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Research article

The Effect of the Pubertal Stage on Irisin Levels in Male Adolescents: A Novel Myokine

Short title: Irisin Levels during Puberty

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What is already known on this topic?
Irisin is a recently discovered peptide and is defined as an adipomyokine. The amount of fat and muscle tissue and gender affect the release of irisin. The relation between irisin levels and pubertal stages (2-5) after the onset of puberty has not been studied.

What this study adds?
Our study stated that irisin levels are not related to stages of puberty in male adolescents.

Abstract
Objective: Irisin is a recently discovered protein and is defined as adipomyokine. The relation of irisin with carbohydrate metabolism and other hormone parameters have been investigated; however, studies evaluating the relationship between irisin and puberty are limited. This study aimed to evaluate the levels of circulating irisin during different pubertal stages in male adolescents.
Methods: The study included pubertal male adolescents between the ages of 13 $^{6}/_{12}$-14 $^{11}/_{12}$ with normal weight, who had entered puberty. Fasting serum irisin levels were evaluated, and bioelectrical impedance analysis (BIA) was used to measure body fat ratio (BFR) and fat-free mass (FFM). BFR was also calculated by measuring the thickness of the triceps subcutaneous fat with a caliper.
Results: Sixty-eight adolescents were enrolled. The number of adolescents in pubertal stage 2, 3, 4 and 5 were n = 17 (25%), n = 13 (19.1%), n = 21 (30.1%) and n = 17 (25%), respectively. The levels of circulating irisin did not differ according to the pubertal stage. Additionally, there was no significant relationship between irisin levels and body fat percentage or FFM.
Conclusions: Irisin levels do not differ after the onset of puberty thereafter with the progressing pubertal maturation. This may have important implications when using this adipomyokine in the future for diagnosis or treatment of obesity-related diseases.
Keywords: Irisin, male adolescent, puberal stage, body fat percentage, muscle mass

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Introduction

Irisin is a myokine recently described by Boström (1) and derived from the extracellular N-terminal domain of fibronectin type III domain-containing-5 (FNDC5), a muscle cell transmembrane protein. The transfer of irisin from muscles to circulation after exercise is regulated by peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α). Irisin also contributes to the regulation of energy consumption and glucose metabolism by influencing the transformation of white fat tissue to brown fat tissue (2).
Some studies (3-5) have suggested that irisin is released from muscles in connection with the exercise, and the muscle mass is the determining factor for the level of irisin in circulation. However, in another study, irisin levels were not found associated with acute or chronic exercise, as well as overweight or impaired glucose tolerance (6). Since high levels of irisin were found in people with obesity, it was also suggested that irisin was released from the adipose tissue (7, 8) which lead it to be considered as an adipomyokine that acts as a hormone since it is released from both muscle and adipose tissues and affects distant organs (9, 10).
Several studies have investigated the relationship between irisin and body mass index (BMI), exercise, thyroid function tests, bone metabolism, regulation of blood glucose, and metabolic syndrome in adults (11-14). Similarly, the relation between irisin and BMI, exercise, weight loss, and metabolic and anthropometric measurements have also been investigated in pediatric age groups (15, 16). All of these studies imply that irisin levels depend on the ratio of body fat and muscle mass. Muscle and body fat mass increases with growth during childhood and varies with gender during puberty. When the increase in the adolescent's body fat and muscle mass exceeds a critical limit, the hypothalamic-pituitary-gonadal axis is stimulated, and puberty begins (17, 18). During puberty, the amount and distribution of muscle and fat masses vary according to gender and pubertal stages. While the fat-free mass, most of which consists of muscle mass, and body fat are not different between girls and boys in the prepubertal period, boys have 1.5 times more muscle mass than girls whereas girls have more body fat

than boys at the end of puberty (19). Since the level of irisin is reported to be associated with both fat and muscle mass (20), irisin levels may also vary along with the pubertal stages in adolescents.
To date, very few studies in children and adolescents have addressed the effects of puberty on irisin levels. In two of the studies claimed that the prepubertal/pubertal stage was not associated with the irisin level. However, in one of the studies, it was reported that the level of irisin was higher in pubertal adolescents than in prepubertals (21, 22, 23).
Considering the utility of irisin in treatment, in relation to many factors such as metabolic or chronic disease and obesity in the future, it may be important to know how the levels of irisin change according to sex and pubertal stages. The objective of this study was to investigate whether irisin levels differ according to pubertal stages in male adolescents.

