Doctoral Thesis/Tesis Doctoral

Genetic polymorphisms associated to obesity and cardiovascular disease risk factors in European adolescents

Polimorfismos genéticos asociados a obesidad y factores de riesgo cardiovascular en adolescentes europeos

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Jose Miguel Pascual Gamarra
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A mis padres
por su apoyo incondicional,
por su comprensión,
por su sacrificio,
por enseñarme tanto
y por ser las mejores personas que he conocido.

Todos tenemos dos vidas.
Y la segunda empieza cuando nos damos cuenta
de que la primera se acaba.

Confucio

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ABSTRACT

ABSTRACT

Obesity and related cardiovascular diseases (CVDs) are the main cause of premature death and chronic disability worldwide. CVD events occur most frequently during or after the fifth decade of life, however, there is evidence indicating that the precursors of CVD have its origin in the first decades of life. Therefore, prevention is fundamental to reduce the incidence of these pathologies, especially in young people. CVDs are a result of complex interactions between environmental and genetic risk factors.

The overall aim of the present Doctoral Thesis was to study the genetics variants of Uncoupling proteins (UCP), cilliary neurotrophic factor (CNTF), lipoprotein lipase (LPL) and adiponectin (ADIPOQ) genes associated to obesity and others CVD risk factors in adolescents and the potential interaction with physical activity (PA).

We observed significant associations of several single nucleotide polymorphisms (SNPs) of CNTF and UCPs to adiposity markers such as body mass index (BMI), waist circumference, waist to height ratio or waist to hip ratio in European adolescents. Moreover, we also observed an interaction between PA and the *UCP1*rs2071415 polymorphism on waist to hip ratio.

Also, others main findings were the significant association of SNPs of *LPL*, *UCP* and *ADIPOQ* with several CVD risk factors such as serum markers, blood pressure or risk score. Also we observed *LPL* alleles of rs1534649 and rs258 related to higher values of adiposity markers tend to be associated with less adiposity under high levels of PA.

Finally, we have used an *in silico* technique to search new potential novel candidate genes of physiology of brown adipose tissue (BAT) as of *UCP1* as a core gene.

In summary, findings of the present doctoral thesis provide more insights about the genetic role in the predisposition to develop a CVD in European adolescents. These findings also provide information about potential effect of PA in attenuating some of the associations observed between genotypes and CVD phenotypes. Finally, we show a list with 102 novel candidate genes, which set up the BAT-connectome. These potential novel genes suggested new research future lines to improve the knowledge of genetic architecture of BAT.

RESUMEN

La obesidad y enfermedades cardiovasculares relacionadas (ECV) son la principal causa de muerte prematura y discapacidad crónica a nivel mundial. La ECV se manifiesta principalmente durante o después de la quinta década de vida, sin embargo, la evidencia científica indica que los precursores de ECV tienen su origen ya en las primeras décadas de la vida. Por tanto, la prevención es fundamental para reducir la incidencia de estas patologías, sobre todo en la población joven. Las ECV son el resultado de una compleja red de interacciones entre factores de riesgo ambientales y genéticos.

El principal objetivo de la presente tesis doctoral fue el estudio de las variantes génicas de los genes de las uncoupling proteins (UCP), del factor neurotrófico ciliar (CNTF), de la lipoproteinlipasa (LPL) y adiponectina (ADIPOQ) asociados a obesidad y otros factores de riesgo de ECV, y la potencial interacción con la actividad física (AF).

Observamos asociaciones significativas de varios polimorfismos genéticos (SNPs) de *CNTF* y *UCPs* con marcadores de adiposidad (como el índice de masa corporal, índice cintura cadera o circunferencia de cintura entre otros) en adolescentes europeos. Además, observamos también una interacción entre la AF y el SNP *UCP1*rs2071415 sobre el índice cintura cadera.

Otros principales resultados de la presente tesis fueron las asociaciones encontradas de SNPs de *LPL*, *UCP* y *ADIPOQ* con varios factores de riesgo de ECV (marcadores séricos, presiones sanguíneas o risk score). También observamos la interacción con AF entre dos SNPs de LPL (rs1534649 and rs258) y marcadores de adiposidad.

Finalmente, hemos utilizado una técnica *in silico* para la búsqueda de nuevos genes candidatos de la fisiología del tejido adiposo pardo (TAP) utilizando *UCP1* como gen núcleo.

En resumen, los resultados de la presente tesis, proporcionan mas información sobre el papel genético en la predisposición a desarrollar una ECV ya en adolescentes europeos. Estos resultados también nos arroja mas información sobre el potencial efecto de la AF mitigando o disminuyendo estas asociaciones observadas entre genotipos y fenotipos de ECV. Finalmente, mostramos una lista de 102 nuevos genes candidatos, los cuales forman el conectoma del TAP. Estos potenciales genes nuevos sugieren nuevas líneas de investigaciones futuras para ayudarnos a comprender mejor la arquitectura genética del TAP.

INTRODUCTION

INTRODUCTION

Obesity: concept, problem and genetics

The World Health Organization (WHO) defines overweight and obesity as abnormal or excessive fat accumulation that presents a risk to health. This disorder is the consequence of energy imbalances related to complex factors (genetics, environment, etc.) and results from an energy intake higher than energy expenditure[1].

Obesity has become a growing epidemic health problem worldwide. Once considered a problem only in high income countries, overweight and obesity are now dramatically on the rise in low- and middle-income countries, particularly in urban settings [1]. The WHO estimates that, 39% of the global population adults present overweight and of these, 13% are obese. Since 1975, obesity has nearly tripled. Over 340 million children and adolescents aged 5-19 were overweight or obese in 2016[2].

A crude measure of obesity is the body mass index (BMI), a person's weight (in kilograms) divided by the square of his or her height (in metres). A BMI of 30 kg/m^2 is generally considered obese, meanwhile a BMI 25≥ kg/m^2 is considered overweight[1]. However, BMI has notorious limitations, such as it gives no information on fat distribution, which is of high significance in cardiovascular risk [3]. But due to it is a very easy measurement to take, it is widely used and accepted.

Obesity and overweight are well-established major risk factors for a number of very prevalent chronic diseases, such as, diabetes, cardiovascular diseases and cancer, which lend urgency to obesity prevention and control research[4].

Obesity and genetics

As mentioned, obesity is a result of a complex interaction between environmental and genetic factors. An environmental factor widely studied and well known related to obesity is physical activity. Several studies showed that physical activity might delete the effect of several gene polymorphisms on obesity-related traits. A genome-wide meta-analysis of 200,452 European adults showed that physical activity may attenuate the deleterious effect of the *FTO* (fat mass- and obesity-associated) gene polymorphisms on obesity[5]. In adolescents, meeting the daily physical activity recommendations (at least 60 minutes/day of moderate to vigorous physical activity) may offset the genetic predisposition to obesity associated with the *FTO* rs9939609 polymorphism in European adolescents[6].

On the other hand, the strong genetic influence in obesity has been described as a non-mendelian way of inheritance. Only few percent of cases are monogenic obesity type. In these cases, monogenic obesity

genes are involved in the control of appetite center and satiety like leptin (LEP), leptin receptor (LEPR), pro-opiomelacortin (POMC) or prohormoneconvertase 1 (PCSK1) [7]. However, most of the cases of obesity involve multiple genes and complex interactions between them [8]. Among the most studied are *FTO* and melanocortin-4 receptor (*MC4R*) polymorphisms [4,9–11].

Recently, others gene polymorphisms have been associated to obesity. Thus, for example, some neurotropic factors such as ciliary neurotrophic factor (CNTF). CNTF is a neurocytokine from the interleukin 6 family related with injury response in the nervous system and seems to play a role in body weight regulation [12,13]. Control satiety mechanism of CNTF seems to be due to similarities with leptin mechanism, due to overlapping in the activation of signalling cascade [14]. Leptin inhibits orexigenic hormones as neuropeptide Y (NPY) and agouti-related peptide (AgRP), which increase food intake, and stimulates anorexigenic hormones as α-melanocyte stimulating hormone (αMSH) that inhibit food intake through activation of pro-opiomelanocortin (POMC)[15]. So genetics of CNTF have been highlighted as an interesting target against obesity. Indeed, we found in our study a solid association between rs2509914, rs2515363 and rs2515362 *CNTF* polymorphisms with adiposity markers such as BMI, waist circumference, waist to height ratio and waist to hip ratio.

Also, several studies have shown an association of uncoupling proteins (UCPs) gene polymorphisms of brown adipose tissue with obesity[16,17]. Broadly speaking, *UCPs* are related with thermogenesis using fatty acids and glucose as substrate, which make them an attractive target against obesity and other diseases. So, it is possible different polymorphisms of *UCPs* gene may lead UCPs proteins with altered efficiency in their function. As a result, thermogenesis process could be decreased, so lipids oxidation and lipids intake as energy substrate could be altered and finally it influence over adipose tissue composition may be modified. Impact of genetics variants of *UCPs* in obesity are demonstrated by some studies[16,18,19].

Brown adipose tissue

Brown adipose tissue (BAT) is making up by thermogenic cells and mainly regulated by sympathetic nervous system (SNS). Cold exposure makes SNS activates BAT through norepinephrine. Then, in brown adipocytes occurs the uncoupling protein (UCP) activation, which produces the uncoupling of mitochondrial electron transport chain. This way, energy of mitochondrial respiration is dissipated in the form of heat through the oxidation of glucose and fatty acids as energetic substrate [20].

Brown adipose tissue is present in almost all mammals, and it has the task of keep an adequate temperature when mammals are

exposed to under thermoneutrality temperature [20]. Until recently, it was strongly believed that in humans, brown adipose tissue (BAT) was present exclusively in new-borns complying with his non-shivering thermogenesis role as heat production mechanism [21]. Also, it was unknown if BAT could have an impact over human metabolism. However, BAT presence in adults such as its metabolic role in human physiology was recognised by Nedergaard *et al*. [22]. Nowadays, there is solid evidence and no doubt that brown adipose tissue is present in adults and it is thermogenically active [23–25].

The most studied *UCP* genes are: i) *UCP1*, which main function is heat production through non-shivering thermogenesis in brown adipose tissue (BAT) [26]; ii) *UCP2*, seems to be involved in the control of reactive oxygen species (ROS) production [27,28], the modulation of insulin secretion[29], the regulation of mitochondrial fatty acid oxidation[30] and may have a regulating role in thermogenesis of BAT [31], and iii) *UCP3,* which role has been related to the coupling regulation of mitochondrial respiration in skeletal muscle mitochondria[32] and fatty acids oxidation[33], and is a mediator of thermogenesis[34].

Therefore BAT capacity to oxidate fatty acids and glucose and increase energy expenditure when humans are exposed to cold or other a stimulus, which produces its activation, makes brown adipose tissue a target tissue against obesity[20]. On the other hand, in mouse there is evidence about regulation of blood lipid profile[35], meanwhile in humans cardioprotective role of BAT is necessary to clear. However, a study show that BAT decreases triglycerides storage due to his fatty acids consumption as energetic substrate[36].

Also, there is the theory that physical activity could activate and regulate BAT. As we explained before, BAT is activated via catecholamines, so it would be logical to think that exercise, through this way, could have an effect on brown adipose tissue activity. Indeed, some studies show feasible pathways through exercise could regulates BAT[37,38]. However, studies investigating the effect of exercise on BAT are controversial. In rodents, some studies observed an increase of BAT mitochondrial activity [39], while others revealed a reduction [40]. In humans, the effect of exercise on BAT is also unclear. Besides studies trend to shown a decrease of BAT activity as response of exercise[41,42], it is possible that other adaptations against exercise are produced in humans. These studies shows exercise decreases cold- or insulin-stimulated glucose uptake in human BAT. In lights of these results, we may think that rodent BAT and human BAT respond differently to exercise.

Studies have shown a solid association between BAT and obesity markers. It is quite known that obesity is strongly associated to

CVD prognosis. As important is obesity degree as how long a person has been obese in CVD prognosis [43]. That highlighted the importance of delaying obesity onset to achieve CV health benefits and therefore how important is prevent it in early stages of life as children and adolescents. Plus, due to own physiological mechanisms of BAT (utilisation of fatty acids and glucose as substrate) make it an interesting target against obesity and CVDs.

Cardiovascular disease: definition, importance and genetics

Cardiovascular diseases (CVDs) are disorders of the heart and blood vessels and include coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions[44].

Every year, 17.9 million people die from CVDs, an estimated 31% of all deaths worldwide[44], becoming in the main cause of premature death and chronic disability worldwide [45]. Of these deaths, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke. Low- and middle-income countries are disproportionally affected: over 80% of CVD deaths take place in low- and middle-income countries and occur almost equally in men and women. High-income countries trends to plateau but they are not decreasing anymore [45].

CVDs are a result of complex interaction between modifiable and non-modifiable risk factors. Modifiable risk factors are tobacco, physical inactivity, high blood levels of low-density lipoprotein (LDL) cholesterol, high-saturated-fat diet, hypertension (HT), diabetes and obesity[46]. Most of these modifiable risk factors are preventable and susceptible to be controlled by treatment. Indeed, 80% of premature deaths from these causes could be avoided by controlling the main risk factors: tobacco, unhealthy diet and physical inactivity[44]. Non-modifiable risk factor is mainly family history. For example, if both parents have suffered from heart disease before the age of 55, your risk of developing heart disease can rise to 50% compared to the general population[46].

Some studies highlighted the importance of genetic heritability in CVDs through the association between some polymorphisms with stroke[47,48], myocardial infarction [49,50] and others cardiovascular complications[51]. In the case of uncoupling protein genes (*UCPs*), they have been associated with risk factors of cardiovascular disease such as prediabetes and type 2 diabetes mellitus (T2DM) [29,52,53], overweight and obesity [16,54–56], plasma levels of cholesterol [19,57,58] or hypertension (HT) [59] , mainly in adults over 50 years of age. Also, it is described a cardiovascular role of some adipocytokines such as Adiponectin (ADIPOQ). Several studies shown the effect of ADIPOQ

in cardiovascular disease outcomes through their effects in lipid and glucose metabolism[60–62]. Finally, lipoprotein lipase (LPL) has a central role in both VLDL and HDL metabolism. It is reported in several studies an association between genetic variations in *LPL* and cardiovascular risk factors[63–65].

So prevention in CVDs has a major role. Identifying those highest risk of CVDs as soon as possible (young population) and achieve an appropriate treatment, can prevent premature deaths by this cause.

Adolescent health

It is commonly accepted that adolescent population (10-19 years old) is healthy and burden-disease free group[66]. Unfortunately, this is not totally true. Despite of global all-cause child and adolescent have been decreased in last decades, there are still a significant burden disease between these population[67]. Kassebaum et al.[67] found that this health improvement is uneven between different Sociodemographic Index (SDI) countries. Mortality was the primary driver of health loss (caused by infectious, nutritional, maternal and neonatal causes) in low SDI countries, meanwhile nonfatal health loss predominates in high SDI countries. In European countries, adolescents are involved in an environment where a lot of risk factors are present, mainly related with lifestyle and behaviour.

Keeping a good status of health and avoid risk factors in early decades of life it is important to prevent future complications, especially those which need a long-time exposure to risk factors such as cardiovascular disease, diabetes type II or obesity. In the case of cardiovascular disease, findings of the Cardiovascular Risk in Young Finns Study shows that the number of ideal cardiovascular health metrics defined by the American Heart Association (AHA)[68] present in adolescents predicts subsequent cardiometabolic health in adulthood[69]. After 21 years of follow-up, they found that the number of ideal cardiovascular health metrics present was significant associated with reduced risk of hypertension, metabolic syndrome, high low-density lipoprotein cholesterol and carotid artery intima-media thickness in adulthood.

Referring to obesity, more than 124 million children and adolescents (6% of girls and 8% of boys) were obese in 2016 [2]. Children and adolescent obesity is a very important early risk factor for adult cardiovascular related morbidity and mortality[70]. Frequently children obesity persists in adulthood: 80% of obese children becomes in obese adults[71]. Plus, some of the most common complications of obesity are already present in the obese adolescent, such as renal insufficiency and hyperinsulinemia, and predicts insulin resistance in adulthood[72,73] .

Therefore, it is quite clear the importance of act over risk factors and some diseases in adolescent population to prevent adult morbidity and mortality, especially taking account the high prevalence of them.

We considered the importance of the study the association and impact of genetics variants with obesity and cardiovascular diseases, especially due to lack of solid information in these areas. Plus, we look upon very interesting study the role of BAT genetics owing to his physiological mechanisms related with these entities.

Physical activity and interaction with SNPs

It is well known that physical exercise, when performed moderate and regularly, protects against the development and progression of hypokinetic diseases. Regular exercise training provides a wide range of health benefits in the general population, including improvements in blood pressure, diabetes (enhancing insulin sensitivity and improve glucose tolerance), lipid profile (increases high-density lipoprotein (HDL), reduce triglycerides (TG), low density lipoprotein (LDL) and cholesterol levels)[74], decrease cardiovascular disease risk[75], osteoarthritis, osteoporosis, neurocognitive function, and is also associated with decreased mortality and age-related morbidity in adults[76]. A number of studies have shown that moderate-intensity physical activity reduces the incidence of all-cause mortality, particularly deaths related to coronary artery disease (CAD)[77].

Also physical activity may to have a significant effect over phenotypes outcomes even though genetic predisposition. Gene-environmental interactions could exist for complex human traits and identifying these gene-environment interactions may potentially improve risk-assessment for disease and clarify underlying biological pathways[78]. For example, increased levels of physical activity are associated with beneficial changes in diabetes-related phenotypes. Moreover, the positive response depends on inter-individual likely due to variation differences in genetic variants[79]. Also, evidence reports a physical activity-gene interaction over overweight/obese phenotypes. Previous studies suggested that physical activity could attenuate the deleterious effect of some genetic polymorphisms over adiposity markers[6,80]. Both studies shown that meeting the daily physical activity recommendations (at least 60 minutes/day of moderate to vigorous physical activity) may offset the genetic predisposition to obesity.

References

1. (WHO) TWHO. Obesity. Obesity. https://www.who.int/topics/obesity/en/. Published 2016.
2. (WHO) TWHO. Obesity and overweight. Obesity Facts.
3. Zeller M, Steg PG, Ravisy J, et al. Relation between body mass index, waist circumference, and death after acute myocardial infarction. *Circulation*. 2008;118(5):482-490. doi:10.1161/CIRCULATIONAHA.107.753483.
4. Scuteri A, Sanna S, Chen WM, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet*. 2007;3(7):1200-1210. doi:10.1371/journal.pgen.0030115.
5. Graff M, Scott RA, Justice AE, et al. Genome-wide physical activity interactions in adiposity — A meta-analysis of 200,452 adults. *PLoS Genet*. 2017;13(4):1-26. doi:10.1371/journal.pgen.1006528.
6. Ruiz JR, Labayen I, Ortega FB, et al. Attenuation of the effect of the FTO rs9939609 polymorphism on total and central body fat by physical activity in adolescents: the HELENA study. *Arch Pediatr Adolesc Med*. 2010;164(4):328-333. doi:10.1001/archpediatrics.2010.29.
7. Farooqi IS, O'Rahilly S. Genetics of obesity in humans. *Endocr Rev*. 2006;27(7):710-718. doi:10.1210/er.2006-0040.
8. Sheikh AB, Nasrullah A, Haq S, et al. The Interplay of Genetics and Environmental Factors in the Development of Obesity. *Cureus*. 2017;9(7):e1435. doi:10.7759/cureus.1435.
9. Sonestedt E, Roos C, Gullberg B, Ericson U, Wirfält E, Orho-Melander M. Fat and carbohydrate intake modify the association between genetic variation in the FTO genotype and obesity. *Am J Clin Nutr*. 2009;90(5):1418-1425. doi:10.3945/ajcn.2009.27958.
10. Loos RJF. The genetic epidemiology of melanocortin 4 receptor variants. *Eur J Pharmacol*. 2011;660(1):156-164. doi:10.1016/j.ejphar.2011.01.033.
11. Labayen I, Ruiz JR, Huybrechts I, et al. Dietary fat intake modifies the influence of the FTO rs9939609 polymorphism on adiposity in adolescents: The HELENA cross-sectional study. *Nutr Metab Cardiovasc Dis*. 2016;26(10):937-943. doi:10.1016/j.numecd.2016.07.010.
12. Heidema AG, Wang P, van Rossum CTM, et al. Sex-

specific effects of CNTF, IL6 and UCP2 polymorphisms on weight gain. *Physiol Behav*. 2010;99(1):1-7. doi:10.1016/j.physbeh.2009.10.002.
13. Cron L, Allen T, Febbraio MA. The role of gp130 receptor cytokines in the regulation of metabolic homeostasis. *J Exp Biol*. 2016;219(2):259-265. doi:10.1242/jeb.129213.
14. Xu B, Xie X. Neurotrophic factor control of satiety and body weight. *Nat Rev Neurosci*. 2016;17(5):282-292. doi:10.1038/nrn.2016.24.
15. Farr OM, Gavrieli A, Mantzoros CS. HHS Public Access. 2016:1-14. doi:10.1097/MED.0000000000000184.Leptin.
16. Brondani LA, Assmann TS, de Souza BM, Bouças AP, Canani LH, Crispim D. Meta-analysis reveals the association of common variants in the uncoupling protein (UCP) 1-3 genes with body mass index variability. *PLoS One*. 2014;9(5):e96411. doi:10.1371/journal.pone.0096411.
17. Acosta A, Camilleri M, Shin A, et al. Association of UCP-3 rs1626521 with obesity and stomach functions in humans. *Obesity*. 2015;23(4):898-906. doi:10.1002/oby.21039.
18. Van Abeelen AFM, de Krom M, Hendriks J, Grobbce DE, Adan RAH, Van der Schouw YT. Variations in the uncoupling protein-3 gene are associated with specific obesity phenotypes. *Eur J Endocrinol*. 2008;158(5):669-676. doi:10.1530/EJE-07-0834.
19. De Luis DA, Aller R, Izaola O, et al. Relationship of -55C/T Polymorphism of Uncoupling Protein 3 (UCP3) Gene with Metabolic Syndrome by ATP III Classification. *J Clin Lab Anal*. 2012;26(4):272-278. doi:10.1002/jcla.21517.
20. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev*. 2004;84(1):277-359. doi:10.1152/physrev.00015.2003.
21. Lean M, James W, Jennings G, Trayhurn P. Brown adipose tissue uncoupling protein content in human infants, children and adults. *Clin Sci*. 1986;71:291-297.
22. Nedergaard J, Bengtsson T, Cannon B. Unexpected evidence for active brown adipose tissue in adult humans. *Am J Physiol Endocrinol Metab*. 2007;293(2):E444-52. doi:10.1152/ajpendo.00691.2006.
23. Virtanen KA, Lidell ME, Orava J, et al. Functional Brown Adipose Tissue in Healthy Adults. *N Engl J Med*. 2009;360(15):1518-1525. doi:10.1056/NEJMoa0808949.
24. Saito M, Okamatsu-ogura Y, Matsushita M, et al. High

Incidence of Metabolically Active Brown Adipose Effects of Cold Exposure and Adiposity. *October*. 2009;58(JULY):1526-1531. doi:10.2337/db09-0530.

25. Cypess AM, Lehman S, Williams G, et al. Identification and Importance of Brown Adipose Tissue in Adult Humans A BS TR AC T. *N Engl J Med*. 2009;360:1509-1526. doi:http://dx.doi.org/10.1056/NEJMoa0810780.
26. Golozoubova V, Cannon B, Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab*. 2006;291(2):E350-E357. doi:10.1152/ajpendo.00387.2005.
27. Moukdar F, Robidoux J, Lyght O, Pi J, Daniel KW, Collins S. Reduced antioxidant capacity and diet-induced atherosclerosis in uncoupling protein-2-deficient mice. *J Lipid Res*. 2009;50(1):59-70. doi:10.1194/jlr.M800273-JLR200.
28. Arsenijevic D, Onuma H, Pecqueur C, et al. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat Genet*. 2000;26(4):435-439. doi:10.1038/82565.
29. Zhang CY, Baffy G, Perret P, et al. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell*. 2001;105(6):745-755. doi:S0092-8674(01)00378-6 [pii].
30. Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F. UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? *IUBMB Life*. 2009;61(7):762-767. doi:10.1002/iub.188.
31. Caron A, Labbé SM, Carter S, et al. Loss of UCP2 impairs cold-induced non-shivering thermogenesis by promoting a shift toward glucose utilization in brown adipose tissue. *Biochimie*. 2017;134:118-126. doi:10.1016/j.biochi.2017.01.006.
32. Vidal-Puig AJ, Grujic D, Zhang C-Y, et al. Energy Metabolism in Uncoupling Protein 3 Gene Knockout Mice. *J Biol Chem*. 2000;275(21):16258-16266. doi:10.1074/jbc.M910179199.
33. Bezaire V, Spriet LL, Campbell S, et al. Constitutive UCP3 overexpression at physiological levels increases mouse skeletal muscle capacity for fatty acid transport and oxidation. *FASEB J*. 2005;19(8):977-979. doi:10.1096/fj.04-2765fje.
34. Riley CL, Dao C, Kenaston MA, et al. The Complementary and Divergent Roles of Uncoupling Proteins 1 and 3 in

Thermoregulation. *J Physiol*. 2016;594(24):7455–7464. doi:10.1113/JP272971.This.
35. Bartelt A, Bruns OT, Reimer R, et al. Brown adipose tissue activity controls triglyceride clearance. *Nat Med*. 2011;17(2):200-206. doi:10.1038/nm.2297.
36. Blondin DP, Labbé SM, Noll C, et al. Selective impairment of glucose but not fatty acid or oxidative metabolism in brown adipose tissue of subjects with type 2 diabetes. *Diabetes*. 2015;64(7):2388-2397. doi:10.2337/db14-1651.
37. Sanchez-Delgado G, Martinez-Tellez B, Olza J, et al. Activating brown adipose tissue through exercise (ACTIBATE) in young adults: Rationale, design and methodology. *Contemp Clin Trials*. 2015;45:416-425. doi:10.1016/j.cct.2015.11.004.
38. Lehnig AC, Stanford KI. Exercise-induced adaptations to white and brown adipose tissue. *J Exp Biol*. 2018;221(Suppl 1):jeb161570. doi:10.1242/jeb.161570.
39. Ignacio DL, Fortunato RS, Neto RAL, et al. Blunted response of pituitary type 1 and brown adipose tissue type 2 deiodinases to swimming training in ovariectomized rats. *Horm Metab Res*. 2012;44(11):797-803. doi:10.1055/s-0032-1314875.
40. Wu M V., Bikopoulos G, Hung S, Ceddia RB. Thermogenic capacity is antagonistically regulated in classical brown and white subcutaneous fat depots by high fat diet and endurance training in rats: Impact on whole-body energy expenditure. *J Biol Chem*. 2014;289(49):34129-34140. doi:10.1074/jbc.M114.591008.
41. Motiani P, Virtanen KA, Motiani KK, et al. Decreased insulin-stimulated brown adipose tissue glucose uptake after short-term exercise training in healthy middle-aged men. *Diabetes, Obes Metab*. 2017;19(10):1379-1388. doi:10.1111/dom.12947.
42. Vosselman MJ, Hoeks J, Brans B, et al. Low brown adipose tissue activity in endurance-trained compared with lean sedentary men. *Int J Obes*. 2015;39(12):1696-1702. doi:10.1038/ijo.2015.130.
43. Ortega FB, Lavie CJ, Blair SN. Obesity and Cardiovascular Disease. *Circ Res*. 2016;118(11):1752-1770. doi:10.1161/CIRCRESAHA.115.306883.
44. (WHO) TWHO. Cardiovascular diseases.
45. Roth GA, Johnson C, Abajobir A, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J Am Coll Cardiol*. 2017;70(1):1-25.

doi:10.1016/j.jacc.2017.04.052.
46. World Heart Federation. Cardiovascular risk factors.
47. Akhter MS, Biswas A, Rashid H, Devi L, Behari M, Saxena R. Screening of the GPX3 gene identifies the "t" allele of the SNP -861A/T as a risk for ischemic stroke in young Asian Indians. *J Stroke Cerebrovasc Dis*. 2014;23(8):2060-2068. doi:10.1016/j.jstrokecerebrovasdis.2014.03.010.
48. Man BL, Baum L, Fu YP, et al. Genetic polymorphisms of Chinese patients with ischemic stroke and concurrent stenoses of extracranial and intracranial vessels. *J Clin Neurosci*. 2010;17(10):1244-1247. doi:10.1016/j.jocn.2010.01.050.
49. Nasibullin TR, Timasheva YR, Sadikova RI, et al. Genotype/allelic combinations as potential predictors of myocardial infarction. *Mol Biol Rep*. 2016;43(1):11-16. doi:10.1007/s11033-015-3933-3.
50. Buysschaert I, Carruthers KF, Dunbar DR, et al. A variant at chromosome 9p21 is associated with recurrent myocardial infarction and cardiac death after acute coronary syndrome: The GRACE genetics study. *Eur Heart J*. 2010;31(9):1132-1141. doi:10.1093/eurheartj/ehq053.
51. Fiatal S, Ádány R. Application of Single-Nucleotide Polymorphism-Related Risk Estimates in Identification of Increased Genetic Susceptibility to Cardiovascular Diseases: A Literature Review. *Front Public Heal*. 2018;5(January). doi:10.3389/fpubh.2017.00358.
52. Su M, Chen X, Chen Y, et al. UCP2 and UCP3 variants and gene-environment interaction associated with prediabetes and T2DM in a rural population: A case control study in China. *BMC Med Genet*. 2018;19(1):1-9. doi:10.1186/s12881-018-0554-4.
53. Hsu Y-H, Niu T, Song Y, Tinker L, Kuller LH, Liu S. Genetic variants in the UCP2-UCP3 gene cluster and risk of diabetes in the Women's Health Initiative Observational Study. *Diabetes*. 2008;57(4):1101-1107. doi:10.2337/db07-1269.
54. Zhang M, Wang M, Zhao ZT. Uncoupling protein 2 gene polymorphisms in association with overweight and obesity susceptibility: A meta-analysis. *Meta Gene*. 2014;2(1):143-159. doi:10.1016/j.mgene.2013.10.009.
55. Kosuge K, Soma M, Nakayama T, et al. Human uncoupling

protein 2 and 3 genes are associated with obesity in Japanese. *Endocrine*. 2008;34(1-3):87-95. doi:10.1007/s12020-008-9111-9.

56. Oktavianthi S, Trimarsanto H, Febinia CA, et al. Uncoupling protein 2 gene polymorphisms are associated with obesity. *Cardiovasc Diabetol*. 2012;11(1):41. doi:10.1186/1475-2840-11-41.
57. Kotani K, Fujiwara S, Tsuzaki K, et al. The Association Between the Uncoupling Protein-1 Gene A-3826G Polymorphism and High-density Lipoprotein Cholesterol in A General Japanese Population: A Consideration of the Obesity Status. *J Clin Med Res*. 2011;3(6):319-324. doi:10.4021/jocmr738w.
58. Kotani K, Sakane N, Saiga K, et al. Relationship between A-3826G Polymorphism in the Promoter of the Uncoupling protein-1 Gene and High-density Lipoprotein Cholesterol in Japanese Individuals: A Cross-sectional Study. *Arch Med Res*. 2008;39(1):142-146. doi:10.1016/j.arcmed.2007.07.002.
59. Kotani K, Sakane N, Saiga K, et al. The uncoupling protein-1 gene -3826A/G polymorphism and hypertension in Japanese subjects. *Clin Chem Lab Med*. 2007;45(9):1186-1189. doi:10.1515/CCLM.2007.253.
60. Woodward L, Akoumianakis I, Antoniades C. Unravelling the adiponectin paradox: novel roles of adiponectin in the regulation of cardiovascular disease. *Br J Pharmacol*. 2017;174(22):4007-4020. doi:10.1111/bph.13619.
61. Hung J, McQuillan BM, Thompson PL, Beilby JP. Circulating adiponectin levels associate with inflammatory markers, insulin resistance and metabolic syndrome independent of obesity. *Int J Obes*. 2008;32(5):772-779. doi:10.1038/sj.ijo.0803793.
62. Zhang X, Cao YJ, Zhang HY, Cong H, Zhang J. Associations between ADIPOQ polymorphisms and coronary artery disease: a meta-analysis. Lafrenie RM, ed. *BMC Cardiovasc Disord*. 2019;19(1):63. doi:10.1186/s12872-019-1041-3.
63. Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med (Berl)*. 2002;80(12):753-769. doi:10.1007/s00109-002-0384-9.
64. Jia JJ, Zhang X, Ge CR, Jois M. The polymorphisms of UCP2 and UCP3 genes associated with fat metabolism, obesity and diabetes: Etiology and pathophysiology. *Obes Rev*. 2009;10(5):519-526.

doi:10.1111/j.1467-789X.2009.00569.x.
65. Cahua-Pablo JÁ, Cruz M, Méndez-Palacios A, et al. Polymorphisms in the LPL and CETP genes and haplotype in the ESR1 gene are associated with metabolic syndrome in women from Southwestern Mexico. *Int J Mol Sci*. 2015;16(9):21539-21554. doi:10.3390/ijms160921539.
66. (WHO) TWHO. Adolescent health. Adolescent health. https://www.who.int/topics/adolescent_health/en/. Published 2018.
67. Kassebaum N, Kyu HH, Zoeckler L, et al. Child and adolescent health from 1990 to 2015: Findings from the global burden of diseases, injuries, and risk factors 2015 study. *JAMA Pediatr*. 2017;171(6):573-592. doi:10.1001/jamapediatrics.2017.0250.
68. Lloyd-Jones DM, Hong Y, Labarthe D, et al. Defining and setting national goals for cardiovascular health promotion and disease reduction: The american heart association's strategic impact goal through 2020 and beyond. *Circulation*. 2010;121(4):586-613. doi:10.1161/CIRCULATIONAHA.109.192703.
69. Laitinen T, Laitinen TT, Pahkala K, et al. Ideal cardiovascular health in childhood and cardiometabolic outcomes in adulthood: The cardiovascular risk in young finns study. *Circulation*. 2012;125(16):1971-1978. doi:10.1161/CIRCULATIONAHA.111.073585.
70. Skinner AC, Perrin EM, Moss LA, Skelton JA. Cardiometabolic Risks and Severity of Obesity in Children and Young Adults. *N Engl J Med*. 2015;373(14):1307-1317. doi:10.1056/NEJMoa1502821.
71. Srinivasan SR, Bao W, Wattigney WA, Berenson GS. Adolescent overweight is associated with adult overweight and related multiple cardiovascular risk factors: The Bogalusa Heart Study. *Metabolism*. 1996;45(2):235-240. doi:10.1016/S0026-0495(96)90060-8.
72. Steinberger J, Moran A, Hong CP, Jacobs DR, Sinaiko AR. Adiposity in childhood predicts obesity and insulin resistance in young adulthood. *J Pediatr*. 2001;138(4):469-473. doi:10.1067/mpd.2001.112658.
73. Caprio S, Perry R, Kursawe R. Adolescent Obesity and Insulin Resistance: Roles of Ectopic Fat Accumulation and Adipose Inflammation. *Gastroenterology*. 2017;152(7):1638-1646. doi:10.1053/j.gastro.2016.12.

