Multilocus Analyses of Seven Candidate Genes Suggest Interacting Pathways for Obesity-Related Traits in Brazilian Populations

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We investigated whether variants in major candidate genes for food intake and body weight regulation contribute to obesity-related traits under a multilocus perspective. We studied 375 Brazilian subjects from partially isolated African-derived populations (quilombos). Seven variants displaying conflicting results in previous reports and supposedly implicated in the susceptibility of obesity-related phenotypes were investigated: β_2-adrenergic receptor (*ADRB2*) (Arg16Gly), insulin induced gene 2 (*INSIG2*) (rs7566605), leptin (*LEP*) (A19G), LEP receptor (*LEPR*) (Gln223Arg), perilipin (*PLIN*) (6209T > C), peroxisome proliferator–activated receptor-γ (*PPARG*) (Pro12Ala), and resistin (*RETN*) (–420C > G). Regression models as well as generalized multifactor dimensionality reduction (GMDR) were employed to test the contribution of individual effects and higher-order interactions to BMI and waist-hip ratio (WHR) variation and risk of overweight/obesity. The best multilocus association signal identified in the quilombos was further examined in an independent sample of 334 Brazilian subjects of European ancestry. In quilombos, only the PPARG polymorphism displayed significant individual effects (WHR variation, $P = 0.028$). No association was observed either with the risk of overweight/obesity (BMI ≥ 25 kg/m^2), risk of obesity alone (BMI ≥ 30 kg/m^2) or BMI variation. However, GMDR analyses revealed an interaction between the *LEPR* and *ADRB2* polymorphisms ($P = 0.009$) as well as a third-order effect involving the latter two variants plus *INSIG2* ($P = 0.034$) with overweight/obesity. Assessment of the *LEPR–ADRB2* interaction in the second sample indicated a marginally significant association ($P = 0.0724$), which was further verified to be limited to men ($P = 0.0118$). Together, our findings suggest evidence for a two-locus interaction between the *LEPR* Gln223Arg and *ADRB2* Arg16Gly variants in the risk of overweight/obesity, and highlight further the importance of multilocus effects in the genetic component of obesity.

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INTRODUCTION

Compelling evidence demonstrate that both nonsyndromic obesity and other related traits have a significant genetic component (1) and that these phenotypes are the result of an interplay between the genetic background and environmental factors (2).

It is often postulated that a polygenic model encompassing mainly the joint effect of individual common variants is the most plausible model of inheritance for several obesity-related disorders (3). However, supportive evidence has been difficult to obtain in practice. In fact, recent insights from large-scale discovery for obesity-causing genes indicate that, when considered individually, only a small number of candidate polymorphisms has been consistently replicated, and even fewer variants achieve sufficient credibility thresholds across two or more populations (3). While this has been primarily attributed to a low statistical power (4), limited genome coverage, and presence of biases that ultimately lead to between-study heterogeneity (5), the low yield of robust associations for obesity-associated markers may be originated by a lack of rigorous (often neglected) testing for synergistic effects among investigated polymorphisms. Indeed, theoretical considerations support the notion that most examined markers may not demonstrate effects individually (i.e., absence of marginal effects or minuscule effect sizes) (6).

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In this regard, the contrast of each candidate polymorphism against the background of other gene variants has been suggested as a crucial strategy to augment the success of detection of truly disease-predisposing markers (6).

Following this rationale, we evaluated both single locus and multilocus effects of polymorphisms in seven major candidate genes previously associated with obesity-related traits (7–15) in a partially genetically isolated African-derived population, and investigated the consistency of the strongest association signal from this study in an independent sample of Brazilian subjects of European ancestry. The variants examined in the present study comprise single-nucleotide polymorphisms in genes involved in the (i) cathecolaminergic pathway: β_2-adrenergic receptor (*ADRB2*, chromosome 5q31-q32) Arg16Gly; (ii) leptin (LEP) signaling pathway: *LEP* (chromosome 7q31.3) A19G and LEP receptor (*LEPR*, chromosome 1p31) Gln223Arg; (iii) insulin signaling pathway: peroxisome proliferator–activated receptor-γ2 (*PPARG2*, chromosome 3p25) Pro12Ala, and resistin (*RETN*, chromosome 19p13.2) −420C > G; and (iv) oxidative and lipid/sterol synthetic pathways: perilipin (*PLIN*, chromosome15q26) 6209T > C and insulin induced gene 2 (*INSIG2*) rs7566605 (chromosome 2q14.2). These variants have been extensively studied over the past years and the biological mechanism by which they might be associated with obesity is discussed in detail elsewhere (7–17).

