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Relationship of irisin expression with metabolic alterations and cardiovascular risk in type 2 diabetes mellitus: a preliminary study

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SUMMARY

OBJECTIVE: The aim of this study was to investigate the role of irisin in type 2 diabetes mellitus and its association with metabolic alterations and obesity.

METHODS: A cross-sectional case-control study was conducted on participants treated at Centro Universitário FMABC between August 2018 and July 2019, by comparing a control group (n=14) with type 2 diabetes mellitus patients (n=16). The control group consisted of participants aged above 21 years with no chronic diseases, diabetes, smoking, or illicit drug use. The type 2 diabetes mellitus group included patients aged above 21 years, who were diagnosed with type 2 diabetes for at least 5 years (glycated hemoglobin>7%). Exclusion criteria were not willing to continue, recent hospitalization, and failure to meet inclusion criteria. Biochemical parameters included blood glucose, glycated hemoglobin, plasma irisin levels, and *irisin* gene expression in peripheral blood.

RESULTS: Type 2 diabetes mellitus patients exhibited significantly higher plasma glucose levels [143 (40) vs. 92 (13) mg/dL, *p<0.05] and glycated hemoglobin levels [7.1% (1.6) vs. 5.6% (0.5), *p<0.05] compared to the control group. *Irisin* gene expression in type 2 diabetes mellitus patients was lower 0.02288 (0.08050) than the control group 8.506e-006 (1.412e-005) (p=0.06). Correlation analysis revealed a positive association between *irisin* expression and body mass index in type 2 diabetes mellitus (Rho=0.5221, 95%CI -0.058 to 0.838, p=0.06), while plasma irisin showed a negative correlation with body mass index (Rho=-0.656, 95%CI -0.836 to 0.215, p=0.03). No significant correlations were found between plasma glucose or glycated hemoglobin levels and irisin expression.

CONCLUSION: The data suggests that body mass index directly influences plasma irisin levels and the regulation of irisin gene expression, possibly linking irisin to adiposity changes observed in obesity-related type 2 diabetes mellitus.

KEYWORDS: Diabetes mellitus. FNDC5 protein, human. Obesity. Cardiovascular system.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a syndrome of multiple etiologies, characterized by a lack of and/or resistance to the action of insulin. In other words, there is an inability of this hormone to properly exert its effects. This syndrome is marked by chronic hyperglycemia and disturbances in carbohydrate, lipid, and protein metabolism. The genesis of hyperglycemia involves a triad of abnormalities that include increased hepatic glucose production, altered insulin secretion, and action. The severity of these anomalies and their degree of contribution vary and are related to the heterogeneity of metabolic alterations in diabetes[1].

The elevated prevalence of vascular disease in individuals with T2DM is not solely attributed to hyperglycemia but is also influenced by metabolic changes associated with the plurimetabolic syndrome. This syndrome encompasses centrally distributed obesity, hypertension, dyslipidemia, insulin resistance, hyperinsulinemia, increased coagulation factors, platelet adhesion and aggregation, reduced fibrinolysis, and hyperuricemia. The identification of markers indicating the progression of clinical conditions resulting from obesity and insulin resistance, as well as the potential development of cardiovascular disease (CVD), holds significant importance. Irisin, a hormone secreted during skeletal muscle contraction, has emerged as a potential marker. It is released after cleavage of the membrane protein FNDC5 and plays a role in converting white adipose tissue to brown adipose tissue, thereby enhancing insulin sensitivity through the regulation of glucose homeostasis[1-3].

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Several studies have indicated that the expression of the *FNDC5* gene is associated with increased glycosylation of irisin, a process that positively influences the hormone's functionality. Moreover, elevated circulating levels of irisin have been linked to insulin resistance[4]. A study based on the Framingham risk score demonstrated a correlation between higher plasma irisin levels and an increased risk of CVD over a 10-year period[5]. Yilmaz et al. suggested a potential influence of irisin on another crucial cardiovascular risk factor, serum lipid concentration, with findings indicating a correlation between high irisin concentrations and dyslipidemia[6].

