David dos Santos Albuquerque

Study of genetic variants associated with obesity in Portuguese children

Tese de doutoramento em Antropologia, no ramo de especialização em Antropologia Biológica, orientada pelo Doutor Licínio Manco, Doutor Clévio Nóbrega e pela Doutora Raquel Rodríguez-López e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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Study of genetic variants associated with obesity in Portuguese children

Estudo de variantes genéticas associadas à obesidade em crianças de origem Portuguesa

David dos Santos Albuquerque

Dissertation submitted to the Department of Life Sciences of the Faculty of Sciences and Technology of the University of Coimbra (FCTUC) in partial fulfillment of the requirements for the Degree of Doctor in Anthropology in the field of Biological Anthropology.

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Departamento de Ciências da Vida

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"Só sei que nada sei, e o facto de saber isso, coloca-me em vantagem sobre aqueles que acham que sabem alguma coisa"

Sócrates

354 a.C.

Cover front:

Picture held by Daniel Álvarez Vaca. Main image, Colombian Family Painting (1999), adapted from Fernando Botero Angulo. Oil on canvas 195x168cm.

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Contents

Abbreviations

ANOVA – one-way analyses

BAT – brown adipose tissue

BMI – body mass index

BMI Z-score – body mass index standard deviation

CEU – Caucasian

CI95% – confidence interval

cm – centimetres

DNA – deoxyribonucleic acid

dNTPs - deoxynucleotide triphosphates

FTO – fat mass and obesity associated gene

GRS – genetic risk score

GWAS – genome wide association studies

HC – hip circumference

HOXB5 – homeobox B5 gene

IMC – Índice de massa corporal

IOTF – international obesity task force

Kb – kilo base pairs

Kg – kilograms

LCT – Lactase gene

LD – linkage disequilibrium

LEP – leptin gene

LEPR – leptin receptor gene

LMS – lambda-mu-sigma

LNP – Lactase non-persistence

LP – Lactase persistence

Mb – mega base pairs

MC4R – melanocortin-4 receptor gene

min – minutes

MSRA – methionine sulfoxide reductase A gene

NGS – next-generation sequencing

nm – nanometre

NRXN3 – neurexin 3 gene

OLFM4 – olfactomedin 4 gene

OMIM – Online Mendelian Inheritance in Man

OR – odds ratio

p – *p-value*

PCR – polymerase chain reaction

PPARGC1A – peroxisome proliferator-activated receptor gamma, coactivator 1 alpha gene

sec – second

SEC16B – SEC16 homolog B (*S. cerevisiae*) gene

SNP – single nucleotide polymorphism

SNPs – single nucleotide polymorphisms

SPSS – statistical package for social statistics

SSCP – single stand conformation polymorphism

TFAP2B – transcription factor AP-2 beta (activating enhancer binding protein 2 beta) gene

TMEM18 – transmembrane protein 18 gene

v – version

vs. – versus

WC – waist circumference

WHO – world health organization

WHR – waist-to-hip ratio

WHtR - waist-to-height ratio

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Summary

The prevalence of obesity is a growing problem worldwide. Such scenario urges for additional efforts in both investment on prevention and research relating to the identification of risk factors that may aid early intervention. It is widely accepted that obesity is a complex multifactorial and heterogeneous condition with an important genetic component in the susceptibility risk. Therefore, the identification of associated gene variants could be essential in the design of prevention strategies and management of individuals genetically predisposed to obesity. In 2007, it was identified the first single nucleotide polymorphism (SNP) located in the *FTO* gene (rs9939609) associated with obesity by genome-wide association studies (GWAS). Until now, more than 52 genetic *loci* have been unequivocally associated with obesity related-traits in several European populations. However, none of these studies was performed before in a sample of Portuguese population.

The main aims of this study were i) to estimate the prevalence of obesity in 6-12 years old children from the central region of Portugal; ii) to investigate whether 14 previously described SNPs in obesity-related genes are associated with the risk of obesity in Portuguese children; iii) to identify *MC4R* gene mutations in children with morbid obesity (BMI ≥99th) that could justify this phenotype.

Anthropometric parameters such as weight, height and waist circumference were measured in a random representative sample of 1433 children (747 girls and 686 boys) between 6-12 years old from several public schools in 2011. The International Obesity Task Force (IOTF) cut-offs were used to define obesity. Children classified with overweight (320) and obesity (154), and 256 randomly selected lean subjects with 18.5<BMI<25 kg/m^2, were selected for genotyping. Polymorphisms were examined in DNA samples by TaqMan® assay. Sequencing of the *MC4R* gene was carried out in all individuals with BMI ≥99th.

The prevalence of overweight/obesity found in the total sample was 33.0%; 10.7% were obese children. Overweight was significantly higher in boys than in girls (25.9% and 19.0% respectively, p=0.04), whereas no gender differences was found for obesity (10.6 % and 10.7% respectively, p=0.57). Comparison with previous studies showed a slightly increase in overweight/obesity in children of central Portugal in the last 10 years, reaching values of 40.0% prevalence in the 7-9 years old children.

For three study polymorphisms located in the *FTO* gene, we found significant associations for SNPs rs9939609 and rs1421085 with weight, BMI, BMI Z-score and waist circumference (p<0.05 in all traits). For rs1861868, marginally significant associations were obtained with weight (p=0.08) and BMI (p=0.09). Logistic regression analysis, in the additive model, revealed both rs9939609 and rs1421085 SNPs significantly associated with obesity (OR=1.41; p=0.02 and OR=1.45, p=0.01, respectively). Haplotype analyses (rs1861868-rs1421085-rs9939609) identified two combinations (ACA and GCA) associated with a higher risk of obesity (OR=1.53; p=0.02 and OR=1.73; p=0.03, respectively).

We tested the association between the -13910C>T polymorphism, located in the lactase (*LCT*) gene, and obesity-related traits, and found indication for an association between the -13910*T allele and abdominal obesity (OR=1.41; p=0.03). Under the dominant model, significant association was observed between the *LCT*-13910 CT/TT genotypes and abdominal obesity (OR=1.65; p=0.02). No association was detected with the risk of obesity (p=0.35) or anthropometrics traits (p>0.05).

Finally, we tested for the association of obesity-related quantitative traits in Portuguese children with ten polymorphisms in the obesity related genes *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5* and *OLFM4*. The *MC4R* rs12970134 polymorphism was nominally associated with BMI (p=0.03), BMI Z-score (p=0.04) and waist circumference (p=0.02), and borderline associated with weight (p=0.05). Near nominal associations were also found for the *PPARGC1A* rs8192678 polymorphism with weight (p=0.06), and for the *MSRA* rs545854 polymorphism with BMI (p=0.05) and BMI Z-score (p=0.05). Furthermore, logistic regression under the additive model showed that *MC4R* rs12970134 and *TFAP2B* rs987237 were nominally, respectively, associated (OR=1.47; p=0.02) and borderline associated (OR=1.47; p=0.05) with the obese phenotype.

Additionally, 32 children who present a BMI ≥99th were screened for *MC4R* gene mutations. We found two previously described polymorphisms at heterozygous state in two children: the -174A>C (rs34114122) polymorphism in the 5´UTR region in a girl with BMI Z-score=2.51; and the common *missense* mutation 307G>A (Val103Ile) in the *MC4R* gene coding region identified in a boy with a BMI Z-score=2.60. No other pathogenic *MC4R* gene mutations were detected.

In conclusion, this study shows a high prevalence of overweight/obesity among Portuguese children, following the trend of other European countries. We highlighted for the first time the possible association of *FTO*, *MC4R*, *PPARGC1A*, *MSRA* and *TFAP2B* SNPs with several obesity-related traits in a sample of Portuguese children. *FTO* SNPs showed the strong association with the risk of obesity in line with previous studies performed in European populations. Our study is a significant contribution to the knowledge of the genetic basis of obesity in the Portuguese population, but further studies are needed to a better understanding of the genetic factors linked to the obesity risk. Such information could be used in the future for the development of new obesity preventive strategies.

Keywords:

Obesity
Body mass index (BMI)
Portuguese children
Genetic polymorphisms
MC4R gene
Association study

Resumo

A prevalência da obesidade tem vindo a aumentar em todo o mundo. Tal cenário implica esforços adicionais no investimento tanto em matéria de prevenção como de investigação relacionados com a identificação de fatores de risco que podem ajudar a intervenção precoce. É amplamente aceite que a obesidade é uma condição multifatorial e heterogênea complexa com uma importante componente genética. Portanto, a identificação de variantes genéticas associadas poderão ser essenciais em estratégias de prevenção em indivíduos geneticamente predispostos à obesidade. Em 2007, foi identificado o primeiro polimorfismo de nucleótido simples (SNP) localizado no gene *FTO* (rs9939609) associado à obesidade em estudos de associação do genoma (GWAS). Mais de 52 *loci* genéticos foram já associados à obesidade em várias populações Europeias. No entanto, nenhum estudo do género foi até agora realizado numa amostra da população Portuguesa.

Os principais objetivos deste estudo foram i) estimar a prevalência de obesidade em crianças dos 6-12 anos da região centro de Portugal; ii) investigar se 14 SNPs previamente descritos em genes relacionados com a obesidade, estão associados com o risco de obesidade em crianças portuguesas; iii) identificar mutações no gene *MC4R* em crianças com obesidade mórbida (IMC ≥99th) que poderão justificar o fenótipo.

Os parâmetros antropométricos, tais como, peso, altura e circunferência da cintura (CC) foram medidos numa amostra aleatória de 1433 crianças (747 raparigas e 686 rapazes) entre os 6-12 anos, de várias escolas públicas em 2011. Os limites do *International Obesity Task Force* (IOTF) foram usados para definir obesidade. Crianças classificadas com excesso de peso (320) e obesidade (154), e 256 indivíduos normais escolhidos aleatoriamente com 18,5<IMC<25 kg/m^2, foram selecionados para a genotipagem. Os polimorfismos foram examinados em amostras de DNA por ensaios com sondas TaqMan®. A sequenciação do gene *MC4R* foi realizada nos indivíduos com IMC ≥99th.

A prevalência de excesso de peso/obesidade obtida na amostra total foi de 33,0%; 10,7% das crianças eram obesas. O excesso de peso foi significativamente maior nos rapazes do que nas raparigas (25,9% e 19,0%, respetivamente, p=0,04), não tendo sido encontradas diferenças entre os sexos para a obesidade (10,6% e 10,7%, respetivamente, p=0,57). A comparação com estudos anteriores mostrou um ligeiro aumento no excesso de peso/obesidade em crianças do

centro de Portugal nos últimos 10 anos, atingindo valores de prevalência de 40,0% nos 7-9 anos de idade.

Dos três polimorfismos estudados localizados no gene *FTO*, foram encontradas associações significativas dos SNPs rs9939609 e rs1421085 com peso, IMC, IMC Z-score e CC (p<0,05 em todos os parâmetros). Para o SNP rs1861868, foram obtidas associações marginalmente significativas com peso (p=0,08) e IMC (p=0,09). A análise de regressão logística, no modelo aditivo, revelou os SNPs rs9939609 e rs1421085 significativamente associados com a obesidade (OR=1,41; p=0,02, e OR=1,45, p=0,01, respetivamente). A análise de haplótipos (rs1861868-rs1421085-rs9939609) identificou duas combinações (ACA e GCA) associadas a um maior risco de obesidade (OR=1,53; p=0,02, e OR=1,73; p=0,03, respetivamente).

Foi testada a associação entre o polimorfismo -13910C>T, localizado no gene da lactase (*LCT*), e a obesidade, tendo sido encontrada indicação para uma associação entre o alelo -13910*T e a obesidade abdominal (OR=1,41; p=0,03). Sob o modelo dominante, foi observada uma associação significativa entre os genótipos CT/TT e obesidade abdominal (OR=1,65; p=0,02). Nenhuma associação foi observada com o risco de obesidade (p=0,35) ou características antropométricas (p>0,05).

Finalmente, foi testada a associação de características quantitativas relacionadas com a obesidade em crianças portuguesas com dez SNPs nos genes relacionados com a obesidade *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5* e *OLFM4*. O SNP *MC4R* rs12970134 foi encontrado nominalmente associado com IMC (p=0,03), IMC Z-score (p=0,04) e CC (p=0,02), e no limite de significância com peso (p=0,05). Foram também encontradas associações nominais para o polimorfismo *PPARGC1A* rs8192678 com peso (p=0,06), e para o polimorfismo *MSRA* rs545854 com IMC (p=0,05) e IMC Z-score (p=0,05). A regressão logística sob o modelo aditivo mostrou que os SNPs *MC4R* rs12970134 e *TFAP2B* rs987237 se encontram, nominalmente, respetivamente, associado (OR=1,47; p=0,02) e no limite de significância associado (OR=1,45; p=0,05) com o fenótipo obeso.

Adicionalmente, 32 crianças que apresentavam um IMC ≥99th foram rastreadas para mutações no gene *MC4R*. Foram encontrados dois SNPs, previamente descritos, em heterozigotia em duas crianças: o SNP -174A>C (rs34114122) na região 5'UTR numa rapariga com IMC Z-score=2,51; e a mutação *missense* comum 307G>A (Val103Ile) na região codificante

do gene num rapaz com um IMC Z-score=2.60. Não foram detetadas outras mutações no gene *MC4R*.

Em conclusão, este estudo mostrou uma alta prevalência de excesso de peso/obesidade nas crianças portuguesas, seguindo a tendência de outros países europeus. Foi encontrada pela primeira vez numa amostra de crianças portuguesas, uma possível associação dos SNPs nos genes *FTO*, *MC4R*, *PPARGC1A*, *MSRA* e *TFAP2B* com várias medidas de obesidade. Os polimorfismos no gene *FTO* mostraram a associação mais forte com o risco de obesidade em linha com estudos previamente realizados em populações europeias. O nosso trabalho é uma importante contribuição para o conhecimento da base genética da obesidade na população portuguesa, mas são necessários mais estudos para melhor compreender estes fatores genéticos associados ao risco da obesidade. Esta informação poderá vir a ser usada no futuro para o desenvolvimento de novas estratégias de prevenção para a obesidade.

Palavras-chave:

Obesidade
Índice de massa corporal (IMC)
Crianças Portuguesas
Polimorfismos genéticos
Gene MC4R
Estudo de associação

Resumen

La prevalencia de la obesidad es un problema creciente en todo el mundo. Tal escenario exige esfuerzos adicionales e inversión, tanto en materia de prevención como en investigación enfocada a la identificación de factores de riesgo que puedan ayudar a la intervención precoz. Está ampliamente aceptado que la obesidad es un complejo trastorno multifactorial y heterogéneo cuyo componente genético supone un importante factor de riesgo. Por lo tanto, la identificación de variantes genéticas asociadas podría ser esencial en el diseño de estrategias de prevención y de manejo de individuos genéticamente predispuestos a la obesidad. En 2007 se identificó el primer polimorfismo de nucleótido simple (SNP) asociado a la obesidad, localizado en el gen *FTO* (rs9939609), por un estudio de asociación del genoma completo (GWAS). Hasta ahora más de 52 *loci* genéticos han sido asociados inequívocamente a rasgos relacionados con la obesidad en varias poblaciones europeas. Sin embargo, ninguno de estos estudios se realizó antes en una muestra de población portuguesa.

Los principales objetivos de este estudio fueron (i) estimar la prevalencia de la obesidad en niños de 6-12 años de edad de la región central de Portugal; (ii) investigar si 14 SNPs previamente descritos en los genes relacionados con la obesidad están asociados con el riesgo de obesidad en niños portugueses; (iii) identificar en niños con obesidad mórbida (IMC ≥ percentil 99) mutaciones en el gen *MC4R* que podrían justificar el fenotipo.

Se midieron parámetros antropométricos tales como peso, altura y circunferencia de la cintura en una muestra aleatoria representativa de 1433 niños (747 niñas y 686 niños) de 6-12 años de varias escuelas públicas en 2011. Para definir la obesidad se utilizaron los puntos de cortes de la *International Obesity Task Force* (IOTF), y con ellos se seleccionaron para el genotipado 320 niños con sobrepeso, 154 con obesidad, y 256 de peso normal escogidos al azar para el grupo control de un total de 928 ($18.5<IMC<25$ kg/m^2). Los SNPs se estudiaron en muestras de ADN mediante ensayos TaqMan®. La secuenciación del gen *MC4R* se realizó en individuos con IMC ≥ percentil 99.

La prevalencia de sobrepeso/obesidad en la muestra total fue de 33,0%; 10,7% eran obesos. El sobrepeso fue significativamente mayor en niños que en niñas (25,9% y 19,0% respectivamente, p=0,04), mientras que no se encontraron diferencias de sexo en los obesos

(10,6% y 10,7% respectivamente, *p*=0,57). La comparación con estudios anteriores mostró un ligero aumento del sobrepeso/obesidad en los niños del centro de Portugal en los últimos 10 años, alcanzando una prevalencia del 40,0% en los 7-9 años de edad.

De los SNPs localizados en el gen *FTO* se encontró una asociación significativa de rs9939609 y rs1421085 con el peso, IMC, IMC Z-score y circunferencia de la cintura (*p*<0,05 en todas las asociaciones), mientras que el SNP rs1861868 presentó una asociación marginalmente significativa con el peso (*p*=0,08) y el IMC (*p*=0,09). El análisis de regresión logística, con el modelo aditivo, reveló una asociación significativa entre los SNPs rs9939609 y rs1421085 y la obesidad (OR=1.41 *p*=0.02; y OR=1.45 *p*=0.01; respectivamente). Los análisis de haplotipos (rs1861868, rs1421085, rs9939609) identificaron dos combinaciones (ACA y GCA) asociadas a un mayor riesgo de obesidad (OR=1,53 *p*=0,02; y OR=1,73 *p*=0,03; respectivamente).

En el SNP -13910C>T, situado en el gen de la lactasa (*LCT*), se encontraron indicios de asociación entre el alelo -13910*T y la obesidad abdominal (OR=1,41 *p*=0,03). Bajo el modelo dominante, se observó una asociación significativa entre los genotipos CT/TT y la obesidad abdominal (OR=1,65 *p*=0,02), pero no se detectó asociación con el riesgo de obesidad (*p*=0,35) o con rasgos antropométricos (*p*>0,05).

Por último, se estudiaron otros diez SNPs de genes relacionados con la obesidad (*MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5* y *OLFM4*). El SNP rs12970134 *MC4R* mostró una asociación nominal con el IMC (*p*=0,03), el valor Z-score (*p*=0,04) y la circunferencia de la cintura (*p*=0,02), y en el límite de significancia con el peso (*p*=0,05). En el límite se encuentran también las asociaciones nominales obtenidas para el SNP rs8192678 *PPARGC1A* con el peso (*p*=0,06), y para el SNP rs545854 *MSRA* con el IMC (*p*=0,05) y el IMC Z-score (*p*=0,05). Por otra parte, la regresión logística bajo el modelo aditivo mostró que los SNPs *MC4R* rs12970134 y *TFAP2B* rs987237, en relación con el fenotipo obeso, estaban nominalmente asociados (OR=1.47; *p*=0.02) y nominalmente en el límite de la asociación (OR=1.47; *p*=0.05), respectivamente.

Además, se realizó el cribado de mutaciones del gen *MC4R* en los 32 niños que presentaban un IMC ≥ percentil 99. Se encontraron dos SNPs descritos anteriormente en estado heterocigoto: el polimorfismo -174A>C (rs34114122) en la región 5'UTR en una niña con IMC Z-score=2,51; y la

mutación común *missense* 307G>A (Val103Ile) en la región codificante del gen *MC4R* de un niño con IMC Z-score=2,60. No se detectaron otras mutaciones patogénicas en el gen *MC4R*.

En conclusión, este estudio muestra una alta prevalencia de sobrepeso/obesidad entre los niños portugueses, siguiendo la tendencia de otros países europeos. Además, se destaca por primera vez la posible asociación de SNPs en los genes *FTO*, *MC4R*, *PPARGC1A*, *MSRA* y *TFAP2B* con varios rasgos relacionados con la obesidad en una muestra de niños portugueses. Los SNPs del gen *FTO* mostraron una fuerte asociación con el riesgo de obesidad, en línea con estudios previos realizados en poblaciones europeas. Este trabajo es una contribución significativa al conocimiento de las bases genéticas de la obesidad en la población portuguesa, pero se necesitan más estudios para una mejor comprensión de este componente genético, lo que podría ser utilizado en el futuro para el desarrollo de nuevas estrategias preventivas frente a la obesidad.

Palabras clave:

Obesidad
Índice de masa corporal (IMC)
Niños portugueses
Polimorfismos genéticos
Gen MC4R
Estudio de asociación

Chapter I

General Introduction

This chapter was partially based on the following review paper: Albuquerque D, Stice E, Rodríguez-López R, Manco L, Nóbrega C. **Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective**. *Mol Genet Genomics*, 2015 [Accepted for publication].

Introduction

For a considerable period of prehistory, hominins were primarily hunter-gatherers. In that period food was severely limited and this resulted in a natural selection for humans who had the capability of storing energy as fat. Today, food is easily available in modern societies, and this environment change could act in an ancient genetic background selected to store energy. On the other hand, the changes in our environment occurred more rapidly than the modifications in our genetic background. In fact, our genetic background is not very different from 12,000 years ago, which correspond to the beginning of the agriculture development [1]. This means that there might be a delay in the adjustment of the genetic profile to environment, and that our genetic background is similar to the one from the time our forefathers were foragers. Therefore, when considering the imbalance in our modern lifestyle and our "ancient" genetic profile, it is understandable that many people gain weight so easily. When human morphology is considered, there are profound individual differences, such as body size, hair color/form, eyes color/form, etc. These human variations were due, in part, to evolutionary forces, genetic drift, environmental conditions, among others. However, in all societies and subpopulations, there are both obese and non-obese individuals. The difference arises primarily as a consequence of genetic factors, as is revealed by the high heritability for body mass index (BMI) (40-70%) [2–5]. A trait can reflect the activity of a single-gene (Mendelian or monogenic) or more than one gene (polygenic); both cases could be influenced by environmental factors. The polygenic multifactorial condition reflects the additive contribution of many genes conferring different degrees of susceptibility. Accordingly, we may understand a polygenic trait as the combined action of several genes producing a "continuously varying" phenotype. With the advent of the Human Genome Project (1990-2003), millions of DNA sequence variants were discovered in the human genome. This large and diverse database of polymorphism markers provided a novel opportunity to study the human genetic basis of several complex diseases through population approaches. In the study design of population approaches, a significant amount of individuals must be screened for a large number of polymorphisms. If a polymorphism increases susceptibility to a specific disease of interest, we should note that it is more common among

individuals affected by this condition than among non-affected individuals. Thus, through the genotyping of significant number of individuals, the population genetics tools are able to highlight the genetic basis of polygenic diseases, such as obesity.

1. What is Obesity?

Human obesity is a global public health concern and results from an excessive accumulation of body fat that can adversely affect health [6]. The global rise of obesity has serious effects, and may contribute for a significant number of diseases including type 2 diabetes mellitus, cardiovascular diseases, metabolic syndrome, and some cancers [6, 7]. Beyond co-morbidities, obesity has an important social impact with direct and indirect costs in healthcare services [7]. Excessive fat accumulation results from a persistent positive energy balance, that is, the amount of energy consumed exceeds the amount of energy expended [5]. So, obesity could be a consequence of an imbalance between energy intake and energy expenditure [8]. The energy balance represents a conglomerate of traits, each one influenced by numerous variables such as behavior, diet, environment, social structures, metabolic factors and genetics [9]. The result of this complex interaction among all of these variables contributes to individual differences in the development of obesity.

Interestingly, at the early 2000s emerged in the literature a considerable number of studies with a controversy surrounding the idea of the "obesity paradox". According to it, some individuals with overweight or obesity can be considered healthy regarding to their metabolic and cardio-respiratory fitness. Concretely, this paradox suggests that individuals with a high BMI have a better prognosis than individuals with normal BMI concerning the risk association with cardio-vascular diseases and many other chronic diseases [10]. For example, and to cite only one study, Romero-Corral *et al*. [11] showed that overweight individuals had a significantly lower risk of all-cause mortality, and a trend towards decreased cardiovascular mortality, comparing to individuals with normal BMI. Some hypotheses were proposed trying to explain this obesity paradox. For example, this could be a result of a possible selection bias and inability to control non-measurable confounding factors [12]. In fact, BMI cannot distinguish between an elevated body weight due to high levels of lean *vs*. fat body mass. Therefore, an excess of body fat is more frequently associated with metabolic abnormalities than a high level of lean body mass [13]. The

genetically inherited leptin or other adipokines deficiency was also investigated and could have an important role in obesity paradox, in which increased levels of leptin could be cardioprotective [14]. However, until now the underlying mechanisms for obesity paradox remain unclear, and the idea controversial. A more detailed discussion about this topic could be found in detail in other studies [10, 12, 15].

1.1. How obesity is defined?

Currently, the BMI, which is a simple index of weight-for-height, is the most commonly measure used to classify overweight and obesity [16]. It is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2).

The BMI is a measure that was devised in the 19^{th} century by Adolphe Quetelet, although only in 1972 it became a world reference for measuring body fat in adults. Decades of research have shown that BMI provides a good estimate for body fat, although more sophisticated and accurate measures are also being used nowadays. Currently, it is widely accepted that BMI is a reliable and easy way to access to body fat, which make an important advantages of its use as an obesity measure. Also importantly several studies related the risk of developing health problems and risk of death with BMI. According to the world health organization (WHO) for a healthy adult the BMI should range from 18.5 to 24.9 kg/m^2. Overweight is defined as a BMI of 25 to 29.9 kg/m^2, whereas obesity is defined as a BMI above 30 kg/m^2 [16]. If for adults these cutoffs are more or less consensual, the definition of children obesity based in BMI was more controversial. Depending of the age and gender it is normal for children and adolescents to have different amounts of body fat, thus several scales based in age and gender are currently used (WHO, United States the Centers for Disease Control and Prevention (CDC), and International Obesity Task Force (IOTF)). Despite the great advantages and the broad use, BMI have also some limitations. For example, being an indirect measure of body fat it does not distinguish between body fat and lean body mass. Also, it is not so accurate in younger ages compared to adults and it does not take into account normal differences between gender or ethnic groups. Thus, other indirect methods were developed to measure body fat (for example waist circumference or waist-to-hip-ratio) and currently more sophisticated direct methods such as magnetic resonance

imaging or dual energy X-ray absorptiometry are being used. However, despite all these methods and techniques, BMI remains the simple, cheap and most used measure of obesity.

1.2. Prevalence of Obesity

Epidemiological studies indicate that adiposity, as reflected by BMI, has increased worldwide over the past decades [17]. Moreover, obesity is more common in some countries than in others, though precise cross-country comparisons can be difficult because not all samples are representative of the relevant populations. Nonetheless, available data suggest that the increase in the prevalence of obesity began to emerge during the 1980s and ever since more countries have joined the global obesity pandemic [17, 18]. Between 1980 and 2008, the global change per decade for age-standardized mean BMI was increased ~0.4 kg/m^2 and ~0.5 kg/m^2 in men and women, respectively [17]. By 2013, the global estimated prevalence of overweight/obesity in men and women was 36.9% and 38.0%, respectively, comparing with 28.8% and 29.8%, respectively, in 1980. In children and adolescents the prevalence of overweight and obesity has increased from 1980 to 2013 in developed countries (23.8% and 22.6%, in boys and girls, respectively), but also in developing countries with an increase around 5% of both boys and girls. In some countries like Kiribati, Federated States of Micronesia, Libya, Qatar, Samoa among others the estimated prevalence of obesity in adults exceeded 50% of the population [18]. In 1989, worldwide estimates for the prevalence of overweight and obesity among adults (>20 years) was around 857 million individuals, comparing to the 2.1 billion in 2013 [18]. These values represent an increase of ~41% in 33 years.

Concerning the Portuguese population, the prevalence of overweight in adults (>18 year-old) were 46.7% and 38.1% in men and women, respectively, and obesity prevalence was reached the 20.0% in both sex [19]. In children aged 3-10 years 28.0% were overweight or obese (19.7% overweight; 8.2% obese) [20]. Among adolescents (11-17 years-old) the prevalence of overweight/obesity was around 20% for boys and 17% for girls between 2002 and 2010 [21].

In modern societies, despite obesity awareness campaigns and efforts to decrease in energy intake and increase in energy expenditure, obesity prevalence is increasing. However, we don't yet understand why not everyone in our societies becomes obese. Obesity has a multi-factorial etiology, involving various non-genetic and genetic factors [6, 22]. Probably most cases of

obesity results of a cluster towards the middle of this spectrum, which can be best described as the outcome of an adverse obesogenic environment, working on a susceptibility genotype. Effectively, the genetic susceptibility can potentially be mediated through defects in several different homeostatic mechanisms. Certainly, the exposure to an obesogenic and other environmental factors should be the main cause of the increase in the prevalence of high BMI over the last 30 years [6, 7, 17].

The field of genetic epidemiology aims to use systematic methods to investigate the influence of human genetic variation on health and disease, and also the relationship between environmental factors and disease.

2. Genetics of obesity

The increase in the obesity prevalence around the world has been broadly attributed to the change in environment, which is more obesogenic, against an evolutionary background, that could be maladaptive in this new obesogenic context. On the other hand, specific features of the energy balance mechanisms can effectively protect against obesity, possibly explaining why one third or more of the population remains lean [23]. The obesity phenotype only emerges if food consumption exceeds the energy expenditure on a lasting basis, resulting in a prolonged positive energy balance. However, there are many risk factors that predict the development of obesity and generally all involve the interaction of biological and social factors. Numerous studies are consistent with the hypothesis that the personal genetic profile could be a cause for individual differences in the predisposition to weight gain. It is, therefore, interesting that most of the genes involved in the susceptibility of obesity are also related to food intake and regulation of energy balance [23]. Based on genetic and phenotypic characteristics, three types of obesity forms can be considered: monogenic syndromic obesity, monogenic non-syndromic obesity and polygenic (common) obesity.

2.1. Evidence for a genetic component to obesity

Over the last 30 years, the increase in the prevalence of obesity could be attributed primarily to environmental changes, or to high-calorie food intake together with the sedentary lifestyle of modern societies [22]. The fact that the prevalence of obesity in many countries has

increased 3-fold over the last 3 decades seems difficult to conjugate with the notion that genetics are the primary cause of obesity, as revealed by twin and adoption studies [2–5]. Nevertheless it is now believed that environmental factors can influence the genetic background contributing to the increase in obesity prevalence. Moreover, epigenetic mechanisms, in which environmental factors cause changes in the expression of genes, could also help explaining the observed increase in obesity prevalence.

Heritability represents the proportion of phenotypic variation among individuals due to genetic contribution. Hence, it is not surprising that one important risk factor for childhood and adolescent obesity is parental obesity. Whitaker *et al*. [24] found that when both parents are obese there is an increase of more than double of the risk for childhood obesity. However, most of the studies found a small to medium effect of parental obesity as risk factor for childhood obesity [25]. Other studies have found a stronger effect for maternal obesity compared to paternal obesity, which may reflect pre- and postnatal environmental factors [26]. Moreover, maternal weight gain in pregnancy has been positively associated with BMI of the children into adulthood [27]. Several studies found that environmental conditions experienced *in utero* are an important factor in programming obesity.

