Universidad de Navarra

Facultad de Farmacia y Nutrición

Cardiometabolic risk after two lifestyle interventions in children with obesity: the role of genetic markers

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Pamplona, 2019

Universidad de Navarra

Memoria presentada por Dña. Lydia Morell Azanza para aspirar al grado de Doctor por la Universidad de Navarra.

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El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación y Fisiología y autorizo su presentación ante el tribunal que lo ha de juzgar.

Pamplona, 7 de Junio de 2019

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Este trabajo ha sido posible gracias a la financiación del programa de proyectos de investigación de la Universidad de Navarra (PIUNA), el Gobierno de Navarra (PI 54/2009) la fundación MERCK, y la beca AEP-ORDESA. La investigación que ha dado lugar a estos resultados ha sido impulsada por la beca predoctoral 2015-2019 de la Asociación de Amigos de la Universidad de Navarra y la Fundación La Caixa y la ayuda predoctoral de movilidad internacional de la Fundación Caja Rural, ADA y La Caixa.

“Se fiel a las cosas pequeñas, ya que es en ellas donde reside la fuerza”

Santa Teresa de Calcuta

AGRADECIMIENTOS

En primer lugar, me gustaría agradecer a la Universidad de Navarra, a la Facultad de Farmacia y Nutrición y al Departamento de Ciencias de la Alimentación y Fisiología por haberme guiado en mi trayectoria académica desde los inicios hasta el final. Me he sentido en los pasillos de la Universidad como si fuesen mi hogar.

Me gustaría agradecer a la Asociación de Amigos de la Universidad de Navarra, por la labor que realizan recaudando fondos para que personas como yo podamos realizar la tesis doctoral y contribuir a la mejora de la sociedad a través de la investigación. Espero haber podido corresponder la confianza que depositaron en mi. Además, me gustaría agradecer a la Caixa, y a la Fundación Caja Rural por su ayuda económica para la realización de mi estancia en el extranjero.

A la Prof. Amelia Marti del Moral, quien ha dirigido la tesis doctoral, quiero agradecerle su apoyo incansable, su cercanía, el conocimiento que me ha transmitido y su ayuda para poner mis ideas y sugerencias en el proyecto. A la Dra. Azcona, mi codirectora, quiero agradecerle su experiencia clínica con los niños, su entusiasmo por la investigación clínica y su cercanía. Espero haber sido una buena doctoranda y haber correspondido a la responsabilidad que me dabais.

I would like to express my gratitude to Dr. Anke Hinney and to all her group at University Hospital Essen, University of Duisburg-Essen. Thank you for your knowledge on the area of MC4R and your hospitality in the laboratory.

Gran parte de mi tesis se la debo al equipo de personas con las que he trabajado en el día a día: a la Dra. Chueca, a las enfermeras de la CUN y de extracciones, a Mª Carmen, a los residentes de la CUN Amaia y Jose, a Ana Catalán, a las técnicos

de laboratorio: Ana, Vero y María. También me gustaría agradecer a todos los alumnos de estancias o de máster que nos han ayudado con el estudio: Alberto, Alicia, Adriana, Diego, Luisa, Isabel, espero que os haya gustado la experiencia de trabajar en nuestro equipo, parte de los resultados en esta tesis son gracias a vuestro trabajo.

La clave para que un trabajo que implica a tantas personas tenga éxito en su desarrollo, son las personas que forman parte de este. Ana es ese gran ejemplo de perfecta compañera de trabajo: una persona constante, dedicada, trabajadora, intuitiva, entusiasta, cariñosa, comprensiva, una persona que sólo suma y en la que he podido confiar y descansar. Estas palabras se quedan cortas para agradecerte todo lo que has hecho. En este equipo de trabajo, se ha incorporado Lucía, y sólo podemos dar gracias de que hayas venido a trabajar con nosotras. Incorporas a nuestro grupo alegría, locura, aptitudes que nos faltaban y que nos completan. No tengo ninguna duda de que vais a conseguir todo lo que os propongáis y que vais a ser dos personas excepcionales.

En este camino de la tesis, ha ido muy de mi mano Ana Arpón. Gracias por esos momentos juntas, por todas las charlas profesionales a las que hemos asistido y las personales que nos hemos impartido. Ya no te queda nada, aunque estoy segura de que lo tienes todo controlado.

Dentro de mis agradecimientos un gran espacio lo deben de ocupar esas personas con las que he estado todos los días, todas las personas del Departamento. A todos los profesores, investigadores, técnicos y demás doctorandos, GRACIAS. Cuando mi familia y mis amistades me preguntaban acerca de mi tesis y de su dureza, les decía siempre: tengo la gran suerte que todas las personas con las que trabajo son geniales, y aunque algo no te vaya bien, los tienes a ellos. En especial me gustaría agradecer a los más mayores, que me iniciaron en la investigación y

me hicieron sentir parte del departamento: Sonia, Leti, Idoia, Miguel, Irene, Mirian, Amaya, Leyre, Marcos, Jorge, Kike. A las nuevas generaciones, que llegáis pisando fuerte: Ana, Elisa, Amanda, Cristina, Angy, Nacho y Lusi, por todas las cosas buenas que hemos vivido y viviremos.

A Félix, mi hogar de Pamplona, a todas las que habéis vivido en él y conmigo durante estos cuatro años. Sabéis que esta tesis os la debo en parte a vosotras y a vuestro apoyo. Marta, Myri, Charlotte, Lucía, Cris, Marián muchas gracias. Gracias también a mis amigas: Cuca, Inma, María, Muntsa, Laura, porque muchas veces no hace falta estar en la misma ciudad para poder estar ahí. Quisiera agradecer a todas las personas que han formado parte de este camino.

Querida familia, espero que este trabajo os haga estar orgullosos de vuestra hija/hermana. Todo lo que soy y tengo os lo debo a vosotros, a vuestra entrega y dedicación en transmitirme los valores que tengo, la cultura del esfuerzo, la alegría en el trabajo diario y el amor en las cosas cotidianas. Por ser exigentes conmigo, pero también saber cómo serlo y hacerlo. Muchas gracias. Esta tesis es vuestra.

ABSTRACT

Childhood obesity has increased in recent years. In Spain about one in four children are overweight or obese, and most of them will carry this status into adulthood. Health consequences derived from obesity are numerous and complex, but the most frequent are those related to cardiometabolic risk and the future development of type 2 diabetes mellitus and cardiovascular disease.

Different strategies have been proposed to reduce adiposity and improve metabolic outcomes in children with obesity. Lifestyle interventions that combined dietary, physical activity and behavioural approaches with family involvement appear to be the best options to prevent weight regain. Since, obesity development stands from the interplay between lifestyle and genetic factors it is important to understand how genetic markers could influence the weight loss response. Thus, the general objective of the present project is to evaluate the effect of two lifestyle interventions on cardiometabolic risk in children with obesity. The role of genetic markers in the individual response to the intervention will also be evaluated. This thesis project includes two different lifestyle programs: 1) the NUGENOI study based on a dietary intensive intervention (10 weeks), and 2) the IGENOI study, an integral intervention with a 2-year duration (2 months + 22 months of follow up).

Both lifestyle interventions were able to reduce adiposity and improve metabolic outcomes. Parameters related to cardiometabolic risk such as inflammatory cytokines and oxidized LDL cholesterol were also diminished in children with obesity. In addition, greater changes on insulin and HOMA-IR derived from the lifestyle intervention were associated with higher cardiotrophin-1 gene expression at baseline.

The integral intervention of IGENOI study includes the promotion of physical activity in children. Concerning this, children with abdominal obesity from the intensive care group were more physically active (+38.5 minutes per week increase in moderate-to-vigorous physical activity assessed by accelerometry) and lowered their cardiometabolic risk after the intervention. In fact, favorable changes in MVPA were related to changes in leptin levels.

Telomere length is influenced by oxidative stress and inflammatory status, these two processes are present in obese subjects. We observed inverse associations between telomere length and adiposity indices. Moreover, baseline telomere length seemed to be a marker of changes in glucose levels. Moreover, no change in TL was observed, despite achieving a successful decrease in BMI-SDS after the integral lifestyle intervention.

Finally, IGENOI participants were screened for functional mutations in Melanocortin 4 Receptor (MC4R) and Lipocalin 2 (LCN2) genes. The prevalence for the two gene variants were 2.42% and 0.84% for MC4R and LCN2, respectively. These genetic variants seem to partially explain eating behaviors. Nevertheless, subjects with those functional mutations were able to decrease adiposity after our integral lifestyle intervention.

In summary, lifestyle interventions conducted in children with obesity were effective in the reduction of adiposity and metabolic outcomes. It appears that telomere length and cardiotrophin-1 gene expression could have a predictive role in glucose metabolism outcomes. Meanwhile, MC4R and LCN2 mutations might influence eating behaviours but did not change the weight loss response.

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropin Hormone
BMI	Body Mass Index
BMI-SDS	Body Mass Index Standard Deviation Score
BP	Blood Pressure
CEBQ	Children Eating Behavior Questionnaire
CRP	C Reactive Protein
CT-1	Cardiotrphin 1
DASH	Dietary Approach to Stop Hypertension
DEXA	Dual Energy X Ray
DNA	Deoxyribonucleic Acid
FFQ	Food Frequency Questionnaire
FTO	Fat mass and Obesity associated gene
GENOI	Grupo de Estudio Navarro de Obesidad Infantil
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
HDL-c	High Density Lipoprotein Cholesterol
IC	Intensive Care
IDF	International Diabetes Federation
IGENOI	Intervención GENOI
IL-6	Interleukin 6
IOTF	International Obesity Task Force
IR	Insulin Resistance
IRS1	Insulin receptor substrate
LCN2	Lipocalin 2
LDL-c	Low Density Lipoprotein Cholesterol
LEP	Leptin
LEPR	Leptin Receptor

MC4R	Melanocortin 4 Receptor
MD	Mediterranean Diet
MetS	Metabolic Syndrome
MHO	Metabolic Health Obese
MMqPCR	Monochrome multiplex real-time quantitative PCR
MVPA	Moderate to Vigorous Physical Activity
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-Alcoholic Fatty Liver Disease
NND	New Nordic Diet
NUGENOI	Nutrición y Obesidad Infantil
OMD	Optimized Mixed Diet
OSA	Obstructive Sleep Apnea
oxLDL	Oxidixed Low Density Lipoproteins
PA	Physical Activity
PBMC	Peripheral Blood Mononuclear Cells
PCOS	Polycystic Ovary Syndrome
POMC	Pro-opiomelanocortin
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SENC	Sociedad Española de Nutrición Comunitaria
T2DM	Type 2 Diabetes Mellitus
TL	Telomere Lenght
TNF- α	Tumor Necrosis Factor alpha
UC	Usual Care
VAT	Visceral Adipose Tissue
WHO	World Health Organization

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1.INTRODUCTION

Childhood Obesity

1.1 Definition and diagnostic criteria

The World Health Organization (WHO) describes obesity as an excess of fat mass that increase the risk of morbidity, altered physical, psychological or social well-being and/or mortality. Is an etiologically disease that was influenced by genetic, metabolic, hormonal, behavioral, environmental, psychological, economic and social factor (Hall, Guyenet, and Leibel, 2018).

As the measurement of the body fat is not always easy to measure with validated methodology such as: dual energy -X ray (DEXA), air displacement plethysmography (BODPOD) or bioelectrical impedance analysis, some anthropometric measurements have been validated for estimating body fat (Kumar and Kelly, 2017). Body mass index (BMI) is the most common adiposity index and results from the division between weight (Kg) and the squared height (m). Subjects with BMI $\geq$ 25 Kg/m^2 are overweight and those with BMI $\geq$ 30 Kg/m^2 could be defined as obese (World Health Organization).

In children BMI is not the gold standard for the diagnosis of obesity because growth can interfere in that measurement by the constant variability in height and body weight (Kumar and Kelly ,2017). For this reason, in pediatric population it is not possible to determine fixed cut-off points as it can be done in adults. The International Obese Task Force (IOTF) have established BMI international growth charts that take into account sex and age in order to match the values of 25 Kg/m^2 and 30 Kg/m^2 at 18 years. These growth charts were initially published in 2000 (Cole et al., 2000) and were update in 2012 (Cole and Lobstein, 2012). Another way for measuring childhood obesity is through BMI

standard deviation score (BMI-SDS). It has been proposed by the WHO and establish specific cut-off points for BMI-SDS and for percentiles. BMI-SDS (can be also called as z value for BMI) is defined as BMI values converted into standard deviation using age and specific cut off points according to national growth charts. The following formula indicates the way for calculating BMI-SDS:

$$\text{BMI} - \text{SDS} = \frac{\text{BMI observed} - 50 \text{ percentile BMI reference population}}{\text{Standard Deviation reference population}}$$

In 2007 the WHO established that school age children could be classified as overweight when BMI-SDS is > +1, and as obese when BMI-SDS > +2 (de Onis et al., 2007). The BMI-SDS has several advantages:

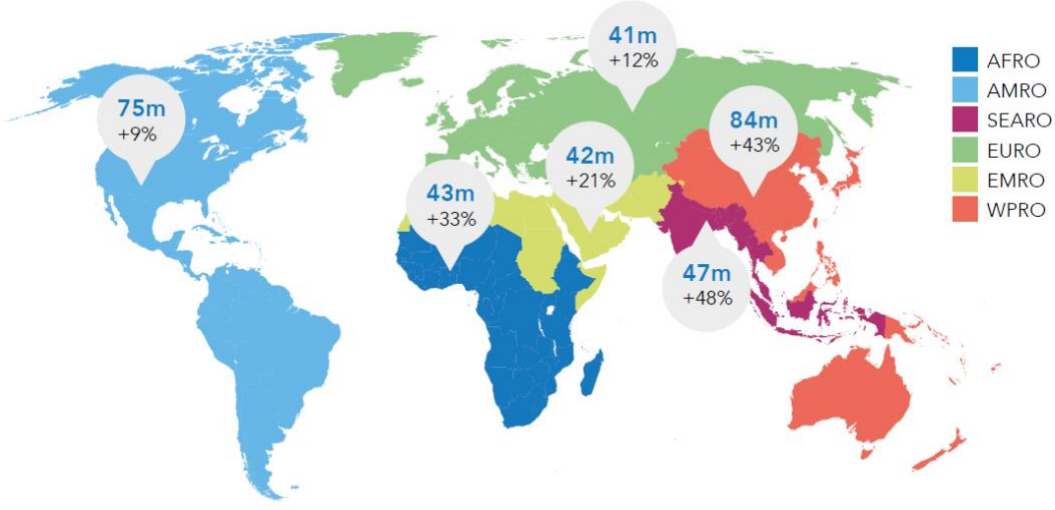
- It is easy to compare with other populations since it is a standardized measure, allowing comparisons between age and sex.
- Each population uses their national growth charts as reference.
- It allows to assess longitudinal changes in growth status.

BMI could have some disadvantages since sometimes it could not discriminate between lean mass and fat mass. For this reason, it is necessary to take that value into consideration with other anthropometric measurements. One of the most valuable indicators of body composition and in particular about the amount of visceral fat is waist circumference. Moreover, waist circumference is an independent predictor for comorbidities related to obesity such as insulin resistance, blood pressure or lipid levels (International Diabetes Federation (IDF), 2017). In this sense, the International Diabetes Federation (IDF) described that those children with a waist circumference higher than 90th percentile are more

likely to suffer cardiovascular disease risk factors. When these children reach the age of 16 years old, specific cut-off point were applied taking into account the sex and the ethnicity of subjects. In Europide subjects, a waist circumference higher than 0.94 cm in man and higher than 80 cm in women was consider as indicator of abdominal obesity.

1.2 Prevalence

Childhood obesity has become one of the most prevalent diseases worldwide, becoming a recognized public health problem. In 2016, over 340 millions of children and adolescents aged 5-19 years were overweight or obese (World Health Organization). The WHO reports that one in ten children aged between 5 to 17 years are overweight or obese worldwide (World Health Organization, 2017). This prevalence reaches higher values in developed countries, even though in developing countries starts to rise (Figure 1).



Source: NCD-RisC (2017)[3]

Figure 1: Number of children aged 5-19 living with overweight or obesity in 2016, and the increase in prevalence from 2010 to 2016, by WHO region. Source NCD-Risk (2017)

The prevalence of childhood overweight or obesity in Europe depends on multiple factors: 1) the diagnostic criteria used, observing higher rates when WHO cut-off were used instead IOTF criteria; 2) the age, increasing the prevalence with the age; 3) the country, it has been described that the prevalence of obesity is usually higher in southern European countries; 4) the sex, boys show higher obesity rates than girls in most countries. Taking all this information into account, there are three big studies conducted in European children (age range 2.0 to 9.9) that reported the prevalence of overweight and obesity (Table 1). The WHO reports on 2017 that the average prevalence of overweight and obesity (combined) in European countries was 19%.

Table 1. Prevalence of overweight and obesity in European and Spanish children and adolescents representative studies.

Study	n	Age	Diagnostic Criteria	% Overweight	% Obese
EUROPE					
IDEFICS (2014) (Ahrens et al. ,2014)	18745	2.0 to 9.9	IOTF	♂= 11.8 ♀= 13.8	♂= 6.8 ♀= 7.3
COSI (2013) (WHO,2017)	280585	6.0 to 9.9	WHO	♂= 12 to 24 ♀= 9 to 27	♂= 6 to 28 ♀= 4 to 20
TOYBOX (2012) (Manios et al. ,2018)	7554	3.5 to 5.5	IOTF	11.3	3.2
SPAIN					
IDEFICS (2014) (Ahrens et al. ,2014)	1541	2.0 to 9.9	IOTF	♂= 13.1 ♀= 17.1	♂= 5.6 ♀= 6.8
COSI (2013) (WHO,2017)	3426	7.0 to 8.9	WHO	♂= 23 ♀= 24	♂= 19 ♀= 17
TOYBOX (2012) (Manios et al. ,2018)	889	3.5 to 5.5	IOTF	12	2.8
ALADINO (2015) (Ortega et al. ,2015)	10899	6.0 to 9.9	WHO	♂= 22.4 ♀= 23.9	♂= 20.4 ♀= 15.8
Sanchez-Cruz et al.(2012)	978	8.0 to 17.9	IOTF	22.3	8.6

In Spain, the prevalence was higher than the mean of the European countries. When IOTF criteria were applied in children between 2.0 to 17.9 years old, it can be observed that the prevalence of overweight and obesity respectively, increased with age from 12% and 2.8% in the younger (3.5 to 5.5 years old) to 22.3% and 8.6% in the older (8.0 to 17.9 years old). Meanwhile, as mentioned before, when the WHO cut-off points were applied, higher rates were observed. Boys between 7.0 to 9.9 years old showed 22.7% and 19.9% and girls 19.9 % and 16.4% rates for overweight and obesity respectively. The most recent data in Spanish population is the ALADINO 2015 study, and its main conclusion is that the prevalence of overweight has decreased significantly in the last years, while the obesity trends are stabilized (Ortega Anta et al., 2015).

1.3 Comorbidities of obesity and related mechanisms

The excess of fat in overweight or obese children is accompanied by multiple comorbidities. It is worthy to clarify that not all obese subjects suffer from metabolic complications or disease risk due to their body weight, and they are known as metabolic healthy obese (MHO) subjects (Elmaogullari, Demirel, and Hatipoglu, 2017).

One of the main problems of childhood obesity is that usually persists into adulthood obesity (Kumar and Kelly, 2017). There are some factors that can lead to that status in adults. It is well known that children with one of their progenitors being obese have higher risk. Moreover, when obesity is present in adolescence, the risk of being obese in adulthood increases. The degree of obesity is also important, 71% of severe obese adolescents maintain that severe obesity into adulthood while only 8% of adolescents with non-severe obesity do it.

1.3.1 Cardiometabolic risk

Children and adolescents are young population and it is very difficult to find a hard end-point of cardiovascular disease. However, obese paediatric subjects start to present cardiovascular and metabolic risk factors at younger ages, which could result in a future cardiovascular event.

The clustering of risk factors for cardiovascular disease was first described in 1988 by Reaven when he observed that obesity, insulin resistance, hypertension and atherogenic dyslipidaemia tended to cluster to form a complex syndrome which he named Syndrome X. This syndrome is defined by a unifying pathophysiology leading to an increased risk for atherosclerotic cardiovascular disease (Reaven, 1988).

Until recently, this syndrome has been described using different terms such as Metabolic Syndrome (MetS), insulin resistance syndrome or cardiometabolic syndrome. This is defined in different ways, but the core concepts remain similar, as indicated by the cardiometabolic health alliance (Sperling et al., 2015): (1) It is a chronic and progressive pathophysiological state, (2) it represents a clustering of risk factors that form a complex syndrome defined by an unifying pathophysiology, and (3) it is associated with an increased risk for atherosclerotic cardiovascular disease, type 2 diabetes (T2DM) and other related disorders. In this sense, the findings of atherogenic products such as oxidized low-density lipoproteins of cholesterol (oxLDL) can be used as a biomarker of cardiometabolic risk (Freitas et al., 2018).

Different definitions have been proposed for the diagnosis of this risk in children and adolescents (Owens and Galloway ,2014; Rupérez et al., 2018). The

criteria for the most frequently used definitions in paediatric population are similar to the adults, but with specific cut-off points for children and adolescents (Table 2).

Table 2. Definitions of abnormal values for risk-factors variables.

Variable	Definition of abnormal value
Total cholesterol	≥ 200 mg/dL
LDL cholesterol	≥ 130 mg/dL
HDL cholesterol	< 35 mg/dL
Triglycerides	≥ 150 mg/dL
Glucose	≥ 100 mg/dL
Glycated haemoglobin	> 5.7 %
Systolic BP	≥ 95th percentile
Diastolic BP	≥ 95th percentile

BP: blood pressure, HDL: high-density lipoprotein, LDL: low-density lipoprotein. Adapted from Skinner et al. 2015

1.3.2 Insulin resistance

Insulin is a hormone produced by the pancreatic β cells that exerts their action when it binds to their receptors located in different tissues (liver, muscle, adipose tissue, or blood vessels among others) allowing the glucose uptake by different cells. Moreover, it is involved in inhibiting liver glucose production, and suppressing lipolysis.

In obesity, there is an insulin resistance (IR) status derived from the expansion of adipose tissue which became more resistant to insulin's metabolic action. The consequence derived from the insulin resistance status is the increase

in blood glucose levels also known as hyperglycaemia. This condition has some effects on body´s health such as: hyperinsulinemia, prediabetes and finally T2DM.

Visceral adipose tissue (VAT) is associated with a pro-inflammatory and adipokine profile that leads to a decrease in insulin resistance (Maffeis and Morandi, 2018). There is a high correlation between visceral fat and insulin resistance (Moschonis et al., 2016); for this reason, waist circumference has been proposed as an anthropometric marker for metabolic disturbances (Ali et al., 2014).

