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Gene polymorphisms and increased DNA damage in morbidly obese women

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ABSTRACT

Obesity is characterized by increased adipose tissue mass resulting from a chronic imbalance between energy intake and expenditure. Furthermore, there is a clearly defined relationship among fat mass expansion, chronic low-grade systemic inflammation and reactive oxygen species (ROS) generation; leading to ROS-related pathological events. In the past years, genome-wide association studies have generated convincing evidence associating genetic variation at multiple regions of the genome with traits that reflect obesity. Therefore, the present study aimed to evaluate the relationships among the gene polymorphisms ghrelin (*GHRL*–rs26802), ghrelin receptor (*GHSR*–rs572169), leptin (*LEP*–rs7799039), leptin receptor (*LEPR*–rs1137101) and fat mass and obesity-associated (*FTO*–rs9939609) and obesity. The relationships among these gene variants and the amount of DNA damage were also investigated. Three hundred Caucasian morbidly obese and 300 eutrophic (controls) women were recruited. In summary, the results demonstrated that the frequencies of the *GHRL*, *GHSR*, *LEP* and *LEPR* polymorphisms were not different between Brazilian white morbidly obese and eutrophic women. Exceptions were the *AA-FTO* genotype and allele A, which were significantly more frequent in obese women than in the controls (0.23% vs. 0.10%; 0.46 vs. 0.36, respectively), and the *TT-FTO* genotype and the T allele, which were less frequent in morbidly obese women ($p < 0.01$). Furthermore, significant differences in the amount of genetic lesions associated with *FTO* variants were observed only in obese women. In conclusion, this study demonstrated that the analyzed SNPs were not closely associated with morbid obesity, suggesting they are not the major contributors to obesity. Therefore, our data indicated that these gene variants are not good biomarkers for predicting risk susceptibility for obesity, whereas ROS generated by the inflammatory status might be one of the causes of DNA damage in obese women, favoring genetically related diseases as obesity comorbidities.

1. Introduction

Overweight and obesity are defined as abnormal or excessive fat accumulation and represent major risk factors for chronic diseases, including diabetes, cardiovascular diseases and cancer [1]. The etiology of obesity is a complex interaction of genetics, diet, metabolic factors, and physical activity [2–5]. Recent advances in genomic technology (e.g., GWAS, genome-wide association studies) have revealed more than 40 candidate genes that predispose individuals to obesity. *FTO* (*fat mass and obesity-associated*), *ghrelin* and *leptin* genes have variants that are associated with obesity and play important roles in maintaining energy homeostasis, food intake, energy expenditure and the lipolysis regulation of body fat [6–9]. In fact, *FTO* is a highly polymorphic gene located on chromosome 16q12.2, and several of its variants have been associated with energy regulation and body mass index (BMI) [10–14]. Among the polymorphisms, rs9939609 has been the most thoroughly studied and annotated to be associated with the obesity phenotype [15,16]. Additionally, ghrelin, a peptide produced in the stomach, has important roles in growth hormone secretion, appetite stimulation, food intake, regulation of gastric motility and acid secretion, adiposity, energy homeostasis and inflammatory processes [17–19]. Leptin is a hormone released by adipocytes, and its circulating levels decrease or increase in response to an acute shortage or excess of food. It is known that leptin is related to the

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direct regulation of adipose tissue metabolism by the inhibition of lipogenesis and the stimulation of lipolysis [20,21]. Furthermore, leptin acts on the hypothalamus to signal when the body has sufficient energy stores, thus inhibiting appetite and acting like a satiety signal [22]. Fasting leptin levels were associated with the 2548G > A single-nucleotide polymorphism (SNP) in the promoter region of the leptin gene (*LEP*) and the 668A > G SNP in the leptin receptor gene (*LEPR*) [23–25].

It is known that adipokines secreted by the adipose tissue promote inflammatory responses and metabolic dysfunction and have both pro- and anti-inflammatory activities. When adipocyte dysfunction occurs as a result of adipose tissue expansion, dysregulation of adipokine production can have local or systemic effects on inflammatory responses [26]. This increased state of oxidative stress is most likely due to the presence of excessive adipose tissue itself. These cytokines are potent stimulators of the production of reactive oxygen and nitrogen by macrophages and monocytes [27]. This inflammatory state leads to overproduction of reactive oxygen species (ROS) that damage cellular structures [28] and are able to interact with DNA, inducing genotoxic damage or mutations that can lead to significant phenotypic changes at the cellular, tissue or body level [29].