051.
74. Physical activity and cardiovascular health. NIH Consensus Development Panel on Physical Activity and Cardiovascular Health. *JAMA*. 1996;276(3):241-246.
75. Sniderman AD, Pedersen T, Kjekshus J. Putting low-density lipoproteins at center stage in atherogenesis. *Am J Cardiol*. 1997;79(1):64-67.
76. Nied RJ, Franklin B. Promoting and prescribing exercise for the elderly. *Am Fam Physician*. 2002;65(3):419-426.
77. Blair SN, Kampert JB, Kohl HW 3rd, et al. Influences of cardiorespiratory fitness and other precursors on cardiovascular disease and all-cause mortality in men and women. *JAMA*. 1996;276(3):205-210.
78. Rask-Andersen M, Karlsson T, Ek WE, Johansson A. Gene-environment interaction study for BMI reveals interactions between genetic factors and physical activity, alcohol consumption and socioeconomic status. *PLoS Genet*. 2017;13(9):e1006977. doi:10.1371/journal.pgen.1006977.
79. Kilpelainen TO, Franks PW. Gene-physical activity interactions and their impact on diabetes. *Med Sport Sci*. 2014;60:94-103. doi:10.1159/000357339.
80. Pascual-Gamarra JM, Salazar-Tortosa D, Martinez-Tellez B, et al. Association between UCP1 , UCP2 , and UCP3 gene polymorphisms with markers of adiposity in European adolescents: The HELENA study. *Pediatr Obes*. 2019;(December 2018):e12504. doi:10.1111/ijpo.12504.

AIMS

AIMS

The **overall aim** of the present Doctoral Thesis was to study the genetics variants associated to obesity and others cardiovascular disease risk factors in adolescents and the potential interaction with physical activity (PA).

The **specific aims** of this Doctoral Thesis were:

AIM 1: to study genetics variants associated to obesity

Chapter 1: to examine the association of *CNTF* gene polymorphisms (i.e. rs2509914, rs17489568, rs2515363, rs1800169, rs2515362) with total and central adiposity markers in European adolescents.

Chapter 2: to examine the association between 18 *UCP1*, *UCP2*, and *UCP3* polymorphisms with total and central adiposity markers in European adolescents and to test if there were gene x PA interactions on adiposity phenotypes.

AIM 2: to study genetics variants associated to cardiovascular disease risk factors

Chapter 3: to examine the association between 14 *ADIPOQ* polymorphisms with CVD risk factors in European adolescents.

Chapter 4: to examine the association of 18 *UCP1*, *UCP2* and *UCP3* polymorphisms with CVD risk factors in European adolescents.

Chapter 5: to examine the association between 13 *LPL* polymorphisms with CVD risk factors in European adolescents. We also examined the interaction effect between PA and *LPL* polymorphisms on CVDs risk factors.

AIM 3: to search for new candidate gene associated with brown adipose tissue.

Chapter 6: to provide a new tool for searching BAT-genes related draw from a core gene. A biological distances approach: connectome.

GENERAL MATERIAL AND METHODS

GENERAL MATERIAL AND METHODS

Chapter	Design	Participants	Variables studied
Aim 1: to study genetics variants associated to obesity and cardiovascular disease			
Chapter 1: to examine the association of *CNTF* gene polymorphisms (i.e. rs2509914, rs17489568, rs2515363, rs1800169, rs2515362) with total and central adiposity markers in European adolescents.	Cross-sectional	1.057 European adolescents (552 girls); BMI: 21.3±3.7kg/m2; age: 12-18 years old	Adiposity markers: Waist and hip circumferences, waist/height, waist/hip, BMI, Body fat percentage, Fat mass index; and Single nucleotides polymorphisms of *CNTF* (genotyping)
Chapter 2: to examine the association between 18 *UCP1, UCP2*, and *UCP3* polymorphisms with total and central adiposity markers in European adolescents and to test if there were gene x PA interactions on adiposity phenotypes.	Cross-sectional	1.057 European adolescents (552 girls); BMI: 21.3±3.7kg/m2; age: 12-18 years old	Adiposity markers: Waist and hip circumferences, waist/height, waist/hip, BMI, Body fat percentage, Fat mass index; Physical activity with accelerometers and Single nucleotides polymorphisms of *UCPs* (genotyping)
Aim 2: to study genetics variants associated to cardiovascular disease risk factors			
Chapter 3: to examine the association between 14 *ADIPOQ* polymorphisms with CVD risk factors in European adolescents.	Cross-sectional	1.057 European adolescents (552 girls); BMI: 21.3±3.7kg/m2; age: 12-18 years old	Cardiovascular markers: serum total cholesterol, HDL, ApoA1, ApoB, leptin, triglycerides, glucose, insulin, blood pressure,

			and calculated HOMA (homeostatic model assessment) and a CVD Risk Score; and Single nucleotides polymorphisms of *ADIPOQ* (genotyping)
Chapter 4: to examine the association of 18 *UCP1*, *UCP2* and *UCP3* polymorphisms with CVD risk factors in European adolescents.	Cross-sectional	1.057 European adolescents (552 girls); BMI: 21.3±3.7kg/m2; age: 12-18 years old	Cardiovascular markers: serum total cholesterol, HDL, ApoA1, ApoB, leptin, triglycerides, glucose, insulin, blood pressure, and calculated HOMA (homeostatic model assessment) and a CVD Risk Score; and Single nucleotides polymorphisms of *UCPs* (genotyping)
Chapter 5: to examine the association between 13 *LPL* polymorphisms with CVD risk factors in European adolescents. We also examined the interaction effect between PA and *LPL* polymorphisms on CVDs risk factors.	Cross-sectional	1.057 European adolescents (552 girls); BMI: 21.3±3.7kg/m2; age: 12-18 years old	Adiposity markers: Waist and hip circumferences, waist/height, waist/hip, BMI, Body fat percentage, Fat mass index; Cardiovascular markers: serum total cholesterol, HDL, ApoA1, ApoB, leptin,

			triglycerides, glucose, insulin, blood pressure, and calculated HOMA (homeostatic model assessment) and a CVD Risk Score; and Single nucleotides polymorphisms of *LPL* (genotyping)
Aim 3: to search for new candidate gene associated with brown adipose tissue			
Chapter 6: to provide a new tool for searching BAT-genes related draw from a core gene. A biological distances approach: connectome	Methodological study.	N/A	168 genes: only the genes in the top 1% UCP1 proximity of all human genes by p-value

RESULTS

GENETIC POLYMORPHISMS ASSOCIATED TO ADIPOSITY MARKERS

CHAPTER 1: Association between *CNTF* gene polymorphisms and adiposity markers in European adolescents: the HELENA study

ABSTRACT

Aims: To examine the association of *CNTF* gene polymorphisms with total and central adiposity markers in European adolescents.

Methods: A cross-sectional study that involves 1.057 European adolescents (12-18 years old) from the Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study. A total of 5 polymorphisms in *CNTF* genes were genotyped. We measured weight, height, waist and hip circumferences and triceps and subscapular skinfold thickness.

Results: The T allele of rs2509914, C allele of rs2515363 and G allele of rs2515362 were significantly associated to high values of several adiposity markers under different genetic models after Bonferroni corrections. Also haplotype CCGGA of *CNTF* (rs2509914, rs17489568, rs2515363 rs1800169 and rs2515362) was significantly associated to lower BMI, waist circumference, waist/height and waist/hip than TCCGG under several genetic models.

Conclusions: Three individual polymorphisms (rs2509914, rs2515363 and rs2515362) and a haplotype of *CNTF* gene were significantly associated with adiposity in European adolescents.

Keywords: CNTF, Obesity, Genetic Susceptibility, Adolescents, SNPs

Introduction

Obesity has become a severe health problem worldwide, particularly in young people[1]. The obese phenotype probably occurs as result of complex interactions between individual's genetic background, environmental, behavioural and socioeconomic factors. In simple terms, obesity is the result of chronic excess of energy intake over energy expenditure. Key factors regulate energy intake (satiety) and energy expenditure (basal metabolism, physical activity, thermoregulation and digestive processes) [2].

Whereas many genes have been related with obesity, special importance take those that are involved in the regulation role of appetite center in the brain. It is well known the role of hormones and other molecules like leptin (LEP), leptin receptor (LEPR), pro-opiomelacortin (POMC) or prohormone convertase 1 (PCSK1) which are related with monogenetic obesity[3]. However, most common is polygenic obesity as result of complex interactions between multiple genes and factors among which polymorphisms of fat mass and obesity gene (FTO) and melanocortin-4 receptor (MC4R)[4,5] are most studied.

It is well known the role of neurotrophic factors as control and developing of synaptic function and synaptic plasticity, while continuing to modulate neuronal survival in the mature nervous system[6]. Plus, a role in control of body weight is also described. Brain-derived neurotrophic factor (BDNF) and BDNF receptor (TrkB) mutations

produces severe obesity in humans[7,8]. Ciliary neurotrophic factor (CNTF) is a neurocytokine from the interleukin 6 family, which main role is related with injury response in the nervous system, indeed lesions in the brain produces a significant increase in CNTF mRNA and CTNF at the hypothalamic level[2]. But also his function is related with body weight control. A placebo-controlled trial described that subcutaneous administration of recombinant CNTF in humans with obesity produces reduction of food intake and weight loss by promoting satiety[9]. Mechanisms underlying satiety-improving of CNTF seems to be related with the similarities in the signalling cascade of leptin and CNTF as they overlap in receptors and mediators. It is well known leptin inhibits orexigenic hormones as neuropeptide Y (NPY) and agouti-related peptide (AgRP), which increase food intake, and stimulates anorexigenic hormones as α-melanocyte stimulating hormone (αMSH) that inhibit food intake through activation of pro-opiomelanocortin (POMC)[10]. This raises the possibility that CNTF acts on leptin-responsive neurons to regulate satiety and body weight[2]. Studies shown that CNTF may to reduce body weight and improve insulin action in both rodents and humans[11]. Nonetheless, there is still poor evidence, especially in humans, that implicates endogenous CNTF signalling in the control of energy balance, neither obesity/hyperphagia[12,13]. Indeed, some studies investigated the effect of natural null mutation of CNTF described by Takahashi *et al*.[12] on adiposity markers. Some of them found an association with BMI or body weight[14], while others not[15–17].

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provide an excellent opportunity to study the association between *CNTF* gene polymorphisms with adiposity makers in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 5 polymorphisms of the *CNTF* gene as well as markers of adiposity. To our knowledge, *CNTF* polymorphisms have not been identified in GWAS of body weight or body composition in adults or other age groups.

The aim of this study was therefore to examine the association of *CNTF* gene polymorphisms (i.e. rs2509914, rs17489568, rs2515363, rs1800169, rs2515362) with total and central adiposity markers in European adolescents.

Material and Methods

Participants

In the present cross-sectional study, we included a total of 1057 adolescents (12-18 year old) with data on *CNTF* gene polymorphisms (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362) and

adiposity phenotypes. HELENA study was conducted between 2006 and 2007. After receiving complete information about the aims and methods of the study, all adolescents and their parents or guardians signed an informed written consent. All participants met the general HELENA inclusion criteria [18]. The study was performed following the ethical guidelines of the Declaration of Helsinki 1961 (revision of Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries. The protocol was approved by the corresponding local human research review committees of the involved centers.

Assessment of adiposity

Weight and height were measured following standard methods [18]. Waist and hip circumference was measured in triplicate with an anthropometric unelastic tape (SECA 200; Seca Deutschland, Hamburg, Germany) and was used as a surrogate measure of central body fat. We calculated waist to height and waist to hip ratios. BMI was calculated as weight in kilograms divided by height in meters squared. Adolescents were classified according to BMI (kg/m^2) as normal weight, overweight or obese according to Cole et al. [19]. Skinfold thickness was measured to the nearest 0.2 mm in triplicate on the left side at the biceps, triceps, subscapularis, suprailium, thigh, and medial calf with a Holtain Caliper (Holtain Ltd, Crymmych, Wales). Body fat percentage was calculated from skinfold thicknesses (triceps and subscapular) using the equations by Slaughter et. al [20]. Finally, fat mass index (FMI) was calculated as fat mass in kilograms divided by height in meters squared.

Genotyping

Samples were genotyped by an Illumina System (Illumina, Inc, San Diego, California) and the software used was GoldenGate (Inc, San Francisco, California). High rate of genotyping success was performed (≥99.9%) and each polymorphism respected the Hardy-Weinberg equilibrium ($P>0.15$ in all cases). All genotype polymorphisms of *CNTF* showed linkage disequilibrium between them (Fig 1).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were determined by means of an exact test and considering a p value of 0.05 as a threshold. Associations between polymorphisms and adiposity markers were assessed through linear models. Five genetic models (dominant, recessive, log-additive, codominant and overdominant) were used for all analyses. Adjustment variables were age, sex and center. For each polymorphism, p values were computed using the likelihood ratio test (LRT) between a model with the polymorphism and a null model without it. These analyses were performed with the "SNPassoc" R package [21]. We considered the associations between all SNPs and each phenotype under a given heritage model as the family test, i.e. the number of test was equal to the

TABLE 1 Characteristics of study population

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
BMI (Kg/m²)	21.3 ± 3.7	21.4 ± 4.0	21.3 ± 3.4
Overweight (%)	22	24	20
Waist circumference (cm)	72 ± 8	74 ± 9	70 ± 8
Waist/Height ratio	0.44 ± 0.05	0.44 ± 0.05	0.44 ± 0.05
Hip circumference (cm)	91 ± 9	90 ± 9	93 ± 8
Waist/Hip ratio	0.79 ± 0.06	0.82 ± 0.05	0.76 ± 0.06
Body fat (%)	23.6 ± 9	20.3 ± 11.0	26.3 ± 6.9
Fat mass index (Kg/m²)	5.3 ± 3.0	4.5 ± 3.6	5.8 ± 2.4

BMI, body mass index; FMI, fat mass index

number of SNPs. Therefore a corrected p value for multiple comparison following the Bonferroni method would be 0.01 (0.05/5). We selected the significant associations to perform further haplotype analysis. Given these associations were used in further analyses and the linkage disequilibrium existent between gene polymorphisms (see below), we performed a exploratory selection of associations using a method to control the expected proportion of false positives (False Discovery Rate [FDR])[22,23] instead the Bonferroni correction, which is more stringent[24]. Therefore, associations with FDR < 0.1 were used in haplotype analyses.

Linkage disequilibrium between polymorphisms and haplotype block structures were evaluated with Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview) and haplo.stats [25]. First, haplotype blocks were generated by the algorithm of four-gamete rules [26]. For each block, we tested if the observed frequencies of haplotypes were deviated from expected under linkage equilibrium. Finally, we assessed the association between haplotypes and phenotypes by means of a permutation procedure. Only additive and dominant models were considered given the low frequency of some haplotypes. For those significant associations we performed regressions between haplotypes and phenotypes with the purpose of testing significant differences between haplotype levels. Given the low number of comparisons (only four haplotypes disregarding those with very low frequency), we did not correct the p values for the comparison between levels of haplotype.

Results

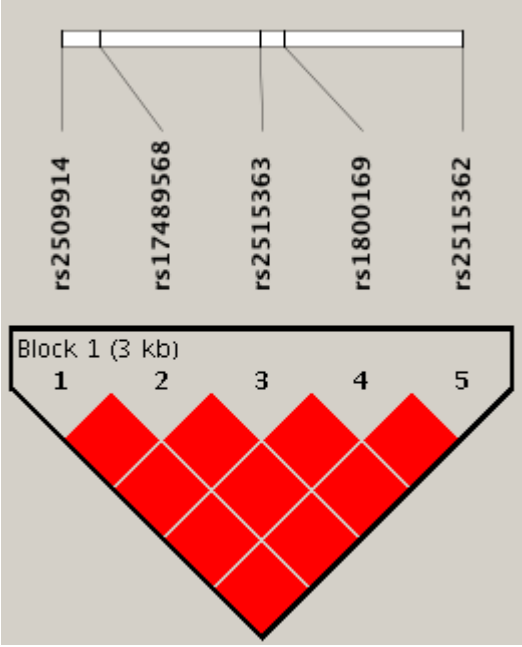
Characteristics of the study sample are shown in Table 1.

Association between CNTF polymorphisms and adiposity markers

Three of the five SNPs analysed showed significant associations with adiposity markers after the Bonferroni correction (rs2509914, rs2515363 and rs2515362; Figures 2,3,4). The significant associations after a less stringent correction (FDR<0.05) for all markers are shown in supplementary appendix (Figures 1-6), along with p values for all associations (Figures 7-14).

We observed an association of minor T allele of rs2509914 polymorphism with higher BMI under recessive model (p=0.00319), higher waist circumference under

FIGURE 1 Haploview result belonging to block 1 of *CNTF* polymorphisms, which contains (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362). Red colour boxes means 100% of linkage disequilibrium (D' = 1) between SNPs according to genotyping data of this study.



codominant, recessive and additive model (p=0.00107, p=0.00034 and p=0.00071 respectively), higher waist to height ratio under codominant, recessive and additive models (p=0.00013, p=5e-05 and p=8e-05 respectively) and with higher waist to hip ratio under dominant, codominant, recessive and additive models (p=0.00305, p=0.00262, p=0.00736 and p=0.00056 respectively) (Figure 2).

Regarding the rs2515363 polymorphism, we observed an association of minor C allele polymorphism with higher BMI under codominant and recessive model (p=0.00841 and p=0.00204 respectively), higher waist circumference under codominant, recessive and additive model (p=0.00082, p=0.00023 and p=0.00065 respectively), higher waist to height ratio under codominant, recessive and additive models (p=0.00012, p=4e-05 and p=8e-05), and higher waist to hip ratio under dominant, codominant, recessive and additive model (p=0.0035, p=0.00303, p=0.00775 and p=0.00066 respectively).

Finally, we observed an association of minor G allele of rs2515362 polymorphism with a higher BMI under codominant and recessive heritage model (p=0.00781 and p=0.00188 respectively), higher waist circumference under codominant, recessive and additive model (p=0.00087, p=0.00027 and p=0.00059 respectively), higher waist to height ratio under codominant, recessive and additive models (p=0.00011, p=4e-05 and p=7e-05 respectively), higher hip circumference (p=0.00961; recessive model) and higher waist to hip ratio under dominant, codominant and additive models (p=0.00254, p=0.00291 and p=0.00065 respectively).

Association between CNTF polymorphism haplotypes and adiposity markers

CNTF block contains rs2509914, rs17489568, rs2515363 rs1800169 and rs2515362 polymorphisms (Fig. 1). Haplotype CCGGA of *CNTF* was significantly associated to lower BMI than TCCGG (global p=0.0098; difference between groups 0.04; 95CI = 0.01 - 0.06; p=0.0019; under

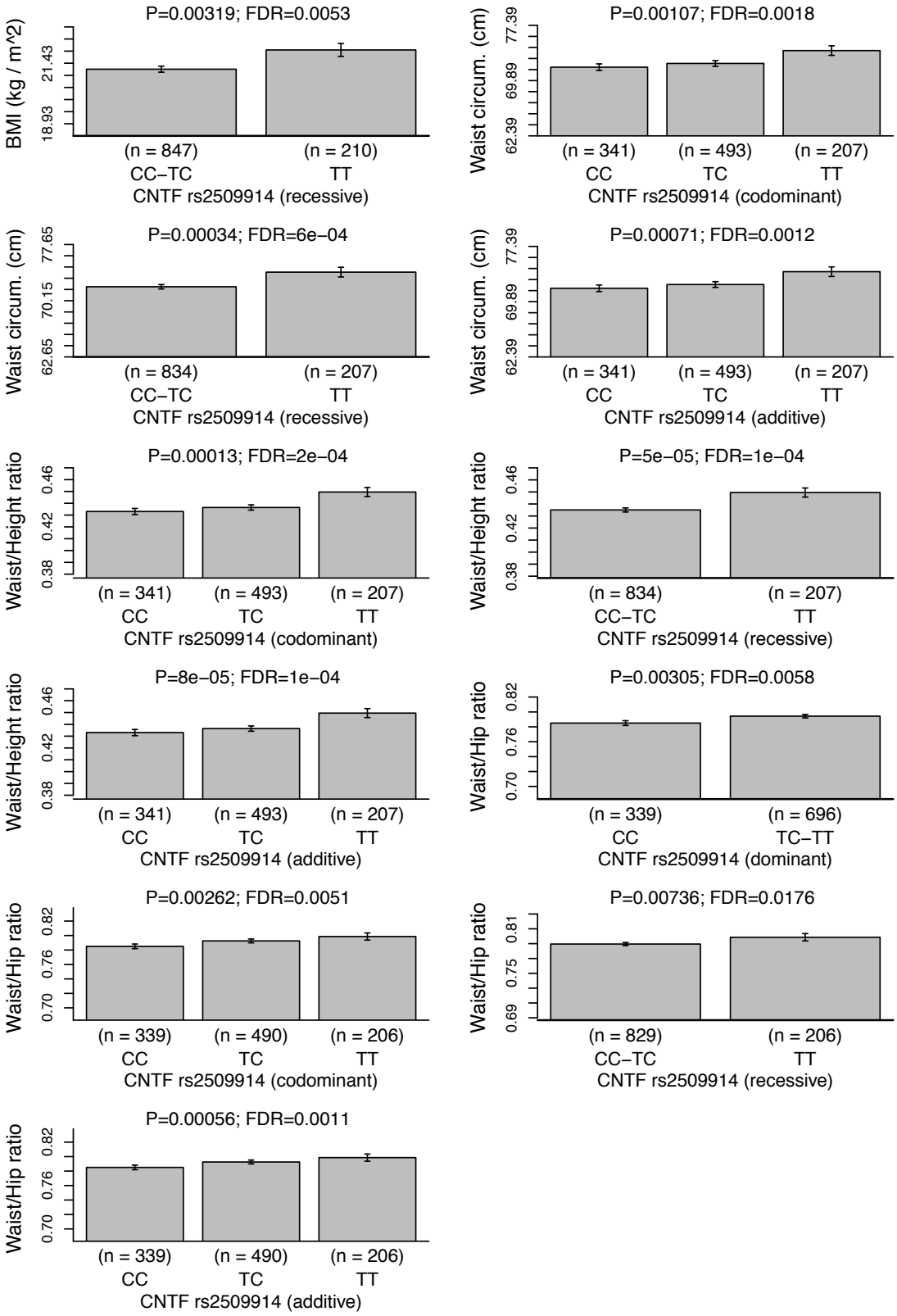


FIGURE 2 Association between rs2509914 (*CNTF*) polymorphism and adiposity markers. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age.

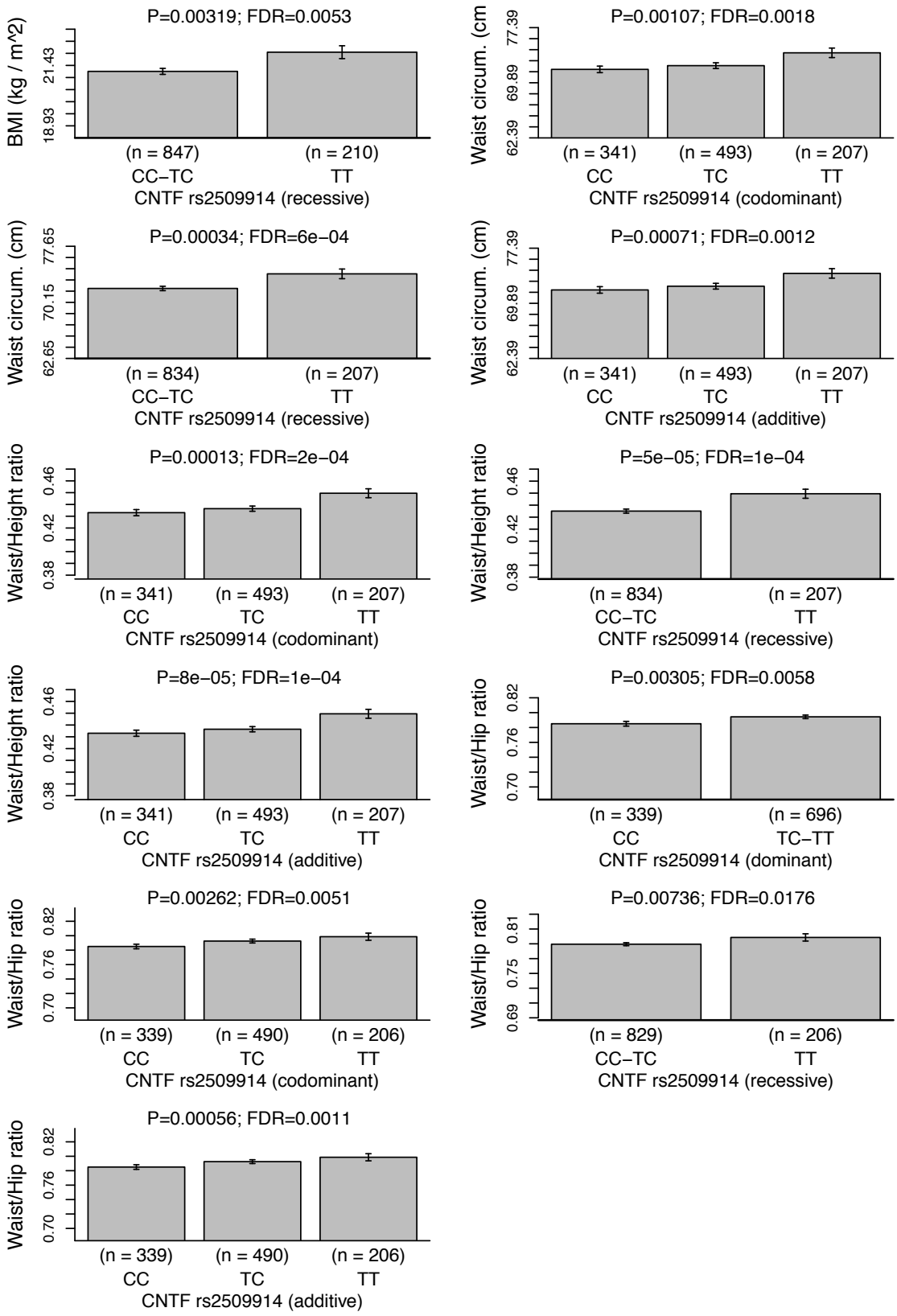


FIGURE 2 (cont.) Association between rs2509914 (*CNTF*) polymorphism and adiposity markers. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age.

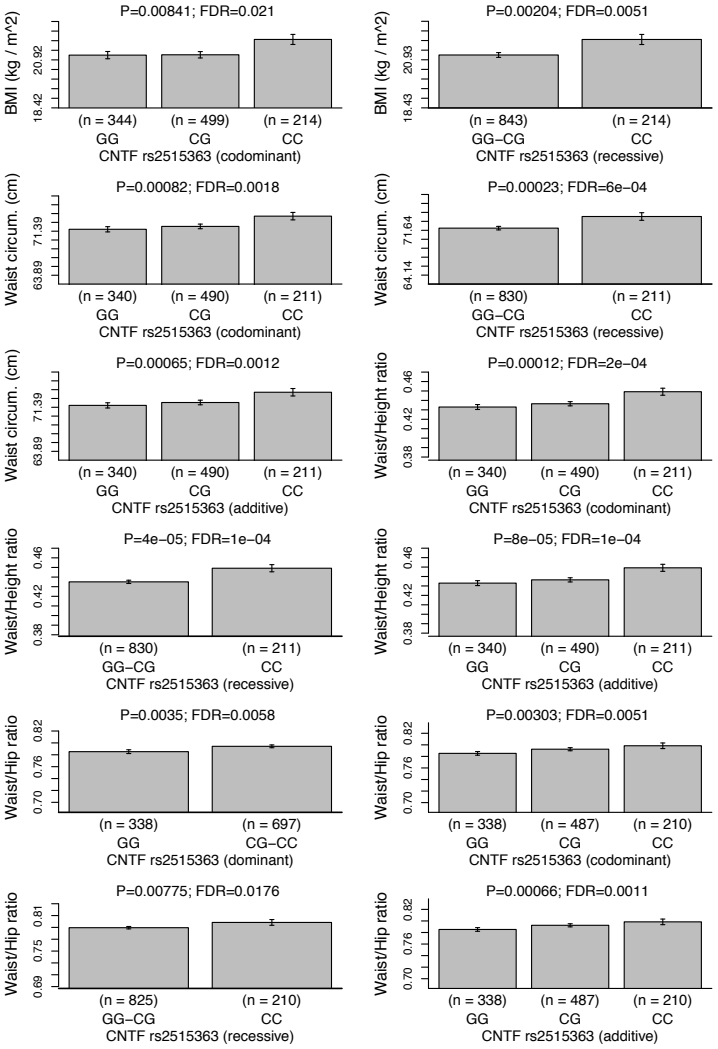


FIGURE 3 Association between rs2515363 (*CNTF*) polymorphism and adiposity markers. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age.

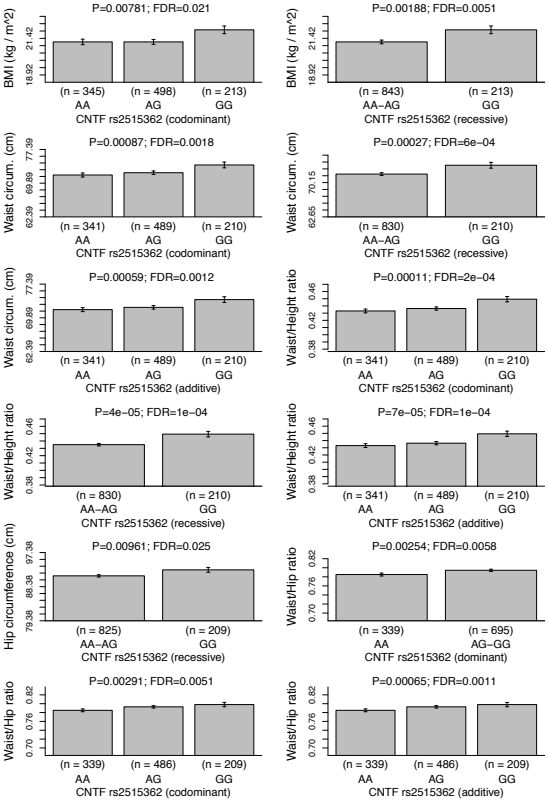
dominant model // global p= 0.04555; difference between groups 0.02; 95CI = 0.00 - 0.03; p=0.018; under additive model; differences between groups obtained from models with the response variable log transformed), to lower waist circumference (global p=0.0049; difference between groups 0.02; 95CI = 0.01 - 0.03; p=0.004: under dominant model // global p=0.00925; difference between groups 0.02; 95CI = 0.01 - 0.03; p=0.0003; under additive model), to lower waist/height (global p=7e-04; difference between groups 0.02; 95CI = 0.01 - 0.03; p=0.0012; under dominant model // global p=0.002; difference between groups 0.02; 95CI = 0.01 - 0.03; p=0.0001; under additive model) and to lower waist/hip (global p=0.01305; difference between groups 0.01; 95CI = 0.01 - 0.02; p= 0.0005; under dominant model // global p=0.01455; difference between groups 0.01; 95CI = 0.01 - 0.02; p=0.0002; under additive model).

