Circulating Irisin Levels in Children With GH Deficiency Before and After 1 Year of GH Treatment

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Purpose: To evaluate circulating irisin levels in children with GH deficiency (GHD) and any relation with clinical and metabolic parameters.

Patients: Fifty-four prepubertal children (mean age, 7.4 ± 0.8 years) with idiopathic GHD treated with GH for at least 12 months and 31 healthy short children as control subjects.

Methods: Body height, body mass index (BMI), waist circumference (WC), IGF-I, HbA1c, lipid profile, fasting and after–oral glucose tolerance test glucose and insulin, insulin sensitivity indices, and irisin levels were evaluated at baseline and after 12 months of GH replacement (GHR).

Results: At baseline, children with GHD, in addition to having lower growth velocity ($P < 0.001$), GH peak after stimulation tests (both $P < 0.001$), and IGF-I ($P < 0.001$), showed significantly lower irisin ($P < 0.001$) and higher BMI ($P < 0.001$) and WC ($P = 0.001$), without any difference in metabolic parameters, than control subjects. After GHR, children with GHD showed a significant increase in height ($P < 0.001$), growth velocity ($P < 0.001$), IGF-I ($P < 0.001$), fasting glucose ($P = 0.002$) and insulin ($P < 0.001$), homeostasis model assessment estimate of insulin resistance ($P < 0.001$), and irisin ($P = 0.005$), with a concomitant decrease in BMI ($P = 0.001$) and WC ($P = 0.003$). In multivariate analysis, the independent variables significantly associated with irisin were BMI ($P = 0.002$) and GH peak ($P = 0.037$) at baseline and BMI ($P = 0.005$), WC ($P = 0.018$), and IGF-I ($P < 0.001$) during GHR.

Conclusions: We report that GHR leads to an increase in irisin levels, strongly related to a decrease in BMI and WC, and to an increase in IGF-I; these changes are among the main goals of GHR. These data confirm the favorable effects of GHR in children. (***J Clin Endocrinol Metab*** **104: 801–808, 2019)**

Irisin, the secreted product of the fibronectin type III domain 5 protein, is a recently identified myokine that was first isolated from muscle tissue in 2012 by Boström *et al.* (1). Data suggest that irisin may play an important metabolic role in regulating adipose tissue metabolism by converting white to brown adipose tissue and may reduce the risk of obesity and diabetes (2, 3). Brown adipose tissue activity is high in the pediatric population, especially in subjects with a lower body mass index (BMI); thus, a reduction in irisin may play a role in the pathogenesis of childhood obesity (4). These data indicate that irisin may be a possible link between skeletal muscle and adipose tissue. Significantly, irisin improves glucose tolerance in mice (3), and circulating irisin levels correlate with glucose tolerance and insulin resistance in humans (5, 6). Because irisin plays a role in both adipose tissue and glucose metabolism, two targets of GH action, changes in irisin may mediate effects of GH deficiency (GHD) and GH replacement (GHR) on these endpoints.

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Abbreviations: ARG, arginine; AUC, area under the curve; BMI, body mass index; GHD, GH deficiency; GHR, GH replacement; GST, glucagon stimulation test; HOMA-IR, homeostasis model assessment estimate of insulin resistance; ISI, insulin sensitivity index; OGTT, oral glucose tolerance test; WC, waist circumference.

Specifically, GHD is characterized by altered body composition (reduced muscle mass and increased adiposity) (7, 8) as well as metabolic alterations (9–11), and GHR may result in a reversal of these effects (12–15).

A direct interplay between irisin and GH has been documented in nonmammalian species. Indeed, fish irisin acts directly at the pituitary level to inhibit GH transcript expression via multiple signaling pathways (16). However, it is not known whether GHD and its replacement can affect irisin levels or whether changes in irisin levels in GHD and replacement are associated with the known changes in body composition and glucose homeostasis.

The aim of this study was to evaluate circulating irisin levels in a cohort of children with GHD at diagnosis, the changes in them during GHR, and any associations of irisin with body composition and metabolic parameters. Based on data indicating that irisin may be a link between muscle and adipose tissue and possible effects of irisin on glucose homeostasis, we hypothesized that GHD is associated with lower irisin levels, which would increase after GHR, and is associated with changes in body composition and metabolic endpoints.

