Universidad de Navarra

Faculty of Pharmacy

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Aurora Pérez Cornago

Pamplona 2014

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Memoria presentada por Dña. **Aurora Pérez Cornago** para aspirar al grado de Doctor por la Universidad de Navarra.

Aurora Pérez Cornago

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

Pamplona, 1 de Septiembre de 2014.

Prof. J. Alfredo Martínez Hernández

Dr. M. Ángeles Zulet Alzórriz

A mis padres

I have never walked alone…

En primer lugar quiero agradecer a la Universidad de Navarra, a la Facultad de Farmacia y en especial al Departamento de Ciencias de la Alimentación y Fisiología y al reciente Centro de Investigación en Nutrición por darme la oportunidad de realizar este trabajo.

No puedo dejar de mencionar a la Asociación de Amigos de la Universidad de Navarra por la financiación recibida durante estos cuatro años. También al Ministerio de Educación por la beca de movilidad recibida para realizar la estancia de mi tesis en University College Dublin (UCD).

En especial quisiera agradecer al Prof. J. Alfredo Martínez y a la Dr. M. Ángeles Zulet, los directores de esta tesis. No ha sido un camino fácil, pero supisteis encauzar mi tesis de la mejor manera posible. Me he enriquecido mucho personal y profesionalmente estando a vuestro lado. Siempre recordaré vuestro apoyo y vuestra confianza en mí, ¡muchas gracias por todo!

I wish to express my gratitude to Dr. Lorraine Brennan and to all her group at UCD for teaching me the interesting field of metabolomics. It was an amazing personal and professional experience.

También quiero agradecer a todo el equipo (personal de la Unidad Metabólica, técnicos y doctores) de SENIFOOD y RESMENA, y en especial a mis compañeras Idoia, Patricia y Rocío con las que tantas cosas he compartido, gracias por hacerlo todo más fácil chicas, ¡qué gran equipo! Y por supuesto, ¡muchísimas gracias a todos los voluntarios!

La ayuda de los departamentos de Medicina Preventiva y de Farmacología ha sido muy importante durante estos años. Muchas gracias por siempre tener vuestras puertas abiertas para cualquier cosa que he necesitado.

Gracias a los compañeros y amigos que han pasado por la salita de ordenadores durante estos años. Deciros que os voy a echar muchísimo de menos, y os quiero dar las gracias por todo vuestro apoyo. Pero sobretodo quiero agradecer al "Granada team", sois las principales responsables de que me dé tanta pena que se acabe esta

etapa, pero sé que esto no es un adiós y que me llevo amigas para siempre. ¡Os voy a echar mucho de menos a todos!

Gracias a Pamplona por todo lo que me ha dado estos años. A todas mis compis de piso, con las que lo he pasado genial. Gracias a mis amigos del master y de la carrera. Pero si algo me llevo de Pamplona es a una "cuadrilla" increíble, gracias a mis "raros", "kukas" y "kukitas" (sentiros todos incluidos) por los "viernes raros", los días de esquí y por todos los grandes momentos vividos. Desde luego que estos años no habrían sido lo mismo sin vosotros.

Gracias a todos mis amigos de Ólvega, Ágreda y Soria, aunque muchos no sepan muy bien lo que hago o no entiendan por qué aún sigo estudiando. Thanks to those that are abroad but who I have always felt by my side; a mis amigas de toda la vida, da gusto que aunque tardemos en vernos cuando nos juntamos parezca que no haya pasado el tiempo. Y muy en especial quiero dar las gracias a Patri, mi mejor amiga desde que éramos unas "enanas". Gracias nena por estar siempre ahí, por ser mi confidente, por apoyarme día a día; realmente no se puede tener una amiga mejor.

A Cesar, quien ha sido fundamental en mi vida estos años de tesis. Gracias por escucharme y aconsejarme, por tu paciencia y por la paz que me transmites. Me has dado fuerza cuando más lo he necesitado, gracias por estar siempre a mi lado y apoyarme en todo lo que hago; gracias por ser tal y como eres, por ser mi compañero perfecto en este viaje de la vida y por hacerme tan feliz.

Y las últimas palabras las quiero dedicar a mi familia, los principales responsables de que esté hoy aquí y a quienes dedico esta tesis. A mis abuelos por apoyarme siempre; en especial a mi abuelo Ángel, quien me enseñó a luchar por mis sueños y quien siempre creyó en mí. A mi hermano Ángel, que como hermano mayor ha sido mi guía en muchos momentos de mi vida y quien siempre está cuando le necesito. Pero quienes se merecen un GRACIAS CON MAYÚSCULAS son mis padres, Mª Ángeles y Manuel. Gracias por darlo todo por mí, por vuestro amor y ayuda infinita, por guiarme y orientarme. Gracias por los tapers mami. Gracias por los valores que me habéis inculcado, estoy muy orgullosa de vosotros. ¡Me atrevo a decir que soy la hija más afortunada del mundo! ¡Os quiero muchísimo!

Abbreviations

- 5-HIAA — 5-hydroxyindoleacetic acid
- 5-HT — Serotonin
- 5-HTR2A — Serotonin 2A receptor
- AA — Amino acid
- ACE — Angiotensin I-converting enzyme
- AHA — American Heart Association
- ALA — α-linolenic acid
- ALADINO — ALimentación, Actividad Física, Desarrollo INfantil y Obesidad - Food, Physical Activity, Child development and Obesity
- AMDIS — Automated Mass Spectral Deconvolution and Identification System
- ASCVD — Atherosclerotic cardiovascular disease
- BBB — Blood-brain barrier
- BCAA — Branched chain amino acid
- BDI — Beck Depression Inventory
- BDNF — Brain-derived neurotrophic factor
- BIA — Bioelectric impedance analysis
- BioSHaRE-EU — Biobank Standardisation and Harmonisation for Research Excellence in the European Union
- BMI — Body mass index
- BS — Biceps skinfold
- CHO — Carbohydrate
- CNS — Central nervous system
- CpG — Cytosine-phosphate-guanine
- CRP — C-reactive protein
- CVD — Cardiovascular disease
- DA — Dopamine
- DASH — Dietary Approaches to Stop Hypertension
- DBP — Diastolic blood pressure

- DEXA — Dual-Energy X-ray Absorptiometry
- DHA — Docosahexaenoic acid
- DiOGenes — Diet, Obesity and Genes
- DNA — Deoxyribonucleic acid
- DNMTs — DNA methyltransferases
- DRD4 — Dopamine receptor D4
- DSM-V — Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders
- EDTA — Ethylenediaminetetraacetic acid
- EGIR — European Group for the Study of Insulin Resistance
- ENRICA — Estudio de Nutrición y Riesgo Cardiovascular en España - Study on Nutrition and Cardiovascular Risk in Spain
- EPA — Eicosapentanoic acid
- ESEMeD — European Study of the Epidemiology of Mental Disorders
- FA — Fatty acid
- FFA — Free fatty acid
- FTO — Fat mass and obesity associated gene
- GAD — Generalized anxiety disorder
- GI — Glycaemic index
- GL — Glycaemic load
- GLP-1 — Glucagon-like peptide-1
- GS/MS — Gas chromatography/mass spectrometry
- GWAS — Genome-wide association studies
- HDL-c — High-density lipoprotein cholesterol
- HEI — Healthy eating index
- HPA-axis — Hypothalamic pituitary adrenal axis
- HPLC — High-performance liquid chromatography
- *HTR2A* — 5-hydroxytryptamine receptor 2A
- ICD-10 — 10th International Classification for Diseases
- IDF — International Diabetes Federation

- IDO Indoleamine 2,3-dioxygenase
- IFG Impaired fasting glucose
- IL Interleukin
- IOTF International Obesity Task Force
- IS Suprailiac skinfold
- LA Linoleic acid
- LDL-c Low-density lipoprotein cholesterol
- MAOIs Monoamine oxidase inhibitors
- MC4R Melanocortin 4 receptor
- MDA Malondialdehyde
- MDD Major depressive disorder
- MedDiet Mediterranean diet
- MetS Metabolic syndrome
- MS Mass spectrometry
- MUFA Monounsaturated fatty acid
- NA Noradrenaline
- NAFLD Non-alcoholic fatty liver disease
- NCEP-ATP III National Cholesterol Education Program Adult Treatment Panel III
- NEFA Non-esterified fatty acids
- NHLBI National Heart, Lung, and Blood institute
- NIST National Institute of Standards and Technology
- NLRP3 Pyrin domain-containing 3
- NMR Nuclear magnetic resonance
- oxLDL Oxidized low density lipoproteins
- PNS Peripheral nervous system
- POMS Profile of Mood States
- POUNDS LOST study The Prevention of Obesity Using Novel Dietary Strategies study
- PREDIMED PREvención con DIeta MEDiterránea
- PUFA Polyunsaturated fatty acid
- PYY Peptide YY

- RCT — Randomized controlled trial
- RESMENA — REducción del Síndrome MEtabólico en Navarra
- RNA — Ribonucleic acid
- ROS — Reactive oxygen species
- SEEDO — Sociedad Española para el Estudio de la Obesidad - Spanish Society for the Study of Obesity
- SFA — Saturated fatty acid
- SLC6A4 — Serotonin transporter
- SNPs — Single nucleotide polymorphisms
- SNRIs — Serotonin and noradrenaline reuptake inhibitors
- SNS — Sympathetic nervous system
- SS — Subscapular skinfold
- SSRIs — Selective serotonin reuptake inhibitors
- STAI — State Trait Anxiety Inventory
- T2DM — Type 2 diabetes mellitus
- TAC — Total antioxidant capacity
- TC — Total cholesterol
- TCA — Tricyclic antidepressant
- TCV — Total caloric value
- TG — Triglycerides
- TNF-α — Tumor necrosis factor-α
- Trp — Tryptophan
- TS — Triceps skinfold
- VAS — Visual analogue scale
- WBC — White blood cells
- WC — Waist circumference
- WHO — World Health Organization
- WHR — Waist-to-hip ratio

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I. INTRODUCTION

1. Obesity

The World Health Organization (WHO) defines obesity as an abnormal or excessive fat deposition that contributes to increase morbidity and mortality (WHO, 2013). This excessive fat accumulation is mainly stored as triglycerides (TG) in the adipose tissue (subcutaneous and intra-abdominal accumulation), which constitute a long-term energy reservoir (Tchernof *et al.*, 2013).

1.1. Aetiology

Generalizing, obesity is the result of a chronic positive energy balance, when individuals consume more calories (e.g. through food and drinks) than they expend (e.g. body functions and physical activity), maintained over time (WHO, 2013; Oliveros *et al.*, 2014).

Several *lifestyle behaviours* have changed considerably in recent decades. Fifty years ago, families took raw products transforming them into comestible food. Since then, a nutrition transition has appeared in both high-income and low-income countries, characterized by industrialization and globalization of the food market (Popkin *et al.*, 2012; Zamora, 2014). Technological innovations, including additives such as preservatives, sweeteners or antioxidants, have enabled the mass preparation of food by companies. Moreover, there has been a rise in the consumption of processed foods, sugar-sweetened beverages, unhealthy fats, and processed foods, at the expense of whole grains, fruits, vegetables and legumes consumption (Moreno *et al.*, 2002). Hence, these *changes in the global food system* and also in *dietary patterns*, have contributed to the obesity epidemic (Gortmaker *et al.*, 2011). In addition, *sedentary behaviours* have dramatically increased in developed countries, with this increase being related to the sedentary work, transportation methods, less physical exercise, and increasing urbanization (Rey-Lopez *et al.*, 2011). Moreover, other environmental factors such as ambient temperature, sleep debt, alcoholism, smoking or side effect of drugs have been shown to have potential implications in obesity (McAllister *et al.*, 2009; Grucza *et al.*, 2010; Fesinmeyer *et al.*, 2013). However,

obesity is a more complex disease with a multifactorial aetiology involving not only unhealthy lifestyle habits (Xia *et al.*, 2013).

The fact that not all individuals exposed to the same environmental risk factors develop obesity, lends support to an underlying *genetic* component to the disorder (Walley *et al.*, 2009). In this context, monogenic obesity considers that obesity is produced by a mutation or deficiency of a single gene (Farooqi *et al.*, 2005). However, it has been proposed that human obesity is mainly polygenic, being caused by the interaction of gene variants with dietary intake that have an influence on body weight (El-Sayed Moustafa *et al.*, 2013). For instance, leptin deficiency was a demonstrated example of monogenic obesity in humans using a candidate-gene approach (Zhang *et al.*, 1994). Another condition of monogenic obesity is the mutation of melanocortin 4 receptor (MC4R) (Razquin *et al.*, 2011). In addition, genome-wide association studies (GWAS) have evidenced that the fat mass and obesity associated gene (FTO) strongly contributes to early onset obesity and type 2 diabetes mellitus (T2DM) (Hinney *et al.*, 2007).

Moreover, recent studies have provided evidence that *epigenetic mechanisms* explain some interactions between genetic and environmental factors (e.g. diet, physical activity or drugs), regulating gene expression over the entire lifetime of the organism (Ordovas *et al.*, 2010). Epigenetic changes include multiple processes such as covalent histone modifications, chromatin folding, micro-ribonucleic acid (RNA) alterations or deoxyribonucleic acid (DNA) methylation of cytosine-phosphate-guanine (CpG) residues (Campion *et al.*, 2009; Marti *et al.*, 2011).

Furthermore, *neuroendocrine factors* have also been suggested to have a major influence on obesity by regulating energy balance (Klok *et al.*, 2007). Numerous hormonal and neural signals are generated in response to a meal, modulating central nervous system (CNS) activities and either afferent or efferent systems (Lustig, 2001; Chambers *et al.*, 2013). In this sense, the previously mentioned molecule leptin, is an anorexigenic hormone that suppresses food intake. Paradoxically, obese individuals may present increased circulating levels of this hormone, which may be associated to leptin resistance (Enriori *et al.*, 2006). Moreover, ghrelin, which is a stomach-derived peptide, modulates energy balance by stimulating appetite, weight

gain, and adiposity (Cummings *et al.*, 2005). Other important molecules that modulate the energy balance are insulin, neuropeptide Y, the agouti-related protein and several neurotransmitters such as dopamine (DA) and serotonin (5-HT) as reviewed elsewhere (Lustig, 2001; Liu *et al.*, 2014).

The use of *certain medications* may increase the risk of obesity by causing weight gain. As an adverse effect of their therapeutic action, some drugs are able to raise body weight by affecting different neurotransmitters (Wofford *et al.*, 2006). Drugs known to promote weight gain include β-blockers, corticosteroids, and antipsychotics (de Morentin-Aldabe *et al.*, 2013).

Many studies have shown that obesity is *socially* distributed. Marriage might predict an increase in body weight, while divorce or widowhood have been associated with weight loss (Dinour *et al.*, 2012). Regarding education status, an inverse relationship of both higher body mass index (BMI) and waist circumference (WC) with lower education level has been found (Hermann *et al.*, 2011). In recent years, lower social classes (or economic status) have experienced a faster growth rate in overweight and obesity (Jones-Smith *et al.*, 2012). In low and middle income countries there is a huge concern since a double burden of disease has emerged, coexisting both undernutrition and obesity in the same population (Gortmaker *et al.*, 2011). Moreover, maternal age and gestational weight gain have been positively related with postpartum weight retention in women, increasing the risk of suffering later obesity (McAllister *et al.*, 2009; Mannan *et al.*, 2013).

In the last years, the *gut microbiota* has been proposed as an environmental factor that may affect the predisposition toward obesity, obesity-associated inflammation and insulin resistance (Shen *et al.*, 2013). The gut microbiota is able to exchange metabolites with the host, and also, may interact with nutrients and with host signalling pathways modulating lipid and amino acid (AA) metabolism and even the host gene expression (Etxeberria *et al.*, 2013b). Also, the type and quantity of dietary fat and carbohydrate (CHO) may alter faecal microbiome (Fava *et al.*, 2013). In this sense, probiotic therapy has been proposed to control body weight (Shen *et al.*, 2013). Yet more studies are required to elucidate whether changes in the gut

microbiota are caused by overweight and obesity, or if they are a consequence of excessive weight (Tagliabue *et al.*, 2013).

Likewise, a bidirectional relationship between *mental disorders* and obesity has been reported (Brumpton *et al.*, 2013). Some of the mechanisms that may link these two highly prevalent diseases are low-grade inflammation, oxidative stress, unhealthy lifestyle habits and monoamine imbalance, among others (Bondy, 2007; Pan *et al.*, 2012a).

Finally, overconsumption of fast food, snacks and sweetened beverages as well as long-time TV watching, low activity level and food advertisements have been specifically proposed as important causes of *childhood obesity* (Moleres *et al.*, 2012; Bemelmans *et al.*, 2014).

In conclusion, obesity is a multifactorial disease where diverse environmental and genetic factors, as well as the combination of both factors (environment x genetics interaction), are involved in its development (McAllister *et al.*, 2009).

1.2. Diagnosis

Obesity diagnosis should be the first step toward treatment. Given that obesity is characterized by an excess of body fat, its diagnosis should be focused on the analysis of body composition in order to detect excessive adiposity.

- Body mass index

Nowadays in clinical practice, the most used tool to detect underweight, overweight and obesity in middle-aged adults is the BMI, defined as the body weight in kilograms divided by height in square metres (BMI = kg/m^2). Both the WHO (WHO, 2013) and the Spanish Society for the Study of Obesity (SEEDO) (SEEDO, 2007) have established the following criteria to classify the nutritional status of the community (**Table 1**).

Table 1. The WHO and SEEDO classifications of the nutritional status by the BMI.

BMI (kg/m²)	Nutritional status	
	SEEDO (Spanish) (SEEDO, 2007)	**WHO** (WHO, 2013)
< 18.5	Underweight	Underweight
18.5 – 24.9	Normal weight (healthy)	Normal weight
25 – 26.9	Overweight level I	Overweight (pre-obesity)
27 – 29.9	Overweight level II (pre-obesity)	
30 – 34.9	Obesity class I (moderately obese)	Obesity class I
35 – 39.9	Obesity class II (severely obese)	Obesity class II
40 – 49.9	Obesity class III (very severely obese)	Obesity class III
≥ 50	Obesity class IV (extreme)	

Abbreviations: BMI, body mass index; SEEDO, Spanish Society for the Study of Obesity; WHO, Word Health Organization.

The BMI interpretation does not differ between sexes and race, and neither distinguishes between degree of fatness, muscle mass, and skeletal mass; therefore it can lead to errors in the estimation of adiposity in, for example, athletes, pregnant women and in metabolic obese normal weight individuals (WHO, 2013; Oliveros *et al.*, 2014).

In relation to the use of BMI in children, the WHO standard and the International Obesity Task Force (IOTF) reference are the two main international datasets to define overweight and obesity in children in terms of BMI. These two criteria estimate differently the prevalence of overweight and obesity in this population, therefore there is clearly a need to harmonize these international standards for childhood obesity (Monasta *et al.*, 2011).

Measurement of overweight and obesity in older adults is controversial. Nowadays, BMI is the most widely used measure for overweight and obesity in the elderly (Decaria *et al.*, 2012). However, this index might be influenced by the reduction in skeletal muscle and height decline associated with ageing. Therefore, the use of body composition measurements to identify patients with high adiposity or low muscle mass has been proposed (Mathus-Vliegen, 2012). Hence, a standard measure and cut-off to determine overweight and obesity in older adults is required.

- Waist circumference

In middle-aged adults, WC has been recognized as a valuable tool to diagnose overweight and obesity. WC is a direct indicator of central obesity, a useful measurement in clinical practice and it has shown to be predictive of cardiovascular risk (Pouliot *et al.*, 1994). Both the International Diabetes Federation (IDF) and the WHO have defined abdominal obesity taking into account different populations, ethnic groups and sexes (Alberti *et al.*, 2009). Moreover, the SEEDO has also proposed WC thresholds stratified by sexes for the Spanish population (SEEDO, 2007) (**Table 2**). In addition, it should be highlighted that when obesity is determined by WC in older adults, the prevalence rates are higher than when defined by BMI (Mathus-Vliegen, 2012).

Table 2. Recommended WC cutoff points for abdominal obesity.

Abdominal Obesity					
IDF (Europid) (Alberti *et al.*, 2005)		**WHO** (Caucasian) (WHO, 2013)		**SEEDO** (Spanish) (SEEDO, 2007)	
Men	Women	Men	Women	Men	Women
≥ 94 cm	≥ 80 cm	≥ 94 cm ≥ 102 cm (higher risk)	≥ 80 cm ≥ 88 cm (higher risk)	> 102 cm	> 88 cm

Abbreviations: IDF, International Diabetes Federation; SEEDO, Spanish Society for the Study of Obesity; WC, waist circumference; WHO, Word Health Organization.

- Waist to hip ratio

The waist-to-hip ratio (WHR) is calculated by dividing the WC by the hip circumference, and it is considered as an additional measure of body fat distribution and disease risk (WHO, 2008). A larger ratio has been related to a higher proportion of visceral fat, and so, to an increased risk of non-communicable diseases, such as myocardial infarction, stroke or premature death (SEEDO, 2000; WHO, 2008). Shown in **Table 3** the WHO and SEEDO classifications of disease risk by the waist to hip ratio.

Table 3. The WHO and SEEDO classifications of disease risk by the waist to hip ratio.

Increased risk of CVD			
WHO (Caucasian) (WHO, 2008)		**SEEDO (Spanish)** (SEEDO, 2000)	
Men	Women	Men	Women
≥ 0.90	≥ 0.85	> 1	> 0.9

Abbreviations: CVD, Cardiovascular Disease; SEEDO, Spanish Society for the Study of Obesity; WHO, Word Health Organization.

- Body composition

The BMI and the WC do not directly measure fat mass, which excess is the main characteristic of obesity. For this reason, other measures to evaluate adiposity have been proposed:

– Skinfolds

Under the assumption that the subcutaneous fat reflects the whole-body fat content, skinfold-thickness measurements are used for the assessment of percentage of body fat mass. For this purpose, the Siri equation was devised (Siri, 1993):

$$\text{Body density (D)} = C - M \times \log (TS + BS + SS + IS)$$

BS = biceps skinfold; C = 1.1143 for men, 1.1278 for women; IS = suprailiac skinfold; M = 0.0618 for men, 0.0775 for women; SS = subscapular skinfold; TS = triceps skinfold.

The strengths of this method are that it is quick, cheap, non-invasive and it has good correlation with total body fat mass (Rodriguez *et al.*, 2005). However, some limitations of skinfold thickness measurements should be considered, such as a high interobserver variation or the difficulty to measure very large fat folds (Stewart *et al.*, 1997).

– Bioelectrical impedance analysis

The bioelectrical impedance analysis (BIA) method determines fat-free mass and total body water in subjects without significant fluid and electrolyte abnormalities. Through this information, whole-body fat content can be determined by standard procedures (Kyle *et al.*, 2004). This method is influenced by hydration, fat mass, physical activity or skin condition

(Kushner *et al.*, 1996). However, it is easy, not very expensive and has shown very good degree of accuracy (Krachler *et al.*, 2013).

– Dual-Energy X-ray Absorptiometry

Dual-Energy X-ray Absorptiometry (DEXA) assesses three compartments (bone mass, fat mass and fat-free mass) and it has the capacity of determining regional body composition. This method is widely considered to be among the most precise methods for determining body composition, however it is expensive and the scanner table might be too small for very obese patients (Genton *et al.*, 2002).

Moreover, there are other effective methods for body composition determination, for instance the whole body air-displacement plethysmography or the hydrostatic weighing (Fields *et al.*, 2000). However, their cost limits their use.

To date, the WHO has not proposed a percentage of body fat threshold for defining obesity (Ho-Pham *et al.*, 2011). However, the Consensus SEEDO 2000 (SEEDO, 2000) has done so for the Spanish population (**Table 4**).

Table 4. The SEEDO classification of the nutritional status by whole-body percentage body fat (SEEDO, 2000).

Nutritional status	Whole-body percentage body fat	
	Men	Women
Normal weight	12-20%	20-30%
Overweight	21-25%	31-33%
Obese	> 25%	> 33%

1.3. Prevalence

The prevalence of overweight and obesity, estimated using the BMI, has reached epidemic proportions in both developed and developing countries (Kelly *et al.*, 2008; WHO, 2013). This high rate has almost doubled since 1980, with half of this increase occurring between 2000 and 2008 (Scully, 2014). In this context, Steven *et al.* revealed that globally, one in three adults were overweight and one in nine were

obese in 2008 (Stevens *et al.*, 2012). However, there are differences in obesity levels and trends among countries (**Figure 1**) (Webber *et al.*, 2014).

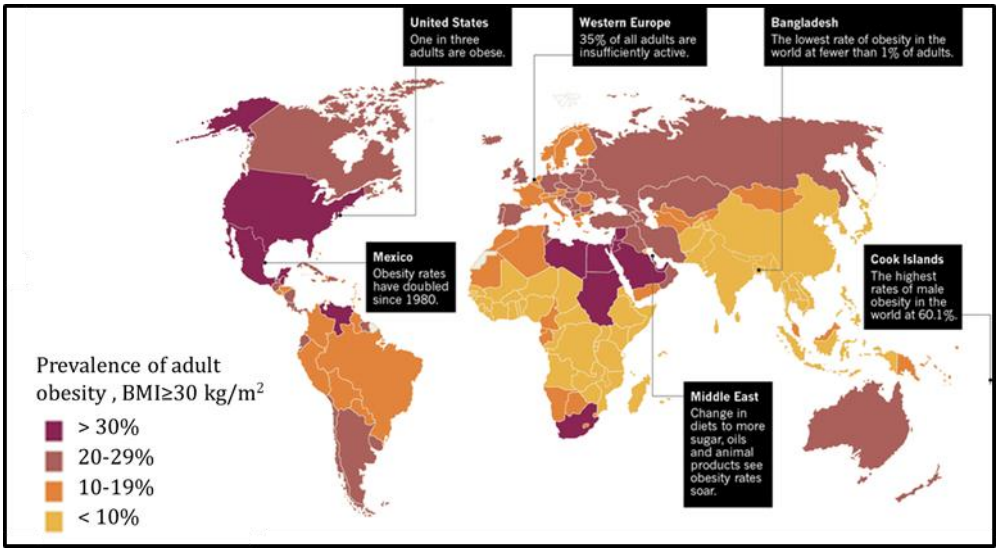

Figure 1. Prevalence of adult obesity by country. Adapted from: (Scully, 2014).

According to the WHO, more than 1.4 billion and over 500 million adults were overweight and obese in 2008, respectively (WHO, 2013). Similarly, the World Obesity Federation (Formerly IASO), estimated that around 475 million adults are obese, with over twice that number overweight (IASO, 2014a). Current trends suggest that by 2030 up to 57.8% of the world's adult population (3.3 billion people) might present obesity (Kelly *et al.*, 2008). Moreover, a recent study has shown that globally, the proportion of adults with a BMI higher or equal to 25 kg/m^2 was 36.9% in men and 38.0% in women in 2013 (Ng *et al.*, 2014).

The prevalence of obesity in children has increased at an alarming rate in the past two decades, especially in western countries (WHO, 2012b). Globally, the prevalence of this disease among preschool children was of 4.2% in 1990, increasing to 6.7% in 2010 (de Onis *et al.*, 2010). In 2010, the IOTF estimated that over 200 million school-age children were overweight or obese (IASO/IOTF, 2010). Moreover, the WHO has calculated that up to 42 million children under the age of five were overweight (WHO, 2012b). The percentage of childhood obesity by region is exhibited (**Table 5**).

Table 5. Percentage of childhood obesity by region according to the World Obesity Federation.

WHO Region	Boys		Girls	
	% Overweight	% Obese	% Overweight	% Obese
Africa	2.8	1.0	4.6	1.1
America	18.4	9.8	17.1	9.5
Eastern Mediterranean	10.7	6.3	12.2	6.3
Europe	15.9	4.4	15.0	4.0
South East Asia	10.4	2.6	6.9	0.5
Western Pacific	5.9	1.9	5.0	1.1

Using IOTF International cut-off points. Age: 5-17 years old. Abbreviations: IOTF, International Obesity Task Force; WHO, World Health Organization. Adapted from: (IASO, 2014b).

Prevalence of obesity in Spain

The Nutrition and Cardiovascular Risk in Spain (ENRICA) study, carried out between 2008 and 2010 in over 12.000 Spanish adults, revealed that Spain is one of the countries with higher rates of obesity in Europe. This investigation showed that over 39% of Spanish adults were overweight and that more than the 22% were obese, being the rates higher in men than in women. Importantly, the ENRICA study (**Figure 2**) evidenced that in Spain the frequency of obesity increased with decreasing education level in both sexes (Gutierrez-Fisac *et al.*, 2012).

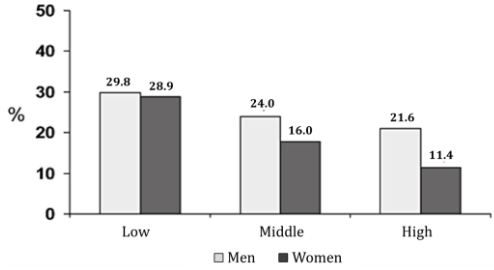

Figure 2. Prevalence of obesity (BMI ≥ 30 kg/m^2) by education level in Spain. Source: (Gutierrez-Fisac *et al.*, 2012).

Furthermore, the Food, Physical Activity, Child development and Obesity (ALADINO) study, which was performed between October 2010 and May 2011, revealed that in Spanish children, the prevalence of obesity in boys ranged from 11.0% to 20.9%, and

in girls from 11.2% to 15.5%, depending on the cut-off criteria employed (Perez-Farinos *et al.*, 2013).

On the other hand, most developed countries have increased their life expectancy, and so, the population is growing older. The age of 60 or 65 is said to be the beginning of old age (Witham *et al.*, 2010; Mathus-Vliegen, 2012). In this context, Spain is composed by 17% of older adults and the number is projected to grow to 33% in 2050 (Gomez-Cabello *et al.*, 2011). A fast increase in the prevalence of overweight and obesity and chronic diseases has been reported in this age group in developed countries (Witham *et al.*, 2010). In Spain, a cohort of 3136 older adults revealed that more than 56% of older adults suffer from central obesity (Gomez-Cabello *et al.*, 2011). Moreover, the ENRICA study showed that 46% of Spanish older adults were overweight and that 35% were obese, obesity being determined using the BMI of the participants (Gutierrez-Fisac *et al.*, 2012).

1.4. Consequences

Obesity has been related to many adverse health consequences, lowering life expectancy (Preston *et al.*, 2011). Among the wide range of health outcomes attributed to obesity are hypertension, dyslipidaemia, stroke, T2DM and cancer. The pathological basis of obesity is an increase in the size (hypertrophy) and number (hyperplasia) of adipocytes, which lead to an increased secretion of pathogenic factors (Bray, 2007).

The disadvantages of obesity can be divided into those caused by the increase of fat mass and those produced by hypersecretion from enlarged fat cells (Bray, 2004). It can be found that sleep apnoea, osteoarthritis or skin illnesses, as well as social discrimination, are among the detrimental effects of increased fat mass. On the other hand, the most important diseases related to hypersecretion from enlarged adipocytes are cancer, low-grade inflammation, non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD) (Bray, 2004; Bray, 2007). Obesity may also be responsible of developing polycystic ovarian syndrome and infertility in women (Motta, 2012). Moreover, excess adiposity has been related to low-grade inflammation, hypertension, dyslipidaemia and increased total cholesterol (TC),

among others (Huang, 2009). All this leads to an increase risk of suffering metabolic syndrome (MetS), which will be explained in section 1.7..

Additionally, childhood obesity is associated with a higher chance of staying obese into adulthood (**Figure 3**), increasing the risk of non-communicable diseases and disability later in life (WHO, 2012b).

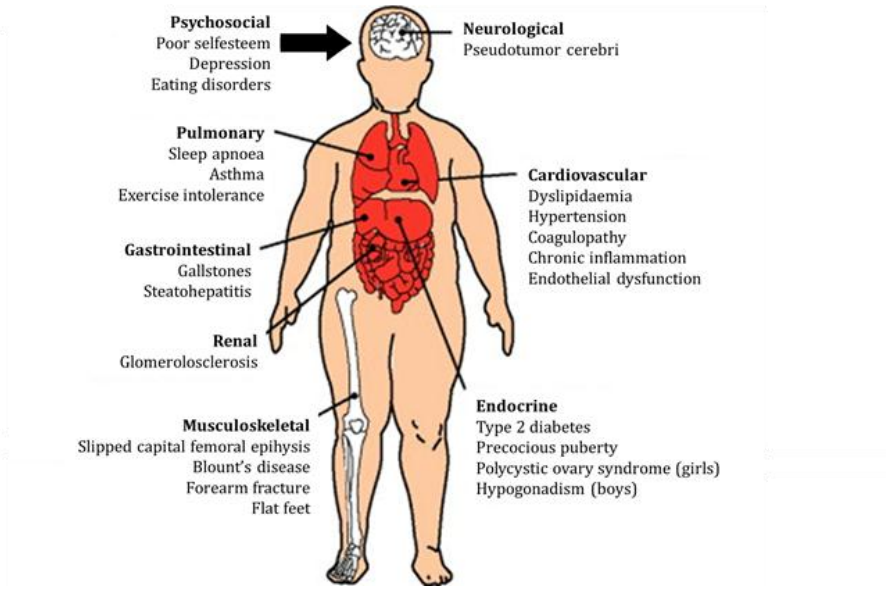

Figure 3. Complications of childhood obesity. Source: (Ebbeling *et al.*, 2002).

Moreover, both aging and obesity represent a considerable economic burden on health-care systems (Mathus-Vliegen, 2012). Aging is associated with significant changes in cardiovascular physiology and also in body composition (Mathus-Vliegen, 2012). With increasing aging, there is an increase of fat deposited in skeletal muscle and in the liver, and also of visceral fat, contributing to glucose intolerance and insulin impairment in the elderly (Mathus-Vliegen, 2012).

Furthermore, aging is accompanied by the decrease of skeletal muscle mass, size and strength (sarcopenia), that implies additional serious health consequences, such as disability and mortality (Bouchonville *et al.*, 2013).

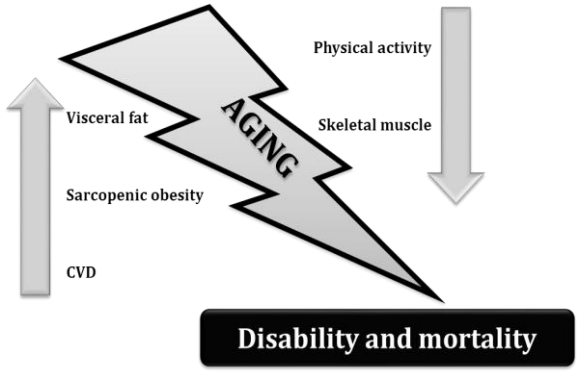

Figure 4. Sarcopenic obesity in older adults. Abbreviation: CVD, cardiovascular disease.

In recent years, there has been a rise of sarcopenic obesity (**Figure 4**), which is characterized by a reduction in skeletal muscle mass and an increase of total fat mass. This disorder has been closely related with MetS and it has also been attributed to a decrease in physical activity with aging (Lu *et al.*, 2013).

1.5. Treatment

The first-line treatment for overweight and obesity includes lifestyle modification through weight loss challenges together with the increase of physical activity with the purpose of balancing energy intake with energy expenditure (Abete *et al.*, 2011). Moreover, it is crucial to evaluate the risk of overweight in the patient, which might be done using the BMI as a guideline and including a laboratory assessment (**Table 6**).

Table 6. Appropriate treatments for overweight and obesity.

	BMI (kg/m²)				
Treatment	**25-26.9**	**27-29.9**	**30-34.9**	**35-39.9**	**> 40**
Lifestyle (diet and exercise)	✔	✔	✔	✔	✔
Pharmacotherapy		With co-morbidities	✔	✔	✔
Surgery				With co-morbidities	✔

Abbreviation: BMI, body mass index. Source: (Bray, 2007; SEEDO, 2007).

Although the first step for overweight management is lifestyle modification, patients with a BMI ≥ 30 kg/m^2 or those with a BMI ≥ 27 kg/m^2 and associated co-morbidities, such as T2DM or CVD, would be eligible for pharmacological treatment (Snow *et al.*, 2005; SEEDO, 2007). In addition, individuals might be suitable for surgery if they have a BMI ≥ 40 kg/m^2 or a BMI ≥ 35 kg/m^2 together with serious co-morbidities (Bray, 2007; SEEDO, 2007).

1.5.1. Energy restriction

Energy restriction, with an adequate intake of macro- and micronutrients, has demonstrated to have a wide range of health benefits for obese individuals (Fontana, 2009; Abete *et al.*, 2011). Moderate weight loss (5-10% below their initial weight) can reduce some risk factors as for example dyslipidaemia, hypertension, fasting insulin levels, several growth factors and cytokines, among others (Bray, 2007). Moreover, accumulating data indicate that energy restriction, together with an adequate nutrition, may even protect against cancer (Fontana, 2009). However, lean mass might be reduced after following a hypocaloric diet, with this being more notable with aging (Mathus-Vliegen, 2012).

On the other hand, many individuals fail to maintain the weight lost after an energy-restricted diet. This failure is commonly be due to hunger, food cravings, dietary monotony, lack of variability of food and adaptations in energy expenditure (Cornier *et al.*, 2013). Subjects following a dietary treatment with very-low-calorie diet (<800 kcal/day) or low-calorie diet (<1200 kcal/day) achieve a greater initial weight loss, but also elicited a higher weight regain compared with moderate restricted diets (below 500-1000 deficit) (Johansson *et al.*, 2014).

1.5.2. Healthy dietary patterns

Healthy dietary patterns have been shown to be effective for the prevention and treatment of obesity. Two of the most widely recognized healthy dietary patterns are the American Heart Association (AHA) guidelines and the Mediterranean diet (MedDiet).

- American heart association guidelines

In 1996, the AHA recommended a cholesterol intake less than 300 mg/day and a macronutrient distribution of ≤ 30% total energy value (TCV) from lipids, 55-60% from CHO and the rest from proteins (Krauss *et al.*, 1996). In 2000, this association presented the following guidelines for reducing the risk of CVD by dietary modification and other healthy lifestyle factors (Krauss *et al.*, 2000):

- *Overall Healthy Eating Pattern*. Include a variety of fruits, vegetables, grains, skimmed dairy products, fish, legumes, poultry, lean meats and ≥ 25 g/day of fibre.

- *Appropriate Body Weight.* Match energy intake (limitation of high caloric density foods) to energy expenditure.

- *Desirable Cholesterol Profile*. Limit foods high in saturated fat and cholesterol; and consume unsaturated fat from vegetables, fish, legumes, nuts.

- *Desirable Blood Pressure*. Limit salt and alcohol consumption; maintain a healthy body weight and a diet with emphasis on vegetables, fruits, and skimmed dairy products.

- The Mediterranean diet

In the 1950s, the Seven Countries Study showed that, despite a high fat intake, the population of the island of Crete (Greece) had a long life expectancy, and very low prevalence of coronary heart disease and certain types of cancer. The good health observed in Crete, and also in other Mediterranean regions, was attributed to the dietary patterns typical of the Mediterranean countries (Keys, 1995; Willett *et al.*, 1995).

The traditional MedDiet is characterized by a low intake of dairy products, red meat, processed food, and sweets; a moderate intake of fish and poultry; a high intake of olive oil, fruit, nuts, vegetables, and cereals; and wine in moderation with meals (Estruch *et al.*, 2013).

Adherence to the MedDiet might be used for the prevention and treatment of CVD (Fung *et al.*, 2009; Estruch *et al.*, 2013). In this sense, the PREvención con DIeta MEDiterránea (PREDIMED) study, based on a MedDiet supplemented with either extra-virgin olive oil or nuts, is the first large randomized trial showing that a

MedDiet is able to diminish the incidence of cardiovascular events among individuals at high cardiovascular risk (Estruch *et al.*, 2013). This study showed that a MedDiet might reduce heart rate (Garcia-Lopez *et al.*, 2014), the incidence of T2DM (Salas-Salvado *et al.*, 2014), and inflammation (Estruch, 2010), as well as increasing plasma total antioxidant capacity (TAC) levels (Zamora-Ros *et al.*, 2013).

In addition, the MedDiet reduces the odds of having MetS (Babio *et al.*, 2009). A recent meta-analysis has shown that a greater adherence to the MedDiet is associated with lower WC, hypertension and TG levels and higher high-density lipoprotein cholesterol (HLD-c) (Kastorini *et al.*, 2011).

These beneficial properties have been attributed to a number of healthy components of the MedDiet. A diet rich in monounsaturated fatty acids (MUFAs) contributes to a more anti-inflammatory gene expression profile. The traditional MedDiet is characterized by being a good source of complex CHO and fibre, which produce a low glycaemic index (GI) decreasing the risk of suffering hyperglycaemia, T2DM, low-grade inflammation, oxidative stress and obesity. Moreover, the MedDiet is a good source of antioxidant vitamins, phytochemicals and minerals (Corella *et al.*, 2014).

1.5.3. Dietary components

- Macronutrient distribution

A low-fat diet (≤ 30% TCV from fat) has been traditionally recommended for weight loss. Specifically the reduction of saturated fatty acids (SFAs) and trans fats are beneficial for T2DM and other obesity comorbidities (ADA, 2009). However, individuals may fail in maintaining adherence to this diet in the long-term (Abete *et al.*, 2010).

On the other hand, low-CHO diets (≤45% of TCV from CHO) have become popular for weight loss in the last years. The lower CHO intake should be replaced by a higher protein consumption. It is known that CHO consumption reduces the use of fat for energy (Marques-Lopes *et al.*, 2001). Excess CHO are converted to fatty acids (FAs) and triacylglycerol through de novo lipogenesis, which increase the formation of TG and cholesterol (Hudgins *et al.*, 2000). Hence, low-CHO diets are associated with favourable changes in CVD risk factors (Foster *et al.*, 2010).

Moreover, better maintenance of blood pressure reduction (Engberink *et al.*, 2014) and weight-loss (Johansson *et al.*, 2014), as well as higher satiety and basal energy expenditure (Bendtsen *et al.*, 2013), have been observed in subjects following low-CHO diets together with increased intake of protein. Additionally, differences between the thermogenic effect of protein, CHO and fat have led to the hypothesis that dietary composition might affect energy expenditure (Bray *et al.*, 2012).

Moreover, the Diet, Obesity and Genes (DiOGenes) study, found that a modest increase in protein content and a reduction in the GI led to an improvement in study completion and maintenance of weight loss (Larsen *et al.*, 2010).

In contrast, the Prevention of Obesity Using Novel Dietary Strategies (POUNDS LOST) study showed that resting energy expenditure fell significantly after weight loss but was not related to diet composition, but it was attributed to the decrease in body weight (de Jonge *et al.*, 2012).

A meta-analysis comparing low-CHO diets with low-fat diets displayed that both dietary strategies were equally effective at reducing body weight and WC. However, those individuals following a low-CHO diet had greater decreases in TG and higher increases in HDL-c, but less reduction in low-density lipoprotein cholesterol (LDL-c) when compared with individuals on low-fat diets (Hu *et al.*, 2012).

- Glycaemic index

The GI is defined as the area under the blood glucose curve after the consumption of 50 g CHO of a test food relative to the consumption of 50 g of CHO from standard food of either white bread or glucose (Wolever *et al.*, 1991). Consistent with this, the GI provides a good summary of postprandial glycaemia and it may predict the peak response, the maximum glucose fluctuation, and other attributes of the response curve (Brand-Miller *et al.*, 2009). Evidence suggests that low-GI diets have a beneficial effect on glycaemic control, low-grade inflammation, body weight and CVD, while high-GI diets increase the risk of suffering obesity, T2DM, hyperglycaemia, insulin resistance and hypertriglyceridemia (Zulet *et al.*, 2011; Mirrahimi *et al.*, 2014). All these comorbidities are well-known risk factors for the development of the MetS (Abete *et al.*, 2011).

Additionally, high-GI meals may rise blood glucose levels after food consumption, inducing hormonal changes that may stimulate hunger and inhibit fat oxidation, favouring the catabolism of lean body mass (Abete *et al.*, 2008).

The foods with the lowest GI are non-starchy vegetables, fruit, legumes, pasta and dairy products (Barclay *et al.*, 2008). Moreover, the MedDiet has been proposed as having the most favourable effect on glycaemic control (Shai *et al.*, 2008), reducing the incidence of T2DM (Rossi *et al.*, 2013).

- Fatty acids

FAs are involved in many cellular processes, serving as energy reservoirs and even regulating gene expression (Warensjo *et al.*, 2006; Bjermo *et al.*, 2010). The FA composition in the human body mirrors both the dietary fat composition and the endogenous synthesis and metabolism of FAs, mainly by FA synthesis from CHO (Warensjo *et al.*, 2006; Bjermo *et al.*, 2010). Dietary fats consist of TG molecules of three individual FAs, each linked by an ester bond to a glycerol backbone and differing in chain length and saturation (Chowdhury *et al.*, 2014). High levels of total FAs in blood have been positively related with CVD, particularly with the development of the MetS (Xie *et al.*, 2012a; Kien *et al.*, 2013). However, evidence suggests that the dietary fat quality rather than quantity might have a greater influence on health and disease (Jakobsen *et al.*, 2009; Kien *et al.*, 2013).

LDL-c, TG and TC are known to be raised by SFAs in the bloodstream, which increase cardiovascular risk (Lopez-Alvarenga *et al.*, 2010; Flock *et al.*, 2013). Saturated fats are found for example in red meat and high-fat dairy products, and are predominant in Western diets (Astrup *et al.*, 2011).

Oleic acid is the most abundant MUFA presented in the diet, which health benefits have been widely demonstrated (Kien *et al.*, 2013). Oleic acid is the major FA in olive oil, and its consumption has been related with lower risk of stroke (Martinez-Gonzalez *et al.*, 2014). However, the role of other MUFAs in human metabolism, as is the case of palmitoleic acid (C16:1), has not been fully clarified, although high levels of this particular FA have been associated with increased risk of suffering CVD (Gong *et al.*, 2011).

In contrast, polyunsaturated fatty acids (PUFAs) have different roles on human health. While ω-6 PUFAs may contribute to an increased expression of lipogenic genes, ω-3 PUFAs are thought to have the opposite effect (Lorente-Cebrian *et al.*, 2013; Muhlhausler *et al.*, 2013; Chowdhury *et al.*, 2014). The potential beneficial effects of long chain ω-3 PUFAs, concretely α-linolenic acid (ALA), eicosapentanoic acid (EPA), and docosahexaenoic acid (DHA), have been exhibited in both epidemiological and clinical studies. The major food source of EPA and DHA is fish (finfish and shellfish), while ALA is mainly found in seeds, nuts, and their oils (Poudyal *et al.*, 2011b).

The anti-inflammatory properties of ω-3 PUFAs have been well-established, although the underlying mechanisms remain poorly understood. Recently, it has been proposed that ω-3 PUFAs may prevent inflammation and metabolic disorders through inhibition of pyrin domain-containing 3 (NLRP3) inflammasome activation (Yan *et al.*, 2013). It has been also stated that the consumption of this long-chain FAs reduces total and LDL-c as well as blood pressure and heart rate. Moreover, ω-3 PUFAs have anti-thrombotic effects and may also improve glucose handling and increase serum total antioxidant levels (Mozaffarian *et al.*, 2011; Poudyal *et al.*, 2011a).

However, supplementation with ω-3 PUFAs was not associated with a lower risk of major cardiovascular outcomes (Rizos *et al.*, 2012; Chowdhury *et al.*, 2014) and its association with weight loss remains controversial (Munro *et al.*, 2012).

- Fibre

Dietary fibre might be defined as a plant cell wall, which is indigestible by endogenous animal enzymes (Van Soest, 1978). The consumption of fibre has been reduced in the past decades since current western intakes provide between 10-20 g/day, while it has been estimated that the Palaeolithic diet supplied > 100 g/day of fibre (Frost *et al.*, 2014).

Fermentation of fibre by the colonic microbiota has been proposed to produce positive effects on human metabolism. Higher dietary fibre intake may modulate glycaemia and insulinemia, and decrease blood lipids (TG, LDL-c and TC) and blood pressure. Moreover, fibre consumption has been related to lower dietary intake,

body weight and adiposity. Fibre is thought to promote the release of the anorectic and antidiabetic gut hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1). Moreover, the positive effect of indigestible CHO on body weight might be also mediated by the short-chain FA acetate, which reduces appetite via a central homeostatic mechanism (Delzenne *et al.*, 2005; Abete *et al.*, 2011; Frost *et al.*, 2014).

- Dietary antioxidants

Dietary TAC represents the sum of antioxidant presented in a food, without distinguishing the contribution from each individual component (Puchau *et al.*, 2010). A diet rich in fruits, vegetables, legumes, olive oil, red wine, green tea and nuts has been proposed to be protective against various diseases, including cancer and CVD. This positive effect is mainly due to their antioxidants properties, which decrease the risk of developing oxidative stress-related diseases (Puchau *et al.*, 2010; Abete *et al.*, 2011).

Thus, the consumption of food rich in antioxidants is proposed for the treatment of CVD and obesity (Lopez-Legarrea *et al.*, 2013). However, the role of antioxidants supplementation on health remains controversial (Alberdi *et al.*, 2014; Murer *et al.*, 2014).

- Meal frequency and chrononutrition

In the past decades, the changes in lifestyle habits such as variations in meal times and deprived of sleep, have altered circadian clocks. The circadian system is connected to energy homeostasis, and can be affected by feeding time as well as by food composition (Garaulet *et al.*, 2014; Oike *et al.*, 2014).

Portion distortion refers to the perception of over-sized portions as appropriate amounts to eat, and may be an important contributor to the obesity epidemic. Thus, portion control at meals is crucial in every weight-loss programme (ADA, 2009).

The consumption of small and frequent meals has been related to weight control. The possible mechanisms are that frequent meal consumption may increase energy expenditure and fat oxidation, as well as maintaining plasma glucose concentrations, leading to better appetite control; however, no consensus has been reached (Ohkawara *et al.*, 2013).

The timing of food intake itself may have a significant role in obesity and in the success of a weight-loss treatment, with changes in the chronotype, genetic background and/or circadian system function playing a crucial role (Garaulet *et al.*, 2014).

Thus, it has been observed that individuals who were allowed to consume the fast day meal at dinnertime, or who divided the meal into smaller meals throughout the day, experienced a higher weight loss with alternate day fasting (Hoddy *et al.*, 2014). Although timing of food has been proposed as a novel therapeutic strategy for obesity and weight loss (Garaulet *et al.*, 2014), research on eating frequency and timing is not extensive and more studies are urgently needed (ADA, 2009; Raynor *et al.*, 2014).

- Personalized nutrition

The fact that not all individuals respond equally to the same dietary treatments, suggest that some of these variations might be due to genetic factors (Konstantinidou *et al.*, 2014).

Recently emerging data from genomics studies have led to identification of some gene–nutrient interactions that may be related to obesity and body change as well as with dietary factors such as the intake of energy and macronutrients (Huang *et al.*, 2014; Qi, 2014). Therefore, the effectiveness of personalized nutrition as a more suitable tool for the treatment of obesity instead of the traditional recommendations is under study (Konstantinidou *et al.*, 2014). Although this theory has just emerged, a personalized nutrition approach for the treatment of obesity might be incorporated to clinical practice in the near future (Perez-Martinez *et al.*, 2012; Konstantinidou *et al.*, 2014).

