Research Article

Genetic Variants in the Activation of the Brown-Like Adipocyte Pathway and the Risk for Severe Obesity

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Keywords

Polymorphism · Irisin · Severe obesity · UCP1 · PPARGC1A

Abstract

Background: Regular physical activity has an important role in energy expenditure and combats the development of obesity. During exercise, *PPARGC1A* is overexpressed, stimulating an increase of the expression of *FNDC5*. This protein is cleaved to release the hormone irisin, which activates a browning process in white adipose tissue through an increase in *UCP1* expression. As a result, irisin leads to mitochondrial heat production and energy expenditure.

Objectives: The aim of this study was to investigate whether genetic variants in genes related to browning are associated with severe obesity and obesity-related features. This case-control study comprised 210 individuals with severe obesity (median body mass index [BMI] 45.6 [range 40.5–52.2]) and 191 normal-weight subjects (BMI 22.8 [21.1–23.9]). **Methods:** Genomic DNA was extracted from peripheral blood and the genotypes of the *PPARGC1A* (rs8192678, rs3736265, rs2970847, and rs3755863) and *UCP1* (rs6536991 and rs12502572) genes were obtained using Taqman[®] assay. For the *FNDC5* gene, screening of exons 3–5 as well as their intron-exon boundaries was performed using automatic sequencing. **Results:** Our results demonstrated that *PPARGC1A* rs2970847 and *UCP1* rs12502572 are associated with severe obesity. Furthermore, these polymorphisms influence anthropometric traits, such as BMI,

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body weight, and body adiposity index. Our findings also showed a dose-effect relationship between *PPARGC1A* rs8192678 and fasting plasma glucose. Finally, 5 rare mutations were identified in *FND5*, and 1 of these is a novel missense mutation. **Conclusion:** This study shows that genetic variants in the activation of brown-like adipocyte pathway play an important role in the susceptibility to severe obesity.

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Introduction

Obesity (body mass index [BMI] ≥ 30) is a worldwide epidemic, affecting populations from developed and developing countries. Recent data show that >1.9 billion adults are overweight and there are at least 650 million people with obesity worldwide [1]. Epidemiological studies have shown that individuals with a very high BMI, e.g., subjects with severe obesity (BMI ≥ 35) and individuals with morbid obesity (BMI ≥ 40), have a substantial increased risk of comorbidities and mortality. Currently, the worldwide prevalence of severe obesity is nearly 3.7% and that of morbid obesity is 1.1%. This continues to increase at alarming rates, with global projections estimating that severe obesity prevalence will reach 7.5% by 2025 [2, 3]. In Brazil, 2.2 million men (3.8%) and 6.7 million women (5.3%) had severe obesity in 2014 [2]. This pathology has emerged as one of the most prominent health care system problem in the world, since subjects with severe and morbid obesity incur between 45–113% higher medical costs compared to normal-weight individuals [4–6]. Extreme BMI has been associated with a loss of life expectancy of 6.5–13.7 years [3].

The elevated prevalence of obesity is mostly due to the lifestyle changes in the last decades. These changes have resulted in an increased consumption of hypercaloric foods and reduced physical activity [7]. Energy expenditure, especially during exercise, plays an important role in combatting the development of obesity and related features. Regular physical activity benefits several tissues and improves quality of life; however, the precise molecular aspects underlying this mechanism are still unknown [8–10]. Boström et al. [11] reported a key role of brown-like adipocytes (“beige cells”) in mice. During exercise, the peroxisome proliferator-activated receptor γ coactivator 1 α gene (*PPARGC1A*) is overexpressed in skeletal muscles, stimulating an increase in fibronectin type III domain containing 5 (*FND5*) gene expression. *FND5* encodes a muscle membrane protein which is cleaved to release a newly identified hormone into the bloodstream, termed irisin. This novel myokine appears to act on white adipose tissue in mice and humans, stimulating browning through an increase in uncoupling protein 1 (*UCP1*) gene expression. This protein promotes the uncoupling of protons (H⁺) used for ATP synthesis during the mitochondrial respiratory chain, dissipating energy in the form of heat [12]. As a result, irisin activates adipocyte thermogenic programs, leading to mitochondrial heat production and energy expenditure [10, 11].

Irisin is a soluble small polypeptide containing 111 amino acids (22 kDa), resulting from *FND5* proteolysis at amino acid positions 30 and 140 [11, 13]. Since irisin was discovered, much research has focused on exploring its role in pathological and physiological conditions. Altered levels of irisin in the circulation have been previously associated with human obesity, but this association is inconsistent throughout the literature [14–17]. Demirpence et al. [17] showed that subjects with morbid obesity have lower levels of irisin compared to normal-weight subjects. Irisin was also found to be reduced in patients with type 2 diabetes and metabolic syndrome [18, 19]. Animal studies have suggested that increased plasma levels of irisin are associated with a decrease in body weight and an increase in energy expenditure [11]. Zhang et al. [13] conducted in vivo

experiments to examine the potential therapeutic effects of irisin. After 14 days of daily injections of human recombinant irisin, fat mice showed reduced body weight and fasting insulin, and improved glucose tolerance. This result was accompanied by an increased number of multilocular subcutaneous adipocytes and the expression of *UCP1*, which suggests brown-like adipocyte development. All these findings indicate that irisin plays a key role in adiposity changes.