Several studies have linked oxidative stress to genotoxicity and various chronic degenerative diseases. However, the relationship between DNA damage and obesity, linked to some gene polymorphisms, has not been described. Therefore, the present study aimed to investigate the frequencies of ghrelin (*GHRL)* and ghrelin receptor (*GHRL*), *LEP* and *LEPR* and *FTO* genotypes and their relationships with primary DNA damage in morbidly obese and eutrophic women.

2. Materials and methods

The Ethical Committee for Human Research from Botucatu Medical School—São Paulo State University (São Paulo, Brazil) approved the protocol used in this study.

2.1. Subjects

Three hundred white morbidly obese women (body mass index > 40 kg/m^2) who underwent bariatric surgery were recruited at the International Center of Excellence for Bariatric Surgery in Piracicaba – São Paulo – Brazil, between 2010 and 2011. All the volunteers gave informed consent and answered a structured questionnaire, including questions about cigarette smoking habits (non-smoker, ex-smoker, or current smoker), alcohol consumption, medical history and use of medicines or dietary supplements. Data, including height, body weight (BW) and body mass index (BMI), were also obtained. Obese women with metabolic syndrome (n = 30) age-matched with thirty without metabolic syndrome (according to the criteria established by the International Diabetes Federation (IDF; http://www.idf.org/metabolic-syndrome) were selected to evaluated the effect of this group of risk factors on the evaluated endpoints. After classified, three hundred healthy, white eutrophic (BMI < 25 kg/m^2) and age-matched women were recruited as a control group. The exclusion criteria included: infectious, chronic and endocrine diseases; pregnancy; and cigarette and alcohol consumption.

2.2. Blood sampling

Peripheral venous blood samples (10 ml) from the obese women were collected in BD Vacutainer® EDTA tubes at least one month before the bariatric surgery. After blood sampling (approximately 4 h), peripheral blood mononuclear cells (PBMCs) were isolated in Ficoll–Paque® gradients and used for DNA genotyping and DNA damage evaluation by the comet assay.

2.3. Comet assay (single-cell gel electrophoresis assay)

The subset of the samples used for the comet assay was randomly selected from subjects with different genotypes (n = 30 per genotype). Immediately after PBMCs isolation, the comet assay was performed according to the protocols described by Singh et al. [30], Tice et al. [31], Collins, 2011 [32] and OECD (2014) [33]. Briefly, 10 μl of the PBMC suspension were embedded in 120 μl of 0.5% low melting point agarose at 37 °C, and the suspensions were layered on slides precoated with 1.5% normal agarose. Then, cells were lysed overnight in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl at pH 10, with 1% Triton X-100 and 10% fresh dimethylsulfoxide). To evaluate oxidative DNA damage, slides were washed in PBS for 5 min and washed again (3 × 5 min each) in a buffer (40 mM HEPES, 100 mM KCl, 0.2 mg/ml bovine serum albumin and 0.5 mM EDTA at pH 8). The slides were incubated for 45 min at 37 °C with endonuclease III (which converts oxidized pyrimidines to strand breaks) or with formamidopyrimidine glycosylase (FPG, which recognizes altered purines) and were subsequently left for 15 min at 4 °C. Afterwards, the slides were exposed to a freshly prepared alkaline buffer (1 mM EDTA, 300 mM NaOH, pH 13) in a horizontal electrophoresis tank. After a 40-min DNA unwinding period, electrophoresis was conducted at 0.8 V/cm and 300 mA for 30 min. Following 15 min of neutralization with 0.4 M Tris (pH 7.5), slides were fixed in absolute ethanol and stored at room temperature for maximum 6 months until analysis. Slides were stained with 50 μl of SYBR Gold (1:2000) and immediately analyzed with a fluorescent microscope at 400x magnification. Images from 100 nucleoids (two replicates coded) per enzyme (FPG or Endo III) treatment for each patient were blindly scored using the Comet Assay II Image System (Perceptive Instruments, Haverhill, Suffolk, UK). Tail intensity (% DNA in the tail) was used to estimate the extent of DNA damage.