1.6. New metabolomic and epigenetic biomarkers in obesity

Clinically, the identification of new peripheral biomarkers combined with available diagnostic criteria, may be helpful for early diagnosis and to predict future onset of a disease (Lopresti *et al.*, 2014).

1.6.1. Omic techniques: metabolomics

The scientific techniques and approaches used in molecular biology noticeably changed in the nineties due to the determination of the human genome (Lindon *et al.*, 2006). This transformation of molecular biology has led to new diagnostic methods and to better targeted therapies.

These new methods have been proposed to be classified together under the general term of "omic" techniques, which includes genomics, transcriptomics, proteomics and metabolomics (Ordovas, 2009). The growth of omics sciences has allowed determinations at various levels, from the cell to the whole organism. However, these technologies generate enormous volume of information, therefore bioinformatics and software and biostatistical methods are of great importance for disease classification and therapy (Ordovas, 2009; Patti *et al.*, 2012).

Many studies have shown that certain environmental factors, among them lifestyle habits, may disturb gene and protein expression and also affect metabolite levels (Patti *et al.*, 2012). The metabolome may provide valuable functional information, since it mirrors the genetic variations, disease or environmental influences that occur in the organism. Thereby, metabolites reflect more accurately the phenotype than genes and proteins, which might be affected by epigenetic and post-translational modifications, respectively (**Figure 5**).

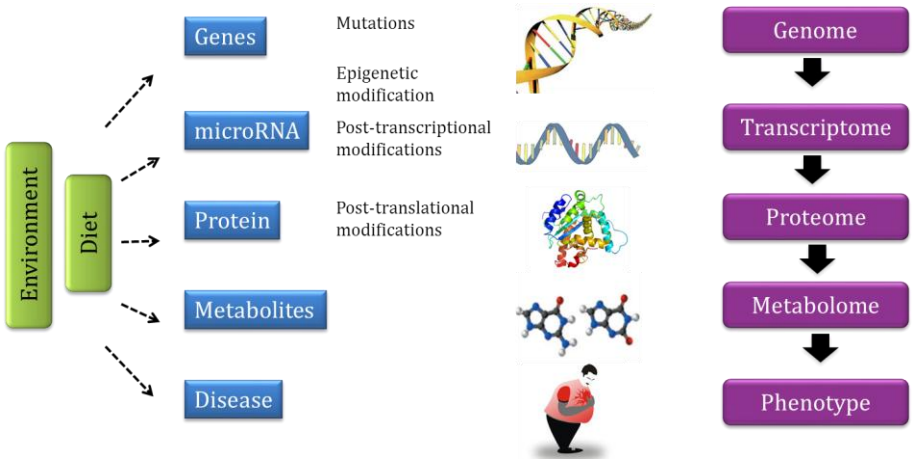

Figure 5. Omic techniques. Abbreviation: RNA, ribonucleic acid. Adapted from: (Patti *et al.*, 2012).

Metabolomics is an important "omic" discipline that aims to identify and quantify the metabolome (Weckwerth *et al.*, 2005). It is the study of a wide range of low molecular weight metabolites occurring in biological samples such as biofluid, cellular extract or culture media, which are intermediates and products of metabolism (Patti *et al.*, 2012). These small molecules are involved in many biochemical processes such as cellular functions, signalling or proteins and DNA formation. Metabolomics provides a wide range of information about the effects caused by changes in gene expression and also those caused by lifestyle factors, allowing to set relationships between phenotype and genetics. Hence, metabolomics is able to display alterations of metabolic pathways linked to phenotypic perturbations (Patti *et al.*, 2012).

Due to the wide variety of endogenous metabolites, it is crucial to determine the number of metabolites to be measured before performing a metabolomics analysis. This choice will determine the sample preparation and the required instrumentation (Patti *et al.*, 2012).

Targeted metabolomics: The targeted approach is focused on one or more related pathways, in which well-defined and expected endogenous metabolites are quantified (Griffiths *et al.*, 2010). It is essential to have a previous hypothesis in order to investigate a particular pathway. This approach allows the quantification of sugars, AAs or FAs, among others.

Untargeted metabolomics: This method is mainly used for global metabolome analysis and it consists on the measurement of as many metabolites as possible from a determined biological sample without bias (Patti *et al.*, 2012). This approach is not used to validate a hypothesis but to generate one. It permits to compare the metabolites between two or more sample groups (for example, men *vs*. women, or healthy *vs*. disease), allowing to discover metabolites that were not known before (Griffiths *et al.*, 2010; Patti *et al.*, 2012).

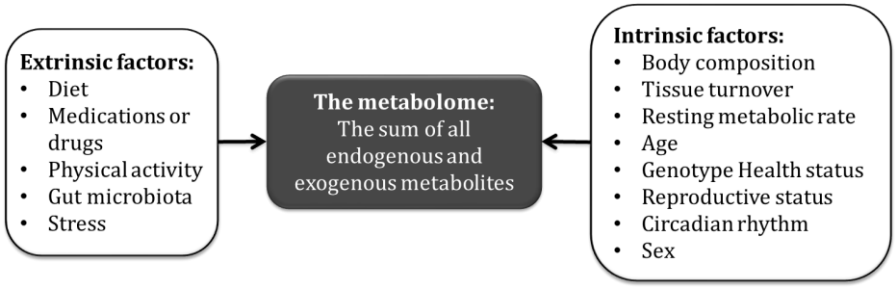

Figure 6. Exogenous and endogenous factors likely to influence the human nutritional metabolome. Adapted from: (Gibney *et al.*, 2005).

There are many factors affecting the human metabolome, that can be divided into intrinsic and extrinsic factors (Gibney *et al.*, 2005). Among them, the diet is an important extrinsic factor that plays a key role in human metabolome (**Figure 6**). For this reason, the use of metabolomics in nutrition research is increasing and applications range from assessing novel biomarkers of dietary intake to its use in dietary intervention studies (Brennan, 2013). The application of metabolomics to assess the response to a dietary intervention generates valuable information on the effect and predisposition of a prescribed diet on metabolic regulation. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the two main methods used in nutritional metabolomics. These approaches have their advantages and disadvantages and currently, there is no unique analytical technique capable of measuring and identifying all metabolites in a single sample simultaneously. Consequently, comprehensive metabolomic data needs to be assessed by bringing

together data from different platforms (Sumner *et al.*, 2003; Walsh *et al.*, 2006; Etxeberria *et al.*, 2013a).

1.6.2. Epigenetics: DNA methylation

The epigenetic mechanisms have been defined as heritable changes in gene expression that cannot be explained by changes in DNA sequence (Milagro *et al.*, 2013). Specifically, they refer to changes on the genome that are copied from one cell generation to the next, which may alter gene expression, but that occur without changes in the DNA sequence (Campion *et al.*, 2009).

The most extensively studied epigenetic mechanisms are covalent histone modifications, microRNA alterations and DNA methylation of CpG residues (Campion *et al.*, 2009; Ordovas *et al.*, 2010).

Nowadays, DNA methylation is the most studied epigenetic mark (van Dijk *et al.*, 2014). DNA methylation is a covalent modification of DNA caused by the addition of methyl groups to cytosine bases in DNA (**Figure 7**). Methyl groups are added to cytosine in DNA by DNA methyltransferases (DNMTs), which catalyse the addition of a methyl group to the 5-position of cytosine ring in 5'- to 3'-oriented CG dinucleotides known as CpGs sites (Craig *et al.*, 2011; Webster *et al.*, 2013).

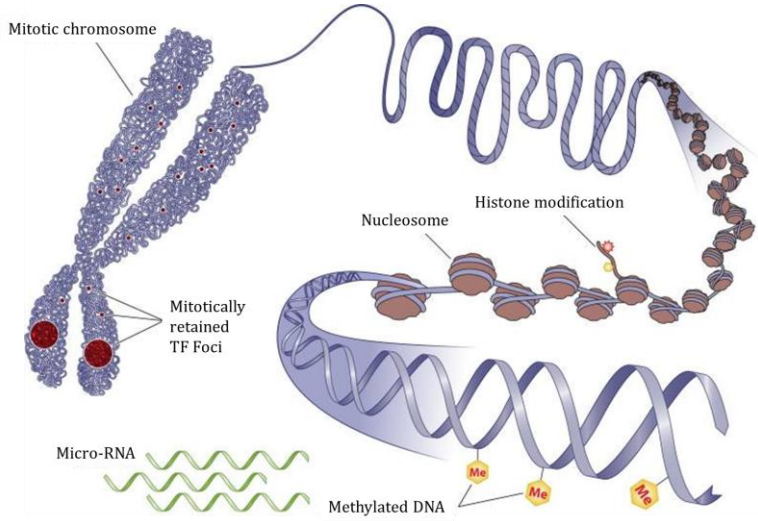

Figure 7. Mechanisms of inheritable epigenetics. Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid. Source: (Zaidi *et al.*, 2010).

The enzymes DNMTs are part of a complex regulatory network, in which they not only interact with each other, but also with histone deacetylases, histone methyltransferases and methylcytosine-binding proteins (Campion *et al.*, 2009). DNMT3a and DNMT3b are responsible for the creation of DNA methylation patterns during embryonic development. In contrast, DNMT1 is thought to maintain methylation marks during DNA replication (Webster *et al.*, 2013).

Methylation of CpG sites in the promoter region of a gene is considered an effective mechanism of silencing gene expression (Craig *et al.*, 2011). This might be due to the fact that methyl-cytosines in the recognition elements of transcription factors could block their binding with the subsequent reduction in the transcriptional activity.

Environmental factors, such as dietary intake and physical activity, are important contributors to changes in DNA methylation (Campion *et al.*, 2009; Hermsdorff *et al.*, 2013). Numerous studies have shown that some nutrients and bioactive compounds affect the pathways involved in DNA methylation (Supic *et al.*, 2013). In this sense, adequate dietary levels of methyl-donor precursors (methionine, choline, betaine, and vitamins B_2, B_6 and B_{12} or folate) have been demonstrated to be important to prevent obesity and psychiatric diseases (Abdolmaleky *et al.*, 2004; Cordero *et al.*, 2013). Dietary methyl-donors deficiency may also induce cancer through DNA hypomethylation (Cordero *et al.*, 2013; Supic *et al.*, 2013).

One of the most studied nutrients that affect DNA methylation is folate (Milagro *et al.*, 2013). The deficiency of this B vitamin during pregnancy increases the risk of neural tube defects, making it crucial for normal fetal development. In this context, an abnormal DNA methylation pattern has been proposed as a possible mechanism, since folate status is associated with decreases in global DNA methylation (Canani *et al.*, 2011; Crider *et al.*, 2012).

1.7. The metabolic syndrome

The MetS, also known as syndrome X, the insulin resistance syndrome and the deadly quartet, is defined as a cluster of mayor cardiovascular risk factors, including central obesity, glucose intolerance, elevated blood pressure and dyslipidaemia (Eckel *et al.*, 2005). Hence, this syndrome emphasizes the co-occurrence of risk factors for CVD.

1.7.1. Characteristics of the metabolic syndrome: aetiology and consequences

Since in many cases it is difficult to difference between the causes and the consequences of the MetS, they need to be considered together.

Like obesity, the MetS might be triggered by both genetic and environmental factors, as well as by their interactions. Nonetheless, the mechanisms underlying the MetS are not fully understood, having a multifactorial aetiology (Huang, 2009).

Insulin resistance and abdominal obesity are considered among the main characteristic of the MetS, and are both well-known risk factors for CVD. Unhealthy lifestyle habits such as lack of physical activity, unbalanced diet, cigarette smoking and alcohol consumption are known to increase the risk of MetS. Other factors as for example aging, hypertension, dyslipidaemia, family history of premature coronary heart disease, elevated TG, oxidative stress, low-grade inflammation and prothrombotic state, are identified as having an untoward effect on CVD (Grundy *et al.*, 2004).

Central obesity is one of the main factors contributing to the development of the MetS (Alberti *et al.*, 2005; Alberti *et al.*, 2009). Excess of central adiposity is known to be more harmful to health than fat located in the subcutaneous region, hips or thighs. Adipose tissue is recognised not only as an energy storage tissue, but also as a multifunctional organ. With the expansion of adipose tissue in obesity there is an excess release of free fatty acids (FFAs) and other damaging molecules into the circulation, increasing the risk of T2DM and CVD (Bray *et al.*, 2006).

The following factors might be considered both as causes and consequences of the MetS:

- *Hyperglycaemia*: impaired fasting glucose, impaired glucose tolerance and insulin resistance play a key role in the development of T2DM, and so, in CVD. This disorder is strongly involved in the progress of other metabolic risk factors, inducing atherosclerosis, hypertension, increased systemic inflammation and TG levels, or pancreatic β-cells dysfunction, through a variety of mechanism (Grundy *et al.*, 2004; Malin *et al.*, 2014).
- *Hypertension*: High blood pressure is a multifactorial vascular disorder highly associated with obesity, T2DM and CVD (Bray, 2007). Overweight individuals with hypertension show altered sympathetic activity and also, a thickening of the ventricular wall and larger heart volume, presenting a higher risk of cardiac failure. In addition, subjects with hypertension have a greater likelihood of stroke and kidney disease than healthy individuals. Importantly, for each decline of 1 mmHg in diastolic blood pressure (DBP), there is a decrease in the risk of myocardial infarction between the 2 and the 3% (Bray, 2007). In Europe, the prevalence of hypertension appears to be around 30-45% of the general population, with the rate increasing with aging (Mancia *et al.*, 2013).
- *Dyslipidaemia*: This disease is primarily characterized by raised TG and total and LDL-c, as well as low concentrations of HDL-c. All these abnormalities increase the risk of atherosclerotic cardiovascular disease (ASCVD), which has emerged as the leading cause of death worldwide (IAS, 2014). Atherosclerosis is a complex inflammatory disease that involves the formation of lesions in the arteries that are characterized by lipid peroxidation, inflammation, cell death and fibrosis, which may lead to foam cells formation and ultimately to atherosclerotic plaque (Hansson *et al.*, 2006).
- *Low-grade inflammation*: Increased accumulation of macrophages due to excess adiposity may lead to dysregulation of cytokine production and the activation of proinflammatory signalling pathways, leading to the increase of inflammatory markers in the circulation and within specific tissues. Low-grade inflammation may play pathogenic roles in insulin resistance, T2DM, and CVD, promoting the development of MetS (Romeo *et al.*, 2012; Fuentes

et al., 2013). Some of the most studied inflammatory markers are C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF)-α, which increases have been related to obesity and the MetS (Zulet *et al.*, 2007; Lopez-Legarrea *et al.*, 2014).

- *Oxidative stress*: Augmented vascular reactive oxygen species (ROS) production disturbs the balance between oxidant and antioxidant factors in the body, which leads to a pro-oxidative condition (de la Iglesia *et al.*, 2013). Accordingly, a positive association between oxidative stress and vascular inflammation, augmented adipogenesis, T2DM, obesity, hypertension and dyslipidaemia has been found. On the other hand, ROS might be augmented by hyperglycaemia and inflammation, forming a harmful vicious circle (Hopps *et al.*, 2010; Youn *et al.*, 2014). Malondialdehyde (MDA), which is a product of lipid peroxidation, and oxidized low density lipoproteins (oxLDL), that consist of LDL-c particles modified by oxidation, are important biomarkers of oxidative stress (de la Iglesia *et al.*, 2013).
- *Prothrombotic state*: Endothelial dysfunction is implicated in the pathogenesis of many diseases, like occlusive thrombus formation in arteries and veins. FFAs and low-grade inflammation have been directly associated with prothrombotic state, increasing the risk of atherosclerosis, hypertension and the MetS (Grundy *et al.*, 2004; Xie *et al.*, 2012b).
- *NAFLD*: This disorder is characterized by excess fat in the liver due to non-alcoholic causes, which contributes to T2DM and dyslipidaemia (Yki-Jarvinen, 2014).

Likewise, sleep apnoea, stigma, psychological disorders and cancer (**Figure 8**) are also known as important consequences of the MetS (Grundy *et al.*, 2004; Pan *et al.*, 2012a).

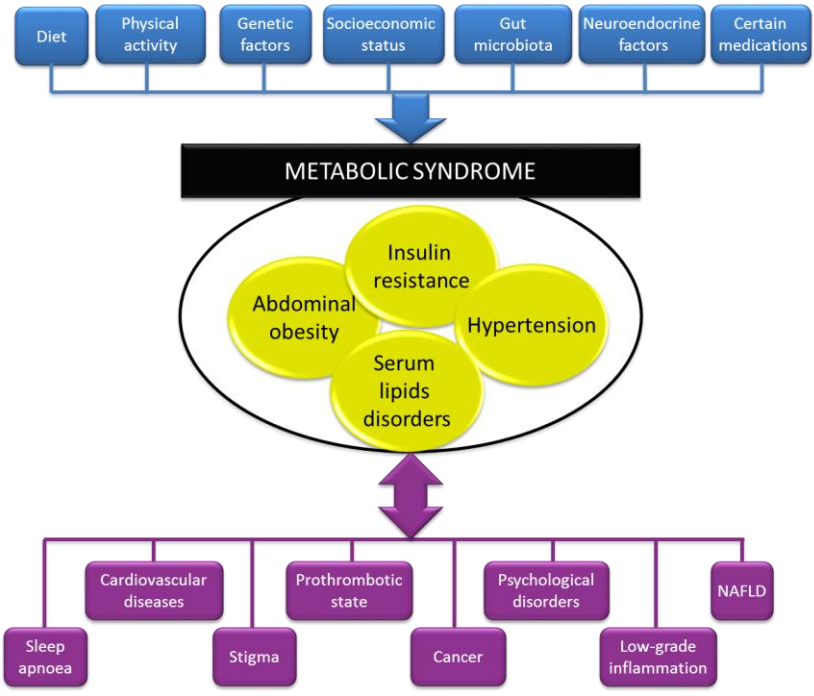

Figure 8. Main causes and consequences of the MetS. Abbreviation: NAFLD, non-alcoholic fatty liver disease.

Thus, the MetS has multiple causes that may also interact with each other and dramatically increase morbidity and mortality (Grundy *et al.*, 2004).

1.7.2. Diagnosis

Nowadays, there are numerous diagnosis methods for identifying the MetS, with a lack of a universal accepted definition (Alberti *et al.*, 2009). The diagnosis of the MetS takes into account the presence and degree of the main features of the disease (**Table 7**); however, different thresholds have been proposed (Huang, 2009).

The WHO was the first international organization that projected a definition for the MetS at the end of the 90s, establishing insulin resistance as the key factor of the disorder (Alberti *et al.*, 1998). This initiative was followed by the European Group for the Study of Insulin Resistance (EGIR), that also considered mandatory to present insulin resistance in order to be diagnosed as having the MetS (Balkau *et al.*, 1999).

In 2001 the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) introduced alternative clinical criteria for the diagnosis of the MetS, which did not require insulin resistance as the primary component (ATP-III, 2001). This definition was revised by the American Heart Association/National Heart, Lung, and Blood Institute (AHA/NHLBI) in 2005, which changed the thresholds for WC and hyperglycaemia proposed by the NCEP-ATP III (Grundy *et al.*, 2005). Finally, the IDF exposed in 2005 that abdominal obesity was an indispensable requirement for the diagnosis (Alberti *et al.*, 2005). In 2009, a meeting between several major organizations in an attempt to unify the MetS criteria established that there should not be an obligatory component, but that waist measurement would continue to be a useful preliminary screening tool (Alberti *et al.*, 2009). Currently, the NCEP-ATP III and the IDF criteria are the most widely accepted definition of the MetS worldwide (Huang, 2009).

Table 7. Criteria for the diagnosis of the MetS.

	WHO (Alberti *et al.*, 1998)	**EGIR** (Balkau *et al.*, 1999)	**NCEP-ATP III** (ATP-III, 2001)	**IDF** (Alberti *et al.*, 2005)	**AHA/NHLBI** (Grundy *et al.*, 2005)
Absolutely required	Insulin resistance defined as IGT, IFG, or T2DM	Insulin resistance (plasma Insulin > 75th percentile) in nondiabetic patients	None	Central obesity WC: ≥ 94 cm (M) ≥ 80 cm (W)	None
Criteria for diagnosis	Insulin resistance plus 2 of the following:	Insulin resistance plus 2 of the following:	Any 3 of the following:	Central obesity plus 2 of the following:	Any 3 of the following:
Central obesity	BMI ≥ 30 kg/m^2 or Waist/hip ratio: > 0.90 (M) > 0.85 (W)	WC: ≥ 94 cm (M) ≥ 80 cm (W)	WC: > 102 cm (M) > 88 cm (W)	Central obesity already required	WC: ≥ 102 cm (M) ≥ 88 cm (W)
Hyperglycemia	Insulin resistance already required	Insulin resistance already required	Fasting glucose ≥ 110 mg/dl	Fasting glucose ≥ 100 mg/dl	Fasting glucose ≥ 100 mg/dl
Hypertension	≥ 140/90 mmHg	≥ 140/90 mmHg	≥ 130/85 mmHg	≥ 130/85 mmHg	≥ 130/85 mmHg
Hypertriglyceridemia	≥ 150 mg/dL	≥ 150 mg/dL	≥ 150 mg/dL	≥ 150 mg/dL	≥ 150 mg/dL
Low HDL-c	< 35 mg/dL (M) < 39mg/dL (W)	< 39mg/dL	< 40 mg/dL (M) < 50 mg/dL (W)	< 40 mg/dL (M) < 50 mg/dL (W)	< 40 mg/dL (M) < 50 mg/dL (W)
Others	Microalbuminuria[1]				

[1] urinary albumin excretion ≥ 20 μg/min or albumin/creatinine ratio ≥ 30 mg/g. Abbreviations: AHA/NHLBI, American Heart Association/National Heart, Lung, and Blood Institute; BMI, Body Mass Index; EIGIR, European Group for the Study of Insulin Resistance; HDL-c, high-density lipoprotein cholesterol; IDF, International Diabetes Federation; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; M, men; NCEP-ATP III, National Cholesterol Education Program Adult Treatment Panel III; T2DM, type 2 diabetes; W, women; WHO, World Health Organization; WC, waist circumference.

1.7.3. Prevalence

As in obesity, the prevalence of MetS is rapidly increasing in most countries. However, the disagreement in the diagnosis of the MetS makes difficult to establish a prevalence of the disorder (Huang, 2009). Using the IDF criteria, a prevalence of 13–30% in developing countries and approximately 30–35% in developed countries has been estimated (Cameron *et al.*, 2004; Tauler *et al.*, 2014).

Accumulating evidence indicates that the prevalence of MetS increases with age and differs between ethnicities (Razzouk *et al.*, 2009). However, controversy exists concerning the prevalence rates of the disorder between sexes, although a recent review suggests that the prevalence is higher in women than in men (Pradhan, 2014).

The MetS is present in approximately one-fourth of the adult European population (Grundy, 2008). According to the Biobank Standardisation and Harmonisation for Research Excellence in the European Union (BioSHaRE-EU) Healthy Obese Project, there is considerable variability in the prevalence of MetS in Europe. For instance, among obese people, the rates are around 33.5% in Italy, while in Finland oscillates the 71%, being higher in men than in women (van Vliet-Ostaptchouk *et al.*, 2014).

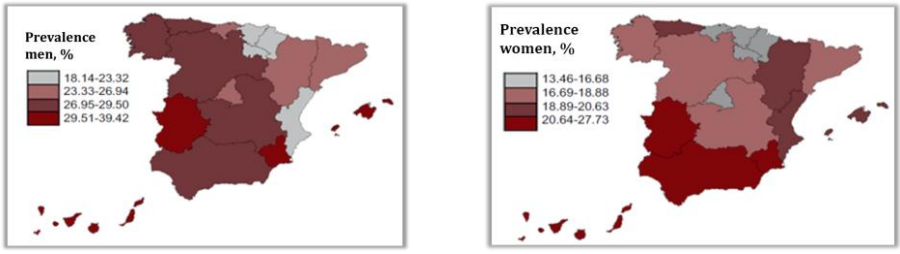

Figure 9. Prevalence of MetS in Spain. 18 years or older. Source: (Guallar-Castillon *et al.*, 2014).

In Spain, two studies have recently reported data of the prevalence of MetS. On the one hand, the ENRICA Study has published that the prevalence of MetS is of 22.7% in adults aged 18 years and older (**Figure 9**) (Guallar-Castillon *et al.*, 2014). On the other hand, another study has shown that Spanish adult workers had a prevalence of 12.4% using ATP III criteria, and 16.5% using IDF criteria (Tauler *et al.*, 2014).

Finally, the prevalence of MetS in children and adolescents is around 3.3% worldwide, suggesting that intervention in early childhood may ameliorate later MetS (Friend *et al.*, 2013).

1.7.4. Treatment

Weight loss alone is often considered the first-line treatment for MetS, since it may improve all its components (Bray, 2007). Consequently, the previously detailed treatment for obesity (section 1.5.) might be also considered of great interest for the MetS.

Specifically, a diet rich in a variety of fruits, vegetables, whole grains, legumes, fish oils, dairy product and nuts has been recommended for the treatment of the MetS (Abete *et al.*, 2011). Legumes may reduce lipid peroxidation as well as LDL-c and TC (Crujeiras *et al.*, 2007). Moreover, it increases satiety and may reduce the negative effect of caloric restriction on lean mass loss and basal metabolic rate.

Additionally, the Dietary Approaches to Stop Hypertension (DASH) diet, based on small amounts of total and saturated fat and cholesterol and larger amounts of potassium, calcium, magnesium, dietary fiber, and protein have proved to be effective in reducing blood pressure (Sacks *et al.*, 2001; Hikmat *et al.*, 2014).

However, treatment must be personalized, and in some cases the individual treatment of each component is mandatory, being necessary the use of anti-hypertensive, hypolipidaemic or hypoglycaemic drugs (Bray, 2007).

2. Mental and behavioural disorders in obesity

2.1. Mental and behavioural disorders

2.1.1. Mood disorders: major depressive disorder

The definition and diagnosis of mood disorders have been controversial due to the multifactorial causation, highly individualized and variable course, and different response to treatment (WHO, 1992; Belmaker *et al.*, 2008). Although both the WHO and the American Psychiatric Association have described the disorder, there is no universal definition (WHO, 1992; DSM-V, 2013). In this context, mood (affective) disorders might be generally defined as those illnesses that have a disturbance in mood or affect (depressed mood or elation) as the predominant feature (WHO, 1992; DSM-IV, 2000).

The 10th International Classification for Diseases (ICD-10) was designed by the WHO in the 90s (WHO, 1992). The 11th revision of this classification is currently being updated and it is expected to appear in 2017 (WHO, 2014). On the other hand, the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) has been recently released based on current scientific evidence, but some of the changes with respect to the previous version (DSM-IV, 2000) have been subject of debate (Nemeroff *et al.*, 2013).

Based on their symptomatic differences, mood disorders are generally categorized into depressive disorders ("unipolar depression"), bipolar disorders, mood disorder due to a general medical condition and substance-induced mood disorder (DSM-IV, 2000). This classification is based on the symptomatic differences since it has not been evidenced that there exist different underlying disease states (Nemeroff *et al.*, 2013).

The two most widely used self-report tools to assess general mood state are the Profile of Mood States (POMS) (McNair, 1971) and the mood thermometer visual analogue scale (VAS) (Mitchell *et al.*, 2012).

The WHO defines unipolar depression or major depressive disorder (MDD) as a common mental disorder characterized by low mood, loss of interest or pleasure,

feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness, and poor concentration. Moreover, it is one of the most commonly diagnosed psychiatric illnesses (Marcus M, 2012).

2.1.1.1. Aetiology

The mood disorder MDD is a multifactorial disease caused by biological, environmental, and psychological factors, or by the interaction of these elements (Belmaker *et al.*, 2008). This disease is likely to be the result of alterations in multiple pathways, and not due to a single one (Stone *et al.*, 2008). Some of the multiple mechanisms implicated in this complex disease are explained below (**Figure 10**). Functional neuroimaging studies carried out in depressed individuals have shown a hypoactivity of brain areas involved in positively motivated behaviour together with a hyperactivity in regions implicated in stress adaptation (Stone *et al.*, 2008).

The *monoamine theory* of depression, proposed by Schildkraut in 1965, has been widely accepted for several decades (Schildkraut, 1965). According to this, depression is caused by dysfunction of serotoninergic or noradrenergic systems in the CNS. This theory is based on two principles: 1) the capacity of some antidepressants (for example tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs)) to enhance monoaminergic transmission by blocking the reuptake of noradrenaline (NA) and 5-HT in the presynaptic neuron or by preventing deamination of substrates, respectively; 2) the capacity of drugs like reserpine to induce depression (Belmaker *et al.*, 2008).

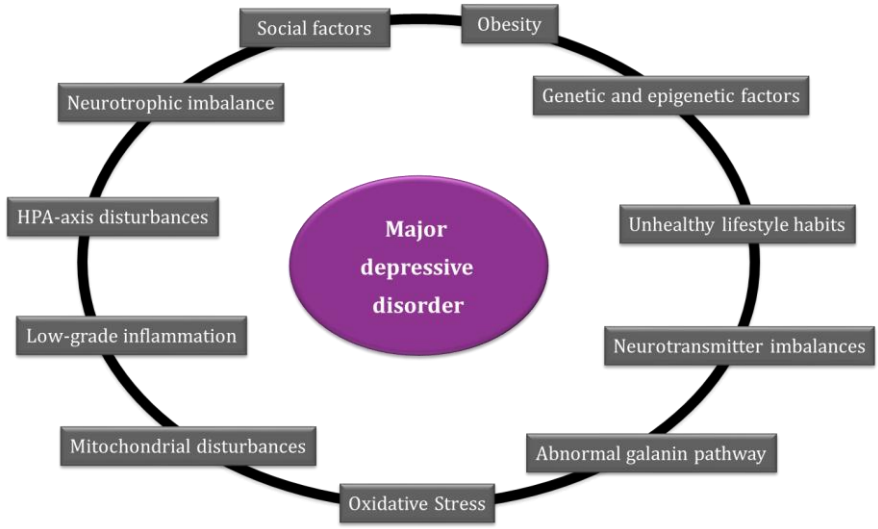

Figure 10. Main causes of MDD. Abbreviation: HPA-axis, hypothalamic pituitary adrenal axis.

First-degree relatives of patients with MDD have two to four times higher risk of suffering this disorder when compared with the general population. *Heritability* in MDD is around 40%, with the personality trait neuroticism (negative affectivity) playing a major role in this genetic liability (DSM-IV, 2000). In these cases, MDD is not caused by a unique gene, but there are many genes involved (Belmaker *et al.*, 2008; Hodgson *et al.*, 2013). Several *polymorphisms* that may predispose individuals to MDD have been identified, as for example the genes encoding for angiotensin I-converting enzyme (ACE), dopamine receptor D4 (DRD4), serotonin 2A receptor (5-HT2A), serotonin transporter (SLC6A4) or brain-derived neurotrophic factor (BDNF), among others (Lopez-Leon *et al.*, 2008). Moreover, other studies have provided evidence that MDD may be also influenced by *epigenetic* marks (Philibert *et al.*, 2008).

Elevated *low-grade inflammation* has been observed in depressed patients, particularly increased levels of citokines and adipokines such as CRP, IL-1, IL-6 and leptin. In excess, these molecules may disturb the serotonergic system and the hypothalamic pituitary adrenal axis (HPA-axis), as well as the function of neurotransmitters in the CNS (Soczynska *et al.*, 2011; Morris *et al.*, 2012).

Accumulating evidence suggests that *oxidative stress* is implicated in MDD since increased levels of lipid peroxidation, including elevations in MDA and increased oxidative damage to DNA has been observed in subjects with depression (Lopresti *et al.*, 2013). These findings might be mediated by inflammation or unhealthy lifestyle habits, as for example tobacco and alcohol consumption (Yager *et al.*, 2010).

Dysfunction of the *HPA-axis*, characterised by increased cortisol secretion, might be found in patients with MDD (Kunugi *et al.*, 2010). The BDNF is the best characterized *neurotrophin*, which is widely spread in the CNS. This molecule is involved in neuronal survival, growth and proliferation, and it has been found in low concentrations in patients with MDD (Autry *et al.*, 2012). Moreover, rates of MDD are increased in patients with *mitochondrial disturbances* (Lopresti *et al.*, 2013).

Unhealthy lifestyle behaviours and *social factors*, for instance low education and socio-economic status, disability, unemployment and marital status (being divorced or widowed) may increase the prevalence of MDD. Moreover, an abnormal galanin pathway has been recently implicated in MDD (Juhasz *et al.*, 2014).

Other illnesses, including dementia, obesity and the MetS, may lead to a higher risk of developing MDD (Belmaker *et al.*, 2008; Pan *et al.*, 2012a).

To sum up, nowadays there is no unique biomarker for MDD, being considered a heterogeneous mental disorder in which many risk factors are involved (Belmaker *et al.*, 2008; Penninx *et al.*, 2013).

2.1.1.2. Diagnosis

The diagnosis of depression is fraught with difficulty and has been a challenge for decades (Richards, 2011). This mental disorder is mainly associated with low mood, but not all patients develop the same symptoms, varying also in severity, frequency and duration (Belmaker *et al.*, 2008). Moreover it should be distinguished from temporal low mood and from depression, which does not disappear when the external cause of these negative emotions vanishes (Belmaker *et al.*, 2008). Mild depression can be treated with psychological interventions, however more severe cases may need antidepressants (Turner *et al.*, 2014).

Diagnosis of depression is mainly based on symptomatic criteria and some guidelines have been created to enable general practitioners to recognize it (Thompson *et al.*, 2001). Nowadays, there are two main diagnostic tools designed to help clinicians in the diagnosis of psychiatric disorders, which have been defined by the WHO (WHO, 1992) and by the American Psychiatric Association (DSM-V, 2013). Both scales distinguish between depression and dysthymia, which is described as a period of at least two years of constant and constantly recurring depressed mood, in which intervening periods of normal mood rarely last for longer than a few weeks (WHO, 1992).

- International Classification for Diseases-10th

According to the ICD-10 classification, depression is categorized as mild, moderate and severe depending on the number and type of symptoms presented (WHO, 1992). The diagnosis is based on a 2-week duration of symptoms, including at least 2 key symptoms, which are summarized in **Table 8**.

Table 8. The ICD-10 categories of MDD according to the WHO.

Key symptoms	Other symptoms
Depressed mood	Sleep disturbances
Loss of interest and enjoyment	Poor or increased appetite
Increased fatigability	Low self-confidence
	Concentration problems or indecisiveness
	Suicidal thoughts or acts
	Agitation or slowing of movements
	Guilt or self-blame

Classification	Number of symptoms	Type of symptoms	
		Key	Other
Mild	4	2	2
Moderate	5-6	2-3	3-4
Severe	≥ 7	3	4 or more

- Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders

The DSM-V has established that for a person to be diagnosed as having MDD he or she must present at least five of the nine symptoms exposed in **Table 9**. For diagnosis, symptoms must be present for at least two weeks, nearly every day and most of the day (with the exception of weight change and suicidal ideation). One out of two essential features (depressed mood or anhedonia) is mandatory. In addition, symptoms should not be attributable to the physiological effects of medication or a medical condition, and the state should cause clinically significant distress or impairment of functioning.

Table 9. Criteria for the diagnosis of MDD according the DSM-V (DSM-V, 2013).

A. Five (or more) of the following symptoms have been present during the same 2-week period and represent a change from previous functioning; at least one of the symptoms is either (1) depressed mood or (2) loss of interest or pleasure. 1. Depressed mood most of the day, nearly every day, as indicated by either subjective report (e.g., feels sad, empty, hopeless) or observation made by others (e.g., appears tearful). 2. Markedly diminished interest or pleasure in all, or almost all, activities most of the day, nearly every day (as indicated by either subjective account or observation). 3. Significant weight loss when not dieting or weight gain (e.g., a change of more than 5% of body weight in a month), or decrease or increase in appetite nearly every day. 4. Insomnia or hypersomnia nearly every day. 5. Psychomotor agitation or retardation nearly every day (observable by others, not merely subjective feelings of restlessness or being slowed down). 6. Fatigue or loss of energy nearly every day. 7. Feelings of worthlessness or excessive or inappropriate guilt (which may be delusional) nearly every day (not merely self-reproach or guilt about being sick). 8. Diminished ability to think or concentrate, or indecisiveness, nearly every day (either by subjective account or as observed by others). 9. Recurrent thoughts of death (not just fear of dying), recurrent suicidal ideation without a specific plan, or a suicide attempt or a specific plan for committing suicide.
B. The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
C. The episode is not attributable to the physiological effects of a substance or to another medical condition.
D. The occurrence of the major depressive episode is not better explained by schizoaffective disorder, schizophrenia, schizophreniform disorder, delusional disorder, or other specified and unspecified schizophrenia spectrum and other psychotic disorders.
E. There has never been a manic episode or a hypomanic episode.

In research, mental disorders are often evaluated by using rating instruments, which measure the severity of the symptoms based on dimensional scores (Wilkinson, 2007). There are two types of rating scales: self-rating, made by the subject, or observer-rating that is completed by an interviewer. Some of the benefits of self-rating questionnaires are that they are more economical and time efficient than personal interviews. However, it might be found that those with lower depressive signs tend to overrate their symptoms, while those with major depression may underrate them (Sharp *et al.*, 2002; Wilkinson, 2007).

Effective self-reported questionnaires should be brief, clear and easy to manage. Two of the most used self-rating scales for depressive symptoms are the Beck Depression Inventory (BDI), and the Hamilton Depression Rating Scale (Hamilton, 1960; Beck *et al.*, 1961). In addition some rating questionnaires have been created for specific patients groups, such as the Hospital Anxiety and Depression Scale (for people with physical health problems) and the Geriatric Depression Scale (EBC, 2011).

2.1.1.3. Prevalence

Globally, the burden of depression is on the rise (WHO, 2012a). The WHO estimated that more than 350 million people suffer from depression in the world in 2012. The prevalence of this mental disorder differs between 0.4 and 15.7% across countries (Rai *et al.*, 2013). Moreover, it has been projected that depression will be the second leading cause of global disability burden by 2020 (WHO, 2001).

The rate of depression in Europe is of 30.3 million (Wittchen *et al.*, 2011). Depression is twice more prevalent in women than in men, however, the prevalence of suicide is almost five times higher in men (WHO, 2012c). Prevalence rates of this illness vary among the European countries, and they are higher in the north than in the south of the continent. These variations are attributed to the hours of sun and also to socioeconomic or cultural factors (Gabilondo *et al.*, 2012; WHO, 2012c). In Spain, the European Study of the Epidemiology of Mental Disorders (ESEMeD-Spain) project revealed that the lifetime prevalence of depression was 10.6% (Gabilondo *et al.*, 2010).

2.1.1.4. Consequences

Twenty years ago, depression was not even diagnosed as a disease, however, nowadays is considered a major cause of suicide and a global burden (Richards, 2011; Wittchen *et al.*, 2011). At its most severe, depression can lead to suicide, which is related to the loss of approximately 850,000 lives every year (Marcus M, 2012). In Europe, it has been suggested to cause the 7.2% of the overall burden of disease (Wittchen *et al.*, 2011). In addition, the IDF has recently identified this mental disorder as a risk factor for adverse medical outcomes in patients with acute coronary syndrome (Lichtman *et al.*, 2014).

Depression increases the risk of overall mortality, particularly depression related suicide. The development of hypertension, stroke, obesity, Alzheimer disease or to a lesser extent even cancer, has been also proposed as consequences of depression (Penninx *et al.*, 2013).

Those subjects affected may also show decreased quality of life, productivity at work, and functional impairment in many other areas of their lives (Richards, 2011). For instance, there is a perceived inability to relate to other people, having a detrimental effect on their personal and professional relationships (EBC, 2011).

2.1.1.5. Treatment

If depression is not properly treated, it may become chronic or recurrent and lead to poor interpersonal problem-solving skills and to difficulty in dealing with everyday responsibilities. Hence, adequate treatment for depression is urgently needed (Turner *et al.*, 2014).

Treatment for depression depends on the severity of the disease, and it is normally treated using antidepressant drugs, psychosocial therapy or by the combination of both treatments (EBC, 2011). Psychotherapy should be the first-line treatment for mild depression; however, moderate and severe depressive patients might need this treatment combined with antidepressant medication (WHO, 2012a).

Nowadays, antidepressant drugs are known as the most effective treatment for depressive disorders (EBC, 2011). Antidepressants are commonly supposed to

normalize naturally occurring brain neurotransmitters, particularly 5-HT, NA and DA. Selective serotonin reuptake inhibitors (SSRIs) and serotonin and noradrenaline reuptake inhibitors (SNRIs) are the more prescribed nowadays. However, some treatments may suit some patients better than others, therefore TCAs and MAOIs must be also taken under consideration.

Due to their slow onset therapeutic effects, antidepressant drugs should be taken for at least three to four weeks. In addition, the treatment should not be interrupted without medical supervision (EBC, 2011; WHO, 2012a).

However, antidepressant therapy is often inadequate with only approximately 50% of patients experiencing a positive response, and with the percentage of patients achieving a response with second-line treatment decreasing to approximately 30% (Turner *et al.*, 2014). Moreover, if the slow onset of appreciable clinical improvement and the associated side effects are considered, the need for the improvement of antidepressant therapy is clear. Hence, pharmacogenetic, due to its capacity for identifying genetic variants that influence the response to a treatment, has been proposed for improving the clinical response to antidepressant treatment (Lanni *et al.*, 2013).

2.1.2. Anxiety disorder: generalized anxiety disorder

Anxiety disorder is a behavioural disturbance associated for instance with tension and hyper-vigilance in preparation for future threat, when no real threat exists (WHO, 1992; DSM-V, 2013). Anxiety disorder might be confused with fear, which is the emotional response to a real or perceived imminent threat. There are several categories of anxiety disorder including generalized anxiety, anxiety disorder due to another medical condition, agoraphobia, panic attack, social anxiety disorder, specific phobia, selective mutism and separation anxiety disorder (DSM-V, 2013).

Generalized anxiety disorder (GAD) is the most common anxiety disorder worldwide and it is characterized by psychological symptoms such as excessive worry, fear, apprehension, and physical symptoms for instance fatigue, heart palpitation and muscular tension (DSM-V, 2013).

2.1.2.1. Aetiology

The fundamental cause of GAD is complex, with many factors being involved. Among them are included personality traits as well as environmental and genetic factors.

Genetic heritability has been related to anxiety disorder, but in a lower extend than in depressive disorders. In this way, genetic factors should be considered together with environmental, psychological and social factors that the individual has experienced during his life (Gross *et al.*, 2004).

Psychological *trauma* experienced during development, may predispose to anxiety when an event similar to the one that triggered the trauma appears. Moreover an individual's deficit in *coping response*, and *negative thoughts and beliefs* are thought to precipitate anxiety reactions (Stein *et al.*, 2010).

Health status, physical changes, other mental illnesses, aging, loss of a job, loss of a loved one or changes in life circumstances may also cause anxiety (Stein *et al.*, 2010). In addition, there are some *substances*, for instance caffeine and amphetamines, that are able to trigger a stress response by affecting the nervous system (Stein *et al.*, 2010).

Abnormalities in the *HPA-axis* seem also to play a substantial role in anxiety disorders. In this sense, subjects with anxiety disorder might present increased levels of cortisol and corticotropin-releasing hormone (Arborelius *et al.*, 1999).

Moreover, *low-grade inflammation* and *oxidative stress* have been related to anxiety disorders, since elevated levels of cytokines and oxidative stress biomarkers have been found in subjects suffering from anxiety (Donev, 2012).

2.1.2.2. Diagnosis

As for depression, both the ICD-10 and the DMS-V classification systems are extensively used for GAD diagnosis (WHO, 1992; DSM-V, 2013).

- International Classification for Diseases-10th

The ICD-10 classification of GAD states the following criteria concerning diagnostic guidelines (WHO, 1992):

The sufferer must have primary symptoms of anxiety most days for at least several weeks at a time, and usually for several months. These symptoms should usually involve elements of:

- Apprehension (worries about future misfortunes, feeling 'on edge', difficulty in concentrating, etc.)
- Motor tension (restless fidgeting, tension headaches, trembling, inability to relax); and
- Autonomic overactivity (light headedness, sweating, tachycardia or tachypnoea, epigastric discomfort, dizziness, dry mouth, etc.)

- Fifth edition of the Diagnostic and Statistical Manual of Mental Disorders

The DSM-V has stated that the essential feature of GAD is excessive anxiety and worry about a number of activities or events (DSM-V, 2013). The diagnoses guidelines are detailed (**Table 10**).

Table 10. Criteria for the diagnosis of GAD according the DSM-V (DSM-V, 2013).

A. Excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months, about a number of events or activities (such as work or school performance).
B. The individual finds it difficult to control the worry.
C. The anxiety and worry are associated with three (or more) of the following six symptoms (with at least some symptoms having been present for more days than not for the past 6 months): 1. Restlessness or feeling keyed up or on edge. 2. Being easily fatigued. 3. Difficulty concentrating or mind going blank. 4. Irritability. 5. Muscle tension. 6. Sleep disturbance (difficulty falling or staying asleep, or restless, unsatisfying sleep).
D. The anxiety, worry, or physical symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning.
E. The disturbance is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition (e.g., hyperthyroidism).
F. The disturbance is not better explained by another mental disorder.

As in the diagnosis of depression, GAD is often evaluated using rating instruments, being the most used rating instrument the State Trait Anxiety Inventory (STAI) (Spielberger *et al.*, 1971).

2.1.2.3. Prevalence

Globally, the prevalence of anxiety disorder is of the 8.7% in women and 4.3% in men (Steel *et al.*, 2014). Anxiety disorders are the most frequent mental disorders in Europe with a rate of the 14.0% (Wittchen *et al.*, 2011).

In Spain, the ESEMeD evidenced that the lifetime prevalence of anxiety disorders is of the 6.2%. This incidence was two fold higher in women compared with men (Haro *et al.*, 2006).

2.1.2.4. Consequences

Anxiety disorders have a detrimental effect on health and social well-being, where high levels of anxiety negatively affect individual's problem solving capacity, challenging work tasks, social relationships and also decreases quality of life (Sareen *et al.*, 2006).

This mental disorder has been associated with *gastrointestinal diseases*, for example, irritable bowel syndrome. There is also a strong relationship between *asthma* and anxiety disorders (Roy-Byrne *et al.*, 2008). Moreover, *allergic* conditions, migraine *headaches, thyroid diseases* and *arthritic* conditions have been also observed in stressed subjects (Sareen *et al.*, 2006).

Additionally, there is an exceed activation of the HPA-axis and the CNS, which increase the change of *CVD*, including a higher risk of developing myocardial infarction, hypertension and low-grade inflammation (Barger *et al.*, 2005; Pitsavos *et al.*, 2006). Anxiety disorders increase the risk of presenting *obesity*, since dysregulation of the HPA-axis is known to contribute to appetite impairment and subsequent weight gain (Gariepy *et al.*, 2010).

2.1.2.5. Treatment

Historically, alcohol was considered to have anxiety-reducing properties (Rang *et al.*, 2012). Nowadays, the global approach for the treatment of anxiety disorders includes both psychological and pharmacological treatments.

Anti-anxiety agents are known to ameliorate anxiety symptomatology. Benzodiazepines have been the most prescribed anxiolytic medications for many years, however SSRIs and SNRIs have recently merged as the gold standard treatment for anxiety disorders. Moreover, other agents such as anticonvulsants antipsychotics, beta-blockers or antihistamines have also been used for anxiety treatment (Stein *et al.*, 2010; Berger *et al.*, 2012).

Since uncertainty increase the possibility of suffering anxiety, the learning theory is considered as an effective behavioural therapy for this illness. An effective fear-conditioning system enhances the organism to response to aversive events by, for example, associating them to determine stimuli (Stein *et al.*, 2010).

2.2. Dietary intake and mental and behavioural disorders

Dietary intake may influence specific molecular systems and mechanisms in the brain, affecting cognitive processes and emotions. Therefore, many scientific efforts are under way to reveal the effects of both dietary patterns and specific nutrients in behavioural disorders (Murakami *et al.*, 2010; Sanchez-Villegas *et al.*, 2013a).

2.2.1. Isolated nutrients

Elevated serum levels of total homocysteine have been implicated in mental illnesses, and so, nutrients involve in the homocysteine pathway may have a role in these disorders. (Kwok *et al.*, 2011; Gu *et al.*, 2012). In this sense, deficiency of folate and other B vitamins, such as vitamins B_2, B_6 or B_{12}, may cause increased levels of homocysteine leading to a higher risk of low mood (Sanchez-Villegas *et al.*, 2009b; Qin *et al.*, 2013). Moreover, vitamin B_6 is also involved in the serotonergic neurotransmission, together with the amino acid tryptophan (Trp) (Shabbir *et al.*, 2013). In addition, low thiamine consumption has been associated with higher risks of depressive symptoms (Zhang *et al.*, 2013).

Due to the involvement of oxidative stress in behavioural disorders, the consumption of dietary antioxidants may have a beneficial effect (Payne *et al.*, 2012). Ascorbic acid may have a positive effect since it acts as a cofactor for NA and also plays a role in the conversion of Trp to 5-HT. Moreover, vitamin E and D as well as carotenoids have been related with lower odds of presenting behavioural disorders (Murakami *et al.*, 2010; Scapagnini *et al.*, 2012). Some of the most frequently investigated minerals involved in psychiatric disorders are selenium, zinc and magnesium, which have been proposed as having a protective effect (Pascoe *et al.*, 2011). Zinc may modulate serotonergic receptors and increase levels of BDNF, producing antidepressant-like effects (Vashum *et al.*, 2014). Besides, both selenium and magnesium have been hypothesized to protect the brain against oxidative damage and low-grade inflammation, acting against mood fluctuations (Jacka *et al.*, 2009; Derom *et al.*, 2013; Johnson *et al.*, 2013).

The impact of FAs on psychiatric disorders has sparked particular interest. In this way, ω-3 PUFAs have been hypothesized to reduce depressive and anxiety symptoms because of their anti-inflammatory properties and their involvement in the functioning of the CNS (Pascoe *et al.*, 2011). Moreover, the consumption of both MUFAs and olive oil may reduce the incidence of depression (Sanchez-Villegas *et al.*, 2011). However, high SFAs as well as total dietary fat consumption are considered as unhealthy dietary patterns, which have been connected to higher odds of presenting depressive and anxiety symptoms, although the effect of total fat and SFAs consumption has not been much studied (Alsio *et al.*, 2009; Murakami *et al.*, 2010; Sanchez-Villegas *et al.*, 2011). Regarding anxiety symptomatology, palatable food such as sugars and fats, may produce an anxiolytic effect by reducing central stress responses (Maniam *et al.*, 2010), however previous dietary experiences and maternal diet during and prior to pregnancy may also modulate anxiety (Sullivan *et al.*, 2010; Murphy *et al.*, 2013; Sasaki *et al.*, 2013). Moreover, a linear relationship between lower consumption of DHA and an increased likelihood of clinically determined anxiety disorders was observed (Jacka *et al.*, 2013).