The rationale behind the choice of these variants is based on the fact that they represent examples of polymorphisms in which the cumulative evidence suggest either lack of effect or conflicting (i.e., heterogeneous) effects across populations. Our hypothesis was that these variants may not display individual effects, but might interact with each other in order to modulate the variance of obesity-related traits.

The first sample of this study encompasses subjects ascertained from peculiar African-derived Brazilian communities. The Brazilian population comprises an admixture of several ethnic groups, including an important Portuguese contribution through colonization, a Native American genetic pool and an African contribution through slavery, and their historical interbreeding. Before and after the abolition of slavery in Brazil in 1888, many communities named quilombos were founded by fled or abandoned African slaves, presently referred to as quilombo remnants. It is estimated that there are thousands of such communities in the Brazilian territory. They can be regarded as relics of the original African genetic contribution to the Brazilian population. Our previous studies on genetic admixture in some of these populations revealed that, besides a prominent African contribution, Europeans and Amerindians also took part in their genetic constitution (18). As partially genetically isolated populations and in a homogeneous rural lifestyle, they represent an interesting subject group to the study of multifactorial diseases. Interestingly, recent changes in their lifestyle, such as a decrease in the intensity of agricultural activities and nutrition transition, are associated with a larger frequency of common diseases such as essential hypertension and obesity.

In the present investigation, we demonstrate evidence for a synergistic effect between the *ADRB2* Arg16Gly and *LEPR* Gln223Arg variants in the risk of overweight plus obesity. By using a combination of logistic regression–based likelihood-ratio tests and dimensionality reduction techniques, we show that this interaction signal initially observed in the quilombo sample is further supported by a re-analysis of an independent sample with remarkable differences in terms of ancestry and environmental factors.

METHODS AND PROCEDURES

Subjects and phenotyping

Quilombo sample. The first study sample consisted of nondiabetic subjects of African ancestry cross-sectionally selected from 10 quilombo populations (Abobral, Galvão, São Pedro, Pedro Cubas, Pilões, Maria Rosa, André Lopes, Nhunguara, Sapatu, and Ivaporunduva) located in the Vale do Ribeira, São Paulo State (Southern Brazil). Clinical evaluation occurred at multiple visits performed between 2003 and 2008. The estimated total number of individuals in these populations is 2,641. From a total of 810 clinically examined individuals aged over 17 years, we selected a sample of 375 randomly unrelated subjects. Further demographic and geographic information on the source populations can be found elsewhere (18,19).

General European-derived sample. Data from a second study population were retrospectively re-analyzed from previous investigations (13,14). This sample is part of a cross-sectional study of individuals of European ancestry from Porto Alegre city (Rio Grande do Sul State). Briefly, nondiabetic subjects (aged ≥15 years) descendants of German, Italian, and Portuguese immigrants were selected. Full detail on subjects' ancestry as well as additional information on demographic characteristics of the studied individuals can be found elsewhere (12–15).

Anthropometrical measurements. Anthropometric measurements including height, weight and waist and hip circumferences were accomplished using standard techniques: waist circumference measured at the natural waist (defined as the minimal circumference between the umbilicus and the xiphoid process) and hip circumference measured at the level of the greater trochanters. This study was approved by the ethics committee of the Instituto de Biociências da Universidade de São Paulo. Informed consent was obtained from all participants or their guardians.

Genotyping determination

DNA was extracted from whole blood using standard procedures. Genotypes for the variants in the *LEP*, *LEPR*, *ADRB2* and *PPARG2* were determined by polymerase chain reaction followed by restriction fragment-length polymorphism analyses as described previously (16,17,20,21). For markers in *PLIN*, *RETN* and *INSIG2* genotyping was carried out in a Mega BACE TM 1000 analyzer (Amersham Biosciences, Buckinghamshire, UK), using the *MegaBace SnuPe Genotyping Kit* (Amersham Biosciences). The *Snupe Primers* used for the automatic genotyping were: *PLIN* CTGTGGGAGGGAAGGTGAGC; *RETN* CCAGTCTCTGGACATGAAGA and *INSIG2* CTTAACAAT GGATATTTGAT. Further information on genotyping determinations are available upon request. Positive (previously tested samples with known genotype) and negative (buffer only) controls were included in each batch as a quality control measure. Genotyping was performed blinded to clinical status.

