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Original Article

FTO gene is related to obesity in Chilean Amerindian children and impairs HOMA-IR in prepubertal girls

Riffo B, Asenjo S, Sáez K, Aguayo C, Muñoz I, Bustos P, Celis-Morales CA, Lagos J, Sapunar J, Ulloa N. FTO gene is related to obesity in Chilean Amerindian children and impairs HOMA-IR in prepubertal girls. Pediatric Diabetes 2012: 13: 384–391.

Objective: The objective of this study was to investigate the allelic frequency of the fat mass and obesity-associated (FTO) gene (rs9939609) and its influences on obesity and metabolic risk biomarkers in a cohort of normal weight and obese Chilean children determining its ethnicity.
Methods: A total of 136 normal weight children and 238 obese children (between 6 and 11 yr old) from an urban setting were recruited for this case–control study. The children were classified as normal weight [body mass index (BMI) $\geq$ 5th and $<$ 85th percentiles] or obese (BMI $>$95th percentile), according to the international age- and gender-specific percentiles defined by the Center for Disease Control and Prevention. The analysis of serum markers was carried out using commercial kits. The FTO polymorphism was determined through a high-resolution melting enabled real time polymerase chain reaction. Ethnicity was determined by analyzing mitochondrial DNA by the restriction fragment length polymorphism method.
Results: As much as 85% of the cohort was Amerindian. The minor A allele of rs9939609 was associated with obesity (odds ratio (OR): 1.422 [95% confidence interval (CI) 1.068–1.868] $p = 0.015$), calculated using an additive model. In sex-stratified analysis we found that the risk variant (A) of rs9939609 was associated with a higher homeostasis model of assessment for insulin (HOMA-IR) in prepubertal obese girls. In male carriers of the A allele, HOMA-IR showed no further deterioration than that already associated with obesity.
Conclusions: In summary, we confirm the association of the FTO gene single-nucleotide polymorphism rs9939609 with obesity in Chilean Amerindian children. Furthermore we show an association between the risk allele (A) and insulin resistance-related markers in prepubertal obese girls.

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Key words: Amerindian – childhood obesity – FTO gene – insulin sensitivity

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Childhood obesity has reached epidemic levels in developed countries (1) and constitutes one of the most important risk factors in the development of metabolic syndrome, type 2 diabetes (T2D) (2), and cardiovascular disease in adulthood (3). In Chile, in the population under 6 yr old the prevalence of childhood obesity and overweight reached 32%, in 2010 (4). Additionally, we have previously reported a high

prevalence of metabolic syndrome (37.5%) in Chilean adolescents; males had a higher risk of developing metabolic syndrome than females in the adolescent population (odds ratio [OR]: 1.58 [95% confidence interval (CI) 1.08–2.03]; $p = 0.01$) (5).

The etiology of obesity is complex; it has become clear that genetics as well as environmental factors, such as excessive energy intake and diminished physical activity, contribute to its development (6). In 2007, a genome-wide association study for T2D detected a strong association between common single-nucleotide polymorphisms (SNPs) in the fat mass and obesity-associated gene (FTO) region and risk of T2D (7). However, subsequent analyses showed that the association between FTO SNPs and T2D was mediated by an association with body mass index (BMI) in white European populations (8). Thus far, genetic variation in FTO has the largest effect of any of the genes found to be associated with BMI in individuals of European descent. Each risk allele was found to increase BMI by 0.26–0.66 kg/m^2 (9). Findings from the studies of non-White European populations were initially inconsistent; however there is increasing evidence that effect sizes are similar to those observed for European populations (10–15). Although the relationship between FTO and T2D was found to be mediated by obesity-related phenotypes in white European populations (7, 8), recent studies suggest that FTO is related to an increased T2D risk (12–42%) independent of adiposity factors in other ethnic groups such as African/Americans, Chinese, South Asians, and Japanese populations (16–19). Associations have not only been demonstrated in adults; FTO genotypes have also been found to be associated with adiposity in children and adolescents of different ethnic backgrounds (20, 21). However, to the best of our knowledge, this study is the first to investigate the association between FTO and obesity in South American children of Amerindian ancestry.

Therefore, the aim of this study was to test the association between the FTO rs9939609 SNP and childhood obesity and to explore whether this variant might be associated additionally with unfavorable metabolic traits in Chilean children.