Interestingly, wine consumption in the range of two to seven glasses/week was inversely associated with depressive symptomatology, while a higher consumption may represent a risk (Gea *et al.*, 2013).

The possible involvement of fibre in mood fluctuations has been less investigated, although a negative association has been observed (Park *et al.*, 2010). This might be due to the fact that food rich in fibre such as whole grains, fruits and vegetables are rich in B vitamins and antioxidants (Logan, 2006; Park *et al.*, 2010).

At the same time, it was observed that dehydration affects human homeostasis, mood and cognitive functions; hence, water intake may be negatively associated with psychological disorders (Pross *et al.*, 2013).

Chocolate consumption has been related to putative mood benefits (Parker *et al.*, 2012), although it has been reported that subjects with higher depressive symptoms showed a greater chocolate consumption (Rose *et al.*, 2010).

2.2.2. Dietary patterns

Recently, the study of the influence of the whole dietary pattern on behavioural illnesses has attracted much attention (Murakami *et al.*, 2010; Sanchez-Villegas *et al.*, 2013a; Rahe *et al.*, 2014).

Firstly, it should be determine whether subjects are happier, presenting less anxiety and depressive manifestations, because they follow a healthier diet, or whether subjects with higher mood adhere more easily to healthier dietary profiles (Trovato *et al.*, 2014).

Most of the studies that have focused on understanding the role of the whole food intake on mental disorders present a cross-sectional design, being few of them longitudinal studies (Sanchez-Villegas *et al.*, 2009a; Jacka *et al.*, 2010; Akbaraly *et al.*, 2013; Rienks *et al.*, 2013; Sanchez-Villegas *et al.*, 2013a; Lai *et al.*, 2014). A longitudinal study showed that a healthy diet rich in fruit, vegetables and fish, and low in processed food, is protective against depressive symptoms 5 years later in middle-age subjects (Akbaraly *et al.*, 2009). In the same line, maintaining a healthy dietary pattern or improving it over a 10-year period was negatively associated with

depressive symptoms in women (Akbaraly *et al.*, 2013). Similarly, the MedDiet might prevent the development of future behavioural disorders (Sanchez-Villegas *et al.*, 2009a; Rienks *et al.*, 2013; Sanchez-Villegas *et al.*, 2013b), while unhealthy dietary habits like fast-food, processed food and trans-unsaturated fat consumption were related to an increased risk of suffering depression (Akbaraly *et al.*, 2009; Akbaraly *et al.*, 2013; Sanchez-Villegas *et al.*, 2013a). On the contrary, a recent study did not find a clear relationship between following a determined dietary pattern and future depression risk (Chocano-Bedoya *et al.*, 2013). Therefore, to date, most current prospective studies did not support a reverse causality between diet and depression, reporting that baseline unhealthy diet may be a risk for future depressive symptoms, but baseline depressive symptoms were not related to poorer eating habits in the long-term (Akbaraly *et al.*, 2009).

Regarding anxiety symptoms, there is not enough data available to make any firm conclusion. In human studies, better diet quality as well as greater adherence to the MedDiet has been related with lower likelihood of anxiety disorders (Jacka *et al.*, 2010; Jacka *et al.*, 2011; Trovato *et al.*, 2014). Pregnant women in the highest tertile of the healthy and the traditional dietary pattern scores were less likely to report high anxiety than those with high scores on a vegetarian pattern or low intake of ω-3 PUFAs (Vaz Jdos *et al.*, 2013).

As for the macronutrient distribution, no association between mood state and different macronutrient content (high-protein vs. high-CHO) has been reported (Lemmens *et al.*, 2011). Likewise, limited data are available on the relationship between low dietary variety and occurrence of mood disorders (Tangney *et al.*, 2002), although fruits and vegetables variety, but not quantity, appears to be important in reducing inflammation (Bhupathiraju *et al.*, 2011).

Furthermore, the impact of weight loss approaches on psychological problems has been a matter of controversy. While early reports linked dieting and weight loss to depression, recent studies have suggested that weight loss in obese subjects may improve body image, physical functioning and quality of life (Fabricatore *et al.*, 2011).

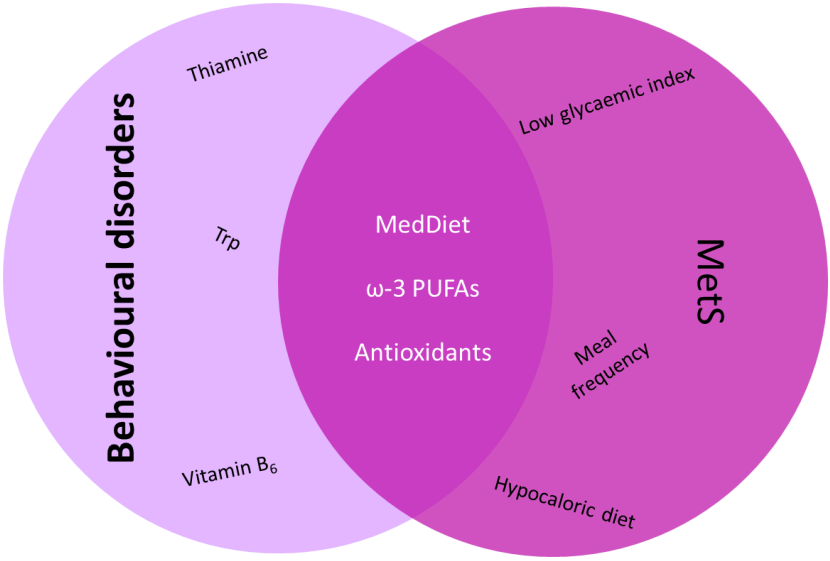

Figure 11. Association between the dietary treatment for MetS and behavioural disorders. Abbreviations: MedDiet, mediterranean diet; MetS, metabolic syndrome; Trp, tryptophan; ω-3 PUFAs, omega-3 polyunsaturated fatty acids.

Finally, it has been proposed that the dietary recommendations for MetS might be helpful for behavioural disorders treatment (**Figure 11**), since both seem to share several common mechanisms (Sanchez-Villegas *et al.*, 2013a).

To sum up, despite the clear association between diet and psychiatric disorders, recent reviews have pointed out that further observational longitudinal studies as well as randomized controlled trials (RCTs) with improved methodology and including participants at high risk of mental disorders, are urgently needed to clarify the diet-mood relations (Murphy *et al.*, 2013; Sanchez-Villegas *et al.*, 2013a).

3. Association of mental and behavioural disorders with the metabolic syndrome

Definitely, the prevalence of both MetS and mental and behavioural disorders is increasing worldwide, and for this reason, the potential association between these two diseases has attracted much attention in recent years (Renn *et al.*, 2011; Pan *et al.*, 2012a). In this sense, several theories have been put forward to explain the causal mechanisms involved in this association (**Figure 12**), which in some cases has been proposed to be bidirectional (Renn *et al.*, 2011; Pan *et al.*, 2012a).

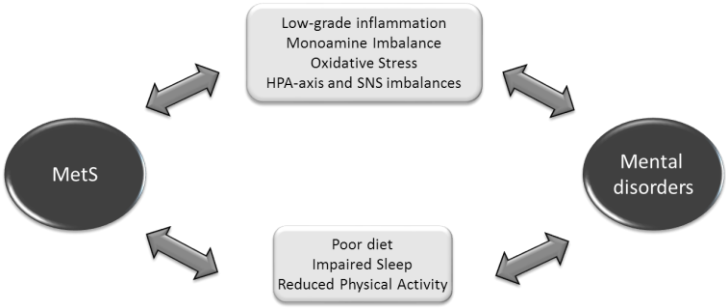

Figure 12. Bidirectional relationship between MetS and mental disorders. Abbreviations: HPA-axis, hypothalamic pituitary adrenal axis; MetS, metabolic syndrome; SNS, sympathetic nervous system.

3.1. Unhealthy behaviours

Individuals with depression and anxiety seem to follow unhealthy patterns, such as the intake of hypercaloric diets, higher smoke and alcohol consumption and lack of exercise. Sleep and appetite disturbances, as well as fatigue and diminished ability to think or concentrate are also common in people with depression and anxiety (Renn *et al.*, 2011; Pan *et al.*, 2012a). Moreover, symptoms of anxiety may trigger overconsumption of palatable food, high in sugar and fat, which increases HPA-axis activity (Gariepy *et al.*, 2010). These unhealthy attitudes are well-known risk factors for the progress of MetS (Yamaoka *et al.*, 2012).

On the other side of the spectrum, individuals with MetS trend to have poor health habits, which is thought to increase the risk of suffering depression and anxiety disorders (Gariepy *et al.*, 2010; Renn *et al.*, 2011; Pan *et al.*, 2012a).

3.2. Low-grade inflammation

In normal physiological conditions, inflammation plays an active role in regulating host defence response to injury and infection, being a crucial part of the immune system (Lopez-Legarrea *et al.*, 2014). However, excessive chronic inflammation becomes harmful and is thought to enhance tissue damage. Relevant to this, increased pro-inflammatory cytokines have been detected in subjects with MetS as well as in patients with depression and anxiety disorders (Salim *et al.*, 2012; Fuentes *et al.*, 2013).

In obesity, excess adiposity increases the production of a wide range of hormones and immune factors. Increased pro-inflammatory cytokines may increase the risk of suffering psychological disorders through several pathways (Shelton *et al.*, 2010). A plausible theory is that persistently elevated levels of low-grade inflammation can enhance atherosclerotic lesions, which may affect the frontal-subcortical circuits of the brain resulting in higher risk of depression or anxiety disorders (Donev, 2012). Pro-inflammatory cytokines are known to affect neurotransmitters metabolism by increasing the expression of the serotonin transporter and also via the activation of the tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) that activates the kynurenine pathway, which decreases Trp levels in plasma (Dantzer *et al.*, 2011). Moreover, an elevated immuno-inflammatory response may produce hyperactivation of the sympathetic nervous system (SNS) as well as dysregulation of the HPA-axis (Donev, 2012). Moreover, these cytokines may produce endothelial cells damage, where BDNF is synthesize and release, increasing the risk of mental illnesses (Sanchez-Villegas *et al.*, 2013a). Additionally, the permeability of the blood-brain barrier (BBB) might be perturbed by chronic exposure to elevated levels of cytokines and by altered glycaemic conditions, what have been related to higher inflammation and oxidative damage in the CNS (Hawkins *et al.*, 2007).

At the same time, depression and anxiety may lead to low-grade inflammation (Renn *et al.*, 2011; Pan *et al.*, 2012a). Negative emotions, the exposure to stressful experiences or psychological stress increase the production of pro-inflammatory cytokines. Inflammatory factors can produce increased SNS and HPA-axis activation, which may cause vasoconstriction, as well as an augmented release of

corticotrophin-releasing hormone, cortisol and FFAs in the bloodstream. These factors are well-known contributors to the development of MetS since they increase abdominal fat accumulation, and may alter glucose metabolism and blood pressure regulation (Gimeno *et al.*, 2009; Do *et al.*, 2010; Pan *et al.*, 2012a; Salim *et al.*, 2012).

3.3. Oxidative stress

Increased oxidative stress, triggered via generation of ROS, has a role in the pathogenesis of a number of diseases, including MetS and mental disorders (Yager *et al.*, 2010; de la Iglesia *et al.*, 2013). When the neutralizing effects of endogenous antioxidants are exceeded by ROS production, these species become toxic. At high levels ROS are involved in various degenerative diseases, via their detrimental effect on cell membrane, DNA, lipids or proteins (Perez-Matute *et al.*, 2009; de la Iglesia *et al.*, 2013).

Both obese individuals and people with depression or anxiety have exhibited lowered plasma concentrations of antioxidants and total antioxidant status in the bloodstream as well as increased lipid peroxidation, comprising elevations in MDA (Yager *et al.*, 2010; Maes *et al.*, 2011; Lopresti *et al.*, 2013).

As previously exposed, individuals with anxiety and depression trend to follow unhealthy lifestyle habits, such as smoking and alcohol consumption or diet poor in antioxidants, which may trigger oxidative damage and increase the risk of suffering obesity. In contrast, people with obesity have showed greater levels of oxidative stress biomarkers, which might be harmful for the brain and the CNS (Salim *et al.*, 2012; Penninx *et al.*, 2013; Lopresti *et al.*, 2014). All in all, oxidative stress might simultaneously be a cause and a consequence of low-grade inflammation and contribute through this mechanism to the bidirectional relationship between MetS and psychiatric diseases.

3.4. Monoamine imbalance

Monoamine imbalance in the CNS and peripheral nervous system (PNS) may also serve as a link between psychological disorders and the MetS (Luppino *et al.*, 2010; Pan *et al.*, 2012a).

In the CNS, the serotonergic and noradrenergic systems are able to modulate the brain areas involved, for example, in behaviour, sleep, appetite, migraine, tension and thoughts (Belmaker *et al.*, 2008). A diet poor in protein and rich in CHO gives Trp advantage in the competition for access to the brain, what lead to an increase 5-HT release, making people feeling better (Wurtman *et al.*, 2003). Hence, people with depression and anxiety disorder might overeat CHO to feel better, which increase the risk of weight gain (Shabbir *et al.*, 2013). By contrast, hyperactive reward system has been also observed in patients with obesity, what makes these subjects prone to eat high-calorie foods (Stoeckel *et al.*, 2008). Moreover, prolonged high fat diet intake is known to modify brain DA levels (Carlin *et al.*, 2013; Kaczmarczyk *et al.*, 2013).

Nevertheless, the role of peripheral monoamines in both MetS and mental disorders has not been fully elucidated. Most 5-HT in the body (close to 95%) exists in the PNS acting as a peripheral hormone that may affect vasoconstriction, liver repair, gastrointestinal and endocrine function, glucose and lipid metabolism (Watanabe *et al.*, 2011) as well as regulating body weight and energy homeostasis (Stunes *et al.*, 2011). Moreover, inverse associations between whole-blood 5-HT and both depression and anxiety symptoms has been reported (Williams *et al.*, 2006; Sekiyama *et al.*, 2013). Fasting conditions stimulate the synthesis of gut-derived 5-HT (Sumara *et al.*, 2012), however, whether the increase in peripheral 5-HT in MetS through energy restriction might result in an improvement of psychiatric illnesses still remain uncertain.

Peripheral DA, which is involved in the control of glucose metabolism, body weight and blood pressure, is mainly released into the blood from neuronal fibres, adrenal gland, and neuroendocrine cells (Pernet *et al.*, 1984; Eliassi *et al.*, 2008; Rubi *et al.*, 2010). However, the role of this peripheral monoamine in mental disorders is unknown.

3.5. Antidepressant treatment

Antidepressant treatment is considered a precipitating factor for the progress of MetS. These drugs may cause insulin resistance by inhibiting insulin signalling, which may lead to an increased risk of developing T2DM (Levkovitz *et al.*, 2007; Pan *et al.*, 2012b). Antipsychotic medication may cause the blockage of D2 DA receptors and induce weight gain in humans. In this way, it was also demonstrated that treatment with an inhibitor of DA uptake, reduced body weight in obese patients (Astrup *et al.*, 2008; Reinholz *et al.*, 2008). Moreover, antidepressants may affect the regulation of the HPA-axis, promoting cortisol release, and thus increasing the risk of developing insulin resistance and subsequent hyperglycaemia (Khoza *et al.*, 2012).

All this goes to show that there exists a bidirectional relationship between MetS and psychiatric disorders, and therefore, it has been proposed that in patients with mental illnesses, the cardiometabolic risk factors should be carefully monitored, and vice versa (Pan *et al.*, 2012a).

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II. Aim And Objectives

1. General aim

The main purpose of this research was to determine the possible metabolic and epigenetic mechanisms behind weight loss in overweight individuals after following different energy-restricted diets, as well as evaluating the impact of the dietary treatments on symptoms of depression and anxiety disorders.

2. Specific objectives

1. To examine metabolomic changes after the exposure to an energy-restricted diet in overweight and obese older adults (*chapter 1*).

2. To evaluate the association between the dietary intake and the overall mood state in obese individuals with metabolic syndrome (*chapter 2*).

3. To assess the effects of a hypocaloric diet designed to reduce metabolic syndrome features on self-perceived depression and the possible dietary, oxidative, and inflammatory underlying factors (*chapters 3 and 4*).

4. To determine the impact of a weight loss intervention on peripheral monoamines concentrations and anxiety symptoms in obese subjects with metabolic syndrome (*chapter 5*).

5. To investigate the relationship of *HTR2A* gene methylation levels with obesity measures and depressive symptoms in metabolic syndrome patients (*chapter 6*).

III. Subjects And Methods

1. SENIFOOD study

1.1. Study design

This trial was designed as a prospective intervention study where subjects followed a personalised and hypocaloric diet for 8 weeks (**Figure 13**). Similarly, participants were asked to continue with their usual physical activity, which was controlled with pedometers (Omron, HJ-152K-E, Japan).

This study was conducted in the Metabolic Unit of the University of Navarra in Pamplona, Spain. It was approved by the Ethics Committee of the University of Navarra (033/2011) and conformed to the principles outlined in the Declaration of Helsinki.

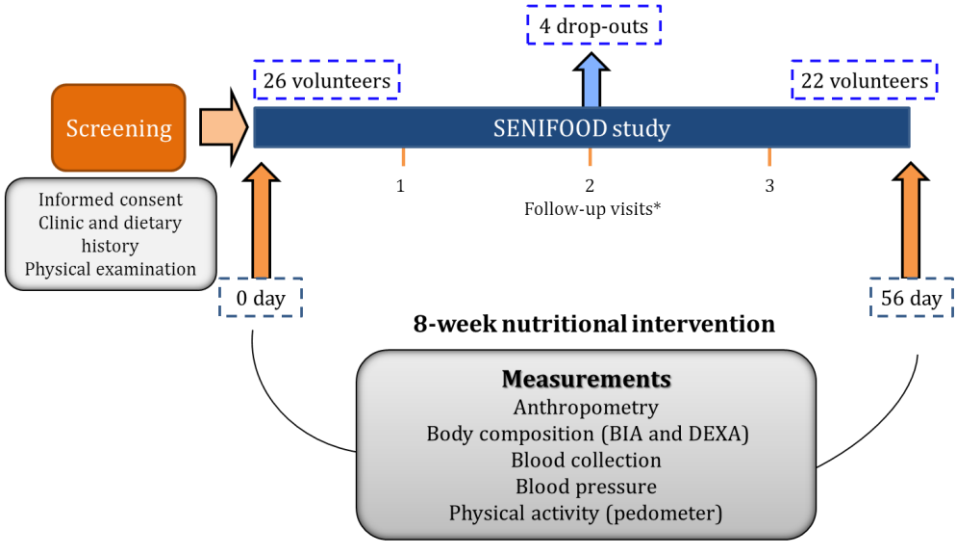

Figure 13. Study design. Abbreviations: BIA, bioelectric impedance analysis; DEXA, Dual-Energy X-ray Absorptiometry. * Assessments of body weight, waist and hip circumference, body composition by BIA, as well as dietary advice.

Prescribed diet

The personalised and hypocaloric diet (-15% of daily energy requirements) followed by the volunteers presented 45% of calories from CHO, 30% from lipids and 25% from proteins. The diet was designed by trained dieticians using a food exchange system and a menu indicating what the volunteers should choose each day of the week in order to follow a healthy diet. In addition, the volunteers were instructed to weight all the food they consumed and were advised to eat 5 meals per day (**appendix 1**).

1.2. Study population

The volunteers were recruited through a local newspaper and from the Department database. Prior to the beginning of the study, subjects attended the Metabolic Unit of the University of Navarra, where the physician informed them in detail about the study conditions and they signed the written informed consent (**appendix 2**). The inclusion and exclusion criteria are explained in **Table 11**.

Table 11. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Healthy Men and Women	BMI: < 27 or ≥ 35 kg/m^2
BMI: 27-34.9 kg/m^2	Having a serious disease
55-80 years of age	Diabetes or having glucose > 110 mg/dl
Stable weight (±3 kg) for the previous 3 months	Drug cholesterol treatment
	Gastrointestinal problems
	Daily smokers
	Vitamin or nutritional supplements
	Following a special Diet

Abbreviation: BMI, body mass index.

A total of twenty-six Caucasian healthy older adults with overweight or obesity (BMI between 27-34.9 kg/m^2) were enrolled in the study. After 8 weeks of dietary treatment, there were four drop-outs and twenty-two subjects finished the study.

1.3. Clinical and biochemical assessments

1.3.1. Anthropometric and biochemical measurements

Anthropometric and body composition measurements were assessed at the beginning and at the end of the study in fasting conditions. Body weight was assessed to the nearest 0.1 kg using a Tanita bioelectrical impedance (SC-330, Tanita, Tokyo, Japan) and height was measured using a wall-mounted stadiometer (Seca 220, Vogel & Halke, Germany) to the nearest 1 mm. BMI was determined as the body weight divided by the squared height (kg/m^2). All measurements were carried out after an overnight fast and with the subjects in their underwear. WC was measured at the narrowest point between the rib cage and the iliac crest and the hip circumference at the widest point over the buttocks. Body composition was measured by a BIA and by DEXA (DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA).

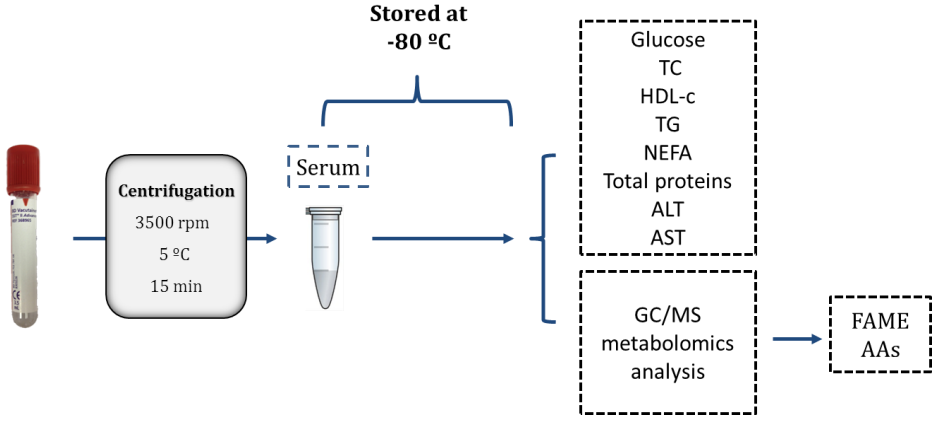

Figure 14. Blood sample collection, processing, storage and biochemical determinations. Abbreviations: AAs, amino acids; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FAME, fatty acid methyl esters; GC/MS, gas chromatography/mass spectrometry; HDL-c: high-density lipoprotein cholesterol; NEFA: non-esterified fatty acids; TC, total cholesterol; TG, triglycerides.

Serum samples were collected at baseline and at the end of the study, after a 12-h overnight fast from each volunteer (**Figure 14**). Serum glucose, TC, HDL-c, TG and non-esterified fatty acids (NEFA) were measured in an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain) with commercially available kits. LDL-c levels were

calculated following the Friedewald formula: LDL-c = TC – (HDL-c + TG/5) (Friedewald *et al.*, 1972).

1.3.2. Metabolite extraction & GC/MS analysis

For analysis of FAs, 300 µl of serum was combined with 50 µl of nonadecanoic acid (C19:0) (2 mg/ml methanol) as an internal standard and extracted using a 1:2 mixture of chloroform:methanol based on the method of Bligh & Dyer (Bligh *et al.*, 1959). Briefly, extracts were derivatised by methylation using methanolic BF_3. Derivatives were re-suspended in 200 µl of hexane and 1 µl was injected into the GC/MS. The GC/MS system comprised of an Agilent 7890A GC coupled with a 5975C MS. The GC temperature was initially 70 °C for 2 min, increased at 15 °C/min to 190 °C and held for 9 min, then increased at 5 °C/min to 230 °C and held for 13 min and finally raised to 320 °C at 20 °C/min and held for 10 min.

Aqueous compounds were isolated using a methanolic extraction (Jiye *et al.*, 2008) following deproteinisation with acetonitrile. An aliquot of 100 µl of serum were combined with 20 µl of ^{13}C myristic acid (2 mg/ml methanol) as an internal standard prior to extraction with 800 µl methanol. Following drying, samples were methoximised using 60 µl of methoxyamine hydrochloride (20 mg/ml pyridine) for 17 hours at room temperature prior to silylation with 60 µl of N-methyl-N-(trimethylsilyl)fluoroacetamide for 1 hour. Samples were diluted with 210 µl of hexane and analysed by GC/MS. The GC temperature was initially 70 °C for 2 min, increased at 5 °C/min to 260 °C, held for 41 min and finally raised to 320 °C at 30 °C/min and held for 3 min. After a solvent delay of 1 min full scan, mass spectra were recorded within a scan range of 45-650 amu (atomic mass units).

<u>Metabolite identification & quantification</u>

Calibration was achieved by comparison of peak areas for amino and FAs with reference to known standards (Amino acid standard A9906 and Supelco 37 component FAME mix, Sigma Aldrich, Ireland) using Agilent Chemstation (MSD E.02.00.493) and by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST) library 2.0. Automatic peak detection was carried out with Agilent Chemstation. Mass spectra deconvolution was

performed with the Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.65). Peaks with a signal to noise (S/N) ratio lower than 30 were rejected, which is an acceptable level to avoid false positives as reported by Norli and colleagues (Norli *et al.*, 2010). To obtain accurate peak areas for internal standard and specific peaks/compounds, one quant mass for each peak was specified as the target ion and three masses were selected as qualifier ions. Each data file was then manually analysed for false positives/negatives in Agilent Chemstation.

1.3.3. Enzyme activity determination

The desaturase activity was calculated using the ratio of individual FAs according to the following criteria: C16 Δ9-desaturase = (C16:1/C16:0), C18 Δ9-desaturase = (C18:1n-9 /C18:0), Δ6-desaturase = (C18:3n-6/C18:2n-6) and Δ5-desaturase = (C20:4n-6/C20:3n-6) (Bjermo *et al.*, 2010). The elongase activity index of FAs was assessed from the ratio C18:0/C16:0 (Pan *et al.*, 1995).

2. RESMENA study

2.1. Study design

The REducción del Síndrome MEtabólico en Navarra (RESMENA) study was designed as a randomized, longitudinal and controlled intervention trial to compare the effects of two hypocaloric dietary strategies on MetS comorbidities over a six-month period (**Figure 15**). Participants were assigned (using the "random between 1 and 2" function in the Microsoft Office Excel 2003 software (Microsoft Iberica, Spain) to follow one of the two energy-restricted diets, the Control diet or the RESMENA diet by trained dieticians (Zulet *et al.*, 2011).

The study lasted a total of six months divided in two sequential periods: one intervention period of 8 weeks in which subjects received nutritional assessment every 15 days (Lopez-Legarrea *et al.*, 2013), and a following 4 months self-control period where subjects followed the first period learned-habits (de la Iglesia *et al.*, 2014a).

Also, they were asked to maintain their usual physical activity, which was controlled by a 24-h physical activity questionnaire administered at the beginning and at the end of the study.

The RESMENA study followed the CONSORT 2010 guidelines, except for blinding. The trial was performed according to the ethical guidelines of the Declaration of Helsinki, and it was registered (www.clinicaltrials.gov; NCT01087086). The study was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009). Details of the design and methods of this trial have been reported elsewhere (Zulet *et al.*, 2011).

In addition, for most of the analyses carried out in this investigation, both control and RESMENA groups were pooled and longitudinally analysed together as a unique observational cohort group.

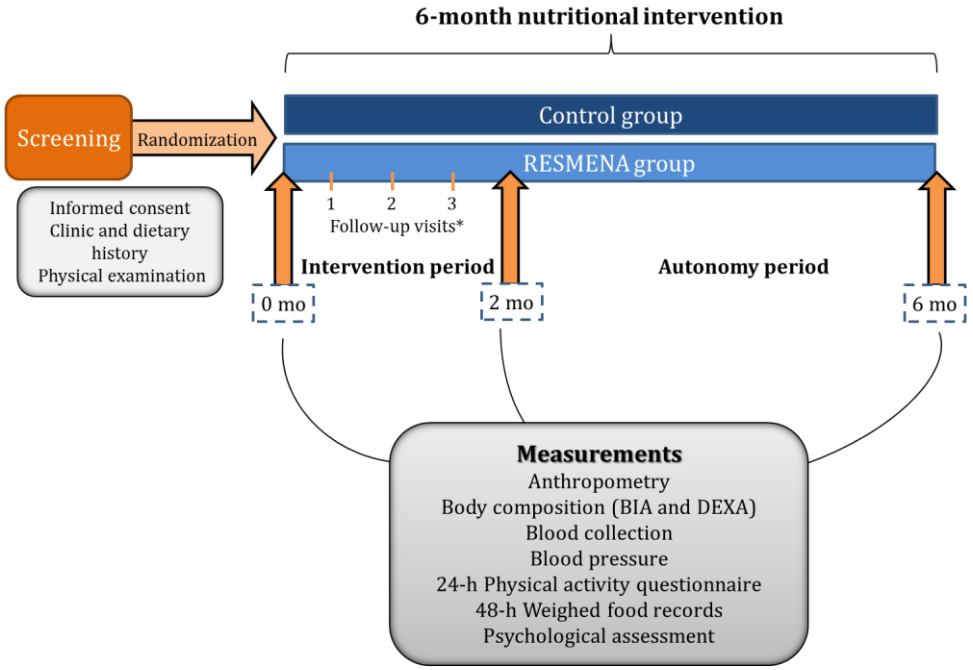

Figure 15. Study design. Abbreviations: BIA, bioelectric impedance analysis; DEXA, Dual-Energy X-ray Absorptiometry. * Assessments of body weight, waist and hip circumference, body composition by BIA, as well as dietary advice.

Prescribed diets

Two-energy-restricted diets, both with the same energy restriction (-30% of the studied requirements), were prescribed and compared. The Control diet was based on the AHA guidelines, including 3-5 meals/day, a macronutrient distribution of 55% TCV from CHO, 15% from proteins and 30% from lipids, a healthy FA profile, a cholesterol consumption lower than 300 mg/day and a fibre intake of 20-25 g/day (**appendix 3**). In contrast, the RESMENA diet was designed with a higher meal frequency, consisting of 7 meals/day, and a macronutrient distribution of 40% TCV from CHO, 30% from proteins and 30% from lipids (**appendix 4**). As was the case with the Control group, the RESMENA diet also maintained a healthy FA profile, a cholesterol content of less than 300 mg/day and a fibre intake of 20-25 g/day (Zulet *et al.*, 2011).

2.2. Study population

This study was conducted in the Metabolic Unit of the University of Navarra in Pamplona, Spain. Subjects were recruited through local advertisements and the Department database.

The inclusion and exclusion criteria are detailed in **Table 12**, however, it should highlighted that those subjects previously diagnosed with psychiatric disorders as well as those who had a current prescription of antidepressant drugs were also excluded (Zulet *et al.*, 2011).

Table 12. Inclusion and exclusion criteria.

Inclusion criteria	Exclusion criteria
Middle-aged adults	Psychiatric or psychological disorders
MetS according IDF criteria	Difficulty for changing dietary habits
Stable weight (±3 kg) for the previous 3 months	Eating disorders
	Specific pharmacological treatment
	Chronic diseases related to the metabolism of energy and nutrients
	Subjects on special diets
	Food allergies or intolerances
	Having a serious disease
	Daily smokers
	Vitamin or nutritional supplements

Abbreviations: IDF, International Diabetes Federation; MetS, metabolic syndrome.

The study details were explained to all recruited volunteers by the dieticians and written informed consent was obtained from all of them before the trial was started (**appendix 5**). In **Figure 16** is shown the flowchart of participants in the RESMENA study.

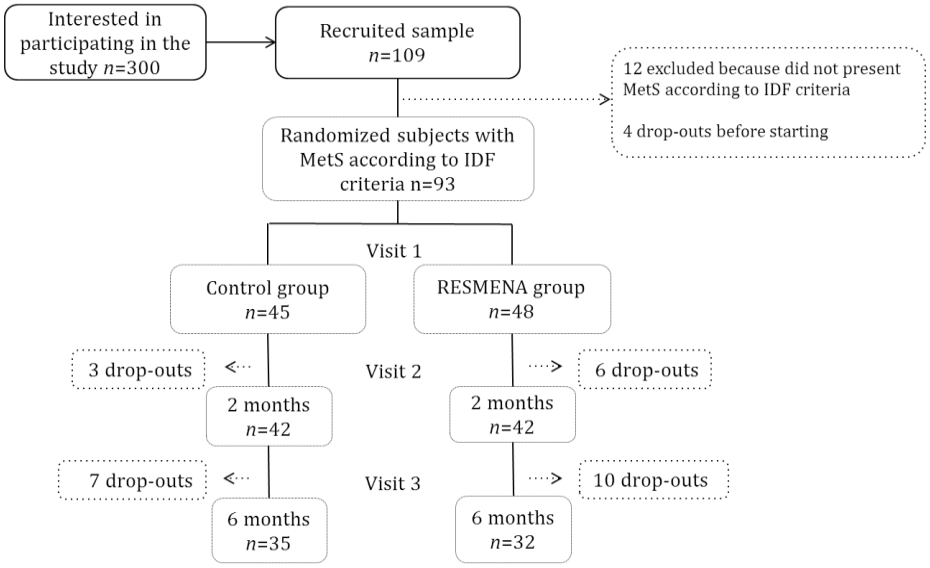

Figure 16. Flowchart of participants in the RESMENA study. Abbreviations: IDF, International Diabetes Federation; MetS, metabolic syndrome.

2.3. Dietary assessment

Compliance to the diet composition of the participants was conducted taking into account 48-h weighed food records at the beginning and at the end of the study. The energy, nutrient content and variety of the diet were determined using the DIAL (Alce Ingenieria, Madrid, Spain) software (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013).

The overall diet quality was assessed using the Healthy Eating Index (HEI) calculated by the DIAL program, which reflects how the diet conforms to the recommendations providing different values between 0 and 100. The final score is classified in 5 categories: > 80 points indicates “excellent diet”, 71-80 points express “very good diet”, 61-70 points is considered a “good diet”, 51-60 “acceptable diet” and a final score between 0 and 50 points indicate an “inadequate diet”. This questionnaire takes into account the daily servings of cereals, vegetables, fruits, dairy products and meat and it also considers the percentage of energy provided by total and saturated fats, the amount of cholesterol and sodium per day and the variety of the diet expressed by the number of different foods consumed daily. Each HEI component

ranges from 0 to 10, representing a high score a healthier dietary approach (de la Iglesia *et al.*, 2013).

2.4. Clinical and biochemical assessments

2.4.1. Anthropometric measurements

Anthropometric and body composition measurements were taken at the beginning and at the end of the study in fasting conditions. Body weight was assessed to the nearest 0.1 kg using a Tanita bioelectrical impedance (SC-330, Tanita, Tokyo, Japan) and height was measured using a wall-mounted stadiometer (Seca 220, Vogel & Halke, Germany) to the nearest 1 mm. BMI was determined as the body weight divided by the squared height (kg/m^2). All measurements were carried out after an overnight fast and with the subjects in their underwear. WC was measured at the narrowest point between the rib cage and the iliac crest and the hip circumference at the widest point over the buttocks (Pérez *et al.*, 2005). Body composition was measured by BIA and by DEXA (DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA).

2.4.2. Biochemical assessment

Blood samples were collected by venepuncture at baseline, after two months and at the end of the study, after a 12-h overnight fast (**Figure 17**). Three serum tubes and six ethylenediaminetetraacetic acid (EDTA) tubes were collected from each volunteer. These tubes were centrifuged at 3,500 rpm, 5 °C, 15 min (Model 5804R, Eppendorf, Germany) in order to obtain plasma, serum and buffy coat from white blood cells (WBC), which were frozen immediately at -80 °C until the analyses (de la Iglesia *et al.*, 2014b).

Serum glucose, TC, HDL-c, TG, NEFA and homocysteine levels were measured in a Pentra C-200 autoanalyser (HORIBA ABX, Madrid, Spain) with specific kits, while homocysteine levels were assessed by an automatized colorimetric assay (COBAS MIRA, Roche, Basel, Switzerland). Serum fasting insulin was measured by an enzyme immunoassay kit (Mercodia, Sweden). Moreover, serum concentrations of leptin

were measured using a RIA-based method (Diagnostic Products Corp., Los Angeles, CA) (Lopez-Legarrea *et al.*, 2013; de la Iglesia *et al.*, 2014a).

From plasma samples MDA, BDNF, cortisol, oxLDL, CRP and peripheral monoamines were determined. MDA levels were colorimetrically determined as a marker of lipid peroxidation using a commercial kit (BIOXYTECH® LPO-586™, Oxis Research™, Portland, OR, USA). Colorimetric assays were read using a laboratory spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). Plasma oxLDL was measured using capture ELISA assay kits from Mercodia (Uppsala, Sweden) (de la Iglesia *et al.*, 2013). BDNF levels using a commercially available ELISA kit (Promega, WI, USA) (Sanchez-Villegas *et al.*, 2011), while cortisol concentrations were measured with a ELISA kit according to the manufacturer's instructions (Demeditec Diagnostics, Kiel, Germany). Moreover, concentrations of CRP were assessed by an Immunodiagnostic AG kit (Bensheim, Germany) using an automated analyser system (Triturus, Grifols, Barcelona) (Lopez-Legarrea *et al.*, 2014).

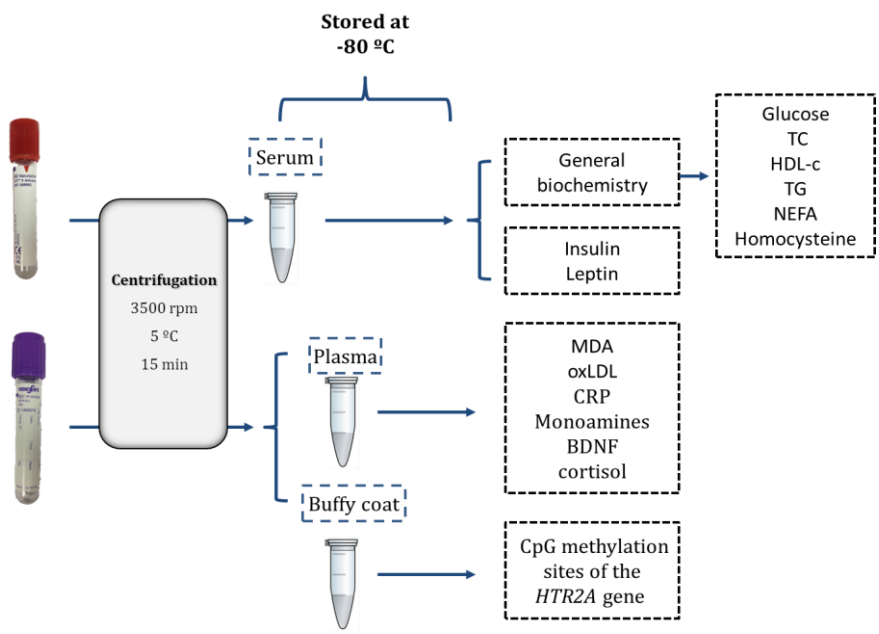

Figure 17. Blood sample collection, processing, storage and biochemical determinations. Abbreviations: BDNF, brain-derived neurotrophic factor; CpG, cytosine-phosphate-guanine; CRP, C-reactive protein; HDL-c: high-density lipoprotein cholesterol; *HTR2A*, 5-hydroxytryptamine receptor 2A; MDA, malondialdehyde; NEFA: non-esterified fatty acids; oxLDL, oxidized low density lipoproteins; TC, total cholesterol; TG, triglycerides.

Peripheral concentrations of DA, dopac, 5-HT, 5-hydroxyindoleacetic (5-HIAA) and NA were analysed using high-performance liquid chromatography (HPLC). Samples were injected using an automatic sample injector (Waters 717 plus) onto a Spherisorb ODS-2 reverse phase column (10 mm, 150 x 4.6 mm, Waters) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of NaH_2PO_4 0.05 M, octanesulphonic acid 0.16 mM, EDTA 0.1 mM and methanol 16% (pH 3), pumped at a flow rate of 1 ml/min (Garcia-Alloza *et al.*, 2005).

2.4.3. Methylation measurements

DNA isolation and DNA methylation study

Genomic DNA from WBC was obtained using the Master Pure kit (Epicenter, Madison, WI, USA), and its quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). A total of 500 ng of DNA were modified using EZ-96 DNA Methylation Kit (Zymo Research Corporation, Orange, CA) according to the manufacturer's instructions, thus converting cytosine into uracil.

Array-based specific DNA methylation analysis was performed with the Infinium Human Methylation 450K bead chip technology (Illumina, San Diego, CA). Bisulfite-treated genomic DNA was whole-genome amplified, hybridized to HumanMethylation450 BeadChips (Illumina, San Diego, CA) and scanned using the Illumina iScanSQ platform. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, San Diego, CA). β-values were computed using the formula [aa1] β-value = M/[U+M] where M and U are the raw “methylated” and “unmethylated” signals, respectively. β-values were corrected for type I and type II bias using the peak based correction. The data were normalized in R using a categorical Subset Quantile Normalization method (SQN) and probes associated to X and Y chromosomes were filtered out using the pipeline developed by Touleimat and Tost (Epigenomics 2012;4:325-41). Probes containing SNPs with a minor allele frequency (MAF) > 0.001 in Iberian population in Spain were removed from the analysis. The methylation status of twenty CpG sites of the

HTR2A gene that codes for the HTR2A receptor, were selected from the Illumina array and analysed separately. Specific CpG sites located in the transcriptional regulatory region (promoter, 5'-untranslated region and exon 1) were included.

Analysis of gene expression by quantitative-real-time PCR

Total RNA from WBC was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The integrity of isolated RNA was also evaluated by Experion (Biorad), following the manufacturer's instructions. Briefly, denatured RNA samples (1 μl) were mixed with sample buffer provided, and 6 μl of each mixed sample were loaded in RNA StdSens chips (BioRad, Hercules, CA) for analysis. In all samples we also evaluated the RNA quality indicator number (RQI) and this was considered as optimal (ranging from 7.9 to 10). Furthermore, *HTR2A* transcript expression levels were assessed using quantitative real-time RT-PCR (qPCR). cDNA was synthesized from total RNA (1 μg) of the entire cohort individuals using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor following the manufacturer's instructions (Life Technologies, Foster city, CA). The transcript levels for *HTR2A* gene and one housekeeper gene were measured using an ABI Prism 7900HT Fast Real time PCR system with a 384-well format and TaqMan Gene Expression Assays (Life Technologies, Foster city, CA) (*HTR2A*: Hs01033524_m1 and *GAPDH*: Hs02758991_g1). The ΔΔCT method was used for quantification (ABI) and the fold changes are reported as $2^{-\Delta\Delta CT}$ (Livak *et al.*, 2001).

In silico sequence analysis

Human genomic DNA sequences, from 2188 bp upstream to +1 pb of the transcription Start Site (TSS) of the *HTR2A* gene, were downloaded from the National Center of Biotechnology Information. http://www.ncbi.nlm.nih.gov/ (12 JAN 2014) database [GenBank: NG_013011.1, 13:47472360-13: 47469640]. Possible transcription factor-binding sites were predicted on genomic DNA sequences using MatInspector software Genomatix Software GmbH, Munich, Germany, which is a specifically designed tool for promoter analysis (Quandt *et al.*, 1995).

2.4.4. Psychological assessment

Psychological assessment was performed at baseline and at the endpoint of each period by using different validated questionnaires.

Assessment of mood

The mood thermometer VAS was used as a general measure of mood state (**appendix 6**). It is a rapidscreening tool consisting of a straight line, 100 mm in length, which represents the mood state in the last week. The scale was anchored with "low mood" at one end and "high mood" at the other end, ranging from 0 to 100, but the subjects ignored the numbers. A decrease in the mood thermometer is associated with worsening in mood. Participants completed this VAS at baseline, marking a line at the appropriate point, being the score the distance between this point and cero as described elsewhere (McCormack *et al.*, 1988; Rampling *et al.*, 2012).

Assessment of depressive symptoms

Symptoms of depression were assessed using the Spanish version of the BDI (Conde *et al.*, 1975), which is considered a validated and reliable measure of depressive symptoms (**appendix 7**) (Beck *et al.*, 1961; Davidson *et al.*, 2006). The BDI is a 21-item test that measures the presence and degree of depressive symptoms in respondents. Scores can range from 0 to 63, with a score of 10 or higher indicating moderate depressive symptoms. The BDI questionnaire was divided into two subscales: the cognitive and the somatic symptom components. Question number 19 of the test, relating to weight loss, was discarded from all the analyses given that losing weight is considered a manifestation of depression. However, in our volunteers it was considered a positive aspect because they were enrolled in a weight loss treatment program.

Assessment of anxiety symptoms

Anxiety symptoms were assessed using the validated Spanish translation of the STAI (**appendix 8**) (Spielberger *et al.*, 1971). This questionnaire consists on 20 brief items answered on a 4-point Likert-type scale (ranging from 1 for "not at all" to 4 for "very much"). Total score was obtained by summing all items and final score was

transformed into percentiles, with higher values indicating greater anxiety (Spielberger *et al.*, 1971).

3. Subjects and Methods references

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IV. RESULTS

CHAPTER 1

Post-print version of the article:

Metabolomics identifies changes in fatty acid and amino acid profiles in serum of overweight older adults following a weight loss intervention

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J Physiol Biochem. 2014 Jun; 70:593–602

doi: 10.1007/s13105-013-0311-2

Impact factor (2013): 2.496

35/81 in Physiology

174/291 in Biochemistry & Molecular Biology

Abstract

The application of metabolomics in nutritional research may be a useful tool to analyse and predict the response to a dietary intervention. The aim of this study was to examine metabolic changes in serum samples following exposure to an energy-restricted diet (−15 % of daily energy requirements) over a period of 8 weeks in overweight and obese older adults (n=22) using a gas chromatography/mass spectrometry (GC/MS) metabolomic approach. After 8 weeks, there were significant reductions in weight (7 %) and metabolic improvement (glucose and lipid profiles). Metabolomic analysis found that total saturated fatty acids (SFAs), including palmitic acid (C16:0) and stearic acid (C18:0) and monounsaturated fatty acids (MUFAs), were significantly decreased after the 8-week intervention. Furthermore, palmitoleic acid (C16:1) was found to be a negative predictor of change in body fat loss. Both the total ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) significantly decreased, although the overall total amounts of PUFAs did not. The branched chain amino acid (BCAA) isoleucine significantly decreased in the serum samples after the intervention. In conclusion, this study demonstrated that the weight loss intervention based on a hypocaloric diet identified changes in the metabolic profiles of serum in overweight and obese older adults, with a reduction in anthropometric and biochemical parameters also found.

Keywords: metabolomics, fatty acids, amino acids, weight loss, obesity, older adults.

1. Introduction

The prevalence of overweight and obesity, established as excessive fat accumulation, has increased rapidly worldwide (Chopra *et al.*, 2002). Fat excess is considered a major predisposing factor for a number of chronic diseases such as Type 2 diabetes mellitus, hypertension, dyslipidemias, cardiovascular disease (CVD) and cancer (Van Gaal *et al.*, 2006). In turn, overweight and obesity prevalence is growing even among older adults (≥ 60 years) in developed countries (Witham *et al.*, 2010). Aging is associated with significant changes in body composition causing a decrease of muscle mass and an increase of total fat mass especially in the abdominal region. (Mathus-Vliegen, 2012).

Essentially, many treatments for overweight and obesity include lifestyle modification through weight loss challenges and exercise with the purpose of balancing energy intake with energy expenditure. Nevertheless, most of the dietary interventions result not only in the desired body fat mass loss but also in a decrease in lean mass which is discouraged especially in older adults (Newman *et al.*, 2005; Santanasto *et al.*, 2011).

Metabolomics is a technique that aims to identify and quantify the metabolome (Weckwerth *et al.*, 2005). It is the study of metabolites present in biological samples such as biofluids/cellular extracts and culture media. Its use in nutrition research is increasing and applications range from assessing novel biomarkers of dietary intake to utilization of metabolomics in intervention studies (Brennan, 2013). Application of this technique to analyse the response to a dietary intervention generates valuable information on the effect and predisposition of the prescribed diet on metabolic regulation. It also allows a connection between dietary intake and a particular metabolic phenotype (Gibney *et al.*, 2005; Rezzi *et al.*, 2007; Smilowitz *et al.*, 2009). The two main approaches employed in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). These approaches both have their advantages and disadvantages and at present there is no unique analytical technique capable of measuring and identifying all metabolites in a single sample simultaneously. Therefore comprehensive metabolomic data needs to be assessed by bringing together data from different platforms (Sumner *et al.*, 2003; Walsh *et al.*,

2006; Etxeberria *et al.*, 2013). A number of nutritional studies have used a gas chromatography/mass spectrometry (GC/MS) based metabolomic approach to analyse fatty acids (FAs) and amino acids (AAs) (Ni Raghallaigh *et al.*, 2012; Morris *et al.*, 2013).

FAs play an important role in metabolic health taking part in many cellular processes, serving as energy reserves or regulating gene expression. Obese subjects report elevated concentrations of serum total FAs, which may have an impact on the development of metabolic syndrome and related disorders (Smilowitz *et al.*, 2009; van Dijk *et al.*, 2009; Kien *et al.*, 2013). However, aside from the amount of total lipids, the type of fat has been suggested to be crucial in the development of obesity (Moussavi *et al.*, 2008). The FA composition in the human body mirrors not only the dietary fat composition but also the endogenous synthesis and metabolism of FAs, mainly by FA synthesis from carbohydrates (CHO), desaturation and elongation (Warensjo *et al.*, 2006; Bjermo *et al.*, 2010). In this way desaturases have been suggested to play a role in the growth of metabolic disorders (Attie *et al.*, 2002; Kroger *et al.*, 2012).

Due to the rising prevalence of overweight and obesity among older adults, the design of effective weight loss interventions in this age group is needed. Therefore, the aim of this study was to examine metabolic changes after the exposure to an energy-restricted diet over a 8-week period in overweight and obese older adults using a GC/MS metabolomic approach and also using anthropometric and biochemical data.

2. Material and methods

2.1. Study population

Twenty-two of the twenty-six enrolled Caucasian healthy older adults with overweight or obesity (BMI between 27-34.9 kg/m^2) finished the study. All participants were non-smokers, followed a diet free of antioxidants or vitamin supplements and presented a stable weight (±3 kg) for the previous 3 months. Diabetes mellitus, history of previous psychiatric disorders or chronic diseases related with the metabolism of nutrients was considered as exclusion criteria. The

volunteers were recruited through a local newspaper and the Department database. Prior to beginning the study, subjects attended the Metabolic Unit of the University of Navarra, where the physician informed them in detail about the study conditions and they signed the written informed consent.

2.2. Study protocol

The present study was designed as a prospective intervention study in which subjects followed a personalised and hypocaloric diet (-15% of daily energy requirements) over 8 weeks. The macronutrient distribution was as follows: 45% of calories from CHO, less than 30% from lipids and 25% from proteins. The diet was designed by trained dieticians using a food exchange system and a menu indicating what the volunteers should choose each day of the week in order to follow a healthy diet. In addition, the volunteers were instructed to weigh all the food they consumed and were advised to eat 5 meals per day. Similarly, they were asked to continue with their usual physical activity which was controlled with pedometers (Omron, HJ-152K-E, Japan).