The evaluation of irisin gene regulation in peripheral blood samples can elucidate the actions of irisin and its metabolic consequences, particularly in diabetic patients[7]. The objective of this study was to further investigate the relationship between plasma levels of irisin and the gene expression of this hormone, as well as the metabolic alterations present in DM. Additionally, the objective was also to examine whether irisin is involved in the adiposity changes observed in patients with obesity-related T2DM and to evaluate whether there are significant platelet alterations in diabetic patients during this phase of the disease.

METHODS

This cross-sectional case-control study was conducted on participants treated at the specialist outpatient clinic of Centro Universitário FMABC, under the supervision of cardiologist Dr. Neif Murad. The study included a total of 30 participants, who were divided into two groups: the control group, consisting of 14 individuals, and the diabetic group (T2DM), comprising 16 patients.

The control group was formed based on specific inclusion criteria, which required participants to be above 21 years of age, with no history of chronic diseases or diabetes, and no smoking or use of illicit drugs. On the contrary, the T2DM group comprised patients aged above 21 years who had been diagnosed with type 2 diabetes (glycated hemoglobin (HbA1C) >7%) for at least 5 years, were non-carriers of cardiovascular, renal, or hepatic disease, and had no HIV or cancer. To ensure the validity of the study, exclusion criteria were applied. The exclusion criteria were not willing to continue, hospitalization for any reason within the past 30 days, and not meeting the inclusion criteria.

The enrollment of patients for this study took place between August 2018 and July 2019. This time frame was essential for collecting relevant data and ensuring that the study's findings were representative of the specified period.

The collection of personal data and medical history from the participants and verification of all medications used in the treatment of diabetes and its comorbidities took place through an interview. The participants' body weight and height were measured to calculate the body mass index (BMI). Body weight (kg) was divided by height squared (m^2) and the result was expressed in kg/m^2, following the criteria of the World Health Organization (WHO).

Evaluation of fasting plasma glucose levels

Blood samples were obtained by vacuum venipuncture after an 8-h fast to determine the quantification of HbA1C, glucose, and plasma irisin. Values above 140 mg/dL were considered altered, and after three repetitions of the test, they were referred for medical evaluation. The determination of fasting plasma glucose levels was performed by an automated enzymatic-colorimetric method with fluoride serum. Values ≥140 mg/dL were considered altered for this parameter.

Evaluation of glycated hemoglobin levels

Glycated hemoglobin was determined through low-pressure liquid chromatography (LPLC) using the DiaSTAT analyzer - Bio-Rad, which expresses the percentage of total hemoglobin and reflects the glycemic state over the past 8–12 weeks. A 5 mL sample of whole blood with 1 mL of hemolyzed reagent was collected. Values above 7% were considered altered.

Study of irisin gene expression in peripheral blood samples

The total RNA extraction process followed the standard protocol for TRIzol. After total RNA extraction, cDNA synthesis was performed: total RNA samples (initial 1µg) obtained from peripheral blood were converted to cDNA using an SSIII first strand qPCR supermix (Invitrogen, cat no. 11752050), according to the protocol from the manufacturer.

The specific primers for each selected gene were designed using the Primer3 Input 0.4.0 software program. The characteristics of specific primers were (*Ribosomal Protein L13a* (*RPL13a*)): forward – gtggtcgtacgctgtgaag and reverse – acagtgcgccagaaaatgc, amplicon: 126 bp. *Irisin*: forward – gatccagccatcaaggacat and reverse – ttgtccaagctagcatttctga, amplicon: 113 bp.

Evaluation of hematological parameters and plasma irisin

Platelet count (PLT) and mean platelet volume (MPV) were used to evaluate the hematological parameters using the automated flow cytometry method by the XN counter equipment (Roche, Switzerland®).