Discussion

Our findings show a solid association between rs2509914, rs2515363 and rs2515362 *CNTF* polymorphisms with adiposity markers such as BMI, waist circumference, waist to height ratio and waist to hip ratio in European adolescents. We also observed a correlation between a *CNTF* haplotype (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362) with BMI, waist circumference, waist to hip and waist to height. Taken together, these findings suggest a role of CNTF in boy weight regulation in adolescents.

To our knowledge, only one study examined the association between CNTF polymorphisms and adiposity markers in humans. Heidema *et al.*[27] followed a cohort of Dutch adult population (N=545) of stable and weight gainers, and found a significant association of A allele of *rs1800169* with weight gain (odds ratio (OR)=2.15, 95%CI: 1.27–3.64, p=0.004) in women. These findings partially concur with our results. We

FIGURE 4 Association between rs2515362 (*CNTF*) polymorphism and adiposity markers. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age.



also showed an association of G allele of rs1800169 with decreased levels of adiposity markers in the context of *CNTF* haplotype. However, we observed this association in both women and men.

A plausible mechanism could be that *CNTF* polymorphisms or haplotypes of them lead to express CNTF proteins with altered function, which produces a potential status of predisposition of weight gain. As we mentioned before, *CNTF* plays a role in the regulation of body weight [2]. It is described that, high levels of *CNTF* either due to response to neuronal injury producing inflammatory response and fever[28] or by administration of recombinant *CNTF*[9], leads to weight loss. This weight loss is most likely trough satiety mechanisms owing to similarities between *CNTF* and leptin signalling cascade such as: i) close relationships between leptin receptor and *CNTF* receptor complex, ii) overlapping in molecules activation between *CNTF* and *leptin*, like *STAT3* and, iii) *CNTF* and *leptin* receptors have overlapping distributions in some hypothalamic nuclei involved in feeding control[2]. So these similarities with leptin may explain the underlying mechanism trough some polymorphisms of *CNTF* could lead to functionally altered CNTF proteins and therefore have an effect in control of satiety and weight control.

Results of our study must to be taken with caution owing to its cross-sectional nature. Another limitation of this study is the unknown of relatedness patterns among the participants such as ethnic/racial make-up of the sample.

In conclusion, we observed an association between rs2509914, rs2515363 and rs2515362 *CNTF* polymorphisms with adiposity markers in adolescents from nine European countries. We also observed a haplotype association a *CNTF* haplotype (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362) with several adiposity markers. These findings suggest that *CNTF* may have an important role in the development of

overweight/obesity predisposition already in adolescents.

References

1. The GBD 2015 Obesity Collaborators, A. F. Health Effects of Overweight and Obesity in 195 Countries over 25 Years. *N. Engl. J. Med.* **377,** 13–27 (2017).
2. Xu, B. & Xie, X. Neurotrophic factor control of satiety and body weight. *Nat. Rev. Neurosci.* **17,** 282–292 (2016).
3. Farooqi, I. S. & O'Rahilly, S. Genetics of obesity in humans. *Endocr. Rev.* **27,** 710–718 (2006).
4. Scuteri, A. *et al.* Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet.* **3,** 1200–1210 (2007).
5. Vogel, C. I. G. *et al.* Common variants near MC4R: Exploring gender effects in overweight and obese children and adolescents participating in a lifestyle intervention. *Obes. Facts* **4,** 67–75 (2011).
6. Huang, E. J. & Reichardt, L. F. Neurotrophins: Roles in Neuronal Development and Function. *Annu. Rev. Neurosci.* **24,** 677–736 (2001).
7. Gray, J. *et al.* Hyperphagia, severe obesity, impaired cognitive function, and hyperactivity associated with functional loss of one copy of the brain-derived neurotrophic factor (BDNF) gene. *Diabetes* **55,** 3366–71 (2006).
8. Yeo, G. S. H. *et al.* A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. *Nat. Neurosci.* **7,** 1187–1189 (2004).
9. Miller, R. G. *et al.* A placebo-controlled trial of recombinant human ciliary neurotrophic (rhCNTF) factor in amyotrophic lateral sclerosis. rhCNTF ALS Study Group. *Ann. Neurol.* **39,** 256–260 (1996).
10. Farr, O. M., Gavrieli, A. & Mantzoros, C. S. HHS Public Access. 1–14 (2016). doi:10.1097/MED.0000000000000184.Leptin
11. Cron, L., Allen, T. & Febbraio, M. A. The role of gp130 receptor cytokines in the regulation of metabolic homeostasis. *J. Exp. Biol.* **219,** 259–265 (2016).
12. Takahashi, R. *et al.* A null mutation in the human CNTF gene is not causally related to neurological diseases. *Nat. Genet.* **7,** 79–84 (1994).
13. Masu, Y. *et al.* Disruption of the CNTF gene results in motor neuron degeneration. *Nature* **365,** 27–32 (1993).
14. O'Dell, S. D. *et al.* Null mutation in human ciliary neurotrophic factor gene confers higher body mass index in males. *Eur. J. Hum. Genet.* **10,** 749–752 (2002).
15. Vogels, N. *et al.* Relation of weight maintenance and

dietary restraint to peroxisome proliferator-activated receptor γ2, glucocorticoid receptor, and ciliary neurotrophic factor polymorphisms. *Am. J. Clin. Nutr.* **82,** 740–746 (2005).
16. Jacob, A. C. *et al.* Ciliary neurotrophic factor (CNTF) genotype and body composition. *Eur. J. Hum. Genet.* **12,** 372–376 (2004).
17. Münzberg, H. *et al.* Screening for variability in the ciliary neurotrophic factor (CNTF) gene: No evidence for association with human obesity. *Exp. Clin. Endocrinol. Diabetes* **106,** 108–112 (1998).
18. Moreno, L. A. *et al.* Design and implementation of the healthy lifestyle in europe by nutrition in adolescence cross-sectional study. *Int. J. Obes.* **32,** S4–S11 (2008).
19. Cole, T. J., Bellizzi, M. C., Flegal, K. M. & Dietz, W. H. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* **320,** 1240–3 (2000).
20. Slaughter, M. *et al.* Skinfold equations for estimation of body fatness in children and youth. *Hum. Biol.* **60,** 709–723 (1998).
21. González, J. R. *et al.* SNPassoc: An R package to perform whole genome association studies. *Bioinformatics* **23,** 644–645 (2007).
22. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B J. R. Stat. Soc. B* **57,** 289–300 (1995).
23. Qu, H.-Q. H.-Q., Tien, M. & Polychronakos, C. Statistical significance in genetic association studies. *Clin. Investig. Med.* **33,** E266–E270 (2010).
24. Sham, P. C. & Purcell, S. M. Statistical power and significance testing in large-scale genetic studies. *Nat. Rev. Genet.* **15,** 335–346 (2014).
25. Sinnwell, A. J. & Maintainer Jason P Sinnwell, S. D. Title Statistical Analysis of Haplotypes with Traits and Covariates when Linkage Phase is Ambiguous. (2016).
26. Wang, N., Akey, J. M., Zhang, K., Chakraborty, R. & Jin, L. Distribution of Recombination Crossovers and the Origin of Haplotype Blocks: The Interplay of Population History, Recombination, and Mutation. *Am. J. Hum. Genet.* **71,** 1227–1234 (2002).
27. Heidema, A. G. *et al.* Sex-specific effects of CNTF, IL6 and UCP2 polymorphisms on weight gain. *Physiol. Behav.* **99,** 1–7 (2010).
28. Shapiro, L., Zhang, X. X., Rupp, R. G., Wolff, S. M. & Dinarello, C. a. Ciliary neurotrophic factor is an endogenous pyrogen. *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8614–8 (1993).

CHAPTER 2: Association between *UCP1*, *UCP2* and *UCP3* gene polymorphisms with markers of adiposity in European adolescents: the HELENA study

ABSTRACT

Aims: To examine the association between *UCP1*, *UCP2* and *UCP3* gene polymorphisms with adiposity markers in European adolescents, and to test if there were gene interactions with objectively measured physical activity and adiposity.
Methods: A cross-sectional study that involves 1.057 European adolescents (12-18 years old) from the Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study. A total of 18 polymorphisms in *UCP1*, *UCP2* and *UCP3* genes were genotyped. We measured weight, height, waist and hip circumferences and triceps and subscapular skinfold thickness. Physical activity was objectively measured by accelerometry during 7 days.
Results: The C allele of the *UCP1* rs6536991 polymorphism was associated with a lower risk of overweight [odds ratio (OR): T/C + C/C vs. T/T) = 0.72; 95% confidence interval (CI): 0.53 to 0.98; P=0.034; false discovery rate (FDR)=0.048)]. There was a significant interaction between *UCP1* rs2071415 polymorphism and physical activity with waist-to-hip ratio (P = 0.006; FDR = 0.026). Adolescents who did not meet the physical activity recommendations (less than 60min/day of moderate to vigorous physical activity) and carrying the C/C genotype had higher waist-to-hip ratio (+ 0.067; 95% CI, 0.028 to 0.106; P = 0.003), while no differences across genotypes were observed in adolescents meeting the recommendations.
Conclusions: Two *UCP1* polymorphisms were associated with adiposity in European adolescents. Meeting the daily physical activity recommendations may overcome the effect of the *UCP1* rs2071415 polymorphism on obesity-related traits.

Keywords: Physical Activity, Brown Adipose Tissue, Genetic Susceptibility, Adolescents, Uncoupling Protein

INTRODUCTION

Obesity is a major public health problem throughout the world. Despite later reports suggest a plateau in children's and adolescent's body mass index (BMI) from high-income countries, there are still little evidences on strategies to stop this pandemic[1].

Obesity is a result of a complex interaction between environmental and genetic factors[2]. Several studies showed that physical activity may overcome the effect of several gene polymorphisms on obesity-related traits. A genome-wide meta-analysis of 200,452 European adults showed that physical activity may attenuate the deleterious effect of the *FTO* (fat mass- and obesity-associated) gene polymorphisms on obesity[3]. In adolescents, we showed that meeting the daily physical activity recommendations (at least 60 minutes/day of moderate to

vigorous physical activity) may offset the genetic predisposition to obesity associated with the *FTO* rs9939609 polymorphism in European adolescents[4].

The strong genetic influence in obesity has been described as a non-mendelian way of inheritance. Only few percent of cases are monogenic obesity type. In these cases, monogenic obesity genes are involved in the control of appetite center and satiety like leptin (LEP), leptin receptor (LEPR), pro-opiomelacortin (POMC) or prohormoneconvertase 1 (PCSK1) [5]. However, 95% of the cases of obesity can be explained by genetic variants of multiple genes and complex gene-gene and gene-lifestyle interactions [2,6,7], among which are uncoupling proteins (UCPs) gene polymorphisms [8].

The most known *UCP* genes include *UCP1*, *UCP2* and *UCP3*. *UCP1* is responsible of heat production through non-shivering thermogenesis in brown adipose tissue (BAT) [9]. Less clear is, however, the role of *UCP2* and *UCP3* genetic variants, which have been related with obesity phenotypes through the potential influence on muscle metabolism due to the high expression of these genes in skeletal muscle [10], diabetes mellitus and lipid/ lipoprotein-related diseases [11]. UCP2 seems to be involved in the control of reactive oxygen species (ROS) production, a modulator of insulin secretion and a regulator of mitochondrial fatty acid oxidation[12]. More recently, Caron et al.[13] reported that *UCP2* may have a regulating role in thermogenesis of BAT. UCP3 role relates with the coupling regulation of mitochondrial respiration in skeletal muscle mitochondria[14] and as mediator of thermogenesis[15]. The *UCP1* rs6536991 polymorphism[16], *UCP2* rs659366[17], rs660339 polymorphisms[18–21] and *UCP3* rs1800849, rs2075577 polymorphisms[21,22] have been associated with obesity traits, but most of them have been studied in adults and the results are so far controversial. Several studies suggested that the rs659366-A allele (UCP-2) might be protective against obesity in 55 age average subjects[17], whereas others found an association of the rs659366-A allele with a borderline increase of fat body mass index (FBMI) [19] and higher risk of central obesity[20].

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provide an excellent opportunity to study the association between *UCP1*, *UCP2* and *UCP3* gene polymorphisms with adiposity makers in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 18 polymorphisms (SNPs) of the *UCP1*, *UCP2* and *UCP3* genes as well as markers of adiposity. To our knowledge, *UCP* polymorphisms have not been identified in GWAS of

body weight or body composition in adults or other age groups.

The aim of this study was therefore to examine the association between 18 *UCP1*, *UCP2* and *UCP3* polymorphisms with total and central adiposity markers in European adolescents, and to test if there were gene x physical activity interactions on adiposity phenotypes.

Material and methods

Participants

Adolescents were part of the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) cross-sectional study. A total of 3865 adolescents (12-18 year old) of 10 centers in nine European countries were recruited between 2006 and 2007. Adolescents were randomly selected from schools by using a proportional cluster sampling method, and age was taken into account. One-third of the classes were randomly selected for blood collection, resulting in a total of 1155 blood samples for the subsequent clinical biochemistry assays and genetic analyses. Among these participants, 1057 individuals (552 girls) with data on *UCP1, UCP2 and UCP3* gene polymorphisms, adiposity phenotypes and physical activity were included in this study. After receiving complete information about the aims and methods of the study, all adolescents and their parents or guardians signed an informed written consent. All participants met the general HELENA inclusion criteria[23]. The study was performed following the ethical guidelines of the Declaration of Helsinki 1961 (revision of Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries. The corresponding local human research review committees of the involved centers approved the protocol.

Assessment of adiposity

Weight and height were measured following standard methods. Waist and hip circumference was measured in triplicate with an anthropometric unelastic tape (SECA 200; Seca Deutschland, Hamburg, Germany) and was used as a surrogate measure of central body fat. We calculated waist to height and waist to hip ratios. BMI was calculated as weight in kilograms divided by height in meters squared. Adolescents were classified according to BMI (kg/m^2) as normal weight, overweight or obese categories according to Cole et al.[24]. The overweight-obese categories were grouped into one category (hereafter called overweight). Skinfold thickness was measured to the nearest 0.2 mm in triplicate on the left side at the biceps, triceps, subscapularis, suprailium, thigh, and medial calf with a Holtain Caliper (Holtain Ltd, Crymmych, Wales). Body fat percentage was calculated from skinfold thicknesses (triceps and subscapular) using the equations by Slaughter et al.[25]. Finally, fat mass index (FMI) was

calculated as fat mass in kilograms divided by height in meters squared.

Assessment of physical activity

Physical activity was assessed during 7 consecutive days with a uniaxial accelerometer (GT1M; ActiGraph, Pensacola, Florida) attached to the lower back[26] . Adolescents were instructed to wear the accelerometer during all time awake and to remove it only during water-based activities. At least 3 days of recording with a minimum of 8 hours registered per day was set as an inclusion criterion[26]. The time-sampling interval (epoch) was set at 15 seconds. We calculated the time engaged in at least moderate physical activity (≥3 metabolic equivalents) based on a standardized cutoff of 2000 counts/min or more. Moderate to vigorous physical activity was dichotomized into less than 60 min/day and 60 min/day or longer[26].

Genotyping

The genotyping was done by an Illumina system (Illumina, Inc, San Diego, California) using the GoldenGate technology (GoldenGate Software, Inc, San Francisco, California). The mean genotyping success rate was 99.84%. All genotype distributions respected Hardy-Weinberg equilibrium (P > 0.2; Appendix S1). Some polymorphisms exhibited linkage disequilibrium between them (Appendix S2, S3).

Data analysis

Deviations from Hardy-Weinberg equilibrium were assessed by means of an exact test and considering a P value of 0.05 as a threshold. Linkage disequilibrium between polymorphisms was evaluated with "genetics" R package. Associations between SNPs and phenotypes were analyzed by means of general linear models (GLM) using Gaussian and Binomial error distributions for continuous and discrete phenotypes, respectively. Interactions between SNPs of different *UCP* genes were assessed using the same models but including an interaction term for each gene pair. Interaction with physical activity was assessed for SNPs that were significantly associated with obesity phenotypes including an interaction term with both factors. Moreover, we performed the analyses stratified by moderate to vigorous physical activity categories (<60 and ≥60 min/day). Five genetic models (dominant recessive, log-additive, codominant and over dominant and additive) were used for all analyses except in those that rs2071416, rs2735572 and rs17132534 were involved. These polymorphisms were analyzed using only dominant model due to the low number of minor homozygotes (MAF < 0.1; Appendix S1). Previous studies highlighted the association between non-additive models with UCPs, which indicates the importance of perform this five models and compare the additive models with non-additives ones [27]. In all analyses,

adjustment variables were age, gender and center. For each polymorphism, P values were computed using the likelihood ratio test (LRT) between a model with the polymorphism or interaction term and a null model without it.

With the purpose of controlling the chance of any false positives, we corrected the significance level of 0.05 by the number of test (polymorphisms) for each genetic model (Bonferroni correction). Therefore, significance threshold was 0.0033 for all models except for dominant model, in which it was 0.0028. Given that some of studied SNPs were in linkage disequilibrium (Appendix S2, S3), the number of independent test would be lower than number of studied polymorphisms and thus Bonferroni correction could be potentially over conservative[28]. Because of this, we also used a less stringent approach, which controls the expected proportion of false positives (False Discovery Rate [FDR]). As in the case of Bonferroni, the family test included all genotyped markers for a given genetic model (18 tests). Significance for the interaction analyses was determined in the same way, i.e. a family test included the interaction between physical activity and the 18 genotyped markers under a given heritage model. All analyses were performed using the "SNPassoc" package in the R environment 3.4.1.

Results

Characteristics of the study sample are shown in Table 1.

Association between UCP polymorphisms and markers of adiposity

Only one of the 18 studied polymorphisms was individually associated with overweight phenotypes after multiple-comparison corrections (Appendix S1 & Fig. 1; Appendix S4:S11). The minor C allele of the *UCP1* rs6536991 polymorphism was associated with a lower risk of overweight [odds ratio (OR): T/C + C/C vs. T/T = 0.72; 95% confidence interval (CI), 0.53 to 0.98; P=0.034; FDR=0.048; Figure 1). The *UCP1* rs6536991 polymorphism was not however nominally associated with BMI (Appendix S5) or body fat percentage (Appendix S10). We found no significant gene-gene interactions, whereas there was an interaction of physical activity with *UCP1* rs2071415 and *UCP3* rs2075577 polymorphisms on waist to hip ratio under codominant model (P<0.0001; FDR=0.004, Appendix S12). However, the number of individuals in the interaction group were rather low.

Interaction between UCP polymorphisms, physical activity and markers of adiposity

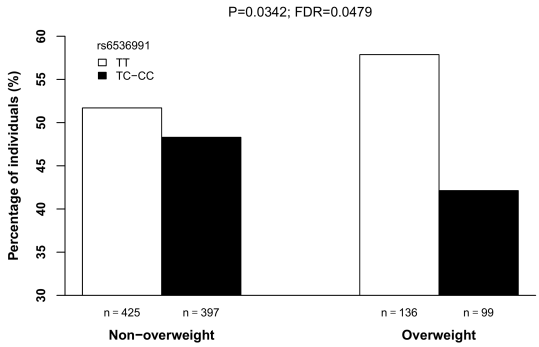
There was a significant interaction between physical activity and the *UCP1* rs2071415 polymorphism on waist to hip ratio (P=0.006; FDR=0.026; Figure 2). The C/C

Table 1: Characteristics of the study population

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
BMI (Kg/m²)	21.3 ± 3.7	21.4 ± 4.0	21.3 ± 3.4
Overweight (%)	22	24	20
Waist circumference (cm)	72 ± 8	74 ± 9	70 ± 8
Waist/Height ratio	0.44 ± 0.05	0.44 ± 0.05	0.44 ± 0.05
Hip circumference (cm)	91 ± 9	90 ± 9	93 ± 8
Waist/Hip ratio	0.79 ± 0.06	0.82 ± 0.05	0.76 ± 0.06
Body fat (%)	23.6 ± 9	20.3 ± 11.0	26.3 ± 6.9
Fat mass index (Kg/m²)	5.3 ± 3.0	4.5 ± 3.6	5.8 ± 2.4

BMI, body mass index; FMI, fat mass index

FIGURE 1 Association between overweight and UCP1 rs6536991 polymorphism under a dominant model. P values are adjusted for centre, sex, and age



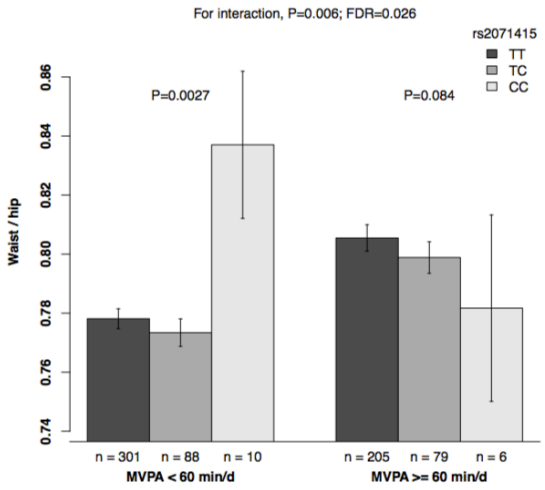
genotype was associated with higher waist to hip ratio (+ 0.067; 95%CI, 0.028 to 0.106; P = 0.003) in adolescents who spent less than 60 min/day of moderate to vigorous physical activity (n = 399). On the contrary, the C/C genotype of the *UCP1* rs2071415 polymorphism was not associated with higher waist to hip ratio (– 0.047; 95%CI, -0.099 to 0.004; P = 0.084) in adolescents who spent at least 60 min/day of moderate to vigorous physical activity (n = 290).

Discussion

The results of the present study show that the C allele of the *UCP1* rs6536991 polymorphism was associated with a lower risk of overweight and obesity (OR=0.72) in European adolescents from 9 countries. We also observed an interaction between physical activity and the *UCP1*rs2071415 polymorphism on waist to hip ratio. Adolescents meeting the daily physical activity recommendations may overcome the effect of the *UCP1* rs2071415 polymorphism on waist to hip ratio. Our results of *UCP1* polymorphisms are in agreement with others[16]. Ramos *et al.*[16] showed a significant association between the *UCP1* rs6536991 polymorphism and obesity and BMI in 352 Brazilian adults. They showed that the C allele was associated with a lower risk of obesity (OR=0.69) and a lower BMI in individuals with no obesity.

We did not find significant associations between *UCP2* or *UCP3* polymorphisms and adiposity markers, which is in contrast with the finding by Van Abeelen *et al.*[22] in Dutch men (40-80 years old). They reported a significant association between homozygosity for the minor allele (C) of *UCP3* rs2075577 polymorphism and BMI but not with other adiposity phenotypes such as waist to hip ratio. Another study[21] reported a significant association between the (*UCP3* rs2075577–*UCP2* rs660339) haplotype with BMI. The frequency of the C–T haplotype in patients with obesity was significantly higher than that

FIGURE 2 Interaction between the UCP1 rs2071415 SNP and levels of moderate to vigorous physical activity (MVPA; <60 min/d vs ≥60 min/d) on waist to hip ratio, under an additive model. Error bars represent ± SE. P values adjusted for centre, sex, and age



seen in subjects without it. However, these results were observed in females only. These association discordances between polymorphism and phenotypes may be due to the fact that they are population dependent, with possibly different allele frequencies and penetrance in these populations. Also differences in age, inter-country differences in lifestyle behaviours and sample sizes are important.

A plausible explanation of the observed associations between polymorphisms in the *UCP1* gene and obesity phenotypes could be because this polymorphism may lead to less functional UCPs proteins. As a result, uncoupling activity could be decreased and therefore reduce heat dissipation as well as lipid oxidation[9], leading to overweight/obesity. Recent studies have described that calcium cycling in the skeletal muscle has a similar role than UCP1 in non-shivering thermogenesis in muscle and BAT of rabbits [29]. Indeed uncoupling ATP hydrolysis in sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) mediated by sarcolipin in mice with overexpression of this small transmembrane proteolipid increased basal metabolic rate and decreased diet-induced obesity risk [30]. In line with this findings, overexpression of UCP2 was associated with an improved fatty acid oxidation [31] suggesting a possible relevant role on the muscle function because *UCP2* and *UCP3* genes are highly expressed in skeletal muscle[10]. More studies are needed to understand if sarcolipin could compensate a functional deficit of UCP1 in overweight individuals such as fatty acid utilization by UCP2.

Findings from our study should be taken with caution owing to its cross-sectional nature. Lifestyle intervention studies in adolescents are needed to determine to what extent the effect of *UCP* genes on obesity-related traits can be modified, especially in genetically predisposed individuals. Results about gene-gene interaction between *UCP1* and *UCP3* polymorphisms must be taken with caution due to low number of individuals in the interaction group with significant differences, and should be confirmed in other studies

with larger sample sizes. Unfortunately, we have no information on relatedness patterns among the participants, and we do not know the ethnic/racial make-up of the sample.

In conclusion, we observed an association between the *UCP1* rs6536991 polymorphism and the risk of overweight in adolescents. Our results also suggest that physical activity could compensate the deleterious effect of the *UCP1* rs2071415 polymorphism on adiposity markers.

References

1. NCD Risk Factor Collaboration (NCD-RisC). Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 19·2 million participants. *Lancet*. 2016;387(10026):1377-1396. doi:10.1016/S0140-6736(16)30054-X.
2. Mäkelä J, Lagström H, Pitkänen N, et al. Genetic risk clustering increases children's body weight at 2 years of age - the STEPS Study. *Pediatr Obes*. 2016;11(6):459-467. doi:10.1111/ijpo.12087.
3. Graff M, Scott RA, Justice AE, et al. Genome-wide physical activity interactions in adiposity — A meta-analysis of 200,452 adults. *PLoS Genet*. 2017;13(4):1-26. doi:10.1371/journal.pgen.1006528.
4. Ruiz JR, Labayen I, Ortega FB, et al. Attenuation of the effect of the FTO rs9939609 polymorphism on total and central body fat by physical activity in adolescents: the HELENA study. *Arch Pediatr Adolesc Med*. 2010;164(4):328-333. doi:10.1001/archpediatrics.2010.29.
5. Farooqi IS, O'Rahilly S. Genetics of obesity in humans. *Endocr Rev*. 2006;27(7):710-718. doi:10.1210/er.2006-0040.
6. Sheikh AB, Nasrullah A, Haq S, et al. The Interplay of Genetics and Environmental Factors in the Development of Obesity. *Cureus*. 2017;9(7):e1435. doi:10.7759/cureus.1435.
7. Zhao H, Wilkinson A, Shen J, Wu X, Chow WH. Genetic polymorphisms in genes related to risk-taking behaviours predicting body mass index trajectory among Mexican American adolescents. *Pediatr Obes*. 2017;12(5):356-362. doi:10.1111/ijpo.12151.
8. Brondani LA, Assmann TS, de Souza BM, Bouças AP, Canani LH, Crispim D. Meta-analysis reveals the association of common variants in the uncoupling protein (UCP) 1-3 genes with body mass index variability. *PLoS One*. 2014;9(5):e96411.

doi:10.1371/journal.pone.0096411.

9. Golozoubova V, Cannon B, Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab*. 2006;291(2):E350-E357. doi:10.1152/ajpendo.00387.2005.
10. Schrauwen P, Hesselink M. UCP2 and UCP3 in muscle controlling body metabolism. *J Exp Biol*. 2002;205(Pt 15):2275-2285.
11. Jia JJ, Zhang X, Ge CR, Jois M. The polymorphisms of UCP2 and UCP3 genes associated with fat metabolism, obesity and diabetes: Etiology and pathophysiology. *Obes Rev*. 2009;10(5):519-526. doi:10.1111/j.1467-789X.2009.00569.x.
12. Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F. UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? *IUBMB Life*. 2009;61(7):762-767. doi:10.1002/iub.188.
13. Caron A, Labbé SM, Carter S, et al. Loss of UCP2 impairs cold-induced non-shivering thermogenesis by promoting a shift toward glucose utilization in brown adipose tissue. *Biochimie*. 2017;134:118-126. doi:10.1016/j.biochi.2017.01.006.
14. Vidal-Puig AJ, Grujic D, Zhang C-Y, et al. Energy Metabolism in Uncoupling Protein 3 Gene Knockout Mice. *J Biol Chem*. 2000;275(21):16258-16266. doi:10.1074/jbc.M910179199.
15. Riley CL, Dao C, Kenaston MA, et al. The Complementary and Divergent Roles of Uncoupling Proteins 1 and 3 in Thermoregulation. *J Physiol*. 2016;594(24):7455–7464. doi:10.1113/JP272971.This.
16. Ramos A V, Bastos-Rodrigues L, Resende BA, et al. The contribution of FTO and UCP-1 SNPs to extreme obesity, diabetes and cardiovascular risk in Brazilian individuals. *BMC Med Genet*. 2012;13(1):101. doi:10.1186/1471-2350-13-101.
17. Esterbauer H, Schneitler C, Oberkofler H, et al. A common polymorphism in the promoter of UCP2 is associated with decreased risk of obesity in middle-aged humans. *Nat Genet*. 2001;28(june):178-183. doi:10.1038/88911.
18. Andersen G, Dalgaard LT, Justesen JM, et al. The frequent UCP2 −866G>A polymorphism protects against insulin resistance and is associated with obesity: a study of obesity and related metabolic traits among 17 636 Danes. *Int J Obes*. 2013;37(2):175-181. doi:10.1038/ijo.2012.22.
19. Iqbal Kring SI, Larsen LH,

Holst C, et al. Genotype-phenotype associations in obesity dependent on definition of the obesity phenotype. *Obes Facts*. 2008;1(3):138-145. doi:10.1159/000137665.

20. Martinez-Hervas S, Mansego ML, de Marco G, et al. Polymorphisms of the UCP2 gene are associated with body fat distribution and risk of abdominal obesity in Spanish population. *Eur J Clin Invest*. 2012;42(2):171-178. doi:10.1111/j.1365-2362.2011.02570.x.
21. Kosuge K, Soma M, Nakayama T, et al. Human uncoupling protein 2 and 3 genes are associated with obesity in Japanese. *Endocrine*. 2008;34(1-3):87-95. doi:10.1007/s12020-008-9111-9.
22. van Abeelen AFM, de Krom M, Hendriks J, Grobbee DE, Adan RAH, van der Schouw YT. Variations in the uncoupling protein-3 gene are associated with specific obesity phenotypes. *Eur J Endocrinol*. 2008;158(5):669-676. doi:10.1530/EJE-07-0834.
23. Moreno LA, De Henauw S, González-Gross M, et al. Design and implementation of the healthy lifestyle in europe by nutrition in adolescence cross-sectional study. *Int J Obes*. 2008;32:S4-S11. doi:10.1038/ijo.2008.177.
24. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ*. 2000;320(7244):1240-1243. doi:10.1136/bmj.320.7244.1240.
25. Slaughter M, Lohman T, Boileau R, et al. Skinfold equations for estimation of body fatness in children and youth. *Hum Biol*. 1998;60:709-723.
26. Ruiz JR, Ortega FB, Martínez-Gómez D, et al. Objectively measured physical activity and sedentary time in european adolescents. *Am J Epidemiol*. 2011;174(2):173-184. doi:10.1093/aje/kwr068.
27. Krishnan M, Thompson JMD, Mitchell EA, et al. Analysis of association of gene variants with obesity traits in New Zealand European children at 6 years of age. *Mol BioSyst*. 2017;13(8):1524-1533. doi:10.1039/C7MB00104E.
28. Sham PC, Purcell SM. Statistical power and significance testing in large-scale genetic studies. *Nat Rev Genet*. 2014;15(5):335-346. doi:10.1038/nrg3706.
29. De Meis L, Oliveira GM, Arruda AP, Santos R, Madeiro Da Costa R, Benchimol M. The thermogenic activity of rat brown adipose tissue and rabbit white muscle Ca2+-ATPase. *IUBMB Life*. 2005;57(4-5):337-345.

doi:10.1080/15216540500092534.

30. Maurya SK, Bal NC, Sopariwala DH, et al. Sarcolipin is a key determinant of the basal metabolic rate, and its overexpression enhances energy expenditure and resistance against diet-induced obesity. *J Biol Chem*. 2015;290(17):10840-10849. doi:10.1074/jbc.M115.636878.

31. Kukat A, Dogan SA, Edgar D, et al. Loss of UCP2 Attenuates Mitochondrial Dysfunction without Altering ROS Production and Uncoupling Activity. *PLoS Genet*. 2014;10(6). doi:10.1371/journal.pgen.1004385.

GENETIC POLYMORPHISMS ASSOCIATED TO CARDIOVASCULAR DISEASE RISK FACTORS

CHAPTER 3: Association between *ADIPOQ* gene polymorphisms and cardiovascular disease risk factors in European adolescents: The HELENA study

Introduction

Almost 18 million people die from cardiovascular disease (CVD) worldwide every year[1]. That means 31% global deaths. Exact cause of CVD is still unknown, but numerous risk factors are described, as smoking, high blood pressure, high cholesterol, diabetes, inactivity, overweight/obesity, and family history among others[2].