Materials and Methods

We prospectively studied 54 prepubertal children (42 male, 12 female; mean age, 7.4 ± 0.8 years; range, 4.3 to 8.5 years) with isolated idiopathic GHD who were consecutively admitted to the Section of Endocrinology of the University of Palermo during the years 2016 and 2017 and treated with GH for at least 12 months. Thirty-one healthy short children without GHD, matched for sex (20 male, 11 female), age (mean age, 7.4 ± 1.1 years; range, 4.6 to 9.9 years), and pubertal status, were recruited among patients referred for the assessment of short stature as a control group of children with GHD at baseline. A subgroup of 15 healthy children was evaluated after 12 months of observation.

Children with a therapeutic follow-up of <12 months or receiving other hormonal replacement treatment due to multiple pituitary hormone deficiency were excluded from the study. To avoid interference of puberty with the clinical and metabolic parameters, all children included in the study were in the first stage of sexual development and maintained their prepubertal clinical and hormonal status during the 12 months of follow-up.

The diagnosis of GHD was established by the clinical, auxological, and biochemical criteria of the GH Research Society (17).

As auxological criteria, we considered height and growth velocity 12 months before diagnosis. As radiological criteria, we considered a bone age delay of at least 1 year with respect to the chronological age, estimated from an X-ray of the left wrist and hand and evaluated according to the Greulich-Pyle method (18).

GHD was biochemically demonstrated by GH peaks below 8 μg/L during the arginine (ARG) and glucagon stimulation test (GST) performed on two different days. Neuroimaging, with magnetic resonance imaging of the hypothalamic-pituitary region, was performed as per internal protocol in children with more severe GHD (*i.e.*, with GH peak ≤3 μg/L; n = 20). No evidence of intrasellar lesions was found. Seven children showed pituitary hypoplasia. All patients received GH once daily at bedtime with a pen injection system. During the follow-up, IGF-I levels and growth velocity allowed us to use in all children the GH dose in line with our internal fixed protocol, with an initial daily dose of 0.025 mg/kg from baseline until month 6 and a mean dose of 0.028 mg/kg from months 6 to 12. During the entire follow-up, IGF-I levels remained within the normal range for age.

Study protocol

In all children at baseline and after 12 months of GHR, we measured body height (expressed as SD), growth velocity, BMI (SD), waist circumference (WC, SD), and bone/chronological age ratio.

The areas under the curve (AUC) of GH (AUC_{GH}) during ARG and GST test were calculated using the trapezoidal rule.

On a different day, a blood sample was drawn after an overnight fast for the measurement of fasting glucose, fasting insulin, hemoglobin A1c (HbA1c), lipid profile (including total and high-density lipoprotein cholesterol and triglycerides), IGF-I, and irisin. Low-density lipoprotein cholesterol levels were evaluated by the following formula: total cholesterol – (high-density lipoprotein cholesterol − triglycerides/5). This sample also served as the baseline sample for an oral glucose tolerance test (OGTT). Glucose (1.75 g/kg body weight, up to a maximum of 75 g) was given orally, and blood samples were collected every 30 minutes up to 2 hours for glucose and insulin measurements.

As surrogate estimates of insulin sensitivity, we considered the homeostasis model assessment estimate of insulin resistance (HOMA-IR) using the following formula: HOMA-IR = (fasting glucose × fasting insulin)/22.5 (19) and the insulin sensitivity index (ISI), a more composite index derived from the OGTT and validated by Matsuda and DeFronzo using the following formula: ISI = $10{,}000/\sqrt{}$ (fasting glucose × fasting insulin × Gmean × Imean), where Gmean is the mean plasma glucose concentration during OGTT and Imean is the mean serum insulin concentration during OGTT (20).

The institutional Ethics Committee of the University of Palermo approved this study. At the time of hospitalization, all patients and their parents gave informed written consent to the study and for scientific use of the data.