Recently it has been observed that IR is also highly correlated with hepatic insulin sensitivity since their main actions consists of: (1) inhibiting the gluconeogenesis by the phosphorylation of forkhead box protein (Fox01); and (2) increasing *de novo* lipogenesis by activating the transcription factor sterol regulatory element binding protein (SREBP-1c) (Malaguarnera et al., 2009). In subjects with insulin resistance, these insulin actions are impaired, and it occurs enhancing of *de novo* lipogenesis pathway. The consequences of that enhancing are: an increase in triglycerides and intrahepatic lipid storage, and a high flux of free fatty acids, all of them factors of the metabolic syndrome. Hepatosteatosis has been considered as a good predictor of metabolic health in obese children (Blüher and Schwarz, 2014). It has been described that after adjusting for BMI-SDS and waist circumference, the absence of liver steatosis is a good predictor of MHO youth (Senechal et al., 2013).

1.3.3 Other complications

In obese children there are some alterations associated to the excess of body weight (Figure 2). They affect most organs and exert a negative effect on childhood health. It is important to reverse this situation, because these pathologies turn into chronic diseases in adulthood.

Obesity and metabolic syndrome have been identified as risk factors for chronic kidney disease. Moreover, since 1974 there are several studies that have described an obesity-related glomerulopathology (Nehus and Mitsnefes, 2019). The potential mechanisms for that association are hypertension, hyperlipidaemia and hyperglycaemia among others (Mount et al., 2015).

Obstructive sleep apnea (OSA) is defined as a disorder for breathing during sleep that leads to a prolonged partial upper airway obstruction or intermittent complete obstruction with the consequence of a disruption of normal ventilation during sleep (Andersen, Holm, and Homøe, 2016). This comorbidity is highly prevalent in obese children where 33% to 76% of them suffer from it in comparison with normal weight children with a prevalence range from 15% to 37%. In this respect, it has been observed that weight loss interventions improved significantly OSA in obese youths (Marcus et al., 2012; Xanthopoulos, Berkowitz, and Tapia, 2018).

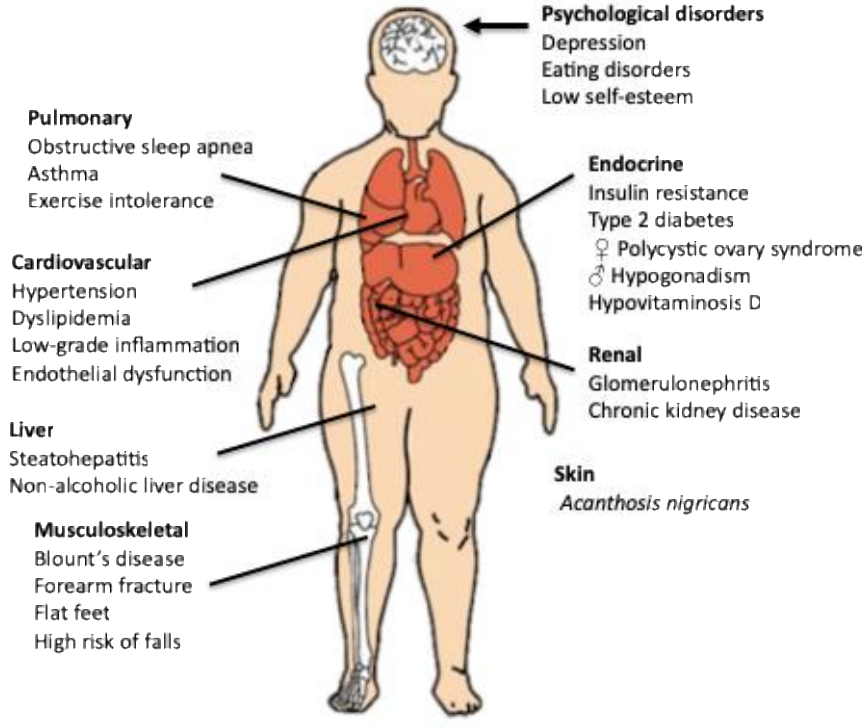


Figure 2. Comorbidities associated with childhood obesity. Adapted from Garver et al. 2013.

Polycystic ovary syndrome (PCOS) has been highly correlated with obesity, being obese more than a half of women with PCOS (Ollila et al., 2016). Moreover, it has been described that when the weight gain occurs during childhood it exacerbates the symptoms of PCOS (Koivuaho et al., 2019). In boys, the presence of excessive body weight has been associated with hypogonadism and gynecomastia (Ahsan and Banu, 2012).

Mental health is a health issue that has been highly correlated with childhood obesity. Weight status (normal weight vs. overweight or obese) was a risk factor for suffering from depression (Sanders et al., 2015). Moreover, overweight or obese children have low self-esteem and are at higher risk to be bullied than normal-weight pairs. Health-related quality of life is diminished in these subjects (Gungor, 2014).

Non-alcoholic fatty liver disease (NAFLD) has been considered as the hepatic manifestation of MetS (Di Sessa, Umano, and Miraglia del Giudice, 2017). It is defined as a liver condition that ranges from hepatic steatosis to steatosis accompanied by inflammation that may lead to non-alcoholic steatohepatitis (Delvin et al., 2014).

Other health consequences on health derived from childhood obesity could be: the major risk of accidents with the consequent risk to the bones, the presence of *Acanthosis Nigricans* in subjects with insulin resistance and other complications that can be observed in Figure 2.

1.3.4 Inflammation and oxidative stress

Obesity is related to a chronic low-grade inflammation derived from the expansion of white adipose tissue. In obesity, there is an increase in adipocyte number (hyperplasia) and in adipocyte size (hypertrophy) derived from an energy imbalance. The adipogenesis process comprises the differentiation of preadipocytes into mature adipocytes. These adipocytes are secretory cells that release cytokines (named as adipokines) into adipose tissue and blood stream. Thus, there is an increase in the expression of adipokines that are

pro-inflammatory such as leptin, interleukin 6 (IL-6), tumour necrosis factor alpha (TNF-α). The release of these adipokines and the increased amounts of free fatty acids in blood, stimulate the production of hepatic C reactive protein (CRP) a well-known marker of inflammatory status (Singer and Lumeng, 2017). CRP has been highly correlated with obesity in youth, where higher levels of CRP were found in children with higher BMI values (Ferrari et al., 2015; González-Gil et al., 2018).

Cardiotrophin-1 (CT-1) is a cytokine from the interleukin-6 family that has been described as a molecule with different physiological roles. Several studies have shown the role of CT-1 in the regulation of body weight and glucose metabolism. Furthermore, CT-1 is involved in the modulation of inflammatory response attenuating the expression of pro-inflammatory cytokines (López-Yoldi, Moreno-Aliaga, and Bustos, 2015).

On the other hand, because of the hyperplasticity of adipocytes a hypoxia status develops in white adipose tissue that increases the secretion of pro-inflammatory mediators (for example: IL-6, TNF-α). Moreover, this process leads to an increase in macrophage's recruitment raising the levels of inflammatory molecules and oxidative stress (Codoñer-Franch et al., 2011).

There is strong evidence supporting that childhood obesity is associated with a low-grade inflammation process accompanied by oxidative stress (Lechuga-Sancho et al., 2018). Oxidative stress could be defined as the imbalance between reactive oxygen species (ROS) and the production of antioxidant defences, leading to oxidative damage of the cells. ROS are chemical species containing free radicals that are: hydroxyl radical (OH^-), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). The production of ROS in adipocytes occurs mainly across the catalytic activity of nicotinamide adenine dinucleotide phosphate

(NADPH) oxidase (NOX) (Han, 2016). The excess of adipocytes on obesity produce the increase of ROS species and as a consequence the increase on oxidative stress. Thus, several studies had correlated childhood obesity with oxidative stress (Correia-Costa et al., 2016; Rendo-Urteaga et al., 2014).

Inflammation and oxidative stress related to obesity could modify telomere length. Telomeres are non-coding regions of DNA that are located at the end of chromosomes. They are repetitive DNA sequences (TTAGGG) whose main function is to protect the stability of genetic material. Telomere DNA adopts a specific structure called t-loop structure that is composed by shelterin complexes which prevent DNA from damage.

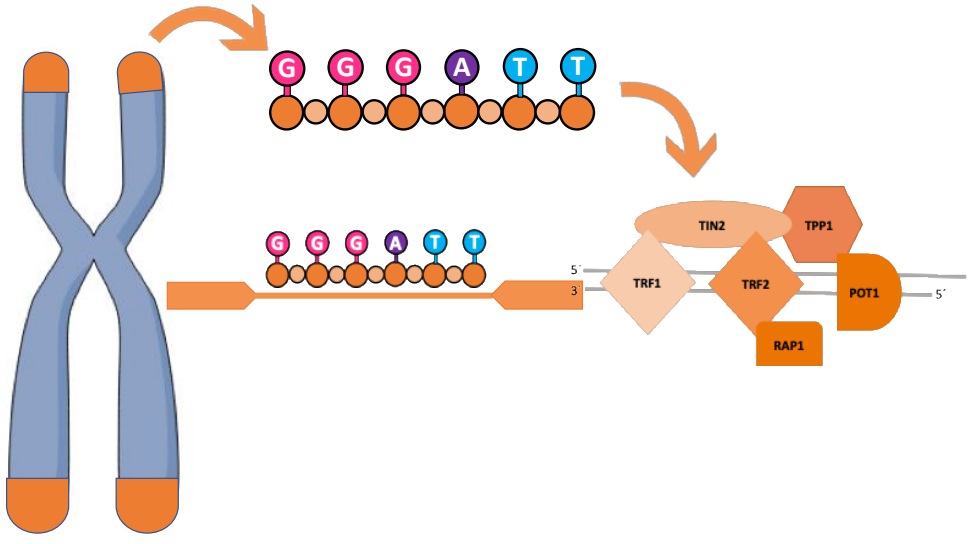


Figure 3. Telomeres in human cells and shelterin complex

Moreover, in a recent meta-analysis it has been found that BMI is negatively associated to telomere length in adult population (Mundstock et al., 2015). Obese children showed shorter telomeres than normal-weight subjects

(Buxton et al., 2011; Kjaer et al., 2018) Previous work of our research team showed that TL is a marker of adiposity (García-Calzón and Marti, 2017) and that could also be related to glucose metabolism (García-Calzón, Moleres, et al., 2016). Telomere length is also influenced by environmental factors such as diet, physical activity, tobacco or social stress (Ojeda-Rodriguez et al., 2018).

1.4 Factors that influence adiposity traits

There are several factors that are correlated and exert their influence in BMI-SDS in children and adolescents. The accumulation of body fat is the result of an imbalance between energy intake and energy expenditure. Many factors exert their influence on this imbalance, and for this reason obesity has been described as a multi-factorial disease. Some of these factors can be modified by different interventions or lifestyle habits, while others cannot be changed.

1.4.1 Genetic factors

It has been widely associated the presence of several genetic factors with BMI and BMI-SDS in children. The predisposition to suffer obesity increases when these factors are found in children. The genetic influence on BMI appears to be stronger during childhood. The presence of parental obesity is one of the strongest predictive factors for childhood obesity (Yeung et al., 2017). In this sense, it has been described that this association mainly comes from genetic factors rather than environmental factors (Hinney, Vogel, and Hebebrand, 2010).

There are few cases of early onset obesity explained by monogenic disorders. The genes responsible for the main cases of monogenic obesity are:

the Melanocortin 4 Receptor gene (*MC4R*), the leptin deficiency and leptin receptor mutations (*LEP* and *LEPR*) and the pro-opiomelanocortin deficiency or mutation (*POMC*).These genes mainly regulate the hunger pathways and the satiety response (Koves and Roth, 2018).

The *MC4R* deficiency is known as the most common monogenic obesity form. Most of the mutations on MC4R exert their effect in inhibiting the action of the alpha-melanocyte stimulating hormone (α-MSH) that is involved in the appetite response (Ochoa et al., 2007). For this reason, the main clinical manifestations of children affected by MC4R mutations are is hyperfagia, early onset severe obesity and hyperinsulinemia among others (Koves and Roth, 2018). It has been estimated that between 2% to 6% of severe obese children harbour functional mutations on *MC4R* (Hinney, Volckmar, and Knoll, 2013). Moreover, there are two polymorphisms that have been demonstrated to exert a protective effect on BMI (Stutzmann et al., 2008).

Lipocalin 2 gene (*LCN2)* has been associated to MC4R receptor. Lipocalin 2 is a cytokine produced mainly by osteoblast but also by adipose tissue and it has been shown that it binds to MC4R receptor, and recent investigations looking on that relation have been reported (Mosialou et al., 2017).

Leptin is a cytokine produced mainly by adipose tissue and their effects remain in the satiety pathway. Mutations in *LEP* or *LEPR* genes led to an impairment of leptin signalling and consequently cause hyperphagia and severe early-onset obesity with their cardiovascular health consequences (Olza et al., 2017). There are few subjects worldwide who have a mutation in the *LEP* gene, and they can be treated with the administration of exogenous leptin with their consequent improvement in BMI (Wasim et al., 2016).

POMC neurons exert their actions as the precursors of the pituitary adrenocorticotropin hormone (ACTH) that cleaves into α-MSH. α-MSH binds to MC4R receptor in the hypothalamus and regulates the inhibition of food intake. When a POMC deficiency or mutation occurs that pathway is altered causing hyperphagia and severe onset obesity (Rubinstein and Low, 2017). Obesity has been described as a polygenic disease since genome-wide association studies have identified more than 97 genetic loci associated with obesity (Fang et al., 2018). Polygenic variants are defined as a group of alleles at different gene loci that can affect the expression of a phenotype (Mărginean, Mărginean, and Meliţ, 2018). It has been stated that those loci exert a small effect on obesity-susceptibility and may explain only a small fraction of total variance. There are multiple genes associated to polygenic obesity as for example: fat mass and obesity-associated protein gene (*FTO*), variants near *MC4R*, the insulin receptor substrate (*IRS1*) and the SH2B adaptor protein 1 (*SH2B1*) among others (Loos, 2012). The *FTO* was the first gene studied to cause polygenic obesity and has the largest effect on obesity-susceptibility. It has been estimated that as much as 21% of BMI variability could be attributed to common genetic variants, while the mean for BMI monogenic forms of obesity was 2.7% (Locke et al., 2015).

1.4.2 Lifestyle factors

Childhood and adolescence are stages of life where children acquire and learn healthy or unhealthy lifestyle habits. In this respect, the environment where these youths live will influence their behaviour. As mentioned before, obesity has been described as the result of an imbalance between energy intake and energy expenditure. Nowadays, it has been described that children find themselves in

an "obesogenic" environment that is characterized by sedentary behaviours an unhealthy dietary pattern (Lanigan, 2018). Moreover, family environment can influence adiposity in children by different pathways: 1) the availability of different types of food at home, 2) the food patterns followed by the family and 3) eating behaviours, where parent's attitude forward children eating behaviour has been demonstrated to have an effect on adiposity levels (Brown and Perrin, 2018; Johannsen, Johannsen, and Specker, 2006). The enKID study has demonstrated that a high intake of fats, pastries, sugar-sweetened drinks and cold meats and a low consumption of vegetables and fruits are associated with a higher risk of obesity in Spanish children (Aranceta-Bartrina and Pérez-Rodrigo, 2016). Recently, it has been described that unhealthy dietary patterns established during infancy (< 2 years old) are track into childhood (Luque et al., 2018).

Regarding physical activity behaviours we can classify them as sedentarism and physical inactivity. In this respect, it has been demonstrated that a greater MVPA has a protective effect on childhood obesity, while sedentary time is a risk factor for adiposity in children (Engel et al., 2018) . Moreover, the promotion of physical activity in a family context is recommended as a preventive strategy for childhood obesity (Foster et al., 2018).

Socioeconomic environment is another factor that contributes to weight in youth population. It is widely described that children from lowest socioeconomic classes have higher rates of obesity (Gurnani, Birken, and Hamilton, 2015). This association has been explained due to the lower access to healthiest foods and to the fewer opportunities for physical activity (Noonan, 2018).

1.5 Treatment

Childhood obesity has increased in the last 50 years reaching high values in many countries. It is considered as a number one health problem of the XXI century, multiple approaches have been proposed in order to diminish the number of obese children. Since obesity in childhood tracks into adulthood, the treatment and prevention in the youth population is a key factor in reducing this health problem.

Intensive lifestyle programs have been demonstrated to be the best approach for reducing the excess of weight in children and adolescents. Obesity is characterized by an excess of energy intake and low energy expenditure. For this reason, strategies aiming to modify those aspects should be considered. They consist of the combination of three areas: dietary treatment, promotion of physical activity and behavioural therapy. Thus, a multidisciplinary team that comprises paediatricians, dieticians, nurses, physical activity experts, and is needed.

On the other hand, it is important when treating obese children not to interfere with normal development and growth. For this reason, the national health system guidelines for children state that in overweight or slightly obese children without metabolic complications it is recommended to maintain body weight. Meanwhile in obese children or adolescents with metabolic complications, a moderate weight loss is advised, but taking into account the obesity degree (Marqués et al., 2012; Sistema Nacional de Salud, 2009).

1.5.1 Dietary treatment

The dietary recommendations for these subjects comprise nutritional counselling such as reduce refined carbohydrates, pastry, meat and saturated fat intakes, and to increase the consumption of fruit, vegetables, whole grains, fish and legumes. Moreover, the Spanish Society for Community Nutrition (SENC), recommend the following pattern for total energy distribution: 50-55% by carbohydrates (being less of 10% provided by sugars); 30-35% by fats where the mainly source were monounsaturated fatty acids and limiting the saturated fatty acids to be less than 10%; and 15% by proteins (Aranceta Batrina et al., 2015).

There are different dietary patterns that have been demonstrated to be effective in weight loss and the improvement of metabolic comorbidities. These patterns are known under the following names: Optimized mixed Diet (OMD), the Mediterranean Diet (MD), the New Nordic Diet (NND) and the Dietary approach to stop hypertension also known as DASH dietary pattern.

The Mediterranean Diet is a healthy dietary pattern typical of the countries bordering the Mediterranean Sea as Greece, Italy or Spain among others. It has been observed that a high adherence to MD is associated with a reduced BMI gain (Tognon et al., 2014).It is characterized by a high consumption of vegetables (2 portions per day), fruits (3 portions per day), whole grains, legumes, nuts and olive oil, a moderate consumption of poultry, fish and dairy products; and a reduced consumption of processed meats. This pattern is accompanied by different lifestyle factors such as the way of cooking foods, using spices and herbs instead of salt, being physically active or enjoying the foods with family or friends. It has been demonstrated that obese children under a MD lifestyle intervention, improve their BMI-SDS status and MetS components as:

triglycerides, HDL cholesterol and glucose levels, decreasing the prevalence of MetS in the 45% of the participants (Velazquez-Lopez et al., 2014). Moreover, in the PREDIMED study, treatment with MD in adult subjects with cardiovascular risk factors has demonstrated to be a prevention strategy to reduce the end-points for cardiovascular disease (Estruch et al., 2018).

1.5.2 Physical activity promotion

As mentioned previously, childhood obesity results from an imbalance between energy intake and energy expenditure, being physical activity a key point in the treatment of obesity. The main problems in obese youths are: 1) the lack of physical activity and 2) high levels of sedentary activity.

On one hand, the WHO advises that children and adolescents should spent more than 60 minutes per day in moderate-to-vigorous physical activity (MVPA) this means a physical activity that leads to an increase in heart rate at least of the 65-70%. This recommendation was based on the evidence that there are consistent favourable associations between PA and adiposity and cardiometabolic biomarkers (Poitras et al., 2016; Jiménez-Pavón et al., 2013). Healthy European children did not achieve this recommendation, while the mean time spent in MVPA was 36 min/day (Konstabel et al., 2014). The 16% of European obese or overweight children and adolescents did not comply the recommendations (Hughes et al., 2006). In a recent meta-analysis it has been demonstrated that obese children are less active than normal-weight pairs (Poitras et al., 2016). In this context, it has been demonstrated that dietary lifestyle interventions combined with the promotion of physical activity or

behavioural therapy are more effective than just dietary interventions (De Miguel-Etayo et al., 2013).

On the other hand, the sedentarism has been considered one of the main risk factors for childhood obesity. In this line, several worldwide organizations have stated the recommendation not to spend more than 2 hours a day on screen-based activities such as: watching TV, playing videogames and time spent with tablets or smartphones. Screen-time is used for extrapolating time in sedentarism because it represents the major part of the sedentary time and because it has been related with adverse health outcomes (Keane et al., 2017; Henriksson et al., 2018)

Moreover, it is important in lifestyle interventions not to focus the physical activity recommendations on a single behaviour (PA, sedentarism or sleep time) but to take into account the 24 hours of the day. In a recent review it has been observed that children and adolescents with lower levels of adiposity were those with a combination of optimal physical activity and behaviour (i.e. high PA, low sedentary time and sufficient sleep) (Chaput, Saunders, and Carson, 2017).

1.5.3 Behaviour therapy

One of the main objectives of lifestyle intervention in children and adolescents are to achieve an improvement in anthropometric and metabolic outcomes, and to maintain (van Hoek et al., 2016) the habits that had led to that improvement. In this context, behaviour therapy during the lifestyle intervention is a key element for achieving the improvement in eating and PA behaviours. Behavioural therapy encompasses all actions that includes counselling on self-

monitoring of PA and eating behaviours, control of stimulus, action planning, goal setting and other modification strategies (van Hoek et al., 2016).

Since children and adolescents live with their families and parents exert an important influence in healthy behaviours, it is important that behavioural interventions include the family in the approach. It has been described that lifestyle interventions that include the family are more successful than those that only focus on children (Wilfley et al., 2017)

2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Obesity is characterized by a chronic low-grade inflammation and increased oxidative stress. Since childhood obesity is one of the main health problems of the XXI century and their health consequences track into adulthood, it is urgent to design effective treatments (Skinner et al., 2015). A number of programs have shown improvements in adiposity indices of obese children and in their metabolic outcomes as well as an attenuation of the inflammatory state and oxidative stress. In this sense, the hypothesis of this work is that a lifestyle intervention in children with obesity can be associated with changes in metabolic, transcriptomic, and genetic profile toward to reduce cardiometabolic risk.

2.2 Objectives

General Objective

The general objective of the present project is to evaluate the effect of lifestyle interventions in cardiometabolic factors, and the role of genetic markers in the individual response to the intervention.

Specific Objectives

1. To evaluate the effect of a dietary intervention on oxidized LDL levels, and its association with cardiometabolic risk in a sample of children and adolescents with obesity (Chapter 1).
2. To examine changes in serum inflammatory markers (IL-6, TNF-α and CT-1) and their transcript levels after a dietary intervention in children with obesity, and to assess their potential association with glucose metabolism (Chapter 2).