The hormone irisin was analyzed from centrifuged plasma using an enzyme-linked immunosorbent assay (ELISA) kit (EK-067-29, Phoenix Pharmaceuticals, Burlin). The kit detects specific peptides and related peptides based on the principle of "competitive" ELISA. The kit's immunoplate was pre-coated with a non-specific secondary antibody with blocked binding sites. The secondary antibody bound to the Fc fragment of the primary antibody (peptide antibody), whose Fab fragment was competitively bound by both biotinylated standard peptides and target peptides in the samples. The biotinylated peptide interacted with streptavidin-horseradish peroxidase (SA-HRP), which catalyzed the substrate solution composed of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide to produce a blue-colored solution. The enzyme-substrate reaction was stopped by hydrochloric acid (HCl), and the solution turned yellow. The intensity of yellow color was directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of peptide in standard solutions or samples. A standard concentration curve was established, and the unknown concentration in samples was determined by extrapolation to the curve. Plasma was collected in EDTA tubes on ice, centrifuged at 4°C, 3,000 rpm for 10 min, and then separated into two aliquots (0.5 mL each) to be stored at -20°C until analysis.

Ethical aspects

Participants were enrolled and treated at the Specialist Outpatient Clinic of Centro Universitário FMABC. This work was submitted and approved by the Ethics Committee of Centro Universitário FMABC (process No. 084592-2017, September 28, 2017). Individuals who agreed to participate signed a free and informed consent form (FICF), with thorough explanations about the adopted protocols, following the guidelines, regulations, and relevant ethical principles of the Declaration of Helsinki.

Statistical analysis

In this study, the sample size calculation was performed using GPower®. The input parameters used for the calculation were as follows: the tail of the test was one-tailed, indicating a directional hypothesis, an effect size (p) of 0.50, a significance level (α) of 0.05 (corresponding to a 5% type I error rate), and a desired statistical power (1-β) of 0.80 (equivalent to an 80% probability of detecting a true effect). Based on these inputs, the noncentrality parameter (δ) was determined to be 2.6457. The critical t-value for a one-tailed test at a significance level of 0.05 with 19 degrees of freedom was calculated as 1.7291. To achieve the desired statistical power of 0.80, the total sample size required for the study was determined to be 21 participants. After conducting the study and analyzing the data, the actual statistical power was calculated to be 0.8172279, which indicates that the study had a high likelihood of correctly detecting the hypothesized effect given the sample size and effect size. The calculated sample size of 21 participants ensured that the study had adequate statistical power to detect the anticipated effect size with a reasonable probability of avoiding type II errors. These findings support the validity and reliability of the study's conclusions and demonstrate the importance of carefully considering sample size calculations in research design to optimize the likelihood of obtaining meaningful and conclusive results.

This study employed the Shapiro-Wilk test to assess the normality (>0.05) of the collected data and to distinguish between parametric and non-parametric data. The results were expressed as the mean±standard deviation (SD). These were compared using the Mann-Whitney U test to non-parametric (*irisin* gene expression) data and the Student's t-test to parametric data. Analyses were performed using the GraphPad Prism computer software (GraphPad, version 7.0, USA). For correlation assessments, Spearman tests were performed. The established significance level was 5% (descriptive value of $p<0.05$).

RESULTS

Initially, 25 participants were selected for each group. However, during the screening process, 35% of the participants in the control group did not meet the inclusion criteria due to the presence of associated comorbidities or their unwillingness to continue with the study. In the T2DM group patients, 42% of the enrolled participants did not meet the inclusion criteria. As a result, the final analysis included 16 participants in the control group and 14 participants in the group with diabetes mellitus. These final sample sizes were considered adequate for the statistical analyses and interpretation of the study's findings.