This study was approved by the Ethics Committee of the University of Navarra (033/2011) and conforms to the principles outlined in the Declaration of Helsinki.

2.3. Anthropometric and biochemical measurements

Anthropometric and body composition measurements were taken at the beginning and at the end of the study. Body weight was assessed to the nearest 0.1 kg using a Tanita bioelectrical impedance (SC-330, Tanita, Tokyo, Japan) and height was measured using a wall-mounted stadiometer (Seca 220, Vogel & Halke, Germany) to the nearest 1 mm. Body Mass Index (BMI) was determined as the body weight divided by the squared height (kg/m^2). All measurements were carried out after an overnight fast and with the subjects in their underwear. Waist circumference was measured at the narrowest point between the rib cage and the iliac crest and the hip circumference at the widest point over the buttocks. Body composition was measured by a dual-energy X-ray absorptiometry (DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA).

Serum samples were collected at baseline and at the end of the study, after a 12-h overnight fast from each volunteer. Serum glucose, total cholesterol, HDL-c, triglycerides and non-esterified fatty acids (NEFA) were measured in an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain) with commercially available kits. LDL-c levels were calculated following the Friedewald formula:
LDL-c = Total cholesterol − HDL-c − TG/5 (Friedewald *et al.*, 1972).

2.4. Metabolite extraction & GC/MS analysis

For analysis of FAs, 300 µl of serum was combined with 50 µl of nonadecanoic acid (C19:0) (2 mg/ml methanol) as an internal standard and extracted using a 1:2 mixture of chloroform:methanol based on the method of Bligh & Dyer (Bligh *et al.*, 1959). Briefly, extracts were derivatised by methylation using methanolic BF_3. Derivatives were re-suspended in 200 µl of hexane and 1 µl was injected into the GC/MS. The GC/MS system comprised of an Agilent 7890A GC coupled with a 5975C MS. The GC temperature was initially 70 °C for 2 min, increased at 15 °C/min to 190 °C and held for 9 min, then increased at 5 °C/min to 230 °C and held for 13 min and finally raised to 320 °C at 20 °C/min and held for 10 min.

Aqueous compounds were isolated using a methanolic extraction (Jiye *et al.*, 2008) following deproteinisation with acetonitrile. An aliquot of 100 µl of serum were combined with 20 µl of ^{13}C myristic acid (2 mg/ml methanol) as an internal standard prior to extraction with 800 µl methanol. Following drying, samples were methoximised using 60 µl of methoxyamine hydrochloride (20 mg/ml pyridine) for 17 hours at room temperature prior to silylation with 60 µl of *N*-methyl-*N*-(trimethylsilyl)fluoroacetamide for 1 hour. Samples were diluted with 210 µl of hexane and analysed by GC/MS. The GC temperature was initially 70 °C for 2 min, increased at 5 °C/min to 260 °C, held for 41 min and finally raised to 320 °C at 30 °C/min and held for 3 min. After a solvent delay of 1 min full scan, mass spectra were recorded within a scan range of 45-650 amu (atomic mass units).

2.5. Metabolite identification & quantification

Calibration was achieved by comparison of peak areas for amino and FAs with reference to known standards (Amino acid standard A9906 and Supelco 37

component FAME mix, Sigma Aldrich, Ireland) using Agilent Chemstation (MSD E.02.00.493) and by comparison of their mass spectra with those in the National Institute of Standards and Technology (NIST) library 2.0. Automatic peak detection was carried out with Agilent Chemstation. Mass spectra deconvolution was performed with the Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.65). Peaks with a signal to noise (S/N) ratio lower than 30 were rejected, which is an acceptable level to avoid false positives as reported by Norli and colleagues (Norli *et al.*, 2010). To obtain accurate peak areas for internal standard and specific peaks/compounds, one quant mass for each peak was specified as the target ion and three masses were selected as qualifier ions. Each data file was then manually analysed for false positives/negatives in Agilent Chemstation.

2.6. Enzyme activity determination

The desaturase activity was calculated using the ratio of individual FAs according to the following criteria: C16 Δ9-desaturase = (C16:1/C16:0), C18 Δ9-desaturase = (C18:1n-9 /C18:0), Δ6-desaturase = (C18:3n-6/C18:2n-6) and Δ5-desaturase = (C20:4n-6/C20:3n-6) (Bjermo *et al.*, 2010). The elongase activity index of FAs was assessed from the ratio C18:0/C16:0 (Pan *et al.*, 1995).

2.7. Statistical analysis

Data are expressed as mean ± standard deviation (SD), unless otherwise specified. The Shapiro Wilk test was used to analyse the normality of the measured variables. The differences between baseline measurements and those taken after the 8 week intervention were assessed using a paired t-test or by using the nonparametric Wilcoxon test when variables followed a non-normal distribution. Correlation analyses were applied to assess the potential relationships between specific metabolites with biochemical and anthropometrical parameters. Linear regression analysis was performed to predict changes in anthropometric variables according to FA levels at baseline. Average weight loss between groups (more weight loss vs less weight loss) was assessed using an independent measure t-test. All statistical procedures were conducted using SPSS version 15 for Windows (SPSS Ibérica, Madrid, Spain). $P<0.05$ was considered statistically significant.

3. Results

After the 8-week weight loss intervention, there were significant reductions in body weight, BMI, waist circumference, total fat mass, lean mass and diastolic blood pressure (**Table 1**). Physical activity did not change during the weight loss intervention (**Table 1**). In addition, the dietary program was effective in reducing total cholesterol, LDL-c and transaminases, however it also decreased HDL-c concentrations (**Table 2**).

The FA concentrations of serum samples at baseline and after the 8-week intervention are reported in **Table 3**. Analysis of the FAs revealed a significant decrease in total SFAs ($p<0.05$), including myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and lignoceric acid (C24:0) after the 8-week intervention. Final serum levels of total MUFAs including oleic acid (C18:1) and cis-11–eicosenoic acid (C20:1) were significantly decreased after the 8-week intervention. Although total PUFA levels did not significantly decrease, linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6), cis-8, 11, 14, 17–eicosatrienoic acid (C20:3n-6), cis-11, 14–eicosadienoic acid (C20:2) and cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n-3) were significantly reduced following the 8 week diet. Total ω-6 and ω-3 PUFAs also significantly decreased, although the ratio of ω-6/ ω-3 did not change following the intervention.

The activity of Δ5-desaturase significantly increased after weight loss, whereas the activity of the remaining investigated enzymes did not significantly change from the beginning of the study to the end of the study (**Table 3**). Analysis of correlation showed significant positive association between the change in elongase activity and the variation of total cholesterol ($r=0.648$, $p=0.003$), HDL-c ($r=0.457$, $p=0.049$) and LDL-c ($r=0.562$, $p=0.012$). Furthermore, the resulting change in percentage of body fat was positively predicted by the baseline circulating concentrations of palmitoleic acid (C16:1) (**Figure 1**).

In this study weight loss was categorised into two groups (greater weight loss (7 .4 kg) vs less weight loss (3.4 kg)) in order to identify whether there were differences in the metabolomic profiles between the groups. In this context, we found that

individuals who achieved greater weight loss also reduced their total MUFA levels (p=0.021), particularly oleic acid (C18:1) (p=0.042) and stearic acid (C18:0) (p=0.024).

A positive association between triglycerides and total SFAs (r=0.517, p=0.023), including myristic acid (C14:0) (r=0.486, p=0.035), pentadecanoic acid (C15:0) (r=0.702, p=0.001), palmitic acid (C16:0) (r=0.456, p=0.050), heptadecanoic acid (C17:0) (r=0.474, p=0.040), behenic acid (C22:0) (r=0.546, p=0.016) and tricosanoic acid (C23:0) (r=0.507, p=0.027) were found at baseline. Likewise, the change in triglyceride levels were positively associated with the variation in pentadecanoic acid (C15:0) (r=0.748, p=<0.001), heptadecanoic acid (C17:0) (r=0.489, p=0.033), behenic acid (C22:0) (r=0.481, p=0.037) and lignoceric acid (C24:0) (r=0.732, p=<0.001).

A total of 4 AAs were identified and semi-quantified in the serum (**Table 4**), of these 3 were BCAAs. Of the BCAAs it was found that isoleucine significantly decreased in the serum following the intervention (p=0.02).

4. Discussion

The effectiveness of the dietary intervention was reflected in the decrease in body weight, BMI, waist circumference, total fat mass and the diastolic blood pressure. However, subjects also showed a decline in lean mass. In general, lean mass reduces after following a hypocaloric diet, with this being more notable with aging (Leidy *et al.*, 2007; Mathus-Vliegen, 2012). In order to avoid losing lean mass the prescribed hypocaloric diet presented a higher percentage of protein (25%). Despite our attempt to prevent lean mass loss, it was significantly decreased in subjects after the dietary intervention.

Subjects were asked to continue with their usual physical activity, which was controlled throughout the weight loss treatment with pedometers so as to control the effect of physical activity on weight loss. Consequently, the variations in anthropometric, biochemical parameters and metabolite concentrations cannot be associated to changes in physical activity, but to the dietary weight loss intervention.

Levels of NEFA in serum decreased, although not significantly after the weight loss intervention. In this sense, high levels of total FAs in blood have been positively related with CVD, particularly with obesity and diabetes (Xie *et al.*, 2012). However, evidence suggests that the dietary fat quality rather than quantity might have a greater influence on disease risk (Moussavi *et al.*, 2008; Jakobsen *et al.*, 2009; Kien *et al.*, 2013). In this context the analysis of the contribution of each FA has emerged indicating that SFAs are positively associated with the development of obesity and diabetes, increasing comorbidities related to metabolic disease (Jakobsen *et al.*, 2009; Kien *et al.*, 2013).

In this study, total SFAs in serum significantly decreased with the hypocaloric diet, among them palmitic acid (C16:0) and stearic acid (C18:0), which have been previously related with the incidence of Type 2 diabetes (Iggman *et al.*, 2010; Kurotani *et al.*, 2012). Also, total SFAs may increase CVD risk by raising levels of LDL-c and total cholesterol (Flock *et al.*, 2013). Total MUFAs and in particular oleic acid serum levels decreased in this study. The health benefits of (C18:1), which represents the most abundant MUFA provided in the diet have been described previously (Ryan *et al.*, 2000; Kien *et al.*, 2013). Both total ω-6 and ω-3 PUFAs decreased significantly in this study. The ω-6 PUFAs are thought to promote adipogenesis and increase expression of lipogenic genes, while the ω-3 PUFAs have been suggested to do the opposite (Lorente-Cebrian *et al.*, 2013; Muhlhausler *et al.*, 2013). Nevertheless, the association between weight loss and the ω-3 PUFAs remains controversial (Munro *et al.*, 2012). Linoleic acid (C18:2n-6) as well as cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (C22:6n-3), which have been suggested to decrease obesity features (Chen *et al.*, 2012a; Chen *et al.*, 2012b), decreased significantly in this study. Mice under calorie restriction have shown increased expression of genes responsible of FA β-oxidation compared with ad libitum-fed controls (Bruss *et al.*, 2010), what may be implicated in the reduction of body fat after weight loss. Therefore, it can be proposed that in this study the energy restriction has increased FA β-oxidation (Labayen *et al.*, 2004), decreasing body fat and for that reason these metabolites are presented in lower amounts in the bloodstream.

This research also found that for individuals who lost more body weight they also had reduced levels of stearic acid (C18:0), total MUFAs and oleic acid (C18:1) levels. This might be explained by a lower FA production or a higher oxidation of these compounds, thus decreasing serum levels leading to a greater body weight reduction. Furthermore, it was observed that subjects with higher circulating values of palmitoleic acid (C16:1) experienced lower reduction in percentage body fat. The role of palmitoleic acid in human metabolism has not been fully clarified. Animal models have shown that adipose-derived palmitoleic acid may contribute to resistance to diet-induced obesity by inhibiting stearoylcoenzyme A desaturase 1 activity in the liver (Cao *et al.*, 2008). However, studies carried out in humans have not observed this effect (Gong *et al.*, 2011), and others have observed a detrimental influence of this MUFA on health (Warensjo *et al.*, 2005; Paillard *et al.*, 2008). High levels of this particular FA have been associated with increased risk of suffering cardiovascular diseases, since it has been positively associated with metabolic syndrome (MetS) (Warensjo *et al.*, 2005), including hypertriglyceridemia (Paillard *et al.*, 2008) and abdominal adiposity (Gong *et al.*, 2011). Mice supplemented with palmitoleate presented higher fat deposition, hepatic steatosis and also increased hepatic expression of sterol regulatory element-binding protein 1c and FA synthase, demonstrating the pro-lipogenic effect of this MUFA (Guo *et al.*, 2012). Moreover, in a Chinese population, high erythrocyte palmitoleic acid concentrations were related with lower plasma adiponectin and higher inflammatory markers (Zong *et al.*, 2012). Palmitoleic acid (C16:1) serum concentrations mostly show *de novo* hepatic FA synthesis from palmitic acid (16:0) by the C16 Δ9-desaturase enzyme (Ntambi *et al.*, 2004; Zong *et al.*, 2012). Therefore, it can be speculated that subjects with higher palmitoleic acid (C16:1) at baseline could be predisposed to present a lower response to the dietary treatment by decreasing less amount of body fat.

The present finding of a positive association between SFAs and triglycerides is consistent with previous data in which serum SFAs have been suggested to increase triglyceride levels (Lopez-Alvarenga *et al.*, 2010), whereas PUFAs are thought to reduce triglycerides levels but we did not find this association.

It is known that exist an inverse balance between CHO and FA β-oxidation (Labayen *et al.*, 2004). The role of CHO in controlling the balance between fat intake and fat oxidation is well-established, since CHO consumption reduces the use of fat for fuel (Marques-Lopes *et al.*, 2001). De novo lipogenesis (DNL) reflects the conversion of excess CHO to new FA and triacylglycerol, which are key substrates for the formation of TG and cholesterol (Hudgins *et al.*, 2000). FA desaturases are enzymes that create MUFAs from SFAs (Chong *et al.*, 2008). The use of desaturase indices is particularly useful when liver tissue samples are not available (Kroger *et al.*, 2012). The activity of the enzymes elongase, Δ6-desaturase, C16 Δ9-desaturase and C18 Δ9-desaturase did not change during the weight loss intervention. Increased levels of the previous enzymes have been detected in subjects with obesity and MetS (Bjermo *et al.*, 2010; Kroger *et al.*, 2012). Nevertheless, Δ5-desaturase significantly increased after the 8 week intervention, indicating that activity appears to be decreased in obese individuals (Warensjo *et al.*, 2006). Therefore, the increase of this desaturase during the study suggests a potential benefit to the participants.

Higher levels of circulating BCAAs have been reported in obese individuals compared to lean individuals (Newgard *et al.*, 2009; Morris *et al.*, 2012), with a number of studies reporting a reduction in BCAAs levels after weight loss (Lien *et al.*, 2009; Laferrere *et al.*, 2011). BCAAs predicted improvements in insulin resistance in patients participating in a weight loss intervention (Shah *et al.*, 2012; Magkos *et al.*, 2013), and a positive association between BCAAs and insulin resistance has been also reported (Shah *et al.*, 2012; Morris *et al.*, 2013). In the current study, insulin levels were not determined. Regarding BCAAs values, only isoleucine serum levels significantly decreased. Existing evidence suggests that the reduction in isoleucine levels promote lipolysis via induction of lipolytic genes and by the suppression of lipogenesis in liver (Du *et al.*, 2012Du *et al.*, 2012).

Although these results are interesting, the study has a number of limitations such as the small number of participants. Future studies in larger cohorts would be required in order to validate these findings. Additionally, dietary intake data was not available: such data might be helpful in further understanding the alterations in the diet following the 8-week intervention.

In conclusion, this study demonstrates that the weight loss intervention based on a hypocaloric diet not only improved anthropometric and biochemical parameters but also metabolite serum levels in overweight and obese older adults. Metabolomic analysis identified a significant decrease in FAs and isoleucine levels and an increase in Δ5-desaturase activity. Moreover, the MUFA palmitoleic acid (C16:1) predicted the change in the percentage of body fat and an association between SFAs and triglycerides levels was observed.

Acknowledgments

The authors wish to thank the volunteers for their participation in the study and the physician Blanca E. Martínez de Morentín, the nurse Salomé Pérez, as well as the technician Verónica Ciaurriz for excellent technical assistance in the University of Navarra. We thank Ciara Morris and Martina Wallace from University College Dublin for their contribution to this study. Aurora Pérez-Cornago gratefully acknowledges the pre-doctoral research grant from the "Asociación de Amigos Universidad de Navarra", as well as the mobility grant from the Spanish Government.

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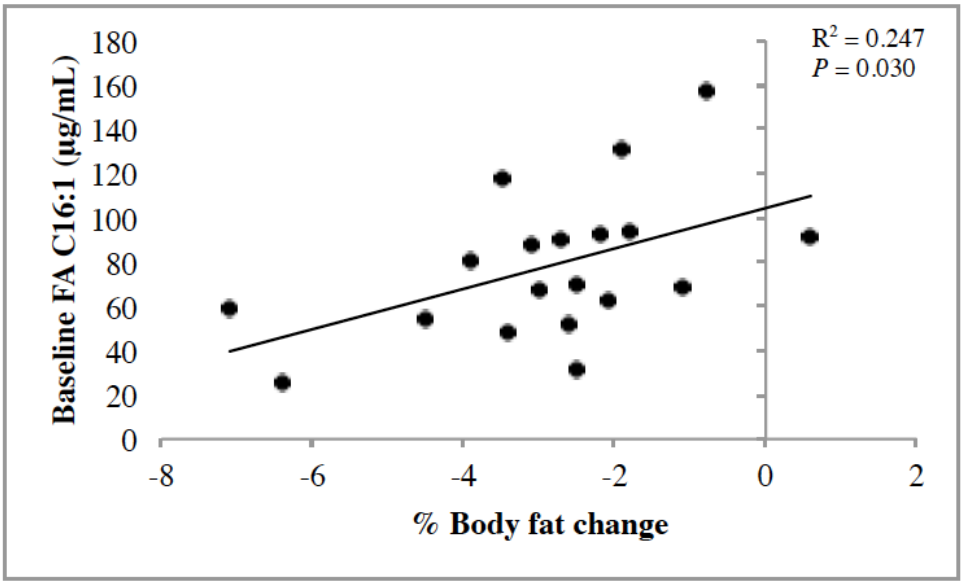

Figure 1. Association between baseline FA C16:1 serum values and % of body fat change in response to the 8-week energy restriction intervention (n=19).

Abbreviation: FA: fatty acid.

Table 1. General characteristics of the study population (n=22) at baseline and at the end of the dietary intervention (8 weeks).

	Baseline	**8 weeks**	***P-value***
Age (years)	60 ± 5		
Female sex. % (n)	68.2% (15)		
Anthropometric variables			
Weight (kg)	76.8 ± 10.3	71.4 ± 8.6	<0.001
BMI (kg/m^2)	29.7 ± 2.0	27.6 ± 1.9	<0.001
Waist circumference (cm)	92.7 ± 7.5	87.7 ± 6.9	<0.001
Total fat mass (kg)	30.6 ± 5.1	26.4 ± 5.3	<0.001
Lean mass (kg)	43.4 ± 9.7	42.4 ± 9.4	<0.001
Other variables			
Systolic Pressure (mmHg)	126.9 ± 22.3	118.1 ± 13.8	ns
Diastolic Pressure (mmHg)	79.3 ± 8.8	72.6 ± 9.2	0.004
Pedometer (steps/day)	12447 ± 6835	11562 ± 5558	ns

Data are expressed as means ± SD. P-values were based on paired t-test.

Abbreviations: BMI: body mass index; SD: standard deviation.

Table 2. Biochemical parameters of the study population (n=22) at baseline and at the end of the dietary intervention (8 weeks).

	Baseline	**8 weeks**	***P-value***
Glucose (mg/dL)	95.2 ± 7.2	91.9 ± 8.2	0.046
Total Cholesterol (mg/dL)	240 ± 37	212 ± 23	<0.001
HDL-col (mg/dL)[a]	55.3 ± 10.6	47.3 ± 8.0	<0.001
LDL-col (mg/dL)	165.6 ± 32.7	147.8 ± 21.7	0.006
Triglycerides (mg/dL)	95.2 ± 33.8	86.5 ± 39.5	ns
NEFA (μg/ml)	138.7 ± 52.6	115.5 ± 38.5	ns
Total proteins (g/L)	68.8 ± 3.3	66.9 ± 4.2	0.034
Alanine aminotransferase (U/L)	22.8 ± 7.0	19.8 ± 5.3	0.017
Aspartate aminotransferase (U/L)	22.7 ± 6.5	19.1 ± 4.7	0.002

Data are expressed as means ± SD. P-values were based on paired t-test or Wilcoxon test.

Abbreviations:HDL-c: high-density lipoprotein cholesterol; LDL-c: low-density lipoprotein cholesterol; NEFA: non-esterified fatty acids; SD: standard deviation.

[a] P-value based on non-parametric Wilcoxon test compared the two time points of the study.

Table 3. Fatty acid composition of serum samples (n=19) taken at baseline and at the end of the dietary intervention (8 weeks).

Fatty acid (µg/ml)	Baseline	8 weeks	*P-value*
SFAs	1783.8 ± 588.7	1449.8 ± 520.8	0.002
Myristic acid (C14:0)[a]	68.16 ± 40.72	49.51 ± 26.46	<0.001
Pentadecanoic acid (C15:0)	7.07 ± 2.41	6.95 ± 2.73	ns
Palmitic acid (C16:0)[a]	1097.44 ± 479.92	901.69 ± 381.39	<0.001
Heptadecanoic acid (C17:0)	8.80 ± 2.77	7.69 ± 3.55	ns
Stearic acid (C18:0)	566.26 ± 220.57	452.94 ± 166.59	0.003
Arachidic acid (C20:0)	8.45 ± 4.39	8.31 ± 3.26	ns
Behenic acid (C22:0)	12.65 ± 4.36	11.30 ± 4.37	ns
Tricosanoic acid (C23:0)	5.67 ± 2.03	4.96 ± 2.06	ns
Lignoceric acid (C24:0)	9.30 ± 3.88	6.45 ± 2.24	0.003
MUFAs	2011.6 ± 497.1	1765.6 ± 528.5	0.001
Palmitoleic acid (C16:1)	78.17 ± 32.88	63.47 ± 25.53	ns
Oleic acid (C18:1n9c)[a]	1902.75 ± 488.09	1676.62 ± 532.75	<0.001
Cis-11 Eicosenoic acid (C20:1)	8.69 ± 6.22	6.43 ± 2.77	0.046
Nervonic acid (C24:1)	21.98 ± 11.16	19.08 ± 8.10	ns
PUFAs	2367.5 ± 383.0	1901.2 ± 391.9	ns
Polyunsaturated ω-6	2204.6 ± 383.9	1775.6 ± 393.3	<0.001
γ-Linolenic acid (C18:3n6)	8.67 ± 4.12	8.16 ± 5.19	ns
Linoleic acid (C18:2n6c)[a]	1548.39 ± 449.04	1264.42 ± 361.06	<0.001
Arachidonic acid (C20:4n6)[a]	551.88 ± 172.93	446.62 ± 128.99	0.004
Cis-8,11,14-Eicosatrienoic acid (C20:3n6)	90.27 ± 39.12	52.23 ± 25.69	<0.001
Cis-11,14-Eicosadienoic acid (C20:2n6)	5.36 ± 2.90	4.15 ± 2.67	0.017
Polyunsaturated ω-3	162.9 ± 61.0	125.6 ± 27.0	0.007
Cis-5,8,11,14,17-Eicosapentaenoic acid	78.93 ± 51.24	58.83 ± 26.27	ns
Cis-4,7,10,13,16,19-Docosahexaenoic acid	84.01 ± 49.22	66.81 ± 34.26	0.004
Ratio ω-6/ ω-3	15.3 ± 6.2	14.9 ± 5.1	ns
C16 Δ9- desaturase[b]	0.088 ± 0.060	0.079 ± 0.036	ns
C18 Δ9- desaturase[c]	3.97 ± 2.45	4.22 ± 2.20	ns
Δ6- desaturase[d]	0.006 ± 0.002	0.007 ± 0.005	ns
Δ5- desaturase[e]	7.48 ± 4.08	10.00 ± 4.35	0.023
Elongase[f]	0.60 ± 0.29	0.54 ± 0.19	ns

Data expressed as mean ± SD. P-values were based on paired t-test or Wilcoxon test.

Abbreviations: MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; SFAs: saturated fatty acids; SD: standard deviation. [a] P-value based on non-parametric Wilcoxon test compared the two time points of the study. [b] C16 Δ9-desaturase = (C16:1/C16:0); [c] C18 Δ9-desaturase (C18:1n-9/C18:0); [d] Δ6-desaturase = (C18:3n-6/C18:2n-6); [e] Δ5-desaturase = (C20:4n-6/C20:3n-6); [f] elongase activity C18:0/C16:0; PUFAs ω-6 = C18:3n6 + C18:2n6c + C20:4n6 + C20:3n6 + C20:2n6; PUFAs ω-3 = C20:5n3 + C22:6n3 .

Table 4. Amino acid composition of serum samples (n=14) taken at baseline and at the end of the dietary intervention (8 weeks).

Amino acids	Baseline	8 weeks	*P-value*
Glycine	92.2 ± 38.0	99.8 ± 41.2	ns
L-leucine	31.5 ± 10.5	29.0 ± 11.4	ns
L-isoleucine	29.6 ± 12.4	21.7 ± 7.2	0.018
L-Valine	155.7 ± 57.8	164.2 ± 73.9	ns

Data expressed as mean ± SD. P-values were based on paired t-test.

Abbreviation: SD: standard deviation.

CHAPTER 2

Association between mood and diet quality in subjects with metabolic syndrome participating in a behavioural weight-loss programme: A cross-sectional assessment

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Nutr Neurosci. 2014 Mar.

doi:http://dx.doi.org/10.1179/1476830514Y.0000000116

Impact factor (2013): 2.114
47/78 in Nutrition & Dietetics
173/251 in Neuroscience

CHAPTER 3

Post-print version of the article:

A decline in inflammation is associated with less depressive symptoms after a dietary intervention in metabolic syndrome patients: a longitudinal study

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Nutr J. 2014 Apr;13:36

doi: 10.1186/1475-2891-13-36

Impact factor (2013): 2.635

32/78 in Nutrition & Dietetics

Abstract

Background: Metabolic syndrome (MetS) and depression have become two prevalent diseases worldwide, whose interaction needs further investigation. Dietary treatment for weight loss in patients with MetS may improve depressive manifestations, however, the precise interactive pathways remain uncertain. Therefore, the aim of this study was to examine the effects of a hypocaloric diet designed to reduce MetS features on self-perceived depression and the possible underlying factors.

Methods: Sixty subjects (Age:50±1 y; BMI:36.1±0.6 kg/m2) with MetS were selected from the RESMENA study (control and intervention) after they completed the 6-months hypocaloric treatment and rated for depressive symptoms using the Beck Depression Inventory (BDI). Anthropometric and biochemical measurements including leptin, C-reactive protein (CRP) and insulin levels were evaluated.

Results: Depressive symptoms decreased during the weight loss intervention, with no differences between both dietary groups (control group -4.2±0.8 vs RESMENA group -3.2±0.6, P=0.490). The number of criteria of the MetS was higher among subjects with more somatic-related depressive symptoms at baseline (B=1.032, P-trend=0.017). After six months of dietary treatment, body weight decreased in all subjects (-8.7%; confidence interval (95%CI)=7.0-9.7) and also self-perceived depression (-37.9%; 95%CI=2.7-4.9), as well as circulating leptin (-20.1%; 95%CI=1.8-6.8), CRP (-42.8%; 95%CI=0.6-3.0) and insulin (-37.7%; 95%CI=4.1-7.2) concentrations. The decrease in BDI was significantly associated with declines in body fat mass (B=0.34, 95%CI=0.11-0.56) and also with the decrease in leptin (B=0.16, 95%CI=0.04-0.28) and CRP (B=0.24, 95%CI=0.01-0.46) concentrations.

Conclusions: The decrease in depressive manifestations after a weight loss intervention was related with adiposity, CRP and leptin in subjects with MetS.

Trial registration: ClinicalTrials.gov: NCT01087086.

Key words: metabolic syndrome, depression, inflammation, leptin, hypocaloric diet, adiposity. .

1. Background

The metabolic Syndrome (MetS) is defined as a cluster of major cardiovascular risk factors including central obesity, glucose intolerance, hypertension and serum lipid disorders, whose prevalence is rapidly increasing worldwide (Yanai *et al.*, 2008; Yamaoka *et al.*, 2012). Similarly, certain MetS features such as excessive adiposity, glucose intolerance and dyslipidemia, have been associated with depression (Murabito *et al.*, 2013), which is considered the fourth leading cause of disease burden in the world (Ustun *et al.*, 2004).

Because of the high prevalence and public health implications of both depression and MetS, the potential association between them has recently received much attention (Pan *et al.*, 2012; Sanchez-Villegas *et al.*, 2013). However, the exact interactive pathways between these diseases still remain uncertain, although they seem to be bidirectional and predisposed by both biochemical and behavioral mediators (Pan *et al.*, 2012). Depression involves dysregulation of the adrenocortical and autonomic nervous systems, which could increase MetS risk by favoring abdominal fat accumulation and insulin resistance (Pan *et al.*, 2012). Furthermore, subjects with MetS present increased levels of inflammatory cytokines and leptin resistance. Thus, C-reactive protein (CRP), a serum marker of systemic inflammation, has been a frequently investigated inflammatory marker in subjects with MetS (Kraja *et al.*, 2007). Also, chronic inflammation could be involved in mood disorders, as a positive relationship between depression and CRP has been reported (Daly, 2013). Regarding the association between depression and leptin, an adipokine mainly secreted by adipocytes with a key role in energy regulation, it seems that resistance to this hormone may contribute to higher depression rates in obese subjects (Morris *et al.*, 2012).

Various strategies have been proposed to counteract MetS manifestations, including lifestyle (diet and exercise) modification and drug therapy based on antihypertensives, insulin sensitizers or therapies for dyslipidemia (Swislocki *et al.*, 2012). One of the most prescribed lifestyle changes is dietary treatment for weight loss, where the Mediterranean diet has been proven to be a useful tool to improve both MetS and depression symptoms (Sanchez-Villegas *et al.*, 2009; Salas-Salvado *et*

al., 2011). Also, the psychological effects of weight loss approaches have been a matter of controversy, mainly regarding how changes in body weight correlate with depressive symptoms (Fabricatore *et al.*, 2011).

This research is based on a subsample of the RESMENA-S study (de la Iglesia *et al.*, 2013a; de la Iglesia *et al.*, 2013b; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013), a randomized, controlled intervention study that aims to reduce MetS features using a hypocaloric diet during six months. In this article, we hypothesized that a hypocaloric treatment designed to reduce MetS features produces a positive effect on depressive symptoms, and we sought to explore the possible underlying mechanisms and interactions of this effect.

2. Subjects and methods

2.1. Subjects

A total of ninety-three subjects (52M/41F) with a body mass index (BMI) of 36.1±0.6 kg/m^2 aged 50±1 years diagnosed with MetS according to the IDF cut-offs (Alberti *et al.*, 2005) started the weight loss treatment. The inclusion and exclusion criteria have been previously reported (de la Iglesia *et al.*, 2013a; de la Iglesia *et al.*, 2013b; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013), but it should be pointed out that subjects following antidepressant treatment were excluded as well as those with past mood disorders, including eating disorders. Also, vitamin or mineral supplements were not allowed.

After six months of weight loss intervention there were twenty-six dropouts due to loss to follow-up or consent withdrawal. Seven of the sixty-seven participants that finished the study did not complete all the Beck Depression Inventories (BDI). Therefore the present longitudinal study assessed the data from those 60 subjects (age: 50±1 y.; 38M/22F), who completed the BDI in the three main visits (baseline, after two months and at the end of the study), as described in the study flowchart (**Figure 1**).

In the RESMENA study, the CONSORT 2010 guidelines (Schulz *et al.*, 2010) were followed, and all the volunteers signed written informed consent before participating in the intervention study. The study protocol was performed in accordance with the

ethical guidelines of the Declaration of Helsinki, and was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009).

2.2. Study protocol

The current study is based on a subsample of the RESMENA-S study, a randomized controlled intervention study aiming to improve clinical criteria and biomarkers associated with MetS through a dietary strategy for weight loss during six months. Briefly, participants were randomly assigned to follow one of the two energy-restricted diets, the control diet (Grundy *et al.*, 2005) or the RESMENA diet (de la Iglesia *et al.*, 2013a; de la Iglesia *et al.*, 2013b; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013), both with the same energy restriction (-30% energy of the calculated requirements) by using the "random between 1 and 2" function in the Microsoft Office Excel 2003 software (Microsoft Iberica, Spain) . The diets composition, as well as the different 48-h dietary records, were analyzed by the DIAL (Alce Ingenieria, Madrid, Spain) software and described elsewhere (de la Iglesia *et al.*, 2013b; Perez-Cornago *et al.*, 2013).

The volunteers were asked to maintain their usual physical activity, which was controlled by a 24-h physical activity questionnaire at the beginning and at the end of the study (Food and Nutrition Board, 1989). A psychological control using the BDI questionnaire was carried out at the main time points previously mentioned. More aspects of this intervention study have been previously detailed (de la Iglesia *et al.*, 2013a; de la Iglesia *et al.*, 2013b; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013).

2.3. Anthropometric and biochemical measurements

Anthropometric and body composition measurements were taken following standardized procedures previously described (de la Iglesia *et al.*, 2013b; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013).

Serum glucose, total cholesterol, HDL-cholesterol, triglycerides and free fatty acids serum concentrations were measured in an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Insulin concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) using

an automated analyser system (Triturus, Grifols, Barcelona). Serum insulin resistance was estimated by the Homeostasis Model Assessment Index (HOMA-IR) calculated with the following formula: HOMA-IR = fasting glucose (mmol/L)xfasting insulin (mU/L)]/22.5 (Matthews *et al.*, 1985).

Plasma concentrations of CRP was assessed by an Immunodiagnostic AG kit (Bensheim, Germany) using an automated analyser system (Triturus, Grifols, Barcelona). Serum concentrations of leptin were measured using a RIA-based method (Diagnostic Products Corp., Los Angeles, CA).

2.4. Assessment of depressive symptoms

Symptoms of depression were assessed at the main three time points of the study (baseline, after two months and at the end of the study) using the Spanish version of the BDI (Conde *et al.*, 1975), which is considered a validated and reliable measure of depressive symptoms (Beck *et al.*, 1961; Davidson *et al.*, 2006). A score ≥ 10 reflects moderate depressive symptoms. The BDI questionnaire was divided into two subscales: the cognitive and the somatic symptom components. Question number 19 of the test, relating to weight loss, was discarded in some of the analyses given that losing weight is considered a depressive symptom, but our volunteers estimated it a positive aspect because they were enrolled in a weight loss treatment program (Perez-Cornago *et al.*, 2013).

2.5. Statistical analyses

The sample size for the main study (the RESMENA study) was calculated based on previous findings (Katcher *et al.*, 2008), where the mean difference in the waist circumference was 4.3 cm and the standard deviation (SD) was 6.8 cm. To test the hypothesis of the present substudy and taking into account previous studies (Brinkworth *et al.*, 2009), a sample size of 29 subjects would be enough to obtain a statistically significant difference in the reduction of BDI score (2.2±2.5 units), with an alpha error of 5% and a power of 90%.

The main results were summarized as mean ± SEM. Following published studies, the BDI score was analyzed as a continuous variable (Morris *et al.*, 2012). The Kolmogorow-Smirnov and the Shapiro Wilk test were used to analyze the normality

of the measured variables. Analysis of covariance (ANCOVA) was used to assess changes between dietary treatments for the main dietary variables of the study, with sex and age as covariates. Changes in anthropometric and biochemical variables, as well as changes in the BDI questionnaire and physical activity level, were evaluated by repeated-measures ANOVA (three time points) or by using the nonparametric Friedman test when variables followed a non-normal distribution. Also, the effect of diet on these variables as well as the time-diet interaction was taken in consideration. A Bonferroni *post hoc* multiple comparison analysis was used when appropriate.

Tests of linear trend by increasing MetS criteria were conducted by assigning the mean value of BDI (somatic and cognitive questions separately) and modeling these values as a continuous variable. The changes in the cognitive and somatic questions (6 months – baseline) were assessed by ANCOVA with sex, age and dietary group as covariates. The association between changes (6 months – baseline) in BDI questionnaire and changes (6 months – baseline) total fat mass (kg) was analyzed using multivariable linear regression analysis adjusted by sex, age and dietary group. For the association between changes in CRP, leptin and insulin with changes in BDI score three models were constructed: Model 1 included sex, age and dietary group variables. Model 2 included model 1 additionally adjusted for changes in activity level, and model 3 consisted of model 1 plus changes in total fat mass (kg). All statistical analyses were carried out using SPSS 15.0 software for Windows (SPSS Ibérica, Madrid, Spain). Differences were considered statistically significant at $P<0.05$.

3. Results

The main dietary characteristics of the two prescribed diets were compared showing that, as expected, the RESMENA group consumed more protein than the Control group during the dietary treatment. Moreover, the RESMENA group showed a greater decrease in lipid intake, but lower intake of ω3 PUFA (**Table 1**). After six months of dietary treatment, subjects' anthropometric and biochemical variables improved significantly, observing a mean weight loss of 8.4±1.2 kg ($P<0.001$). However, there were no differences ($P\geq0.10$) between both dietary intervention

groups in neither anthropometric nor biochemical variables, as well as activity level of the volunteers. Also, both Control and RESMENA diets proved to be equally effective on improving the BDI score (**Table 2**). Therefore, as no significant differences between dietary groups were found in any of the variables analyzed in this study, henceforth the two groups were merged and analyzed together as a unique experimental group.

The comparative analysis between completers (n=62) and dropouts (n=25) concerning BDI score available at baseline, showed no significant differences (*P*=0.255). Nonetheless, completers evidenced a lower mean value of BDI score at baseline (7.6±0.7) in relation to those who did not finish the dietary treatment (9.3±1.0).

The total number of subjects presenting a BDI score ≥10 units at baseline was 25% (9M/6F). After two months of weight loss treatment, this number decreased to 8.3% of the volunteers (3M/2F). At the end of the study (6 months later), only 6.6% of the subjects reported a BDI score ≥ 10 (2M/2F).

A test of linear trend revealed that the higher the number of components of MetS according to IDF criteria (3, 4 or 5 components), the higher somatic BDI score at baseline. Interestingly, this association was not observed in the cognitive questions of the questionnaire (**Figure 2**).

In turn, losing more weight led to greater reductions (*P*<0.013) in depressive symptoms (more weight loss ΔBDI = -5.2±0.9; vs less weight loss ΔBDI = -2.5±0.6). As expected, when we compared all the somatic-related questions separately between both groups (more weight loss vs less weight loss), significant differences were observed in the weight loss question, but also in the fatigability and loss of libido questions. Subjects reported losing more weight at 6 months than at baseline in the BDI questionnaire, hence the score of this question was higher at the end of the study. However, subjects who lost more weight did not show a greater reduction in the cognitive-related questions compared to individuals with lower body weight change (**Figure 3**).

Additionally, a positive association between changes in depressive symptoms and changes in kg of total fat mass was observed (**Figure 4**). We also found an association between changes in BDI score during the intervention period and changes in CRP, leptin and insulin levels. Further adjustment for body fat change eliminated the relation between depressive symptoms and insulin levels, however, the association of BDI with CRP and leptin remained statistically significant (**Table 3**).

4. Discussion

The present study reports an association of the reduction in depressive manifestations with the decrease in CRP and leptin after a dietary treatment for weight loss in subjects with MetS. Moreover, the reduction in fat mass was also involved in the decrease of depressive symptoms, but it was not implicated in the association of this variable with CRP and leptin. Previous investigations have reported a decrease in depressive manifestations after a weight loss treatment (Fabricatore *et al.*, 2011; Somerset *et al.*, 2011), however, this study specifically demonstrates a relationship of the decrease in CRP and leptin with the reduction in self-perceived depression in subjects suffering MetS.

The domains of self-perceived depression were divided in cognitive and somatic questions in order to better interpret the primary cause of patient depression (Kupper *et al.*, 2012). At baseline, there was a ranked relationship between increasing number of MetS components and a higher rate of somatic questions but not of cognitive components. These findings were similar to those observed in a previous study also based on self-reported BDI questionnaire (Skilton *et al.*, 2007).

Question number 19 of the BDI test, related to weight loss, was apparently distorting the total BDI score. This observation was confirmed because the weight loss was associated with the decrease in body weight at the end of the dietary treatment, showing that those subjects who lost most weight, had a higher score on the weight loss question of the test. Therefore, the weight loss question was removed from the total BDI score in some analyses. Moreover, the improvement in the fatigability and loss of libido questions (somatic-related questions) might be considered as benefits of the weight loss.

Noteworthy, a positive association between decreases in CRP values and reductions in depressive symptoms was observed as has been reported in previous studies (Daly, 2013). Inflammation seems to be, in part, responsible for the link between depressive symptoms and MetS. However, the precise nature of this relationship is not clear (Daly, 2013). Theoretically, the association of leptin with depression may be related both to its metabolic properties and neurobiological activities (Morris *et al.*, 2012). Leptin affects cognition and mood in the hippocampus, the cortex and other brain areas associated with cognition (Morrison, 2009; Morris *et al.*, 2012). Obesity is related to higher levels of circulating leptin reflecting, in part, an increased formation of adipocytes, and causing leptin resistance in some obese subjects. A positive association between the decline in BDI score and the drop in leptin values was found, in line with results from previous studies (Milaneschi *et al.*, 2012; Morris *et al.*, 2012). Furthermore, in an unadjusted model, a positive association was noted between insulin and depressive symptoms, being this link well-established (Kullmann *et al.*, 2012; Silva *et al.*, 2012). However, this association was no longer statistically significant when change in body fat was introduced in the model.

A previous weight loss intervention study showed that depression scores at baseline predicted adherence to a dietary treatment (Somerset *et al.*, 2011). In accordance with this outcome, a higher BDI score in drop-outs at baseline was observed, although this value did not reach statistical significance. A positive association between lower BDI score and decreases in body weight was found, which is in agreement with previous studies (Fabricatore *et al.*, 2011). In addition, there is some evidence suggesting that adiposity is directly related with depression (Murabito *et al.*, 2013). In this study, the higher decrease in depressive symptoms was noticeable in those participants with a greater decline in total fat mass. Since adipose tissue is known to secrete inflammatory cytokines and leptin (Clement *et al.*, 2004), it might be hypothesized that the decrease in body fat mass may have contributed to reduce CRP and leptin and subsequently decrease depressive symptoms.

Furthermore, the Mediterranean diet has been associated with reduced prevalence and incidence of MetS and depressive symptoms (Sanchez-Villegas *et al.*, 2009; Salas-Salvado *et al.*, 2011). A healthy dietary pattern has been associated with better mood

(Perez-Cornago *et al.*, 2014) and diets rich in ω3 PUFA may decrease serum cytokine production and depressive signs (Swenne *et al.*, 2011). Also, an inverse association between both vitamin D and vitamin C levels with depressive symptoms has been observed (Bertone-Johnson *et al.*, 2011; Amr *et al.*, 2013). In this context, we have previously shown that folate intake during this dietary intervention was well correlated with the decrease in depressive symptoms (Perez-Cornago *et al.*, 2013).

Because both MetS and depressive manifestations seem to share several common mechanisms, it has been suggested that the dietary recommendations for MetS might be helpful for depression treatment (Sanchez-Villegas *et al.*, 2013). The present study strengthens this hypothesis, as the dietary treatment for MetS manifestations also reduced depressive symptoms. Moreover, several unhealthy lifestyle habits have been related with depressive symptoms, such as fast food consumption, high fat intake or low physical activity (Rosenberg *et al.*, 2013; Sanchez-Villegas *et al.*, 2013). In this study, physical activity was not directly associated with depressive symptoms, being in agreement with previous investigations (Appelhans *et al.*, 2012).

The control diet based on the AHA guidelines is a well-design strategy for weight loss (Grundy *et al.*, 2005), what may explain that no differences between dietary groups were observed in the variables analyzed in this study. As both dietary groups proved to be effective in reducing depressive symptoms and no differences between them were found, both groups were merged and analyzed together as a unique experimental group. A longitudinal observational analysis comparing the BDI scores before and after intervention was conducted, serving the volunteers as their own control. The clear differences between the beginning and the end of the study lend support to the soundness of the analysis. One of the strengths of within-subjects (paired) analyses is the reduction in error variance associated with individual differences, which increases statistical power. Moreover, in order to control the possible confounding role of the intervention group, this variable was included in the multiple-adjusted models investigating the association of anthropometric and biochemical variables with depressive manifestations in the complete sample (Perez-Cornago *et al.*, 2013).

The study has some limitations. Firstly, depressive symptoms were evaluated using a self-report questionnaire, the Beck Depression Inventory, which is not designed as a diagnostic tool but as a screening method (Beck *et al.*, 1961). However, this test was chosen as it is widely recognized, it has been shown to be valid for clinical assessment (Davidson *et al.*, 2006) and it has previously been used to record depressive symptoms in weight loss studies (Fabricatore *et al.*, 2011; Somerset *et al.*, 2011). Secondly, this study only aimed to evaluate the association between the variables included and it cannot be determined any conclusions on causality between changes in body weight, fat mass, leptin and CRP and changes in depressive symptoms. In addition, the prevalence of depressive symptoms in our sample was low, which can be explained with the fact that subjects presenting psychiatric disorders at enrollment were not allowed to participate in the study. Also, the number of participants in this study is not very high, but it may be proposed that type-II errors were overcome since important statistical differences were found.

5. Conclusions

In conclusion, this study shows an association of the reduction in depressive manifestations with CRP and leptin in subjects with MetS after following a weight loss treatment. Interestingly, the decrease in fat mass was also related with the reduction of depressive symptoms. More studies are needed to explore the mechanisms underlying the MetS-depression relationship, which may be decisive for the prevention and treatment of both conditions.

Competing interests

None declared.

Author's contributions

The authors contributions were as follows: A.P.C. performed the research, analysed data and wrote the manuscript; R.I., P.L.L., I.A. and S.N.C. conducted research; F. L. and C.I.L. selected and contributed to the interpretation of the psychological test; M.A.M contributed to the statistical analysis. J.A.M. and M.A.Z. designed and managed the research, and had primary responsibility for final content. All the authors read and approved the final version of the manuscript.

Acknowledgements

We wish to thank the volunteers of this study and Blanca E. Martínez de Morentín, Salomé Pérez, as well as Verónica Ciaurriz for excellent technical assistance in the University of Navarra. We would like to thank Dr Paul W. Miller from the Institute of Modern Languages of the University of Navarra for careful reading the manuscript.

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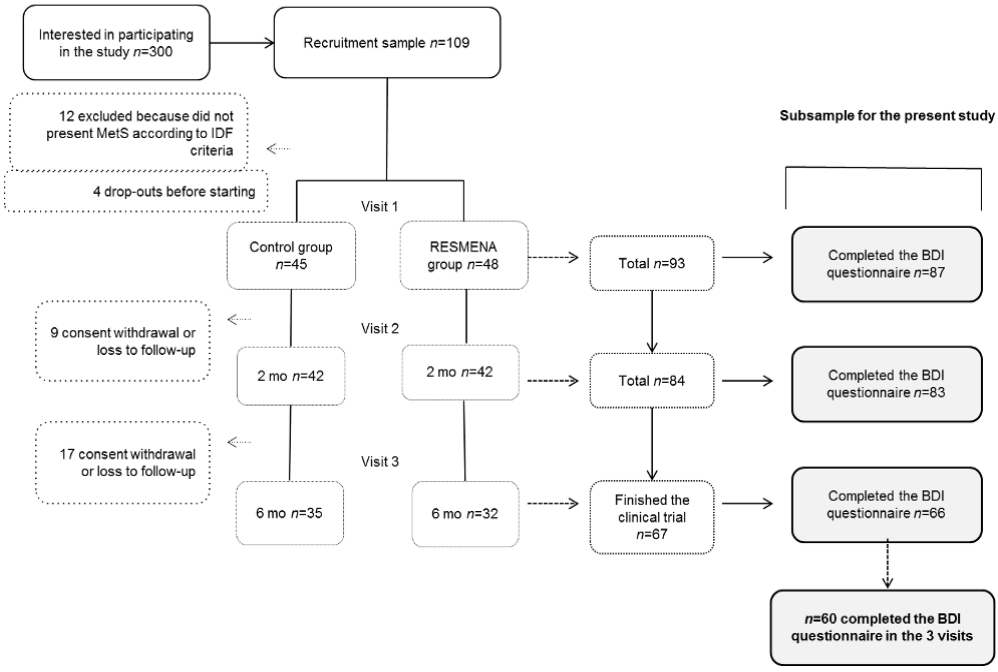

Figure 1. Flowchart of the randomized and controlled dietary intervention for adults with MetS. The 26 volunteers that dropped out did so for personal reasons not related to the study.

Abbreviations: BDI, Beck Depression Inventory; IDF, International Diabetes Federation; MetS, Metabolic Syndrome.

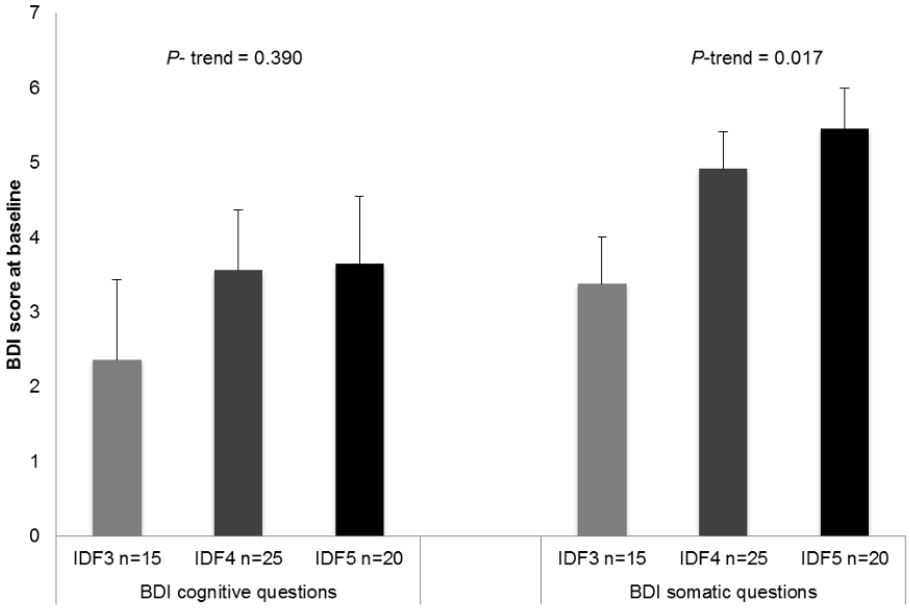

Figure 2. Association between the number of MetS components assessed by IDF criteria and the BDI score at baseline. Values are mean ± SEM, n = 60. BDI score divided by somatic and cognitive questions. Abbreviations: BDI, Beck Depression Inventory; IDF, International Diabetes Federation.

