Universidad de Navarra
Facultad de Farmacia

EVALUATION OF A NEW DIETARY STRATEGY FOR THE TREATMENT OF OBESITY AND ASSOCIATED INFLAMMATION: ENDOCRINE AND EPIGENETIC MECHANISMS

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Discovery consists of seeing what everybody has seen and thinking what nobody has thought.

Albert Szent-Gyorgyi, Nobel Prize Winner for Medicine 1937

ABBREVIATIONS

AACE	American Association of Clinical Endocrinologists
ADA	American Diabetes Association
AHA	American Heart Association
ALADINO	*Alimentación, actividad física, desarrollo infantil y obesidad*
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adult treatment panel
AVENA	*Alimentación y valoración del estado nutricional en los adolescentes españoles*
BMI	Body mass index
CLYDIA	*Prevalencia del síndrome metabólico en pacientes con enfermedad cardiovascular en España*
CoA	Coenzyme A
CONSORT	Consolidated standards of reporting trials
CRP	C-reactive protein
CVD	Cardiovascular disease
DARIOS	Dyslipaemia, atherosclerosis risk and increased hsCRP and inflammatory and oxidative status in the Spanish population
DASH	Dietary approaches to stop hypertension
DHA	Docosahexaenoic acid
DiOGenes	Diet, obesity and genes
DM	Diabetes mellitus
DNMT	DNA methyltransferase

DXA	Dual-energy X-ray absorptiometry
EGIR	European group for the study of insulin resistance
ELISA	Enzyme-linked immunosorbent assay
EnKID	*Estudio de alimentación infantil y juvenil*
ENRICA	*Estudio de nutrición y riesgo cardiovascular*
EPA	Eicosapentaenoic acid
EPIC	European prospective investigation of cancer-Norfolk
EVASYON	*Desarrollo, aplicación y evaluación de la eficacia de un programa terapéutico para adolescentes con sobrepeso y obesidad: educación integral nutricional y de actividad física*
FAO	Food and Agriculture Organization
FNDC5	Fibronectin type III domain containing 5
GI	Glycemic index
GL	Glycemic load
HDL-c	High-density lipoprotein-cholesterol
HLBI	Heart, lung and blood institute
HOMA-IR	Homeostasis model assessment for insulin resistance
HT	Hypertension
IDF	International Diabetes Federation
IL-6	Interleukin-6
LDL-c	Low density lipoprotein-cholesterol
MADRIC	*Madrid riesgo cardiovascular*
MedDiet	Mediterranean diet

MESYAS	Metabolic syndrome in active subjects
MetS	Metabolic syndrome
NCEP	National cholesterol education program
OmniHeart	Optimal macronutrient intake trial to prevent heart disease
PAI-1	Plasminogen activator inhibitor type 1
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1 alpha
PREDIMED	*Prevención con dieta mediterránea*
PUFA	Polyunsaturated fatty acids
RESMENA	*Reducción del síndrome metabólico en Navarra*
SEEDO	*Sociedad española para el estudio de la obesidad*
SUN	*Seguimiento Universidad de Navarra*
SUVIMAX	*Supplementation on vitamines et mineraux antioxydants*
TAC	Total antioxidant capacity
TNF-α	Tumor necrosis factor-α
WBC	White blood cells
WHO	World Health Organization

INDEX

1. INTRODUCTION

1.1. Obesity

1.1.1. Etiology

Obesity is defined as an excessive body fat accumulation as a consequence of a chronic positive energy balance, which is often associated to detrimental effects for health (Brown 2012; Salas-Salvado *et al.* 2007). Excessive caloric intake due to important changes in food habits in the past decades, together with a decrease in physical activity level due to less physically demanding jobs and the increase of a sedentary lifestyle are important factors implicated in the onset and development of this disease (McAllister *et al.* 2009; Martinez 2000).

Nevertheless, numerous epidemiological studies have revealed the existence of several factors that contribute to increase the risk of suffering obesity, and which are responsible to determine the disparate prevalence rates found in different countries around the world. Sociocultural factors including education level, marital status and economic level or occupation have been widely reported as linked to obesity (Daviglus *et al.* 2012). Thus, this disease prevalence is higher among people with lower educational level, marriage is related with a rise in overweight and obesity is associated with low-income strata. Indeed, increases in Body Mass Index (BMI) have been related to a country income, being obesity rates among countries with medium-high incomes about 24% compared with those with medium-low incomes, which presented 7% prevalence (WHO 2010). In the same way, obesity rates have been observed to vary depending on demographic factors such as age or ethnicity (Daviglus *et al.* 2012) as evidenced in several studies from the United States where the Puerto Ricans showed higher obesity rates as compared with individuals with Cuban, Dominican, Mexican, Central or South American backgrounds (Daviglus *et al.* 2012).

In addition, biological factors, such as sex or the number of children show a relationship with weight gain (Hermsdorff *et al.* 2012). In every region included in the World Health Organisation (WHO) report from 2010, the prevalence of obesity was higher among women as compared with men rates (WHO 2010). Also behavioral and environmental agents, such as nutrition, tobacco (stop smoking trend to increase weight), alcohol (high intake of alcoholic beverages is associated with greater BMI values) and physical activity (people physically active trend to be leaner than those with

sedentary behaviors) have been observed to relate with the presence of overweight and obesity (Mesas *et al.* 2012). In this sense, several studies have reported that a higher fruit and vegetable availability is associated with lower mean values of BMI, as compared with disadvantaged populations that have difficulties to accede to this kind of products (McAllister *et al.* 2009; Casagrande *et al.* 2009). Moreover, recent data indicate that the total dietary fat is proportional to the country incomes (WHO 2010).

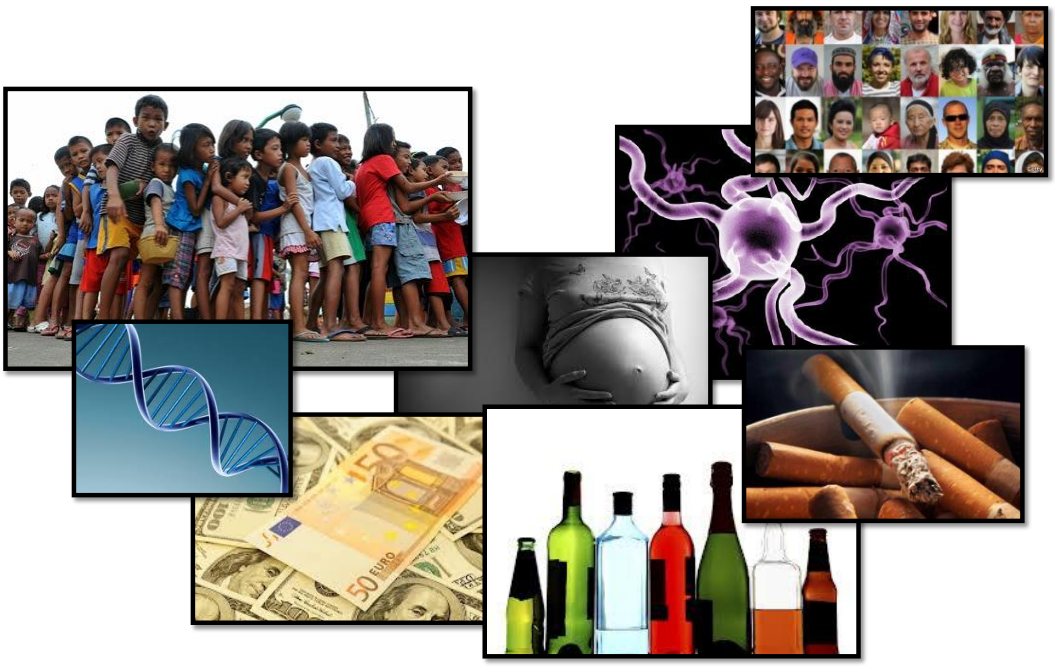

Figure 1. Obesity determinants.

On the other hand, endocrine disruptions (Crujeiras *et al.* 2010), the individual capacity of substrate oxidation (Veldhorst *et al.* 2010), the gestational feed (Thompson *et al.* 2007) and the genetic predisposition (Marti *et al.* 2008) have been revealed as influential agents on the development of obesity. Genetics is, among these factors, an important element that can determine the presence of the disease and the distribution of the adipose tissue (Haidar and Cosman 2011). To date, over 600 genes or chromosomal regions have been directly or indirectly involved in the regulation of body weight and the development of obesity (Rankinen *et al.* 2006). Regarding the genetic mechanisms associated with obesity, in recent years there has been growing evidence that genetic variations can result in differing biological responses to specific nutrients, but also it has

been proposed that different nutrient intake can trigger discriminated gene expression (Brahe *et al.* 2013; Phillips 2013; Martinez *et al.* 2008). In the last years, epigenetic regulation of gene expression has also emerged as an important contributor (Campion *et al.* 2010). Epigenetic describes the study of mitotically heritable alterations in gene expression potential that are not caused by changes in DNA sequence (McAllister *et al.* 2009). Nevertheless, genetic factors by themselves do not determine the presence of obesity; in many cases an interaction between genetic factors and social/environmental factors is necessary. Therefore, genetic factors predispose to the disease but environmental cues are decisive to determine the final effect on the metabolism (Ordovas 2008).

Recently, the gut microbiota has been proposed to contribute to obesity and incidence associated metabolic related disorders (Diamant *et al.* 2011). There are not many human interventional studies because of methodological difficulties. However, relevant outcomes have been approaching this issue suggesting that the composition of the gut microflora may influence harvesting energy from food, mucosal immunity, intestinal permeability, transit time and systemic inflammation, which all relate to the obesity state (Ley 2010). A balanced gut microbiota composition confers benefits to the host, whereas microbial imbalances are associated with metabolic and immune-mediated disorders. In agreement with these statements, the EVASYON (*Desarrollo, aplicación y evaluación de la eficacia de un programa terapéutico para adolescentes con sobrepeso y obesidad: educación integral nutricional y de actividad física*) recent investigation in Spanish children revealed that specific gut bacteria were related to body weight changes in adolescents under lifestyle intervention, suggesting interactions between diet, gut microbiota and host metabolism in obesity (Nadal *et al.* 2009).

The obesity condition leads to several adverse metabolic effects such as the increase of blood pressure, the increment of cholesterol and triglycerides serum levels or inducing glycemic dysfunctions, features which are accompanying the risk of initiating and developing other diseases such as insulin resistance, type 2 diabetes mellitus (DM), cardiovascular diseases (CVD), metabolic syndrome (MetS) and even some type of cancers (WHO 2012) (Misra and Khurana 2008; Prieto-Hontoria *et al.* 2011). Furthermore, white adipose tissue in the obese is known to present a low but chronic inflammatory state, and there is evidence that this phenomenon plays a crucial

role in the initiation and development of the obesity-associated comorbidities (Bondia-Pons *et al.* 2012).

A number of studies have evidenced that obesity is related to a reduction in the quality of life linked to health (Fontaine and Barofsky 2001) and to a decrease in life expectancy (Olshansky *et al.* 2005; Peeters *et al.* 2003). Moreover, this disease accounts for between 0.7% and 2.8% of a country's total healthcare expenditures (Withrow and Alter 2011), and obese individuals have medical costs that are approximately 30% greater than their normal weight peers (Hill *et al.* 2009; Janssen *et al.* 2009).

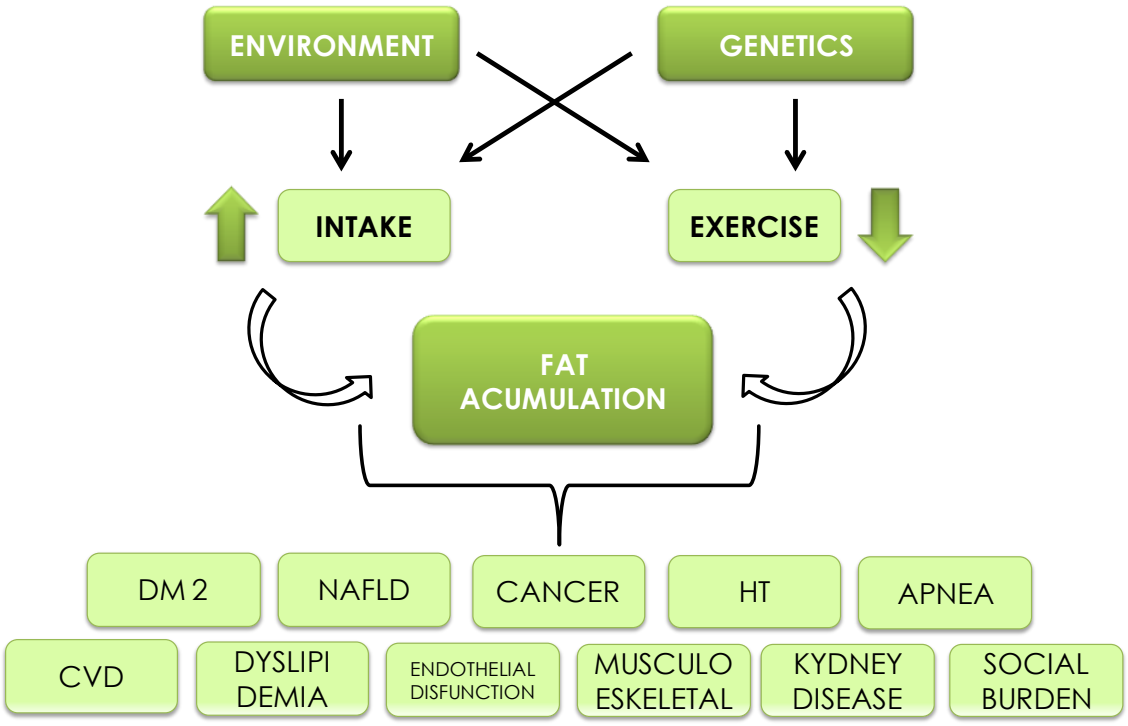

Figure 2. Interaction between environment and genetics as determinants of obesity and associated comorbidities. DM: Diabetes Mellitus; NAFLD: non-alcoholic fatty liver disease, HT: hypertension; CVD: cardiovascular disease. (Modifified from Bray 2004).

It is evident, therefore, that obesity is a multifactorial disorder with important economic, social and health implications. Obesity is not only an aesthetic problem, but also the establishment of this disease has become a major public health concern, not only by itself but mainly because of the cluster of associated comorbidities (Marchetti *et al.* 2012; Cox *et al.* 2011), which lead to an important increase of mortality rates (Bartolomucci *et al.* 2012).

1.1.2. Obesity assessment

In adults, overweight and obesity are often defined using the BMI criteria calculated as the ratio of the weight in kilograms and the height in squared meters [$BMI=w/h^2$]. Thus, overweight is defined as a BMI between 25.0 and 29.9 kg/m², and obesity is conferred with a BMI value higher than 30.0 kg/m² (WHO 2012) (Table 1). However, BMI makes an assumption about the distribution of adipose tissue and lean muscle mass of an individual using only weight and height, over or underestimating adiposity depending on subject complexion, age or ethnicity (Lara-Esqueda *et al.* 2004). BMI score may overestimate adiposity of an individual with a large proportion of lean muscle mass and underestimates the adiposity in an individual with low lean muscle mass. Similarlity, with aging body fat naturally increases and muscle mass decreases without a corresponding change in BMI. Thus, BMI often is underestimated in older individuals. Moreover, BMI can neither accurately measure adiposity in children or in pubertal adolescents, where weight can double while the height only increases by 20%. The last major limitation of this ratio is due to ethnicity-derived differences in height, muscle mass and adipose tissue distribution (Haidar and Cosman 2011).

In order to establish a more accurate diagnosis of obesity the utilization of other measurements has been proposed as an indicator of abdominal fat. In this sense, waist circumference is considered a good indicator of visceral fat (Anuradha *et al.* 2012). Although all adipose tissue increases the risk of obesity-related disorders, not all fat depots equally contributes to disease state. Visceral obesity is associated with a higher risk of CVD, hypertension (HT), insulin resistance and type 2 DM (Saunders *et al.* 2013). So that, lot of authors consider waist circumference as a better indicator of obesity and associated health risks (Anuradha *et al.* 2012). Waist circumference values higher than 102 cm in men and than 88 cm in women have been set up as indicative of cardiovascular risk by the *Sociedad española para el estudio de la obesidad* (SEEDO) (Salas-Salvado *et al.* 2007), in accordance with the WHO threshold for Caucasians (Alberti *et al.* 2009).

Table 1. Comparison of Body Mass Index (BMI) categories between the World Health Organisation (WHO) and the *Sociedad española para el studio de la obesidad* (SEEDO) classifications.

BMI (kg/m²)	WHO	SEEDO	
Underweight	<18.5	<18.5	
Normal weight (healthy)	18.5 - 24.9	18.5 - 24.9	
Overweight (increased health risk)	25.0 – 29.9	25.0 – 29.9	
		25.0 – 26.9	Type I overweight
		27.0 – 29.9	Type II overweight
Obesity (substantially increased health risk)	>30	>30	
		30.0 – 34.9	Type I obesity
		35.0 – 39.9	Type II obesity
Morbid obesity	>40	>40	
		40.0 49.9	Type III obesity
		>50	Type IV obesity (Extreme)

Since obesity is defined as an excessive body fat accumulation, different methodologies have been developed in the last decades in order to better estimate the body fat mass. The measurement of the skinfolds thickness has been one of the most employed, applying different equations such as Durnin and Wormersley (1974) or Siri (1956). Nevertheless, the reliability of this indirect method has some limitations due to the high inter-observer variability and the difficulty of assessing this kind of measurements in patient with a important obesity degree (Salas-Salvado *et al.* 2007).

Alternatively, bioelectrical impedance analysis is widely use in clinics and research (Navas-Carretero *et al.* 2011). This approach is based on a bicompartimental model and takes into consideration the resistance offered by the different body components to a low intensity and high frequency alternating current flow (Sun *et al.* 2005). Thus, bioelectrical impedance measures extracellular water and, indirectly, determines the amount of total fat mass. However this technique is sensitive to water content variations and therefore some specific criteria must be considered to apply this technique (Hsieh *et al.* 2013).

Dual-energy X-ray absorptiometry (DXA) is a relatively new reference method that can provide accurate results. It was initially designed for bone density study but also allows assessing fat mass and fat-free mass accurately radiating very slightly the individual for about ten minutes. Additionally, it is capable to differentiate the diverse fat depots within the body (Sun *et al.* 2005). Nonetheless this method is costly and often inaccessible to the public.

Finally, other precise tools exist, although less used because of complexity such as air displacement plethysmography, better known as Bod-Pod method, which is the gold standard approach for body fat measurement (Peeters 2012); the neutron activation technique (Wang and Pierson 2010) or the underwater weighting strategy (Moon *et al.* 2011).

In general, anthropometry and related indexes as well as bioelectrical impedance or DXA have advantages and limitations; however, they are very useful for determining adiposity and its distribution and consequently, they allow investigating adiposity relation with clinical and metabolic parameters.