Statistical analyses

Data were expressed as means ± s.d. or counts (percentage) when appropriate. Deviations from the Hardy–Weinberg equilibrium were tested by an exact approach. One-way analysis of variance followed by the Scheffe's procedure was used to detect differences in continuous variables among and between groups of genotypes, respectively. Because the putative genetic model of action of each examined marker is unknown, genotype frequencies between overweight plus obesity

(BMI ≥ 25 kg/m^2) and lean (BMI < 25 kg/m^2) subjects were first compared by means of the max-statistic, which selects the largest test statistic from the dominant, recessive and multiplicative models (22). A similar approach was applied to the waist-to-hip ratio (WHR), in which subjects were classified as cases when WHR ≥ 0.81 and WHR ≥ 0.96 for female and male subjects, respectively, or controls otherwise. The max-statistic null distribution was obtained by multiple integrations (22). Next, multiple logistic regression and multiple linear regression models assuming additive models were also fit to assess the contribution of each genotype to the risk of overweight plus obesity, obesity, BMI and WHR, while adjusting for age, gender, and physical activity. To examine potential gene–gene interactions, we applied the generalized multifactor dimensionality reduction (GMDR) approach, a MDR-derived data mining technique discussed in detail elsewhere (23). Similarly to the standard MDR (24), the GMDR enables the evaluation of a *n*-dimensional space formed by *k* markers, which is ultimately reduced into a single dimension with two levels: combinations of genotypes of high and low risk, respectively. However, under the GMDR framework the cases-to-controls ratio is replaced by cumulative maximum-likelihood–derived scores. This technique is applicable to both continuous and binary phenotypes, and permits the adjustment for confounding variables (23). We used tenfold leave-one-out cross-validation and an exhaustive search of all possible one- to four-loci interactions only to avoid model overfitting. Permutation was employed to obtain empirical *P* values of prediction error–based on 5,000 shuffles. We further explored departures from additive effects for two-locus models with suggestive evidence of interaction from GMDR analyses by using multiple logistic regression–based likelihood-ratio tests. Briefly, for each possible two-locus combination a full model including age and gender, additive plus dominance as well as interaction terms (model 1: interaction model) was fit. Next, a nested model (model 0: null) with main effects only (i.e., without interaction) as well as age and gender was constructed.

The hypothesis of gene–gene interactions was tested by means of a likelihood-ratio test that assumes the following form: $\Delta = -2(l_0 - l_1)$, where l_1 and l_0 are the natural log-likelihoods under the model with and without interaction, respectively. We used permutation to test whether the full model fits the data significantly better than the noninteraction model by comparing the observed test statistic Δ to those obtained in 5,000 randomly generated shuffles. Data analysis was performed using the R package (version 2.81, http://www.r-project.org/) and GMDR (version 0.7, http://sourceforge.net/projects/gmdr/). Statistical significance was set at the 5% level (one-tailed for GMDR-based models, and two-tailed for the remaining tests).

RESULTS

Sample characteristics

The demographic and clinical characteristics of the *quilombo* sample are shown in **Table 1**. Assuming a BMI ≥ 25 kg/m^2 the overall prevalence of overweight plus obesity was 40.2% in the *quilombos*, an estimate that is comparable to findings from recent epidemiological investigations in Brazilian populations (25). In this sample, obesity defined as a BMI ≥ 30 kg/m^2 was observed in 8.7% of the studied subjects. In both phenotypes, the odds for women was significantly higher than the odds for men; odds ratio (exact 95% confidence intervals (CI)) for women vs. men equal to 3.87 (2.40–6.290) and 6.88 (2.31–27.5) for overweight/obesity and obesity alone, respectively. In the second studied sample of European ancestry (median age of 39 years, interquartile range from 29 to 49 years; 54.2% women), the prevalence of overweight plus obesity was 55%, whereas obesity was observed in 18% of the subjects. No differences in the prevalence of overweight plus obesity (51% in women and 59% in men) or obesity only (17% in women and 19% in men) were observed between genders.

Single locus associations

Table 2 summarizes the distribution of genotype and allele frequencies for the seven examined polymorphisms studied in the *quilombo* population. Overall, univariate single locus analyses did not reveal any significant association between the examined variants and BMI or WHR as continuous variables. Likewise, the distribution of seven polymorphisms was found to be similar between overweight and lean subjects (**Table 2**). When analyses considered a more extreme phenotype (e.g., BMI ≥ 30 kg/m^2 vs. BMI < 30 kg/m^2), there was still no association (data not shown). In a multiple linear regression model, gender (β = −0.082, 95% CI: −0.095 to −0.069, $P < 0.001$), age (β = 0.001, 95% CI: 0.0006 to 0.014, $P < 0.001$) and the *PPARG2* Pro12Ala polymorphism (β = 0.015, 95% CI: 0.0016 to 0.028, $P = 0.028$) proved to be the only significant predictors for WHR values. No variant showed significant effects either on waist or hip circumference.