3. To evaluate the effects of a lifestyle interventions (Usual Care vs. Intensive Care) in changes in BMI-SDS, biochemical parameters and physical activity in children with abdominal obesity (Chapter 3).
4. To determine telomere length in children with abdominal obesity after a lifestyle intervention and to assess their potential association with cardiometabolic risk factors (Chapter 4).
5. To screen for mutations in *MC4R* and *LCN2* genes and to evaluate their effects on eating behavior and weight loss response in children with abdominal obesity (Chapter 5).

3.METHODOLOGY

3.1 NUGENOI study

The NUGENOI study (Nutrigenómica y obesidad infantil) was conducted during 2010 by the GENOI (Grupo de Estudio Navarro de Obesidad Infantil) members in Pamplona, Navarra, Spain. It consists of a 10-week dietary intervention for obese or overweight children. The project was performed following the Helsinki declaration and was approved by the ethics committee of the University of Navarra (Reference Number 038/2009).

3.1.1 Study population

Children between 7 to 15 years old and classified as obese following the Cole et al. criteria (Cole et al.,2000) were invited to participate in the study. In Table 3, the inclusion and exclusion criteria are described. It was conducted in the University of Navarra and was registered (NCT01329367). Participants were recruited from the endocrinology units at *Clinica Universidad de Navarra* and *Complejo Hospitalario de Navarra*.

From the initially seventy-one recruited participants, fifty-four accepted to participate and underwent baseline visit. Finally, forty-four subjects concluded the 10-week intervention. The drop-out rate was 18.5% and the main reasons for dropping-out were discouragement, social reasons, being in an exam period or the inability to be accompanied by one of the members of the family.

Table 3. Inclusion and exclusion criteria for NUGENOI participants.

Inclusion Criteria	Exclusion criteria
√ Age 7-15 years old	X Eating disorders
√ To be classified as obese or overweight under Cole et al. criteria	X Major cardiovascular or respiratory disease
√ To be Spanish or foreign residing in Spain for at least 1 year	X Major psychiatric illness
	X Familiar hypercholesterolemia
	X Pharmacological treatment for obesity

NUGENOI: "Nutrigenómica y obesidad infantil"

3.1.2 Dietary intervention

The 10-week intervention was conducted by a multidisciplinary team conformed by: registered dietitians, pediatricians, nurses and laboratory technicians. The dietary treatment consists of a moderate calorie-restricted diet, calculated according to children's obesity degree (Marqués et al.,2012). Energy expenditure was obtained across the Scholfield equation adapted for sex and age (Scholfield, 1985). Participants were given a fixed full-day meal plan with their energy restriction. Caloric diets content ranged between 1300 kcal and 2200 kcal.

During the intervention period, children accompanied by one of their parents or legal guardians assisted to a weekly individual session with the registered dietitian. In those sessions, the adherence to diet, a weight control and a nutritional education was performed.

3.1.3 Data collection

Anthropometric, biochemical and clinical outcomes were assessed at baseline and at the end of the intervention (10 weeks) by trained personnel. All measurements were carried out following standard procedures.

Moreover, venous blood samples were obtained after an overnight fast by a trained nurse. Three EDTA tubes of 4.5 mL were collected, and within a 1 hour of collection blood was centrifuged during 15 minutes at 3500 rpm at 4ºC. Two of the tubes were aliquoted in order to obtain plasma for inflammatory cytokines and other determinations, and peripheral blood mononuclear cells, to measure gene expression. The other tube was used for standard blood analytics: glucose, insulin, total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides.

3.2. IGENOI study

The IGENOI study ("Intervención del Grupo de Estudio Navarro de la Obesidad Infantil") is a lifestyle intervention carried out in Pamplona, Navarra, Spain that was performed from January 2015 to January 2019. It consists of a 2-year lifestyle intervention in children and adolescents with abdominal obesity. The project was approved by University of Navarra Humans´ Investigation Ethics Committee (Reference number 044/2014). It was conducted following the ethical standards of the Helsinki Declaration (Fortaleza, Brazil, 2013).

3.2.1 Study population

Children and adolescents diagnosed with abdominal obesity, between 7 to 16 years old from the endocrinology units at *Clínica Universidad de Navarra, Complejo Hospitalario de Navarra*, and from Pamplona health care centers, were invited to participate in the IGENOI study. Abdominal obesity was defined as having a waist circumference higher than 90th centile from national growth charts (enKID study) (Serra Majem et al., 2000). Moreover, participants were screened about the inclusion and exclusion criteria described in Table 4.

Table 4. Inclusion and exclusion criteria of IGENOI study.

Inclusion Criteria	Exclusion criteria
√ Age 7-16 years old	X Eating disorders or psychiatric disease
√ Waist circumference higher than 90th centile	X Previous diabetes
√ Availability to attend the established visits	X Presence of other diseases beside diabetes
√ Open to be assigned to any of the designed groups.	X Pharmacological treatment
√ Committed to do their best to follow the assigned protocol	X Food allergies or intolerances
	X Follow special diets
	X Non-access to phone calls or internet.

IGENOI: "Intervención del Grupo de Estudio Navarro de Obesidad infantil".

The study is a 2-year randomized control trial (NCT03147261) where participants were assigned in two different groups (Usual care and Intensive care) in a 1:3 ratio. The study was designed in order to follow two phases. The intensive phase that was carried out in the first two months, and the follow-up phase that lasted up to 22 months.

From the initially 126 recruited volunteers, 121 started the intervention, and 114 complete the intensive phase. At the end-point of the intervention 61 participants concluded the follow-up phase.

The drop-out rate was 6.5% in the intensive phase. The main reasons for leaving the study were: social problems, discouragement, inability to attend the visits and change of address or phone number given for the notifications.

3.2.2 Lifestyle intervention

IGENOI study consists of a family-based lifestyle intervention. It was carried out by a multidisciplinary team that includes registered dietitians, pediatricians, physical activity experts and nurses. Participants were randomly assigned in two different treatments: usual care (UC) and intensive care (IC). The randomization was carried out in a 1:3 ratio by computer-generate randomization. The rationale for the difference in the size of both groups relies on the fact that a high number of subjects could benefit from the intensive care. The intervention was divided in two different phases. First, an intensive period of 2-months, and second a follow-up period of 22 months. During all the intervention, all participants were encouraged to increase their physical activity on 200 minutes of PA per week at 60-75% of their maximum heart rate.

Intensive phase

During the intensive phase, the IC participants received a moderate hypocaloric Mediterranean Diet. The energy restriction was applied on total daily energy expenditure and was calculated according to the obesity degree of each participant, and not disturbing the normal growth of children. Total energy expenditure was estimate using Schofield et al equation for basal metabolism and taking 1.3 as physical activity factor.

The dietary intervention consists on a fixed full-day meal plan of five meals. Total daily energy was distributed among the day following the pattern: 20% on breakfast, 5-10% on morning snack, 30-35% on lunch, 10-15% on afternoon snack and 20-25% on dinner. The dietary plan was based in Mediterranean pattern; thus, it includes a high consumption of fruits, vegetables, whole grains, legumes, olive oil and minimally processed foods; a moderate consumption of dairy products, fish and poultry; and a low consumption of red meat (Ojeda-Rodríguez et al., 2018). IC participants and their parents received six 30-min. individual sessions with the dietitian in order to assess the accomplishment of the diet. Moreover, one parallel group session was organized for IC participants and their parents. In that session children were taught about different topics such as energy balance, portion size, food groups and the importance of being physically active. Meanwhile, parents were explained about their role in the intervention and the obesity related comorbidities.

On the other hand, UC participants received standard pediatric recommendations on healthy diet, following the national guidelines of the Spanish association of communitarian nutrition (SENC) (Aranceta Batrina et al., 2015). A 30-min individual session with the dietitian was performed in order to

get those recommendations. They have five monitoring visits to assess anthropometric parameters by the research team.

Follow-up phase

After the intensive phase period, participants were follow-up for 22 months. They attended 8 visits in the following months: 3, 4, 5, 6, 9, 12, 18 and 24 months. During the visits anthropometric and dietary assessment was performed in all participants. Furthermore, IC participants and their families received specific nutritional and behavioral education in those visits.

Month 3: a game with healthy foods flash-cards.

Month 4: a 1-hour group session with children and their parents informing about the different food groups and their portion size and frequency recommendation

Month 5: individual session about healthy breakfast.

Month 6, 9, 12 and 24: to assess dietary and behavior patterns that participant followed.

Month 18: 1-hour group session about Mediterranean dietary pattern.

3.2.3 Data collection

Dietary, physical activity, anthropometric, clinical and biochemical outcomes were assessed at baseline, 2, 12 and 24 months. Moreover, eating behavior was measured at intervention baseline. All the measurements were obtained following standard procedures or protocols.

Anthropometric and clinical outcomes were measured by trained personnel following the protocol. Dietary data were collected by registered dietitians using a baseline semi-quantitative 136-item Food-Frequency Questionnaire (FFQ) which was validated in a Spanish population (Martin-Moreno et al., 1993)

Physical activity was measured in children and adolescents using physical activity questionnaires and accelerometry (Actigraph wGT3X-BT). Participants were instructed to wear the accelerometer during 4 consecutive days, including 2 weekend days.

Eating behavior was measured by the validated questionnaire Children Eating Behavior Questionnaire (CEBQ) that includes 35 items that evaluate two different dimensions: food approach or food avoidance behaviors. These dimensions are comprised by different sub-scales: Food responsiveness, food enjoyment, emotional overeating, desire to drink, satiety responsiveness, slowness in eating, emotional undereating and food fussiness.

Moreover, venous blood samples were obtained after an overnight fast by a trained nurse. Two EDTA tubes of 4.5 mL were collected, and within 30 minutes of collection blood was centrifuged during 15 minutes at 3500 rpm at 4ºC. The tubes were aliquoted in order to obtain plasma for other determinations, and buffy-coat cells, that were used to DNA extraction.

4.RESULTS

Chapter 1

Serum oxidized low-density lipoprotein levels are related to cardiometabolic risk and decreased after a weight loss treatment in obese children and adolescents

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Pediatric Diabetes,2016
DOI: 10.1111/pedi.12405
Impact factor (2016): 4.267
4/121 in Pediatrics, Q1
30/138 in Endocrinology and Metabolism, Q2

Morell-Azanza L, García-Calzón S, Rendo-Urteaga T, Martin-Calvo N, Chueca M, Martínez JA, Azcona-Sanjulián MC, Marti A. Serum oxidized low-density lipoprotein levels are related to cardiometabolic risk and decreased after a weight loss treatment in obese children and adolescents. Pediatr Diabetes. 2017 18(5):392-398. http://doi.org/10.1111/pedi.12405

Chapter 2

Serum and gene expression levels of CT-1, IL-6, and TNF-α after a lifestyle intervention in obese children

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Pediatric Diabetes,2017
DOI: 10.1111/pedi.12405
Impact factor (2017): 3.172
14/124 in Pediatrics, Q1
63/142 in Endocrinology and Metabolism, Q2

Marti A, Morell-Azanza L, Rendo-Urteaga T, García-Calzón S, Ojeda-Rodríguez A, Martín-Calvo N, Moreno-Aliaga MJ, Martínez JA, Azcona-San Julián MC. Serum and gene expression levels of CT-1, IL-6, and TNF-α after a lifestyle intervention in obese children. Pediatr Diabetes. 2018, 19(2):217-222. http://doi.org/10.1111/pedi.12561

Chapter 3

Changes in objectively measured physical activity after a multidisciplinary lifestyle intervention in children with abdominal obesity: a randomized control trial

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BMC Pediatrics,2019
DOI: 10.1186/s12887-019-1468-9
Impact factor (2018): 2.042
48/124 in Pediatrics, Q2

Morell-Azanza *et al. BMC Pediatrics* (2019) 19:90
https://doi.org/10.1186/s12887-019-1468-9

BMC Pediatrics

RESEARCH ARTICLE Open Access

Changes in objectively measured physical activity after a multidisciplinary lifestyle intervention in children with abdominal obesity: a randomized control trial

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Abstract

Background: Physical activity (PA) is associated with changes in body composition that affect insulin sensitivity and leptin levels. Few studies have assessed the effect of lifestyle interventions on changes in objectively measured PA levels in obese children. To evaluate the effects of a multidisciplinary lifestyle intervention on anthropometric indices, biochemical parameters and accelerometer measured PA in abdominal obese children.

Methods: A randomized control trial was performed in 106 children and adolescents with abdominal obesity. Participants were randomly assigned to usual or intensive care group for 8-week. PA was measured by accelerometry over four days including, at least, two weekdays in all participants. Both groups were encouraged to accumulate an extra time of 200 min per week in their PA.

Results: At baseline, 75% of subjects do not fulfill the WHO recommendation of being more than 60 min/day on moderate-to-vigorous PA (MVPA). The intensive care group achieved a significant reduction in anthropometric indexes compared to the usual care but no significant change was found in biochemical or PA parameters. Both groups achieved a significant reduction in light PA. Interestingly, intensive care participants significantly increased MVPA in 5.5 min/day. Moreover, an inverse association between changes in MVPA and leptin levels was found.

Conclusion: The two lifestyle intervention reduced anthropometric indexes and lowered light PA in abdominal obese children. No significant differences were observed between intensive care and usual care in regard to PA. Intensive care participants significantly increase physical activity (MVPA) and, changes in MVPA were inversely associated with changes in leptin levels after the intervention.

Trial registration: ClinicalTrials.gov, Identifier: NCT03147261. Registered 10 May 2017. Retrospectively registered.

Keywords: Obesity children, MVPA, Accelerometer, Metabolic risk, Leptin

Background

Dyslipidemia, hypertension, insulin resistance or type 2 diabetes, are the main alterations that derive from obesity and contribute to aggravate cardiometabolic risk in pediatric populations [1]. Obese children with waist circumference at/or above the 90th percentile are at higher risk for dyslipidemia and insulin resistance than obese children with normal waist circumference [2, 3]. The factors that contribute to childhood obesity are complex, and include an excessive energy intake, a decrease in physical activity and an increase in sedentary behaviors, for example, the screen-time activities [4].

Sedentary lifestyle, the fourth leading cause of global mortality, is becoming more frequent in pediatric populations [5, 6]. "Global Recommendations on Physical Activity for Health" by the WHO state that children and

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youth aged 5–17 should accumulate a minimum of 60 min of moderate-to-vigorous intensity physical activity (MVPA) every day [5]. But, in Spain, only a 35.2% of children and 11.9% of adolescents (over 13 years) achieved that recommendation [7]. Different approaches for obesity treatment have been proposed over the last decades, but the evidence suggests that successful intervention does include diet and physical activity recommendations, behavioral therapy and family implication [8, 9]. Physical activity is associated with changes in body weight and body fat that ultimately affect insulin sensitivity [7, 10]. In obesity, the described expansion of adipose tissue leads to an increase in leptin levels. This hyperleptinemia has been associated with a pro-inflammatory status with deleterious effects on children's health [11]. In regard to this, several intervention studies reported a decrease of leptin levels after aerobic PA in obese adolescents [11–13]. Thus, increased physical activity, specifically MVPA, may lead to a decrease of leptin levels [14]. To our knowledge, few studies have evaluated the effect of lifestyle interventions on changes in objectively measured PA levels in obese children at high metabolic risk [4, 15–19]. We hypothesized that a successful lifestyle intervention based on PA recommendations is able to modify BMI-SDS, metabolic parameters and objectively measured PA. Hence, the aims of this RCT were: [1] to assess the effectiveness of the two interventions (usual care vs. intensive care group) based on BMI-SDS changes (primary outcome), [2] to evaluate changes on biochemical and PA parameters (secondary outcome) between the two lifestyle interventions. Specifically, in our study we will assess: differences between the two lifestyle interventions and also changes (pre vs. post intervention) in the measured variables in each group.

Methods

Participants

The IGENOI (*Intervention Grupo Estudio Navarro de Obesidad Infantil*) study is a randomized control trial (NCT03147261) conducted in Pamplona, Navarra (Spain). It is a 2-year family-based lifestyle intervention program for children with abdominal obesity. Seven to sixteen year-old children were recruited from the Endocrinology Pediatric Units of the University of Navarra Clinic, Navarra's Hospital Complex and Primary Health Care Centers in Pamplona. General inclusion criteria for enrollment was waist circumference above the 90th percentile, according to national data [20, 21]. Subjects with pre-diabetes or food intolerance, following special diets, regular alcohol consumption, major psychiatric illness, eating disorders or medical therapy were excluded. The study protocol was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki (Fortaleza, Brasil, October 2013) and was approved by the Ethics Committee of the University of Navarra (Reference number 044/2014). The parents and/or legal guardians and children involved in the trial received detailed explanations about the aim of the study. Informed assent was obtained from every child and all parents and/or legal guardians signed an informed consent according to the Helsinki declaration.

121 of the 126 recruited participants met the inclusion criteria, and 114 successfully concluded the 8-week program. The dropout rate was 6.5% and the main reasons were discouragement, social problems, inability to comply the weekly visits (school exam periods, and change of address for the notifications), as described in other trials with pediatric populations [22]. One hundred and six participants with completed data of objectively measured physical activity both at baseline and after the 8-week (Fig. 1).

The primary endpoint of the IGENOI study was to assess the effectiveness of the lifestyle interventions based on BMI-SDS values. Changes in BMI-SDS are the main outcome since successful interventions should decrease BMI-SDS near 0.5 units in order to reduce cardiometabolic risk [23]. Taking this information into consideration, sample size was estimated with the assumption of: an error of 5%, a power of 90%, a 1:3 ratio and a mean difference of 0.50 (SD 0.47) units in BMI-SDS after the lifestyle intervention. The sample size calculation indicated that 13 and 39 subjects were needed for usual care and intensive care group, respectively. The rationale for the difference in the size of both groups relies on the fact that a high number of subjects could benefit from intensive intervention as indicated in other program for obese children [24].

Study design/ lifestyle intervention

The IGENOI study is a family-based lifestyle program carried out by a multidisciplinary team (dietitians, pediatricians, psychologist, physical activity experts, and nurses) in a clinical setting. It consists of a two-year program that comprises an 8-week phase with individual and group sessions and a follow-up period of 22 months. Our research group has different weight loss interventions where we have observed that in a short time period (8 to 10 weeks) there are substantial changes in weight loss and other metabolic parameters [8, 9]. In this study we present data from the treatment period corresponding to the first 8-weeks, since the study is still on going.

Participants were randomly assigned to the usual or intensive care group with a ratio of 1:3. The randomization was performed using a computer-generate randomization. The intensive care group was advised to follow a fully-day meal plan during the intensive phase. This diet consists on a moderate hypocaloric Mediterranean diet to not to

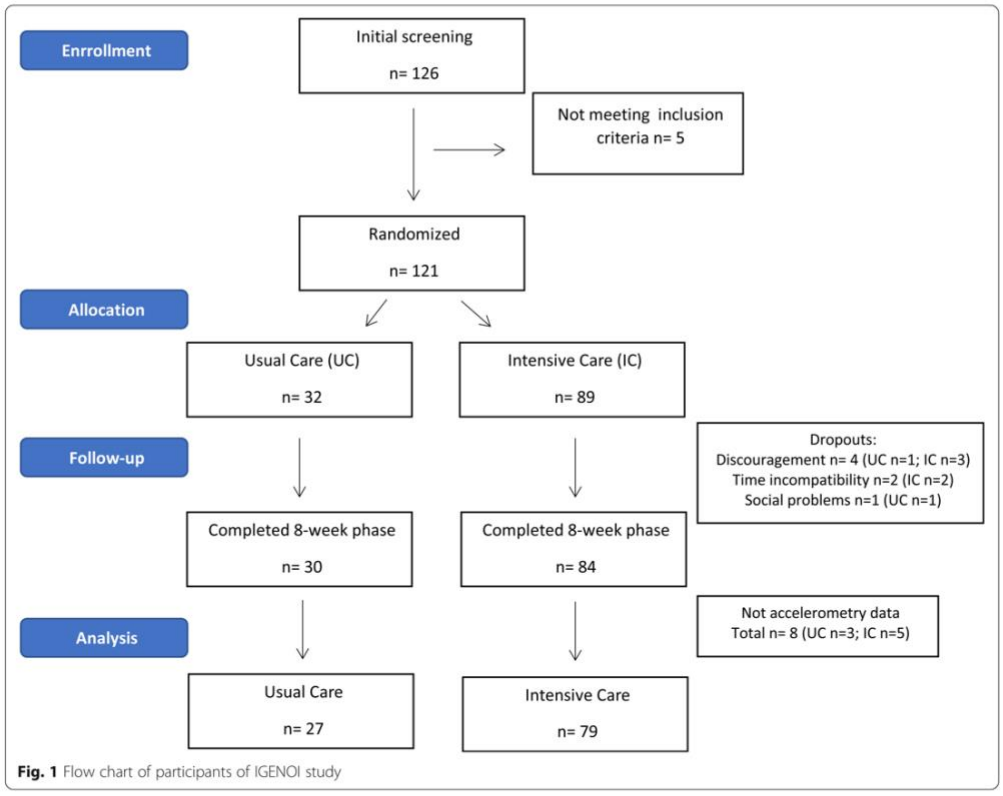

Fig. 1 Flow chart of participants of IGENOI study

interfere with children's growth. Energy restriction (10 to 40%) was calculated according to obesity degree and physical activity levels, as described elsewhere [9, 25]. The dietary pattern was based on a high consumption of fruits (3 portions per day) and vegetables (2 portions per day), legumes, whole grains and olive oil; moderate consumption of dairy products, poultry and fish, and the reduction of processed and red meats, limiting them to 1 portion per week. Standard paediatric recommendations on healthy diet were given to usual care subjects. Participants of both groups were aimed to accumulate an extra time per week of 200 min of PA at a 60–75% of their maximum heart rate.

Intensive care participants and their parents received six 30 min-sessions lead by the dietitian during the 8-week period in order to monitor the accomplishment of the diet. One parallel group sessions was organized for 1) intensive care participants and 2) their parents or legal tutors. During the group sessions parents were told their role in the intervention and the obesity related co-morbidities, while children were taught about different topics such as energy balance, portion sizes, groups of foods, the importance of the breakfast and physical activity [25]. On the other hand, usual care participants and their parents received one 30-min individual session with the dietitian and five monitoring visits to assess anthropometric parameters.

Anthropometric, clinical and biochemical measurements

Anthropometric measurements (body weight, height) were evaluated by trained personnel following standard procedures. Body mass index (BMI) was calculated as weight divided by squared height (Kg/m^2), and these values were converted into standard deviations (BMI-SDS) using age and sex-specific cut-off points according to Spanish reference growth charts [20]. Waist and hip circumferences were assessed with a non-stretchable measuring tape (Type SECA 200) following standard procedures.

Clinical outcomes such as pubertal stage (Tanner stage) and the presence of *acanthosis nigricans* were examined by pediatricians of the team [26].

Venous blood samples were obtained after an overnight fast. Glucose, insulin and lipid profiles were determined by standard autoanalyzer techniques. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting glucose and insulin values. Leptin levels were measured by ELISA (R&D Systems, Minneapolis, MN). All the measurements were taken at baseline and after the 8 week period.