Twin studies have been used to model the genetic component of a given trait, due to the fact that monozygotic (MZ) twins are genetically identical, while non-identical dizygotic (DZ) twins share only 50% of their genetic material [22]. In 1977, Feinleib *et al*. [2] studied the correlations for weight in 250 MZ and 264 DZ male veteran twin pairs, and established for the first time that familial aggregation for obesity results mainly from genetic influence. In 1986, Stunkard *et al*. [4] confirmed these results in a 25-year follow-up study using more than 4000 MZ and DZ twin pairs. High heritability values for BMI were observed for the same subjects at 20 years (h^2=0.77) and at 45 years (h^2=0.84). The heritability of fat mass among MZ twins has been reported to range from 70-90%, while in DZ twins it is 35-45%. Adoption studies have strengthened the evidence of a strong genetic influence on human body weight. Body corpulence of adopted children correlates more strongly with BMI of their biologic parents versus the BMI of their adoptive parents [3]. Recently, Silventoinen *et al*. [5] conduct a review of studies in twins and adopted children, suggesting that genetic factors could have a much stronger effect than environmental factors on the BMI trends in children up to the age of 18

years.

Another genetic component for obesity is highlighted through the different prevalence between racial groups. For example, it was found that obesity prevalence in Caucasian and Asian populations is of about 35% or less compared to 50% or more found among Pima Indians living in New Mexico [28]. However, the search for underlying genotypes that cause of obesity has been challenging due to the complex interactions involved in the regulation of adiposity. Indeed, many of individual genotypes (especially those obtained with a lower odds ratio) that have been associated with elevated body mass have not been replicated in a reliable fashion. Moreover, environmental factors and cultural diversity also account for the different obesity prevalence found across ethnicities.

Studies have shown that genetic population substructure, economic disadvantages, psychosocial stress, or access to medical care could have an important impact in obesity development and prevalence. The cultural context could also influence obesity, by defining for example the type and quality of food intake. Also, in some cultural contexts the obesity phenotype could represent a signal of wealth and high social status. Another important factor is the genetic architecture, which is different across population (population substructure) and might be differentiated in ethnic clusters. This could presuppose that disease-causing alleles are more likely to be present in some groups or even be specific to other groups. This point could be considered as the population genetic predisposition to develop the disease. For example, it is well demonstrated that in the US black-white population occur disparities in the risk of developing cardiovascular disease [29]. Also, social factors such as economics disadvantage or psychosocial stress between groups could have a real impact in causes of ill health. For example, disparities originated by the limited access of quality medical care between different ethnics groups living in the same population might influence health, causing different disease rates.

The role of maternal nutrition and stress suffered during pregnancy, mostly due to social disparities or cultural differences could also influence biological processes and responses across the life cycle that will be discussed later. All these factors affect the intrauterine environment reflecting differences in birth weight. However, nowadays is still evident that genetic factors play a considerable role in obesity. Three distinct forms of obesity could be found: monogenic, syndromic and polygenic obesity.

2.2. Mendelian forms of obesity

Monogenic forms of obesity result from an alteration of a single gene and are rare, affecting about 5% of the population, and severe [30, 31]. There are more than 200 described cases of human obesity associated with homozygous forms of a single gene mutation [32, 33]. Two forms of Mendelian inheritance of obesity could be found: syndromic and non-syndromic. Most of these monogenic forms of obesity are characterized by an early-onset of the disease and an extreme phenotype [30]. In the search for homologous mutations in mice, several human forms of obesity have been identified [34]. Thus, murine models appear useful to understand the molecular pathogenesis of human obesity [35]. Family studies based on individuals with extreme obesity, also proved to be very successful in the detection of obesity-related mutations [36]. Below is presented a brief and general review about the two syndromic forms of obesity.

2.2.1. Non-syndromic form of obesity

Over the past 15-20 years, several gene mutations have been shown to cause autosomal recessive or co-dominant forms of obesity. More than 200 single-gene mutations have been found to cause human obesity [37]. Interestingly, all these mutations can be found in only ten genes [33]. However, these mutations are rare and lead to extreme obesity with an early-onset obesity and other endocrine disorders [31]. There are eight well-known genes in monogenic non-syndromic form of obesity explaining up to 10% of cases with early-onset extreme obesity, affecting *LEP*, *LEPR*, *POMC*, *PCSK1*, *MC4R*, *BDNF*, *NTRK2* and *SIM1* (Table 1.1) [30, 31, 38]. All these genes code for proteins with a central role in the leptin-melanocortin signaling pathway present in the hypothalamus, and therefore affect regulation of food intake and energy expenditure [31]. This pathway is activated when *LEP* is secreted by the adipose tissue, binds to its receptor, localized in the surface neurons in the arcuate nucleus of the hypothalamus [39]. The signal that regulates satiety and energy homeostasis is then propagated through the *POMC*/cocain and amphetamine related transcript (CART) and melanocortin system [31]. While *POMC*/CART neurons synthesize anorexigenic peptide alpha-melanocyte-stimulating hormone (α-MSH), a distinct group of neurons synthesizes the orexigenic peptide neuropeptide Y (*NPY*) and agouti related protein (AGRP), which act as inhibitors of *MC3* and *MC4* receptors [40].

Table 1.1. Monogenic forms (syndromic and non-syndromic) of obesity.

Non-syndromic forms

Gene name	*Gene symbol*	*Chromosome location*	*Mutations*	*Obesity Phenotype*
Leptin	*LEP*	7q32.1	ΔG133, Arg105Trp	Extreme, early-onset obesity, hyperphagia.
Leptin receptor	*LEPR*	1p31.3	Exon 16 splice donor G→A	Extreme, early-onset obesity, hyperphagia.
Pro-opiomelanocortin	*POMC*	2p23.3	G7013T, 7133delC, C3804A, A6851T, 6906delC, 6996del, 7100insGG, 7134delG	Early onset obesity.
Proconvertase 1	*PCSK1*	5q15	Gly483Arg, A→C+4 intron 5 donor splice site, Glu250Stop, Del213Ala	Childhood onset obesity, elevated proinsulin, hypocortisolemia, depressed POMC, reactive hypoglycemia.
Melanocortin-4 receptor	*MC4R*	18q21.32	>150	Early onset obesity, hyperphagia, increased fat mass, increased lean mass.
Brain-derived neurotrophic factor	*BDNF*	11p13	46, XX, inv(11)(p13p15.3)	Severe obesity, Hyperphagia, body weight.
Neurotrophic tyrosine kynase receptor type 2	*NTRK2*	9q22.1	Y722C	Severe early onset obesity, hyperphagia.
Single-minded homolog 1	*SIM1*	6q16.3	*de novo* balanced translocation 1p22.1 and 6q16.2.	Early-onset obesity, hypotonia, developmental delay.

Syndromic forms

Syndrome	*Gene*	*Chromosome location*	*Obesity Phenotype*
Prader Willi syndrome (PWS)	*Contiguous gene disorder*	15q11-13	Neonatal hypotonia, poor feeding, evolving into extreme hyperphagia, central obesity.
Bardet-Biedl syndrome (BBS)	*BBS1-BBS12*	11q13.2	progressive late childhood obesity
Alstrom syndrome	*ALMS1*	2p13.1	Mild truncal obesity
WAGR syndrome	*BDNF*	11p14.1	Obesity
16p11.2 deletion		16p11.2	Progressive obesity

The derived peptide nature of *POMC* depends of the endoproteolytic type enzyme present, specific in brain region. In the anterior pituitary, the *PCSK1* enzyme produces adrenocorticotropic hormone (ACTH) and β-lipotropin (β-LPH), while the combined presence of *PCSK1* and *PCSK2* in the hypothalamus control the production of α-, β-, γ- MSH and β-endorphins [31]. Individuals carrying mutations in the *MC4R*, *LEP* and *LEPR* genes represents the most extreme phenotype and become obese at a very young age.

The protein encoded by the *MC4R* gene, is a membrane-bound receptor and a member of the melanocortin receptor family [41]. The protein interacts with adrenocorticotropic and MSH hormones and is mediated by G proteins. The *MC4R* gene is composed by a single exon, and is located in the chromosome 18q21.3, encoding for the 332-amino acid seven-transmembrane G-protein-linked receptor, critically involved in regulating energy balance [42]. It is expressed mainly in the central nervous system, including in the hypothalamus, contributing to food intake and energy expenditure regulation [42, 43]. In 1998, two independent groups reported a mutation in the *MC4R* gene, which result in a non-functional receptor causing severe early-onset obesity [44, 45]. In morbidly obese individuals, deficiency in the *MC4R* gene activity represents the most common cause (1 to 6%) for the obese phenotype [45–47]. More than 150 variants of this gene have been described, usually classified into five classes depending of their molecular effects [41].

The *LEP* gene (chromosome 7q31.2) encodes a protein that is secreted by white adipocytes, which plays a central role in body weight regulation [39]. This protein, acts as part of a signaling pathway that can inhibit food intake and/or regulate energy expenditure to maintain constancy of adipose mass. In 1997, in a screening for serum level concentrations in severely obese subjects, two children of the same family were found with undetectable levels of leptin [48]. Subsequently research revealed that leptin deficiency is inherited and produces extreme early onset obesity [49]. This deficiency can be caused by a frameshift mutation (del G133), which produces a truncated protein that is not secreted [49] or a missense mutation Arg105Trp, which is associated with low levels of circulating leptin [50].

The protein encoded by the *LEPR* gene (chromosome 1p31.3) belongs to the gp130 family of cytokine receptors, which stimulate gene transcription via activation of cytosolic STAT proteins, predominantly in the hypothalamic neurons [51]. This protein is a receptor for leptin

and is involved in regulation of fat metabolism. A splice site mutation in the exon 16 is associated with leptin receptor deficiency, producing extreme obesity [52].

2.2.2. Syndromic form of obesity

Syndromic forms refer to obesity cases that occur in a distinct set of associated clinical phenotypes, such as mental retardation or organ-specific developmental abnormalities [53]. There are more than 30 Mendelian disorders that result in obesity [37]. Research is beginning to determine the genetic basis of some of these syndromes, thus elucidating the pathogenesis of the chronic positive energy balance. The genetic basis of these disorders is extremely heterogeneous. Table 1.1 presents the most common forms of early-onset syndromic obesity for which the genetic basis is, at least, partially understood, including WAGR (Wilm´s tumor, aniridia, genitourinary anomalies and mental retardation), Prader-Willi, Bardet-Bield, Altröm and Cohen syndromes.

WAGR syndrome is a rare genetic disorder characterized by a deletion at chromosome 11p13 in a region containing the Wilm´s tumor 1 (*WT1*) and paired box 6 (*PAX6*) genes [30]. A specific type of WAGR has been associated with a deletion in the brain-derived neurotrophic factor (*BDNF*) gene, which results in an obese phenotype.

Prader-Willi syndrome (PWS) can have several etiologies, characterized by central obesity, neonatal hypotonia, hyperphagia, hypothalamic hypogonadism and mild mental retardation, with such abnormalities as short stature and peculiar facial features [30]. Most of the cases were associated with loss of expression from paternal deletions of the 15q11.2-q12 chromosomal region [31].

Bardet-Biedl syndrome (BBS) is characterized by early-onset obesity, which is associated with progressive cone-rod dystrophy, morphological finger abnormalities, dyslexia, learning disabilities, and progressive renal disease [30]. BBS has extensive genetic heterogeneity with at least 14 *loci*, (often called BBS gene) and several mutations identified within these *loci* [31].

Alström (ALMS) and Cohen syndromes are associated with childhood mild truncal obesity and small stature [30, 31]. Both of them are autosomal recessive and genetically homogenous. ALMS is caused by a balanced translocation of chromosome 2p13 that disrupts *ALMS1* gene or by a small number of mutations in this gene. Cohen syndrome results from mutations in the

COH1 gene, located at chromosome 8q22, which encodes a transmembrane protein of unknown function [30].

Finally, we can also found ciliary dysfunction, collectively termed “ciliopathies”. These comprise a group of several disorders associated with genetic mutations encoding defective proteins, affecting normal function or formation of cilia [54]. Ciliary dysfunction can manifest as a set of heterogeneous features including retinal degeneration, renal disease, cerebral anomalies, congenital fibrocystic diseases of the liver and pancreas, diabetes, obesity and skeletal dysplasias [55]. Due to the heterogeneous phenotype, ciliopathies have been associated with mutations in more than 40 genes including the genes involved in BBS and ALMS syndromes.

2.3. Polygenic or common obesity

In most modern societies, the environment favors weight gain rather than loss, due to food abundance and lack of physical activity. Furthermore, the increase of common obesity in both adults and children has increased in the last decade worldwide. However, the genetic and molecular mechanisms involved in body weight regulation are complex. The genetic profile of polygenic obesity results from the effects of several altered genes. In theory, the genetic basis of polygenic obesity implies that the specific set of variants relevant for obesity vary considerably from one obese person to the next [36]. For this reason, the study of common obesity is far more complex. However, the advent of new techniques facilitated this study by allowing the analysis of several *loci* at the same time.

2.3.1. Genetic approach for common obesity

The study of common obesity is based in the analysis of gene variation in genomic DNA (single nucleotide polymorphism, or microsatellites) situated within or near candidate genes. In contrast with monogenic obesity, in polygenic obesity each variant confers susceptibility, requiring additionally the presence of other variants and an obesogenic environment to determine the obese phenotype [56]. There are some approaches used in the detection and analysis of a candidate gene in body weight regulation: linkage studies, candidate gene association studies and GWA studies. Their objective is to determine whether an association between a genetic variation and an obesity-related trait do exist. Until now, GWAS had

identified more than 52 *loci* associated with obesity-related traits [57].

Recently, with the advent of automated DNA sequencing instruments, involving advances in engineering, chemistry, molecular biology, and software, open a number of new opportunities [58]. Currently, molecular diagnosis based on Sanger's sequencing is restricted to only a few genes as this technology is expensive, time consuming, and labor intensive. The advent of next-generation sequencing (NGS) technology provides a new method for molecular diagnosis, allowing the sequencing of whole genomes or exomes, or several genes at the same time [59]. NGS promises to change the landscape of genetic testing with innovative cost-efficient methods for sensitive obesity multi-gene screening.

Only a few studies have used NGS technology to study obesity. Saeed *et al*. [60] analyzed 26 susceptible genes for obesity in a sample of 39 Pakistani children with early-onset obesity. They found two new *LEPR* mutations at the homozygous state: a splice site mutation in exon 15 (c.2396-1 G>T), and a nonsense mutation in exon 10 (c.1675 G>A). Sällman *et al*. [61] amplified the entire region of the fat mass and obesity-associated (*FTO*) gene (412 kilo base pairs), from 524 severely obese and 527 lean Swedish children. They detected 705 single nucleotide polymorphisms, from which 19 were novel obesity-associated polymorphisms within the first intron of the *FTO* gene. An interesting finding was the fact that 10 of them have a stronger association with obesity (p<0.007) when comparing with the commonly studied rs9939609 polymorphism (p<0.012). This study concluded that within the entire region of the *FTO* gene the first intron was the only one associated with obesity. Bonnefond *et al*. [62] searched for mutations with NGS in 40 patients, with a monogenic form of diabetes (n=19) or obesity (n=21), in which the causing mutation was already known. The study found the same mutations described as the phenotype cause, except for one variant (mean of 98.6%). On the other hand novel mutations were found in 3 patients with a putative deleterious effect.

The NGS approach could be used as an efficient tool with highly sensitive screening for mutations in genes associated with obesity or other diseases. Further, sequencing the human genome can now be accomplished in the data-generation phase within two weeks at a cost of approximately US $5,000 [58]. However, the price for genome sequencing continues to decrease; in 2014 Illumina announced that would produce a new system called HiSeq X Ten that can deliver full coverage of human genomes for less than US $1,000. However, until now the

majority of *loci* associated with obesity susceptibility were found by GWAS. For this reason we described the *loci* found by this technique in more detail and in a chronological order.

2.4. Common *loci* associated with obesity-susceptibility discovered through GWAS

The GWAS approach is the most commonly methodology used, allowing geneticists to scan numerous polymorphisms (~0.1-5 million of polymorphisms) across the entire genome using powerful statistical methods to identify *loci* associated with a particular phenotype. Since the start of the GWAS era in 2005, there have been five waves of GWAS' discoveries for BMI. The first *loci* identified through GWAS was the *FTO* gene, and until now more than 50 genetic *loci* have been identified as being associated with at least one obesity-related trait [8, 22, 63] (Figure 1.1).

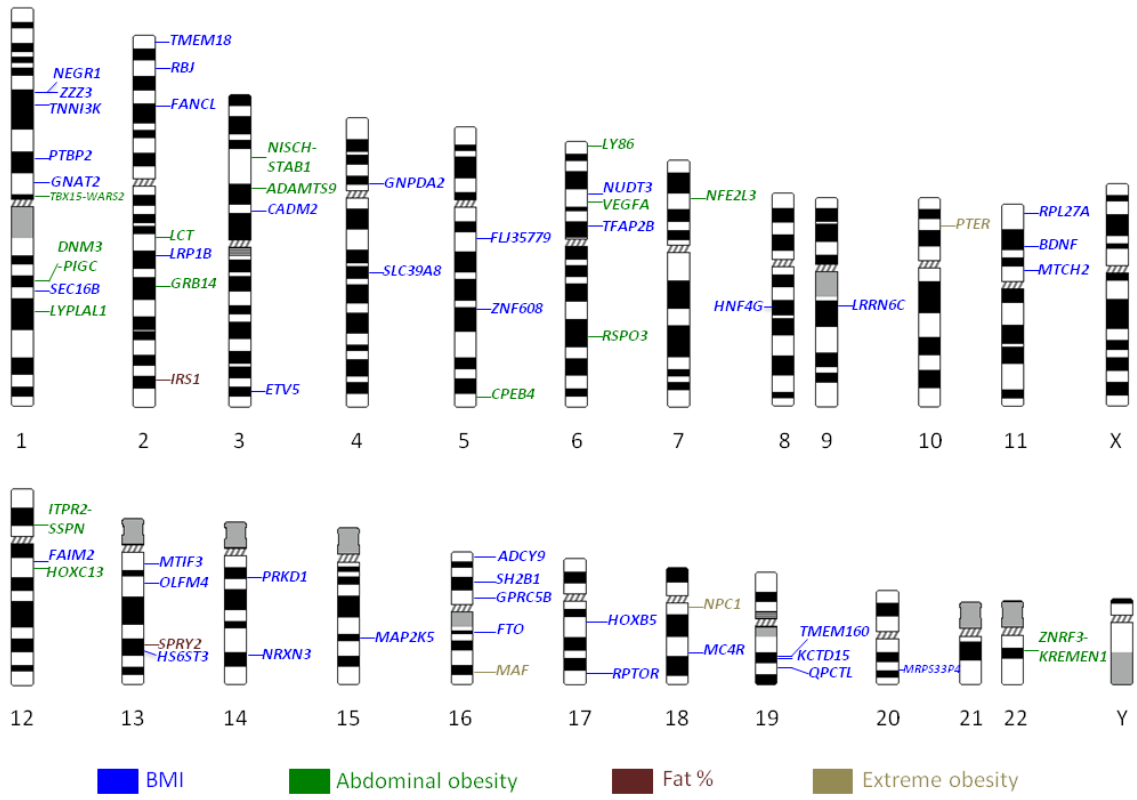

Figure 1.1. *Loci* associated with obesity-related phenotypes. Almost in every human chromosome it was found a locus linked to predisposition to obesity-phenotype (BMI, abdominal fat, fat percentage or extreme obesity).

2.4.1. First discoveries by GWAS: the *FTO* gene

The first locus associated with obesity was the insulin-induced gene 2 (*INSIG2*) [64]. However, replication studies demonstrated very inconsistent results. So, the first locus unequivocally associated with obesity by a GWA study was the *FTO* gene [65]. Initially, Frayling *et al*. [65] conducted a GWA study to test the correlation between polymorphisms across the entire human genome and type II diabetes (T2D). They found that the rs9939609 polymorphism, located in the first intron of the *FTO* gene was strongly associated with T2D and increased BMI. However, after adjustment for BMI, the apparent association of the polymorphism with T2D was not maintained. The effect size of *FTO* polymorphism on BMI is modest, with homozygous individuals for the risk allele (in this case the "A-allele") weighing on average 3 kg more than those homozygous for the protective allele (in this case the "T-allele"), with the difference representing approximately 0.36 kg/m^2 [22].

These findings have been independently replicated and have consistently confirmed the association of rs9939609 polymorphism with the etiology of common obesity in several populations: European [65–68], Asian [69–72] and African [73–75], both in children and adults. Two following studies reported other polymorphisms in the intronic *FTO* region also consistently associated with severe early-onset childhood and adult obesity (rs1421085 and rs17817449) [76], and have extended the association to other obesity-related traits including body weight and waist-to-hip circumference ratio (WHR) (rs9930506) [67]. The *FTO* polymorphisms were also associated with abdominal obesity, waist circumference and waist-to-hip ratio (WHR) [77, 78], and also with body fat percentage [79]. Although these posterior reports replicate well the initial findings, the *FTO* polymorphisms explain only 1-3% of the variance in BMI [65, 67].

To date, over 500 studies have been performed concerning the association of *FTO* polymorphisms with obesity in several populations worldwide, and more than 60 polymorphisms in this gene were significantly associated with obesity [61]. All these polymorphisms were found within a 47 kb linkage disequilibrium (LD) block encompassing parts of the first two introns as well as exon 2 of the *FTO* gene [80]. This is a region where the sequence is strongly conserved across species, with polymorphisms highly correlated (LD r^2 > 0.80 in CEU of the HapMap) in Caucasian populations [81].

The functional mechanism underlying *FTO* role in obesity remains unknown, as well as the

pathway underlying that role. The *FTO* is a very large gene with 9 exons spanning more than 400 kilobase (kb) in the chromosome 16q12.2 [82]. It was originally identified in 1999 in the Fused toes (*Ft*) homologue mutants, in a deletion of 1.6 megabase (Mb) on chromosome 8 [83]. Homozygosity of *Ft* mutants is embryonically lethal. To investigate the biological function of *FTO* gene, two mouse models were used. Homozygous *FTO*-/- mice introduced by Fischer *et al*. [84] show postnatal growth retardation, significant reduction in fat and lean body mass compared to the wild-type animals [85]. In other mice model, Church *et al*. [86] observed a lean phenotype in mice carrying a missense mutation in exon 6 of *FTO* (*FTO*I367F mice). These results seem to indicate that *FTO* could play a role in food intake control, energy expenditure and homeostasis.

The predicted human protein consists of 505 amino acids, characterized as a 2-oxoglutarate-dependent enzyme that is localized in the cell nucleus, belonging to the (2OG) oxygenases AlkB family of proteins [87]. The AlkB is a DNA repair enzyme, which catalyzes Fe(II)- and 2OG-dependent demethylation of damaged DNA substrates [88]. Recently, Jia *et al*. [89] indicated that *FTO* also demethylates N6-methyladenosine (m6A) residue in nuclear RNA. *FTO* variation appears to lead to an increase in energy intake [90] by modifying hypothalamic control of appetite [81]. The crystal structure of *FTO* has recently been published and reveals the basis for its substrate specificity [91].

Moreover, it was found that the *FTO* gene is also a transcriptional co-activator [92] and a possible regulator of telomere length [93]. A recent study found that BMI-associated *FTO* variants interact with the promoter region of iroquois homeobox 3 (*IRX3*) gene in the human, mouse and zebrafish genomes [94]. They also found that in Irx3-deficient mice, there is a reduction in body weight of 25 to 30%. This study suggests that *IRX3* gene is a functional long-range target of obesity-associated polymorphisms within *FTO*. On the other hand, the *FTO* deficiency remains still poorly understood in the context of obesity development and confirm the complexity of the genetics underlying common obesity.

2.4.2. Five waves of GWAS

Following the discovery of the *FTO* locus, investigators enhanced GWA studies by increasing the sample size improving statistical power to uncover additional obesity-susceptibility *loci* (Table 1.2). Subsequently, a large-scale international consortium, called the Genetic

Investigation of Anthropometric Traits (GIANT) emerged. The association data of 16,876 Caucasians from seven GWAS for BMI were combined in a meta-analysis [95]. This study confirmed the strong association of obesity with polymorphisms in the *FTO* gene, and identified one new locus near the *MC4R* gene which mutations are known to be the common cause of extreme childhood obesity [30]. The *MC4R* was the second gene significantly associated with common obesity [95, 96]. The rs17782313 polymorphism near the *MC4R* gene was associated with obesity among both adults and children [95]. Another polymorphism (rs12970134) near the *MC4R* gene also appears to increase the risk of obesity among Europeans [97]. Several polymorphisms near the *MC4R* gene have subsequently been found and replicated in various European populations, as well as in Asians [98], African-American [98], both in children and adolescents [73, 75].

In the third wave of discoveries, a meta-analysis was performed using 15 GWAS for BMI in Caucasians (n > 32,000) and replicated in another 14 studies for a second-stage sample of 59,082 individuals [99]. They confirmed the association of the *FTO* and *MC4R* genes, and found six new genes positively associated with obesity: *MTCH2*, *GNPDA2*, *KCTD15*, *SH2B1*, *NEGR1* and *TMEM18*. At the same time, a GWAS of 31,392 individuals, predominantly from Iceland population, found seven new genetic *loci* near or in *BDNF*, *SEC16B*, *ETV5* and *FAIM2*, as well as *FTO* and *MC4R* genes associated with BMI [97]. Four of the seven newly identified *loci* were common with the results from Willer *et al*. [99].

In 2010, the fourth wave, the GIANT consortium expanded its GWAS stage to comprise 249,796 individuals of European origin, and reveal 18 new *loci* associated with BMI near or in: *PRKD1*, *SLC39A8*, *GPRC5B*, *MAP2K5*, *QPCTL*, *RBJ*, *LRRN6C*, FLJ35779, *CADM2*, *TMEM160*, *FANCL*, *LRP1B*, *TNNI3K*, *MTIF3*, TFAP2B, *ZNF608*, NRXN3, *RPL27A*, *PTBP2* and *NUDT3* [100]. By 2011, GWAS had identified 32 genetic *loci* unequivocally associated with BMI.

The most recent and fifth wave expanded the GIANT meta-analysis, to comprise 263,407 individuals of European ancestry [57]. Besides confirming all 32 BMI-associated *loci* previously identified by the fourth wave, they found seven new *loci*, *ZZZ3*, *RPTOR*, *ADCY9*, *GNAT2*, *MRPS33P4*, *HS6ST3* and *HNF4G*, explaining an additional 0.09% of the variability in BMI [57].

Table 1.2. Currently established *loci* associated with BMI in GWAS.

Wave	Gene symbol	Gene name	SNP ID	Effect size BMI (OR 95%CI)*	Discovery study
First	*FTO*	Fat mass and obesity associated	rs9939609	1.31 (1.23-1.39)	[65, 67]
Second	Near *MC4R*	Melanocortin-4 receptor	rs17782313	1.12 (1.08-1.16)	[95]
Third	Near *TMEM18*	Transmembrane protein 18	rs7561317	1.20 (1.13-1.27)	[97, 99]
			rs6548238	1.19 (1.10-1.26)	
	FAIM2	Fas apoptotic inhibitory molecule 2	rs7138803	1.14 (1.09-1.19)	
	Near *GNPDA2*	Glucosamine-6-phosphate deaminase 2	rs10938397	1.12 (1.07-1.17)	
	SEC16B	S. cerevisiae Sec16	rs10913469	1.11 (1.05-1.18)	
	BDNF	Homolog of brain-derived neurotrophic factor	rs925946	1.11 (1.05-1.16)	
	Near *ETV5*	Ets variant 5	rs7647305	1.11 (1.05-1.17)	
	SH2B1	SH2B adaptor protein 1	rs7498665	1.11 (1.06-1.17)	
	Near *NEGR1*	Neuronal growth regulator 1	rs2568958	1.07 (1.02-1.12)	
	Near *KCTD15*	Potassium channel tetramerization domain containing 15	rs29941	1.10 (1.04-1.15)	
			rs11084753	1.04 (0.98-1.10)	
	MTCH2	Mitochondrial carrier 2	rs10838738	1.03 (0.98-1.08)	
Fourth	Near *PRKD1*	Protein kinase D1	rs11847697	1.10 (1.03-1.17)	[100]
	SLC39A8	Solute carrier family 39, member 8	rs13107325	1.10 (1.05-1.15)	
	TFAP2B	Transcription factor AP-2 beta	rs987237	1.09 (1.05-1.12)	
	QPCTL	Glutaminyl-peptide cyclotransferase-like	rs2287019	1.09 (1.05-1.12)	
	NRXN3	neurexin 3	rs10150332	1.09 (1.05-1.12)	
	Near *GPRC5B*	G protein-coupled receptor, family C, group 5, member B	rs12444979	1.08 (1.04-1.11)	
	Near *RBJ-DNAJC27*	DnaJ (Hsp40) homolog, subfamily C, member 27	rs713586	1.07 (1.05-1.09)	

	MAP2K5	Mitogen-activated protein kinase 5	rs2241423	1.07 (1.04-1.10)	
	Near *TMEM160*	Transmembrane protein 160	rs3810291	1.06 (1.03-1.08)	
	Near *FANCL*	fanconi anemia, complementation group L	rs887912	1.06 (1.03-1.08)	
	Near *FLJ35779-POC5*	centriolar protein	rs2112347	1.05 (1.03-1.08)	
	Near*LRP1B*	low density lipoprotein receptor-related protein 1B	rs2890652	1.05 (1.02-1.08)	
	MTIF3	mitochondrial translational initiation factor 3	rs4771122	1.05 (1.01-1.08)	
	LRRN6C	leucine rich repeat neuronal 6C	rs10968576	1.04 (1.02-1.06)	
	TNNI3K	interacting kinase	rs1514175	1.04 (1.02-1.07)	
	CADM2	cell adhesion molecule 2	rs13078807	1.03 (1.00-1.06)	
	NUDT3	nucleoside diphosphate linked moiety X type motif 3	rs206936	1.03 (1.01-1.06)	
	Near *RPL27A*	ribosomal protein L27a	rs4929949	1.03 (1.01-1.05)	
	Near *ZNF608*	izinc finger protein 608	rs4836133	1.03 (1.01-1.05)	
	Near *PTBP2*	polypyrimidine tract binding protein 2	rs1555543	1.02 (0.99-1.04)	
Fifth	*GNAT2*	guanine nucleotide binding protein (G protein) alpha transducing activity	rs17024258	1.27 (*p*=0.02)	
	HS6ST3	heparin sulphate 6-O-sulfotransferase 3	rs7989336	1.09 (*p*=0.0001)	
	HNF4G	hepatocyte nuclear factor 4, gamma	rs4735692	1.09 (*p*=1.97x10-5)	
	RPTOR	regulatory associated protein of MTOR, complex 1	rs7503807	1.08 (*p*=7.07x10-5)	[57]#
	MRPS33P4	mitochondrial ribosomal protein S33 pseudogene 4	rs13041126	1.08 (*p*=0.001)	
	ZZZ3	zinc finger, ZZ-type containing 3	rs17381664	1.08 (*p*=0.001)	
	ADCY9	adenylate cyclise 9	rs2531995	1.06 (*p*=0.01)	

Abbreviations: BMI, body mass index; OR, odd ratio; 95%CI, confidence interval; SNP ID, polymorphism identification.
*Effect size from first discovery study.
This study not reported confidence intervals, but rather *p*-values.