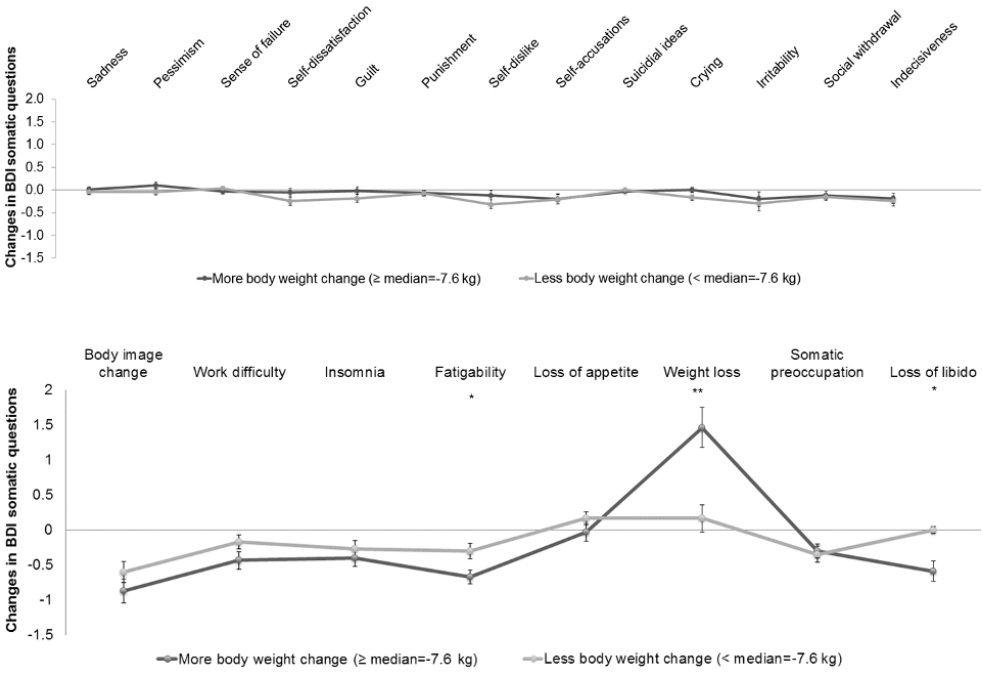

Figure 3. Changes in BDI cognitive and somatic questions scores (6 months – baseline). n = 60. Range from -3 to 3. Subjects with MetS were divided into more (≥ median=-7.6 kg) or less (< the median=-7.6 kg) body weight change after the dietary treatment. Abbreviations: BDI, Beck Depression Inventory; MetS, Metabolic Syndrome.

* $p < 0.05$

** $p < 0.010$

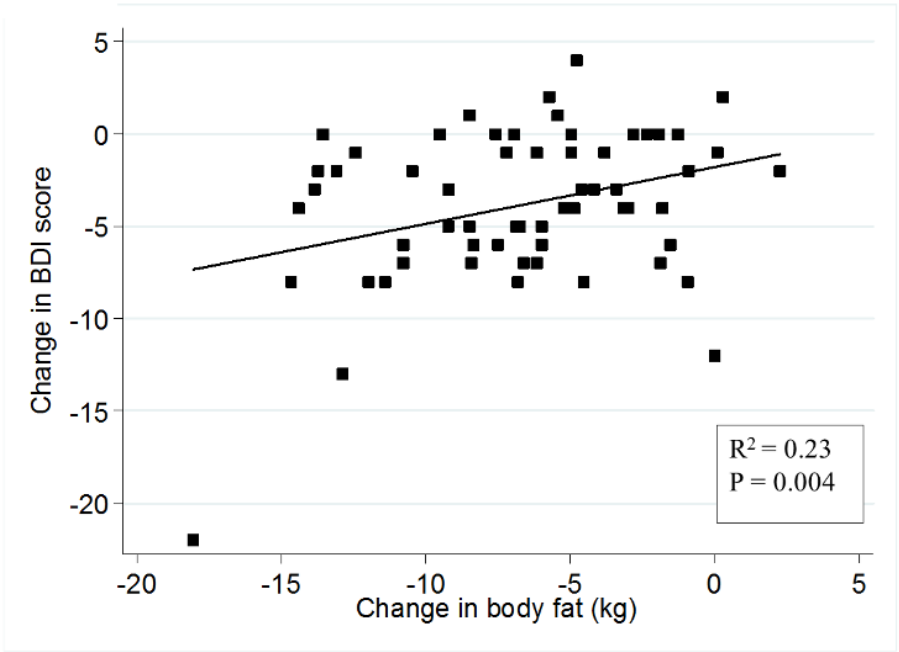

Figure 4. Association between changes in BDI score and changes in body fat (kg). Changes (6 months – baseline), n = 60. Abbreviations: BDI, Beck Depression Inventory. Adjusted by sex, age and dietary group.

Table 1. Comparison of the two dietary treatments, control diet and RESMENA diet.

	Control group (n=32)		RESMENA group (n=28)		*P* value
Dietary intake	**Baseline**	**6 months**	**Baseline**	**6 months**	
Total energy intake, *Kcal/d*	2108 ± 69	1535 ± 54*	2279 ± 99	1573 ± 72*	0.105
Proteins, *g/d*	95.9 ± 3.5	66.9 ± 3.4*	95.6 ± 3.4	79.3 ± 3.4*	0.012
Carbohydrates, *g /d*	187.3 ± 9.8	142.6 ± 8.1*	196.4 ± 11.5	138.9 ± 6.2*	0.579
Lipids, *g /d*	94.7 ± 4.3	69.1 ± 2.9*	108.7 ± 5.9	66.98 ± 3.8*	0.026
Fiber, *g/d*	21.4 ± 1.8	17.8 ± 1.7	22.6 ± 1.1	19.4 ± 1.2	0.748
Glycemic load	106.6 ± 8.6	72.8 ± 5.8*	112.1 ± 7.4	69.7 ± 5.1*	0.491
ω3 PUFAs , *g/d*	0.28 ± 0.01	0.28 ± 0.02	0.35 ± 0.16	0.08 ± 0.02*	0.002
Meal frequency, *meals/d*	4.6 ± 0.1	4.5 ± 0.1	5.5 ± 0.2	5.9 ± 0.2	0.100

Data are mean±SEM, n = 60. *Different from baseline in each dietary group, P <0.05; P value: comparison between dietary group differences (6months-baseline). Abbreviations: ω3 PUFAs, omega-3 polyunsaturated fatty acids.

Table 2. Anthropometric and biochemical variables in adults with MetS.

				P value		
Variables	Baseline	2 months	6 months	Time	Diet	TimexDiet
Weight, *kg*	102.8 ± 2.2[a]	95.4 ± 2.1[b]	94.4 ± 2.2[b]	< 0.001	0.834	0.666
BMI, *kg/m²*	36.1 ± 0.6 [a]	33.5 ± 0.5[b]	33.2 ± 0.6[b]	< 0.001	0.997	0.474
WC, *cm*	114.2 ± 1.6[a]	106.8 ± 1.5[b]	105.7 ± 1.6[b]	< 0.001	0.999	0.102
DXA Measurements						
Fat mass, *kg*	42.9 ± 1.2[a]	37.5 ± 1.2[b]	36.3 ± 1.2[c]	< 0.001[1]	0.469	0.432
Muscle mass, *kg*	56.6 ± 1.5[a]	54.5 ± 1.4[b]	54.8 ± 1.5[b]	< 0.001	0.694	0.239
Biochemical variables						
Cholesterol, *mg/dL*	219 ± 5[a]	197 ± 5[b]	219 ± 5[a]	< 0.001	0.568	0.751
Glucose, *mg/dL*	128 ± 5[a]	111 ± 3[b]	116 ± 4[b]	0.001[1]	0.786	0.377
TG, *mg/dL*	195 ± 13[a]	146 ± 10[b]	154 ± 12[b]	< 0.001[1]	0.730	0.955
FFA, *mmol/L*	0.56 ± 0.21[a]	0.52 ± 0.24[a,b]	0.47 ± 0.20[b]	0.016	0.122	0.461
CRP, *mg/L*	4.3 ± 0.7 [a]	3.1 ± 0.4[a,b]	2.5 ± 0.4[b]	0.005[1]	0.597	0.183
HOMA index, *mM/L*	4.9 ± 0.4[a]	2.8 ± 0.3[b]	3.0 ± 0.4[b]	< 0.001	0.956	0.288
Leptin, *ng/mL*	21.5 ± 2.1[a]	14.5 ± 1.5[c]	17.2 ± 1.9[b]	< 0.001	0.816	0.837
Insulin, *μU/mL*	15.4 ± 1.1[a]	9.7 ± 0.9[b]	9.6 ± 1.0[b]	< 0.001[1]	0.964	0.634
BDI questionnaire	7.6 ± 0.8[a]	4.4 ± 0.6[b]	3.8 ± 0.7[c]	< 0.001	0.777	0.798
Activity level[2]	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.154	0.697	0.184

Values are mean ± SEM n=57-60. Effects of time, diet (control diet vs RESMENA diet) and time-diet interactions were analysed with repeated-measures ANOVA or Friedman test. Values in a row with different superscript letters (a, b, c) are significantly different, P<0.05 by Bonferroni post hoc test, being a>b>c. Abbreviations: BDI, Beck Depression Inventory; BMI, body mass index; CRP, C-reactive protein; DXA, dual-energy X-ray absorptiometry; FFA, free fatty acids; MetS, Metabolic Syndrome; TG, triglycerides; WC, waist circumference.

[1] P-value based on non-parametric Friedman test compared the three time points of the study.

[2] Average daily exercise calculated by twenty-four physical activity questionnaire.

Table 3. Association between changes in BDI score and changes in CRP, leptin and insulin.

	Change in BDI score		
Changes in:	**B**	**95 % CI**	**p**
CRP *mg/L*			
Unadjusted model	0.25	0.01 to 0.49	0.043
Model 1	0.27	0.03 to 0.51	0.029
Model 2	0.26	0.01 to 0.50	0.044
Model 3	0.28	0.06 to 0.51	0.015
Leptin *ng/mL*			
Unadjusted model	0.18	0.07 to 0.29	0.002
Model 1	0.21	0.10 to 0.32	0.001
Model 2	0.22	0.08 to 0.36	0.012
Model 3	0.17	0.04 to 0.30	0.013
Insulin $\mu U/mL$			
Unadjusted model	0.21	0.02 to 0.40	0.026
Model 1	0.25	0.07 to 0.42	0.006
Model 2	0.33	0.15 to 0.52	0.001
Model 3	0.18	-0.01 to 0.36	0.059

The table shows B coefficients (95%CI) and p-value. Changes (6months-baseline), n=60. Abbreviation: BDI, Beck Depression Inventory; CRP, C-reactive protein.

Model 1: Adjusted by sex, age and dietary group. Model 2: Model 1 additionally adjusted for changes in activity level. Model 3: Model 1 additionally adjusted for changes in body fat (kg).

CHAPTER 4

Post-print version of the article:

Longitudinal relationship of diet and oxidative stress with depressive symptoms in patients with metabolic syndrome after following a weight loss treatment: the RESMENA project

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Clin Nutr. 2013 Nov. [Epub ahead of print]

doi: 10.1016/j.clnu.2013.11.011.

Impact factor (2013): 3.940

13/78 in Nutrition & Dietetics

ABSTRACT

Background & aim: Metabolic syndrome and depression seem to share some common underlying mechanisms, although less is known about the impact of metabolic syndrome dietary treatments on depression. This study examined the association between a hypocaloric treatment designed to reduce metabolic syndrome features in self-perceived depression and the potential involvement of dietary components and oxidative stress changes.

Methods: Analyses were based on volunteers (n=55) with metabolic syndrome (age 50±1 y.o.; 38M/17F), where depressive symptoms were assessed using the Beck Depression Inventory. Participants followed two hypocaloric diets (control diet and RESMENA diet) with the same energy restriction (-30% TCV) for six months. Depressive symptoms, dietary records, anthropometrical measurements, biochemical parameters and oxidative stress levels were analysed.

Results: Both diets improved self-perceived depression similarly (p=0.528). Participants with lower depressive symptoms at baseline reported a significantly higher intake of omega-3 polyunsaturated fatty acids (p trend=0.002). Interestingly, after adjusting for potential confounders, the increase in folate consumption (p=0.011) and the decrease in plasma malondialdehyde levels (p=0.012) throughout the intervention, were associated with the improvement in depressive symptoms.

Conclusions: A higher intake of folate and a decline in malondialdehyde plasma levels during a weight loss intervention, were related to improvements in manifestations of depression. (www.clinicaltrials.gov; NCT01087086).

Keywords: depression, caloric restriction, omega-3, folate, malondialdehyde, metabolic syndrome.

1. Introduction

The prevalence of depression, an emotional disability and a major public health problem worldwide, has increased substantially in recent years (Pan *et al.*, 2012). Several studies have evaluated the potential links between depression and metabolic syndrome (MetS), which have revealed that both diseases share several underlying metabolic pathways (van Reedt Dortland *et al.*, 2013). In this sense, inflammatory processes play a significant role in both MetS and depression, and this has led to an increased interest in the study of pro-inflammatory cytokines (Pascoe *et al.*, 2011). Also, a relationship between MetS and oxidative stress has been established (de la Iglesia *et al.*, 2013), and some evidence suggests that oxidative stress is also linked to depression as higher concentrations of oxidative stress biomarkers have been found in depressed subjects (Yager *et al.*, 2010; Talarowska *et al.*, 2012).

Given that nutrients may affect brain development and functioning, the importance of the role of diet in depression has attracted much interest, and there is information concerning the potential beneficial effects that isolated nutrients play in depression (Sanchez-Villegas *et al.*, 2013). One of the most frequently investigated single nutrients is omega-3 polyunsaturated fatty acids (PUFA). It has been suggested that these reduce depressive symptoms mediated by their anti-inflammatory properties and by their involvement in the functioning of the central nervous system (Pascoe *et al.*, 2011). Further, folate and other B vitamins, such as vitamins B_6 and B_{12}, are involved in homocysteine metabolism and a deficiency of these vitamins may cause increased levels of homocysteine leading to a higher risk of low mood (Qin *et al.*, 2013). Moreover, a higher intake of ascorbic acid (Payne *et al.*, 2012) and the essential amino acid tryptophan (Markus *et al.*, 2010) have been related to lower depressive symptoms and also the intake of certain minerals, including magnesium and selenium have been associated with mood (Sanmartin *et al.*, 2011; Derom *et al.*, 2013).

Despite all these well-established approaches, research focusing on understanding the role of the whole food intake on depression have just emerged, most of which are observational studies (Sanchez-Villegas *et al.*, 2009; Akbaraly *et al.*, 2013; Rienks *et al.*, 2013; Sanchez-Villegas *et al.*, 2013). A recent prospective study demonstrated

that the adherence to healthy dietary guidelines has a protective effect against future depressive symptoms in women (Akbaraly *et al.*, 2013). Accordingly, it has also been demonstrated that subjects following a Mediterranean dietary pattern were at lower risk of suffering depressive symptoms (Sanchez-Villegas *et al.*, 2009; Rienks *et al.*, 2013). Furthermore, it has been suggested that unhealthy dietary habits such as fast-food, processed food and trans-unsaturated fat consumption increase the risk of developing depression (Akbaraly *et al.*, 2009; Sanchez-Villegas *et al.*, 2013).

On the other hand, dieting has proved to be effective at reducing MetS comorbidities, since most individuals with the MetS have central obesity. In this sense, much scientific efforts has focused on designing new dietary strategies that might help to reduce MetS manifestations. One of these is the Mediterranean diet, which has been associated with reduced prevalence and incidence of MetS (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013). However, less is known about the impact of MetS dietary treatment on relieving depression.

Thus, the aim of the present study was to determine the putative association between an energy-restricted diet and self-perceived depression in participants diagnosed with MetS. Also, we sought to investigate whether the changes in the intake of some specific nutrients, from the prescribed energy-restricted diet, influenced symptomatic depression, as well as the relationship between oxidative stress and depressive symptoms after weight loss.

2. Material and methods

2.1. Study design

The current study is based on a subsample of the RESMENA-S study, which was designed as a randomized, controlled intervention trial to compare the effects of two hypocaloric dietary strategies on MetS comorbidities over a six-month period. Participants were assigned (using the "random between 1 and 2" function in the Microsoft Office Excel 2003 software (Microsoft Iberica, Spain) (Zulet *et al.*, 2011)) to follow one of the two energy-restricted diets, the Control diet or the RESMENA diet by trained dietitians. Also, they were asked to maintain their usual physical activity, which was controlled by a 24-h physical activity questionnaire administered at the

beginning and at the end of the study. The intervention lasted six months during which psychological monitoring of the participants was carried out using the BDI questionnaire.

The RESMENA study followed the CONSORT 2010 guidelines, except for blinding. It was performed according to the ethical guidelines of the Declaration of Helsinki, and it was registered (www.clinicaltrials.gov; NCT01087086). The study was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009). Additional aspects of this intervention trial have been previously detailed (Zulet *et al.*, 2011).

2.2. Subjects

This study was conducted in the Metabolic Unit of the University of Navarra in Pamplona, Spain, over a period of 23 months (from January 2010 to November 2011). Subjects were recruited through local advertisements and the Department database.

The inclusion and exclusion criteria have been previously detailed (Zulet *et al.*, 2011). Briefly, subjects should be adults presenting MetS according to IDF criteria. However, all subjects with chronic diseases related to nutrient metabolism, those following special diets and those experiencing body weight changes in the last three months were excluded from the trial. Those subjects previously diagnosed with psychiatric disorders as well as those who had a current prescription of antidepressant drugs were also excluded. The study details were explained to all recruited volunteers by the dietitians and written informed consent was obtained from all of them before the trial was started.

2.3. Prescribed diets

Two-energy-restricted diets, both with the same energy restriction (-30% of the studied requirements), were prescribed and compared. The Control diet was based on the American Heart Association (AHA) guidelines, including 3-5 meals/day, a macronutrient distribution of 55% Total Caloric Value (TCV) from carbohydrates, 15% from proteins and 30% from lipids, a healthy fatty acids (FA) profile, a cholesterol consumption lower than 300 mg/day and a fiber intake of 20-25 g/day.

In contrast, the RESMENA diet was designed with a higher meal frequency, consisting of 7 meals/day, and a macronutrient distribution of 40% TCV from carbohydrates, 30% from proteins and 30% from lipids (Zulet *et al.*, 2011). As was the case with the Control group, the RESMENA diet also maintained a healthy FA profile, a cholesterol content of less than 300 mg/day and a fiber intake of 20-25 g/day.

A 48-h weighed food record was collected at the beginning and at the end of the study, which was used to assess the volunteer's adherence to the prescribed diet. The energy and nutrient content of these questionnaires were determined using the DIAL software (Alce Ingenieria, Madrid, Spain) (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013).

2.4. Anthropometric and biochemical measurements

Anthropometric measurements were taken in fasting conditions and following standardized procedures previously described (Zulet *et al.*, 2011). Serum glucose and total cholesterol were measured in an Pentra C-200 autoanalyser (HORIBA ABX, Madrid, Spain) with specific kits. Plasma malondialdehyde (MDA) levels were colorimetrically determined as a marker of lipid peroxidation using a commercial kit (BIOXYTECH® LPO-586™, Oxis Research™, Portland, OR, USA). Colorimetric assays were read using a laboratory spectrophotometer (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). Serum homocysteine levels were assessed by an automatized colorimetric assay (COBAS MIRA, Roche, Basel, Switzerland).

2.5. Assessment of depressive symptoms

The BDI was the screening tool used to assess depressive symptoms at the beginning and at the end of the study. The BDI is a self-administrated validated 21-item scale that measures depressive symptoms providing a continuous score ranging from 0-63. Each item is rated on 0-3 scale with a score ≥10 reflecting moderate depressive symptoms (Beck *et al.*, 1961). Question number 19 of the test, concerning weight loss, was removed from the analysis because one of the aims of the participants of this study was to lose weight and therefore cannot be considered a depressive symptom in our sample.

2.6. Outcome evaluation

The primary outcomes were measurements associated with metabolic syndrome features, such as anthropometric parameters and lipid and glucose profile assessments. Secondary endpoints were self-perceived depression and plasma MDA levels (Zulet *et al.*, 2011).

2.7. Statistical analyses

In the RESMENA study, sample size was calculated based on previous studies (Katcher *et al.*, 2008). Therefore, a sample size of 100 was estimated to be sufficient to obtain a statistically significant difference ($p < 0.05$) in the reduction of the waist circumference (main variable) of 4.3±6.8cm, with a power of 80% and an estimated dropout rate of 25%.

Statistical analysis was performed using the SPSS 15.0 software for Windows (SPSS Ibérica, Madrid, Spain). The main results were summarized as mean ± SE, unless otherwise specified. Following published studies, the BDI score was analysed as continuous variable (Morris *et al.*, 2012). Drop-out analyses were performed using the χ^2 test. Changes between the beginning and the end of the study in within each dietary group were analyzed using a paired t-test. The differences in changes between both groups were assessed by analysis of covariance (ANCOVA) using a general linear model, with sex and age as covariates, and also with total grams or total energy intake when appropriate. Participants were categorized into tertiles of BDI at baseline. Tests of linear trend across increasing tertiles of the depressive symptoms questionnaire were conducted for the different nutrient intakes, which mean was adjusted for total energy intake, sex and age.

Multiple linear regression analysis was used to assess the contributions of increasing the intake of specific nutrients (6 months – baseline) to the changes occurred in BDI, and were represented by B coefficients, 95% Confidence Intervals (CI) and *p*-value. Four models were constructed: Model 1 consisted of an unadjusted model. Model 2 included the first unadjusted model plus age, sex and both the depressive symptoms inventory and the specific nutrient intake at baseline. Model 3 included model 2 additionally adjusted for dietary group, and model 4 consisted of model 2 plus

weight loss. Partial correlation analysis was performed to assess the association between the change in MDA and the change in BDI (adjusted by their initial values, sex, age and weight loss). Differences were considered statistically significant at $p < 0.05$.

3. Results

A total of 109 Caucasian adults were initially enrolled in the study, however, 12 did not present MetS according to the IDF criteria when the study began, and another 4 subjects decided not to start the dietary treatment after signing the written informed consent. Therefore, 93 subjects presenting MetS started the intervention trial. After six months of weight loss intervention, there were twenty-six dropouts, and sixty-seven subjects finished the study. Dropouts were due to loss to follow-up or consent withdrawal. The present study assessed the data from a subsample of 55 volunteers (age: 50±1 y.o.; 38M/17F) who fully completed all the 48-hour weighed food records and Beck Depression Inventory (BDI) questionnaires, as described in the flowchart (**figure 1**).

The main characteristics of the 55 participants with MetS according to the nutritional intervention group are summarised in **Table 1**. Both Control and RESMENA dietary strategies were effective at improving anthropometric and biochemical variables, with the exception of total cholesterol and homocysteine, which did not decrease significantly in either group. In addition, both Control and RESMENA diets proved to be effective at improving the BDI score. However, no differences were observed among dietary groups in any of the variables analysed in **Table 1**. There was a relatively higher number of drop-outs in the RESMENA (n=16) group compared with the Control group (n=10), although this difference was not statistically significant (p=0.233). Furthermore, from those participants whose baseline BDI score was available, no statistical differences were found between treatment completers (n=62) and dropouts (n=25) with respect to the initial BDI score (P=0.255).

The dietary records after the 6 months of the trial revealed that as designed, the RESMENA group consumed more protein during the dietary treatment than the control group. However, no differences were observed between groups in the intake

of the remaining macronutrients. As for food intake, the RESMENA group consumed more meat, fish and eggs, and fewer cereals than the control group. In addition, no differences were found in the micronutrient intake with the exception of omega-3 PUFA (**Table 2**).

As no significant differences were found between dietary groups in the main variable of this study (BDI score), the two groups were merged and analysed together as a single observational experimental group. Nevertheless, in order to control for the possible confounding role of the intervention group, this variable was included in the following adjusted models.

Approximately 29% of the subjects presented self-perceived depression (BDI score ≥ 10) at the beginning of the study (BDI score: 14.2±1.6). The baseline relations between the BDI and different nutrient consumption are presented in **Table 3**. Remarkably, participants with a lower BDI score reported a significantly higher intake of omega-3 PUFA (p trend=0.002). Moreover, in those subjects with a low BDI there was a trend toward statistical significance in favour of a higher fiber (p trend=0.076) and folate (p trend=0.093) consumption.

There was no statistical difference in the consumption of total grams of vegetables between dietary groups (p=0.430). However, it must be highlighted that there was a trend toward significance for the association between a higher intake of total grams of vegetables during the dietary intervention and a greater decline in depressive symptoms (B=-0.005, 95% CI=0.003 to -0.235, p=0.070).

In a longitudinal analysis, a positive association between the decline in depressive symptoms and the change in body weight (B=-0.297, 95% CI=0.110 to -0.484, p=0.003) was noted. As a result, the change in body weight was included in some of the following adjusted models. Due to the BDI score improvement throughout the weight loss treatment, additional analyses were carried out to assess the nutrient consumption that most influenced this outcome (**Table 4**). Model 1 (unadjusted model) showed that the improvement in BDI might be mediated by a higher intake of fiber (p=0.001), thiamine (p=0.038), vitamin B_6 (p=0.047), folate (p<0.001), omega-3 PUFA (p=0.022), ascorbic acid (p=0.031) and magnesium (p=0.004). Model 2 (adjusted for sex, age, BDI score at baseline and the initial nutrient intake) revealed

that only fiber (p=0.038), folate (p=0.002) and magnesium (p=0.006) consumption were involved in the decline of depressive symptoms. Model 3 (model 2 additionally adjusted for dietary group) was carried out to evaluate the impact of the dietary group, demonstrating that this variable was not involved in the influence that the nutrient consumption had on the BDI decline. Interestingly, model 4 (Model 2 additionally adjusted for weight loss), revealed the importance of weight loss in the improvement of depressive symptoms, with fiber (p=0.170) and magnesium (p=0.056) losing the significant effect that they had in the previous models. Irrespective of adjustments, a higher consumption of folate during the dietary intervention was involved with the BDI decline in all four models (p=0.011).

Furthermore, partial correlation analysis revealed that the decline in depressive symptoms was positively associated (r=0.357, p=0.012) with the reduction in MDA plasma levels after the 6 months of dietary treatment (**Figure 2**).

4. Discussion

In this study, we investigated the relationship between diet, oxidative stress and depressive symptoms in subjects with MetS who followed an energy-restricted diet for weight loss, using both initial and follow-up data. It was found that both dietary treatments, the AHA guidelines based diet and the RESMENA diet, achieved a decrease in depressive symptoms after six months of weight loss. In addition, independent of adjustments, folate was the nutrient that had the greatest positive influence on depressive symptoms, with MDA plasma levels also being involved in this improvement.

To the best of our knowledge this is the first interventional study analysing the effect of diet and oxidative state on symptomatic depression in subjects with MetS before and after a weight loss intervention. Both Control and RESMENA diets led to a decrease in depressive symptoms although neither of them proved to be better than the other. In this context, as both diets were designed following a healthy pattern, it is not surprising that we did not observe differences between dietary groups.

At baseline, an association between omega-3 PUFA consumption and depressive symptoms was observed. The impact of this nutrient on depressive symptoms has

sparked particular interest, leading to a number of reviews and meta-analyses that suggest a potential beneficial effect on depressive illness, but data are scarce and often inconsistent (Pascoe *et al.*, 2011; van de Rest *et al.*, 2012).

As for nutrient consumption during the trial, which was obtained through the dietary records, and their association with the BDI decline, the unadjusted model revealed that an increase in the consumption of fiber, thiamine, vitamin B_6, folate, omega-3 PUFA, ascorbic acid, and magnesium during the six month of dietary treatment were all associated with a decrease in BDI. The possible involvement of fiber in depression has received little attention, although a lower intake of fiber has been observed in depressive subjects (Park *et al.*, 2010). With regard to B vitamins, low vitamin B_6 and folate consumption has been associated with depression (Shabbir *et al.*, 2013). Low thiamine consumption has been associated with higher risks of depressive symptoms (Zhang *et al.*, 2013). In addition, it has been suggested that ascorbic acid may also influence depression (Payne *et al.*, 2012). Finally, low magnesium levels have been related to negative mood scores, although more studies are needed to draw firm conclusions (Derom *et al.*, 2013). Nevertheless, it should be pointed out that in this study, after adjustments, most of these relationships were no longer statistically significant. The adjusted model revealed that the improvements in depressive symptoms were mirrored by a higher fiber, magnesium and folate intake during the dietary treatment, with folate being the nutrient that had most influence on this improvement.

Moreover, whole grains, fruits and vegetables, which are large contributors to dietary fiber, contain B vitamins and antioxidants, and have been demonstrated to improve mood (Logan, 2006). Our results showed a trend toward significance for the association between a higher intake of total grams of vegetables during the dietary intervention and a greater decline in depressive symptoms. This finding may be explained by the fact that within the food exchange system diets, consumption of vegetables very low in calories such as lettuce, celery, zucchini, onion or cucumber, was allowed ad libitum in both dietary groups in order to increase satiety and given their low caloric value. This higher consumption of vegetables, which are known for

being rich in B vitamins and fiber, may have contributed to the decline in depressive symptoms.

In this trial, homocysteine levels did not decrease after the dietary treatment although elevated serum levels of total homocysteine have been related to depressive symptoms (Gu *et al.*, 2012). In contrast, a recent published study revealed that homocysteine lowering induced by B vitamins did not significantly reduce global cognitive decline (Kwok *et al.*, 2011). Despite the increase in folate intake during the dietary treatment in this study, a decline in serum homocysteine levels was not detected. This outcome can be explained because our participants did not receive vitamin B supplementation, just a hypocaloric diet, and it is reasonable to assume that this amount was not sufficient to decrease homocysteine levels.

Oxidative stress has a role in the pathogenesis of a number of diseases, including MetS and depression (de la Iglesia *et al.*, 2013). Since the brain is sensitive to oxidative damage that might alter neural signals, there is evidence that oxidative stress is involved in depression (Yager *et al.*, 2010). MDA is a product of lipid peroxidation, and thus is considered a biomarker of oxidative stress (de la Iglesia *et al.*, 2013). In this sense, increased levels of this biomarker have been reported in patients with depressive symptoms and MetS (Talarowska *et al.*, 2012; de la Iglesia *et al.*, 2013). Our findings are in agreement with this statement because the greater the decrease in MDA levels, the more marked the decline in depressive symptoms.

Several limitations of the current study should be noted. First, depressive symptoms were assessed using a self-reported questionnaire, the BDI, and not a clinical diagnosis. Nevertheless, good reliability and validity for the BDI have been reported and the instrument is widely used in psychiatric research (Beck *et al.*, 1961; Davidson *et al.*, 2006). It is also worth mentioning that this tool has been previously utilized to assess depressive symptoms in weight loss studies (Somerset *et al.*, 2011). In addition, the objective of this trial was to explore depressive symptoms and not to examine a diagnosed depression, thus participants previously identified as having mental disorders were excluded. Also, our aim was to offer a psychological monitoring as a part of the dietary intervention, analysing the degree of depressive symptoms according to weight loss and dietary components. Second, the use of

weighed food records may lead to bias. However, diary records are more precise than other dietary assessments, for example food frequency questionnaires (Akbaraly *et al.*, 2013). Third, although the RESMENA diet promoted a higher intake of omega-3 PUFA, the weighed food records revealed that the control group consumed higher amounts of this fatty acid. Finally, the authors acknowledge that the number of drop-outs and the missing data from some volunteers have decreased the sample size of this trial. However, the fact that important statistical differences were found despite the smaller sample size, suggests that potential type-II errors were overcome.

5. Conclusions

In summary, the results of this study indicate that the increase in folate consumption, and also the decline in MDA levels, during a weight loss treatment program by subjects with MetS, decreased depressive symptoms. Also, both the Control diet and RESMENA diets may be recommended to improve depressive symptoms. The findings here reinforce that oxidative stress and some dietary compounds have an impact on depressive symptoms. However, to date, this is the only study to report these results in a MetS population and therefore further investigation is needed to confirm our findings.

Acknowledgements

The authors are grateful to the volunteers of the study as well as to the physician Blanca E. Martínez de Morentín, the nurse Salomé Pérez, and the technician Verónica Ciaurriz for excellent technical assistance in the University of Navarra. We would like to thank Dr Paul W. Miller from the Institute of Modern Languages of the University of Navarra for careful reading of the manuscript. The pre-doctoral research grant to Aurora Pérez-Cornago from the Asociación de Amigos Universidad de Navarra is gratefully acknowledged.

Statement of authorship

The authors' contributions were as follows: A.P.C. contributed to the data collection, analysis and writing of the manuscript; P.L.L. and R.I. were involved in the fieldwork. F. L. contributed with the choice and the interpretation of the psychological test.

J.A.M., project co-leader, was responsible of the follow-up, design, financial management and editing of the manuscript. M.A.Z. was responsible for the general coordination, follow-up, design, and financial management. All the authors actively participated in the manuscript preparation, as well as read and approved the final manuscript.

Source of funding

This study was supported by the Health Department of the Government of Navarra (48/2009) and the Linea Especial about Nutrition, Obesity and Health (University of Navarra LE/97). The support from CIBERobn and RETICS schemes is gratefully accredited.

Conflict of interest

None declared.

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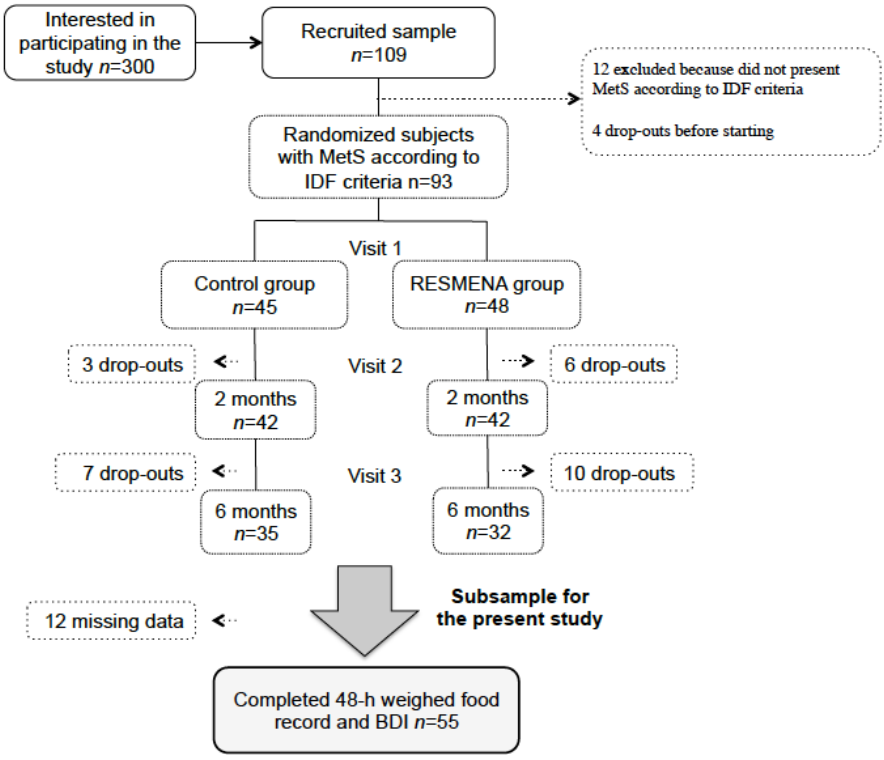

Figure 1. Flowchart of participants in the present analysis.

Abbreviations: BDI, Beck Depression Inventory; MetS, Metabolic Syndrome; IDF, International Diabetes Federation.

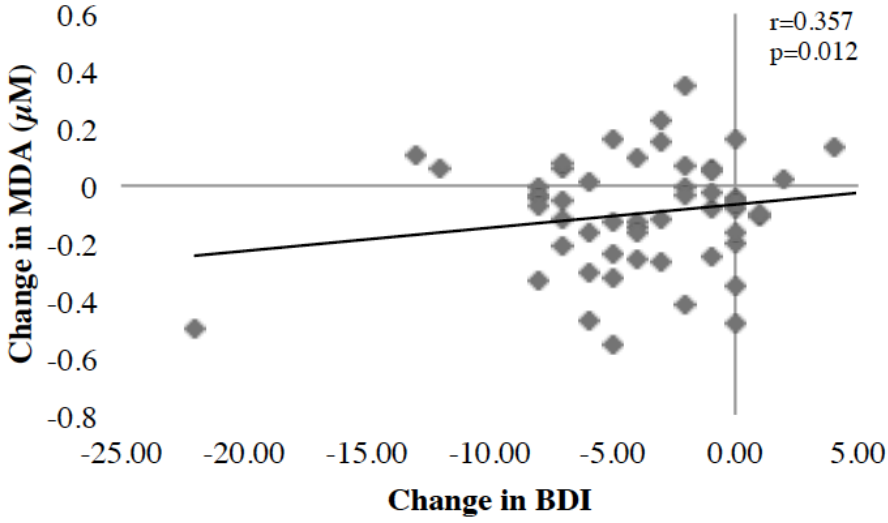

Figure 2. Association between changes (6months – baseline) in MDA and changes (6months – baseline) in BDI score.

Abbreviations: BDI, Beck Depression Inventory; MDA, malondialdehyde.

Adjusted by sex, age, weight loss and MDA and BDI score at baseline.

Table 1. General characteristics and changes in anthropometric and biochemical variables and scores of BDI.

	Control group (n=30)		**RESMENA group (n=25)**		***P***
Variables	**Baseline**	**6 months**	**Baseline**	**6 months**	
Age (years)	49.4±1.7		50.4±2.0		-
Male sex % (n)	70 (21)		68 (17)		-
Weight (kg)	102.6±3.1	94.2±3.2**	104.8±3.4	95.7±3.5**	0.679
Waist circumference (cm)	113.6±2.3	105.8±2.3**	116.2±2.5	106.0±2.4**	0.092
Total fat mass (kg)	42.0±1.5	35.6±1.7**	43.3±2.0	35.9±2.0**	0.470
Lean mass (kg)	57.8±2.3	55.4±2.1**	57.8±2.1	56.4±2.2*	0.120
MetS components	4.0±0.1	3.0±0.2**	4.1±0.1	3.5±0.2*	0.224
Total cholesterol (mg/dl)	219.9±7.7	220.1±7.8	214.6±8.3	213.8±7.9	0.956
Glucose (mg/dl)	126.9±7.0	119.7±5.2	126.4±6.4	112.7±5.7*	0.280
Homocysteine (μmol/L)	15.5±0.7	15.6±0.8	14.1±0.7	17.6±1.5	0.160
MDA (μM)	0.92±0.08	0.80±0.08*	0.81±0.07	0.75±0.06	0.170
BDI score	8.1±1.2	3.8±1.1**	7.0±1.0	3.4±0.6**	0.528

Data expressed as mean±SE. Symbols: * $p<0.05$; ** $p<0.001$ (comparison between baseline and 6 months within each dietary group); *P*: Changes after 6 months of dietary treatment were analysed using ANCOVA with age and sex as covariates. Abbreviations: MDA, malondialdehyde; MetS, metabolic syndrome; BDI, Beck Depression Inventory.

Table 2. Comparison of the two dietary treatments, control diet and RESMENA diet, followed by the subjects with MetS at the endpoint.

	Control group (n=30)	**RESMENA group (n=25)**	***P* value**
Total Energy Intake (Kcal/day)	1533.6 ± 59.0	1565.1 ± 64.7	0.720
Macronutrients			
Proteins (% TCV/day)[a]	17.4 ± 0.6	20.5 ± 0.7	0.002
Lipids (% TCV/day)[a]	40.7 ± 1.1	38.2 ± 1.2	0.131
CHO (% TCV/day)[a]	37.1 ± 1.2	35.9 ± 1.3	0.508
Micronutrients			
Fiber (g/day)[b]	18.6 ± 1.4	18.2 ± 1.5	0.863
Thiamine (g/day)[b]	1.2 ± 0.1	1.3 ± 0.1	0.234
Vitamin B_6 (g/day)[b]	1.9 ± 0.1	1.8 ± 0.1	0.584
Folate (µg/day)[b]	301.2 ± 22.4	269.6 ± 24.7	0.362
Omega 3 PUFA (g/day)[b]	0.28 ± 0.02	0.07 ± 0.03	<0.001
Ascorbic acid (mg/day)[b]	155.2 ± 13.1	160.7 ± 14.5	0.784
Tryptophan (mg/day)[b]	673.3 ± 32.0	692.2 ± 35.2	0.701
Magnesium (mg/day)[b]	258.7 ± 11.0	270.8 ± 12.1	0.474
Selenium (µg/day)[b]	84.6 ± 5.0	75.1 ± 5.5	0.222
Vitamin B_{12} (µg/day)[b]	5.2 ± 1.1	6.0 ± 1.2	0.641
Foods			
Meat + fish + eggs (Kcal/day)[a]	269.7 ± 23.3	365.3 ± 25.6	0.008
Cereals (Kcal/day)[a]	349.4 ± 27.9	231.9 ± 30.6	0.007
Fruits + vegetables (Kcal/day)[a]	226.8 ± 24.9	276.8 ± 27.3	0.182
Dairy products (Kcal/day)[a]	219.8 ± 21.6	257.5 ± 23.7	0.247
Legumes (Kcal/day)[a]	34.1 ± 12.1	42.8 ± 13.2	0.630
Sugar, sweets and bakery (Kcal/day)[a]	7.73 ± 6.0	25.2 ± 6.5	0.054

Data expressed as mean±SE. Abbreviations: CHO, carbohydrates; PUFA, Polyunsaturated fatty acids.

Adjusted for sex, age and the total calorie value[a] or total grams intake[b] at the end of the study.

Table 3. Association of the consumption of different nutrients with the BDI questionnaire at baseline.

	Tertiles of BDI at baseline			
	Low	**Moderate**	**High**	***P* for trend**
Fiber (g/day)	25.04 ± 2.06	21.97 ± 2.07	19.97 ± 1.86	0.076
Thiamine (g/day)	1.81 ± 0.12	1.35 ± 0.13	1.56 ± 0.11	0.147
Vitamin B_6 (g/day)	2.31 ± 0.16	2.17 ± 0.16	2.07 ± 0.14	0.261
Folate (μg/day)	12.81 ± 4.53	6.60 ± 4.57	2.27 ± 4.10	0.093
Omega 3 PUFA (g/day)	0.40 ± 0.03	0.28 ± 0.03	0.27 ± 0.03	0.002
Ascorbic acid (mg/day)	150.75 ± 22.84	138.04 ± 23.04	130.04 ± 20.68	0.508
Tryptophan (mg/day)	956.92 ± 56.03	991.41 ± 56.52	880.30 ± 50.74	0.319
Magnesiun (mg/day)	340.12 ± 19.48	308.49 ± 19.64	322.03 ± 17.63	0.498
Selenium (μg/day)	124.28 ± 9.17	138.73 ± 9.25	120.52 ± 8.30	0.764
Vitamin B_{12} (μg/day)	6.12 ± 1.89	10.67 ± 1.91	6.30 ± 1.71	0.944

Data expressed as mean±SE. Abbreviation: BDI, Beck Depression Inventory.

Adjusted for sex, age and the total calorie value at baseline.

Table 4. Association between the change of individual nutrients intake during the dietary treatment and the change in BDI.

	Change in BDI											
	Model 1			**Model 2**			**Model 3**			**Model 4**		
Changes in:	**B**	**95 % CI**	**P**	**B**	**95 % CI**	**p**	**B**	**95 % CI**	**p**	**B**	**95 % CI**	**p**
Fiber (g/day)	-0.162	-0.257 to -0.068	0.001	-0.132	-0.257 to -0.008	0.038	-0.135	-0.261 to -0.009	0.036	-0.083	-0.204 to 0.037	0.170
Thiamine (g/day)	-1.816	-3.531 to -0.102	0.038	-1.658	-3.861 to 0.545	0.137	-1.796	-4.121 to 0.529	0.127	-0.696	-2.771 to 1.380	0.504
Vitamin B_6 (g/day)	-1.363	-2.705 to -0.021	0.047	-1.271	-2.720 to 0.178	0.084	-1.346	-2.821 to 0.129	0.073	-0.638	-1.980 to 1.017	0.372
Folate (µg/day)	-0.058	-0.082 to -0.034	<0.001	-0.039	-0.063 to -0.015	0.002	-0.039	-0.063 to -0.014	0.003	-0.031	-0.054 to -0.007	0.011
Omega 3 PUFA (g/day)	-6.323	-11.704 to -0.941	0.022	-2.065	-7.925 to 3.795	0.482	-4.712	-12.788 to 3.365	0.247	-3.081	-8.568 to 2.407	0.265
Ascorbic acid (mg/day)	-0.011	-0.021 to -0.001	0.031	-0.008	-0.019 to 0.004	0.181	-0.008	-0.020 to 0.003	0.164	-0.004	-0.015 to 0.007	0.447
Tryptophan (mg/day)	-0.001	-0.006 to 0.003	0.534	0.001	-0.005 to 0.006	0.776	0.001	-0.005 to 0.006	0.834	0.001	-0.004 to 0.006	0.753
Magnesium (mg/day)	-0.018	-0.029 to -0.006	0.004	-0.019	-0.032 to -0.006	0.006	-0.021	-0.035 to -0.007	0.004	-0.013	-0.027 to 0.000	0.056
Selenium (µg/day)	-0.010	-0.035 to 0.015	0.441	-0.010	-0.045 to 0.026	0.596	-0.009	-0.041 to 0.033	0.615	-0.012	-0.044 to 0.021	0.478
Vitamin B_{12} (µg/day)	0.045	-0.074 to 0.165	0.452	0.070	-0.088 to 0.228	0.378	0.067	-0.095 to 0.229	0.409	0.059	-0.085 to 0.204	0.415

The table shows B coefficients (95%CI) and p-value. Abbreviation: BDI, Beck Depression Inventory.

Model 1: Unadjusted model. Model 2: Adjusted by sex, age, BDI score at baseline and the initial intake of each nutrient. Model 3: Model 2 additionally adjusted for dietary group. Model 4: Model 2 additionally adjusted for weight loss.

CHAPTER 5

Post-print version of the article:

Effect of dietary restriction on peripheral monoamines and anxiety symptoms in obese subjects with metabolic syndrome

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Psychoneuroendocrinology. 2014 Sep; 47:98-106

doi: 10.1016/j.psyneuen.2014.05.003

Impact factor (2013): 5.591

13/135 in Psychiatry

16/123 in Endocrinology & Metabolism

33/251 Neuroscience

Summary

Reduced circulating monoamines may have a role in the development of the metabolic syndrome (MetS), which is becoming a major health problem worldwide. Moreover, an association between anxiety disorder and MetS has been reported; however, it is not clear whether weight loss can diminish anxiety. This investigation is aimed to examine the effects of a weight loss intervention on peripheral monoamines levels and anxiety symptoms in subjects with metabolic syndrome (MetS). The study population encompassed subjects with MetS (Age:50±10 y.o. and BMI:35.8±4.3 kg/m2) selected from the RESMENA study after they had completed the 6-month weight loss intervention (-30% energy). Anthropometric measurements, dietary records, anxiety symptoms, and blood monoamines levels were analysed before and after the intervention. Dopamine (DA) (+18.2%; 95% confidence interval (CI):-51.2 to -0.5) and serotonin (5-HT) (+16.1%; 95% CI:-26.3 to -2.2) blood levels were significantly increased after the intervention. Higher DA blood concentrations at the end of the study were inversely related with the carbohydrate intake during the study (B=-3.3; 95% CI:-8.4 to -0.4) and basal DA levels predicted a greater decrease in body weight and anthropometric parameters. Subjects with higher 5-HT concentrations after the weight loss intervention also showed a lower energy intake during the intervention (B=-0.04; 95% CI:-0.07 to -0.01). Additionally, anxiety symptoms decreased after the weight loss treatment (-28.3%; 95% CI:6.2 to 20.4), which was parallel to a greater decrease in body weight and anthropometric markers, being related to lower 5-HT basal levels. Dietary restriction in patients with MetS may help in reducing anxiety symptoms, and also in increasing 5-HT and DA blood levels. These results provide further insights regarding emotional and neurological factors behind weight loss.

Keywords: peripheral monoamines, anxiety symptoms, serotonin, dopamine, metabolic syndrome, weight loss, diet.

1. Introduction

The prevalence of the metabolic syndrome (MetS), characterized by excessive adiposity, hypertension, impaired glucose tolerance and hyperlipidemia, has become a major public health problem in most developing countries (Alberti *et al.*, 2005). Lowering body weight and improving abnormalities in lipid and glucose metabolism have been applied as useful strategies, however intensive scientific effort is still required to prevent and treat this syndrome (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013). Moreover, subjects suffering from MetS also present a higher prevalence of mental disorders, in particular greater symptoms of depression, anxiety and eating disorders (Pan *et al.*, 2012). This MetS-mood disorders relationship is likely to be mediated by several mechanisms and it has been suggested to be bidirectional (Pan *et al.*, 2012). One of the mechanisms that may link these two disorders is the hypothesis of monoamine imbalance in the central (CNS) and peripheral nervous system (PNS) (Luppino *et al.*, 2010; Pan *et al.*, 2012).

In this context, serotonin (5-HT) is an endogenous amine synthesized from the dietary amino acid tryptophan (Trp), which controls central and peripheral functions (Berger *et al.*, 2009). A diet rich in carbohydrate (CHO) and poor in protein gives Trp advantage in the competition for access to the brain, due to the raise of the plasma Trp to the sum of the other large neutral amino acids (Trp/LNAA) ratio (Wurtman *et al.*, 2003). This monoamine is involved in multiple brain processes such as feeding behaviour, psychiatric disorders and the regulation of circadian rhythm (Berger *et al.*, 2009). However, most 5-HT in the body (close to 95%) exists outside the CNS, being mainly released into the gut by enterochromaffin cells and stored in blood platelets, whose action in peripheral tissues and organs has been less extensively investigated (Berger *et al.*, 2009). Peripheral 5-HT works as a peripheral hormone affecting vasoconstriction, liver repair, gastrointestinal and endocrine function, glucose and lipid metabolism (Watanabe *et al.*, 2011) as well as regulating body weight and energy homeostasis (Berger *et al.*, 2009; Stunes *et al.*, 2011). Many studies have reported that 5-HT does not cross the blood-brain barrier (BBB), however, it has been also demonstrated that augmented 5-HT levels within the brain crosses the BBB through the 5-HT transporter to the circulating blood (Nakatani *et*

al., 2008). Therefore, blood 5-HT has been used as an indicator of mood disorders in previous investigations, finding negative associations between whole-blood 5-HT and both depression and anxiety symptoms (Williams *et al.*, 2006; Sekiyama *et al.*, 2013).