As most diseases, CVDs development are the result of a complex interplay of genetics and environmental factors. Some studies have shown a strong genetic cause of CVDs as coronary artery disease[3] or sickle cell anemia[4]. Genetic susceptibility to CVDs is commonly set up by multiple genes and polymorphisms acting together. A recent review[5] collected a list of genetic markers that influence HDL levels, LDL levels, triglycerides levels an others, as well as some heritable conditions (e.g. coronary artery disease, atrial fibrillation, myocardial infarction). Others studies have described influence of some genetic polymorphisms with stroke [6,7], myocardial infarction [8,9] and other cardiovascular complications [10].

Adiponectin (ADIPOQ) is an adipocytokine secreted almost exclusively from adipocites[11]. This hormone has wide physiological benefits in the organism. Berg *et al*.[12] found that adiponectin improves insulin resistance, suggesting that this molecule inhibits glucose production and/or improves glucose assimilation independent of insulin levels. Others have described a role in lipid metabolism, showing an increase of beta-oxidation and an inhibition of lipid accumulation in the liver[13]. Indeed, low levels of adiponectin may contribute to the increased risk for cardiovascular complications in obesity, insulin resistance and diabetes[14,15]. A recent meta-analysis[16] studied the association between *ADIPOQ* single nucleotides polymorphism (SNPs) and CAD. They found that the *rs1501299* polymorphism is associated with the susceptibility to coronary artery disease in Caucasians, East Asians and South Asians, meanwhile the *rs2241766* polymorphism affects only in East Asians. Kanu *et al*.[17] performed another meta-analysis looking for association between three *ADIPOQ* SNPs (*rs266729*, *rs2241766*, and *rs1501299*) and CVD (atherothrombotic cerebral infarction or *ACI*, acute Coronary Syndrome or *ACS*, atherosclerosis, coronary artery disease[3], coronary heart disease, *CVD* cardiovascular disease and myocardial infarction or *MI*). They found a significant increased CVD risk associated with *rs266729* and *rs2241766*, but not associated with *rs1501299*. Another study described that rs266729, rs822395, rs1501299 and rs2241766 polymorphisms were all significantly associated with the susceptibility to coronary artery disease[3] in certain populations[18]. Therefore, still some inconsistencies have existed in

research findings on the association between CVD and single nucleotide polymorphisms (SNPs) of *ADIPOQ*.

Currently, most of medical actions to prevent cardiovascular episodes are pharmacological or life-style behaviour changes. However genetic studies to know genetic susceptibility to suffer CAD and therefore create risk groups within a population and make individualized prevent and treatments are not performed.

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provide an excellent opportunity to study the association between *ADIPOQ* gene polymorphisms with cardiovascular risk factors in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 14 polymorphisms (SNPs) of the *ADIPOQ* gene as well as cardiovascular risk factors.

The aim of this study was therefore to examine the association between 14 *ADIPOQ* polymorphisms with CVD risk factors in European adolescents.

Material and methods

Participants

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS) attempted to report the lifestyle and nutritional status of European adolescents. A total of 3865 participants (12-18 year old) of nine European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria and Spain) were selected to be part of this study. They were randomly selected from public and private schools in each city between October 2006 and December 2007. We collected blood samples of one-third of these participants (N=1155) with the consequent genetic analysis and clinical biochemistry assays. Finally 1057 (552 girls) adolescents with *ADIPOQ* gene polymorphisms and CVD risk factors data were included in this study. Adolescents and corresponding parents/guardians were fully informed about aims and methods of the study such as

TABLE 1 Minor allele frequency (MAF) and results of exact test to assess deviations from Hardy-Weinberg equilibrium (HWE).

	Major allele	**Minor allele**	**MAF**	**HWE**
ADIPOQ				
rs182052	G	A	0.32	0.14
rs822391	T	C	0.19	0.09
rs822393	C	T	0.21	0.51
rs16861210	G	A	0.09	0.86
rs822395	A	C	0.35	0.16
rs822396	A	G	0.18	0.12
rs12495941	G	T	0.38	0.74
rs7649121	A	T	0.15	0.27
rs2241766	T	G	0.13	0.78
rs1501299	C	A	0.29	0.01
rs3821799	C	T	0.48	0.24
rs3774261	G	A	0.42	0.05
rs17366743	T	C	0.02	0.1
rs1063537	C	T	0.12	0.39

Table 2 Characteristics of the study population.

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
Age (years)	14.71 ± 1.22	14.74 ± 1.25	14.68 ± 1.2
Weight (kg)	58.72 ± 12.67	61.86 ± 14.29	55.85 ± 10.17
Height (cm)	165.46 ± 9.34	169.5 ± 9.91	161.76 ± 6.98
BMI	21.34 ± 3.67	21.39 ± 3.99	21.3 ± 3.37
Cholesterol (mmol/l)	160.74 ± 27.69	154.03 ± 26.13	166.88 ± 27.68
HDL(mmol/l)	55.26 ± 10.67	53.17 ± 10.12	57.17 ± 10.81
LDL (mmol/l)	94.49 ± 25.09	90.78 ± 24.32	97.89 ± 25.33
Triglycerides (mmol/l)	69 ± 35.09	64.13 ± 31.65	73.46 ± 37.45
LDL/HDL	1.78 ± 0.63	1.78 ± 0.65	1.78 ± 0.6
Cholesterol/HDL	2.99 ± 0.66	2.98 ± 0.69	2.99 ± 0.63
Triglycerides/HDL	1.33 ± 0.88	1.29 ± 0.83	1.37 ± 0.92
ApoA1	1.5 ± 0.22	1.46 ± 0.21	1.55 ± 0.23
ApoB	0.65 ± 0.16	0.63 ± 0.15	0.68 ± 0.16
ApoB/ApoA1	0.44 ± 0.13	0.44 ± 0.13	0.45 ± 0.13
apoB/LDL	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Leptin (ng/ml)	19.61 ± 22.19	9.55 ± 14 21	28.36 ± 24.11
Insulin	10.31 ± 7.79	10.16 ± 8.82	10.46 ± 6.7
HOMA	2.35 ± 1.96	2.36 ± 2.24	2.34 ± 1.65
QUICKI	0.35 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
SBP (mm Hg)	120.03 ± 13.3	124.16 ± 13.93	116.29 ± 11.5
DBP (mm Hg)	68.03 ± 8.84	67.52 ± 8.91	68.49 ± 8.7
CVD Risk score	-0.01 ± 0.61	-0.03 ± 0.66	0.02 ± 0.56

BMI: Body mass index; HDL: High density lipoprotein; LDL: Low density lipoprotein; Apo: Apolipoprotein; HOMA: Homeostatic Model Assessment; QUICKI: Quantitative Insulin Sensitivity Check Index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure

inclusion criteria [19,20], and signed an informed written consent. Ethical guidelines of the Declaration of Helsinki 1961 (revision of Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries were respected by the study. Human research committees of each center involved approved the protocol [21].

Assessment of Cardiovascular Disease Risk factors

A total of 30 ml of venous blood was extracted between 8 and 10 am in fasting conditions (ten hours after last meal). Samples were collected in heparinized tubes, maintained in ice and centrifuged (3.500rpm/15min) within 30 min. After centrifugation they were stored and transported (4-7ºC) to the central laboratory (Bonn, Germany) where they were deposited at -80ºC. Serum concentrations of cardiovascular risk factors were measured in centralized laboratories.

The CVD risk factors analysed included serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density cholesterol (LDL), ApoA1, ApoB, leptin,

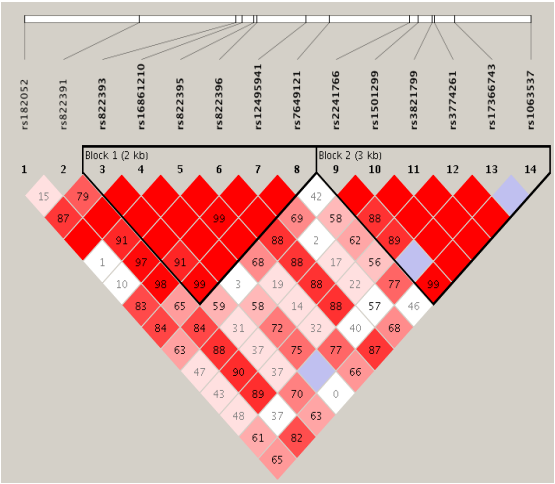


FIGURE 1 Blocks 1 and 2 of *ADIPOQ* polymorphisms, which contains rs822393, rs16861210, rs822395, rs822396, rs12495941 and rs7649121 for block 1; rs2241766, rs1501299, rs3821799, rs3774261, rs17366743 and rs1063537 for block 2, according to genotyping data of this study. Boxes number referred to linkage disequilibrium (D') between SNPs, boxes with no number means 100% linkage (D' = 1). Colour legend: i) Bright red = high D'; White = low D'; iii) Purple = High D' but low LOD score (see Haploview documentation for further details; http://www.broad.mit.edu/mpg/haploview).

triglycerides and glucose, which were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods. Insulin was measured by a solid-phase two-site chemiluminescent immunometric assay with an Immulite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany). Homeostasis model assessment (HOMA) was calculated (glycaemia X insulin/22.5) as resistance to insulin indicator as well as the Quantitative Insulin Sensitivity Check Index (QUICKI). Blood pressure was measured with an automatic oscillometric device (OMRON M6). Adolescents quietly sat for 5 min before the measurements, conducted on the right arm in an extended position. Two measures were taken 5 min apart, and the mean of both values (in mmHg) was used in analyses. We computed a CVD risk score with the mean of the standardize value [(value-mean)/standard deviation] of the following variables: Total cholesterol/HDL, triglycerides, HOMA, systolic blood pressure, and triceps and subscapular skinfolds[22].

Genotyping

Samples were genotyped by an Illumina System (Illumina, Inc, San Diego, California) and the software used was GoldenGate (Inc, San Francisco, California). High rate of genotyping success was performed (≥99.8%) and each polymorphism respected the Hardy-Weinberg equilibrium (P≥0.01 in all cases; Table 1). Several polymorphisms of the *ADIPOQ* gene showed linkage disequilibrium between them (Figure 1).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were determined by means of an exact test and considering a p value of 0.01 as a threshold. Associations between genetic markers and CVD risk factors were assessed through linear models. Five genetic models (dominant, recessive, log-additive, codominant and overdominant) were used for all analyses, except in those where rs16861210 and rs17366743 polymorphisms were involved. These polymorphisms were analysed using only a dominant model due to the low

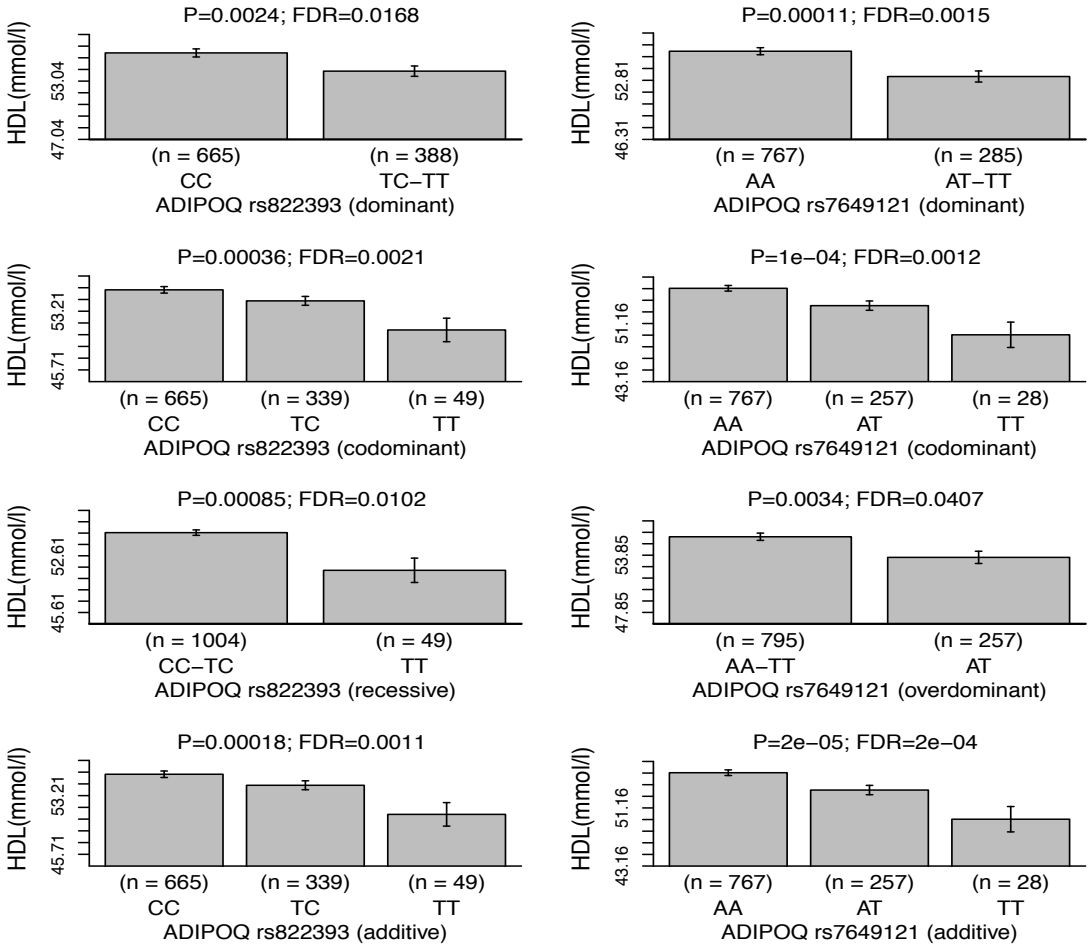


FIGURE 2 Association between *ADIPOQ* polymorphisms and high density lipoprotein cholesterol (HDL). Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for body mass index, center, sex, and age.

number of minor homozygotes (MAF < 0.1; Table 1). Adjustment variables were body mass index (calculated as weight in kilograms divided by height in meters squared), age, gender and center. For each polymorphism, p values were computed using the likelihood ratio test (LRT) between a model with the polymorphism and a null model without it. These analyses were performed with the "SNPassoc" R package[23]. We considered the associations between all SNPs and each phenotype under a given heritage model as the family test, i.e. the number of test was equal to the number of SNPs. Therefore a corrected p value for multiple comparison following the Bonferroni method would be 0.0036 (0.05/14). We selected the significant associations to perform further haplotype analysis. Given these associations were used in further analyses and the linkage disequilibrium existent between gene polymorphisms (see below), which reduces the number of independent tests, we performed a

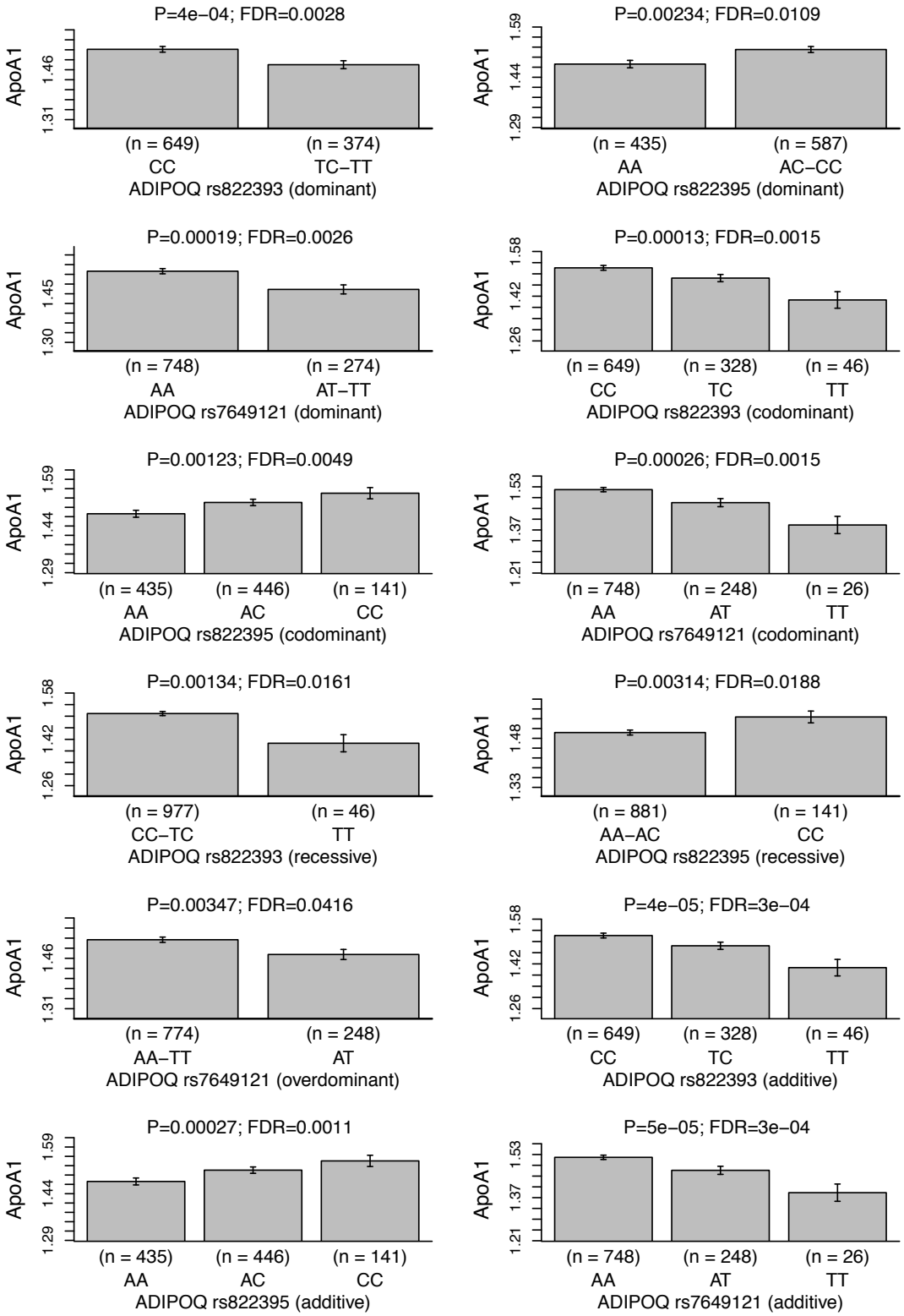


FIGURE 3 Association between *ADIPOQ* polymorphisms and apolipoprotein (Apo)A1. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for body mass index, center, sex, and age.

exploratory selection of associations using a method to control the expected proportion of false positives (False Discovery Rate [FDR])[24,25] instead the Bonferroni correction, which is more

stringent[26]. Therefore, associations with FDR < 0.1 were used in haplotype analyses.

Linkage disequilibrium between polymorphisms and haplotype block structures were evaluated with Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview) and haplo.stats[27] . First, haplotype blocks were generated by the algorithm of four-gamete rules[28]. For each block, we tested if the observed frequencies of haplotypes were deviated from expected under linkage equilibrium. Finally, we assessed the association between haplotypes and phenotypes by means of a permutation procedure. Only additive and dominant models were considered given the low frequency of some haplotypes. For those significant associations we performed regressions between haplotypes and phenotypes with the purpose of testing significant differences between haplotype levels. Again, the FDR was calculated from the p values for differences between the reference haplotype (the most frequent) and other haplotypes.

Results

The characteristics of the study sample are shown in Table 2.

Association between ADIPOQ polymorphisms and CVD Risk Markers

Three of the fourteen SNPs analysed showed significant associations with CVD risk factors after the Bonferroni correction (rs822393, rs822395 and rs7649121; Figures 2,3,4). The significant associations after a less stringent correction (FDR<0.05) for all markers are shown in supplementary appendix (Figures S1-3), along with p values for all associations (Figures S4-21).

We observed an association of minor T allele of the rs822393 polymorphism with lower HDL under dominant, codominant, recessive and additive model (p=0.0024, p=0.00036, p=0.00085 and p=0.00018, respectively) and with lower ApoA1 under dominant, codominant, recessive and additive model (p=4e−04, p=0.00013, p=0.00134 and p=4e−05 respectively) (Figures 2 and 3).

Regarding the rs822395 polymorphism, we observed an association of major A allele polymorphism with lower ApoA1 under dominant, codominant, recessive and additive model (p=0.00234, p=0.00123, p=0.00314 and p=0.00027, respectively), higher SBP under dominant and additive model (p=0.00087 and p=0.00231, respectively) and with higher risk score under recessive and additive model (p=0.00234 and p=0.00238, respectively) (Figures 3 and 4).

Finally, we observed an association of minor T allele of the rs7649121 polymorphism with a lower HDL under dominant, codominant overdominant and additive heritage model (p=0.00011, p=1e−04, p=0.0034 and p=2e−05 respectively), and with lower ApoA1 under dominant, codominant,

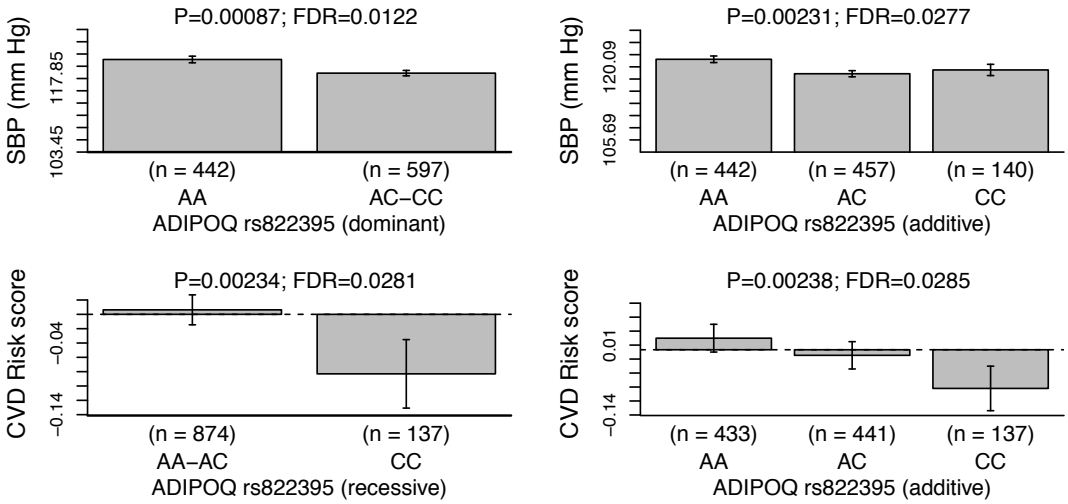


Figure 4 Association of *ADIPOQ* polymorphisms with systolic blood pressure (SPB) and risk score. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for body mass index, center, sex, and age.

overdominant and additive model (p=0.00019; p=0.00026; p=0.00347 and p=5e−05, respectively) (Figures 2 and 3).

Association between ADIPOQ polymorphism haplotypes and CVD risk factors

ADIPOQ block 1 contains rs822393, rs16861210, rs822395, rs822396, rs12495941 and rs7649121 polymorphisms (Figure 1). Haplotype TGAAGT of *ADIPOQ* was significantly associated to lower HDL than CGAATA (global p=0.00335; difference between groups 0.04; 95CI = 0.07 - 0.01; P = 0.0042; FDR=0.02940; under dominant model / / global p=0.0088; difference between groups 0.04; 95CI = 0.06 - 0.02; P = 0.0011; FDR=0.0077; under additive model; differences between groups obtained from models with the response variable log transformed). The TGAAGT haplotype was also associated with lower ApoA1 levels than the CGAATA haplotype (global p=0.0095; difference between groups 0.03; 95CI = 0.05 - 0.01; P = 0.0039; FDR=0.0273; under dominant model / / global p=0.00375; difference between groups 0.03; 95CI = 0.05 - 0.01; P = 0.0026; FDR=0.0182; under additive model).

Discussion

The primary findings of this study show a significant association between the rs822393, rs822395 and rs7649121 polymorphisms of *ADIPOQ* gene and several CVD risk factors (i.e. HDL, Apo1, SBP, Risk Score) in European adolescents. We also found a significantly association of the TGAAGT *ADIPOQ* haplotype (rs822393, rs16861210, rs822395, rs822396, rs12495941 and rs7649121) with HDL and Apo1 serum levels. Taken together, these findings highlight the relevance of genetic influence over the

cardiovascular profile in adolescents.

To our knowledge, this is the first study investigating the association between *ADIPOQ* gene polymorphisms and CVD risk factors in European adolescents. He et al.[29] did not find a significant association between four *ADIPOQ* polymorphisms (i.e. rs182052, rs2241766, rs266729, rs822393) and cardio-metabolic traits, neither circulating adiponectin concentration in Mexican children. However, they found a strong association between circulating adiponectin concentration and cardio-metabolic traits in Mexican children. In our study we also analysed the rs182052 and rs2241766 polymorphisms and we did not observe significant association either. However, in contrast with their study, we found a strong association between the rs822393 polymorphism and HDL and Apo1 serum levels under several genetic models (dominant, codominant, recessive and additive model). Both studies have similar size and age study population, so these discordances may be due to the fact that these polymorphisms are population dependent, with possibly different allele frequencies and penetrance in these populations. Their study sample was based of urban population of central Mexico, while children of nine different European countries set up our study.

A meta-analysis investigated the association between adiponectin gene polymorphisms and coronary artery disease, and showed that the A allele of the rs822395 (-4034A>C) polymorphism was significantly associated with polymorphism under the additive model (OR= 1.20, 95% CI = 1.02–1.43) in Caucasians [30]. These findings concur with our results. Our data show that the A allele of the rs822395 polymorphism is associated to lower Apo1 levels, higher SBP and a higher Risk Score. Another study based on a Chinese Han population[31], showed that homozygous carriers of the T allele of the rs1501299 polymorphism and the G allele of the rs2241766 polymorphism were associated with increased coronary heart disease risk, and the homozygous carriers T allele of the rs7649121 polymorphism was associated with decreased coronary heart disease risk. Moreover, they found an interaction between smoking and GG or GT genotypes of the rs1501299 polymorphism with higher risk of coronary heart disease. In our study we did not find significant association between the rs1501299 and rs2241766 polymorphisms and CVD risk factors, yet we observed a solid association of minor the T allele of the rs7649121 polymorphism with a lower HDL and with lower Apo1. For a long time epidemiological studies have consistently supported an inverse association between plasma HDL levels and coronary heart disease[32], which makes incoherent these results. In the same line, numerous studies have demonstrated that Apo1 provide

identical or even improve our ability to identify patients at risk for future coronary heart disease and prognostic information as HDL[33]. However, causality of this association is still unclear despite data from human genetic and pharmacological studies. It is possible that this phenomenon might be influenced by other confounding factors that are causally associated with coronary heart disease risk as well as HDL and are primarily responsible for the epidemiologic observation[34]. Nevertheless, genetics heritability population dependent, with possibly different allele frequencies and penetrance in these populations, could explain these discordances. Finally, age differences between both studies are remarkable (mean age of 61.8 ± 12.1 years).

Fewer and unclear results about haplotypes of *ADIPOQ* and cardiovascular risk have been performed to our knowledge. Pischon *et al*.[35] did not suggest an important role of a five polymorphisms haplotype (rs266729, rs822395, rs822396, rs2241766 and rs1501299) in the development of coronary heart disease in men and women. However, we found that the haplotype TGAAGT of *ADIPOQ (*rs822393, rs16861210, rs822395, rs822396, rs12495941 and rs7649121) was significantly associated to lower HDL and lower Apo1 serum levels. Sample size, age, genetic variants of the haplotype and the follow up period (6-8 years) could explain some differences with our results.

Adiponectin regulates glucose and lipid metabolism, influencing in the development of multiple metabolic disorders including obesity/overweight and diabetes mellitus. Therefore, it is evident that these two common metabolic disorders were associated with an increased risk of CVD[36]. A possible mechanism that may partially explain the observed associations in our study is that polymorphisms or haplotypes of *ADIPOQ* could alter adiponectin functions and therefore predispose to an increased cardiovascular risk. Adiponectin increases serum HDL and lowers serum triglycerides (TG)[37]. Adiponectin increases HDL-C through several mechanisms: i) via increase the hepatic production of Apo1, main apolipoprotein of HDL[38], and ii) via activation of lipoprotein lipase (LPL) and ATP-binding cassette transporter A1 (ABCA1) and decreases hepatic lipase, which can also reduce TG[39]. Taken together, adiponectin plays a role in the development CVD. Therefore, functional *ADIPOQ* genetic polymorphisms, which may alter the expression level of adiponectin or the functional capability of the expressed protein, may also alter individual susceptibility to CVD.

Some limitations of this work should also be acknowledged when interpreting our findings. First limitation it is the cross-sectional nature of our study so causality associations could not be

determined. Despite our positive findings, future studies are still needed to examine if there is direct causal correlation between *ADIPOQ* polymorphisms and CVD risk factors. Second, the associations between *ADIPOQ* polymorphisms and CVD risk factors could be modified by gene-gene and gene-environmental interactions. Third, we have no information on relatedness patterns among the participants, and we do not know the ethnic/racial make-up of the sample. Our results should be considered carefully and studies with larger sample size could help to further confirm this possible genetic predisposition.

In conclusion, we observed an association between single rs822393, rs822395, rs7649121 polymorphisms and six polymorphisms haplotype of the *ADIPOQ* gene and CVD risk factors in European adolescents.

References

1. (WHO) TWHO. Cardiovascular diseases.
2. NHS. Cardiovascular disease. https://www.nhs.uk/conditions/cardiovascular-disease/. Published 2018.
3. Won HH, Natarajan P, Dobbyn A, et al. Disproportionate Contributions of Select Genomic Compartments and Cell Types to Genetic Risk for Coronary Artery Disease. *PLoS Genet*. 2015;11(10):1-20. doi:10.1371/journal.pgen.1005622.
4. Pauling L, Itano H, Singer S, Wells I. Sickle Cell Anemia, a Molecular Disease. *Science (80-)*. 1949;110(2865). doi:10.1126/science.110.2865.543.
5. Shukla H, Mason JL, Sabyah A. Identifying genetic markers associated with susceptibility to cardiovascular diseases. *Futur Sci OA*. 2019;5(1):FSO350. doi:10.4155/fsoa-2018-0031.
6. Akhter MS, Biswas A, Rashid H, Devi L, Behari M, Saxena R. Screening of the GPX3 gene identifies the “t” allele of the SNP -861A/T as a risk for ischemic stroke in young Asian Indians. *J Stroke Cerebrovasc Dis*. 2014;23(8):2060-2068. doi:10.1016/j.jstrokecerebrovasdis.2014.03.010.
7. Man BL, Baum L, Fu YP, et al. Genetic polymorphisms of Chinese patients with ischemic stroke and concurrent stenoses of extracranial and intracranial vessels. *J Clin Neurosci*. 2010;17(10):1244-1247. doi:10.1016/j.jocn.2010.01.050.
8. Nasibullin TR, Timasheva YR, Sadikova RI, et al. Genotype/allelic combinations as potential predictors of myocardial infarction. *Mol Biol Rep*. 2016;43(1):11-16. doi:10.1007/s11033-015-

3933-3.
9. Buysschaert I, Carruthers KF, Dunbar DR, et al. A variant at chromosome 9p21 is associated with recurrent myocardial infarction and cardiac death after acute coronary syndrome: The GRACE genetics study. *Eur Heart J*. 2010;31(9):1132-1141. doi:10.1093/eurheartj/ehq053.
10. Fiatal S, Ádány R. Application of Single-Nucleotide Polymorphism-Related Risk Estimates in Identification of Increased Genetic Susceptibility to Cardiovascular Diseases: A Literature Review. *Front Public Heal*. 2018;5(January). doi:10.3389/fpubh.2017.00358.
11. Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF. A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem*. 1995;270(45):26746-26749. doi:10.1074/jbc.270.45.26746.
12. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE. The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med*. 2001;7(8):947-953. doi:10.1038/90992.
13. Yamauchi T, Kamon J, Waki H, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med*. 2001;7(8):941-946. doi:10.1038/90984.
14. Woodward L, Akoumianakis I, Antoniades C. Unravelling the adiponectin paradox: novel roles of adiponectin in the regulation of cardiovascular disease. *Br J Pharmacol*. 2017;174(22):4007-4020. doi:10.1111/bph.13619.
15. Hung J, McQuillan BM, Thompson PL, Beilby JP. Circulating adiponectin levels associate with inflammatory markers, insulin resistance and metabolic syndrome independent of obesity. *Int J Obes*. 2008;32(5):772-779. doi:10.1038/sj.ijo.0803793.
16. Zhang X, Cao YJ, Zhang HY, Cong H, Zhang J. Associations between ADIPOQ polymorphisms and coronary artery disease: a meta-analysis. Lafrenie RM, ed. *BMC Cardiovasc Disord*. 2019;19(1):63. doi:10.1186/s12872-019-1041-3.
17. Kanu JS, Qiu S, Cheng Y, et al. Associations between three common single nucleotide polymorphisms (rs266729, rs2241766, and rs1501299) of ADIPOQ and cardiovascular disease: a meta-analysis. *Lipids Health Dis*. 2018;17(1):126. doi:10.1186/s12944-018-0767-8.
18. Wang Z, Diao J, Yue X, Zhong J.

Effects of *ADIPOQ* polymorphisms on individual susceptibility to coronary artery disease: a meta-analysis. *Adipocyte*. 2019;00(00):1-7. doi:10.1080/21623945.2019.1595270.

