UNIVERSIDADE D
COIMBRA

Simão Artur Sousa Coutinho Santiago Pinho

A STUDY OF GENETIC VARIANTS ASSOCIATED WITH OBESITY AMONG PORTUGUESE CHILDREN

Dissertação de Mestrado em Evolução e Biologia Humanas, orientada por Doutora Cristina Padez e Doutor Licínio Manco e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Setembro de 2018

A study of genetic variants associated with obesity among Portuguese children

Simão Artur Sousa Coutinho Santiago Pinho

Dissertação de Mestrado em Evolução e Biologia Humanas, orientada por Doutora Cristina Padez e Doutor Licínio Manco e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Setembro de 2018

UNIVERSIDADE D
COIMBRA

Acknowledgments/Agradecimentos

A realização desta tese contou com o apoio de inúmeras pessoas, às quais jamais encontrarei as palavras suficientes para agradecer. Quero deste modo, expressar a minha profunda gratidão a todos aqueles que, pelo apoio e amizade, direta ou indiretamente, contribuíram para a realização deste trabalho.

Ao meu coorientador, o Doutor Licínio Manuel Mendes Manco, por me ter acolhido no seu laboratório, por toda a sua orientação e ajuda na realização deste trabalho. As palavras serão sempre poucas para expressar a minha gratidão, por todo o tempo que gastou, e sabedoria que partilhou para a realização deste trabalho.

Ao Centro de Investigação em Antropologia e Saúde (CIAS) e em particular à minha orientadora a Professora Doutora Cristina Padez, por todo o apoio e ajuda que prestou.

Agradeço também aos meus amigos que sempre me apoiaram e estiveram comigo desde a minha licenciatura até ao final do meu mestrado, meras palavras nunca serão o suficiente para expressar a minha gratidão pelos anos que passamos juntos. Mas do fundo do meu coração obrigado.

À Beatriz por todo o apoio, coragem, força e momentos felizes que tivemos agradeço por todo o amor que me deste.

Aos meus pais nomeadamente a minha mãe, que me guiou e cuidou de mim, dando-me sempre o seu apoio e que fez sempre o possível para me deixar feliz e certificar de que tinha tudo o que precisava. Do fundo do meu coração agradeço todos os sacrifícios e horas que gastou em cuidar de mim.

Finalmente, e por não poder alongar-me mais, agradeço a todos os que de algum modo tornaram possível a realização desta dissertação

Abstract

Obesity has become a serious public health problem, and its impact is more salient in more industrialized countries. It is widely accepted that obesity is a complex multifactorial condition, with an important genetic component. In 2007, it was identified the first single nucleotide polymorphism (SNP) in the *FTO* gene (rs9939609) associated with obesity in humans through a genome-wide association study (GWAS). Since then, many other obesity-associated loci have been found.

The main aim of this work was to investigate the association of the *FTO* SNP rs9939609 and the two *SLC6A4* polymorphisms (5-HTTLPR and STin2) with obesity and other anthropometric variables, such as weight, height, BMI, BMI z-score, waist circumference, hip circumference, waist-to-height ratio, and skinfold measurements in a sample of Portuguese children. Moreover, we also tested for interaction effects between the study polymorphisms and physical activity in relation to obesity.

Anthropometric parameters and physical activity were measured in a random sample of 645 children (321 girls and 324 boys) between 3-11 years-old, of several public schools from the central region of the country. Physical activity was measured objectively during 7 days with *Actigraph* accelerometers. The International Obesity Task Force (IOTF) cut-offs were used to define obesity. Genotyping of the two *SLC6A4* (5-HTT) gene polymorphisms, 5-HTTLPR and STin2, was performed by Polymerase Chain Reaction (PCR) followed by agarose gel electrophoresis. The *FTO* SNP rs9939609 genotyping was performed by real time PCR using TaqMan probes. For statistical association analyses the population sample was stratified in two groups aged 3-6 years-old and 7-11 years-old. Subjects defined as overweight or obese were merged in one case group.

In children aged 7-11 years-old, the *FTO* rs9939609 showed a statistically significant association with BMI Z-score ($P = 0.04$), and marginal associations were found with BMI ($P = 0.07$) and WHtR ($P = 0.07$). Also, a marginal association was found between rs9939609 and risk of overweight/obesity ($P = 0.087$). A strong significant association was observed with risk of abdominal obesity ($P = 4.5 \times 10^{-4}$) in the same age group. When stratified by physical activity, several statistically significant associations were observed with obesity-related anthropometric parameters in non-active individuals aged 7-11 years, including for BMI ($P = 0.02$), BMI Z-score ($P = 0.027$), waist

circumference (P = 0.035), WHtR (P = 0.045), subscapular skinfold (P = 0.015) and sum of skinfolds (P = 0.046), but not in active individuals. In concordance, a statistically significant association between the *FTO* polymorphism and risk of obesity and abdominal obesity was observed in non-active individuals (P = 0.037 and P = 0.003 respectively), but not in active individuals. No associations with obesity or related anthropometric parameters were found in children aged 3-6 years-old.

For the two *SLC6A4* polymorphisms (5-HTTLPR and STin2), no significant associations were found in both age groups (except in children aged 7-11 years-old between 5-HTTLPR and subscapular or suprailiac skinfold measures; P = 0.02 and P = 0.04, respectively), even when the population was divided in active and non-active individuals.

In conclusion, the *FTO* SNP rs9939609 showed association with several anthropometric measures and the risk of obesity or abdominal obesity in Portuguese children, in line with previous studies performed in European populations. Moreover, we observed an interaction effect between the *FTO* gene and physical activity in obesity. Our results also suggest that the association between the *FTO* SNP rs9939609 and obesity becomes evident only after the age of seven.

This work contributed to a better knowledge of the genetics of obesity in Portuguese children, but further studies are needed to improve the understanding of the genetic factors underlying the obesity risk in children.

Key-words: Abdominal obesity; FTO; STin2; 5-HTTLPR genes; Physical activity

Resumo

A obesidade tornou-se um grave problema de saúde pública, e o seu impacto é mais saliente em países industrializados. É amplamente aceite que a obesidade é uma condição complexa e multifatorial, com um importante componente genético. Em 2007, foi identificado o primeiro polimorfismo de nucleótido simples (SNP) no gene *FTO* (rs9939609) através de um estudo de associação genómica (GWAS). Desde então, muitos outros loci têm sido encontrados associados à obesidade.

O principal objetivo deste trabalho foi investigar a associação com obesidade e outras variáveis antropométricas como o peso, altura, IMC, IMC z-score, circunferência da cintura, relação cintura / estatura e mediadas de dobras cutâneas do SNP *FTO* rs9939609 e de dois polimorfismos no gene *SLC6A4* (5-HTTLPR e STin2), numa amostra de crianças portuguesas. Além disso, foram também testados efeitos da interação entre os polimorfismos genéticos e atividade física para a obesidade.

Os parâmetros antropométricos e a atividade física foram medidos numa amostra aleatória de 645 crianças (321 sexo feminino e 324 sexo masculino) entre os 3-11 anos de idade, provenientes de várias escolas públicas da região centro do país. A atividade física foi medida durante 7 dias com o acelerômetro *Actigraph*. Os pontos-de-corte do *International Obesity Task Force* (IOTF) foram usados para definir obesidade. A genotipagem dos dois polimorfismos do gene SLC6A4 (5-HTT), 5-HTTLPR e STin2, foi realizada pela Reação em Cadeia da Polimerase (PCR) seguida de eletroforese em gel de agarose. O SNP *FTO* rs9939609, foi genotipado por PCR em tempo real com sondas TaqMan. Para os estudos de associação, a população foi dividida em dois grupos de acordo com a idade: um grupo com idade entre os 3-6 anos e um segundo grupo com idades entre os 7-11 anos. Os indivíduos com obesidade e excesso de peso foram incluídos num único grupo.

O polimorfismo *FTO* rs9939609 mostrou uma associação estatisticamente significativa com o IMC Z-Score (P = 0,04) e associações marginais foram encontradas com o IMC (P = 0,07) e WHtR (P = 0,07) em crianças com idade entre 7-11 anos. Além disso, foi encontrada uma associação marginal com o risco de excesso de peso/obesidade (P = 0,087), e uma forte associação com o risco de obesidade abdominal ($P = 4{,}5 \times 10^{-4}$) em crianças com idade 7-11 anos. Quando a população foi estratificada por atividade física, várias associações estatisticamente significativas foram observadas com

parâmetros antropométricos como o IMC (P = 0,02), IMC Z-score (P = 0,027), circunferência da cintura (P = 0,035), WHtR (P = 0,045), pele subescapular (P = 0,015) e soma de dobras cutâneas (P = 0,046), em indivíduos não ativos com idade entre 7-11 anos. Além disso, o polimorfismo *FTO* mostrou uma associação estatisticamente significativa com o risco de obesidade (P = 0,037) e obesidade abdominal (P = 0,003) em indivíduos inativos, mas não em indivíduos ativos. Em crianças com 3-6 anos de idade não foi encontrada qualquer associação com obesidade ou parâmetros antropométricos.

Para os dois polimorfismos *SLC6A4* (5-HTTLPR e STin2), nenhuma associação significativa foi encontrada em ambos os grupos etários (exceto no grupo 7-11 anos entre 5-HTTLPR e pele subescapular ou pregas suprailíacas; P = 0,02 e P = 0,04, respetivamente), mesmo quando a população foi dividida em indivíduos fisicamente ativos e não ativos.

Em conclusão, o SNP *FTO* rs9939609 mostrou associação com o risco de obesidade e obesidade abdominal em crianças portuguesas, em consonância com os estudos realizados anteriormente noutras populações europeias. Além disso foi observado um efeito de interação entre o gene *FTO* e a atividade física na obesidade. Os nossos resultados também sugerem que a associação entre o gene *FTO* e a obesidade se torna evidente apenas após os 7 anos de idade.

Este trabalho contribuiu para um melhor conhecimento da genética da obesidade em crianças portuguesas, mas são necessários outros estudos para uma melhor compreensão dos fatores genéticos subjacente ao risco de obesidade em crianças.

Palavras-chave: Obesidade Abdominal; Genes FTO; 5-HTTLPR; STin2; Atividade Física

Index

Table Index

1- Introduction

1.1. What is obesity?

Obesity is a major public health problem that has experienced an increase in the world population, and its impact is more salient in more industrialized countries (Xia & Grant, 2013). According to the definition from the World Health Organization (WHO), obesity is defined as abnormal or excessive fat accumulation that may impair health. The most commonly used measure of obesity and overweight is Body Mass Index (BMI), which is defined as a person's weight in kilograms divided by the square of the person's height in meters (kg/m^2). Obesity results from the interaction of several family, environmental and genetic factors (Srivastava et al., 2016; Xia & Grant, 2013). Numerous environmental factors are associated with obesity, including sedentary behaviors, breastfeeding, and sleep duration, just to mention some of them. Nevertheless, it is generally accepted that this increase in obesity prevalence results mainly from an increase in the consumption of energy-dense food and, on the other hand, a reduction in physical activity (PA) levels (Xia & Grant, 2013).

However, it is also clear that some individuals are more susceptible to these "obesogenic" environmental changes (Sandholt et al., 2012; Tung & Yeo, 2011). Indeed, studies using monozygotic twins (MZ) and dizygotic twins (DZ) (genetically equal individuals and individuals sharing 50% similarity respectively) have estimated that the concordance for fat mass in MZ twins ranges between 70-90% while in DZ twins ranges between 35-45%, demonstrating that obesity has a great genetic component and that characteristics such as body weight are strongly hereditary (Tung & Yeo, 2011; Xia & Grant, 2013). In fact, it is now accepted that obesity constitutes a complex nonmendelian trait that results from numerous susceptibility *loci* (Srivastava et al., 2016). Therefore the identification of obesity-associated *loci* could enable the ability to distinguish individuals who have a strong genetic predisposition to obesity, allowing the development of prevention or treatment strategies (Sandholt et al., 2012)

1.2. Genetics of obesity: the *FTO* and *SLC6A4* genes

Since polygenic obesity is the most common form of obesity (Tung & Yeo, 2011), human genome-wide association studies (GWAS) through the study of thousands of single nucleotide polymorphisms (SNPs) across all the genome, allowed the identification of a large number of *loci* associated with obesity (Speliotes et al., 2010;

Wen et al., 2012; Loos & Yeo, 2014). The fat mass and obesity-associated (*FTO*) gene, located on chromosome 16q12.2, was the first obesity-associated *locus* identified by GWAS (Dina et al., 2007; Frayling et al., 2007).