1.1.3. Epidemiology

Along with malnourishment, underweight and infectious disease obesity has become a major public health burden worldwide, mainly due to the huge social and economic impact derived from its related comorbidities (Brown 2012). The WHO has classified it as the 21st century epidemic since instead of reducing, its rates are expected to increase for the next years. Although prevalence rates vary from country to country, this organisation estimates that over 1500 million adults around the world are

overweight and 312 million are obese. Regarding the whole world population, obesity has increased from 8 to 14% in men and from 5 to 10% in women in the last two decades (WHO 2012). Furthermore, the situation does not appear to improve because a recent report from the same institution estimates that by the year 2015, 700 million people will be obese all over the world (WHO 2010).

Rates of obesity have double in the last 20 years in the developing world as they become more urbanized and adopted a Western lifestyle of higher caloric intake, derived from overconsumption of cheap energy-dense food combined with decreased physical activity levels (McAllister *et al.* 2009). The Middle East, Pacific Islands, Southeast Asia and China are facing the greatest challenges (Hossain e*t al.* 2007). Obesity is highly prevalent in the middle-income countries of Eastern Europe, Latin America and Asia, where obesity is the fifth most common cause of disease burden, ranking just below underweight (WHO 2010). Overweight and obesity have been estimated to account for the 16% of the global burden disease (Hossain *et al.* 2007). About 18 million people worldwide die every year from CVD, for which diabetes and HT, both obesity-related comorbidities, are major predisposing factors (Haidar and Cosman 2011).

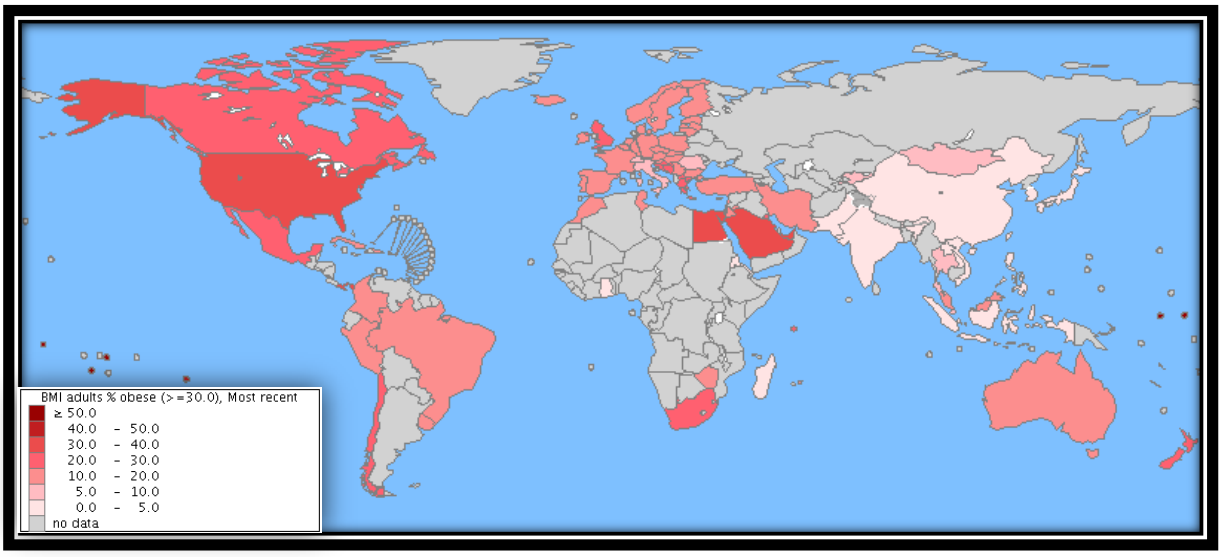

Figure 3. Worldwide prevalence of obesity distributed by countries. Percentage of adults with a Body Mass Index (BMI) higher than 30 kg/m² (World Health Organisation 2012).

In Europe overweight and obesity prevalence vary substantially, being Spain one of the countries with the higher rates (23%) in adults older than 18 years old (Martinez *et al.* 2004). In this sense, one of the most recent and important research of the Iberian country is the ENRICA (*Estudio de Nutrición y Riesgo Cardiovascular*) project, which was performed between 2008 and 2010. Considering measurements of weight, height and waist circumference this work has evidenced that 21.4% Spanish women adults and 24.4% men presented obesity, while overweight rates were 32.5% and 46.4% for women and men respectively. On the contrary, when referring to abdominal obesity the prevalence was higher in women (39.2%) than in men (31.7%), being the mean total rate 35.5% (Gutierrez-Fisac *et al.* 2011). Regarding geographical distribution, the ENRICA study revealed a higher prevalence in the Canary Islands and in the Southern Spain (Figure 3), in accordance to previous studies (Grau *et al.* 2011). Finally, when comparing with other European countries rates the prevalence of obesity in Spain is similar to that in England (23%) or Finland (21%), but much higher than those of Portugal (14%) (Gutierrez-Fisac *et al.* 2011).

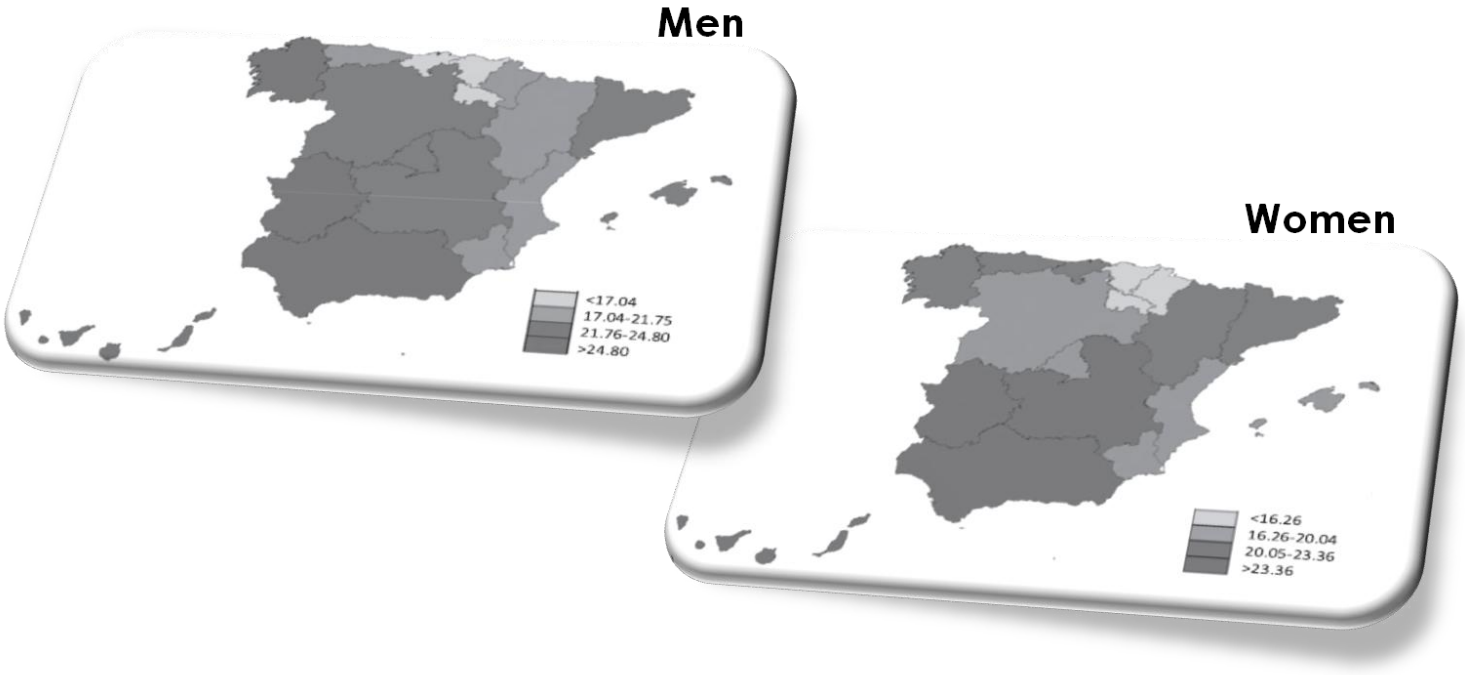

Figure 4: Regional prevalence of obesity in Spanish men and women.
(Modified from Gutierrez-Fisac *et al.* 2011).

Although preventing obesity and related comorbidities will require fundamental social and political changes, including public health initiatives for making healthful foods available and affordable or for encouraging and facilitating exercise, the changes in lifestyle that lead to weight loss significantly reduce the incidence.

1.1.4. Childhood obesity

Childhood obesity is considered a complex, multifactorial origin, with multiple associated comorbidities syndrome in like manner to adult obesity (Raj and Kumar 2010). However, it is more difficult to establish diagnosis criteria for determining obesity among children since in childhood optimal fat depots are determined by age and sex (1981). The more frequently employed tool is the BMI-SDS or z-value, obtained by the following equation (Cole *et al.* 2005):

$$\text{BMI-SDS} = \frac{\text{Children BMI} - \text{Population average BMI}}{\text{Population BMI SD}}$$

Childhood obesity equally affects developed and developing countries and every economic and social group, being independent of age, sex or ethnicity (Kosti and Panagiotakos 2006). Concurrently with the increase in adult obesity, childhood obesity has significantly rised in the last decades (Valdes Pizarro and Royo-Bordonada 2006). A recent report from the WHO estimated that in 2010 there were 43 million of children under five presenting overweight or obesity, form which approximately 35 million lived in developed countries (WHO 2012). Obesity prevalence among children rose from 4.2% to 6.7% between 1990 and 2010 (de Onis *et al.* 2010).

In Europe, rates at early ages are higher than among adolescents, while sex pattern varies depending on the country (Lobstein and Jackson-Leach 2006). In Spain, one of the most important performed studies is the AVENA (*Alimentación y Valoración del Estado Nutricional de los Adolescentes españoles*) project which enrolled 2320 adolescents between 13 and 14 years old and evidenced that overweight and obesity prevalence was 45% betwee the years 2000-2002 (Moreno *et al.* 2005). Recently, the ALADINO (*Alimentación, Actividad física, Desarrollo Infantil y Obesidad*) study

carried out by the Spanish Agency for Food Safety and Nutrition in 2011 involved 8000 Spanish children between 6 and 10 years old and showed a prevalence of obesity of the 31.4% lightly higher as compared with the EnKID (*Estudio de alimentación infantil y juvenil*) project performed 10 years before, which prevalence data ranged 30.4% (Serra Majem *et al.* 2003).

As a consequence of the excessive body weight in childhood and adolescence some complications are often developed mainly related to orthopedic alterations, type 2 DM, CVD, sleep alterations, certain types of cancer, deficient immune function or skin problems (Han *et al.* 2010). These comorbidities contribute to a reduced quality of life during the childhood, but also lead to other problems that manifest at a later age increasing morbid-mortality throughout adulthood (Reilly and Kelly 2011).

To implement prevention strategies during pregnancy, childhood and adolescence is a key point for counteracting the obesity outbreak (Must 2003). On the other hand, intervention programs have been shown to be effective once the disease has established, involving time-maintained light body weight losses, as evidenced in the EVASYON project (Marques *et al.* 2012). This study revealed that losses of a 0.5 of the BMI-SDS score lead to important improvements both on adiposity and the metabolic profile of the adolescents.

In summary, obesity represents a complex health burden among childhood in like manner as in adults, with the aggravating factor of increasing comorbidities not only when a child but also in the adulthood. Therefore nutritional intervention strategies in order to prevent and treat this disease should be developed.

1.2. Metabolic syndrome

1.2.1. Etiology

The importance of obesity lies not only of itself, but also of the wide range of associated comorbidities (Marchetti *et al.* 2012). In this context, the MetS encompasses a cluster of risk factors for dislipemia, CVD and type 2 DM (Alberti *et al.* 2009). This clinical condition is widely diffused in western countries and diagnosis criteria have only been better defined recently, even though they are still ambiguous (Marchetti *et al.* 2012). MetS is defined by the presence of central obesity (expressed by waist circumference) in combination with at least two of the following disturbances: HT, dyslipidemia -raised triglycerides and lowered high-density lipoprotein cholesterol (HDL-c)- and/or impaired glucose tolerance (Alberti e*t al.* 2009). With these risk factors, it has been clearly demonstrated that the syndrome is common and has a rising prevalence worldwide, which can be related to changes towards more sedentary lifestyles and increase on obesity. Therefore, MetS is both a public health and a clinical problem being important to identify individuals with the MetS at the clinical level.

Despite its growing prevalence worldwide, there is still a lack of an uniformly accepted definition. Numerous organisations have proposed several clinical definitions in order to determine the presence of MetS. They are based on the same parameters, but differ on the limits/cut offs that they considered as pathogenic. This disagreement over the terminology and diagnostic criteria makes very difficult to establish reliable data about prevalence. The basic elements for MetS were described for first time in the 1920s by Kylin, a Swedish physician, as the clustering of HT, hyperglycemia and gout. Later, in 1947 Vague drew attention to upper body adiposity (android or male type adiposity) as the obesity phenotype that was commonly associated with metabolic abnormalities. Then, in the 90`s decade Reaven called the cardiovascular risk factors associated to an excess of body weight and fat mass as X Syndrome. It was also called "the insulin resistance syndrome" or the "deadly quartet" and nowadays it is known as MetS (Alberti *et al.* 2009). Over the past two decades, a striking increase in the number of people suffering the syndrome worldwide has taken place, associated with the global epidemic of obesity. Thus, in the 90s, there was an initiative to develop an internationally recognized definition. The WHO proposed the first classification in 1999

(Eckel *et al.* 2005), emphasizing insulin resistance as the major underlying risk factor and required evidence of insulin resistance for diagnosis. In the same year, the European Group for the Study of Insulin Resistance (EGIR) established its own set of criteria (Eckel *et al.* 2005). The other major criteria came from the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) in 2001, which are actually one of the most used (Balkau *et al.* 2003). In these criteria, neither insulin resistance demonstration nor a single factor was required for diagnosis, but instead the presence of 3 among 5 proposed factors was the basis for establishing the diagnosis (The main clinical criteria for MetS diagnosis are listed in Table 2). In 2005, the International Diabetes Federation (IDF) proposed its own definition criteria (Alberti *et al.* 2009). The IDF proposal dropped out the insulin resistance requirement, but made abdominal obesity necessary as 1 of 3 factors required for the diagnosis, with emphasis on waist circumference measurement as a simple screening tool. The remainder risk factors were the same to those provided by the ATP III criteria. The European Association for the study of Diabetes and the European Atherosclerosis Society also adopted the IDF definition. Yet another attempt at a definition came from the National Heart, Lung and Blood Institute (HLBI) together with the American Heart Association (AHA). They differ with the IDF criteria in the cut off point for waist circumference. Recently, IDF and HLBI/AHA held discussions to attempt to resolve the differences between both MetS definitions (Alberti *et al.* 2009). It would result on a common definition in which abdominal obesity should not be a prerequisite for diagnosis but that it is one of the 5 criteria, so that, the presence of any 3 of the 5 risk factors would constitute a diagnosis of MetS. The American Association of Clinical Endocrinologists (AACE), as well as the American Diabetes Association (ADA) also employs their own definitions.

In any case, general agreement exists in the health science community that obesity and its medical complications including MetS deserve great attention (Marchetti *et al.* 2012). Patients suffering MetS are at twice the risk of developing CVD over the next 5 to 10 years as individuals without the syndrome. Furthermore, the MetS confers a 5-fold increase in risk for type 2 DM (Alberti *et al.* 2009). Additionally, it has been reported that MetS represented in Spain a total annual cost of 1909 million Euros in 2008 and according to the same report it is expected to rise a 179% by 2020 (Scholze *et al.* 2010).

Table 2. Main clinical criteria for metabolic syndrome.

WHO (1999)	NCEP ATP III (2001)	IDF (2005)
One of this	**Any ≥3 of**	**Mandatory**
1. Fasting glucose >110 mg/dl **2.** Diagnosis of type 2 DM **3.** Impaired glucose tolerance (2 h. glucose ≥ 142 mg/dl)	**1.**Central obesity, expressed as waist circumference: >102 cm in men > 88 cm in women **2.** TG > 150 mg/dl or specific treatment for hypertriglyceridemia	**1.** Central obesity, expressed as waist circumference: *In men:* ≥94 cm American origin ≥90 cm Hispanic/Chinese origin ≥85 cm Japanese origin *In women:* ≥80 cm (except for Japanese women ≥ 90 cm)
Plus ≥2 of		**Plus ≥2 of**
1. TG > 150 mg/dl and/or HDL-c <35mg/dl for men and < 39 mg/dl for women **2.** Microalbuminuria: urine albumin excretion >20 g/min or albumin:creatinin >30 mg/g **3.** SBP ≥140 mmHg or DBP ≥90 mmHg or treatment for HT previously diagnosed **4.** Central obesity: Waist:hip in men >0.90, in women >0.85 and/or BMI 30 Kg/m²	**3.** HDL-c <40 mg/dl for men and < 50 mg/dl for women or specific treatment **4.** SBP ≥130 mmHg or DBP ≥85 mmHg or treatment for HT previously diagnosed **5.** Fasting glucose >110 mg/dl or type 2 DM previously diagnosed	**2.** TG > 150 mg/dl or specific treatment for hypertriglyceridemia **3.** HDL-c <40 mg/dl for men and < 50 mg/dl for women or specific treatment **4.** SBP ≥130 mmHg or DBP ≥85 mmHg or treatment for HT previously diagnosed **5.** Fasting glucose >100 mg/dl or type 2 DM previously diagnosed

WHO: World Health Organisation; NCEP ATP III: National Cholesterol Education Program Adult Treatment Panel III; IDF: International Diabetes Federation; DM: diabetes mellitus; TG: triglycerides; HDL-c: high density lipoprotein-cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; HT: hypertension; BMI: Body mass index.

1.2.2. Prevalence

Comparisons of published prevalence for different populations are difficult despite attempts to reach an agreement on the definition of the MetS. Many studies compare prevalence using different criteria, and most of the times the main achievement is to reinforce the need for a standardized international definition. The prevalence of MetS is highly dependent on the criteria used for diagnosis and the existing disagreement regarding diagnostic criteria difficult the determination of reliable prevalence rates. According to some works, prevalence in Europe ranges from 13% in France (Balkau *et al.* 2003) to 33% in Turkey (Ozsahin *et al.* 2004).

The prevalence of MetS in Spain is largely unknown. Several small population studies have been published. However, their results cannot be easily compared owing to the type of samples selected and the different methods used. In addition, it is well-known that geographic heterogeneity with respect to cardiovascular disease-related mortality and morbidity exists between and even within countries (Müller-Nordhorn 2008). Consequently, there is a considerable regional variation concerning also MetS prevalence.