To date, more than 35 *loci* have been found associated with the increase of BMI (explaining ~1-4% of the variance in BMI), while other *loci* correlate with abdominal obesity, establishing 13 *loci* associated with it, assessed by the WHR [101]. Other *loci*, such as the Lactase gene (*LCT*) have been associated with BMI and abdominal obesity, but more studies are required to confirm associations [102–104]. A study identified two new *loci* with body fat percentage: *IRS1* and the other near *SPRY2* [105]. There is a gap between the explained variance of BMI due to known common polymorphisms (1-4%), and the estimated heritability (40-70%). One of the main problems pointed out in GWAS is the failure to detect *loci* that are associated with traits whose effect sizes are too small to reach genome-wide statistical significance (false negative rate). To circumvent this "missing heritability" the genome-wide complex trait analysis (GCTA) method appears to show a multitude of low penetrance common polymorphisms, each with causal effects but too small to allow detection by GWA studies. Using this approach, Yang *et al*. [106] estimated in 17% the BMI variance due to common genetic variation and a recent analysis of twin studies revealed that additive effects of multiple common polymorphisms could explain 37% of BMI [107]. Most GWAS were performed in population samples of Caucasians adults, and only a few in children. However, it seems important to determine the genetic predisposition in children, as obesity tends to develop from childhood into adult life.

2.5. Testing adult-discovered *loci* in children

Childhood obesity is a major health problem in developing countries throughout the world. Most of obesity susceptible genes were found in studies with adults, which prompted an effort to replicate findings in studies with children [75, 108]. Knowledge of the genetic risk factors of obesity in children could be used as a first step to develop possible prevention measures. The *FTO* locus remains the most replicated gene and the strongest gene associated with obesity susceptibility, both in adults and children [75, 109]. Results from longitudinal studies suggested a possible age-related change in the association between the *FTO* rs9939609 polymorphism and higher BMI. Sovio *et al*. [110] studied subjects of European ancestry aged from early infancy to 13 years-old. In that sample, individuals' carriers of the minor A-alleles of this polymorphism showed lower BMI in infancy and higher BMI later in childhood. In another study, Hallman *et al*. [111] analyzed a sample of non-Hispanic white children and adolescents (8-18 years). It was found a significant age-by-genotype

interaction predicting that in individuals with AA genotype the BMI would be ~0.7 kg/m^2 higher at age 8, and ~1.6 kg/m^2 higher at age 17, comparing to those with AT or TT genotypes. The results reported in these studies might help to reveal mechanisms regulating body mass in humans during a critical period of development. Genes *TMEM18* and *GNPDA2* were also associated with obesity susceptibility, with a similar effect of the *FTO* gene [112]. Remaining *loci* with evidence for association with obesity in children were *INSIG2*, *MC4R*, *NEGR1*, *BDNF* and *KCTD15* [112, 113].

In a GIANT meta-analysis, Zhao *et al*. [108] examined 32 genetic *loci* in 1,097 obese cases and 2,760 lean controls, aged between 2 and 18 years old, in a pediatric European American sample. They found evidence of associations with nine of these *loci*, namely *FTO*, *TMEM18*, *NRXN3*, *MC4R*, *SEC16B*, *GNPDA2*, *TNNI3K*, *QPCTL*, and *BDNF*. Overall, 28 of the 32 *loci* showed directionally consistent effects to that of the adult BMI meta-analysis.

Another similar report by the Early Growth Genetics (EGG) consortium investigated the effect of established adult BMI with two recently associated *loci* with childhood obesity (*HOXB5* and *OLFM4* genes) [114] in a Greek adolescents cohort [115]. The genetic risk score of the 34 (GRS-34) variants was calculated and found that variants at the *FTO*, *TMEM18*, *FAIM2*, *RBJ*, *ZNF608* and *QPCTL loci* produced nominal evidence for association with BMI and/or obesity risk. Overall, 27 out 34 variants showed consistent effects with those reported by large-scale meta-analyses adult BMI.

These results showed clearly that these obesity-conferring variants operate early in life, suggesting that individual preventative lifestyle intervention in childhood could be important to obesity development.

2.6. GWAS-related investigations in other ethnicities

There are remarkable disparities in the prevalence of obesity between ethnic groups. To date most of GWAS published reports have been performed in populations of European origin. Only one study identified, at the first discovery stage, a locus near *MC4R* gene associated with waist circumference and insulin resistance in a cohort of South Asian population [96]. This could be partly due to the fact that some susceptible *loci* only affect a specific ethnic group, while others might affect any ethnic group. Indeed, the human genetic architecture differs across ethnicities, which is well illustrated by differences in linkage disequilibrium (LD), whereas haplotype blocks vary only somewhat among human populations [116].

As a case in point, *FTO locus* also have consistently correlated with BMI and risk of obesity in populations of African [73–75, 117], Asian [69–72] and Pacific-Islander [118] ancestry. Despite the fact that effect sizes were similar to those observed in white European populations, the risk allele frequency varies substantially: around 45% in white Europeans, 25% in Asian, and range of 7 to 18% in African origin [119]. In the case of *FTO* gene, Peters *et al*. [120] genotyped 3,756 polymorphisms across a 646 kb region, encompassing the large *FTO* gene (16q12.2) and the flanking gene *RPGRIP1L* in 20,488 African Americans. Authors reported the rs56137030 polymorphism as the most significantly associated with BMI. Interestingly, they found that in individuals of European ancestry, this polymorphism represents a cluster of 103 polymorphisms (r^2>0.50), whereas in African Americans this cluster includes only 29 polymorphisms (at r^2>0.50).

Two recent independent meta-analysis were performed in both East Asian and African populations [121, 122]. Wen *et al*. [121] performed a meta-analysis using 27,715 individuals, followed by *in silico* and *de novo* replication studies in a further 37,691 and 17,642 individuals of East Asian origins, respectively. Seven previously identified *loci* were detected (*FTO*, *SEC16B*, *MC4R*, *GIPR-QPCTL*, *ADCY3-RBJ*, *BDNF* and *MAP2K5*) and three new *loci* were uncovered, near or in *CDKAL1*, *PCSK1* and *GP2* genes. Data also implicated three *loci*, *GNPDA2*, *TFAP2B* (previously identified) and *PAX6*, which all reached the genome-wide significance threshold. A recent meta-analysis was conducted to examine the association of >3.2 million polymorphisms with BMI in 39,144 adults of African ancestry [122]. It identified one new *locus* at 5q33 (*GALNT10*, rs7708584 polymorphism) and another at 7p15, when data from the GIANT consortium was included (*MIR148A-NFE2L3*, rs10261878 polymorphism). They also found evidence of an association at 6q16 (*KLHL32*, rs974417 polymorphism) in African-ancestry sample. Overall, 32 of the 36 previously established BMI variants showed consistent effect in this GWAS. The 36 known BMI *loci* explain in average 1.30% of the variance in BMI of African ancestry compared with 1.67% and 1.25% in European and Asian ancestry populations, respectively [122]. More recently, Tan *et al*. [123] replicated six confirmed obesity genes (*FTO*, *CTNNBL1*, *ADRB2*, *LEPR*, *PPARG* and *UCP2* genes) in eight different population samples from different ancestries (five Caucasian, one Chinese, one African-American and one Hispanic population). The main goal of this study was to explore whether the same genes contribute differentially to obesity susceptibility in populations of different ancestries. Regarding the *FTO* gene they found 35 polymorphisms significantly associated with obesity in Caucasian populations.

However, none of them showed evidence of associations with obesity in another ethnic group.

Association studies across different populations can help us to define more precisely which *loci* or variants could play a role in the obesity etiology, and help to understand the genetic and environmental factors contributing to obesity. As we can see, polymorphisms in the *FTO* gene are associated with obesity in several ethnicities. However, the allele frequencies of the BMI-associated *FTO* polymorphisms vary substantially across the different ethnicities. The highest prevalence of minor risk allele is observed in Europeans and the smaller frequency in Asian and African populations. The LD cluster of *FTO* polymorphisms was clearly demonstrated by Loos and Yeo [82], in which Europeans present the larger cluster, followed by Asian populations that do not overlap with the African cluster (probably without association with obesity-related traits). The discovery of new *loci* in replication studies at established *loci* found in other populations reflect differences in allele frequency and effect size. Further studies will be needed to test the biological function at the associated *loci* and take into account difference observed regarding LD.

2.7. Obesity risk-allele scores

As noted, several GWAS have identified a large number of obesity susceptibility *loci*. Nevertheless, the major part of these studies only identified single genetic *loci* associated with obesity. It has indeed been demonstrated that combining information from all these obesity *loci* into genetic risk-allele scores (GRS) could be a convenient way to summarize risk-associated variations across the genome [124] and more useful when individual genetic effects are moderate [125]. The simplest way to calculate a GRS is by summing the number of accumulated risk alleles associated with the disease. Using this approach, Zhu *et al*. [126] analyzed 28 BMI-associated polymorphisms in a sample of Han Chinese and found 26 nominally associated with BMI. To assess the combined effect of all polymorphisms studied with BMI, they create a GRS which was associated with increased risk of obesity (OR= 1.06; CI95%: 1.03-1.10), and each additional BMI-increasing allele in the GRS was associated with 0.11 kg/m^2 higher BMI (p=1.54x10^{-7}). Willer *et al*. [99] found effect sizes between 0.06 kg/m^2 to 0.33 kg/m^2 per allele in BMI changes and that account for 0.40% of the variance of BMI analyzing six *loci* together (*TMEM18*, *KCTD15*, *GNPDA2*, *SH2B1*, *MTCH2* and *NEGR1*). When they included the *FTO* and *MC4R* genes in the combined effect the variance increases to 0.84%. Similar results have also been found in other studies trying to explain the variance of BMI. Combining 12

polymorphisms in a sample of 20,431 of European descent, the GRS obtained by Li *et al*. [127] explained 0.9% of BMI variation. Apart the nominally association between 15 polymorphisms located in or near the *INSIG2*, *FTO*, *MC4R*, *TMEM18*, *GNPDA2*, *NEGR1*, *BDNF*, *KCTD15*, and 1q25 with BMI, Zhao *et al*. [128] explained 1.12% of the total variation for BMI z-score in a sample of children of European ancestry. In other sample of European descent González *et al*. [129] create a GRS including six polymorphisms located in the *FTO*, *TFAP2B*, *SEC16B*, *ETV5* and *SH2B1* genes and found that individuals carrying ≥7 risk-alleles had 3.1 (OR=3.11; CI95%: 1.58-6.61) times increase in the odds of developing the obese phenotype. Individually, each risk allele conferred an estimated increased risk of 1.69 (OR= 1.69; CI95%: 1.46-1.97) times to develop obesity.

This is particularly more evident when the allele score consists either of many common polymorphisms with small effects, or of rare polymorphisms [125]. Generally, when several polymorphisms are combined, the estimated genetic score may explain a considerable proportion of variation in the risk factor, even if none of the polymorphisms individually does. This is partially due to the fact that the "signal" obtained from a GRS is more robust to imperfect linkage than each polymorphism individually [125]. In complex diseases it is likely that the effects of different genetic *loci* related to obesity operate in an interactive fashion. Future research should investigate this possibility using classification or regression tree analyses, which are well suited to detecting complex non-linear interactions.

The identification of the complex interplay among all genes in the genome-wide context is essential to unravel the molecular mechanisms in the obesity etiology. However, as previously demonstrated there are differences between populations regarding to allele frequencies. Belsky *et al*. [125] developed a GRS for obesity using results obtained in 16 previously published GWAS in European descent samples. Analyzing 32 *locus* they found a significantly predictor of BMI and obesity among Europeans. However, the predictive effects for this GRS did not replicate among African Americans due particularly to the differences in risk-allele distributions.

In less than 10 years we assisted to the discovery of several genes associated with obesity-related traits. Despite the discoveries, all these genes only explain a small percentage of obesity susceptibility. Of course, several genes remain to be found and certainly in the next year's novel

candidate *loci* will appear. However, and more recently, new fields called epigenetic emerge as a new potential factor influencing the obese phenotype and helping to found differences in obesity risk based in the environment that surrounds us.

2.8. Epigenetics

Epigenetic regulation of gene expression emerged in the last few years as a potential factor that might explain individual differences in obesity risk [130]. Epigenetics can be defined as heritable changes that are mitotically stable (and potentially meiotically) and affect gene function but do not involve changes in the DNA sequence [131]. At the molecular level, epigenetic markers include genomic DNA methylation, changes in chromatic organization by histone modifications, the non-coding microRNAs (miRNA), genomic imprinting, non-covalent mechanisms, and other nuclear proteins that are critical for epigenetic gene regulation [132]. Currently, there is a growing interest in the study of the relations between genetic variation, epigenetic variation, and disease simultaneously.

Emerging studies have characterized the potential mechanisms by which epigenetic factors could increase the risk for obesity (Table 1.3).

Moreover, unlike DNA genotypes, epigenetic markers can change during lifetime, and have a heterogeneous distribution in tissues. DNA methylation is the most well know epigenetic marker, which has been proposed as a new generation of biomarkers. It is a biologic process that consists of the addition of a methyl group at the carbon-5 position of cytosine, in the context of the CpG dinucleotides, and usually associated with gene silencing in the promoter regions [131, 133]. The universal methyl donor is DNA methyltransferases (Dnmts) that maintain the cellular DNA methylation patterns [130]. Despite the high number of DNA methylation candidate genes and some epigenome-wide association studies (EWAS), most of the associations have not yet been replicated in other samples to further confirm and establish whether those *loci* are reliably associated with obesity.

Table 1.3. Some human genes related to obesity through epigenetic mechanisms.

Gene symbol/EWAS	Associated genes	Epigenetic mechanisms	Tissue	Study sample	Role in obesity	References
EWAS	*UBASH3A*, *TRIM3*	DNA methylation	Peripheral blood leukocytes	14 African-American men (14-18) Replication: 46 Obese (14-18) and 46 lean (14-30) African-American men	Obesity	[134]
EWAS in individuals carriers *FTO* risk allele (rs9939609)	*KARS, TERF2IP, DEXI, MSI1, STON1, BCAS3*	DNA methylation	Whole blood	33 obese and 24 normal-weight preadolescent girls Caucasian (Greek) (9-13 years)	Obesity	[135]
SLC6A4	*SLC6A4*	DNA methylation	Peripheral blood leukocytes	84 MZ twin pairs Caucasian (~55.1 year)	BMI, body weight, waist circumference	[136]
PPARGC1A, PPARG, Tfam	*PPARGC1A*	DNA methylation	Umbilical cord tissue and white blood cells	88 healthy pregnant women (~29.7 year) and their babies	Maternal BMI	[137]
MC4R	*MC4R*	DNA methylation	Brain tissues	Berlin fat mouse (*Mus musculus*)	Fat diet	[138]
EWAS	*HSP90B3P, NAV1, NR5A2, CCDC48, GPR125, SNCA, EHMT2, IER3,*	DNA methylation, mRNA expression	Adipose tissue	31 healthy Caucasian men (Sweden) (~37.4)	Adipocyte metabolism	[139]

	SERPINB1, STX1A, PVT1, LHX6, ENKUR, CTTN, HCCA2, PKNOX2, ANO2, ITPR2, RB1, PACS2, CRTC3, KIFC3, MIR1910, ZFHX3, MSI2, RPTOR, TRPM4, C20orf160, LOC647979, MLC1, CDX4, KCND1					
EWAS	Diferences between number of differentially methylated CpG sites and number of differentially variable CpG sites	DNA methylation	Peripheral blood leukocytes	48 obese and 48 lean African-American (14-20)	Obesity	[140]
LEP	*LEP*	DNA methylation	Troncal blood and retroperitoneal adipose tissue	Male Wistar rats	diet	[141]
RXRA, eNOS, SOD1, IL8, PI3KCD	*RXRA, eNOS*	DNA methylation	Umbilical cord tissue	78 Caucasian women (≥16) Replication: 239 children	Fat mass and %fat mass	[142]

Using a genome wide approach, obesity has been related to changes in DNA methylation status in peripheral blood leukocytes of lean and obese adolescents for two genes. In the ubiquitin-associated and SH3 domain-containing protein A (*UBASH3A*) gene, a CpG site showed higher methylation levels in obese cases, and one CpG site in the promoter region of Tripartite motif-containing 3 (*TRIM3*) gene, showed lower methylation levels in the obese cases [134]. In a recent work, Godfrey *et al*. [142] measured the methylation status of 68 CpGs 5' from five candidate genes in umbilical cord tissue DNA from healthy neonates, and found that methylation higher levels within promoter region of retinoid X receptor-a (*RXRA*) gene, measured at birth, was strongly correlated with greater adiposity in later childhood [142]. A positive correlation between maternal BMI and promoter methylation in peroxisome proliferator-activated receptor-gamma co-activator 1alpha (*PPARGC1A*), a gene encoding a transcriptional coactivator of the peroxisome proliferator-activated receptor (*PPAR*) α and У, playing an essential role in energy homeostasis, was observed when analyzing promoter genomic DNA from umbilical cord newborns [137].

The obesity risk allele of *FTO* has been associated with higher methylation of sites within the first intron of the *FTO* gene, suggesting an interaction between genetic and epigenetic factors [137]. Moreover, Almén *et al*. [135] determined the methylation profile on a genome-wide scale by sampling DNA from peripheral whole blood in female preadolescents. The sample included obese and a normal weight groups, both of which contains homozygous carriers of both the *FTO* normal and risk alleles (rs9939609). They analyzed how the risk allele for rs9939609 polymorphism affects the methylation status of sites related to other genes (*KARS*, *TERF2IP*, *DEXI*, *MSI1*, *STON1* and *BCAS3*), showing that the *FTO* gene may influence the methylation level of other genes [135].

A study examined the *MC4R* gene, which is associated with common and morbid obesity and encodes for a protein that is a membrane-bound receptor and member of the melanocortin receptor family controlling food intake and energy expenditure. Mouse genomic DNA of brain tissue was examined to determine the methylation status of the *MC4R* exon. Results indicated that methylation of the CpGs was decreased in response to high-fat diet [138]. A study examining whether a high-energy diet may affect promoter methylation of *LEP* gene, encoding an adipokine involved in body weight and food intake regulation, showed in DNA isolated from retroperitoneal adipocytes in rats that leptin methylation pattern can be influenced by diet-induced obesity [141]. Zhao *et al*. [136] demonstrated that promoter hypermethylation in the serotonin transporter gene (*SLC6A4*) was

associated with an increase in BMI, body weight and waist circumference. Xu *et al*. [140] studied 470,000 CpG sites from 48 obese and lean youth African-American (14-20 years-old); they found a differential variability in CpG sites which was more variable in obese than lean subjects, constituting an important feature of obesity related with methylation changes. In another recent EWA study, Rönn *et al*. [139] analyzed 476,753 CpG sites to evaluate the possible alteration of DNA methylation patterns after a six-month exercise intervention. A global DNA methylation changes were found in 17,975 individual CpG sites altering the levels of DNA methylation in response to physical activity [139].

Thus, most of these DNA methylation sites need to be confirmed as being associated with obesity, taking into account the tissue sampled, obesity history, and eating behaviors. However, the high number of new studies concerning obesity epigenetics will undoubtedly permit the confirmation some of these associations, thereby establishing an epigenetic basis for human obesity. Interestingly, one recent work of genome wide analysis revealed that carriers of the *FTO* risk allele (rs9939609) had a significant differential methylation level in 6 *loci* (*KARS*, *TERF2IP*, *DEXI*, *MSI1*, *STON1* and *BCAS3*) compared to non-carriers controls [138]. This work could elucidate the mechanisms underlying the association of obesity with genetic variants, possibly due to epigenetic factors.

Studies based on pre-conceptual, *in utero*, and postnatal developmental environment showed an impact on long-term risk for adult-onset obesity by a set point of adaptive changes. It could be understood as a "critical period" where environmental conditions experienced *in utero* may have a life-long effect on the propensity to develop the obese phenotype. The *Agouti* mouse viable yellow (A^{vy}) model is one of the best examples on how early environmental exposures interact with epigenetic gene regulation influencing the phenotype [143]. Briefly, the murine *agouti* gene influences DNA methylation in early development, affecting coat color, which correlates with adult body weight. Varying the mother´s diet tends to produce offspring with a wide variation in individual coat color and obese phenotype as epigenetic modifications of *agouti* gene were established in early development [144]. These variations in phenotypes are caused by DNA methylation patterns, which were acquired during early embryonic development and passed through the female germline that results in stable intergenerational transmission [145].

In a recent report, Relton *et al*. [146] evidenced that DNA methylation patterns in 9 of 24 (37.5%) genes at birth, show association with at least one index of body composition (BMI, fat mass,

lean mass, height) at age of 9 years. This observation suggests that variation in DNA methylation patterns at birth in multiple target genes may influence body size in childhood. Moreover, maternal diet can alter later child´s adiposity, accompanied by epigenetic changes in genes controlling the energy homeostasis. Parental pre-conceptional environmental exposures could also have an effect in the health status of the offspring in later life. In two recent studies regarding parental obesity conducted by Soubry *et al*. [147, 148] it has been observed an association between DNA methylation profiles at human imprinted genes, such as *MEST*, *PEG3*, and *NNAT*, in children born from obese parents, when compared with children born from non-obese parents. Changes related to maternal obesity were also detected at *loci PLAGL1*, *MEG3* and *H19* [147, 148]. Hypomethylation at the *IGF2* gene was associated with paternal obesity [148]. These results points to a pre-conceptional influence of parental life-style or over-nutrition on the reprogramming of imprint marks during gametogenesis and early development [147, 148]. The trans-generational effects of parental obesity can influence the offspring's future health status. These reports evidenced that peri-natal events are important in defining the epigenetic marks that will persist until the adult age. In the future it might be used as early prognostic markers to identify those individuals with more risk to develop obesity. However, the knowledge of mechanisms by which maternal nutritional environment induces such changes remains largely unknown.

microRNAs

Another type of epigenetic mechanisms is microRNAs (miRNAs). The gene expression in humans is precisely controlled in cellular, temporal, and condition specific manner. Because miRNAs have been shown to be important in gene regulation, it is not surprising that they have been implicated in the development of obesity [149]. Therefore, the understanding of the regulatory mechanisms of gene expression can shed some light on the underlying mechanisms causing obesity. miRNAs are endogenous short single-stranded non-protein-coding RNAs with about 21/25 nucleotides in length which are involved in post-transcriptional regulation of gene expression by partially complementary binding to the 3' untranslated region (3' UTR) of target mRNAs [150, 151].

Several miRNAs expression patterns have been profiled during adipocyte differentiation [152–154], others have been linked to adipocyte phenotype, and other obesity parameters [152–160] (Figure 1.2). For example, miR-21 was strongly expressed in human adipose tissue and positively

correlated with BMI [158]. These studies revealed that miRNAs may represent biomarkers for obesity, and could also be implicated in the molecular mechanisms leading to this disease. However, further studies are needed to elucidate the effect of miRNAs and other epigenetic mechanisms in the etiology of obesity.

Continuous advances in research show promising results about the implication of epigenetics mechanisms in the etiology of obesity. Epigenetics has shown that our genes are not the only factor to determine our phenotype and that our behaviors can alter the expression of our genotypes. However, additional research is needed, particularly with regard to which cell types should be explored in EWAS.

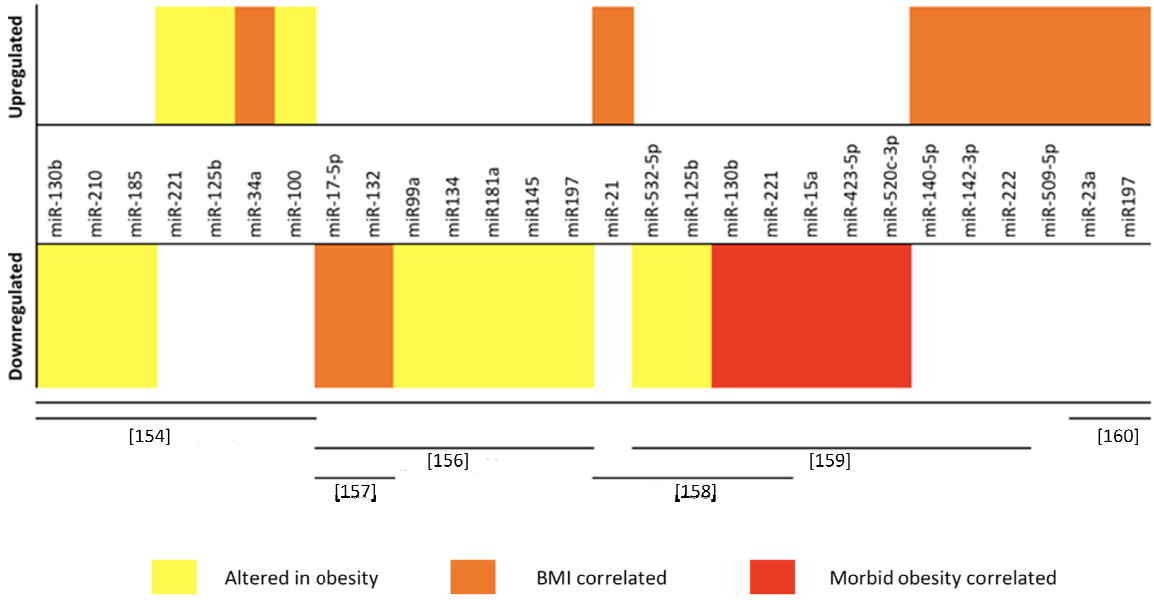

Figure 1.2. Gene expression profile in obesity-related phenotypes. Several microRNAs were found altered in obesity, and others were found significantly correlated with BMI and morbid obesity.

3. Evolutionary explanations for obesity

The evidence for a genetic component of obesity has been well established in recent years [32]. The question about the evolution of obesity is: how has natural selection favored the spread of genes that increase risk for an obese phenotype and how has this predisposition to obesity evolved? Answers to these questions should advance the understanding of the etiology of obesity. However,

our knowledge about the evolution of body-weight regulation mechanisms in humans remains incomplete. Nevertheless, four different types of evolutionary perspective have been proposed in an attempt to address these questions [161, 162].

The first hypothesis, called “thrifty gene”, is that the modern genetic predisposition to obesity was adaptive in the past, when storing large amounts of fat could have been selectively advantageous [163]. It explain the prevalence of obesity and diabetes in modern societies due to a change in lifestyle from that of Paleolithic hunters-gatherers to a subsistence based on agriculture, a pattern characterized by more sedentary occupations. The basis of this hypothesis states that during evolution of the modern humans, genes that promoted efficient fat accumulation would be extremely advantageous for primitive humans, because they allow their holders to survive famine periods [164]. In modern societies, where food supply is always available, such genes are disadvantageous and the result is the widespread obesity [162, 164].

Studies were conducted to try and identify genes under positive selection that have a role on obesity. A study lead by Myles *et al*. [165], suggested that the high frequency of the risk allele of the Gly482Ser variant in the *PPARGC1A* gene in Polynesians populations remains a thrifty allele in the Pacific populations. Another variant, the *PC-1* Gln121, was also considered as a possible thrifty gene supported by studies in African and other groups [166]. A recent study provides evidence for a positive selection of *TRIB2* gene, which influences visceral fat accumulation in East Asians [167]. In addition to these few examples showing a possibly positive selection in our evolutionary history with metabolic traits, other *loci* have been extensively studied, one of them being the lactase (*LCT*) gene, at ~7,000 years bp, which is considered a prototypic example of selective advantage leading to rapid human evolution compatible with the agricultural innovations [168]. In European populations, the -13910C>T polymorphism, located ~14 kb upstream of the *LCT* gene, has been associated with the persistence of the lactase enzyme in adulthood: individuals carrying the CC genotype possess insufficient enzyme activity in intestinal cells and are classified as lactase non-persistence (i.e., show lactose intolerance), which is considered the ancestral condition in humans, whereas individuals carrying at least one T allele are considered lactase persistent [169]. Nevertheless, this adaptive hypothesis reveals some problems: if accumulating extra adipose tissue was advantageous in the past populations, many people with these thrifty genotypes in modern society do not develop the obese phenotype, despite the environmental change favoring fat storage. On the other hand,

population genetic models predict that thrifty genes would not have sufficient advantage or even time to spread in the human population [170].

A second explanation, for the evolutionary perspective favoring obesity is that most mutations in the obesity susceptibility genes are neutral and have been drifting over evolutionary time [162, 171]. The neutral theory of molecular evolution, postulates that most evolutionary changes at the molecular level is not caused by natural selection, but by genetic drift [172]. According to this theory, the majority of genetic variation observed within and between species is selectively neutral, i.e. does not affect the fitness of individuals, in contrast to the theory of natural selection for which most of the genetic variation observed in populations affect the fitness of individuals and thus is subject to selection [173]. This new "drifty genes" hypothesis is a non-adaptive scenario providing an explanation for why some individuals get obese while others remain obesity resistant [162, 171].

The maladaptive viewpoint is another hypothesis that suggests that obesity is not adaptive and may never even have existed in human evolution history, except in some individuals with unusual genetic modifications such as the monogenic forms of obesity [162]. Nevertheless, genes that actually predispose to obesity could be favored as a maladaptive by-product of positive selection on some other advantageous trait. One example of this maladaptive interpretation is a work suggesting that obesity could result from individual differences in brown adipose tissue (BAT) [161]. This recent point of view emerged highlighting the differences in genetic susceptibility to develop an obese phenotype based on BAT.