2.4. Single-nucleotide polymorphism genotyping

Genomic DNA was isolated from whole blood using the Illustra™ blood genomicPrep Kit (Amersham Pharmacia Biotech Inc, USA), according to the protocol recommended by the manufacturer. *Ghrelin* (*GHRL*, -501A > C, rs26802), *ghrelin receptor* (*GHSR*, 477G > A, rs572169), *leptin* (*LEP*, -2548G > A, rs7799039) and *leptin receptor* (*LEPR*, 668A > G, rs1137101) and *fat mass and obesity-associated* (*FTO* A > T, rs9939609) gene polymorphisms were analyzed using the TaqMan fluorescent allelic discrimination assay (Applied Biosystems) with standard procedures. The genotyping call rate exceeded 95% per SNP, and no discordant genotypes were observed in 10% of the duplicate samples. No deviation from the Hardy–Weinberg equilibrium was observed for any SNP.

2.5. Statistical analysis

Data were expressed as the means ± standard deviations, and $p < 0.05$ was considered to be significant. All data were tested using the PROC-GENMOD module of the SAS v.9.2 statistical software package (SAS Institute Inc., Cary, NC, USA). DNA damage data were not normally distributed. Therefore, a gamma distribution was conducted prior to the analyses. Student's t-test and an analysis of variance (ANOVA) followed by Tukey's test were used for demographic data, and chi-square tests were used to identify any significant departure from Hardy–Weinberg equilibrium and for comparing gene frequencies.

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Table 1
Demographic characteristics of the eutrophic ($n = 300$) and obese women ($n = 300$).

Group	Age (years)	Weight (kg)	Height (m)	BMI (kg/m^2)
Eutrophic	32.45 ± 6.83	59.20 ± 6.71	1.64 ± 0.06	21.96 ± 1.85
Obese	33.41 ± 6.51	119.05 ±15.84*	1.61 ± 0.06	45.69 ± 5.80*

Data are expressed as the means ± standard deviations; (*$p < 0.01$); BMI–body mass index.

3. Results

Table 1 shows the demographic characteristics (mean age, weight, height and BMI) from the morbidly obese ($n = 300$) and eutrophic women ($n = 300$). Metabolic syndrome was detected in 99/300 (33%) of the obese subjects (data not shown).

The genotype and allele frequencies of the *GHRL*, *GHSR*, *LEP*, *LEPR* and *FTO* genes are presented in Table 2. None of the genotype distributions showed significant deviation from Hardy-Weinberg equilibrium, and none of the allele frequencies or the genotype distributions differed between patients and controls ($p > 0.05$). Exceptions were the *AA-FTO* genotype and allele A, which were significantly more frequent in obese women than in the control group (0.23% vs. 0.10%; 0.46 vs. 0.36, respectively). In contrast, *TT-FTO* and the T allele were less frequent in morbidly obese women ($p < 0.01$). No relationship was found between body weight or BMI and any gene variant (data not shown).

The amounts of DNA damage (tail intensity) in both the control and obese populations are shown in Table 3. Obese women presented higher levels of DNA strand breaks and alkali-labile sites than eutrophic group ($p < 0.01$). Similarly, increased amounts of oxidized purines and pyrimidines were detected in PBMCs of obese women ($p < 0.01$). Moreover, the data revealed no significant differences between obese women with ($n = 30$) and without ($n = 30$) metabolic syndrome (Table 4).