One of the most recent information regarding MetS prevalence in Spain comes from the DARIOS (Dyslipaemia, Atherosclerosis Risk and increased hsCRP and Inflammatory and Oxidative status in the Spanish population) study (Fernandez-Berges *et al.* 2012). This survey was carried out considering the WHO criteria, which exclude subject suffering from DM or CVD, because a primary prevention is not possible among them. The sample was of 24670 adult subjects, approximately equivalent to the 70% of the Spanish population, the biggest MetS sample analyzed in Spain until the moment. MetS prevalence was 32% in men and 29% in women. After the age of 65 years old, the prevalence became greater among females. Differences were also evidenced when comparing between regions. Cataluña, Navarra and Madrid showed the lowest rates. Contrariwise, Canarias and Baleares had the highest values. Furthermore, Leon-Latre *et al.* (2009) analyzed clinical and laboratory data obtained during health check-ups carried out in 17837 Spanish workers during 2003 with the aim of determining whether geographic variations exist in the prevalence of MetS in the Spanish working population according to the ATP III classification. Again, some differences were obtained regarding region distribution. The southern and western

regions showed a greater prevalence of MetS, (e.g. 22.15% and 20.60% in men in Extremadura and Galicia, respectively) reaching up to twice of that seen in the center and north regions (e.g. in the Pais Vasco and Castilla y Leon). This agrees with the pattern described for HT, diabetes, and obesity in Spain, both in adults and children (Leon Latre *et al.* 2009). The mean prevalence of MetS was 17% in men and 6.5% in women. The MADRIC (*MADrid RIesgo Cardiovascular*) study aimed at estimating the prevalence of MetS in Madrid taking into consideration both ATP III and IDF criteria (Martinez *et al.* 2008). The age and sex adjusted prevalence of MetS was 24.6% using the ATP III definition and 30.9% using the IDF definition. Prevalence by both criteria was higher in men than in women and increased with age.

On the other hand, the CLYDIA (*Prevalencia del síndrome metabólico en pacientes con enfermedad cardiovascular en España*) study recruited 1342 participants diagnosed of cardiovascular disease and considered the ATP III criteria (Palma-Gamiz *et al.* 2007). As relevant outcomes they revealed that the 37% of the population presented MetS. In this case, they found a higher prevalence among women (51.5% *vs.* 31.8% in men). A work derived from the ESOPOH study, evidenced a MetS prevalence of 44.6% according to ATP III criteria and 61.7% when considering IDF classification (de la Sierra 2006) among hypertensive patients. Data from the MESYAS (MEtabolic SYndrome in Active Subjects) study, performed in 2005 in working men and taking into consideration the ATP III criteria showed a mean prevalence of MetS of 10.2%, significantly higher in men than in women (8.7% *vs.* 3.0%, respectively) (Alegria *et al.* 2005).

1.3. Inflammation

1.3.1. Inflammation as a link between obesity and associated comorbidities

Inflammation is a physiological response that contribute to protect the organism from harmful physical, chemical or biological stimuli (Monteiro and Azevedo 2010). The process initiates pathogen killing as well as tissue repair processes and helps to restore homeostasis at infected or damaged sites (Monteiro and Azevedo 2010).

Pathological inflammation involves a loss of tolerance and/or of regulatory processes. When these events become excessive, irreparable damage to host tissues and disease can occur (Calder *et al.* 2011). The existence of inflammatory diseases has been widely recognized, nonetheless the condition of low-grade inflammation has just recently received attention (Monteiro and Azevedo 2010).

Low-grade chronic inflammation has been hypothesized to be a major mechanism in the pathogenesis and progression of obesity related disorders and the link between adiposity, insulin resistance, MetS and CVD (Bondia-Pons *et al.* 2012). The inflammatory process that characterizes obesity seems to have its own unique features, being distinguished by raised concentrations of inflammatory markers in the systemic circulation (Calder *et al.* 2011). The up-regulation of systemic indicators of inflammation such as leucocyte count, and serum and plasma concentrations of acute-phase proteins, pro-inflammatory cytokines, chemokines, soluble adhesion molecules and prothrombotic mediators is modest, usually less than 2-fold above what is observed in control (Calder *et al.* 2011).

Nevertheless, the mechanisms that initiate the inflammatory signaling are partly unknown. Hotamisligil *et al.* proposed for first time in 1993 a mechanistic link between obesity and low-grade inflammation. These researchers evidenced that white adipose tissue synthesizes and releases the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α), and that its expression is elevated in adipocytes of obese and insulin resistant mice. Subsequently, other adipose tissue-derived proteins were described. Thus, it was suggested that adipose tissue plays an important immune role and may be a major source of proinflammatory mediators, which initiate the development of chronic inflammation, insulin resistance and atherosclerosis, characteristic of the metabolic dysregulation associated to obesity (Hotamisligil *et al.* 1993).

Other hypothesis and theories have arisen, considering nutrients themselves as inflammatory, and consequently a physiological slight immune response may be activated while they are metabolized (Gregor and Hotamisligil 2011). Alternatively, it has been proposed that the nutrient *per se* is not inflammatory, but overfeeding and high fat intake could be identified as an external factor triggering the inflammatory response (Shi *et al.* 2006). It has been also suggested that the greater intestinal permeability in obese subjects after feeding may facilitate the entrance of more inflammatory signals

with nutrients (Gregor and Hotamisligil 2011). Finally, another proposed possibility is that the increased adipocyte death in obese tissue recruits macrophages to clear away dead cells and repair tissue function eliciting a pro-inflammatory phenomenon (Baker *et al.* 2011). Additionally, oxidative stress (Zulet *et al.* 2007) and the hypoxia of the adipose tissue (Quintero *et al.* 2010) have been proposed as potential inductors of the inflammatory status.

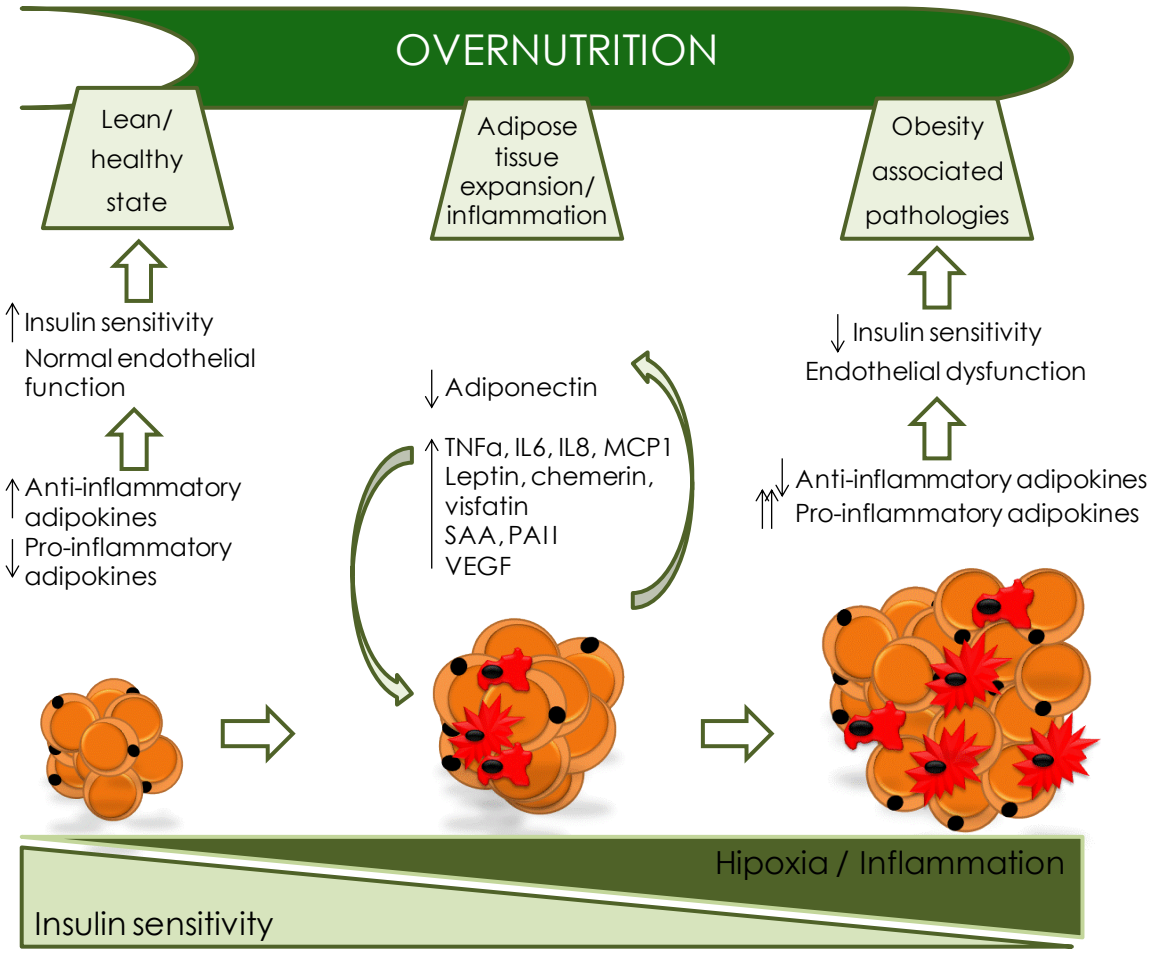

Figure 5. Representation of the alterations in adipose tissue that accompany body-weight gain. TNF-α: tumor necrosis factor-alpha; IL-6: interleuquin-6; IL-8: interleuqin-8; MCP-1: monocyte chemoattractant protein-1; SAA: serum amyloid; PAI-1: plasminogen activator inhibitor-I; VEGF: vascular endothelial growth factor. (Modified from Calder *et al.* 2011).

The view of adipose tissue has changed importantly over the last two decades, with the discovery of leptin in 1994 (Zhang *et al.* 1994). This finding modified the perception of adipose tissue as an organ for energy storage to be the largest endocrine gland in the body. The discovery of leptin introduced the concept of adipocytokines or adipokines, substances produced and secreted by adipose tissue and which circulate in the bloodstream, acting as hormones (Trayhurn and Wood 2004). It is established that macrophages travel and accumulate in the adipose tissue in obesity and that may represent major contributors to the production of adipokines (Trujillo and Scherer 2006).

The expression of macrophage markers in human adipose tissue is higher in subjects with obesity and insulin resistance, and also correlated with the expression of TNF-α and interleukin-6 (IL-6) (Rasouli and Kern 2008). There are a number of possible mechanisms underlying the infiltration of macrophages into adipose tissue. One possibility is the elaboration of chemokines by adipocytes, which would then attract resident macrophages. Adipocytes express low levels of monocyte chemoattractant protein-1, and increased expression is found in obese subjects (Rasouli and Kern 2008). On the other hand, some studies have suggested that macrophages infiltrate adipose tissue as part of a scavenger function in response to adipocyte necrosis (Strissel *et al.* 2007; Cinti *et al.* 2005).

Hence, the regulation of secretion of these proinflammatory substances is mediated by increasing adiposity, supporting the hypothesis for a low-grade inflammatory process during obesity (Bondia-Pons *et al.* 2012). Nevertheless, it is difficult to measure the inflammatory process linked to obesity directly; no current image techniques can assess inflammatory changes, although they will probably be available in the future, and arterial biopsy to monitor such alterations or therapeutic interventions is neither practical nor ethical. For that reason, there is a growing interest on finding and validating biomarkers of inflammation, plasma proteins that can be quantified in peripheral blood.

1.3.2. Inflammation markers

C-reactive protein (CRP)

Acute reactant phase proteins from the liver, such as C-reactive protein (CRP), have been linked to the development of inflammatory processes. This reactant protein is hepatically synthesized in response to various proinflammatory cytokines derived either from monocyte and/or macrophages or the adipose tissue, such as TNF-α and IL-6 (Zulet *et al.* 2007). Unlike other acute phase markers, the levels of CRP are relatively stable, with no significant diurnal variation and therefore, can be accurately measured (Silva and Pais de Lacerda 2012).

There is evidence that, when determined by high-sensitivity methods, CRP can act as a marker of low-grade chronic inflammation, being also an important independent predictor of cardiovascular events and coronary heart diseases (Jialal and Devaraj 2003). Elevated levels are linked to body weight, BMI, visceral fat accumulation (Hermsdorff *et al.* 2011; Khera *et al.* 2009; Warnberg *et al.* 2006; Rexrode *et al.* 2003), insulin resistance and type 2 DM (Ridker 2007). This was firstly established in 1997 on a report from the Physicians Health Study (Ridker *et al.* 1998) and since then, it has been confirmed by more than 20 diverse population cohorts (Cushman *et al*. 2005; Pai *et al.* 2004; Ridker *et al.* 1998). Moreover numerous studies have confirmed that CRP levels are increased in patients with MetS (Ridker *et al.* 2008; Jialal and Devaraj 2003).

On the other hand, it has been reported that physical activity, weight loss and a reduction of abdominal obesity lead to an important decrease of CRP levels (Gogebakan *et al.* 2011; Bruun *et al.* 2006; Dvorakova-Lorenzova *et al.* 2006). Thus, the cardio-protective effects derived from a healthier lifestyle could be explained, at least in part, by the reduction on the levels of CRP attributed to them.

Tumor necrosis factor alpha (TNF-α)

TNF-α is one of the main inflammatory and immune response mediator cytokines (Wieser *et al.* 2013). Indeed, it had been the first pro-inflammatory cytokine being associated with obesity and related insulin resistance (Hotamisligil *et al.* 1993). TNF-α is expressed in the adipose tissue, mostly secreted by macrophages, as well as in

leukocyte, endothelial and muscle cells. It has been reported to be elevated in obesity and correlations between serum TNF-α concentrations and BMI have been evidenced (Hermsdorff *et al.* 2012). In the same way, TNF-α levels were elevated in obese patients with MetS when compared with obese without MetS (Xydakis *et al.* 2004). On the other hand, energy restriction resulting in a weight loss has been shown to reduce TNF-α levels (Forsythe *et al.* 2008), although these results differ from some other published studies (Johnson and Fritsche 2012).

Elevated levels of TNF-α are associated with an increase of the secretion of leptin, plasminogen activator inhibitor type 1 (PAI-1), IL-6 and CRP (Rajala and Scherer 2003). Additionally, some studies have stated that TNF-α could promote the onset of insulin resistance in obese subjects due to the inhibition of the insulin signaling pathway, resulting on a reduction of the synthesis and translocation of the glucose transporter GLUT-4 (Rajala and Scherer 2003).

Interleukin 6 (IL-6)

Together with TNF-α, IL-6 is the most common studied cytokine in relation to obesity (Moreira *et al.* 2013). This protein is secreted by macrophages and adipocytes, being the adipose tissue responsible for the 30% of plasma circulating levels. In this regard, although the increase of total fat mass is related to higher total plasma IL-6 levels, there is evidence that this feature is exacerbated if such increase is due to visceral fat rather than subcutaneous fat (Zulet *et al.* 2007).

On the other hand, research concerning humans has evidenced elevated IL-6 levels in overweight and obesity when comparing with normal weight, which are positively correlated with waist circumference, waist-to-hip ratio or BMI (Goyenechea *et al.* 2005). It has been also demonstrated that dietary and physical activity interventions improve that condition (Goyenechea e*t al.* 2005). Effects of IL-6 on insulin sensitivity in skeletal muscle are controversial (Kristiansen and Mandrup-Poulsen 2005), but it is established that IL-6 enhances formation and release of CRP and also induce PAI-1 production in the adipose tissue (Zulet *et al.* 2007).

Plasminogen Activator Inhibitor type I (PAI-1)

PAI-1 is a prothrombotic factor secreted by adipocytes and other cell types (Zulet *et al.* 2007). This protein regulates thrombus formation by inhibiting the activity of tissue-type plasminogen activator, an anticlotting factor (Tschoner *et al.* 2012). In humans, visceral adipose tissue mass has been shown to be a primary determinant of PAI-1 levels (Belalcazar *et al.* 2011). Several studies have demonstrated a preferential synthesis and mobilization of PAI-1 by visceral adipose tissue, potentially due to a higher amount of stromal cells in visceral than in subcutaneous fat (Tschoner *et al.* 2012; Cox *et al.* 2011). However, this is not unequivocal as no such association or even higher PAI-1 expression in subcutaneous fat have been reported (Alessi *et al.* 2000).

A relationship between PAI-1 and cardiovascular disturbances is firmly established and circulating PAI-1 levels are predictive of incident CVD in general population (Belalcazar *et al.* 2011). Recent data also suggest that PAI-1 to be an acute-phase protein that may contribute directly to increase the risk of obesity-related complications, including the development of type 2 DM, independent of insulin resistance and proinflammatory states (Tschoner *et al.* 2012). Since elevated PAI-1 concentrations have consistently been reported in obese subjects, some authors have considered it as an additional criteria for the diagnosis of MetS (Alessi and Juhan-Vague 2008). Increased levels of PAI-1 are found in subjects presenting MetS and on the contrary, those levels significantly decrease with weight loss (Belalcazar *et al.* 2011).

Leptin

Leptin is a 167-amino acid hormone secreted largely by the adipose tissue, preferentially by the subcutaneous depot, that control food intake and energy expenditure (Rasouli and Kern 2008). Circulating levels of leptin parallel fat cell stores, increasing with overfeeding and decreasing with starvation (Zulet *et al.* 2007). Indeed, dietary interventions leading to body weight loss entails a reduction in leptin circulating levels (Sumithran *et al.* 2011). Additionally, a predictive role of higher baseline leptin levels on weight regain has been suggested (Crujeiras *et al.* 2010).

The effects of leptin are mediated by receptors mainly located in the central nervous system and other tissues including adipocytes and endothelial cells (Marti *et al.* 2009). Leptin receptor engages different pathways and therefore it is involved in mediating food intake, liver glucose production and gonadotropin secretion (Gomez Abellan *et al.* 2011; Romeo *et al.* 2011; Buettner *et al.* 2008). Other potential roles for leptin such as T-cells immune response modulation, proliferation of T-helper cell stimulation and increase of the production of pro-inflammatory cytokines have been described (Rasouli and Kern 2008). In this sense, leptin and its receptor exhibit structural and functional similarity with IL-6. Although controversy exists some studies suggest an increase in human leptin level after TNF-α treatment and it has been also shown to be increased in response to inflammation mediators such as interleukin-1 (Zulet *et al.* 2007). Also, Tilg and Moschen (1996) reported a role for leptin in many aspects of inflammation and immunity, thereby redefining adipose tissue as a key component not only of the endocrine system, but also of the immune system (Tilg and Moschen 2006).

Adiponectin

Adiponectin was discovered during gene-expression profiling of human adipose tissue conducted by the human cDNA project (Di Chiara *et al.* 2012). It is exclusively expressed in the adipose tissue and is abundant in human plasma, although contrariwise to the other cytokines reported, circulating levels are decreased in obesity-induced insulin resistance (Di Chiara *et al.* 2012). The physiological role of this adipokine remains unclear, but it appears to possess insulin sensitizing (Patane *et al.* 2013) and potentially antiinflammatory and antiatherogenic properties (Zhang *et al.* 2010; Moreno-Aliaga *et al.* 2010). These antiinflammatory and antiatherogenic roles are in part attributed to the adiponectin-mediated reduction in vascular cell adhesion molecule 1 (Moreno-Aliaga *et al.* 2010). The plasma range of adiponectin in humans is 3–30 μg/ml, accounting for 0.01% of total plasma protein (Kadowaki and Yamauchi 2005). The primary indication that adiponectin might have a role in human obesity derives from a report of Hu *et al.* (1996), where they observed a reduction of adiponectin expression in the adipose tissue of obese mice and humans. Accordingly, plasma adiponectin levels are found higher in women than in men and in non obese than in obese subjects (Scaglione *et al.* 2010).