Methods

Study design and population

This was a case–control study; 136 normal weight children and 238 obese children (aged between 6 and 11 yr old) from an urban setting of the city of Hualpén, Región del Bio Bio, Chile were recruited. Children who suffered from any chronic pathology were excluded. This study was approved by the Bioethical Committee of the Universidad de Concepción. Parents provided written informed consent prior to their child's inclusion in the study.

Children measurements

All instruments were validated following the manufacturers' protocols. Height was measured without shoes using wall-mounted stadiometers to the nearest 0.1 cm (Seca, model 208). Body weight was measured with light clothes and no shoes to the nearest 0.1 kg using a Tanita scale (model TBF-300). BMI (kg/m^2) was calculated as body weight divided by height squared. The BMI *z*-score, based on age and sex, was calculated according to the methods recommended by the World Health Organization (22). Children were classified as normal (BMI $\geq$ 5th and $<$85th percentiles) or obese (BMI $>$ 95th percentile) according to the international age- and gender-specific percentiles defined by the Center for Disease Control and Prevention (23). Waist circumference was measured midway between the lowest rib and the superior border of the iliac crest with an inelastic measuring tape to the nearest 0.1 cm (Seca, model 201). Body Composition was assessed by bioelectrical impedance analysis (TANITA TBF-300). All measurements were taken by trained nutritionists. Children were examined by physicians who defined their pubertal status according to Tanner criteria (24). Clinical data and blood sample collections were carried out between March and June 2008.

Laboratory methods

For metabolic analysis, 4 mL of fasting venous blood was collected, after an overnight (8–12 h) fast. Concentrations of total serum cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol, triglyceride, and glucose levels were determined by colorimetric methods using commercially available kits (Cobas C111 Roche, Indianápolis IN, USA). Plasma insulin and adiponectin were measured using a commercial ELISA kit (Linco Research, St. Charles, MO, USA) with the use of multireader, Synergy 2 (Biotek, Winooski, VT, USA). Homeostasis model of assessment for insulin (HOMA-IR) was calculated from fasting glucose and insulin concentrations according to previous definitions (25).

Genotyping

The FTO rs9939609 SNP polymorphism was determined using genomic DNA extracted from leukocytes using the QIAamp DNA Blood Mini Kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Polymerase chain reaction (PCR) amplifications were performed in a total volume of 25 μL on

a Rotor-Gene 6500 realtime PCR Thermocycler (Corbett Research, Sydney, Australia) and the reaction mixture contained 100 ng of genomic DNA, 1× buffer, 3.0 mM $MgCl_2$, 0.2 mM dNTP, 600 nM primers, 1.5 μM Eva green fluorescent nucleic acid stain, and 0.5 U Platinum Taq DNA polymerase (Quantance, Taunton, MA, USA). The samples were amplified using the following primers: forward 5′d AACTGGCTCTTGAATGAAATAGGATTCAGA 3′ and reverse 5′d AGAGTAACAGAGACTATCCAAGTGCATCAC 3′, which were previously defined by Lopez-Bermejo et al. (26). The amplification cycles and the melting data were achieved according to the manufacturer's protocols (SensiMix HRM™, Quantance). Genotype identification was done by comparison (95% CI) of the melting data with known genotypes, previously identified by sequencing analysis in the Departamento de Ecología, Facultad de Ciencias biológicas, Pontificia Universidad Católica de Chile. In order to confirm our genotype method, we took 20 random samples analyzed by high-resolution melting, following sequencing analysis, and found identical results. Finally, to confirm a single PCR product, electrophoresis using 3% agarose was performed.

Characterization of mtDNA haplogroups

The Amerindians' mtDNA haplogroups were characterized through the amplification of the polymorphic sites that define Amerindian mtDNA lineages (27), using the PCR technique according to Moraga et al. (28). This protocol allows fragments of the mitochondrial genome to be amplified; a 145-bp fragment containing the distinctive HaeIII site at position 663 for A haplogroup, a 157-bp segment with the polymorphic HincII at position 13.259 for C haplogroup, a 179-bp fragment for the polymorphic AluI site at positions 5176 for D haplogroup, and a 122-bp fragment for B haplogroup. The presence or absence of restriction sites for A, C, and D haplogroups was verified by specific endonucleases. To detect the 9-bp deletion, which identifies the haplogroup B, the PCR products were directly sized by electrophoresis. All gels were re-read blindly; there were no inconsistencies between readings. Furthermore, 20% of the analyses were selected at random to be repeated, as has previously been reported by Guzman et al. (29).