In this context, genetic variations in *PPARGC1A*, *UCP1*, and *FNDC5* may be associated with obesity or related obesity-traits. The chromosomal region (4p15.1) in which *PPARGC1A* is located, has been linked with BMI in Mexican Americans [20] and abdominal fat in the Québec Family Study [21]. Furthermore, *PPARGC1A* polymorphisms have been associated with the development of obesity in different populations [22, 23]. Franks et al. [23] reported that the *PPARGC1A* rs8192678 polymorphism is associated with the accumulation of subcutaneous adiposity. Albuquerque et al. [24] observed a near nominal association between rs8192678 and body weight among Portuguese children. Interestingly, Pihlajamäki et al. [25] observed two haplotype blocks in *PPARGC1A*, one of which was located in the coding region and 3'UTR region and included the rs2970847 (Thr394 =), rs8192678 (Gly482Ser), rs3755863 (Thr528 =), and rs3736265 (Thr612Met) polymorphisms. They found that this haplotype block was associated with glucose tolerance, BMI, and insulin sensitivity in patients with type 2 diabetes.

Ramos et al. [26] investigated the association between the *UCP1* rs6536991 and rs12502572 polymorphisms and morbid obesity in a cohort of Brazilian population. They reported that rs6536991 was associated with BMI. Interestingly, both polymorphisms are located in the intronic region and it is unclear whether they affect *UCP1* expression. Nishimura et al. [27] found that rs12502572 has an effect on nonshivering thermogenesis, which might contribute to body weight regulation. To date, only a few studies have investigated the association between *FNDC5* polymorphisms and obesity. Al-Daghri et al. [28] showed that the *FNDC5* rs3480 polymorphism is associated with a decreased risk of obesity and a lower BMI in the Saudi population. Tang et al. [29] found that the rs16835198 polymorphism is associated with high-density lipoprotein (HDL)-cholesterol levels in individuals with overweight/obesity. These studies have focused on previously described common variants. However, there is a lack of information about rare and/or novel mutations.

Therefore, the aim of this study was to evaluate whether genetic variants in *PPARGC1A*, *UCP1*, and *FNDC5* are associated with severe obesity and related obesity traits in a cohort of adults from Rio de Janeiro, Southeast of Brazil. We postulated that these polymorphisms may affect protein functions or gene expression efficiency and could contribute to several pathophysiological conditions in severe obesity.

Subjects

This case-control, cross-sectional study comprised 401 adult individuals (70.8% female and 29.2% male), aged 18–65 years (median 35.0 [range 26.0–44.0] years), from the Southeast of Brazil. The participants were divided into 2 groups according to their BMI. Exclusion criteria were pregnancy, lactation, and the use of medication to lose or gain weight. The group with severe obesity (BMI ≥ 35.0) consisted of 210 individuals recruited from a nongovernmental organization (the “Grupo de Resgate à Autoestima e Cidadania do Obeso” [GRACO]). These patients were candidates for bariatric surgery. The group of normal-weight subjects (BMI ≥ 18.5 and ≤ 24.9) included 191 participants who were volunteers from public hospitals in Rio de Janeiro.

Materials and Methods

Anthropometric Parameters

The measurement of height, body weight, and waist and hip circumference was made according to classical methods. Briefly, waist circumference was evaluated at the midpoint between the iliac crest and the last costal arch, and hip circumference was measured at the level of the greater trochanters. BMI, body adiposity index (BAI), waist-to-weight ratio (WWR), and waist-to-hip ratio (WHR) were then calculated for each participant. BAI is a parameter used to estimate the percentage of body fat for adult men and women and is calculated using the formula: $\text{hip circumference}/(\text{height}^{1.5}) - 18$ [30].

Biochemistry

Glucose, total cholesterol (TC), HDL-cholesterol (HDL-c), and triglycerides (TG) were measured by oxidase-peroxidase method (BioSystems), after an overnight fast. C-reactive protein (CRP) was evaluated using the latex agglutination method and glycated hemoglobin was measured by turbidimetric inhibition immunoassay (TINIA). Low-density lipoprotein cholesterol (LDL-c) was calculated by Friedewald formula ($\text{LDL-c} = \text{TC} - \text{HDL-c} - \text{TG}/5$). Biochemical measurements influenced by medications taken by participants were not used in this statistical analysis.

