in the management of insulin sensitivity, could play a role in this process. This study aimed to investigate the association between irisin and reduced insulin sensitivity induced by weight regain.

*Materials/Methods.* Insulin sensitivity was evaluated according to the homeostasis model assessment of insulin resistance (HOMA-IR) in 136 obese patients who followed an eight-week hypocaloric diet (30% reduced energy expenditure) to lose weight and were re-evaluated four or six months after treatment. Irisin plasma levels, as well as the levels of leptin, adiponectin, ghrelin and TNF-α, were quantified in a sub-cohort (n=73) from the initially studied patients at baseline (T0), at the diet endpoint (T1) and after the follow-up period (T2).

*Results.* After a successful dietary intervention to lose weight, 50% of the patients who regained the lost weight during the follow-up period were categorized as insulin resistant (HOMA-IR≥2.5) compared with only 25% of patients who maintained the weight loss (p=0.018). Importantly, in addition to the well-studied hormones leptin and adiponectin, irisin plasma levels were statistically associated with several risk factors for insulin resistance. Indeed, the increased risk of insulin resistance during the follow-up period was related to high irisin levels at baseline (odds ratio=4.2; p=0.039).

*Conclusions.* Circulating irisin predicts the insulin resistance onset in association with weight regain. Therefore, irisin could be secreted as an adaptive response to counteract the deleterious effect of excess adiposity on glucose homeostasis.

*Keywords:* FNDC5, hypocaloric diet , insulin sensitivity, weight regain

**Abbreviations**

AHEAD, Action for Health Diabetes

BMI, Body mass index

FNDC5, Fibronectin type III domain-containing 5

HOMA-IR, Homeostasis assessment model of insulin resistance

RESMENA-S, MEtabolic Syndrome REduction in Navarra Study

TNF-α, Tumor Necrosis Factor-alpha

WAT, White adipose tissue

## 1. Introduction

Nutritional interventions, such as caloric restriction diets, are currently the most suitable noninvasive therapeutic approach to promote weight loss in the obese patients [1]. Most therapeutic trials involving dietary treatments report mean weight losses greater than 5% of body weight [1]. In addition to reducing body weight, dietary treatments are able to counteract comorbidities of obesity, including insulin resistance. However, long-term data regarding the effect of dietary interventions on mortality are disappointed, as was recently determined by the Look AHEAD (Action for Health Diabetes) study [2]. This fact could be because less than 20% of individuals who attempt to lose weight are able to achieve and maintain a 10% reduction over a year [3]. This adverse dietary outcome has relevant health consequences because weight regain may contribute to all-cause mortality [4].

Importantly, it was recently proposed that the deleterious metabolic features associated with excess body weight are largely related to the presence of insulin resistance [5] because it is associated with obesity and may increase the risk of all-cause, cancer, and cardiovascular disease mortality [6, 7]. Therefore, research examining the detrimental effects of weight regain on insulin sensitivity to elucidate the potential mechanisms involved in this process is an important priority to develop more personalized therapeutic approaches to counteract obesity and its comorbidities.

Insulin resistance is a pathological condition characterized by a decrease in the efficiency of insulin to regulate blood sugar levels that occurs in response to a complex interplay of metabolic and inflammatory mediators of energy balance. In addition to well-studied energy metabolism and insulin resistance-related proteins, such as leptin, adiponectin, ghrelin and tumor necrosis alpha [8], an exercise-induced peptide known as irisin has recently been identified [9]. Relevantly, irisin was reported to improve obesity states and glucose homeostasis and to prolong life expectancy [9]. This protein was initially described as a

cleavage product of the type I membrane protein fibronectin type III domain-containing 5 (FNDC5) [9]; however, recent crystal structure and biochemical characterization studies of the FNDC5 ectodomain corresponding to the irisin myokine, indicated that irisin consists of an N-terminal fibronectin III(FNIII)-like domain attached to a flexible C-terminal able to form dimers independently of glycosylation [10]

Since its discovery, irisin garnered great attention as it was noted that this peptide could play an important role in animal and human physiology and biology [11]. Nevertheless, the  beneficial role attributed to irisin in humans is unclear [12], and the effects of exercise training on FNDC5 gene expression and irisin levels remain unspecified [9, 13-18].