The *FTO* gene is expressed mainly in the brain and hypothalamus playing a role in the regulation of energy homeostasis (Tung & Yeo, 2011; Dina et al., 2007). The *FTO* gene product is a nuclear protein 2-oxoglutarate Fe (II) dependent demethylase, although little is known about the physiological function of this protein. Recent studies suggest that the enzyme can remove methyl groups from DNA and RNA nucleotides *in vitro*, with highest affinity for single stranded RNA molecules (Leonska - Duniec et al., 2016; Loos & Yeo, 2014; Tung & Yeo, 2011). The *FTO* gene has been the focus of many studies, covering a large number of distinct populations of European (González-Sánchez et al., 2009; Hubacek et al., 2008; Loos & Yeo, 2014), African (Adeyemo et al., 2010; Hassanein et al., 2010; Loos & Yeo, 2014), and Asian (Cha et al., 2008; Chang et al., 2008; Loos & Yeo, 2014; Tan et al., 2008) ancestries, that have confirmed the association of *FTO* with obesity. The SNP rs9939609 located in intron 1 of the *FTO* gene is the most widely studied among populations showing the highest levels of association with obesity and BMI (Xia & Grant, 2013). Each additional minor rs9939609 A risk allele was found to be associated with a 20%–30% increase in the risk of obesity and 1–1.5 kg increase in body weight (Frayling et al., 2007). Studies in the Portuguese population also have shown a strong association between this SNP and obesity in both children and adults (Albuquerque et al., 2013; Ferreira Carlos et al., 2013; Muc et al., 2015).

It is worth noting that many of the variants discovered showing an association with obesity are predominantly expressed in the central nervous system (Sandholt et al., 2012). An example is the serotonin transporter (5-HTT) which is translated by the solute carrier family 6 member 4 (*SLC6A4*) gene on chromosome 17q11.2 (Miranda et al., 2017; Sookoian et al., 2007). The 5-HTT is an integral membrane protein localized in presynaptic neuronal membranes (Calati et al., 2011), implicated in the transport of serotonin (5-hydroxytryptamine or 5-HT) from the synaptic cleft into the pre-synaptic neurons, with the purpose of terminating serotonin action and recycling it (Bah et al., 2010; Calati et al., 2011). The *SLC6A4* gene has two well studied polymorphic regions, the serotonin transporter linked polymorphic region in the promoter region (5-HTTLPR) and a variable number of tandem repeats (VNTR) in the second intron (known as STin2) (Dias et al., 2016).

The 5-HTTLPR polymorphism has two common alleles: a short (S) and a long (L) with 14 and 16 copies, respectively, of a 20 to 23 base pair (bp) repeat sequence (Bonnet et al., 2017; Peralta-leal et al., 2012). Individuals homozygous (SS) and heterozygous (LS) compared to homozygous individuals (LL), have a reduced expression of *SLC6A4*, resulting in a reduced reuptake of serotonin from the synaptic cleft into presynaptic neurons (Peralta-leal et al., 2012). The second *SLC6A4* polymorphic region STin2 is a multiallelic 17-bp VNTR with two common 10 and 12 repeat alleles, and two rare alleles with 9 and 7 repeats (Lesch et al., 1994; MacKenzie & Quinn, 1999). The intronic polymorphism STin2 may act as a positive transcriptional regulatory element, the 12-allele having a higher transcriptional activity (Fiskerstrand et al., 1999; MacKenzie & Quinn, 1999).

Until now, there is no consensus in the literature about the association with obesity for 5-HTTLPR polymorphic region. Several studies in different populations have reported associations of both S and L alleles with obesity or obesity related traits (Bah et al., 2010; Borkowska et al., 2015; Fuemmeler et al., 2008; Iordanidou et al., 2010; Lan et al., 2009; Miranda et al., 2017; Peralta-leal et al., 2012; Sookoian et al., 2007; Sookoian et al., 2008), while other reports showed no such associations (Hameed et al., 2015; Mergen et al., 2007). In the Portuguese population a recent study demonstrated an association between the 5-HTTLPR L allele and overweight/obesity in a sample of Portuguese young adults (Dias et al., 2016). Almost all the studies conducted thus far, have focus mainly on adults. The few studies that have been performed to investigate association of this variant with obesity in children have shown an association between the S allele and increased BMI and other obesity-related parameters (Miranda et al., 2017; Sookoian et al., 2007). Regarding STin2, to our knowledge, only two studies have been performed to investigate the association between this polymorphism and obesity. The first study found no significant association between this polymorphism and obesity in Turkish adults (Uzun et al., 2015) and a second study in Portuguese young adults, showed the STin2 10 allele and L/10 haplotype associated with overweight/obesity (Dias et al., 2016).

1.3. Gene environment interactions

A gene-environment interaction (GxE) exists where the risk conveyed by a specific genotype depends on one or more environmental exposure levels. Modulation of *FTO*-obesity associations by self-reported physical activity and by diet, the two major

factors contributing to body weight regulation, are the most replicated gene-environmental interactions in obesity. Many individual observations in European ancestry populations suggests that moderate to vigorous physical activity attenuates the effect of *FTO* genetic susceptibility to obesity (Albuquerque et al., 2017). It was shown that low physical activity accentuates the effect of the *FTO* on obesity risk, as reported in both adults (Andreasen et al., 2008; Rampersaud et al., 2008) and children (Ruiz et al., 2010; Xi et al., 2011). Studies in the Portuguese population also have shown the influence of higher physical activity (sport practice) on the attenuation of the FTO rs9939609 genetic susceptibility to obesity in young adults (Muc et al., 2015). For the *SLC6A4* polymorphisms, a recent study in the Portuguese population showed that in less active individuals, overweight/obesity was significantly associated with the 5-HTTLPR L-allele and nominally associated with STin2 10-allele, demonstrating that physical inactivity accentuates the influence of the *SLC6A4* polymorphisms on obesity risk (Dias et al., 2016). However, to our knowledge no other study conducted thus far assessed for interactions between *SLC6A4* polymorphisms and physical activity on obesity risk.

1.4. Objectives

The main aim of this work was to investigate possible associations between polymorphisms located at the two *loci FTO* and *SLC6A4* with obesity and obesity-related variables (weight, BMI, BMI z-score, waist circumference, hip circumference, waist-to-height ratio, and skinfold measurements) in a sample of Portuguese children aged 3-11 years-old.

In particular, our study aimed:

I. to investigate the association between the *FTO* SNP rs9939609 and overweight/obesity and other anthropometric variables;

II. to investigate the possible association of the two *SLC6A4* polymorphisms, 5-HTTLPR and STin2, with obesity and other anthropometric variables;

III. to test for interaction effects between the study polymorphisms and physical activity in relation to obesity.

2 - Material and methods: fundamentals and protocols

2.1. Selection of polymorphisms

For this study three polymorphisms of two *loci*, that have been previously identified from the literature as being related with obesity or obesity-related traits, were selected:

- the *FTO* SNP rs9939609 (position: chr16:53820527), the most widely studied locus in terms of obesity, that has previously also been associated with obesity and obesity related traits in both children (Albuquerque et al., 2013) and adults (Muc et al., 2015) of the Portuguese population;

- the two *SLC6A4* (chr17q11.2) polymorphisms STin2 and 5-HTTLPR, that have been previously associated with obesity in a sample of Portuguese young adults (Dias et al., 2016).

2.2. Study subjects and anthropometric measures

The study population included 645 Portuguese children (321 females, 324 males; age 3-11 years old), randomly selected from several public schools in the central region of Portugal (Coimbra).

This study was conducted under a project approved by the Ministry of Education - *Direcção Geral de Inovação e de Desenvolvimento Curricular*. The study was performed anonymously and according to the guidelines laid down in the Declaration of Helsinki. Written informed consent was obtained from all children's parents.

All anthropometric measurements were taken barefoot and in minimal clothing for weight, height, waist circumference (WC), hip circumference (HC), triceps, subscapular and suprailiac skinfolds. The following anthropometric variables were considered for this study:

i) BMI was calculated as the weight in kilograms divided by the square of height in meters (kg/m^2), and was used to classify children as normal weight, overweight or obese according the age and sex specific BMI cut-off points provided by the International Obesity Task Force (IOTF).

ii) Body mass index z-scores, also called BMI standard deviation (s.d.) scores, are measures of relative weight adjusted for child age and sex. Given a child's age, sex, BMI, and an appropriate standard reference, a BMI z-score (or its equivalent BMI-for-age percentile) can be determined. It should be noted that BMI z-scores are calculated relative to an external reference (whether national or international) and not to an internal

reference. Body mass index Z-scores correspond to growth chart percentiles, and can be converted into their equivalent BMI-for-age percentiles by comparison to a normal distribution table (Must & Anderson, 2006).

iii) Waist-to-height ratio (WHtR) constitutes an anthropometric tool used for measuring central adiposity (body fat), calculated by dividing WC (cm) by height (cm). WHtR has been gaining more attention and has been described has a more sensitive universal screening tool than BMI to detect health risks (Yoo, 2016).

iv) Skinfold measurements is a common method to estimate body fat composition of the body. The sum (mm) of the triceps, subscapular and suprailiac values is then converted to a percentage of body weight according to age and sex.

2.3. Physical activity measurement

Physical activity (PA) was measured for consecutive 7 days using a w*GT3X-BT Actigraph* accelerometer (ActiGraph LLC, Pensacola, FL, USA). This accelerometer is a triaxial motion sensor and, it is one of the most commonly used devices for assessing PA. The dimensions of the sensor are 4.6x3.3x1.5 cm with a weight of 19 grams. The accelerometer was placed on an elastic belt above the right anterior superior iliac spine and it was selected a sample rate of 100 Hz (range 30–100 Hz). The filtered acceleration signal is digitized, and the magnitude is summed over a user-specified period of time (an epoch interval) which was set at 5 seconds as in other studies of children which has been shown to be more accurate for the assessment of the spontaneous and intermittent activities of young children. The accelerometer was placed over the hip for seven consecutive school days. Accelerometer data were electronically downloaded using the *ActiLife 6 software*. Subsequently, data were reduced to an Excel file containing movement counts. The output also included the total time that the accelerometer was worn (minutes) on each day.

The total amount of daily moderate-to-vigorous physical activity (MVPA) was expressed in minutes/day. Participants who did not complete a minimum of 600 minutes of accelerometer data per day after removing sequences of 20 or more consecutive zero counts (Andersen et al., 2006; Bringolf-Isler et al., 2009) were excluded from subsequent analyses.

Data processing and inclusion criteria were the same as in the *European Youth Heart Study* (Riddoch et al., 2004), the *Avon Longitudinal Study of Parents and Children*

(Riddoch et al., 2007), the *National Health and Nutrition Examination* (Troiano et al., 2008), *and the Midlands Adolescent Lifestyle Study* (Machado-Rodrigues et al., 2014; Machado-Rodrigues et al., 2011a; Machado-Rodrigues et al., 2011b).

For 6-10 years-old children accelerometer output was interpreted using intensity-based cut-points, which categorizes activity counts as sedentary, light, moderate, or vigorous physical activity. Time spent in moderate-to-vigorous physical activity (MVPA) was calculated using a specific pediatric cut-point for preschool-aged children (Evenson et al., 2008). Children were classified as active if they accumulated at least 60 minutes of MVPA, and non-Active if they do not reach these recommended values.