Methods

This cross-sectional analytical study was conducted with eligible participants from the adolescent outpatient clinic. The study was approved by the Research Ethics Committee at Hacettepe University. Written informed consent was obtained from all participants and their parents. Male adolescents aged between $13^{6/12}$ - $14^{11/12}$ years, of healthy weight, with no chronic illness, who had entered puberty were enrolled in the study at well-child care visits. The study focused on male adolescents between $13^{6/12}$ - $14^{11/12}$ years to control for the age variable and to ensure that all paricipants had entered puberty. Additionally peak height velocity, minimum BF ratio and maximum FFM are all observed at the age of 14 in male adolescents (24). Those who had a psychiatric, or endocrine disease, were using chronic medication for any reason, were underweight (BMI equal to or under than the 5th percentile), overweight or obese (BMI equal to or higher than the 85th), had exercised a day before the study, were elite athletes, had a special diet, or took food supplements were excluded from the study. In addition, patients with acute infection during the examination, and those with pathological findings related to lipid or glucose metabolism were excluded from the study. Maturation of sexual development was based on Tanner (25) stages according to pubic hair stages and testis volume in the males. Patients were examined by the same clinician for pubertal staging.
Body weight was measured to the nearest 0.1 kg using a body composition analyzer (Tanita SC-330). Height was measured using a fixed wall-scale to the nearest 1 mm. BMI (kg/m^2) was used to define healthy weight (5th to 85th percentile), according to age and sex-specific growth reference data (26). Fat-free mass (FFM) (kg) and BFR were measured by bioelectric impedance analysis (BIA-BFR) technique with Tanita SC-330. BIA was performed without socks, shoes, and heavy clothing in the morning after 8 hours of fasting.
Additionally, the BFR was calculated by triceps skinfold thickness measurement. Skinfold thickness was measured with Harpenden caliper at triceps, at the middle point between the acromion process and olecranon process on the left arm (27). Subcutaneous adipose tissue was measured by gently pulling the skin and subcutaneous fatty tissue upwards while the patient was standing upright and arms drooping on both sides. The measurement was completed twice and repeated if the difference was more than 1 mm. All skinfold measurements were performed by the same specialist. The body fat percentage was calculated byTriceps Skinfold Thickness (TricepsBFR) measurement, using the reference values for Turkish children and adolescents (28).

Irisin measurement

Adolescents who met the inclusion criteria were invited to the clinic at 08.30-09.00 am after 8 hours of fasting to obtain their serum irisin measurements. The blood serum was isolated and stored at -80 °C until the time of analysis, which was three months at maximum. Quantitative measurements of irisin were performed with Human FNDC5 (Fibronectin type III domain-containing protein 5) enzyme-linked immunosorbent assay (ELISA) kit Catalog No: E-EL-H2254 Elabscience, Wuhan, China- assay, sensitivity 0.10 ng/mL and detection range were 0.16–10 ng/mL. The irisin levels were expressed as ng/mL. The lowest detectable dose of irisin was 0.16 ng/mL, with intra- and interassay coefficients of variation were <6%.

Statistical Analysis

Data from the study were analyzed using SPSS 23.0 for Windows(IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp.). Descriptive statistics were presented as mean ± standard deviations, frequency distributions, and percentages. Chi-square test was used to analyze categorical variables. The normal distribution of variables was tested using visual (histogram and probability graphs) and analytical methods (Kolmogorov-Smirnov / Shapiro-Wilk Test). The variance equation was controlled by the Levene test. One-way analysis of variance where parametric test preconditions are provided to determine whether there is a significant difference between the three groups (One –Way ANOVA) and Bonferroni test is used for post-hoc tests for double comparisons, Wallis-H test was used when the assumption was not met. The relationship between the variables was evaluated by the Pearson Correlation Coefficient and Spearman Correlation Coefficient when it did not provide the parametric test prerequisites. $p<0.05$ was accepted, for the significance level of the tests.