Sellayah *et al*. [161] proposed the thermogenesis hypothesis, in which climatic selection pressures in the evolutionary history could exert a strong influence on genes. Effectively, climate changes played a key role in our evolution, representing the principal engine of evolutionary change (climate is an important factor in determining anatomical differences among different geographical populations). In fact, we can see around the world several differences among warm-adapted and cold-adapted species. Regarding human populations there is a significant variation in their body form, by an adaptation to different climates, suggesting a link between body shape and climate, probably related to thermoregulation. In warm climates, individuals have a large surface area relative to body mass (e.g. slim, long trunk) that facilitate heat loss, whereas in cold climates individuals have a small surface area relative to body mass (e.g. bulky, short trunk) allowing heat retention. In most eutherian mammals, BAT is an essential factor in thermogenesis helping to maintain their body

temperature regardless of the ambient temperatures. The uncoupling protein 1 (*UCP1*) gene was found with a key role to maintain body temperature in cold climates, which is highly expressed in BAT [174]. Some polymorphisms were found in the *UCP1* gene associated with body fat accumulation, body weight gain and BMI in response to a high-fat diet [175]. Feldmann *et al*. [176] demonstrated in mice exempt from thermal stress that *UCP1* ablation induced itself obesity, even in mice fed with control diet. They conclude that ambient temperature has an important role in UCP1 in mediating diet-induced adrenergic thermogenesis. They suggest that, UCP1 activity could be determinative for obesity in mice, and possibly in humans. As it was stated by Sellayah *et al*. [161], epidemiological studies found different rates of obesity (including other metabolic disorders) across certain ethnic groups. These studies demonstrated that in United States there are ethnically differences; with blacks, Hispanics, and people of Native American ancestry being more prone to develop an obese phenotype than European Caucasians and people of East Asian ancestry (Chinese, Japanese, and Koreans). When we look to these ethnic population distributions it could be observed that people living in warm climates have a higher obesity prevalence compared to people living in cold temperatures. In evolution history, genes with an essential role to survival, especially in newborn and young children, were positively selected.

So, Sellayah *et al*. [161] proposed in the thermogenesis hypothesis that migration to colder climates could result in a more efficient BAT and *UCP1* gene function. This fact, would endow the capacity of higher energy expenditure and energy-burning capacity, providing a higher metabolic rate, which then could reduce body fat. At the opposite side, Africans and South Asians whose ancestors had no need to evolve efficient BAT and *UCP1* function due to the warm climate, show an increased propensity for obesity when subjected to sedentary and hypercaloric lifestyle.

Regarding the thrifty and drifty genotype hypotheses that attempt to explain how human obesity evolved, at least one fact remains unclear. Obesity emerged in industrialized countries, which then exported their sedentary and hypercaloric lifestyle. One of the principal drawbacks in these two hypotheses is that it cannot explain the clear evidence for ethnic differences in susceptibility to develop obesity. On the opposite side, the thermogenesis point of view it will be interesting; as during the *out of Africa* migration, genes involved in the BAT thermogenic function could be positively selected to a better cold adaptation, although it has also some drawbacks. So, further investigation in the *UCP1* gene and other in genes involved in metabolic regulation could help

unraveling the causes of obesity susceptibility, and to explain the differences among populations and why not all people in the same environment seem to have the same predisposition for obesity. However, it should be mentioned that these hypotheses are not mutually exclusive and it is possible that all have some valid arguments explaining the evolutionary origin of obesity. Thus, understanding human evolution could help us to understand modern human behavior and traits.

4. Prevention and treatment based on genotyping

4.1. Nutrigenetics

Nutrition is one of the lifestyle factors contributing to the development and progression of obesity. An appropriate intake of energy and nutrients has been commonly accepted to prevent weight gain. Furthermore, epigenetics studies have demonstrated that several nutrients and bioactive food could play a role in the complex machinery involving the interaction between genome and the epigenome, which regulate gene expression [177]. The ingestion of these nutrients introduces some bioactive components that have signal molecules that carry information from the external environment [178]. Many dietary components can modulate epigenetic phenomena by inhibiting enzymes such as DNA methyltransferases and histone deacetylases [177], with the most well know vitamin B-12 and folate providing methyl groups for DNA methylation reaction [179, 180]. New research has attempted to understand the variability in metabolic responses to diet and food components, which could affect health. Nutrigenetics and nutrigenomics are defined of the effect of genetic variation on dietary response and the role of nutrients and bioactive food compounds in gene expression, respectively [181]. These areas aim to develop diagnostic tools that can “read” genetic susceptible *loci* in order to offer a personalized diet, taking into account the individual needs. Interactions among genetic *loci* and diet were found for obesity in *IL-6*, with daily food intake, *PPAR-gama2* and *FTO* with fat intake [182]. The Mediterranean diet is known to be rich in folates, which is crucial for the DNA methylation status. Ortega-Azorín *et al*. [183] found a significant gene-diet interaction of the *FTO* rs9939609 and *MC4R* rs17782313 polymorphisms with type 2 diabetes depending on diet, in which the Mediterranean diet counteracts the genetic predisposition. A cross-sectional study found that individuals carrying both AA risk allele of the rs9939609 polymorphism were positively associated with a high intake of total fat (>34% energy) and low fiber consumption

(<16 g/day), independently of BMI [184]. It has also been reported in a recent study that obesity susceptibility genes (*FAIM2*, *FLJ35779*, *FTO*, *LRRN6C*, *RBJ*, and *SEC16B*) were found to interact with dietary carbohydrates (sugar-sweetened beverages) to increase BMI when one or more servings are consumed per day [185]. Other two genes, β-adrenergic receptor 2 (*ADRB2*) and *MC4R*, were also suggested being related with carbohydrate intake [182].

During pregnancy and early postnatal life, an individual can be programmed for nutritional thrift to adapt and survive in an environment scarce in resources. In 2008, Heijmans *et al*. [186] studied the degree of methylation at five DNA sites in the insulin-like growth factor 2 (*IGF2*) gene on the population exposed to the Dutch famine of 1944-1945. Prenatal exposure to the Dutch famine was associated with the risk of obesity. Kucharski *et al*. [187], provided evidence that epigenetic information could be differentially altered by the nutritional input in honeybee (*Apis mellifera*). Moreover, they found that epigenetic modifications could provoke profound changes in developmental fates with implications in reproductive and behavioral status. When bee larvae are fed royal jelly, it turns off the expression of DNA Dnmt3 and other genes are expressed, turning some of them into a queen, whereas bee larvae that are not fed royal jelly, Dnmt3 remains active and the larval development produces the worker variety of bees.

In the past few years, a new window of research opened concerning the possible influence of the diversity of human gut microbiota (microorganisms that populate the adult intestines) in obesity [188]. Some studies found that the gut microbiota of nonobese individuals is more diverse than that of obese individuals [189]. Furthermore, several studies found an increase or decrease of different phyla between diet modification (e.g. reduced-carbohydrate, fiber, high-fat, etc.) and weight gain/loos [188, 189]. However, the mechanisms underlying gut microbiota affecting obesity in humans remain largely unknown. Thus, new studies and discoveries about how the gut microbiota affects the host metabolism could provide a more comprehensive understanding on how it affects obesity. A dietary intervention could be helpful in prevention as a potential instrument that can complement dietary advice. However, there are some limitations concerning nutrigenetics applications, such as, the lack of studies analyzing the evidence of common polymorphisms, polymorphisms differ on ethnic background, and the high cost of the genetic analyses. More generally, compliance with nutrient based recommendations, such as reducing intake of fat and sugar, has been very poor.

4.2. Physical activity–genotype interactions in obesity

Physical activity is another important component involved in the heterogeneous set of factors influencing obesity. Regular exercise is one of the most promising behavioral candidates for preventing and reducing weight gain, with other health and psychological benefits [190]. The most extensively studied example of a gene interaction with physical activity in obesity is the *FTO locus*; evidencing that physical activity attenuates the association of *FTO* variants with obesity-related traits [190–195]. A meta-analysis by Kilpeläinen *et al*. [79] observed that the estimated effect of the A risk allele of rs9939609 increased the odds ratio of obesity by 1.23-fold/allele, but this effect is attenuated by 27% in physical active adults ($p_{interaction}$ =0.001). Similarly, the meta-analysis conducted by Ahmad *et al*. [196] showed a statistical significant GRS and physical activity interaction effect in obesity ($p_{interaction}$ =0.015). In this analysis of 111,421 adults of European ancestry, data support the interaction effect between physical activity and a genetic risk score (combining 12 polymorphisms) in obesity disposition ($p_{interaction}$ =0.015). So, higher levels of physical activity may attenuate the influence of obesity susceptibility polymorphisms on BMI during adolescence.

However, several studies have provided evidence that the propensity to be physically active has also a strong genetic component in both animals and humans [197]. In humans, physical activity has been shown to aggregate in families; more active parents have more active children relative to inactive parents [198]. The physical activity heritability ranges from 9% in Mexican-American families, to almost 80% in European twins [197]. Common polymorphisms in the *MC4R* gene were also found associated with self-reported physical inactivity in French-Canadian families and Mexican-Americans [199, 200]. Another variant, the Gln223Arg polymorphism located in *LEPR* gene, was found to be associated with lower 24h energy expenditure and physical activity levels in individual homozygotes for the Arg223 allele compared to Gln homozygotes in Pima Indians population [201]. Summarizing, it appears that some variation in our DNA could contribute to the variation in the physical activity level. Thus, new studies and the identification of new loci implicated in this interaction could better enligth and help to understand the causes contributing to the development of obesity.

4.3. Drug genotype interaction

The use of drugs as a treatment option for obesity could be indicated for individuals with a BMI >30 kg/m^2 with existing co-morbidities such as diabetes, dyslipidemia or hypertension [202]. In the

last decade, with the discovery that some drugs were affected by hereditarily variation, the concept of "pharmacogenetics" emerged [203]. This new field focuses on the study of polymorphisms within one or more candidate genes for associations with pharmacologic phenotypes. So, common polymorphisms may alter the response to pharmacotherapy affecting drug metabolism, drug transport or drug targets [202, 203]. Relating to obesity, at least 35 *loci* were validated as being associated with BMI and the advent of GWAS and next generation sequencing will likely lead to the identification of additional genetic biomarkers. Until now, only three obesity-related drugs were approved for continuous use in the United States of America (USA): orlistat (Xenical®, Alli®), lorcaserin HCL (Belviq®), and phentermine and topiramate extended release (Qsymia™) [202]. Orlistat is a drug that alters metabolism by inhibiting the gastro-intestinal absorption of triglycerides [204]. Lorcaserin HCL and Phentermine are drugs that act centrally as an appetite suppressant [202, 205]. At the end of 2014, the US Food and Drug Administration (FDA) approved a new drug the Saxenda® (Novo Nordisk), an agonist of a glucagon-like peptide 1 (*GLP-1*).

In the future, it may be possible to determine which sub-populations will respond optimally to particular doses of drugs, allowing more effective personalized pharmacologic intervention. To achieve this end, it would be ideal if pharmacogenetic studies could identify differences in drug response and tolerability, and investigate gene regulation, epigenetic modifications, and DNA-protein interactions that could explain individual differences in responses to drugs beyond genetic variation. Ultimately, it will also be necessary for clinical trials to evaluate pharmacologic interventions that are guided by genetic tests.

4.4. Surgical intervention

For patients with morbid obesity (BMI ≥40 kg/m^2) and overweight (BMI ≥35 kg/m^2), suffering also of obesity-related comorbidities, which failed diet, exercise, and drug therapy, a surgical intervention could be the only option for the resolution of obesity problem. This approach could be a definitive way in many situations to reduce loss weight. However, some patients present a significant weight gain after chirurgical intervention. There are several guidelines and procedures that surgeons/gastroenterologists need to follow [206]. In this section we will not detail about surgical intervention strategies, which have been reviewed elsewhere [207], but about a possible relation between some genetic variants and the success to maintain weight loss after surgical intervention.

Throughout this introduction we discuss the importance that genetics factors play in the etiology of obesity, and for that it should be a factor taking into account in patients undergoing surgery. Effectively, there is a high degree of inter-subject variability for surgical outcomes [208]. Generally, patients submitted at a surgical intervention have a durable weight loss [209]. However, despite its effectiveness not all people lose the same amount of weight or obtain the same clinical benefits after the intervention. Some studies emerged associating specific polymorphisms with the response to bariatric surgery. Still *et al*. [210] used a summative allele risk score to found the presence of an association between several polymorphisms (including the *FTO*, and *MC4R* genes) with postoperative weight loss. De Luis *et al*. [211] investigated the role of rs6923761 polymorphism at *GLP-1R* gene on outcomes after biliopancreatic diversion. They found that homozygous individuals for the rs6923761 G allele showed higher weight loss 12 and 18 months after bariatric surgery than individuals A allele carriers. In another study, Hatoum *et al*. [209] found that a 15q26.1 locus was significantly associated with weight loss after Roux-en-Y gastric bypass surgery. Using the same surgery intervention, in 2013, a GWAS identified 17 polymorphisms whose frequencies were significantly different between two phenotypic extremes of weight loss at two years after surgery [212].

There are some evidences for the use of genomics to identify response to surgical procedures [209, 213]. Thus, the identification of genetic contributors could be useful to select those individuals who will obtain a better benefit from a weight-loss surgery. However, these results need to be interpreted with some caution due to the few number of replication studies.

5. Objectives

The main goal of this study was to investigate for the first time the genetics of common obesity in Portuguese children, which could allow in the future the identification of a genetic predisposition to obesity, and help developing possible approaches to treat this condition.

From this general goal, we pointed out more detailed objectives, specifically:

- To assess prevalence of overweight and obesity in a sample of 6-12 years old children with Portuguese origin.
- To assess the association between three *FTO* polymorphisms with obesity-related traits in the sample previously established.
- To test the association in the sample of Portuguese children, the -13910C>T (*LCT*) polymorphism with obesity-related traits.
- To investigate the nominal association of ten obesity-related polymorphisms within or near genes *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5* and *OLFM4* in the previously established sample.
- To search in children with morbid obesity (BMI ≥99th) for possible mutations in *MC4R* gene.

Chapter II

General laboratory methods: fundamentals and protocols

1. Selection of polymorphisms

In the beginning of this PhD project, in 2009, eleven single nucleotide polymorphisms, identified from the literature as being related to obesity or obesity-related traits in populations of European origin, were selected for this study. Throughout the development of the study, three new polymorphisms were included in the analysis, totaling fourteen polymorphisms.

FTO gene

Three polymorphisms located within the first intron of the *FTO* gene were selected: two that have been closely associated with obesity and prominent in the literature, rs9939609 (position: chr16:53820527), described by Frayling *et al*. [65], rs1421085 (position: chr16:53800954), reported in the work of Dina *et al*. [76], and the yet poorly studied rs1861868 polymorphism (position: chr16:53790402), described in only two studies [66, 192].

MC4R gene

Two polymorphisms located in the *MC4R* gene, prominent in the literature, rs17782313 (position: chr18:57851097) and rs12970134 (position: chr18:57884750), were chosen.

NRXN3, ***PPARGC1A***, ***TMEM18***, ***SEC16B*** genes

Four poorly studied polymorphisms comprising rs10146997 (position: chr14:79945162) in *NRXN3*, rs8192678 (position: chr4:23815662) in *PPARGC1A*, rs7561317 (position: chr2:644953) in *TMEM18* and rs10913469 (position: chr1:177913519) in *SEC16B*, were chosen.

MSRA and ***TFAP2B*** genes

Two polymorphisms associated in adult´s populations but never replicated in sample children, rs545854 (position: chr8:9860080) in *MSRA* and rs987237 (position: chr6:50803050) in *TFAP2B* [214], were chosen.

LCT gene

The rs4988235 (-13910C>T) (position: chr2:136608646) polymorphism located ~14 kb upstream from the *LCT* gene coding region, reported in 2010 as a new candidate related with obesity in adults of European origin [102–104], was chosen.

HOXB5 and ***OLFM4*** genes

The two remaining polymorphisms were chosen due to their recent (2012) association with childhood obesity, but never replicated in an independent study: rs9299 (position: chr17:46669430) in *HOXB5* and rs9568856 (position: chr13:54064981) in *OLFM4* [114].

2. Ethical procedures

The study protocol was approved by *Direcção-Geral de Inovação e de Desenvolvimento Curricular*, the ethical committee of the *Ministério da Educação e Ciência* (Portugal). The authorization can be found at the http://mime.gepe.min-edu.pt homepage, using the identification number process nº: 0151100001. The study protocol was also conducted in accordance with the institutional guidelines of the University of Coimbra (Portugal).

After having obtained the written informed consent of children´s parents/guardians, children with positive consent were included in this study.

3. Study subjects

Subjects were selected in 2011 (April-June) from several public schools in the central region of Portugal. The study subjects were derived from five grouping of schools: *Agrupamento de escolas Dr. Manuel Fernandes* (Abrantes), *Agrupamento de escolas Pêro da Covilhã* (Covilhã), *Agrupamento de escolas da Pedrulha* (Coimbra), *Agrupamento de escolas Carolina Beatriz Ângelo* (Guarda) and *Agrupamento de escolas das Dairas* (Vale de Cambra). All children from these groups of schools, aged between 6 to 12 years old, were chosen to integrate this study. From a total of 4028 initially selected children, 1468 parents gave their written informed consent. Thirty-five children were excluded from the study due to non-Portuguese origin: African (*n*=8), Asian (*n*=2), or other European origins (*n*=15). After this exclusion, the final sample was composed by 1433 children with Portuguese ancestry, comprising 747 girls and 686 boys.

4. Anthropometric measures

Some anthropometric measures are used by anthropologists to assess variation in physical size and shape of the body in humans. These analyses can provide data and clues about the cause of human variation. A trait can be described and reflect the activity of a single-gene (Mendelian or monogenic) or more than one gene (polygenic). Both of them can be multifactorial influenced in the same time by environment factors. A polygenic multifactorial condition reflects additive contribution of several genes conferring different degrees of susceptibility.

4.1. Anthropometry

Anthropometry arises as a branch of biological science that aims the study of body size. The principal study object is the different character measurable in human morphology, such as height, weight, waist circumference, and subcutaneous fold. It has the particularity to be a universally applicable, inexpensive and non-invasive method to assess the size and composition of human body. This field was developed mostly in the early nineteenth century and in other phase in the early of the twentieth century. Initially constituted an attempt, through the physical dimensions of man, to subdivide and classify human in "race". In the last decade, its focus was mainly devoted to human growth and physical classification.

4.2. Body mass index (BMI) in adults

To define and classify the body weight in adults, we used the Body Mass Index (BMI) [215]. The BMI is a weight parameter corrected for height. This is calculated by dividing body weight (in kg) by height (in square meters) [215]. Table 2.1 presents the values for BMI for adults, according to the classification of the World Health Organization (WHO). BMI between 18.6 and 24.9 kg/m^2 are considered with normal weight. However, values above $25kg/m^2$ mean excess of weight [16].

Table 2.1. World Health Organization classification for BMI in adults.

Classification	Body Mass Index (BMI), kg/m^2
Underweight	<18.5
Normal range	18.5 – 24.9
Overweight	25 – 29.9
Obese	30.0 - 34.9
Severely obese (Class I)	35.0 – 39.9
Morbidly obese (Class II)	40.0 – 49.9
Super obese (Class III)	>50.0

One of the main advantages of the BMI is to be relatively inexpensive, easy to use, non-invasive, and does not cause any discomfort to people. This has numerous advantages in the clinical setting, making BMI generally used as a measure of total body fat. Despite this advantage, there are some limitations to its use as BMI does not measure the amount of body fat directly, therefore can lead to an inaccurate assessment of adiposity. Nevertheless, it has clinical validity and acceptable, but must be used with caution [216, 217].

A major problem of fat deposits in the body that can lead to metabolic risk, especially with regard to the central abdominal fat, is measured by waist circumference and can be associated with the development of metabolic risk factors, diabetes, cholesterol and cardiovascular diseases (hypertension) [218]. Thus, the waist circumference has become a good indicator of central obesity, since it quantifies the accumulated body fat in the abdomen. Understanding pathogenic central fat distribution may help in understanding the relationship between adiposity and cardiovascular disease risk. It is through the ratio of the circumference of waist / hip ratio that the indicator of abdominal obesity and associated risk is obtained. According to the National Institute of Health (USA), men and women with a waist circumference greater than 100 cm and 80 cm, respectively, are more likely to become obese [219].

4.3. Body mass index in children and adolescents

Body mass index Z-scores, also called BMI standard deviation (s.d.) scores (BMI Z-score), are measures of relative weight adjusted for child age and sex [220]. Given a child's age, sex, BMI, and an appropriate reference standard, 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 a national or international 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 [221] (Figure 2.1). For example, using the US BMI-for-age reference, a 5-year-old boy with a BMI of 20 kg/m^2 has a BMI Z-score of approximately 2.5 (BMI >99th percentile) and a 15-year-old boy with a BMI of 20 kg/m^2 has a BMI Z-score of approximately 0.0 (BMI= 50th percentile). In the United States, BMI-for-age percentiles above the 95th percentile in children and adolescents are labeled "obese" and BMI-for-age percentiles between the 85th and 95th percentiles are labeled "overweight" [222]. Therefore, although both the 5- and 15-year-old boys described above have a BMI equal to 20 kg/m^2, only the 5-year-old would be considered obese.

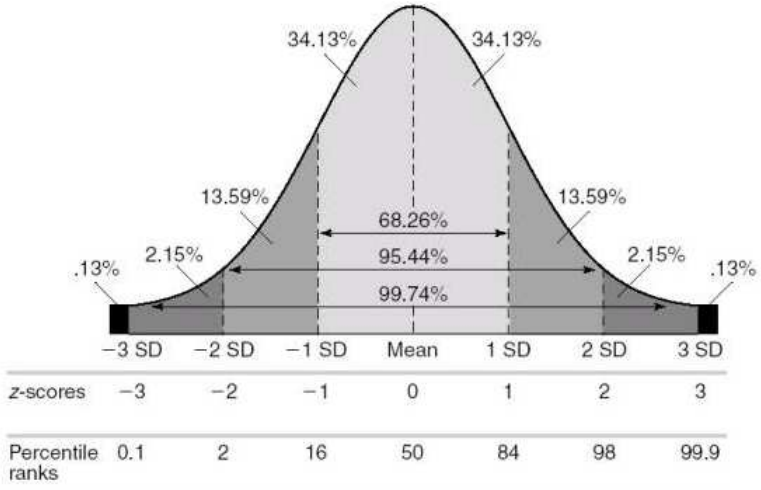

Figure 2.1. Percentile ranks and standard scores in relation to the normal curve (SD, standard deviation). Adapted from [223].

4.4. Anthropometric measurements

All children participants in this study underwent anthropometric measurements of height (cm), weight (kg), waist and hip circumference (cm) using a standardized protocol by the same operator. The equipments used were a compact digital weighing scale (Seca, model 872, Germany), a portable stadiometer (Seca, model 213, Germany) and a flexible measurement tape (Seca, model 201, Germany).

Body weight (kg) and height (cm) were taken with participants dressed in lightweight clothing without shoes. Waist circumference (WC) was measured midway between the lowest rib and the iliac

crest to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference.

The BMI was calculated as the weight in kilograms (kg) divided by the square of height in meters (kg/m^2). Overweight and obesity were defined according to the International Obesity Task Force (IOTF) reference data, making a correspondence between the traditional adult cutoff (BMI in adult's cut-points of 25 kg/m^2 and 30 kg/m^2 respectively for overweight and obesity) and specific values for children according to gender and age [221]. A Z-score was calculated for each child using the LMS (lambda-mu-sigma) method and the calculation was determined using the LMS growth add-in for Microsoft Excel [224].

5. Genotyping

5.1. Collecting buccal samples

For each child, individuals packaged containing a sterile brush was used (Sarstedt, Nümbrecht, Germany) to collect buccal cells. After 1-2 minutes rubbing into mouth, buccal swab sample were released in an *eppendorf* tubes containing 1ml of ethanol (absolute) for conservation, for subsequent DNA extraction, and PCR analysis. All tubes were properly blindly numbered for *a posteriori* identification with the child phenotype.

5.2. DNA extraction

From the original sample of 1433 subjects, a total of 730 children were selected for genotyping including 154 subjects as obese, 320 subjects as overweight, and 256 for the control group random chosen from the total of 928 children with a normal BMI ($18.5>BMI<25.0$ kg/m^2).

The genomic DNA was extracted from buccal cells using the PureLink Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the instructions of the manufacturer.

5.3. DNA quantification

After the DNA extraction we performed the quantification of the sample to determine the average concentration of DNA present, as well as the purity. For this purpose we used the NanoDrop™ 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) according to the instructions of the manufacturer. This technology is based on the absorption of ultraviolet light at

260 nm by nucleic acids, and a photo-detector measures the light passes through the sample. When more light is absorbed by the sample, higher is the nucleic acid concentration in the sample. In molecular biology is common for nucleic acid samples to be contaminated with other molecules (i.e. proteins, organic compounds, other). To assess the purity of the sample, the ratio of absorptions at 260 nm *vs.* 280 nm is used. Generally, pure nucleic acids yield a 260/280 ratio of ~1.8 for DNA. If the sample present values out of range it will probably require further optimization before the isolation technique used.

Due to the higher number of quantified samples we only present the mean values obtained in our sample collection:

12 ng/µl A260/280nm = 1.88 A260/230 nm = 1.80

5.4. Allelic discrimination using TaqMan® probes approach

The detection of polymorphisms in the genome is a growing approach due to the development of new technologies. The real-time polymerase chain reaction urged as a more promising PCR method for high through put assays. The real time PCR also called quantitative polymerase chain reaction (qPCR) method is a technique that involves simultaneously amplification, detection and quantification of a specific nucleic acid target in a biological sample. The introduction of a fluorescent reporter molecule in the PCR reaction allows a quicker detection of polymorphisms in large scale.

TaqMan® (Applied Biosystems, Foster City, USA) probes are oligonucleotide fragments labeled with two fluorophores at the 5´and 3´ends. The 5' nuclease activity of *Taq* polymerase is used to cleave a non-extendable oligonucleotide hybridization probe during the extension phase of PCR. This methodology use dual-labeled fluorogenic hybridization probes for two alleles, including a reporter (R) dye FAM™ (6-carboxyfluorescein) for one allele and a reporter dye VIC® for the second allele covalently linked to the 5' end, whose emission spectra is quenched (Q) by a second dye TAMRA (6-arboxytetramethylrhodamine), covalently linked to the 3' end. During a PCR cycle, the probe specifically hybridizes to the corresponding template, cleaves via the 5' to 3' exonuclease activity of

Taq DNA polymerase and subsequently increases the FAM™ and VIC® fluorescent emission (Figure 2.2).

Except for the rs4988235 (-13910C>T at the *LCT* gene) polymorphism all the polymorphisms were amplified for allelic discrimination assays, using TaqMan® probes (Applied Biosystems, Foster City, USA). For the rs4988235 polymorphism we used previously reported primers and labeled probes for genotyping [225]. The TaqMan® probes used are described in Table 2.2.

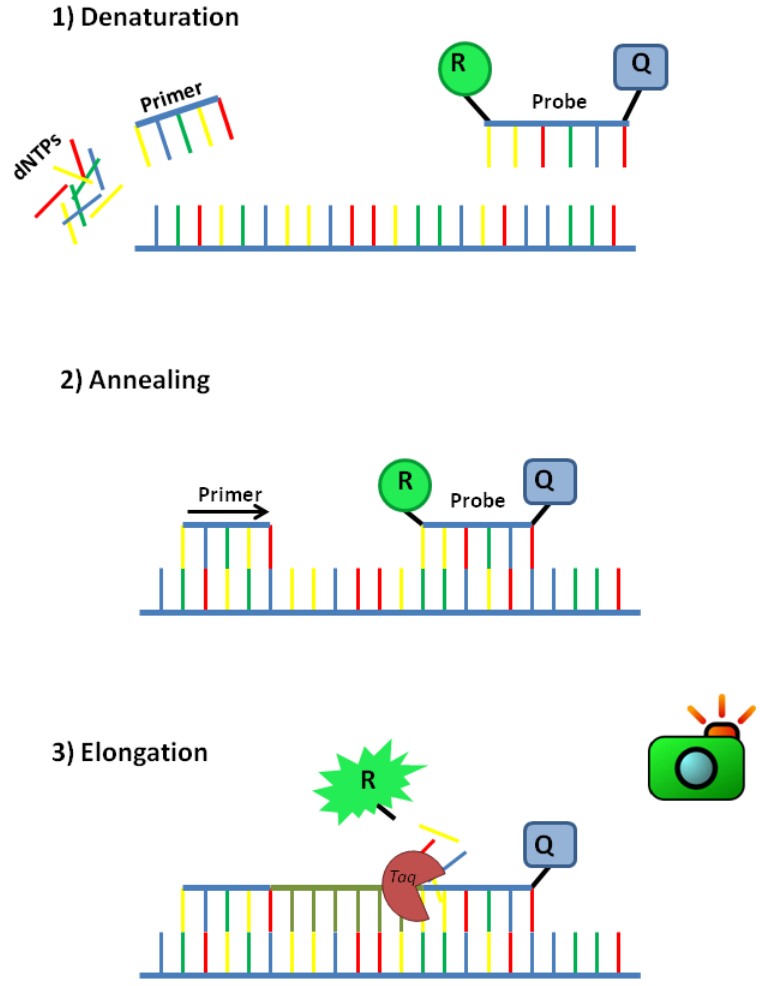

Figure 2.2. Representation of the TaqMan® PCR based on the 5'-3'-exonuclease activity of *Taq* polymerase. This method uses a probe (additional primer), which also binds specifically to the target DNA sequence. Probes have a fluorescent 'reporter' dye (R) at one end and a 'quencher' dye (Q), which inhibits fluorescence at the other. During the extension stage (phase 3) the probe is broken apart by the DNA-polymerase and begins to fluoresce more strongly. The fluorescence can be measured at each cycle and increases in proportion to the number of target sequence copies produced.

Table 2.2. Description of TaqMan® probes (20X) used for amplification of DNA target.

Chromosome	Gene	Polymorphism	TaqMan® probes*
1q25	*SEC16B*	rs10913469	C__3193319_10
2q21	*LCT*	rs4988235	[225]#
2p25	*TMEM18*	rs7561317	C__11804554_10
4p15	*PPARGC1A*	rs8192678	C__1643192_20
6p12	*TFAP2B*	rs987237	C__9489781_10
8p23	*MSRA*	rs545854	C__27120820_10
13q14	*OLFM4*	rs9568856	C__30191235_20
14q31	*NRXN3*	rs10146997	C__30288512_10
16q12.2	*FTO*	rs9939609	C__30090620_10
16q12.2	*FTO*	rs1421085	C__8917103_10
16q12.2	*FTO*	rs1861868	C__11717119_10
17q21	*HOXB5*	rs9299	C__2906037_20
18q21	*MC4R*	rs12970134	C__3058722_10
18q22	*MC4R*	rs17782313	C__32667060_10

*Applied Biosystems, Foster City, USA
#Regarding the *LCT* gene the previously described probes in [225] were used, VIC®-labelled probe ATAATGTAGTCCCTGGCCT to detect the T-allele and the 6-FAM™-labelled probe ATAATGTAGCCCCTGGC to detect the C-allele.

All polymerase chain reactions (except for the rs4988235 polymorphism) were carried out in a total volume reaction of 20 µl containing 1x of SsoFast™ Probes Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µl of specific TaqMan® polymorphism Genotyping Assays (20x) (Applied Biosystems, Foster City, USA) and 2 µl (~12 ng/µl) of genomic DNA, according to the manufacturer´s instructions.

For the rs4988235 polymorphism of the *LCT* gene, the reaction was carried out in a total volume of 20 µl containing 1x of SsoFast™ Probes Supermix (Bio-Rad, Hercules, CA, USA), 0.4 µM of each primers, 0.2 µM of each probe, and 2 µl (~12 ng/µl) of genomic DNA (Table 2.3).