Genotyping

Peripheral blood was collected from each participant and genomic DNA was extracted using a commercial DNA extraction kit (QIAamp Blood Kit, Qiagen, Valencia, CA, USA). Genotypes for the *PPARGC1A* (rs8192678, rs3736265, rs2970847, and rs3755863) and *UCP1* (rs6536991 and rs12502572) polymorphisms were analyzed by real-time PCR using TaqMan® assays (ThermoFisher, Foster City, CA, USA). Amplification was performed in a StepOne® Plus real-time PCR system (ThermoFisher) using the manufacturer's protocol. Negative (all components excluding DNA) and positive controls were included for the genotyping quality conformation. We duplicated the test in 10% of the sample and there was 100% consistency.

For the *FNDC5* gene, screening of exons 3–5 as well as their intron-exon boundaries was performed using Sanger automatic sequencing. These regions encode important structural regions of *FNDC5*, including the transmembrane domain, the targeting signal, and the site cleaved by irisin. These exons were amplified by PCR using 3 sets of custom-designed primers: exon 3 forward primer, 5'-TGGTCACCGAGTGTGACAG-3' and reverse 5'-GGATA-AGGGGGAGGAGACAG-3'; exon 4 forward primer, 5'-TGTGTGGGCACCTGTAGAAA-3' and reverse 5'-TCCAAACCCCTTACCCTTTT-3'; and exon 5 forward primer, 5'-AGAGACCATTG-GCAAGCACT-3' and reverse 5'-GTCCAGGGATTACCAGAGCA-3'. Reactions were performed in a total volume of 25 µL, which included 20–50 ng of genomic DNA, 1.5 units of AmpliTaq Gold (ThermoFisher), 1× AmpliTaq buffer, 0.2 mmol/L of each dNTP, 0.3 µM of each primer and 2 mmol/L MgCl₂. Amplification of exon 3 was carried out under the following conditions: 95 °C for 10 min, followed by 35 cycles of 94 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min; then an elongation step at 72 °C for 10 min. For exons 4 and 5, the only difference was in the temperature used for annealing, i.e., 60 °C. PCR products were visualized on 1.5% agarose gels and purified with an Exosap kit (ThermoFisher) according to the manufacturer's protocol. Finally, sequencing was performed using reactions with 10 µL, containing 10–40 ng of PCR products, sequencing buffer 1×, 1.0 µL big dye terminator kit v3.1 and 0.32 µM of primer. The sequencing of products was carried out with the following conditions: 40 cycles of 94 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequencing analysis was performed on an ABI Prism 3730xl analyzer (ThermoFisher). The amplicons were sequenced bidirectionally and aligned with the sequence provided by the National Centre for Biotechnology Information (accession No. NM_001171940) using the software SeqScape v2.1 (ThermoFisher).

Table 1. Anthropometrical, biochemical, and demographic characteristics

	N	All	N	Controls	N	Cases	p
Age, years	401	35.0 (26.0–44.0)	191	29.0 (24.0–37.0)	210	39.0 (31.0–48.3)	<0.001
Gender							
Female	401	284 (70.8)	191	117 (61.3)	210	167 (79.5)	<0.001
Male		117 (29.2)		74 (38.7)		43 (20.5)	
Physical activity							
Yes	397	251 (63.2)	191	99 (51.8)	206	47 (22.8)	<0.001
No		146 (36.8)		92 (48.2)		159 (77.1)	
Weight, kg	401	91.9 (63.1–126.5)	191	63.0 (57.0–70.0)	210	124.8 (106.8–142.3)	<0.001
Height, m	401	1.65 (1.59–1.72)	191	1.68 (1.61–1.74)	210	1.62 (1.58–1.69)	<0.001
BMI	401	35.9 (22.9–46.3)	191	22.8 (21.1–23.9)	210	45.8 (40.6–52.3)	<0.001
BAI	401	36.4 (27.1–49.1)	191	27.0 (24.1–29.6)	210	48.7 (42.9–54.6)	<0.001
WWR	401	1.20 (1.10–1.30)	191	1.3 (1.2–1.4)	208	1.1 (1.0–1.2)	<0.001
Waist circumference, cm	401	106.0 (82.0–135.0)	191	81.0 (75.0–85.5)	208	133.7 (123.3–145.0)	<0.001
Hip circumference, cm	401	119.0 (98.0–141.0)	191	98.0 (93.0–101.0)	208	141.0 (130.0–151.0)	<0.001
WHR	401	0.90 (0.83–0.97)	191	0.83 (0.78–0.89)	208	0.96 (0.90–1.01)	<0.001
Glucose, mmol/L	331	5.11 (4.77–5.72)	184	4.88 (4.77–5.26)	147	5.66 (5.11–6.27)	<0.001
Total cholesterol, mg/dL	361	187.0 (160.5–215.5)	184	178.5 (156.0–200.0)	177	194.0 (168.0–225.5)	<0.001
HDL-c, mg/dL	361	52.0 (44.0–63.0)	184	59.0 (48.0–69.0)	177	48.0 (41.5–54.0)	<0.001
LDL-c, mg/dL	354	109.0 (90.0–132.0)	183	102.0 (86.0–123.0)	171	121.0 (96.0–142.0)	<0.001
Triglycerides, mg/dL	361	99.0 (70.0–139.5)	184	77.0 (61.0–101.8)	177	127.0 (94.5–185.0)	<0.001
Glycated hemoglobin, %	340	5.3 (4.9–5.9)	165	5.1 (4.8–5.4)	175	5.8 (5.1–6.3)	<0.001
CRP, mg/dL	336	0.43 (0.12–1.11)	164	0.14 (0.08–0.28)	172	1.0 (0.55–1.59)	<0.001