Although irisin was initially described as an exercise-induced hormone secreted by muscle [9], and it was found related to other myokines [19], it was also described to behave as an adipokine expressed and secreted by white adipose tissue (WAT). In particular it was reported the secretion of irisin mainly by subcutaneous adipocytes in rats and humans depending on exercise and nutritional status [20]. In addition, it was found that irisin is expressed in glutamate decarboxylase-positive Purkinje cells of the cerebellum in the brain [21], and an important role in the neurogenic regulation was suggested [22, 23]. Interestingly, it appears to have no in vitro effect on cell proliferation and malignant potential of obesity-related cancer cell lines in physiological and higher physiological/pharmacological concentrations [24]. Moreover, this peptide could influence the regulation of metabolic pathways, and the postnatal growth and development of different organs in the newborn because it is present in human breast milk [25].

Lower circulating irisin was associated with the onset of type 2 diabetes [26-28] with the risk of non-alcoholic fatty liver disease [29], chronic kidney disease [30] and heart failure [31]. Additionally, oxidative stress and inflammation, which are two mechanisms involved in the onset of insulin resistance [32], were associated with an increase in irisin levels, as was

reflected in human muscle [33] and liver [34]. However, the irisin effect appears to be exerted in a nutrition-independent fashion because its circulating levels were not associated with dietary indices [35].

The goals of this study were to evaluate the impact of weight regain after a successful hypocaloric diet-induced weight loss on the prevalence of insulin resistance classified according to the homeostasis assessment model of insulin resistance (HOMA-IR) and to explore the association among circulating levels of irisin and the detrimental effect of weight regain on insulin sensitivity, as well as changes in hormones that can affect insulin resistance such as leptin, adiponectin, ghrelin and tumor necrosis factor-alpha (TNFα).

## 2. Patients and Methods

### 2.1. Study patients and design

The study protocol included a group of obese patients (n=136; body mass index 33.3±4.4 kg/m$^2$; 74 men/62 women; 43.0±11.2 years) who followed an eight-week hypocaloric diet (-30% energy expenditure) to lose weight and were re-evaluated four or six months after treatment (Fig. 1). The therapy program was based on a nutritional intervention controlled by trained dieticians from the Department of Nutrition, Food Sciences and Physiology of the University of Navarra.

Among the total participants, 63 patients (cohort 1; 32 men/31 women) were enrolled in a nutritional weight loss program that consisted of an eight-week balanced hypocaloric diet containing 55% of the energy supply as carbohydrates, 15% as proteins, and 30% as fat. Upon completion of the dietary intervention (week 8; T1), volunteers were given general dietary guidelines to maintain the weight loss but without calorie restrictions or specific follow-up instructions. Six months after dieting ended (week 32; T2), the patients returned to the clinical research unit for further assessment.

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The additional 73 participants (cohort 2; 42 men/31 women) followed a therapy program based on the RESMENA-S (MEtabolic Syndrome REduction in Navarra) study, which was a randomized controlled intervention trial aiming to improve clinical criteria and biomarkers associated with metabolic syndrome (Met Synd) through a dietary strategy for weight loss during six months [36, 37]. Briefly, the study lasted six months in two sequential periods: one intervention period of two months (-30% energy restriction, 40-55%E carbohydrate/30%E lipids/15-30%E protein) in which subjects were randomly assigned to two energy-restricted diets (control diet and RESMENA diet) followed by a self-control period of four months in which subjects were advised to follow the learned-habits in the first period, which is further described elsewhere [36, 37]. Four months (week 24; T2) upon completion of the dietary intervention (week 8; T1), subjects returned to the clinical research unit for further assessment.

In both cohorts, participants were asked to maintain their normal physical activity during the study and received nutritional assessment every 15 days during the energy restriction period. Both protocols were approved by the Ethics Committee of the University of Navarra in accordance with the Declaration of Helsinki (ref 54/2006 and ref 065/2009), and all participants provided written informed consent.

Before and after the nutritional treatment, selected anthropometric measurements were taken. Dietary compliance was assessed through 3-days weighed food records [38]. Calculations of the energy and nutrient intake were performed using appropriate software (DIAL software; Alce Ingenieria, Madrid, Spain) based on the recognized Spanish food composition tables. Food questionnaires were completed during the week before the beginning of the intervention and 1 week before the completion of the hypocaloric diet. These reports provided information regarding the baseline intake and the adherence to the prescribed diets.