Multilocus associations: evidence for synergistic effects between *LEPR* and *ADRB2*

In the sample from *quilombos*, GMDR-based predictive models for the risk of overweight plus obesity (defined as a BMI ≥ 25 kg/m^2) adjusted for age and gender suggested a statistically significant two-locus interaction between *LEPR* and *ADRB2* as well as a three-locus interaction that involved *LEPR*, *ADRB2*

Table 1 Demographic characteristics of the *quilombo* sample

	Women		Men		All	
	N	Mean ± s.d.	*N*	Mean ± s.d.	*N*	Mean ± s.d.
Age (years)	197	43.65 ± 18.06	178	44.78 ± 17.00	375	44.18 ± 17.55
Weight (Kg)	197	60.50 ± 11.61	178	64.53 ± 9.78	375	62.41 ± 10.95
Height (m)	197	1.54 ± 0.06	178	1.66 ± 0.07	375	1.60 ± 0.09
BMI (Kg/m^2)	197	25.41 ± 4.59	178	23.36 ± 2.95	375	24.43 ± 4.02
WC (cm)	164	84.37 ± 10.83	143	81.63 ± 7.43	307	83.09 ± 9.48
HC (cm)	162	97.33 ± 9.69	144	85.94 ± 7.38	306	91.97 ± 10.37
WHR	162	0.87 ± 0.06	143	0.95 ± 0.05	305	0.91 ± 0.07

HC, hip circumference; WC, waist circumference; WHR, waist-hip ratio.

Table 2 Genotype frequencies for the seven studied polymorphisms in overweight/obese and lean *quilombo* subjects

Gene/status	Genotype, *n* (%)			*P* HWE	MAF	*P* MAX[a]
LEP (A19G)	G/G	G/A	A/A			
BMI ≥ 25	64 (44.4)	62 (43)	18 (13)	0.70	0.34	
BMI < 25	104 (47.3)	90 (41)	26 (12)	0.36	0.32	0.83
LEPR (Gln223Arg)	Arg/Arg	Arg/Gln	Gln/Gln	0.29	0.37	0.77
BMI ≥ 25	17 (12)	75 (51)	54 (37)	0.89	0.37	
BMI < 25	31 (14)	102 (46)	90 (40)			
ADRB2 (Arg16Gly)	Gly/Gly	Arg/Gly	Arg/Arg	0.24	0.46	0.55
BMI ≥ 25	37 (26)	77 (55)	26 (19)	0.79	0.45	
BMI < 25	69 (31)	107 (48)	45 (20)			
PPARG2 (Pro12Ala)	Pro/Pro	Ala/Pro	Ala/Ala	0.64	0.10	0.24
BMI ≥ 25	113 (81)	25 (18)	2 (1)	0.002	0.12	
BMI < 25	173 (79)	36 (17)	9 (4)			
PLIN1 (6209T > C)	C/C	T/C	T/T	0.24	0.42	0.88
BMI ≥ 25	52 (36)	64 (44)	30 (21)	0.02	0.43	
BMI < 25	82 (36)	94 (41)	51 (22)			
RETN (–420C > G)	C/C	G/C	G/G	0.99	0.33	0.79
BMI ≥ 25	64 (45)	64 (45)	15 (10)	0.44	0.32	
BMI < 25	107 (48)	91 (41)	25 (11)			
INSIG2 (rs7566605)	G/G	C/G	C/C	0.48	0.14	0.07
BMI ≥ 25	107 (75)	32 (22)	4 (3)	0.83	0.20	
BMI < 25	140 (64)	69 (32)	9 (4)			

ADRB2, β_2-adrenergic receptor; HWE, Hardy–Weinberg equilibrium; *INSIG2*, insulin induced gene 2; *LEP*, leptin; *LEPR*, leptin receptor; MAF, minor allele frequency; MAX, max-statistic; *PLIN*, perilipin; *PPARG2*, peroxisome proliferator–activated receptor-γ2; *RETN*, resistin.
[a]*P* values for the max-statistic.

Table 3 Best predictive models from GMDR analyses

Population	*N*	Model	Training balanced accuracy (%)	Testing balanced accuracy (%)	Cross-validation consistency	*P*[a]
Quilombo (all)	331	*LEPR, ADRB2*	60.4	59.7	10/10	0.009
		LEPR, ADRB2, INSIG2	64.7	55.1	8/10	0.034
European-derived (all)	334	*LEPR, ADRB2*	58.2	55.4	—	0.072
European-derived (men)	153	*LEPR, ADRB2*	67.5	61.8	—	0.012
European-derived (women)	181	*LEPR, ADRB2*	64.3	58.3	—	0.052

ADRB2, β_2-adrenergic receptor; INSIG2, insulin induced gene 2; LEPR, leptin receptor.
[a]Based on 5,000 permutations. Results are adjusted for age and gender or age only when appropriated.

and *INSIG2* (Table 3). Interestingly, no statistically significant interaction was found for the risk overweight/obesity defined according to the WHR or for the remaining obesity-related traits as quantitative variables (data not shown). Figure 1a depicts the best-identified model (*LEPR–ADRB2*) for the risk of overweight plus obesity in the *quilombo* sample, defined as the model with the smallest number of attributes, and the highest consistency and testing accuracy.