19. Moreno LA, De Henauw S, González-Gross M, et al. Design and implementation of the healthy lifestyle in europe by nutrition in adolescence cross-sectional study. *Int J Obes*. 2008;32:S4-S11. doi:10.1038/ijo.2008.177.
20. Moreno LA, González-Gross M, Kersting M, et al. Assessing, understanding and modifying nutritional status, eating habits and physical activity in European adolescents: the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) Study. *Public Health Nutr*. 2008;11(3):288-299. doi:10.1017/S1368980007000535.
21. Béghin L, Castera M, Manios Y, et al. Quality assurance of ethical issues and regulatory aspects relating to good clinical practices in the HELENA Cross-Sectional Study. *Int J Obes (Lond)*. 2008;32 Suppl 5:S12-S18. doi:10.1038/ijo.2008.179.
22. Lobelo F, Pate RR, Dowda M, Liese AD, Ruiz JR. Validity of cardiorespiratory fitness criterion-referenced standards for adolescents. *Med Sci Sports Exerc*. 2009;41(6):1222-1229. doi:10.1249/MSS.0b013e318195d491.
23. González JR, Armengol L, Solé X, et al. SNPassoc: An R package to perform whole genome association studies. *Bioinformatics*. 2007;23(5):644-645. doi:10.1093/bioinformatics/btm025.
24. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B J R Stat Soc B*. 1995;57(1):289-300. doi:10.2307/2346101.
25. Qu H-QH-Q, Tien M, Polychronakos C. Statistical significance in genetic association studies. *Clin Investig Med*. 2010;33(5):E266-E270.
26. Sham PC, Purcell SM. Statistical power and significance testing in large-scale genetic studies. *Nat Rev Genet*. 2014;15(5):335-346. doi:10.1038/nrg3706.
27. Sinnwell AJ, Maintainer Jason P Sinnwell SD. Title Statistical Analysis of Haplotypes with Traits and Covariates when Linkage Phase is Ambiguous. 2016. http://www.mayo.edu/research/labs/statistical-genetics-genetic-epidemiology/.
28. Wang N, Akey JM, Zhang K, Chakraborty R, Jin L.

Distribution of Recombination Crossovers and the Origin of Haplotype Blocks: The Interplay of Population History, Recombination, and Mutation. *Am J Hum Genet*. 2002;71(5):1227-1234. doi:10.1086/344398.

29. He J, Stryjecki C, Reddon H, et al. Adiponectin is associated with cardio-metabolic traits in Mexican children. *Sci Rep*. 2019;9(1):3084. doi:10.1038/s41598-019-39801-2.
30. Hou H, Ge S, Zhao L, et al. An Updated Systematic Review and Meta-analysis of Association Between Adiponectin Gene Polymorphisms and Coronary Artery Disease. *Omi A J Integr Biol*. 2017;21(6):340-351. doi:10.1089/omi.2017.0007.
31. Liang C, Yanqing C, Yawei X, Aihong M, Qinwan W, Jingying Z. Association of AdipoQ single-nucleotide polymorphisms and smoking interaction with the risk of coronary heart disease in Chinese Han population. *Clin Exp Hypertens*. 2017;39(8):748-753. doi:10.1080/10641963.2017.1324479.
32. The Emerging Risk Factors Collaboration*. Major Lipids, Apolipoproteins, and Risk of Vascular Disease. *JAMA*. 2009;302(18):1993. doi:10.1001/jama.2009.1619.
33. Khuseyinova N, Koenig W. Apolipoprotein A-I and risk for cardiovascular diseases. *Curr Atheroscler Rep*. 2006;8(5):365-373. doi:10.1007/s11883-006-0033-9.
34. Vitali C, Khetarpal SA, Rader DJ. HDL Cholesterol Metabolism and the Risk of CHD: New Insights from Human Genetics. *Curr Cardiol Rep*. 2017;19(12). doi:10.1007/s11886-017-0940-0.
35. Pischon T, Pai JK, Manson JAE, et al. Single nucleotide polymorphisms at the adiponectin locus and risk of coronary heart disease in men and women. *Obesity*. 2007;15(8):2051-2060. doi:10.1038/oby.2007.244.
36. Wang Z V., Scherer PE. Adiponectin, the past two decades. *J Mol Cell Biol*. 2016;8(2):93-100. doi:10.1093/jmcb/mjw011.
37. Christou GA, Kiortsis DN. Adiponectin and lipoprotein metabolism. *Obes Rev*. 2013;14(12):939-949. doi:10.1111/obr.12064.
38. Feng Y, Tschöpe C, Kintscher U, et al. Impact of HDL on adipose tissue metabolism and adiponectin expression. *Atherosclerosis*. 2010;210(2):438-444. doi:10.1016/j.atherosclerosis.2010.01.001.
39. Yanai H, Yoshida H. Beneficial Effects of Adiponectin on Glucose and Lipid Metabolism

and Atherosclerotic Progression: Mechanisms and Perspectives. *Int J Mol Sci*. 2019;20(5):1190. doi:10.3390/ijms20051190.

CHAPTER 4: Association of *UCP1*, *UCP2* and *UCP3* gene polymorphisms with cardiovascular disease risk factors in European adolescents: the HELENA study

ABSTRACT

Objective: To examine the association of *UCP1*, *UCP2* and *UCP3* gene polymorphisms with cardiovascular disease (CVD) risk factors in European adolescents.

Study design: A cross-sectional study that involves 1.057 European adolescents (12-18 years old) from the HELENA Study. A total of 18 polymorphisms of *UCP1*, *UCP2* and *UCP3* genes were genotyped. We measured serum total cholesterol, HDL, ApoA1, ApoB, leptin, triglycerides, glucose, insulin, blood pressure, and calculated HOMA (homeostatic model assessment) and a CVD Risk Score.

Results: The G allele of *UCP2* rs2735572 and T allele of *UCP2* rs17132534 were associated with higher diastolic blood pressure (p=0.0019; FDR=0.017 and p=0.0017; FDR=0.0017, respectively). We observed that the AATAG haplotype of *UCP1* was associated with higher serum ApoB/ApoA1 (p = 0.008; FDR = 0.031) and ApoB levels (p = 0.008; FDR = 0.031). Moreover, the ACC haplotype of *UCP3* was associated with a higher CVD risk score (p = 0.003; FDR = 0.009).

Conclusions: Two *UCP2* polymorphisms and haplotypes of *UCP1* and *UCP3* were associated with cardiovascular disease risk factors. These findings suggest that *UCPs* may have a role in the development of cardiovascular diseases already in adolescents.

Introduction

Cardiovascular diseases (CVDs) are the main cause of premature death and chronic disability worldwide [1]. CVD events occur most frequently during or after the fifth decade of life, however, there is evidence indicating that the precursors of CVD have its origin in the first decades of life [2]. Therefore, prevention is fundamental to reduce the incidence of these pathologies, especially in young people.

CVDs are a result of complex harmful interactions between environmental and genetic risk factors. Environmental factors include unhealthy diet, tobacco use or physical inactivity [3]. However, efforts have been insufficient to decrease the prevalence and new pathogenic dimensions come into play. Recent studies have described the association between some genetic polymorphisms with stroke [4,5], myocardial infarction [6,7] and other cardiovascular complications [8]. For example, uncoupling protein genes (*UCPs*) have been associated with risk factors of cardiovascular disease such as prediabetes and type 2 diabetes mellitus (T2DM) [9,10], overweight and obesity [11–13], plasma levels of cholesterol [14] or hypertension (HT) [15] , mainly in adults over 50 years of age.

The most studied *UCP* genes are: i) *UCP1*, which main function is heat production through non-shivering thermogenesis in brown adipose tissue (BAT) [16]; ii) *UCP2*, seems to be involved in the control of reactive oxygen species (ROS)

production [17], the modulation of insulin secretion[18], the regulation of mitochondrial fatty acid oxidation[19] and may have a regulating role in thermogenesis of BAT [20], and iii) *UCP3,* which role has been related to the coupling regulation of mitochondrial respiration in skeletal muscle mitochondria[21] and fatty acids oxidation[22], and is a mediator of thermogenesis[23]. The playing role of uncoupling proteins in human physiology makes UCPs ideal targets against cardiovascular-associated pathologies. Indeed, several polymorphisms of *UCP2* (rs660339, rs659366)[9,24,25] and *UCP3* (rs2075577, rs3781907, rs1800006, rs1800849) [9,26–28] have been associated with T2DM, overweight/obesity, serum total and LDL-cholesterol and others cardiovascular risk markers. Nevertheless, poor evidence of CVD risk factors, especially in youth has been described.

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provide an excellent opportunity to study the association of *UCP1*, *UCP2* and *UCP3* gene polymorphisms with CVD risk factors in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 18 polymorphisms (SNPs) of *UCP1*, *UCP2* and *UCP3* genes as well as a number of CVD risk factors. To our knowledge, *UCP* polymorphisms have not been identified in GWAS of body weight or body composition in adults or other age groups

The aim of this study was therefore to examine the association of 18 *UCP1*, *UCP2* and *UCP3* polymorphisms with CVD risk factors in European adolescents.

Material and methods

Participants

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS) attempted to report the lifestyle and nutritional status of European adolescents. A total of 3865 participants (12-18 year old) of nine European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria and Spain) were selected to be part of this study. They were randomly selected from public and private schools in each city between October 2006 and December 2007. We collected blood samples of one-third of these participants (N=1155) with the consequent genetic analysis and clinical biochemistry assays. Finally 1057 (552 girls) adolescents with *UCP* gene polymorphisms and CVD risk factors data were included in this study. Adolescents and corresponding parents/guardians were fully informed about aims and methods of the study such as inclusion criteria, and signed an informed written consent. Ethical

guidelines of the Declaration of Helsinki 1961 (revision of

TABLE 1 Minor allele frequency (MAF) and results of exact test to assess deviations from Hardy-Weinberg equilibrium (HWE).

	Major allele	Minor allele	MAF	pHWE
UCP1				
rs2071415	T	C	0.15	0.39
rs7688743	G	A	0.19	0.92
rs2071416	T	G	0.08	0.31
rs6818140	A	G	0.19	0.32
rs6822807	T	C	0.27	0.59
rs11932232	A	G	0.19	0.32
rs12502572	G	A	0.36	0.74
rs6536991	T	C	0.27	1
UCP2				
rs2735572	G	A	0.06	0.58
rs660339	C	T	0.39	0.65
rs17132534	T	C	0.06	0.79
rs659366	C	T	0.36	0.84
UCP3				
rs7930460	A	G	0.21	0.78
rs2075577	T	C	0.47	0.22
rs2734828	C	T	0.25	0.81
rs3781907	T	C	0.27	0.75
rs1800006	T	C	0.24	0.5
rs1800849	C	T	0.22	0.42

Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries were respected by the study. Human research committees of each center involved approved the protocol [29].

Assessment of Cardiovascular Risk Factors

A total of 30 ml of venous blood was extracted between 8 and 10 am in fasting conditions (ten hours after last meal). Samples were collected in heparinized tubes, maintained in ice and centrifuged (3.500rpm/15min) within 30 min. After centrifugation they were stored and transported (4-7ºC) to the central laboratory (Bonn, Germany) where they were deposited at -80ºC. Serum concentrations of cardiovascular risk factors were measured in centralized laboratories.

The CVD risk factors analysed included serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), ApoA1, ApoB, leptin, triglycerides and glucose, which were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods. Insulin was measured by a solid-phase two-site chemiluminescent immunometric assay with an Immulite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany). Homeostasis model assessment (HOMA) was calculated (glycaemia X insulin/22.5) as resistance to insulin indicator as well as the Quantitative Insulin Sensitivity Check Index (QUICKI). Blood pressure was measured with an automatic oscillometric device (OMRON M6). Adolescents quietly sat for 5 min before the measurements, conducted on the right arm in an extended position. Two measures were taken 5 min apart, and the lowest value was recorded in mmHg.

We computed a CVD risk score with the mean of the standardize value [(value-mean)/standard deviation]

of the following variables: Total cholesterol/HDL, triglycerides,

TABLE 2 Mean ± SD of cardiovascular risk factors.

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
Cholesterol (mmol/l)	160.74 ± 27.69	154.03 ± 26.13	166.88 ± 27.68
HDL(mmol/l)	55.26 ± 10.67	53.17 ± 10.12	57.17 ± 10.81
LDL (mmol/l)	94.49 ± 25.09	90.78 ± 24.32	97.89 ± 25.33
VLDL (mmol/l)	13.8 ± 7.02	12.83 ± 6.33	14.69 ± 7.49
Triglycerides (mmol/l)	69 ± 35.09	64.13 ± 31.65	73.46 ± 37.45
LDL/HDL	1.78 ± 0.63	1.78 ± 0.65	1.78 ± 0.6
Cholesterol/HDL	2.99 ± 0.66	2.98 ± 0.69	2.99 ± 0.63
Triglycerides/HDL	1.33 ± 0.88	1.29 ± 0.83	1.37 ± 0.92
ApoA1	1.5 ± 0.22	1.46 ± 0.21	1.55 ± 0.23
ApoB	0.65 ± 0.16	0.63 ± 0.15	0.68 ± 0.16
ApoB/ApoA1	0.44 ± 0.13	0.44 ± 0.13	0.45 ± 0.13
apoB/LDL	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Leptin (ng/ml)	19.61 ± 22.19	9.55 ± 14.21	28.36 ± 24.11
Insulin	10.31 ± 7.79	10.16 ± 8.82	10.46 ± 6.7
HOMA	2.35 ± 1.96	2.36 ± 2.24	2.34 ± 1.65
QUICKI	0.35 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
SBP (mm Hg)	180.88 ± 20.52	187.13 ± 21.46	175.22 ± 17.85
DBP (mm Hg)	102.43 ± 13.65	101.63 ± 13.49	103.16 ± 13.77
Risk score	1.99 ± 0.61	1.97 ± 0.66	2.02 ± 0.56

HDL: High density lipoprotein; LDL: Low density lipoprotein; VLDL: very low-density lipoprotein; Apo: Apolipoprotein; HOMA: homeostatic model assessment; QUICKI: Quantitative Insulin Sensitivity Check Index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure

HOMA, systolic blood pressure, and triceps and subscapular skinfolds[30].

Genotyping

Samples were genotyped by an Illumina System (Illumina, Inc, San Diego, California) and the software used was GoldenGate (Inc, San Francisco, California). High rate of genotyping success was performed (97,8%) and each polymorphism respected the Hardy-Weinberg equilibrium (P>0.2 in all cases; Table 1). Several polymorphisms of the same genes showed linkage disequilibrium between them (Figures 1 and 2).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were determined by means of an exact test and considering a p value of 0.05 as a threshold. Associations between genetic markers and CVD risk factors were assessed through linear models. Five genetic models (dominant, recessive, log-additive,

codominant and overdominant) were used for all analyses, except in

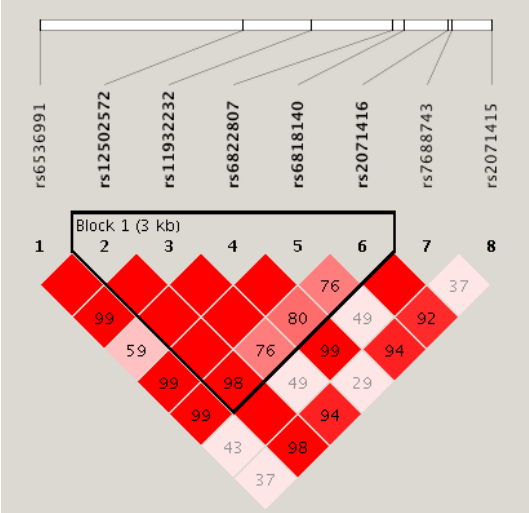


FIGURE 1 Haploview result belonging to block 1 of *UCP1* polymorphisms, which contains (rs12502572, rs11932232, rs6822807, rs6818140 y rs2071416), according to genotyping data of this study. Boxes number referred to correlation level (linkage disequilibrium; D') between SNPs. Red colour boxes with no number means 100% correlation (D' = 1).

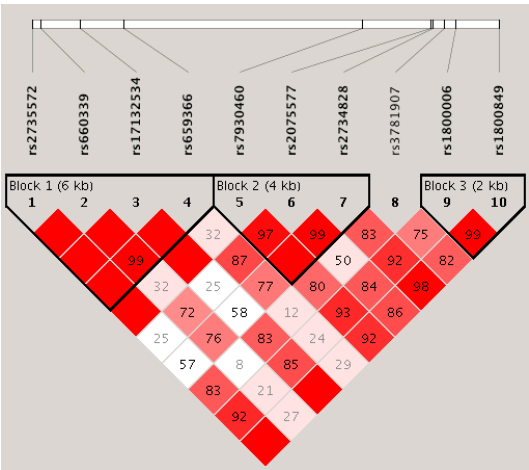


FIGURE 2 Blocks 1, 2 and 3 of *UCP2* and *UCP3* polymorphisms, which contains rs2735572, rs660339, rs17132534 and rs659366 for block 1 (*UCP2*); rs7930460, rs2075577 and rs2734828 for block 2 (*UCP3*); rs1800006 and rs1800849 for block 3 (*UCP3*), according to genotyping data of this study. Boxes number referred to correlation level (linkage disequilibrium; D') between SNPs. Red colour boxes with no number means 100% correlation (D' = 1).

those where rs2071416, rs2735572 and rs17132534 polymorphisms were involved. These polymorphisms were analysed using only a dominant model due to the low number of minor homozygotes (MAF < 0.1; Table 1). Previous findings shown the association between non-additive models with UCPs, which indicates the interest of perform this five models and compare the additive models with non-additives ones[31]. Adjustment variables were body mass index (calculated as weight in kilograms divided by height in meters squared), age, gender and center. For each polymorphism, p values were computed using the likelihood ratio test (LRT) between a model with the polymorphism and a null model without it. These analyses were performed with the "SNPassoc" R package. We selected the significant associations to perform further analysis (haplotype analysis). Given the exploratory nature of these analyses and the linkage disequilibrium existent between markers (see below), the number of independent test would be lower than number of studied polymorphisms, so Bonferroni correction could be likely over conservative [32]. We used a method to control the expected proportion of false positives (False Discovery Rate [FDR])[33], which is less stringent than Bonferroni correction. 18 tests were perfomed in order to include all genotypes markers for each genetic model, as the same as Bonferroni.

Therefore, associations with FDR < 0.1 were used in haplotype analyses.

Linkage disequilibrium between polymorphisms and haplotype block structures were evaluated with Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview) and haplo.stats. First, haplotype blocks were generated by the algorithm of four-gamete rules. For each block, we tested if the observed frequencies of haplotypes were deviated from expected under linkage equilibrium. Finally, we assessed the association between haplotypes and phenotypes by means of a permutation procedure. Only additive and dominant models were considered given the low frequency of some haplotypes. For those significant associations we performed regressions between haplotypes and phenotypes with the purpose of testing significant differences between haplotype levels. Again, the FDR was calculated from the p values for differences between the reference haplotype (the most frequent) and other haplotypes.

Results

The characteristics of the study sample are shown in Table 2.

Association between UCP polymorphisms and CVD Risk Factors

Two *UCP2* polymorphisms were individually associated with CVD risk factors after multiple-comparison corrections (Figure 3) with a FDR<0.05 threshold (previous analysis with FDR<0.1 was also performed as screening method to further haplotype analysis). All individual comparison between genotypes and CVD risk factors are presented in supplementary file. We observed that the G minor allele of rs2735572 and the T minor allele of rs17132534 were associated with higher DBP (beta coefficient=0.04, p=0.0019; FDR=0.017 and beta coefficient=0.04, p=0.0017; FDR=0.0017 respectively; beta coefficients obtained from models with the response variable log transformed) under a dominant genetic model (Figure 4).

Association between UCP polymorphism haplotypes and CVD risk factors

UCP1 block contains the rs12502572, rs11932232, rs6822807, rs6818140, and rs2071416 polymorphisms (Fig. 1). The AATAG haplotype of *UCP1* was significantly associated with a higher ApoB/ApoA1 ratio than the reference GATAT haplotype (global p=0.046; difference between groups =0.06; 95CI = 0.02 - 0.10; p = 0.008; FDR = 0.031; under additive model; differences between groups

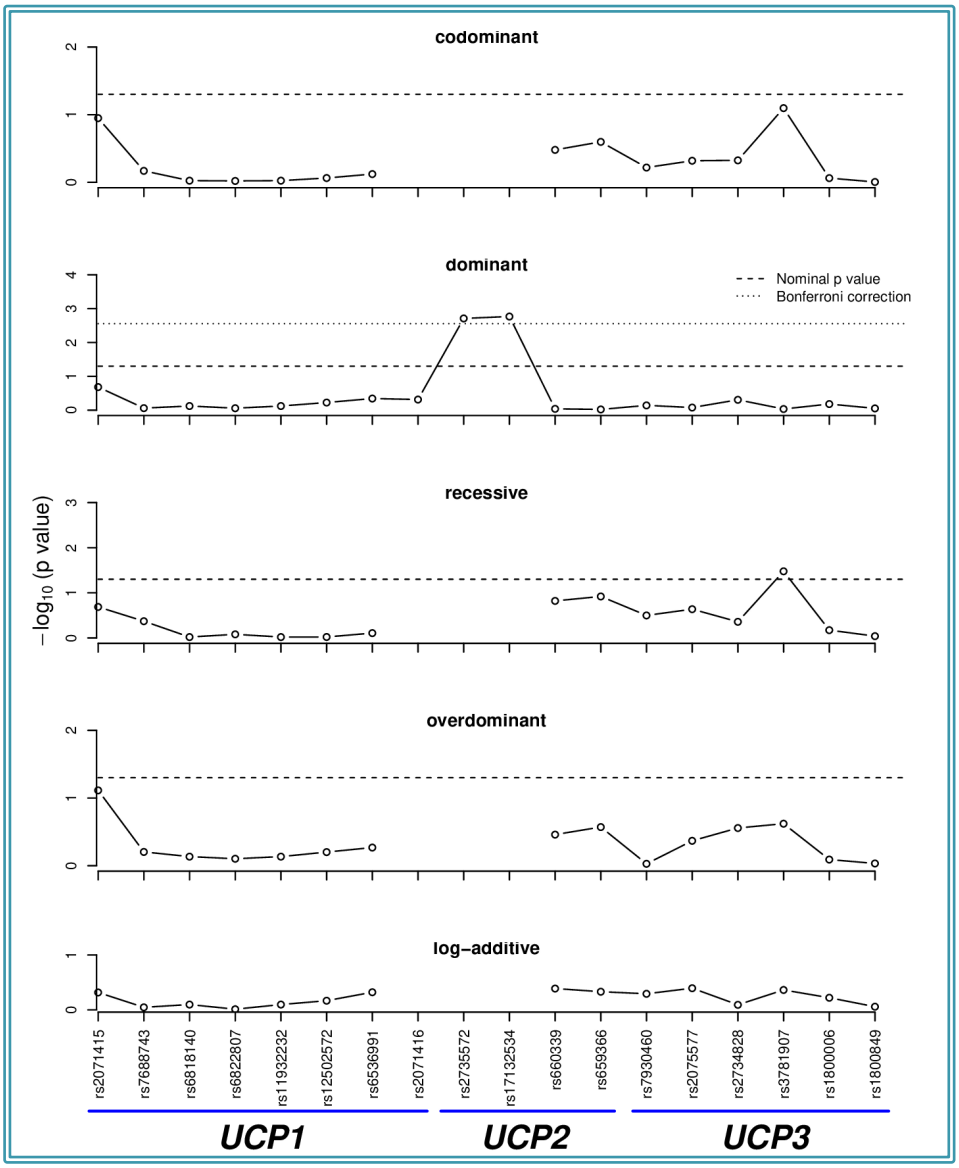


FIGURE 3 The log p values from likelihood ratio test from polymorphisms and diastolic blood pressure are shown for each genetic model. The horizontal dotted lines indicate two different thresholds. One threshold based on Bonferroni correction, and another on the nominal p-value (0.05). Note that missing cases correspond to polymorphisms with MAF < 0.1, which were only analysed for dominant model.

obtained from models with the response variable log transformed). The AATAG haplotype was also associated with higher ApoB levels than the reference GATAT haplotype (global p=0.045; difference between groups =0.06; 95CI = 0.02 - 0.10; p = 0.008; FDR = 0.031; under additive model). Also an association between the *UCP3* block 2 (rs7930460, rs2075577 and rs2734828, Fig.2) and the risk score was observed. The ACC haplotype was associated with a higher risk score compared with the ATC haplotype (global p=0.01; difference between groups= 0.06; 95CI = 0.02 - 0.10; p = 0.003; FDR = 0.009; under dominant model).

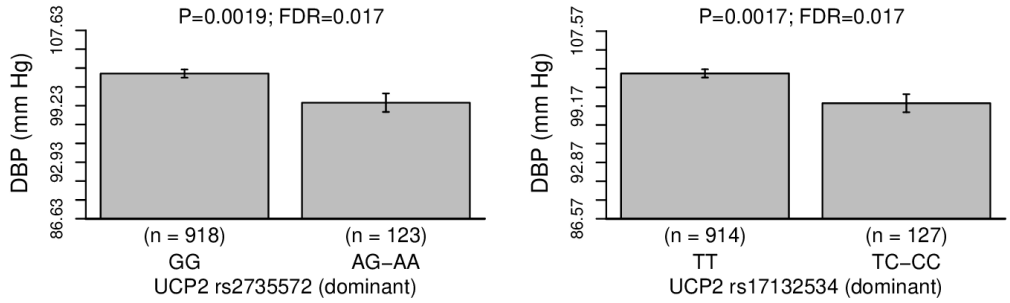


FIGURE 4 Association between rs2735572 (*UCP2*) and rs17132534 (*UCP2*) with diastolic blood pressure (DBP) under a dominant model. P value and false positive discovery rate are shown for this association. Values are adjusted for body mass index, center, sex, and age.

Discussion

We observed that the *UCP2* G and T alleles of the rs2735572 and rs17132534 polymorphisms, respectively, were associated with higher diastolic blood pressure in European adolescents. Moreover, we found that the *UCP1* AATAG haplotype was associated with higher serum ApoB/ApoA1 and ApoB levels. Finally, the *UCP3* ACC haplotype was associated with a higher CVD risk score. Taken together, these findings suggest that *UCPs* may have an important role in cardiovascular health already in the first decades of life.

To our knowledge, this is the first study investigating the association between *UCPs* and CVD risk factors in European adolescents. Several studies with smaller sample sizes [10,27,28,34] reported an association of single polymorphisms of *UCP3* or combined haplotypes *UCP2/UCP3* with several CVD risk factors such as serum total and LDL-cholesterol levels, insulin or HOMA in adults. In contrast, we observed no association of these *UCP* polymorphisms with CVD risk factors in adolescents. These discordances may be due to the fact that they are population dependent, with possibly different allele frequencies and penetrance in these populations. Also differences in age, inter-country differences in lifestyle behaviours and sample sizes are important and could lead to differences across studies.

A plausible mechanism to partially explain the observed associations is that polymorphisms or haplotypes of *UCP*s could alter UCP functions and predispose to cardiovascular risk. This dysfunction may explain the phenotypes observed with CVD risk through, i) dysfunction of the process of oxidation of fatty acids, leading to altered serum lipid levels as total cholesterol, LDL, HDL or triglycerides [19,35], ii) in relation to ROS regulation mediated by UCP2; studies show that knockout mice deletion of the *UCP2* gene

contributes to atherosclerosis lesion development and a significantly shorter lifespan [36]. Several studies in humans and cultured cells suggested that excessive ROS production is involved in the atherosclerotic plaque formation and progression [37,38]. Therefore, evidence suggests that decreasing ROS production is a remarkable target to prevent the atherosclerotic process. *UCP2* negatively regulates intracellular ROS production[17,39] making it a potential therapeutic target for the treatment of vascular diseases, iii) UCP proteins have been also related to blood pressure control. Dhamrait et al.[40] described the role of UCPs in the regulation of angiotensin-converting enzyme (ACE). This enzyme is a pivotal component of the endocrine renin–angiotensin system (RAS) playing a key role in the regulation of the human circulation. ACE favours the rise of angiotensin II and aldosterone, leading to salt and water retention by the kidney and to constriction of small blood vessels in the arterial tree, actions that, together, serve to elevate blood pressure. This study [40] shows that some polymorphisms of *UCP3* and *UCP2* were associated with higher age-adjusted ACE activity, which could contribute to a hypertension status, which is also consistent with our findings. Cardiovascular diseases are pathologies with a long-term latency period and modifiable cardiovascular risks factors, which makes prevention fundamental, especially in these populations with genetic predisposition.

A limitation of our study is its cross-sectional nature. Our results should be considered carefully and studies with larger sample size could help to further confirm this possible genetic predisposition.

In conclusion, we observed an association between the *UCP2* rs2735572 and rs17132534 polymorphisms with higher diastolic blood pressure in adolescents from nine European countries. We also observed a haplotype association of *UCP1* and *UCP3* with higher blood apolipoproteins levels and risk score, respectively. These findings suggest that *UCPs* may have an important role in the development of CVD predisposition already in adolescents.

References

1. Roth GA, Johnson C, Abajobir A, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J Am Coll Cardiol*. 2017;70(1):1-25. doi:10.1016/j.jacc.2017.04.052.
2. Shah S. Primary prevention of Cardiovascular Disease. *World Heal Organ*. 2011;364:937. doi:10.1093/innovait/inr119.
3. World Health Organization. Global status report on noncommunicable diseases 2010. *World Health*. 2010:176. doi:978 92 4 156422 9.
4. Akhter MS, Biswas A, Rashid

H, Devi L, Behari M, Saxena R. Screening of the GPX3 gene identifies the “t” allele of the SNP -861A/T as a risk for ischemic stroke in young Asian Indians. *J Stroke Cerebrovasc Dis*. 2014;23(8):2060-2068. doi:10.1016/j.jstrokecerebrovasdis.2014.03.010.

5. Man BL, Baum L, Fu YP, et al. Genetic polymorphisms of Chinese patients with ischemic stroke and concurrent stenoses of extracranial and intracranial vessels. *J Clin Neurosci*. 2010;17(10):1244-1247. doi:10.1016/j.jocn.2010.01.050.
6. Nasibullin TR, Timasheva YR, Sadikova RI, et al. Genotype/allelic combinations as potential predictors of myocardial infarction. *Mol Biol Rep*. 2016;43(1):11-16. doi:10.1007/s11033-015-3933-3.
7. Buysschaert I, Carruthers KF, Dunbar DR, et al. A variant at chromosome 9p21 is associated with recurrent myocardial infarction and cardiac death after acute coronary syndrome: The GRACE genetics study. *Eur Heart J*. 2010;31(9):1132-1141. doi:10.1093/eurheartj/ehq053.
8. Fiatal S, Ádány R. Application of Single-Nucleotide Polymorphism-Related Risk Estimates in Identification of Increased Genetic Susceptibility to Cardiovascular Diseases: A Literature Review. *Front Public Heal*. 2018;5(January). doi:10.3389/fpubh.2017.00358.
9. Su M, Chen X, Chen Y, et al. UCP2 and UCP3 variants and gene-environment interaction associated with prediabetes and T2DM in a rural population: A case control study in China. *BMC Med Genet*. 2018;19(1):1-9. doi:10.1186/s12881-018-0554-4.
10. Hsu Y-H, Niu T, Song Y, Tinker L, Kuller LH, Liu S. Genetic variants in the UCP2-UCP3 gene cluster and risk of diabetes in the Women’s Health Initiative Observational Study. *Diabetes*. 2008;57(4):1101-1107. doi:10.2337/db07-1269.
11. Zhang M, Wang M, Zhao ZT. Uncoupling protein 2 gene polymorphisms in association with overweight and obesity susceptibility: A meta-analysis. *Meta Gene*. 2014;2(1):143-159. doi:10.1016/j.mgene.2013.10.009.
12. Brondani LA, Assmann TS, de Souza BM, Bouças AP, Canani LH, Crispim D. Meta-analysis reveals the association of common variants in the uncoupling protein (UCP) 1-3 genes with body mass index

variability. *PLoS One*. 2014;9(5):e96411. doi:10.1371/journal.pone.0096411.