For pre-school children (aged 3-5 years-old) accelerometer output was interpreted using intensity-based cut-points, which categorizes activity counts as sedentary, light, moderate, or vigorous physical activity. Time spent in MVPA was calculated using a specific pediatric cut-point for preschool-aged children (Pate et al., 2006). Children were classified as active if they accumulated at least 60 minutes of MVPA, and non-Active if they do not reach these recommended values.

2.4. Genotyping

Buccal swabs of volunteer individuals were submitted to DNA extraction, using the FavorPrep^{TM} Genomic DNA Mini Kit (Favorgen® Biotech Corp, Taiwan), according to the instructions of the manufacturer. Briefly, buccal cells were subjected to a lysis buffer and proteinase K. The lysed sample was transferred to a column containing a silica resin that selectively binds DNA. The silica membrane with DNA was then washed to remove impurities (as proteins and salts). The final step in the DNA extraction protocol is the release of pure DNA from the silica membrane with 50 µl of Elution Buffer. Final product was transferred and stored at 4ºC or -20ºC.

Genotyping of the two *SLC6A4* (5-HTT) gene polymorphisms, 5-HTTLPR and STin2, was performed by Polymerase Chain Reaction (PCR) followed by electrophoresis in 2% agarose gels, visualized under ultraviolet light after ethidium bromide staining. Primers for the 5-HTTLPR polymorphism are described in Gelernter et al. (1997): forward JP 5'-ATGCCAGCACCTAACCCCTAATGT-3'and reverse GR 5'-GGACCGCAAGGTGGGCGGGA-3'. Primers for the STin2 polymorphism are described in Uzun et al. (2015): forward 5'-GGTCAGTATCACAGGCTGCGAGTAG-3' and reverse 5'-TGTTCCTAGTCTTACGCCAGTGAAG-3'. PCR amplification was performed in 25 µl reaction volume containing ~50 ng of genomic DNA, 1X PCR buffer,

3.0 mmol $MgCl_2$, 0.5 mmol of each dNTP, 10.0 pmol of each primer and 1 unit of Taq DNA polymerase (Thermo Scientific ™). PCR was carried out in a Biometra TProfessional Thermocycler (Biometra, Goettingen Germany) as follows: 5 min at 94ºC followed by 35 cycles of denaturation at 94ºC for 30sec, annealing at 60ºC for 30sec, extension at 72ºC for 30sec, and a final extension of 5 min at 72ºC.

For the *FTO* SNP rs9939609, samples were genotyped by allelic discrimination assays using the TaqMan® SNP Genotyping Assay C_30090620_10 (Applied Biosystems, Foster City, USA). The detection of polymorphisms in the genome through the real-time polymerase chain reaction, also called quantitative polymerase chain reaction (qPCR), combines amplification, detection and quantification of a specific nucleic acid into a single step. Each predesigned TaqMan SNP Genotyping Assay includes two allele-specific TaqMan MGB probes containing distinct fluorescent dyes (dye FAM™ and dye VIC®) to detect specific SNP targets and a PCR primer pair. During a PCR cycle, the probe hybridizes specifically to the corresponding template, cleaves via the 5′ to 3′ exonuclease activity of Taq DNA polymerase and subsequently increases the FAM™ and VIC® fluorescent emissions. Real-time PCR instruments measure the accumulation of fluorescent signal during the exponential phase of the reaction for precise quantification of PCR products and objective data analysis.

The qPCR amplification was carried out in 20 µl of a total reaction volume containing 1.5 µl (~40 ng) of DNA, 0.2 µM of TaqMan probes in 1x of SsoFast™ Probes Supermix (Bio-Rad, Hercules, CA, USA). PCR conditions were an initial denature step at 95°C for 10 minutes, followed by 40 cycles of 1 minute at 62°C and 15 seconds at 95°C. Fluorescence was visualized through a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). To assess genotyping reproducibility, a random 10% selection of samples were re-genotyped or submitted to automatic sequencing by Sanger's dideoxy chain termination reaction using the Big-Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and the ABI 3130 automatic sequencer (Applied Biosystems).

2.5. Statistical analysis

Genotype and allele frequencies, Hardy-Weinberg equilibrium probability values were calculated for *FTO* rs9939609, 5-HTTLPR and STin2 polymorphisms in the whole population.

For statistical association analyses the population sample was stratified in two groups aged 3-6 and 7-11 years-old. Subjects defined as overweight or obese were merged in one case group. Linear regression models were used to test the association of the studied polymorphisms with obesity-related quantitative traits height (m), weight (kg), BMI (kg/m2), BMI z-score, WC (cm), WHtR, triceps (mm), subscapular (mm), suprailiac (mm) and sum of skinfolds (mm). The association between genotype distributions and both risk of overweight/obesity and risk of abdominal obesity was tested by logistic regression, unadjusted and adjusted for sex and age, and presented as odds ratios (OR) with 95% confidence intervals (CI) and p values. Genotypes with rare variants of the 5-HTTLPR and STin2 polymorphism were not included in the association analysis due to their low frequency in the population. All these statistical analyses were done by using the set-based tests implemented on PLINK software v.1.07 (Purcell et al., 2007; http://pngu.mgh.harvard.edu/purcell/plink/).

Normality of the quantitative data was assessed using the Kolmogorov-Smirnov test. The nonparametric Kruskal-Wallis or Mann-Whitney tests were used to compare quantitative variables between groups. These statistical analyses were performed using the IBM SPSS Statistics software, version 24.0 (SPSS, Inc., Chicago, IL)

3 - Results

3.1. Population characteristics

General characteristics of the study population are summarized in Table 3.1. From a total of 645 participants, 321 (49.8%) were girls and 324 (50.2%) were boys. Significant gender-related differences in several anthropometric measurements were observed ($P < 0.05$). Females showed a significant higher value of triceps, subscapular, suprailiac and sum of skinfolds. Nevertheless, males showed significant higher values of waist circumference and waist to height ratio (WHtR) as well as higher values of physical activity.

According the IOTF cut-offs, the prevalence of excess weight, including overweight and obesity, reach 18.9% in the study sample. Ninety-nine individuals (15.3%) were classified as overweight and 23 (3.6%) as obese (81.1 % of subjects were classified as normal-weight). Boys and girls showed identical prevalence of obesity (3.7% and 3.4%, respectively) and prevalence of overweight is higher in females (17.1% in girls and 13.7% in boys) (Table 3.1).

For the genetic association analyses, the study population was divided into two groups based on age: one group aged 3-6 years-old and a second group aged 7-11 years-old. Children with 3 to 6 years-old include 241 individuals, 112 girls (46.5%) and 129 boys (53.5%), while children between 7 to 11 years-old include 404 individuals, 209 girls (51.7%) and 195 boys (48.3%). General characteristics of the population according the two age groups are detailed in Table 3.2.

Significant age-related differences were observed in the whole population as well as in males and females, except for BMI z-score (Table 3.2). In almost all characteristics, children aged 7-11 years-old showed higher values in comparison with individuals aged 3-6 years-old, except for WHtR (Table 3.2). Curiously, children aged 3-6 years-old had higher values of physical activity, 69.61 *vs.* 53.35 minutes of moderate-to-vigorous physical activity (MVPA) per week.

Table 3.2. General characteristic of the sampled children.

Characteristics	Overall		Female		Male		P
	N	Mean (±SD)	N	Mean (±SD)	N	Mean (±SD)	
Height (cm)	645	125.82 (±0.12)	321	124.48 (±0.12)	324	125.16 (±0.12)	0.470
Weight (kg)	645	26,54 (±7,12)	321	26.48 (±7.44)	324	26.06 (±6.79)	0.573
BMI (kg/m²)	645	16.77 (±2.23)	321	16.81 (±2.38)	324	16.72 (±2.07)	0.874
BMI z-score	638	0.44 (±0.93)	318	0.46 (±0.94)	320	0.42 (±0.92)	0.480
Waist C (cm)	643	57.36 (±6.09)	320	56.85 (±6.25)	323	57.86 (±5.88)	**0.004**
WHtR	643	0.461 (±0.04)	320	0.458 (±0.04)	323	0.464 (±0.04)	**0.04**
Triceps (mm)	641	13.27 (±5.93)	319	14.64 (±6.25)	322	11.92 (±5.26)	**<0.001**
Subscapular (mm)	641	8.82 (±4.93)	319	9.59 (±5.44)	322	8.05 (±4.23)	**<0.001**
Suprailiac (mm)	641	9.78 (±6.97)	319	10.82 (±7.13)	322	8.75 (±6.66)	**<0.001**
Sum of skinfolds (mm)	641	31.87 (±16.85)	319	35.05 (±17.72)	322	28.72 (±15.33)	**<0.001**
PA 7d (min)	436	59.5 (±21.9)	214	54.31 (±20.46)	222	64.51 (±22.12)	**<0.001**
Normal*	645	523 (81.1)	321	255 (79.4)	324	268 (82.7)	χ^2=1.57 P=0.455
Overweight*		99 (15.3)		55 (17.1)		44 (13.6)	
Obese*		23 (3.6)		11(3.4)		12 (3.7)	

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; WHtR, Waist-to-Height Ratio; Waist C, Waist circumference; PA, physical activity.
Data are presented as mean ± standard deviation (SD) for continuous variables and N (%) for categorical variables (*). Quantitative variables were compared between females and males through the Mann-Whitney test. Significant results (P <0.05) are in bold.

Table 3.2. Characteristics of the study population sample stratified according two age groups (3-6 and 7-11 years old) and comparison between the sexes.

Characteristics	Overall					Female					Male				
	3-6 years		7-11 years		P	3-6 years		7-11 years		P	3-6 years		7-11 years		P
	N	Mean (±SD)	N	Mean (±SD)		N	Mean (±SD)	N	Mean (±SD)		N	Mean (±SD)	N	Mean (±SD)	
Height (cm)	241	113.38 (±0.08)	404	131.49 (±0.08)	**<0.001**	112	112.04 (±0.08)	209	130.97 (±0.08)	**<0.001**	129	114.55 (±0.08)	195	132.05 (±0.08)	**<0.001**
Weight (kg)	241	20.95 (±3.83)	404	29.87 (±6.51)	**<0.001**	112	20.44 (±3.78)	209	29.71 (±6.88)	**<0.001**	129	21.4 (±3.83)	195	30.04 (±6.11)	**<0.001**
BMI (kg/m²)	241	16.15 (±1.48)	404	17.13 (±2.51)	**<0.001**	112	16.15 (±1.46)	209	17.17 (±2.69)	**0.002**	129	16.16 (±1.5)	195	17.09 (±2.31)	**<0.001**
BMI z-score	235	0.40 (±0.85)	403	0.46 (±0.98)	0.581	109	0.46 (±0.81)	209	0.47 (±0.81)	0.907	126	0.36 (±0.88)	194	0.46 (±0.95)	0.408
Waist C (cm)	240	53.42 (±3.92)	403	59.4 (±6.22)	**<0.001**	111	53.13 (±4.01)	209	58.82 (±6.34)	**<0.001**	111	54.59 (±3.73)	194	60.03 (±6.05)	**<0.001**
WHtR	240	0.48 (±0.04)	403	0.45 (±0.04)	**<0.001**	129	0.47 (±0.04)	209	0.45 (±0.04)	**<0.001**	129	0.47 (±0.03)	194	0.45 (±0.04)	**<0.001**
Triceps (mm)	238	11.22 (±3.64)	403	14.49 (±6.65)	**<0.001**	110	12.11 (±3.82)	209	15.97 (±6.86)	**<0.001**	128	10.46 (±3.29)	194	12.89 (±6.05)	**<0.001**
Subscapular (mm)	238	7.26 (±2.51)	403	9.73 (±5.72)	**<0.001**	110	7.82 (±2.79)	209	10.52 (±6.22)	**0.002**	128	6.79 (±2.14)	194	8.88 (±5.01)	**<0.001**
Suprailiac (mm)	238	7.81 (±4.19)	403	10.94 (±7.96)	**<0.001**	110	8.59 (±4.43)	209	11.99 (±7.97)	**<0.001**	128	7.14 (±3.86)	194	9.81 (±7.81)	**0.001**
Sum of skinfolds (mm)	238	26.29 (±9.44)	403	35.16 (±19.24)	**<0.001**	110	28.52 (±9.89)	209	38.48 (±19.86)	**<0.001**	128	24.39 (±8.63)	194	31.58 (±17.93)	**<0.001**
PA 7d (min)	165	69.61 (±24.84)	271	53.35 (±17.25)	**<0.001**	71	63.3 (±24.31)	143	49.85 (±16.62)	**<0.001**	94	74.38 (±24.29)	128	57.26 (±17.16)	**<0.001**
Normal *	241	201 (83.4)	404	322 (79.7)	χ^2=1.41 P=0.493	112	91 (81.3)	209	164 (78.5)	χ^2=0.46 P=0.792	129	110 (85.3)	195	158 (81.0)	χ^2=0.98 P=0.612
Overweight *		33 (13.7)		66 (19.3)			18 (16.1)		37 (17.7)			15 (11.6)		29 (14.9)	
Obese *		7 (2.9)		16 (4.0)			3 (2.7)		8 (3.8)			4 (3.1)		8 (4.1)	

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; WHtR, Waist-to-Height Ratio; Waist C, Waist circumference; PA, physical activity. Data are presented as mean ± standard deviation (SD) for continuous variables and N (%) for categorical variables (*). Quantitative variables were compared between the two age groups through the Mann-Whitney test. Significant results (P <0.05) are in bold.