Physical activity and sleep duration

Physical activity and sedentary time were objectively assessed using triaxial accelerometry over four days, including, at least, two weekdays in all participants. Participants and parents were instructed on wearing the accelerometer (Actigraph wGT3X-BT, Actigraph LLC,Penascola,Florida, USA) around the non-dominant waist all the time, including sleep time, and removing it just for water-related activities (bathing or showering). The monitors were initialized using 60-s epochs, as described elsewhere [15].

Accelerometry data were analyzed using ActiLife 6.0 software (Actigraph LLC, Penascola, Florida, USA). Continuous 24-h accelerometer data were recorded from weekdays and weekend days and were analyzed separately. Total PA was obtained by weighting 5 times weekdays plus two times weekend days by two and dividing the result by seven, as previously reported [27]. Data were expressed as counts per minute (CPM). PA intensity was categorized using validated cut-points (Evensons) for children and adolescents. CPM were under 100 was considered sedentary time. CPM between 101 and 2295 was considered light PA. Moderate and vigorous physical activity were combined into moderate-to-vigorous PA (MVPA) when counts were over 2296 CPM.

Sleep was assessed using the accelerometer Actigraph wGT3X-BT, and the data was analysed using the Sadeh algorithm derived from fundamental research performed by Avi Sadeh et al. [28] .This algorithm was commonly used in younger adolescents [29](10 to 11 years old) .

Statistical analysis

All statistical analyses were two-tailed and a p value < 0.05 was considered as statistically significant. Variables were described using mean ± SD. Student's t test was used for the comparison between groups (two independent groups test) and for the comparison within group (paired test) before and after the intervention. The change in each variable was calculated as the difference between post- and pre- intervention values for each subject.

Analyses of covariance (ANCOVA) were performed to assess the changes in anthropometric, biochemical and PA variables between usual care and intensive care groups after the adjustment for potential confounders: the studied variable at baseline, baseline BMI-SDS, sex and Tanner stage." Stata 12.0 (StataCorp, USA) for Windows was used in all the analyses.

Furthermore, we fitted a multivariable adjusted linear regression to examine the association of changes in leptin with changes in metabolic parameters and PA intensity.

Results

The study population includes 106 children with obesity (BMI-SDS 2.89 ± 1.05) (37.7% boys), with waist circumference over the sex and age-specific 90th percentile and a mean age of 11.31 ± 2.47 years old. At baseline, participants spent, on average, 997.9 (101.6) min/day on sedentary activities, including sleep time, 43.9 (23.4) min/day in MVPA activities, and 394.2 (95.9) min/day on light PA.

Most abdominal obese children (74.5%) do not fulfilled the WHO recommendations of being more than 60 min/day on MVPA at baseline. Participants were more active during weekdays compared to weekend days (Additional file 1).

Effectiveness of the lifestyle intervention

As expected, due to the randomization, participants in the two groups were similar for most of the clinical parameters at baseline, except for blood glucose levels (Table 1). The usual care group (n = 27) had significantly higher glucose levels compared with the intensive care group (n = 79) (p = 0.016). We did not find significant differences between groups regarding age, sex or Tanner stage.

The decrease in BMI-SDS (p = 0.029) and hip circumference (p = 0.019) was significantly higher in the intensive care group compared to the usual care group after adjusting for potential confounders (Table 1). Notably, participants in both groups achieved a significant reduction in waist circumference (Δ − 4.42 cm for usual care vs. Δ − 3.93 cm for intensive care group).

Regarding biochemical parameters, no differences were found between the two lifestyle interventions. A reduction in glucose (p = 0.004), insulin (p = 0.010), and leptin (p < 0.001) levels were observed in the intensive care group. Meanwhile, subjects in the usual care group did also significantly decrease glucose (p < 0.001) and leptin (p < 0.001) levels.

Changes in physical activity after the lifestyle intervention

As this is a randomized study, no differences were found on PA levels at baseline between groups (Additional file 2). No differences were found between the two lifestyle interventions in PA levels (Table 2). Interestingly, light PA significantly decreased in both the intensive and usual care group after the intervention. Participants from the intensive care group MVPA significantly increased from 43.5 min to 49.1 min (p = 0.024) in the intensive care group (Table 2). Interestingly, when all participants were analyzed in a

Table 1 Changes in anthropometric and biochemical measures after the lifestyle intervention in children with abdominal obesity ($N = 106$)

	Usual care group ($n = 27$)			Intensive care group ($n = 79$)			P^2
	Baseline	8 week	P^1	Baseline	8 week	P^1	
Age	10.74 (2.39)			11.50 (2.48)			
Sex (male/female) (%)	33/67			39/61			
Tanner Stage (I,II,III,IV,V) (%)	37.4/ 4.1/ 29.16/ 4.1/ 25			32.9/21.1/14.4/6.6/25			
Waist circumference (cm)	86.97 (11.53)	82.54 (10.51)	**< 0.001**	86.30 (10.98)	82.37 (11.15)	**< 0.001**	0.926
Weight (Kg)	64.81 (17.32)	62.92 (16.41)	**< 0.001**	66.96 (19.21)	64.29 (19.13)	**< 0.001**	0.057
Height (cm)	149.28 (12.64)	150.47 (12.66)	**< 0.001**	151.77 (13.18)	152.63 (13.03)	**< 0.001**	0.063
BMI (Kg/m^2)	28.54 (4.35)	27.31 (4.09)	**< 0.001**	28.40 (4.46)	26.91 (4.62)	**< 0.001**	0.075
BMI-SDS	3.07 (1.24)	2.62 (1.29)	**0.002**	2.83 (0.98)	2.32 (1.03)	**< 0.001**	**0.016**
Hip circumference (cm)	98.34 (12.02)	96.71 (10.92)	**0.012**	99.07 (12.77)	96.42 (13.37)	**< 0.001**	**0.024**
Waist to hip ratio	0.88 (0.07)	0.85 (0.06)	**< 0.001**	0.87 (0.06)	0.85 (0.06)	**< 0.001**	0.066
Waist to height ratio	0.58 (0.05)	0.54 (0.05)	**< 0.001**	0.56 (0.04)	0.53 (0.04)	**< 0.001**	0.971
Glucose (mg/dL)	92.18 (6.45)†	86.40 (5.09)	**< 0.001**	88.10 (6.08)†	85.59 (6.28)	**0.004**	0.738
Insulin (μU/mL)	20.34 (20.29)	17.93 (15.35)	0.329	15.30 (7.15)	13.09 (6.01)	**0.010**	0.147
HOMA-IR	4.65 (4.82)	3.92 (3.35)	0.271	3.37 (1.73)	2.80 (1.33)	**0.006**	0.187
Leptin (ng/mL)	38.82 (19.24)	23.49 (14.95)	**< 0.001**	30.58 (15.22)	22.35 (20.63)	**< 0.001**	0.505

Values are means (SD) or %. Abdominal obesity was defined as WC above the sex and age-specific 90th percentile. † Baseline differences p = 0.016; P^1 paired ttest for the group comparison after intervention program; The change in each variable was calculated as the difference between post- and pre- intervention values for each subject (usual care or intensive care subjects); P^2 is for the comparison between groups (usual care vs. intensive care) of the mean change in each variable adjusted for the corresponding variable at baseline, baseline BMI-SDS, sex and Tanner stage
Abbreviations: *BMI* Body mass index, *BMI-SDS* Standard deviation score for body mass index
P values below 0.05 are written in boldface

multivariable-adjusted model, an inverse association between the percentage of change in MVPA and the percentage of change in leptin levels was found (*B*: -2.17; 95% CI: -3.73 to – 0.61). Moreover, changes in leptin were also associated with changes in anthropometric and metabolic parameters (Table 3).

Discussion

In this study the two lifestyle interventions reduced anthropometric indexes and lowered light PA in abdominal obese children. No significant differences were observed between intensive care and usual care in PA levels. Intensive care participants significantly increase MVPA levels after the intervention. Moreover, changes in MVPA were inversely associated with changes in leptin levels after the intervention.

Concerning objectively measurement PA levels, our participants spent 44 min/day on MVPA at baseline. These data are similar to those found in overweight and obese pediatric populations from Madrid, Spain (9 years old: 58.6 min/day in MVPA, and 15 years old: 49.3 min/day) [7]. Both studies reported an appropriate

Table 2 Objectively measured physical activity before and after the lifestyle intervention in children with abdominal obesity

	Usual care group ($n = 27$)			Intensive care group ($n = 79$)			P^2
	Baseline	8-week	P^1	Baseline	8-week	P^1	
CPM	641.75 (183.23)	589.37 (233.69)	0.257	569.60 (181.93)	577.64 (203.18)	0.666	0.348
Sleep time (min)	510.33 (65.76)	516.62 (141.58)	0.825	538.51 (82.52)	526.42 (72.53)	0.344	0.814
Sedentary PA (min)	456.31 (114.48)	490.79 (160.13)	0.285	467.71 (135.22)	495.33 (117.83)	0.119	0.971
LPA (min)	420.34 (79.17)	369.69 (110.41)	**0.003**	385.34 (99.93)	361.55 (96.26)	**0.040**	0.668
MVPA (min)	45.17 (22.98)	44.89 (25.26)	0.955	43.56 (23.79)	49.08 (23.90)	**0.024**	0.217
Steps (number)	10,540 (3105)	9953 (3774)	0.391	10,151 (3083)	10,288 (2981)	0.740	0.332

Numbers are means (SD). Abdominal obesity was defined as WC above the sex and age-specific 90th percentile. P^1 paired ttest for the group comparison after the intervention program; The change in each variable was calculated as the difference between post- and pre- intervention values for each subject (usual care or intensive care subjects); P^2 is for the comparison between groups (usual care vs. intensive care) of the mean change in each variable adjusted for the corresponding variable at baseline, baseline BMI-SDS, sex and Tanner stage
Abbreviations: *CPM* Counts per minute, *LPA* Light physical activity, *MVPA* Moderate-to-vigorous physical activity, *PA* Physical activity
P values below 0.05 are written in boldface

Table 3 Association between changes in leptin levels with changes in metabolic and PA parameters: multivariable linear regression analysis in children with abdominal obesity

	% Δ leptin			
	Crude regression		Adjusted regression[1]	
	β	*P*	β	*p*
% Δ BMI-SDS	0.176	**< 0.001**	0.146	**0.001**
% Δ waist circumference	0.040	**< 0.001**	0.051	**< 0.001**
% Δ Waist-to-height ratio	0.041	**< 0.001**	0.050	**< 0.001**
% Δ glucose	0.009	0.639	0.040	0.077
% Δ insulin	0.194	0.049	0.244	0.055
% Δ HOMA-IR	0.187	0.077	0.271	**0.050**
% Δ Sleep time	−0.035	0.573	−0.119	0.083
% Δ Sedentary PA	0.003	0.973	0.111	0.241
% Δ LPA	0.020	0.670	0.001	0.994
% Δ MVPA	−0.088	0.888	−2.173	**0.007**

Abdominal obesity was defined as WC above the sex and age-specific 90th percentile. [1] The regression model was adjusted for the corresponding variable at baseline, BMI-SDS, sex and Tanner stage
Abbreviations: *BMI-SDS* Standard deviation score for body mass index, *LPA* Light physical activity, *MVPA* Moderate-to-vigorous physical activity, *PA* Physical activity
P values below 0.05 are written in boldface

management of the accelerometers concerning the cut-off points and epoch rate used. It is worth mentioning that our participants were 11.31 years old and had abdominal obesity.

Evidence shows that PA levels are different between normal-weight and obese subjects. Besides, PA levels are higher in children than in adolescents and in boys than in girls, with accelerometer-measured MVPA ranging from 49.1 to 85.1 min/day in normal-weight children and adolescents [7, 30–32].

We addressed the achievement of WHO recommendations on MVPA levels in our population. Only 25% of participants accumulated more than 60 min/day of MVPA at baseline. When considering the day of the week, 32% of the participants achieved the recommendations during the weekdays but only 16% of them did it at weekends. The evidence regarding this point is controversial. Some authors recommend the promotion of PA during the weekdays because children have PA lessons at the school [33, 34], while many others have observed that children are more physically active during the weekends, probably due to the fact that they have more spare time [7, 35] at the weekends. In our study, we observed that abdominal obese children are more physically active during the weekdays and more sedentary during the weekends. This could be explained because during the weekdays children attend school-based activities and sport games after the classes as well. However, in the weekends they may not have that many organized activities, the opportunities of being physically active are less and thus they spend more time in sedentary activities [36].

In the literature we found several lifestyle intervention studies that examined both adiposity indexes (i.e. BMI-SDS) and objectively measured PA levels [15, 16, 18] in obese children. First, a family-based behavioral program in 210 families of obese children achieved a reduction in − 0.1 units of BMI-SDS but did not report changes in MVPA after 12-week intervention [15]. Another study performed in 41 Latino families of obese children did not observe changes in either BMI-SDS or MVPA levels after a 6 months intervention [16]. In contrast, Hughes et al. (2008) performed a controlled trial with an intervention based on a traffic light diet and advice to increase PA on 1 h per day [18]. They found significant changes in sedentary behavior and light PA after 6 months of follow-up in the intervention group, but no changes were found in MVPA levels. Two more trials reported no changes in MVPA despite using novel approaches, such as active video games and motivational interviewing treatment [4, 17].

To our knowledge, this is the first intervention program that achieved a significantly increase in MVPA (+ 5.5 min/day,) in intensive care subjects. We did not found differences in PA between intervention groups. Intensive care subjects were under a moderate hypocaloric Mediterranean diet and achieved a successful weight loss (Δ BMI-SDS = − 0.51) that was higher than usual care group. It has been described that the combination of PA and Mediterranean diet might provide greater health benefits that those acquired separately in a recent meta-analysis [37]. In addition, a close follow-up of the participants (i.e number of visits and duration) and family enrolment are important issues in regard to the effectiveness of a paediatric intervention [38].

Leptin is a peptide hormone secreted by adipose tissue which plays a central role in regulating human energy homeostasis [24, 25]. Leptin levels are higher in obese adult and children, but physical activity might effectively reduce adipose tissue and lower leptin levels [26]. Obese children in this study reduced leptin levels after the intervention. Moreover, an inverse association between changes in MVPA and leptin levels was observed. Similar findings were reported in other intervention studies using objectively measured PA levels in adults [14].

The strengths of our study include: [1] the longitudinal design; [2] the effectiveness of the intervention with obese participants achieving a substantial weight loss; [3] the use of objectively measured physical activity. On the other hand, the diversity in important variables such as age and pubertal stage of the studied population is a limitation of this study. In order to control these potential confounders, sex, age and

Tanner stages were included in the statistical models. Another limitation could be a possible lack of statistical power for PA analysis between the two lifestyle interventions.

Conclusion

In conclusion, the two lifestyle interventions were successful, since a reduction in anthropometric indexes and light PA in abdominal obese children was achieved. No significant differences were observed between intensive care and usual care groups in regard to PA. Intensive care participants following a hypocaloric Mediterranean diet significantly increased MVPA. Changes in MVPA were inversely associated with changes in leptin levels after the intervention.

Additional files

Additional file 1: Table S1. Physical activity characteristics measured by accelerometry before lifestyle intervention in children with abdominal obesity ($N = 106$). (DOCX 13 kb)

Additional file 2: Table S2. Baseline characteristics in participants with abdominal obesity divided by type of intervention. (DOCX 14 kb)

Abbreviations

BMI: body mass index; BMI-SDS: body mass index standard deviation; CPM: counts per minute; HOMA-IR: homeostasis model assessment for insulin resistance; MVPA: moderate to vigorous physical activity; PA: physical activity; WHO: world health organization

Acknowledgments

We thank all the children and families in the trial for their enthusiastic and maintained collaboration, and other investigators of IGENOI group for their participation in the recruitment process and the acquisition of the data. AOR is fully acknowledged for the fellowships to Asociación de Amigos de la Universidad de Navarra (ADA). LMA and the research leading to these results has received funding from "la Caixa" Banking Foundation.

Funding

The IGENOI study was supported by the MERCK foundation grant and the Laboratories ORDESA (Sant Boi de Llobregat; Barcelona, España)-FEI-AEP grant. None of the sponsors participated in the design and conduct of the study; collection, management, analysis, and interpretation of the data; or preparation, review, or approval of the manuscript.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The authors' contributions were as follows: LMA contributed to the data collection, performed the statistical analyses and wrote the manuscript; AOR contributed to the data collection. AOE contributed to the data analysis; NMC, MC, AM, CAS were responsible of the follow-up, design, financial management and editing of the manuscript. All the authors actively participated in the manuscript preparation, as well as revise and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki (Fortaleza, Brasil, October 2013) and was approved by the Ethics Committee of the University of Navarra (Reference number 044/2014). Informed assent was obtained from every child and all parents and/or legal guardians signed an informed consent according to the Helsinki declaration.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 18 December 2018 Accepted: 24 March 2019
Published online: 04 April 2019

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Supplementary Table 1. Physical activity characteristics measured by accelerometry before lifestyle intervention in children (N = 106) with abdominal obesity.

Values are means (SD). Abdominal obesity was defined as WC above the sex and age-specific 90th percentile.

	Weekdays	**Weekend days**	**p**
METS	1.55 (0.20)	1.48 (0.20)	**<0.001**
CPM	607.17 (193.93)	519.68 (194.05)	**<0.001**
Sedentary PA (min)	986.97 (107.31)	1030.72 (112.70)	**<0.001**
LPA (min)	402.30 (101.59)	374.15 (103.24)	**<0.001**
MVPA (min)	48.04 (26.69)	34.92 (25.21)	**<0.001**
Steps (number)	10832 (3470)	8795 (3894)	**<0.001**

Abbreviations: CPM, counts per minute; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; PA, physical activity

Supplementary Table 2. Baseline characteristics in abdominal obese participants taking into account the arm of intervention.

	Usual Care	Intensive Care	p
Age	10.74 (2.39)	11.50 (2.48)	0.166
Sex (male/female) (%)	33/67	39/61	0.585
Tanner Stage (I,II,III,IV,V) (%)	37.4/ 4.1/ 29.16/ 4.1/ 25	32.9/21.1/14.4/6.6/25	0.236
Waist circumference (cm)	86.97 (11.53)	86.30 (10.98)	0.788
Weight (Kg)	64.81 (17.32)	66.96 (19.21)	0.608
Height (cm)	149.28 (12.64)	151.77 (13.18)	0.395
BMI (Kg/m^2)	28.54 (4.35)	28.40 (4.46)	0.889
BMI-SDS	3.07 (1.24)	2.83 (0.98)	0.309
Hip circumference (cm)	98.34 (12.02)	99.07 (12.77)	0.797
Waist to hip ratio	0.88 (0.07)	0.87 (0.06)	0.397
Waist to height ratio	0.58 (0.05)	0.56 (0.04)	0.213
Glucose (mg/dL)	92.18 (6.45)	88.10 (6.08)	**0.016**
Insulin (µU/mL)	20.34 (20.29)	15.30 (7.15)	0.193
HOMA-IR	4.65 (4.82)	3.37 (1.73)	0.170
Leptin (ng/mL)	38.82 (19.24)	30.58 (15.22)	0.183
CPM	641.75 (183.23)	569.60 (181.93)	0.078
Sleep time (min)	510.33 (65.76)	538.51 (82.52)	0.211
Sedentary PA (min)	456.31 (114.48)	467.71 (135.22)	0.144
LPA (min)	420.34 (79.17)	385.34 (99.93)	0.101
MVPA (min)	45.17 (22.98)	43.56 (23.79)	0.759
Steps (number)	10540 (3105)	10151 (3083)	0.573

Values are means (SD) or %. Abdominal obesity was defined as WC above the sex and age-specific 90th percentile. P^1 ttest is for the comparison between groups (usual care vs. intensive care).

Abbreviations: BMI, body mass index; BMI-SDS, standard deviation score for body mass index; CPM, counts per minute; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity.

Chapter 4

Telomere length in abdominal obese children under a lifestyle intervention.

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Nutrition, Metabolism and Cardiovascular Disease,2019
Under-review
Impact factor (2017): 3.318
47/128 in Cardiac and Cardiovascular Systems, Q2
57/142 in Endocrinology and Metabolism, Q2
63/142 in Nutrition and Metabolism, Q2

Abstract

Background and Aims: It has been observed that children with obesity have shorter telomeres than normal weight pairs, that could be explained because the excess of adipose tissue lead to a low-grade chronic inflammation that affect telomere length (TL). Thus, lifestyle interventions aiming to reduce weight have been proposed as a good strategy to maintain TL. The main objectives of the present study were to measure TL in children with abdominal obesity after an 8-week lifestyle intervention, and to assess their potential associations with anthropometric and metabolic outcomes.

Methods and results: We assess anthropometric and biochemical outcomes at baseline and after 8-week lifestyle intervention in 106 children with abdominal obesity (11.30±2.49 years old, 63% girls).TL were measured by monochrome multiplex real-time quantitative PCR. After the lifestyle intervention, participants significantly decreased anthropometric parameters and glucose metabolism indicators. No significant difference was found when TL was assessed. A negative correlation between baseline telomeres and BMI, body weight or waist circumference was observed. Furthermore, baseline TL predicted changes in blood glucose levels after the lifestyle intervention.

Conclusions: we observed a significant inverse correlation between TL and obesity traits. The 8-week intervention did not modify TL. Interestingly, we reported that baseline TL could be used as a biomarker for predicting changes in blood glucose levels.

Keywords: weight loss; BMI-SDS; glucose metabolism; adolescents.

Introduction

Telomeres are repetitive DNA sequences (TTAGG) located at the end of chromosomes. Their main function is to stabilize and protect chromosomes from being recognized as double-strand by DNA repair proteins [1]. With each cell division telomeres get shorter due to the "end-replication problem" [1,2]. Some factors that could contribute to telomere shortening are TL at birth, genome instability or aging [3]. It has been observed that telomere shortening is independent of chronological age suggesting that telomere attrition is a modifiable factor [4]. A recent study performed in the Nurse's Health Study have demonstrated that a healthy lifestyle in combination with the adherence to the Mediterranean Diet did delay telomere shortening [5].

Obesity is characterized by a low-grade chronic inflammation that is accompanied by oxidative stress [6]. It has been suggested that obesity may accelerate telomere shortening [7–10]. Therefore, obese subjects showed shorter telomeres than normal weight age and sex pairs [11]. Furthermore, it is reported that TL was negatively associated with Body Mass Index (BMI), hip and waist circumferences [9,12]. Also, Revesz et al. associated baseline TL with unfavorable metabolic syndrome outcomes in an adult follow up study [13]. Shorter telomeres are also found in obese children and adolescents, in addition they showed higher cellular aging than healthy subjects [11,14]. Interestingly, weight loss is a good strategy for reducing excess adipose tissue, the inflammatory and oxidative stress status derived from it [7] and could help to prevent telomere attrition.