Fig. 1 shows the levels of DNA damage according to genotype in the obese and control groups. For all the genes and genotypes, the

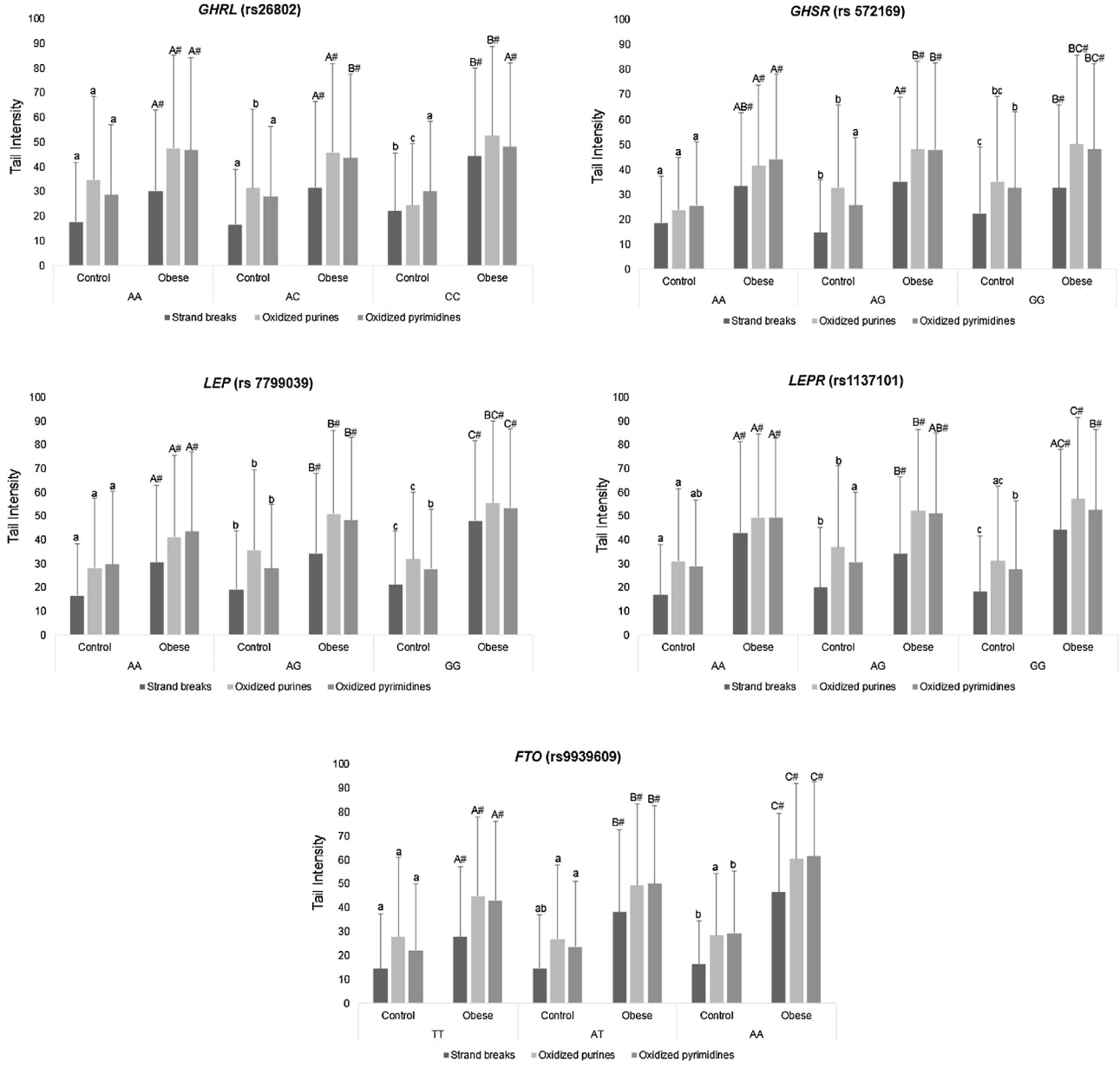

Fig. 1. DNA damage (tail intensity) in obese and eutrophic (control) women according to genotypes ($n = 30$ per genotype). # $p < 0.05$ (obese vs. control with same genotype and same type of DNA damage); different letters indicate significant differences ($p < 0.05$) among the three genotypes in control (small letters) and in obese (capital letters) groups.

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Table 2
Genotype and allele frequencies in eutrophic ($n = 300$) and obese ($n = 300$) populations.