3.2. Population genetics

Genotypes and allele frequencies for the three studied polymorphisms are detailed in Table 3.3. The genotyping success rate in the total sample of 645 subjects was 96.6% for 5-HTTLPR and 87.9% for STin2. The *FTO* SNP was genotyped in 436 subjects with information for physical activity, with a genotyping success rate of 96.6%. The genotype distributions in the whole population were in Hardy-Weinberg equilibrium for polymorphisms *FTO* SNP rs9939609 (P = 1) and 5-HTTLPR (P = 0.1). Polymorphism STin2 showed a significant HWE p-value (P = 0.03) that could be explained by the two rare genotypes (10/9 and 9/9) identified in the sample.

Table 3.3. Genotype and allele frequencies for the three polymorphism 5-HTTLPR, STin2 and FTO in the sample of Portuguese children.

	Polymorphisms	**N (Freq)**		**P HWE**
		Genotypes	Alleles	
General population (645)	FTO (421)	TT: 163 (0.387) TA: 198 (0.470) AA: 60 (0.143)	T: 0.622 A: 0.378	1
	5-HTTLPR (623)	LL: 188 (0.302) LS: 289 (0.464) SS: 144 (0.231) LSv: 2 (0.003)	L: 0.535 S: 0.463 Sv: 0.002	0.1
	STin2 (567)	12 12: 219 (0.386) 12 10: 267 (0.471) 10 10: 72 (0.127) 12 9: 7 (0.012) 10 9: 1 (0.002) 9 9: 1 (0.002)	12: 0.627 10: 0.363 9: 0.01	0.03

Abbreviations: N, number of genotyped samples; Freq, frequency; HWE, Hardy–Weinberg Equilibrium.

3.3. Associations with obesity-related quantitative traits

In Tables 3.4, 3.5 and 3.6 we detail the results of the association analysis between the three polymorphism and obesity-related quantitative traits in the two age groups, 3-6 years-old and 7-11 years-old.

Regarding the 5-HTTLPR polymorphism, a statistically significant association was found in children aged 7-11 years-old for subscapular and suprailiac skinfold measures (P = 0.02 and P = 0.04, respectively) (Table 3.4). No other associations were found in the remaining anthropometric parameters in the overall sample. Moreover, when the population was split by physical activity, the previous association was lost both in physical active and non-active individuals (P >0.05). No significant associations were found in children aged 3-6 years-old.

For the STin2 polymorphisms no statistically significant associations were found among the analyzed parameters (P >0.05) in both age groups 3-6 years old and 7-11 years-old, although a near significant association was found in children aged between 7-11 years-old for suprailiac skinfold thickness (P = 0.06) (Table 3.5). Also, when the population was split by physical activity no significant associations were found both in active and non-active individuals (P >0.05).

The *FTO* polymorphism showed a statistically significant association with BMI Z-score (P = 0.04) in children aged between 7-11 years (Table 3.6). Although no other significant associations were found, a marginal association was found for WHtR and BMI (P = 0.07) in the same age group. When we tested association split by physical activity, statistically significant associations were found for several obesity-related anthropometric parameters in non-active individuals aged 7-11 years, including for BMI (P = 0.02), BMI Z-score (P = 0.027), waist circumference (P = 0.035), WHtR (P = 0.045), subscapular skinfold (P = 0.015) and sum of skinfolds (P = 0.046). No significant associations were found in children aged 3-6 years in whole sample or in both active and non-active groups (P >0.05) (Table 3.6).

Table 3.4. Association of 5-HTTLPR polymorphism with obesity-related quantitative traits.

Trait	5-HTTLPR			P	P*	P**
	LL	LS	SS			
3-6 years						
N	61	121	48	230	66	94
Height (cm)	114.05 (±0.07)	113.22 (±0.09)	112.43 (±0.09)	0.89	0.46	0.74
Weight (kg)	21.15 (±3.19)	20.93 (±3.94)	20.73 (±4.12)	0.82	0.94	0.55
BMI (kg/m^2)	16.16 (±1.39)	16.17 (±1.54)	16.19 (±1.32)	0.94	0.76	0.73
BMI z-score	0.45 (±0.82)	0.25 (±0.88)	0.42 (±0.76)	0.97	0.72	0.70
Waist C (cm)	54.33 (±3.57)	53.72 (±4.04)	53.93 (±3.6)	0.35	0.57	0.73
WHtR	0.48 (±0.03)	0.48 (±0.04)	0.48 (±0.03)	0.58	0.09	0.81
Triceps (mm)	11.37 (±3.3)	11.41 (±3.82)	10.69 (±3.52)	0.31	0.55	0.22
Subscapular (mm)	6.98 (±1.92)	7.48 (±2.82)	7.09 (±2.15)	0.96	0.41	0.21
Suprailiac (mm)	7.45 (±3.17)	7.95 (±4.45)	7.81 (±4)	0.92	0.56	0.43
Sum of skinfolds (mm)	25.8 (±7.59)	26.84 (±10.09)	25.59 (±9.09)	0.69	0.59	0.379
7-11 years						
N	127	168	96	391	171	91
Height (cm)	131.97 (±0.08)	131.34 (±0.08)	130.95 (±0.07)	0.58	0.74	0.25
Weight (kg)	30.22 (±6.36)	30.07 (±6.84)	28.97 (±6.36)	0.21	0.92	0.33
BMI (kg/m^2)	17.23 (±2.46)	17.26 (±2.58)	16.8 (±2.51)	0.14	0.31	0.95
BMI z-score	0.50 (±0.96)	0.53 (±0.96)	0.32 (±1)	0.14	0.17	0.99
Waist C (cm)	59.79 (±6.27)	59.41 (±6.07)	58.67 (±6.59)	0.14	0.83	0.47
WHtR	0.45 (±0.04)	0.45 (±0.04)	0.45 (±0.04)	0.27	0.44	0.58
Triceps (mm)	14.78 (±6.55)	14.61 (±6.46)	13.99 (±7.36)	0.15	0.85	0.52
Subscapular (mm)	10.44 (±6.29)	9.59 (±5.38)	9 (±5.6)	**0.02**	0.16	0.33
Suprailiac (mm)	11.47 (±8.32)	10.74 (±7.26)	10.38 (±8.69)	**0.04**	0.64	0.36
Sum of skinfolds (mm)	36.69 (±20.15)	34.94 (±18.04)	33.38 (±20.51)	0.06	0.60	0.49

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; WHtR, Waist-to-Height Ratio; Waist C, Waist circumference. Data are presented as mean ± standard deviation.

P-values were obtained using the Kruskal–Wallis test. P-values nominally significant ($P < 0.05$) are in bold. P* is for non-active individuals and P** is for active individuals.

Table 3.5. Association of STin2 polymorphism with obesity-related quantitative traits.

Trait	STin2			P	P*	P**
	12 12	12 10	10 10			
3-6 years						
N	91	96	32	219	61	94
Height (cm)	113.67 (±0.08)	112.25 (±0.08)	114.71 (±0.08)	0.29	0.98	0.34
Weight (kg)	21.14 (±3.65)	20.64 (±3.84)	21.63 (±4.39)	0.44	0.59	0.29
BMI (kg/m^2)	16.22 (±1.48)	16.2 (±1.49)	16.19 (±1.61)	0.83	0.18	0.57
BMI z-score	0.43 (±0.85)	0.47 (±0.86)	0.39 (±0.86)	0.61	0.14	0.28
Waist C (cm)	54.2 (±3.57)	53.7 (±3.91)	54.36 (±4.84)	0.46	0.62	0.88
WHtR	0.48 (±0.03)	0.48 (±0.04)	0.47 (±0.03)	0.81	0.77	0.30
Triceps (mm)	10.89 (±3.5)	11.57 (±4.06)	11.63 (±3.43)	0.32	0.51	0.17
Subscapular (mm)	6.96 (±2.01)	7.57 (±2.94)	7.48 (±2.68)	0.36	0.05	0.55
Suprailiac (mm)	7.81 (±3.97)	8.16 (±4.66)	8.06 (±4.34)	0.78	0.25	0.48
Sum of skinfolds (mm)	25.67 (±8.75)	27.29 (±10.68)	27.17 (±9.86)	0.55	0.32	0.34
7-11 years						
N	128	171	40	339	145	78
Height (cm)	130.87 (±0.08)	131.45 (±0.07)	131.78 (±0.08)	0.19	0.31	0.87
Weight (kg)	29.6 (±6.95)	29.58 (±5.79)	31.93 (±9.11)	0.72	0.55	0.90
BMI (kg/m^2)	17.1 (±2.56)	17.02 (±2.37)	18.09 (±3.66)	0.89	0.60	0.73
BMI z-score	0.45 (±0.98)	0.44 (±1.)	0.68 (±1.13)	0.92	0.57	0.99
Waist C (cm)	59.33 (±6.96)	59.05 (±5.7)	61.09 (±7.32)	0.76	0.72	0.68
WHtR	0.45 (±0.04)	0.45 (±0.04)	0.46 (±0.05)	0.17	0.23	0.46
Triceps (mm)	14.26 (±7.36)	14.39 (±6.48)	16.53 (±7.11)	0.25	0.29	0.44
Subscapular (mm)	9.94 (±6.36)	9.24 (±5.04)	12.14 (±7.93)	0.36	0.36	0.51
Suprailiac (mm)	10.9 (±8.64)	10.55 (±7.02)	13.49 (±10.85)	0.06	0.44	0.78
Sum of skinfolds (mm)	35.11 (±21.46)	34.18 (±17.27)	42.16 (±24.88)	0.25	0.26	0.57

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; WHtR, Waist-to-Height Ratio; Waist C, Waist circumference. Data are presented as mean ± standard deviation.

P-values were obtained using the Kruskal–Wallis test. P-values nominally significant ($P < 0.05$) are in bold. P* is for non-active individuals and P** is for active individuals.

Table 3.6. Association of FTO polymorphism with obesity-related quantitative traits.