The mean age of the participants in this study was 51±15 years for the control group (n=14) and 64±10 years for the T2DM group (n=16). Regarding the biochemical parameters, the following results were obtained: glucose (control group 92±13, n=14 vs. T2DM 143±40 mg/dL, n=16, $p<0.05$) and HbA1C (control group 5.6±0.5%, n=14 vs. T2DM 7.1±1.6%, n=16, $p<0.05$) (Table 1). Participants with T2DM had increased blood glucose and HbA1C values, as expected. Regarding the results of hematological parameters, the T2DM group had lower MPV values compared to the control group (Table 1). There was no statistical difference in mean corpuscular volume (MCV) between groups.

Table 1 presents the expression profile of irisin in the control and T2DM groups, revealing a noticeable trend toward increased irisin expression in the T2DM group. Furthermore, upon applying the correlation test between plasma irisin quantification and BMI in the T2DM group, a negative correlation was observed. Conversely, there was a trend toward a positive correlation between irisin gene expression and BMI. However, no correlation was found between glucose levels and HbA1C with irisin expression (Table 2). Plasma irisin values were similar between the control and T2DM groups. In the T2DM group, there was no correlation between plasma irisin concentration and *irisin* expression.

DISCUSSION

Among the main findings of this study, it is worth highlighting that there was a trend toward increased gene expression of *irisin* in diabetic patients compared to the control group. A positive correlation was observed between *irisin* expression and BMI in diabetic patients. Plasma irisin values were similar between the control group and T2DM group. There was a negative correlation between BMI and plasma irisin levels.

In this study, the observations revealed a positive modulation between *irisin* expression and BMI in diabetic patients, suggesting a link between irisin and adiposity changes associated with obesity in T2DM. In contrast to prior research, our findings demonstrated a negative correlation between BMI and plasma irisin levels. These results indicate that elevated blood glucose levels in T2DM may initially induce an increase in irisin levels as a compensatory response, but as the disease progresses, reduced sensitivity to irisin's effects may develop, suggesting the presence of irisin resistance in insulin-resistant conditions like metabolic syndrome and T2DM[8].

Reiterating the compensatory role of irisin in the context of insulin resistance, obesity, and T2DM, it can be inferred that this hormone effectively ameliorates some of the metabolic disorders present in early T2DM, in addition to its functions in promoting browning of white adipose tissue, enhancing glucose uptake in skeletal muscle and heart, improving hepatic glucose and lipid metabolism, and stimulating pancreatic β-cell function[9]. The direct relationship between irisin and adiposity, as demonstrated in this study[10], strongly suggests that adipose tissue serves as a secondary inducer of irisin secretion, thereby justifying the increase in gene expression in patients with higher BMI and the observed elevation in plasma irisin concentration.

Increased *irisin* expression, found in some studies, may be related to the initiation of compensatory metabolic processes generated by metabolic stress[11-13] In contrast, Tu et al.,

Table 1. Evaluations of demographic characteristics of the study participants (control group and type 2 diabetes mellitus group), quantifications of biochemical parameters, and irisin gene expression.

Variables	Control group (n=14)	T2DM group (n=16)	p
Age (years, mean±SD)*	51 (15)	64 (10)	**0.008**
Glucose (mg/dL, mean±SD)*	92 (13)	143 (40)	**<0.0001**
HbA1C (%, mean±SD)*	5.6 (0.5)	7.1 (1.6)	**0.002**
Irisin gene expression ($2^{-\Delta Ct}$, mean±SD)#	8.506e-006 (1.412e-005)	0.02288 (0.08050)	0.06
Plasma irisin (mg/dL, mean±SD)*	44.86 (19.83)	49.83 (7.63)	0.44
MCV (mcm^3, mean±SD)*	85.55 (6.91)	89.29 (3.35)	0.06
MPV (mcm^3, mean±SD)*	10.50 (1.18)	7.74 (2.02)	**0.004**

Note: SD: standard deviation; HbA1C: glycated hemoglobin; MCV: mean corpuscular volume; MPV: mean platelet volume. #Mann-Whitney U test. *Student's t-test. Statistically significant values are denoted in bold.