Adiponectin is therefore downregulated in relation to weight gain, and it has been suggested that visceral fat accumulation might contribute to this alteration through secretion of some adipokines that inhibit factors for adiponectin synthesis or secretion, such as TNF-α (Di Chiara *et al.* 2012). Lower plasma levels of adiponectin are also predictive of type 2 DM and are found in diabetic subjects and in patients with hypertriglyceridemia, low HDL-c, and HT (Lezhenko and Gladun 2013; Chang *et al.* 2012). Therefore, human subjects with cardiovascular risk factors, or with MetS, would be expected to have lower plasma adiponectin levels (Razquin *et al.* 2010; Shargorodsky *et al.* 2009). On the contrary, other prospective studies have not reported a significant cardio-protective effect (Sattar *et al.* 2006). These conflicting results raise the possibility that adiponectin may have different prognostic implications in population at different vascular disease risk.

1.4. Hormonal influences on obesity: Irisin

1.4.1. The recently discovered myokine

Beneficial consequences of diet and exercise have been extensively documented and it is widely accepted that they are the cornerstones for the prevention and treatment of obesity and related metabolic disorders (Dunstan 2011). Nonetheless, underlying mechanisms through which exercise exerts positive effects remain unexplained.

In 2012 Boström and collaborators identified irisin, thus named after the Greek messenger Goodness Iris. Irisin is a signaling protein release to blood by skeletal muscle after proteolysis of the membrane protein fibronectin type III domain containing 5 (FNDC5) regulated by peroxisome proliferator-activated receptor-γ coactivator-1 alpha (PGC-1α) (Bostrom *et al.* 2012). PGC-1α is a transcriptional coactivator that regulates genes in response to nutritional and physiological cues. Exercise is accompanied by increased muscle expression of PGC-1α, whereas type 2 DM or sedentary lifestyles are associated with reduced expression (Castillo-Quan 2012). Increasing the muscle expression of PGC1-α has been evidenced in mice to protect against weight gain, inflammation, oxidative stress, muscle wasting and bone loss. Additionally, augmented PGC-1α expression improves metabolic parameters such as

insulin sensitivity and insulin signaling (Wenz *et al.* 2009). Subsequently, it has been also confirmed FNDC5 gene expression in human skeletal muscle (Timmons *et al.* 2012; Huh *et al.* 2012; Lecker *et al.* 2012). The myokine irisin has been proposed as the mediator of these benefits of muscular exercise on metabolic diseases by triggering a process known as "browning" which consists on the induction of white fat into a brown fenotype by stimulating the expression of the uncoupling protein 1 and hence caloric expenditure (Bostrom *et al.* 2012). Boström and colleagues found irisin in the plasma of wild-type mice and showed that muscle-specific knockout of PGC-1α decreased irisin levels by 72%. After 3 weeks of free wheel running, plasma irisin levels had increased by 65% in mice and, correspondingly, plasma irisin levels were found to double in healthy humans after 10 weeks of endurance exercise (Bostrom *et al.* 2012). There is a report from Timmons *et al.* (2012) that contradict this statement and rest relevance to irisin role since they found muscle FNDC5 induction only in a minority of subjects whereas all types of exercise training programmes, in the vast majority of people, yield some gain in cardiovascular or metabolic health. Nonetheless, there were some methodological differences, to which Boström team attributed Timmons failure results.

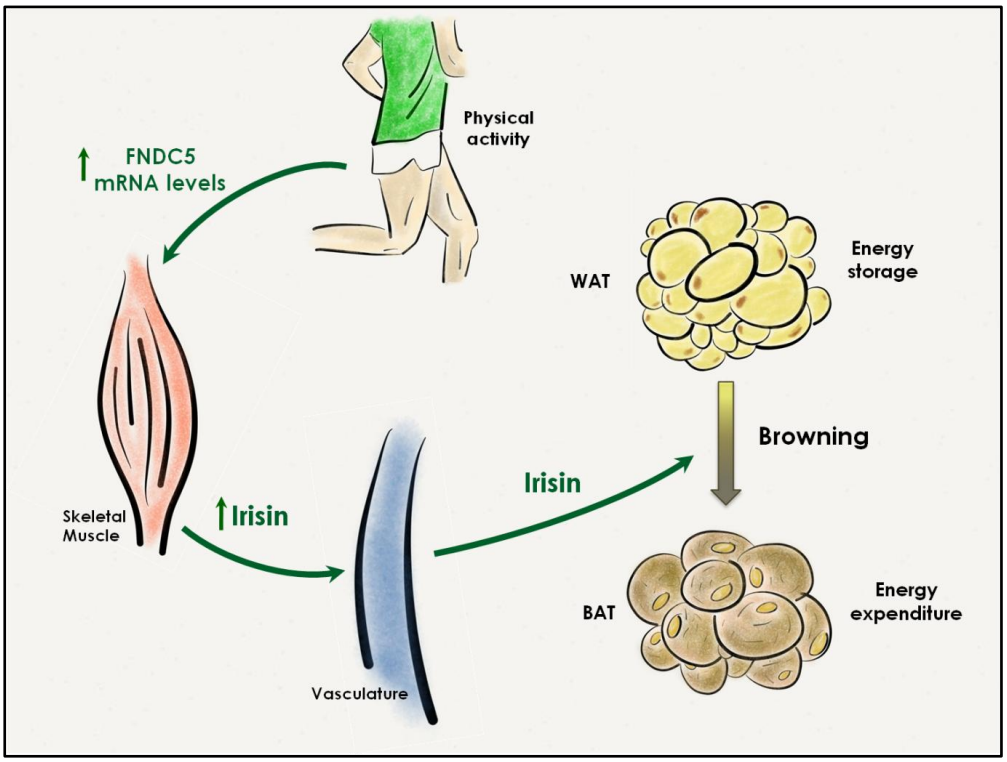

Figure 6. Physical activity-induced irisin action. FNDC5: fibronectin type III domain containing 5; WAT: white adipose tissue; BAT: beige adipose tissue.

The browning effect improved the tissue metabolic profile and increased whole body-energy expenditure and thermogenesis in mice making irisin a potential new target for the treatment of human metabolic diseases, since although browning of white adipose tissue has not been described in humans, the existence of functional brown adipose tissue depots suggest that the conversion observed in rodents might be conserved (Castillo-Quan 2012).

1.4.2. Early researches on irisin

After the laid down landmark by Boström and collaborators several researches have been carried out assessing specific potential roles for irisin in humans by quantifying circulating irisin and examining its associations with different parameters. Huh *et al.* (2012) evidenced that FNDC5 gene is predominantly expressed in muscle, suggesting that despite adipose tissue has been found to be affected by irisin, this effect may probably be endocrine. In agreement with this issue, a latter study evidenced a 200-fold increased expression of FNDC5 in muscle relative to adipose tissue (Moreno-Navarrete *et al.* 2012). Nonetheless, a study carried out by Roca-Rivada *et al.* (2013) was the first to reveal that white adipose tissue also liberated FNDC5, hence irisin may also behave as an adipokine. Age and muscle mass were found in Huh´s work to be the primary predictors of circulating irisin, which was positively correlated with biceps circumference, BMI, glucose, ghrelin and insulin-like growth factor 1 (Huh *et al.* 2012). Moreover, irisin concentrations were negatively correlated with age (Moreno-Navarrete *et al.* 2012), insulin, cholesterol and adiponectin (Huh *et al.* 2012). Given that some authors have proposed irisin as the "anti-obesity" hormone, its role on body weight regulation has been one of the most studied topics. Stengel and colleagues (2013) compared circulating irisin levels in patients within a wide range of BMI, from anorexia nervosa to different stages of obesity, and reinforced the previously reported correlation of irisin with BMI (Huh *et al.* 2012). This research also showed an association of irisin with body weight and fat mass. However, other studies have shown an inverse association BMI-irisin, being the latter lower in the obese subjects (Moreno-Navarrete *et al.* 2012).

Interestingly, a positive association of FNDC5 gene expression and brown adipose tissue markers and insulin-pathways related genes has been described (Choi *et al.* 2013). Moreover, it has been also found that both muscle FNDC5 mRNA (Moreno-Navarrete *et al.* 2012) and serum irisin levels (Choi *et al.* 2013) were significantly decreased in subjects with type 2 DM. In turn, insulin levels were positively correlated with irisin concentrations (Stengel *et al.* 2013). Collectively, these data suggest that decrease serum irisin levels may be associated with the development of insulin resistance and type 2 DM. In accordance with this statement, Choi *et al.* (2013) showed in their research that elevated irisin levels were associated with reduced new onset of type 2 DM.

Regarding inflammation, not many assessments have been carried out, but controversial results have been obtained when analising irisin relationships with inflammatory markers. Whereas some authors found negative correlations between FNDC5 gene expression and TNF-α (Moreno-Navarrete *et al.* 2012), others did not evidence any association of serum irisin concentrations and CRP (Stengel *et al.* 2013).

In summary, although much work is still needed and many gaps remain to be investigated concerning irisin specific action mechanisms and regulatory pathways, this novel myokine may represent an encouraging tool in the treatment of metabolic disturbances such as obesity or diabetes, but also in a wide range of pathological conditions related to variable imbalances of energy demand and expenditure.

1.5. Dietary strategies/components and its role on obesity and inflammation

As a complex multifactorial disorder, obesity is very difficult to treat. Therefore, prevention strategies must be implemented in first place. Lifestyle changes affecting dietary habits are essential to promote weight loss (Abete *et al.* 2010). Indeed, dietary strategies have traditionally been the main employed tool in order to prevent and counteract obesity and related comorbidities. Since obesity is the consequence to a positive energy balance, nutritional intervention studies have been mainly focused on reducing calorie intake. Thus, it has been repeatedly demonstrated that obese adults can

lose 0.5-1.0 kg per week by decreasing their daily intake by 500 to 1000 kcal below the energy required to maintain their weight (Richard *et al.* 2012; Lopez-Fontana *et al.* 2003).

Several health institutions or associations have proposed different dietary guidelines aiming at reducing body weight and consequently, improving obesity-associated cormorbidities. Among them, the AHA pattern is considered as a reference in the treatment of obesity and reduction of the risk of CVD (Krauss *et al.* 2000). In the same way the Mediterranean countries eating-pattern, established as the Mediterranean Diet (MedDiet), has been linked to not only body weight reduction, but also to numerous health benefits (Medina-Remon *et al.* 2012; May *et al.* 2012; Mitjavila *et al.* 2012). A body of evidence suggests that the contrasted benefits of the Mediterranean model are the consequence of specific food and dietary components consumed within this pattern (Medina-Remon *et al*. 2012; Urpi-Sarda *et al.* 2012).

In this sense, while dietetic programs have focused for decades on calorie restriction, new nutritional alternatives are nowadays being investigated, considering the specific role of selected dietary components, since they may show additional benefits that the absolute weight loss. They entail macronutrient distribution (Larsen *et al.* 2010), meal frequency (Cameron *et al.* 2009), bioactive compounds (Grube *et al.* 2013) (Peairs *et al.* 2011), or specific parameters in order to assess the quality of the diet such as the dietary total antioxidant capacity (TAC) (Puchau *et al.* 2010), the glycemic index (GI) and the glycemic load (GL) (Gogebakan *et al.* 2011).

1.5.1. American Heart Association guidelines

The AHA designed its dietary recommendations, appropriate for all individuals > 2 years of age, with the aim of preventing the development of recurrent heart and blood vessel diseases and for promoting overall well-being (Krauss *et al.* 2000). The major highlighted aspects of AHA recommendations are:

- To include food from all major food groups. To consume a variety of fruits and vegetables (≥5 servings per day) and grain products (≥6 servings per day) including whole grains. To include also fat-free and low dairy products, fish, legumes, poultry, and lean meats. These rules may allow reaching an

intake of fiber >25 g per day, although according to the AHA experts there are insufficient data to recommend a specific target for fiber intake.

- To maintain a healthy body weight, matching the energy intake to overall energy needs, limiting the consumption of foods with a high calorie density and/or low nutritional quality. A macronutrient distribution of 30% energy from total fats (<30% for weight reduction), 55% from carbohydrates and 15% of total energy from protein is recommended, as well as to maintain a level of physical activity that achieves fitness and balances energy expenditure with energy intake.

- To maintain a desirable blood cholesterol and lipoprotein profile, limiting the consumption of high-saturated fatty acids (<10% of total energy) and cholesterol (<300 mg per day) food and substituting them by grains and unsaturated fatty acids from vegetables, fish, legumes, and nuts.

- To maintain a desirable blood pressure, limit the salt intake (<6 g per day) and alcohol consumption (no more than 1 drink a day for women and 2 drinks per day for men).

In summary, the dietary guidelines from the AHA aim at achieving the goals of maintaining normal plasma lipoprotein levels, body weight, and blood pressure and consequently, reducing the risk of CVD and ensuring an overall balanced dietary pattern in general population (Krauss *et al.* 2000).

1.5.2. Mediterranean diet

In 1993 on the occasion of the International Conference on the Diets of the Mediterranean, international experts on diet, nutrition and health met with the aim of reviewing research on the composition and health implications of Mediterranean diets consumed during the past half century (Willett *et al.* 1995). The term "Mediterranean diet" reflects food typical patterns in the early 1960s from the regions bordering the Mediterranean Sea. These geographical areas and specific time were selected based on the evidence that adults life expectancy in those sites was among the highest in the world and rates of CVD, certain cancers and other diet-related chronic diseases were among the lowest in the world; data on food availability and dietary intake describe

dietary patterns with many common characteristics and that those common patterns were associated with low rates of chronic diseases and high adult life expectancy (Willett *et al.* 1995).

The MedDiet of the 1960s can be described by an abundance of plant food (fruit, vegetables, breads, other forms of cereals, potatoes, beans, nuts and seeds); minimally processed, seasonally-fresh, and locally growth foods; fresh fruit as the typical daily dessert; olive oil as the principal source of fat; dairy products (principally cheese and yoghurt) consumed daily in moderate amounts; fish and poultry consumed in low to moderate amounts; no more than 4 eggs consumed weekly; red meat consumed in low amounts; and wine consumed in low to moderate amounts, normally with meals. As far as can be determined this diet was low in saturated fat (≤7-8% energy) with total fat ranging from <25% to >35% of energy depending on the area. Data also suggest that work on the field and kitchen resulted on an active lifestyle (Willett *et al.* 1995).

Over the last two decades a wide number of nutritional investigations have assessed the potential benefits of the MedDiet on weight loss, but also on the modification of some biochemical parameters and the improvement of diverse diseases. In this sense, the PREDIMED (*Prevención con Dieta Mediterránea*) study, carried out in Spain, is one of which has generated more relevant contributions (Estruch *et al.* 2013). Body weight, glucose and lipid parameters, as well as blood pressure have been evidenced to reduce after undergoing a MedDiet (May *et al.* 2012; Ajala *et al.* 2013; Medina-Remon *et al.* 2012). In the same way, an inverse association between the adherence to the MedDiet and the incidence of MetS (Kesse-Guyot *et al.* 2012; Di Daniele *et al.* 2012; Mitjavila *et al.* 2012), type 2 DM (Ajala *et al.* 2013; Abiemo *et al.* 2012) and CVD (Hoevenaar-Blom *et al.* 2012; Estruch *et al.* 2013) has been demonstrated. Additionally, it has been also reported a benefit impact of the MedDiet in a diverse range of diseases such as non-alcoholic fatty liver disease (Perez-Guisado and Munoz-Serrano 2011), certain types of cancer (Couto *et al.* 2013; Buckland *et al.* 2012), mental disease (Ye *et al.* 2013), osteoporosis (Rivas *et al.* 2013), asthma (Arvaniti *et al.* 2011) and kidney disease (Diaz-Lopez *et al.* 2012).

In regard to the role of MedDiet on inflammation, a higher adherence has been negatively associated to inflammation markers concentrations such as CRP, IL-6 or TNF-α (Richard *et al.* 2012; Ahluwalia *et al.* 2012; Estruch 2010; Hermsdorff *et al.*

2009) both considering the whole pattern and some of its components separately (it would be more detail explained in the following sections). Given that inflammation is now recognized as a key etiological factor in the pathogenesis of atherosclerosis, CVD and MetS among other comorbidities linked to obesity, the described anti-inflammatory role, could explain, at least in part, the beneficial impact of the MedDiet on the prevention, treatment and mortality rates of these diseases.

1.5.3. Macronutrient distribution

In diets designed to prevent and treat obesity by manipulating energy content, macronutrient distribution was commonly set at 15% energy from protein, ≤30% from lipids, and 50–55% from carbohydrates, with increases in fiber favoured (Abete *et al.* 2010). This recommendation seemed to be effective for decreasing energy density and promoting weight loss in the short term, but the low level of observed satiety it achieved decreased dietary adherence over longer periods (Astrup 2008). Monitoring of low-energy diets with these macronutrient proportions revealed that they were often not sustainable for long periods of time (Abete *et al.* 2010). For that reason, some years ago alternative macronutrient distribution began to be evaluated. Increasing protein content, in detriment of carbohydrates, has been the most assessed variation in weight lowering plans, due to the effect on enhancing satiety with a reduction of energy intake in subsequent meals and the higher thermogenic effect attributed to them (Westerterp-Plantenga *et al.* 2012; Larsen *et al.* 2010). In addition, other potentially beneficial effects of increasing protein content accompanied by a reduction in carbohydrates content have been reported (Figure 7).

It has been revealed that controlled energy intake in combination with a moderately elevated (30% energy) protein intake may represent an effective weight loss and weight maintenance strategy, better than normal-protein diets (Brehm and D'Alessio 2008). Thus, a dietary strategy with elevated protein content showed higher 24-h satiety, thermogenesis, sleeping energy expenditure, protein balance and fat oxidation (Abete *et al.* 2009; Lejeune *et al.* 2006). Another trial evidenced better effectiveness for fat mass loss and body composition improvement during the weight loss and the weight maintenance period when following a diet with 30% energy from protein, compared with a conventional 15% protein diet (Layman *et al.* 2009). In addition to these protein-

beneficial findings, the authors found that the compliance was better among the moderately-high protein group, demonstrating also the better adherence to this kind of treatments, as also reported in other investigations (Larsen *et al.* 2010).

Despite the cited benefits of moderately-high protein diets on body weight and body composition, the role of protein intake on obesity-related inflammation is still controversial (Santesso *et al.* 2012). Given that inflammation has been recognized as the key etiological factor in the pathogenesis of obesity-related comorbidities, improvement of the inflammatory status must be also considered as a potential target. In this sense, it has been reported that lower protein content is associated with lower serum inflammation markers concentrations (Gogebakan *et al.* 2011). Moreover, a recent meta-analysis reported, although not in a significant manner, a trend also suggesting an association between higher protein intake and deterioration on inflammation (Santesso *et al.* 2012). Nevertheless, some authors suggest that the type of protein (rather animal or vegetable) in addition to the total protein intake must be also considered (Hermsdorff *et al.* 2011; Azadbakht and Esmaillzadeh 2009).