Thermal cycling conditions for all polymorphisms were 10 minutes at 95°C, and 35 cycles of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence was observed through a MiniOpticon real time PCR system (Bio-Rad, Hercules, CA, USA).

6. Genotyping reproducibility

6.1. Amplification of DNA target by Polymerase Chain Reaction (PCR)

To assess genotyping reproducibility, about 10% random samples were re-genotyped for all polymorphisms by the Single Strand Conformation Polymorphism (SSCP) method or sequencing by the Sanger's dideoxy chain termination reaction, using oligonucleotides detailed in Table 2.3.

The PCR technique allows obtaining a large number of copies of a specific segment of DNA, called DNA target. The principle of this technique consists in using two primers (small sequences of DNA with about 18-24 nucleotides) that will bind to the complementary DNA strands of the two sites bounding the region to be amplified. The elongation of complementary strands from the primers is ensured by a polymerase enzyme. This technical concept was developed by Kary Mullis in 1983 [226], which earned him the Nobel Prize for Chemistry in 1993. Although the principle of PCR have been described in 1986, it was in 1988 that the better PCR performance was obtained using *Taq* polymerase derived from *Thermus aquaticus* bacteria isolated from the hot springs of Yellowstone Park, which has the property of being stable at heat temperatures (~100°C) [227]. This revolutionary method becomes one of the most universal techniques used in the field of molecular biology.

In this study, the PCR conditions were performed in a final reaction volume of 25 µl, containing 50 ng of PCR primers, 0.2 mM of each dNTPs, 3 mM of $MgCl_2$, 1x *Taq* buffer [750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20], 0.5 U *Taq* DNA Polymerase (Thermo Scientific, Fermentas) and about 20 ng of genomic DNA. Amplification conditions consisted in 35 cycles of 45 seconds denaturation at 94°C, 45 seconds annealing at 56-62°C and 45 seconds extension at 72°C. A 5 minutes initial denaturation at 95°C and a 5 minutes final extension at 72°C were performed (Table 2.3).

Table 2.3. Description of primers and PCR conditions used for amplification of DNA target.

Gene	Poly. ID	Pr.	Primers sequences (5´- 3´)	Amplification conditions (x35 cycles)			Size (pb)
				Denaturation	*Annealing*	*Elongation*	
SEC16B	rs10913469	F R	AGGACGTTCAAACATCAGCA TCTACTGAACTTTTCCTCATTAGCTT	94°C – 45´´	57°C – 45´´	72°C – 45´´	154
*LCT**	rs4988235	F R	AAATGCAACCTAAGGAGGAGAGTTC CTGCGCTGGCAATACAGATAAG	94°C – 45´´	58°C – 45´´	72°C – 45´´	71
TMEM18	rs7561317	F R	CCTTCCCAGAGGTGAGGTCT CAGGGCTCATTCACGCTTAT	94°C – 45´´	58°C – 45´´	72°C – 45´´	153
PPARGC1A	rs8192678	F R	CCTTGCAGCACAAGAAAACA CTTCGCTGTCATCAAACAGG	94°C – 45´´	58°C – 45´´	72°C – 45´´	211
TFAP2B	rs987237	F R	ACCGCCGCTCATATGAATTA AAGTGTGCCCCATTCTTCC	94°C – 45´´	58°C – 45´´	72°C – 45´´	206
MSRA	rs545854	F R	CCCCATCACATGGTTTAAGG CGGTTGCCTTTCGTAGAGAC	94°C – 45´´	56°C – 45´´	72°C – 45´´	196
OLFM4	rs9568856	F R	TGTGCATATTGTGTTTGGGATT TTTGCTTGTGTGATTAGGCATC	94°C – 45´´	58°C – 45´´	72°C – 45´´	169
NRXN3	rs10146997	F R	ATGCCGTGTCATCATTGAAA CAACAGCTTACAGGGTCCAG	94°C – 45´´	58°C – 45´´	72°C – 45´´	242
FTO	rs9939609	F R	CATCAGTTATGCATTTAGAATGTCTG TCCCACTCCATTTCTGACTGT	94°C – 45´´	58°C – 45´´	72°C – 45´´	132
FTO	rs1421085	F R	AATCTCATTGTTCCTCCTGCT ACAGTGGAGGTCAGCACAGA	94°C – 45´´	58°C – 45´´	72°C – 45´´	179
FTO	rs1861868	F R	CGCATCTCTGCAACTCTTTT CCCCTTGCATCAGAGTGTTT	94°C – 45´´	58°C – 45´´	72°C – 45´´	171
HOXB5	rs9299	F R	GGGAGCATGGAAGGAAAATA CTGGCCCCTCCAATCCTC	94°C – 45´´	62°C – 45´´	72°C – 45´´	187
MC4R	rs17782313	F R	AAGGGCATAAGCAAGTTCTACC GCTACCTCAATCCCAGATGC	94°C – 45´´	58°C – 45´´	72°C – 45´´	211
MC4R	rs12970134	F R	CAGATTATTTCGGTTCTAAGCAA CAGGTACTAACAAGCACCCTTC	94°C – 45´´	56°C – 45´´	72°C – 45´´	99

*Primers previously described in [225].
All primers described (except for the *LCT* gene) were designed for this study based on the Primer3 program, version 0.4.0 [228] (freely available in http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

All amplifications were performed in a Biometra TProfessional Thermocycler (Biomedizinische Analytik GmbH, Göttingen, Germany).

The amplification efficiency was confirmed by and electrophoresis run with 5 µl of the amplified product in a gel of 1.5% agarose containing ethidium bromide (final concentration 4 µg/ml) and visualized under ultraviolet (UV) light. A negative control was also used in all amplifications to verify a possible contamination, as well as a molecular weight marker (pBR322/Hinf1) to compare the size of the amplified band.

6.2. Allelic discrimination by SSCP

The Single-Stranded Conformation Polymorphism (SSCP) method was used to corroborate the results obtained by TaqMan® probes for rs1421085, rs1861868, rs7561317, rs8192678, rs987237, rs545854, rs10146997, rs12970134 polymorphisms. The SSCP technique developed by Orita *et al*. [229] allows the detection of polymorphisms based on the three-dimensional configuration of a single chain DNA. It relies on the fact that single stranded DNA acquires a particular structure depending on their nucleotide composition. A variation of one or more nucleotides directly translates into a modification of the 3D structure of the chain, which can be detected.

The SSCP method involves denaturation of the two strands of DNA amplified by PCR at elevated temperatures (± 94°C) in the presence of formamide, followed by rapid cooling preventing that these single strands become annealed again. The two chains independently acquire a three-dimensional structure itself. The PCR products are separated by electrophoresis in non-denaturing gel. Depending on their structure, migration will also be different in the electrophoresis run. The polymorphism detection is made by comparison of the different electrophoretic profiles from different individuals.

The amplification reaction was made using primers previously described in Table 2.3. Then, it was added a denaturing solution with 95% deionized formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA, 0.1% SDS 1:1 giving a final volume of 6 µl/tube. The mixture was denatured by heat (94°C for 5 min) in a thermocycler (40 Robocycler, Stratagene) and subsequently placed in a vertical polyacrylamide gel. After, 4 µl of the solution was applied to a mini-gel (7.2 x 10.2 cm) constituted by: 12% acrylamide-bisacrylamide (C = 1.3%), 10% glycerol and 200 mM TBE buffer (89 mm Tris, 89 mm boric acid, 2 mM EDTA, pH 8.3).

Electrophoresis was performed at 60 volts (V) for 16 hours.

The DNA bands were visualized by staining with silver nitrate using the following solutions and steps [230]:

1) 10% of acetic acid, for 20 minutes;

2) Distilled water, twice, for 2 minutes;

3) 0.1% silver nitrate (100ml) with 37% formaldehyde (150 µl), for 30 min;

4) Distilled water, for 30 second;

5) 3% Sodium carbonate (100ml), with 37% formaldehyde (150 µl) and 10% sodium thiosulfate solution (20 µl), for 2 to 5 minutes;

6) 10% acetic acid, for 30 seconds;

7) Distilled water.

6.3. Automated DNA sequencing

Automated DNA direct sequencing was used to corroborate the results obtained by TaqMan® probes for rs9939609, rs10913469, rs17782313, rs9568856, rs9299, and rs4988235 polymorphisms, using an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA). The BigDye® Terminator v1.1 Cycle Sequencing kit Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) was used to sequence DNA fragments. The amplified PCR products (4 µl) were purified with 1 µl of ExoSAP enzyme (Pharmacia Biotech) (conditions: 37°C for 15 minutes followed by 80°C for 15 minutes) and used directly in reactions of cyclic dideoxynucleotide sequencing (Sanger et al, 1977): 2 µl of the purified PCR product, 1 µl of reagent BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 0.5 µl (100 ng/µl) of primer (Table 2.3) and 6.5 µl of bi-distilled water, for a total volume of 10 µl. The conditions used for the sequencing reaction were 25 successive cycles of denaturation at 96°C for 20 seconds and annealing and extension at 60°C for 2 minutes. The sequenced samples were then purified using Centri-Sep Columns (DyeEx 2.0 Spin Columns Qiagen, Hilden, Germany) according to the manufacturer instructions, and applied directly on the ABI PRISM 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using the Sequencing Analysis v5.2 software (Applied Biosystems, Foster City, CA, USA).

Nevertheless, for all the studied polymorphisms the 3 different genotypes were confirmed by automatic sequencing of selected samples based on the allelic discrimination by TaqMan® assay, using primers described in Table 2.3.

7. Direct sequencing of the *MC4R* gene

In all children that present a morbid obese phenotype (BMI ≥99th), the entire sequence of *MC4R* gene was screened.

DNA was amplified by polymerase chain reaction (PCR), using primers and conditions previously published for the *MC4R* coding sequence [231]. For the promoter region, primers were designed with the Primer3 software, v0.4.0 [228] (freely available in http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 2.4).

Table 2.4. Description of primers and PCR conditions used for amplification of *MC4R* DNA fragments.

Frag.	Pr.	Primers sequences (5´- 3´)	Amplification conditions (x35 cycles)		
			Denaturation	***Annealing***	***Elongation***
MC4R-Prom#	F	CGCCTACAGCCCCTAACACT	94°C – 45´´	57°C – 45´´	72°C – 45´´
	R	CCTCCTGGGTCAGGGAGT			
*MC4R-A**	F	ATCAATTCAGGGGGACACTG	94°C – 45´´	57°C – 45´´	72°C – 45´´
	R	CATGGGTGAATGCAGATTCT			
*MC4R-B**	F	GTGATTGTGGCAATAGCCAA	94°C – 45´´	58°C – 45´´	72°C – 45´´
	R	TCCACTGCAATTGAAAGCAG			
*MC4R-C**	F	TGTAGCTCCTTGCTTGCATC	94°C – 45´´	60°C – 45´´	72°C – 45´´
	R	GGCCATCAGGAACATGTGGA			
*MC4R-D**	F	ACCATGTTCTTCACCATGCTG	94°C – 45´´	55°C – 45´´	72°C – 45´´
	R	GAGACATGAAGCACACACAA			
*MC4R-E**	F	CCATTCTTCCTCCACTTAAT	94°C – 45´´	55°C – 45´´	72°C – 45´´
	R	TGCATGTTCCTATATTGCGTG			

Abbreviations: Frag., fragment; Pr., primer; F, forward; R, reverse; °C, Celsius degree; ´, minute; ´´, second.
*Primers previously described in [231].
#Primers for the promoter region were designed for this study based on the Primer3 program, version 0.4.0 [228] (freely available in http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The PCR conditions were performed in a final reaction volume of 25 µl, containing 50 ng of PCR primers, 0.2 mM of each dNTPs, 3 mM of $MgCl_2$, 1x *Taq* buffer [750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20], 0.5 U *Taq* DNA Polymerase (Thermo Scientific, Fermentas) and about 20 ng of genomic DNA. Amplification consisted in 35 cycles of 45 seconds denaturation at 94°C, 45 seconds annealing at 55-60°C and 45 seconds extension at 72°C. A 5 minutes initial denaturation at 95°C and a 5 minutes final extension at 72°C were performed

All amplified fragments were purified with the enzyme ExoSAP-IT (GE Healthcare, New Jersey, USA). The conditions used for purification were one cycle at 37°C for 2 hours follow by one cycle at 95°C for 5 minutes (21 µl of PCR product add with 1.0 µl of ExoSap-IT).

For the sequencing reaction 5 µl of purified product with 1.0 µl BigDye® Terminator v3.1 Reagent Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 1.5 µl buffer and 1.0 µl (100 ng/µl) of

primer (F or R) was added to 1.5 µl of double distilled water to a total volume of 10 µl. This step was realized for both forward and reveres strands. The conditions used for the sequencing reaction were 25 successive cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 15 seconds and extension at 60°C for 2 minutes. The sequenced samples were then purified using the conditions described above:

1) Addition of 1 µl of 125 mM EDTA and 40 µl 1:25 3M NaOAc: 100% ethanol during 15 minutes at room temperature;
2) Centrifugation at 4000 rpm during 30 minutes at 4°C;
3) Discard the flow;
4) Washed with 60 µl of 70% ethanol;
5) Centrifugation at 1000rpm during 2 minutes at 4°C (2 times);
6) Discard the flow;
7) Dry the pellet;
8) Add 15 µl of formamide and wait 15 minutes (to re-suspend the pellet);
9) Denaturation at 95°C for 5 minutes.

After this purified step, samples were applied directly on an automated sequencer ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequencing data were analyzed with the Staden Package software [232] and the SeqScape software v2.5 (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared with the standard *MC4R* gene sequence (ENSG00000166603).

8. Statistical analysis

The quantitative variables were expressed as means and standard deviation (SD), whereas qualitative variables were expressed as absolute numbers and frequencies. For the rs4988235 polymorphism (*LCT* gene), a dominant model was used in all the statistical analyses: children with CT or TT genotypes were grouped and compared with CC children. This was performed because individuals of European populations carrying the T allele possess sufficient activity in intestinal cells and are classified as lactase persistence, whereas individuals carrying the CC genotype are classified as lactase non-persistence.

All SNP allele, genotype and haplotype frequencies were estimated by direct counting. The software package Arlequin, v3.11 [233] (freely available in http://cmpg.unibe.ch/software/arlequin3/), was used to:

- Estimate allele, genotype and haplotype frequencies;
- Estimate statistical significance levels (p-values) for the Hardy-Weinberg equilibrium and population differentiation;
- Test of non-random association of alleles at different *loci*, *D´*and r^2 values for linkage disequilibrium (LD);
- Assess haplotype phase by statistical inference via the ELB algorithm;

8.1. Association of quantitative traits

Normality of the data was examined using the Kolmogorov–Smirnov test. Due to lack of the normal distribution, non-parametric tests have been used in the statistical analyses. For each obesity-related quantitative parameters (BMI, BMI Z-score, weight, height, and waist circumference), the nonparametric Kruskal-Wallis test was used to evaluate differences among the three genotypes in all polymorphisms (except for the *LCT* gene). Regarding the *LCT* gene the Mann-Whitney test was used to compare means of obesity-related traits between genotypes (CT/TT *vs*. CC).

All these statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, for windows version 18.0; Chicago, IL, USA), as well as graphical analysis. A *p*-value below 0.05 was considered as statistical significant.

8.2. Case-control study

A case-control study is used to determine if an exposure is associated with an outcome (i.e. disease). Is an analytical study, which compares individuals who have a specific disease, “cases”, with a group of individuals without the disease, “control”. Frequencies of each of the measured variables in each of the two groups were calculated. As a measure of the strength of the association between an exposure and the outcome, case-control studies yield an odd ratio (OR). An OR is the ratio of the odds of an exposure in the case group to the odds of an exposure in the control group. A 95% confidence interval (CI) is also calculated for each OR. A 95% CI that not includes 1.0 could be associated between the exposure and the disease at the 0.05 significance level. Logistic regression is

regularly used to analyze relationship between a dichotomous (or binary) dependent variable (phenotypes) and metric or dichotomous independent variables (genotypes), as in the case of most obesity genetic studies. It focuses instead upon the relative probability (odds) of obtaining a given category.

In our study sample, children were classified into three groups based in their phenotype "obese", "overweight", and "normal-weight" (as control group). Logistic regression, under an additive genetic model (except for the *LCT* gene), was used to test the risk of being overweight (normal *vs.* overweight subjects) and the risk of being obese (normal *vs.* obese subjects) calculating OR, 95% CI and p-values. The association between polymorphisms and the risk of developing obesity was adjusted for age and sex.

All these statistical analyses were done using the set-based tests implemented on PLINK software v1.07 [234] (freely available in http://pngu.mgh.harvard.edu/purcell/plink/). A significant *p*-value was considered below 0.05.

8.3. Power calculation

The power of a statistical test represents the chance that the study will be successful in detecting a true effect and is dependent on a number of factors, including sample size, study design, and the specified false-positive rate. In case-control study, performing a power calculation it is important because the association with a functional *locus* can vary due to the random sampling process (each replicated sample differs slightly in its estimated odd ratio) [235]. The chance of detecting true effects brings two types of inferential error: the type I error (or false-positive) that detect a nonexistent effects; and the type II error (or false-negative) that do not detect the true effects [236]. In many studies, investigators have adopted by convention the value of 80% power as representing a realistic and adequate trade-off.

To estimate the power calculation for each polymorphisms studied, QUANTO version 1.1 power calculator (freely available in http://hydra.usc.edu/gxe/) for genetic association studies was used to estimate the power of association as a function of the frequency of the effect allele assuming an additive model [237].

Chapter III

Assessment of obesity and abdominal obesity among Portuguese children

This chapter was mainly based on the following original paper: Albuquerque D, Nóbrega C, Samouda H, Manco L. **Assessment of obesity and abdominal obesity among Portuguese children**. *Acta Med Port* 2012, 25(3): 167-173.

1. Abstract

Background: Childhood obesity is a major public health issue in developed countries, and frequently proceeds into adulthood. The aim of this study was to estimate the prevalence of obesity and abdominal fat distribution in 6-12 years old children from the central region of Portugal, providing new data about trends on prevalence, epidemiology and evolution in obesity.

Methods: Weight, height and waist circumference were measured in a random representative sample of 1433 children (747 girls and 686 boys) from public schools in 2011. International Obesity Task Force (IOTF) cut-offs were used to define overweight and obesity. Abdominal obesity was estimated using the sex and age-specific ≥90th waist circumference percentile and waist-to-height ratio cut-off.

Results: The prevalence of overweight and obesity among children was 33.0%; 10.7% were obese. Overweight was significantly higher in boys than in girls (p=0.044), whereas no gender differences was found in obesity (10.6 % in boys and 10.7% in girls, p=0.571). The prevalence of abdominal obesity based on waist circumference was similar in girls and boys (3.8% *vs.* 3.9% respectively; p=0.924), but significantly higher in boys than in girls based on waist-to-height ratio (28.1% *vs.* 19.4%, respectively; p=0.009). Comparison with previous studies showed a slightly increase in overweight/obesity in children of central Portugal in the last 10 years, reaching values of 40.0% prevalence in the 7-9 years old.

Conclusion: In conclusion, this study shows a very high prevalence of overweight/obesity and abdominal obesity among Portuguese children, following the trend of other southern European countries. Thus, it is of the utmost importance the development of preventive and treatment strategies.

2. Introduction

The prevalence of overweight and obesity in childhood, according to the World Health Organization (WHO), is a growing problem worldwide, in many European countries as well as in developing countries [16, 238]. The health consequences of overweight and obesity during childhood are strongly associated with risk factors for cardiovascular diseases, diabetes, orthopedic problems and it is an important predictor of adult obesity [239]. In fact, it tends to develop from childhood into adult life, resulting in an elevated risk of illness and premature mortality [239]. In Portugal, the majority of obesity and overweight prevalence studies were carried in adults, although it is reported a tendency for weight increase in children along the last 30 years [240].

The definition of obesity during childhood and adolescence is controversial due to the gender differences, and the variability in growth rate. The standard measure of adult overweight and obesity is Body Mass Index (BMI): weight (kg) / height $(m)^2$. It started being used for measuring children obesity after the development of a BMI for age and sex as an international standard for the assessment of childhood overweight and obesity [221]. However, this alone could be insufficient as an obesity indicator because it is limited to give an approximation of the total adiposity in the body [218]. The use of other complementary obesity measures can overcome this problem, as it gives a better approximation of overweight and obesity in children.

Waist circumference (WC) can be defined as an excessive accumulation fat around the organs inside the abdominal cavity and it is calculated between the lowest rib and the superior border of the iliac crest [219]. It can also be a good indicator of abdominal fat reported in the development of cardiovascular risk factors [219]. Recently, waist-to-height ratio (WHtR) emerged as a good predictor for abdominal obesity and cardiovascular risk factors [241]. This measure is very simple to use, and it can be applied to both genders and ages with a cut-off of WHtR ≥0.50 defining those with excess abdominal fatness [241].

This study aimed to estimate the prevalence of overweight, obesity and abdominal fat distribution among 6-12 years old Portuguese children from the central region of Portugal, providing new data based on different obesity measures, from a recent sampling in 2011.

3. Material and Methods

3.1 Study Subjects

Subjects were selected in 2011 from several public schools in the central region of Portugal. Children derived from five grouping of schools; *Agrupamento de escolas Dr. Manuel Fernandes* (Abrantes), *Agrupamento de escolas Pêro da Covilhã* (Covilhã), *Agrupamento de escolas da Pedrulha* (Coimbra), *Agrupamento de escolas Carolina Beatriz Ângelo* (Guarda) and *Agrupamento de escolas das Dairas* (Vale de Cambra), and all children from these groups of schools, aged between 6 to 12 years old, were chosen to integrate this study. From a total of 4028 initially selected children, only 1468 parents gave their written informed consent. Thirty-five children were excluded from the purpose due to had African (*n*=8), Asian (*n*=2), or other European origins (*n*=15). After these exclusions, the final sample was composed by 1433 children with Portuguese ancestry, comprising 747 girls and 686 boys.

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the *Ministério da Educação* (available at http://mime.gepe.min-edu.pt/ with the identification number process: 0151100001), and was conducted in accordance with the institutional guidelines of the University of Coimbra.

3.2 Anthropometric measurements and analyses

Height (cm) and weight (kg) were measured with participants dressed in lightweight clothing and without shoes. Waist circumference (cm) was measured midway between the lowest rib and the iliac crest to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. The BMI was calculated as the weight in kilograms divided by the square of height in meters (kg/m^2). The definition of overweight and obesity were defined using the International Obesity Task Force (IOTF) cut-offs [221], derived from the BMI in adults cut-points of 25 kg/m^2 and 30 kg/m^2 respectively. Abdominal obesity was defined using the sex and age-specific $\geq 90^{th}$ waist circumference percentile [242] and waist-to-height ratio was calculated as the ratio of waist and height using the cut-off value of ≥0.5 [218]. Comparisons between groups for all the characteristics were made using the student t-

test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, for windows version 18.0; Chicago, IL).

4. Results

The children characteristics are shown in Table 3.1.

Table 3.1. Characteristics of the sampled Portuguese children.

Characteristics	Overall (*n*=1433)	Boys (*n*=686)	Girls (*n*=747)	*p*-value
Age (years)	9.3 ± 1.77	9.2 ± 1.77	9.4 ± 1.76	0.952
Height (cm)	136.6 ± 11.90	136.3 ± 11.30	136.9 ± 12.43	**0.002**
Weight (kg)	34.3 ± 10.00	34.1 ± 9.59	34.6 ± 10.38	**0.033**
BMI (kg/m^2)	18.1 ± 3.08	18.0 ± 3.04	18.1 ± 3.12	0.370
BMI Z-score	0.40 ± 1.02	0.45 ± 1.05	0.35 ± 0.99	0.080
WC (cm)	63.9 ± 7.20	64.7 ± 7.21	63.1 ± 7.11	0.619
HC (cm)	76.0 ± 9.41	75.4 ± 8.86	76.5 ± 9.84	**0.006**
WHR	0.84 ± 0.06	0.86 ± 0.05	0.83 ± 0.06	**0.003**
WHtR	0.47 ± 0.05	0.48 ± 0.05	0.46 ± 0.05	0.953

Data are presented as mean ± standard deviation.
Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation score; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; WHtR, waist-to-height ratio.
p-value significant ($p \leq 0.05$) in bold.

Significant statistical differences were found between boys and girls regarding height (*p*=0.002), weight (*p*=0.033), hip circumference (*p*=0.006) and waist-to-hip ratio (*p*=0.003). The prevalence of overweight and obesity found in the sample ranged 33.0% (36.5% in boys and 29.7% in girls), with 22.3% children classified as overweighed and 10.7% as obese (Table 3.2).

Table 3.2. Prevalence of overweight, obesity and abdominal obesity among Portuguese children by gender according to the International Obesity Task Force (IOTF) cut-offs of BMI.

		Body mass index*, % (*n*)			Abdominal obesity, % (*n*)	
	n	Overweight and obese	Overweight	Obese	WC ≥90th	WHtR ≥0.50
Overall	1433	33.0 (473)	22.3 (320)	10.7 (154)	7.8 (111)	23.6 (338)
Boys	686	36.5 (250)	25.9 (179)	10.6 (71)	3.9 (52)	28.1 (193)
Girls	747	29.7 (223)	19.0 (141)	10.7 (82)	3.8 (59)	19.4 (145)
p-value		**0.002**	**0.044**	0.571	0.924	**0.009**

* According to the International Obesity Task Force (IOTF) cut-offs of BMI.
Abbreviations: WC, waist circumference; WHtR, waist-to-height ratio.
p-value significant ($p \leq 0.05$) in bold.

Boys presented higher prevalence of overweight than girls (25.9% for boys and 19.0% for girls, $p \leq 0.05$), but obesity prevalence was statistically similar between genders. Regarding the prevalence of abdominal obesity it was found that 7.8% of the children had a waist circumference ≥ 90th, and 23.6% had a waist-to-height ratio (WHtR) ≥ 0.50 (Table 3.2). The WHtR was significantly higher among boys (28.1%) than in girls (19.4%) (p=0.009). The prevalence of overweight and obesity increased with age until the 10 years-old in boys, while in girls no trend was detected across different ages (Figure 3.1). From the 959 children with a BMI < 25 kg/m^2 (derived from the BMI in adults cut-points), 928 were classified as normal weight (64.8%) and 31 (2.2%) were classified as underweight (BMI < 18.5 kg/m^2, derived from the BMI in adults cut-points).

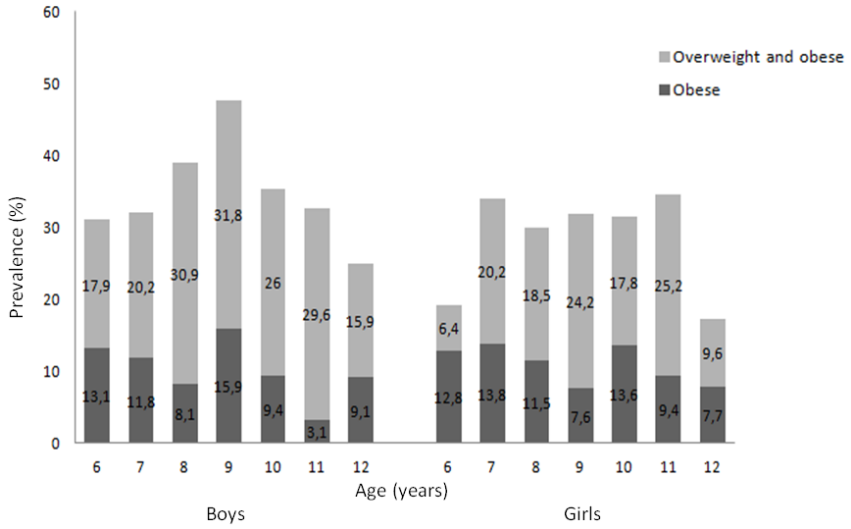

Figure 3.1. Prevalence of overweight and obesity among Portuguese children by age and gender.

5. Discussion

We found that overweight and obesity are common among Portuguese children. Comparing our data with a study of 2004 (data collected from October 2002 to June 2003) [240], we observe a slightly higher prevalence (1.5%) in overweight and obesity, but a decrease in children classified as obese (10.7% in this study and 11.3% in the 2004 study). However, if we consider only the children with 7-9 years old (like in the 2004 study) the prevalence of overweight and obesity increased during the last 10 years (overweight and obesity was estimated 40.7%, and 12.6% were classified as obese), which represents an increase of 10% in overweight and obesity comparing to 2004 study. These values revealed a high prevalence of overweight and obesity in this Portuguese region, mainly in the 7-9 years old age category, being these values similar to those reported for Spain, Greece and Italy [243, 244]. This data indicates that, in Europe, 31.8% of children are estimated to be overweight and of these 7-9% are obese [244]. Our recent data (collected in 2011) reveals an increase in prevalence for overweight and obesity in central region of Portugal during this last 10 years [245], with values particularly dramatic for 7-9 years old children, as observed in Greek children [243]. This study provides further evidence that there were strong increases in BMI among Portuguese children between 2002/2003 and 2011.

The changes in the social and economic structures in Portugal in the last three decades led to a global improvement of the living conditions [240]. These changes also had some negative effects leading to higher percentages of sedentary lifestyle for adults [240]. Several studies shown that the prevalence of obesity is common in families with low socio-economic status [246, 247]. Different studies have proved that healthier diets are more expensive than low quality diets, and could affect the acquisition of healthy foods both for parents and schools with lower budgets [246].