Trait	FTO			P	P*	P**
	T T	T A	A A			
3-6 years						
N	61	75	20	151	81	76
Height (cm)	113.89 (±0.09)	114.10 (±0.09)	115.72 (±0.06)	0.63	0.27	0.61
Weight (kg)	21.28 (±4.22)	21.77 (±4.11)	22.32 (±2.93)	0.44	0.31	0.81
BMI (kg/m^2)	16.29 (±1.46)	16.5 (±1.61)	16.51 (±1.82)	0.78	0.41	0.50
BMI z-score	0.43 (±0.83)	0.59 (±0.86)	0.69 (±1.04)	0.42	0.70	0.47
Waist C (cm)	54.14 (±4.27)	54.94 (±4.1)	55.14 (±4.06)	0.27	0.35	0.11
WHtR	0.48 (±0.03)	0.48 (±0.04)	0.48 (±0.04)	0.74	0.52	0.09
Triceps (mm)	11.48 (±3.55)	11.49 (±4.17)	11.67 (±3.79)	0.99	0.50	0.61
Subscapular (mm)	7.39 (±2.55)	7.51 (±2.62)	8.02 (±4.06)	0.93	0.56	0.83
Suprailiac (mm)	8.37 (±4.99)	8.19 (±4.35)	7.84 (±3.14)	0.99	0.29	0.50
Sum of skinfolds (mm)	27.23 (±10.37)	27.19 (±10.23)	27.54 (±10.06)	0.98	0.34	0.60
7-11 years						
N	102	123	40	265	164	82
Height (cm)	131.92 (±0.07)	132.60 (±0.08)	132.12 (±0.09)	0.49	0.511	0.270
Weight (kg)	30.71 (±6.24)	30.47 (±7)	32.3 (±7.98)	0.36	0.092	0.305
BMI (kg/m^2)	17.51 (±2.43)	17.16 (±2.63)	18.33 (±3.25)	0.07	**0.020**	0.405
BMI z-score	0.66 (±0.95)	0.44 (±0.95)	0.92 (±1.18)	**0.04**	**0.027**	0.704
Waist C (cm)	59.88 (±5.99)	59.9 (±6.13)	62.38 (±7.95)	0.11	**0.035**	0.347
WHtR	0.45 (±0.03)	0.45 (±0.04)	0.47 (±0.05)	0.07	**0.045**	0.521
Triceps (mm)	15.59 (±6.96)	14.47 (±6.29)	16.49 (±8.45)	0.44	0.056	0.495
Subscapular (mm)	10.19 (±5.16)	9.67 (±5.54)	12.39 (±8.78)	0.18	**0.015**	0.560
Suprailiac (mm)	11.59 (±7.65)	11.19 (±7.54)	14.63 (±11.42)	0.32	0.087	0.292
Sum of skinfolds (mm)	37.37 (±18.74)	35.34 (±18.35)	43.52 (±27.49)	0.33	**0.046**	0.339

Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation; WHtR, Waist-to-Height Ratio; Waist C, Waist circumference. Data are presented as mean ± standard deviation.

P-values were obtained using the Kruskal–Wallis test. P-values nominally significant ($P < 0.05$) are in bold. P* is for non-active individuals and P** is for active individuals.

3.4. Associations with overweight/obesity

We merged overweight and obese subjects in one group and the association with overweight/obesity was tested in a case-control model in both groups of children aged 3-6 years-old (Table 3.7) and 7-11 years-old (Table 3.8), in the whole samples and split by physical activity.

In the age group 3-6 years-old, the binary logistic regression model, revealed no statistically significant associations with overweight/obesity ($P > 0.05$) for both the *SLC6A4* polymorphisms (5-HTTLPR and STin2) or the *FTO* polymorphism, in the whole sample as well as in both non-active and active subsamples (Table 3.7).

In children aged 7-11 years-old, logistic regression, in the recessive model, revealed a marginal association of the *FTO* rs9939609 A allele with overweight/obesity ($P = 0.09$) for the whole population (Table 3.8). When the population was split by physical activity, a statistically significant association with risk of obesity was observed for the *FTO* rs9939609 A allele in non-active individuals (OR 2.619; 95% CI, 1.058-6.483; $P = 0.037$) but not in active individuals ($P = 0.99$) (Table 3.8). In concordance, in the low physical activity group, children carrying the homozygous minor allele genotype rs9939609 AA ($N = 24$) had a statistically significant higher BMI (18.67 ± 3.21 kg/m^2) compared with non-AA homozygous TT+TA ($N = 140$) (17.22 ± 2.45 kg/m^2) ($P = 0.026$). There was no statistically significant difference in the BMI between the rs9939609 genotypes AA *vs.* TT+TA ($P = 0.48$) in the group with a high level of physical activity ($N = 11$; BMI 16.45 ± 1.98 kg/m^2 *vs.* $N = 71$; BMI 17.05 ± 2.6 kg/m^2, respectively). No significant association was found with overweight/obesity for the 5-HTTLPR and STin2 polymorphisms ($P > 0.05$) in children aged 7-11 years-old, for the whole sample as well as in both non-active and active subsamples (Table 3.8).

3.5. Associations with abdominal obesity

We also tested for the association between polymorphisms and risk of abdominal obesity as defined by a conventional cut-off for WHtR ≥ 0.50. Sixty-nine subjects 3-6 years old and 51 subjects 7-11 years old were classified with abdominal obesity (WHtR ≥ 0.50).

Logistic regression showed no statistically significant associations ($P > 0.05$) with abdominal obesity in children aged 3-6 years-old for any polymorphic site (Table 3.9).

In children aged 7-11 years-old, a strong significant association with risk of abdominal obesity was observed for the *FTO* rs9939609 A allele for the whole sample (OR 3.93; 95% CI, 1.83-8.44; $P = 4.5x10^{-4}$) (Table 3.10). When the sample was stratified according physical activity, the statistically significant association was maintained in non-active individuals (OR 4.341; 95% CI, 1.65-11.45; $P = 0.003$) but, in contrast, was lost in physical active children ($P = 0.165$). No statistically significant association with abdominal obesity was found for the *SLC6A4* polymorphisms (5-HTTLPR and STin2) in children aged 7-11 years old, for the whole sample as well as in both non-active and active subsamples (Table 3.10).

Table 3.7. Association of polymorphisms in genes SLC6A4 (5HTTLPR and STin2) and FTO (rs9939609) with risk of overweight/obesity in the sample of Portuguese children aged between 3 and 6 years old.

Groups	Locus (N)	Alleles	Normal N (freq)	OB/OW N (freq)	OR (95% CI)	P
Overall (241)	FTO (156)	A	85 (0.357)	30 (0.405)	1.452 (0.5147-4.094)	0.481
		T	153 (0.643)	44 (0.595)		
	5-HTTLPR (230)	S	183 (0.477)	34 (0.447)	0.8316 (0.3416-2.025)	0.685
		L	201 (0.523)	42 (0.553)		
	STin2 (219)	10	132 (0.367)	28 (0.359)	1.077 (0.4107-2.824)	0.880
		12	228 (0.633)	50 (0.641)		
Active (97)	FTO (88)	A	45 (0.336)	19 (0.475)	2.857 (0.7948-10.27)	0.108
		T	91 (0.664)	21 (0.525)		
	5-HTTLPR (94)	S	71 (0.473)	16 (0.421)	0.3945 (0.08272-1.881)	0.243
		L	79 (0.527)	22 (0.579)		
	STin2 (94)	10	51 (0.342)	13 (0.325)	0.6263 (0.127-3.088)	0.565
		12	97 (0.658)	27 (0.675)		
Non-active (67)	FTO (60)	A	40 (0.408)	8 (0.364)	0.0000002364 (0-inf)	0.998
		T	58 (0.592)	14 (0.636)		
	5-HTTLPR (66)	S	54 (0.491)	11 (0.5)	1.344 (0.308-5.863)	0.694
		L	56 (0.509)	11 (0.5)		
	STin2 (61)	10	38 (0.373)	9 (0.45)	1.344 (0.2398-7.53)	0.737
		12	64 (0.657)	11 (0.55)		

Data are presented as mean ± standard deviation

Abbreviations: OB/OW, obese/overweight; freq, frequency; OR, odds ratio; CI, confidence interval; N, number of samples/alleles The OR and p values were obtained by logistic regression under a recessive model. The nominal significant results ($p<0.05$) are in bold and underlined.

Table 3.8. Association of polymorphisms in genes SLC6A4 (5HTTLPR and STin2) and FTO (rs9939609) with risk of overweight/obesity in the sample of Portuguese children aged between 7 and 11 years old.

Groups	Locus (N)	Alleles	Normal N (freq)	OB/OW N (freq)	OR (95% CI)	P
Overall (404)	FTO (265)	A	146 (0.374)	57 (0.407)	1.855 (0.913-3.767)	0.087
		T	244 (0.626)	83 (0.593)		
	5-HTTLPR (391)	S	288 (0.463)	72 (0.45)	0.946 (0.532-1.683)	0.851
		L	334 (0.537)	88 (0.55)		
	STin2 (339)	10	194 (0.363)	57 (0.396)	1.707 (0.820-3.553)	0.152
		12	340 (0.637)	87 (0.604)		
Active (94)	FTO (82)	A	47 (0.351)	11 (0.367)	0.991 (0.191-5.142)	0.991
		T	87 (0.649)	19 (0.633)		
	5-HTTLPR (91)	S	77 (0.5)	11 (0.393)	0.391 (0.081-1.889)	0.242
		L	77 (0.5)	17 (0.607)		
	STin2 (78)	10	48 (0.364)	10 (0.417)	1.867 (0.4293-8.116)	0.405
		12	84 (0.636)	14 (0.583)		
Non-active (177)	FTO (164)	A	93 (0.375)	37 (0.463)	2.619 (1.058-6.483)	**0.037**
		T	155 (0.625)	43 (0.537)		
	5-HTTLPR (171)	S	120 (0.458)	36 (0.45)	1.122 (0.492-2.557)	0.783
		L	142 (0.542)	44 (0.55)		
	STin2 (145)	10	88 (0.4)	29 (0.414)	1.862 (0.634-5.469)	0.258
		12	132 (0.6)	41 (0.586)		

Data are presented as mean ± standard deviation

Abbreviations: OB/OW, obese/overweight; freq, frequency; OR, odds ratio; CI, confidence interval; N, number of samples/alleles The OR and p values were obtained by logistic regression under a recessive model. The nominal significant results ($p<0.05$) are in bold and underlined.

Table 3.9. Association of polymorphisms in genes SLC6A4 (5HTTLPR and STin2) and FTO (rs9939609) with risk of abdominal obesity in the sample of Portuguese children aged between 3 and 6 years old.

Groups	Locus (N)	Alleles	Normal N (freq)	Abdominal N (freq)	OR (95% CI)	P
Overall (240)	FTO (155)	A	76 (0.362)	38 (0.38)	0.886 (0.319-2.463)	0.817
		T	134 (0.638)	62 (0.62)		
	5-HTTLPR (229)	S	150 (0.4601)	65 (0.492)	1.538 (0.7799-3.034)	0.214
		L	176 (0.5399)	67 (0.508)		
	STin2 (218)	10	112 (0.366)	47 (0.361)	0.908 (0.3954-2.087)	0.821
		12	194 (0.634)	83 (0.639)		
Active (97)	FTO (87)	A	35 (0.324)	28 (0.424)	1.199 (0.3472-4.141)	0.774
		T	73 (0.676)	38 (0.576)		
	5-HTTLPR (93)	S	53 (0.457)	32 (0.457)	1.068 (0.3712-3.073)	0.903
		L	63 (0.543)	38 (0.543)		
	STin2 (93)	10	40 (0.351)	23 (0.319)	0.427 (0.1091-1.673)	0.222
		12	74 (0.649)	49 (0.681)		
Non-active (67)	FTO (60)	A	41 (0.418)	7 (0.318)	0.0000236 (0-inf)	0.998
		T	57 (0.582)	15 (0.682)		
	5-HTTLPR (66)	S	54 (0.4909)	11 (0.5)	1.344 (0.308-5.863)	0.694
		L	56 (0.5091)	11 (0.5)		
	STin2 (61)	10	38 (0.373)	9 (0.45)	1.344 (0.239-7.530)	0.737
		12	64 (0.627)	11 (0.55)		

Data are presented as mean ± standard deviation

Abbreviations: freq, frequency; OR, odds ratio; CI, confidence interval; N, number of samples/alleles The OR and p values were obtained by logistic regression under a recessive model. The nominal significant results ($p<0.05$) are in bold and underlined.