To our knowledge there are three studies (one in adolescents) that have performed lifestyle interventions based on both dietary and physical activity recommendations, and its effects on TL are not clear [15–17]. Furthermore, the role of telomere length as a biomarker of the effectiveness of lifestyle interventions has been assessed in adults and pediatric subjects [14,17,18]. In a previous work, we demonstrated that higher baseline TL in obese adolescents,

significantly predicted a higher decrease in body weight, and in standard deviation score of BMI (BMI-SDS) and in blood glucose levels after a 2-month lifestyle intervention [17,19].

We hypothesized that TL could be associated with adiposity, metabolic traits and may change with weight loss. Thus, the aim of the study was to determine TL in abdominal obese children during a lifestyle intervention: (1) to evaluate the relationship between adiposity or metabolic traits with TL; and (2) to assess the potential role of TL as biomarkers of the effectiveness of weight loss interventions.

Materials and Methods

Participants

The study population includes 121 children and adolescents participants of the Intervention Grupo Estudio Navarro de Obesidad Infantil (IGENOI) from Pamplona, Spain. IGENOI study consists of a 2-year family-based lifestyle intervention (NCT03147261). Participants are girls and boys with ages between 7 to 16 years old with a waist circumference above the 90th percentile from the National growth charts [20]. Children were recruited from the Endocrinology Pediatric Units from Navarra's Hospital and University of Navarra Clinic, and from primary health care centers in Pamplona. The exclusion criteria were: major psychiatric illness such as eating disorders, pharmacology treatment, subjects with pre-diabetes, following special diets, regular consumption of alcohol or suffering from food intolerance.

The study protocol was approved by the Ethics Committee of the University of Navarra (reference number 044/2014) and was performed in accordance with the ethical standards laid down in the 2013 Declaration of Helsinki (Fortaleza, Brasil, October 2013). At the recruitment visit, the parents and/or legal guardians received all the information about the aim of the study. Each participant gave

the informed assent and an informed consent was obtained from all parents and/or legal guardians according to the Helsinki declaration.

Lifestyle intervention

The study is a 2 year-lifestyle program that was implemented in two different stages: the first two months were the intensive period, and afterwards the patients were followed up to 22 months. The study was conducted in the clinical setting by pediatricians, dieticians, physical activity experts and nurses. In the present study we have measured TL in 116 subjects at baseline and 108 subjects after the 8-week intervention. The drop-out rate was of 6.9 % for the 8-week intervention and the main reason were discouragement, changes of address for the notifications, social problems or inability to comply the visits (school exam periods or difficulty of parents to accompany the children to the visits). These reasons were similar to those reported in other trials with obese children [21].

Participants were randomly assigned in two arms: Usual care group received healthy diet advice following national guidelines, while intensive care group received moderately hypocaloric Mediterranean diet, as described elsewhere [22]. Both groups were encouraged to accumulate an extra time of 200 minutes of PA per week at a 60-75% of their maximum heart rate.

The primary endpoint of the on-going study was to achieve a successful weight loss and as consequence to improve the metabolic status of abdominal obese children. The present study involved a secondary analysis of telomere length. The sample size was calculated taking into account the primary outcome, thus under the assumptions for an error of 5%, a power of 90%, a 1:3 ratio and a mean difference of 0.50 (±0.47) in BMI-SDS after the lifestyle intervention [23].

Anthropometric, clinical and biochemical parameters.

All anthropometric and biochemical parameters were measured at baseline and after the intensive phase of the intervention by trained personnel following standard procedures. Body weight was measured using an electronic scale (BC-418, TANITA, Tokyo, Japan). Participants were asked to stare in barefoot with light clothes. Height was measured using a stadiometer of 1 mm precision (Seca 220, Vogel&Halke, Hamburg, Germany). Waist and hip circumferences were assessed with a non-stretchable measuring tape (Type SECA 200). Body mass index (BMI) was calculated as the ratio of weight by squared height (Kg/m2). BMI-SDS were obtained after converting BMI into standard deviations using age and sex specific cut-off points derived from Spanish reference growth charts [24]. The paediatrician team assessed pubertal stage using the Tanner stage and also the presence of *Acanthosis nigrigans* [25].Venous blood samples were obtained by trained nurses at the hospital after an overnight fast. Glucose and insulin were determined by standard autoanalyzer techniques.

Telomere length assessment

A monochrome multiplex quantitative PCR (MMqPCR) method described elsewhere [26] was used to assess telomere length. Briefly, this method performs in a single reaction the quantification of the relative copy numbers of telomeres (T) and a single copy gene (S). Telomere length was expressed as a T/S ratio. A calibration curve of a reference DNA samples (150-2.34 ng/µL in 2-fold dilutions) was included in each 384 plate and was used for the relative quantification.

The master mix used contained: QuantiTect Syber Green PCR kit (Qiagen, Valencia, CA, USA), telomere primers pairs, albumin primer pairs and ultrapure water to complete the final volume. The final primer concentrations were for telomere amplification telg and telc 900 nM; and for the amplification of the

single copy gene albu and albd 900 nM. The primer sequences were telg (5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'), telc (5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'), albu (5'-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG-3') and albd (5'-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT-3'). All primers were from Sigma Aldrich (St. Louis, MO, USA), and were purified by the manufacturer using high-performance liquid chromatography.

We carried out the following MMqPCR in a CFX384 Touch Real-Time PCR system (BioRad, CA, USA) following the protocol: 15 min at 95 °C for enzyme activation followed by two cycles of 95 °C at 15 seconds and 49 °C at 15 seconds, and 35 cycles of 15 s at 95 °C, 10 s at 63 °C, 15 s at 74 °C (first signal acquisition) and 15 s at 88 °C (second signal acquisition). For each sample, we generated a melting curve from 45 °C to 95 °C, ramped at 0.2 °C/s. Experiments were conducted in a high-throughput 384-well plate and all samples were run in triplicate for quality control. When samples showed a high variation (more than 10%) were rerun and reanalysed. The intra-assay coefficient of variation was 8.52% and the inter-assay coefficient of variation was 13.66%.

Statistical analysis

All statistical analyses were two-tailed and a p value of <0.05 was considered to be as statistically significant. Clinical and biochemical data from 116 participants were described using mean ± SD. Paired t-test was used to compare the studied variables before and after the intervention. Telomere length data was log transformed to fit a normal distribution.

We calculated Pearson's correlation coefficients to describe the associations between baseline TL with age, anthropometric indices and glucose metabolism outcomes.

Furthermore, we fitted a multivariable adjusted linear regression to examine whether baseline TL were able to predict changes in fasting glucose levels after the lifestyle intervention after fitting the model for sex, age, plate, and baseline glucose levels.

Results

We analyzed telomere length in abdominal obese children (BMI-SDS 2.95 $\pm$ 1.07) with a mean age of 11.30 (2.49) years old, 63 % females. Telomeres at baseline were no different between girls and boys (p=0.341, data not shown). We found a negative association between telomere length at baseline and age (r= -0.194, p=0.037) (Figure 1). We analyzed the entire population (intensive + usual care groups) because no differences were found in the studied variables between groups.

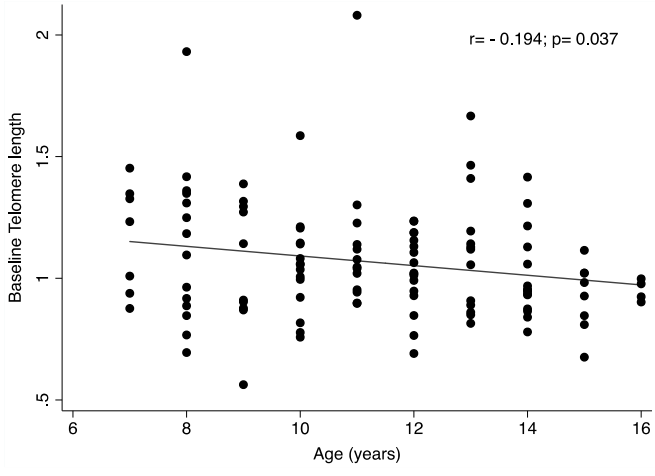

Figure 1. Correlation between baseline TL and age.

The correlation between baseline TL and anthropometric indices and biochemical parameters was shown in Table 1. It can be observed that those children with shorter TL showed higher values of body weight, BMI and waist circumference. When biochemical parameters were examined, we did not find any significant correlation.

The effectiveness of the weight loss was assessed in all participants, since no differences between intervention groups were found (Supplementary Table 1). All participants achieved a successful weight loss (Δ BMI-SDS = - 0.48, p<0.001) and anthropometric indices and glucose metabolism indicators (Δ glucose = -2.91, p<0.001) were reduced after 8 weeks of lifestyle intervention (Table 2). We did not find differences in TL (p=0.540).

Table 1. Changes in anthropometric, biochemical and telomere length after lifestyle intervention in abdominal obese children.

	Baseline n=116	8-week n=108	p
Age (years)	11.30 (2.49)	-	
Sex (M/F)	43/73	-	
Tanner stage (I/II/III/IV/V)	39/19/21/8/29	-	
Weight (Kg)	66.85 (19.39)	64.25 (1.81)	**<0.001**
Height (cm)	150.96 (13.20)	151.92 (13.09)	**<0.001**
BMI (Kg/m2)	28.64 (4.50)	27.17 (4.52)	**<0.001**
BMI-SDS	2.95 (1.07)	2.43 (1.10)	**<0.001**
Waist circumference (cm)	86.89 (11.38)	82.65 (11.23)	**<0.001**
Hip circumference (cm)	99.16 (12.46)	96.80 (12.68)	**<0.001**
Waist to Height ratio	0.57 (0.05)	0.54 (0.05)	**<0.001**
Glucose (mg/dL)	88.91 (6.63)	86.23 (5.75)	**<0.001**
Insulin (μU/mL)	17.60 (12.54)	14.56 (9.61)	**0.006**
HOMA-IR	3.91 (2.96)	3.14 (2.09)	**0.005**
Telomere length (T/S)	1.06 (0.23)	1.04 (0.22)	0.540

Values are means (SD) or %. Abdominal obesity was defined as WC above the sex and age-specific 90th percentile.

Abbreviations: BMI, body mass index; BMI-SDS, standard deviation score for body mass index.

We observed a correlation between baseline TL and changes in blood glucose levels (Figure 2). A multivariable regression model was performed in order to assess the predictive value of baseline telomere length on changes in anthropometric variables and glucose metabolism indicators. As described in Table 3, we found that a higher TL at baseline significantly predicted a greater reduction in blood glucose levels, in the crude model and after adjusting for potential confounders (β= - 7.982; 95%CI (-14.61 to -1.34) and p=0.019). We did not find any association between baseline telomere and changes in any anthropometric measurement (data not shown).

Table 2. Correlation coefficients at baseline between telomere length and clinical and biochemical outcomes.

	All participants	
	r	p
Weight (Kg)	-0.205	**0.027**
BMI (Kg/m2)	-0.187	**0.044**
BMI-SDS	-0.048	0.610
Waist circumference (cm)	-0.230	**0.013**
Waist to height ratio	-0.165	0.077
Glucose (mg/dL)	0.107	0.274
Insulin (μU/mL)	-0.005	0.958
HOMA-IR	-0.002	0.982

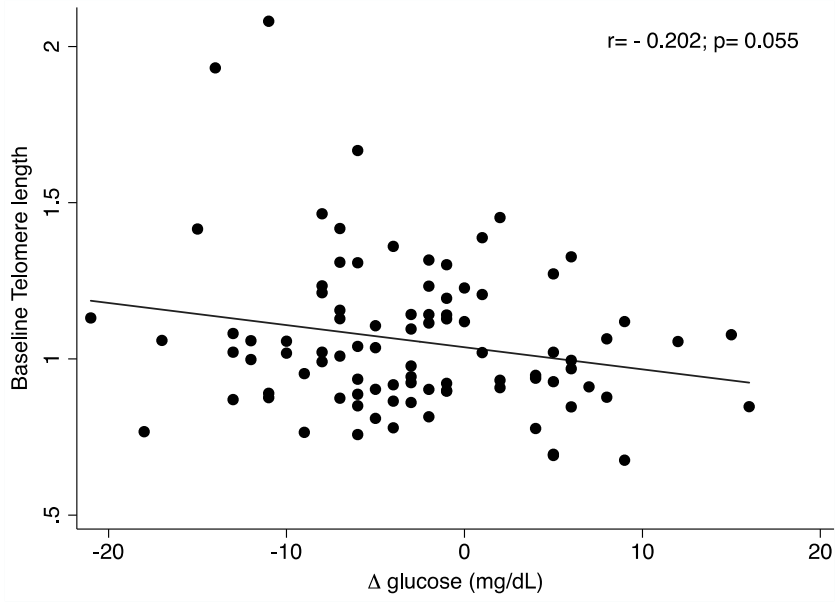

Figure 2. Correlation between baseline TL and changes in glucose levels after 8-week lifestyle intervention.

Table 3. Changes in glucose after 8 weeks of lifestyle intervention, according to the increase of 1 SD in baseline TL.

	All participants n=108	
Δ glucose (mg/dL)	β (95% CI)	p
Crude model	- 6.286 (-12.45 to -0.11)	0.046
Model 1	- 8.071 (-14.63 to -1.51)	0.017
Model 2	- 8.268 (-15.08 to -1.45)	0.018
Model 3	- 7.982 (-14.61 to -1.34)	0.019

Linear regression analysis with adjustments. Model 1: plate; Model 2: plate and tanner stage; Model 3: plate, tanner stage and baseline glucose levels.

Discussion

This study was performed in obese children at a high metabolic risk, since they presented abdominal obesity (waist circumference ≥ 90th). The anthropometric and glucose metabolism indicators significantly decreased after the 8-week lifestyle intervention in all participants. No significant difference was found when telomere length was assessed. We observed a significant inverse correlation between baseline TL and adiposity indices (BMI, body weight and waist circumference). Furthermore, we found that baseline TL predicted changes in blood glucose levels after the lifestyle intervention.

Telomeres get shorter with age and there are several studies that have demonstrated an inverse association between TL and age in the pediatric population [11,27,28]. This observation was confirmed in our study with abdominal obese children, where TL did decline with age. TL have been described as the markers of biological age since they get shorter with each cell division and when they are critically shorter, they trigger senescence [3].

As our study population was children with abdominal obesity, we performed an 8-week lifestyle intervention in order to achieve an improvement of anthropometric and metabolic parameters related to insulin resistance. All anthropometrics parameters and glucose metabolism indicators were reduced by the lifestyle interventions. These results were in accordance with other studies performed in obese children and adolescents (age range: 5 to 17 years old) involved in lifestyle interventions carried out by multidisciplinary teams [29–33].

We assessed changes in TL after the lifestyle intervention. To our knowledge, there are three studies that have evaluated the effects of lifestyle interventions in TL in obese subjects, two of them were conducted in adults while the other one was carried out in adolescents. It is worthy to mention that all of them measured TL in leukocytes and following the Cawthon's 2002 technique [34]. In the present

study, we have measured leukocyte TL using monochrome multiplex real-time quantitative PCR described by Cawthon et al. in 2009, and that improves the previous methodology, eliminating the variability due to potentially variable amounts of DNA pipetted in the separate Telomere and Single copy reactions [26].

Mason et al. 2018 performed an RCT of 5.5 months of duration in 162 adults, participants achieved a significant reduction of weight loss higher than 5% [15]. They did not find any association between weight loss and changes in TL. However, they described that those participants classified as weight loss maintainers (had maintained 10% or more than their initial body weigh) had longer TL 12 months later. Previously, in 439 obese postmenopausal women that were under an intervention with only diet, only exercise or the combination of both of them (weight loss higher than 10%) Mason et al. (2013) concluded that there was change in TL after 12 months follow up [16]. Finally, the third study evaluating weight loss and TL in obese participants, was performed by members of our group and was a multidisciplinary lifestyle intervention carried out in 74 obese or overweight Spanish adolescents (49% males) [17]. The program aimed to improve dietary habits, physical activity and cognitive areas. Participants achieved a successful weight loss (Δ BMI-SDS♂= -0.91; Δ BMI-SDS♀= -0.7) that was accompanied by lowering TL attrition. The results presented in the present study (37% males) indicated that despite achieving a successful weight loss (Δ BMI-SDS= -0.48), telomeres did not change after the 8-week intervention. Differences in the design of the study, characteristic of participants, TL methods of measurement and so on may explain the discrepancy. Thus, more research work needs to be done to clarify the effect of weight loss on telomere length.

Telomere length has been previously associated with anthropometric outcomes such as: weight, BMI, BMI-SDS, waist circumference and waist to height ratio in adults and in children [8,9,11,14,35]. In accordance with those results,

participants of the present study were children with abdominal obesity, and we found that baseline TL was inversely correlated with body weight, BMI and waist circumference. These inverse associations may be explained because adiposity (measured as elevated BMI, waist circumference or body weight) is linked to a low-grade inflammatory state and oxidative distress, and these two factors may contribute to telomere attrition [36].

Moreover, we did not find any association between glucose metabolism indicators and TL at baseline. In previous studies conducted in adults, there are controversy about those associations, while there are some authors that have found that longer TL were significantly associated with lower blood glucose levels [13,37,38] there are other authors who did not observe any association [39].

García-Calzón et al. reported in a study in obese and overweight adolescents that longer TL at baseline significantly predicted a higher decrease in body weight and BMI-SDS after an intensive multidisciplinary study [17]. Another study in adults subjects at high cardiovascular risk that followed Mediterranean Diet pattern, described that TL at baseline significantly predicted a greater decrease in adiposity indices after 5 years of follow up [18]. Moreover, in a lifestyle intervention in obese adolescent longer TL at baseline significantly predicted a greater reduction in blood glucose levels after 2 and 6 months of follow up [19]. In our study, we did not find that TL could predict changes in anthropometric measurements. But to the best of our knowledge, this study confirmed the previous findings that higher baseline TL significantly predict more favorable changes in blood glucose during a lifestyle intervention. The plausibility of this association could be partially explained because our participants -children with abdominal obesity- had abdominal obesity that has been associated with inflammation, oxidative distress, insulin resistance and shorter telomeres [35].

Our study has several strengths: 1) the longitudinal study design with pre- and post- intervention measures, 2) a successful lifestyle intervention in which

participants reduced their adiposity indices; 3) it is performed in abdominal obese pediatric subjects that are not affected by chronic-obesity related problems. On the other hand, some limitations should be addressed: telomere length is a measure which has a wide range of variability, for this reason we have applied a new method (MMqPCR) where telomere and single gene copy were measured on the same well to reduce variability due to pipetting. It is worthy to recognize that in our study the age group is wide (7 to 16 years) there are different pubertal stages, in order to take that into account we have adjusted all the statistical models by Tanner stage.

Conclusions

We found an inverse association between TL and body weight, BMI and waist circumference in a population of children with abdominal obesity. Age was shown to be associated to TL. No changes in TL were found after an 8-week lifestyle, despite achieving a significant reduction in adiposity traits and blood glucose levels. Furthermore, we observed that a higher reduction in glucose levels after the intervention were found in those participants with higher telomeres at baseline

Author Contributions

Conceptualization: MC.A.S, G.Z, and A.M; Methodology: L.M.A, A.O.R, G.Z, MC.A.S and A.M; Formal analysis: LMA and G.Z , Writing-Original Draft Preparation: LMA; Writing- Review and Editing: A.O.R, MC.A.S, G.Z and A.M, Project administration: MC.A.S and A.M; Funding acquisition: MC.A.S and A.M

Funding

The IGENOI study was supported by the MERCK foundation, ORDESA-AEP and PIUNA (Universidad de Navarra) grants.

Acknowledgments

We thank all the children and families in the trial for their enthusiastic and maintained collaboration, and other investigators of IGENOI group for their participation in the recruitment process and the acquisition of the data. LMA and AOR have a predoctoral fellowship from "la Caixa" Banking Foundation.

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Chapter 5

Melanocortin-4 receptor and lipocalin 2 gene variants in Spanish children with abdominal obesity: effects on weight loss after a lifestyle intervention

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Nutrients,2019
DOI: 10.3390/nu11050960
Impact factor (2018): 4.196
18/83 in Nutrition and Dietetics, Q1

nutrients

MDPI

Article

Melanocortin-4 Receptor and Lipocalin 2 Gene Variants in Spanish Children with Abdominal Obesity: Effects on BMI-SDS After a Lifestyle Intervention

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Received: 11 March 2019 / Accepted: 24 April 2019 / Published: 26 April 2019

Abstract: Mutations leading to a reduced function of the melanocortin-4 receptor (MC4R) exert a major gene effect on extreme obesity. Recently it was shown that the bone derived hormone lipocalin 2 (LCN2) binds to the MC4R and activates a MC4R dependent anorexigenic pathway. We identified mutations in both genes and screened the effects of *MC4R* and *LCN2* mutations on eating behavior and weight change after a lifestyle intervention. One hundred and twelve children (11.24 ± 2.6 years, BMI-SDS 2.91 ± 1.07) with abdominal obesity participated in a lifestyle intervention. *MC4R* and *LCN2* coding regions were screened by Sanger sequencing. Eating behavior was assessed at baseline with the Children Eating Behavior Questionnaire (CEBQ). We detected three previously described non-synonymous *MC4R* variants (Glu42Lys, Thr150Ile, and Arg305Gln) and one non-synonymous polymorphism (Ile251Leu). Regarding *LCN2*, one known non-synonymous variant (Thr124Met) was detected. Eating behavior was described in carriers of the *MC4R* and *LCN2* mutation and in non-carriers. *MC4R* and *LCN2* mutations were detected in 2.42% and 0.84%, respectively, of Spanish children with abdominal obesity. A number of subjects with functional mutation variants in *MC4R* and *LCN2* were able to achieve a reduction in BMI-SDS after a lifestyle intervention.

Keywords: childhood obesity, CEBQ, eating behavior and Ile251Leu

1. Introduction

Obesity has been defined by the World Health Organization (WHO) as an abnormal or excessive body fat accumulation that may impair health [1]. Both environmental and genetic factors have an influence on weight gain [2]. The impact of genetics on obesity is heterogeneous. For a small number of subjects obesity is caused by mutations in single genes, for the majority of the population obesity has a polygenic nature [2,3].

One of the most common single genes harboring variants associated with obesity is the melanocortin-4 receptor gene (*MC4R*) [4,5]. It is known as a regulator of energy homeostasis due to its effect on food intake and energy expenditure via neuronal melanocortinergic pathways [6]. More than 369 mutations including non-synonymous, nonsense, and frameshift mutations have been identified mainly in obese individuals [7,8]. Most of the non-synonymous mutations lead to partial or complete loss of function of the *MC4R* [9]. The polymorphism Ile251Leu leads to an increased function of MC4R and is associated with a reduced BMI [7].