Anthropometric measurements and venous blood samples were collected after a 12-hour overnight fast performed according to standardized procedures at baseline (T0), the endpoint (T1) and the follow-up period (T2). The patient's EDTA-plasma and serum was separated from whole blood and immediately frozen at -80ºC until assayed.

2.2.Anthropometric and body composition measurements

Body weight measurements were performed using a digital balance accurate to 0.1 kg (Tanita bioelectrical impedance; SC-330, Tanita, Tokyo, Japan), and height was measured using a wall-mounted stadiometer (Seca 220; Vogel & Halke). The waist circumference was measured at the site of the smallest circumference between the rib cage and the iliac crest. Body composition was measured by bioelectrical impedance (SC-330, Tanita, Tokyo, Japan). All measurements were taken with the subjects in underwear and after an overnight fast.

2.3.Biochemical and hormonal analysis

Glucose and triacylglyceride serum concentrations were measured in an autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Insulin concentrations were assessed using an enzyme-linked immunosorbent assay (ELISA) kit available from Mercodia, AB (Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was indirectly determined by the homeostatic model assessment index (HOMA-IR), which was calculated following the formula [fasting plasma glucose (mg/mL) x fasting plasma insulin (μU/mL)/405], as described elsewhere [39]. Furthermore, patients were divided into two categories according to estimated insulin resistance (insulin sensitive: HOMA-IR<2.5; insulin resistant HOMA-IR≥2.5) following previously reported data [5, 40].

The quantitative measurement of irisin in human plasma samples was performed using a commercial ELISA kit directed against amino acids 31-143, which include the extracellular part of the protein of the FNDC5 protein (Irisin ELISA Kit EK-067-52; Phoenix Pharmaceuticals, INC., CA) according to the manufacturer's instructions. Absorbance from each sample was measured in duplicate using a spectrophotometric microplate reader at wavelength of 450 nm (Versamax Microplate Reader; Associates of Cape Cod Incorporated, East Falmouth, MA).

Overnight fasting plasma levels of leptin, adiponectin and ghrelin were measured by ELISA using a commercially available kit (Millipore, MA, USA). Fasting serum levels of TNFα were determined by the Quantikine High-Sensitivity Human TNFα Enzymatic Immunoassay (R&D Systems, Minneapolis, MN) in a Triturus autoanalyzer (Grifols SA).

2.4.Statistical analysis

The sample size of these interventional trials was estimated after accounting for the weight loss after treatment (main variable) and was calculated for an $\alpha=0.05$ and a power of 80% according to the equation reported by Mera et al. [41]. Thus, to detect differences, the sample size was established at a minimum of 66 obese patients who finished the nutritional intervention. The data from the obese patients were analyzed by the protocol and merged, as they followed a hypocaloric diet with the same energy restriction, and no differences were observed in the main variable analyzed (weight loss).

Diet-induced changes in body weight and hormone values were calculated (as percentages) as the difference between the endpoint (T1) and baseline (T0) measurements and related to the baseline (T0). Weight regain (percent) was calculated as the difference between the follow-up period (T2) and endpoint (T1) with respect to the endpoint values (T1). Successful weight-loss maintenance was assessed using the criterion of less than 10% weight

regain according to previously published reports [42]. The patients were subsequently sorted in two groups: those who regained ≥10% of the weight loss ("regainers") and those who maintained the weight loss ("non-regainers"). The values of anthropometric and biochemical parameters at T0, T1 and T2 were compared between the two groups.

Previously, the normal distribution of variables was explored using the Kolmogorov-Smirnov and the Shapiro Wilk tests. An ANCOVA was used to study differences between groups adjusted for gender and cohort as applicable. Moreover, a repeated-measures ANCOVA was used to study the effects of the time of the nutritional therapy program and groupings on body weight and biochemical parameters in the obese patients adjusted for gender and cohort. The Chi-square ($\chi^2$) test was used to compare the prevalence of insulin resistance between regainers and non-regainers. The potential associations between anthropometric and biochemical parameters were evaluated using the Spearman coefficient test. To analyze the effect of high or low hormone levels on insulin sensitivity, the median (above and below the 50th percentile) cutoff values in the irisin, leptin, adiponectin, ghrelin and TNFα were considered in the analyzed population, as previously applied [42] and based on a validated method to assign the studied population into two groups of disease risk [43]. In addition, a logistic regression analysis was applied to assess the potential predictive factors of insulin resistance risk during the weight maintenance period (T2). Insulin resistant categories at T2 were used as the dependent variable, and median cut-off values of hormone levels at baseline (T0) or at follow-up (T2) were used as independent variables. The logistic regression models were adjusted for gender, age, body weight at follow-up (T2), insulin sensitivity state (according to the HOMA-IR≥2.5 cut-off criteria) at baseline (T0) and change (%) in fat mass.