Statistical analyses

Analyses were performed using the statistical software SAS (version 9.1-release Institute, Cary, NC, USA). Prior to analyses, quantitative data were tested for normality using Shapiro–Wilk tests. To avoid skewed distributions, log-transformations were performed as appropriate. Differences between groups were assessed by *t*-test or analysis of variance (ANOVA) when more than two groups were compared. If variables still had a non-normal distribution following transformation, comparisons were carried out by Mann–Whitney test for two groups and Kruskal–Wallis test for more than two groups. Categorical variables were represented as percentages and were tested by the chi-square test.

Goodness-of-fit chi-square test was performed to confirm whether the observed genotype counts were in Hardy–Weinberg equilibrium. Differences in obesity-related phenotypes and metabolic risk markers between genotyping groups were tested by ANOVA. The associations of FTO rs9939609 with obesity and metabolic traits, as well as the per-allele effect size on these traits, were estimated using linear regression assuming an additive genetic model. The genotypes were coded as 0, 1, or 2 corresponding to the number of copies of the risk allele (A). Logistic regression was performed to determine if the odds of obesity differed between the FTO genotypes. Potential confounder factors were adjusted for where appropriate. All values were expressed as mean ± SD, and for all analyses significance was defined as $p < 0.05$.

Results

Characteristics of subjects

The clinical and anthropometric characteristics for normal weight and obese girls and boys are described in Table 1. All groups are comparable in age and pubertal stage. Anthropometric measurements (BMI *z*-score, fat mass percentage, and waist circumference) were significantly higher in obese children, ($p < 0.0001$). Obese children had significantly lower HDL-C levels ($p < 0.0001$) and significantly higher concentrations of total triglycerides ($p < 0.0001$), basal insulin and HOMA-IR ($p < 0.0001$). Additionally, the adiponectin level was significantly lower in obese children ($p < 0.05$). Thus, markers associated with insulin resistance were significantly altered in obese children compared to normal weight children, in both, girls and boys.

Ethnic classification

Table 1 shows the frequency of haplogroups A, B, C, and D in the children. Of the recruited cohort, 85% of children had one of the four basic ancestral Amerindian lineages. Haplotypes B and C were the most prevalent in the cohort. The children who did not have any of Amerindians haplogroups (15%) were classified as non-Amerindian. The proportion of Amerindian:non-Amerindian children did not differ significantly between the normal weight and obese groups, as in girls as boys ($p = 0.276$).

Table 1. Descriptive characteristics of the school children cohort by nutritional condition

	Girls			Boys		
	Normal weight (mean ± SD)	Obese (mean ± SD)	p-Value	Normal weight (mean ± SD)	Obese (mean ± SD)	p-Value
Age	8.41 ± 1.50	8.51 ± 1.41	n.s.	8.43 ± 1.44	8.58 ± 1.46	n.s.
Sample size (n)	67	119		69	119	
Tanner stage (n prepubertal/n pubertal)	52/15	86/33		58/11	103/16	
Ethnicity (Am: Amerindian; NAm: non-Amerindian)	Am: 83% NAm: 17%	Am: 86% NAm: 14%		Am: 84% NAm: 16%	Am: 86% NAm: 14%	
	(Am haplogroup: %) A: 0% B: 35% C: 31% D: 34%	(Am haplogroup: %) A: 5% B: 31% C: 46% D: 18%	n.s.	(Am haplogroup: %) A: 3% B: 31% C: 50% D: 16%	(Am haplogroup: %) A: 2% B: 38% C: 26% D: 35%	n.s.
Waist circumference (cm)	61.7 ± 6.3	78.8 ± 8.7	<0.0001	59.5 ± 5.5	77.8 ± 8.6	<0.0001
BMI *z*-score	0.45 ± 0.37	2.04 ± 0.27	<0.0001	0.42 ± 0.33	2.11 ± 0.30	<0.0001
Fat percentage (%)	20.1 ± 5.8	35.5 ± 4.9	<0.0001	17.8 ± 3.1	32.2 ± 6.7	<0.0001
Lean body mass (kg)	22.7 ± 4.2	27.3 ± 4.8	<0.0001	22.7 ± 4.7	28.9 ± 5.5	<0.0001
Total-cholesterol (mg/dL)	181.5 ± 33.8	184.5 ± 37.2	n.s.	176.6 ± 39.7	184.9 ± 32.7	n.s.
HDL-C (mg/dL)	56.7 ± 12.8	46.7 ± 11.4	<0.0001	58.0 ± 11.9	50.3 ± 10.5	<0.0001
LDL-C (mg/dL)	105.9 ± 26.6	109.4 ± 31.2	n.s.	104.6 ± 30.5	113.8 ± 26.9	<0.05
Triglycerides (mg/dL)	99.4 ± 48.0	139.2 ± 72.5	<0.0001	74.4 ± 27.2	124.6 ± 76.3	<0.0001
TG/HDL-C	1.95 ± 1.30	3.39 ± 2.68	<0.0001	1.34 ± 0.56	2.78 ± 2.41	<0.0001
Fasting glucose (mg/dL)	86.2 ± 7.2	87.2 ± 9.7	n.s.	88.3 ± 9.5	90.6 ± 7.7	n.s.
Fasting insulin (μU/mL)	5.9 ± 4.8	10.6 ± 8.3	<0.0001	5.1 ± 5.6	8.5 ± 4.9	<0.0001
HOMA-IR	1.28 ± 1.06	2.24 ± 1.58	<0.0001	1.13 ± 1.31	1.90 ± 1.07	<0.0001
Adiponectin (μg/mL)	16.7 ± 5.6	13.5 ± 4.6	<0.0001	17.4 ± 6.9	13.6 ± 5.7	<0.0001

BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; HOMA-IR, homeostasis model of assessment for insulin.
Non significant results are indicated as n.s.

Prevalence of FTO rs9939609 and its association with obesity

In all study subjects, the genotype distributions were in Hardy–Weinberg equilibrium ($p = 0.061$). The frequency of the minor allele (A) was 29% in the normal weight children and 39% in the obese children, and did not differ significantly between haprogroups A, B, C, D, and non-Ameridian. For the normal weight children, there was no difference by FTO genotype in mean BMI *z*-score (Table 2). However, the mean BMI *z*-score of obese children carrying two copies of the risk allele (A) was significantly higher ($p < 0.01$) than obese non-carriers (TT) (Table 2). Percentage fat mass was not significantly different across the FTO genotypes for either normal weight or obese children (Table 2). Birth weight was not significantly different across the FTO genotypes in both normal weight and obese children (Table 2).

The odds ratio for obesity for each extra copy of the risk allele was 1.422 (95% CI 1.068–1.868; $p = 0.015$) (Table 3). Further adjustment for sex and age did not alter this finding.

Table 3. Odd ratio to obesity

	Percentage of FTO rs9939609 genotype frequency (n)		
	T/T	T/A	A/A
Normal weight (n = 136)	53.7 (73)	34.6 (47)	11.7 (16)
Obese (n = 238)	46.4 (111)	28.2 (67)	25.2 (60)
$OR_{(additive\ model)} = 1.422$ (95% CI 1.068–1.868; $p = 0.015$)			

CI, confidence interval; FTO, fat mass and obesity associated; OR, odds ratio.

Influences of FTO rs9939609 SNP on insulin resistance-related markers

Levels of plasma markers related to insulin sensitivity were analyzed for each FTO genotype. After stratifying the data by gender and nutritional status (normal/obese), we found that HOMA-IR and fasting insulin were significantly higher in obese females who carried the A allele compared with obese females who carried the T allele ($p < 0.001$); however, this behavior was not observed within the normal weight girls, which did not demonstrate any change across FTO genotypes (Fig. 1). Further adjustment for BMI and age did not alter this finding, although the significance level was reduced ($p = 0.009$ and $p = 0.019$; for fasting insulin and HOMA-IR, respectively). In contrast, boys did not show any significant difference in HOMA-IR nor fasting insulin when related to FTO genotype. Neither boys nor girls showed significant differences in adiponectin levels across FTO genotypes (Fig. 1), even if adjusting by BMI and age.

In addition, we stratified this population in relation with pubertal status measured by Tanner (Fig. 2). HOMA-IR was almost twice higher in prepubertal obese girls homozygous for the A allele compared with the TT obese carriers ($p < 0.001$), reaching similar HOMA-IR level than pubertal obese girls carrying any genotype. Normal weight girls did not demonstrate any change across FTO genotypes (Fig. 2A). Further adjustment for BMI and age did not alter these findings. On the contrary, prepubertal obese or normal weight boys did not show any increase in HOMA-IR across FTO genotypes (Fig. 2B). This same behavior was observed in fasting insulin, but not in any other markers.