The protein lipocalin 2 has been associated with obesity [10,11] and was thought to be secreted from adipose tissue as an adipokine [12]. However recent studies discovered a ten-fold higher expression in osteoblasts than in adipose tissue. In this line, recent studies have shown that lipocalin 2 (LCN2) binds to the MC4R to activate MC4R-dependent anorexigenic pathways [13]. It has also been observed that obese participants heterozygous for *MC4R* mutations leading to impaired function have higher levels of plasma LCN2 than BMI-matched MC4R wild-type controls [13].

Our aim was to screen for mutations in the *MC4R* and *LCN2* genes in a Spanish pediatric population with abdominal obesity. Moreover, we investigated the effects on eating behavior and weight change after participating in a one-year lifestyle intervention for carriers versus non-carriers of *MC4R* and *LCN2* variants.

2. Materials and Methods

2.1. Subjects

For this study, a total of 112 children between 7 to 16 years of age and with abdominal obesity defined as a waist circumference higher than the 90th percentile [14] participate in a lifestyle intervention. The IGENOI study is a 2-year lifestyle intervention program for children with abdominal obesity carried out by GENOI group (*"Grupo de Estudio Navarro de la Obesidad infantil"*). This study is a randomized control trial (NCT03147261) conducted in Pamplona, Spain. Children were recruited from the Pediatric Endocrinology at "Clinica Universidad de Navarra" and "Complejo hospitalario de Navarra", and from health care centers around Pamplona and its neighborhoods.

Participants with previous diabetes, presence of other diseases beside obesity, major psychiatric illness including bulimia nervosa, pharmacological treatment, food intolerance, or treatment with special diets, or frequent alcohol or drug consumption were excluded. Children and their parents signed a written informed consent in the screening visit. The study protocol was performed in accordance with the ethical standards of the Declaration of Helsinki (Fortaleza, Brasil, October 2013), and was approved by the ethics committee of the University of Navarra (Reference number 044/2014).

2.2. Experimental Design

The lifestyle intervention comprises an 8-week intensive phase (N = 104 subjects) and a follow-up period of 10 months (N = 85 subjects, 1 year). The dropout rate was 7% at week 8 and 24% at 1 year of follow up.

A multidisciplinary team conformed by dietitians, pediatricians, nurses, physical activity experts, and laboratory technicians were involved in the development of the study protocol. Participants were randomly assigned into two different groups: intensive care and usual care group, following a 3:1 ratio. The first one was given a moderate hypocaloric Mediterranean diet, as previously described [15], while the usual care group received usual pediatric advice with healthy diet recommendations [16]. Specifically, intensive care participants were prescribed with a moderate hypocaloric diet based on a fixed full-day meal plan. The restriction, not to interfere with growth, was calculated depending on the degree of obesity (from −10% to −40% of total energy intake). A Mediterranean-style diet based consists of high consumption of fruit, vegetables, whole grains, legumes, nuts, seeds, and olive oil, minimally processed foods; moderate consumption of dairy products, fish, and poultry; and low consumption of red meat [15]. Both groups were encouraged to increase their physical activity to at least 200 minutes per week as recommended by The American College of Sports Medicine to prevent weight gain [17].

The intervention group participants had 30-minute individual sessions with the dietitian every two weeks during the 8-week treatment phase. In these sessions a follow-up about the accomplishment of the diet and anthropometric measurements was performed. In addition, the parents or legal guardians had one parallel group session where they received education about their role in the study and obesity related problems, while intensive care participants were encouraged to make healthy lifestyle decisions about food choices, eating behavior, sedentary activities, and physical activity. On the other hand, usual care participants and their parents received one 30-min individual session with the dietitian and five monitoring visits to assess anthropometric parameters.

During the follow-up period, participants had monitoring visits at 3, 4,5,6,9, and 12 months from the baseline visit.

2.3. Anthropometric, Clinical and Biochemical Measurements

All anthropometric measurements were performed at baseline, after 8 weeks, and at 1 year of follow up according to standard procedures and calibrated tools in the paediatric population [18]. Measurements were performed by trained personnel in a wide space, while the participants were asked to stand barefoot and wore light clothing, without hair ornaments or jewels.

Body weight and body fat were determined using a digital scale BC-418 Segmental Body Composition Analyzer according to manufacturer instructions (Tanita, Tokyo, Japan). To measure height, participants were asked to stand on stadiometer with the feet placed parallel and slightly apart, and heels, buttocks, scapula, and occipital head area touching the vertical board at the same time. A non-stretchable measuring tape (type SECA 200) was used for measuring waist circumference (WC) and hip circumference (HC) by standard procedures (Type SECA 200). The waist-to-hip and waist-to-height ratios were also calculated.

Body mass index was calculated as weight divided by squared height (Kg/m^2). BMI-standard deviation (BMI-SDS) is BMI values converted into standard deviation using age and specific cut-points according to Spanish reference growth charts [19]. Pubertal status was determined using Tanner stage and was evaluated by a paediatrician. The presence of *Acanthosis Nigricans* was diagnosed by pediatricians of the team at baseline. Blood pressure was measured following standard procedures, as described elsewhere [20].

Venous blood samples were obtained by specialized trained nurses at the Hospital after an overnight fast. Glucose, insulin leptin, and lipid profiles were determined by standard autoanalyzer techniques at baseline. Insulin resistance was calculated from the homeostasis model assessment of insulin resistance (HOMA-IR).

2.4. Physical Activity

Moderate-to-vigorous physical activity was assessed at baseline using triaxial accelerometry (Actigraph wGT3X-BT, Actigraph LLC, Penascola, Florida, USA). Briefly, participants wore the accelerometer around the non-dominant waist for four days, including, at least, two weekend days, as described elsewhere [21]. Accelerometry data were analysed using ActiLife 6.0 software (Actigraph LLC, Penascola, Florida, USA) as describe elsewhere [22].

2.5. Children Eating Behavior Questionnaire (CEBQ)

The Children Eating Behaviour Questionnaire (CEBQ) is a multidimensional parent-reported questionnaire about their children's eating behaviour [23]. It includes 35 items regarding eating styles that are clustered into eight subscales. The eight subscales are classified into two dimensions: food approach or food avoidance. The food approach dimension comprises the following subscales: "Emotional overeating", "Enjoyment of food", "Desire for drink", and "Food responsiveness". Food avoidance is represented by the following: "Slowness in eating", "Satiety responsiveness", "and Emotional undereating", and "Food fussiness". It was fulfilled by parents or legal guardians at baseline.

For each behaviour subscale parents report their children's behaviour on a five-point Likert Scale, that ranges from never, rarely, sometimes, often, or always (1 to 5). A ratio was calculated between the sums of the food approach vs. the sum of the food avoidance subscales.

2.6. DNA Extraction

Venous blood samples were obtained on ethylendiaminetetraacetic acid (EDTA) tubes, which were centrifuged 30 minutes after the extraction at 3500 rpm for 15 minutes at 4 °C. DNA was extracted from the buffy coat fraction using a commercial kit MasterPure DNA purification kit for Blood Version II (Epicenter Biotechnologies, Madison, WI, USA). Its quality and quantity were determined with a Nanodrop spectrometer ND-1000 (Nanodrop Technologies, Wilmington, Delware, USA) and it was stored at −80 °C until processing.

2.7. Mutation Screen

MC4R and *LCN2* genes were screened for mutations. All subjects were heterozygous for the mutations. *MC4R* was analyzed with one PCR fragment, while *LCN2* was divided into four PCR fragments. Methodological details can be obtained from the authors.

All samples were commercially Sanger sequenced by LGC Genomics (Berlin, Germany). Analyses of the sequences were performed by two experienced individuals in Essen (Germany). Samples with discrepancies were re-sequenced by Seqlab laboratories (Göttingen, Germany).

2.8. Statistical Analysis

Statistical analyses were performed using STATA version 12 (StataCorp, College Station, TX, USA). Normality was assessed by Shapiro-Wilk. All the tests were two-sided and the significance level was set at $\alpha = 0.05$. We did not correct for multiple testing.

We used Wilcoxon rank-sum test for the comparison between subjects with Ile 251Leu MC4R mutation and without MC4R mutation for the variables studied at baseline.

Paired t-test was applied for assessing changes in BMI-SDS between baseline vs. week 8, and baseline vs. 1 year. Furthermore, we performed a comparison between subjects with Ile 251Leu MC4R mutation and without MC4R mutation for changes in BMI-SDS (week 8 and one year, Wilcoxon rank-sum test).

3. Results

One hundred and twelve children with abdominal obesity (mean age 11.24 years , males 38%, BMI-SDS 2.93) participated in the study.

3.1. Description of Identified Variants

Mutation screen of the coding region of MC4R identified a total of four variants, three known nonsynonymous variants, rs776051881 (Glu42Lys), rs766665118 (Thr150Ile), and rs775382722 (Arg305Gln), and one nonsynonymous polymorphism rs52820871 (Ile251Leu) (Table 1). First, we classified variants as leading to reduced receptor function or similar to the wild type MC4R function. Three previously reported variants (Glu42Lys, Thr150Ileu, and Arg305Gln) lead to a reduced MC4R function as classified by in-silico predictors [24] that claim them to be disease causing. Furthermore, some in-vitro analyses had described reduced function for the mutations Thr150Ileu and Arg305Gln [9,25,26]. Our study shows a frequency of 2.52% of MC4R mutations leading to a reduced function in our sample of Spanish of children with abdominal obesity.

Table 1. MC4R and LCN2 genetic variants in Spanish children with abdominal obesity.

Gene Subject	N° of Subjects	Aminoacid Exchange	rs Number	In-silico Prediction*	Function	Reference of Functional Analysis
MC4R Mutations						
Glu42Lys	1	p.Glu42Lys	rs776051881	Disease causing	Not known	-
Thr150Ile	1	p.Thr150Ile	rs766665118	Disease causing	Reduced	[9,25]
Arg305Gln	1	p.Arg305Gln	rs775382722	Disease causing	Reduced	[26]
Polymorphisms						
Ile251Leu	5	p.Ile251Leu	rs52820871	Disease causing	Like wild type	[27–31]
LCN2 Mutations						
Thr124Met	1	p.Thr124Met	rs79993583	Probably harmless	-	-

Abdominal obesity was defined as waist circumference (WC) above the sex and age-specific 90th percentile. * In-silico prediction was performed by mutation taster www.mutationtaster.org.

The mutation screen of the coding region of *LCN2* identified a total of twelve variants, eleven are located in intronic regions (rs2232632, rs202024127, rs11794980, rs2232629, rs2232625, rs2232626, rs116745581, rs2232628, rs568419305, rs2232631, rs2232632) and one is a known nonsynonymous variant: rs79993583 (Thr124Met). The analysis of all detected variants by in-silico predictors show that three of them could be disease causing (Supplementary Table S1).

3.2. Phenotypic Description of Mutation Carriers

In Table 2 phenotypic characteristics of participants with mutations at MC4R and LCN2 genes at baseline are described in detail. We also provide data from obese participants of IGENOI study without mutations in the coding regions of these genes.

For 103 children (37.8% males) with a mean age of 11.32 years no mutations in MC4R and LCN2 genes were found, they are wild type carriers. Mutated subjects are compared with them in the subsequent analyses. All wild type carriers were also obese (BMI-SDS: 2.92 ± 1.10) and 41.7% showed clinical evidence for insulin resistance in the presence of Acanthosis Nigricans that was accompanied by a HOMA-IR of 4.03 ± 3.17.

A 14 year-old girl was heterozygous for the Glu42Lys mutation at the MC4R gene. She suffers from severe-obesity (BMI-SDS:4.04) reflected in a fat mass of 40.8%. The main clinical features of this participant were leptin levels higher than expected for the BMI [32], and the presence of insulin resistance observed as hyperinsulinemia, increased HOMA-IR (4.23), and Acanthosis Nigricans.

Mutation Thr150Ileu at the MC4R gene was observed in an eight year-old boy with a BMI-SDS of +3.5. Biochemical parameters were normal and the participant was physically active (50.65 min/day). One participant carries two variants at the MC4R gene: Arg305Gln and the polymorphism Ile251Leu. She is a 12 year-old girl with a BMI-SDS of 2.91, and a fat mass of 43.2% (measured by bioimpedance). Five independent children also carried the polymorphism Ile251Leu. They had a mean age of nine years and a mean BMI-SDS of 2.69. In comparison with the participants with the wild type receptor, none of the participants carrying this polymorphism showed Acanthosis Nigricans.

The polymorphism Ile251Leu of MC4R was observed in five participants with a mean age of nine years old. We found increased levels of total cholesterol and LDL-cholesterol in comparison to participants without MC4R mutation (p = 0.004 and p = 0.013; Wilcoxon rank-sum test). These

differences persist when subjects with the Ile251Leu MC4R mutation were compared to age and sex matched subjects without MC4R mutation (Supplementary Table S2).

One male 15 year-old heterozygously carried the mutation Thr124Met at the LCN2 gene. Clinical characteristics of this participant included severe obesity (BMI-SDS = +4.01) accompanied by an elevated percentage of fat mass (38.7%). Also, a remarkable difference of higher levels of MVPA was detected in comparison with the wild type population. The carrier of this mutation was much more active than the wild-type controls (120.92 min/day vs. 44.82 min/day, $p = 0.088$). We also evaluated the siblings (three girls) of the index patient, all of them were obese. Two of them were also carriers of Thr124Met (BMI-SDS: 3.47 and BMI-SDS: 3.76). The MAF for this variant in the LCN2 gene in the study population is 0.44%.

Table 2. Baseline characteristics of heterozygous MC4R and LCN2 variant carriers and wild type Spanish children with abdominal obesity.

		MC4R Mutations				LCN2 Mutation
	Wt Population	Glu42Lys	Thr150Ile	Arg305Gln+Ile251Leu	Ile251Leu	Thr124Met
N	103	1	1	1	5	1
Age (years)	11.32 (2.46)	14	8	12	9 (1)	15
Sex (Male/Female)	39/64	Female	Male	Female	2/3	Male
Tanner (I/II/III/IV/V)	31/17/18/6/24	V	I	II	4/-/1/-/-	V
Height (cm)	151.23 (12.72)	168	137.8	151	137.3 (13.86)	175.9
Weight (Kg)	66.71 (18.77)	97	49.1	67.2	49.94 (17.87)	112.9
BMI (Kg/m^2)	28.55 (4.51)	34.4	25.85	29.5	25.64 (3.53)	36.5
BMI-SDS	2.92 (1.10)	4.04	3.5	2.91	2.69 (1.05)	4.01
WHR	0.88 (0.06)	0.90	0.89	0.93	0.86 (0.04)	0.82
% fat mass	37.22 (6.33)	40.8	32.1	43.2	33.98 (9.28)	38.7
Acantosis nigricans (+/-)	43/52	+	-	-	0/5	+
Glucose (mg/dL)	89.04 (6.58)	85	88	78	88.25 (6.84)	87
Insulin (μu/mL)	17.92 (13.29)	20.2	7	13.5	11.87 (6.43)	11.4
HOMA-IR	4.03 (3.17)	4.23	1.52	2.6	2.66 (1.68)	2.44
Total Colesterol (mg/dL)	162.65 (24.97)	116	162	157	198.75 (14.88)*	160
HDL-colesterol (mg/dL)	46.70 (9.96)	42	53	46	56.75 (12.25)	41
LDL-colesterol (mg/dL)	97.67 (21.15)	64	96	97	125.75 (16.82)*	99
Triglycerides (mg/dL)	94.48 (44.73)	49	64	68	81.25 (41.65)	98
Leptin (ng/mL)	36.41 (18.60)	90.8	14	NA	33.52 (13.11)	8.1
MVPA (min/day)	44.88 (23.69)	37.5	50.65	30.93	44.55 (18.37)	120.92
CEBQ ratio	1.22 (0.42)	0.82	1.25	NA	1.01 (0.12)	0.85

Data are expressed as mean (SD), * $p < 0.05$ for the comparison between Ile 251Leu MC4R mutation subjects and subjects without MC4R mutation, Wilcoxon rank-sum test was applied Abdominal obesity was defined as WC above the sex and age-specific 90th percentile. BMI-SDS, standard deviation score for body mass index; CEBQ, Children Eating Behavior Questionnaire Ratio; MVPA, moderate to vigorous physical activity; WHR: waist to height ratio.

3.3. Children Eating Behavior

The eating behavior scores with four dimension charts of children with abdominal obesity at baseline is described in Figure 1. Wild type subjects (no mutations in MC4R and LCN2 genes) had a score of 1.19. Carriers of the MC4R Thr150Ileu and LCN2 Thr124Met variants had nominally higher scores than wild type subjects, while participants with Ile251Leu polymorphism have lower score.

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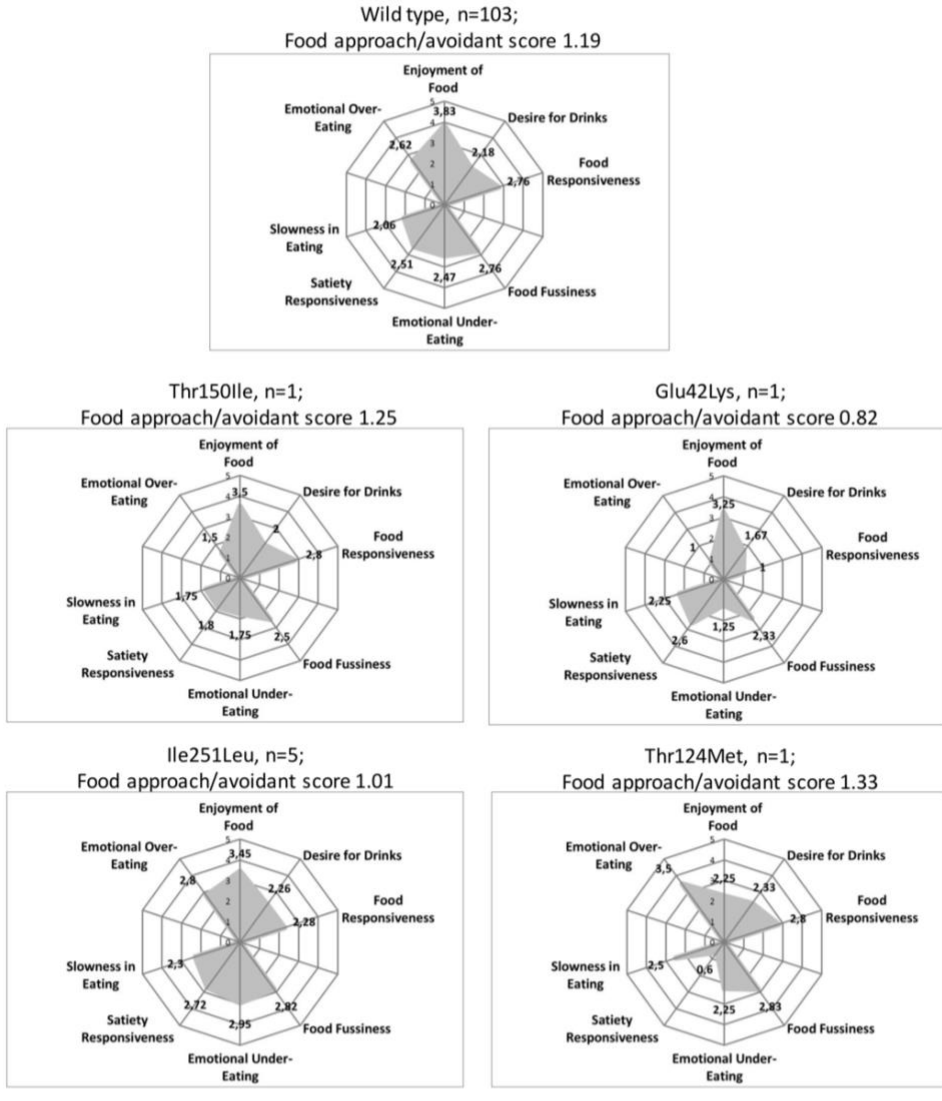

Figure 1. Childhood Eating Behavior Score (CEBQ) in Spanish children with abdominal obesity. The Food approach/avoidant score refers to the quotient between the sums of scores of the "food approach" subscales divided by the sum of the scores of the "food avoidant" subscales. Subjects with mutations in MC4R (Thr124Met, Thr150Ileu) and LCN2 (Thr124Met) genes were evaluated.

3.4. Change in BMI-SDS and Mutations in MC4R and LCN2 After 8-Week and 1-Year of Follow up

Carriers of mutations that lead to a reduced function in MC4R and LCN2 genes showed a disparity of responses to the lifestyle intervention (Table 3). Changes in BMI-SDS of the five subject carriers of the Ile251Leu polymorphism are quite variable with two of them not showing any improvement at 1 year. Moreover, no significant differences in BMI-SDS changes were found after eight weeks or one-year of follow up in mutated carriers vs. non carriers subjects in analysis conducted in matched age and sex subjects with and without a functional mutation in MC4R (Supplementary Table S3).

Table 3. Changes in BMI-SDS according to MC4R and LCN2 variants after an 8-week and 1-year lifestyle intervention.

		ΔBMI-SDS			
		8 Week		1 Year	
		Mutation Carriers	Non Carriers	Mutation Carriers	Non Carriers
Usual Care Group			*n* = 27 −0.44 (0.66)***		*n* = 22 −0.47 (0.52)***
MC4R: Glu42Lys		− 0.51		Drop out	
MC4R: Thr150Ile		−0.67		−0.90	
Intensive Care group			*n* = 68 −0.51 (0.38)***		*n* = 56 −0.60 (0.72)***
MC4R: Arg305Gln + Ile251Leu		−0.13		- 0.81	
MC4R: Ile251Leu	Mean (*n* = 5)	−0.74 (0.41)**		−1.02 (1.21)	
	Carrier 1	−0.95		−1.47	
	Carrier 2	−0.10		0.07	
	Carrier 3	−0.56		0.38	
	Carrier 4	−1.14		−1.56	
	Carrier 5	−0.97		−2.53	
LCN2: Thr124Met		−0.59		Drop out	

Data are expressed as mean (SD). Paired t-test for changes between baseline vs. 8 week, and baseline vs. 1 year was applied (** < 0.010,*** < 0.001).

4. Discussion

To our knowledge, this is the first study evaluating *MC4R* and *LCN2* gene variants, in a population of children and adolescents with abdominal obesity. In addition, we measured the eating behavior in all participants with the CEBQ. Finally, we reported changes in BMI-SDS achieved after eight weeks and 1 year of follow-up according to the two different strategies (usual care or intensive care) and the presence of *MC4R* and *LCN2* gene variants.

The rate of participants carrying a *MC4R* mutation leading to a reduced function was 2.67 %. This frequency is in accordance with the reported values in Czech children with obesity being 2.4% [33]. Previously, a wide variability (ranging from 0.5 to 5.8%) in the frequency of *MC4R* mutations had been described in children and adolescents with obesity in different populations [33–36].

In our population, we found one extremely obese participant (BMI-SDS +4.04) carrying the *MC4R* mutation Glu42Lys. This mutation was previously reported in 5.6% of Turkish children with obesity [37]. In our case, the minor allele frequency (MAF) for this mutation was 0.89%.