13. Pascual-Gamarra JM, Salazar-Tortosa D, Martinez-Tellez B, et al. Association between UCP1 , UCP2 , and UCP3 gene polymorphisms with markers of adiposity in European adolescents: The HELENA study. *Pediatr Obes*. 2019;(December 2018):e12504. doi:10.1111/ijpo.12504.
14. De Luis DA, Aller R, Izaola O, et al. Relationship of -55C/T Polymorphism of Uncoupling Protein 3 (UCP3) Gene with Metabolic Syndrome by ATP III Classification. *J Clin Lab Anal*. 2012;26(4):272-278. doi:10.1002/jcla.21517.
15. Kotani K, Sakane N, Saiga K, et al. The uncoupling protein-1 gene -3826A/G polymorphism and hypertension in Japanese subjects. *Clin Chem Lab Med*. 2007;45(9):1186-1189. doi:10.1515/CCLM.2007.253.
16. Golozoubova V, Cannon B, Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab*. 2006;291(2):E350-E357. doi:10.1152/ajpendo.00387.2005.
17. Moukdar F, Robidoux J, Lyght O, Pi J, Daniel KW, Collins S. Reduced antioxidant capacity and diet-induced atherosclerosis in uncoupling protein-2-deficient mice. *J Lipid Res*. 2009;50(1):59-70. doi:10.1194/jlr.M800273-JLR200.
18. Zhang CY, Baffy G, Perret P, et al. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell*. 2001;105(6):745-755. doi:S0092-8674(01)00378-6 [pii].
19. Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F. UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? *IUBMB Life*. 2009;61(7):762-767. doi:10.1002/iub.188.
20. Caron A, Labbé SM, Carter S, et al. Loss of UCP2 impairs cold-induced non-shivering thermogenesis by promoting a shift toward glucose utilization in brown adipose tissue. *Biochimie*. 2017;134:118-126. doi:10.1016/j.biochi.2017.01.006.
21. Vidal-Puig AJ, Grujic D, Zhang C-Y, et al. Energy Metabolism in Uncoupling Protein 3 Gene Knockout Mice. *J Biol Chem*. 2000;275(21):16258-16266. doi:10.1074/jbc.M910179199.
22. Bezaire V, Spriet LL, Campbell S, et al. Constitutive UCP3 overexpression at physiological levels increases mouse skeletal muscle

capacity for fatty acid transport and oxidation. *FASEB J*. 2005;19(8):977-979. doi:10.1096/fj.04-2765fje.

23. Riley CL, Dao C, Kenaston MA, et al. The Complementary and Divergent Roles of Uncoupling Proteins 1 and 3 in Thermoregulation. *J Physiol*. 2016;594(24):7455–7464. doi:10.1113/JP272971.This.
24. Gamboa R, Huesca-gómez C, López-pérez V, et al. The UCP2 -866G / A , Ala55Val and UCP3 -55C / T polymorphisms are associated with premature coronary artery disease and cardiovascular risk factors in Mexican population. 2018.
25. Huriyati E, Luglio HF, Ratrikaningtyas PD, Tsani AFA, Sadewa AH, Juffrie M. Dyslipidemia, insulin resistance and dietary fat intake in obese and normal weight adolescents: The role of uncoupling protein 2 -866G/A gene polymorphism. *Int J Mol Epidemiol Genet*. 2016;7(1):67-73.
26. Hsu YH, Niu T, Song Y, Tinker L, Kuller LH, Liu S. Genetic variants in the UCP2-UCP3 gene cluster and risk of diabetes in the Women's Health Initiative Observational Study. *Diabetes*. 2008;57(4):1101-1107. doi:10.2337/db07-1269.
27. Mottagui-Tabar S, Hoffstedt J, Brookes AJ, Jiao H, Arner P, Dahlman I. Association of ADRB1 and UCP3 gene polymorphisms with insulin sensitivity but not obesity. *Horm Res*. 2007;69(1):31-36. doi:10.1159/000111793.
28. Salopuro T, Pulkkinen L, Lindström J, et al. Variation in the UCP2 and UCP3genes associates with abdominal obesity and serum lipids: The Finnish Diabetes Prevention Study. *BMC Med Genet*. 2009;10(1):94. doi:10.1186/1471-2350-10-94.
29. Béghin L, Castera M, Manios Y, et al. Quality assurance of ethical issues and regulatory aspects relating to good clinical practices in the HELENA Cross-Sectional Study. *Int J Obes (Lond)*. 2008;32 Suppl 5:S12-S18. doi:10.1038/ijo.2008.179.
30. Lobelo F, Pate RR, Dowda M, Liese AD, Ruiz JR. Validity of cardiorespiratory fitness criterion-referenced standards for adolescents. *Med Sci Sports Exerc*. 2009;41(6):1222-1229. doi:10.1249/MSS.0b013e318195d491.
31. Krishnan M, Thompson JMD, Mitchell EA, et al. Analysis of association of gene variants with obesity traits in New Zealand European children at 6 years of age. *Mol BioSyst*. 2017;13(8):1524-1533. doi:10.1039/C7MB00104E.
32. Sham PC, Purcell SM. Statistical power and significance testing in large-

scale genetic studies. *Nat Rev Genet*. 2014;15(5):335-346. doi:10.1038/nrg3706.
33. Qu H-QH-Q, Tien M, Polychronakos C. Statistical significance in genetic association studies. *Clin Investig Med*. 2010;33(5):E266-E270.
34. Ochoa MC, Santos JL, Azcona C, et al. Association between obesity and insulin resistance with UCP2-UCP3 gene variants in Spanish children and adolescents. *Mol Genet Metab*. 2007;92(4):351-358. doi:10.1016/j.ymgme.2007.07.011.
35. Kotani K, Fujiwara S, Tsuzaki K, et al. The Association Between the Uncoupling Protein-1 Gene A-3826G Polymorphism and High-density Lipoprotein Cholesterol in A General Japanese Population: A Consideration of the Obesity Status. *J Clin Med Res*. 2011;3(6):319-324. doi:10.4021/jocmr738w.
36. Pierelli G, Stanzione R, Forte M, et al. Uncoupling protein 2: A key player and a potential therapeutic target in vascular diseases. *Oxid Med Cell Longev*. 2017;2017. doi:10.1155/2017/7348372.
37. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: An overview. *Free Radic Biol Med*. 2000;28(12):1815-1826. doi:10.1016/S0891-5849(00)00344-0.
38. Mallat Z, Nakamura T, Ohan J, et al. The relationship of hydroxyeicosatetraenoic acids and F2-isoprostanes to plaque instability in human carotid atherosclerosis. *J Clin Invest*. 1999;103(3):421-427. doi:10.1172/JCI3985.
39. Arsenijevic D, Onuma H, Pecqueur C, et al. Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat Genet*. 2000;26(4):435-439. doi:10.1038/82565.
40. Dhamrait SS, Maubaret C, Pedersen-Bjergaard U, et al. Mitochondrial uncoupling proteins regulate angiotensin-converting enzyme expression: crosstalk between cellular and endocrine metabolic regulators suggested by RNA interference and genetic studies. *BioEssays*. 2016;38:S107-S118. doi:10.1002/bies.201670909.

CHAPTER 5: Association between *LPL* single nucleotide polymorphisms and cardiovascular disease risk factors in European adolescents: The HELENA study

Introduction

The World Health Organization (WHO) reported that cardiovascular diseases (CVDs) are the most common causes of death globally[1]. In 2009, costs related to CVD amounted to E106 billion, representing 9% of the total healthcare expenditure across the European Union (EU). A reduction in cardiovascular risk factors in adolescence would lead to a reduction in the number affected by CVDs later in life. In the last three decades, more than half of the reduction in CV mortality has been attributed to reduction in cholesterol and blood pressure (BP) levels and smoking. This favourable trend is partly offset by an increase in other risk factors, mainly obesity and type 2 diabetes mellitus (T2DM)[2].

Despite CVDs main risk factors are well studied (socio-economic status, smoking, high BMI and central obesity, physical inactivity, dyslipidaemia, DM2, hypertension, etc), they are the result of a complex interplay of genetics and environmental factors. In one hand, many genes have been related with obesity, especially those that are involved in the regulation role of appetite center in the brain. As some genes related with monogenic obesity as leptin (LEP), leptin receptor (LEPR), pro-opiomelacortin (POMC) or prohormone convertase 1 (PCSK1)[3]. Also genes related with polygenic obesity which is the most common type, as polymorphisms of fat mass and obesity gene (FTO) and melanocortin-4 receptor (MC4R)[4,5]. Regular exercise training provides a myriad of health benefits in the general population, including improvements in blood pressure, diabetes (enhancing insulin sensitivity and improve glucose tolerance), lipid profile (increases high-density lipoprotein (HDL), reduce triglycerides (TG), low density lipoprotein (LDL) and cholesterol levels)[6] and decrease cardiovascular disease risk[7]. A number of studies have shown that moderate-intensity physical activity reduces the incidence of all-cause mortality, particularly deaths related to coronary artery disease (CAD)[8]. In adolescents, we showed that meeting the daily physical activity recommendations (at least 60 minutes/day of moderate to vigorous physical activity) may offset the genetic predisposition to a main cardiovascular risk factor, such as obesity, associated with the *FTO* rs9939609[9] and *UCP1* rs2071415[10] polymorphism in European adolescents. On the other hand, evidence supported a strong genetic cause of CVDs as CAD[11]. Genetic susceptibility to CVDs is mainly make up by multiple genes and polymorphisms acting together. A recent review of Shukla et al.[12] collect a list of genetic markers that influence HDL levels, LDL levels, triglycerides levels an others, as well as some heritable conditions (CAD, atrial fibrillation, myocardial infarction, etc).

The use of triglycerides (TG) for energy production or as stored fat in adipose tissue is one of the many important beneficial biologic functions of lipids. Lipoprotein lipase (LPL) is a key enzyme in hydrolysis of chylomicron and very low-density lipoprotein (VLDL) triglyceride[13]. The heart, skeletal muscle and adipose tissue are the principal sites of LPL synthesis. Chylomicrons are large, buoyant triglyceride-rich particles that are not thought to be associated with increased risk for coronary artery disease [14]. VLDL formed in the liver reacts with LPL, the results are hydrolysis of the TG core and the formation of VLDL remnants and intermediate-density lipoprotein (IDL), the precursor of LDL[15]. IDL is also believed atherogenic because of evidence that type III hyperlipidemia (elevated TG and LDL-C due to increased of IDL) appears to place an individual at very high risk. LPL has a central role in both VLDL and HDL metabolism. LPL deficiency results in an accumulation of chylomicrons and other triglyceride-rich lipoproteins in the plasma, producing hypertriglyceridemia[16]. Plus, abnormalities in LPL is associated to atherosclerosis, chylomicronemia, obesity, Alzheimer's disease, and the dyslipidemia related to diabetes and insulin resistance[17].

Several studies have shown an association between genetic variations in *LPL* and cardiovascular risk factors. A study of Zhang *et al*.[18] searched gene expression profiling based upon their proximity to SNPs with strong and significant associations in prior GWAS for CAD, subclinical atherosclerosis, blood lipids and other blood risk factors. They found a significant association between *LPL* gene expression and lipid levels. Others found an association between some polymorphisms of *LPL* and metabolic syndrome[19] and cerebral infarction[20]. Some works have been suggested a possible association between rs285 *LPL* polymorphism and ischemic stroke. Despite of some discordances between this association, some recent meta-analysis observed a significant relationship between this genetic variant and ischemic stroke risk[21,22]. However, to our knowledge, few studies have identified in a consistent way genetic variations in *LPL* that are directly associated with CVDs.

Data obtained within the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study provide an excellent opportunity to study the association between *LPL* gene polymorphisms with cardiovascular risk factors in European adolescents. The HELENA study was designed to provide reliable data on nutrition and health-related variables in a relatively large sample of European adolescents from 9 different countries and includes information on 13 single nucleotide polymorphisms (SNPs) of the *LPL* gene as well as adiposity markers and cardiovascular risk factors.

The goal of this study was therefore to examine the association between 13 *LPL* polymorphisms with cardiovascular risk factors in European adolescents. Also, test if any interaction with physical activity occurs objectively assessed by accelerometry on adiposity markers or CVDs markers.

Material and methods

Participants

The Healthy Lifestyle in Europe by Nutrition in Adolescence Cross-Sectional Study (HELENA-CSS) attempted to report the lifestyle and nutritional status of European adolescents. A total of 3865 participants (12-18 year old) of nine European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria and Spain) were selected to be part of this study. They were randomly selected from public and private schools in each city between October 2006 and December 2007. We collected blood samples of one-third of these participants (N=1155) with the consequent genetic analysis and clinical biochemistry assays. Among these participants, 1057 individuals (552 girls) with data on *LPL* gene polymorphisms, adiposity markers, CVD risk factors and physical activity were included in this study. Adolescents and corresponding parents/guardians were fully informed about aims and methods of the study such as inclusion criteria [23,24], and signed an informed written consent. Ethical guidelines of the Declaration of Helsinki 1961 (revision of Edinburgh 2000), Good Clinical Practice, and legislation about clinical research in humans in each of the participating countries were respected by the study. Human research committees of each center involved approved the protocol [25].

Assessment of adiposity

Weight and height were measured following standard methods[23]. Waist and hip circumference was measured in triplicate with an anthropometric unelastic tape (SECA 200; Seca Deutschland, Hamburg, Germany) and was used as a surrogate measure of central body fat. We calculated waist to height and waist to hip ratios. BMI was calculated as weight in kilograms divided by height in meters squared. Adolescents were classified according to BMI (kg/m^2) as normal weight, overweight or obese according to Cole *et al*.[26]. Skinfold thickness was measured to the nearest 0.2 mm in triplicate on the left side at the biceps, triceps, subscapularis, suprailium, thigh, and medial calf with a Holtain Caliper (Holtain Ltd, Crymmych, Wales). Body fat percentage was calculated from skinfold thicknesses (triceps and subscapular) using the equations by Slaughter *et al*.[27]. Finally, fat mass index (FMI) was calculated as fat mass in kilograms divided by height in meters squared.

Assessment of Cardiovascular Risk Factors

A total of 30 ml of venous blood was extracted between 8 and 10 am in fasting conditions (ten hours after last meal). Samples were collected in heparinized tubes, maintained in ice and centrifuged (3.500rpm/15min) within 30 min. After centrifugation they were stored and transported (4-7ºC) to the central laboratory (Bonn, Germany) where they were deposited at -80ºC. Serum concentrations of cardiovascular risk factors were measured in centralized laboratories.

The CVD risk factors analysed included serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density cholesterol (LDL), ApoA1, ApoB, leptin, triglycerides and glucose, which were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods. Insulin was measured by a solid-phase two-site chemiluminescent immunometric assay with an Immulite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany). Homeostasis model assessment (HOMA) was calculated (glycaemia X insulin/22.5) as resistance to insulin indicator as well as the Quantitative Insulin Sensitivity Check Index (QUICKI). Blood pressure was measured with an automatic oscillometric device (OMRON M6). Adolescents quietly sat for 5 min before the measurements, conducted on the right arm in an extended position. Two measures were taken 5 min apart, and the mean of both values (in mmHg) was used in analyses. We computed a CVD risk score with the mean of the standardize value [(value-mean)/standard deviation] of the following variables: Total cholesterol/HDL, triglycerides, HOMA, systolic blood pressure, and triceps and subscapular skinfolds[28].

TABLE 1 Minor allele frequency (MAF) and results of exact test to assess deviations from Hardy-Weinberg equilibrium (HWE).

	Major allele	**Minor allele**	**MAF**	**HWE**
LPL				
rs1534649	G	T	0.44	0.62
rs13266204	A	G	0.19	0.12
rs3779788	C	T	0.15	0.04
rs343	C	A	0.1	0.62
rs256	C	T	0.16	0.04
rs258	C	G	0.46	0.5
rs270	C	A	0.15	0.55
rs283	G	A	0.2	0.78
rs285	C	T	0.49	0.39
rs316	C	A	0.13	0.68
rs320	T	G	0.29	0.55
rs328	C	G	0.12	0.77
rs10099160	T	G	0.22	0.32

Assessment of physical activity

Physical activity was assessed during 7 consecutive days with a uniaxial accelerometer (GT1M; ActiGraph, Pensacola, Florida) attached to the lower back[29]. Adolescents were instructed to wear the accelerometer during all time awake and to remove it only during water-based activities. At least 3 days of recording with a minimum of 8 hours registered per day was set as an inclusion criterion[29]. The time-sampling interval (epoch) was set at

15 seconds. We calculated the time engaged in at least moderate physical activity (≥3 metabolic equivalents) based on a standardized cutoff of 2000 counts/min or more. Moderate to vigorous physical activity was dichotomized into less than 60 min/day and 60 min/day or longer[29].

Genotyping

Samples were genotyped by an Illumina System (Illumina, Inc, San Diego, California) and the software used was GoldenGate (Inc, San Francisco, California). High rate of genotyping success was performed (≥99.8%) and each polymorphism respected the Hardy-Weinberg equilibrium (P≥0.01 in all cases; Table 1). Several polymorphisms of the *LPL* gene showed linkage disequilibrium between them (Figure 1).

Statistical analysis

Deviations from Hardy-Weinberg equilibrium (HWE) were determined by means of an exact test and considering a p value of 0.01 as a threshold. Associations between genetic markers, adiposity variables and CVD risk factors were assessed through linear models. Five genetic models (dominant, recessive, additive, codominant and overdominant) were used for all analyses. Adjustment variables were age, gender and center. In the case of CVD risk factors, body mass index was also included as a covariable in the models. We also considered interactions between SNPs and physical activity under the same models but including an interaction term (gene*physical activity). For each polymorphism, p values were computed using the likelihood ratio test (LRT) between a model with the polymorphism/interaction and a null model without it. Associations in which any level (genotype or physical activity) had lower than 10 samples were discarded. These analyses were performed in the R environment[30]. We considered the associations between all SNPs and each phenotype under a given heritage model as the family test, i.e. the number of test was equal to the number of SNPs analysed for a given phenotype. We selected the significant genotype-phenotype associations to perform interaction and haplotype analysis, i.e. only SNPs and phenotypes significant associated were considered for next analyses. Given these associations were used in further analyses and the linkage disequilibrium existent between gene polymorphisms (see below), which reduces the number of independent tests, we performed a exploratory selection of associations using a method to control the expected proportion of false positives (False Discovery Rate [FDR])[31,32] instead the Bonferroni correction, which is more stringent[33]. Therefore, associations with FDR < 0.1 were used in interaction and haplotype analyses.

Linkage disequilibrium between polymorphisms and haplotype block structures were

TABLE 2 Characteristics of the study population.

Phenotype	All (n=1057)	Male (n=505)	Female (n=552)
Age (years)	14.71 ± 1.22	14.74 ± 1.25	14.68 ± 1.2
Weight (kg)	58.72 ± 12.67	61.86 ± 14.29	55.85 ± 10.17
Height (cm)	165.46 ± 9.34	169.5 ± 9.91	161.76 ± 6.98
BMI (kg / m^2)	21.34 ± 3.67	21.39 ± 3.99	21.3 ± 3.37
Percent. individuals (%)	22.23	24.75	19.93
Waist circum. (cm)	72.4 ± 8.69	74.38 ± 9.09	70.6 ± 7.9
Waist/Height ratio	0.44 ± 0.05	0.44 ± 0.05	0.44 ± 0.05
Hip circum. (cm)	91.56 ± 8.78	90.36 ± 9.04	92.66 ± 8.4
Waist/Hip ratio	0.79 ± 0.06	0.82 ± 0.05	0.76 ± 0.06
Body fat (%)	23.57 ± 9.5	20.3 ± 11.03	26.3 ± 6.91
Fat mass index (kg / m^2)	5.29 ± 3.04	4.69 ± 3.56	5.79 ± 2.42
Cholesterol (mmol/l)	160.74 ± 27.69	154.03 ± 26.13	166.88 ± 27.68
LDL (mmol/l)	94.49 ± 25.09	90.78 ± 24.32	97.89 ± 25.33
HDL (mmol/l)	55.26 ± 10.67	53.17 ± 10.12	57.17 ± 10.81
Cholesterol/HDL	2.99 ± 0.66	2.98 ± 0.69	2.99 ± 0.63
LDL/HDL	1.78 ± 0.63	1.78 ± 0.65	1.78 ± 0.6
TG (mmol/l)	69 ± 35.09	64.13 ± 31.65	73.46 ± 37.45
TG/HDL	1.33 ± 0.88	1.29 ± 0.83	1.37 ± 0.92
ApoA1	1.5 ± 0.22	1.46 ± 0.21	1.55 ± 0.23
ApoB	0.65 ± 0.16	0.63 ± 0.15	0.68 ± 0.16
ApoB/ApoA1	0.44 ± 0.13	0.44 ± 0.13	0.45 ± 0.13
apoB/LDL	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.03
Insulin	10.31 ± 7.79	10.16 ± 8.82	10.46 ± 6.7
Leptin (ng/ml)	19.61 ± 22.19	9.55 ± 14.21	28.36 ± 24.11
HOMA	2.35 ± 1.96	2.36 ± 2.24	2.34 ± 1.65
QUICKI	0.35 ± 0.03	0.35 ± 0.03	0.35 ± 0.03
SBP (mm Hg)	120.03 ± 13.3	124.16 ± 13.93	116.29 ± 11.5
DBP (mm Hg)	68.03 ± 8.84	67.52 ± 8.91	68.49 ± 8.76
CVD Risk score	-0.01 ± 0.61	-0.03 ± 0.66	0.02 ± 0.56

BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); FMI, fat mass index (calculates as fat mass in kilograms divided by height in meters squared); HDL: high density lipoprotein; LDL: low density lipoprotein; Apo: Apolipoprotein; HOMA: homeostatic model assessment; QUICKI: quantitative insulin sensitivity check index; SBP: systolic blood pressure; DBP: diastolic blood pressure

evaluated with Haploview 4.2 (http://www.broad.mit.edu/mpg/haploview), haplo.stats[34] and the "SNPassoc" R package[35]. First, haplotype blocks were generated by the algorithm of four-gamete rules[36]. For each block, we tested if the observed frequencies of haplotypes were deviated from expected under linkage equilibrium. Finally, we assessed the association between haplotypes and phenotypes by means of a permutation procedure. Only additive and dominant models were considered given the low frequency of some haplotypes. For those significant associations we performed regressions between

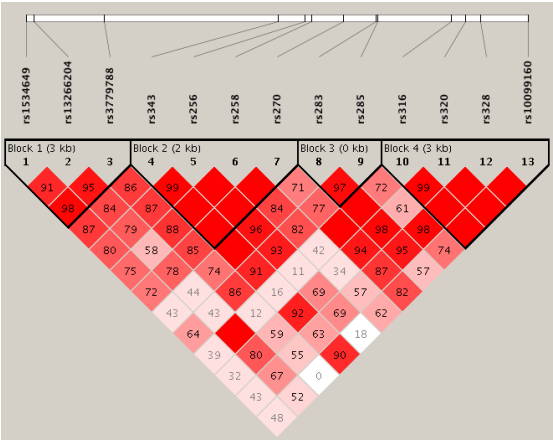


FIGURE 1 Blocks 1, 2, 3 and 4 of *LPL* polymorphisms, which contains rs1534649, rs13266204 and rs3779788 for block 1; rs343, rs256, rs258 and rs270 for block 2; rs283 and rs285 for block 3; rs316, rs320, rs328 and rs10099160 for block 4, according to genotyping data of this study. Boxes number referred to linkage disequilibrium (D') between SNPs, boxes with no number means 100% linkage (D' = 1). Colour legend: i) Bright red = high D'; White = low D' (see Haploview documentation for further details; http://www.broad.mit.edu/mpg/haploview).

haplotypes and phenotypes with the purpose of testing significant differences between haplotype levels. Again, the FDR was calculated from the p values for differences between the reference haplotype (the most frequent) and other haplotypes. In the case of gen*physical activity interactions, correction for multiple comparison was also performed through the False Discovery Rate, with a threshold at 0.05.

Results

The characteristics of the study sample are shown in Table 2.

Association between LPL polymorphisms and CVD Risk Factors

Four of the thirteen SNPs analysed showed significant associations with adiposity markers or CVD Risk Factors after the Bonferroni correction (rs1534649, rs258, rs320, rs328; Figures 2 and 3). The significant associations after a less stringent correction (FDR<0.1) for all markers are shown in supplementary appendix (Figures 1-4), along with p values for all associations (Figures 5-30).

We observed an association of the minor T allele of rs1534649 polymorphism with higher BMI under dominant and additive model (p=0.00252 and 0.00373; FDR= 0.0164 and 0.0446). The minor G allele of rs258 was also associated with higher BMI under dominant model (p=0.00193; FDR=0.0164). Regarding rs320, the major T allele of this marker was associated under additive model with higher triglycerides (p=0.00275; FDR=0.0179), along with higher leptin under codominant and overdominant model (p=0.00104 and 0.00035; FDR= 0.0136 and 0.0046).

Finally, the major C allele of rs328 was associated under recessive model with higher cholesterol/HDL (p=0.00375; FDR=0.0488) and higher LDL/HDLratio (P=0.00234; FDR=0.0304), with higher triglycerides under codominant, dominant, overdominant and additive model (p=0.00022, 0.00023, 0.00279 and 6e−05; FDR=0.0028, 0.003, 0.0363 and 7e−04), with higher triglycerides/HDL ratio

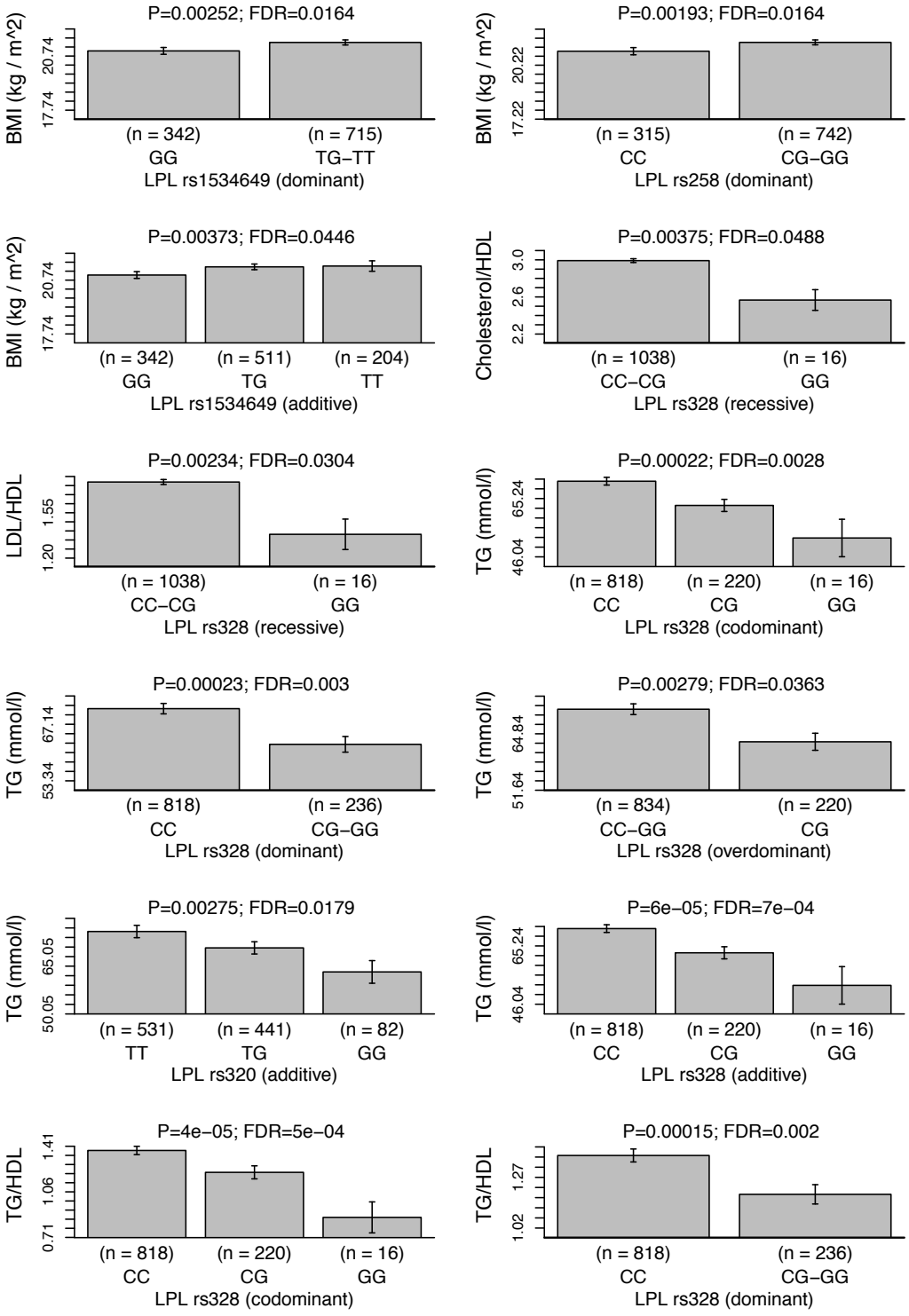


FIGURE 2 Significant associations between *LPL* polymorphisms and phenotypes according to Bonferroni correction. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age (also BMI for cardiovascular risk factors).

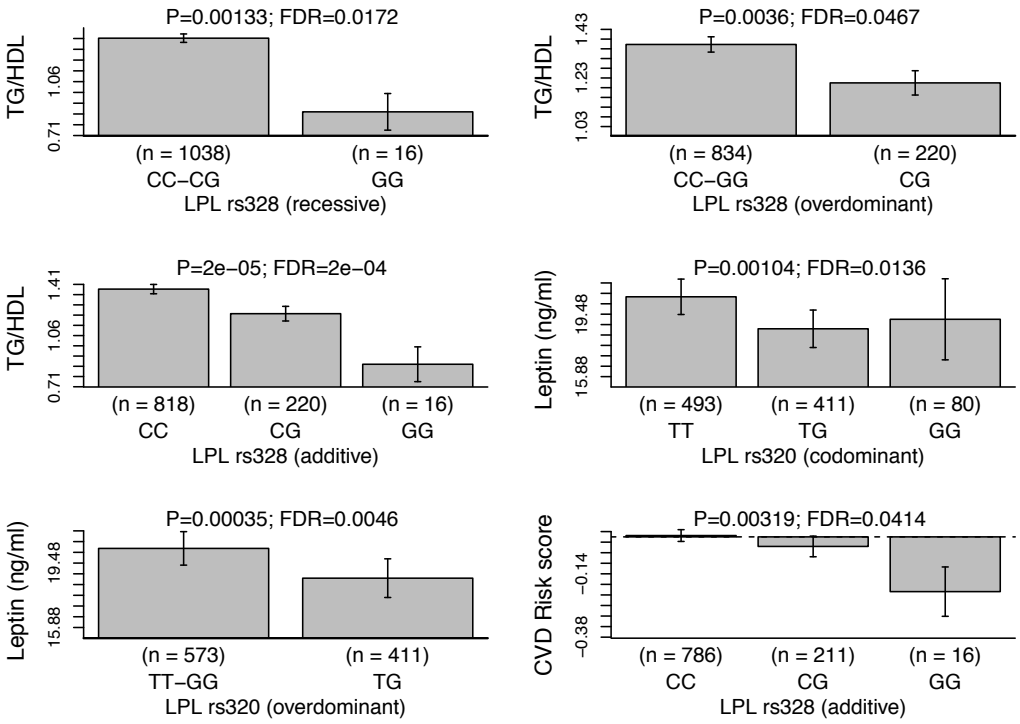


FIGURE 2 (continuation) Significant associations between *LPL* polymorphisms and phenotypes according to Bonferroni correction. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age (also BMI for cardiovascular risk factors).

under codominant, dominant, recessive, overdominant and additive model (p=4e−05, 0.00015, 0.00133, 0.0036, 2e−05; FDR=5e−04, 0.002, 0.0172, 0.0467 and 2e−04) and with higher risk score (p=0.00319; FDR=0.0414).

Association between LPL polymorphism haplotypes and CVD risk factors

LPL block 3 contains rs283 and rs285 polymorphisms (Figure 1). The haplotype GC of this block was significant associated with higher triglycerides than the GT haplotype (global p=0.0134; difference between groups 0.08; 95CI=0.03-0.13; p=0.0023; FDR=0.0046 under dominant model // global p=0.01055; difference between groups 0.08; 95CI=0.04-0.12; p=0.0003; FDR=0.0005 under additive model; differences between groups obtained from models with the response variable log transformed) and with higher triglycerides/HDL ratio (global p=0.02745; difference between groups 0.09; 95CI=0.03-0.15; p=0.0048; FDR=0.0097 under dominant model // global p=0.02015; difference between groups 0.08; 95CI=0.04-0.13; p=0.0007; FDR=0.0014 under additive model).

We also found significant associations between cardiovascular risk factors and the *LPL* block 4, which contains rs316, rs320, rs328 and rs10099160 polymorphisms (Figure 1). The CTCT haplotype of

this block was significant associated with higher triglycerides than the CGGT haplotype (global p=0.0012; difference between groups 0.11; 95CI=0.05-0.18; p=0.0004; FDR=0.0014 under dominant model // global p=0.00055; difference between groups 0.11; 95CI=0.05-0.17; p=0.0001; FDR=0.0005 under additive model), with higher triglyceride/HDL ratio (global p=0.00175; difference between groups 0.14; 95CI=0.06-0.21; p=0.0003; FDR=0.0013 under dominant model // global p=0.00065; difference between groups 0.14; 95CI=0.07-0.21; p=4.5138e−05; FDR=0.0002 under additive model) and with higher risk score (global p=0.03135; difference between groups 0.04; 95CI=0.01-0.07; p=0.0046; FDR=0.0184 under additive model).

Interaction between LPL polymorphisms, physical activity and adiposity markers

We found that alleles of rs1534649 and rs258 related to higher values of adiposity markers tend to be associated with less adiposity under high levels of physical activity (Figure 3). Carriers of the minor T allele of rs1534649 showed higher values of BMI only for those performing less than 60 min/day of moderate to vigorous physical activity (under recessive and codominant model; p=0.011015 and 0.015437; FDR=0.022031 and 0.030874). A similar pattern was found for rs258. The minor G allele of this marker was associated with higher hip circumference and BMI only for those individuals performing low levels of physical activity (under overdominant and codominant model for hip circumference [p=0.002028 and 0.00541; FDR= 0.02636 and 0.032459]; under recessive, overdominant and codominant model for BMI [p= 0.000546, 0.00692 and 0.001761; FDR= 0.001639, 0.044978 and 0.005282]).