Gene		Control	Obese
GHRL (rs26802)	Genotype		
	AA	0.473[a]	0.445[A]
	AC	0.449[a]	0.448[A]
	CC	0.078[b]	0.106[B]
	Allele		
	A	0.696[a]	0.669[A]
	C	0.303[b]	0.330[B]
	Genotype		
	GG	0.581[a]	0.578[A]
	AG	0.338[b]	0.344[B]
GHSR (rs572169)	AA	0.079[c]	0.077[C]
	Allele		
	G	0750[a]	0.750[A]
	A	0.250[b]	0.250[B]
	Genotype		
	AA	0.379[a]	0.376[A]
	AG	0.418[a]	0.474[B]
LEP (rs7799039)	GG	0.202[b]	0.175[C]
	Allele		
	A	0.588[a]	0.613[A]
	G	0.411[b]	0.386[B]
	Genotype		
	AA	0.207[a]	0.219[A]
	AG	0.483[b]	0.472[B]
LEPR (rs1137101)	GG	0.309[c]	0.300[C]
	Allele		
	A	0.449[a]	0.455[A]
	G	0.550[b]	0.544[B]
	Genotype		
	TT	0.40[a]	0.30[A*]
	AT	0,50[b]	0,47[B]
FTO (rs9939609)	AA	0.10[c]	0.23[A*]
	Allele		
	T	0.64[a]	0.53[A*]
	A	0.36[b]	0.46[B*]

Different letters mean significant differences ($p < 0.01$) between genotypes and between alleles in control (small letter) and obese (capital letter) groups. * $p < 0.01$ – between the obese and control groups.

morbidly obese women had more DNA damage (i.e., strand breaks, oxidized purines and pyrimidines) than the controls. The increases in DNA damage according to genotype were usually similar in obese and control women. However, for *GHRL-CC* and *LEP-GG*, oxidized purine and oxidized pyrimidines, respectively, were decreased in the controls, but in obese women, these two genotypes were related to the highest level of damage. For *FTO*, differences between control and obese women were also observed. In the controls, the homozygous *AA* genotype was associated with increased frequencies of DNA strand breaks and oxidized pyrimidines, and in obese women, the presence of the A allele (*AT* and *AA*) was related to increased DNA damage (strand breaks and oxidized purines and pyrimidines).

4. Discussion

Fasting levels of several (an)orexigenic hormones are strongly genotype-dependent, suggesting a genetic component in the

Table 3
DNA damage (tail intensity) in peripheral blood mononuclear cells from obese ($n = 70$) and eutrophic women ($n = 70$).

Group	Strand breaks[a]	Oxidized purines	Oxidized pyrimidines
Control	16.94 ± 23.51	33.29 ± 33.46	29.79 ± 29.81
Obese	28.96 ± 33.12[b]	46.5 ± 37.06[b]	44.74 ± 35.5[b]

[a] DNA single- and double-strand breaks and alkali-labile sites
[b] $p < 0.01$ (obese vs. control).

Table 4
DNA damage (tail intensity) in peripheral blood mononuclear cells of morbidly obese women according to the presence of metabolic syndrome.

Group	Strand breaks[a]	Oxidized purines	Oxidized pyrimidines
Without MS ($n = 30$)	39.04 ± 33.67	50.30 ± 34.62	47.02 ± 34.22
With MS ($n = 30$)	38.58 ± 34.96	52.01 ± 34.34	46.61 ± 32.77

MS - metabolic syndrome.
[a] DNA single- and double-strand breaks and alkali-labile sites.

physiological regulation of food intake. Therefore, considering the obesity epidemic from a genomic perspective, studies of molecular epidemiology may have the potential to improve the effectiveness of intervention strategies and obesity prevention [34]. Based on these premises, we investigated whether the *GHRL*, *GHSR*, *LEP*, *LEPR*, and *FTO* genotypes were different between obese and eutrophic subjects. Additionally, we assessed whether some of these gene variants affected the level of DNA damage in PBMCs. In fact, the relationship between genotoxic events and some diseases has been extensively reported in the literature. However, little is known about obesity.