Data are presented as median (interquartile range) for continuous traits and *n* (%) for categorical traits. Data were analyzed by Mann-Whitney U test (for nonnormally distributed variables) or χ^2 test (for categorical variables). BMI, body mass index; BAI, body adiposity index; WWR, waist-to-weight ratio; WHR, waist-to-hip ratio; HDL/LDL-c, high-/low-density lipoprotein cholesterol; CRP, C-reactive protein. The *p* value was for the difference between cases and controls.

Bioinformatic Tools

FND5 sequences (genomic, transcript, and protein) were obtained from the National Centre for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/>) and the Ensembl database (<http://www.ensembl.org/>). Additionally, the UniProt database was used to acquire information on the protein structure (<http://www.uniprot.org/>). PolyPhen and SIFT were selected to examine the potential pathogenicity of missense variants identified in this study (<http://www.ensembl.org/info/docs/tools/vep/>). Synonymous mutations were evaluated using FAS-ESS software which predicts the potential exonic splicing elements (<http://genes.mit.edu/fas-ess/>). Finally, intronic mutations of *FND5* were explored using a splice-site prediction interface in NNSplice (http://www.fruitfly.org/seq_tools/splice.html).

Statistical Analysis

Normality of continuous parameters was tested with the Kolmogorov-Smirnov and Shapiro-Wilk tests. All continuous parameters were shown as nonnormal distribution. Differences in clinical, biochemical, and anthropometric characteristics between the case and control groups were calculated with the Mann-Whitney U and χ^2 tests.

Genotype and allele distributions were calculated by gene counting. The Hardy-Weinberg equilibrium (HWE) was verified for each polymorphism using the χ^2 test. Logistic regression analyses were performed to evaluate the association between *PPARGC1A* and *UCP1* polymorphisms with severe obesity susceptibility, adjusted for gender and age. These association analyses were carried out in the additive, dominant, and recessive models. Moreover, the *FND5* variants found in the case and control groups had association analyses performed with the χ^2 test.

Continuous variables with nonnormal distribution were log-transformed before linear regression. The traits of interest (biochemical and anthropometric parameters) were chosen as dependent variables and polymorphism genotypes as independent variables (additive model). Gender and age were used as possible confounding variables for BMI, body weight, BAI, and WWR. Gender, age, and BMI were used as possible confounding variables for all other biochemical and anthropometric measurements. The analyses were performed using the SPSS statistical package (IBM, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

In order to evaluate the sample size, our group used an iterative process to compute the minimum number of participants required to test the difference between 2 groups of qualitative variables (cases vs. controls). In this study, different polymorphisms were analyzed by selection of a conservative and a convenience sample (80% of statistical power) [31].

Results

Clinical characteristics of the 401 participants are shown in Table 1. The cohort consisted of 210 subjects with severe obesity (i.e., cases; median BMI 45.6 [range 40.5–52.2]) and 191 individuals of normal-weight (i.e., controls; BMI 22.8 [21.1–23.9]). Generally, as expected, cases had higher anthropometric and biochemical data values than controls; exceptions were height, HDL-c, and WWR, for which the control group had higher values.

All samples were genotyped for polymorphisms in *PPARGC1A* (rs8192678, rs3736265, rs2970847, and rs3755863) and *UCP1* (rs6536991 and rs12502572). The sample details about the genotype and allele frequencies are shown in Table 2. Genotypes of the polymorphisms were in HWE ($p > 0.05$) for both case and control groups. Association analyses showed that genotype and allele frequencies of *PPARGC1A* rs2970847 and *UCP1* rs12502572 differed

Table 2. Genotype and allele frequencies of *PPARGC1A* and *UCP1* polymorphisms, and the risk for severe obesity