Discussion

The results of the present research showed a significant association T allele of rs1534649, G allele of rs258, T allele of rs320 and C allele of rs328 as risk alleles, with several markers of CVDs (i.e. BMI, TG, Leptin, cholesterol/HDL, LDL/HDL, TG/HDL) under different genetics models. Also, haplotypes block 3 GC (rs282, rs285) and block 4 CTCT (rs3126, rs320, rs328, rs10099160) were significant associated to higher levels of TG, TG/HDL. Plus, block 4 haplotype was associated to these phenotypes in addition to a higher risk score. Finally, we observed alleles of rs1534649 and rs258 related to higher values of adiposity markers tend to be associated with less adiposity under high levels of physical activity.

Findings in Cho *et al*. study[37] in Korean population (N=944, including 474 T2DM subjects and 470 normal healthy controls) demonstrate that individuals who have the A allele of rs343 *LPL* polymorphism, appear to be

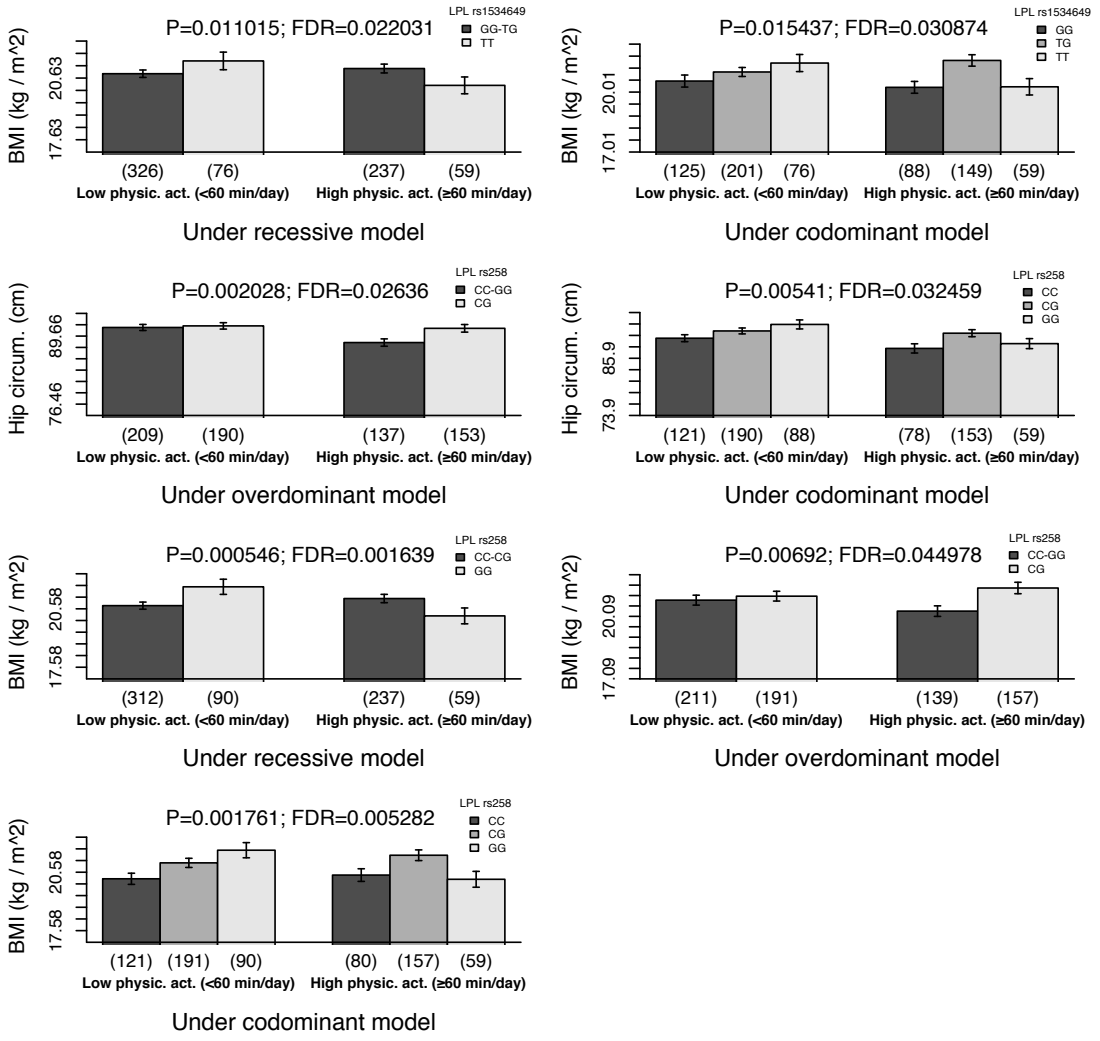


FIGURE 3 Significant interactions (FDR<0.05) between the studied SNPs and level of physical activity. Heritage model, P value and false positive discovery rate are shown for each association. Values are adjusted for center, sex, and age.

protected against T2DM. Moreover, SNP rs343 was also marginally associated with some of T2DM-related phenotypes including total cholesterol, high density lipoprotein cholesterol (HDLc), and log transformed glycosylated hemoglobin in normal controls, although no significant association was detected by multiple tests. Our findings also have not shown significant association between rs343 single nucleotide polymorphism and cardiovascular risk serum markers (neither HOMA or insulin levels) under FDR tests, neither Bonferroni corrections. Gao *et al.*[38] found that chinese obese adolescents of Han ethnicity (N=55) with GG genotype of the rs283 polymorphism were more sensitive to exercise-induced parameter changes (body fat percentage, HOMA and triglycerides levels). We did not observed significant association of rs283 isolated with cardiovascular serum parameters. We only found association TG and TG/HDL parameters with block 3 (rs283-rs285) haplotype GC. Both studies

have similar age study population, so these discordances may be due to the fact that these polymorphisms are population dependent, with possibly different allele frequencies and penetrance in these populations. Their study sample was based of Han Chinese obese adolescents, while children of nine different European countries set up our study. Moreover, differences in sample size are remarkable. *HindIII* or rs320 genetic variant is one the most common polymorphism of *LPL*. *HindIII* is a transition of intronic bases of thymine (T) to guanine (G) on position 495 of intron 8 of the LPL gene, which eliminates the restriction site for the *HindIII* enzyme[39]. Several studies have shown association between rs320 and CVDs or CV risk factors and others not, so data are controversial. Data from a meta-analysis of Nejati *et al*. indicated that allele G of rs320 significantly decreased the risk of stroke[21] (also significant association was observed with rs285). However, some discordances like this case-control research where was not found any association between rs285 and rs320 *LPL* gene polymorphism and acute ischemic stroke in Colombian population[40]. A very recent review[39], found
that *LPL* gene *HindIII* polymorphism (rare allele H- or G) has a protective function due to its role in producing an improved lipid profile (low TG and LDL-c and high HDL-c). On the other hand, the presence of common allele (T or H+) is associated with pro-atherogenic dyslipidemias and raised cardiovascular risk. Moreover, an Iranian multicentre study[41] found rs320 interactions with other SNPs affects HDL-C levels, but not an isolated effect. They suggested that an interaction of not TT genotype of LPL rs320 or not TT genotype of CETP rs708272 is associated with higher odds of low HDL-C levels. This other study[42], also found a significant lowering effect on TG levels of the *LPL*-HindIII and S447X polymorphisms interaction. Our findings are consistent with these results. We observed a very significant association between rs320 allele T or H+ with higher blood levels of TG. Moreover we observed that association in its isolated form unlike previous studies mentioned, which emphasized the importance of rs320 polymorphism in cardiovascular protective role. Discordances with some works, may due to heritability differences because population-dependent penetrance, age and sample size. Finally, we also found a significant association between rs328 and some cardiovascular risk parameters, but some studies not[21,43]. A research of Bentley *et al*[44]. suggested that interethnic differences in the distribution of serum lipids might be due to some genetic determinants differences. Their results found that among African Americans (AA) with only European ancestry at this locus, carriers of the minor or G allele rs328 had higher HDL-c than those homozygous for the C allele. Plus

minor allele had lower TG levels than non-carriers. Meanwhile, among those with African ancestry at this locus not any or reduced effect was observed. That could help to explain differences observed between studies of different ethnic population samples. In this case, our results are consistent with findings in African Americans with European ancestry, where C allele performs a risk genetic variant against cardiovascular health. To our knowledge, any interaction with physical activity either haplotypes of these polymorphisms of LPL are described previously in literature associated to CVD risk markers.

Dyslipidaemia and obesity are major risk factors for CVDs. Obesity/overweight, high TC, TAG and LDL-C, as well as decreased serum HDL-C, are frequently associated with sedentary and poor eating habits, but there is also an effect of genetic interplay and single nucleotide polymorphism related to a specific protein dysfunction, like *LPL*. A plausible mechanism to partially explain the observed associations is polymorphisms or haplotypes of *LPL* could alter lipoprotein lipase enzyme functions and predispose to cardiovascular risk. *LPL* gene product is a major regulator of triglyceride clearance in the blood. LPL is a multifunctional glycoprotein which catalyzes the hydrolysis of triglycerides of circulating chylomicrons and VLDL, regulating plasma triglyceride levels and provides fatty acids for consumption in the heart and liver or storage in adipose tissue. Indeed, deficiencies in LPL activity alters lipid metabolism producing hyperlipidaemia and atherosclerosis, which are the main risk factors for myocardial infarction and stroke[45]. So, if some alleles of *LPL* codifying an enzyme with abnormalities of their biological functions could occur that cardiovascular parameters become altered, and become the onset of a CVD.

A possible explanation for our physical activity and adiposity markers interaction results, it is rs258 and rs1534649 *LPL* polymorphisms may play a low or high-response gene role. Our results found that mainly homozygous carriers (GG and TT) of these polymorphisms are associated with some beneficial effect in adiposity markers in high physical activity groups. There is evidence that physical activity attenuates the genetic susceptibility to obesity when compared to a sedentary lifestyle[9,10]. But very few studies showed a response modulation of specifics genetic variants by physical activity. In the 80s, several studies in monozygotic twins[46–48] found that there was less variation within pairs of twins than between pairs of twins for several response phenotypes, suggesting that certain training responses were indeed genotype-dependent. Timmons et al.[49] suggests that high or low response to physical activity may be mediated through the expression profile of certain RNAs and/or the expression

profile of certain micro RNAs[50]. Therefore, expression of some polymorphisms of *LPL* could be more effectiveness in their physiological role (i.e. lipid oxidation) within metabolic-pool effects of physical activity.

Some limitations should be considered when interpreting our findings. First, our work is an observational study. Despite our positive findings, future experimental studies are still needed to examine if there is direct causal correlation between *LPL* polymorphisms and CVD risk factors. Second, the associations between *LPL* polymorphisms and CVD risk factors could be modified by gene-gene and other gene-environmental interactions. Third, we have no information on relatedness patterns among the participants, and we do not know the ethnic/racial make-up of the sample. Our results should be considered carefully and studies with larger sample size could help to further confirm this possible genetic predisposition.

In conclusion, we observed an association between single polymorphisms rs1534649, rs258, rs320 and rs328 and two polymorphisms haplotypes (rs282, rs285 and rs3126, rs320, rs328, rs10099160) of the *LPL* gene and CVD risk factors in European adolescents. Also, our results suggest that physical activity can ameliorate the deleterious effect of the *LPL* rs1534649 and rs258 *LPL* polymorphisms on body fat estimates in adolescents.

References

1. (WHO) TWHO. Cardiovascular diseases.
2. Piepoli MF, Hoes AW, Agewall S, et al. 2016 European Guidelines on cardiovascular disease prevention in clinical practice. *Eur Heart J*. 2016;37(29):2315-2381. doi:10.1093/eurheartj/ehw106.
3. Farooqi IS, O'Rahilly S. Genetics of obesity in humans. *Endocr Rev*. 2006;27(7):710-718. doi:10.1210/er.2006-0040.
4. Scuteri A, Sanna S, Chen WM, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet*. 2007;3(7):1200-1210. doi:10.1371/journal.pgen.0030115.
5. Vogel CIG, Boes T, Reinehr T, et al. Common variants near MC4R: Exploring gender effects in overweight and obese children and adolescents participating in a lifestyle intervention. *Obes Facts*. 2011;4(1):67-75. doi:10.1159/000324557.
6. Physical activity and cardiovascular health. NIH Consensus Development Panel on Physical Activity and

Cardiovascular Health. *JAMA*. 1996;276(3):241-246.
7. Sniderman AD, Pedersen T, Kjekshus J. Putting low-density lipoproteins at center stage in atherogenesis. *Am J Cardiol*. 1997;79(1):64-67.
8. Blair SN, Kampert JB, Kohl HW 3rd, et al. Influences of cardiorespiratory fitness and other precursors on cardiovascular disease and all-cause mortality in men and women. *JAMA*. 1996;276(3):205-210.
9. Ruiz JR, Labayen I, Ortega FB, et al. Attenuation of the effect of the FTO rs9939609 polymorphism on total and central body fat by physical activity in adolescents: the HELENA study. *Arch Pediatr Adolesc Med*. 2010;164(4):328-333. doi:10.1001/archpediatrics.2010.29.
10. Pascual-Gamarra JM, Salazar-Tortosa D, Martinez-Tellez B, et al. Association between UCP1 , UCP2 , and UCP3 gene polymorphisms with markers of adiposity in European adolescents: The HELENA study. *Pediatr Obes*. 2019. doi:10.1111/ijpo.12504.
11. Won HH, Natarajan P, Dobbyn A, et al. Disproportionate Contributions of Select Genomic Compartments and Cell Types to Genetic Risk for Coronary Artery Disease. *PLoS Genet*. 2015;11(10):1-20. doi:10.1371/journal.pgen.1005622.
12. Shukla H, Mason JL, Sabyah A. Identifying genetic markers associated with susceptibility to cardiovascular diseases. *Futur Sci OA*. 2019;5(1):FSO350. doi:10.4155/fsoa-2018-0031.
13. Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. *Atherosclerosis*. 2003;170(1):1-9. doi:10.1016/S0021-9150(03)00014-5.
14. Gotto AM. Triglyceride as a risk factor for coronary artery disease. *Am J Cardiol*. 1998;82(9A):22Q-25Q. doi:10.1093/oxfordjournals.aje.a115130.
15. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. *Science*. 1986;232(4746):34—47. doi:10.1126/science.3513311.
16. Okubo M, Horinishi A, Saito M, et al. A novel complex deletion-insertion mutation mediated by Alu repetitive elements leads to lipoprotein lipase deficiency. *Mol Genet Metab*. 2007;92(3):229-233. doi:10.1016/j.ymgme.2007.06.018.
17. Mead JR, Irvine SA, Ramji DP. Lipoprotein lipase: structure, function, regulation, and role in disease. *J Mol Med (Berl)*. 2002;80(12):753-769. doi:10.1007/s00109-002-0384-9.
18. Zhang X, Johnson AD,

Hendricks AE, et al. Genetic associations with expression for genes implicated in GWAS studies for atherosclerotic cardiovascular disease and blood phenotypes. *Hum Mol Genet*. 2014;23(3):795-782. doi:10.1093/hmg/ddt461.

19. Cahua-Pablo JÁ, Cruz M, Méndez-Palacios A, et al. Polymorphisms in the LPL and CETP genes and haplotype in the ESR1 gene are associated with metabolic syndrome in women from Southwestern Mexico. *Int J Mol Sci*. 2015;16(9):21539-21554. doi:10.3390/ijms160921539.
20. Shimo-Nakanishi Y, Urabe T, Hattori N, et al. Polymorphism of the lipoprotein lipase gene and risk of atherothrombotic cerebral infarction in the Japanese. *Stroke*. 2001;32(7):1481-1486.
21. Nejati M, Atlasi MA, Karimian M, Nikzad H, Azami Tameh A. Lipoprotein lipase gene polymorphisms as risk factors for stroke: a computational and meta-analysis. *Iran J Basic Med Sci*. 2018;21(7):701-708. doi:10.22038/IJBMS.2018.29009.7001.
22. Cao L, Li Q, Chen X. The HindIII and PvuII polymorphisms of lipoprotein lipase (LPL) gene reduce the risk of ischemic stroke (IS): A meta-analysis. *Medicine (Baltimore)*. 2018;97(18):e0483. doi:10.1097/MD.0000000000010483.
23. Moreno LA, De Henauw S, González-Gross M, et al. Design and implementation of the healthy lifestyle in europe by nutrition in adolescence cross-sectional study. *Int J Obes*. 2008;32:S4-S11. doi:10.1038/ijo.2008.177.
24. Moreno LA, González-Gross M, Kersting M, et al. Assessing, understanding and modifying nutritional status, eating habits and physical activity in European adolescents: the HELENA (Healthy Lifestyle in Europe by Nutrition in Adolescence) Study. *Public Health Nutr*. 2008;11(3):288-299. doi:10.1017/S1368980007000535.
25. Béghin L, Castera M, Manios Y, et al. Quality assurance of ethical issues and regulatory aspects relating to good clinical practices in the HELENA Cross-Sectional Study. *Int J Obes (Lond)*. 2008;32 Suppl 5:S12-S18. doi:10.1038/ijo.2008.179.
26. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ*. 2000;320(7244):1240-1243. doi:10.1136/bmj.320.7244.1240.
27. Slaughter M, Lohman T, Boileau R, et al. Skinfold equations for estimation of

body fatness in children and youth. *Hum Biol*. 1998;60:709-723.

28. Lobelo F, Pate RR, Dowda M, Liese AD, Ruiz JR. Validity of cardiorespiratory fitness criterion-referenced standards for adolescents. *Med Sci Sports Exerc*. 2009;41(6):1222-1229. doi:10.1249/MSS.0b013e318195d491.
29. Ruiz JR, Ortega FB, Martínez-Gómez D, et al. Objectively measured physical activity and sedentary time in european adolescents. *Am J Epidemiol*. 2011;174(2):173-184. doi:10.1093/aje/kwr068.
30. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2017.
31. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J R Stat Soc Ser B J R Stat Soc B*. 1995;57(1):289-300. doi:10.2307/2346101.
32. Qu H-QH-Q, Tien M, Polychronakos C. Statistical significance in genetic association studies. *Clin Investig Med*. 2010;33(5):E266-E270.
33. Sham PC, Purcell SM. Statistical power and significance testing in large-scale genetic studies. *Nat Rev Genet*. 2014;15(5):335-346. doi:10.1038/nrg3706.
34. Sinnwell AJ, Maintainer Jason P Sinnwell SD. Statistical Analysis of Haplotypes with Traits and Covariates when Linkage Phase is Ambiguous. 2016. http://www.mayo.edu/research/labs/statistical-genetics-genetic-epidemiology/.
35. González JR, Armengol L, Solé X, et al. SNPassoc: An R package to perform whole genome association studies. *Bioinformatics*. 2007;23(5):644-645. doi:10.1093/bioinformatics/btm025.
36. Wang N, Akey JM, Zhang K, Chakraborty R, Jin L. Distribution of Recombination Crossovers and the Origin of Haplotype Blocks: The Interplay of Population History, Recombination, and Mutation. *Am J Hum Genet*. 2002;71(5):1227-1234. doi:10.1086/344398.
37. Cho YS, Go MJ, Han HR, et al. Association of lipoprotein lipase (LPL) single nucleotide polymorphisms with type 2 diabetes mellitus. *Exp Mol Med*. 2008;40(5):523-532. doi:10.3858/emm.2008.40.5.523.
38. Gao R-R, Wang M, Hu Y, et al. Impact of LPL gene rs283 polymorphism on exercise-induced changes in metabolism of obese adolescents and the

regulatory mechanisms behind it. *Exp Physiol*. 2015;100(6):698-707. doi:10.1113/EP085127.

39. Palacio Rojas M, Prieto C, Bermúdez V, et al. Dyslipidemia: Genetics, lipoprotein lipase and HindIII polymorphism. *F1000Research*. 2018;6(0):2073. doi:10.12688/f1000research.12938.2.
40. Velasquez Pereira LC, Vargas Castellanos CI, Silva Sieger FA. Polymorphisms of the lipoprotein lipase gene as genetic markers for stroke in colombian population: a case control study. *Colomb medica (Cali, Colomb*. 2016;47(4):189-195.
41. Moghadasi M, Kelishadi R, Marateb HR, et al. Logic Regression Analysis of Gene Polymorphisms and HDL Levels in a Nationally Representative Sample of Iranian Adolescents: The CASPIAN-III Study. *Int J Endocrinol Metab*. 2017;15(3):e14037. doi:10.5812/ijem.14037.
42. Ariza M-J, Sanchez-Chaparro M-A, Baron F-J, et al. Additive effects of LPL, APOA5 and APOE variant combinations on triglyceride levels and hypertriglyceridemia: results of the ICARIA genetic sub-study. *BMC Med Genet*. 2010;11:66. doi:10.1186/1471-2350-11-66.
43. Shahid SU, Shabana, Cooper JA, et al. Genetic risk analysis of coronary artery disease in Pakistani subjects using a genetic risk score of 21 variants. *Atherosclerosis*. 2017;258:1-7. doi:10.1016/j.atherosclerosis.2017.01.024.
44. Bentley AR, Chen G, Shriner D, et al. Gene-based sequencing identifies lipid-influencing variants with ethnicity-specific effects in African Americans. *PLoS Genet*. 2014;10(3):e1004190. doi:10.1371/journal.pgen.1004190.
45. Frizzell JP. Acute stroke: pathophysiology, diagnosis, and treatment. *AACN Clin Issues*. 2005;16(4):421-428.
46. Hamel P, Simoneau JA, Lortie G, Boulay MR, Bouchard C. Heredity and muscle adaptation to endurance training. *Med Sci Sports Exerc*. 1986;18(6):690-696.
47. Prud'homme D, Bouchard C, Leblanc C, Landry F, Fontaine E. Sensitivity of maximal aerobic power to training is genotype-dependent. *Med Sci Sports Exerc*. 1984;16(5):489-493.
48. Despres JP, Bouchard C, Savard R, Prud'homme D, Bukowiecki L, Theriault G. Adaptive changes to training in adipose tissue lipolysis are genotype dependent. *Int J Obes*. 1984;8(1):87-95.

49. Timmons JA, Knudsen S, Rankinen T, et al. Using molecular classification to predict gains in maximal aerobic capacity following endurance exercise training in humans. *J Appl Physiol*. 2010;108(6):1487-1496. doi:10.1152/japplphysiol.01295.2009.
50. Davidsen PK, Gallagher IJ, Hartman JW, et al. High responders to resistance exercise training demonstrate differential regulation of skeletal muscle microRNA expression. *J Appl Physiol*. 2011;110(2):309-317. doi:10.1152/japplphysiol.00901.2010.

SEARCHING FOR NEW CANDIDATE GENES: CONNECTOME

CHAPTER 6: Novel brown adipose tissue candidate genes predicted by the human gene connectome.

Abstract

Brown adipose tissue (BAT) seems to be a promising therapeutic target against obesity. Therefore, research on genetic architecture of BAT could be key for the development of successful therapies against this complex disease. Hypothesis-candidate gene association studies are a useful tool for studying genetic determinants of complex traits, however they are dependent of the previous knowledge to select candidate genes. In this study, we predict *in silico* new 102 novel-BAT candidate genes, using for that the uncoupling protein one (UCP1), which is undoubtedly involved in BAT activity. We first identified the top 1% of human genes predicted by the human genes connectome to be biologically close to the UCP1, calculating the BAT connectome (168 genes). We then validated this prediction by showing that 65 genes that have been reported or suggested to be related with BAT were included in the connectome ($p<10^{-7}$), being the rest of genes (102) potential candidates. The resulting new list of predicted BAT genes should be useful for the discovery of novel BAT gene.

Introduction

Brown adipose tissue (BAT) has been recognise as a possible therapeutic target against obesity and related disorders due to its ability to oxidize glucose and lipids and to dissipate energy as heat[1]. Therefore, research on genetic architecture of BAT could be key for the development of successful therapies against obesity. Hypothesis-candidate gene association studies (CGAS) are a useful approach to study the genetic determinants of complex traits [2]. However this approach depends on the previous knowledge to formulate and test association hypothesis. Here, we used the human gene connectome (HGC) to predict novel-candidate genes for brown adipose tissue. The HGC is a set of biological distances and routes, predicted *in silico* by shortest distance algorithm applied to the full human genome network, conceptually similar to GPS navigation [3]. The HGC and its associated server [4] could be used to detect new candidate BAT-related genes, by ranking al human genes on the basis of their biological proximity to already known BAT-related genes, assuming the most highly ranked genes to be the most likely to be related with BAT. However, a list of novel potential BAT-related gene candidates, rigorously identified on the basis of biological relevance to BAT phenotype, would be useful and easy for most BAT investigators to use.

We generated a list of *in silico*-predicted novel BAT-related gene candidates, which we describe here. We considered UCP1 as the unique core gene, this gene encodes a protein that plays a key role in BAT activity. UCP1 is involved in the exclusive feature of BAT: The uncoupling of the electron-transport chain in the mitochondria inner membrane, which increases energy expenditure and heat production [1]. Therefore its

relevance and exclusivity on BAT activity is beyond doubt. The procedure followed was very similar to that followed by Itan *et al.* (2015), although with slightly differences because we used only one instead of a set of core genes. For example, they recommended filtering of gene lists according to biological function because the prediction algorithm of HGC is based on protein–protein binding interactions rather than biological functions associated with the gene. However, we did not consider necessary this step because of the exclusivity and relevance of UCP1 for BAT. Genes biologically close to UCP1 should be tightly related with BAT, although their function would not be related with uncoupling of electron-transport chain. Therefore, we used the following procedure: We first used the HGC to extract the top 1% of all human genes biologically closest to UCP1, and then we determined the biological clustering level of selected genes. We also showed that 65 genes known or suggested to be related with BAT were included in that list, being the rest of them (102) the set of novel candidates.

Material and methods

Extraction of BAT connectome

We used, as an input, the connectome of UCP1, being the set of all human genes ranked according to their biological proximity to UCP1. From this connectome, we extracted only the genes in the top 1% of all human genes by p-value, to obtain a list of 168 genes (167 without including UCP1 itself). We considered these genes as the connectome of brown adipose tissue (BAT connectome hereafter).

Biological proximity of BAT genes: Statistical simulations

We determined whether the 168 genes included in the BAT connectome were significantly closer to each other, biologically, than to other genes, by estimating biological distances between all these genes (a total 14028 biological distance values) with the HGC [3]. We then randomly sample one-million biological distances from the set of all human genes [6], and determined, for both BAT and all-gene sets, the proportion of distances falling into the following categories: i) small biological distances (<10); ii) small-medium biological distances (between 10 and 20, being 17 the median biological distance between all human genes); ii) medium-large biological distances (between 20 and 30); iv) large-very large biological distances (between 30 and 40); and iv) very large-extremely large biological distances (>40). We then compared the biological density of BAT genes with that of other human genes, by first calculating the median distance between all BAT genes, and the simulating sets of 168 randomly chosen genes, calculating the median distance between these genes, and estimating a p-value by determining the proportion of simulated random gene set with a median biological distance equal or smaller than that for the BAT genes.

Filtering of BAT genes candidates and assessment the predictive power

We considered as novel candidates those genes for which there is not evidence of relationship with BAT (Appendix S1), thus the final list of candidate include 102 genes. We assessed the predictive power of BAT connectome testing if the number of known BAT genes included in BAT connectome would be expected by chance (60 already known and 5 suggested by literature; Appendix S1, S2). For that, we simulated sets of 60 and 5 randomly chosen genes respectively, and estimating a p-value by determining the proportion of simulated random gene sets that were completely included in the BAT connectome. We are aware that some genes with a relationship described with BAT could have been not included in our revision of the literature, however an increase of the number of known BAT genes into the connectome would support even more its predictive power.

Phylogeny of BAT genes

The biological-interrelatedness between BAT genes was estimated with the functional genomics alignment (FGA) phylogeny [3]. We first created a biological distance matrix between all BAT genes; we then applied a neighbour-joining algorithm by means of the nj function of the R package APE [7]. Last, we plotted a phylogenetic fan-shaped tree based on HGC-predicted biological distances between BAT genes, using for that the plot function of APE package [7].

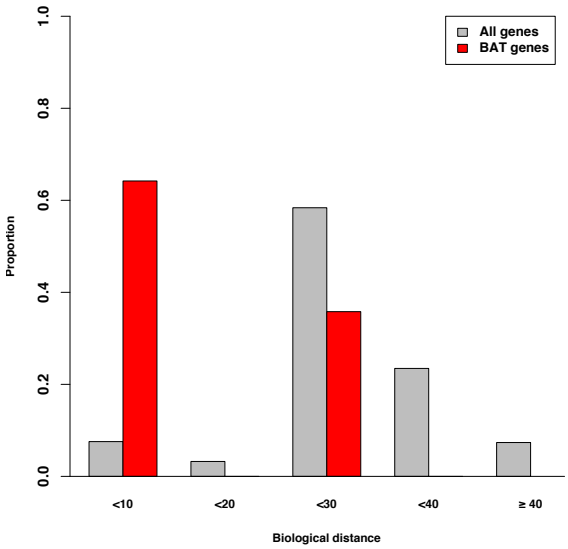


Figure 1: Comparison of biological distance between BAT genes and all human genes. Biological distances between BAT genes (red) and all human genes (gray) are distributed according to the proportion of distances in five categories: i) small biological distance (<10); ii) small-medium biological distance (between 10 and 20); iii) medium-large biological distance (between 20 and 30); iv) large-very large biological distance (between 30 and 40); v) extremely large biological distance (>40).

Computing resources and programming languages

This project was performed on a cluster with 48 cores and 131 GB RAM. Biological distances between genes were calculates by the HGC. Data extraction and statistical simulations, along with FGA phylogenetic analyses and visualizations were performed with the R programming language [8]. The programs and online server used in this study (in particular for ranking

candidate genes by biological distance from UCP1 and FGA trees generation) are freely available to non-commercial users with step-by-step instruction at http://lab.rockefeller.edu/casanova/HGC and http://hgc.rockefeller.edu, and the scripts for the minor technical procedures in this study are available upon request.

Results

Small biological distances between BAT genes

We tested the hypothesis that BAT genes are functionally close to each other (which would be prerequisite for identifying candidate BAT genes on the basis of biological proximity), by comparing the biological distance between BAT genes and all human genes, in terms of the proportions of biological distances assigned to five categories, from the smallest to the larges (Figure 1). Most intra-BAT gene distances belonged to the very small category (64%), the proportion of BAT genes falling into this category being larger than for all human genes (7%). We found that the median biological distance between BAT genes was 4.72, while that between simulated sets of 168 genes was 17.78. None of the simulated sets of 168 random genes had a median equal or smaller than that of BAT genes ($p < 10^{-5}$; 95 CI: 0 - 0.00036), consistent with the hypothesis of tight functional interrelatedness between BAT genes. While it was expected that genes belonging to the same pathway would display small biological distances between each other, confirming this hypothesis make it possible to infer novel genes related with BAT based on HGC-predicted biological distance to genes known related with this tissue.

Final set of proposed novel BAT-candidates genes and assessment of predictive power

The final list of candidates includes 102 genes, which were included in a hierarchical clustering along with known or suggested BAT genes [3,7]. This analysis showed that the candidate BAT genes identified in this study were evenly distributed over the whole range of known or suggested BAT genes (Figure 2). Moreover, we demonstrated the utility of this approach for identify novel candidate BAT genes. 60 genes related with BAT according to literature (Appendix S1) were included in the BAT connectome. This is a 35.92% of total connectome genes, which is higher than expected by chance with $p < 10^{-7}$ (95 CI: 0 - 4.79×10^{-6}). In addition, 5 genes suggested as potential candidates by literature were also include in the connectome $p < 10^{-7}$; 95 CI: 0 - 4.79×10^{-6}). These results support the predictive power of the BAT connectome.