Hormone and biochemical assays

All biochemical data were collected after overnight fasting. Glucose and lipid profiles were measured in the centralized accredited laboratories of the University of Palermo with the standard methods. HbA1c levels were determined by HPLC with an ion-exchange resin (BioRad D10; BioRad, Milan, Italy). Serum insulin was measured by electrochemiluminescence (ECLIA; Elecsys Insulin, Roche, Milan, Italy). The sensitivity of the method was 0.4 μU/mL. The normal range was 2.6 to 24.9 μU/mL. Serum GH levels were measured by electrochemiluminescence (ECLIA). The lower limit of detection of the

assay was 0.030 μg/L. The intra- and interassay coefficients of variation were 0.6% to 5.0% and 3.8% to 5.0%, respectively. We reported GH concentrations in μg/L of International Standard 98/574. Serum IGF-I levels were measured by means of a chemiluminescent immunometric assay (Immulite 2000; Diagnostic Products Corp., Los Angeles, CA) using murine monoclonal anti–IGF-I antibodies. The standards were calibrated against the World Health Organization second International Standard 87/518. The sensitivity was 1.9 μg/L. The intra- and interassay coefficients of variation were 2.3% to 3.9% and 3.7% to 8.1%, respectively.

Serum samples were analyzed for irisin concentration using a commercial enzyme immunoassay kit (EK-067-29; Phoenix Pharmaceuticals, Karlsrue, Germany). The lowest detectable concentration of irisin was 7.0 ng/mL, and the highest was 32.3 ng/mL. The kit used in the current study was validated against Western blotting and mass spectrometry (21). Samples were assayed following the manufacturer's instructions without a prior extraction step.

Statistical analysis

SPSS version 19 was used for data analysis. The baseline characteristics were presented as mean ± SD for continuous variables. Rates and proportions were calculated for categorical data. Normality of distribution for quantitative variables was assessed with the Kolmogorov-Smirnov test. The differences between the two independent groups (children with GHD *vs.* control subjects) were evaluated by Student *t* test, and differences between paired continuous variables (before and after 12 months of therapy in children with GHD) were analyzed using paired *t* tests. Correlation between variables was evaluated with the Pearson test. Multiple linear regression analysis was performed to identify independent predictors of the dependent variable irisin at baseline and at 12 months. The decision to keep the variables in the multivariate model was based on clinical and statistical significance. Variables having a potential clinical impact on irisin levels and significantly associated with irisin on univariate analysis (Pearson correlation) were included (*i.e.*, BMI, WC, and GH peak at baseline and BMI, WC, and IGF-1 at 12 months). A P value <0.05 was considered statistically significant.

Results

At baseline (children with GHD and control subjects)

Clinical and hormonal parameters

At baseline, children with GHD showed significantly lower growth velocity ($P < 0.001$), bone/chronological age ratio ($P < 0.001$), GH peak and AUC after ARG (both $P < 0.001$), and GST ($P < 0.001$ and 0.007, respectively) and IGF-I SD ($P < 0.001$), with higher BMI ($P < 0.001$) and WC ($P = 0.001$), than control subjects (Table 1).

Table 1. Clinical, Hormonal, and Metabolic Parameters of Control Subjects and Children With GHD at Diagnosis and After 12 Months of GH Replacement