Statistical analysis was performed using SPSS version 15.0 software (SPSS Inc., Chicago, IL) for Windows XP (Microsoft, Redmond, WA). P≤0.05 was considered to be statistically significant.

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## 3. Results

As designed, the hypocaloric diet induced a statistically significant weight loss (-6.31±0.195%; p<0.001, adjusted for gender and cohort) and a reduction in body mass index (BMI), waist circumference and body fat mass in the patients as a group (data not shown). After the follow-up period (T2), as a group, patients continued to decrease their body weight compared with the endpoint (T1) of the energy restriction treatment (89.0±1.0 kg vs. 87.2±1.1 kg; p<0.001, adjusted for gender and cohort). However, when the participants in the current study were categorized according to the 10% of weight regain classification criterion, 75 subjects maintained the weight loss (non-regainers), and 61 subjects regained (regainers) at least 10% of the weight loss at T2. The analysis of the clinical characteristics of the patients revealed no significant differences in age, body weight, body fat mass or waist circumference between regainers and non-regainers at baseline (Table 1). Nevertheless, an expected, statistically significant (p<0.05), higher body weight was observed in regainers compared with non-regainers, along with a higher BMI, waist circumference and fat mass at T2 (Fig. 2). No differences were observed in fat free mass between both groups (58.79±1.24 kg non-regainers vs. 60.85±1.23 kg regainers; p=0.252, adjusted for gender and cohort). These anthropometric differences between both groups were concomitant with statistically significant (p<0.05) elevated levels of glucose and insulin, as well as HOMA-IR, in regainers compared with non-regainers (Fig. 2).

As expected, the percentage change in HOMA-IR from T0 to T2 was statistically correlated with the percentage of weight regain (r=0.41; p<0.001), suggesting that unsuccessful weight maintenance is associated with a reduced insulin sensitivity. To investigate the association between insulin sensitivity and weight regain further, patients were first classified according to the changes in HOMA-IR from T0 to T2 into three groups; "improving group" (ΔHOMA-IR <-0.01), "worsening group" (ΔHOMA-IR>0.01) and "non-change group" (-

0.009>ΔHOMA-IR<0.009). Under these conditions, approximately 32% (n=43/136) of the subjects exhibited reduced insulin sensitivity from T0 to T2 as demonstrated by an HOMA-IR increase greater than 0.01 units (Fig. 3A). This reduced insulin sensitivity was observed in 34.4% (n=21/61) of regainers and in 29.3% (22/75) of non-regainers.

Importantly, when patients were categorized as insulin resistant or insulin sensitive according to the HOMA-IR≥2.5 cut-off criteria [5, 40], 18.0% of regainers exhibited worsened insulin sensitivity from a state of insulin sensitivity at T0 to insulin resistance at T2, and only 16.4% of regainers improved their insulin sensitivity during the therapy program (Fig. 3B). In contrast, among the non-regainers, only 9.3% of insulin sensitive patients at T0 were classified as insulin resistant at T2, whereas 30.7% of non-regainers improved their insulin sensitivity at T2 from their insulin resistance condition at T0 (p=0.018, compared with regainers; Fig. 3B).

Taking into account that insulin sensitivity is influenced by several energy metabolism-related proteins and that the newly discovered myokine irisin is proposed to be involved in the management of insulin sensitivity [9], the circulating levels of irisin as well as the levels of leptin, adiponectin, ghrelin and TNFα were evaluated as potential factors associated to the decrease in insulin sensitivity. Due to insufficient blood sample for this aim in overall patients included in this study this objective was performed specifically in the cohort 2 (n=73; Fig. 1).

Interestingly, although there was no statistical correlation with glucose levels, irisin plasma levels at T0 and at T2 were statistically associated with the circulating levels of insulin and with the HOMA-IR, as well as with the circulating triacylglycerides (Table 2). Indeed, at baseline (T0), circulating insulin was identified an independent factor affecting the irisin plasma levels, after adjusting for gender and BMI, (β=6.21; 95%CI: 0.06-12.35; p=0.048).