Regarding genotype frequencies, in general, we did not observe differences between the two studied Brazilian populations (obese and eutrophic women). Exceptions were the *FTO-AA* genotype and allele A, which were significantly more frequent in obese women than in the controls, whereas *FTO-TT* and the T allele were less frequent in morbidly obese women than in the controls. Recently, GWAS have provided evidence that several common variants in the *FTO* gene are significantly associated with obesity in populations of European origin. In fact, similar findings were observed in populations from Spain [35,36], France [37], Finland [38] and Sweden [39]. Therefore, our data are in accordance with those previous studies because the Brazilian populations recruited were 100% white women. According to the genetic profile of the Brazilian population, the literature shows a major contribution of European ancestry (0.771), followed by African (0.143) and Amerindian contributions (0.085) [40]. However, no association was identified between the common *FTO* variants (including rs8050136, rs9939609 and rs9930506) and obesity in a Chinese Han population, suggesting that these polymorphisms are not major contributors to obesity [41].

For *GHRL* and *GHSR*, the data in the literature are not conclusive. Although the *GHRL* (rs4864677) and *GHSR* (rs572169) polymorphisms were positively associated with obesity in a French population, they were not in a German population [42]. In a larger study with 1464 Canadian subjects, no associations were identified among five *GHRL* SNPs (including the rs26802), body fat and serum lipid levels [43]. In contrast, a study in Finland, including 1045 subjects, showed that -501A > C CC genotypes presented higher fasting ghrelin plasma concentrations than the AA carriers. However, the CC carriers had lower BMIs and waist circumferences than the AC and AA genotypes [44]. For den Hoed et al. (2008), this controversial genotype-related association could be due to a physiological adaptation to facilitate normal homeostasis in homozygous C allele carriers because they are at an increased risk for overeating. Furthermore, the authors show the importance of exploring SNP–SNP interactions to elucidate relationships that could remain inexplicable with conventional SNP associations [45].

Overall, our data showed greater amount of DNA strand breaks and oxidized purines and pyrimidines in obese than in eutrophic women. Because obesity is characterized by a chronic low-grade inflammatory state, ROS generated due to this condition could be responsible for the DNA damage [28]. It has been reported that the increase of fat storage in obesity promotes adipocyte

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hypertrophy, resulting in adipose tissue hypoxia, which promotes altered secretion of proinflammatory and anti-inflammatory cytokines by adipocytes and pre-adipocytes [46]. These cytokines induce the release of reactive oxygen and nitrogen species from immune cells (macrophages, monocytes, T and B-cells) infiltrated in adipose tissue, contributing to systemic oxidative stress [47] and, consequently, for increasing mostly oxidative DNA damage. Indeed, evaluating another subset of morbidly obese women we observed an elevated level of circulating pro-inflammatory cytokines (IL-6, TNF-α, eotaxin and MCP-1) and low of the anti-inflammatory IL-10 (data still not published).

It is well known that oxidative stress can lead to oxidative DNA damage, causing strand breaks, altered gene expression and ultimately mutagenesis [48]. A recent study showed that both lipid peroxidation and 8-OHdG (8-hydroxydeoxyguanosine, a biomarker for oxidative damage) were positively correlated with BMI, blood pressure, waist/hip circumference and C-reactive protein [49]. Increased amounts of 8-OHdG in diabetic and pre-diabetic patients were also positively correlated with BMI [50]. Similarly, high levels of 8-OHdG were detected in the skeletal muscle of overweight patients, suggesting obesity as a contributing factor to increased oxidative stress [51]. In addition, a recent study suggested that DNA damage caused by ROS occurs more frequently in morbidly obese patients than in age-matched, healthy non-obese controls [48].

Herein, we did not find an interference of metabolic syndrome (MS) on the amount of DNA damage in morbidly obese women. Differently, it has been reported increased DNA damage and decreased total antioxidant capacity in normal-weight patients with MS compared to those without MS [52]. The authors suggest this might occur because of the increase in the imbalance between the production of oxidants and antioxidant defenses in subjects with MS. Perhaps, in our study the ROS generated due to the inflammatory status in obesity has masked the effect of MS, i.e., an additional factor (MS), which can potentially stimulates oxidative stress might not further increase damage. Similar findings were previously reported [53] indicating that contribution of MS components to the oxidative stress burden generated by high blood pressure is minimal in patients with essential hypertension. Recently, no increase of DNA damage, as depicted by the three versions (alkaline, neutral and hOGG1-modified) of the comet assay, was also detected in subjects with MS compared to health individuals (without MS) [54].