	Control Subjects (n = 31)	Subjects With GHD		*P* Value[a]	*P* Value[b]
		Baseline (n = 54)	12 mo (n = 54)		
Gender				0.142	—
Male, n (%)	20 (64.5)	42 (78)			
Female, n (%)	11 (35.5)	12 (22)			
Age, y	7.4 ± 1.1	7.4 ± 0.8	—	0.913	—
Height, (SD)	−1.9 ± 0.3	−2.1 ± 0.7	−1.4 ± 0.7	0.159	<0.001
BMI	−0.9 ± 0.2	−0.5 ± 0.2	−0.6 ± 0.3	<0.001	0.001
Waist circumference, (SD)	−0.4 ± 0.2	0.2 ± 0.1	0.02 ± 0.08	0.001	0.003
Growth velocity, (SD)	0.2 ± 0.7	−0.9 ± 0.5	0.1 ± 0.4	<0.001	<0.001
Bone/chronological age ratio	0.8 ± 0.09	0.7 ± 0.1	0.8 ± 0.08	<0.001	<0.001
GH peak during ARG test, μg/L	11.1 ± 4	3.6 ± 2	—	<0.001	—
AUC_{GH} during ARG test, μg/L	598 ± 376	219 ± 148	—	<0.001	—
GH peak during GST, μg/L	13.5 ± 4	3.7 ± 2.2	—	<0.001	—
AUC_{GH} during GST, μg/L	931 ± 397	390 ± 154	—	0.007	—
IGF-I	0.7 ± 0.3	−0.5 ± 0.5	0.5 ± 0.1	<0.001	<0.001
Fasting glucose, mmol/L	4.1 ± 0.4	4.3 ± 0.5	4.6 ± 0.3	0.157	0.002
Fasting insulin, μU/mL	5.3 ± 2.7	5.8 ± 3.3	9 ± 3.8	0.370	<0.001
HbA1c, %	5.2 ± 0.3	5.1 ± 0.3	5.2 ± 0.3	0.328	0.686
HOMA-IR	0.9 ± 0.4	1 ± 0.7	1.9 ± 0.7	0.680	<0.001
ISI	14.1 ± 10.5	11.8 ± 4.1	10.1 ± 1.5	0.499	0.055
Total cholesterol, mmol/L	4.1 ± 0.6	4 ± 0.7	3.9 ± 0.8	0.650	0.401
HDL cholesterol, mmol/L	1.7 ± 0.4	1.6 ± 0.3	1.6 ± 0.2	0.257	0.260
LDL cholesterol, mmol/L	2 ± 0.5	2.1 ± 0.6	1.9 ± 0.7	0.713	0.104
Triglycerides, mmol/L	1.6 ± 0.5	1.4 ± 0.4	1.5 ± 0.4	0.213	0.270
Irisin, ng/mL	22.3 ± 4.4	14.8 ± 6.9	17.5 ± 6.1	<0.001	0.005

Data are presented as mean ± SD unless otherwise indicated.

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a]Difference between control subjects and children with GHD at baseline.

[b]Difference between children with GHD at baseline and after 12 mo of GH replacement.

Irisin and metabolic parameters

At baseline, children with GHD showed significantly lower irisin levels ($P < 0.001$) than control subjects. No significant difference was found in other metabolic parameters (Table 1).

In children with GHD, baseline irisin levels were significantly and negatively correlated with BMI ($r = -0.528$, $P < 0.001$) and WC ($r = -0.400$, $P = 0.003$) and positively correlated with stimulated GH peak ($r = 0.275$, $P = 0.044$) without correlation with other clinical and metabolic parameters (Table 2).

These correlations at baseline were confirmed in the control group, where the irisin levels were correlated negatively with the BMI ($r = -0.427$, $P = 0.017$) and positively with the stimulated GH peak ($r = 0.370$, $P = 0.043$).

Multivariate analysis showed that the independent variables significantly associated with irisin levels in children with GHD were BMI ($P = 0.002$) and GH stimulated peak ($P = 0.037$) (Fig. 1a). In the control group, irisin was independently associated with BMI ($P = 0.012$) and peak stimulated GH concentration ($P = 0.023$).

At 12 months of GHR (children with GHD)

Clinical and hormonal parameters

After 12 months of GHR, children with GHD showed a significant increase in height ($P < 0.001$), growth velocity ($P < 0.001$), bone/chronological age ratio ($P < 0.001$), and IGF-I ($P < 0.001$), with a concomitant decrease in BMI ($P = 0.001$) and WC ($P = 0.003$).