In summary, energy restriction deficit is the key factor for weight loss, but the macronutrient composition also produces changes in body composition and influences long-term compliance. Therefore, more studies are needed to establish higher protein recommendations during periods of energy restriction in order to improve weight loss and long-term weight maintenance.

1.5.4. Meal frequency

When dealing with dietary patterns for improving control over body weigh it is important to consider hunger and fullness sensations. A number of reports have proposed the increase of feeding frequency to produce favorable effects on energy intake, body weight and adiposity (Toschke *et al.* 2009; Ruidavets *et al.* 2002; Kant *et al.* 1995), mainly mediated by an increased fullness which derives on the compliance to the energy restricted diet enhancedment. Nevertheless, controversy exists with this respect (Farshchi *et al.* 2005; Summerbell *et al.* 1996). Although it has been hypothesized that the favorable effects of increased meal frequency could derived from

a more sustained release of gastrointestinal hormones, studies demonstrating mechanistic effects are lacking.

An 8-week intervention trial comparing low (3 meals/day) *vs.* high (6 meals/day) meal frequency with the same calorie restriction did not find differences regarding weight loss or adiposity indices, appetite measurements and gut peptides (Cameron *et al.* 2009). Contrariwise, it has been reported different behavior of lower *vs.* high meal frequency concerning triacylglycerol (significantly lower in the low-meal frequency condition) and insulin (significantly higher in the high-meal frequency condition) concentrations (Heden *et al.* 2012). Interestingly, hunger and desire to eat have been also reported to increase together with the augment in meal frequency from 3 to 6 meals per day (Ohkawara *et al.* 2012).

On the other hand, there is emerging literature demonstrating a relationship between the timing of feeding and weight regulation in animals, although whether the timing of food intake influences the success of a weight-loss diet in humans is unknown (Garaulet *et al.* 2013). A pioneer study in this sense evidenced that people having lunch after 15.00 h. lost less weight during a 20-weeks treatment as compared with early eaters (before 15.00 h.) indicating that novel therapeutic strategies should incorporate not only the calorie intake and macronutrient distribution, but also timing of food (Garaulet *et al.* 2013).

1.5.5. Omega-3 Fatty Acids

Polyunsaturated fatty acids (PUFA) play wide ranging roles in cell metabolism, signaling and inflammation (Vaughan *et al.* 2012). Specifically, the beneficial properties in the prevention and management of CVD of the very long chain omega-3 PUFA have been widely investigated (Munro and Garg 2013; Micallef *et al.* 2009; Krebs *et al.* 2006; Wang *et al.* 2006). The omega-3 PUFA of particular interest for that purpose include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are found predominantly in oily fish and fish oils (Wang *et al.* 2006). Regular consumption of fish and fish oil is known to have pleiotropic effects, including actions against platelet aggregation, hypertension and hyperlipidemia (Harris *et al.* 2008). These beneficial effects may be mediated through several distinct mechanisms such as

alterations in cell membrane composition and function, gene expression modulation or eicosanoid production (Calder 2013; Micallef *et al.* 2009; Reiner *et al.* 2007).

In addition to these beneficial effects, it has been suggested that the inclusion of oily fish or omega-3 PUFA in dietary strategies could increase fat oxidation, improve body weight reduction and prevent weight gain (Munro and Garg 2013; Micallef *et al.* 2009; Flachs *et al.* 2009; Thorsdottir *et al.* 2007). Indeed, obesity has been linked to low levels of omega-3 PUFA (Munro and Garg 2013).

The role of on inflammation is probably one of the best studied behaviors in relation to omega-3 PUFA, being also one the most highlighted beneficial properties of these fatty acids. Diets high in omega-3 PUFA are typically associated with lower systemic levels of markers of inflammation (de Mello *et al.* 2011; Calder *et al.* 2011; Peairs *et al.* 2011; Ramel *et al.* 2010). Omega-3 PUFA influence inflammation trough a variety of mechanisms many of them associated with changes in fatty acids composition of cell membrane. Cells involved in the inflammatory response are typically rich in the omega-6 PUFA arachidonic acid, but the content of arachidonic acid and EPA and DHA can be altered through oral administration (Calder 2010). Eicosanoids produced from arachidonic acid have roles on inflammation (Ramel *et al.* 2010). EPA also gives rise to eicosanoids and these have different properties from those arachidonic acid-derived eicosanoids. On the other hand, EPA and DHA give rise to resolvins which are anti-inflammatory (Serhan 2008). Increased membrane content of EPA and DHA results in a changed pattern of production of eicosanoids and resolvins and also affects production of other peptide mediators of inflammation, as NF-kB (Calder 2010). In conclusion, the anti-inflammatory properties of seafood omega-3 PUFA suggest that they may be useful as therapeutic agents against the obesity-related inflammatory process.

1.5.6. Dietary Total Antioxidant Capacity (TAC)

It is widely accepted that diets rich in fruits, vegetables, legumes, and other nutrient-rich plant food are associated with a lowered incidence of various chronic diseases (Del Rio *et al.* 2011; Pitsavos *et al.* 2005). Given that the concentration of single antioxidants may not reflect the total antioxidant power of food, the concept of TAC was introduced (Serafini and Del Rio 2004). The dietary TAC can be defined as

the antioxidant capacity derived from the synergic action of all the food-contained antioxidants (Carlsen *et al.* 2010). Since it is has been positively associated with plasma total antioxidant capacity (Bahadoran *et al.* 2012) it is gaining importance as a valuable tool to investigate the relationship between diet and oxidative stress-related diseases (Puchau *et al.* 2009).

Increased dietary TAC has been associated with higher diet quality scores (Puchau *et al.* 2009) as well as with improvements concerning glucose (Psaltopoulou *et al.* 2011) and lipids (Hermsdorff *et al.* 2011) metabolism and lower risk for ischemic stroke (Del Rio *et al.* 2011). Moreover, dietary TAC values are inversely associated with central adiposity measurements (Hermsdorff *et al.* 2011; Puchau *et al.* 2010) and it has been also reported to prevent weight and abdominal fat gain during a 3-year follow up (Bahadoran *et al.* 2012). Interestingly, individuals consuming a high-TAC diet showed a lower prevalence of overweight and obesity (Chrysohoou *et al.* 2007).

In the literature, investigations assessing the influence of dietary TAC on the inflammatory status evidenced and inverse association with serum CRP concentrations (Yang *et al.* 2013; Kobayashi *et al.* 2012; Brighenti *et al.* 2005) as well as with expression in peripheral blood mononuclear cells of some relevant proinflammatory markers such as IL-6 or TNF-α (Hermsdorff *et al.* 2010). Moreover, diets with high antioxidant capacity have been related to increase adiponectin levels (Detopoulou *et al.* 2010).

1.5.7. Glycemic Index (GI) / Glycemic Load (GL) and fiber

GI and GL [GI x dietary carbohydrates content] are two relatively recent concepts referring to the absorption rate of carbohydrates (Scazzina *et al.* 2013). The GI measures the ability of a carbohydrate-containing food to raise the blood glucose level (Goto *et al.* 2012). Food items with high GI quickly elevate blood glucose levels, which results in a higher insulin demand and secretion compared with low-GI foods (Scazzina *et al.* 2013). The use of the GI method for classifying carbohydrate-rich foods was endorsed in 1997 by the Food and Agriculture Organization (FAO) and the WHO and it has been proven to be a more useful nutritional and quality-assessment concept than is the chemical classification of carbohydrates (simple or complex, sugars or starches, or available and unavailable), allowing new insights into the relation between the

physiologic effects of carbohydrate-rich foods and health (Foster-Powell *et al.* 2002). Diets with a low GI have been associated with reduced risks of chronic diseases such as, type 2 DM (Dong *et al.* 2011; Liu and Chou 2010), CVD (Dong *et al.* 2012; Mente *et al.* 2009) and certain types of cancers (Dong and Qin 2011). The acting mechanism between low-GI diets and chronic disease has been assumed to be related to the decrease of postprandial blood glucose concentrations and subsequent insulin responses (Goto *et al.* 2012). In addition, it has been proposed that a low GI may also result in improved nutrient adequacy because foods that are naturally high in nutrients often have a low GI (Louie *et al.* 2012).

Data from clinical trials suggest that low-GI diets based on high amounts of fruits, vegetables, legumes and whole grains are better than conventional diets for weight loss (Abete *et al.* 2008) and maintenance (Rubio-Aliaga *et al.* 2012; Goyenechea *et al.* 2011; Larsen *et al.* 2010). However, there is controversy since no differences between low and high-GI patterns (Buscemi *et al.* 2012) or even better results for the high-GI strategies (Mediano and Sichieri 2012) have been also reported. Despite different conclusions have been stated for the efficacy of low-GI diets in body weight loss, appetite suppression and reduction of metabolic rate, it is known that many starchy foods produce high glycemic responses that elicit a sequence of hormonal changes that alter fuel partitioning and cause overeating (Brand-Miller *et al.* 2002). On the contrary, diets based on low-GI foods may enhance weight control because they promote satiety, minimize postprandial insulin secretion and maintain insulin sensitivity (Abete *et al.* 2010). It is important to highlight that low-GI diets are generally characterized by high fiber content, which may also contribute to the cited benefits.

With regard to the potential role of GI/GL on the inflammation status linked to obesity, studies carried out found that low-GI carbohydrates may specifically reduce the low-grade chronic inflammation state in obese adults, through further reducing high sensitivity (hs)CRP (Neuhouser *et al.* 2012; Gogebakan *et al.* 2011) or modestly increasing adiponectin (Neuhouser *et al.* 2012).

The most relevant strategy that has been applied so far for decreasing the GI is the addition of fiber (Scazzina *et al.* 2013). Dietary fiber is deemed to be a key component in healthy eating. It includes a broad category of non-digestible food ingredients that comprehend non-starch polysaccharides, oligosaccharides, lignin and

analogous polysaccharides (Raninen *et al.* 2010). Several health benefits of dietary fiber have been described including the prevention and mitigation of type 2 DM, CVD and colon cancer by reducing the risk of hyperlipidemia, hypercholesterolemia and hyperglycemia and decreasing the contact time of carcinogens within the intestinal lumen (Satija and Hu 2012; Kaczmarczyk *et al.* 2012; Eshak *et al.* 2010; Papathanasopoulos and Camilleri 2010). Moreover, diverse clinical studies have examined the role of this dietary component in weight reduction, and a strong relationship has been established (Kristensen *et al.* 2012; Du *et al.* 2010).

Different mechanisms by which dietary fiber intake can influence body weight have been proposed. Data suggest that fiber can reduce the risk of obesity by promoting satiety and reducing energy intake (Brownawell *et al.* 2012; Wanders *et al.* 2011; Burton-Freeman 2000). In this sense, numerous studies have been carried out to clarify the effects of dietary fiber on satiety suggesting the following actions: given that the metabolisable energy content of fiber is less than that of other nutrients (Livesey 1992) and meal intake volume is relatively constant (Poppitt and Prentice 1996), consuming foods rich in fiber decreases total energy intake; moreover, high dietary fiber intakes can increase chewing activity or oral exposure time to foods, which may result in earlier satiation (Zijlstra *et al.* 2009). Furthermore, fiber can slow down gastric emptying and concurrently increase stomach distension which also leads to satiation (de Graaf *et al.* 2004).

By and large, dietary fiber has been inversely correlated with most of the inflammatory markers (Johansson-Persson *et al.* 2013; Satija and Hu 2012; de Mello *et al.* 2011).

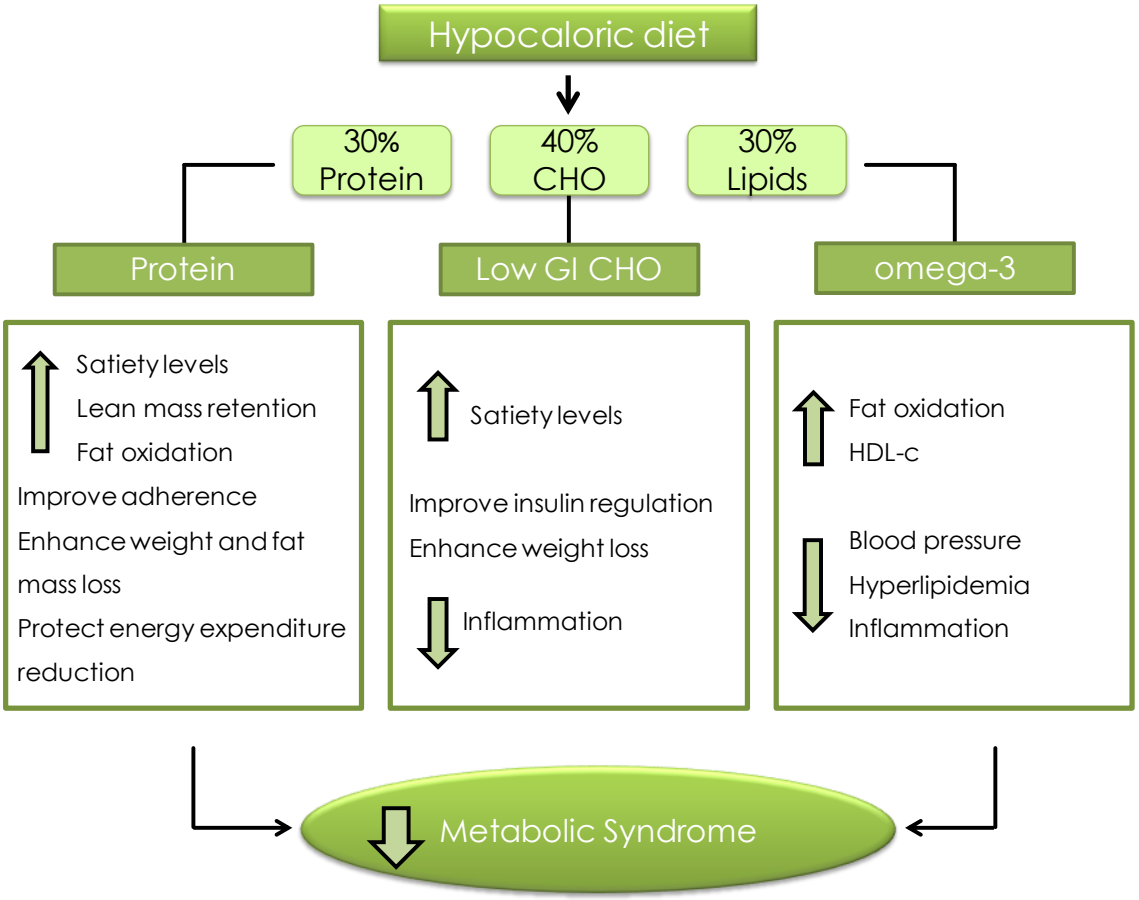

Figure 7. Metabolic changes that could be achieved with an energy-restricted diet combining moderate protein content with low glycemic index carbohydrates and high omega-3 fatty acids intake. CHO: carbohydrates; HDL-c: high density lipoprotein-cholesterol. (Modified from Abete *et al.* 2010).

1.6. Epigenetics and nutrition

1.6.1. Obesity epigenetics

The epigenetics concept was for first time described at the beginning of the 40´s, by Conrad Waddington. It comes from the Greek "beyond the genetic" (Marti and Ordovas 2011) and relates to gene expression patterns that remain stable and occur without changes in the DNA nucleotide sequence (Campion *et al.* 2010). Thus, the same nucleotide sequence within two individuals can be expressed or not depending on specific epigenetic marks (Fraga *et al.* 2005). In this way, epigenetics contributes to explain at least in part the variability that has not been clarified by the Human Genome Project and which aims to explain the Human Epigenome Project (Jones and Martienssen 2005).

The main modifications involved in the epigenetics control include DNA chain methylation and changes in histones terminal tails, mainly, methylations and acetylations. These epigenetics marks are not permanent along time and different factors such as nutrition, oxidative stress, hypoxia, inflammation or age can affect the epigenome modification contributing to its plasticity during life (Cordero *et al.* 2011).

1.6.2. Epigenetic modifications

DNA methylation

DNA methylation is an epigenetic process that participates in the regulation of gene expression throughout two different pathways: directly, by preventing the union of transcription factors and indirectly, by enhancing the chromatin "closed" structure (Herrera *et al.* 2011). The methylation of CpG sites, defined as genome regions containing a high frequency of cytosine-guanine dinucleotides, results on cytosine conversion into 5-methylcytosine. When happening in the promoter regions this fact is often associated with gene silencing (Marti and Ordovas 2011). Three enzymes are involved in the establishment and maintenance of DNA methylation patterns: DMNT3A and DMNT3B, which are *de novo* methyltransferases, and DNMT1 which ensures that methylation patterns are faithfully copied through each cellular division (Walton *et al.*

2011). These enzymes cooperate in order to both establish and maintain DNA cellular methylation patterns and they also interact with deacetylases and methyltransferases of methylcytosine union histones and proteins within a complex regulation system. CpG sites methylation suppresses gene transcription and is a control element for gene expression (Figure 8).

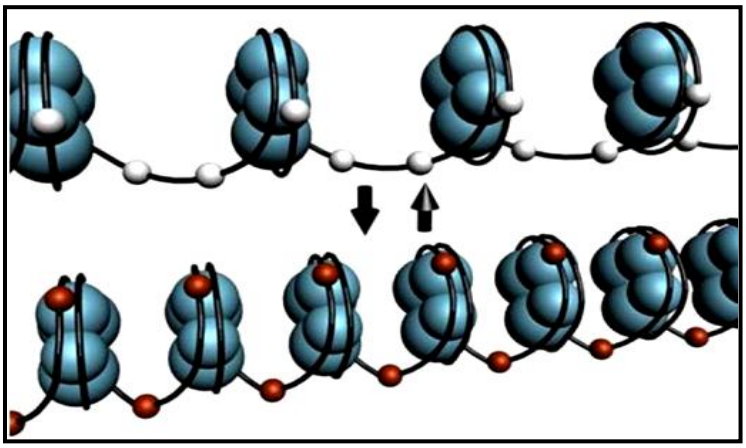

Figure 8.
DNA methylation process. (Above) The gen is uncompacted and this fact favours its expression. (Below) When genes are methylated chromatin is compacted and gene expression is reduced.

In human cells most of the CpG sites placed in no promoter regions are hypermethylated, fact that is related to the transcription suppression, whereas CpG sites located in promoter regions of active genes are not methylated (Marti and Ordovas 2011). It has been proposed that DNA methylation is a reversible process and that methylation patterns depend on a dynamic balance between methylation a demethylation processes (Kouzmenko *et al.* 2010).

In the last years, the analysis of methylated DNA represents an interesting tool for disease diagnosis, therapy and prognosis, as well as for the pharmacogenetics field (Cordero *et al.* 2011; Kacevska *et al.* 2011; Campion *et al.* 2009).