Table 3.20. Association of polymorphisms in genes SLC6A4 (5HTTLPR and STin2) and FTO (rs9939609) with risk of abdominal obesity in the sample of Portuguese children aged between 7 and 11 years old.

Groups	Locus (N)	Alleles	Normal N (freq)	Abdominal N (freq)	OR (95% CI)	P
Overall (403)	FTO (264)	A	158 (0.353)	45 (0.549)	3.929 (1.830-8.436)	**4.5×10^{-4}**
		T	288 (0.646)	37 (0.451)		
	5-HTTLPR (390)	S	316 (0.465)	44 (0.44)	0.962 (0.481-1.927)	0.914
		L	364 (0.535)	56 (0.56)		
	STin2 (339)	10	215 (0.368)	36 (0.383)	1.667 (0.716-3.879)	0.236
		12	369 (1.632)	58 (0.617)		
Active (93)	FTO (81)	A	51 (0.34)	7 (0.583)	3.667 (0.585-22.97)	0.165
		T	99 (0.66)	5 (0.417)		
	5-HTTLPR (90)	S	76 (0.458)	10 (0.714)	4.2 (0.8658-20.37)	0.075
		L	90 (0.542)	4 (0.286)		
	STin2 (78)	10	52 (0.361)	6 (0.5)	2.773 (0.4516-17.02)	0.271
		12	92 (0.639)	6 (0.5)		
Non-active (177)	FTO (164)	A	100 (0.362)	30 (0.577)	4.341 (1.646-11.45)	**0.003**
		T	176 (0.638)	22 (0.423)		
	5-HTTLPR (171)	S	133 (0.456)	23 (0.46)	1.332 (0.5123-3.462)	0.557
		L	159 (0.544)	27 (0.54)		
	STin2 (145)	10	100 (0.413)	17 (0.354)	1.092 (0.2882-4.136)	0.897
		12	142 (0.587)	31 (0.646)		

Data are presented as mean ± standard deviation

Abbreviations: freq, frequency; OR, odds ratio; CI, confidence interval; N, number of samples/alleles The OR and p values were obtained by logistic regression under a recessive model. The nominal significant results ($p<0.05$) are in bold and underline.

4- Discussion

In this study we investigated in a sample of Portuguese children the possible associations with several obesity-related parameters, the risk of overweight/obesity and abdominal obesity for the *FTO* polymorphism rs9939609 and the two *SLC6A4* variants 5-HTTLPR and STin2. Moreover, we also tested for interaction effects between the study polymorphisms and physical activity in relation to obesity.

4.1. *SLC6A4* polymorphisms

For the 5-HTTLPR polymorphism, we found a statistically significant association with two skinfold measurements (subscapular P = 0.02 and suprailiac P = 0.04) in children aged 7-11 years-old. No other associations were found between 5-HTTLPR and the remaining anthropometric variables in both age groups 3-6 years-old and 7-11 years-old. Moreover, when tested for the association with overweight/obesity and risk of abdominal obesity no significant results were found for 5-HTTLPR.

To our knowledge, this is the first study in Portuguese children testing for the possible association of the 5-HTTLPR polymorphism with obesity. Previous works in children from other populations, found that S allele carriers were associated with a higher risk of obesity. A study by Sookoian et al. (2007) reported that Argentinean children/adolescents with the S allele presented a greater risk of being obese/overweight, and a recent study by Miranda et al. (2017) demonstrated that Brazilian children homozygotes (SS) had higher anthropometric parameters and higher food intake than homozygous (LL).

Most association studies between 5-HTTLPR and obesity were conducted in adults and found significant association between the S allele and obesity. A study by Iordanidou et al. (2010) found that the 5-HTTLPR S allele was strongly associated with the presence of T2D in Greeks independent of obesity status, but failed to demonstrate an association with obesity. Other study of Sookoian et al. (2008) found an association between the S allele and obesity in young adult men, similar to their previous work in children (Sookoian et al., 2007). Another study in young adults, reported a strong association between the 5-HTTLPR S allele and BMI (Fuemmeler et al., 2008). Furthermore, Lan et al (2009) demonstrated that the SS genotype was associated with a significant increase in BMI and risk obesity in non-elderly, but not in elderly patients with stroke. However, some studies found no such association results between 5-HTTLPR and categories of BMI. Mergen et al. (2007) found no significant association between the 5-HTTLPR and obesity in Turkish adults, and Hameed et al. (2015) failed to find any association with T2D and obesity in the Pakistani population. On the other

hand, Bah et al. (2010) showed opposite results in significant associations, as they reported homozygotes SS to be more frequent in underweight subjects. Also Peralta-leal et al. (2012) showed a direct association between BMI and the L allele, similar to Borkowska et al. (2015) that found the L allele associated with obesity in Polish adults. Interesting enough, in the same work, they detect that the S allele was associated with the development of depressive temperament. Finally, in a recent work conducted in Portuguese young adults, an association between L-allele and risk of obesity was also found, but only in less active individuals (Dias et al., 2016)

The association of 5-HTTLPR polymorphism with obesity has been largely investigated because of its role in controlling serotonin transmission in regions of the brain associated with mood regulation, ingestion of food, energy expenditure and weight adjustment (Borkowska et al., 2015). Authors of the studies that found the L allele associated with obesity, argue that the S allele is associated with higher availability of 5-HT in central serotonergic synapses, which should intensify satiety, reducing food intake and inducing lower BMI and fat mass (Peralta-leal et al., 2012).

For the STin2 polymorphism, located in the second intron of the *SLC6A4* gene, the present study reports no significant association with obesity-related quantitative traits, risk of overweight/obesity or abdominal obesity in both age groups of children, even when stratified by physically activity. Our study is the first to test for the possible association between *SLC6A4* STin2 polymorphism and obesity in Portuguese children. Only two studies until now have tested this polymorphism for association with obesity, and none have used children. Uzun et al. (2015) found no significant effects on Turkish adults. Dias et al. (2016) found no significant association in a population sample of Portuguese young adults, but when stratified by physical activity a significant association was observed between the STin2 10 allele and overweight/obesity in less active subjects.

When the study population was stratified by physical activity no significant association results were found between the SLC6A4 polymorphisms (5-HTTLPR and STin2) and obesity risk in non-active or active subjects in both children aged 3-6 years or 7-11 years. Thus, our study revealed no interaction effects between physical activity and the SLC6A4 polymorphisms (5-HTTLPR and STin2) on obesity risk, which is in contrast with results obtained in Portuguese young adults where physical activity seems to accentuate the influence of SLC6A4 polymorphisms on risk of obesity (Dias et al., 2016). However, no other study conducted thus far assessed for interactions between *SLC6A4* polymorphisms

and physical activity on obesity risk to allow for comparisons with data from other populations.

4.2. *FTO* rs9939609 polymorphism

The *FTO* gene has been the focus of several studies, and its strong association with obesity has been confirmed in European and other different populations (Albuquerque et al., 2013; González-Sánchez et al., 2009; Hubacek et al., 2008; Loos & Yeo, 2014). In children aged 7-11 years old, we have found significant association of the *FTO* SNP rs9939609 with only one anthropometric measure (BMI Z-sore P = 0.04) for the whole group, in contrast with a report of Albuquerque et al. (2013) that found associations with several obesity related parameters in a previous study in Portuguese children. In contrast, a recent report in Portuguese children showed that the *FTO* rs9939609 polymorphism has no effect, or eventually a recessive very weak increasing on BMI z-score (Almeida et al., 2018). Nevertheless, some studies in other populations have reported no association results between *FTO* SNPs and obesity related quantitative traits (H. Li et al., 2008; Ohashi et al., 2007).

When testing for the risk of overweight/obesity in the whole sample, we found a marginal association between the *FTO* polymorphism and overweight/obesity in children aged 7-11 years-old (P = 0.087). Albuquerque et al. (2013) reported in Portuguese children a significant association between the *FTO* rs9939609 AA genotype with obesity (P = 0.026) but not with overweight. Similarly, previous studies performed in other European populations reported the association of *FTO* rs9939609 A-allele with obesity (Dina et al., 2007; Frayling et al., 2007; González-Sánchez et al., 2009; Scuteri et al., 2007).

The rs9939609 polymorphism is the most replicated polymorphism associated with obesity across the world. We failed to find strong significant associations in our study general population of children which could be due to the low statistical power (because of a low sample size, low carrier cases frequency or small genetic effects).

4.3. Gene Vs. Physical Activity interaction

When the study population was stratified by physical activity, we found statistically significant associations for several obesity-related traits in non-active individuals aged 7-11 years-old. Moreover, in the same age stratus, a statistically significant association was found between rs9939609 and the risk of overweight/obesity (P = 0.037) as well as for BMI between AA vs. TT+TA genotypes (P = 0.026). This provides evidence that physical

inactivity accentuates the influence of *FTO* gene on the risk of obesity, as described in previous studies for adults and adolescents (Ahmad et al., 2013; Kilpeläinen et al., 2011; Kim et al., 2016; S. Li et al., 2010; Muc et al., 2015). The meta-analysis by Kilpeläinen et al. (2011) showed that the effect of FTO rs9939609 on BMI, waist circumference, body fat percentage and obesity risk is approximately 30% lower in physically active than in sedentary adults, but no such interaction was observed for children and adolescents. In a previous study, Lee et al. (2010) showed that in a low physical activity subgroup of 7 to 10 years-old children of Korean ethnicity, rs9939609 A allele carriers had a higher BMI than TT carriers (P=0.0147). Thus, to our knowledge, our study is the first clearly suggesting in children of European ancestry an interaction effect between the FTO genotype and physical activity on obesity.

4.4. Association with abdominal obesity

In our study we also tested association for the risk of abdominal obesity, which is more strongly correlated with metabolic risk factors in children than BMI, as indicated by higher value of WHtR (Khoury et al., 2013). We report a strong association between the *FTO* SNP rs9939609 and risk of abdominal obesity (OR 3.93) in children aged 7-11 years-old, and that the *FTO* influence on risk of abdominal obesity seems to increase in non-physical active individuals (OR 4.341) as previous observed for obesity. However, to our knowledge no other study conducted thus far assessed for associations between *FTO* rs9939609 polymorphisms and risk of abdominal obesity.

4.5. Age-related associations

Interestingly, we noted that the associations between the FTO polymorphism and risk of obesity changed across the two age groups. Several significant associations were found in children aged 7-11 years-old in the non-active group (as well as a marginal association in the whole population), while in children aged 3-6 years-old no such associations were found. This age-dependent association was previously reported in a longitudinal study showing that the association of *FTO* rs9939609 with BMI and weight increases during childhood up to age 20 years and then weakened during later adulthood (Hardy et al., 2010). Also a study revealed that the *FTO* genotype only influence BMI after the age of 7 years (Hakanen et al., 2009). A recent study in Chinese individuals revealed that association between *FTO*

rs9939609 and obesity-related traits may change from childhood to adolescence, and the association may start as early as age 12 years (Zhang et al., 2014).

Also, our results revealed that, at least in Portuguese children, the two *SLC6A4* polymorphisms do not have the same effect on risk of obesity than in adults as reported in Dias et al. (2016). This result could be related with age of individuals under study since the 5-HTTLPR effects in obesity have been previous described to be age dependent (Lan et al., 2009).

5- Conclusion

Our results replicate, at least in non-active individuals, the well-known association, both in children and adults, between the *FTO* rs9939609 polymorphism and obesity or obesity-related parameters. We observed an interaction effect between the *FTO* gene and physical activity in obesity, since genetic associations were observed in non-active but not in active subjects. This result is in accordance with previous studies, both in children and adults, suggesting that that moderate to vigorous physical activity attenuates the effect of *FTO* genetic susceptibility to obesity.

We also observed that the *FTO* rs9939609 association with risk of obesity is age-dependent. Children aged 7-11 years-old showed associations with risk of obesity, abdominal obesity and several obesity related parameters, but such significant results were not observed in children aged 3-6 years-old. Thus, our results suggest that the association between *FTO* gene and obesity becomes evident only after 7 years-old, as observed in previous studies.