Results

Sixty-eight adolescents were included in this study. The number of adolescents in pubertal stage 2, 3, 4 and 5 were n = 17 (25%), n = 13 (19.1%), n = 21 (30.1%) and n = 17 (25%), respectively. According to the pubertal stages; mean body weigt, mean height, mean BMIpersentile (BMIp), irisin, BFR and FFM values are given in Table 1.
A significant positive correlation was found between Triceps-BFR and BIA-BFR (r=0.444**; p=0.01), Triceps-BFRand FFM (kg) (r=0.446**; p=0.01).
The change of FFM, according to the pubertal stage, was significant while the change of irisin level, according to the pubertal stage was not statistically significant (Table 2).
Correlation analysis of BMIp, BIA-BFR, FFM, and Triceps-BFR variables in each pubertal stage are given in Table 3.
A significant correlation was found between BIA-BFR and Triceps-BFR and BMIp. There was a significant correlation between FFM and Triceps-BFR, BMIp, and pubertal stage. Irisin was not found correlated with any of the parameters. The inter-correlations between the parameters investigated in this study are seen in Table 4.

Discussion

To the best of our knowledge, this was the first study to evaluate the circulating irisin levels according to pubertal stages (Stage 2-5) after the onset of puberty in adolescents, in male adolescents. In the literature, there are only four clinical investigations evaluating circulating irisin levels in which the participants consisted of adolescents, but these studies did not

specifically interpret the changes according to pubertal stages. These are briefly reviewed below to build up the background for the discussion of the results of our study.

Al-Daghri et al. (29) conducted their research with the adolescents between 12 and 15 years of age with healthy body weight and found positive relationships between irisin and fasting blood sugar and HDL cholesterol. The circulating irisin levels of female adolescents were found to be higher than male adolescents and in a multivariate regression analysis for potential confounders, the irisin levels were independently associated with fasting blood glucose levels predominantly in girls who lead the authors to the conclusion that irisin is a predictor of glucose metabolism which has sexually dimorphic effects in adolescence. The participants were not separated according to pubertal stage and the relationship between irisin and puberty was not mentioned.

Blüher et al. (22) evaluated the levels of the baseline and follow-up irisin levels of children and adolescents between 7-18 years of age with obesity after a yearlong intervention. At baseline, they did not find any significant relationships between irisin levels and age, gender, BMI, or other adipokines. Participants were also classified according to Tanner stages as pre-/early pubertal (stage 1 and 2), pubertal (stage 3 and 4) and post-pubertal (stage 5 values) and they did not find any evidence for differences depending on the pubertal status. However, the pubertal stages of these adolescents were not analyzed separately for males and females, which we believe is not accurate. Overall, circulating irisin levels at baseline were increased by 12% after the intervention for obesity.

Jang et al. (21) evaluated the relationships between circulating irisin and metabolic profiles and anthropometric indices in adolescents between 12-15 years of age in two groups, one with healthy body weight and one with obesity. They found that circulating irisin was positively correlated with adiposity indices, including percent body fat, fat mass, and the ratio of fat mass to fat-free mass. Again, girls had higher irisin levels than boys after adjusting for confounders in the normal-weight but not obese adolescents. Adolescents were further classified as prepubertal (stage 1 and 2), pubertal (stage 3 and 4) and postpubertal (stage 5 and 6) in both normal weight and obese groups but again not differentiating for girls and boys and serum irisin levels did not differ significantly between the groups. They also analyzed the levels of irisin in two groups as pre-menarche or post-menarche in girls and did not find any difference. They found that elevated circulating irisin in adolescents was associated with obesity, whereas irisin increased in adolescents with healthy body weight after exercise but not in the obese group.

Lastly, Reinehr et al. (23) investigated irisin and its relation to pubertal status in children and adolescents. Pubertal developmental status was categorized into two groups based on breast and genital stages determined according to Marshall and Tanner (prepubertal, boys with genital stage I and girls with breast stage I; and pubertal, boys with genital stage ≥II and girls with breast stage ≥II). The irisin concentrations differed significantly between the prepubertal and pubertal children. Analyzing only obese children demonstrated the same findings: the irisin concentrations differed significantly between the obese prepubertal and obese pubertal children.