Irisin and metabolic parameters

After 12 months of GHR, we found a significant increase in fasting glucose ($P = 0.002$), fasting insulin ($P < 0.001$), HOMA-IR ($P < 0.001$), and irisin ($P = 0.005$), with a trend toward decrease, although not statistically significant, in ISI ($P = 0.055$) and without significant changes in Hba1c and lipid profile (Table 1). The mean increased irisin levels in children with GHD observed after GHR did not reach mean values observed in control subjects ($P < 0.001$). The increase in irisin levels from baseline to 12 months of GHR was also documented by grouping children with GHD according to GH peak ≥3 or <3 μg/L. Specifically, irisin levels increased from 15.5 ± 7.0 to 18.4 ± 6.5 ng/mL ($P = 0.030$) in children with GH peak ≥3 μg/L and from 13.2 ± 6.7 to 16.1 ± 5.3 ng/mL ($P = 0.041$) in children with GH peak <3 μg/L.

In the subgroup of 15 healthy children, no significant difference in irisin levels from baseline to 12 months of observation was documented (data not shown).

In the GHD group, after 12 months of GHR, irisin levels were correlated negatively with BMI ($r = -0.753$, $P < 0.001$) and WC ($r = -0.497$, $P < 0.001$) and positively with IGF-I ($r = 0.865$, $P < 0.001$).

Multivariate analysis showed that the independent variables were significantly associated with irisin levels in GHD children were BMI ($P = 0.005$), WC ($P = 0.018$), and IGF-I ($P < 0.001$) (Fig. 1b).

Discussion

This prospective study demonstrates that children with GHD show lower irisin levels than control subjects and

Table 2. Correlation Between Irisin and Clinical, Hormonal, and Metabolic Parameters (Univariate Analysis) in Children With GHD at Diagnosis and After 12 Months of GH Replacement

	Irisin			
	Baseline		12 mo	
	r	*P* Value	*r*	*P* Value
Height (SD)	−0.062	0.657	−0.099	0.494
BMI (SD)	−0.528	<0.001	−0.753	<0.001
Waist circumference (SD)	−0.400	0.003	−0.497	<0.001
GH peak during ARG test, μg/L	0.275	0.044	—	—
AUC_{GH} during ARG test, μg/L	0.207	0.133	—	—
IGF-I	−0.187	0.175	0.865	<0.001
Fasting glucose, mmol/L	−0.259	0.079	−0.095	0.514
Fasting insulin, μU/mL	−0.136	0.338	0.162	0.275
HOMA-IR	0.002	0.992	0.106	0.497
ISI	−0.152	0.355	0.008	0.961
Total cholesterol, mmol/L	−0.206	0.170	−0.086	0.575
HDL cholesterol, mmol/L	0.050	0.744	0.242	0.114
LDL cholesterol, mmol/L	−0.247	0.098	−0.209	0.184
Triglycerides, mmol/L	−0.118	0.433	−0.245	0.109