Moreover, an eight year-old boy with a BMI-SDS of +3.5 was a heterozygous carrier of the *MC4R* Thr150Ileu variation. This mutation had previously been described in an obese Chilean child with a BMI-SDS of +2.79 [38]. In the Chilean population the MAF for this variant was 0.45% similar to the observed value in our Spanish population (0.84%).

Mutation Arg305Gln of the *MC4R* gene was characterized in functional studies as a variation that causes a decrease in MC4R constituently activity and in the response to the agonist [9]. We found this mutation in a 12 year-old girl with obesity (43.2% body fat mass and BMI-SDS +2.91). This participant also carries the Ile251Leu polymorphism. There is also a German obese boy harboring this Arg305Gln variation (BMI-SDS +2.5) [39]. The frequency of this variation in our population is 0.84% while in the German population was 0.19%.

Two polymorphisms in the *MC4R* (Val103Ileu and Ile251Leu) had been demonstrated to reduce the risk of obesity [40–42]. Val103Ileu was described in white Europeans with frequencies between 1–4% [2]. In our study, we did not observe carriers of this variation. In other independent studies in Spanish children the frequencies of this polymorphism were lower than in other European countries [43–45]. The frequency of the Ile251Leu variation in our studied sample was 5.04 % (6 children with

abdominal obesity), which is higher than the observed values in other Spanish and Polish pediatric populations [28,43,44]. Heterozygous subjects for the Ile251Leu SNP showed higher levels of total cholesterol and LDL-cholesterol. There are studies on the association between *MC4R* variants and other lipid markers in several populations [31,46].

To our knowledge this is the first study evaluating non-common variants in the *LCN2* gene in children with abdominal obesity. We found one heterozygous carrier of the Thr124Met variant that was severely obese (BMI-SDS: + 4.01) despite being physically active (more than 2 hours per day on moderate-to-vigorous physical activity).

It has been demonstrated that LCN2 suppresses appetite by signaling trough MC4R [13]. For this reason we evaluated eating behavior in our population with the children eating behavior questionnaire (CEBQ), for which solid reproducibility and high internal consistency had been reported [47]. In particular we represent graphically the multidimensionality of participants carrying *MC4R* and *LCN2* mutations. We observed that carriers of the Thr150Ile (*MC4R*) and Thr124Met (*LCN2*) variants showed slightly higher eating behavior scores than obese individuals without *MC4R* variants. This tentatively suggests an effect of these mutations on eating behavior, as mutation carriers showed lower scores on food avoidance subscales. However, as the number of mutation carriers is very low, meaningful statistical analyses are not possible.

Regarding the mutation Thr150Ile of the *MC4R* gene, an association between this variant and eating behavior in three Chilean obese carriers was reported. These participants had a cognitive restraint measured by the TFEQ-R18 questionnaire [48].

The participant carrying the *LCN2* Thr124Met allele showed lower satiety responsiveness (0.6 out of 5). This could be explained by a potential effect of LCN2 on appetite-suppressing activities [13]. Participants carrying the Ile251Leu polymorphism of the *MC4R* had lower scores in satiety responsiveness than the wild type population (1.01 vs. 1.19). Nevertheless, carrying the infrequent allele at *MC4R* Ile251Leu seems not to have an influence on eating behavior compared to children with obesity and variations in the *MC4R* gene. A previous study had described association between *MC4R* polymorphism rs17782313 (in the 3′ region of the gene) and childhood eating behavior. Satiety responsiveness dimension was decreased and enjoyment of food was increased in carriers of the CC allele [38]. However, the lack of significance in our results should be seen under the limited statistical power as we analyzed rare variants in a relatively small study group.

In our population, participants carrying mutations in *MC4R* and *LCN2* that lead to a reduced function were able to achieve similar or greater reduction in BMI-SDS than children without mutation in these genes after a lifestyle intervention. These results are in concordance with previous weight loss MC4R studies [39,4]. It had been demonstrated that children with mutations in *MC4R* had a significantly greater beneficial effect from the short lifestyle interventions than wild type carriers [49]. However, in our study one carrier of Arg305Gln only achieved a successful reduction in BMI-SDS after one year of intervention. When the polymorphism Ile251Leu of the *MC4R* gene was analyzed, some participants showed a reduction in BMI-SDS similar to that of wild type subjects after the intervention. Limitations of our study comprise of a relatively small sample size, the lack of a normal-weight study group, that no functional in vitro tests were performed, and also that we cannot exclude the presence of mutations in other genes involved in monogenic obesity.

5. Conclusions

In summary, *MC4R* and *LCN2* mutations were detected in 2.42% and 0.84%, respectively, of Spanish children with abdominal obesity. Our data suggests a putative association between profiles of eating behavior and functional mutations in *MC4R* gene. Specifically, *MC4R* and *LCN2* mutation carriers having abdominal obesity were able to reduce BMI-SDS after a lifestyle intervention.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6643/11/5/960/s1, Table S1: *LCN2* genetic variants in Spanish children with abdominal obesity. Table S2: Baseline characteristics from a subpopulation of matched age and sex subjects with the Ile251Leu MC4R mutations and without the mutation. Table S3: Changes in anthropometric and biochemical from a subpopulation of matched age and sex subjects with the Ile251Leu MC4R mutations and without the mutation.

Author Contributions: Conceptualization, MC.A.S, A.H and A.M.; methodology, L.M.-A, A.O.-R and J.G.; formal analysis, L.M.-A., J.G., A.O.R and A.H.; resources, M.C.A.-S., J.H., A.H., and A.M.; data curation, L.M.A and A.H.; writing—original draft preparation, L.M.A.; writing—review and editing, ,A.O.R, J.G., MC.A.S., J.H., A.H and A.M.; supervision, A.H.; project administration, M.C.A.-S and A.M.; funding acquisition, M.C.A.-S, J.H., A.H. and A.M.

Funding: The IGENOI study was supported by a MERCK foundation grant and Laboratories ORDESA (Sant Boi de Llobregat; Barcelona, España)-FEI-AEP grant. AH and JH were supported by the Deutsche Forschungsgemeinschaft (DFG; HI 865/2-1) and the BMBF (01GS0820). AH and JG were supported by the 'Landesprogramm für Geschlechtergerechte Hochschulen - Programmstrang Förderung von Denominationen in der Genderforschung.

Acknowledgments: We thank all the children and families in the trial for their enthusiastic and maintained collaboration, and other investigators of the IGENOI group for their participation in the recruitment process and the acquisition of the data. We are indebted to S. Düerkop and J. Andrä for technical support. L.M-A and A.O-R. acknowledge their fellowships from the "la Caixa" Banking Foundation

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Table 1. *LCN2* genetic variants in Spanish children with abdominal obesity

Subject	Nº of families	Location	Altered codon	rs number	*In-silico* prediction
Mutations					
	2	Intron c.375+88	-	rs2232632	Probably harmless
	1	Intron c.376-9	-	rs202024127	Disease causing
	3	Intron c.713-28	-	rs2232629	Probably harmless
	1	Intron c.510-119		rs2232625	Probably harmless
	1	Intron c.593+8	-	rs2232626	Disease causing
	1	Intron (splice región) c.593-3	-	rs116745581	Disease causing
	1	Intron c.713+50	-	rs2232628	Probably harmless
	1	Intron c.593+77		rs56841305	Probably harmless
	1	Intron c.848+69		rs2232631	Probably harmless
	1	Intron c.848+90	-	rs2232632	Probably harmless
Thr124Met	1	Exon	ACG→ATG	rs7999358	Probably harmless
Polymorphisms					
	10	Intron c.593-15		rs11794980	Probably harmless

**In-silico* prediction was performed by mutation taster.

Supplementary Table 2. Baseline characteristics from a subpopulation of matched age and sex subjects with the Ile251Leu MC4R mutations and without the mutation.

	Ile251Leu MC4R mutation (n=5)	No MC4R mutation (n=22)	P[1]
Age	9(1)	9.36 (0.78)	0.382
Sex (Male/Female)	2/3	7/15	0.726
Tanner (I/II/III/IV/V)	4/-/1/-/-	13/6/3/-/-	0.451
Height (cm)	137.38 (13.86)	143.63 (8.28)	0.191
Weight (Kg)	49.94 (17.87)	52.97 (9.51)	0.592
BMI (Kg/m^2)	25.64 (3.53)	25.49 (2.47)	0.914
BMI-SDS	2.69 (1.05)	2.45 (0.76)	0.556
WHR	0.558 (0.02)	0.552 (0.03)	0.713
% fat mass	33.98 (9.28)	35.71 (3.62)	0.495
Acanthosis nigricans (+/-)	5/-	13/9	0.080
Glucose (mg/dL)	88.25 (6.84)	86.5 (5.62)	0.593
Insulin (μU/mL)	11.87 (6.43)	15.29 (10.58)	0.549
HOMA-IR	2.66 (1.68)	3.32 (2.47)	0.620
Total cholesterol (mg/dL)	198.75 (14.88)	164 (23.64)	**0.011**
HDL-cholesterol (mg/dL)	56.75 (12.25)	49.11 (2.58)	0.229
LDL-cholesterol (mg/dL)	125.75 (16.82)	97.08 (22.31)	**0.027**
Triglycerides (mg/dL)	81.25 (41.65)	94.5 (53.89)	0.651
Leptin (ng/mL)	33.52 (13.11)	27.54 (7.83)	0.320
MVPA (min/day)	2.6 (1.14)	2.21 (1.08)	0.486
CEBQ	1.00(0.12)	1.19 (0.44)	0.356

Data are mean (SD), p[1] Unpaired T –Test for the comparison between the two groups.

Supplementary Table 3. Changes in anthropometric and biochemical from a subpopulation of matched age and sex subjects with the Ile251Leu MC4R mutations and without the mutation.

	Ile251Leu MC4R mutation	No MC4R mutation	
8-wek	N=5	N=21	p[1]
1 year	N=4	N=13	
D Height (cm)			
8-wek	1.42 (0.46)**	1.13 (0.50)***	0.257
1 year	6.44 (2.80)**	5.82 (0.63)***	0.437
D Weight (Kg)			
8-wek	-2.54 (2.54)	-2.66 (2.14)***	0.909
1 year	2.47 (6.52)	1.73 (6.44)	0.843
D BMI (Kg/m^2)			
8-wek	-1.8 (1.29)**	-1.68 (1.06)***	0.837
1 year	-1.2 (3.14)	-1.19 (3.18)	0.996
D BMI-SDS			
8-wek	-0.74 (0.41)*	-0.57 (0.479)***	0.477
1 year	-1.02 (1.21)	-0.69(0.85)*	0.523
D WHR			
8-wek	-0.02 (0.01)*	-0.03 (0.02)***	0.234
1 year	-0.04 (0.03)	-0.02 (0.02)***	0.163
D Glucose (mg/dL)			
8-wek	2.33 (5.68)	-2.47 (6.67)	0.258
1 year	6.01 (0)	-1.54 (7.77)	0.214
D Insulin (∝U/mL)			
8-wek	3.16	-0.57 (4.48)	0.266
1 year	6.80 (1.27)	-3.34 (10.89)	0.234
D HOMA-IR			
8-wek	0.71 (1.71)	-0.16 (1.02)	0.245
1 year	1.65 (0.22)	-0.82 (2.68)	0.238
D Total cholesterol(mg/dL)			
8-wek	-29 (11.53)*	-15.05 (24.97)*	0.363
1 year	25.00 (1.41)*	7.81 (26.51)	0.832
D HDL-cholesterol (mg/dL)			
8-wek	-3.66 (6.65)	-5.05 (7.85)	0.776
1 year	-5 (1.41)	0.44(5.15)	0.187
D LDL-cholesterol (mg/dL)			
8-wek	-24 (4.35)**	-7.4 (18.51)	0.148
1 year	-21 (5.65)	-2.92 (25.81)	0.373
DTriglycerides (mg/dL)			
8-wek	-6.33 (10.01)	-8.05 (35.83)*	0.963
1 year	6 (11.31)	-26.63 (54.89)	0.435
D Leptin (ng/mL)			
8-wek	-2.43 (15.85)	-11.57 (10.42)*	0.289
1 year	19.15 (16.47)	0.42 (17.91)	0.262

Data are mean (SD). Paired T-test for changes between baseline vs 8 week, and baseline vs. 1 year. (* <0.05, ** <0.010,***<0.001); p[1] Unpaired T –Test for the comparison between the two groups.

5. GENERAL DISCUSSION

Obesity has been considered as a major worldwide disease and its prevalence has increased in youths in the last years. Therefore, the study of its consequences on health in the pediatric populations and the evaluation of different interventions has a remarkable interest in the clinical practice. Moreover, during infancy and adolescence the eating behaviors and lifestyle habits are still developing. Thus, changes in unhealthy lifestyle factors and the acquisition of healthy habits may be easier to achieve in young people than in older ones. Furthermore, the environment is a main factor for development of obesity, hence in the case of the children and adolescents it is necessary the implication of their families in the prevention and treatment of this disease. The effect of different lifestyle factors, such as diet or physical activity, has been related to changes in the expression of some genes or changes in DNA structure, in particular in telomere length. In addition, it has been stated that between 40 to 70 % of obesity susceptibility is due to genetic factors (Fang et al., 2018).

In this research, we have conducted different approaches in order to assess the effect of lifestyle intervention in children and adolescents with obesity or abdominal obesity. We have examined different health consequences of the excess of adiposity and their improvement across the interventions. Moreover, we investigated the role of *MC4R* genetic variants in the weight loss response and in health comorbidities.

5.1 Cardiometabolic risk in childhood obesity

Several studies have shown an association between childhood obesity and independent risk factors for cardiometabolic risk such as high glucose levels, elevated either total or LDL cholesterol levels, low HDL cholesterol levels, high

triglycerides levels and elevated blood pressure (Chung, Onuzuruike, and Magge, 2018; Gurnani, Birken, and Hamilton, 2015). In children and adolescents there is not a specific consensus for defining cardiometabolic risk, therefore in 2015 Skinner et al. build a cardiometabolic risk score including all the before mentioned parameters (Skinner et al., 2015). One of the aims of our research was to assess the cardiometabolic health of the obese children. For this reason, we selected a biomarker of atherogenesis state (oxLDL) and we examined whether it was associated with a cardiometabolic risk score in obese children (Chapter 1). We observed that those participants with higher levels of oxLDL had also higher scores of the cardiometabolic risk. Oxidized LDL cholesterol is a marker of oxidative stress in the body, because the enzyme called myeloperoxidase (MPO) is involved in the oxidation of the LDL-cholesterol particles making them more atherogenic. This enzyme was produced by the macrophages recruited in the white adipose tissue contributing to the low-grade inflammation of obesity status. In the same line as our results, a recent meta-analysis reported that elevated serum oxLDL levels are associated with an increased risk of cardiovascular diseases (Gao et al., 2017).

The excess of adiposity has been associated with atherosclerosis lesions in different longitudinal analysis (Chung, Onuzuruike, and Magge, 2018). Visceral or abdominal obesity has been highly stated as one of the greatest predictors of cardiovascular and metabolic risk in children and adolescents (Aguilar-Morales et al. ,2018). Since one of the main health problems of obesity is the cardiometabolic risk and this risk tracks into adulthood, the IGENOI study was designed in order to evaluate the effect of lifestyle intervention (diet and physical activity recommendations) in children with high metabolic risk.

5.2 Lifestyle intervention and their effects on cardiometabolic risk factors in obese subjects

In our project we have two different study populations: NUGENOI subjects who are obese children, and IGENOI participants who have abdominal obesity.

The two lifestyle interventions were able to modify lifestyle factors (Rendo-Urteaga et al., 2014; Ojeda-Rodríguez et al., 2018) and consequently participants significantly reduce anthropometric and biochemical parameters.

In NUGENOI study we evaluated the effects of a 10-week dietary intervention in obese children. We demonstrated that in those participants that are classified as high responders (they achieved a BMI-SDS reduction higher than 0.5 units) the studied anthropometric and biochemical parameters significantly decreased, and these reductions were accompanied by a decrease in oxLDL serum levels (Chapter 1). On the other hand, those participants considered as low responders, did not change their oxLDL levels despite decreasing their adiposity slightly. We described that change in oxLDL levels depend on changes in total cholesterol and LDL-c. The literature evaluating the effect of weight loss in oxLDL levels is scarce. A study performed in 35 obese adolescents following a 6-week weight loss program also showed a significantly decrease in oxLDL levels (Kelishadi et al., 2008). A recent study performed in obese adults who underwent bariatric surgery has observed that a weight loss of at least 12% of their initial body weight was accompanied by a decrease in oxLDL levels. In contrast, there is one 3-month weight loss program conducted in 26 obese adolescents that did not find any changes in oxLDL, nor did manage to reduce weight (Tjønna et al., 2008). In any case, the literature and our results seem to indicate that a successful weight loss is accompanied by a reduction of oxLDL particles, decreasing the cardiometabolic risk.

The effect of NUGENOI intervention in inflammatory markers was studied in Chapter 2. Firstly, we have measured IL-6, CRP, TNF-α and cardiotrophin 1 (CT-1) serum levels at baseline and after the 10 weeks. The intervention significantly decreased CRP and TNF-α, but no changes were observed with to respect to IL-6 and CT-1. The association between obesity and low-grade inflammation in children has been widely described since being obese during childhood and adolescence have been described to show higher inflammatory markers that may raise cardiovascular risk (Caminiti, Armeno, and Mazza, 2016). CRP is proposed as a marker of early diagnosis of cardiometabolic risk (Carmona-Montesinos et al., 2015), and is also considered as a marker of weight loss since its levels decreased with weight loss and increase with weight regain (Lausten-Thomsen et al., 2015). Another pro-inflammatory cytokine was TNF-α that showed high levels in obese children in comparison with normal weight pairs (Rivera et al., 2019). The significant reduction in TNF-α levels was previously described in a 16-week lifestyle intervention in pre-scholar children (Bocca et al., 2014). In that same study, IL-6 was measured and did not change. There are other studies that did not observe changes in IL-6 despite the weight reduction (Romeo et al., 2011; Gong et al., 2014; S. García-Calzón et al., 2016). A successful weight loss program (with a reduction in BMI-SDS greater than 0.5) in children from 6 to 9 years of age was able to decrease IL-6 serum levels (Martos et al., 2009), and there are other studies that confirm this reduction (Izadpanah et al., 2012; Nascimento et al., 2016). The characteristics of the sample (age and sex), the duration of the follow-up and the type of intervention (ie, dietary intervention, PA intervention) could explain the observed discrepancies. Moreover, cardiotrophin 1, has been described as a cytokine of the IL-6 family that plays an important role in the control of energy metabolism. In a previous study conducted in the entire NUGENOI population, a significantly decrease was reported in high responder

subjects. In the results described in chapter 2 we did not find changes in CT-1 levels and this may be due to the small sample size.

Secondly, in Chapter 2, it has been addressed the study of inflammatory cytokines and their gene expression in Peripheral Blood Mononuclear Cells (PBMC) after following a lifestyle intervention in NUGENOI population. We described that after a weight reduction, serum levels of TNF-α and CRP significantly decreased in the participants. In addition, gene expression levels of IL-6, TNF-α and CT-1 were analyzed and a reduction of transcript levels of CT-1 was found. Changes in serum levels of CT-1 were positively correlated with changes in CT-1 gene expression, but this association was not found in IL-6 and TNF-α. There are several studies that have reported that PBMC transcript did not reflect serum cytokine levels (Miranda et al., 2014; O'Rourke et al., 2006). While there are others that observed significant associations between gene expression of some inflammatory markers in the visceral adipose tissue of obese adults and the circulating levels of that cytokines, but not in IL-6 and TNF-α (Lasselin et al., 2014). Furthermore, changes in CT-1 and IL-6 gene expression were positively associated, suggesting that CT-1 was able to induce changes in IL-6 transcript. In other study carried out in monocytes, it has been described that this is dependent on time and concentration manner (Jiménez-González et al., 2013).

Moreover, CT-1 has been described as an important molecule with a significant role in glucose metabolism, being involved in beta-cell viability and also improving glucose-stimulated insulin secretion (Moreno-Aliaga et al., 2011). In Chapter 2 we showed that baseline CT-1 gene expression was able to predict post-intervention insulin values and HOMA-IR. Thus, our results are in accordance with the regulatory effect of CT-1 in glucose metabolism outcomes.

It is important to note that IGENOI participants are subjects with high cardiometabolic risk because they have a waist circumference higher than the 90th percentile. In this context, an integral 2-year lifestyle intervention has been designed to reduce the metabolic risk in these subjects (Chapter 3). One of the components is the promotion of MVPA, since higher levels are able to decrease cardiometabolic risk (Poitras et al., 2016). Participants were instructed to increase their MVPA up to 200 minutes per week. There are different protocols to objectively determine PA by accelerometry. We followed the methodology used in the IDEFICS study conducted in European children and adolescents (Konstabel et al., 2014). We observed that only the 25% of abdominal obese children fulfill the WHO recommendations which indicate that daily they should perform more than 60 minutes of MVPA. Moreover, the daily average of MVPA was 44 minutes. These results are in agreement with other national studies conducted on overweight children (Laguna et al., 2013). The outcome of IGENOI intervention was a significantly reduction in BMI-SDS in both groups, which was higher in the intensive care group. Moreover, in the intensive care subjects anthropometric and biochemical parameters studied decreased significantly. In relation to PA, the lifestyle intervention resulted in a decrease in light PA in both groups, this result was also observed by other authors in a RCT conducted in obese children (Hughes et al., 2008). Furthermore, in IGENOI study we demonstrated that intensive care participants significantly increased their MVPA by more than 38.5 minutes per week, while usual care participants did not change. It is important to mention that both groups received the same PA recommendation. It has been described that a close follow-up of participants (i.e. duration of the visits) and the family involvement are key issues to improve the effectiveness in pediatric interventions (Berge and Everts, 2011). In this sense, intensive care participants had a close follow-up compared to UC participants, a fact that could partially explained that difference. Moreover, a recent meta-analysis showed that the combination of PA

and Mediterranean diet pattern provides greater health benefits than those acquired with PA or diet alone (Malakou et al., 2018).

Leptin, a hormone produced by adipose tissue that is involved in the regulation of the appetite and plays a central role in the energy homeostasis (Rosenbaum and Leibel, 2014). It has been highly correlated with cardiovascular and metabolic outcomes in obese and non-obese children (Gonzaga et al., 2014; Stakos et al., 2014). The IGENOI participants significantly decreased their leptin levels in the intensive phase. That change in leptin was explained by changes in adiposity traits and MVPA (Chapter 3). The decrease in adiposity traits such as BMI-SDS, waist circumference and waist-to-height ratio is accompanied by a reduction in adipose tissue and consequently in the leptin secretion (Siegrist et al., 2013; Müller, Enderle, and Bosy-Westphal, 2016). On the other hand, the association between MVPA and leptin has been previously described in obese adults. An increase in PA (and the hypocaloric diet) leads to a decrease in adiposity and consequently in leptin levels (Nurnazahiah, Lua, and Shahril, 2016).