Gene	Polymorphism	Controls (n = 191)	Cases (n = 210)	Odds ratio (95% confidence interval)		p	p1
				unadjusted	adjusted		
<i>PPARGC1A</i>	rs8192678						
	<i>Genotype</i>						
	GG	100 (52.4)	114 (54.3)	1.00 (ref.)	1.00 (ref.)	–	–
	GA	78 (40.8)	82 (39.0)	0.92 (0.61–1.39)	0.98 (0.63–1.53)	0.699	0.927
	AA	13 (6.8)	14 (6.7)	0.94 (0.42–2.10)	0.81 (0.34–1.96)	0.889	0.648
	<i>Dominant model</i>						
	GG	100 (52.4)	114 (54.3)	1.00 (ref.)	1.00 (ref.)	–	–
	GA + AA	91 (47.6)	96 (45.7)	0.92 (0.62–1.37)	0.95 (0.62–1.46)	0.699	0.830
	<i>Recessive model</i>						
	GG + GA	178 (93.2)	196 (93.3)	1.00 (ref.)	1.00 (ref.)	–	–
	AA	13 (6.8)	14 (6.7)	0.98 (0.45–2.13)	0.82 (0.35–1.94)	0.956	0.655
	<i>Allele</i>						
	G	278 (72.8)	310 (73.8)	1.00 (ref.)	1.00 (ref.)	–	–
	A	104 (27.2)	110 (26.2)	0.95 (0.69–1.30)	0.94 (0.66–1.33)	0.738	0.724
	rs3736265^a						
	<i>Genotype</i>						
	GG	166 (87.0)	188 (89.5)	1.00 (ref.)	1.00 (ref.)	–	–
	GA + AA	25 (13.0)	22 (10.5)	0.78 (0.42–1.43)	0.82 (0.43–1.57)	0.417	0.553
	<i>Allele</i>						
	G	355 (92.9)	398 (94.8)	1.00 (ref.)	1.00 (ref.)	–	–
	A	27 (7.1)	22 (5.2)	0.73 (0.41–1.30)	0.77 (0.42–1.42)	0.287	0.408
	rs2970847						
	<i>Genotype</i>						
	CC	142 (74.3)	180 (85.7)	1.00 (ref.)	1.00 (ref.)	–	–
	CT	46 (24.1)	29 (13.8)	0.50 (0.30–0.83)	0.48 (0.27–0.84)	0.008	0.010
	TT	3 (1.6)	1 (0.5)	0.26 (0.03–2.56)	0.27 (0.03–2.83)	0.250	0.276
	<i>Dominant model</i>						
	CC	142 (74.3)	180 (85.7)	1.00 (ref.)	1.00 (ref.)	–	–
	CT + TT	49 (25.7)	30 (14.3)	0.48 (0.29–0.80)	0.46 (0.27–0.80)	0.005	0.006
	<i>Recessive model</i>						
	CC + CT	188 (98.4)	209 (99.5)	1.00 (ref.)	1.00 (ref.)	–	–
	TT	3 (1.6)	1 (0.5)	0.31 (0.03–3.23)	0.30 (0.03–2.91)	0.328	0.299
	<i>Allele</i>						
	C	330 (86.4)	389 (92.6)	1.00 (ref.)	1.00 (ref.)	–	–
	T	52 (13.6)	31 (7.4)	0.50 (0.31–0.80)	0.48 (0.29–0.81)	0.004	0.006
	rs3755863						
	<i>Genotype</i>						
	GG	78 (41.0)	94 (44.8)	1.00 (ref.)	1.00 (ref.)	–	–
	GA	90 (47.0)	84 (40.0)	0.78 (0.51–1.18)	0.84 (0.53–1.32)	0.236	0.443
	AA	23 (12.0)	32 (15.2)	1.15 (0.63–2.13)	1.16 (0.59–2.27)	0.647	0.666
	<i>Dominant model</i>						
	GG	78 (41.0)	94 (44.8)	1.00 (ref.)	1.00 (ref.)	–	–
	GA + AA	113 (59.2)	116 (55.2)	0.85 (0.57–1.27)	0.90 (0.59–1.39)	0.428	0.639
	<i>Recessive model</i>						
	GG + GA	168 (88.0)	178 (84.8)	1.00 (ref.)	1.00 (ref.)	–	–
	AA	23 (12.0)	32 (15.2)	1.31 (0.74–2.33)	1.27 (0.68–2.38)	0.354	0.456

Table 2 (continued)