By investigating whether some gene variants were positively or negatively associated with levels of primary DNA damage, we demonstrated that for all the genotypes, the obese women always showed higher amounts of damage than the control women. In fact, overweight individuals with increased BMIs have an increased risk of DNA damage due to oxidative stress [55]. Furthermore, our data also showed that some gene polymorphisms were associated with high levels of damage in both obese and control populations. However, exceptions occurred for the *GHRL-CC* and *LEP-GG* genotypes, which were associated with increased levels of oxidized purines and pyrimidines, respectively, though only in obese women. In contrast, these two genotypes were related to less DNA damage in eutrophic women. The literature does not contain enough data to confirm or explain possible associations between *GHRL* and genotoxicity. It has been reported that the *GHRL* products are closely associated with chronic systemic inflammation [22,28] and that C allele carriers show higher ghrelin concentrations [44]. Therefore, it is possible that obese women with the *GHRL-CC* genotype generate more ROS able to reach and damage DNA. Similarly, although our findings did not show any association between *GHSR* variants and body weight or BMI, obese women with the GG genotype exhibited increased levels of oxidized DNA purines and pyrimidines. The 477G > A (rs572169) *GHSR* variant is located in the coding region of the gene; therefore, it can cause changes of the corresponding amino acid, leading to structural alterations in the ghrelin receptors and, consequently, affecting their interactions with ligands [44,56]. The increased level of oxidized DNA damage detected in obese *LEP-GG* women might also be due to the amount of ROS generated. A previous study demonstrated that a SNP in the promoter region (-2548G > A) of *LEP* is associated with increased levels of leptin in GG obese girls [23]. Because leptin is a proinflammatory cytokine, its increased availability can stimulate inflammatory processes and, consequently, ROS generation [57]. Indeed, leptin has been linked to increased oxidative stress because circulating leptin is associated with increased levels of oxidized low-density lipoprotein (LDL), independent of BMI and insulin resistance [58]. Furthermore, leptin increases ROS production in endothelial cells, which have important roles in the initiation and maintenance of inflammatory processes and tissue injuries. Moreover, hyperleptinemia-related oxidative stress seemed to be involved in the excessive obesity-related inflammatory response [59].

We observed remarkable differences in the amount of genetic damage in obese women, but not in the controls, depending on the *FTO* genotype, with increased damage (i.e., strand breaks and oxidized purines and pyrimidines) being related to the presence of allele A (*AT* and *AA*). The literature shows that the *FTO-AA* variant may lead to increased circulating C-reactive protein levels and the inflammatory state in adipose tissue, with a consequent increase in systemic inflammation [60].

In summary, our results demonstrated that the frequencies of the *GHRL* (-501A > C, rs26802), *GHSR* (477G > A, rs572169), *LEP* (-2548G > A, rs7799039), *LEPR* (668A > G, rs1137101) and *FTO* (rs9939609) polymorphisms were not different between Brazilian morbidly obese women and eutrophic women. Additionally, our data showed more DNA strand breaks and oxidative DNA lesions in PBMCs of obese women than in those of eutrophic women, independent of the genotype. In general, some gene variants were related to increased DNA damage in both obese and control subjects. Exceptions were the *GHRL-CC* and *LEP-GG* genotypes, which were associated with large amounts of DNA damage only in obese women. Furthermore, remarkable differences in the amounts of genetic lesions associated with *FTO* variants were observed only in obese women. In conclusion, this study demonstrated that the SNPs analyzed were not closely associated with morbid obesity, suggesting they are not major contributors to obesity. Therefore, our data indicated that these gene variants are not good biomarkers for predicting risk susceptibility for obesity, whereas ROS generated by the inflammatory status seemed to be the main cause of DNA damage in obese women, favoring genetically related diseases as obesity comorbidities.

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Conflict of Interest

The authors declare that there are no conflicts of interest, except for Irineu Rasera Junior, who declares he is a medical consultant for Johnson & Johnson in Brazil.

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