Table 2. Descriptive anthropometric characteristics of the school children cohort by genotype and nutritional condition

	Genotype			
	T/T	T/A	A/A	p-Value
Sample size (n)				
Normal weight	73	47	16	
Obese	111	67	60	
BMI *z*-score				
Normal weight	0.43 ± 0.38	0.46 ± 0.28	0.38 ± 0.39	0.704
Obese	2.01 ± 0.24	2.12 ± 0.33	2.14 ± 0.28	0.003
Body composition				
Normal weight				
Fat mass (%)	19.4 ± 4.9	19.0 ± 4.6	16.8 ± 3.7	0.193
Lean body mass (kg)	22.9 ± 4.5	22.3 ± 4.4	23.0 ± 4.4	0.711
Obese				
Fat mass (%)	33.1 ± 5.3	34.0 ± 6.4	35.2 ± 6.9	0.061
Lean body mass (kg)	27.8 ± 5.0	28.0 ± 5.0	28.6 ± 6.0	0.376
Birth weight (kg)				
Normal weight	3.3 ± 0.5	3.5 ± 0.4	3.5 ± 0.5	0.169
Obese	3.4 ± 0.5	3.4 ± 0.5	3.6 ± 0.5	0.258

BMI, body mass index.

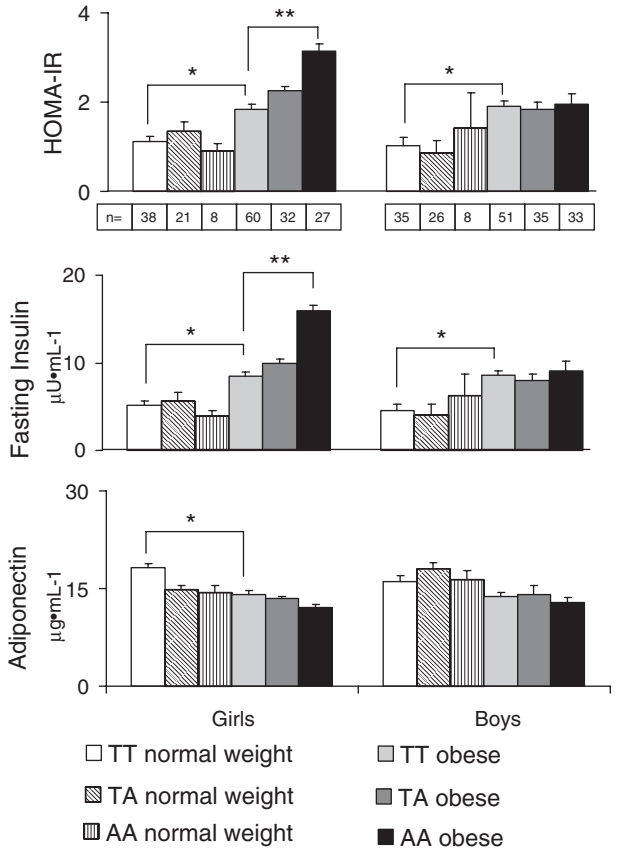

Fig. 1. Association between FTO rs9939609 and markers related to insulin sensitivity, classified by genotype and nutritional conditions. Data is presented as mean and its respective SE. The Ancestral genotype is denoted as (TT), heterozygote as (TA), and homozygote as (AA). Significant results, $p < 0.05$ and $p < 0.001$, are highlighted as * and **, respectively. Non significant results are indicated as n.s.

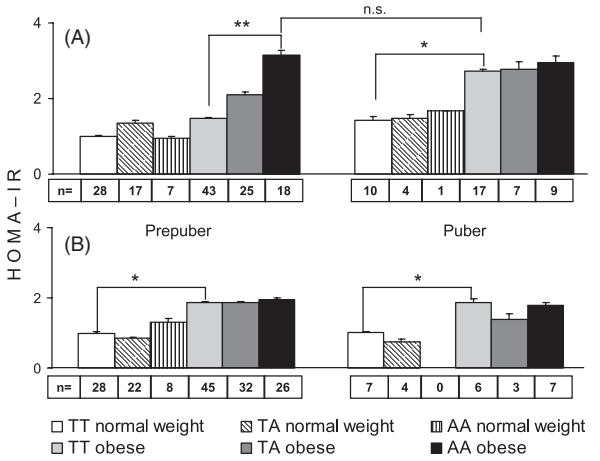

Fig. 2. Association between FTO rs9939609 and HOMA-IR stratified by genotype, pubertal stage and weight status for (**A**) girls and (**B**) boys. Data is presented as the mean and its respective SE. The Ancestral genotype is denoted as (TT), heterozygote as (TA), and homozygote as (AA). Significant results, $p < 0.05$ and $p < 0.001$, are highlighted as * and **, respectively. Non significant results are indicated as n.s.