In addition, circulating levels of leptin, adiponectin, ghrelin and TNFα at T0 and T2 were also statistically correlated with several risk factors of insulin resistance, such as waist circumference and glucose, insulin, triacylglycerides and HOMA-IR levels (Table 2). Thus, the

studied insulin resistance risk factors were directly associated with the circulating levels of irisin, leptin and TNFα and were inversely correlated with adiponectin. An inverse association was also observed between ghrelin levels and waist circumference (Table 2). The cross correlation of the hormones showed that irisin levels at T0 were inversely associated with ghrelin at T0 and TNFα at T2 (Table 2).  No association was detected between irisin and leptin or adiponectin.

To further investigate the influence of irisin levels on the impairment of insulin sensitivity, the patients were categorized into two groups according to the median baseline (T0) irisin plasma levels. Patients exhibiting irisin plasma levels above the median showed statistically higher HOMA-IR in the weight maintenance period (p=0.012) than those with irisin levels below the median after adjusting for age and gender (Fig. 4). In addition, the effects of adiponectin, leptin, ghrelin and TNFα were explored in the same manner, observing that baseline circulating leptin levels above the median were accompanied by increased follow-up HOMA-IR (p=0.005), and no differences in HOMA-IR were observed according to the baseline adiponectin, ghrelin and TNFα levels (Fig. 4). These results were also observed when patients were divided according to the median irisin, adiponectin, leptin, ghrelin and TNFα levels at follow-up (T2), with significant differences detected specifically according to irisin and leptin levels (Fig. 4).

Reinforcing these findings, a logistic regression analysis (Table 3) revealed that high irisin levels at baseline (odds ratio=4.2; p=0.039) and a trend at follow-up (odds ratio=3.6; p=0.088), as well as high follow-up leptin levels (odds ratio=10.8; p=0.008), predicted an increased risk of insulin resistance during the follow up period (T2) independently of gender, age, body weight at T2, and insulin sensitivity state at T0 (Table 3). These statistically significant associations remained after adjusting for the change in fat mass from the end of the dietary treatment to follow-up (Table 3).

When patients were categorized according to their success in weight maintenance, no statistically significant differences (p>0.05) were observed at baseline (T0) between regainers and non-regainers. At follow up (T2), regainers, who were more often associated with decreased insulin sensitivity, exhibited statistically higher irisin and leptin levels than did non-regainers (Table 4). Although adiponectin did not predict insulin sensitivity with statistical significance in the patients as a group, statistically significant lower adiponectin levels were observed in regainers compared with non-regainers at follow–up, whereas no differences were observed in ghrelin and TNFα between the two groups.

## 4. Discussion

The current findings indicate that a high proportion of patients who follow a dietary intervention to lose weight are prone to decreased insulin sensitivity over the period of free-living despite successful weight loss and an improvement in insulin sensitivity induced by the dietary weight-lowering program. This decreased insulin sensitivity was especially relevant in those patients who regained their lost weight, which reinforces the main concern of identifying robust biological markers able to detect patients who are more likely to regain diet-induced weight loss early [42, 44] and to guide appropriate therapy and thereby improve clinical outcomes. Among the potential factors associated with this insulin resistance progress, irisin appears to play a role in addition to leptin and adiponectin, both of which are proteins with a highly demonstrated insulin resistance relationship. Therefore, irisin could play a role in promoting insulin resistance or be an adaptive response to counteract the glucose homeostasis disturbance in obesity.

The application of a balanced hypocaloric diet for weight loss in the current work improved glucose, insulin and HOMA-IR levels. This insulin sensitivity improvement during an energy restriction phase was previously associated with a lower subsequent weight regain

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[45]. However, when the subjects included in this study were categorized into two groups according to their success in body weight maintenance, those subjects who regained at least 10% of the lost weight experienced an increase in their glucose, insulin and HOMA-IR levels, whereas the subjects who maintained or continued to decrease their body weight exhibited decreasing values of the glucose regulatory parameters evaluated, which is in agreement with previous findings [4]. This deleterious effect observed during a free-living phase of a nutritional intervention to lose weight appears to be prevented when patients followed behavioral strategies for maintaining weight loss and they were in continuous supervision [46], instead of a free-living phase after dieting.