While evaluating the changes of circulating irisin levels during puberty, it should not be overlooked that irisin is an adipomyokine (10) and its levels may depend on the ratio of body fat and muscle mass (9) which varies with gender and pubertal stages during adolescence. The levels of irisin in girls were already documented to be higher than boys (21, 29) and we believe that comparing the irisin levels in the pre-early puberty versus mid-puberty versus post-puberty can only be done accurately by analyzing the data separately for females and males, which is not the case for the above studies (21, 22). Relevantly, the study by Reinehr et al. (23) has shown that the levels of irisin significantly increase with the onset of puberty in both sexes and the study by Jang et al. (21) reported that irisin levels do not change in girls before and after menarche. Thus, we investigated the irisin levels in male adolescents after the onset of puberty, which we believe was the uncovered area of the irisin-puberty research.

We investigated circulating irisin levels only in boys keeping the age constant to discard the changes due to age differences and to test the hypothesis that the irisin levels may vary in the course of sexual maturation. The age 14 years (>13 $^{6/12}$ years) was chosen to exclude pre-pubertal boys and to have adolescent boys at different stages of pubertal development since puberty in boys has to be started before 13 $^{6/12}$ years of age to stay in the normal ranges. We also only included healthy boys with normal body weight that had not exercised the day before, this way we were able to ensure the only independent variable in grouping the participants of the study was the Tanner stage.

In this study, we evaluated BMI, body fat with two different methods, namely BIA and triceps skinfold thickness and fat-free mass of male adolescents while investigating the irisin levels. The significant correlation found between Triceps-BFR and BIA-BFRhave validated the measurements with different methods. The BMI and body fat mass percentages did not differ significantly between the pubertal stages, whereas the fat-free mass was the only parameter which significantly increased with the progressing stages. These results are in concordance with the previous studies reporting that the lean body mass changes from approximately 80 % of body weight in early puberty to 90 % at maturity which primarily reflects increased muscle mass in male adolescents while the percentage of body fat during puberty decreases from stage 1 to stage 2 and remains unchanged in stages 3, 4 and 5 in males (30, 31). Also, in male adolescents, gain in muscle mass reaches its maximum velocity in accordance with the peak height velocity which occurs at Tanner Stage 4 in boys (32) and fat-free mass increase in our study group was found to be maximum from stage 3 to 4. Thus, the size of our study population was large enough to document any physiological changes during pubertal development if present.

However, we did not find any significant difference between the pubertal stages for irisin levels. We investigated the correlations between irisin levels and BMI, body fat and fat-free mass for the whole study population and further, separately in each pubertal stage and did not find any correlation.

Study Limitations

Serum irisin was measured by a commercial ELISA method, and more reliable levels can be measured by immunoblotting (33) and mass spectroscopy (34), but still, ELISA method allows us to compare between the stages.

Conclusion

Although recent findings indicate that irisin levels might be different between prepubertal and pubertal boys, this study suggests that levels do not differ thereafter with the progressing pubertal maturation in male adolescents.

Ethics Committee Approval: The study was approved by the Research Ethics Committee at Hacettepe University (Protocol number: GO 16/721-08, date of approval: 24.11.2016).
Informed Consent: Written informed consent was obtained from all participants and their parents.
Authorship contributions Consept: Demet Taş, Alkım Akman Öden, Sinem Akgül, Ziya E Metin, Aslı Pınar, Nuray Kanbur
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Table 1. BMIp, irisin, BF (%) and FFM values according to pubertal stages

	Pubertal Stage			
	2	3	4	5
Body Weight (mean±sd)	45.6±6.22	46±8	49±7	57±6
Height (mean±sd)	155±4.35	155±6	160±5	170±6
BMIp (mean±sd)	40.71±27.54	52.38±24.81	53.57±25.32	51.88±25.31
Irisin (ng/ml) (mean±sd)	10.50 ±10.50	12.41±11.48	14.05±14.98	12.12±12.95
BF (BIA) (%) (mean±sd)	16.34±4.76	16.69±4.72	15.22±3.56	16.49±4.17
FFM (kg) (mean±sd)	35.58±3.30	37.67±5.22	44.34±7.40	46.91±3.82
BF (Triceps) (%) (mean±sd)	19.29±4.54	20.92±3.09	19.95±3.26	18.94±2.01