Discussion

The main findings of this study were as follows: (i) genetic variation of the FTO rs9939609 was associated with an increase in BMI (*z*-score) in obese children independent of age and sex; (ii) in sex-stratified analysis we found that the risk allele (A) of rs9939609 was associated with a higher HOMA-IR in prepubertal girls compared to non-carriers of the risk allele; this finding was independent of age and BMI.

To the best of our knowledge, our study reports for the first time a significant association between rs9939609 and obesity in obese Chilean children. The risk allele of the FTO SNP rs9939609 polymorphism is frequently found throughout all populations, but their impact on obesity is modified by ethnicity (30, 31). Latin American populations are defined as Amerindian by the identification of mitochondrial DNA markers, defined by four haplogroups designated as; A, B, C, and D (32). A previous study reported a high frequency of these genetic markers in the Chilean population (33), revealing that more than 80% of the population in the Bio-Bio and Araucania region of Chile have Amerindian backgrounds (34). This study confirmed these previous results, reporting that 85% of the population recruited was Amerindian, with B and C the predominant haplotypes. Although the ethnicity in our study was mainly Amerindian, the frequency of the risk allele (A) was similar to that reported for European populations (35, 36). We demonstrated a discrete increase in BMI *z*-score units for each extra copy of the risk allele in the obese children, but not in the normal weight children (Table 2). The obesity risk, which was calculated according to the additive model and expressed as an odd ratio (Table 3), was comparable to the others' reports (37, 38).

It is known that girls have more fat mass and a distinct pattern of fat distribution during infancy and childhood compared to boys (39–41). Among normal weight children, girls have lower insulin sensitivity than boys before puberty, and it has been speculated that this gender difference may contribute to the higher prevalence of T2D mellitus in women compared to men (42). Our study performed an exploration of the insulin-sensitivity metabolic trait (HOMA-IR, fasting insulin, and adiponectin), with regard to the FTO polymorphism. We found a detrimental effect of the rs9939609 FTO variant on insulin-sensitivity markers in obese prepubertal girls. Jacobsson et al. (43) genotyped 450 obese adolescents and compared them with 512 healthy controls. They found that the FTO variant (SNP rs9939609) was associated with higher BMI and a diminished insulin sensitivity in girls, but not in boys. Interestingly, our analysis revealed that the influence of the FTO gene on insulin resistance was observed early in prepubertal girls, while in the males

the same markers showed no further deterioration than those caused by obesity, in agreement with the Jacobsson report. However, our study failed to demonstrate the same behavior in pubertal girls, probably because the sample size of this group was insufficient, in other words, the pubertal girls' subgroups might not contain enough subjects in order to see this association. The impairment of HOMA-IR associated with rs9939609 was not observed in normal weight girls or boys (data not shown).

Additionally, this study demonstrates that impairment of insulin sensitivity in girls carrying two copies of the risk allele was not associated with a lower adiponectin concentration (compared to the T carriers). Therefore, this polymorphism does not seem to act on insulin sensitivity by reducing the total plasma adiponectin. Future studies are needed to explore other mechanisms by which FTO reduces insulin sensitivity at an early age in females.

Birth weight did not vary between carriers and non-carriers of the risk allele (Table 2); this is in agreement with previously reported data (44). Recent studies suggest that genetic effects of the FTO gene do not start to influence adiposity before the age of 7 yr (45). The design of our study has allowed us to demonstrate the effects in children aged between 7 and 11 yr old.

This study has some limitations. First, the small sample size limits the statistical power of some results, although it was sufficient to detect the association between FTO genotype and obesity risk in this Amerindian children. Second, the case–control study design does not allow for any conclusions about causality to be made. Third, only one SNP rs9939609 of FTO was assessed in this study; however this variant is part of a large cluster of >40 SNPs that are highly correlated [linkage disequilibrium (LD) $r^2 > 0.80$ in Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain collection, (CEU population) of the HapMap]. Similar results would be expected for other SNPs that fall in a region of strong LD in intron 1 (6, 46).

In summary, we confirm the association of the rs9939609 risk A allele with early-onset obesity in obese Chilean Amerindian children and demonstrate the association of this SNP with impaired insulin sensitivity in prepubertal stage, just in obese girls.

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