Histone modification

Histones suffer acetylation and deacetylation processes, especially in the N-terminal tail of linin residues (Figure 9). This regulatory mechanism is catalyzed by two types of enzymes: histone acetyltransferases and histone deacetylases, which act not only on histone substrates but also on no histone proteins. In the acetylation process the acetyl coenzyme A (CoA) is the acetyl group giver and, actually, nuclear synthesis of

acetyl-CoA is a limiting step for histone acetylation (Selvi and Kundu 2009). Thus, acetyl-CoA metabolism is directly related to chromatin regulation and can affect diverse cell processes in which acetylation and metabolism are interrelated such as disease and aging (Takahashi *et al.* 2006). H3 and H4 histones acetylation and deacetylation is involved in chromatin structure and in the accessibility of the transcription factors. Given this fact, it is hypothesized that some histone deacetylases inhibitors could be plausible as drugs for treating obesity (Lawless *et al.* 2009).

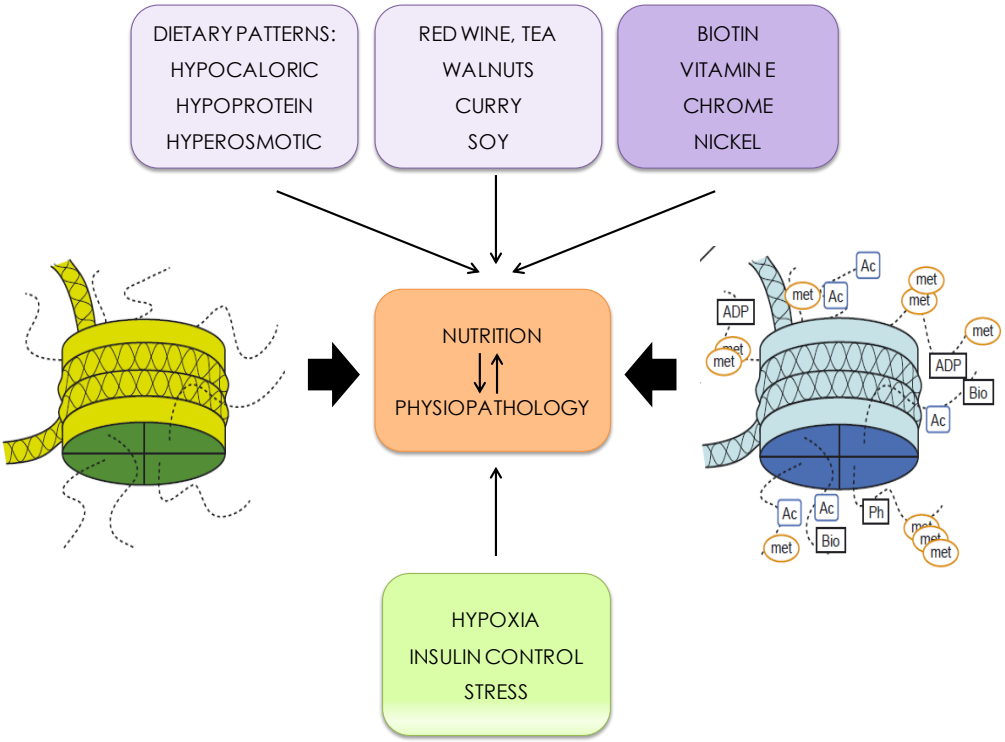

Figure 9. Epigenetic modifications of histone aminoacidic tails mediated by nutrition and physiopathology conditions. Ac: acetylation, ADP: ADP-ribosylation; Bio: biotinylation; met: methylation; Ph: phosphorylation. (Modified from Cordero *et al.* 2010).

Histone methylation represents a good potential combination with respect to other modifications since lisin residues can harbor mono, di or tri-methyl fractions in the amino group, whereas arginine residues can carry mono or dimethyl groups in their guanidine group (Cordero *et al.* 2010). Methylations in both lisin or arginine can act as

activators or inhibitors of gene transcription (Moleres and Marti 2008). Histone phosphorylation, although less known than methylation and acetylation, is thought to play a direct role on mitosis, cell death, reparation, replication and recombination, and it has been also related to gene transcription activation (Prigent and Dimitrov 2003).

Other histone modification with effects on gene expression are histone biotinylation, which depends on biotin availability (Hassan and Zempleni 2006) and might play an important role on cell response against DNA damage (Cordero *et al.* 2010) or H2A and H2B histone ubiquitination which is inhibited by nickel concentrations (Karaczyn *et al.* 2006).

1.6.3. Diet Influence on epigenetic modifications

Diet can directly affect DNMT or influence the availability of molecule givers of methyl groups or implicated on its metabolism. Some nutrients such as folic acid, betaine, choline or vitamin B12 promote homocysteine conversion to methionine and subsequently to S-adenosylmethionine, which is the final molecule given the methyl group to the DNA chain (Cordero *et al.* 2010).

The influence of nutrition on DNA methylation has been mainly evidenced by animal experimental models. In mice, obesogenic (Zhang *et al.* 2009) and hypoprotein (van Straten *et al.* 2010) diets during pregnancy have been shown to induce changes in DNMT metabolism, as well as in the expression and methylation of gene promoters involved in lipid metabolism.

In adult mice, obesogenic diets also affected DNA expression and methylation (Lomba *et al.* 2010). On the other hand, changes on the intake of selenium and folic acid also influence the variation of total DNA methylation (Uthus *et al.* 2006). In concern to histone modification it has been shown that both calorie (Li *et al.* 2011) and protein (Sohi *et al.* 2011) restriction produce changes in H3 methylation. In like manner, the intake of some minerals such as chrome or nickel also affects histone methylation levels (Zhou *et al.* 2009).

Human studies have observed that glucose metabolism affect histone methylation level. Thus, hyperglycemia can produce changes in several H3 residues

methylation (Pirola *et al.* 2011), whereas insulin can reduce this histone methylation (Hall *et al.* 2007). In relation to histone acetylation different activators or inhibitors of acetyltranferases action can be consumed through the diet. Glucose (Friis *et al.* 2009) and ethanol (Shepard and Tuma 2009) are included among the activators, whereas in the latest group we can found the anacardic acid from walnuts (Sung *et al.* 2008), the garcinol from *Garcinia indica* fruit (Nishino *et al.* 2011) and the curry curcumin (Reuter *et al.* 2011). The tea teophyline (Ito *et al.* 2002), the red wine resveratrol and a hypocaloric diet (Ford *et al.* 2011) enhance as dietary mediators for histone deacetylases activation. Contrariwise, garlic-derived sulfur compounds (Druesne-Pecollo and Latino-Martel 2011), vitamin E-derived metabolites, biotin, butyrate or broccoli sprouts act as inhibitors (Dashwood and Ho 2007).

Nowadays there are two main issues in concern to dietary factors influencing epigenetic. On one hand, to identify at an early age people presenting gene methylation specific profiles that suggest a higher susceptibility to different metabolic diseases, including excessive body weight or type 2 diabetes, in order to prevent and control their evolution. On the other hand, the use of dietary supplementation as a tool for counteracting adverse epigenomic profiles, as happens with histone deacetylases or DNMTs inhibitors for cancer therapy (Campion *et al.* 2009).

Human studies have found several genes whose methylation is related to obesity so far. Thus, mehtylation levels of the serotonin transporter gene promoter are positively correlated with BMI, body weight and waist circumference values (Zhao *et al.* 2012). In obese women it has been observed that the methylation percentage of dipeptidyl peptidase-4 gene is associated with plasma lipid profile (Turcot *et al.* 2011). A recent study has shown a relationship of the methylation percentage of some genes at birth with adiposity measurements at the age of nine. In fact, methylation levels together with gender were able to predict more than 25% of the variation in adiposity levels (Godfrey *et al.* 2011). Studies for analyzing a potential association between some genes methylation patterns and adiposity changes in response to a dietary intervention have been also carried out. Thus, these genes could act as biomarkers in order to predict the response to a dietary treatment. Bouchard *et al.* (2010) observed more than 35 *loci* differentially methylated before and after an intervention in accordance to the grade of follow up of the dietary treatment (responders or no responders).

In summary, since obesity has turned one of the most prevalent diseases among all-ages and worldwide population, to investigate a potential interaction between epigenetic and environmental factors such as diet represent a research area of great interest.

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2. HYPOTHESIS AND AIMS / HIPÓTESIS Y OBJETIVOS

2.1. Hypothesis

Obesity has become a worldwide major public health burden, being dietary approaches the most prescribed treatments for both preventing and counteracting this disease. In this context, the novel dietary strategy RESMENA (*REducción del Síndrome MEtabólico en NAvarra*), based on traditional food, with a modified macronutrient profile (40% of the total calorie value as carbohydrates, 30% as protein and 30% as lipids), an increased meal frequency (7 meals/day), enhanced content of bioactive compounds such as omega-3 PUFA, promoting low glycemic index/load food and encouraging a high antioxidant capacity and the adherence to the MedDiet is hipothesized to be effective in improving adiposity, biochemical and inflammation markers on obese subjects presenting MetS, as compared with a pattern based on the AHA guidelines, which includes a macronutrient distribution of 55% carbohydrates, 15% proteins and 30% lipids and 3-5 meals per day.

2.2. Aims

2.2.1. General aim

This research was designed to evaluate the effectiveness of a nutritional intervention with a new energy-restricted dietary strategy (RESMENA) during 8 weeks on improving anthropometrical, biochemical and inflammation markers of obese subjects presenting MetS manifestations and to compare the outcomes with the AHA dietary pattern, assessing some possible mechanisms underlying the actual effects.

2.2.2. Specific aims

The study was devised to achieve the following specific aims:

- To evaluate changes on weight and body composition as well as on lipid and glucose metabolisms throughout the nutritional intervention and to assess the differences on effectiveness between the two dietary hypocaloric treatments.

- To examine changes on the obesity-associated inflammatory status throughout the nutritional trial and to assess the potential differences between both dietary strategies.

- To determine the potential influence of specific dietary components included on the dietary patterns on the modification of anthropometrical, biochemical and inflammation markers.

- To further explore the specific role of protein intake (amount and type) within both dietary approaches on the obesity-related inflammatory status.

- To investigate the influence of the recently discovered hormone irisin on anthropometrical and biochemical measurements.

- To research the potential role of methylation levels of the *SERPINE1* gene, which codes for PAI-1, as a tool to better understand the influence of weight-reducing approaches on the epigenetic mechanisms implicated on obesity.

Hipótesis

La obesidad se ha convertido en una importante carga para salud pública, siendo las estrategias dietéticas el tratamiento más empleado tanto para su prevención, como para su tratamiento. En este sentido, la nueva estrategia dietética RESMENA (REduccion del Síndrome MEtabólico en NAvarra), basada en la alimentación tradicional, con un perfil de macronutrientes modificado (40% de calorías totales en forma de hidratos de carbono, 30% en forma de proteínas y 30% en forma de lípidos), una frecuencia de tomas aumentada (7 al día), un incremento del contenido de compuestos bioactivos, como ácidos grasos omega-3, favoreciendo alimentos con bajo índice/carga glucémica y con una elevada capacidad antioxidante y adherencia a la Dieta Mediterránea podría ser efectiva para mejorar los parámetros de adiposidad, bioquímicos e inflamatorios de sujetos obesos con manifestaciones de síndrome metabólico, en comparación a un patrón basado en la recomendaciones de la Asociación Americana del Corazón (AHA), que incluye una distribución de macronutrientes de 55% de las calorías totales en forma de hidratos de carbono, 15% en forma de proteínas y 30% en forma de lípidos, con un frecuencia de tomas de entre 3 y 5 al día.

Objetivos

Objetivo general

Este trabajo ha sido diseñado con el fin de evaluar la eficacia de una intervención nutricional con una nueva estrategia dietética hipocalórica (RESMENA) durante 8 semanas en la mejora de marcadores antropométricos, bioquímicos e inflamatorios de sujetos obesos con signos de síndrome metabólico y para comparar su efectividad con la del patrón dietético propuesto por la AHA, analizando posibles mecanismos implicados en los efectos observados.

Objetivos específicos

El estudio fue ideado para alcanzar los siguientes objetivos específicos:

- Evaluar cambios en el peso y en la composición corporal, así como en los metabolismos lipídico y glucídico a lo largo de la intervención y analizar las posibles diferencias en cuanto a efectividad de los dos tratamientos dietéticos hipocalóricos.

- Examinar los cambios en el estado inflamatorio asociado a la obesidad a lo largo de la intervención nutricional y evaluar las posibles diferencias entre las dos estrategias dietéticas.

- Evaluar la posible influencia de componentes dietéticos específicos incluidos en los patrones dietéticos analizados en la modificación de los parámetros antropométricos, bioquímicos e inflamatorios analizados.

- Explorar más a fondo el papel específico de la ingesta y tipo de proteína en ambas dietas en el estado inflamatorio asociado a la obesidad.

- Investigar la influencia de la hormona irisina, recientemente descubierta, en las determinaciones antropométricas y bioquímicas.

- Valorar el potencial papel de los niveles de metilación del gen *SERPINE*, que codifica para PAI-1, como herramienta de cara un mejor pronóstico del efecto de las estrategias dietéticas para la pérdida de peso en los mecanismos epigenéticos implicados en la obesidad.

3. METHODS

3.1. Study design

In order to test the laid down hypothesis and achieve the proposal goal a randomized intervention trial of 6-months duration was designed, in which the effects on obese participants with MetS of two different dietary strategies were evaluated and compared. The RESMENA trial was devised in two sequential periods. In the first 2-month intervention participants received nutritional assessment with fortnightly follow-up visits at the Metabolic Unit of the University of Navarra. Subsequently, participants underwent a 6-month self-control period in which they had to continue with the previously learnt habits. For this thesis work, results from the intervention period were considered.

In every visit, weight and waist and hip circumferences were taken, together with skinfold thickness, body composition by bioelectrical impedance (Tanita SC-330, Tanita corp, Japan) and blood pressure. In addition to these measurements, at the beginning and at the end of each period body composition by DXA (Lunar Prodigy, software version 6.0, Madison, WI) was also assessed and blood and urine samples collected. In order to evaluate dietary intake 48-h food records were performed at baseline and at the endpoint of each period. The study was approved by the Ethics Committee of the University of Navarra (065/2009) and appropriately registered at www.clinicaltrials.gov; NCT01087086. Consequently, all the participants gave written informed consent for participation in agreement with the Declaration of Helsinki. This work was performed following the Consolidated standards of reporting trials (CONSORT) 2010 guidelines (Turner *et al.* 2012).

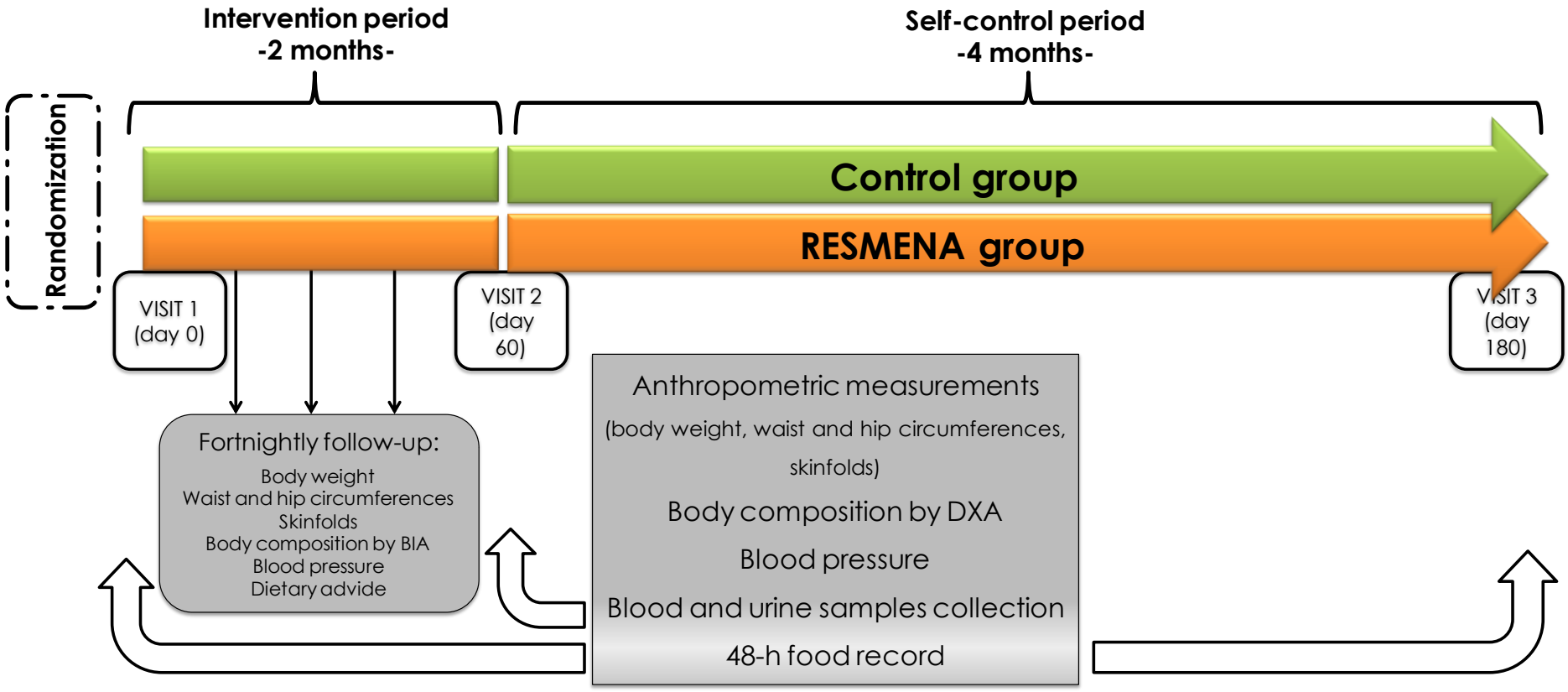

Figure 10. Chronogram of the study representing the Intervention and Self-control periods as well as the measurements taken at each point. DXA: dual-energy X-ray absorptiometry; BIA: bioimpedance.

3.2. Subjects

3.2.1. Inclusion criteria

The IDF definition for MetS was considered as inclusion criteria (Alberti *et al.* 2009) (Table 3). Contrariwise, psychiatric and eating disorders, chronic diseases related to nutrients metabolism, major body weight changes (≥3kg) in the last three months, as well as difficulties for changing food habits were taken into consideration as exclusion criteria (Table 3).

Table 3: Inclusion and exclusion criteria applied in the study.

INCLUSION CRITERIA	Presence of central obesity: ≥90 cm in men ≥80 cm in women Together with 2 or more of the following: o TG > 150 mg/dl or specific treatment for hypertriglyceridemia o HDL-c <40 mg/dl for men and < 50 mg/dl for women or specific treatment o SBP ≥130 mmHg or DBP ≥85 mmHg or treatment for HT previously diagnosed o Fasting glucose >100 mg/dl or type 2 DM previously diagnosed	Presence of : o Psychiatric disturbances o Eating disorders o Chronic diseases related with the metabolism of nutrients o Major body weight changes in the last three months o Difficulties in changing food habits	EXCLUSION CRITERIA

TG: triglycerides; HDL-c: high density lipoprotein-cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; HT: hypertension; DM: diabetes mellitus.