Gene	Polymorphism	Controls (n = 191)	Cases (n = 210)	Odds ratio (95% confidence interval)		p	p ¹
				unadjusted	adjusted		
	<i>Allele</i>						
	G	246	272	1.00 (ref.)	1.00 (ref.)	–	–
	A	136	148	0.98 (0.74–1.30)	1.00 (0.74–1.37)	0.916	0.974
UCP1	rs12502572						
	<i>Genotype</i>						
	GG	67 (35.1)	54 (25.7)	1.00 (ref.)	1.00 (ref.)	–	–
	GA	89 (46.6)	106 (50.5)	1.48 (0.94–2.33)	1.44 (0.87–2.36)	0.093	0.152
	AA	35 (18.3)	50 (23.8)	1.77 (1.01–3.10)	1.82 (0.99–3.35)	0.046	0.055
	<i>Dominant model</i>						
	GG	67 (35.1)	54 (25.7)	1.00 (ref.)	1.00 (ref.)	–	–
	GA + AA	124 (64.9)	156 (74.3)	1.56 (1.02–2.40)	1.54 (0.97–2.46)	0.042	0.070
	<i>Recessive model</i>						
	GG + GA	156 (81.7)	160 (76.2)	1.00 (ref.)	1.00 (ref.)	–	–
	AA	35 (18.3)	50 (23.8)	1.39 (0.86–2.26)	1.45 (0.86–2.46)	0.181	0.167
	<i>Allele</i>						
	G	223 (58.4)	214 (51.0)	1.00 (ref.)	1.00 (ref.)	–	–
	A	159 (41.6)	206 (49.0)	1.34 (1.02–1.78)	1.36 (1.00–1.84)	0.037	0.049
	rs6536991						
	<i>Genotype</i>						
	TT	80 (41.9)	72 (34.3)	1.00 (ref.)	1.00 (ref.)	–	–
	TC	84 (44.0)	104 (49.5)	1.38 (0.90–2.11)	1.22 (0.77–1.95)	0.145	0.397
	CC	27 (14.1)	34 (16.2)	1.40 (0.77–2.54)	1.30 (0.68–2.48)	0.270	0.431
	<i>Dominant model</i>						
	TT	80 (41.9)	72 (34.3)	1.00 (ref.)	1.00 (ref.)	–	–
	TC + CC	111 (58.1)	138 (65.7)	1.38 (0.92–2.07)	1.24 (0.80–1.92)	0.118	0.336
	<i>Recessive model</i>						
	TT + TC	164 (85.9)	176 (83.8)	1.00 (ref.)	1.00 (ref.)	–	–
	CC	27 (14.1)	34 (16.2)	1.17 (0.68–2.03)	1.16 (0.64–2.10)	0.568	0.624
	<i>Allele</i>						
	T	244 (63.9)	248 (59.0)	1.00 (ref.)	1.00 (ref.)	–	–
	C	138 (36.1)	172 (41.0)	1.16 (0.85–1.58)	1.22 (0.92–1.63)	0.354	0.164

Odds ratios and p value were calculated using logistic regression. ¹ Adjusted for gender and age.

^a No homozygous AA in case subjects was identified.

significantly between the groups. The frequency of rs2970847 wild-type genotype (CC) was significantly higher in the case group than in the control group. In order to explore this association, dominant and recessive models were performed. *PPARGC1A* rs2970847 was significantly associated with severe obesity in a dominant model. Allelic analysis showed that the rs2979847 (C) allele was more frequent in the subjects with severe obesity than in the individuals of normal weight (7.4 vs. 13.7; $p = 0.004$). Furthermore, individuals carrying the rs2979847 (C) allele had a 2.01-fold increased risk of severe obesity compared to subjects without this allele.

The frequency of *UCP1* rs12502572 (AA) genotype was higher in the cases than in the controls. Moreover, our result demonstrated that this polymorphism was associated with obesity in a dominant model. After adjustment for age and gender, these associations were

Fig. 1. A Electropherograms of the *FNDC5* gene. **B** *FNDC5* structure and position of the 5 mutations identified by Sanger sequencing. **C** A portion of the amino acid sequence of *FNDC5* in diverse species. The position of the new mutation is showed in the red squares, suggesting it is highly conserved in different organisms. The nonconserved position in specific species is shown in the white squares.

no longer observed. However, allelic analysis showed that the minor allele (A) frequency was higher in subjects with severe obesity than in controls (49.0 vs. 41.6; $p = 0.037$). Therefore, for individuals carrying the *UCP1* rs12502572 (A) allele, the risk of developing severe obesity increased 1.34-fold. No association was found between obesity and *PPARGC1A* (rs8192678, rs3736265, and rs3755863) or *UCP1* (rs6536991).

The influence of *PPARGC1A* and *UCP1* polymorphisms on anthropometric and biochemical measurements in our sample was analyzed (online suppl. data, Table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000505666). *PPARGC1A* rs2970847 and *UCP1* rs12502572 were associated with body weight, BMI, and BAI in an additive model. *PPARGC1A* rs2970847 was also associated with WWR. In addition, *PPARGC1A* rs8192678 had an influence on plasma glucose measurements. The additive effect on variables was found for the *UCP1* rs12502572 and *PPARGC1A* rs8192678 polymorphisms. Our results showed that the median value increased according to the number of risk alleles, so that subjects carrying 2 risk alleles had higher median values. Our results indicated no significant effects of *PPARGC1A* rs3755863, *PPARGC1A* rs3736265, and *UCP1* rs6536991 polymorphisms on anthropometric and biochemical levels.