It is also well established that dopamine (DA), a key neurotransmitter in the brain synthesized from the amino acid tyrosine, plays a critical role in the regulation of food reward, eating behaviours, learning and memory (Carlin *et al.*, 2013). Prolonged high fat diet intake is known to alter brain DA levels (Carlin *et al.*, 2013; Kaczmarczyk *et al.*, 2013) while an inverse association between body mass index (BMI) and DA D2 receptors in the striatum of obese individuals have also been reported (Wang *et al.*, 2001). However, while DA brain functions are well-known, the identification of DA as an important peripheral monoamine has just emerged (Rubi *et al.*, 2010). Peripheral DA is mainly released into the blood from neuronal fibers, adrenal gland, and neuroendocrine cells (Rubi *et al.*, 2010). This monoamine is involved in a wide variety of peripheral functions, including the control of both glucose metabolism and body weight, the modulation of blood pressure, the inhibition of gastric acid secretion as well as apoptosis of tumoral cells (Pernet *et al.*, 1984; Eliassi *et al.*, 2008; Rubi *et al.*, 2010).

On the other hand, the monoamine noradrenaline (NA), which is also synthesized from the amino acid tyrosine, is found in both the CNS and the PNS (Morton *et al.*, 2006). Low NA in the CNS may raise the risk of suffering from obesity by inducing an increase of food intake in order to restore the reward system (Morton *et al.*, 2006). Plasma NA mainly derives from overspill of NA from sympathetic nerve endings (Esler *et al.*, 1990). This monoamine has multiple functions; it triggers thermogenic actions since it regulates lipolysis decreasing fat mass (Zhao *et al.*, 1999) and it is also implicated in the immune response, mood and sleep regulation. However, very high plasma NA levels may have a detrimental effect, as it may cause heart failure due to the extreme activation of sympathetic nervous system (Kimura *et al.*, 2010).

Given these findings and the scarcity of studies in this area, there is a need to further investigate the association of peripheral monoamines blood levels with MetS features, dietary intake, weight loss and anxiety symptoms. Therefore, the primary

aim of the present study was to examine the impact of weight loss on indices of peripheral monoamines and anxiety status in obese MetS subjects. A secondary aim was to assess the putative relationships between blood monoamines levels, dietary intake and anxiety levels.

2. Methods

2.1. Study protocol

The present study deals with a subsample of the RESMENA (Metabolic Syndrome Reduction in Navarra) project (ClinicalTrials.gov: NCT01087086), a randomised controlled trial designed to compare the effect of two dietary strategies, a control and RESMENA diets, for weight loss on improving clinical criteria and biomarkers associated with MetS. The study lasted a total of six months in which participants were randomly allocated to follow the control or the RESMENA diet, both with the same energy restriction (-30% energy of the studied requirements) by using the "random between 1 and 2" function in the Microsoft Office Excel 2003 software (Microsoft Iberica, Spain). Compliance to the diet composition of the participants was conducted taking into account 48h weighed food records according to previously described procedures (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013). To control for Hawthorne effect, both control and RESMENA groups were treated under exactly the same conditions except for the prescribed diet (McCarney *et al.*, 2007). Participants were instructed to practice their usual physical activity during the intervention, which was monitored using a 24-h physical activity recall (Perez-Cornago *et al.*, 2013). Further details about the diets, design and methods of this study are available in detail elsewhere (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013).

2.2. Subjects

The study population encompassed women (n=25) and men (n=37) with MetS according to IDF criteria (Alberti *et al.*, 2005) derived from the RESMENA study after they completed the 6-month weight loss intervention (Age:50±1 y.o.; BMI:35.8±0.5 kg/m^2, 95% confidence interval (CI): 34.7 to 36.9). Subjects were carefully chosen within those who had completed monoamines data of both the beginning and the

end of the study from the two arms of the study: control group (n=32) and RESMENA group (n=30) (**Figure 1**). Details of the screening inclusion and exclusion criteria have been previously reported (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013), but it should be highlighted that subjects suffering psychiatric disturbances were not allowed to participate in the study (Zulet *et al.*, 2011). In addition, for most of the analyses carried out in this paper, both control and RESMENA groups were pooled and longitudinally analysed together as a unique observational cohort group (Perez-Cornago *et al.*, 2013).

The RESMENA study followed the CONSORT 2010 guidelines for randomised controlled trials and was registered at the ClinicalTrials.gov (identifier: NCT01087086) (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013; Perez-Cornago *et al.*, 2013). All participants gave written informed consent. This study complied with all provisions of the Declaration of Helsinki and it was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009).

2.3. Assessment of anxiety symptoms

Anxiety symptoms were assessed using the validated Spanish translation of the State-Trait Anxiety Inventory (STAI) (Spielberger *et al.*, 1971). This questionnaire consists on 20 brief items answered on a 4-point Likert-type scale (ranging from 1 for “not at all” to 4 for “very much”). Total score was obtained by summing all items and final score was transformed into percentiles, with higher values indicating greater anxiety (Spielberger *et al.*, 1971).

2.4. Anthropometric and biochemical measurements

Anthropometry and body composition measurements were carried out in fasting conditions under standard conditions as previously described (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2013). Serum glucose, HDL-cholesterol and triglycerides serum concentrations were determined in an autoanalyser Pentra C-200 (HORIBA ABX) with specific kits (Perez-Cornago *et al.*, 2013).

Fasting plasma was collected by venepuncture into an EDTA tube (Vacutainer) and frozen immediately at -80ºC until the analysis of monoamines concentrations was performed. Peripheral concentrations of DA, dopac, 5-HT, 5-hydroxyindoleacetic (5-

HIAA) and NA were analyzed using high-performance liquid chromatography (HPLC). Samples were injected using an automatic sample injector (Waters 717 plus) onto a Spherisorb ODS-2 reverse phase column (10 mm, 150 x 4.6 mm, Waters) connected to a DECADE amperometric detector (Antec Leyden, Zoeterwoude, The Netherlands), with a glassy carbon electrode maintained at 0.7 V with respect to a Ag/AgCl reference electrode. The mobile phase consisted of NaH_2PO_4 0.05 M, octanesulphonic acid 0.16 mM, EDTA 0.1 mM and methanol 16% (pH 3), pumped at a flow rate of 1 ml/min.

2.5. Statistical analyses

This study is a longitudinal subanalysis within the RESMENA study. A normality test (Kolmogorov–Smirnov) was performed to determine normal distribution of the variables. A paired t-test was used to analyse changes in different variables after the nutritional intervention in the whole-study sample. A matched case control was conducted comparing both cases of MetS and each of its components at the beginning and at the end of the study. Differences between dietary groups were assessed by using an analysis of covariance (ANCOVA) adjusting for sex, age and baseline value. A chi-squared was conducted to test differences between groups of intervention regarding categorical changes in MetS features. Because no differences between groups of intervention were found for the main variables in this study, most results are presented for the whole population studied, including group of intervention as covariate in the multiple-adjusted models. In addition, multivariable linear regression models were fitted, with sex and age as covariates, to assess the following associations: 1) Changes in body weight as well as changes in BMI, waist circumference and fat mass according to DA concentrations at baseline. 2) The association between the decrease in anxiety symptoms with changes in body weight, BMI, waist circumference and fat mass. 3) Changes in anxiety symptoms according to 5-HT concentrations at baseline. 4) The association between final 5-HT and DA concentrations and changes in calories and CHO intake, respectively. Potential cofounders included as covariates in the multiple-adjusted models were: age, sex, physical activity, group of intervention and baseline values. A P-value < 0.05 was

considered statistically significant. Analyses were performed using STATA version 12.0 (StataCorp, College Station, TX, USA).

3. Results

A total of 26 subjects (27.9%) dropped out during the 6-month weight loss period due to loss to follow-up or consent withdrawal: 10 participants from the control group and 16 participants from the RESMENA group, without finding differences between groups (p=0.233). Therefore, the RESMENA subsample assessed in this study included a total of 62 participants (**Figure 1**). Characteristics of participants including components of MetS according to IDF criteria, monoamines blood levels, anxiety symptoms, as well as the dietary intake at baseline and at the end of the study are presented (**Table 1**). As expected, the comparative analysis between dietary groups showed a higher protein intake of the RESMENA group and also lower CHO intake, but no differences between dietary groups were detected in the rest of nutritional markers analysed. Consequently, because the group of intervention did not contribute to the outcomes examined in this study, both dietary groups were combined for all subsequent analyses. It is noteworthy that after six-months of weight loss intervention, subjects significantly improved several MetS features, including waist circumference, glucose intolerance, hypertension and hypertriglyceridemia but not HDL-c (**Table 1**). Regarding the monoamines levels in blood, a significant increase was observed in DA and 5-HT values. In contrast, no change in the rest of monoamines analysed was observed. Moreover, anxiety symptoms were significantly decreased after the 6-month weight loss treatment. The evaluation of the dietary intake showed that there were no significant differences between the beginning and the end of the study concerning the percentage of energy derived from CHO, lipids and proteins (**Table 1**). Moreover, no differences between dropouts and completers were found at baseline with the exception of serotonin, which values were 11.3% higher in the completers group compared with dropouts (data not shown).

In fully adjusted analyses, participants who reduced more of their body weight, BMI, waist circumference and total fat mass also showed a greater decline in anxiety symptoms as well as lower anxiety score at the end of the nutritional intervention in

the whole-study subjects (**Table 2**). It is noteworthy that a trend toward significance was found in the relationship between anxiety symptoms and 5-HT plasma values at baseline (B=-0.22, 95% CI=-0.49 to 0.04, p=0.098). Furthermore, it was found that the reduction in anxiety symptoms after the 6-month weigh loss treatment was also influenced by the initial 5-HT plasma values (**Figure 2**). However, we failed to find a tripartite relationship between the decrease in anxiety symptoms, weight loss and monoamine levels, possibly due to the fact that the changes in anxiety symptoms were not related with changes in any of the peripheral monoamine concentrations (data not shown).

A greater reduction in body weight, BMI, waist circumference and total fat mass was significantly predicted by higher DA blood values at baseline, after adjustments for age, sex, group of intervention, change in physical activity and the corresponding anthropometric variable at baseline (**Table 3**).

Interestingly, after adjustments for sex and age, individuals who reduced more of their energy intake during the dietary treatment also showed higher 5-HT plasma levels at the end of the study. However, this association did not reach statistical significance in the multiple-adjusted model (sex, age, activity level, group of intervention and basal values). Moreover, the fully adjusted model showed that those subjects consuming less CHO during the dietary treatment also showed higher DA peripheral values after the 6-month intervention (**Figure 3**).

4. Discussion

The 6-month dietary treatment for weight loss increased plasma DA and 5-HT levels and also improved anxiety symptoms in subjects suffering MetS. The increase in 5-HT blood levels was related to a lower energy intake during the nutritional intervention. Moreover, a lower CHO intake might be implicated in the higher DA blood values at the end of the study. Regarding the decrease in anxiety symptoms, it was positively related with weight loss and the decrease in other anthropometric measurements. These findings indicate apparently for the first time that an energy-restricted diet could result in an increase in peripheral monoamines concentrations and also in a reduction of anxiety manifestations. Moreover, this study shows the

usefulness of measuring blood monoamines levels as prognostic factors for predicting the response to a dietary intervention in MetS patients.

The functional roles of peripheral monoamines have not been much investigated. In this sense, a relationship between gut-derived 5-HT (main source of peripheral 5-HT) and weight loss has been established (Sumara *et al.*, 2012). Fasting conditions promote synthesis of gut-derived 5-HT, which in turn favours both lipolysis and liver gluconeogenesis by signalling in adipocytes and hepatocytes through the same receptor (Sumara *et al.*, 2012). Therefore, it can be suggested that, energy restriction has promoted the production of gut-derived 5-HT and, consequently, it may have contributed to weight loss by stimulating lipolysis in the adipose tissue.

A growing body of evidence links peripheral DA to obesity, since this monoamine seems to be involved in the regulation of carbohydrate metabolism, body weight and blood pressure, among others (Rubi *et al.*, 2010). In mice, high-fat diet induced gastrointestinal dysfunctions, which caused DA deficiency (Tellez *et al.*, 2013). Peripheral DA seems to inhibit insulin release through D2 receptors in the pancreas and the blockade of DA receptors would enhance insulin release, promoting adipogenesis, weight gain and insulin resistance (Rubi *et al.*, 2005; Rubi *et al.*, 2010). In addition, in vivo experiments support the notion that the insulin-signalling pathway has a detrimental effect on the dopaminergic system (Williams *et al.*, 2007). In this study, an inverse association between CHO intake and blood DA levels was found, being supported by a previous work (Agharanya *et al.*, 1985). In this sense, high carbohydrate consumption has been related with insulin resistance. (Daly *et al.*, 1997). Therefore, it might be speculated that lower CHO intake led to lower insulin release and this might have increased DA peripheral values. However, we failed to find a direct association between peripheral DA levels and insulin values (data not shown). Moreover, this study emphasize the importance of high levels of DA plasma levels at baseline in determining the magnitude of weight loss, BMI, waist circumference and total fat mass in response to the dietary treatment. This finding might be supported by the previous reasoning. Moreover, the blockage of D2 DA receptors caused by antipsychotic medication, induce weight gain in humans. In this way, it was also demonstrated that treatment with an inhibitor of DA uptake,

reduced body weight in obese patients (Astrup *et al.*, 2008; Reinholz *et al.*, 2008). All this evidence proves the importance of DA on body weight management.

Concerning the monoamine NA and the dopac/DA and 5-HIAA/5-HT ratios, no remarkable changes were observed throughout the weight loss treatment. Moreover, they were not related with anxiety symptoms in any of the time points of the study (data not shown).

Anxiety disorders, characterized by both psychological (excessive worry, fear and apprehension) and physical symptoms (fatigue, heart palpitation and tension), have been associated with the MetS (Narita *et al.*, 2008; Gariepy *et al.*, 2010; Lambert *et al.*, 2010). There are several theories for explaining this association; one of them is that stress may disturb the regulation of the hypothalamic pituitary adrenal (HPA) axis, which may enhance adipocyte accumulation causing chronic inflammation, obesity, insulin resistance and dyslipidemia. Simultaneously, obesity is characterized by increased low-grade inflammation, which might trigger HPA axis dysfunction, creating a harmful vicious circle (Kyrou *et al.*, 2006; Gariepy *et al.*, 2010). Recently, a review paper has pointed out that there is no strong evidence to indicate that energy-restricted diets have a beneficial effect on anxiety (Eyres *et al.*, 2014). However, in this study, anxiety symptoms significantly decreased after the nutritional intervention, being this reduction associated with positive change in body weight and anthropometric variables. This finding is in agreement with previous studies showing that a dietary treatment for weight loss may improve anxiety symptoms (Bas *et al.*, 2009; Swencionis *et al.*, 2013). Thus, it might be suggested that the energy-restricted diet induced a reduction in fat mass contributing to decreased inflammation, and consequently, improved stress management through a better HPA axis functioning.

Many monoamines have been implicated in the modulation of anxiety and other mood disorders (Zweifel *et al.*, 2011; Maron *et al.*, 2012). These molecules have multiple effects on the overall stress response due to the fact that they are capable of regulating the HPA axis at numerous levels (Berger *et al.*, 2009). In the present study, the reduction in anxiety symptoms was higher in those participants who had lower basal 5-HT blood values. There was a trend toward significance between anxiety

symptoms and 5-HT plasma values at baseline, showing lower 5-HT concentrations in participants with higher anxiety symptoms. Therefore, it is possible that subjects with poor 5-HT plasma levels at baseline may have benefited most from the intervention by decreasing more their anxiety symptoms. Moreover, since dropouts had lower 5-HT levels at baseline, higher 5-HT concentrations cannot be discarded as being involved in the follow-up. Blood 5-HT has been used as an indicator of brain 5-HT in previous human investigations (Williams *et al.*, 2006; Sekiyama *et al.*, 2013), however, there is a debate as to whether this monoamine can cross the BBB and to date, only one study has showed that augmented 5-HT within the brain crosses the BBB (Nakatani *et al.*, 2008).

On the other hand, depression has been suggested to be in part due to monoamine imbalance. In this context, in the RESMENA study depressive manifestations were also measured (Perez-Cornago *et al.*, 2013), however we did not find a relationship between this psychological disorder and monoamine levels (data not shown). In accordance with previous studies (Fabricatore *et al.*, 2011), we observed that after weight loss subjects had significantly improved depressive symptoms (Perez-Cornago *et al.*, 2013). Furthermore, previous weight loss intervention studies demonstrated that depressive symptoms scores at the beginning of the study significantly predicted adherence to a dietary treatment (Fabricatore *et al.*, 2009; Somerset *et al.*, 2011), nevertheless we did not find the same results neither with depressive or anxiety symptoms (data not shown).

This study is novel in that it is the first time that peripheral monoamines were measured in subjects with MetS in the context of a weight loss intervention, however, some limitations should be acknowledged. First, anxiety symptoms were measured using a self-reported questionnaire, the STAI. Nevertheless, this survey has been validated and previously used to assess anxiety symptoms in weight loss studies (Bas *et al.*, 2009). Second, peripheral monoamines do not necessarily indicate the concentrations in the CNS, however, a strength of this research is that it shows that peripheral monoamines may play a role in weight loss and anxiety symptoms in a less invasive way. Third, the number of drop-outs and also the missing data from some volunteers has reduced the sample size of this study. However, the likelihood

of type-II errors is low because important statistical differences were found despite the smaller sample size. Moreover, an effort has been made to analyse all the monoamines under the same experimental and chromatographic conditions. Finally, observational cohort studies might be subject to bias and confounding, leading to problems in determining causality. However, this limitation might be partly solved by controlling for different confounders, such as the group of intervention. Moreover, it has been shown that the results of observational studies are remarkably similar to those from RCTs on the same topic (Concato *et al.*, 2000).

In conclusion, this study evidenced a reduction in anxiety symptoms and an increase in 5-HT and DA blood levels after the weight loss treatment in obese patients with MetS, with this increase being related with lower energy and CHO intakes, respectively. An association between baseline DA concentrations and obesity-related parameters was found, proposing the assessment of DA as a marker of weight loss and adiposity. However, more studies are needed to confirm these findings and to better understand the possible biological mechanisms explaining these associations.

Role of the funding sources

This study was supported by the Health Department of the Government of Navarra (48/2009) and the Linea Especial about Nutrition, Obesity and Health (University of Navarra LE/97). The pre-doctoral research grant to Aurora Perez-Cornago from the Asociacio´n de Amigos Universidad de Navarra is gratefully acknowledged. The funding sources had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

Conflict of interest statement

None declared.

Acknowledgements

We wish to thank the volunteers of this study and Blanca E. Martinez de Morentin, Salomé Pérez, Maluz Muro, Sandra Lizaso as well as Veronica Ciaurriz for excellent technical assistance in the University of Navarra. We acknowledge Shauna Drumm (native English speaker) for reviewing the final version of the manuscript.

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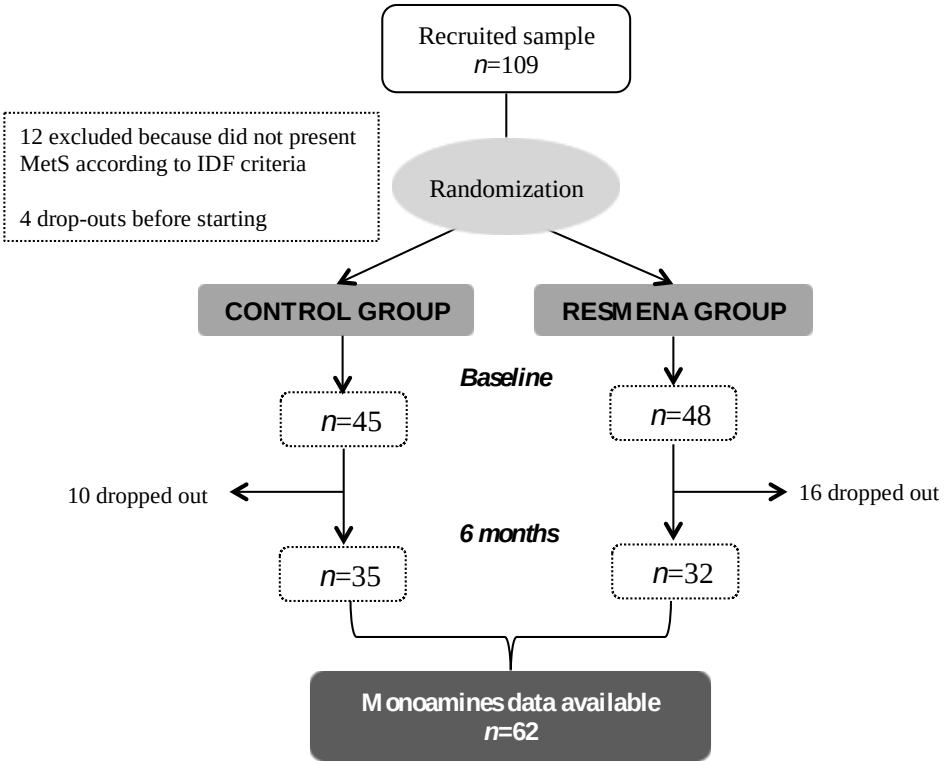


Figure 1. Flowchart of participants in the RESMENA study. Abbreviations: IDF, International Diabetes Federation; MetS, Metabolic Syndrome.

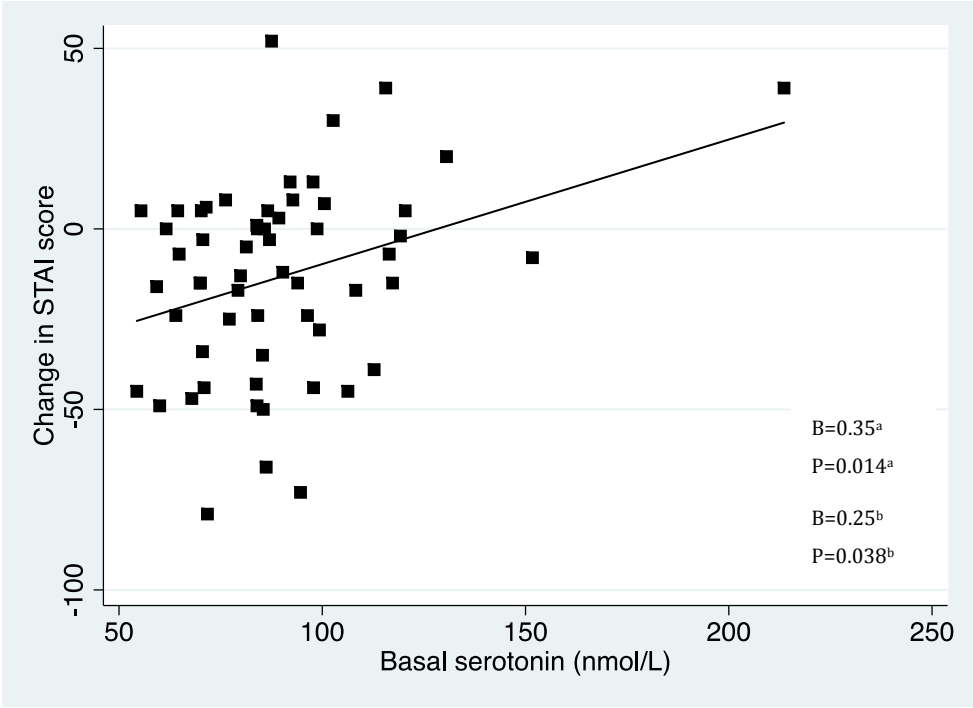
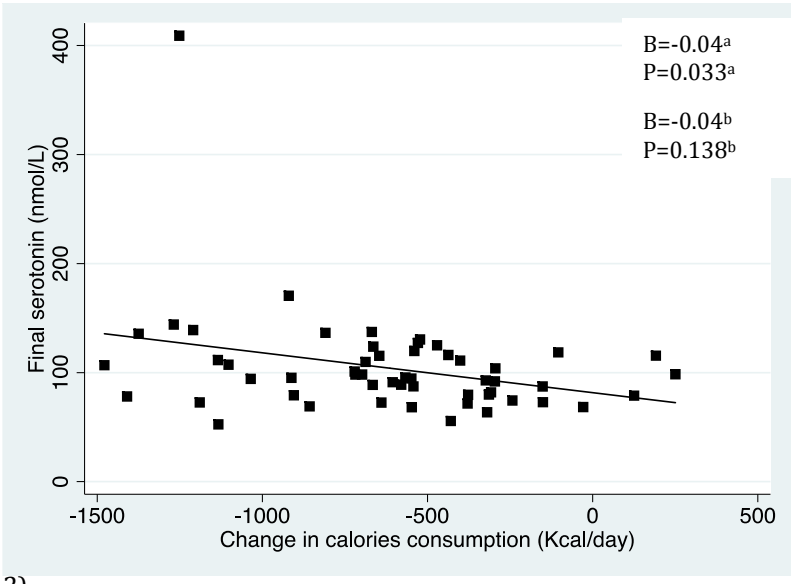


Figure 2. Association between basal serotonin and changes in anxiety symptoms in the whole population. [a] adjusted for sex and age. [b] adjusted for sex, age, basal STAI score, physical activity, and group of intervention. Abbreviations: CHO, carbohydrate; STAI, state-trait anxiety inventory.

1)



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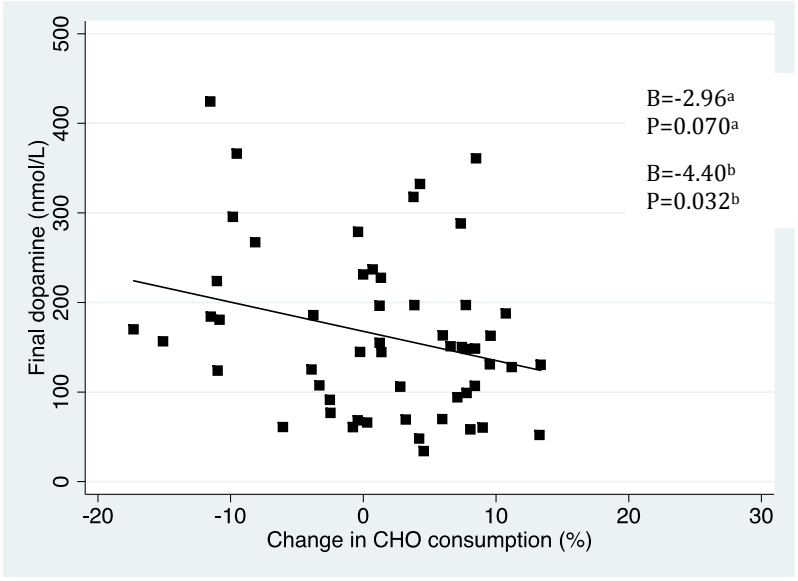

Figure 3. Associations between: 1) final serotonin and changes in total caloric intake; 2) final dopamine and carbohydrate consumption; in the whole population. [a] adjusted for sex and age. [b] adjusted for sex, age, basal values, physical activity, and group of intervention. Abbreviations: CHO, carbohydrate.

Table 1. Changes in anthropometric, biochemical, anxiety and dietary parameters after the 6-month weight loss treatment in subjects with MetS .

	Baseline (n=62) (mean ± SE)	6-months (n=62) (mean ± SE)	P-value[1]	P-value[2]
Age (year)	50.1 ± 1.2		-	-
Male sex, %	59.7%		-	-
BMI (kg/m^2)	35.8 ± 0.5	32.8 ± 0.6	<0.001	0.590
MetS features	n (%)	n (%)		
MetS (IDF criteria), %	62 (100)	44 (70.9)	<0.001[3]	0.871[4]
WC, %	62 (100)	58 (93.5)	<0.001[3]	0.947[4]
Glucose intolerance, %	53 (85.5)	41 (66.1)	<0.001[3]	0.323[4]
Hypertension, %	59 (95.2)	48 (77.4)	<0.001[3]	0.176[4]
Low HDL-c, %	34 (54.8)	26 (41.9)	0.878[3]	0.078[4]
Hypertriglyceridemia , %	45 (72.5)	33 (53.2)	0.021[3]	0.122[4]
Monoamines	(mean ± SE)	(mean ± SE)		
Dopamine, nmol/L	141.6 ± 11.2	167.4 ± 12.3	0.046	0.904
Dopac, nmol/L	58.1 ± 4.2	62.7 ± 3.1	0.285	0.942
Serotonin, nmol/L	88.5 ± 3.2	102.8 ± 5.9	0.020	0.418
5-HIAA, nmol/L	130.6 ± 5.6	134.2 ± 5.5	0.580	0.899
Noradrenaline, nmol/L	3323.4 ± 177.1	3276.0 ± 166.2	0.554	0.779
Ratio Dopac/Dopamine	0.52 ± 0.04	0.51 ± 0.05	0.881	0.917
Ratio 5-HIAA/Serotonin	1.56 ± 0.08	1.43 ± 0.08	0.230	0.876
Dietary intake				
Total energy intake	2163 ± 58	1542 ± 42	<0.001	0.761
CHO, % TEV/day	35.6 ± 0.8	36.9 ± 0.9	0.240	0.893
Lipids, % TEV/day	41.1 ± 0.9	39.2 ± 0.8	0.094	0.034
Proteins, % TEV/day	17.8 ± 0.4	18.6 ± 0.5	0.229	0.004
Activity level [5]	1.6	1.58	0.450	0.136
STAI, score	39.4 ± 3.5	26.1 ± 3.1	<0.001	0.475

The table shows mean ± SE or n (%). Bold numbers indicate statistical significance. Abbreviations: BMI, body mass index; CHO, carbohydrates; HLD-c, high-density lipoprotein cholesterol IDF, International Diabetes Federation; MetS, metabolic syndrome; TEV, total energy value; WC, waist circumference.

[1] Differences between the beginning and the end of the study in the whole-study sample.

[2] Differences between dietary groups (control group and RESMENA group) adjusted by sex, age and basal value of each variable.

[3] Matched case control analyses.

[4] Chi-squared analyses.

[5] Average daily exercise calculated by twenty-four physical activity questionnaire

Table 2. Associations between changes and final anxiety score with changes in body weight and anthropometric variables after 6 months of nutritional intervention in the whole population.

	Δ STAI score		**Final STAI score**	
	B (95% CI)	**p**	**B (95% CI)**	**p**
Δ Body weight, kg				
Age and sex-adjusted model	1.26 (-0.14 to 2.67)	0.077	0.81 (-0.34 to 1.96)	0.158
Multiple-adjusted model[1]	1.63 (0.54 to 2.72)	0.004	1.61 (0.46 to 2.77)	0.007
Δ BMI, kgm^{-2}				
Age and sex-adjusted model	3.95 (0.02 to 7.88)	0.049	2.43 (-0.79 to 5.67)	0.137
Multiple-adjusted model[1]	3.33 (0.35 to 6.31)	0.029	3.86 (0.62 to 7.11)	0.021
Δ WC, cm				
Age and sex-adjusted model	0.38 (-0.92 to 1.68)	0.562	0.57 (-0.43 to 1.56)	0.254
Multiple-adjusted model[1]	1.25 (0.17 to 2.34)	0.024	1.21 (0.11 to 2.32)	0.032
Δ Fat mass, kg				
Age and sex-adjusted model	1.61 (-0.07 to 3.3)	0.060	1.09 (-0.31 to 2.49)	0.126
Multiple-adjusted model[1]	1.85 (0.50 to 3.20)	0.008	1.81 (0.40 to 3.22)	0.013

Bold numbers indicate statistical significance. Abbreviations: BMI, body mass index; STAI, state-trait anxiety inventory; WC, waist circumference.

[1] Adjusted for sex, age, basal BMI, basal WC or basal fat mass, basal STAI score, physical activity, and group of intervention.

Table 3. Association between baseline dopamine levels and changes in anthropometric variables after 6 months of nutritional intervention in the whole population.

	Baseline Dopamine		
	B	**95 % CI**	**p**
Δ Body weight, kg			
Age and sex-adjusted model	-0.013	-0.029 to 0.002	0.093
Multiple-adjusted model[1]	-0.016	-0.032 to -0.001	**0.042**
Δ BMI, kgm^{-2}			
Age and sex-adjusted model	-0.005	-0.010 to 0.001	0.066
Multiple-adjusted model[1]	-0.006	-0.012 to -0.001	**0.045**
Δ WC, cm			
Age and sex-adjusted model	-0.014	-0.032 to 0.003	0.120
Multiple-adjusted model[1]	-0.018	-0.036 to -0.001	**0.047**
Δ Fat mass, kg			
Age and sex-adjusted	-0.013	-0.025 to -0.001	**0.046**
Multiple-adjusted model[1]	-0.015	-0.028 to -0.003	**0.020**

Bold numbers indicate statistical significance. Abbreviations: BMI, body mass index; WC, waist circumference.

[1] Adjusted for sex, age, basal BMI, basal WC or basal fat mass, physical activity, and group of intervention.

CHAPTER 6

Post-print version of the article:

DNA hypermethylation of the Serotonin Receptor Type-2A Gene is associated with a worse response to a weight loss intervention in subjects with metabolic syndrome

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Nutrients. 2014 Jun; 6:2387-2403

doi:10.3390/nu6062387

Impact factor (2013): 3.148

23/78 in Nutrition & Dietetics

Abstract

Understanding the regulation of gene activities depending on DNA methylation has been the subject of much recent study. However, although polymorphisms of the *HTR2A* gene have been associated with both obesity and psychiatric disorders, the role of *HTR2A* gene methylation on these illnesses remains uncertain. The aim of this study was to evaluate the association of *HTR2A* gene promoter methylation levels in white blood cells (WBC) with obesity traits and depressive symptoms in individuals with metabolic syndrome (MetS) enrolled in a behavioural weight-loss programme. Analyses were based on 41 volunteers (mean age 49±1 y) recruited within the RESMENA study. Depressive symptoms (as determined using the Beck Depression Inventory), anthropometric and biochemical measurements were analysed at the beginning and after 6 months of weight-loss treatment. At baseline, DNA from WBC was isolated and cytosine methylation in the *HTR2A* gene promoter was quantified by a microarray approach. In the whole-study sample, a positive association of *HTR2A* gene methylation with waist circumference and insulin levels was detected at baseline. Obesity measures significantly improved after 6 months of dietary treatment, where a lower mean *HTR2A* gene methylation at baseline was associated with major reductions in body weight, BMI and fat mass after the treatment. Moreover, mean *HTR2A* gene methylation at baseline significantly predicted the decrease in depressive symptoms after the weight-loss treatment. In conclusion, this study provides newer evidence that hypermethylation of the *HTR2A* gene in WBC at baseline is significantly associated with a worse response to a weight loss intervention and with a lower decrease in depressive symptoms after the dietary treatment in subjects with MetS.

Keywords: DNA methylation, *HTR2A* gene, obesity, metabolic syndrome, energy restriction, depressive symptoms, adiposity, epigenetics.

1. Introduction

Depressive disorders are a significant cause of disability in developed countries and their prevalence is expected to increase in the coming years (Richards, 2011). This psychiatric disease has been suggested to be often related to the metabolic syndrome (MetS), a state characterized by a combination of central obesity, peripheral insulin resistance, hypertension and serum lipid abnormalities (Alberti *et al.*, 2009; Lichtman *et al.*, 2014). Different theories exist concerning the MetS-depression relationship but most suggest that it is bidirectional and mediated by several common mechanisms, such as low-grade inflammation, unhealthy dietary habits and genetic factors (Bondy, 2007; Pan *et al.*, 2012).

Indeed, lifestyle as well as genetic factors affects both depression and MetS (Campion *et al.*, 2009; Schroeder *et al.*, 2010). Moreover, recent studies have provided evidence that the pathogenesis of these diseases may be also influenced by epigenetic marks (Philibert *et al.*, 2008; de la Iglesia *et al.*, 2014). In this context, epigenetics is defined as the study of heritable changes in gene expression that, unlike polymorphisms, occur without changes in the DNA sequence and are not permanent (Campion *et al.*, 2009). Therefore, epigenetic mechanisms may be capable of explaining some interactions between genetic and environmental factors (e.g. diet, physical activity or drugs), regulating gene expression over the entire lifetime of the organism (Ordovas *et al.*, 2010). Epigenetic changes include multiple processes such as covalent histone modifications, chromatin folding or microRNA abnormalities or DNA methylation of Cytosine-phosphate-guanine (CpG) residues, which is probably the issue which has most extensively been studied (Campion *et al.*, 2009; Ordovas *et al.*, 2010). Whole-genome analysis has often shown an inverse association between DNA methylation and gene expression, especially in the promoter region (Terry *et al.*, 2011). Moreover, DNA methylation has been demonstrated to be a predictive tool in the assessment of responses to a nutritional intervention (Moleres *et al.*, 2013; de la Iglesia *et al.*, 2014).

Serotonin (5-HT) is a monoamine neurotransmitter involved in the regulation of important functions such as body temperature, sleep, pain, mood or energy balance (Berger *et al.*, 2009; Stunes *et al.*, 2011). Thus, disturbances of the serotonergic

pathway have been implicated in both MetS and depressive disorders (Berger *et al.*, 2009). There are multiple 5-HT receptors, each with several subtypes and different biological roles (Fidalgo *et al.*, 2013). Among them the serotonin 2A receptor, which is encoded by the 5-hydroxytryptamine receptor 2A (*HTR2A*) gene (Fabbri *et al.*, 2013), has been involved in the pathogenesis of major psychiatric disorders and obesity (Falkenberg *et al.*, 2011; Li *et al.*, 2013). In addition to several single nucleotide polymorphisms (SNPs) that have been reported for the *HTR2A* gene (Falkenberg *et al.*, 2011), DNA methylation factors have also been implicated in the regulation of *HTR2A* gene expression (Polesskaya *et al.*, 2006) However, results have been both diverse and inconclusive (Abdolmaleky *et al.*, 2011; Ghadirivasfi *et al.*, 2011; Fabbri *et al.*, 2013).

The goal of the present study was to assess the association of *HTR2A* gene promoter methylation levels with obesity measures (e.g. BMI, body weight or fat mass) and depressive symptoms. We tested this hypothesis by determining *HTR2A* gene promoter methylation levels in white blood cells (WBC) of subjects with MetS enrolled in a behavioural weight loss programme, and then by comparing these methylation levels with both baseline and follow-up obesity traits and depressive symptoms.

2. Materials and Methods

2.1. Subjects and study protocol

The present research is a secondary analysis of the RESMENA (Metabolic Syndrome Reduction in Navarra) project which is a randomized controlled trial, focused on improving MetS features through two dietary strategies (the control and the RESMENA diets) designed for weight loss during a 6-month period (Perez-Cornago *et al.*, 2013; de la Iglesia *et al.*, 2014; Perez-Cornago *et al.*, 2014a; Perez-Cornago *et al.*, 2014b; Perez-Cornago *et al.*, 2014c) Both diets were designed following the same energy restriction (-30% of the studied requirements), cholesterol (>300 mg/day) and fibre content (20-25 g/day). The Control diet was based on the American Hearth Association (AHA) guidelines, including 3-5 meals/day, a macronutrient distribution of 55% Total Caloric Value (TCV) from carbohydrates, 15% proteins and 30% lipids. On the other hand, the RESMENA diet was designed with a higher meal frequency,

consisting of 7 meals/day, and a macronutrient distribution of 40% TCV from carbohydrates, 30% proteins and 30% lipids, as described elsewhere (Perez-Cornago *et al.*, 2013).

In this analysis within the RESMENA study, forty-one Caucasian adults of the two arms of the study were pooled (Perez-Cornago *et al.*, 2013; Perez-Cornago *et al.*, 2014a; Perez-Cornago *et al.*, 2014b). The study was approved by the Ethics Committee of the University of Navarra (065/2009) and appropriately registered at www.clinicaltrials.gov; NCT01087086. Each participant gave written informed consent for participation in agreement with the Declaration of Helsinki. This research was performed following the CONSORT 2010 guidelines. This study was conducted in the Metabolic Unit of the University of Navarra in Pamplona, Spain, over a period of 23 months (from January 2010 to November 2011) (Perez-Cornago *et al.*, 2013). Details of the design and methods of this trial have been reported elsewhere (Zulet *et al.*, 2011).

2.2. Anthropometry and blood pressure

Anthropometric measurements were taken with participants in fasting conditions and wearing only their underwear and using previously validated procedures (Zulet *et al.*, 2011). Body Mass Index (BMI) was determined as the body weight divided by the squared height (kg/m^2). Systolic (SBP) and diastolic (DBP) blood pressures were measured following standardized World Health Organization criteria (Whitworth *et al.*, 2004). Body composition was specifically measured by a dual-energy X-ray absorptiometry (DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA). Anthropometric measurements as well as blood pressure determinations were carried out at the beginning and at the end point of the intervention.

2.3. Biochemical analysis

Venous blood samples were taken at baseline and at the end of the study after a 12-h overnight fast period by venipuncture. The EDTA–plasma and serum samples as well as WBC were separated from whole blood by centrifugation at 3,500 rpm, 5 ºC, 15 min (Model 5804R, Eppendorf, Germany), and were frozen immediately at -80 ºC until assay [WBC in buffy-coat with and without Trizol reagent (Invitrogen, Carlsbad,

CA)] as described elsewhere (Hermsdorff *et al.*, 2013; Lopez-Legarrea *et al.*, 2013; de la Iglesia *et al.*, 2014).

Serum glucose, total cholesterol, triglycerides, and non-esterified fatty acids (NEFAs) were measured in a Pentra autoanalyser C-200 (HORIBA ABX, Madrid, Spain) with commercially available kits. Serum fasting insulin was measured by an enzyme immunoassay kit (Mercodia, Uppsala,Sweden).

2.4. Psychological assessment

Symptoms of depression were assessed at the beginning and at the end of the study using the validated Spanish version of the Beck Depression Inventory (BDI) as published elsewhere (Conde *et al.*, 1975). The BDI is a 21-item test that measures the presence and degree of depressive symptoms in respondents. Scores can range from 0 to 63, with a score of 10 or higher indicating moderate depressive symptoms. Question number 19 of the test, relating to weight loss, was discarded from all the analyses given that losing weight is considered a manifestation of depression. However, in our volunteers it was considered a positive aspect because they were enrolled in a weight loss treatment program (Perez-Cornago *et al.*, 2013).

2.5. DNA isolation and DNA Methylation Study

Genomic DNA from WBC was obtained using the Master Pure kit (Epicenter, Madison, WI, USA), and its quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). A total of 500 ng of DNA were modified using EZ-96 DNA Methylation Kit (Zymo Research Corporation, Orange, CA) according to the manufacturer's instructions, thus converting cytosine into uracil.

Array-based specific DNA methylation analysis was performed with the Infinium Human Methylation 450K bead chip technology (Illumina, San Diego, CA). Bisulfite-treated genomic DNA was whole-genome amplified, hybridized to HumanMethylation450 BeadChips (Illumina, San Diego, CA) and scanned using the Illumina iScanSQ platform. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, San Diego, CA). β-values were computed using the formula [aa1] β-value = M/[U+M] where M and U are the raw “methylated” and “unmethylated” signals, respectively. β-values were

corrected for type I and type II bias using the peak based correction. The data were normalized in R using a categorical Subset Quantile Normalization method (SQN) and probes associated to X and Y chromosomes were filtered out using the pipeline developed by Touleimat and Tost (Epigenomics 2012;4:325-41). Probes containing SNPs with a minor allele frequency (MAF) > 0.001 in Iberian population in Spain were removed from the analysis. The methylation status of twenty CpG sites of the *HTR2A* gene that codes for the HTR2A receptor, were selected from the Illumina array and analysed separately. Specific CpG sites located in the transcriptional regulatory region (promoter, 5'-untranslated region and exon 1) were included (**Figure 1**). Reference names and characteristics of the selected CpG were reported (**Table 1**).

2.6. Analysis of gene expression by quantitative-real-time PCR

Total RNA from WBC was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The integrity of isolated RNA was also evaluated by Experion (Biorad), following the manufacturer's instructions. Briefly, denatured RNA samples (1 µl) were mixed with sample buffer provided, and 6 µl of each mixed sample was loaded in RNA StdSens chips (BioRad, Hercules, CA) for analysis. In all samples we also evaluated the RNA quality indicator number (RQI) and this was considered as optimal (ranging from 7.9 to 10). Furthermore, *HTR2A* transcript expression levels were assessed using quantitative real-time RT-PCR (qPCR). cDNA was synthesized from total RNA (1 µg) of the entire cohort individuals using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor following the manufacturer's instructions (Life Technologies, Foster city, CA). The transcript levels for *HTR2A* gene and one housekeeper gene were measured using an ABI Prism 7900HT Fast Real time PCR system with a 384-well format and TaqMan Gene Expression Assays (Life Technologies, Foster city, CA) (*HTR2A*: Hs01033524_m1 and *GAPDH*: Hs02758991_g1). The ΔΔCT method was used for quantification (ABI) and the fold changes are reported as $2^{-\Delta\Delta CT}$ (Livak *et al.*, 2001).

2.7. In silico sequence analysis

Human genomic DNA sequences, from 2188 bp upstream to +1 pb of the transcription Start Site (TSS) of the *HTR2A* gene, were downloaded from the National

Center of Biotechnology Information. http://www.ncbi.nlm.nih.gov/ (12 JAN 2014) database [GenBank: NG_013011.1, 13:47472360-13: 47469640]. Possible transcription factor-binding sites were predicted on genomic DNA sequences using MatInspector software Genomatix Software GmbH, Munich, Germany, which is specifically designed tool for promoter analysis (Quandt *et al.*, 1995).

2.8. Statistical analyses

Results are shown as mean±SEM. Data normality was determined by the Shapiro-Wilk test. The mean methylation value of the *HTR2A* gene promoter region, including the 20 CpGs, was calculated. Baseline characteristics of participants according to tertiles of mean *HTR2A* gene methylation were compared. Means and SEM for each variable across the *HTR2A* gene methylation tertiles were calculated, with the differences among them assessed using one-way analysis of variance. Pearson correlations were fitted to evaluate the potential correlations of *HTR2A* transcriptional regulatory region methylation with all sites across the promoter region, gene expression and changes in anthropometric measurements and depressive symptoms. Body weight, WC, BMI and fat mass were divided by medians and the differences between groups were analysed using a Student's t-test. Moreover, multiple testing correction (Benjamini–Hochberg) analyses were performed when appropriate. Statistical analyses were performed using STATA version 12.0 (StataCorp, College Station, TX, USA). P-value <0.05 was considered as statistically significant.

3. Results

The CpG sites were located within the *HTR2A* promoter region, a 2198-bp region positioned from −2188 to +10 (**Figure 1**). The transcription factor binding sites in the activation regions of the *HTR2A* promoter were screened by using MatInspector. These analyses showed that the promoter region contained a consensus binding motif in the studied CpG sites for seven transcription factors (AHRR, HIF, CREB, HESF, NRSF, XCPE, E2FF) (**Figure 1** and **supplementary data S1**). Mean DNA methylation levels revealed a high correlation with some of the CpG sites for the HTR2A gene (**supplementary data S2**). Moreover, DNA methylation levels of CpG

sites within the promoter region were associated in some different regions. These distinct areas might imply the presence of specific response elements of gene regulatory machinery in the gene promoter.

The baseline anthropometric, biochemical and psychological characteristics of the whole-study sample (n=41) stratified by tertiles of baseline mean *HTR2A* gene promoter methylation levels are presented (**Table 2**). In this context, those individuals in the upper category of mean *HTR2A* methylation levels had a higher waist circumference (WC) and insulin levels. Nevertheless, no association between *HTR2A* methylation levels and depressive symptoms was observed at the beginning of the study.

The 20-selected CpG methylation sites of the *HTR2A* gene showed few associations with baseline body weight, WC, BMI and fat mass (**Supplementary data S3**).

After the 6-month weight loss treatment, body weight, WC, BMI, fat mass and depressive symptoms significantly decreased in all subjects (**Figure 2**). Changes in body weight, WC, BMI and fat mass were divided by medians showing that those participants with a greater response to the dietary treatment had lower *HTR2A* gene promoter methylation levels (**Figure 3**). Moreover, Pearson correlation analyses between mean *HTR2A* gene methylation levels and changes in anthropometric parameters confirmed these findings, since positive correlations between mean *HTR2A* gene methylation levels and changes in body weight, BMI and fat mass but not in WC were found.

Pearson correlation analyses were performed to assess the relationship between baseline *HTR2A* gene methylation levels and changes in body weight, WC, BMI and fat mass after the 6-month dietary treatment. The analysis of the 20-selected CpG methylation sites of the *HTR2A* gene showed that CpG 4, CpG 7 and CpG 17 sites were also positively related to changes in body weight, BMI and fat mass (**Table 3**). However, after applying a multiple comparison correction the statistical significance was attenuated. In addition, no association between *HTR2A* gene methylation with changes in insulin and triglyceride levels was observed.

Interestingly, higher methylation levels of both the HTR2A gene promoter and HTR2A CpG17 site at baseline were related to a less marked decrease in depressive symptoms after the nutritional intervention (**Figure 4**).

The gene expression analysis showed very low concentrations in WBC and only revealed values for 20 participants. No association was found between gene expression and the *HTR2A* gene promoter methylation levels (r=-0.257, p=0.274), or the methylation of *HTR2A* CpG 17 site (r=0.094, p=0.693).

4. Discussion

This study showed a positive association between baseline methylation levels of the HTR2A gene in peripheral WBC and both initial and subsequent changes in anthropometric variables, suggesting a regulatory action of this gene on methylation levels in the improvement of obesity measures (e.g. body weight). Moreover, the baseline methylation levels of the *HTR2A* gene promoter region were associated with a decrease in depressive manifestations after the weight-loss treatment.

In order to detect putative consensus transcription factor binding sites in the entire *HTR2A* coding region, a computer analysis of this region using MatInspector was carried out (Quandt *et al.*, 1995). This analysis showed that the *HTR2A* CpG 17 site matches a core-binding motif for CREB, HESF and HIF. A growing literature has related environmental factors to serotonin release acting on diverse serotonin receptors, by the mediation of CREB, which may activate the expression of many genes to produce different proteins involved in neural growth, synapse formation, and long-lasting structural changes, which may be related to depression (Abdolmaleky *et al.*, 2011; Ghadirivasfi *et al.*, 2011). As for the HESF transcription factor, it has been involved in neuronal excitability in the brain (Jiang *et al.*, 2008). Moreover, HIF is a key regulator of oxygen homeostasis and has been reported to play a role in the transcriptional regulation of low-grade inflammation, tissue-protective signalling pathways or weight loss (Rosenberger *et al.*, 2009; Urdampilleta *et al.*, 2012). Since it has been proposed that low-grade inflammation promotes both obesity and depression (Pan *et al.*, 2012), it might be hypothesised that methylation of *HTR2A* could interact with obesity and depressive disorders by hindering the binding of HIF, HESF and CREB to the *HTR2A* gene promoter region.

Interestingly, the CpG methylation of a promoter region may be a mechanism implicated in gene inactivation by blocking its transcription (Milagro *et al.*, 2013a). Nonetheless, in this study, we failed to find an association between promoter methylation levels and gene expression, although this might be due to the fact that not all samples could be read. One factor that might explain this unexpected finding is that the promoter region CpG 17 site, an important transcription factor binding site of *HTR2A* gene, was highly methylated and may have caused a very low *HTR2A* gene expression in our sample. Moreover, other studies have found that DNA methylation is involved in the regulation of *HTR2A* expression (Polesskaya *et al.*, 2006). On the other hand, a previous study has reported very low concentrations of *HTR2A* gene expression in peripheral blood mononuclear cells, and also, an increase of HTR2A gene expression after clinical improvement of depressive symptoms (Belzeaux *et al.*, 2010). Indeed, this research proposed HTR2A gene as a potential biomarker for clinical improvement of depression.