Insulin sensitivity is modulated by several adipose-derived hormones, such as adiponectin and leptin. Adiponectin is considered an insulin sensitizer, has antidiabetic properties and decreased adiponectin levels are associated with obesity and insulin resistance [47]. Leptin collaborates with insulin to regulate glucose and lipid metabolism, in addition to regulating food intake and metabolic rate via the central nervous system [48]. However, increased leptin concentration is associated with obesity and insulin-resistant states, suggesting that obese individuals display leptin resistance [49].

Recently, a muscle-secreted peptide named irisin was identified and proposed to play an important role in improving obesity and glucose homeostasis [9, 50]. Like leptin, irisin has attracted considerable attention because it has been proposed to have therapeutic potential for obesity and diabetes and to improve life expectancy. However, in a manner similar to leptin, irisin circulating concentrations [51-53] as well as its expression in muscle and adipose tissue [20, 54] is increased in obesity, which suggests potential irisin resistance [55] as occur with leptin.

Given this prior knowledge, the present study explored the association between the insulin sensitivity in a weight regain condition with irisin plasma levels and other hormones

related to body weight homeostasis. The findings identified irisin and leptin as relevant factors that may contribute to the onset of insulin resistance. The risk of insulin resistance at follow-up was higher in those patients with high levels of irisin and leptin. Although adiponectin has been demonstrated to have an insulin-sensitizing activity [47], no association was observed between the insulin resistance during the weight maintenance period and adiponectin levels. In fact, only high irisin plasma levels at baseline were able to predict the classification of patients as insulin resistant during the weight maintenance period, independent of gender, body weight at follow-up and insulin sensitivity state at baseline and even when the change in fat mass from the end of the dietary treatment to follow-up was included as a confounder factor.

Interestingly, when the patients were categorized according to their successes in weight maintenance at follow-up, the decreased insulin sensitivity that was observed more frequently in the regainer group, was accompanied by a greater increase in irisin and leptin levels as well as smaller increases in adiponectin than did non-regainers during the follow-up period. Therefore, in addition to the expected contribution of leptin and adiponectin changes, the insulin resistance observed in regainers occurred concomitantly with increased levels of irisin during the free-living follow-up period.

The current results are in agreement with recent reports in which a positive association was observed between circulating irisin levels or *FNDC5* expression in human myotubes and fasting insulin concentrations and HOMA-IR [18, 56]. Additionally, during the peer-review process of the current work, an independent study reported a significant positive correlation between baseline circulating irisin levels and insulin resistance as assessed by HOMA-IR [57].

In contrast, three previous reports [26-28] observed a decreased level of circulating irisin in patients with type 2 diabetes compared with normal glucose tolerance subjects. However, the subjects included in these studies exhibited a low degree of obesity, whereas in the current work, the mean of body mass index was 33.5 kg/m$^2$. In fact, circulating irisin

correlated positively with most known markers of insulin resistance in non-diabetic individuals [27]. All together the current and previous results support the hypothesis that increased circulating irisin is an adaptive response to compensate for the decreasing insulin sensitivity and disturbances in metabolism associated with obesity [51, 58]. Though irisin is also secreted by adipose tissue [20], the reduced irisin levels displayed in type 2 diabetes [26-28] may be due to the decrease of body fat stores in uncontrolled insulin-deficient diabetes as occur with leptin [59]. On the other hand, it could be speculated that in obesity a long-term increase in irisin becomes a pathological condition that promotes insulin insensitivity. The potential resistance to irisin displayed in obesity could promote insulin secretion resulting in hyperinsulinemia and leading to insulin resistance. However, these hypotheses remain to be demonstrated and it cannot be elucidated by the current work.

The strength of this study is its longitudinal design, which allows the evaluation of the time-course of changes of insulin resistance risk factors in parallel with irisin circulating levels and other insulin sensitivity-related hormones, as well as differences between regainers and non-regainers of body weight. In fact, this study provides, for the first time, evidence revealing that insulin resistance associated with weight regain during the free-living period after a diet-induced weight loss can be predicted by the newly discovered myokine irisin.