It was not possible to replicate previous results in children regarding the association of 5-HTTLPR polymorphism with risk of obesity. Possible explanations could be the low statistical power, age differences on the analysed samples or ethnicity.

Although being a small population-based genetic association study, focusing only in three candidate polymorphisms from two genes, we fell that our findings contribute to the knowledge of the genetic obesity in Portuguese children, replicating previous data observed in other European populations. Further research on the role of the *SLC6A4* gene on obesity risk, with longitudinal studies in children and considering the impact of environmental factors such as physical activity should be improved.

6 - References

Adeyemo, A., Chen, G., Zhou, J., Shriner, D., Doumatey, A., Huang, H., & Rotimi, C. (2010). FTO genetic variation and association with obesity in West Africans and African Americans. *Diabetes*, *59*(6), 1549–1554. https://doi.org/10.2337/db09-1252

Ahmad, S., Rukh, G., Varga, T. V., Ali, A., Kurbasic, A., Shungin, D., … Franks, P. W. (2013). Gene × Physical Activity Interactions in Obesity: Combined Analysis of 111,421 Individuals of European Ancestry. *PLoS Genetics*, *9*(7), 1–9. https://doi.org/10.1371/journal.pgen.1003607

Albuquerque, D., Nóbrega, C., & Manco, L. (2013). Association of FTO Polymorphisms with Obesity and Obesity-Related Outcomes in Portuguese Children. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0054370

Albuquerque, D., Nóbrega, C., Manco, L., & Padez, C. (2017). The contribution of genetics and environment to obesity. *British Medical Bulletin*, *123*(1), 159–173. https://doi.org/10.1093/bmb/ldx022

Almeida, S. M., Furtado, J. M., Mascarenhas, P., Ferraz, M. E., Ferreira, J. C., Monteiro, M. P., … Ferraz, F. P. (2018). Association between LEPR, FTO, MC4R, and PPARG-2 polymorphisms with obesity traits and metabolic phenotypes in school-aged children. *Endocrine*, *60*(3), 466–478. https://doi.org/10.1007/s12020-018-1587-3

Andersen, L. B., Harro, M., Sardinha, L. B., Froberg, K., Ekelund, U., Brage, S., & Anderssen, S. A. (2006). Physical activity and clustered cardiovascular risk in children: a cross-sectional study (The European Youth Heart Study). *Lancet (London, England)*, *368*(9532), 299–304. https://doi.org/10.1016/S0140-6736(06)69075-2

Andreasen, C. H., Stender-Petersen, K. L., Mogensen, M. S., Torekov, S. S., Wegner, L., Andersen, G., … Hansen, T. (2008). Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation. *Diabetes*, *57*(1), 95–101. https://doi.org/10.2337/db07-0910

Bah, J., Westberg, L., Baghaei, F., Henningsson, S., Rosmond, R., Melke, J., … Eriksson, E. (2010). Further exploration of the possible influence of polymorphisms in HTR2C and 5HTT on body weight. *Metabolism: Clinical and Experimental*, *59*(8), 1156–1163. https://doi.org/10.1016/j.metabol.2009.11.007

Bonnet, G., Gómez-Abellán, P., Vera, B., Sánchez-Romera, J. F., Hernández-Martínez, A. M., Sookoian, S., … Garaulet, M. (2017). Serotonin-transporter promoter polymorphism modulates the ability to control food intake: Effect on total weight loss. *Molecular Nutrition and Food Research*, *61*(11), 1–10. https://doi.org/10.1002/mnfr.201700494

Borkowska, A., Bieliåski, M., Szczęsny, W., Szwed, K., Tomaszewska, M., Kałwa, A., … Akiskal, H. (2015). Effect of the 5-HTTLPR polymorphism on affective temperament, depression and body mass index in obesity. *Journal of Affective Disorders*, *184*, 193–197. https://doi.org/10.1016/j.jad.2015.05.061

Bringolf-Isler, B., Grize, L., Mäder, U., Ruch, N., Sennhauser, F. H., & Braun-Fahrländer, C. (2009). Assessment of intensity, prevalence and duration of everyday activities in Swiss school children: A cross-sectional analysis of accelerometer and diary data. *International Journal of Behavioral Nutrition and Physical Activity*, *6*, 1–10. https://doi.org/10.1186/1479-5868-6-50

Calati, R., De Ronchi, D., Bellini, M., & Serretti, A. (2011). The 5-HTTLPR polymorphism and eating disorders: A meta-analysis. *International Journal of Eating Disorders*, *44*(3), 191–199. https://doi.org/10.1002/eat.20811

Cha, S. W., Choi, S. M., Kim, K. S., Park, B. L., Kim, J. R., Kim, J. Y., & Shin, H. D. (2008).

Replication of genetic effects of FTO polymorphisms on BMI in a Korean population. *Obesity (Silver Spring)*, *16*(9), 2187–2189. https://doi.org/10.1038/oby.2008.314

Chang, Y. C., Liu, P. H., Lee, W. J., Chang, T. J., Jiang, Y. Der, Li, H. Y., … Chuang, L. M. (2008). Common variation in the fat mass and Obesity-Associated (FTO) gene confers risk of obesity and modulates BMI in the chinese population. *Diabetes*, *57*(8), 2245–2252. https://doi.org/10.2337/db08-0377

Dias, H., Muc, M., Padez, C., & Manco, L. (2016). Association of polymorphisms in 5-HTT (SLC6A4) and MAOA genes with measures of obesity in young adults of Portuguese origin. *Archives of Physiology and Biochemistry*, *122*(1), 8–13. https://doi.org/10.3109/13813455.2015.1111390

Dina, C., Meyre, D., Gallina, S., Durand, E., Körner, A., Jacobson, P., … Froguel, P. (2007). Variation in FTO contributes to childhood obesity and severe adult obesity. *Nature Genetics*, *39*(6), 724–726. https://doi.org/10.1038/ng2048

Evenson, K. R., Catellier, D. J., Gill, K., Ondrak, K. S., & McMurray, R. G. (2008). Calibration of two objective measures of physical activity for children. *Journal of Sports Sciences*, *26*(14), 1557–1565. https://doi.org/10.1080/02640410802334196

Ferreira Carlos, F., Silva-Nunes, J., Flores, O., Brito, M., Doria, G., Veiga, L., & Baptista, P. V. (2013). Association of FTO and PPARG polymorphisms with obesity in Portuguese women. *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, *6*(July 2013), 241–245. https://doi.org/10.2147/DMSO.S45779

Fiskerstrand, C. E., Lovejoy, E. A., & Quinn, J. P. (1999). An intronic polymorphic domain often associated with susceptibility to affective disorders has allele dependent differential enhancer activity in embryonic stem cells. *FEBS Letters*, *458*(2), 171–174. https://doi.org/10.1016/S0014-5793(99)01150-3

Frayling, T. M., Timpson, N. J., Weedon, M. N., Freathy, R. M., Lindgren, C. M., Perry, J. R. B., … Mccarthy, M. I. (2007). Index and Predisposes to Childhood and Adult Obesity. *Science*, *316*(5826), 889–894. https://doi.org/10.1126/science.1141634.A

Fuemmeler, B. F., Agurs-collins, T., Mcclernon, F. J., Kollins, S. H., Kail, M., Bergen, A. W., & Ashley-koch, A. E. (2008). *Obesity*, *16*(2), 348–355. https://doi.org/10.1038/oby.2007.65.Genes

Gelernter, J., Kranzler, H., & Cubells, J. F. (1997). Serotonin transporter protein (SLC6A4) allele and haplotype frequencies and linkage disequilibria in African- and European-American and Japanese populations and in alcohol-dependent subjects. *Human Genetics*, *101*(2), 243–246. https://doi.org/10.1007/s004390050624

González-Sánchez, J. L., Zabena, C., Martínez-Larrad, M. T., Martínez-Calatrava, M. J., Pérez-Barba, M., & Serrano-Ríos, M. (2009). Variant rs9939609 in the FTO gene is associated with obesity in an adult population from Spain. *Clinical Endocrinology*, *70*(3), 390–393. https://doi.org/10.1111/j.1365-2265.2008.03335.x

Grant, S. F. A., Li, M., Bradfield, J. P., Kim, C. E., Annaiah, K., Santa, E., … Hakonarson, H. (2008). Association analysis of the FTO gene with obesity in children of Caucasian and African ancestry reveals a common tagging SNP. *PLoS ONE*, *3*(3), 1–6. https://doi.org/10.1371/journal.pone.0001746

Hakanen, M., Raitakari, O. T., Lehtima, T., Peltonen, N., Pahkala, K., & Lagstro, H. (2009). FTO Genotype Is Associated with Body Mass Index after the Age of Seven Years But Not with Energy Intake or Leisure-Time Physical Activity. T*he Journal of Clinical Endocrinology & Metabolism, 94*(June), 1281–1287. https://doi.org/10.1210/jc.2008-1199

Hameed, A., Ajmal, M., Nasir, M., & Ismail, M. (2015). Genetic association analysis of serotonin transporter polymorphism (5-HTTLPR) with type 2 diabetes patients of Pakistani population. *Diabetes Research and Clinical Practice*, *108*(1), 67–71. https://doi.org/10.1016/j.diabres.2015.01.009

Hardy, R., Wills, A. K., Wong, A., Elks, C. E., Wareham, N. J., Loos, R. J. F., … Ong, K. K. (2010). Life course variations in the associations between FTO and MC4R gene variants and body size. *Human Molecular Genetics*, *19*(3), 545–552. https://doi.org/10.1093/hmg/ddp504

Hassanein, M. T., Lyon, H. N., Nguyen, T. T., Akylbekova, E. L., Waters, K., Lettre, G., … Hirschhorn, J. N. (2010). Fine mapping of the association with obesity at the FTO locus in African-derived populations. *Human Molecular Genetics*, *19*(14), 2907–2916. https://doi.org/10.1093/hmg/ddq178

Hubacek, J. A., Bohuslavova, R., Kuthanova, L., Kubinova, R., Peasey, A., Pikhart, H., … Bobak, M. (2008). The FTO gene and obesity in a large eastern European population sample: The HAPIEE study. *Obesity*, *16*(12), 2764–2766. https://doi.org/10.1038/oby.2008.421

Iordanidou, M., Tavridou, A., Petridis, I., Arvanitidis, K. I., Christakidis, D., Vargemezis, V., & Manolopoulos, V. G. (2010). The serotonin transporter promoter polymorphism (5-HTTLPR) is associated with type 2 diabetes. *Clinica Chimica Acta*, *411*(3–4), 167–171. https://doi.org/10.1016/j.cca.2009.10.022

Khoury, M., Manlhiot, C., Bs, C., & Mccrindle, B. W. (2013). Role of the Waist / Height Ratio in the Cardiometabolic Risk Assessment of Children Classified by Body Mass Index. *Journal of the American College of Cardiolog*y, *62*(8), 742-751. https://doi.org/10.1016/j.jacc.2013.01.026

Kilpeläinen, T. O., Qi, L., Brage, S., Sharp, S. J., Sonestedt, E., Demerath, E., … Loos, R. J. F. (2011). Physical activity attenuates the influence of FTO variants on obesity risk: A meta-analysis of 218,166 adults and 19,268 children. *PLoS Medicine*, *8*(11). https://doi.org/10.1371/journal.pmed.1001116

Kim, J. Y., DeMenna, J. T., Puppala, S., Chittoor, G., Schneider, J., Duggirala, R., … Coletta, D. K. (2016). Physical activity and FTO genotype by physical activity interactive influences on obesity. *BMC Genetics*, *17*(1), 1–7. https://doi.org/10.1186/s12863-016-0357-6

Lan, M., Chang, Y., Chen, W., Kao, Y., Lin, H., & Liu, J. (2009). Serotonin transporter gene promoter polymorphism is associated with body mass index and obesity in non-elderly stroke patients. *Journal of Endocrinological Investigation*, *32*, 119–122.