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

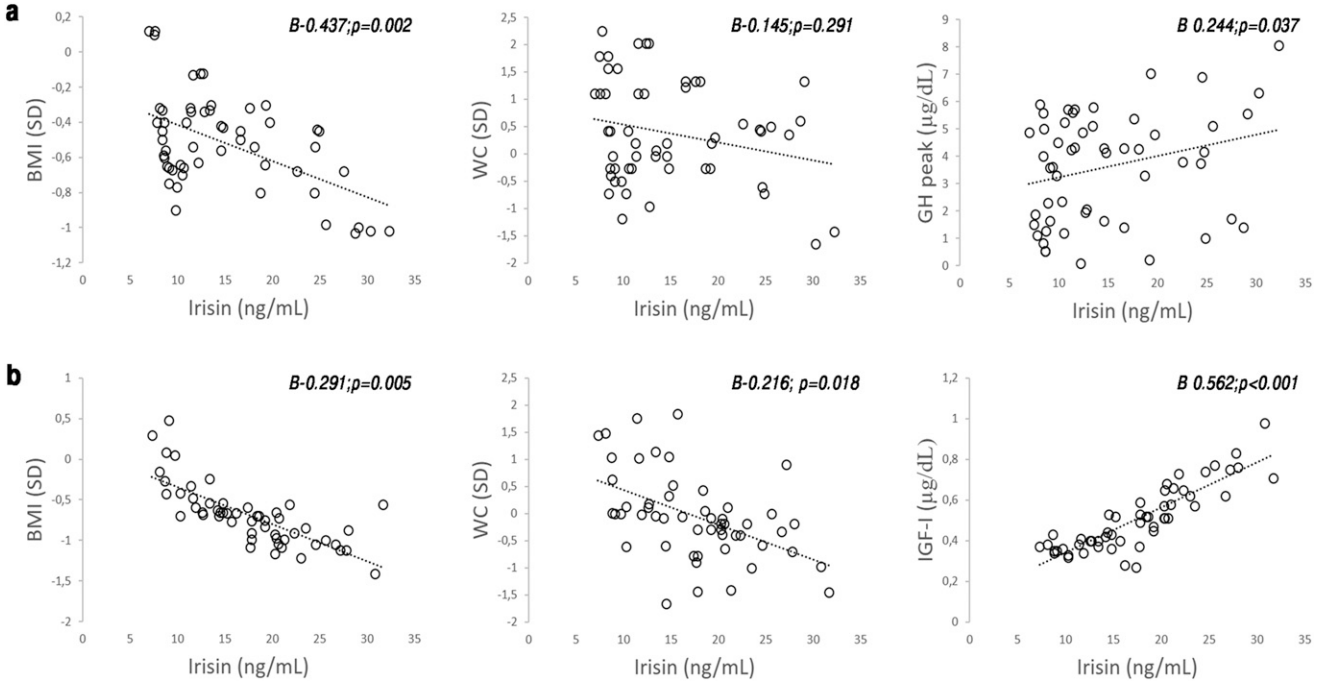

Figure 1. Independent variables associated with irisin levels at (a) baseline and (b) 12 months of GH replacement in multivariate analysis.

that GHR is able to increase these levels, which are associated with BMI, WC, and IGF-I levels.

Very few studies have evaluated irisin in patients with GHD. Taking these aspects into account, the evaluation of circulating irisin could be of interest in these patients.

Contradictory data have been found regarding the production of irisin in human tissues. In some studies, the amount of muscular mass has been reported as the main predictor for circulating irisin, whereas in other studies adipose mass has been found to be the major factor influencing irisin levels (5, 22). Irisin has been found to be significantly related to body composition and insulin sensitivity, although the role of irisin in obesity and its metabolic consequences are controversial, especially regarding its relationship with insulin resistance and BMI.

In our study, we found lower irisin levels in the GHD group than in control subjects, concomitant with the higher BMI and WC. These data confirmed the relationship between irisin and body composition. Indeed, we documented a significant correlation between irisin and the clinical indexes of adiposity.

In obese children, irisin levels have been found to be positively related to insulin resistance (23, 24), providing evidence regarding the role of irisin in insulin sensitivity in childhood obesity (25), and in the authors' hypothesis increased irisin levels can be interpreted as an adaptive response that compensates for the decreasing insulin sensitivity and metabolic disturbances associated with obesity (5, 26). Conversely, Al-Daghri *et al.* (6) showed a negative correlation between serum irisin levels and insulin resistance.

The results of the current study are in agreement with the study by Binay *et al.* (23), who showed that in obese children irisin levels were positively correlated with BMI and WC. Indeed, it has been suggested that circulating irisin levels could be an indicator of body fat mass, showing a positive correlation with BMI (5, 27–30). Similarly, body fat mass was the main independent factor associated with irisin levels in a large cohort of Korean adolescents (31) and in patients with hypopituitarism (32). Recently, in a cohort of children with obesity, lower mean irisin concentrations were found compared with children with normal weight, with a significant inverse correlation between irisin and BMI and WC, and children with metabolic syndrome exhibited lower irisin concentrations than those without metabolic syndrome. The authors of this study concluded that irisin might be a biomarker for metabolic syndrome in prepubertal children (33), and these data are concordant with our study.

In agreement with other studies, we found that irisin levels were correlated with BMI and WC, without association with metabolic parameters (34).