BMIp: body mass index percentile, BF: body fat ratio, BIA: bioelectric impedance analysis,
FFM: free fat mass

Table 2. Statistical analysis of variables according to pubertal stage

Pubertal stage	BMIp median (25th-75th centile)	Irisin (ng/ml) median (25th-75th centile)	BF (BIA) (%) mean±sd	FFM(kg) mean±sd	BF (Triceps) (%) Median (25th-75th centile)
2	39 (15.50-64)	8.80 (5.88-10.12)	16.34± 4.76	35.58± 3.30[a]	19 (14.50-24.00)
3	63 (27.50-73)	8.20 (6.32-15.91)	16.69± 4.72	37.67± 5.22[a]	20 (19.00-24.00)
4	62 (24.00-75.50)	9.15 (6.13-21.38)	15.22± 3.56	44.34± 7.40[b]	19 (18-23.50)
5	62 (24.00-75.50)	7.24 (4.50-14.63)	16.49± 4.17	46.91± 3.82[c]	19 (18.00-20.00)
p	0.467	0.917	0.796	<0.001*	0.440

*Statistical differences between the pubertal stages with different letters are significant for FFM.
BMIp: body mass index percentile, BF: body fat ratio, BIA: bioelectric impedance analysis,
FFM: free fat mass

Table 3. Correlation analysis of BMIp, BF (BIA) (%), FFM (kg) and BF (Triceps) (%) variables according to pubertal stages

Pubertal Stage			BMIp	BF (BIA)(%)	FFM (kg)	BF (Triceps)(%)
2	Irisin (ng/ml)	r	-.259	.192	-.233	-.273
		p	.316	.530	.443	.288
	BMIp	r		.476	.732[a]	.814[a]
		p		.100	.004	.000
	BF (BIA)(%)	r			.212	.112
		p			.488	.716
	FFM (kg)	r				.574[b]
		p				.040
3	Irisin (ng/ml)	r	.300	.233	-.106	.016
		p	.319	.545	.786	.959
	BMIp	r		.814[a]	.450	.531
		p		.008	.224	.062
	BF (BIA)(%)	r			.058	.604
		p			.883	.085
	FFM (kg)	r				.611
		p				.081
4	Irisin (ng/ml)	r	.056	-.194	-.059	-.146
		p	.810	.472	.827	.529
	BMIp	r		.528[b]	.857[a]	.773[a]
		p		.035	.000	.000
	BF (BIA)(%)	r			.323	.663[a]
		p			.223	.005
	FFM (kg)	r				.665[a]
		p				.005
5	Irisin (ng/ml)	r	-.140	.260	-.278	-.002
		p	.592	.368	.336	.994
	BMIp	r		.670[a]	.515	.712[a]
		p		.009	.059	.001
	BF (BIA)(%)	r			.351	.816[a]
		p			.218	.000
	FFM (kg)	r				.516
		p				.059

a:$p<0.01$, b:$p<0.05$
BMIp: body mass index percentile, BF: body fat, BIA: bioelectric impedance analysis, FFM: free fat mass

Table 4. The inter-correlations between the parameters studied

		FFM (kg)	BF (Triceps)(%)	BMIp	Irisin(ng/ml)	Pubertal[a]Stage
BF (BIA)(%)	r	.137	.444[c]	.551[c]	.060	-.015
	p	.334	.001	.000	.672	.914
FFM (kg)	r		.446[c]	.627[c]	-.012	.692[c]
	p		.001	.000	.934	.000
BF (Triceps)(%)	r			.702[c]	-.113	-.102
	p			.000	.361	.408
BMIp	r				-.003	.142
	p				.980	.250
Irisin (ng/ml)	r					.010
	p					0.938

a: Spearman Correlation Coefficient,c:p<0.01
BMIp: body mass index percentile, BF: body fat, BIA: bioelectric impedance analysis,
FFM: free fat mass