Table 2. Body mass index values and correlation analysis between body mass index values and irisin expression in the peripheral blood

	Control group (14)	T2DM group (16)	p
BMI	26.4 (4.5)	27.6 (4.3)	0.46
Correlation test*			
Irisin expression vs. BMI	Rho (CI) 0.522 (-0.058 to 0.838)		0.06
Plasma irisin vs. BMI	-0.656 (-0.836 to 0.215)		**0.03**

Note: BMI: body mass index; CI: confidence interval; T2DM: type 2 diabetes mellitus. *Spearman correlation test. Statistically significant values are denoted in bold.

and Kałużna et al., demonstrated a reduction in plasma irisin in diabetics, when the low levels of irisin were directly related to cachexia, present in decompensated diabetics[14,15]. AlKhairi et al., demonstrated a significant rise in plasma irisin levels among diabetic individuals, particularly in those with obesity[13]. The authors highlighted that plasma irisin levels may also vary across different ethnicities and genders, underscoring the importance of considering these parameters when quantifying irisin levels. Nevertheless, the precise role of irisin in diabetic individuals requires further investigation, particularly in diverse nutritional contexts.

The regulation of serum irisin levels is influenced by various factors, which have been investigated to understand the metabolic processes in both normal individuals and different pathologies. However, the direct relationship between increased irisin expression and plasma levels of this hormone is complex, as multiple genes are involved in its activation cascade. Additional studies are needed to completely elucidate this metabolic pathway in DM. In an animal model of DM, Varela-Rodríguez et al.[17] observed an upregulation of the *FNDC5* gene in various tissues while protein expression remained unchanged, consistent with the findings of our study. Additionally, the same study established a connection between glycemic metabolism and irisin synthesis and action. Fasting in healthy rodents for 48 h resulted in reduced irisin synthesis, release, and *FNDC5* expression. Conversely, in this study, *FNDC5* expression was decreased in the muscles of diabetic rats, with this reduction directly linked to the animals' nutritional status. In contrast, our study examined *irisin* expression in peripheral blood without changes in nutritional status. Consequently, the regulation of *irisin* gene expression appears to be more influenced by glucose and insulin levels[15,16-22].

In individuals with T2DM, alterations in platelet metabolism, function, and morphology were observed in this study. Increased MPV and MCV indicate changes in thrombopoiesis and suggest micro and macrovascular complications, worsening the disease. However, diabetic patients undergoing drug treatments, particularly those with antiatherogenic effects targeting glycemic and/or hyperlipidemic control, show a decrease in these hematological factors. This hematological profile differs in more severe pathological conditions, such as hemodialysis patients. The direct association between high irisin levels, known for their cardioprotective effects, appears to be lost in disordered metabolic states such as T2DM. Thus, it is suggested that irisin acts as a protective factor for the cardiovascular system only in situations of homeostasis and in healthy individuals.

This study has limitations in terms of the sample size. Due to this reason, we describe the study as preliminary. A new study with the participation of a larger number of volunteers could confirm our findings. It is known that studies with a small sample size may introduce a bias in the interpretation of the data. However, as there are limited studies that have evaluated the gene expression of irisin and its relationship with metabolic alterations in diabetics, we believe that the data presented can guide future research in this area.

CONCLUSION

The data suggest that BMI directly influences plasma irisin levels and the regulation of irisin gene expression, possibly linking irisin to adiposity changes observed in obesity-related T2DM.

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AUTHORS' CONTRIBUTIONS

GLV: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – review & editing. **VLM:** Conceptualization, Data curation, Writing – review & editing. **MISM:** Conceptualization, Data curation, Writing – review & editing. **FLAF:** Conceptualization, Methodology, Investigation, Validation, Writing – review & editing. **BCAA:** Investigation, Methodology, Validation. **DPS:** Investigation. **CGCA:** Investigation. **MMP:** Investigation. **JFAE:** Investigation. **JRSR:** Investigation.

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