Next, we sought to corroborate the results from GMDR-based models (nonparametric) with analyses based on logistic regression, a parametric approach. To this end, we used multiple logistic regression models followed by likelihood-ratio tests to infer departures from additive effects. While the null model assumes no interaction, the full model assumes presence of synergistic effects beyond what would be expected if both variants in *LEPR* and *ADRB2* acted independently. The log-likelihood for the null model was $l_0 = -220.28$, whereas the log-likelihood for the model with interaction was $l_1 = -213.06$. Minus twice the log-likelihood difference was used to construct a statistic to test the hypothesis of synergistic effects. Using 5,000 permutations, we noted that differences equal or larger than the one observed between the two models ($\Delta = -2(l_0 - l_1) = -2[-220.28 - (-213.06)] = 14.43$) have a small probability to occur by chance ($P = 0.0068$), indicating that the model with interaction fits the data significantly better than the one without interaction. Similar results were obtained when subjects were stratified by gender ($P = 0.0122$ and $P = 0.0462$ for men and women, respectively). Given our relatively small sample size, logistic regression was not practical for modeling the third-order interaction (*LEPR-ADRB2-INSIG2*) due to overfitting, collinearity, and presence empty or nearly empty genotype combinations that ultimately lead to poor parameter estimates (data not shown).

Our next goal was to investigate the *LEPR–ADBR2* interaction signal in an independent sample composed by 334 individuals of European-derived ancestry. The distribution of the

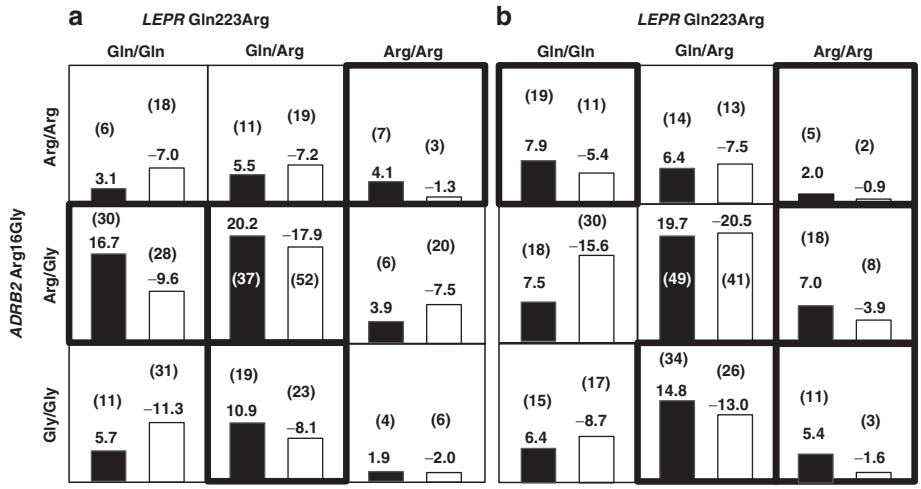

Figure 1 Best predictive models from generalized multifactor dimensionality reduction analyses. Results are adjusted for age and gender or age only when appropriated. Results for (**a**) the quilombo sample are based on 331 subjects (overweight plus obesity to lean subjects ratio equal to 0.65), whereas results for (**b**) the European-derived sample are based on 334 subjects (overweight plus obesity to lean subjects ratio equal to 1.21). Thick-line boxes correspond to genotype categories classified as high-risk, whereas thin-line boxes represent low-risk combinations. Black and white bars within each box are proportional to the sum of scores in overweight/obese and lean subjects, respectively. Values in parentheses indicate the number of individuals in each of these categories. Balanced accuracy is a function used here for multilocus modeling due to the cross-sectional nature of the investigated samples (i.e., imbalanced data sets), and is defined as (specificity + specificity)/2. *ADRB2*, β_2-adrenergic receptor; *LEPR*, leptin receptor.