Lee, H. J., Kim, I. kyoung, Kang, J. H., Ahn, Y., Han, B. G., Lee, J. Y., & Song, J. (2010). Effects of common FTO gene variants associated with BMI on dietary intake and physical activity in Koreans. *Clinica Chimica Acta*, *411*(21–22), 1716–1722. https://doi.org/10.1016/j.cca.2010.07.010

Leonska-Duniec, A., Jastrzebski, Z., Zarebska, A., Maciejewska, A., Ficek, K., & Cieszczyk, P. (2016). Assessing effect of interaction between the FTO A/T polymorphism (rs9939609) and physical activity on obesity-related traits. *Journal of Sport and Health Science*, (March), 6–11. https://doi.org/10.1016/j.jshs.2016.08.013

Lesch, K. P., Balling, U., Gross, J., Strauss, K., Wolozin, B. L., Murphy, D. L., & Riederer, P. (1994). Organization of the human serotonin transporter gene. *Journal of Neural Transmission. General Section*, *95*(2), 157–162.

Li, H., Wu, Y., Loos, R. J., Hu, F. B., Liu, Y., Wang, J., … Lin, X. (2008). Variants in the fat

mass- and obesity-associated (FTO) gene are not associated with obesity in a Chinese Han population. *Diabetes*, *57*(1), 264–268. https://doi.org/10.2337/db07-1130.H.L.

Li, S., Zhao, J. H., Luan, J., Ekelund, U., Luben, R. N., Khaw, K. T., … Loos, R. J. F. (2010). Physical activity attenuates the genetic predisposition to obesity in 20,000 men and women from EPIC-Norfolk prospective population study. *PLoS Medicine*, *7*(8), 1–9. https://doi.org/10.1371/journal.pmed.1000332

Loos, R. J. F., & Yeo, G. S. H. (2014). The bigger picture of FTO - The first GWAS-identified obesity gene. *Nature Reviews Endocrinology*, *10*(1), 51–61. https://doi.org/10.1038/nrendo2013227

Machado-Rodrigues, A. M., Coelho-E-Silva, M. J., Mota, J., Cumming, S. P., Riddoch, C., & Malina, R. M. (2011). Correlates of aerobic fitness in urban and rural Portuguese adolescents. *Annals of Human Biology*, *38*(4), 479–484. https://doi.org/10.3109/03014460.2011.554865

Machado-Rodrigues, A. M., Coelho-E-Silva, M. J., Mota, J., Cyrino, E., Cumming, S. P., Riddoch, C., … Malina, R. M. (2011). Agreement in activity energy expenditure assessed by accelerometer and self-report in adolescents: Variation by sex, age, and weight status. *Journal of Sports Sciences*, *29*(14), 1503–1514. https://doi.org/10.1080/02640414.2011.593185

Machado-Rodrigues, A. M., Coelho-E-Silva, M. J., Mota, J., Padez, C., Martins, R. A., Cumming, S. P., … Malina, R. M. (2014). Urban-rural contrasts in fitness, physical activity, and sedentary behaviour in adolescents. *Health Promotion International*, *29*(1), 118–129. https://doi.org/10.1093/heapro/das054

MacKenzie, A., & Quinn, J. (1999). A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(26), 15251–15255. https://doi.org/10.1073/pnas.96.26.15251

Mergen, H., Karaaslan, C., Mergen, M., Deniz Ozsoy, E., & Ozata, M. (2007). LEPR, ADBR3, IRS-1 and 5-HTT genes polymorphisms do not associate with obesity. *Endocrine Journal*, *54*(1), 89–94. https://doi.org/10.1507/endocrj.K06-023

Miranda, R. C. K., Genro, J. P., Campagnolo, P. D. B., Mattevi, V. S., Vitolo, M. R., & Almeida, S. (2017). Biallelic and triallelic approaches of 5-HTTLPR polymorphism are associated with food intake and nutritional status in childhood. *The Journal of Nutritional Biochemistry*, *43*, 47–52. https://doi.org/10.1016/j.jnutbio.2017.01.015

Muc, M., Padez, C., & Manco, L. (2015). Influence of physical activity on the association between the FTO variant rs9939609 and adiposity in young adults. *American Journal of Human Biology*, *27*(5), 734–738. https://doi.org/10.1002/ajhb.22712

Must, A., & Anderson, S. E. (2006). Body mass index in children and adolescents: Considerations for population-based applications. *International Journal of Obesity*, *30*(4), 590–594. https://doi.org/10.1038/sj.ijo.0803300

Ohashi, J., Naka, I., Kimura, R., Natsuhara, K., Yamauchi, T., Furusawa, T., … Ohtsuka, R. (2007). FTO polymorphisms in oceanic populations. *Journal of Human Genetics*, *52*(12), 1031–1035. https://doi.org/10.1007/s10038-007-0198-2

Pate, R. R., Almeida, M. J., McIver, K. L., Pfeiffer, K. A., & Dowda, M. (2006). Validation and calibration of an accelerometer in preschool children. *Obesity*, *14*(11), 2000–2006. https://doi.org/10.1038/oby.2006.234

Peralta-leal, V., Leal-ugarte, E., Meza-espinoza, J. P., Dávalos-rodríguez, I. P., Bocanegra-

alonso, A., Acosta-gonzález, R. I., … Durán-gonzález, J. (2012). Association of a serotonin transporter gene (SLC6A4) 5-HTTLPR polymorphism with body mass index categories but not type 2 diabetes mellitus in Mexicans.*Genetic Molecular Biology, 35(3)*, 589–593.

Pereira, P. de A., Alvim-Soares, A. M., Sandrim, V. C., Lanna, C. M. M., Souza-Costa, D. C., Belo, V. de A., … Miranda, D. M. de. (2016). Falta de associação entre o polimorfismo genético do FTO, AKT1 e AKTIP e o sobrepeso e a obesidade infantis. *Jornal de Pediatria*, *92*(5), 521–527. https://doi.org/10.1016/j.jped.2015.12.007

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., … Sham, P. C. (2007). PLINK: A Tool Set for Whole-Genome Association and Population-Based Linkage Analyses. *The American Journal of Human Genetics*, *81*(3), 559–575. https://doi.org/10.1086/519795

Rampersaud, E., Mitchell, B. D., Pollin, T. I., Fu, M., Shen, H., O'Connell, J. R., … Snitker, S. (2008). Physical activity and the association of common FTO gene variants with body mass index and obesity. *Archives of Internal Medicine*, *168*(16), 1791–1797. https://doi.org/10.1001/archinte.168.16.1791

Riddoch, C. J., Andersen, L. B., Wedderkopp, N., Harro, M., Klasson-Heggebø, L., Sardinha, L. B., … Ekelund, U. (2004). Physical Activity Levels and Patterns of 9- and 15-yr-Old European Children. *Medicine and Science in Sports and Exercise*, *36*(1), 86–92. https://doi.org/10.1249/01.MSS.0000106174.43932.92

Riddoch, C. J., Mattocks, C., Deere, K., Saunders, J., Kirkby, J., Tilling, K., … Ness, A. R. (2007). Objective measurement of levels and patterns of physical activity. *Archives of Disease in Childhood*, *92*(11), 963–969. https://doi.org/10.1136/adc.2006.112136

Ruiz, J. R., Labayen, I., Ortega, F. B., Legry, V., Moreno, L. A., Dallongeville, J., … Meirhaeghe, A. (2010). Attenuation of the effect of the FTO rs9939609 polymorphism on total and central body fat by physical activity in adolescents: The HELENA study. *Archives of Pediatrics and Adolescent Medicine*, *164*(4), 328–333. https://doi.org/10.1001/archpediatrics.2010.29

Sandholt, C. H., Hansen, T., & Pedersen, O. (2012). Beyond the fourth wave of genome-wide obesity association studies. *Nutrition and Diabetes*, *2*(JULY), e37-10. https://doi.org/10.1038/nutd.2012.9

Scuteri, A., Sanna, S., Chen, W. M., Uda, M., Albai, G., Strait, J., … Abecasis, G. R. (2007). Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genetics*, *3*(7), 1200–1210. https://doi.org/10.1371/journal.pgen.0030115

Sookoian, S., Gemma, C., García, S. I., Gianotti, T. F., Dieuzeide, G., Roussos, A., … Pirola, C. J. (2007). Short allele of serotonin transporter gene promoter is a risk factor for obesity in adolescents. *Obesity (Silver Spring, Md.)*, *15*(2), 271–6. https://doi.org/10.1038/oby.2007.519

Sookoian, S., Gianotti, T. F., Gemma, C., Burguẽo, A., & Pirola, C. J. (2008). Contribution of the functional 5-HTTLPR variant of the SLC6A4 gene to obesity risk in male adults. *Obesity (Silver Spring)*, *16*(2), 488–491. https://doi.org/10.1038/oby.2007.64

Speliotes, E. K., Willer, C. J., Berndt, S. I., Monda, K. L., Thorleifsson, G., Jackson, A. U., … Kaakinen, M. (2010). Association analyses of 249,796 individuals reveal eighteen new loci associated with body mass index. *Nature Genetics*, *42*(11), 937–948. https://doi.org/10.1038/ng.686.Association

Srivastava, A., Srivastava, N., & Mittal, B. (2016). Genetics of Obesity. *Indian Journal of*

Clinical Biochemistry, *31*(4), 361–371. https://doi.org/10.1007/s12291-015-0541-x

Tan, J. T., Dorajoo, R., Seielstad, M., Sim, X. L., Ong, R. T. H., Chia, K. S., … Tai, E. S. (2008). FTO variants are associated with obesity in the chinese and malay populations in Singapore. *Diabetes*, *57*(10), 2851–2857. https://doi.org/10.2337/db08-0214

Troiano, R. P., Berrigan, D., Dodd, K. W., Mâsse, L. C., Tilert, T., & Mcdowell, M. (2008). Physical activity in the United States measured by accelerometer. *Medicine and Science in Sports and Exercise*, *40*(1), 181–188. https://doi.org/10.1249/mss.0b013e31815a51b3

Tung, Y. C. L., & Yeo, G. S. H. (2011). From GWAS to biology: Lessons from FTO. *Annals of the New York Academy of Sciences*, *1220*(1), 162–171. https://doi.org/10.1111/j.1749-6632.2010.05903.x

Uzun, M., Saglar, E., Kucukyildirim, S., Erdem, B., Unlu, H., & Mergen, H. (2015). Association of VNTR polymorphisms in DRD4, 5-HTT and DAT1 genes with obesity. *Archives of Physiology and Biochemistry*, *121*(2), 75–79. https://doi.org/10.3109/13813455.2014.985686

Wen, W., Cho, Y. S., Zheng, W., Dorajoo, R., Kato, N., Qi, L., … Okada, Y. (2012). Meta-analysis identifies common variants associated with body mass index in East Asians. *Nature Genet*, *44*(3), 307–311. https://doi.org/10.1038/ng.1087.Meta-analysis

Xi, B., Wang, C., Wu, L., Zhang, M., Shen, Y., Zhao, X., … Mi, J. (2011). Influence of physical inactivity on associations between single nucleotide polymorphisms and genetic predisposition to childhood obesity. *American Journal of Epidemiology*, *173*(11), 1256–1262. https://doi.org/10.1093/aje/kwr008

Xia, Q., & Grant, S. F. A. (2013). The genetics of human obesity. *Annals of the New York Academy of Sciences*, *1281*(1), 178–190. https://doi.org/10.1111/nyas.12020

Yoo, E. G. (2016). Waist-to-height ratio as a screening tool for obesity and cardiometabolic risk. *Korean Journal of Pediatrics*, *59*(11), 425–431. https://doi.org/10.3345/kjp.2016.59.11.425

Zhang, M., Zhao, X., Cheng, H., Wang, L., Xi, B., Shen, Y., … Mi, J. (2014). Age- and sex-dependent association between FTO rs9939609 and obesity-related traits in Chinese children and adolescents. *PLoS ONE*, *9*(5). https://doi.org/10.1371/journal.pone.0097545