The prevalence of overweight and obesity was particularly high in boys (36.5%) when compared to girls (29.7%) (p=0.002). These differences remain significant when comparing the prevalence of overweight (p=0.044). However the same cannot be said about obesity, where prevalence values are similar in both genders (p=0.571). This trend is also observed across different age children, with a higher prevalence of overweight and obesity in boys comparing with girls. This prevalence is reverse when compared to the 2004 study [240], where prevalence of overweight and obesity was higher in girls. This fact could be due to the lack of physical activities in detriment of spending more time

playing electronic games/computer, which is more typical in boys than in girls. It was previously shown a relation between Portuguese children who spend more time playing electronic games/computer with higher values of BMI [248]. It is possible that Portuguese children especially boys display also a highly sedentary behavior, contributing to their increase in overweight/obesity. If we compare this results to other southern European countries, Portugal follows the trend found in Spain and Greece [249, 250] and the frequency of obesity in the last decade has increased more in boys than in girls [250]. A trend study from 1997-2007 in 8-9 years old Greek children, showed an increase of overweight and obesity in boys (26.5% and 12.2%, respectively), and in girls (26.7% and 11.2%, respectively) [243]. In that study, the prevalence obtained in 2007 for overweight/obesity was estimated in 38.3%, and from these 11.7% were obese [243]. In our study, the prevalence of obesity is similar in boys and in girls, but overweight is higher in boys than in girls. We also analyzed abdominal obesity in the children, as it could be a better predictor for cardiovascular disease risk in children than BMI [219], and also important in weigh management [251]. Furthermore according to our data, BMI seems a better tool than WC for discriminating obesity prevalence. We also looked to WHtR as it might be a useful index to identify metabolic risk in overweight children [252]. This index showed a similar trend to BMI in both gender, and these could indicate that WHtR is a good tool for discriminating the prevalence of abdominal obesity, and also a possible measure of total obesity prevalence. All these measures are useful in measuring overweight and obesity prevalence, but they all have limitations and the use of all depicts a more accurate picture of the prevalence in a sample. Traditionally, BMI has been used to predict body composition whereas WC is a measure of adipose tissue, but both are important in prediction of health risks in children [219, 241]. Recently, WHtR also emerged has a good tool for evaluating obesity prevalence and health risk in children [252]. Our data are in agreement with this, as we detected a higher prevalence (above ≥ 0.50) in boys and girls using this index, than using BMI or WC. The higher value for WHtR than for WC could be due to the fact that WHtR takes into account differences in body height. Curiously, the trend of adults in European countries such as Spain shows that abdominal obesity is more frequent in women than in men [253]. We found that abdominal obesity in Portuguese children has a similar frequency in boys and girls using waist circumference percentile, but it is more frequent in boys than in girls using waist-to-heigh ratio cut-off. Nevertheless, data on secular trends in WC and WHtR are scarce in Portuguese children,

thus it is impossible to compare these results with previous studies. Future studies concerning overweight and obesity trends in children should also consider WC and WHtR as screening tools.

Children obesity and overweight is a major public health issue, not only in developed but also in emerging countries. Our data in central Portugal showed a prevalence of childhood overweight and obesity similar to other southern European countries. However, our data also shown an increase in overweight and obesity prevalence in the last decade in the Portuguese children, indicating that the rate of prevalence could be increasing. It seems important to reverse this scenario, making prevention since childhood, and by that helping to reduce the higher incidence of cardiovascular diseases, that are the major cause of death in Portugal.

Chapter IV

Association of *FTO* polymorphisms with obesity and obesity-related outcomes in Portuguese children

This chapter was mainly based on the following original paper: Albuquerque D, Nóbrega C, Manco L. **Association of *FTO* polymorphisms with obesity-related outcomes in Portuguese children**. *PLoS one* 2013, 8(1): e54370.

1. Abstract

Background: Several studies have reported an association between single nucleotide polymorphisms in the first intron of the *FTO* gene and body mass index (BMI) or obesity. However, this association has not yet been studied among the Portuguese population. This study aims to assess the association of three *FTO* polymorphisms (rs1861868, rs1421085 and rs9939609) with obesity-related outcomes in a sample of Portuguese children.

Methods: We examined a total of 730 children, 256 normal-weight, 320 overweight and 154 obese, aging from 6 to 12-years-old, recruited randomly from public schools in the central region of Portugal. DNA samples were genotyped for the three polymorphisms by allelic discrimination TaqMan® assay. Association of the *FTO* polymorphisms with several anthropometric traits was investigated. Additionally, we tested association with the risk of obesity using overweight and obese *vs.* normal-weight children group.

Results: We found significant associations of rs9939609 and rs1421085 polymorphisms with weight, BMI, BMI Z-score, and waist circumference ($p<0.05$ in all traits). For rs1861868 polymorphism, significant associations were obtained with weight ($p=0.004$), BMI ($p=0.011$), and waist circumference ($p=0.024$). In case-control studies, both rs9939609 and rs1421085 polymorphisms were significantly associated with obesity (OR=1.41; 95% CI, 1.05-1.89; $p=0.023$; OR=1.45; 95% CI, 1.08-1.95; $p=0.012$, respectively) but not with overweight ($p>0.05$). Haplotype analyses (rs1861868-rs1421085-rs9939609) identified two combinations (ACA and GCA) associated with a higher risk of obesity (OR=1.53; 95% CI, 1.06-2.22; $p=0.023$; OR=1.73; 95% CI, 1.06-2.87; $p=0.030$, respectively).

Conclusions: This study provides the first evidence for the association of *FTO* polymorphisms with anthropometric traits and risk of obesity in Portuguese children.

2. Introduction

Overweight and obesity are a major health issue associated with risk factors for the development of hypertension, type 2 diabetes and cardiovascular diseases [254]. This complex phenotype results from the interaction of environmental and multiple genetic factors influencing body mass index (BMI), with heritability estimated at 40-70% [255].

The advent of Genome Wide Association Studies (GWAS) emerged as a powerful approach to identify genetic variants associated with common diseases [256]. Until now, GWAS deliver the identification of at least 52 genetic loci robustly associated with obesity [63]. In 2007, a strong association was detected between common single nucleotide polymorphisms in the first intron of the fat mass and obesity-associated gene (*FTO*), on the chromosome 16q12.2, and risk of obesity [65, 67]. Of those polymorphisms, the rs9939609 is one of the most extensively studied, explaining about 1% of BMI heritability [65]. Each rs9939609-A allele in this gene increases body weight by 1.5 kg in adult, with similar effects observed in children and adolescents [65]. Subsequently, several other studies have consistently confirmed the association of a cluster of polymorphisms within the first intron of the *FTO* gene with obesity-related traits in several European [65–68, 76, 192], Asian [69–72, 257] and African [73, 74] populations.

Knowledge of the genetic risk factors associated with common childhood obesity, can be helpful to design prevention strategies. Although in the Portuguese population several studies were made concerning the prevalence of overweight and obesity [240, 258], until now, no studies reporting the association of genetic variants with the risk of common obesity have been generated. Thus, the aim of this study was to evaluate the association between three *FTO* polymorphisms, including rs9939609 and rs1421085, prominent in the literature, and rs1861868, yet poorly studied, with the susceptibility to obesity in a sample of Portuguese children.

3. Material and Methods

3.1. Study subjects

Children aging 6 to 12 years old were randomly selected from several public schools in the central region of Portugal. A total of 1433 Portuguese children of European descent comprising 747

girls and 686 boys were recruited [258], and were classified using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF) [221]. From the 1433 analyzed children, three BMI groups were formed: 320 subjects were classified as overweight (resulting from the BMI in adult's cut-points between ≥25 kg/m^2 and <30 kg/m^2), 154 as obese (BMI ≥30 kg/m^2), 928 as normal weight (18.5 ≥ BMI <25 kg/m^2), [31 children were classified as underweight (BMI <18.5 kg/m^2) and excluded of this study].

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the Portuguese Ministry of Education (available at http://mime.gepe.min-edu.pt/ with the identification number process: 0151100001), and was conducted in accordance with the institutional guidelines of the University of Coimbra. Written informed consent was previously obtained from the children´s parents.

3.2. Anthropometric Measurements

Height (cm) and weight (kg) were taken with participants dressed in lightweight clothing without shoes. Waist circumference (cm) was measured midway between the lowest rib and the iliac crest, to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. The BMI was calculated with the weight in kilograms divided by the square of height in meters (kg/m^2). Abdominal obesity was defined using the sex and age-specific ≥90th waist circumference percentile [259]

3.3. Selected and genotyping of the FTO polymorphisms

Samples were analyzed for three polymorphisms located within first intron of the *FTO* gene: two that have been closely associated with obesity and prominent in the literature, rs9939609 (position: chr16:53820527), described by Frayling *et al*. [65], and rs1421085 (position: chr16:53800954), reported in the work of Dina *et al*. [76], and the yet poorly studied rs1861868 polymorphism (position: chr16:53790402), described in two studies [66, 192].

A buccal swab sample was collected from each child for genetic studies. The genomic DNA was extracted from buccal cells using the PureLink Pro 96 Genomic DNA Kit (Invitrogen Corporation,

Carlsbad, CA, USA), according to the instructions of the manufacturer, and was only used for the polymorphisms genotyping.

Samples were genotyped for the three *FTO* polymorphisms by allelic discrimination assays using TaqMan® probes (C_30090620_10, C_11717119_10 and C_8917103_10; Applied Biosystems, Foster City, CA, USA). The PCR amplification was carried out in 25 µl of a total reaction volume containing 2 µl (~20 ng) of DNA, 0.5 µl TaqMan® probes (Applied Biosystems, Foster City, CA, USA) in 1x of SsoFast™ Probes Supermix™ (Bio-Rad, Hercules, CA, USA). The PCR conditions were an initial denature step for 10 minutes at 95°C, followed by 40 cycles consisting of 1minute at 60°C and 15 seconds at 95°C. Fluorescence was visualized through a MiniOpticon real time PCR system (Bio-Rad, Hercules, CA, USA).

To identify the allele associated with the FAM probes, the automatic sequencing by Sanger's method was used. After the PCR product amplification, using oligonucleotides 5´-CATCAGTTATGCATTTAGAATGTCTG-3´ (forward) and 5´-TCCCACTCCATTTCTGACTGT-3´ (reverse) for rs9939609, 5´-AATCTCATTGTTCCTCCTGCT-3´(forward) 5´-ACAGTGGAGGTCAGCACAGA-3´(reverse) for rs1421085, and 5´-CGCATCTCTGCAACTCTTTT-3´ (forward) and 5´-TGCTTTGTTAAGGCCATAGG-3´ (reverse) for rs1861868, PCR fragments were purified with the enzyme ExoSAP-IT (GE Healthcare, New Jersey, USA) and subsequently subjected to Sanger´s dideoxy chain termination sequencing reaction using BigDye® Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), according to protocols recommended by the manufacturer and analyzed in an ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Base calling was performed with Sequencing Analysis software v5.2 (Applied Biosystems, Foster City, CA, USA).

To assess genotyping reproducibility a selection of 10% random samples was re-genotyped for all SNPs with 100% concordance by the Single Strand Conformation Polymorphism (SSCP) method, using oligonucleotides previously described (see conditions in chapter II).

3.4. Statistical analysis

The allele and haplotype frequencies were estimated by direct gene counting. The software package Arlequin, v3.5. (http://cmpg.unibe.ch/software/arlequin35/) [233], was used to calculate allele frequencies, Hardy-Weinberg equilibrium probability values and *D'* and r^2 values for linkage disequilibrium (LD). Haplotype phase was determined by statistical inference via the ELB algorithm

implemented in Arlequin, v3.5. The quantitative variables were expressed as means and standard deviation, and qualitative variables were presented as absolute numbers and frequencies. Normality of data was assessed using the Kolmogorov-Smirnov test. For each obesity-related quantitative parameters (BMI, BMI Z-score, weight and waist circumference), the nonparametric Kruskal-Wallis test was used to evaluate differences among the three genotypes in all polymorphisms. These statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences for Windows, version 18.0, SPSS inc., Chicago, IL, USA). Logistic regression under an additive genetic model, allowing for analysis of a binary outcome (a case–control *status*), was used to test obesity and overweight phenotype polymorphism associations, adjusted for age and sex, by calculating odds ratios (OR) with 95% of confidence intervals (CI) and *p*-values. This statistical analysis was done by using the set-based tests implemented on PLINK software v.1.07. (http://pngu.mgh.harvard.edu/purcell/plink/) [234]. Statistical significance was taken at *p*-values <0.05 for all comparisons. QUANTO, v1.1. power calculator (http://biostats.usc.edu/software) was used to estimate the power of association as a function of the frequency of the effect allele assuming an additive model [237].

4. Results

The analyzed children were divided into three groups according to the definition of BMI specified by IOTF cut-offs [221]. From a total of 1433 children measured for anthropometric traits, genotyping was performed in a total of 730 children comprising 320 subjects classified as overweight (≥25 kg/m^2 BMI <30 kg/m^2), 154 classified as obese (BMI ≥30 kg/m^2) and a control group of 256 subjects randomly selected from the total normal weight children (*n*=928, 18.5≤ BMI <25 kg/m^2). A descriptive study of the total genotyped sample, stratified by phenotype distribution, is shown in Table 4.1.

Table 4.1. General characteristic of the sampled children by phenotype distribution.

Characteristics	Overall	Phenotype distribution*		
		Normal	Overweight	Obese
N	730	256	320	154
Girls (%)	50.7	55.9	45.3	53.2
Age (years)	9.1 ± 1.7	8.6 ± 1.6	9.5 ± 1.6	9.0 ± 1.7
Height (cm)	136.2 ± 11.7	131.1 ± 11.1	139.5 ± 11.1	137.9 ± 10.6
Weight (kg)	37.2 ± 11.3	28.1 ± 6.6	40.2 ± 9.3	46.1 ± 11.0
BMI (kg/m^2)	19.6 ± 3.4	16.1 ± 1.5	20.3 ± 1.8	23.8 ± 2.5
BMI Z-score	0.93 ± 0.97	-0.15 ± 0.78	1.3 ± 0.23	1.99 ± 0.23
Waist circumference (cm)	67.2 ± 7.8	60.3 ± 4.5	68.9 ± 5.4	75.1 ± 6.6
Hip circumference (cm)	79.0 ± 10.3	70.4 ± 6.5	81.9 ± 8.0	87.1 ± 9.4
WHR	0.85 ± 0.06	0.86 ± 0.06	0.85 ± 0.06	0.87 ± 0.05

Data are presented as mean ± standard deviation.
*Phenotype distribution was determined using age and gender specific BMI cut-offs provided by the International Obesity Task Force (IOTF).
Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation score; WHR, waist-to-hip ratio.

The genotyping success rate of the three selected polymorphisms varied between 93.3% and 99.6%. Genotype frequencies for the total sampled population were in accordance with Hardy-Weinberg equilibrium (p=1.000 for rs9939609, p=0.598 for rs1421085, and p=0.937 for rs1861868). The minor allele frequency observed for the three polymorphisms in the total sample was 44.8% for the rs9939609-A allele, 45.4% for the rs1421085-C allele, and 46.1% for the rs1861868-G allele. The estimated power of association observed was above 95% for the rs9939609 and rs1421085 polymorphisms and, 56% for the rs1861868 polymorphism.

We analyzed anthropometric traits among different genotypes of *FTO* polymorphisms and found statistical significant differences in the mean score for rs9939609 and rs1421085 polymorphisms for increasing weight, BMI, BMI Z-score, and waist circumference (WC) ($p \leq 0.05$ for all traits) (Table 4.2). The strongest associations were found with weight (p=0.002) and waist circumference (p=0.003) (Table 4.2). The rs9939609 per-A allele increases was ~0.6 kg/m^2 in BMI, ~1.2 cm in waist circumference and ~1.7 kg in weight; similar values were obtained for each rs1421085-C allele: 0.55 kg/m^2, 1.25 cm and 1.55 kg, for BMI, waist circumference and weight, respectively. The rs1861868 polymorphism also showed associations with weight, BMI, and waist circumference (Table 4.2).

Table 4.2. Minor allele frequencies and Hardy-Weinberg equilibrium test of the 3 studied *FTO* polymorphisms in the sampled Portuguese children and their associations with obesity-related quantitative traits.

Polymorphism	Chr.	Gene	Alleles*	*n*	MAF	HWE	No. 11/12/22	Genotype (mean ± SD)			*p*-value
								11	12	22	
rs9939609	16q12.2	*FTO*	T:A	721	0.45	1.000	220/357/144				
BMI (kg/m^2)								19.0 ± 3.4	19.7 ± 3.4	20.2 ± 3.5	***0.005***
BMI Z-score								0.78 ± 1.1	0.97 ± 0.9	1.1 ± 0.9	***0.011***
Weight (kg)								35.5 ± 11.7	37.7 ± 11.1	38.9 ± 10.9	***0.003***
Waist C (cm)								65.9 ± 7.9	67.7 ± 7.6	68.4 ± 7.6	***0.002***
rs1421085	16q12.2	*FTO*	T:C	727	0.45	0.598	213/368/146				
BMI (kg/m^2)								19.1 ± 3.5	19.6 ± 3.4	20.2 ± 3.5	***0.013***
BMI Z-score								0.76 ± 1.0	0.95 ± 0.9	1.1 ± 0.9	***0.012***
Weight (kg)								35.6 ± 11.7	37.4 ± 11.2	38.7 ± 10.9	***0.012***
Waist C (cm)								65.9 ± 7.8	67.5 ± 7.6	68.5 ± 7.8	***0.006***
rs1861868	16q12.2	*FTO*	A:G	668	0.47	0.937	195/342/144				
BMI (kg/m^2)								18.8 ± 3.2	19.6 ± 3.5	20.0 ± 3.5	***0.011***
BMI Z-score								0.75 ± 1.0	0.95 ± 0.9	1.0 ± 0.8	0.162
Weight (kg)								34.5 ± 10.3	37.4 ± 11.6	38.6 ± 11.3	***0.004***
Waist C (cm)								65.6 ± 7.5	67.4 ± 7.8	68.1 ± 7.6	***0.024***

Abbreviations: Chr., chromosome; *n*, number of genotyped children; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; BMI, body mass Index; BMI Z-score, body mass index standard deviation; SD, standard deviation; Waist C, waist circumference.
* Alleles: Major (1): Minor (2).
p-values were obtained using the Kruskal-Wallis test.
p-value significant ($p<0.05$) are in bold and italic.

We performed nominal association analysis under the additive model using BMI case-control groups (Table 4.3). When compared obese *vs.* normal-weight groups the rs9939609 minor A-allele showed significant association with risk of obesity (OR=1.41; 95% CI, 1.05-1.89; *p*=0.023). Accordingly, 23.4% of the obese individuals were AA homozygotes compared to 16.2% of the control subjects. Significant association was also observed with increased risk of being obese for the rs1421085 minor C-allele (OR=1.45; 95% CI, 1.08-1.95; *p*=0.012). For this polymorphism, 26.6% of the obese individuals had the CC genotype, against 17.7% with normal weight. For the rs1861868 polymorphism, it was not found a significant association with obesity (OR=0.85; 95% CI, 0.63-1.15; *p*=0.318) (Table 4.3). Association analysis under an allelic model, comparing obese *vs.* normal-weight groups, showed similar significant results for the rs9939609 A-allele (OR=1.44; 95% CI, 1.08-1.91; *p*=0.012) and rs1421085 C-allele (OR=1.48; 95% CI, 1.12-1.98; *p*=0.007), but not for rs1861868 (OR=1.20; 95% CI, 0.89-1.61; *p*=0.228). We detected no significant association when comparing overweight *vs.* normal-weight groups ($p \geq 0.05$) (Table 4.3).

We further investigated the difference in the genotype distribution between cases and controls for abdominal obesity by logistic regression analysis. In the total of 730 children, 111 revealed abdominal obesity (using sex and age-specific $\geq 90^{th}$ waist circumference percentile). From the three polymorphisms studied, none of them showed association with increased abdominal obesity.

Table 4.3. Allele frequencies of the 3 studied *FTO* polymorphisms in the Portuguese children, and their associations with risk of obesity among phenotypic groups.

SNP ID/ Gene	Allele		Obese *vs.* normal			Overweight *vs.* normal		
		Normal*	Obese*	OR CI 95%	*p-value*	Overweight*	OR CI 95%	*p-value*
rs9939609	T	0.591	0.503	1.41 (1.05-1.89)	***0.023***	0.546	1.15 (0.89-1.47)	0.274
FTO T>A	A	0.409	0.497			0.454		
rs1421085	T	0.572	0.474	1.455 (1.08-1.95)	***0.012***	0.560	1.00 (0.78-1.29)	0.944
FTO T>C	C	0.428	0.526			0.440		
rs1861868	A	0.504	0.552	0.859 (0.63-1.15)	0.318	0.557	0.886 (0.68-1.14)	0.350
FTO A>G	G	0.496	0.448			0.443		

Abbreviations: SNP ID; single nucleotide polymorphism identification; OR, odds ratio; CI, confidence interval; *vs.*, versus.
*Phenotypic groups was determined using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF).
Logistic regression was used to compare genotype distribution. *p*-values (asymptotic *p*-value for t-statistic) shown are for an additive model and are adjusted for age and sex. OR is shown for the minor allele.
p-value significant ($p<0.05$) are in bold and italic.

Haplotype analysis associating the three studied *FTO* polymorphisms (rs1861868-rs1421085-rs9939609), revealed all the eight possible haplotypes, being the most commons GTT (33%), ACA (32%), ATT (19%) and GCA (12%) (Table 4.4). Three haplotypes had an estimated frequency below 1% (GCT, ATA and GTA). Compared with the most common and non-risk haplotype (GTT), two haplotypes (ACA and GCA) were significantly associated with a higher risk of being obese (OR=1.534; 95% CI, 1.06-2.22; p=0.023; OR=1.739; 95% CI, 1.06-2.87; p=0.030, respectively).

Regarding allelic combinations, polymorphisms rs9939609 (position: chr16:53820527) and rs1421085 (position: chr16:53800954), distant from one another about 19.6 kb, were found in high LD (D'= 0.91; r^2=0.82). The rs1861868 polymorphism (position: chr16:53790402) was found in low LD (D'= 0.39; r^2=0.11) with rs9939609, distant about 30.1 kb, as well with rs1421085 (D'= 0.44; r^2=0.13), distant about 10.6 kb.

Table 4.4. Haplotype frequencies associating *FTO* rs1861868-rs1421085-rs9939609 polymorphisms in the sampled Portuguese children.

Haplotype	Frequency	OR	95% CI	*p*-value
GTT	0.33	Reference		
ATT	0.19	1.133	0.73-1.75	0.572
ACA	0.32	1.534	1.06-2.22	***0.023***
GCA	0.12	1.739	1.06-2.87	***0.030***
ACT	0.02	2.000	0.68-5.89	0.209
Rare	0.02	1.200	0.42-3.42	0.733

Rare: haplotypes with a frequency under 1% (GCT, ATA and GTA).
Abbreviations: OR, odd ratio; CI, confidence interval.
p-value significant (p<0.05) in bold and italic form.

5. Discussion

Recently, the growth in studies regarding the association of obesity, or obesity-related traits, with SNPs in the *FTO* gene has been reported for several populations across the world [65–74, 76, 192, 257]. Most studies confirmed that *FTO* polymorphisms are strongly associated with BMI and/or obesity [65–74, 76, 192, 257]. However, in Portugal there are no studies to confirm the association between genetic variants and common obesity, which could permit the comparison with data from other European populations. Despite the similar genetic background between European populations, it is known that for several polymorphisms, frequencies can vary within different Caucasian populations [168, 260]. Moreover, a few studies failed to associate some *FTO* polymorphisms and obesity [261, 262], highlighting the need of more studies in different populations to better understand the role of *FTO* gene in obesity. The present study is the first to test whether common *FTO* gene polymorphisms are associated with obesity or to related anthropometric traits in children of Portuguese origin.

Our research showed a significant genetic association of rs9939609 and rs1421085 polymorphisms, in strong linkage disequilibrium (r^2=0.82), with the risk of obesity in Portuguese children. Consistently, we also observed significant association with several anthropometric measurements including weight, BMI, waist circumference and hip circumference. These results are similar to those found in previous studies performed in other European populations reporting the association of *FTO* polymorphisms with obesity [65, 67, 76, 263]. In our study, the effect obtained for each copy of rs9939609-A allele was ~0.6 kg/m^2 in BMI, ~1.2 cm in waist circumference and ~1.7 kg in weight, similar to the effect stated by Frayling *et al*. [65].

Regarding rs1861868 polymorphism, association with BMI was first described in a sample of Old Order Amish with low physical activity [192] and replicated in a sample of Spanish children [66]. However, in this last study, it was not found a significant association with BMI or obesity. Our study showed an association with weight, BMI, waist circumference and hip circumference.

Our results show that in Portuguese children the rs9939609 and rs1421085 polymorphisms are in association with obesity, with no differences between girls and boys, and in line with previously reported studies in other European populations [65–68, 76, 192]. We found an odd ratio of 1.41 for the rs9939609 polymorphism under the additive model. This result appears similar to the effects

reported by Frayling *et al*. [65] in UK children (OR=1.35; 95% CI, 1.14-1.61), and Hinney *et al*. [264] in German children/adolescents (OR=1.57; 95% CI, 1.30-1.90). We also found an odd ratio of 1.45 for the rs1421085 polymorphism similar to that reported by Dina *et al*. [76] in French children (OR=1.43; 95% CI, 1.25-1.64), and Meyre *et al*. [265] in German children (OR=1.50; 95% CI, 1.25-1.79). The rs9939609 polymorphism was the most replicated polymorphism associated with obesity across the world, nevertheless, in our study the strongest association was obtained with the rs1421085 polymorphism (OR=1.45; 95% CI, 1.08-1.95; p=0.012, additive model), similar to the result obtained by Price *et al*. [266] in a sample of Caucasian women when analyzing both polymorphisms.

None of the three study polymorphisms showed evidence of association with overweight in the sample. This means that the *FTO* risk allele predominates in individuals with higher BMI; hence the association was detected in severe obesity rather than in overweight population, similarly to the results obtained by Liu *et al*. [257].

The *FTO* risk allele frequencies observed in our study are within range of reported values in European populations [65–68, 76, 192]. Both rs9939609 and rs1421085 polymorphisms were found in high LD (r^2=0.82) in our study reflecting the high LD across the 19.6 kb region within the intron 1 of *FTO* gene. Polymorphisms rs9939609 and rs1421085 are both part of a set of BMI-associated polymorphisms within a 47 kb LD block encompassing parts of the first two introns as well as exon 2 of the *FTO* gene [80] suggesting that they all tag a same genetic signal in that region. The low LD (r^2=0.13) observed in our study between rs1861868 5' apart 10.6 kb from rs1421085, complement the lower genetic predictive power of rs1861868 for the studied obesity related parameters, suggesting that LD block decline between these two polymorphisms. As we show (Table 4.4) the only two common haplotypes that seem to confer risk to obesity were ACA (p=0.023) and GCA (p=0.030), which include both risk alleles A and C for rs9939609 and rs1421085, respectively. For the haplotype ACT, presenting only one risk allele, no association (p=0.209) with obesity was found. This seems to reflect that haplotypes combining the risk alleles for the two SNPs rs9939609 and rs1421085 have increased risk of obesity.

In 1962, Neel proposed the thrifty gene hypothesis [163] suggesting that populations whose ancestral environments were characterized by periods of feast and famine, experienced positive selection for thrifty alleles that promote the storage of fat and energy. Thus, under modern conditions, populations with such thrifty alleles are expected to have high rates of obesity. Regarding

the ancestral alleles for polymorphisms rs9939609 and rs1421085 comparing sequence similarity with non-human primates, the ancestral rs9939609-A allele is associated with the obesity risk but not the ancestral rs1421085-T allele. This different genetic association pattern is not consistent with the thrifty gene hypothesis, as also suggested in a previous report [118], because under this hypothesis we should expect a similar pattern regarding ancestry of the risk alleles.

This is the first study reporting allele and genotype frequencies of the *FTO* polymorphisms in the Portuguese population. We found evidence that the previously reported common polymorphisms rs9939609 and rs1421085 in *FTO* gene increase the risk of obesity in the Portuguese children. Further studies on other polymorphisms from *FTO* and other genes are needed, to establish the genetic basis contributing to the risk of obesity in the Portuguese population.

Chapter V

The lactase persistence -13910C>T polymorphism shows indication of association with abdominal obesity among Portuguese children

This chapter was mainly based on the following original paper: Albuquerque D, Nóbrega C, Manco L. **The lactase persistence -13910C>T polymorphism shows indication of association with abdominal obesity among Portuguese children**. *Acta Paediatr* 2013, 102(4): e153-157.

1. Abstract

Aim: The -13910C>T (rs4988235) single nucleotide polymorphism located upstream of the lactase gene (*LCT*) was found tightly associated with lactase persistence in European populations. Recently, it was also associated with body mass index (BMI) and obesity in European adults. The aim of this study was to test the association of -13910C>T polymorphism, with obesity-related traits and risk of obesity in children.

Methods: We genotyped 580 Portuguese children (6-12 years-old) for the -13910C>T polymorphism using Dual-labeled probes by real-time PCR. Anthropometric measurements were assessed in all children. Obesity was defined according to the IOTF cut-offs and abdominal obesity using the sex and age-specific ≥90th waist circumference percentile.

Results: We found indication for association between the -13910*T allele and children abdominal obesity (OR=1.41; 95% CI: 1.03-1.94; *p*=0.030). Under the dominant model, the indicative association was observed between the *LCT* -13910 CT/TT genotypes and abdominal obesity, remaining significant after adjustment for age and gender (OR=1.65; 95% CI: 1.04-2.60; *p*=0.029). No association was detected with the risk of obesity (*p*=0.350).

Conclusion: Our results suggest that the -13910C>T polymorphism may predispose to abdominal obesity in Portuguese children. The association with BMI or obesity risk, previously observed in adults, was not confirmed.

2. Introduction

The lactase gene (*LCT*; MIM 603202, chromosome 2q21) was recently reported as a new candidate related with BMI and obesity in adults of European origin. Three independent studies reported a strong association of the -13910C>T (rs4988235) single nucleotide polymorphism, located ~14kb upstream from the *LCT* coding region, with BMI and obesity: the -13910*T allele carriers (CT and TT genotypes) had higher weight, BMI and risk of obesity [102–104].

All newborns display an adequate expression of the lactase enzyme that decline significantly in quantity following weaning, and this condition is apparently the major reason for avoiding milk in diet [267]. However, substantial numbers of individuals maintain the ability to digest milk and other dairy products into adulthood. This lactase persistence (LP) phenotype is an autosomal dominant condition that reaches its highest values in north-western Europe (80-90%), declining to the south and east (~50%) [268, 269]. The lactase non-persistence (LNP) is considered the ancestral condition in humans and these individuals are unable to digest significant amounts of lactose, suffering from adverse unspecific abdominal symptoms, including bloating, abdominal pain and diarrhea, after ingestion of milk [270]. In European populations the -13910C>T polymorphism was found tightly associated with the persistence of the lactase enzyme in adulthood: TT or TC individuals possess sufficient enzyme activity in intestinal cells to be classified as LP, and individuals carrying the CC genotype are classified as LNP [169]. The prevalence of the -13910*T allele vary across Europe, reaching 70-80% in Northern European populations, and 5-10% in Southern European populations from Greece and Italy [271]. In Northern Portugal the -13910*T allele frequency was estimated 37.0% [272].

Until now, the genetic background of *LCT* -13910C>T polymorphism in obesity has not yet been examined among children of European descent. Thus, the aim of this study was to test the association between *LCT* -13910C>T polymorphism and obesity or obesity-related traits in a sample of Portuguese children.

3. Material and Methods

3.1. Subjects and measures

The study comprised 580 children with Portuguese ancestry (6-12 years old) randomly selected from several public schools in a Northern-Central region of Portugal located between *Mondego* and *Douro* rivers. Samples were collected from three geographic areas: municipalities of Coimbra (*n*=266), Vale de Cambra (*n*=147) and Guarda/Covilhã (*n*=167).

Anthropometric measurements were assessed in all children. Children were distributed in three groups: 140 with obesity, 233 with overweight and 207 with normal weight. The definition of overweight and obesity was obtained using the International Obesity Task Force (IOTF) cut-offs [221], resulting from the BMI in adult's cut-points of 25 kg/m^2 and 30 kg/m^2, respectively. Abdominal obesity was defined using the sex and age-specific ≥90th waist circumference percentile [259].