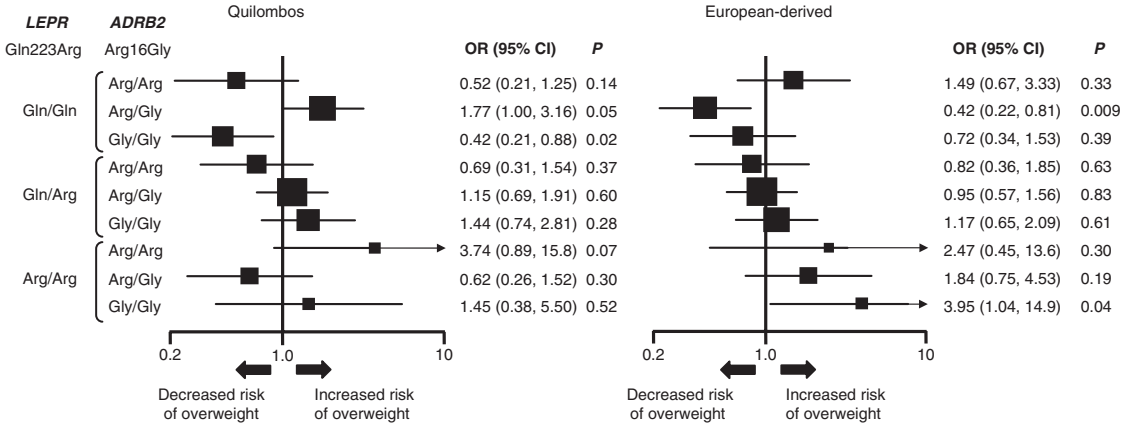

Figure 2 Forest plots depicting estimated odds ratios (ORs) for each genotype combination formed by the leptin receptor (*LEPR*) (Gln223Arg) and β_2-adrenergic receptor (*ADRB2*) (Arg16Gly) variants. ORs are calculated using a multiple regression model adjusted for age and gender. The OR for each category was calculated in relation to all of the remaining genotype combinations. Square sizes are proportional to the inverse of the within-group variance (e.g., larger squares are given for comparisons with more precision). Results are depicted in a logarithm scale. CI, confidence intervals.

LEPR GlnGln, GlnArg and ArgArg genotypes were 52 (28%), 97 (53%), and 34 (19%), respectively, among overweight/obese subjects (with a minor allele frequency equal to 0.45), and 58 (38%), 80 (53%), and 13 (9%), respectively, among lean individuals (minor allele frequency = 0.35). For the *ADRB2* ArgArg, ArgGly and GlyGly genotypes, the distribution were 38 (21%), 85 (46%), and 60 (33%), respectively, among subjects with a BMI ≥ 25 kg/m^2 (minor allele frequency = 0.56), whereas in lean individuals the correspondent distribution were 26 (17%), 79 (52%), and 46 (30%), respectively (minor allele frequency = 0.57). Based on this second data set, we were able to marginally replicate this two-locus effect for the risk of overweight plus obesity (BMI ≥ 25 kg/m^2) in a GMDR model adjusted for age and gender ($P = 0.0724$). Further analyses considering GMDR predictions stratified by gender revealed a stronger association signal in men ($P = 0.0118$) compared to women ($P = 0.0516$). **Figure 1b** summarizes the two-locus *LEPR* and *ADRB2* genotype combinations in this independent validation sample.

Surprisingly, parametric results from multiple logistic regression–based likelihood-ratio tests did not furnish evidence for departures of multiplicative effect in the European-derived sample as whole: the log-likelihood for the null model was $l_0 = -212.81$ and the log-likelihood for the model with interaction was $l_1 = -210.84$, yielding a $P = 0.436$ after 5,000 permutations. Nevertheless, an in-depth analysis indicated that the synergistic effect was largely confined to men ($P = 0.0022$),

but not to women (P = 0.7852), corroborating results from GMDR-based analyses. **Figure 2** depicts effect sizes adjusted for age and gender for each genotype combination vs. all of the remaining categories in both studied samples.

DISCUSSION

Main findings

Our study serves as an example of the potential benefit of considering gene–gene interactions in genetic association studies of obesity-related traits. Specifically, by using a combination of parametric and nonparametric approaches, we provide evidence for a synergistic effect between the *ADRB2* Ar16Gly variant and the *LEPR* Gln223Arg polymorphism on the risk of obesity in Brazilian populations. Our data also indicate that this potential interaction might be further modulated by gender effects.

Potential mechanism of action

To the best of our knowledge, no previous reports on synergistic effects between *ADRB2* and *LEPR* variants on obesity-related traits are available. However, both investigated *ADRB2* and *LEPR* variants poses appealing biological bases for being associated with adiposity. Indeed, the LEP-signaling pathway is a ubiquitous route not only for the regulation of energy homeostasis, but also for the human metabolism. This pathway includes the hormone LEP, which is released from the adipose tissue and exerts its effects in the central nervous system. In the brain, LEP provides information on body energy stores as well as nutritional status, reducing food intake. Both metabolic and endocrine effects exerted by the LEP depend on a tight interaction between its receptor, named the LEPR, a member of the class I cytokine receptor family (2,3,7,8).