5.3 Effects of an integral lifestyle intervention on telomere length in abdominal obese children

In Chapter 4, telomere length was measured by a technique modified by Cawthon et al. in 2009. The method is called “monochrome multiplex quantitative PCR (MMqPCR)”. Briefly, in the same well-plate reaction both telomere and single copy gene are measured. It is more accurate than telomere length measurement by single plex quantitative PCR. Telomere length was measured in DNA samples from IGENOI participants at baseline and after 8-week intensive phase of the lifestyle intervention. Baseline TL was inversely correlated with adiposity indices

such as BMI, body weight and waist circumference. Other studies showed that obese children had shorter TL than normal weight children (Buxton et al., 2011). This observation has also been shown in Arab youths, where it has been observed only in obese boys that have shorter telomeres than normal weight pairs, this association was not found in girls (Al-Attas et al., 2010). Moreover, in a recent study performed in Latino children, authors have observed that TL is a predictor of childhood obesity (Kjaer et al., 2018). In contrast, there are other studies that found no association between adiposity indices and TL in children and/or adolescents (Needham et al., 2012; Masi et al., 2012). The mechanisms involved in the association between adiposity and TL could be explained by the low-grade inflammatory status and the oxidative stress linked to obesity, that could contribute to telomere attrition (Guyatt et al., 2018).

Moreover, we examined the effect of the lifestyle intervention on changes in TL. We did not observed changes in TL attrition, despite achieving a successful decrease in BMI-SDS. In this sense, a previous study conducted by García-Calzón et al. had demonstrated that TL attrition was reduced after a successful 3-month lifestyle intervention in Spanish adolescents (García-Calzón et al., 2014). There are few studies that evaluate the effect of a lifestyle interventions on TL, most of them were conducted in adults and some of them reported TL elongation (Mason et al., 2013), while others did not find any change (Mason et al., 2018). There are other studies in which bariatric surgery was the treatment to reduce weight. It seems that in extremely obese adults who underwent a bariatric surgery, telomere shortening tends to decrease (Carulli et al., 2016; Laimer et al., 2016; Dershem et al., 2017) However, as with lifestyle interventions, a recent systematic review has concluded that there is an inconsistent effect of weight loss on TL (Himbert, Thompson, and Ulrich, 2018).

Telomere length has been proposed as a biomarker of cardiometabolic risk. Several studies have associated TL with metabolic and cardiovascular factors in both adults (Pusceddu et al., 2018) and in children (Barraclough et al., 2019). In our study, baseline TL was not associated with any cardiometabolic risk factor. Nevertheless, we observed that longer TL at baseline, significantly predict greater changes in glucose levels after the intensive phase of IGENOI study (Chapter 4). Our results agree with those previously reported by our research group in obese adolescents, in which longer telomeres at baseline significantly predict glucose changes at months 2 and 6 of follow-up (García-Calzón et al., 2016). One potential explanation for that association is that subjects with longer TL at baseline are less affected by inflammation and oxidative stress and could benefit more from the lifestyle intervention.

5.4 Effects of *MC4R* and *LCN2* genetic variants on the response to an integral lifestyle intervention in abdominal obese children

The Chapter 5 was performed in order to investigate whether carrying a mutation in the transcript regions of *MC4R* and *LCN2* genes could affect the effectiveness of the lifestyle intervention. Therefore, IGENOI participants were screened for mutations in those genes. In the event that two or more members of the family were participating in the study, those with the highest BMI-SDS were selected. The 2.67% of IGENOI participants were carriers of functional *MC4R* mutations. In particular they carried the following mutations: Glu42Lys, Thr150Ile and Arg305Gln. Moreover, the 5.06% (6 participants) of participants were carriers of the polymorphism Ile251Leu that has been associated with a lower risk of obesity. On the other hand, only one participant was a carrier of the *LCN2* variant. It is important to mention that only one participant was carrier of each mutation,

so it is impossible to perform statistical analysis to find differences with the wild type subjects for *MC4R* and *LCN2* genes. That variants had been previously described in different populations (Valladares et al., 2010; Demiralp, Berberoğlu, and Akar, 2011; Santos et al., 2008). The anthropometric and biochemical profile of the participants carrying the variants was shown in Chapter 5. It can be observed that all participants with functional mutations in *MC4R* and *LCN2* genes had higher BMI-SDS values than wild type subjects, except for the participant carrying the Arg305Gln mutation who also carries the Ile251Leu protective polymorphism. In this line, carriers of the polymorphism, Ile251Leu presented lower BMI-SDS than wild type subjects.

The influence of carrying a functional mutation on weight loss achieved by a lifestyle intervention was evaluated in IGENOI sample. Our results showed that participants with *MC4R* and *LCN2* mutations were able to achieve similar or greater weight losses than children without mutation in those genes. A systematic review had addressed this issue and its results are in accordance with ours, confirming that children with *MC4R* single nucleotide polymorphism (SNP) can lose more body weight than non-carriers (Koochakpoor et al., 2016). Reinher et al. have observed in a study conducted in 514 overweight children that carriers of a mutation in *MC4R* gene that lead to a reduced receptor function, can achieve a successful BMI-SDS reduction, but had difficulties to maintain the weight loss reached (Reinehr et al., 2009).

On the other hand, since *MC4R* and *LCN2* exert their actions in the regulation of appetite and satiety pathways, we assessed eating behavior of all participants. CEBQ was used as a validated questionnaire that classifies children's eating behaviors in two dimensions: food approach and food avoidance. A ratio between the media of food approach and food avoidance could be useful to

compare the behaviors among participants (González, Martínez, and Santos-Martínez, 2011). It can be observed in Chapter 5 that sub dimensions of food avoidance behaviors were lower in mutation carriers than in wild type participants. Meanwhile, carrying Ile251Leu variant does not seem to have an influence on the eating behavior compared to the obese subjects without variation in the *MC4R*. The scientific literature that evaluates the relationship eating behavior and *MC4R* or *LCN2* genes in obese or overweight children is scarce. In a cross-sectional study performed in 226 Chilean obese children, the *MC4R* rs17782313 polymorphism was screened. The authors found that the CC carriers of the allele had the satiety responsiveness dimension decreased and the enjoyment of food increased (Valladares et al., 2010). Another study performed in 151 Chinese children had associated food responsiveness with a mutation near *MC4R* gene (rs12970134) (Wang et al., 2017).

In summary, lifestyle interventions conducted in children with obesity were effective in the reduction of adiposity and metabolic outcomes. *MC4R* and *LCN2* mutations seemed to modify eating behaviors but did not change the weight loss response. Meanwhile, some biomarkers such as telomere length and cardiotrophin-1 gene expression could have a predictive role in glucose metabolism outcomes.

6. CONCLUSIONS

6. Conclusions

1. Children with obesity decreased anthropometric indices after the two lifestyle interventions

2. Higher baseline oxidized LDL levels were associated with higher cardiometabolic risk score. After a dietary intervention oxidized LDL levels diminished in participants with a greater reduction in BMI-SDS.

3. Serum CRP and TNF-a levels showed a decline after a dietary intervention in children with obesity. Furthermore, baseline CT-1 transcript levels were able to predict a reduction in HOMA index and insulin levels in children with obesity.

4. The lifestyle intervention in children with abdominal obesity increased their moderate-to-vigorous PA in 38.5 minutes per week (Intensive care participants). Moreover, changes in moderate-to-vigorous PA were significantly associated with changes in serum leptin levels.

5. Children with abdominal obesity showed an inverse correlation between TL and obesity traits. The wight loss program did not modify telomere length. Interestingly, baseline TL predicted changes in blood glucose levels in this population with abdominal obesity.

6. MC4R and LCN2 mutations were detected in 2.42% and 0.84% respectively, of Spanish children with abdominal obesity. Subjects with functional mutations were able to reduce BMI-SDS after one-year of lifestyle intervention. Eating behavior seemed to partially explain the effect of these genetic variants.

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8. APPENDICES

Appendix 1

Aging, Telomere Integrity, and Antioxidant Food

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Book Chapter

Gene-Environment Interactions and Human Diseases

Ed. Lu Qi

Nova Science Publishers (2016)

ISBN: 978-1-63483-680-7

Chapter 12

Aging, Telomere Integrity, and Antioxidant Food

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1. AGING AND CELLULAR SENESCENCE

A major public health aim is the prevention of age-related pathologies, since the proportion of elder population is increasing in almost all countries [1]. Aging is a multifactorial, time-dependent decline in physiological functions of multiple organs and tissues that occurs in biological systems [2]. It is the result of molecular interactions, cellular responses, and protein actions in different tissues and multicellular organisms, associated with mechanisms such as deregulated autophagy, mitochondrial dysfunction, telomere shortening, oxidative stress, systemic inflammation, metabolic dysfunction [3], and senescence (Fig. 12.1). These hallmarks of aging are divided into three categories: the causes of age-associated damage; the responses to the damage; and the consequences of the responses and culprits of the aging phenotype [4]. In the last 2 decades there has been intensive study of telomere shortening, which is influenced by genetic and lifestyle factors [5]. Aging occurs at all levels (molecular, cellular, and organ levels) and involves a large number of genes [6]. The effects of aging are reflected as deoxyribonucleic acid (DNA) damage in human cells [5].

Cellular senescence, the main aging-related event [4], is defined as the irreversible cell cycle arrest (usually in G_0 state) to experiment phenotypic changes [3]. Senescence arrest is irreversible because there is no stimulus able to rejoin the cell cycle [6]. Cell cycle arrest requires the overexpression of p53, pRB, p21, or $p16^{INK4a}$ [6]. Throughout life humans produce these senescent cells, which are accumulated in mitotic tissues, causing tissue degradation and leading to the aging process [6]. It has been known since 1970 that every time a cell divides, enters randomly into a senescent state and this chance is increased with each division [7].

Hayflick et al. (1961) found 5 decades ago that cells have a limited proliferation in human fibroblasts [6]. Now it is known that the senescence observed by Hayflick and colleagues was due to telomere shortening [8]. Moreover, senescence involves changes in chromatin organization and gene expression, resulting in cells that overproduce a large variety of proinflammatory cytokines, interleukins, chemokines, proteases, and growth factors [7]. This process, called the senescence-associated secretory phenotype (SASP), has potent autocrine and paracrine activity [9], tissue remodeling, and immune cell recruitment [4].

Moreover, many pathologies have been associated with senescence. Some studies have shown that senescent cells could lead to pathological states [3,7], including sarcopenia, atherosclerosis, heart failure, osteoporosis, macular degeneration, pulmonary insufficiency, renal failure, diabetes, neurodegeneration, cancer, and cardiovascular disease, among others [6].

There are two recognized types of cellular senescence: replicative senescence and stress-induced premature senescence (SIPS) [10]. The first refers to telomere attrition, capping the end of chromosomes; and the second is triggered by external factors, mitogenic oncogenes, and irradiation, leading to SIPS [9] which is not related to telomere shortening [10].

Obesity. https://doi.org/10.1016/B978-0-12-812504-5.00012-X

Appendix 2

Reduced serotonin levels after a lifestyle intervention in obese children: association with glucose and anthropometric measurements

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Nutrición Hospitalaria,2018
DOI: 10.20960/nh.1439
Impact factor (2018): 0.845
70/83 in Nutrition and Dietetics, Q4

ISSN (electrónico): 1699-5198 - ISSN (papel): 0212-1611 - CODEN NUHOEQ S.V.R. 318

ARÁN

Nutrición Hospitalaria

Trabajo Original

Pediatría

Reduced serotonin levels after a lifestyle intervention in obese children: association with glucose and anthropometric measurements

Disminución de los niveles de serotonina tras una intervención de estilo de vida en niños obesos: asociación con glucosa y medidas antropométricas

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Abstract

Background: serotonin signaling participates in body weight regulation and glucose metabolism. However, little information is available on circulating serotonin levels in obese subjects after a weight loss program. We aimed to assess the effect of a lifestyle intervention on serotonin levels in obese children and possible associations with anthropometric and blood glucose measurements.

Methods: forty-four obese children were enrolled in a ten-week lifestyle intervention consisting of a moderate caloric restriction diet, nutritional education and familial involvement. They were distributed according to the weight loss response. Subjects who lost > 0.5 BMI-SDS were considered as high responders (HR; n = 22) and those who lost ≤ 0.5 BMI-SDS, as low responders (LR; n = 22). Anthropometric, biochemical parameters and plasma serotonin levels were measured as pre and post-intervention values.

Results: obese children (HR and LR groups) were able to reduce anthropometric indices and to improve glucose profile after the intervention. Interestingly, plasma serotonin levels were significantly ($p < 0.05$) reduced in all subjects (-35.14 nmol/l HR group and -30.63 nmol/l LR group). Moreover, multiple-adjusted regression models showed a significant association between pre-intervention ($R^2 = 0.224$, B = 0.047; p = 0.004) and post-intervention ($R^2 = 0.140$; B = 0.055; p = 0.042) plasma serotonin and glucose levels. In addition, in HR subjects changes in plasma serotonin were associated with changes in glucose levels ($R^2 = 0.292$; b = 0.04; p = 0.045). Interestingly, pre and post-intervention plasma serotonin levels were inversely associated ($p < 0.05$) with anthropometric measures.

Conclusions: serotonin levels were reduced after a lifestyle intervention independently of the program response. Moreover, plasma serotonin levels were associated with glucose and anthropometric measures in obese children.

Key words:

Serotonin. Lifestyle. Intervention. Obese. Children.

Resumen

Introducción: la señalización de la serotonina está involucrada en la regulación del peso corporal y el metabolismo de la glucosa. Sin embargo, existe poca información disponible sobre los niveles de serotonina circulantes en sujetos obesos tras un programa de pérdida de peso. Nuestro objetivo fue evaluar el efecto de una intervención de estilo de vida en los niveles plasmáticos de serotonina en niños obesos y su posible asociación con medidas antropométricas y de glucosa en sangre.

Métodos: cuarenta y cuatro niños obesos participaron en una intervención de estilo de vida durante diez semanas consistente en una dieta con restricción calórica moderada, educación nutricional y participación familiar. Los sujetos que perdieron > 0,5 z-IMC fueron considerados como altos respondedores (AR; n = 22) y aquellos que perdieron ≤ 0,5 z-IMC, como bajos respondedores (BR; n = 22). Los parámetros antropométricos y bioquímicos y los niveles plasmáticos de serotonina se midieron antes y después de la intervención.

Resultados: los niños obesos (grupos AR y BR) pudieron reducir los índices antropométricos y mejorar el perfil lipídico y glucémico tras la intervención. Los niveles plasmáticos de serotonina fueron significativamente ($p < 0,05$) reducidos en todos los sujetos (-35,14 nmol/l grupo de AR y -30,63 nmol/l grupo de BR). Por otra parte, los modelos de regresión múltiple ajustada mostraron una asociación significativa entre los niveles plasmáticos de serotonina y glucosa previos a la intervención ($R^2 = 0,224$; B = 0,047; p = 0,004) y tras la intervención ($R^2 = 0,140$; B = 0,055; p = 0,042). Además, en los sujetos AR los cambios en la serotonina plasmática se asociaron con cambios en los niveles de glucosa ($R^2 = 0,292$; b = 0,04; p = 0,045). Los niveles de serotonina plasmática antes y después de la intervención se asociaron inversamente ($p < 0,05$) con índices antropométricos.

Conclusiones: los niveles plasmáticos de serotonina se redujeron tras una intervención de estilo de vida en todos los grupos. Además, los niveles plasmáticos de serotonina se asociaron con niveles de glucosa e índices antropométricos en niños obesos.

Palabras clave:

Serotonina. Estilo de vida. Intervención. Obesos. Niños.

Received: 17/07/2017 • Accepted: 31/10/2017

**Other members of the GENOI study group are: Tara Rendo-Urteaga, María Chueca.*
Funding: Navarra Government (PI 54/209), PIUNA and CIBERobn (project CIBER, CB06/03/1017).

Ojeda-Rodríguez A, Morell-Azanza L, Azcona-Sanjulián MC, Martínez JA, Ramírez MJ, Marti A; and GENOI members*. Reduced serotonin levels after a lifestyle intervention in obese children: association with glucose and anthropometric measurements. Nutr Hosp 2018;35:279-285

DOI: http://dx.doi.org/10.20960/nh.1439

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Appendix 3

Improved Diet Quality and Nutrient Adequacy in Children and Adolescents with Abdominal Obesity after a Lifestyle Intervention

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Nutrients,2018
DOI: 10.3390/nu10101500
Impact factor (2018): 4.196
19/83 in Nutrition and Dietetics, Q1

nutrients

MDPI

Article

Improved Diet Quality and Nutrient Adequacy in Children and Adolescents with Abdominal Obesity after a Lifestyle Intervention

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Received: 13 September 2018; Accepted: 11 October 2018; Published: 13 October 2018

Abstract: High rates of childhood obesity require integral treatment with lifestyle modifications that achieve weight loss. We evaluated a lifestyle intervention on nutrient adequacy and diet quality in children and adolescents with abdominal obesity. A randomized controlled trial was performed on 107 participants, assigned either to a usual care group or to an intensive care group that followed a moderate hypocaloric Mediterranean diet and received nutritional education. Intake adequacy was evaluated using Dietary Reference Intakes and diet quality through the Diet Quality Index for Adolescents (DQI-A), the Healthy Lifestyle Diet-Index (HLD-I) and the Mediterranean Diet Quality Index (KIDMED). Both groups achieved a significant reduction in BMI standard deviation score (BMI-SDS), glucose and total cholesterol levels. Intake of Calcium, Iodine and vitamin D were higher in the intensive care group, with enhanced compliance with recommendations. Higher dietary scores were associated with lower micronutrient inadequacy. DQI-A and HLD-I were significantly higher in the intensive care group vs. usual care group after the treatment. In conclusion, we observed that an intensive lifestyle intervention was able to reduce BMI-SDS in children with abdominal obesity. Furthermore, participants significantly improved dietary indices getting closer to the nutritional recommendations. Therefore, these diet quality indices could be a valid indicator to evaluate micronutrient adequacy.

Keywords: dietary intervention; childhood obesity; Mediterranean diet; nutritional requirements

1. Introduction

Obesity is a multifactorial disease and its treatment requires a multidisciplinary approach. Although weight gain could have a genetic component, lifestyle factors are the most important modifiable risk factors [1,2]. In recent years, there has been an increase in portion size and consumption of high-energy foods and a decrease in fruit and vegetable consumption in paediatric populations,

Appendix 4

Pistachio consumption modulates DNA oxidation and genes related to telomere maintenance: a crossover randomized clinical trial

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American Journal of Clinical Nutrition,2019
DOI: 10.1093/ajcn/nqz048.
Impact factor (2018): 6.549
4/83 in Nutrition and Dietetics, Q1

Pistachio consumption modulates DNA oxidation and genes related to telomere maintenance: a crossover randomized clinical trial

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ABSTRACT

Background: Telomere attrition may play an important role in the pathogenesis and severity of type 2 diabetes (T2D), increasing the probability of β cell senescence and leading to reduced cell mass and decreased insulin secretion. Nutrition and lifestyle are known factors modulating the aging process and insulin resistance/secretion, determining the risk of T2D.

Objectives: The aim of this study was to evaluate the effects of pistachio intake on telomere length and other cellular aging-related parameters of glucose and insulin metabolism.

Methods: Forty-nine prediabetic subjects were included in a randomized crossover clinical trial. Subjects consumed a pistachio-supplemented diet (PD, 50% carbohydrates and 33% fat, including 57 g pistachios/d) and an isocaloric control diet (CD, 55% carbohydrates and 30% fat) for 4 mo each, separated by a 2-wk washout period. DNA oxidation was evaluated by DNA damage (via 8-hydroxydeoxyguanosine). Leucocyte telomere length and gene expression related to either oxidation, telomere maintenance or glucose, and insulin metabolism were analyzed by multiplexed quantitative reverse transcriptase-polymerase chain reaction after the dietary intervention.

Results: Compared with the CD, the PD reduced oxidative damage to DNA (mean: −3.5%; 95% CI: −8.07%, 1.05%; $P = 0.009$). Gene expression of 2 telomere-related genes (*TERT* and *WRAP53*) was significantly upregulated (164% and 53%) after the PD compared with the CD ($P = 0.043$ and $P = 0.001$, respectively). Interestingly, changes in *TERT* expression were negatively correlated to changes in fasting plasma glucose concentrations and in the homeostatic model assessment of insulin resistance.

Conclusions: Chronic pistachio consumption reduces oxidative damage to DNA and increases the gene expression of some telomere-associated genes. Lessening oxidative damage to DNA and telomerase expression through diet may represent an intriguing way to promote healthspan in humans, reversing certain deleterious metabolic consequences of prediabetes. This study was registered at clinicaltrials.gov as NCT01441921. *Am J Clin Nutr* 2019;0:1–0.

Introduction

Telomere attrition is a natural phenomenon widely recognized as one of the hallmarks of aging. A large number of population-based studies have observed a decrease in leukocyte telomere length (LTL) in parallel with increased age (1). However, over the last decade a growing body of evidence has indicated that short telomeres are a relevant modifier of type 2 diabetes (T2D) risk and may be essential biomarkers that identify individuals at high future risk of T2D in clinical settings (2). Despite the fact that the mechanism(s) involved are not clear (3), several

The Western Pistachio Association (USA) and Paramount Farms supported the trial. SC was supported by an RYC-2013-12598 grant; SG was supported by a predoctoral fellowship from AGAUR (2018FI_B_00444); JM received funding from the European Union's Horizon 2020 research and innovation program under Marie Skłodowska-Curie grant agreement 713679 and from the Universitat Rovira i Virgili (URV); JG was awarded a predoctoral grant (PFIS FI17/00255) from the Instituto de Salud Carlos III (ISCIII); and LMA received her fellowship from the "La Caixa" Foundation. The study was funded by the Spanish Ministry of Science, Innovation and Universities (RYC-2013-12598), the Navarra Government (PI 54/2009), PIUNA, and CIBERobn (project CIBER, CB06/03). CIBERobn is an initiative of the ISCIII, Spain, which is supported by FEDER funds (CB06/03). None of the funding sources played a role in the design, collection, analysis, or interpretation of the data, or in the decision to submit the manuscript for publication.

Supplemental Tables 1–4 and Supplemental Figure 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: 8-OHdg, 8-hydroxydeoxyguanosine; CD, control diet; E%, energy percentage; IR, insulin resistance; LTL, leucocyte telomere length; miRNA, microRNA; ROS, reactive oxygen species; PD, pistachio-supplemented diet; T2D, type 2 diabetes; TL, telomere length.

Received December 5, 2018. Accepted for publication March 11, 2019.

First published online 0, 2019; doi: https://doi.org/10.1093/ajcn/nqz048.