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the Portuguese Ministry of Education (available at http://mime.gepe.min-edu.pt/ with the identification number process: 0151100001), and was conducted in accordance with the institutional guidelines of the University of Coimbra. Written informed consent was previously obtained from all the children´s parents.

3.2. Genotyping

Genomic DNA was extracted from buccal swabs using the PureLink Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the instructions of the manufacturer.

The -13910C>T polymorphism (Chr.2: 135851076 position) was genotyped using dual-labeled probes for real-time polymerase chain reaction (PCR) on a MiniOpticon instrument (Bio-Rad, Hercules, CA, USA) using primers and labelled probes previously reported [225]. The PCR amplification was carried out in 20 µl of a total reaction volume containing 2 µl (~20 ng) of DNA, 0.4 µM primers, 0.2 µM 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 60°C and 15 seconds at 95°C. To assess genotyping reproducibility, a random 10% selection of samples was re-genotyped with 100% concordance (see conditions in chapter II).

3.3. Statistical analysis

Allelic and genotypic frequencies of the -13910C>T polymorphism were estimated by direct counting. Hardy-Weinberg equilibrium probability value, heterozygosity and exact *p* values for population differentiation [273] were achieved using the software package Arlequin, v3.11 (http://cmpg.unibe.ch/software/arlequin3/) [233].

In statistical analyses we follow a dominant model, and subjects with CT and TT genotypes were grouped and compared with CC subjects. Normality of the data was examined using the Kolmogorov–Smirnov test. Due to lack of the normal distribution, non-parametric tests have been used in the statistical analyses. The Mann-Whitney test was used to compare means of obesity-related traits between genotypes. Logistic regression was used to estimate *p*-values, odds ratio (OR), and 95% confidence intervals (CI), assessing the association of -13910C>T polymorphism with risk of obesity and abdominal obesity. Statistical analyses were performed using the SPSS software (Statistical Package for the Social Sciences for Windows, version. 18.0, SPSS inc., Chicago, IL, USA). A significant *p*-value was considered at <0.05 for all comparisons.

4. Results

The anthropometric characteristics of the study subjects distributed by phenotype are shown in Table 5.1.

The *LCT* -13910C>T genotype distributions regarding whole sample (*n*=580) were: CC 42.8%, CT 44.8% and TT 12.4%. Individual LP -13910*T allele frequency was 34.8% (Table 5.2). The similar allele frequencies across the three sampled subgroups (-13910*T allele frequency values of 34.6%, 32.3% and 37.4% in Coimbra, Vale de Cambra and Guarda/Covilhã municipalities, respectively) indicate absence of population stratification for the -13910C>T polymorphism (*p*=0.466 on the exact test of sample differentiation). No deviations from the Hardy-Weinberg equilibrium were observed in any population subgroup, neither in the whole sample (*p*>0.05).

Table 5.1. Body size and genetic characteristics of the children participants according to phenotype.

Characteristics	Overall	Phenotype*		
		Normal	Overweight	Obese
N	580	207	233	140
Age (years)	9.0 ± 1.7	8.6 ± 1.6	9.6 ± 1.6	9.0 ± 1.8
Girls (%)	51.9	54.1	48.1	55.0
Height (cm)	136.1 ± 11.8	130.8 ± 11.1	139.8 ± 11.3	137.8 ± 10.8
Weight (kg)	37.4 ± 11.6	27.9 ± 6.6	40.5 ± 9.5	45.9 ± 11.2
BMI (kg/m^2)	19.7 ± 3.5	16.1 ± 1.4	20.4 ± 1.8	23.8 ± 2.6
BMI Z-score	0.95 ± 0.9	-0.11 ± 0.8	1.28 ± 0.24	1.98 ± 0.2
Waist circumference (cm)	67.4 ± 7.8	60.4 ± 4.4	69.1 ± 5.4	74.9 ± 6.6
Hip circumference (cm)	79.2 ± 10.5	70.4 ± 6.3	82.3 ± 8.3	87.1 ± 9.5
Waist-to-Height Ratio	0.86 ± 0.06	0.86 ± 0.06	0.84 ± 0.06	0.86 ± 0.05

Abbreviations: *n*, total subjects; BMI, body mass index; BMI Z-score, body mass index standard deviation.
*Phenotype was defined using the International Obesity Task Force (IOTF) cut-offs.
Data are presented as mean ± standard deviation.

Testing the association with anthropometric variables, we found no association between the -13910C>T polymorphism and obesity-related traits, as BMI, weight or waist circumference (p>0.05 for all traits) (Table 5.3).

Logistic regression analysis was used to test the association of the -13910C>T polymorphism with the risk of obesity and abdominal obesity (Table 5.4). We observe indication of an association between the LP -13910*T allele and abdominal obesity (OR=1.41; 95% CI: 1.03-1.94; p=0.030). In a dominant model of genetic effect, the -13910 CT/TT genotypes show also indication of an association with abdominal obesity (unadjusted OR=1.64; 95% CI: 1.04-2.62; p=0.034), remaining significant after adjustment for age and gender (OR=1.65; 95% CI: 1.04-2.60; p=0.029). No association was found between the -13910 CT/TT genotypes and obesity (OR=1.24; 95% CI: 0.79-1.92; p=0.350) or overweight (OR=0.86; 95% CI: 0.59-1.28; p=0.769).

Table 5.2 The *LCT* -13910C>T polymorphism genotype and allelic distribution among municipalities.

Municipalities	*N*	Genotype distribution, % (*n*)			Allele frequencies		HWE	He
		CC	CT	TT	C	T		
Coimbra	266	43.6 (116)	43.6 (116)	12.8 (34)	0.654	0.346	0.589	0.453
Vale de Cambra	147	45.6 (67)	44.2 (65)	10.2 (15)	0.677	0.323	1.000	0.439
Guarda/Covilhã	167	38.9 (65)	47.3 (79)	13.8 (23)	0.626	0.374	1.000	0.469
Total	580	42.8 (248)	44.8 (260)	12.4 (72)	0.652	0.348	0.788	0.454

Abbreviations: *n*, total subjects; HWE, exact *p*-value for the Hardy-Weinberg equilibrium (*p* significant <0.05); He, expected heterozygosity.

Table 5.3. Comparison of anthropometric parameters among different genotypes of *LCT* -13910C>T polymorphism.

	LCT -13910C>T genotypes		*p*-value
	CC (*n*=248)	CT/TT (*n*=332)	
Age (years)	9.0 ± 1.7	9.1 ± 1.7	0.706
Height (cm)	135.3 ± 11.8	136.8 ± 11.7	0.328
Weight (kg)	36.5 ± 11.4	37.9 ± 11.7	0.252
BMI (kg/m^2)	19.5 ± 3.4	19.8 ± 3.6	0.308
BMI Z-score	0.91 ± 0.9	0.98 ± 0.9	0.316
Waist circumference (cm)	67.0 ± 7.7	67.7 ± 8.0	0.350
Hip circumference (cm)	78.6 ± 10.6	79.6 ± 10.4	0.393
Waist-to-Height Ratio	0.85 ± 0.04	0.86 ± 0.05	0.977

Data are presented as mean ± standard deviation.
p-values were obtained using the Kruskal-Wallis test.
p-values significant <0.05 in italic and bold.

Table 5.4. Associations of *LCT* -13910C>T polymorphism with risk of obesity and abdominal obesity [OR (95% CI)].

	Genotype distribution, % (*n*)					OR (95% CI)		
	Normal *n*=207	Overweight *n*=233	Obese *n*=140	Gluteofemoral *n*=483	Ab obesity *n*=97	Normal *vs.* Obese	Normal *vs.* Overweight	Gluteofemoral *vs.* Ab obesity
Dominant Model								
CC	43.0 (89)	45.5 (106)	37.9 (53)	44.7 (216)	33.0 (32)	1	1	1
CT/TT	57.0 (118)	54.5 (127)	62.1 (87)	55.3 (267)	67.0 (65)	1.24 (0.79-1.92) *p*=0.350	0.86 (0.59-1.28) *p*=0.769	1.65 (1.04-2.60) *p*=**0.032**
Allelic Model								
C	65.6 (273)	65.7 (307)	61.7 (174)	66.4 (641)	58.2 (113)	1	1	1
T	34.4 (141)	34.3 (159)	38.3 (106)	33.6 (325)	41.8 (81)	1.17 (0.86-1.62) *p*=0.305	1.00 (0.76-1.32) *p*= 1.00	1.41 (1.03-1.94) *p*=**0.029**

Abbreviations: Ab obesity; abdominal obesity defined using the sex and age-specific ≥90th waist circumference percentile. Gluteofemoral, children under the <90th waist circumference percentile. OR, odds ratio; CI, confidence interval.
Overweight and obesity was defined using the International Obesity Task Force (IOTF) cut-offs.
p-value under the dominant and allelic model was adjusted for age and gender (*p*-value significant <0.05 in bold).

5. Discussion

Several genetic polymorphisms have been reported to be associated with obesity or obesity-related phenotypes both in children and adults [274], which is a growing problem worldwide including Portugal [240, 258]. To better understand the genetic basis of obesity it is important to replicate these results in different populations across the world. Recently it was reported that *LCT* -13910C>T polymorphism is strongly associated with BMI and obesity in European adults by three studies that found adult's carriers of the -13910 CC genotype with lower weight and BMI [102–104].

There are some controversial data regarding effects of dairy products intake on body weight and fat [275]. Several studies in adults support for an increase in body weight associated with dairy products intake [276–278]. One possible explanation for this association could be that the extension of consumption of dairy products in daily diet, often high in energy content, potentially increase calorie intake in adulthood [278]. In children, relationship between dairy products consumption and weight/body composition indicate either a beneficial or a neutral effect [279, 280]. However, no studies were performed to see if individual *LCT* genetic profiles influence the relationship between dairy products consumption and body weight.

We conducted a population study to test whether the -13910C>T polymorphism, tightly associated with LP in individuals of European descent, was associated with obesity and/or obesity-related traits in Portuguese children. The exact test of sample differentiation based on allele frequencies showed no significant differences between the three study geographic areas of Coimbra, Vale de Cambra and Guarda/Covilhã, excluding population substructure and possible bias in association signals for the -13910C>T polymorphism, a locus that was shown to be prone to population stratification [281]. We found indication of an association between the LP -13910 CT/TT genotypes and abdominal obesity (OR=1.65; p=0.032), however, we did not find evidence for the association of the -13910C>T polymorphism with the children risk of obesity or other anthropometric measurements.

Considering previous studies showing that obesity risk in adulthood is significantly higher in T-allele carriers (TT and CT genotypes) than in CC subjects [102–104], our findings suggest that, by a continuous intake of rich fat dairy products, individuals with -13910 CT/TT genotypes could have a

more predisposition to develop obesity into adulthood than individuals with -13910 CC genotype, associated with adult hypolactasia.

This study suggests that LP -13910 CT/TT genotypes may predispose to abdominal obesity in Portuguese children. Association of the -13910C>T polymorphism with BMI or risk of obesity, previously observed in adults, was not confirmed in children. Further studies are needed i) to replicate the present results in children from other populations; ii) to see whether individual *LCT* genetic profiles influence the relationship between dairy products consumption and obesity-related traits.

Chapter VI

Association study of common polymorphisms in *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5*, and *OLFM4* genes with obesity-related traits among Portuguese children

This chapter was mainly based on the following original paper: Albuquerque D, Nóbrega C, Rodríguez-López R, Manco L. **Association study of common polymorphisms in *MSRA*, *TFAP2B*, *MC4R*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5*, and *OLFM4* genes with obesity-related traits among Portuguese children**. *J Hum Genet* 2014, 59(6): 307-313.

1. Abstract

Background: At least 52 genetic *loci* were associated with obesity-related traits. However, little is known about the genetic basis of obesity among children. This study aims to test whether 10 polymorphisms in obesity-related genes *MSRA, TFAP2B, MC4R, NRXN3, PPARGC1A, TMEM18, SEC16B, HOXB5* and *OLFM4* are associated with the risk of obesity in Portuguese children.

Methods: A total of 730 children aging from 6 to 12 years-old, recruited randomly from public schools in Portugal, were analyzed. Anthropometric measurements were obtained and children were classified into three phenotypic groups, normal-weight (*n*=256), overweight (*n*=320), and obese (*n*=154), according to the IOTF cut-offs. Polymorphisms were genotyped by allelic discrimination TaqMan® assays.

Results: The *MC4R* rs12970134 polymorphism was nominally associated with BMI (*p*=0.035), BMI Z-score (*p*=0.043) and waist circumference (*p*=0.020), and borderline associated with weight (*p*=0.053). Near nominally associations were also found for the *PPARGC1A* rs8192678 polymorphism with weight (*p*=0.061), and for the *MSRA* rs545854 polymorphism with BMI (*p*=0.055) and BMI Z-score (*p*=0.056). Furthermore, logistic regression showed that *MC4R* rs12970134 and *TFAP2B* rs987237 were nominally, respectively, associated (*p*=0.029) and borderline associated (*p*=0.056) with the obese phenotype.

Conclusion: This study highlighted the possible association of *MC4R*, *PPARGC1A*, *MSRA* and *TFAP2B* polymorphisms with several obesity-related traits in a sample of Portuguese children. The two significant associated *TFAP2B* rs987237 and *MC4R* rs12970134 polymorphisms showed an opposite direction of effect to that in the original reports.

2. Introduction

The obesity phenotype has been increasing in the last decades and the causes for this complex disorder are thought to be related with an imbalance between energy intake and energy expenditure due to changes in lifestyles including exposure to an obesogenic environment [282]. Furthermore, it is estimated that the heritable predisposition to obesity may range from 40 to 70% [5, 283, 284].

In less than seven years, genome wide association studies (GWAS) have successfully identified more than 52 genetic *loci*, which were unequivocally associated with obesity-related traits [63]. The first *locus* associated with obesity was the fat mass and obesity associated (*FTO*) gene by Frayling *et al*. [65] which is the most replicated gene across the world, both in adult and children samples [66, 69, 73], including in the Portuguese population [285]. Subsequently, several other studies emerged associating single nucleotide polymorphisms in several genes across the genome, most of them in adults [214, 286], and a few in children [114]. Nevertheless, candidate and replication studies of obesity *loci* among different populations emerge as an important step to identify and clarify which variants are indeed associated with obesity. The frequency of obesity-susceptibility alleles varies between populations, and these allele distributions could be a consequence of population-specific obesogenic environments associated with specific demography and cultural histories. Replication studies are also relevant to determine which polymorphisms previously associated with obesity in adults are also linked in children, and, in a final instance, to better understand the complexity of obesity susceptibility.

In this study, a sample of Portuguese children was tested for the association of obesity and obesity-related quantitative traits with ten polymorphisms in nine candidate genes including, methionine sulfoxide reductase A (*MSRA*), transcription factor AP-2 beta (*TFAP2B*), melanocortin 4 receptor (*MC4R*), neurexin 3 (*NRXN3*), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*), transmembrane protein 18 (*TMEM18*), homolog of *S. cerevisiae* Sec16 (*SEC16B*), homeobox B5 (*HOXB5*) and olfactomedin 4 (*OLFM4*).

3. Material and Methods

3.1. Study subjects

The original sample consisted of 1433 Portuguese children of European descent, aging between 6-12 years old, randomly selected from several public schools in the central region of Portugal [258]. From this original sample, three body mass index (BMI) groups, using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF) [221], were attained in a total of 730 children, including: i) 154 obese subjects (resulting from the BMI in adult's cut-points ≥30 kg/m^2); ii) 320 overweight subjects (resulting from the BMI in adult's cut-points between 25 kg/m^2 and 29 kg/m^2); and iii) 256 lean controls randomly selected from the initial group of 928 children with 18.5≥ BMI <25 kg/m^2 [31 children were classified as underweight (BMI<18.5) and excluded of this study].

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the Portuguese Ministry of Education (available at http://mime.gepe.min-edu.pt/ with the identification number process: 0151100001), and was conducted in accordance with the institutional and ethical guidelines of the University of Coimbra. Written informed consent was previously obtained from the children´s parents.

3.2. Anthropometric measurements

All children underwent anthropometric measurements of height, weight, waist and hip circumference using a standardized protocol. Body weight (kg) and height (cm) were taken with participants dressed in lightweight clothing without shoes to determine the BMI. Waist circumference (WC) (cm) was measured midway between the lowest rib and the iliac crest, to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. The BMI was calculated by dividing weight (in kilograms) by height (in meters) squared (kg/m^2). Abdominal obesity was defined using the sex and age-specific ≥90th waist circumference percentile [259].

3.3. Selection and Genotyping of polymorphisms

Ten single nucleotide polymorphisms identified from the literature significantly associated to obesity or obesity-related traits in children of European origin were selected: rs17782313 (Chr 18:60183864 position) and rs12970134 (Chr 18:60217517 position) near *MC4R*, prominent in the literature; rs10146997 (Chr 14:79478819 position) in *NRXN3*, rs8192678 in (Chr 4:23814039 position) *PPARGC1A*, rs7561317 (Chr 2:644953 position) near *TMEM18* and rs10913469 (1:177944384 position) in *SEC16B*, poorly studied; rs545854 (Chr 8:10002570 position) in *MSRA* and rs987237 (Chr 6:50835337 position) in *TFAP2B*, associated in adult's populations [214] but never replicated in children; and rs9299 (Chr17:48592068 position) in *HOXB5* and rs9568856 (Chr 13:53490846 position) in *OLFM4*, recently associated with childhood obesity [114] but never replicated.

The genomic DNA was extracted from buccal cells using the PureLink® Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the instructions of the manufacturer.

Samples were genotyped for all SNPs by allelic discrimination assays using TaqMan® probes (Applied Biosystems, Foster City, CA, USA). All polymerase chain reactions (PCRs) were done in a total volume of 20 µl containing 1x SsoFast™ Probes Supermix (Bio-Rad, Hercules, CA, USA), 0.5 µl of specific TaqMan® SNP Genotyping Assays (20x) (Applied Biosystems, Foster City, CA, USA) and about 20 ng of genomic DNA, according to the manufacturer´s instructions. Thermal cycling conditions were 10 minutes at 95°C, and 35 cycles each of 95°C for 15 seconds and 60°C for 1 minute. The fluorescence was observed through a MiniOpticon real time PCR system (Bio-Rad, Hercules, CA, USA). To assess genotyping reproducibility, a selection of 10% random samples was re-genotyped for all SNPs with 100% concordance by the Single Strand Conformation Polymorphism (SSCP) method or sequencing by the Sanger's dideoxy chain termination reaction using Big-Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, USA) and the ABI 310 sequencer (Applied Biosystems, Foster City, USA).

3.4. Statistical analysis

The allelic and genotypic frequencies of all polymorphisms were estimated by direct counting. Hardy-Weinberg equilibrium probability values were achieved using an exact test [287]. Logistic regression under an additive genetic model, allowing for analysis of a binary outcome (a case–control *status*), was used to test obesity and overweight phenotype polymorphism associations, adjusted for

age and sex, by calculating odds ratios (OR) with 95% of confidence intervals (CI) and *p*-values. For *MC4R* rs17782313 and rs12970134 polymorphisms, linkage disequilibrium (r^2) values and a case/control (normal *vs.* obese) haplotype association were assessed. All these statistical analyses were done by using the set-based tests implemented on PLINK software v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [234]. Normality of the data was assessed using the Kolmogorov-Smirnov test. For each obesity-related quantitative parameters (BMI, BMI Z-score, weight and waist circumference), the nonparametric Kruskal-Wallis test was used to evaluate differences among the three genotypes in all the polymorphisms. This statistical analysis was performed using the SPSS software (for windows, version 18.0, SPSS inc., Chicago, IL, USA). A significant *p*-value was considered below 0.005 (0.05/10) by applying a Bonferroni correction for multiple testing, and a *p*-value between 0.005 and 0.05 has been considered as nominally significant.

QUANTO, v.1.1 power calculator (freely available http://hydra.usc.edu/gxe/) was used to estimate the power of association as a function of the frequency of the effect allele assuming an additive model [237].

4. Results

The anthropometric characteristics of the study subjects distributed by phenotype are shown in Table 6.1.

The genotyping success rate varied between 96.3% and 99.7%. The minor allele frequencies observed for all polymorphisms in the total sample were: 15% for rs7561317-A (*TMEM18*), 16% for rs10913469-C (*SEC16B*), 36% for rs8192678-A (*PPARGC1A*), 18% for rs987237-G (*TFAP2B*), 16% for rs545854-C (*MSRA*), 12% for rs9568856-A (*OLFM4*), 18% for rs10146997-G (*NRXN3*), 32% for rs9299-C (*HOXB5*), 21% for 17782313-C (*MC4R*) and 22% for rs12970134-A (*MC4R*) (Table 6.2). These frequencies are in accordance with those found in the HapMap CEU population (http://www.ensembl.org/). Genotype distributions among the control group were in agreement with Hardy-Weinberg equilibrium for all the studied polymorphisms ($p>0.05$).

Table 6.1. General characteristics of the Portuguese children participants.

	Total	Phenotype distribution*		
		Normal	Overweight	Obese
Subjects	730	256	320	154
Girls, %	50.7	55.9	45.3	53.2
Age, years	9.1 ± 1.7	8.6 ± 1.6	9.5 ± 1.6	9.0 ± 1.7
Height (cm)	136.2 ± 11.7	131.1 ± 11.1	139.5 ± 11.1	137.9 ± 10.6
Weight (kg)	37.2 ± 11.3	28.1 ± 6.6	40.2 ± 9.3	46.1 ± 11.0
BMI (kg/m^2)	19.6 ± 3.4	16.1 ± 1.5	20.3 ± 1.8	23.8 ± 2.5
BMI Z-score	0.93 ± 0.97	-0.15 ± 0.78	1.3 ± 0.23	1.99 ± 0.23
WC (cm)	67.2 ± 7.8	60.3 ± 4.5	68.9 ± 5.4	75.1 ± 6.6
HC (cm)	79.0 ± 10.3	70.4 ± 6.5	81.9 ± 8.0	87.1 ± 9.4
WHR	0.85 ± 0.06	0.86 ± 0.06	0.85 ± 0.06	0.87 ± 0.05

Data are presented as mean ± standard deviation.
*Phenotype distribution was determined using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF).
Abbreviations: BMI, body mass index; BMI Z-score, body mass index standard deviation score; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio.

We analyzed the obesity-related quantitative traits BMI, BMI Z-score, weight and waist circumference among different genotypes for each of the studied polymorphisms. The mean values for the anthropometric traits among the three different genotypes are detailed in Table 6.2. The *MC4R* rs12970134 major G-allele was found nominally associated with increase in BMI (p=0.035), BMI Z-score (p=0.043) and waist circumference (p=0.020), and borderline associated with weight (p=0.053). Near nominal associations were also found for the *PPARGC1A* rs8192678 minor A-allele with weight (p=0.061) and waist circumference (p=0.093), and for the *MSRA* rs545854 major G-allele with BMI (p=0.055) and BMI Z-score (p=0.056). After correction for multiple testing no statistically significant associations were found.

Genotype distributions of obesity-related parameters for the *MC4R* rs12970134 polymorphism, which showed the highest statistical significant associations with the obesity-related traits, are detailed in Figure 6.1. Homozygotes for the minor A-allele have the lowest value distributions for all the analyzed quantitative parameters (BMI, BMI Z-score, waist circumference and weight).

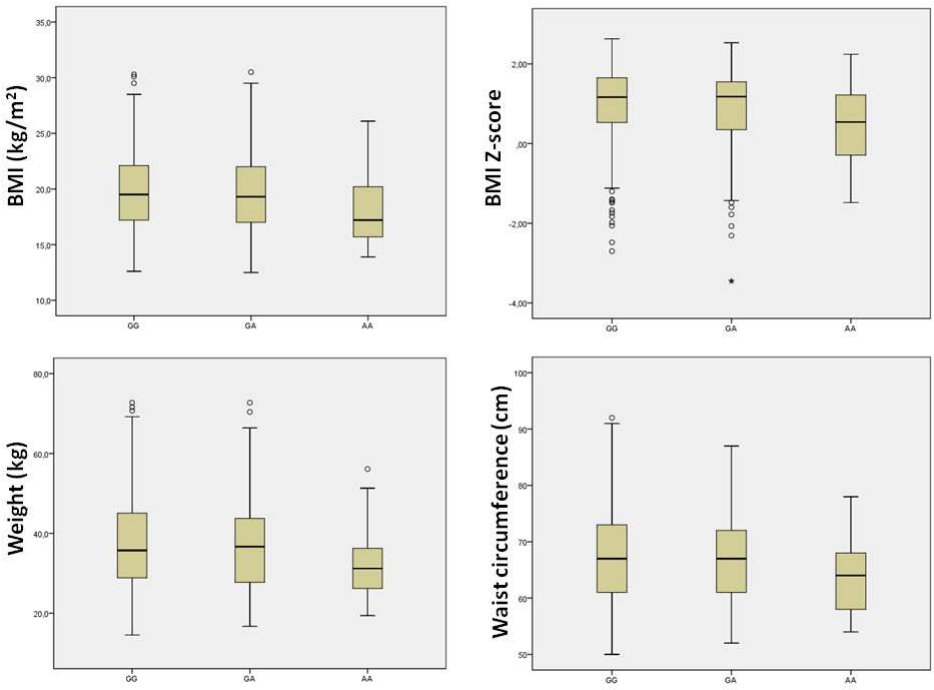

Figure 6.1. Box plot demonstrating the distribution of untransformed BMI, BMI Z-score, weight and waist circumference within each genotype group of *MC4R* rs12970134 polymorphism. Each box represents the anthropometric traits values between the 25th and 75th quartiles, and the dark line within the boxes indicates the median values.

Table 6.2. Minor allele frequencies and Hardy-Weinberg equilibrium test of the 10 studied polymorphisms in the sampled Portuguese children and their associations with obesity-related quantitative traits.

Polymorphism	Chr.	Gene	Alleles*	*n*	MAF	HWE	No. 11/12/22	Genotype (mean ± SD)			*p*-value
								11	12	22	
rs10913469	1q25	*SEC16B*	C:T	728	0.16	0.479	20/186/522				
BMI (kg/m^2)								19.6 ± 3.6	19.5 ± 3.5	19.6 ± 3.4	0.930
BMI Z-score								0.99 ± 0.9	0.92 ± 1.0	0.93 ± 1.0	0.902
Weight (kg)								37.5 ± 13.4	36.9 ± 11.9	37.0 ± 11.1	0.736
Waist C (cm)								67.9 ± 8.6	66.9 ± 8.3	67.3 ± 7.5	0.709
rs7561317	2p25	*TMEM18*	A:G	721	0.15	0.886	16/191/514				
BMI (kg/m^2)								19.2 ± 3.6	19.2 ± 3.3	19.7 ± 3.5	0.141
BMI Z-score								0.81 ± 1.1	0.84 ± 0.9	0.97 ± 1.0	0.120
Weight (kg)								36.7 ± 13.1	36.3 ± 11.4	37.6 ± 11.2	0.306
Waist C (cm)								66.1 ± 7.7	66.3 ± 7.5	67.6 ± 7.8	0.121
rs8192678	4p15	*PPARGC1A*	A:G	703	0.36	1.000	89/323/291				
BMI (kg/m^2)								20.0 ± 3.6	19.8 ± 3.4	19.4 ± 3.4	0.198
BMI Z-score								0.99 ± 1.0	0.97 ± 0.9	0.89 ± 1.0	0.359
Weight (kg)								38.9 ± 12.3	38.0 ± 11.3	36.1 ± 10.9	0.061
Waist C (cm)								68.9 ± 8.7	67.5 ± 7.6	66.6 ± 7.5	0.093
rs987237	6p12	*TFAP2B*	G:A	725	0.18	0.615	21/218/486				
BMI (kg/m^2)								19.0 ± 2.9	19.3 ± 3.2	19.7 ± 3.5	0.392
BMI Z-score								0.87 ± 1.0	0.89 ± 1.0	0.94 ± 1.0	0.536
Weight (kg)								34.1 ± 9.4	36.8 ± 11.2	37.5 ± 11.5	0.353
Waist C (cm)								64.9 ± 7.8	66.6 ± 7.3	67.6 ± 7.9	0.130
rs545854	8p23	*MSRA*	C:G	717	0.16	0.334	22/187/508				
BMI (kg/m^2)								18.3 ± 3.7	19.8 ± 3.6	19.7 ± 3.4	0.055
BMI Z-score								0.51 ± 1,1	0.96 ± 1.1	0.95 ± 1.0	0.056
Weight (kg)								34.3 ± 10.6	38.0 ± 11.7	37.1 ± 11.4	0.240
Waist C (cm)								65.1 ± 6.9	67.5 ± 8.3	67.3 ± 7.5	0.282
rs9568856	13q14	*OLFM4*	A:G	725	0.12	0.059	17/141/567				

BMI (kg/m^2)								19.2 ± 3.6	19.9 ± 3.4	19.5 ± 3.4	0.374
BMI Z-score								0.92 ± 0.8	0.97 ± 0.9	0.92 ± 1.0	0.719
Weight (kg)								35.6 ± 12.2	38.1 ± 11.1	37.0 ± 11.4	0.286
Waist C (cm)								65.7 ± 8.4	68.0 ± 7.5	67.1 ± 7.8	0.248
rs10146997	14q31	*NRXN3*	G:A	716	0.18	0.798	21/212/483				
BMI (kg/m^2)								19.1 ± 3.5	19.6 ± 3.3	19.7 ± 3.5	0.672
BMI Z-score								0.91 ± 1.0	0.92 ± 1.0	0.95 ± 1.0	0.579
Weight (kg)								34.3 ± 11.9	37.5 ± 11.3	37.4 ± 11.3	0.436
Waist C (cm)								64.6 ± 7.6	67.3 ± 7.7	67.5 ± 7.8	0.260
rs9299	17q21	*HOXB5*	C:T	727	0.32	0.672	78/313/336				
BMI (kg/m^2)								19.5 ± 3.4	19.7 ± 3.6	19.5 ± 3.3	0.710
BMI Z-score								0.87 ± 1.0	0.94 ± 1.0	0.92 ± 0.9	0.751
Weight (kg)								37.3 ± 11.6	37.5 ± 11.3	37.0 ± 11.4	0.792
Waist C (cm)								66.9 ± 7.0	67.4 ± 7.9	67.2 ± 7.8	0.896
rs12970134	18q21	*MC4R*	A:G	729	0.22	0.163	29/266/434				
BMI (kg/m^2)								18.1 ± 3.3	19.5 ± 3.5	19.7 ± 3.9	***0.035***
BMI Z-score								0.53 ± 1.0	0.89 ± 1.0	0.98 ± 0.9	***0.043***
Weight (kg)								32.5 ± 9.1	37.1 ± 11.3	37.7 ± 11.5	0.053
Waist C (cm)								63.6 ± 5.8	67.1 ± 7.6	67.6 ± 7.9	***0.020***
rs17782313	18q22	*MC4R*	C:T	716	0.21	0.432	28/247/441				
BMI (kg/m^2)								18.6 ± 3.6	19.7 ± 3.4	19.6 ± 3.5	0.269
BMI Z-score								0.61 ± 1.1	0.95 ± 1.0	0.94 ± 1.0	0.257
Weight (kg)								34.4 ± 10.6	37.7 ± 11.0	37.2 ± 11.5	0.260
Waist C (cm)								65.6 ± 7.5	67.4 ± 7.3	67.3 ± 8.0	0.430

Abbreviations: Chr., chromosome; *n*, number of genotyped children; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; BMI, body mass Index; BMI Z-score, body mass index standard deviation; WC, waist circumference.
*Alleles: Minor (1): Major (2).
p-values were obtained using the Kruskal-Wallis test.
p-value nominally significant ($p<0.05$) are in bold and italic.