3.2.2. Flowchart

Participants were recruited from the Nutrition, Food Science and Physiology Department database and through advertisements in local newspapers. Approximately 300 subjects contacted us after the advisement period. From them, 180 people were appointed for the screening visit. Once applied the inclusion criteria 109 subjects were eligible for participating in the study and finally, among them, 105 volunteers started the intervention, being randomly assigned to the Control or the RESMENA group. Nine participants during the 2-month intervention period and 19 during the 4-month self-control period dropped out the study. Analyses for this thesis work were performed considering the 96 subjects that finished the intervention period.

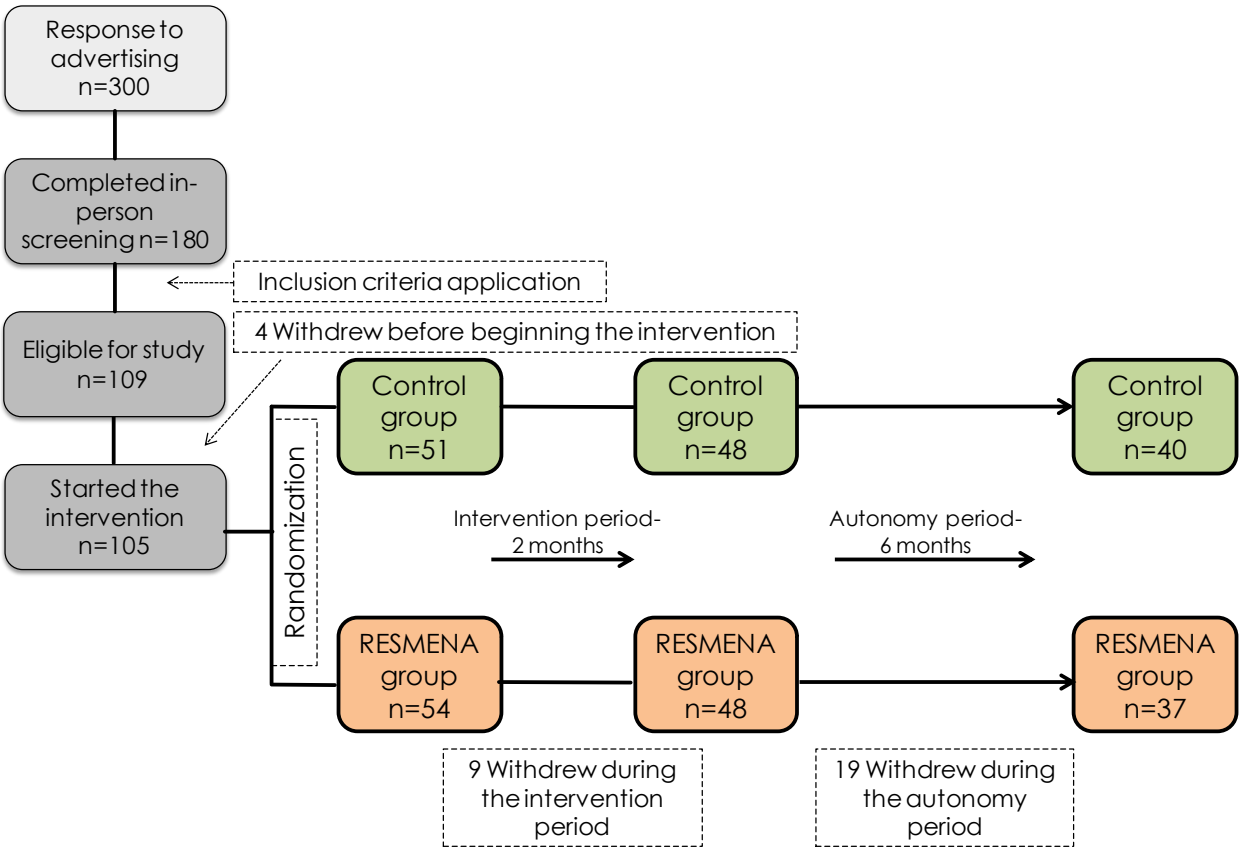

Figure 11. Flowchart of the study subjects from advertisement through to the end of the study.

3.3. Dietary strategies

Two energy-restricted dietary strategies were prescribed and compared. The Control diet was designed following the AHA guidelines (Krauss *et al.* 2000) which is considered as a reference in the treatment of obesity and related comorbidities. The second diet, named as RESMENA diet, was designed taking into consideration the last advances in nutritional research regarding macronutrient distribution and dietary bioactive compounds, such as omega-3 PUFA, as well as increasing meal frequency, promoting high dietary TAC and low GI/GL food and enhancing the adherence to the MedDiet (Zulet *et al.* 2011). Main characteristics of the two prescribed diets are listed in table 4. Diet composition together with 48-h food records were evaluated using the DIAL software (Alce ingenieria, Madrid, Spain).

Table 4: Nutritional profiles of the two dietary strategies evaluated and compared in the study.

	CONTROL DIET	RESMENA DIET
Energy	-30%	-30%
Carbohydrates (%TCV)	50-55	40
Protein (%TCV)	15	30
Total fats (%TCV)	30	30
SFA (%TCV)	<10	<10
MUFA (%TCV)	10-15	10-15
PUFA (%TCV)	8-10	8-10
Cholesterol (mg/day)	<300	<300
Fiber (g/day)	20 - 25	20 - 25
Meal frequency	3-5	7
Other components		High adherence to the Mediterranean diet High dietary total antioxidant capacity Low glycemic index/load

TCV: total caloric value; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

3.4. Samples collection, biochemical determinations and methylation analysis

Blood samples were taken at baseline and at the endpoint of each period after fasting overnight, as described elsewhere (Abete *et al.* 2008). Samples were processed as reported in figure 12, and storage at -80 ºC. Twenty-four hour-urine samples were collected the day before to the corresponding visit, including the last micturition in the morning before coming. Participants were asked to save urine in freezer bags and until the moment to be processed and storage at -80 ºC.

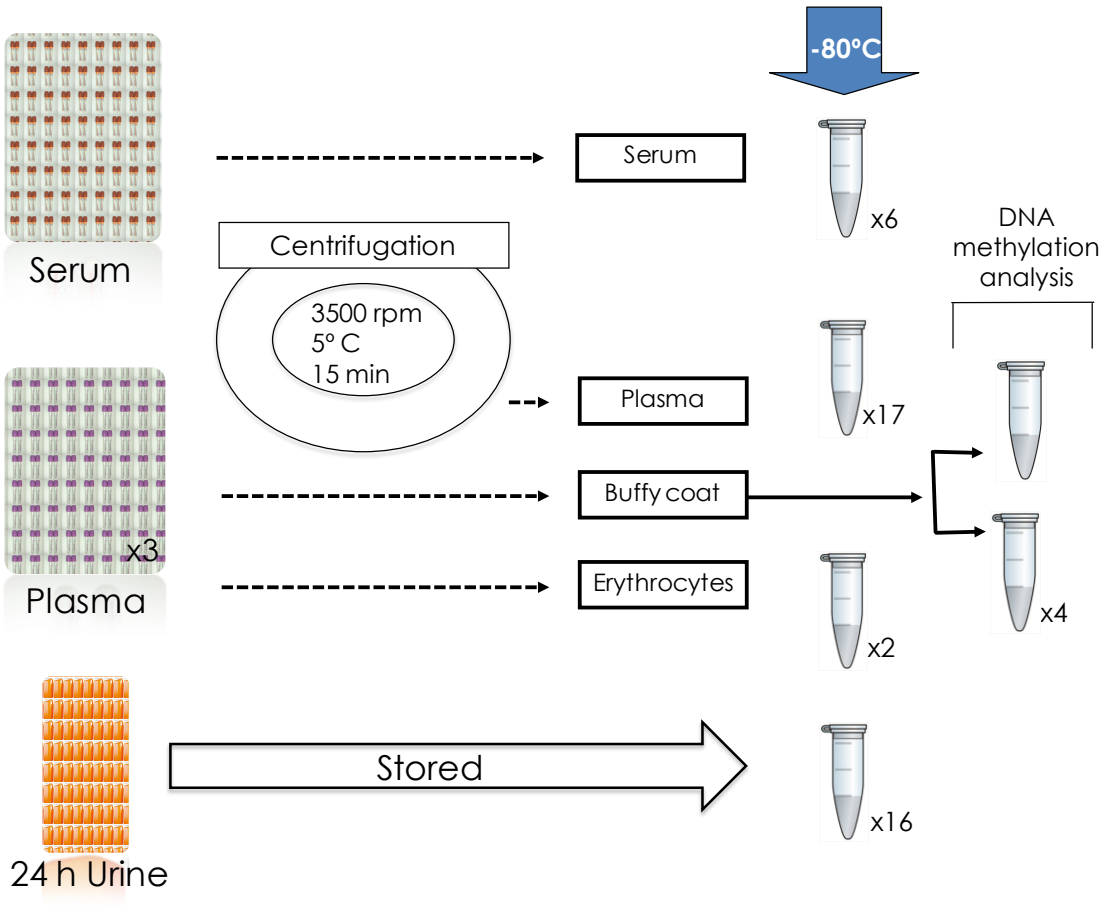

Figure 12. Blood and urine samples processing and storage.

Regarding biochemical analyses, glucose, total cholesterol, HDL-c, triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) serum concentrations were measured with specific kits in an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain), as previously described (Hermsdorff *et al.* 2011). Insulin levels were determined with an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was estimated by the HOmeostasis Model Assessment for insulin resistance (HOMA-IR) index {HOMA-IR = [glucose (mmol/L) × insulin (μU/ml)] / 22.5} (Aller *et al.* 2011). Low-density lipoprotein-cholesterol (LDL-c) levels were calculated following the Friedewald formula: LDL-c = TC-HDL-c – TG/5 (Friedewald *et al.* 1972). Apolipoproteins AI and B were measured with specific kits (Tina-quant Apolipoprotein A-I ver.2 and Tina-quant Apolipoprotein B ver.2) using an autoanalyzer Roche/Hitachi 904/911/912/917/MODULAR (Wang *et al.* 2011).

Plasma concentrations of hsCRP (Demeditec), IL-6 (R&D Systems) TNF-α (R&D Systems) and PAI-1 (BioVendor) were measured with specific enzyme-linked immunosorbent assay kits from specified suppliers using an autoanalyzer system (Triturus, Grifols SA, Barcelona, Spain) according to the manufacturer's instructions (Richard *et al.* 2012).

Leptin and adiponectin were measured using a specific enzyme-linked immunosorbent assay (ELISA) kit (Millipore, MA, USA) (Crujeiras *et al.* 2010). Irisin concentrations were determined in human plasma using a commercial ELISA kit directed against amino acids 31-143 of FNDC5 protein (Irisin ELISA kit EK-067-52; Phoenix Pharmaceuticals, INC, CA), following the manufacturer's instructions. Absorbance from each sample was measured in duplicate using a spectrophotometric microplate reader at wavelength of 450 nm (Versamax Microplate Reader, Associates of Cape Cod Incorporated, East Falmouth, MA) (Roca-Rivada *et al.* 2013).

In relation to urine samples, although no analyses have been performed so far, they will be use for the assessment of metabolomic markers in the near future.

Genomic DNA from white blood cells (WBC) was extracted using the Master Pure kit (Epicenter, Madison, WI, USA), whose quality was assessed with PicoGreen dsDNA Quantitation Reagent (Invitrogen, Carlsbad, CA, USA). A total of 500 ng of

DNA was modified by using EZ-96 DNA Methylation Kit (Zymo Research Corporation, USA) according to the manufacturer's instructions, converting thus cytosine into uracil (Mansego *et al.* 2013). Array-based specific DNA methylation analysis was performed with the Infinium Human Methylation 450K bead chip technology (Illumina, USA). Bisulfite-treated genomic DNA was whole-genome amplified, hybridized to HumanMethylation450 BeadChips (Illumina, USA) 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, USA) (Mansego *et al.* 2013).

3.5. Statistical analyses

Statistical analyses were carried out using SPSS 15.1 software for Windows (SPSS Inc, Chicago, USA). The sample size of this study was calculated for an α=0.05 and a power of 80% based on the waist circumference reduction. Values of $p < 0.05$ were considered as statistically significant and the results are expressed as mean ± SD or median (interquartile range), according to variable distribution, which was determined by the Shapiro-Wilk test. To compare the changes due to the treatment before and after each period a paired *t-test* was used. Only those completing the study were analised. The between group analysis comparing both dietary treatments (Control *vs.* RESMENA) was performed applying an independent measures *t-test.* In order to evaluate potential relationships and associations between variables linear and multiple regression models were performed, including as co-variables those estimated necessary for adjusting the model. Two-way ANOVA tests were carried out in order to assess diet and sex interactions. Pearson correlations were fitted to evaluate the potential correlations between dietary components and anthropometric, biochemical and inflammatory markers, as well as, mehtylation levels (Martinez-Gonzalez *et al.* 2006).

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4. RESULTS

4.1. CHAPTER 1

Short-term role of the dietary total antioxidant capacity in two hypocaloric regimes on obese with metabolic syndrome symptoms: the RESMENA randomized controlled trial

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Abstract

Background

Dietary strategies seem to be the most prescribed therapy in order to counteract obesity regarding not only calorie restriction, but also bioactive ingredients and the composition of the consumed foods. Dietary total antioxidant capacity (TAC) is gaining importance in order to assess the quality of the diet.

Methods

Ninety-six obese adults presenting metabolic syndrome (MetS) symptoms completed an 8-week intervention trial to evaluate the effects of a novel dietary program with changes in the nutrient distribution and meal frequency (RESMENA diet) and to compare it with a dietary pattern based on the American Heart Association (AHA) guidelines. Anthropometric and biochemical parameters were assessed at baseline and at the endpoint of the study, in addition to 48-hours food dietary records.

Results

Both diets equally ($p > 0.05$) improved MetS manifestations. Dietary TAC was the component which showed the major influence on body weight ($p = 0.034$), body mass index ($p = 0.026$), waist circumference ($p = 0.083$) and fat mass ($p = 0.015$) reductions. Transaminases (ALT and AST) levels ($p = 0.062$ and $p = 0.004$, respectively) were associated with lower TAC values.

Conclusion

RESMENA diet was as effective as AHA pattern for reducing MetS features. Dietary TAC was the most contributing factor involved in body weight and obesity related markers reduction.

Trial registration

http://www.clinicaltrials.gov; NCT01087086

Keywords: Antioxidant, Weight loss, Energy restriction, Macronutrient distribution, Dietary components, Nutritional profile

Background

The World Health Organization (WHO) estimates that at least 300 million people are obese nowadays (Ejike and Ijeh 2012). Obesity, is strongly associated with comorbidities such as impaired glucose tolerance or diabetes, insulin resistance, dyslipidemia, hypertension, nonalcoholic fatty liver disease, hyperuricemia, and prothrombotic and proinflammatory states, which are related to the onset of metabolic syndrome (MetS) (Marchetti *et al.* 2012; Hermsdorff *et al.* 2009, Eckel *et al.* 2005). Also according to the WHO estimations, obesity prevalence rates will tend to increase in the next years. So that new effective proposals are need in order to prevent/counteract the obesity onset and spread.

Dietary strategies are one of the most prescribed therapies to prevent/counteract overweight and obesity (Aller *et al.* 2011). While dietetic programs have traditionally focused on calorie restriction, new nutritional alternatives are nowadays being investigated. They entail macronutrient distribution (Larsen *et al.* 2010), meal frequency (Cameron *et al.* 2009), consumption of bioactive ingredients, such as fiber (Grube *et al.* 2012) and n-3 fatty acids (Peairs *et al.* 2011), glycemic index (GI)/glycemic load (GL) (Gogebakan *et al.* 2011) or the dietary total antioxidant capacity (TAC) (Abete *et al.* 2010). Dietary TAC is considered an appropriate approach to measure the cumulative antioxidant properties of food (Pellegrini *et al.* 2006), despite its controversial use at evaluating the role of antioxidants *in vivo* (Ghiselli *et al.* 2000). Oxidative stress is suggested to be involved in the onset of several obesity-related disorders such as hypertension, dyslipidemia, type-2 Diabetes Mellitus and MetS (Abete *et al.* 2010). In this context, dietary TAC is gaining importance as a valuable tool to investigate the relationship between diet and oxidative stress-related diseases (Puchau *et al.* 2009). Furthermore, the influence of the dietary TAC has been poorly investigated in the context of MetS.

Many studies have separately examined the impact of different dietary components, such as macronutrient distribution (Abete and Martinez 2009), meal frequency (Cameron *et al.* 2009), fiber (Grube *et al.* 2012), n-3 fatty acids (Ramel *et al.* 2010), GI/GL (Goto *et al.* 2012) or dietary TAC (Del Rio *et al.* 2011). However, to date, they have not been integrated together on a dietetic plan based on habitual foods intake to combat excessive fat deposition. In this context, the RESMENA-S (MEtabolic

Syndrome REduction in NAvarra-Spain) study (http://www.clinicaltrials.gov; NCT01087086) (Zulet *et al.* 2011) aimed at evaluating the effect of a novel dietary strategy involving a modified macronutrient distribution, higher meal frequency, increased fiber and n-3 fatty acids consumption, low GI/GL and high TAC food and at comparing it with the American Heart Association (AHA) guidelines, which is currently considered as a reference dietary pattern to reduce fat mass content and improve MetS markers (Krauss et al. 2000).

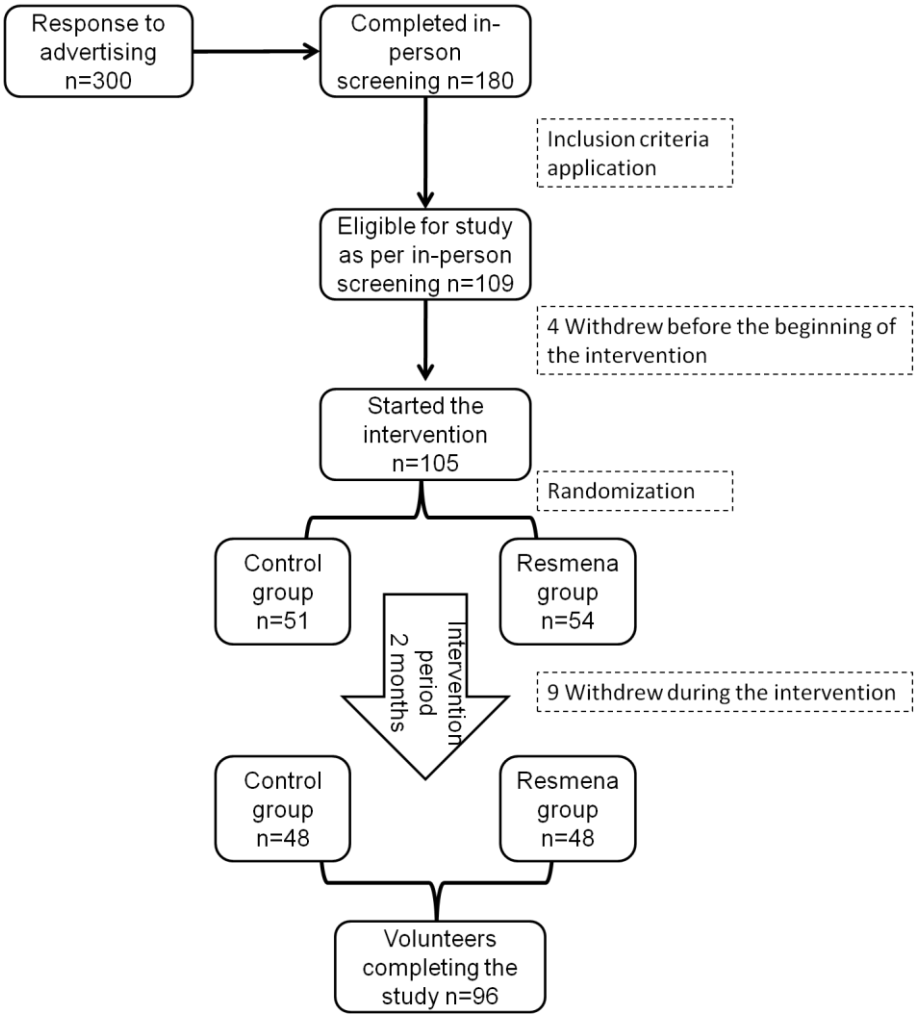

Figure 1. Flowchart of the study subjects from advertisement through to the end of the 8-week intervention.