To the best of our knowledge, the only study on the relationship between GHR and irisin levels was performed in patients with Turner syndrome. Wikiera *et al.* (35) evaluated the anthropometric and metabolic data of 36 young girls before and after supraphysiological doses of GHR (0.05 mg/kg/d). After GHR, an increase in irisin was observed, with a concomitant positive association between irisin and IGF-I levels. These results are in agreement with those of our study. Indeed, we found a significant increase in irisin levels after 12 months of

GHR. In addition, we documented a significant correlation between irisin levels and hormonal parameters (*i.e.*, stimulated GH levels at baseline and IGF-I during GHR). Recent data suggest a relationship between irisin and the GH/IGF-I axis. Indeed, in the skeletal muscle of 15 obese men with reduced GH levels, the local expression of fibronectin type III domain 5 was associated with mRNA expression of IGF-I (36), although fasting irisin levels were found to be similar in normal and hypopituitaric adults with GHD (32).

In our study, no correlation was found between irisin and metabolic parameters. An independent association of irisin with fasting blood glucose levels has been documented in adults (27, 37–39) and children (6, 24, 25). However, we did not find a significant correlation between irisin and glucose levels probably because fasting glucose, HbA1c, and HOMA-IR, even if they increase, almost always remain within the normal range during GHR. In addition, in children with GHD an increase in insulin levels, and consequently in HOMA-IR, or a decrease in ISI, during GHR probably does not indicate a real condition of insulin resistance, and there may be a failure of basal indexes to reliably assess insulin sensitivity. Indeed, the increase in HOMA-IR documented in our study, as well as the trend to decrease in ISI, may represent an expected consequence of GH-induced hyperinsulinemia (15). Similarly, we did not find a significant change in lipid profile during GHR or a correlation between irisin and circulating lipids.

To support the controversial involvement of irisin in metabolic disturbances, in a group of children from the southern Italy, Nigro *et al.* (40) documented that irisin was significantly higher in obese children than in control children and was inversely correlated with adiponectin but had no direct association with insulin resistance.

In our opinion, the discrepancies among the studies may be related to the lack of evaluation of the physical activity levels of the enrolled subjects, and this point represents the main limitation of our study, in addition to the lack of information about nutritional behavior. Indeed, irisin secretion is dependent on the nutritional status and physical activity of the body (41, 42), and a lifestyle intervention program is associated with a rise in irisin levels in obese children (43), although other reports on changes in irisin in response to exercise have been discordant. Indeed, initial data about the improvement in irisin synthesis and secretion after physical exercise have not been confirmed by other studies (30). The acute rise in exercise-induced irisin is most likely accounted for by skeletal muscle release, and basal irisin levels could be accounted for by adipose tissue production (44); this could explain the discrepancies among the studies. In addition, the majority of human studies have produced contradictory results, and data may also be affected by exercise intensity (*i.e.*, acute speed/strength *vs*. chronic or resistance exercise activity) (45). However, no child evaluated in this study practiced agonistic sports, and all subjects performed moderate standard school sports activities, with a frequency of no more than twice a week.

In addition to the lack of information about the physical activity levels and nutritional behavior of the children, other limitations of this study may be represented by the lack of data for the control group after 12 months of follow-up and for the short-term follow-up. Indeed, a longer follow-up period may be useful to obtain more reliable data in both subjects with GHD and control subjects. In addition, adding data on body composition evaluated by bioimpedence and their comparison with irisin levels will confirm these results in future studies. Indeed, whereas data about the correlation between irisin and adiposity or insulin resistance remain quite controversial, the relationship between irisin and muscle mass seems more robust.

Recent reports have questioned the presence of circulating irisin, claiming that the measured human irisin arises from artifacts of poor antibody specificity (46, 47), and the existence of this protein and its role in humans is still a matter of debate (48).

In summary, although IGF-I is the main biochemical marker of GHR, circulating irisin levels could represent a metabolic marker of treatment. Indeed, this study confirms the beneficial metabolic effects of GHR in children with GHD, demonstrated by the increase in irisin levels that could reflect the body composition changes that occur during GHR.

Although the irisin levels observed after GHR were significantly increased over baseline, they did not reach the values of control subjects, probably due to the short-term course of treatment. However, because there are no other similar studies on irisin in this population, we believe that these data should be considered as preliminary and whether the association between GH and irisin is casual or not remains to be demonstrated by future studies with larger populations and a longer follow-up period.

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