Logistic regression analysis, in an additive model, revealed that *MC4R* rs12970134 major G-allele was nominally associated with the obesity risk (OR = 1.477; *p*=0.029) and that *TFAP2B* rs987237 major A-allele and *TMEM18* rs7561317 major G-allele are near nominally associated with the risk of obesity (OR = 1.455; *p*=0.056; and OR = 1.416; *p*=0.092, respectively) (Table 6.3). Only the *PPARGC1A* rs8192678 polymorphism was found nominally associated with the overweight phenotype (OR = 1.297; *p*=0.041) (Table 6.3).

We further investigated the difference in the genotype distribution between cases and controls for abdominal obesity by logistic regression analysis. In the total of 730 children, 111 revealed abdominal obesity (using sex and age-specific ≥90th waist circumference percentile). From the ten polymorphisms studied, only *TMEM18* rs7561317 (major G allele), showed nominal association with increased abdominal obesity (OR = 1.589; 95% CI, 1.02-2.50; *p*=0.042).

The haplotype analysis for the two *MC4R* rs17782313 and rs12970134 polymorphisms, located at a distance of 33.5 Kb and in high linkage disequilibrium (r^2 = 0.74), revealed that the common TG haplotype was associated with the risk of obesity (*p*=0.043) (with frequency of 81.2% in the obese group *versus* 75.0% in the control group).

Table 6.3. Allele frequencies of the 10 studied polymorphisms in the Portuguese children, and their associations with risk of obesity among phenotypic groups.

SNP ID/ Gene	Allele		Obese *vs.* normal			Overweight *vs.* normal		
		Normal*	Obese*	OR CI 95%	*p*	Overweight*	OR CI 95%	*p*
rs10913469 *SEC16B* T>C	T C	0.855 0.145	0.847 0.153	1.058 (0.71-1.57)	0.780	0.825 0.175	1.154 (0.83.1.59)	0.387
rs7561317 *TMEM18* G>A	G A	0.825 0.175	0.869 0.131	0.706 (0.47-1.06)	0.092	0.850 0.150	0.829 (0.60-1.13)	0.248
rs8192678 *PPARGC1A* G>A	G A	0.678 0.322	0.642 0.358	1.176 (0.86-1.59)	0.293	0.619 0.381	1.297 (1.00-1.66)	***0.041***
rs987237 *TFAP2B* A>G	A G	0.804 0.196	0.856 0.144	0.687 (0.46-1.01)	0.056	0.817 0.183	0.915 (0.67-1.23)	0.559
rs545854 *MSRA* G>C	G C	0.820 0.180	0.836 0.164	0.960 (0.65-1.41)	0.838	0.847 0.153	0.878 (0.63-1.20)	0.425
rs9568856 *OLFM4* G>A	G A	0.882 0.118	0.866 0.134	1.156 (0.75-1.77)	0.504	0.884 0.116	0.985 (0.68-1.41)	0.935
rs10146997 *NRXN3* A>G	A G	0.818 0.182	0.860 0.140	0.731 (0.49-1.08)	0.119	0.808 0.192	1.07 (0.79-1.45)	0.645
rs9299 *HOXB5* T>C	T C	0.679 0.321	0.654 0.346	1.122 (0.83-1.51)	0.453	0.687 0.312	0.962 (0.74-1.23)	0.762

rs12970134 *MC4R* G>A	G A	0.749 0.251	0.815 0.185	0.677 (0.47-0.96)	***0.029***	0.782 0.218	0.829 (0.63-1.09)	0.183
rs17782313 *MC4R* T>C	T C	0.781 0.212	0.817 0.183	0.799 (0.55-1.14)	0.221	0.780 0.220	1.005 (0.75-1.33)	0.972

Abbreviations: SNP ID; single nucleotide polymorphism identification; OR, odds ratio; CI, confidence interval.
*Phenotypic groups was determined using age and sex specific BMI cut-offs provided by the International Obesity Task Force (IOTF).
Logistic regression was used to compare genotype distribution. *p*-values (asymptotic *p*-value for t-statistic) shown are for an additive model and are adjusted for age and sex. OR is shown for the minor allele.
p-value significant ($p<0.05$) are in bold and italic.

5. Discussion

Understanding the genetic basis of obesity in children could be used as a first step to develop possible preventive measures. Recent GWAS have identified many (more than 50) different genetic variants conferring susceptibility to obesity [57]. However, the modest association with the obesity risk observed for most variants implies that replication studies in different populations are required to detect and confirm such signals of association, eliminating false positives that may arise by chance or systematic bias.

Focused on ten polymorphisms across the genome (located in or near the *MSRA*, *TFAP2B*, *NRXN3*, *PPARGC1A*, *TMEM18*, *SEC16B*, *HOXB5*, *OLFM4* and *MC4R* genes), previously associated by GWAS with obesity-related outcomes in populations of European origin, we conducted a genetic association study to investigate their role in the susceptibility of obesity in a sample of Portuguese children. Using obesity-related quantitative traits to assess whether the genotypes predicts the trait value, we identified the *MC4R* rs12970134 *loci* nominally associated with several obesity-related traits, and two *loci* (*PPARGC1A* and *MSRA*) near nominally associated with at least one anthropometric parameter (Table 6.2). In addition, using logistic regression analyses, the *MC4R* rs12970134 polymorphism was found nominally associated (p=0.029) with the risk of obesity and the *TFAP2B* rs987237 polymorphism showed borderline significant association (p=0.056) with the obese phenotype. Thus, this study highlights these four polymorphisms as potential genetic markers of the obesity phenotype in this Portuguese children sample.

The *MC4R* gene is known to be the most common cause of monogenic obesity in extreme childhood obesity [63], but also its flanking genomic region has been the third strongest implicated in polygenic obesity. The expression of *MC4R* is restricted to the hypothalamus involved in food intake regulation [43]. Until now, several variations of this gene were established with BMI and/or waist circumference, showing an independent role in body variation. Polymorphisms rs12970134 and rs17782313, located 154 kb and 188 kb, respectively, downstream of the *MC4R* gene, were found associated with obesity and obesity-related traits in several studies in Asian and European populations, both in children and adults [95, 96, 128, 288, 289]. In the present study, nominal significant associations were found between *MC4R* rs12970134 and BMI, BMI Z-score and waist circumference (p<0.05), as also with the risk of obesity (p=0.029). However, our findings do not

replicate previous reports that show the minor A-allele associated with increased risk in BMI and waist circumference in children of European [95, 96, 128, 288, 290] or Asian [289] descent. Instead, in the present study, Portuguese children showed the minor rs12970134-A allele significantly associated with lower BMI (p=0.035), BMI Z-score (p=0.043), waist circumference (p=0.020), and also with a lower risk for the obesity phenotype (OR = 0.677; p=0.029). For this polymorphism, the minor A-allele frequency was 18.5% in the obese group *versus* 25.1% in the control group. For the second *MC4R* polymorphism, rs17782313, previous studies showed the C-allele associated with childhood obesity (increasing BMI in ±0.22kg/m^2) [95], however, in the Portuguese children, no statistical association was found with any obesity-related trait. The haplotype analysis showed the *MC4R* rs17782313 / rs12970134 TG haplotype associated with the risk of obesity, confirming the potential role of rs12970134 major G-allele in the etiology of obesity in our sample of Portuguese children.

A GWA scan meta-analysis conducted by Lindgren *et al*. [78] found that the G allele of the *MSRA* rs545854 polymorphism was associated with waist circumference (p=8.9x10^{-9}) in adults. Bille *et al*. [214] also found significant association between this polymorphism and waist circumference (OR = 1.08; p=0.02). In our study, nominal borderline significant associations with BMI (p=0.055) and BMI Z-score (p=0.056) were observed. The biological function between *MSRA locus* and adiposity remain unclear [78].

PPARGC1A is a transcriptional co-activator that has been implicated in the regulation of genes involved in energy metabolism [291]. The Gly482Ser missense mutation (rs8192678), predicted by a G-to-A transition at position +1,564 in exon 8 of the *PPARGC1A* gene, was found associated with obesity indices in middle-aged women of a cross-sectional Austrian population [291] and with abdominal obesity in Chinese adults [292]. In our study, near nominal associations were obtained with weight (p=0.061) and waist circumference (p=0.093) for the 482Ser variant.

The molecular function of the *TMEM18* gene product is to bind DNA to suppress transcription; it could be differently expressed in the hypothalamus and is possibly involved in the regulation of feeding [293]. Polymorphism rs7561317, located about 22kb downstream of *TMEM18*, is the second best associated *locus* with BMI after *FTO* gene [63]. A GWA study conducted by Thorleifsson *et al*. [97] found that the rs7561317 GG genotype increases BMI in ±0.70kg/m^2 and is associated with obesity. In the present study, significant associations were not found between any obesity-related trait and the *TMEM18* rs7561317 polymorphism, however, our findings are directionally consistent

with previous studies conducted in children and adolescents, as the rs7561317 major G-allele was found marginally associated with the risk of obesity (OR = 0.706; *p*=0.092).

The *TFAP2B* gene is suggested to be involved in global adipocyte response to positive energy balance [78]. The minor G-allele of rs987237 polymorphism was previously found associated with increased waist circumference ($p=1.9x10^{-11}$) and BMI ($p=7.0x10^{-12}$) in a meta-analysis of 16 GWAS within adults of European ancestry [78]; and also in children it was found associated with increased waist circumference ($p=3.5x10^{-2}$) and BMI (*p*=0.06) [290]. Our data in Portuguese children do not replicate previous findings while we observed a nominal borderline significant association of the major rs987237 A-allele with the risk of obesity (OR = 0.692; *p*=0.056).

In the present study, the major *MC4R* rs12970134-G and *TFAP2B* rs987237-A alleles showed, respectively, nominal and near-nominal significant associations with the risk of obesity, but the direction of effect was reverse when comparing to that in the original reports. Despite opposite direction on the effect of a risk allele is highly unlikely, this was observed in several studies including between different populations [294] but also inside a same population [295]. For the *TFAP2B* rs987237 polymorphism, the original significant association was found in adults [78], and to the authors knowledge, this study with Portuguese participants is the first replication report involving children. Therefore, the opposite direction of association for rs987237-A risk allele could be due to differences between children and adults regarding natural physiological differences.

All genes studied in this work are considered as candidates for the risk of developing obesity. Most of them are involved in homeostasis and energy metabolism, nevertheless the casual effect of these polymorphisms in the pathogenesis of obesity remains unclear. In the present study with Portuguese children, only the *MC4R* rs12970134 polymorphism showed nominal significant association ($p<0.05$) with obesity and most of obesity-related traits. In a previous study for the *FTO* gene using the same cohort of individuals [285], significant associations were also found between the rs9939609 minor A-allele and increased risk for several anthropometric traits, including weight (*p*=0.019), BMI (*p*=0.018), BMI Z-score (*p*=0.011) and waist circumference (*p*=0.016), in concordance with reports worldwide. Thus, both *loci FTO* and *MC4R* appear to play a key role in the obesity phenotype in Portuguese children. Most of the analyzed polymorphisms in this present study showed no nominal effects in obesity-related traits, but this difference with the previous findings may be due partly to the sample size, which may have been insufficient to replicate the original findings. The

estimated power of association observed ranges from 6% to 72%, but at least for *MC4R* rs12970134 polymorphism, the variant most associated to obesity in our sample, the obtained power (72%) is close to values (≥80%) commonly considered sufficient to detect genetic variant interaction effects.

Among the 10 *loci* reported in this study, polymorphisms in or near *MC4R*, *PPARGC1A*, *MSRA* and *TFAP2B* genes could be assumed to play a role in the risk of obesity in this population sample of Portuguese children. While we could not replicate the original findings concerning the direction effect of the *MC4R* rs12970134 and *TFAP2B* rs987237 risk alleles our results deserve confirmatory studies in other populations. Moreover, our data may show that the polymorphisms provided here could play a modest role in the obesity etiology in children, at least when comparing with the *FTO* gene, suggesting the existence of others unknown *loci* involved in the obesity susceptibility. Our replication study could also have public health significance while genes playing an essential role in energy homeostasis, such as *PPARGC1A* or *TFAP2B*, suggested by our data as obesity-related genes, may be used as targets for obesity treatment. Further investigations in the near future regarding genetic associations and functional roles of these polymorphisms should be helpful to confirm its implication in the development of obesity and if they could be attractive targets for therapeutic agents.

Chapter VII

Molecular screening for melanocortin-4 receptor mutations in Portuguese severely obese children.

This chapter was partially based on the following original paper: Albuquerque D, Estévez MN, Víbora PB, Giralt PS, Balsera AM, Cortés PG, López MJ, Luego LM, Gervasini G, Hernández SB, Arroyo-Díez J, Vacas MA, Nóbrega C, Manco L, Rodríguez-López R. **Novel variants in the *MC4R* and *LEPR* genes among severely obese children from the Iberian population**. *Ann Hum Genet* 2014, 78(3): 195-207.

1. Abstract

Background: The melanocortin-4 receptor (*MC4R*) gene is the most common cause of monogenic obesity and could be a first step to unravel genetic causes of obesity. The aim of this study was to screen for *MC4R* gene mutations in a sample of Portuguese children with severe obesity.

Methods: A total of 32 severely obese children from Portugal, with a BMI ≥ 99th (ranging 6-10 years-old), were studied. The *MC4R* gene promoter and the entire coding region were analyzed by direct bidirectional sequencing.

Results: Two *MC4R* gene mutations were found at heterozygous state: the previously described 5'UTR single nucleotide polymorphism -178A>C (rs34114122), identified in a girl with a BMI Z-score= 2.51; and the common missense mutation 307G>A (Val103Ile) (rs2229616) in the MC4R gene coding region, identified in a boy with a BMI Z-score= 2.60. The carrier frequency of the Val103Ile mutation in the study sample was (3.1%), similar to other studies in European populations (ranging 1 to 5%). No other pathogenic *MC4R* gene mutations were detected in our study sample.

Conclusion: These results suggest that mutations in the *MC4R* gene might not be a common cause of severe obesity in Portuguese children.

2. Introduction

The melanocortin-4 receptor (*MC4R*) gene (OMIM 155541), located in the chromosome 18q21.3, is composed by a single exon encoding for the 332-amino acid seven-transmembrane G-protein-linked receptor, critically involved in regulating energy balance [42]. The *MC4R* gene is expressed mainly in the central nervous system, including the hypothalamus, and has been implicated in the regulation of food intake and energy expenditure [42, 296].

Deficiency in the *MC4R* activity associated with *MC4R* gene mutations is the most common cause of monogenic obesity, representing about 6% of severe human obesity [47]. In 1998, two independent groups report for the first time a mutation in *MC4R* gene, which abolishes the receptor function, associated with severe obesity [44, 45]. Until now, more than 150 mutations of this gene were described decreasing *MC4R* activity associated with human obesity, and usually classified into different classes depending of their molecular effects [41].

Most of study screenings of *MC4R* gene were focused in the coding sequence. Nevertheless, a few studies included the *MC4R* promoter region, which could be helpful to provide further insight in the identification of transcriptional mutations affecting *MC4R* gene expression [297–299].

The main goal of this study was to screen for mutations in the promoter and entire coding regions of *MC4R* gene in a sample of severely obese Portuguese children to determine possible genetic causes.

3. Material and Methods

3.1. Study subjects

In a previous study, 1433 Portuguese children were randomly selected from public schools, and classified using age and sex specific body mass index (BMI) cut-offs provided by the International Obesity Task Force (IOTF) [258]. A total of 32 unrelated children, with a severely obese phenotype, which pass the 99 percentile, were identified and selected to screen for mutations at the *MC4R* promoter and entire coding region.

The study protocol was approved by *Direção-Geral de Inovação e de Desenvolvimento Curricular,* the ethical Committee of the Portuguese Ministry of Education (available at http://mime.gepe.min-edu.pt/ with the identification number process: 0151100001), and was conducted in accordance with the institutional guidelines of the University of Coimbra. Written informed consent was previously obtained from the children´s parents.

3.2. Anthropometric Measurements

Height (cm) and weight (kg) were taken with participants dressed in lightweight clothing without shoes. Waist circumference (cm) was measured midway between the lowest rib and the iliac crest, to the nearest 0.1 cm after inhalation and exhalation. Hip circumference (cm) was measured at the point over the buttocks yielding the maximum circumference. The BMI was calculated with the weight in kilograms divided by the square of height in meters (kg/m^2).

3.3. Direct sequencing of the MC4R gene

A buccal swab sample was collected from each sampled child for genetic studies. The genomic DNA was extracted from buccal swabs using the PureLink® Pro 96 Genomic DNA Kit (Invitrogen Corporation, Carlsbad, CA, USA), according to the instructions of the manufacturer.

DNA was amplified by polymerase chain reaction (PCR), using primers and conditions previously published for the *MC4R* coding sequence [231], and primers 5'-CGCCTACAGCCCCTAACACT-3' (forward) and 5-CCTCCTGGGTCAGGGAGT-3' (reverse), for the promoter region. This primer pair was designed with the Primer3 software, v0.4.0 [228] (freely available in http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Bidirectional sequence analysis of genomic DNA was performed in 32 unrelated children. Sequencing was performed on an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using BigDye® Terminator v3.1 Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA), according to protocols recommended by the manufacturer. Base calling was performed with Sequencing Analysis v5.2 (Applied Biosystems, Foster City, CA, USA).

The sequences were analyzed with the Staden package software [232] and the SeqScape software v2.5 (Applied Biosystems, Foster City, CA, USA). The obtained sequences were compared with the standard *MC4R* gene sequence (ENSG00000166603).

3.4. Bioinformatics analysis

For potential transcription factor binding site analysis the TFSearch software (http://www.cbrc.jp/research/db/TFSEARCH.html), was used based on the TRANSFAC database [300]. DNA sequences encompassing approximately 20bp upstream and downstream the polymorphic site were provided as input sequence data. Taxonomy matrix was entered as "vertebrate" and the threshold score was set at 85.0 for the analysis. For the missense variant, PolyPhen2 (Polymorphism Phenotyping v2) software, available online at (http://genetics.bwh.harvard.edu/pph2/) was used to assess the phenotypic effect [301]. The amino acid substitution is predicted damaging when the score is >0.95.

4. Results

A total of 32 severely obese (BMI≥99th) children from Portuguese origin were screened for mutations in the *MC4R* gene, including the promoter region. The characteristics of the sampled children are summarized in Table 7.1. Genomic DNA sequencing revealed two *MC4R* gene variations in two different subjects: the nucleotide change -178A>C (rs34114122) in the promoter region, found at heterozygous state in a 8 years-old girl with a BMI Z-score= 2.51 (Height: 128.4 cm; weight: 48.6 kg); and the missense mutation c.307G>A (Val103Ile) in the coding region, found at the heterozygous state in a 6 years-old boy with a BMI Z-score= 2.60 (Height: 134.4 cm; weight: 42.7 kg). The Val103Ile (rs2229616) was previously described as a common variant, and the obtained frequency for heterozygous subjects in our sample was 3.1% (1/32), similar to previous studies from other European countries (Table 7.2).

Table 7.1. Characteristics of the sampled Portuguese children screened for *MC4R* mutations.

Characteristics	Children
N	32
Girls/Boys	11 / 21
Age, years	8.4 ± 1.6
Height, cm	135.2 ± 9.9
Weight, kg	47.3 ± 11.2
BMI, kg/m^2	25.5 ± 2.6
BMI Z-score*	2.3 ± 0.2
Waist circumference, cm	77.8 ± 5.8
Hip circumference, cm	89.0 ± 8.8
WHR	0.87 ± 0.0

Data are presented as mean ± standard deviation.
Abbreviations: *n,* number of subjects; BMI, body mass index; BMI Z-score, body mass index standard deviation score; WHR, waist-to-hip ratio.
*BMI Z-score was determined using age and gender specific BMI cut-offs provided by the International Obesity Task Force (IOTF).

Bioinformatics analysis was used to predict the possible effect of the two identified variants in molecular pathway. For the promoter nucleotide change -178A>C, the TFSearch software used to screen for putative transcription factor binding sites, indicated an AML-1a binding site with 88.7 score at the (ACCT***C***A) sequence when the minor C allele is present. Regarding the Val103Ile missense variant, analysis with the PolyPhen2 software indicates a low score (0.021) predicting non pathogenicity for this variant.

Table 7.2. Frequencies of obese individuals carrying the common Val103Ile missense mutation at heterozygous state among several populations.

Population	Cohort characteristics	*n* (carrier/total)	Frequency*	Reference
Sweden	Unrelated probands	13/284	4.6%	[302]
UK	Extremely obese adults	8/190	4.2%	[303]
Malaysia	Obese adults	5/118	4.2%	[304]
Sweden	Extremely obese adults	9/217	4.1%	[305]
France	Morbidly obese adults and adolescents	8/209	3.8%	[231]
European/USA	Extremely/obese adults	5/140	3.6%	[306]
Serbia	Obese adults	2/62	3.2%	[307]
Portugal	***Severely obese children***	***1/32***	***3.1%***	[308]
France	Morbid Obese adults	23/875	2.6%	[309]
UK	HERTS cohort	28/1089	2.6%	[299]
Denmark	Men with juvenile-onset obesity	18/750	2.4%	[310]
France	Obese children	18/746	2.4%	[309]
Switzerland	Severely obese adults	11/469	2.3%	[311]
Belgium	Morbidly obese adults and obese children/adolescents	5/218	2.3%	[47]
Germany	Obese children	6/291	2.1%	[312]
Japan	Extremely obese adults	1/50	2.0%	[313]
UK	Children with severe early-onset obesity	10/500	2.0%	[314]
France	Severely obese children	1/63	1.6%	[315]
Germany	Extremely obese children/ adolescents	13/808	1.6%	[316]
Italy	Obese children/adolescents	4/240	1.6%	[317]
Germany	Overweight and obese children/adolescents	8/510	1.6%	[318]
Germany	Extremely obese children/adolescents	4/306	1.3%	[319]
UK	Probands with early-onset obesity	3/284	1.2%	[314]
Italy	Obese children and adults	4/120	0.8%	[320]
Finland	Children with early-onset obesity and adults obese	2/308	0.7%	[321]
USA/France	Severely obese adults and children	3/431	0.7%	[297]
Spain	Obese adults	1/159	0.6%	[322]
Italy	Obese children/adolescents	1/208	0.5%	[323]
USA	Severely obese adults	0/47	0%	[305]

*Frequency of individuals with Val103Ile mutation / total number screened (%).

5. Discussion

To our knowledge, this is the first study screening for mutations in the *MC4R* gene in a population sample of severely obese Portuguese subjects. The melanocortin-4 receptor deficiency is widely accepted as the most prevalent form of monogenic obesity [41]. Hence, in children whit early-onset obesity, screening for mutations in the *MC4R* gene could be a first step to determine genetic causes of obesity. We screened the promoter 5' untranslated region (5'UTR) and coding region of *MC4R* gene to investigate possible genetic causes in severely obese Portuguese children.

Only one *MC4R* coding region sequence variation was found at the heterozygous state in a Portuguese obese subject: a G to A nucleotide transition at position 307 resulting in the amino-acid substitution valine (Val) to isoleucine (Ile) at codon 103 (Val103Ile). This missense mutation was first described in two obese British adults at the heterozygous state [303]. In our study, the frequency of the rare allele (103Ile) was 1.56% (1/64) and is in line with those reported in previous studies (Table 7.2). No other mutations were found in the *MC4R* coding region of our study sample.

Across world populations, carrier frequencies for the 103Ile in obese individuals range from 0% (Black women, USA) to 4.6% (Sweden) (sample size between 40 to 1100 subjects; see Table 7.2). Our study sample has a lower population size comparing with previous studies; nevertheless the frequency obtained for the Val103Ile carrier individuals is consistent with the findings in other European countries. In Southern Europe, carrier frequencies ranged between 0.6% (Spain) to 1.6% (Italy), and in Northern European countries this frequency is higher ranging from 1.6% to 4.6% (France, Belgium, Germany, UK and Sweden).

Regarding this polymorphism there are inconsistent findings and ambiguous association with obesity in population studies making the functional effect unresolved. Gotoba *et al*. [303] presumed that this mutation was not related to obesity. Moreover, Rosmond *et al*. [302] has explicitly reported that individuals with the 103Ile allele have lower BMI, lower waist-to-hip ratio, and lower abdominal obesity. Geller *et al*. [324], in a sample comprising extremely obese children or adolescents and both

parents revealed a reduced transmission of the Ile103 allele to obese offspring: carrier individuals of the Ile103 allele have an approximately reduction of 1.6 kg of body weight in a 1.8-m-tall individual. A Genome-wide association study (GWAS) conducted by Meyre *et al*. [265] in a sample on early-onset extreme obesity adults, found a negative association of the 103Ile polymorphism with obesity. Moreover, several case-control studies found similar 103Ile frequencies between extreme obese and non-obese individuals, consistent with previous studies showing no association with obesity [46, 231, 304, 306, 314, 319, 325]. The low score obtained using the PolyPhen2 software (0.021) corroborated a benign effect for this amino acid substitution.

A second nucleotide substitution, the -178A>C transversion, in the 5'UTR region of *MC4R* gene, located 178 nucleotides upstream of the initiation methionine codon, was identified at heterozygous state in one severely obese subject. This polymorphism was first described by Jacobson *et al*. [305] in Swedish obese subjects. This single nucletotide change (rs34114122) was described with a minor allele frequency of 1% (C-allele) in the general European population and 4% in Iberian populations (ENST00000299766). A study conducted by Alharbi *et al.* [299] found a rs34114122-C allele frequency of 0.01% in a UK population and they don't found any effect on BMI. Another study conducted by Cole *et al*. [326] found this polymorphism at a minor allele frequency of 0.018% in the Hispanic population (USA) and concluded that rs34114122 likely has a functional effect on the appetite hormone ghrelin. Bioinformatics analysis indicates that -178A>C substitution creates one putative transcription factor binding site for AML-1a which might suggest functional significance. Thus, the possibility that this nucleotide change in the promoter region of *MC4R* could be implicated on the etiology of obesity cannot be discarded.

This study is the first to screen for *MC4R* gene mutations in a sample of Portuguese children with severe obesity. Only two severely obese subjects shown sequence variations in a heterozygous state: the –178A>C substitution, in the *MC4R* promoter region, and the missense mutation Val103Ile, in the *MC4R* coding region. Further investigation about the obesity association and the functional

function of these two polymorphisms is needed to confirm its implication in the role of obesity. We conclude that *MC4R* gene could not be assumed as an important genetic cause of severe obesity in the Portuguese population.

Chapter VIII

Conclusions and future perspectives

1. Conclusions

General conclusions from results presented in this study are summarized below.

- In 2011, the prevalence of overweight and obesity among 6-12 years-old children from central region of Portugal was 33.0%, from which 10.7% were obese.

  These values represent an increase in the prevalence of overweight/obesity in Portuguese children in the last years:

  - Comparing these results with a previous study using data from 2002, we found a slightly higher prevalence in overweight/obesity (~1.5%), however, the prevalence of obesity decrease (~0.6%).
  - Prevention and campaigns awareness seems to be insufficient, and Portugal remains on the list of European countries with higher prevalence in children obesity.
- The rs9939609 and rs1421085 polymorphisms located in the *FTO* gene in high LD (r^2=0.82) were found associated with the risk of obesity (*p*=0.02 and *p*=0.01, respectively), also with obesity-related traits, weight, BMI, BMI Z-score and waist circumference (*p*<0.05 for both and all traits) in the Portuguese children. Concerning the rs1861868 polymorphism we found association with BMI, weight and waist circumference (*p*<0.05 for all traits), however, no association was found with risk of obesity (*p*=0.31).
  - When performing a Haplotype analysis (rs1861868-rs1421085-rs9939609), two combinations (ACA and GCA) were found associated with a higher risk of obesity (*p*=0.02 and *p*=0.03, respectively).
- The -13910C>T polymorphism, located ~14 kb upstream of the *LCT* gene and associated with lactase persistence in European populations, may predispose to abdominal obesity however the association with BMI was not confirmed in our sample.
- The *MC4R* rs12970134 polymorphism was found associated with BMI, BMI Z-score and waist circumference, and nearly associated with weight (*p*=0.05). An association with the risk of obesity was also found (*p*=0.02).
- Near nominal association was found between the *PPARGC1A* rs8192678 polymorphism and weight (*p*=0.06).

- The *MSRA* rs545854 polymorphism was also nearly associated with BMI (p=0.055) and BMI Z-score (p=0.05).
- The *TFAP2B* rs987237 polymorphism was found nearly associated (p=0.05) with the obese phenotype.
- Regarding the association of *MC4R* rs12970134 and *TFAP2B* rs987237 polymorphisms the signal detected with obesity showed an opposite direction of effect to that previously found in other populations. This result was uncommon, but similar findings were found in several studies regarding other conditions.
- In our sample, 32 children were classified with morbid obesity (BMI ≥9^{9th}). We detected no mutations in the *MC4R* gene associated with this phenotype. However, we found a heterozygous individual with the Val103Ile common polymorphism, ranging similar frequency to that obtained in other European countries. We also found an heterozygous individual with the promoter substitution -178A>C previously described as a non-pathogenic variant.

Overall, the results gathered in the present study demonstrate the possible association of several genes with common obesity in a sample of Portuguese children. Our results also show that allele and genotype frequencies obtained were generally similar to those obtained in other European populations.

This study is a significant contribution to the knowledge of the genetic susceptibility of obesity in Portuguese children, but could also to help in future meta-analysis studies clarifying which variants are truly associated with the predisposition to develop an obese phenotype. This study also helps to better understand the genetic diversity that could be associated with obesity in the Portuguese population and compare it with other populations. Further studies are needed with larger samples to elucidate the real association or not of polymorphisms found nearly associated with obesity. The polygenic obesity remains complex because it is determined by the interaction of multiple additional factors. Despite eight years of investigation in genetic association studies since the discovery of the *FTO* rs9939609 polymorphism, our knowledge about genetic predisposition to polygenic obesity remains unsolved. Today, several other mechanisms were identified as playing a role in obesity

predisposition. Epigenetics, nutrigenetics, microbiota, etc. are new promising research branches which may explain parts of the missing heritability.

Chapter IX

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