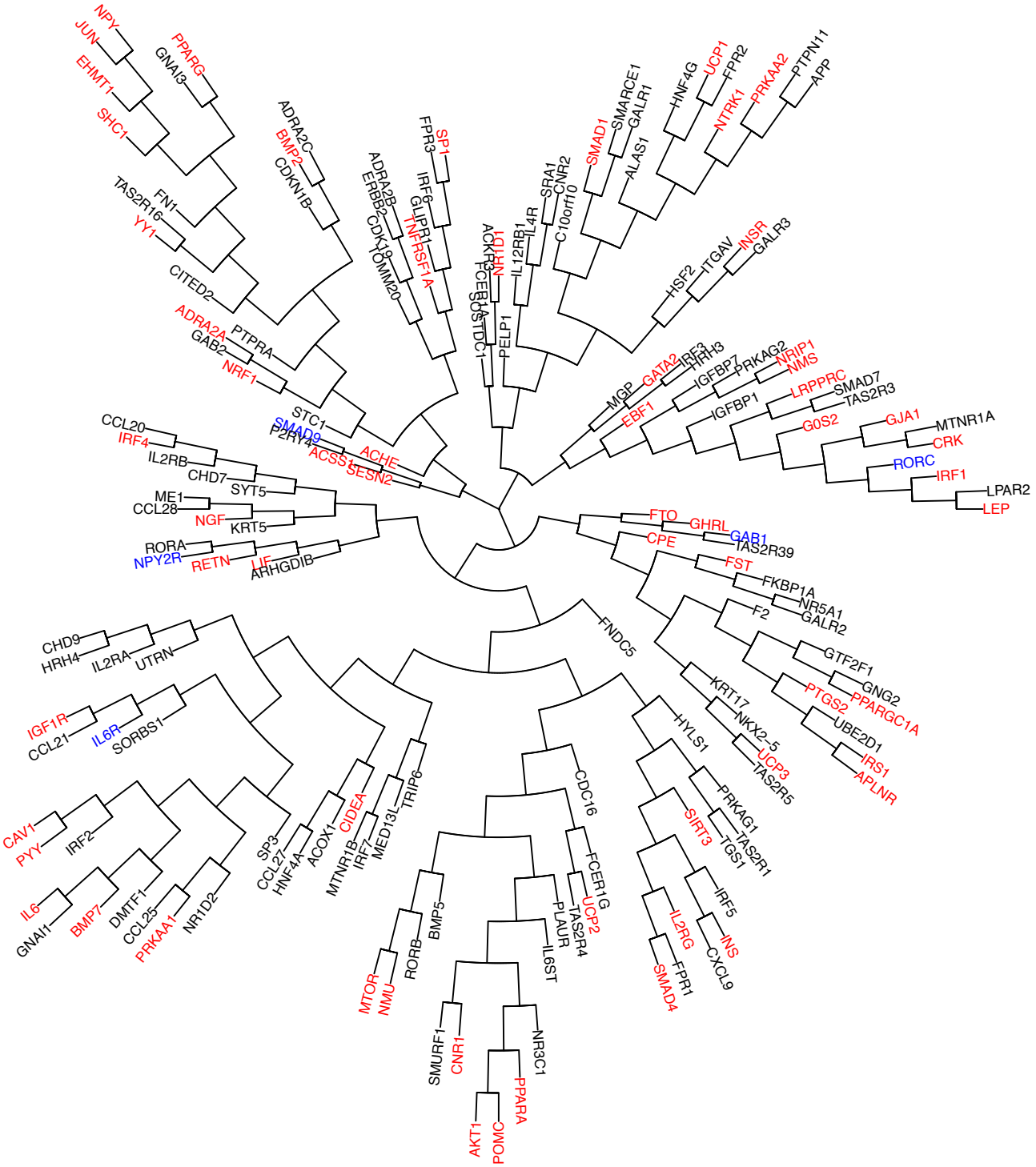


Figure 2: Functional genomic alignment phylogeny of BAT genes. A phylogenetic tree of biological distances generated by the functional genomic alignment method, showing hierarchical clustering of BAT genes. Genes related with BAT according to the literature are shown in red (known) and blue (suggested), whilst predicted-novel candidate genes are in black.

Discussion

We described here a list of 102 novel BAT-candidate genes, initially predicted *in silico* with the HGC biological distance concept. We extracted the 1% of genes biologically closest to UCP1, we then showed that selected genes (BAT connectome) were significantly closer to each other biologically than other human genes. From this connectome we selected those genes for which there is not evidence of relationship with BAT. We generated a final *in silico*-predicted set of 102 human genes, which may be considered reliable candidate BAT genes. In other words, we predict that there will be a high proportion of BAT-related genes among these 102 genes. We then demonstrate the predictive power of BAT connectome by means of checking that the number of known or suggested BAT-related genes included in the connectome was higher than expected by chance.

It is important to note that as in any high-throughput genome-wide analysis, the choice of input data, algorithm, and even small fine-tuning is likely to strongly affect the outcome. However, we attempted here to provide a reliable prediction where false positives (i.e., false BAT genes that are included in the final provided list of candidates genes) are minimized – an aim that is expected to be achieved by selecting only one core gene strongly implicated with the functioning of brown adipose tissue (UCP1). This approach has as counterpart a higher probability of false negatives (i.e., true BAT genes that are not included in the final list), but we preferred to prioritizes exclusion of false positives given the type of study for which this connectome is mainly aimed: candidate gene association studies. These studies use a limited list of genes whose relationship with interest phenotype have to be suggested by previous knowledge [2]. Therefore, a list of 102 novel-candidate genes that are predicted to be biologically close to known BAT genes increases the potential list of genes to be used in CGAS, and hence could be very useful to improve our knowledge about the genetic architecture of brown adipose tissue.

References

1. Cannon B, Nedergaard J. Brown Adipose Tissue: Function and Physiological Significance. *Physiol Rev*. 2004;84(1):277-359. doi:10.1152/physrev.00015.2003.
2. Jorgensen TJ, Ruczinski I, Kessing B, Smith MW, Shugart YY, Alberg AJ. Hypothesis-driven candidate gene association studies: Practical design and analytical considerations. *Am J Epidemiol*. 2009;170(8):986-993. doi:10.1093/aje/kwp242.
3. Itan Y, Zhang S-Y, Vogt G, et al. The human gene connectome as a map of short cuts for morbid allele discovery. *Proc Natl Acad Sci U S A*. 2013;110(14):5558-5563. doi:10.1073/pnas.1218167110.
4. Itan Y, Mazel M, Mazel B, et al.

HGCS: an online tool for prioritizing disease-causing gene variants by biological distance. *BMC Genomics*. 2014;15(1):256. doi:10.1186/1471-2164-15-256.
5. Itan Y, Casanova J-L, Candotti F, Rosenzweig S, Giliani SC. Novel primary immunodeficiency candidate genes predicted by the human gene connectome. *Front Inmunol*. 2015;6. doi:10.3389/fimmu.2015.00142.
6. Flicek P, Ahmed I, Amode MR, et al. Ensembl 2013. *Nucleic Acids Res*. 2013;41(D1):48-55. doi:10.1093/nar/gks1236.
7. Paradis E, Claude J, Strimmer K. APE: analyses of phylogenetics and evolution in {R} language. *Bioinformatics*. 2004;20:289-290.
8. R Core Team. R: A Language and Environment for Statistical Computing. 2017.

GENERAL DISCUSION

DISCUSSION

Currently, obesity and CVD are high-prevalent disorders that cause a huge amount of co-morbidities and deaths worldwide. Besides several decades of research focused in these pathologies, now we just beginning to understand the underlying mechanisms. It seems to be clear multifactorial etiology in the onset of them and the importance of the interplay between genetics and environmental factors. Last decades study of genetic predisposition and impact different genetics variants within population to develop these diseases have increased.

To gain more insights into this field, we investigated polymorphisms associated to obesity firstly (**Chapter 1** and **Chapter 2**), polymorphisms associated to cardiovascular disease risk factors (**Chapters 3, 4** and **5**) and finally we performed a BAT-Connectome in order to search new candidate genes that could guide us in further investigations (**Chapter 6**).

SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED TO OBESITY

Nowadays, a large number of genes and their polymorphisms are well known associated to obesity such as *FTO, MC4R, LEP* or *POMC*[1,2]. However, except some genes responsible of monogenic obesity (very low percentage of total obesity), influence of multiple genes and their interaction with lifestyle behaviour and others environmental factors is major cause of obesity in general population. Therefore, interest in discovering new genes that could partially affect to development of this disorder have increased last years. We performed an intense research of genetic variants associated to adiposity markers in our database of European adolescent (n=1057) of a multi-centric study: the HELENA study.

Firstly, we found a very solid association between *CNTF* and adiposity markers **(Chapter 1)**. Until now, very few studies have related *CNTF* with obesity and only one work of Heidema et al.[3] have found any association of genetic variants of *CNTF* with adiposity markers in humans. Our findings shown an association between rs2509914, rs2515363 and rs2515362 *CNTF* polymorphisms with adiposity markers (BMI, waist circumference, waist to height ratio and waist to hip ratio). We also observed a haplotype association between CNTF haplotype (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362) BMI, waist circumference, waist to hip and waist to height.

On the other hand, we also found a relationship between adiposity markers and *UCP* genes (**Chapter 2**). We found an association between the *UCP1* rs6536991 polymorphism and the risk of overweight in adolescents. We also found an interaction between *UCP1* rs2071415 polymorphism and PA regarding waist to hip ratio, suggesting that meeting the daily PA

recommendations (at least 60 minutes/day of moderate to vigorous physical activity) may offset the genetic predisposition to obesity associated with this polymorphism in European adolescents. Several studies have investigated the effect of polymorphisms of *UCP* on obesity and overweight, but results were controversial and this questions were not totally clarified[4–8].

To understand our results related with obesity: i) in **Chapter 1**, we described that *CNTF* has a role as neurotrophic factor (control, modulation and developing of nervous system and their functions[9]) and also has a role in control of body weight[10,11]. CNTF promotes weight loss is most likely trough satiety mechanisms owing to similarities between *CNTF* and leptin signalling cascade (close relationships between leptin receptor and *CNTF* receptor complex, overlapping in molecules activation between *CNTF* and *leptin*, like *STAT3* and *CNTF* and *leptin* receptors have overlapping distributions in some hypothalamic nuclei involved in feeding control[12]). So these similarities with leptin may explain the underlying mechanism trough some polymorphisms of *CNTF* could lead to functionally altered CNTF proteins and therefore have an effect in control of satiety and weight control; ii) in **Chapter 2**, uncoupling proteins has several physiological functions. *UCP1* is well known as responsible of heat production through non-shivering thermogenesis in brown BAT[13], meanwhile *UCP2* and *UCP3* genes have been related with obesity phenotypes through muscle[14], diabetes mellitus and lipid/ lipoprotein-related diseases [15]. More specifically, *UCP2* is involved in the control of ROS production, a modulator of insulin secretion, a regulator of mitochondrial fatty acid oxidation[16] and may have a regulating role in thermogenesis of BAT[17]. So, uncoupling activity could be altered regarding different alleles of each polymorphism, and therefore reduce heat dissipation as well as lipid oxidation[13], leading to overweight/obesity.

In conclusion, *CNTF* and *UCP* genes are able to produces an effect over adiposity markers through different mechanisms (satiety modulation or lipids oxidation respectively). And different polymorphisms of them may have an altered efficiency in their role.

SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED TO CARDIOVASCULAR DISEASE RISK FACTORS

CVDs main risk factors are well studied, especially those related with lifestyle behaviour. However the research of gene pool associated to these factors have increased in last years, searching genetic susceptibility to suffer or develop a CVD risk factor[18].

In **Chapter 3**, we showed our findings about *ADIPOQ* polymorphisms and CVD risk factors. To our knowledge, this is the first study investigating the association between *ADIPOQ* gene

polymorphisms and CVD risk factors in European adolescents. *ADIPOQ* polymorphisms rs822393, rs822395 and rs7649121 were significant associated to several CVD risk factors (i.e. HDL, Apo1, SBP, Risk Score). We also found a significantly association of the *ADIPOQ* haplotype (rs822393, rs16861210, rs822395, rs822396, rs12495941 and rs7649121) with HDL and Apo1 serum levels.

In **Chapter 4**, we also found a relationship with uncoupling proteins. Two *UCP2* polymorphisms (rs2735572 and rs17132534) and haplotypes of *UCP1* and *UCP3* were associated with cardiovascular disease risk factors (higher diastolic blood pressure, ApoB/ApoA1, ApoB levels and Risk Score). Until now, very few studies suggested any link-up between cardiovascular risk factors and gene polymorphisms of uncoupling proteins. Just some reported an association of single polymorphisms of *UCP3* or combined haplotypes *UCP2/UCP3* with several CVD risk factors such as serum total and LDL-cholesterol levels, insulin or HOMA in adults[19–21].

In **Chapter 5**, *LPL* gene polymorphisms were associated to cardiovascular risk factors, but also to adiposity markers and PA. Results of this chapter showed a significant association between alleles of rs1534649, rs258, rs320 and rs328 with several markers of CVDs (i.e. BMI, TG, Leptin, cholesterol/HDL, LDL/HDL, TG/HDL). Also, haplotypes of *LPL* polymorphisms rs282-rs285 and rs3126-rs320-rs328-rs10099160 were significant associated to TG, TG/HDL. Haplotype rs3126-rs320-rs328-rs10099160 was also associated to risk score. Finally, we observed alleles of rs1534649 and rs258 related to higher values of adiposity markers tend to be associated with less adiposity under high levels of PA. A remarkable amount of studies found a relationship between *LPL* polymorphisms and CVDs or cardiovascular risk factors [22–29]. Nevertheless, multiple discordances and unclear associations generate lack of reliable data in this field. With our results, we hope in this chapter help to clarify information about *LPL* and its role in cardiovascular disease and adiposity markers.

A biological explanation to these findings includes: i) in **Chapter 3**, adiponectin is the most abundant peptide secreted by adipocytes. Decreased adiponectin levels are thought to play a central role in the development of type 2 diabetes, obesity and cardiovascular disease in humans[30]. Adiponectin acts downstream over insulin receptor substrates, over lipids oxidation and energy expenditure through regulation of AMP activated protein kinase (AMPK), PPAR-alpha (thus CoA oxidase and uncoupling proteins), improves glucose metabolism through APPL1, and others effects[30]; ii) in **Chapter 4**, we mentioned yet in **Chapter 2** physiological functions of uncoupling proteins and their impact of human metabolism. Uncoupling proteins use glucose and fatty acids as energetic substrate, and therefore may play a

role in lipid/ lipoprotein-related diseases onset or development[15]; iii) in **Chapter 5**, *LPL* is a key player in lipid metabolism, especially over VLDL and LDL control. Indeed, *LPL* deficiency results in an accumulation of chylomicrons and other triglyceride-rich lipoproteins in the plasma, producing hypertriglyceridemia[31].

In conclusion *ADIPOQ*, *UCPs* and *LPL* genes may have a role in the onset, control or development of CVD risk factors through different biological procedures. Various polymorphisms of a singular gene could associate in different ways to their resultant cardiovascular phenotypes.

SEARCHING FOR NEW CANDIDATE GENES: CONNECTOME

Novel candidate genes could be approached through different tools. Here, we used the human gene connectome (HGC) to predict novel-candidate genes for brown adipose tissue. Results from this doctoral thesis showed a possible relationship between uncoupling proteins and adiposity markers and cardiovascular risk factors (**Chapter 2** and **Chapter 4**, respectively). Taken together, this findings support the hypothesis of BAT as a possible therapeutic target against obesity and related disorders due to its ability to oxidize glucose and lipids and to dissipate energy as heat[32]. So, perform a tool to predict related genes to uncoupling proteins makes an interesting procedure to discover BAT-related genes that could help us to understand the physiology of this tissue.

We described in **Chapter 6**, a list of 102 novel BAT-candidate genes (1% of genes biologically closest to UCP1) initially predicted *in silico* with the HGC biological distance concept. After that we showed that these 102 novel BAT-candidate genes (BAT connectome) were significantly closer to each other biologically than other human genes. From this connectome we observed a high amount of genes, which there is not previous evidence of relationship with BAT. We generated a final *in silico*-predicted set of 102 human genes, which may be considered reliable candidate BAT genes.

In conclusion, BAT connectome could be a very useful tool to increase our knowledge about the genetic architecture of BAT.

PHYSICAL ACTIVITY-GENE INTERACTION

It is well known that cardiovascular disease risk factors, including obesity, are a result of a complex interaction between genetic and environmental factors. In one hand, genetics has a powerful role in obesity and cardiovascular disease development. Indeed, a large number of genes have been associated to them[4,23,28,33,34]. In most cases, genetic predisposition comes from the interplay between multiple genes, and not from a single one (except some cases of monogenic obesity with leptin, leptin receptor, pro-

opiomelanocortin, and prohormone convertase 1 genes)[33]. So, it is really interesting to know the largest possible number of genes or genetic variants that implicates a certain disorder. The more we are capable to know about the genetic architecture of a determined disorder, more we approximate to the real genetic predisposition of population against him. On the other hand, environmental factor has also a key role in obesity and cardiovascular diseases development. Evidence suggest that regular exercise training provides a wide range of health benefits in the general population, including improvements in blood pressure, diabetes, lipid profile (increases high-density lipoprotein, reduce triglycerides, low density lipoprotein and cholesterol levels)[35] and decrease cardiovascular disease risk[36]. In adolescents, we showed in previous studies that meeting the daily physical activity recommendations (at least 60 minutes/day of moderate to vigorous physical activity) may offset the genetic predisposition to a main cardiovascular risk factor, such as obesity, associated with the *FTO* rs9939609[9] and *UCP1* rs2071415[10] polymorphism in European adolescents. We found in this doctoral thesis a PA-gene interaction in **Chapter 2** and **Chapter 5**. In **Chapter 2** we shown that an interaction between the *UCP1*rs2071415 polymorphism and PA regarding waist to hip ratio. Adolescents meeting the daily physical activity recommendations may overcome the effect of the *UCP1* rs2071415 polymorphism on waist to hip ratio. In **Chapter 5**, we observed that *LPL* alleles of rs1534649 and rs258 related to higher values of adiposity markers tend to be associated with less adiposity under high levels of physical activity. So we found that some polymorphisms were associated to adiposity traits and PA could ameliorate this negative effect.

LIMITATIONS

The present Doctoral Thesis has several limitations that should be acknowledged: i) the studies are chapters are observational, so future experimental studies are still needed to examine if there is direct causal correlation between polymorphisms of these genes and the studied phenotypes; ii) the associations between the study polymorphisms and the phenotypes could be altered by gene-gene and other gene-environmental interactions; iii) unfortunately, we have no information on relatedness patterns among the participants, and we do not know the ethnic/racial make-up of the sample.

Therefore, results of the present doctoral thesis should be considered carefully and experimental studies could help to further confirm this possible genetic predisposition.

REFERENCES

1. Farooqi IS, O'Rahilly S. Genetics of obesity in humans. *Endocr Rev*. 2006;27(7):710-718. doi:10.1210/er.2006-0040.
2. Scuteri A, Sanna S, Chen WM, et al. Genome-wide association scan shows genetic variants in the FTO gene are associated with obesity-related traits. *PLoS Genet*. 2007;3(7):1200-1210. doi:10.1371/journal.pgen.0030115.
3. Heidema AG, Wang P, van Rossum CTM, et al. Sex-specific effects of CNTF, IL6 and UCP2 polymorphisms on weight gain. *Physiol Behav*. 2010;99(1):1-7. doi:10.1016/j.physbeh.2009.10.002.
4. Ramos A V, Bastos-Rodrigues L, Resende BA, et al. The contribution of FTO and UCP-1 SNPs to extreme obesity, diabetes and cardiovascular risk in Brazilian individuals. *BMC Med Genet*. 2012;13(1):101. doi:10.1186/1471-2350-13-101.
5. Salopuro T, Pulkkinen L, Lindström J, et al. Variation in the UCP2 and UCP3 genes associates with abdominal obesity and serum lipids: the Finnish Diabetes Prevention Study. *BMC Med Genet*. 2009;10(1):94. doi:10.1186/1471-2350-10-94.
6. Martinez-Hervas S, Mansego ML, de Marco G, et al. Polymorphisms of the UCP2 gene are associated with body fat distribution and risk of abdominal obesity in Spanish population. *Eur J Clin Invest*. 2012;42(2):171-178. doi:10.1111/j.1365-2362.2011.02570.x.
7. Ochoa MC, Santos JL, Azcona C, et al. Association between obesity and insulin resistance with UCP2-UCP3 gene variants in Spanish children and adolescents. *Mol Genet Metab*. 2007;92(4):351-358. doi:10.1016/j.ymgme.2007.07.011.
8. Kosuge K, Soma M, Nakayama T, et al. Human uncoupling protein 2 and 3 genes are associated with obesity in Japanese. *Endocrine*. 2008;34(1-3):87-95. doi:10.1007/s12020-008-9111-9.
9. Huang EJ, Reichardt LF. Neurotrophins: Roles in Neuronal Development and Function. *Annu Rev Neurosci*. 2001;24(1):677-736. doi:10.1146/annurev.neuro.24.1.677.
10. Shapiro L, Zhang XX, Rupp RG, Wolff SM, Dinarello C a. Ciliary neurotrophic factor is an endogenous pyrogen. *Proc Natl Acad Sci U S A*. 1993;90(18):8614-8618. doi:10.1073/pnas.90.18.8614.
11. Miller RG, Petajan JH, Bryan WW, et al. A placebo-controlled trial of recombinant human ciliary neurotrophic (rhCNTF)

factor in amyotrophic lateral sclerosis. rhCNTF ALS Study Group. *Ann Neurol*. 1996;39(2):256-260. doi:doi.org/10.1111/j.1460-9568.1993.tb00201.

12. Xu B, Xie X. Neurotrophic factor control of satiety and body weight. *Nat Rev Neurosci*. 2016;17(5):282-292. doi:10.1038/nrn.2016.24.
13. Golozoubova V, Cannon B, Nedergaard J. UCP1 is essential for adaptive adrenergic nonshivering thermogenesis. *Am J Physiol Endocrinol Metab*. 2006;291(2):E350-E357. doi:10.1152/ajpendo.00387.2005.
14. Schrauwen P, Hesselink M. UCP2 and UCP3 in muscle controlling body metabolism. *J Exp Biol*. 2002;205(Pt 15):2275-2285.
15. Jia JJ, Zhang X, Ge CR, Jois M. The polymorphisms of UCP2 and UCP3 genes associated with fat metabolism, obesity and diabetes: Etiology and pathophysiology. *Obes Rev*. 2009;10(5):519-526. doi:10.1111/j.1467-789X.2009.00569.x.
16. Pecqueur C, Alves-Guerra C, Ricquier D, Bouillaud F. UCP2, a metabolic sensor coupling glucose oxidation to mitochondrial metabolism? *IUBMB Life*. 2009;61(7):762-767. doi:10.1002/iub.188.
17. Caron A, Labbé SM, Carter S, et al. Loss of UCP2 impairs cold-induced non-shivering thermogenesis by promoting a shift toward glucose utilization in brown adipose tissue. *Biochimie*. 2017;134:118-126. doi:10.1016/j.biochi.2017.01.006.
18. Shukla H, Mason JL, Sabyah A. Identifying genetic markers associated with susceptibility to cardiovascular diseases. *Futur Sci OA*. 2019;5(1):FSO350. doi:10.4155/fsoa-2018-0031.
19. Hsu YH, Niu T, Song Y, Tinker L, Kuller LH, Liu S. Genetic variants in the UCP2-UCP3 gene cluster and risk of diabetes in the Women's Health Initiative Observational Study. *Diabetes*. 2008;57(4):1101-1107. doi:10.2337/db07-1269.
20. Salopuro T, Pulkkinen L, Lindström J, et al. Variation in the UCP2 and UCP3genes associates with abdominal obesity and serum lipids: The Finnish Diabetes Prevention Study. *BMC Med Genet*. 2009;10(1):94. doi:10.1186/1471-2350-10-94.
21. Mottagui-Tabar S, Hoffstedt J, Brookes AJ, Jiao H, Arner P, Dahlman I. Association of ADRB1 and UCP3 gene polymorphisms with insulin sensitivity but not obesity. *Horm Res*. 2007;69(1):31-36. doi:10.1159/000111793.
22. Gao R-R, Wang M, Hu Y, et al. Impact of LPL gene rs283 polymorphism on exercise-induced changes in metabolism of obese adolescents and the

regulatory mechanisms behind it. *Exp Physiol*. 2015;100(6):698-707. doi:10.1113/EP085127.

23. Palacio Rojas M, Prieto C, Bermúdez V, et al. Dyslipidemia: Genetics, lipoprotein lipase and HindIII polymorphism. *F1000Research*. 2018;6(0):2073. doi:10.12688/f1000research.12938.2.
24. Cho YS, Go MJ, Han HR, et al. Association of lipoprotein lipase (LPL) single nucleotide polymorphisms with type 2 diabetes mellitus. *Exp Mol Med*. 2008;40(5):523-532. doi:10.3858/emm.2008.40.5.523.
25. Velasquez Pereira LC, Vargas Castellanos CI, Silva Sieger FA. Polymorphisms of the lipoprotein lipase gene as genetic markers for stroke in colombian population: a case control study. *Colomb medica (Cali, Colomb*. 2016;47(4):189-195.
26. Moghadasi M, Kelishadi R, Marateb HR, et al. Logic Regression Analysis of Gene Polymorphisms and HDL Levels in a Nationally Representative Sample of Iranian Adolescents: The CASPIAN-III Study. *Int J Endocrinol Metab*. 2017;15(3):e14037. doi:10.5812/ijem.14037.
27. Ariza M-J, Sanchez-Chaparro M-A, Baron F-J, et al. Additive effects of LPL, APOA5 and APOE variant combinations on triglyceride levels and hypertriglyceridemia: results of the ICARIA genetic sub-study. *BMC Med Genet*. 2010;11:66. doi:10.1186/1471-2350-11-66.
28. Shahid SU, Shabana, Cooper JA, et al. Genetic risk analysis of coronary artery disease in Pakistani subjects using a genetic risk score of 21 variants. *Atherosclerosis*. 2017;258:1-7. doi:10.1016/j.atherosclerosis.2017.01.024.
29. Bentley AR, Chen G, Shriner D, et al. Gene-based sequencing identifies lipid-influencing variants with ethnicity-specific effects in African Americans. *PLoS Genet*. 2014;10(3):e1004190. doi:10.1371/journal.pgen.1004190.
30. Achari AE, Jain SK. Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. *Int J Mol Sci*. 2017;18(6). doi:10.3390/ijms18061321.
31. Okubo M, Horinishi A, Saito M, et al. A novel complex deletion-insertion mutation mediated by Alu repetitive elements leads to lipoprotein lipase deficiency. *Mol Genet Metab*. 2007;92(3):229-233. doi:10.1016/j.ymgme.2007.06.018.
32. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev*. 2004;84(1):277-

359. doi:10.1152/physrev.00015.2003.
33. Sheikh AB, Nasrullah A, Haq S, et al. The Interplay of Genetics and Environmental Factors in the Development of Obesity. *Cureus*. 2017;9(7):e1435. doi:10.7759/cureus.1435.
34. Moukdar F, Robidoux J, Lyght O, Pi J, Daniel KW, Collins S. Reduced antioxidant capacity and diet-induced atherosclerosis in uncoupling protein-2-deficient mice. *J Lipid Res*. 2009;50(1):59-70. doi:10.1194/jlr.M800273-JLR200.
35. Physical activity and cardiovascular health. NIH Consensus Development Panel on Physical Activity and Cardiovascular Health. *JAMA*. 1996;276(3):241-246.
36. Sniderman AD, Pedersen T, Kjekshus J. Putting low-density lipoproteins at center stage in atherogenesis. *Am J Cardiol*. 1997;79(1):64-67.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

MAIN CONCLUSIONS

The main conclusions of the present doctoral thesis are, i) different polymorphisms of a same gene could predispose in European adolescents to develop phenotypes associated to metabolic disorders, ii) in certain polymorphisms that predisposing to altered obesity traits, physical activity could ameliorate this deleterious effect, and iii) BAT connectome could be an advantageous tool to discover unknown BAT-related novel genes.

PART 1: **SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED TO OBESITY**

Chapter 1: The rs2509914, rs2515363 and rs2515362 *CNTF* polymorphisms are associated with adiposity markers in adolescents from nine European countries. We also observed a haplotype association a *CNTF* haplotype (rs2509914, rs17489568, rs2515363, rs1800169 and rs2515362) with several adiposity markers.

Chapter 2: The *UCP1*rs6536991 polymorphism is associated with risk of overweight in adolescents. Our results also suggest that physical activity could compensate the deleterious effect of the *UCP1*rs2071415 polymorphism on adiposity markers.

PART 2: **SINGLE NUCLEOTIDE POLYMORPHISMS ASSOCIATED TO CARDIOVASCULAR DISEASE RISK FACTORS**

Chapter 3: The rs822393, rs822395, rs7649121 polymorphisms and six polymorphisms haplotype of the *ADIPOQ* gene are associated with CVD risk factors in European adolescents.

Chapter 4: The *UCP2* rs2735572 and rs17132534 polymorphisms are associated with higher diastolic blood pressure in adolescents from nine European countries. We also observed a haplotype association of *UCP1* and *UCP3* with higher blood apolipoproteins levels and risk score, respectively.

Chapter 5: The single polymorphisms rs1534649, rs258, rs320 and rs328 and two polymorphisms haplotypes (rs282, rs285 and rs3126, rs320, rs328, rs10099160) of the *LPL* gene are associated with cardiovascular disease risk factors in European adolescents. Also, our results suggest that physical activity can ameliorate the deleterious effect of the *LPL* rs1534649 and rs258 *LPL* polymorphisms on body fat estimates in adolescents.

PART 3: **SEARCHING FOR NEW CANDIDATE GENES: CONNECTOME**

Chapter 6: A list of 102 novel-candidate genes that are predicted to be biologically close to known BAT genes increases the potential list of genes to be used in Hypothesized-candidate gene association studies, and hence could be very useful to improve our knowledge about the

genetic architecture of brown adipose tissue.

FUTURE RESEARCH LINES

- Further experimental studies to test the predisposition effect of these gene polymorphisms against metabolic disorders.
- Future genome-wide association studies to search new polymorphisms associated.
- Further connectome performing applied to different core genes of main metabolic disorders as obesity or cardiovascular disease, to help us to discover new candidate novel gene of their genetic architecture.
- In medical environment: evidence suggests changes in an individual's genes or DNA can cause some form of CVDs, CVDs risk factors and obesity traits, highlighting a complex relationship between genes and the environment. Genotyping, a process used to determine genetic differences within an individual's DNA, can provide doctors with relevant information to identify individuals who are at high risk of developing CVDs. This would allow treatment to begin early and encourage individuals to adopt a healthy lifestyle to reduce their risk.

ANEXES

SHORT CV

- **COLABORATION SCOLARSHIP (Ministry of education, culture and sport) (2013-2014)** "Commitment between growth and immune system response in *Pica pica* nestlings". Dr. Gregorio Moreno Rueda. Animal Biology Department. *University of Granada*

- **INITIATION TO RESEARCH SCOLARSHIP INITIATION (University of Granada) (2012-2013)**. " Molecular markers and genetic basis in arteriosclerosis and cancer". Prof. Ana Linares Gil. Biochemistry and Molecular Biology Department. *University of Granada*

- **CICERONE SCOLARSHIP (Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC)) (2012)**. "Development of the epicardium and its role during regeneration". Nadia Mercader Huber, Junior group leader. *CNIC (Madrid).*

- **INTERNSHIP (2011-2012)**. "Carbohydrates: synthesis and reactivity". Prof. Francisco Santoyo González. Organic chemistry department – *University of Granada.*

- **INTERNSHIP (2011)**. "Gene expression in the rizhosphere", Dr Mª Isabel Ramos González. Zaidín experimental station (EEZ) –*CSIC.*

6. **Lab skill**

- *In situ hibridation (sections)*
- *In situ hibridation (whole mount)*
- *PCR*
- *DNA extraction*
- *Western Blot*
- *Inmunohistochemistry*
- *Eukariotic cultures*
- *Zebrafish handling (cryoinjury, egg microinjection)*
- *Cells culture*

7. Congress participations

1. SALAZAR TORTOSA, D., PASCUAL GAMARRA, JM. and RUIZ RUIZ, J. (2017). "Selection signatures of climate on brown adipose tissue and implications on obesity susceptibility". In: *empseb23*. Krasiczyn (Poland): Maria Niklinska.

2. PASCUAL GAMARRA, JM. and RUIZ RUIZ, J. (2018). "Association of UCPs genetics variants with markers of adiposity in European adolescents". In: CEIBS. Granada (Spain)

8. PUBLICATIONS

1. Pascual-Gamarra JM, Salazar-Tortosa D, Martinez-Tellez B, et al. "*Association between UCP1 , UCP2 , and UCP3 gene polymorphisms with markers of adiposity in European adolescents: The HELENA study".* Pediatr Obes. 2019. doi:10.1111/ijpo.12504

2. Pascual-Gamarra JM, Salazar-Tortosa D, Labayen I, et al. "*Association of UCP1, UCP2 and UCP3 gene polymorphisms with cardiovascular disease risk factors in European adolescents: the HELENA study".* Pediatr Res. 2019.

3. Pascual-Gamarra JM, Salazar-Tortosa D, Ruiz JR. "*Association between CNTF gene polymorphisms and adiposity markers in European adolescents: the HELENA study".* Submitted

4. Pascual-Gamarra JM, Salazar-Tortosa D, Ruiz JR. "*Association between ADIPOQ gene polymorphisms and cardiovascular disease risk factors in European adolescents: The HELENA study".* Submitted

5. Pascual-Gamarra JM, Salazar-Tortosa D, Ruiz JR. "*Association between LPL single nucleotide polymorphisms and cardiovascular disease risk factors in European adolescents: The HELENA study".* Submitted

6. Salazar-Tortosa D, Pascual-Gamarra JM, Yuval I. "*Novel brown adipose tissue candidate genes predicted by the human gene connectome.".* Submitted

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