Possible limitations of the current study warrant consideration before interpretation of the findings. First, the sample size of this study limited the inclusion of potentially relevant co-variables such as physical activity, smoking status, or dietary variables that could help to build a best prediction model of the insulin resistance risk during the free-living period after dieting. However, the statistical significance found when using small populations usually indicates that there is a real difference between the experimental groups. In fact, although the sample size of this trial was not estimated based on the irisin levels because it was a secondary outcome in response to the potential role of irisin as a therapeutic target of metabolic disorders in the last

months, this study had over 93% power to detect the differences in irisin circulating levels between regainers and non-regainers. Secondly, because this report describes an observational association study, this work can only propose hypotheses and mechanisms potentially underlying the association between insulin resistance and irisin, adiponectin and leptin, rather than demonstrating causality. On the other hand, there has been controversy regarding the stability and range of the commercially available irisin ELISA kits, as this field needs a validated antibody that can quantify levels of FNDC5/irisin protein in plasma [60]. In the current work, the ELISA kit employed was directed against amino acids 32 to 143 which include the extracellular part of the protein according to manufacturer information (Phoenix pharmaceuticals); this kit was the same one employed in previous studies in humans [51, 52, 57]. In addition, all of the samples in this study were analyzed using the same kit lot number to avoid bias. Therefore, the current results of the irisin plasma levels are robust and comparable with previous findings in humans.

In summary, the current findings adds consistent information concerning the association between circulating irisin levels and the prevalence of insulin resistance in weight regain patients after a dietary weight-lowering program in obese humans. These observations are of foremost relevance, as they demonstrate that energy restriction treatment could be harmful for an important number of obese treated patients, promoting insulin resistance in a short time, which, consequently, could lead to type 2 diabetes mellitus onset in the future. More novelty, the translational potential of the current findings is that evaluating the circulating irisin values at baseline could predict future disturbance in glucose homeostasis during the free-living time after a dietary treatment. Further mechanistic studies are needed to elucidate whether irisin is a promoter of insulin resistance or whether irisin levels represent an adaptive response induced by insulin signaling to counteract the glucose homeostasis disturbance induced in obesity.

**Disclosure statement**

The authors have nothing to declare.

**Authors' contributions**

ABC, MAZ, JAM and FFC designed the study. ABC, PLL, RdI, MCC and MP, contributed to the acquisition of the data and performed the analyses. ABC performed the statistical analysis. ABC and FCC wrote the first draft of the paper and MAZ, MCC, MP, and JAM, contributed to the interpretation of data and critical revision of the manuscript. All authors were involved in the writing of the manuscript and approved the final version of this article.

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**FIGURE LEGENDS**

**Fig. 1.** Schematic overview of the study protocol. CHO, carbohydrates; prot., proteins; lip., lipids; RESMENA, MEtabolic Syndrome REduction in NAvarra; TNFα, tumor necrosis factor-alpha.

**Fig. 2.** Body mass index (BMI), waist circumference, percent fat mass, plasma glucose, insulin levels and Homeostatic model assessment index-insulin resistance (HOMA-IR) during the nutritional treatment (T0-T1) and after the 4-6 months follow-up period (T2) as a function of the success in weight maintenance (non-regainers, n=75 and regainers, n=61). The data are presented as the mean (SE). ‡, statistically significant differences ($p<0.001$) from T0 to T1 as evaluated by means of a repeated-measures ANCOVA adjusted for gender and cohort. §, statistically significant ($p<0.05$) changes over the duration of the nutritional program (from T0 to T1 and T2). †, statistical significance for the interaction between time x regaining group (non-regainers, regainers) as evaluated by means of a repeated-measures ANCOVA adjusted for gender and cohort. Statically significant differences between regainers and non-regainers were evaluated by ANCOVA adjusted for gender and cohort and are indicated as *$p<0.05$.

**Fig. 3.** (A) Distribution of the patients (n=136) classified according to the changes in HOMA-IR from baseline (T0) to follow-up (T2) in three groups: improving group (ΔHOMA-IR $<-0.01$), worsening group (ΔHOMA-IR$>0.01$) and non-change group (-ΔHOMA-IR$\pm0.009$). (B) Prevalence of insulin resistance according to the HOMA-IR $\geq2.5$ classification criterion at T2 compared with that at T0 among regainers (n=61) and non-regainers (n=75) of at least 10% of lost weight. Statistical significance was evaluated by the $\chi^2$ test.

**Fig. 4.** Differences in HOMA-IR values at follow-up (T2) of cohort 2 patients (n=73) categorized according to the median of the irisin, adiponectin, leptin, ghrelin and tumor necrosis factor-alpha (TNFα) levels at baseline (T0) and at follow-up (T2). Data are presented

as the mean (SE). Statistically significant differences between both groups were evaluated by

ANCOVA adjusted for gender and diet (control and RESMENA) are indicated as *p<0.05.