Diet is considered an important environmental factor that affects DNA methylation (Campion *et al.*, 2009; Hermsdorff *et al.*, 2013). In this sense, the dietary levels of methyl-donor precursors (vitamins of the B complex or certain amino acids) have been shown to be important in the prevention of psychiatric diseases and obesity (Abdolmaleky *et al.*, 2004; Cordero *et al.*, 2013). In this sense, a balance of DNA methylation levels is important for the correct functioning of the central nervous system (Abdolmaleky *et al.*, 2004). Moreover, DNA methylation changes in peripheral WBC have been proposed as a useful biomarker for different diseases (Milagro *et al.*, 2013a; de la Iglesia R, 2014).

On the other hand, one of the most widely studied SNPs of the *HTR2A* gene is rs6311 (-1438G>A), which has been related to many diseases and conditions such as schizophrenia, alcohol dependence, diabetes and obesity (Kring *et al.*, 2009; Cao *et al.*, 2014). For example, it has been suggested that this SNP influences glucose homeostasis (Kring *et al.*, 2009) and also obesity traits (BMI and abdominal obesity) in obese subjects (Sorli *et al.*, 2008). In the present study, we found that hypermethylation of the *HTR2A* gene was associated with higher baseline body weight, WC, insulin and triglycerides levels. Interestingly, those subjects with higher

methylation of *HTR2A* gene and also the *HTR2A* CpG 17 site, showed a less marked decrease in body weight, BMI and fat mass after the 6-month dietary treatment. Apparently, no previous study has analysed DNA methylation levels of *HTR2A* gene in a population with metabolic syndrome enrolled in a weight-loss treatment. Therefore these results could not be compared. On the other hand, SNPs have not been genotyped in this study. Hence, if the methylation levels are tagging a SNP, this may explain why DNA methylation was not associated with gene expression, as this SNP influences translation efficiency (Abdolmaleky *et al.*, 2011; Ghadirivasfi *et al.*, 2011).

In addition, the *HTR2A* gene has been directly associated with major depressive disorder (Mandelli *et al.*, 2013). Some studies have reported that *HTR2A* expression is decreased in the brain of patients with schizophrenia and in those who have committed suicide (Garbett *et al.*, 2008; Hurlemann *et al.*, 2008; Abdolmaleky *et al.*, 2011). Moreover, antipsychotic treatment has been linked with lower DNA methylation and increased expression of the *HTR2A* gene (Abdolmaleky *et al.*, 2011). Unfortunately, methylation of cytosines of the *HTR2A* promoter gene *i*n major depressive disorders has not been sufficiently investigated to date (Fabbri *et al.*, 2013). In this sense, it might be suggested that the average methylation of both *HTR2A* gene and *HTR2A* CpG 17 site predicted the decrease in depressive symptoms.

Under the assumption of an inverse association between DNA methylation and gene expression (Milagro *et al.*, 2013a), the density of 5-HT_{2A} receptors might mirror, in part, *HTR2A* methylation. In this sense, lower density of platelet 5-HT_{2A} receptors has been observed in adolescents with eating disorders (Sigurdh *et al.*, 2013). Moreover, progressive reductions of brain 5-HT_{2A} receptor density have been proposed as an indicator of schizophrenia (Hurlemann *et al.*, 2008). In contrast, results from animal models found that higher 5-HT_{2A} receptor gene expression was linked to obesity (Nonogaki *et al.*, 2006) and also, elevated 5-HT_{2A} receptors in frontal cortex specimens were observed in depressed subjects (Shelton *et al.*, 2009). Hence, the involvement of 5-HT_{2A} receptors in depressive disorders remains to be elucidated.

Some limitations of the study should be mentioned. Firstly, since the sample size is not very large, the risk of type II errors (failing to detect real differences) was high.

Type II errors are frequent when adjustments are used in conjunction with a small effect or a small sample (Smith *et al.*, 2002). Therefore, with the aim of avoiding type II errors, no covariates were included in the analysis carried out in this study. Our approach is consistent with previous investigations that reported that when it is important to discover new facts, as is our case, we may be willing to accept more type I errors in order to avoid type II errors (Keppel *et al.*, 2004; Cohen *et al.*, 2013). Hence, in this study, the probability of type II errors is low because important statistical differences were found despite the small sample size. However, further studies with a large sample size are clearly needed to verify our findings. Secondly, *HTR2A* methylation levels were not measured at the end of the intervention and so it is not possible to confirm whether the dietary treatment had an effect on *HTR2A* gene methylation levels. Thirdly, we failed to find an association between promoter methylation levels and gene expression in WBC. Gene expression varies depending on the tissue or cell type (Milagro *et al.*, 2013b), and it is possible that *HTR2A* gene is not widely expressed in WBC. Moreover, other studies have found that DNA methylation is involved in the regulation of *HTR2A* expression (Polesskaya *et al.*, 2006). In addition, validation of these results with other reliable standards such as pyrosequencing or bisulfite sequencing might be considered in future studies.

On the other hand, the strengths of this study are its novelty, the techniques used for DNA methylation quantification, which are known to be the gold standard for epigenetic studies, and its relevance for future dietary intervention trials.

5. Conclusion

In summary, this study provides novel evidence that hypermethylation of the *HTR2A* gene in WBC at baseline is significantly associated with a worse response to a weight loss intervention and a less marked decrease in depressive symptoms in subjects with MetS. These results, if confirmed, would suggest that *HTR2A* gene methylation in WBC could serve as a useful biomarker to predict weight loss and improvement of depressive symptoms after an energy-restricted dietary treatment. However, replication in a larger cohort is warranted in order to confirm these findings and to better understand the possible biological mechanisms explaining these associations.

Acknowledgements

We wish to thank our physician Blanca E. Martinez de Morentin, our nurse Salome Perez and the technician Veronica Ciaurriz for excellent technical assistance as well as the volunteers who participated in this study. We acknowledge Shauna Drumm (native English speaker) and Dr. Paul Miller (from the institute of Modern Languages of the University of Navarra) for reviewing the final version of the manuscript.

This work was supported by Health Department of the Government of Navarra (48/2009) and the Linea Especial about Nutrition, Obesity and Health (University of Navarra LE/97). Also CIBERobn and RETICS schemes are gratefully credited. The pre-doctoral research grant to A.P.C. from the Asociación de Amigos Universidad de Navarra is gratefully acknowledged. M.L.M. holds a Juan de la Cierva fellowship from Spanish Ministry of Economic and Competitiveness.

Author Contributions

A.P.-C: contributed to the data collection, design, analysis, and writing of the manuscript. M.L.M.: was involved in the design, analysis, and editing of the manuscript, M.A.Z. and J.A.M.: were responsible for the general coordination, design, financial management and editing of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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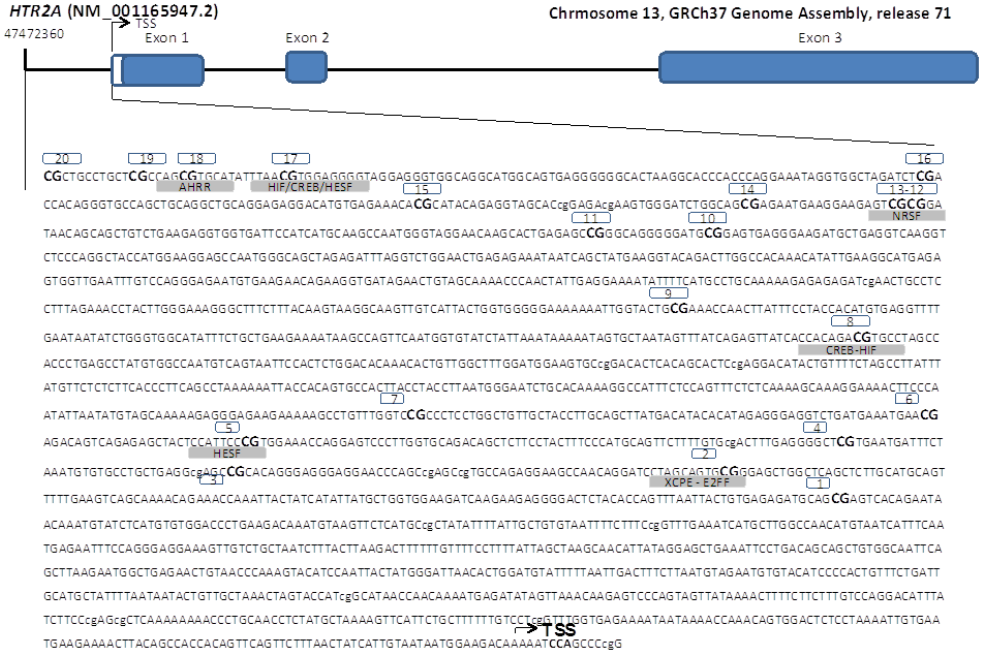

Figure 1. Genomic localization and nucleotide sequence of the 20 CpGs sites covered by the Illumina array for the study of DNA methylation levels of 5-hydroxytryptamine (serotonin) receptor 2A promoter (from - 2188 to +10 pb). Transcription Start Site (TSS). Putative consensus sequences for nine transcriptional factors (AHRR: AHR-related factors, HIF: Hypoxia inducible factor, CREB: cAMP-responsive element binding proteins, HESF: Vertebrate homologues of enhancer of split complex (Basic helix-loop-helix domain containing, class B, 2 (secondary DNA binding preference)), NRSF: Neuron-restrictive silencer factor, XCPE: X gene core promoter element 1, E2FF: E2F transcription factor 6, ZF57: KRAB domain zinc finger protein 57, DMTE: Drosophila motif ten element), found with MatInspector.

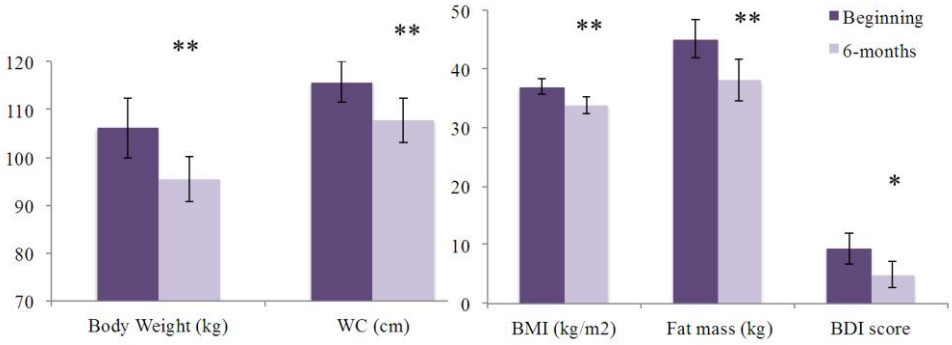

Figure 2. Anthropometric, body composition measurements and in depressive symptoms in the whole-study sample before and after the dietary treatment n=32-34. Data are expressed as mean (CI 95%). Abbreviations: BDI, Beck Depression Inventory; BMI, Body Mass Index; WC, waist circumference.

* = $p < 0.05$. ** = $p < 0.001$.

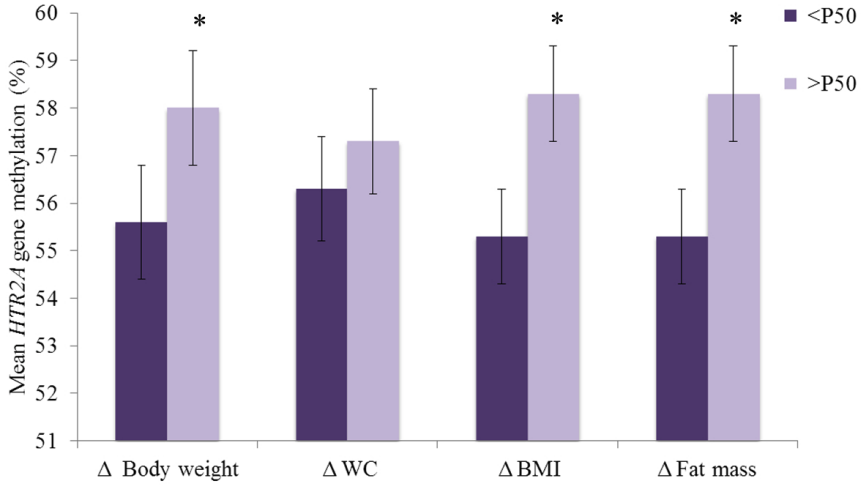

Figure 3. Association of baseline mean HTR2A gene methylation (%) with changes (6 months-baseline) in anthropometric and body composition measurements divided by their medians in the whole-study sample n=34. Δ = 6 months-baseline. Data are expressed as mean (CI 95%). Abbreviations: BMI, Body Mass Index; WC, waist circumference. < P50 = high responders; > P50 = low responders to the dietary treatment. * = $p < 0.05$.

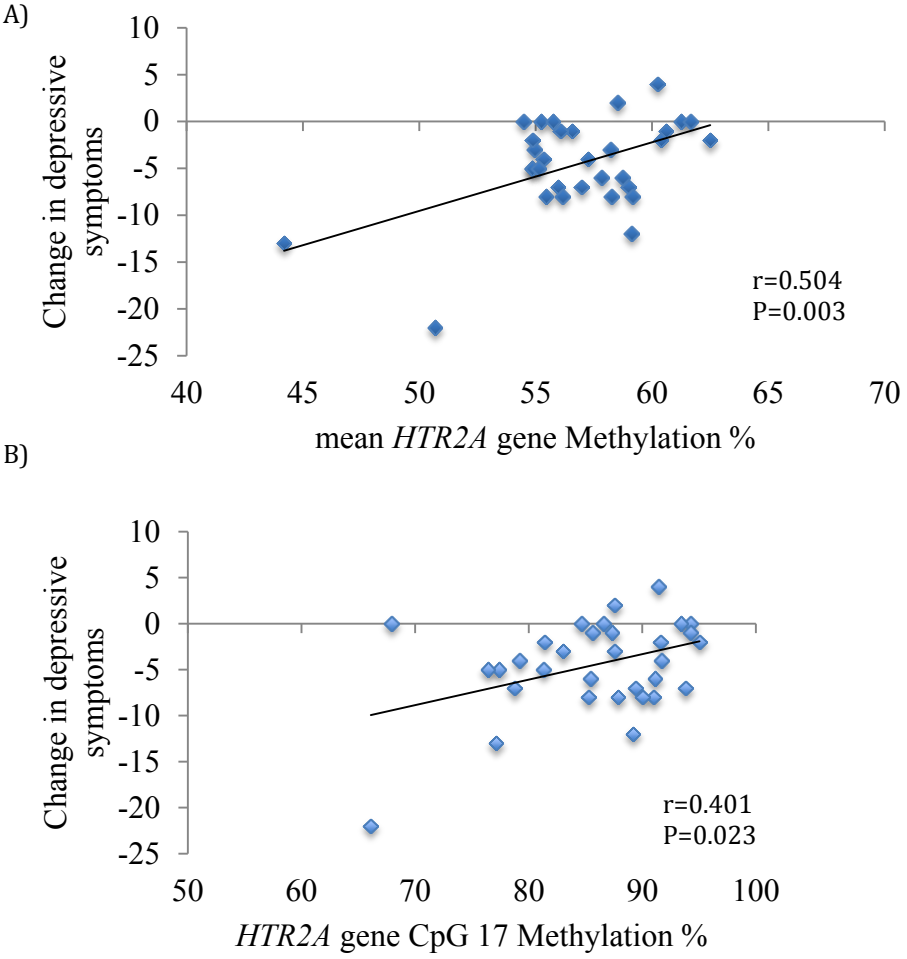

Figure 4. Association of the decrease (6 months-baseline) in depressive symptoms with baseline: A) mean HTR2A methylation; B) methylation levels of HTR2A CpG17 site; in the whole-study sample n=32. Change = 6 months-baseline. Abbreviation: HTR2A; 5-hydroxytryptamine receptor 2A.

Table 1. Information of the selected CpG sites for *HTR2A* gene.

CpG ID[1]	Illumina ID	CHR position[2]	Reference[3]
1	cg15894389	13:47470857	c.-688
2	cg02250787	13:47470989	c.-820
3	cg06476131	13:47471052	c.-883
4	cg16188532	13:47471090	c.-921
5	cg09361691	13:47471169	c.-1000
6	cg11514288	13:47471197	c.-1028
7	cg27068143	13:47471264	c.-1095
8	cg10323433	13:47471562	c.-1393
9	cg02027079	13:47471705	c.-1536
10	cg01192538	13:47472050	c.-1881
11	cg01620540	13:47472064	c.-1895
12	cg06020661	13:47472138	c.-1969
13	cg09798090	13:47472140	c.-1971
14	cg24320398	13:47472158	c.-1989
15	cg18200810	13:47472200	c.-2031
16	cg15692052	13:47472250	c.-2081
17	cg24118521	13:47472330	c.-2161
18	cg23881368	13:47472343	c.-2174
19	cg05506829	13:47472349	c.-2180
20	cg07075299	13:47472360	c.-2191

1, Studied CpG identifier. 2, Genome assembly: GRCh37, Ensemble release 73.37. 3, It begins in the first nucleotide of exon 1.

Table 2. Anthropometric, biochemical and psychological characteristics of the whole-study sample stratified by tertiles of mean *HTR2A* methylation levels at baseline.

	Mean HTR2A gene methylation %			**P *trend***
Variables	**Low (n=14)**	**Medium (n=14)**	**High (n=13)**	
Body weight (kg)	97.6 ± 4.6	106.4 ± 4.6	110.7 ± 4.8	0.059
BMI (kg/m2)	37.0 ± 1.0	37.0 ± 1.0	37.0 ± 1.0	0.995
Waist circumference (cm)	110.8 ± 3.0	114.0 ± 3.0	120.5 ± 3.1	0.027
Total fat mass (kg)	43.8 ± 2.4	45.9 ± 2.4	45.4 ± 2.5	0.682
Truncal fat mass (kg)	26.1 ± 1.8	27.6 ± 1.8	26.2 ± 1.9	0.993
SBP (mmHg)	150.3 ± 4.8	151.2 ± 4.8	148.1 ± 4.9	0.733
DBP (mmHg)	85.4 ± 2.2	87.3 ± 2.2	84.8 ± 2.3	0.784
Glucose (mg/dL)	9.8 ± 1.5	7.4 ± 1.5	11.1 ± 1.6	0.498
Insulin (μU/mL)	11.0 ± 2.3	18.1 ± 2.3	21.7 ± 2.4	0.004
TC (mg/dL)	225.1 ± 12.4	211.1 ± 12.9	205.1 ± 12.9	0.280
Triglycerides (mg/dL)	166.8 ± 29.9	224.6 ± 31.1	247.3 ± 31.1	0.079
NEFA (nmol/L)	0.58 ± 0.05	0.50 ± 0.05	0.56 ± 0.05	0.705
BDI score	9.8 ± 1.9	8.3 ± 1.9	10.9 ± 1.9	0.636

Data expressed as mean±SEM. Bold numbers indicate statistical significance ($P < 0.05$). Abbreviations: BDI, Beck Depression Inventory; BMI, body mass index; DBP, diastolic blood pressure; HTR2A, 5-hydroxytryptamine receptor 2A; NEFA, non-esterified fatty acids; SBP, systolic blood pressure; STAI, State Trait Anxiety Inventory; TC, total cholesterol. Low ≤ 0.556; Medium = 0.557-0.585; High ≥ 0.587.

Table 3. Pearson correlation analyses between changes (6 months-baseline) in body weight, BMI and fat mass with the baseline methylation (%) of *HTR2A* gene.

		Δ Body Weight		**Δ WC**		**Δ BMI**		**Δ Fat Mass (kg)**	
Baseline	**CpG Sites**	***r***	**Uncorrected *p*-Value**	***r***	**Uncorrected *p*-Value**	***r***	**Uncorrected *p*-Value**	***r***	**Uncorrected *p*-Value**
cg15894389	1	0.110	0.537	−0.117	0.510	0.127	0.474	0.167	0.346
cg02250787	2	−0.063	0.725	−0.083	0.639	−0.063	0.723	0.008	0.963
cg06476131	3	0.284	0.103	0.080	0.652	0.286	0.100	0.381	**0.026 ***
cg16188532	4	0.379	**0.027 ***	0.124	0.483	0.383	**0.025 ***	0.415	**0.015 ***
cg09361691	5	−0.195	0.268	−0.188	0.287	−0.198	0.261	−0.153	0.386
cg11514288	6	−0.143	0.418	−0.154	0.383	−0.144	0.414	−0.145	0.412
cg27068143	7	0.369	**0.032 ***	0.116	0.513	0.397	**0.020 ***	0.382	**0.025 ***
cg10323433	8	0.292	0.094	0.052	0.769	0.350	**0.042**	0.249	0.155
cg02027079	9	0.011	0.951	0.160	0.365	0.009	0.957	0.042	0.815
cg01192538	10	0.225	0.201	0.193	0.274	0.230	0.190	0.214	0.225
cg01620540	11	−0.070	0.694	−0.251	0.152	−0.014	0.937	−0.073	0.680
cg06020661	12	0.177	0.316	0.206	0.243	0.196	0.267	0.196	0.267
cg09798090	13	−0.042	0.814	−0.054	0.762	−0.037	0.837	−0.059	0.740
cg24320398	14	0.338	0.051	0.266	0.128	0.377	**0.028 ***	0.343	**0.047**
cg18200810	15	0.310	0.075	0.222	0.206	0.360	**0.037 ***	0.322	0.063
cg15692052	16	0.091	0.608	−0.025	0.890	0.121	0.494	0.103	0.563
cg24118521	17	0.342	**0.047**	0.280	0.108	0.381	**0.026 ***	0.414	**0.015 ***
cg23881368	18	0.297	0.087	0.269	0.124	0.319	0.066	0.291	0.095
cg05506829	19	0.280	0.108	0.252	0.151	0.297	0.088	0.268	0.125
cg07075299	20	0.296	0.089	0.250	0.153	0.307	0.078	0.301	0.084

Data are shown as r and p values from Pearson correlations analysis. Δ = 6 months-baseline. Abbreviations: BMI, body mass index; HTR2A; 5-hydroxytryptamine receptor 2A; WC, waist circumference. *P-value < 0.05 after correcting for Benjamini–Hochberg multiple comparisons.

Supplementary Information

Table S1. The transcription factors that bind to the region of the HTR2A promoter using MatInspector

Matrix Family [1]	Detailed Family Information	Detailed Matrix Information	Start Position/End Position	Sequence [2]	CpG ID [3]
V$AHRR	AHR-arnt heterodimers and AHR-related factors	DRE (dioxin response elements), XRE (xenobiotic response elements) bound by AHR/ARNT heterodimers	13:47472367/13:47472391	ctgctcgcca**GCGTg**catatttaac	18
V$HIF	Hypoxia inducible factor, bHLH/PAS protein family	AhR nuclear translocator homodimers	13:47472383/13:47472399	atattt**aaCGTG**gaggg	17
V$CREB	cAMP-responsive element binding proteins	X-box-binding protein 1	13:47472382/13:47472402	catattta**ACGTg**gaggggta	17
V$HESF	Vertebrate homologues of enhancer of split complex	Basic helix-loop-helix domain containing, class B, 2 (secondary DNA binding preference)	13:47472385/13:47472399	att**aaCGTG**gaggg	17
V$NRSF	Neuron-restrictive silencer factor	Neural-restrictive-silencer-element	13:47472571 / 13:47472601	aag**gaag**a**gtc**g**CGGA**ta**ac**a**gc** agctgtct	13–12

Table S1. Cont.

V$HIFF	Hypoxia inducible factor, bHLH/PAS protein family	Hypoxia inducible factor, bHLH/PAS protein family	13:47473151/13:47473167	accacaga**CGTG**cctag	8
V$CREB	cAMP-responsive element binding proteins	X-box-binding protein 1	13:47473150/13:47473170	caccacag**ACGTg**cctagcca	8
V$HESF	Vertebrate homologues of enhancer of split complex	Basic helix-loop-helix domain containing, class B, 2 (secondary DNA binding preference)	13:47473546/13:47473560	catt**ccCGTG**gaaac	5
O$XCPE	Activator-, mediator- and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	X gene core promoter element 1	13:47473729/13:47473739	**gtGCGGga**g**c**t	2
V$E2FF	E2F-myc activator/cell cycle regulator	E2F transcription factor 6	13:47473726/13:47473742	gcagt**GCGGga**gctggc	2

[1] The "V$" prefixes to the individual matrices are representative of the Vertebrate MatInspector matrix library, while "O$" prefixes to general core promoter elements. [2] Bold: consensus index vector-value > 60 and capital letters: core sequence; [3] Studied CpG identifier.

Table S2. Pearson correlation analyses between the selected twenty CpG sites for *HTR2A* gene.

CpG	Mean	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Mean	1.00																				
1	0.01	1.00																			
2	−0.35	0.67	1.00																		
3	0.19	0.13	0.20	1.00																	
4	0.49	004	-0.05	0.62	1.00																
5	−0.59	0.70	0.78	-0.01	-0.31	1.00															
6	−0.47	0.73	0.80	0.04	−0.23	0.89	1.00														
7	0.73	−0.28	−0.68	0.21	0.41	−0.60	−0.70	1.00													
8	0.63	0.04	−0.30	0.30	0.03	−0.34	−0.24	0.73	1.00												
9	0.51	−0.22	−0.45	0.01	0.36	−0.63	−0.58	0.68	0.36	1.00											
10	0.65	−0.62	−0.78	0.08	0.40	−0.97	−0.89	0.82	0.43	0.70	1.00										
11	0.00	0.44	0.36	−0.18	−0.22	0.45	0.38	−0.33	−0.19	−0.47	−0.48	1.00									
12	0.61	−0.02	−0.11	−0.27	0.02	−0.36	−0.27	0.15	−0.01	0.27	0.28	0.23	1.00								
13	−0.16	0.64	0.67	−0.27	−0.37	0.71	0.69	−0.61	−0.33	−0.44	−0.76	0.64	0.34	1.00							
14	0.86	−0.32	−0.61	0.19	0.44	−0.48	−0.66	0.73	0.60	0.46	0.79	−0.19	0.36	−0.48	1.00						
15	0.78	−0.08	−0.38	0.13	0.28	−0.41	−0.35	0.52	0.53	0.07	0.45	0.11	0.32	−0.15	0.83	1.00					
16	0.43	0.37	0.22	−0.24	−0.06	0.17	0.19	−0.10	−0.03	−0.03	−0.20	0.60	0.70	0.71	0.12	0.32	1.00				
17	0.76	0.09	−0.03	−0.01	0.13	0.32	−0.16	0.23	0.25	0.08	0.22	0.35	0.80	0.32	0.58	0.67	0.71	1.00			
18	0.81	−0.51	−0.66	0.12	0.66	−0.60	−0.81	0.77	0.48	0.58	0.93	−0.35	0.44	−0.60	0.90	0.66	0.01	0.47	1.00		
19	0.76	−0.56	−0.75	0.11	0.45	−0.95	−0.84	0.79	0.46	0.62	0.96	−0.41	0.40	−0.67	0.88	0.60	−0.07	0.39	0.98	1.00	
20	0.71	−0.59	−0.74	0.15	0.47	−0.95	−0.88	0.80	−0.46	0.63	0.98	−0.46	0.32	−0.73	0.84	0.53	−0.16	0.30	0.96	0.98	1.00

Mean = mean *HTR2A* methylation; Flled grey = significantly correlated as identified by Pearson test, $p < 0.05$.

Table S3. Pearson correlation analyses between body weight, BMI and fat mass with the methylation (%) of *HTR2A* gene at baseline.

Baseline	CpG sites	Body Weight		WC		BMI		Fat Mass (kg)	
		r	*P*-Value	*r*	*P*-Value	*r*	*P*-Value	*r*	*P*-Value
cg15894389	1	0.286	0.070	0.509	**0.001**	0.297	0.059	0.320	**0.042**
cg02250787	2	0.207	0.195	0.393	**0.011**	0.327	**0.037**	0.294	0.062
cg06476131	3	0.079	0.623	0.197	0.216	0.077	0.624	–0.028	0.861
cg16188532	4	0.262	0.099	0.247	0.119	0.153	0.340	0.122	0.445
cg09361691	5	0.180	0.260	0.350	**0.025**	0.355	**0.023**	0.327	**0.037**
cg11514288	6	0.122	0.448	0.344	**0.027**	0.304	0.053	0.311	0.047
cg27068143	7	–0.045	0.780	–0.142	0.374	–0.317	**0.045**	–0.305	0.052
cg10323433	8	0.002	0.987	–0.018	0.910	–0.339	**0.030**	–0.268	0.090
cg02027079	9	0.033	0.835	–0.037	0.082	–0.064	0.691	–0.030	0.854
cg01192538	10	–0.108	0.503	–0.278	0.078	–0.295	0.061	–0.280	0.076
cg01620540	11	0.139	0.384	0.241	0.130	0.057	0.725	0.250	0.120
cg06020661	12	0.106	0.510	0.082	0.609	0.046	0.775	0.136	0.396
cg09798090	13	0.153	0.340	0.327	**0.037**	0.251	0.113	0.335	**0.032**
cg24320398	14	0.017	0.913	–0.082	0.612	–0.288	0.067	–0.255	0.108
cg18200810	15	0.047	0.770	–0.045	0.778	–0.230	0.148	–0.198	0.214
cg15692052	16	0.225	0.157	0.361	**0.020**	0.231	0.145	0.307	0.051
cg24118521	17	0.137	0.393	0.137	0.394	–0.026	0.871	0.012	0.941
cg23881368	18	–0.043	0.792	–0.173	0.280	–0.276	0.080	–0.284	0.072
cg05506829	19	–0.064	0.683	–0.217	0.172	–0.273	0.085	–0.268	0.090
cg07075299	20	–0.078	0.626	–0.239	0.133	–0.289	0.067	–0.301	0.056

Data are represented as *r* and *p* values from Pearson correlations analysis. Abbreviations: BMI, body mass index; *HTR2A*; 5-hydroxytryptamine receptor 2A; WC, waist circumference.

Summary of the results

Chapter	Study	Subjects	Journal	Design	Main findings
1	SENIFOOD	Older adults with overweight or obesity	J Physiol Biochem. 2014	Prospective	- A metabolomic analysis showed that after the weight loss intervention participants significantly reduced serum FAs and isoleucine levels. - Palmitoleic acid (C16:1) was found to be a negative predictor of change in body fat loss.
2	RESMENA	Middle-aged subjects with MetS	Nutr Neurosci 2014	Cross-sectional	- A positive association between both the overall dietary pattern (HEI) and isolated nutrients (water, fibre, vitamin B_6, ascorbic acid, trp, magnesium and selenium) with mood state was observed in this population.
3	RESMENA	Middle-aged subjects with MetS	Nutr J. 2014	Cross-sectional	- The number of criteria of the MetS was higher among subjects with more somatic-related depressive symptoms.
				Prospective	- Manifestations of depression decreased after the dietary treatment. This decline was related with changes in body weight, adiposity, CRP and leptin in subjects with MetS.
4	RESMENA	Middle-aged subjects with MetS	Clin Nutr. 2013	Cross-sectional	- Participants with lower depressive symptoms at baseline reported a significantly higher intake of ω-3 PUFAs.
				Prospective	- A higher intake of folate and a decline in MDA plasma levels during the intervention, were related to improvements in depressive symptoms.
5	RESMENA	Middle-aged subjects with MetS	Psychoneuro-endocrinology 2014	Prospective	- Anxiety symptoms decreased after 6 months of nutritional intervention, which was parallel to a greater decrease in body weight and anthropometric markers. - An increase in 5-HT and DA blood levels after the weight loss treatment in obese patients with MetS was detected, with this increase being related with lower energy and CHO intakes, respectively. - Baseline DA concentrations were associated with obesity-related parameters, proposing the assessment of DA as a marker of weight loss and adiposity.
6	RESMENA	Middle-aged subjects with MetS	Nutrients 2014	Cross-sectional	- A positive association of *HTR2A* gene methylation with WC and insulin levels was detected.
				Prospective	- Hypermethylation of the *HTR2A* gene in WBC at baseline was significantly associated with a worse response to the weight loss intervention and with a lower decrease in depressive symptoms after the dietary treatment.

V. General discussion

1. Justification of the study

The rates of non-communicable diseases have reached epidemic proportions worldwide, with obesity and psychiatric disorders being among the most prevalent (WHO, 2011, 2012, 2013). For this reason, the prevention and treatment of these diseases, as well as the investigation of the potential association between them, has attracted much attention in recent years (Bray, 2007; Renn *et al.*, 2011; Pan *et al.*, 2012; Sanchez-Villegas *et al.*, 2013a).

Obesity plays a crucial role in the onset and development of the MetS, where several risk factors for CVD concur (Bray, 2007). As a result, different weight loss strategies have been put forward to combat the obesity epidemic and its comorbidities (Abete *et al.*, 2011). However, most dietary interventions in addition to the desired decrease in fat mass also result in lean mass loss, especially discourage in older adults (Bopp *et al.*, 2008). Moreover, energy-restricted diets may lead to a decrease in basal metabolic rate and satiety, together with the difficulty of maintaining the acquired prescribed diet in the long term. Therefore, the design of effective weight loss interventions is required (Abete *et al.*, 2011), where new genomic tools might be useful to better define key pathways and molecules, and deeper evaluate its efficacy (Konstantinidou *et al.*, 2014).

Diet may also take part in the prevention and treatment of psychiatric disorders (Murakami *et al.*, 2010; Murphy *et al.*, 2013). Nutrients participate in brain development and functioning, affecting cognitive processes and emotions (Pascoe *et al.*, 2011). In this sense, there is information concerning the potential effects that both isolated nutrients and dietary patterns play in psychiatric disorders (Akbaraly *et al.*, 2013; Murphy *et al.*, 2013; Sanchez-Villegas *et al.*, 2013a).

Moreover, a bidirectional relationship of mental and behavioural disorders with obesity and the MetS has been reported (Renn *et al.*, 2011; Pan *et al.*, 2012; Brumpton *et al.*, 2013). Some of the mechanisms that may link them are low-grade inflammation, oxidative stress, unhealthy lifestyle habits and monoamine imbalance, among others (Bondy, 2007; Pan *et al.*, 2012). Therefore, it has been suggested that the dietary recommendations for obesity and its comorbidities might be helpful for

behavioural disorders treatment, since both diseases seem to share several underlying metabolic pathways (Sanchez-Villegas *et al.*, 2013a).

Consequently, the present study was devised to assess the possible metabolic and epigenetic mechanisms underlying weight loss in overweight individuals under two different longitudinal nutritional intervention studies, the SENIFOOD study and the RESMENA study, evaluating also the impact of the dietary treatment to reduce MetS features on symptoms of depression and anxiety.

2. Dietary intake and mental disorders

Available scientific evidence shows that dietary intake, both isolated nutrients and dietary patterns, may have potential effects on depression and anxiety disorders (Akbaraly *et al.*, 2013; Murphy *et al.*, 2013; Sanchez-Villegas *et al.*, 2013a), since diet and food components are related to inflammation, oxidative stress, and brain plasticity and functioning (Sanchez-Villegas *et al.*, 2013a). A protective role of healthy dietary patterns, including the MedDiet, with regards to the prevention of depressive manifestations has been reported (Akbaraly *et al.*, 2009; Sanchez-Villegas *et al.*, 2009a; Akbaraly *et al.*, 2013; Rienks *et al.*, 2013; Sanchez-Villegas *et al.*, 2013b), while unhealthy dietary habits are proposed to have the opposite effect (Jacka *et al.*, 2010; Le Port *et al.*, 2012). However, little information is available on the relationship between dietary intake and psychological state in individuals with MetS.

Self-reported psychological state data was only collected in the RESMENA trial and not the SENIFOOD study. In a cross-sectional (baseline) analysis, a significant positive relationship between overall mood state (using the mood thermometer VAS) and healthy dietary pattern (using the HEI) was observed, where a high intake of vegetables, fruits and dietary variety as well as low calories from lipids and SFAs consumption contributed more to the association, corroborating previous findings (Akbaraly *et al.*, 2009; Alsio *et al.*, 2009; Murakami *et al.*, 2010; Park *et al.*, 2010; Bhupathiraju *et al.*, 2011; Sanchez-Villegas *et al.*, 2011; Payne *et al.*, 2012). In contrast, no relationships between whole dietary pattern (HEI, control diet or RESMENA diet) with either depressive or anxiety symptoms was observed in this cohort.

Furthermore, a higher consumption of B vitamins (specially thiamine, folate and vitamins B_6 and B_{12}), Trp, antioxidants (ascorbic acid, magnesium, zinc and selenium), fibre, water and ω-3 PUFAs could be protective against mental disorders (Firk *et al.*, 2009; Park *et al.*, 2010; Payne *et al.*, 2012; Qin *et al.*, 2012; Derom *et al.*, 2013; Pross *et al.*, 2013; Shabbir *et al.*, 2013).

In our study, those participants with MetS who consumed more water, fibre, vitamin B_6, ascorbic acid, Trp, magnesium and selenium at the beginning of the study presented better mood state. In this sense, dehydration affects human homeostasis, mood and cognitive functions, hence, water intake may be negatively associated with psychological disorders (Pross *et al.*, 2013). The possible involvement of fibre in mood fluctuations has been less investigated, although a negative association has been observed (Park *et al.*, 2010). This might be due to the fact that food rich in fibre such as whole grains, fruits and vegetables are rich in B vitamins and antioxidants (Logan, 2006; Park *et al.*, 2010). Moreover, deficiency of vitamin B_6 and other B vitamins, may cause increased levels of homocysteine leading to a higher risk of low mood (Sanchez-Villegas *et al.*, 2009b; Qin *et al.*, 2013). Vitamin B_6 and the amino acid Trp are also involved in the serotonergic neurotransmission (Shabbir *et al.*, 2013). Furthermore, ascorbic acid acts as a cofactor for NA and also plays a role in the conversion of Trp to 5-HT (Scapagnini *et al.*, 2012). Besides, both selenium and magnesium have been hypothesized to protect the brain against oxidative damage and low-grade inflammation, acting against mood fluctuations (Jacka *et al.*, 2009; Derom *et al.*, 2013; Johnson *et al.*, 2013).

In addition, a positive association between depressive symptoms and ω-3 PUFAs intake was observed at baseline, whose anti-inflammatory properties and their involvement in the functioning of the CNS have been hypothesized to positively affect mood (Pascoe *et al.*, 2011).

These cross-sectional results do not allow us to draw conclusions on cause and effect. Previous prospective studies have shown that an unhealthy diet was predictive of future mental illness, but not the opposite (Akbaraly *et al.*, 2009; Sanchez-Villegas *et al.*, 2009a). However, although it was also reported that individuals with depression consumed poorer quality diets, it was not proven

whether this population followed an unhealthy diet prior presenting the depressive symptoms (Kilian *et al.*, 2006).

In the longitudinal analysis concerning the association between dietary intake and the decrease of depressive symptoms after the RESMENA study, an increase in folate consumption during this weight-loss intervention was involved in the improvement of depressive manifestations. Despite this increase in folate intake, a decline in serum homocysteine levels was not detected. The fact that our participants did not receive vitamin B supplementation, but just a hypocaloric diet, may explain this outcome (Kwok *et al.*, 2011).

Nevertheless, the role of diet on anxiety disorders has been less explored, and most of the research that has shown a relationship between them have been in animal studies (Murphy *et al.*, 2013). While some studies support the anxiolytic effect of palatable food, such as sugars and fats (Maniam *et al.*, 2010), others have reported that unhealthy dietary patterns, that imply higher palatable food, are related with higher likelihood of anxiety disorders (Jacka *et al.*, 2010; Jacka *et al.*, 2011; Trovato *et al.*, 2014). Moreover, previous dietary experiences and maternal diet during and prior to pregnancy may also modulate anxiety (Sullivan *et al.*, 2010; Murphy *et al.*, 2013; Sasaki *et al.*, 2013). In this study, no association between anxiety disorders and dietary intake was observed, being supported by other studies (Bjelland *et al.*, 2003).

3. Metabolomics, epigenetics and weight loss

The fact that not all individuals respond equally to similar dietary treatments, suggest that some of these variations might be due to the genetic make-up (Konstantinidou *et al.*, 2014). Thereby, research in epigenetics and metabolomics has recently emerged as new tools that may provide an understanding at the cellular and molecular level of the underlying processes behind a dietary intervention (Milagro *et al.*, 2013a; Scalbert *et al.*, 2014).

Particularly, the application of metabolomics in nutritional research may be a useful approach to analyse and predict the individualized response to a dietary

intervention. Due to the greater influence of FAs composition and BCAA on health and disease (Morris *et al.*, 2012b; Kien *et al.*, 2013), we sought to research the effect of the dietary restriction on FAs and BCAA serum levels using a GC/MS metabolomic approach within the SENIFOOD study. The most relevant results of the metabolomic analysis were that total SFAs, which have been previously related with an increased cardiovascular risk (Flock *et al.*, 2013), were significantly reduced after the 8-week intervention. Moreover, both the total ω-6 and ω-3 PUFAs significantly decreased although the overall total amounts of PUFAs did not. The ω-6 PUFAs are thought to promote adipogenesis and increase expression of lipogenic genes, while ω-3 PUFAs have been suggested to do the opposite (Lorente-Cebrian *et al.*, 2013; Muhlhausler *et al.*, 2013). Interestingly, palmitoleic acid (C16:1) was found to be a negative predictor of change in body fat loss. The role of palmitoleic acid in human metabolism has not been fully clarified (Cao *et al.*, 2008), although high levels of this particular FA have been associated with increased risk of suffering CVD (Warensjo *et al.*, 2005; Paillard *et al.*, 2008; Gong *et al.*, 2011). In general, the decrease in most of the FAs serum levels might be explained by a lower FA production, a higher oxidation of these compounds, or by a lower fat intake, which could not be confirmed in this study. Additionally, the BCAA isoleucine significantly decreased in the serum samples after the intervention, which reduction has been suggested to promote lipolysis via induction of lipolytic genes and by the suppression of lipogenesis in liver (Du *et al.*, 2012).

Aging is accompanied by the decrease of skeletal muscle mass, size and strength (sarcopenia), that implies additional serious health consequences, such as disability and mortality (Bouchonville *et al.*, 2013). In order to minimize lean mass losses in the overweight population of the SENIFOOD study, the prescribed hypocaloric diet presented a 25% of protein (Krieger *et al.*, 2006; Ibero-Baraibar *et al.*, 2014), however, lean mass was significantly decreased after the dietary intervention, being in line with previous studies (Santanasto *et al.*, 2011). Interestingly, participants significantly decreased body weight, BMI, WC, total fat mass and showed a metabolic improvement (glucose and lipid profile).

On the other hand, epigenetics has emerged as a novel approach to understand gene–environment interactions (Campion *et al.*, 2009). Among the possible epigenetic processes, DNA methylation is considered a predictive tool to assess the response to a nutritional intervention (Moleres *et al.*, 2013; de la Iglesia R, 2014). Additionally, disturbances in the serotonin 2A receptor, which is encoded by the *HTR2A* gene (Fabbri *et al.*, 2013), have been involved in the pathogenesis of major psychiatric disorders and obesity (Falkenberg *et al.*, 2011; Li *et al.*, 2013). Hence, it was assessed the association of *HTR2A* gene promoter methylation levels, in WBC, with obesity measures and depressive symptoms in individuals with MetS enrolled in the RESMENA weight-loss trial over a period of 6 months. In this study, we failed to find an association between gene expression and promoter methylation levels, however, the CpG methylation of a promoter region is usually negatively associated with gene expression, since it may be a mechanism implicated in gene inactivation by blocking its transcription (Milagro *et al.*, 2013a). This finding might be attributed to the fact that not all samples could be read. Interestingly, those subjects with higher methylation levels of *HTR2A* gene at baseline showed a lower decrease in body weight, BMI and fat mass after the 6-month dietary treatment. Apparently, no previous study has analysed DNA methylation levels of *HTR2A* gene in a population with MetS enrolled in a weight-loss treatment. Therefore these results could not be compared, although it has been suggested that the SNP rs6311 (-1438G>A) of the *HTR2A* gene influences glucose homeostasis (Kring *et al.*, 2009) and also obesity traits (BMI and abdominal obesity) in obese subjects (Sorli *et al.*, 2008). However, SNPs have not been genotyped in this study. Hence, if the methylation levels are tagging a SNP, this may explain why DNA methylation was not associated with gene expression, as this SNP influences translation efficiency (Abdolmaleky *et al.*, 2011; Ghadirivasfi *et al.*, 2011).

Moreover, higher methylation levels of the *HTR2A* gene promoter at baseline were related with a lower decrease in depressive symptoms after the nutritional intervention. Some studies have reported that *HTR2A* expression is decreased in the brain of patients with schizophrenia and in those who have committed suicide (Garbett *et al.*, 2008; Hurlemann *et al.*, 2008; Abdolmaleky *et al.*, 2011). Moreover, antipsychotic treatment has been linked with lower DNA methylation and increased

expression of the *HTR2A* gene (Abdolmaleky *et al.*, 2011). However, although *HTR2A* gene has been directly associated with MDD (Mandelli *et al.*, 2013), methylation of cytosines of the *HTR2A* promoter gene on this illness has not been sufficiently investigated yet (Fabbri *et al.*, 2013).

4. Effect of the energy-restricted diet on depressive and anxiety symptoms: underlying mechanisms

The impact of weight loss on psychological problems has been a matter of controversy, however, recent studies support the theory that obese subjects that lose weight may improve body image, physical functioning and quality of life, and consequently relieve psychological stress (Fabricatore *et al.*, 2011). Moreover, it has been proposed that the dietary recommendations for MetS might be helpful for behavioural disorders treatment, since both seem to share several common underlying mechanisms (Sanchez-Villegas *et al.*, 2013a). The RESMENA study strengthens this hypothesis, as the dietary treatment for MetS manifestations also reduced depressive and anxiety symptoms.

Noteworthy, the decrease in depressive symptoms after the RESMENA nutritional intervention positively correlated with body weight and fat mass change, as well as with the decrease in MDA, CRP and leptin. Previous investigations have reported a decrease in depressive manifestations after a weight loss treatment (Fabricatore *et al.*, 2011; Somerset *et al.*, 2011), however, the present study is apparently the first to show it in a population with MetS. Furthermore, the higher decrease in depressive symptoms was more noticeable in those participants with a greater decline in total fat mass. Since adipose tissue is known to secrete inflammatory cytokines and leptin (Clement *et al.*, 2004), it might be hypothesized that the decrease in body fat mass may have contributed to reduce CRP and leptin and subsequently decrease depressive symptoms. In this context, leptin is known to affect the hippocampus, the cortex and other brain areas associated with cognition and mood (Morrison, 2009; Morris *et al.*, 2012a), while CRP has also been highly related with MDD (Daly, 2013).

Since the brain is sensitive to oxidative damage that might alter neural signals, higher levels of the biomarker of lipid peroxidation MDA has been reported in MDD

(Yager *et al.*, 2010; Talarowska *et al.*, 2012). Our findings are in agreement with this statement since the greater the decrease in MDA levels, the more marked the decline in depressive symptoms.

On the other hand, the decrease in anxiety symptoms after the 6-month weight-loss treatment was positively related with weight loss and the decrease in other anthropometric measurements, corroborating previous investigations (Bas *et al.*, 2009; Swencionis *et al.*, 2013). However, it should be noted that a recent review has pointed out that there is no clear evidence to indicate that energy-restricted diets have a direct beneficial effect on anxiety (Eyres *et al.*, 2014). Moreover, the reduction in anxiety symptoms was higher in those participants who had lower basal 5-HT blood values, suggesting that subjects with poor 5-HT plasma levels at baseline may have benefited most from the intervention by decreasing more their anxiety symptoms (Williams *et al.*, 2006; Sekiyama *et al.*, 2013).

Thus, it might be suggested that the energy-restricted diet induced a reduction in fat mass contributing to decrease inflammation, oxidative stress and leptin, which consequently, improved stress and mood management through a better HPA axis and CNS functioning. However, this hypothesis could not be confirmed through BDNF and cortisol blood levels, perhaps because these molecules present a characteristic daily variation (Begliuomini *et al.*, 2008; Owens *et al.*, 2014). Therefore, future human intervention studies focused on psychiatric disorders may benefit from collecting early morning salivary cortisol and BDNF in order to reduce variability due to circadian rhythms (Mandel *et al.*, 2011; Herbert, 2013).

5. Relationship between weight loss and peripheral monoamines

Reduced circulating monoamines may have a role in the development of obesity and psychiatric diseases (Williams *et al.*, 2006; Hodge *et al.*, 2012; Sekiyama *et al.*, 2013). However, while monoamine brain functions are well-known, the identification of these molecules as potentially important peripheral markers has just emerged (Berger *et al.*, 2009; Rubi *et al.*, 2010; Carlin *et al.*, 2013). Hence, there is a scarcity of studies analysing the association between peripheral monoamines, dietary intake,

weight loss and psychological factors. Peripheral 5-HT functions as a peripheral hormone affecting vasoconstriction, glucose and lipid metabolism and energy homeostasis, among others (Berger *et al.*, 2009; Stunes *et al.*, 2011; Watanabe *et al.*, 2011). Moreover, blood 5-HT has been used as an indicator of mood disorders in previous investigations, finding negative associations between whole-blood 5-HT and both depression and anxiety symptoms (Williams *et al.*, 2006; Sekiyama *et al.*, 2013). Concerning peripheral DA, it has been involved in a wide variety of peripheral functions, including the control of both glucose metabolism and body weight, the modulation of blood pressure, the inhibition of gastric acid secretion as well as apoptosis of tumoral cells (Pernet *et al.*, 1984; Eliassi *et al.*, 2008; Rubi *et al.*, 2010). As for plasma NA, very high levels may have a detrimental effect, as it may cause heart failure due to the extreme activation of the SNS (Kimura *et al.*, 2010).

In our research, both DA and 5-HT blood levels were significantly increased after the intervention, with this increase being related with lower energy and CHO intakes, respectively. In this context, energy restriction might have promoted the production of gut-derived 5-HT and, consequently, it may have contributed to weight loss by stimulating lipolysis in the adipose tissue, since it has been shown that fasting conditions promote synthesis of gut-derived 5-HT, which in turn favours both lipolysis and liver gluconeogenesis by signalling in adipocytes and hepatocytes (Sumara *et al.*, 2012). Concerning the increase in DA plasma levels, it might be speculated that lower CHO intake led to lower insulin release and this might have increased DA peripheral values due to the role that peripheral DA plays in inhibiting insulin release through D2 receptors in the pancreas (Rubi *et al.*, 2005). Likewise, the blockade of DA receptors would enhance insulin release, promoting adipogenesis, weight gain and insulin resistance (Rubi *et al.*, 2010). However, we failed to find a direct association between peripheral DA levels and insulin values.

Moreover, an association between higher baseline DA concentrations and greater reduction in body weight and anthropometric parameters was found, which levels might play an important role in body weight management (Astrup *et al.*, 2008; Reinholz *et al.*, 2008; Rubi *et al.*, 2010). The blockage of D2 DA receptors caused by antipsychotic medication induces weight gain in humans, thereby, treatment with an

inhibitor of DA uptake reduced body weight in obese patients (Astrup *et al.*, 2008; Reinholz *et al.*, 2008).