On the other hand, in the cathecolaminergic pathway, cathecolamines such as norepinephrine and epinephrine regulate energy expenditure by stimulating the ADRB2 which are considered the major lipolytic receptors in human fat cells (2,3,9). Overall, our findings provide evidence that effectors from different pathways might interact to augment the risk of obesity, emphasizing the concept of interacting pathways of susceptibility for obesity-related traits.

A potential mechanism underlying the *ADRB2–LEPR* interaction may be a complex interplay among gender, energy expenditure, and food intake. From **Figure 2**, it can be noticed as a trend in the interaction effects. Genotype combinations with fewer risk-alleles display somewhat smaller odds of obesity compared to genotype combinations with a higher number of risk-alleles. These observations might indicate that detrimental effects from one variant (for instance, the *LEPR* 223Arg allele leading to impaired satiety signals that ultimately promote altered feeding behaviors) might be annulled/neutralized by a higher energy expenditure (for example, an augmented susceptibility to a faster breakdown of fat in adipose tissue caused by the *ADRB2* Arg16 allele). As discussed below, these highly speculative mechanisms need to be interpreted in light of several additional confounding factors, such as sampling error, linkage disequilibrium, and putative (yet unknown) gene–nutrient interactions, but provide interesting glimpses for further investigations.

Might the heterogeneity in reported effects across studies be a result of gene–gene interactions?

Except for a small main effect of the *PPARG2* (Pro12Ala) polymorphism on BMI as a continuous trait, our data did not disclose any statistically significant association with obesity-related traits when each marker was considered individually. These observations are consistent with meta-analysis–based summary estimates for the *ADRB2* (Arg16Gly), *LEPR* (Gln223Arg) and *LEP* (A19G) variants that suggest a lack of association between these markers and obesity-related phenotypes (7–9). The credibility of the cumulative evidence (26) for these associations ranges from weak to modest, whereas for both *PLIN* (6209T > C) and *RETN* (−420C > G) variants the unavailability of systematic analyses precludes convincing statements on their correspondent associations. On the other hand, the cumulative epidemiological credibility for the role of both *PPARG2* (Pro12Ala) and *INSIG2* (rs7566605) polymorphisms in the susceptibility for obesity-related traits is strong, but the totality of the evidence suggest statistical heterogeneity (10,11).

Statistical heterogeneity (a scenario also known as between-study heterogeneity) is a common finding in several genetic settings (27–29). It is defined as a situation in which the variability in the observed effects is larger than what would be expected by chance (i.e., sampling error alone) (30). When sufficiently large, between-study heterogeneity may annul genuine genetic effects (i.e., the meta-analysis–derived summary effect loses its nominal statistical significance) (4), or may induce the appearance of *flip-flops* associations (31), that is, the emergence of small protective effects in some populations and susceptibility effects in others (4). For example, a recent meta-analysis indicated that the *INSIG2* (rs7566605) variant (under a recessive model of inheritance) may be a predisposing factor for obesity in general population-based studies (n = 15, fixed-effects model summary odds ratio = 1.10 (95% CI: 1.02–1.18). On the other hand, this same marker may be protective factor for obesity in subjects ascertained from apparently health populations: fixed-effects model summary odds ratio = 0.80 (95% CI: 0.65–0.98) (11).

Potential sources for between-study heterogeneity include differential nongenetic environmental factors across populations (30), linkage disequilibrium between the typed variant and the causal one (31), phenotype misclassification, and any sort of possible factors (e.g., study design (11)) that may lead to differential nonrandom fluctuations in the point estimates across studies (4). Here, we speculate that at least part of the inconsistency among study results may also be originated by the presence of gene–gene interactions. Indeed, interacting variants in the background may modulate individual marker effects, diminishing or increasing marginal associations of the studied polymorphisms (32). As a result, our observations provide additional grounds for the feasibility and the importance to revisit earlier single locus associations that yielded statistical heterogeneity and/or suggested lack of relevance of the *ADRB2*

(Arg16Gly) and *LEPR* (Gln223Arg) variants in obesity-related phenotypes.

Strengths and limitations

Our study has several strengths that should be acknowledged. First, the *quilombos* sample has primarily an African ancestry. African-derived populations have received little attention in previous (33) and recent association studies (34), whereas data on the contribution of candidate polymorphisms to BMI variation in populations of African descent are largely sparse (9,11,35). Second, because we observed the same interaction signals in two samples with noticeable differences in both genetic and environmental backgrounds, our results provide a credible association signal for further replication (36). Third, it is worth mentioning that investigations in isolated populations hold the promise to reduce the signal-to-noise ratio (i.e., sharing of a more homogeneous environmental component), and have gained strong theoretical (37) and empirical (38) support in recent years. Since the *quilombo* sample analyzed here was ascertained from a partially isolated, population with peculiar genetic (i.e., African-derived populations with inbreeding) and environmental characteristics (i.e., nonurbanized, rural communities), the present study strengthens further the feasibility of this strategy.