The *FNDC5* gene was screened in order to investigate the prevalence of mutations in the severe obesity and normal-weight groups. We identified 5 rare mutations in the heterozygous state (Fig. 1A). The prevalence of these variations in the case and control groups is shown in Table 3. Three single-nucleotide substitutions were located at the intron-exon boundaries (rs113173936, rs72882318, and rs1284368). These variants have been described in the literature and were found in both cases and controls. Intronic mutations may not affect the protein sequence but have a potential pathogenic effect if they disrupt splicing mechanisms. These variants were thus examined using a splice-site prediction interface. It demonstrated

that these mutations were not located in acceptor or donor sites. The association between the variants and severe obesity was evaluated in our sample, but no significant result was found.

Only 2 subjects with severe obesity showed variants in the coding region, one with a synonymous mutation (rs138727728) and the other with a novel missense mutation (Fig. 1B). The synonymous mutation is localized at exon 4 of the *FNDC5* gene, where cytosine is substituted for thymine at the base position 466 (Leu81 =). Despite not affecting the amino acid, this type of mutation may disturb important regulatory elements. We therefore examined the potential exonic splicing enhancers or silencers in the *FNDC5* gene. The results showed that the mutation was not localized in these regions.

The novel mutation is localized at exon 5 of *FNDC5*, namely c.629G>A (Ser210Asn). The potential pathogenicity of this mutation was evaluated using in silico software. The PolyPhen software suggested that the mutation was probably damaging (score: 0.916). The SIFT software result indicated that variation was deleterious with low confidence (score: 0.03). Furthermore, this residue is highly conserved between different species (from humans to zebrafish) (Fig. 1C).

Discussion

In this study, we explored the genetic variations in the *PPARGC1A*, *UCP1*, and *FNDC5* genes as potential risk factors for the development of severe obesity in a Brazilian population. Additionally, we evaluated the association between these polymorphisms and obesity-related traits. To this end, we genotyped individuals with severe obesity ($n = 210$) and normal-weight controls ($n = 191$) for common polymorphisms in the *PPARGC1A* and *UCP1* genes. Our results indicated that *PPARGC1A* rs2970847 and *UCP1* rs12502572 are associated with a susceptibility to severe obesity and obesity-related parameters. Moreover, *PPARGC1A* rs8192678 is associated with an increase in glucose plasma levels. We also screened exons 3–5 (including their intron-exon boundaries) of the *FNDC5* gene, and identified 5 rare mutations, 1 of them a novel missense mutation (Ser210Asn).

The *PPARGC1A* gene encodes a transcriptional coactivator protein, which plays a key role in energy metabolism via mitochondrial biogenesis, fatty acid oxidation, hepatic gluconeogenesis, glucose uptake, and lipid metabolism [32–35]. In this study, we examined 4 common polymorphisms, and found that the *PPARGC1A* rs2970847 polymorphism is associated with severe obesity susceptibility. Our findings showed that individuals carrying the *PPARGC1A* rs2970847 (CC) genotype have an increased risk of becoming severely obese. Moreover, subjects with this genotype had higher median body weight, BMI, BAI, and WWR values.

PPARGC1A rs2970847 is a synonymous mutation (Thr394Thr), and it is unclear how this polymorphism could affect body metabolism to alter the risk for obesity in our sample. Interestingly, there is evidence that synonymous mutations are important for different aspects of human disease and therefore therapeutic approaches. These mutations may disturb basic cellular processes such as gene regulation, mRNA secondary structure, stability or splicing, and protein synthesis, folding, or functionality [36]. They could also be in complete or near-complete linkage disequilibrium with a pathogenic variant [37]. In the literature, there are only a few studies on *PPARGC1A* rs2970847 and obesity. Vimalaswaran et al. [37] reported that this polymorphism influences adiposity in Asian Indian subjects. They observed that visceral and subcutaneous fat are lower in subjects with the *PPARGC1A* rs2970847 (CC) genotype. In contrast, in our sample, individuals carrying the *PPARGC1A* rs2970847 (CC) genotype had higher adiposity values. The discrepancy may be explained by the differences in genetic backgrounds and sample criteria.

We also showed an association between *PPARGC1A* rs8192678 and fasting plasma glucose. *PPARGC1A* rs8192678 is a missense mutation which changes glycine to serine at codon 482 (Gly482Ser). Individuals carrying the risk allele (482Ser) have reduced levels of *PPARGC1A* mRNA compared to homozygous subjects [38]. Povel et al. [39] reported that the presence of the risk allele was associated with lower glucose levels in normal-weight individuals (BMI ≤ 25.0). However, their results also suggested an association with the opposite trend in subjects with a higher BMI (BMI ≥ 28.0), similar to our findings. Since the PGC1- α protein, encoded by *PPARGC1A*, acts on energy expenditure and fuel uptake, this polymorphism may affect energy balance. In this context, previous functional studies showed that PGC1- α regulates glucose uptake and oxidation via expression of the glucose transporter type 4 (*GLUT4*) gene in muscle [38, 40]. Therefore, we hypothesized that the higher glucose levels in our patients carrying the Ser482 allele could be explained by the reduced abundance of PGC1- α and GLUT4, which consequently resulted in decreased glucose uptake and metabolism. However, functional studies are necessary to confirm this hypothesis.