6. Strengths and limitations

This research has successfully displayed the beneficial effects of dietary treatment for weight loss in overweight and obese older adults as well as in middle-aged subjects with MetS participating in two different studies. The improvement of depressive and anxiety symptoms, together with the use of novel technological approaches and the discovery of new biomarkers for weight loss, are among the most relevant strengths of this investigation. However, some limitations of both the SENIFOOD and RESMENA studies should be declared.

Firstly, it should be stated that in all the analyses carried out in the RESMENA study, both the Control and the RESMENA groups were merged and analysed together as a unique experimental group. This decision was taken due to the fact that no statistical differences between dietary groups were found in any of the variables analysed in this investigation. Hence, we conducted longitudinal observational analyses comparing the variables before and after intervention, serving the volunteers as their own control (Martínez-González *et al.*, 2006). One of the strengths of within-subjects (paired) analyses is the reduction in error variance associated with individual differences, which increases statistical power. However, observational cohort studies might be subject to bias and confounding, leading to problems in determining causality. However, this limitation might be partly overcome by controlling for different confounders, such as the group of intervention. Moreover, it has been shown that the results of observational studies are remarkably similar to those from RCT on the same topic (Concato *et al.*, 2000).

Secondly, in the RESMENA study, mood state and anxiety symptoms were evaluated using the BDI (Beck *et al.*, 1961), the STAI (Spielberger *et al.*, 1971), and the mood thermometer VAS (McCormack *et al.*, 1988; Rampling *et al.*, 2012) self-report questionnaires, which are widely recognized screening methods, have been shown to be valid for clinical assessment, and have previously been used to record depressive and anxiety symptoms in weight loss studies (Bas *et al.*, 2009; Fabricatore *et al.*,

2011; Mitchell *et al.*, 2012). In addition, our aim was to offer a psychological monitoring as a part of the dietary intervention, thus participants previously identified as having mental disorders were excluded, what may have decrease the number of subjects presenting depressive or anxiety symptoms. In contrast, psychological symptoms were not collected in the SENIFOOD study, what might have enriched this investigation.

Thirdly, the number of participants in these studies is not very large, what may have increased the risk of type II errors (failing to detect real differences), which are frequent when adjustments are used in conjunction with a small effect or a small sample (Smith *et al.*, 2002). Therefore, with the aim of avoiding type II errors, covariates were limited in the analyses carried out in this study. Our approach is consistent with previous investigations that reported that when it is important to discover new facts, we may be willing to accept more type I errors in order to avoid type II errors (Keppel *et al.*, 2004; Cohen *et al.*, 2013). Henceforth, the probability of type II errors is low because important statistical differences were found despite the small sample size. However, further studies with a larger sample size are needed to verify our findings.

Fourthly, dietary intake data was not available in the SENIFOOD study, what could have been helpful in further understanding the changes in metabolites following the 8-week intervention. By contrast, weighed food records were collected in the RESMENA study. This questionnaire may lead to bias, however, diary records are more precise than other dietary assessments, for example food frequency questionnaires (Akbaraly *et al.*, 2013).

Fifthly, peripheral monoamines do not necessarily indicate the concentrations in the CNS (Mann *et al.*, 1992), however, a strength of this research is that it shows that peripheral monoamines may play a role in weight loss and anxiety symptoms in a less invasive way.

Lastly, *HTR2A* methylation levels were not measured at the end of the intervention, being not possible to confirm whether the dietary treatment has an effect on *HTR2A* gene methylation levels. Also, we failed to find an association between promoter methylation levels and gene expression in WBC. Gene expression varies depending

on the tissue or cell type (Milagro *et al.*, 2013b), and it is possible that *HTR2A* gene is not widely expressed in WBC. Moreover, other studies have found that DNA methylation is involved in the regulation of HTR2A expression (Polesskaya *et al.*, 2006). In addition, validation of these results with other reliable standard such as pyrosequencing or bisulfite sequencing might be considered in future studies.

7. Corollary

This thesis proposes new underlying processes behind body weight reduction after following different dietary treatments for weight loss in overweight and obese older adults as well as in middle-aged subjects with MetS. To follow a hypocaloric diet designed to reduce MetS features in middle-aged subjects was effective for decreasing depressive and anxiety symptoms. The decrease in depressive manifestations was positively related to body weight and fat mass changes, as well as with the decrease in CRP, MDA and leptin and with a higher folate intake during the intervention, while the decline in anxiety symptoms was parallel to a greater decrease in body weight and anthropometric markers. Moreover, both the overall dietary pattern and isolated nutrients were related with mood state.

In overweight and obese older adults palmitoleic acid (C16:1) concentrations were found to be a negative predictor of changes in adiposity. Moreover, baseline DA and *HTR2A* methylation levels were proposed as new biomarkers for the response to a nutritional intervention in subjects with MetS. These results provide further insights regarding emotional and metabolic factors behind weight loss, where metabolomics and epigenetics approaches were very useful for assessing the underlying mechanisms.

8. General discussion references

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VI. Conclusions

Conclusions

1. A GC/MS metabolomic analysis evidenced that a weight loss intervention in older adults with overweight and obesity significantly reduced serum fatty acids and isoleucine levels.

2. Palmitoleic acid (C16:1) was found to be a negative predictor of change in body fat loss in overweight older adults.

3. A positive cross-sectional association between a healthy dietary pattern and some specific nutrients (water, fibre, vitamin B_6, ascorbic acid, tryptophan, magnesium and selenium) with mood state was observed, while a negative relationship between depressive symptoms and intake of ω-3 PUFAs was evidenced in middle-aged subjects with metabolic syndrome.

4. Manifestations of depression decreased after the dietary treatment in volunteers suffering from metabolic syndrome. The decrease in depressive symptoms positively correlated with body weight and fat mass changes, as well as with the decrease in C-reactive protein, malondialdehyde and leptin and with a higher folate intake during the intervention.

5. Anxiety symptoms decreased after 6 months of nutritional intervention in patients with metabolic syndrome, which was parallel to a greater decrease in body weight, BMI, waist circumference and fat mass.

6. An increase in serotonin and dopamine blood levels after the weight loss treatment in obese patients with metabolic syndrome was demonstrated, with this increase being related with lower energy and carbohydrates intakes, respectively.

7. Baseline dopamine concentrations were associated with obesity-related variables, suggesting that the assessment of dopamine may be a predictor of weight loss and adiposity in metabolic syndrome subjects.

8. A positive association of *HTR2A* gene methylation with waist circumference and insulin levels was detected in participants with metabolic syndrome.

9. Hypermethylation of the *HTR2A* gene in white blood cells at baseline was significantly associated with a worse response to the weight loss intervention and with a lower decrease in depressive symptoms after the dietary treatment.

VII. APPENDICES

Appendix 1: an example of a SENIFOOD diet (1500 Kcal)

Dieta 1500 kcal

Toma	Alimentos	Cantidad	Ración
Desayuno	Lácteo	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	 1 tazón 2 unidades
	Cereal	*Elegir entre:* -30 g pan (blanco/molde) -30 g biscotes -20 g galletas tipo María -30 g cereales	 1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Proteico	-45 g queso blanco/fresco desnatado	1 ½ loncha
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g kiwi, manzana, piña, ciruela -75 g plátano, 100g uva	 1 unidad pequeña/ mediana
Media Mañana	Lácteo	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	 1 vaso pequeño 1 unidad
Comida	Primer Plato	*Elegir según menú:*	
		-200 g crudo ó 240 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano
		-40 g crudo ó 140 g cocido de pasta + 100 g verdura	
		-40 g crudo ó 115 g arroz + 100 g de verdura	
	Patata (cuando no se elige pasta ni arroz)	-160 g crudo o 180 g cocinado (acompañando a la verdura o de guarnición del segundo plato)	1 unidad mediana
	Segundo Plato	*Elegir según menú:*	
		-170 g crudo ó 150 g en cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…)	1 filete mediana
		-200 g crudo ó 180 g cocinado de pescado blanco ó azul	1 porción mediana
		-80 g crudo ó 240 g cocido de alubias, garbanzos, lentejas -240 g crudo ó 300 g cocido de guisantes frescos	1 plato grande
	Pan	30 g pan blanco/ molde	1 rebanada
	Grasa	5 g de aceite de oliva	1 cucharilla

Merienda	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g kiwi, manzana, piña, ciruela -75 g plátano, 100g uva	1 unidad pequeña/ mediana
	Frutos Secos	-10 g nueces, almendras o avellanas	4-5 unidades
Cena	Primer Plato	-100 g de verdura en crudo o 120 g en cocido	1 plato pequeño
	Segundo Plato	*Elegir según menú:*	
		-60 g huevo	1 unidad
		-90 g queso blanco/fresco desnatado ó 60 g queso semigrasos	3 lonchas/ 2 lonchas finas
		-60 g jamón cocido o serrano magro	2 lonchas
		-120 g crudo ó 90 g cocido de pescado	1 ración pequeña
		-90 g crudo ó 60 g en cocido de pollo o pavo	1 filete pequeño
	Pan	*Elegir entre:* -30 g pan (blanco/molde) -30 g biscotes	 1 rebanada pequeña 3 unidades pequeñas
	Grasa	-5 g de aceite de oliva	1 cucharilla

Postre – Elegir entre:		
Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g kiwi, manzana, piña, ciruela -75 g plátano, 100g uva	1 unidad pequeña/ mediana
Lácteo	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	 1 vaso 1 unidad

Menú

	Lunes	Martes	Miércoles	Jueves	Viernes	Sábado	Domingo
Comida	Verdura Pescado Patata Fruta	Pasta+Verdura Carne magra Lácteo	Verdura Legumbre Patata Fruta	Verdura Carne magra Patata Lácteo	Verdura Legumbre Patata Fruta	Verdura Pescado Patata Lácteo	Arroz+Verdura Carne magra Lácteo
Cena	Ensalada Jamón serrano Lácteo	Ensalada -200g Pescado Fruta	Ensalada Huevo Lácteo	Ensalada Queso Fruta	Ensalada Jamón cocido Lácteo	Ensalada Carne magra Fruta	Ensalada- 200 g Queso Fruta

NOTA IMPORTANTE

EL PARTICIPANTE DEBERÁ EXCLUIR DE SU DIETA:

Suplementos nutricionales con vitaminas y/o minerales.

Productos de herboristería, naturales y/o fitoquímicos, a base de compuestos ricos en polifenoles o antioxidantes

Condimentos y especias como mostaza, canela, azafrán, perejil, tomillo, orégano, hinojo, etc., así como bayas de goji.

Infusiones como las de rooibos, frutas rojas, té verde, té rojo, menta poleo, manzanilla, etc.

Todos los productos (lácteos, galletas, huevos, harinas, cafés solubles, margarinas, cereales, etc.) enriquecidos con algún antioxidante.

RECOMENDACIONES GENERALES

Usted deberá realizar todas las 05 tomas.

Tanto los postres de comida y cena, como las medias mañanas y meriendas, son intercambiables, dentro del mismo día.

Pesar los alimentos en crudo y en limpio, siempre que sea posible. Ej: acelga, después de limpiar pero antes de cocinar.

Respetar las cantidades especificadas en la dieta.

Carne magra: retirar la grasa visible antes de cocinar, ya que es mucho más fácil e higiénico. En el caso del jamón serrano, antes de comer.

Si consume nata, margarina, mantequilla o mayonesa (5 g – 1cucharilla), restar los 5 g de aceite de oliva

Si consume nueces, almendras o avellanas (8 g), restar los 5 g de aceite de oliva

El consumo de embutidos, charcutería, carnes grasas (costillas, patas de cerdo, morcilla, vísceras…), así como de fritos, rebozados y productos conservados en aceite no está permitido. Desgrasar los caldos. Hacer los guisos y estofados sin aceite adicional.

Utilizar los siguientes modos de cocción: se aconseja el hervido, asado, plancha, parrilla, papillote, cocina al vapor, microondas y estofados sin grasa añadida.

Evite el consumo de alcohol en la toma de la comida al medio día.

Consuma como bebida, principalmente, agua, cerveza sin alcohol, gaseosa y refrescos *light*.

Appendix 2: participants written informed consent-SENIFOOD study

HOJA DE INFORMACIÓN AL VOLUNTARIO

Esta hoja informativa le invita a participar en el ESTUDIO: Cambios en la composición corporal de personas mayores con sobrepeso-obesidad tras la ingesta de un extracto de romero incluido dentro de una dieta hipocalórica, equilibrada y controlada.

Se trata de un estudio de intervención nutricional que se llevará a cabo en la Unidad Metabólica del Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra. En este estudio se incluirán alrededor de 76 voluntarios como usted. Este estudio forma parte de un proyecto de investigación en alimentación muy amplio llamado CENIT (Investigación industrial de dietas y alimentos con características específicas – Acrónimo: SENIFOOD). El estudio está financiado por la empresa NATUREX S.A. como promotor.

Es importante que lea y comprenda en su totalidad la información que se le facilita en este documento. Si usted no entendiera alguna parte del documento, debe preguntar al médico que le ha entregado esta información antes de firmar el formulario de Consentimiento Informado.

Su participación en este estudio es totalmente voluntaria y depende de su elección.

¿Cuál es el objetivo del estudio?

Investigar el posible efecto beneficioso de un extracto vegetal, dentro de una dieta hipocalórica y equilibrada, sobre el estado nutritivo de personas entre 55 y 80 años, cuyo consumo podría resultar beneficioso.

¿Cuáles son los procedimientos del estudio?

Para participar en este proyecto usted tiene que estar de acuerdo en acudir a la Unidad Metabólica del Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra en 6 ocasiones.

En la primera cita se le hace entrega de la presente hoja informativa para que usted la lea y pregunte dudas sobre el proyecto. A continuación se le hace entrega de la hoja de consentimiento informado, por duplicado y aprobado por el Comité de Ética de la Investigación de la Universidad de Navarra, para que muestre su conformidad. Si usted acepta participar en el estudio, la médico del equipo le realizará una breve entrevista y una historia clínica con exploración física. En caso de que usted no haya presentado una analítica reciente (últimos 3 meses), la enfermera procederá a la extracción de una muestra de sangre. Para ello, será necesario recoger la sangre en 1 tubo de 4 ml. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio.

La nutricionista-dietista se ocupará de hacer una breve entrevista sobre los alimentos que usted consume habitualmente y sobre sus hábitos de vida. También le entregará y explicará algunos cuestionarios que tendrá que cumplimentar en casa y entregar en la próxima cita:

1) Un registro de pesada de 72 horas: usted tendrá que pesar todos los alimentos que tome a lo largo de 3 días y registrar los valores en el cuestionario dado.

2) Un cuestionario sobre el estado de hambre-saciedad: usted tendrá que contestar en una comida (el día le indicaremos nosotros) a unas preguntas sobre hambre-saciedad (tiempo previsto para contestación – un minuto), justo antes de empezar la comida al igual que 1 hora, 2 horas y 3 horas después de comer.

3) Un registro del número de pasos al día: usted llevará un contador de pasos (podómetro) durante 7 días consecutivos y al final del día, registrará el número de pasos en el cuaderno facilitado.

Para ello, se le entregará una balanza para pesar los alimentos, un podómetro para medir los pasos y una hoja con todas las instrucciones necesarias.

Este estudio tiene una duración total de 8 semanas consecutivas durante las cuales deberá seguir la dieta pautada y consumir 3 cápsulas al día, inmediatamente antes de las tomas principales (desayuno, comida y cena). Las cápsulas contendrán el extracto vegetal en estudio (334 mg) o un placebo, que le será asignado de manera aleatoria.

En el caso de que usted cumpla los criterios de inclusión, se le seleccionará para la primera visita del estudio (Día 0) y se le tomarán medidas de peso, talla, pliegues cutáneos, circunferencia de la cintura y cadera y de composición corporal. A continuación la enfermera le tomará la presión arterial y le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina relacionados con el metabolismo glucídico, lipídico y proteico y otros análisis relacionados al estudio. Para ello será necesario recoger la sangre en 8 tubos de bioquímica, 4 ml en cada tubo. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio.

Pasada esta primera visita se realizará un seguimiento quincenal –Visita 2 (día 15), Visita 3 (día 30), Visita 4 (día 45)-, al que deberá acudir a nuestras instalaciones para realizar una breve entrevista de seguimiento y pesarle. Esta visita le llevará no más de 30 minutos. Asimismo, durante el seguimiento, el participante tendrá que rellenar cuestionarios de ingesta dietética, del estado de hambre-saciedad y del registro de pasos entre la cuarta y quinta semanas del estudio, así como en la última semana.

La última visita (Visita 5) se realizará el día 56, en el que le realizaremos las mismas medidas que en la primera visita (día 0).

¿Qué debe hacer si quiere participar en este estudio?

Su participación es voluntaria y no remunerada. Si quiere participar en el estudio debe saber que puede retirarse del estudio cuando quiera sin tener que dar ningún tipo de explicación. En ningún caso afectará a su atención médica posterior.

Para participar en este estudio deberá usted firmar el consentimiento informado y una vez evaluemos su analítica y confirmemos que no existe ningún impedimento para su participación, le llamaremos al teléfono que nos haya facilitado para citarle y comenzar las 8 semanas de estudio.

Posibles problemas para su salud

El producto en estudio ha pasado absolutamente todos los controles sanitarios oportunos. Asimismo, el hecho de participar en el estudio y consumir el extracto vegetal puede implicar alguna molestia digestiva, además de una reducción modesta de los niveles de glucosa (azúcar) e insulina en sangre.

Durante la extracción de sangre puede sentir alguna molestia y pueden aparecer hematomas en la zona del pinchazo, y excepcionalmente puede sufrir mareo. Ninguna otra prueba de las que le realizaremos implica un riesgo para su salud.

Seguro

Todos los estudios de intervención nutricional, llevados a cabo en la Unidad Metabólica del Dpto. de Ciencias de la de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra, respecto a la responsabilidad profesional de su investigación científica, están aseguradas por la póliza ICT nº 457978. Además existe otro seguro gestionado por el promotor del estudio.

Confidencialidad de los datos

Toda la información que nos proporcione así como los resultados de los análisis de sangre se tratará según la Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal, utilizando códigos para asegurar la confidencialidad y garantizar el anonimato. Sólo los miembros del equipo investigador encargados de contactar con usted para cualquier evento relacionado con el estudio conocerán sus datos personales. El resto de miembros del equipo trabajarán con códigos, ignorando a qué voluntario le corresponde cada

código. El Comité de Ética de la Investigación de la Universidad de Navarra ha revisado los objetivos y procedimientos del estudio y ha dado la aprobación favorable para su realización.

Formulario de consentimiento (COPIA 1)

Cambios en la composición corporal de personas mayores con sobrepeso-obesidad tras la ingesta de un extracto de romero incluido dentro de una dieta hipocalórica, equilibrada y controlada.

Yo (nombre y apellidos) .....

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He hablado con: (nombre del investigador)..... .....

Entiendo que mi participación es voluntaria.

Entiendo que puedo retirarme del estudio:

1. Cuando quiera.
2. Sin tener que dar explicaciones.

Presto libremente mi conformidad para participar en el estudio.

Fecha Firma del participante

Fecha Firma del investigador

Appendix 3: an example of a control diet (1500 Kcal)

DIETA

TOTAL DE ACEITE EN EL DÍA: 35g.
(Por cada 5g. de margarina, mantequilla o mayonesa, restar 5g. de aceite.)

DESAYUNO:

- Fruta: 175g. fresas, 125g. kiwi, 150g. mandarina, 1250g. manzana, 250g. melón, 175g. naranja, 150g. pera, 125g. piña, 75g. plátano, 350g. sandía, 125g. ciruela, 175g. melocotón, 150g. albaricoque…
- 250ml. de leche semidesnatada con café/té (opcional) o 2 yogures desnatados.
- 1 cucharilla de postre de mermelada light (opcional).

+ elegir entre:
- 1 rebanada de pan (30g.) o 2 biscotes (20g.)
- 3 galletas tipo María (15g.)
- 30g. cereales.

COMIDA:

1er plato:
- 200g. de verdura en crudo (240g. cocida): acelgas, alcachofas, berenjenas, borraja, brócoli, cardo, champiñones, setas, coliflor, espinacas, judías verdes, nabos, puerros, remolacha, tomate, zanahoria…
- 1 rebanada de pan (30g.) o 2 biscotes (20g.)

+ elegir entre:
- 40g. de pasta en crudo (100g. en cocinado).
- 40g. de arroz en crudo (90g. en cocinado).
- 160g. de patata en crudo (200g. cocinada).
- 60g. de legumbre (160g. cocinada): lentejas, garbanzos, alubias… → **Sin 2º plato.**
- 200g. de guisantes (460g. cocinados) → **Sin 2º plato.**

2º plato, elegir entre:
- 30g. de carne magra en crudo (25g. en cocinado): ternera, pollo, pavo, cerdo, conejo…
- 40g. de pescado en crudo (35g. en cocinado): bacaladilla, bacalao, besugo, congrio, dorada, gallo, lenguado, lubina, merluza, mero, pescadilla, pez espada, rape, rodaballo, trucha, calamares, sepia, gambas o langostinos, pulpo, bonito, atún, sardinas, salmón, anchoas frescas…
- 30g. de jamón serrano magro, pavo o jamón york.
- 60g. queso fresco desnatado o 40g. de quesitos desnatados o de requesón.

Postre:
- 1 yogur desnatado o ½ vaso de leche semidesnatada (125ml.).
- Fruta: 175g. fresas, 125g. kiwi, 150g. mandarina, 125g. manzana, 250g. melón, 175g. naranja, 150g. pera, 125g. piña, 75g. plátano, 350g. sandía, 125g. ciruela, 175g. melocotón, 150g. albaricoque…

CENA:

1er plato:
- 200g. de verdura cruda en ensalada.
- 1 rebanada de pan (30g.) o 2 biscotes (20g.)

2º plato, elegir entre:
- 80g. carne en crudo (65g. en cocinado): magra de ternera, pollo, pechuga de pollo, pavo, cerdo magro, conejo…
- 105g. de pescado en crudo (90g. en cocinado): bacaladilla, bacalao, besugo, congrio, dorada, gallo, lenguado, lubina, merluza, mero, pescadilla, pez espada, rape, rodaballo, trucha, calamares, sepia, gambas o langostinos, pulpo, bonito, atún, sardinas, salmón, anchoas frescas…
- 80g. de jamón serrano magro, pavo o jamón york.
- 160g. de queso fresco desnatado o 105g. de quesitos desnatados o de requesón.
- 1 huevo duro o una tortilla de 1 huevo con 30g de jamón serrano, pavo o jamón york o con 2 quesitos desnatados o con 40g. de atún o de gambas.

Postre:
- 1 yogur desnatado o ½ vaso de leche semidesnatada (125ml.)
- Fruta: 175g. fresas, 125g. kiwi, 150g. mandarina, 125g. manzana, 250g. melón, 175g. naranja, 150g. pera, 125g. piña, 75g. plátano, 350g. sandía, 125g. ciruela, 175g. melocotón, 150g. albaricoque…

VERDURAS DE CONSUMO LIBRE:

Achicoria, apio, berro, calabacín, cebolla, endibias, escarola, espárragos, lechuga, pepino, pimientos, rábano.

RECOMENDACIONES GENERALES:

- Realizar todas las tomas a lo largo del día.
- Utilizar los siguientes modos de cocción: hervido, asado, plancha, parrilla, papillote, cocina al vapor, microondas y estofados sin grasa añadida.
- Respetar las cantidades especificadas en la dieta, en especial frutas y grasas. Durante los primeros días conviene pesar los alimentos para hacerse una idea de lo que puede comer.
- Consuma como bebida, principalmente, agua. De modo ocasional puede elegir también cerveza sin alcohol, gaseosa y refrescos lights.
- Evite el consumo de alcohol. Vino tinto (250cc) los fines de semana.
- Puede tomar infusiones sin azúcar (con sacarina) siempre que quiera.

Appendix 4: an example of a RESMENA diet (1500 Kcal)

LUNES

ACEITE TOTAL PARA TODO EL DÍA: 12g. (de oliva virgen extra).

DESAYUNO

- 175g de naranja (unidad mediana).

Elegir entre:

- 1 vaso de leche semidesnatada (240g) con té o café.
- 2 yogures desnatados.
- 1 yogur desnatado (125g) y ½ vaso de leche (125g) semidesnatada con té o café.

MEDIA MAÑANA I

- 4 lonchas de jamón serrano, pavo o jamón york (60g) o quesitos desnatados (80g).
- 1 biscote de pan tostado integral (10g).

MEDIA MAÑANA II

- 125g manzana con piel (1 unidad pequeña).

COMIDA

- 1 biscote de pan tostado integral (10g).
- 200g de verdura cruda o bien 240g cocinada: coles de Bruselas, acelgas, alcachofas, berenjenas, borraja, brócoli, calabaza, cardo, champiñones/setas, col lombarda, col rizada, coliflor, espinacas, judías verdes, puerros, remolacha, tomate, zanahoria…
- 40g de pasta integral (100g cocida).

2º plato elegir entre:

- 200g de pescado en crudo (170g cocinado): bacaladilla, bacalao, besugo, congrio, dorada, gallo, lenguado, lubina, merluza, mero, pescadilla, pez espada, rape, rodaballo, trucha, calamares, sepia, gambas o langostinos, pulpo, bonito, atún, sardinas, salmón, anchoas frescas…
- 150g en crudo de carne magra de cerdo (120g cocinada), conejo, pavo, pollo, ternera, jamón serrano, jamón york.

O elegir entre:

- 150g de queso fresco desnatado.
- 100g de quesitos desnatados o requesón.

+ 100g de pescado o 75g de carne (pesado en crudo).

Especias para aliñar: clavo, orégano, jengibre, canela, albahaca, mostaza, perejil, pimienta….

Postre: 125g kiwi (1 unidad pequeña).

MERIENDA I

- 1 yogur desnatado o ½ vaso de leche semidesnatada (125g) con té o café.
- 10g nueces.

MERIENDA II

- 4 lonchas de jamón serrano, pavo o jamón york (60g) o quesitos desnatados (80g).
- 1 biscote de pan tostado integral (10g).

CENA

- 1 biscote de pan tostado integral (10g).
- 200g de verdura cruda en ensalada.
  Especias para aliñar: orégano, albahaca, mostaza, perejil, pimienta….
- Tortilla de 2 huevos (o 2 huevos cocidos) con 40g de quesitos desnatados o 30g de jamón serrano, pavo o jamón york o 40g de atún o gambas.

Postre: 1 yogur desnatado o ½ vaso de leche semidesnatada (125g).

VERDURAS DE CONSUMO LIBRE:

Achicoria, apio, berro, calabacín, cebolla, endibias, escarola, espárragos frescos, lechuga, pepino, pimientos, rábanos.

RECOMENDACIONES GENERALES:

- Realizar todas las tomas a lo largo del día.
- Utilizar como única grasa aceite de oliva virgen extra.
- Utilizar los siguientes modos de cocción: hervido, asado, plancha, parrilla, papillote, cocina al vapor, microondas y estofados sin grasa añadida.
- Respetar las cantidades especificadas en la dieta.
- Sustituya el azúcar por edulcorantes no calóricos (sacarina, aspartamo…).
- Evite el consumo de patata.
- Utilice condimentos y especias como: pimienta, mostaza, ajo, vinagre, limón, canela, azafrán. perejil, tomillo, orégano, hinojo, etc. Le darán un mejor sabor a sus platos sin tener que utilizar tanta sal, además de aportarle sus grandes beneficios antioxidantes
- Evite el consumo de alcohol. A excepción de vino tinto (250cc) los fines de semana (opcional).
- Consuma como bebida principalmente agua. Como alternativas elige cerveza sin alcohol, gaseosa y refrescos lights.
- Consuma un vaso de agua del tiempo (no del frigorífico) antes de cada comida principal.
- Puede tomar infusiones sin azúcar (con sacarina) siempre que quiera.

Appendix 5: participants written informed consent-RESMENA study

HOJA INFORMATIVA PARA EL PARTICIPANTE

TÍTULO: Proyecto de intervención nutricional para pacientes con síndrome metabólico

RESPONSABLE DEL ESTUDIO: Marian Zulet Alzórriz

Esta hoja informativa le invita a participar de forma totalmente voluntaria en un proyecto sobre intervención nutricional para pacientes con síndrome metabólico.

El objetivo global del estudio es contribuir a la mejora de su salud siguiendo una dieta saludable, sin recibir ningún tipo de producto dietético adicional.

El estudio se llevará a cabo en la Unidad de intervención nutricional del departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra, y será atendido por un equipo integrado por una enfermera, una médico-dietista, una dietista-nutricionista.

En esta investigación se van a ensayar dos tipos de dietas personalizadas, una dieta control que cumple con las normas establecidas por la Asociación Americana del Corazón (AHA), o una dieta variada y saludable confeccionada a base de alimentos tradicionales, como el consumo de alimentos propios de la dieta mediterránea. La asignación a un grupo dietético u otro se realizará de modo aleatorio.

En esta primera cita, de aproximadamente media hora de duración, se le hace entrega de esta hoja informativa para que usted la lea y pregunte sus posibles dudas sobre el proyecto. A continuación se le hace entrega de la hoja de consentimiento informado, por duplicado y aprobado por el Comité de ética de la investigación de la Universidad de Navarra, para que muestre su conformidad. El estudio comenzará con una breve historia clínica con exploración física llevada a cabo por una Licenciada en Medicina. En tal caso, la enfermera procederá a la extracción de una muestra de sangre. Este procedimiento puede conllevar algunas molestias para usted como ligera molestia en la zona de punción o presencia posterior de hematoma en esta misma zona y en casos excepcionales lipotimias. La finalidad de tomar estas muestras es llevar a cabo análisis bioquímicos de rutina relacionados con el colesterol, la glucosa, y las proteínas para comprobar que usted cumple todos los criterios establecidos para formar parte de este estudio.

En el caso de que usted cumpla los criterios de inclusión, se le citará para una sesión de grupo en la que se le dará las pautas generales para llevar a cabo la dieta, de una hora de duración aproximadamente. En esta cita, la Dietista le realizará la Historia dietética haciéndole entrega de un cuestionario de hábitos de vida (SUN) y un registro de pesada de 48 horas con las aclaraciones correspondientes acerca de cómo se deben cumplimentar y errores frecuentes que se cometen a la hora de rellenarlos. Igualmente, recibirá citación para comenzar con el estudio de intervención (día 0) de 60 días de duración y se le proporcionará un bote de orina estéril para que recoja orina de primera hora de la mañana el día citado.

En la cita del día 0 se le volverá a recordar en qué consiste su participación y se le tomarán medidas de peso, talla, perímetro cintura y de composición corporal. Al mismo tiempo se le realizará una serie de preguntas relacionadas con el estado anímico y el grado de ansiedad. A continuación se le tomará la tensión arterial y la enfermera le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina (colesterol, glucosa, etc) y otros más específicos de relacionados con síndrome metabólico, entre ellos un análisis de la expresión de

determinados genes. La dietista le proporcionará su dieta personalizada para comenzar el estudio de intervención nutricional. Deberá ajustarse a las pautas que se le establezcan, como la ingesta de alimentos, forma de preparación y a las recomendaciones de estilo de vida.

Durante el estudio, acudirá quincenalmente (días 15, 30 y 45) para comprobar el seguimiento de la dieta, reforzar el cumplimiento de la dieta y resolver las dudas que se le vayan planteando. Además, se le controlará el peso y composición corporal, el apetito y el estado de ánimo y ansiedad; con una duración aproximada de media hora. El día 45 se le proporcionará un bote de orina estéril para que recoja orina de primera hora de la mañana del último día de intervención nutricional (día 60). Además de, un registro de pesada de 48 horas y un test de personalidad para su entrega el último día de intervención (día 60).

El estudio de intervención concluirá tras 2 meses con la valoración de la composición corporal, historia dietética, estado anímico y grado de ansiedad y con la extracción de una muestra de sangre.

El estudio continuará con un periodo de autonomía durante 4 meses más. Durante este tiempo usted no recibirá asesoramiento, pero deberá aplicar lo aprendido previamente.

Al finalizar estos 4 meses se le dará cita para acudir a la Universidad de Navarra y que se le evalúe de nuevo su estado nutricional, en una entrevista de una hora aproximadamente. Tras el procesamiento de los datos, se le informará de los resultados de las pruebas realizadas y se mantendrá la confidencialidad propia de todo procedimiento médico.

Toda la información que nos proporcione así como los resultados de los análisis de sangre se tratarán según la Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal, utilizando códigos para asegurar la confidencialidad y garantizar el anonimato. Sólo dos miembros del equipo investigador conocerán sus datos personales, ya que serán los encargados de contactar con usted para cualquier evento relacionado con el estudio. El resto de miembros del equipo trabajarán con códigos, ignorando a qué voluntario le corresponde cada código. Usted puede abandonar el estudio en cualquier momento, sin dar explicaciones y sin que esto repercuta en su asistencia médica.

SU PARTICIPACIÓN EN EL ESTUDIO NO ESTÁ REMUNERADA.

Formulario de consentimiento (COPIA 1)

REducción de Síndrome MEtabólico en NAvarra-Spain (RESMENA-S) mediante una estrategia multidisciplinar e innovadora, basada en la crononutrición y la educación nutricional, junto con control dietético y psicológico.

Yo (nombre y apellidos)

- He leído la hoja de información que se me ha entregado.
- He podido hacer preguntas sobre el estudio.
- He recibido suficiente información sobre el estudio.
- He hablado con: (nombre del investigador)

Entiendo que mi participación es voluntaria.

Entiendo que puedo retirarme del estudio:

1. Cuando quiera.
2. Sin tener que dar explicaciones.
3. Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

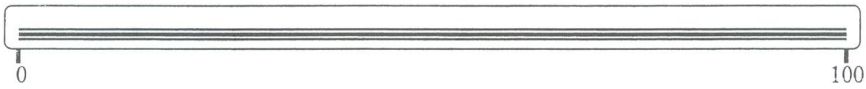
Fecha Firma del participante

Fecha Firma del investigador

Appendix 6: thermometer visual analogue scale

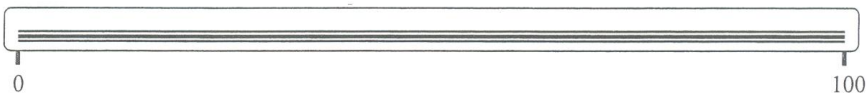
TERMÓMETRO DE LA ANSIEDAD

Valore su grado de ansiedad en la última semana en esta escala, considerando que el "0" es la ausencia total de ansiedad y "100" es el punto máximo que haya experimentado nunca. Señale lo correspondiente con una X.



TERMÓMETRO DEL ESTADO DE ÁNIMO

Valore su estado de ánimo en la última semana en esta escala, considerando que el "0" es el más bajo estado de ánimo que haya experimentado en su vida y "100" es el mejor estado de ánimo que haya experimentado. Señale lo correspondiente con una X.



Appendix 7: Beck Depression Inventory

BDI. INVENTARIO DE BECK

1.
_____0. No me siento triste.
_____1. Me siento triste.
_____2. Me siento triste continuamente y no puedo dejar de estarlo.
_____3. Me siento tan triste o desgraciado que no puedo soportarlo.

2.
_____0. No me siento especialmente desanimado de cara al futuro.
_____1. Me siento desanimado de cara al futuro.
_____2. siento que no hay nada por lo que luchar.
_____3. El futuro es desesperanzador y las cosas no mejorarán.

3.
_____0. No me siento fracasado.
_____1. he fracasado más que la mayoría de las personas.
_____2. Cuando miro hacia atrás lo único que veo es un fracaso tras otro.
_____3. Soy un fracaso total como persona.

4.
_____0. Las cosas me satisfacen tanto como antes.
_____1. No disfruto de las cosas tanto como antes.
_____2. Ya no obtengo ninguna satisfacción de las cosas.
_____3. Estoy insatisfecho o aburrido con respecto a todo.

5.
_____0. No me siento especialmente culpable.
_____1. Me siento culpable en bastantes ocasiones.
_____2. Me siento culpable en la mayoría de las ocasiones.
_____3. Me siento culpable constantemente.

6.
_____0. No creo que esté siendo castigado.
_____1. siento que quizás esté siendo castigado.
_____2. Espero ser castigado.
_____3. Siento que estoy siendo castigado.

7.
_____0. No estoy descontento de mí mismo.
_____1. Estoy descontento de mí mismo.
_____2. Estoy a disgusto conmigo mismo.
_____3. Me detesto.

8.

_____0. No me considero peor que cualquier otro.
_____1. me autocritico por mi debilidad o por mis errores.
_____2. Continuamente me culpo por mis faltas.
_____3. Me culpo por todo lo malo que sucede.

9.

_____0. No tengo ningún pensamiento de suicidio.
_____1. A veces pienso en suicidarme, pero no lo haré.
_____2. Desearía poner fin a mi vida.
_____3. me suicidaría si tuviese oportunidad.

10.

_____0. No lloro más de lo normal.
_____1. ahora lloro más que antes.
_____2. Lloro continuamente.
_____3. No puedo dejar de llorar aunque me lo proponga.

11.

_____0. No estoy especialmente irritado.
_____1. me molesto o irrito más fácilmente que antes.
_____2. me siento irritado continuamente.
_____3. Ahora no me irritan en absoluto cosas que antes me molestaban.

12.

_____0. No he perdido el interés por los demás.
_____1. Estoy menos interesado en los demás que antes.
_____2. He perdido gran parte del interés por los demás.
_____3. He perdido todo interés por los demás.

13.

_____0. tomo mis propias decisiones igual que antes.
_____1. Evito tomar decisiones más que antes.
_____2. Tomar decisiones me resulta mucho más difícil que antes.
_____3. Me es imposible tomar decisiones.

14.

_____0. No creo tener peor aspecto que antes
_____1. Estoy preocupado porque parezco envejecido y poco atractivo.
_____2. Noto cambios constantes en mi aspecto físico que me hacen parecer poco atractivo.
_____3. Creo que tengo un aspecto horrible.

15.

_____0. Trabajo igual que antes.
_____1. Me cuesta más esfuerzo de lo habitual comenzar a hacer algo.
_____2. Tengo que obligarme a mí mismo para hacer algo.
_____3. Soy incapaz de llevar a cabo ninguna tarea.

16.

_____0. Duermo tan bien como siempre.
_____1. No duermo tan bien como antes.
_____2. Me despierto una o dos horas antes de lo habitual y ya no puedo volver a dormirme.
_____3. Me despierto varias horas antes de lo habitual y ya no puedo volver a dormirme.

17.

_____0. No me siento más cansado de lo normal.
_____1. Me canso más que antes.
_____2. Me canso en cuanto hago cualquier cosa.
_____3. Estoy demasiado cansado para hacer nada.

18.

_____0. Mi apetito no ha disminuido.
_____1. No tengo tan buen apetito como antes.
_____2. Ahora tengo mucho menos apetito.
_____3. he perdido completamente el apetito.

19.

_____0. No he perdido peso últimamente.
_____1. He perdido más de 2 kilos.
_____2. He perdido más de 4 kilos.
_____3. He perdido más de 7 kilos.

20.

_____0. No estoy preocupado por mi salud
_____1. Me preocupan los problemas físicos como dolores, malestar de estómago, catarros, etc.
_____2. Me preocupan las enfermedades y me resulta difícil pensar en otras cosas.
_____3. Estoy tan preocupado por las enfermedades que soy incapaz de pensar en otras cosas.

21.

_____0. No he observado ningún cambio en mi interés por el sexo.
_____1. La relación sexual me atrae menos que antes.
_____2. Estoy mucho menos interesado por el sexo que antes.
_____3. He perdido totalmente el interés sexual.

Appendix 8: State Trait Anxiety Inventory

N.º 124

STAI

A / E | PD = 30 + − =
A / R | PD = 21 + − =

AUTOEVALUACION A (E/R)

Apellidos y nombre ..... *Edad* ..... *Sexo* ..... V ó M

Centro ..... *Curso/Puesto* ..... *Estado civil* .....

Otros datos ..... *Fecha* .....

A-E

INSTRUCCIONES

A continuación encontrará unas frases que se utilizan corrientemente para describirse uno a sí mismo. Lea cada frase y señale la puntuación 0 a 3 que indique mejor cómo se *SIENTE* Vd. *AHORA MISMO*, en este momento. No hay respuestas buenas ni malas. No emplee demasiado tiempo en cada frase y conteste señalando la respuesta que mejor describa su situación presente.

		Nada	Algo	Bastante	Mucho
1.	Me siento calmado	0	1	2	3
2.	Me siento seguro	0	1	2	3
3.	Estoy tenso	0	1	2	3
4.	Estoy contrariado	0	1	2	3
5.	Me siento cómodo (estoy a gusto)	0	1	2	3
6.	Me siento alterado	0	1	2	3
7.	Estoy preocupado ahora por posibles desgracias futuras	0	1	2	3
8.	Me siento descansado	0	1	2	3
9.	Me siento angustiado	0	1	2	3
10.	Me siento confortable	0	1	2	3
11.	Tengo confianza en mí mismo	0	1	2	3
12.	Me siento nervioso	0	1	2	3
13.	Estoy desasosegado	0	1	2	3
14.	Me siento muy «atado» (como oprimido)	0	1	2	3
15.	Estoy relajado	0	1	2	3
16.	Me siento satisfecho	0	1	2	3
17.	Estoy preocupado	0	1	2	3
18.	Me siento aturdido y sobreexcitado	0	1	2	3
19.	Me siento alegre	0	1	2	3
20.	En este momento me siento bien	0	1	2	3

COMPRUEBE SI HA CONTESTADO A TODAS LAS FRASES CON UNA SOLA RESPUESTA

Ahora, vuelva la hoja y lea las Instrucciones antes de comenzar a contestar a las frases.

Appendix 9: congress communications

Oral communication

20th International Congress of Nutrition (IUNS)

Ann Nutr Metab. 2013; 63(1): 173; Granada (Spain), 15th-20th Sept 2013

Association between improvement in anxiety symptoms and weight loss in subjects with metabolic syndrome

Perez-Cornago A.[1], de la Iglesia R.[1], López-Legarrea P.[1], Abete I.[1], Navas-Carretero S.[1,2], Lacunza C.S.[3], Lahortiga F.[3], Martínez J.A.[1,2], Zulet M.A.[1,2]

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[2]CIBER Fisiopatología Obesidad y Nutrición (CIBERObn), Instituto de Salud Carlos III, Spain.

[3]Department of Psychiatry and Clinical Psychology, University Clinic of Navarra, Pamplona, Spain.

Background and objectives: Metabolic syndrome is a combination of medical disorders whose prevalence is increasing worldwide, leading to an increased incidence of CVD. An association between anxiety disorder and metabolic syndrome has recently been reported, however, the precise interactive pathways between these diseases still remains unclear. This study examined the effects of a hypocaloric treatment to reduce metabolic syndrome features on anxiety status.

Methods: A total of sixty-two non-demented males (n=39) and females (n=23) subjects with metabolic syndrome according to IDF criteria were enrolled in a randomized controlled clinical trial (Age:50±10 y; BMI:36.1±4.3 kg/m^2) . Subjects followed two hypocaloric diets (control diet and RESMENA diet) with the same energy restriction (-30% TCV) for six months. The 20-items State-Trait Anxiety Inventory (STAI) was used to measure anxiety symptoms. STAI questionnaire as well as anthropometric and biochemical variables were analysed at the beginning and at the end of the intervention.

Results: The subjects mean weight loss was 8.5±5.0 kg. Anxiety symptoms decreased during the weight loss intervention (ΔSTAI: -14.7±27.0) with no differences between dietary groups (p>0.10). Moreover, it was found that those subjects losing more weight during the dietary intervention showed a greater decline in anxiety symptoms (ΔSTAI: -22.6±24.8) than those who lost less weight (ΔSTAI: -7.5±27.2) (p = 0.024).

Conclusions: This study demonstrated that an effective hypocaloric diet designed to reduce metabolic syndrome features, also improved anxiety symptoms, being this improvement higher in those subjects losing more weight during the weight loss intervention.

Keywords: anxiety symptoms, metabolic syndrome, weight loss

Poster

XVI Congress of the Spanish Nutrition Society (SEÑ)

Nutr Hosp. 2014;30(1):34; Pamplona (Spain), 3rd-5th Jul 2014

Ausencia de asociación entre BDNF y cortisol plasmáticos con síntomas depresivos y de ansiedad en pacientes con síndrome metabólico tras la pérdida de peso

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Poster

21st European Congress on Obesity (ECO)

Obesity Facts. 2014;7(1):70; Sofia (Bulgaria), 28th -31st May 2014

Longitudinal changes in peripheral neurotransmitters levels after dietary restriction in subjects suffering from metabolic syndrome

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Introduction: Reduced circulating neurotransmitters may have a role in the development of the metabolic syndrome (MetS), which is becoming a major health problem worldwide. This investigation aimed to examine the effect of a weight loss intervention on peripheral neurotransmitters levels in subjects with MetS.

Methods: The study population encompassed women (n=25) and men (n=37) with MetS according to IDF criteria (Age:50±10 y; BMI:35.8±4.3 kg/m^2) selected from the RESMENA study after they completed the 6-months weight loss intervention (-30% of daily energy requirements). Anthropometric parameters, dietary records, as well as blood neurotransmitters levels (dopamine, serotonin and noradrenaline) determined by HPLC with fluorescence detection, were analysed before and after the 6-month-long study.

Results: Dopamine (+18.2%; p=0.046) and serotonin (+16.1%; p=0.020) blood levels significantly increased at the end of the study, while no changes in peripheral noradrenaline levels were found. Higher dopamine blood concentrations at the end of the study were inversely related with the carbohydrate intake during the intervention (B=-3.26; p=0.045). Moreover, those subjects presenting higher serotonin levels after the weight loss intervention also showed a lower caloric intake during the treatment (B=-0.04; p=0.025).

Conclusion: This study evidenced an increase in serotonin and dopamine blood levels after the weight loss treatment in patients with MetS, being this increase related with lower caloric and carbohydrate intakes, respectively.

1. Conflict of Interest: None.

2. Funding: Research related to this abstract was funded by the Health Department of the Government of Navarra (48/2009), the Linea Especial about Nutrition, Obesity and Health, CIBERobn and RETICS.

Poster

12th International Congress of Obesity (ICO)

Obes Rev. 2014: 15(2): 119; Kuala Lumpur (Malaysia), 17th-20th Mar 2014

A longitudinal assessment of oxidative stress and anxiety symptoms in subjects with metabolic syndrome following a weight loss intervention

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Poster

World Forum for Nutrition Research Conference

Ann Nutr Metab. 2013; 62(2): 83; Reus (Spain), 20th-21st May 2013

Association between weight loss and fatty acids profile in overweight elderly subjects: a metabolomic analysis

Perez-Cornago A.[1], Brennan L.[2], Ibero-Baraibar I.[1], Hermsdorff H.H.[1], O Gormans A.[2], Zulet M.A.[1,3], Martinez J.A.[1,3]

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Background and objectives: Obesity is defined as an excessive fat accumulation which prevalence is increasing worldwide. The application of metabolomics to nutritional research is growing due to its capacity to quantify and identify small molecules present in the biological system. The aim of the present study was to examine changes produced in the fatty acids profile, non-esterified fatty acids (NEFA) and fatty acid methyl esters (FAME), after a weight loss intervention in overweight elderly subjects.

Methods: A total of nineteen overweight elderly volunteers participated in a controlled clinical trial (Age:60±1 y; BMI:29.7±0.47 kg/m^2) for 8 weeks. Subjects followed a personalized and hypocaloric diet (-15% energy of the studied requirements) with a macronutrient distribution of 45% total caloric value from carbohydrates, 30% lipids and 25% proteins. Anthropometric and biochemical variables were analysed at the beginning and at the end of the intervention. Levels of FAME in serum were determined by gas chromatography-mass spectrometry (GC-MS).

Results: After 8 weeks of weight loss intervention subjects mean weight loss was 5.4±0.6 kg. Levels of NEFA in serum decreased during the weight loss intervention without reaching significance ($p=0.071$). Analysis of FAME in serum revealed a significant decrease ($p<0.05$) in myristic acid (14:0), palmitic acid (16:0), linoleic acid (18:2n-6), oleic acid (18:1), stearic acid (18:0), arachidonic acid (20:4n-6), cis-8, 11, 14, 17–eicosatrienoic acid (20:3n-6), cis-11, 14–eicosadienoic acid (20:2), cis-11–eicosenoic acid (20:1), cis-4, 7, 10, 13, 16, 19-docosahexaenoic acid (22:6n-3) and lignoceric acid (24:0).

Conclusions: The weight loss treatment based on a hypocaloric diet reduced body weight and fatty acids levels in overweight elderly subjects. Indeed, GC-MS seems to be a potential technique to effectively detect concentrations of specific fatty acids in serum.

Keywords: metabolomics, fatty acids, weight loss, overweight, elderly.

Poster

20th European Congress on Obesity (ECO)

Obes Facts. 2013: 6(1): 179; Liverpool (UK), 12th-15th May 2013

Omega-3 fatty acids intake is associated with a decline in depressive symptoms in subjects with metabolic syndrome

Perez-Cornago A.[1], López-Legarrea P.[1], de la Iglesia R.[1], Abete I.[2], Navas-Carretero S.[1], Lacunza C.I.[3], Lahortiga F.[3], Zulet MA.[1] y Martínez J.A.[1]

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Introduction: Metabolic syndrome (MetS) and depression are both important public health problems. It has been demonstrated that MetS may be an important predisposing factor for the development of depression, but less is known about the impact of MetS dietary treatment on relieving depression. Mediterranean diet has been associated with reduced prevalence and incidence of both MetS and depressive symptoms. The aim of the present study was to examine the effects of an energy-restricted diet, with Mediterranean dietary components, for reducing metabolic syndrome on self-perceived depression.

Methods: A total of sixty-one male and female subjects (Age:49±10 y; BMI:36.1±4.3 kg/m^2) with metabolic syndrome (according to the IDF criteria) were rated for depressive symptoms using the Beck Depression Inventory (BDI). Subjects followed a hypocaloric diet (-30% energy restriction) with Mediterranean dietary components for six months. BDI questionnaire, adherence to the diet, anthropometric and biochemical variables were analysed.

Results: About 28% of the subjects presented self-perceived depression (BDI score≥10) at baseline (BDI score: 14.5±6.5). After six months of dietary treatment and with the subsequent weight loss (8.4±5.3 kg; p<0.001), only 7% of the subjects reported a BDI score≥10. The decline in depressive symptoms was significantly associated with a higher consumption of omega-3 fatty acids during the dietary treatment (adjusted R^2=0.078, p=0.022).

Conclusions: This study evidenced that an effective hypocaloric diet with Mediterranean dietary components, not only decreases body weight, but also improves self-perceived depression traits in subjects with MetS, being this improvement related with a higher consumption of omega-3 fatty acids.

Poster

XIV Congress of the Spanish Nutrition Society (SEÑ)

Nutr hosp. 2012;27(5):3-66; Zaragoza (Spain), 27-29 sept 2012

La restricción energética en pacientes con síndrome metabólico mejora síntomas depresivos

Perez-Cornago A.1, de la Iglesia R.1, López-Legarrea P.1, Abete I.1, Navas-Carretero S.1, Lacunza C.I.2, Lahortiga F.2, Zulet M.A.1, Martínez J.A.1

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