However, our study also possesses a number of limitations that must be discussed. First, although we have investigated the INSIG2 rs7566605 polymorphism, our study did not cover other recent genome-wide derived markers that have been consistently associated with adiposity across studies. While the lack of analysis of recent genome-wide "top hits" variants may be a limitation of our study, we believe that the choice of the seven variants investigated here does not invalidate our main findings: (i) a potential statistical interaction between the *LEPR* Gln223Arg and *ADRB2* Arg16Gly variants in the risk of overweight/obesity and (ii) the importance of considering gene–gene interactions in studies investigating the genetic component of obesity. Larger investigations covering a wide range of markers obtained by genome-wide studies are warranted, and are likely to expound a more complex view of the role of the epistasis in obesity-related phenotypes. Second, our samples are underpowered to detect typical main effects of genetic variants associated with common traits. With our sample size and at an $\alpha = 5\%$, simulation-based statistical power estimates ranged from 38 to 55% depending on the genetic effect (e.g., odds ratio = 1.3), minor allelic frequency (10 to 45%) and true underlying genetic model. Thus, our results neither rule out putative small effect sizes of the investigated markers when considered individually nor invalidate the hypothesis of epistasis among the remaining studied polymorphisms. Third, we did not attempt to replicate the second GMDR-derived multilocus effect observed in the *quilombo* sample (*ADRB2-LEPR-INSIG2*). This was illustrated by the difficulty we found during the modeling of this third-order interaction through logistic models (i.e., only GMDR was practical), indicating that much larger samples would be required to replicate that signal with confidence. This apparent advantage of model-free dimensionality reduction techniques over parametric approaches may have broader implications for the design and interpretation of future association studies aimed to replicate similar interaction signals. Importantly, GMDR covers not only single but also interactive effects. As a result, both logistic- and GMDR-based tests are not identical and a connection between their results is not obvious. However, both approaches rendered comparable results, and their results can be viewed as complementary.

Fourth, it is important to take into account the difference between biological and statistical epistasis before interpreting our findings. The methods applied here are focused on the detection of statistical interactions and not biological interactions. The former may or may not be a result of the latter, and their relation is not clear-cut (6). In addition, the biological interpretation of statistical interactions may be further complicated when the genotyped variant is a surrogate for the causal variant and there is incomplete linkage disequilibrium between them. This is particularly an important issue in our study given the quite distinct levels and patterns of linkage disequilibrium between African and European-derived populations (39). Furthermore, the presence of genetic heterogeneity in the *LEPR* and/or *ADRB2* loci (e.g., distinct variants within the same gene that exert genuine effects on the risk of obesity) could be also an important factor influencing the consistency between results from different samples (1). Also, for both uncommon and rare genotype combinations, the role of sampling error in the magnitude of effects as well as stability of the dimensional reduction across populations may be substantial in scenarios of small sample sizes. These limitations along with additional unidentified interacting loci might explain why the magnitude and direction of effects within each genotypic composite may invariably not be identical across studies. As a result, conclusions on whether or not our observed statistical interactions reflect truly biological interactions may be premature without compelling experimental data. Further additional studies with larger sample sizes will be crucial to provide a more harmonious perspective on whether certain *LEPR* (Gln223ARg)–*ADRB2* (Arg16Gly) composites have higher or lower risk than other genotype combinations. Finally, our inferences from the European-derived sample are drawn based on a re-analysis of data from previous studies. This sample has been used earlier in separate investigations suggesting small, but independent susceptibility effects of both *LEPR* and *ADRB2* variants on obesity-related phenotypes (13,14). Although simulation studies indicate that the power to detect multilocus effects may be improved by searching first markers with small marginal effects (40), which are then examined for a significant interaction model, it remains an open question as to whether this retrospective analysis may have biased our findings. We need more empirical evidence on how much bias we can introduce by revisiting earlier association studies that showed no or small individual marker effects only, but in which no clearly defined hypothesis about epistasis was previously tested.

Perspectives for future research

Our results emphasize the importance of multilocus analysis in obesity-related traits, providing further support for a polygenic

etiology of body weight regulation in Brazilian populations. Because recent studies demonstrate that replication of association signals arising from exhaustive searches for multilocus effects is as essential as the replication of main effects (36), prospective planned attempts to confirm these results taking into account potential gender-specific interactions are warranted. If replicated, our findings may represent an important further step toward a clearer understanding of the genetic basis of obesity-related traits.

DISCLOSURE

The authors declared no conflict of interest.

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