UCP1 is located in the inner mitochondrial membrane of brown and brown-like adipocytes, where it has a key role in thermogenesis and energy expenditure [11, 12, 41]. In this context, *UCP1* is a strong candidate gene for obesity susceptibility, and several genetic studies have evaluated this association [26, 42, 43]. Our findings demonstrate that the *UCP1* rs12502572 (A) allele is a risk factor for severe obesity. We observed an additive effect of this allele in the anthropometric parameters (body weight, BMI, and BAI).

To date, studies on the *UCP1* rs12502572 polymorphism are scarce in the literature. Ramos et al. [26] selected 239 adult patients from Brazil (126 subjects with morbid obesity and 113 individuals with normal obesity) but found no association between the *UCP1* rs12502572 polymorphism and obesity or related traits. This polymorphism is located at intron 2 and it is unknown whether it affects the gene expression of *UCP1*. Nishimura et al. [27] found that it has an effect on nonshivering thermogenesis caused by the generation of heat via the inhibition of mitochondrial ATP synthesis in brown tissues. Since thermoregulatory mechanisms contribute to the regulation of body weight and our results showed an association between *UCP1* rs12502572 (A) allele and increased body adiposity, we suggest that *UCP1* rs12502572 might affect gene expression and, consequently, energy expenditure. Further functional studies are required to examine how this polymorphism could affect *UCP1* expression.

Finally, the main region of the *FNDC5* gene, which encodes the transmembrane domain, targeting signal, and irisin-cleaved site, was sequenced in the case and control groups. Five rare mutations were identified; 3 were located at the intron-exon boundary and 2 in the coding region. Our results showed that intronic mutations as well as the synonymous mutation were not located in potential splicing elements, suggesting that they do not disturb this cellular mechanism. A novel missense mutation was found at exon 5, which led to a serine-to-asparagine substitution at codon 210. The substituted amino acid is highly conserved in different species from human to zebrafish, and both Polyphen and SIFT software have predicted that it can have pathogenic effects.

FNDC5 is a type 1 transmembrane protein. This novel mutation is positioned in the cytoplasmic region, which has an important role in the microbody-targeting signal (UniProtKB database, ID: Q8NAU1). The C-terminal region of *FNDC5* contains an important sequence to transport proteins into their correct microbody compartment. Moreover, some studies have reported that such regions are evolutionary conserved, demonstrating their important role in protein transportation [44, 45]. Interestingly, the new mutation located in this region was found only in a female patient with extreme obesity (BMI 56.1), who had developed severe obesity during pregnancy. Since *FNDC5* is cleaved and released as irisin, a hormone that has a key role in the activation of the browning pathway [11], we suggest that this novel mutation

may impact on protein transport which could decrease energy expenditure and contribute to a susceptibility to severe obesity.

This study had some limitations that should be considered when interpreting the results. First, it had a cross-sectional study design and we were unable to exclude the fluctuations of biochemical and anthropometric traits. Second, the interaction between genes and lifestyle were not investigated and could be a confounding factor. Finally, a lack of data on the mRNA or protein expression of the studied genes meant we could not confirm that the genetic variants are in fact associated with modifications in expression.

Conclusion

Obesity is a complex disease resulting from an interaction of environmental and genetic factors. A number of previous studies have highlighted the important role of polymorphisms in some energy balance genes and their association with susceptibility to developing obesity, which might be helpful for establishing a personalized therapy and/or prevention. We investigated genetic variants in *PPARGC1A*, *UCP1*, and *FNDC5*, genes that are related to the activation of the brown-like adipocyte pathway. We identified that the *PPARGC1A* rs2970847 and *UCP1* rs12502572 polymorphisms modulate the risk of developing severe obesity and obesity-related traits in a Brazilian population. Our findings also indicated an association of *PPARGC1A* rs8192678 and glucose plasma levels. The sequencing of the *FNDC5* gene identified 5 rare variants, 1 a novel missense mutation located in the microbody-targeting signal. This mutation needs further functional analysis to clarify its potential pathogenic effect on *FNDC5* transport and metabolism.

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Statement of Ethics

The study protocol was performed according to the Declaration of Helsinki (1964) and approved by the Ethics Committee of the Oswaldo Cruz Foundation. All participants provided written consent prior to enrollment.

Disclosure Statement

There were no conflicts of interest.

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Availability of Data

The study data are available on request.

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