Methods

Study population

One hundred and five (56 Male and 49 Female) caucasian adults (49 ± 10 years old) presenting obesity determined by a Body Mass Index (BMI) higher than 30 Kg/m^2 (mean BMI = 35.85 ± 4.67 kg/m^2) and at least two MetS signs according to the International Diabetes Federation criteria (Alberti *et al.* 2005) were enrolled in the study and 96 of them completed the trial (Figure 1). The presence of psychiatric disturbances, eating disorders, chronic diseases related with the metabolism of nutrients, major body weight changes in the last three months and difficulties in changing food habits were considered as exclusion criteria. Subjects were recruited through local newspaper advertisements and the Department database. All subjects gave written informed consent (http://www.clinicaltrials.gov; NCT01087086) as approved by the Ethics Committee of the University of Navarra (065/2009) and in accordance with the Declaration of Helsinki. There were 9 dropouts along the study period. Baseline characteristics are presented in (Table 1).

Table 1. Selected anthropometric characteristics of the whole sample and cathegorized by gender at baseline.

Variable	Total (n=96)	Male	Female	p
Sex	-	51	45	-
Age (years)	49±10	48±9	50±10	0.194
Weight (kg)	99.73±17.85	108.28±15.94	90.03±14.76	<0.001
Height (m)	1.67±0.11	1.74±0.08	1.58±0.07	<0.001
BMI (kg/m^2)	35.84±4.67	35.75±4.38	35.96±5.02	0.822
Waist circumference (cm)	111.10±12.80	116.27±10.04	105.24±13.16	<0.001
Waist/Hip ratio	0.96±0.10	1.03±0.07	0.89±0.08	<0.001

BMI: Body Mass Index.
For the sex variable it is reported the frequency of men and females.
Mean and standard deviation data are shown concerning the remaining variables.
p-value: Comparison between men and women baseline characteristics.
$p < 0.05$ was set-up as statistically significant.

Study protocol

A randomized, controlled trial was designed to compare the effect of two dietary strategies for weight loss with different macronutrient distribution on anthropometric measurements and biochemical markers in obese subjects with MetS manifestations. Participants were randomly assigned to the control or the experimental diet (Figure 1). The study was of six months duration in two sequential periods: one intervention period of 8 weeks in which subjects received nutritional assessment every 15 days followed by a self-control period of 4 months in which subjects followed the first period learned-habits. This work reports on the 8-weeks findings.

At each visit, anthropometric assessments and body composition by bioimpedance were measured. Fasting blood and 24-h urine samples were collected and body composition by Dual-energy X-ray Absorptiometry (DXA) was measured at baseline and at the endpoint of the intervention period.

Diets

Two energy-restricted diets were prescribed and compared. An energy restriction of 30% was applied to the total energy requirements of each patient. Resting metabolic rate was calculated using the Harris-Benedict equation where the Wilkens adjusted weight was applied. Then, physical activity factor was considered in order to calculate total energy requirements according to the "Food and Nutrition Board, National Research Council: Recommended Dietary Allowances: 10th ed." (Council 1989). The Control diet was based on the AHA guidelines (Krauss *et al.* 2000), including 3–5 meals/day, a macronutrient distribution of 50–55% total caloric value from carbohydrates, 15% from proteins and 30% from lipids, a healthy fatty acids profile, an intake of fiber of 20–25 g/day and a cholesterol recommendation of < 300 mg/day (Table 2). The RESMENA diet was composed of 7 meals/day including breakfast, lunch, dinner, two snacks in the morning and other two in the afternoon. The macronutrient distribution was as following: 40% total caloric value from carbohydrates, 30% from proteins and 30% from lipids. This pattern also maintained a healthy fatty acids profile, an input of fiber of 20–25 g/day and a cholesterol content of < 300 mg/day. It included an increased input of n-3 fatty acids, an increased amount of natural antioxidants and focused on low GI/GL carbohydrates (Table 2).

Table 2. Comparisons of the habitual intake, the scheduled diets, the final intake and the adherence.

	Control group (n=48)			RESMENA group (n=42)					
	Habitual intake (day 0)	**Scheduled diet**	**Final intake (day 60)**	**Habitual intake (day 0)**	***p*[a]**	**Scheduled diet**	***p*[b]**	**Final intake (day 60)**	***p*[c]**
Energy (kcal/day)	2103±451	1412±177	1352±284	2277±566	0.099	1395±188	0.649	1337±289	0.808
CHO (g/day)	186.74±58.90 (35.52%)	178.58±20.15 (50.59%)	132.37±35.33$^{\$\$\$}$ (39.16%)	201.66±65.30 (35.43%)	0.243	128.65±15.97 (36.89%)	<0.001	114.55±31.10$^{\$\$}$ (34.26%)	0.013
Protein (g/day)	93.58±21.63 (17.80%)	57.01±5.78 (16.14%)	60.83±17.16 (18.00%)	95.01±20.06 (16.69%)	0.738	99.54±13.43 (28.54%)	<0.001	78.20±17.46$^{\$\$\$}$ (23.39%)	<0.001
PQ	0.30±0.05	0.31±0.01	0.30±0.05$^{\$}$	0.30±0.05	0.594	0.34±0.01	<0.001	0.28±0.05$^{\$\$\$}$	0.070
Protein/CHO	0.54±0.18	0.32±0.01	0.48±0.18$^{\$\$\$}$	0.50±0.13	0.224	0.77±0.01	<0.001	0.70±0.15$^{\$\$}$	<0.001
Total fats (g/day)	97.29±27.00 (41.64%)	46.02±8.69 (29.33%)	59.09±15.51$^{\$\$\$}$ (39.33%)	110.26±31.84 (43.58%)	0.034	46.98±7.07 (30.31%)	0.554	56.58±17.28$^{\$\$\$}$ (38.08%)	0.497
SFA (g/day)	28.89±10.13 (12.36%)	14.02±2.44 (8.94%)	14.48±5.47 (9.64%)	34.85±13.64 (13.76%)	0.017	12.70±1.86 (8.19%)	0.004	16.08±5.80$^{\$\$\$}$ (10.82%)	0.181
MUFA (g/day)	47.35±12.76 (20.26%)	20.59±4.51 (13.12%)	32.25±9.18$^{\$\$\$}$ (21.47%)	51.90±15.42 (20.52%)	0.118	18.96±3.11 (12.23%)	0.042	24.65±7.80$^{\$\$\$}$ (16.59%)	<0.001
PUFA (g/day)	12.58±3.80 (5.38%)	7.54±1.20 (4.81%)	7.65±2.87 (5.09%)	13.92±3.79 (5.50%)	0.088	10.77±1.31 (6.95%)	<0.001	10.91±5.56 (7.34%)	0.001
n3-FA	0.46±0.54	0.20±0.05	0.20±0.44	0.28±0.43	0.079	0.83±0.30	<0.001	0.37±0.49$^{\$\$\$}$	0.096
Cholesterol (mg/day)	363.43±142.46	157.94±18.44	206.68±140.82$^{\$\$\$}$	424.43±162.31	0.053	275.75±46.51	<0.001	241.21±105.00	0.196
Fiber (g/day)	20.07±8.77	27.57±1.33	18.75±8.50$^{\$\$\$}$	21.66±9.69	0.400	22.84±3.48	<0.001	20.61±9.01	0.316
Glycemic Index (U)	579.55±179.16	487.29±12.21	367.48±131.06$^{\$\$\$}$	685.13±226.21	0.013	332.01±38.04	<0.001	326.70±121.11	0.131
Glycemic Load (U)	105.21±43.52	63.77±5.47	70.24±35.70	117.32±43.92	0.178	43.37±5.04	<0.001	52.03±26.40$^{\$}$	0.008
TAC (mmol/day)	7.36±3.66	10.85±0.36	8.88±2.72$^{\$\$\$}$	8.22±4.41	0.302	17.09±0.62	<0.001	13.90±5.05$^{\$\$\$}$	<0.001
HEI score	60.28±11.92	86.21±0.99	70.90±12.75$^{\$\$\$}$	56.05±11.11	0.075	91.39±1.80	<0.001	74.41±10.07$^{\$\$\$}$	0.836
MF (meals/day)	4.65±0.59	3-5	4.59±0.67	5.30±1.25	0.001	7	N.A	6.51±1.06	<0.001

CHO, Carbohydrates; PQ, Protein Quality; SFA, Saturated Fatty Acids; MUFA, Mono Unsaturated Fatty Acids; PUFA, Poly Unsaturated Fatty Acids; n-3 FA, n-3 Fatty Acids; TAC, Total Antioxidant Capacity; HEI, Healthy Eating Index; NA, Not Applicable. $p < 0.05$ was set-up as statistically significant. p^a Differences between Control and RESMENA groups intake before starting the intervention. p^b Differences between Control and RESMENA scheduled diets. p^c Differences between Control and RESMENA groups intake at the end of the intervention. $^{\$}$ Differences between the scheduled diets and the real intake at final day in both Control and Resmena groups. $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$.

Participants were provided a 7-day menu plan in the RESMENA group and a food exchange system plan in the Control group, as previously described (Hermsdorff *et al.* 2009). Usual diet was assessed with a semiquantitative 136-item food frequency questionnaire previously validated in Spain for energy and nutrient intake (Puchau *et al.* 2009). A 48-hour weighed food record was required at the beginning and at the end of the study. Diet composition was analyzed using the DIAL software (Alce Ingenieria, Madrid, Spain). The sum of eicosapentaenoic fatty acid and docosahexaenoic fatty acid (EPA + DHA) obtained by the DIAL program was used to estimate n-3 fatty acids consumption. The Healthy Eating Index (HEI) was calculated using also the DIAL software as described elsewhere (Basiotis 2002). The program gives different values between 0 and 100 considering the servings per day of cereals, vegetables, fruits, dairy products and meat. It also takes into account the percentage of energy provided by total and saturated fats, the amount of cholesterol and sodium per day and the variety of the diet. The final score was classified in five categories: > 80 points indicates "excellent diet"; 71–80 points = "very good diet"; 61–70 points = "good diet"; 51–60 = "acceptable diet" and 0–50 points = "inadequate diet". Protein Quality (PQ) was defined as the ratio of essential amino acid to total dietary protein (Loenneke *et al.* 2012). Dietary TAC was calculated using the list from (Carlsen et al. 2010), which takes into consideration raw or cooked food preparations. They provide a list of the total antioxidant content (mmol/100 g) of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. The TAC value corresponding to the different scheduled/ingested servings per day was calculated. GI and GL were obtained from (Foster-Powell *et al.* 2002) (University of Sydney updated website database 2012).

Participants were asked to maintain their normal physical activity during the study, estimated with a 24-h recall at the beginning and the end of the intervention.

Anthropometric and biochemical assessments

Anthropometric measurements (body weight, height, waist and hip circumferences) were carried out with the subjects in their underwear. Body weight was measured to the nearest 0.1 kg using a Tanita SC-330, (Tanita corp, Japan). Height was estimated with a stadiometer (Seca 713 model, Postfach, Germany) to the nearest 1 mm. BMI was calculated as the body weight divided by the squared height (kg/m^2). Waist and hip circumferences were measured with a commercial measure tap following

validated protocols (Zulet *et al.* 2011). Total body fat was measured by a bioelectric impedance Tanita SC-330 (Tanita corp, Japan) and by DXA (Lunar Prodigy, software version 6.0, Madison, WI) as described elsewhere (Zulet *et al.* 2011).

Glucose, total cholesterol, high density lipoprotein-cholesterol (HDL-c), triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) 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 kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was estimated by the HOMA index {HOMA-IR = [glucose (mmol/L) × insulin (μU/ml)]/22,5} (Aller *et al.* 2011). Low-density lipoprotein-cholesterol (LDL-c) levels were calculated following the Friedewald formula: LDL-c = TC-HDL-c - VLDL (Gogebakan *et al.* 2011).

Statistical analyses

The results are expressed as mean ± SD. Normality distributions of the measured variables were determined according to the Shapiro–Wilk test. Differences between the beginning and the end of the period were analyzed by a paired *t*-test. Only those completing the study were analysed. The analysis between both diets (RESMENA *vs.* Control) was performed through an independent measures *t*-test. A linear regression analysis was applied to assess the potential relationships and associations among the different components of the diet and anthropometrical and biochemical parameters variation. Chi-square test was carried out to compare the percentage of participants from Control and RESMENA dietary groups included in the high-TAC group. A two-way ANOVA was performed in order to assess diet and sex interactions. Analyses were carried out using SPSS 15.1 software for Windows (SPSS Inc, Chicago, USA). Values of $p < 0.05$ were considered as statistically significant.

Results

Food intake assessment

There were available data about food intake of 90 participants (48 from Control group and 42 from RESMENA group). No differences were found in food energy intake between the experimental groups at the study baseline, except for total fat and saturated fat intake, GI and meal frequency (Table 2).

The RESMENA group reported a significantly higher protein and lower carbohydrate intake and the protein/carbohydrate ratio was also higher in this group at the end of the study (Table 2). There were no significant differences between groups either for PQ or total fat intake, but significant differences were found regarding fatty acids profile (Table 2). There were no significant differences in cholesterol intake after the intervention. Concurrently, no differences were found in fiber intake, neither in GI or HEI score. GL was significantly lower ($p = 0.008$) and TAC significantly higher ($p < 0.001$) in RESMENA group, with respect to the AHA group. As designed, consuming the RESMENA diet had an average intake (6.5 meals/day) significantly higher than the control one (4.5 meals/day).

The analysis of 48-h food records showed that in both groups the energy intake was in accordance with the prescribed patterns (Table 2). Protein, saturated fatty acid, polyunsaturated fatty acid (PUFA) and n-3 fatty acids intakes, as well as GL and meal frequency of the Control diet were in agreement with the scheduled pattern (Table 2). RESMENA group adjusted to PUFA, cholesterol and fiber intake and also the GI and meal frequency. RESMENA group did not reach completely the expected values of some components although it achieved an improvement comparing to baseline values.

Anthropometric and biochemical parameters

Energy restriction resulted in a mean body weight loss of 6.73 ± 0.71 kg in the Control diet and 7.09 ± 0.82 kg in the RESMENA diet, with no statistical differences between groups (Table 3). Consequently, BMI was significantly lowered in both groups. Every anthropometrical parameter was significantly reduced after the slimming treatments, with no differences between both dietary groups (Table 3).

Table 3. Changes in selected parameters in Control and RESMENA groups after 8 weeks of nutritional intervention.

Variable	Control group (n=48)			Resmena group (n=48)			
	Visit 1 (day o)	Visit 2 (day 60)	p^a	Visit 1 (day o)	Visit 2 (day 60)	p^b	p^c
Weight (kg)	99.45±19.21	92.72±18.50	<0.001	100.00±16.58	92.91±15.76	<0.001	0.555
BMI (kg/m²)	36.15 ± 4.95	33.70±4.80	<0.001	35.55±4.40	33.03±4.24	<0.001	0.732
Waist circumference (cm)	110.94±13.41	104.18±12.29	<0.001	111.25±12.30	103.78±11.66	<0.001	0.432
Waist/hip ratio	0.96±0.10	0.94±0.09	<0.001	0.96±0.10	0.93±0.10	<0.001	0.355
Bioimpedance Fat mass (kg)	38.97±10.87	33.68±10.22	<0.001	39.23±9.50	33.84±9.09	<0.001	0.886
DXA Fat mass (kg)	42.13±10.18	37.50±10.39	<0.001	42.56±9.18	37.30±8.95	<0.001	0.208
TC (mg/dl)	221±39	204±39	0.001	219±48	203±46	0.020	0.943
HDL-c (mg/dl)	46±10	42±9	0.001	43±10	41±10	0.050	0.158
LDL-c (mg/dl)	140±36	133±35	0.085	137±41	131±40	0.374	0.888
TC/HDL-c ratio	4.94±1.02	4.96±0.94	0.856	5.18±1.24	5.04±1.24	0.398	0.419
LDL-c/HDL-c ratio	3.09±0.77	3.22±0.78	0.178	3.20±0.83	3.23±0.92	0.803	0.623
TG (mg/dl)	176±10	145±70	0.005	194±123	151±99	<0.001	0.421
Glucose (mg/dl)	121.02±33.87	107.98±13.71	0.006	123.81±37.82	110.22±26.18	0.016	0.939
Insulin (µU/ml)	15.30±11.46	9.32±7.18	<0.001	14.36±8.30	9.14±6.13	<0.001	0.557
HOMA index	4.69±3.77	2.61±2.31	<0.001	4.48±3.01	2.60±2.00	<0.001	0.686
ALT (U/L)	37.60±21.04	27.03±10.70	<0.001	29.13±11.59	29.28±14.20	0.936	0.001
AST (U/L)	25.81±10.84	20.68±6.18	0.001	21.90±6.01	22.20±6.14	0.740	0.002

BMI, Body Mass Index; DXA, Dual-energy X-ray Absorptiometry; HDL-c, High Density Lipoprotein-cholesterol; LDL-c, Low Density Lipoprotein-cholesterol; TG,Triglycerides; HOMA, Homeostasis Model Assessment; ALT, Alanine aminotransferase; AST, Aspartate aminotranferase. $p < 0.05$ was set-up as statistically significant. p^a differences between baseline and final values in Control group. p^b differences between baseline and final values in RESMENA group. p^c differences between changes in Control group as compared with RESMENA group at the end of the intervention.

Control and RESMENA groups significantly reduced total cholesterol and triglycerides but there was no significant reduction in LDL-c. Only the AHA group had a significant decrease of the HDL-c values ($p = 0.001$). No changes were observed in the atherogenic ratios TC:HDL-c and LDL-c:HDL-c in none of the groups. Both groups significantly improved glucose metabolism (Table 3). The Control diet, but not the RESMENA one induced a significant reduction in ALT ($p < 0.001$ *vs.* $p = 0.936$) and AST ($p = 0.001$ *vs.* $p = 0.740$) transaminases levels, obtaining significant differences

among groups (Table (Table 3). No differences between groups were found in any of the other biochemical parameters (Table 3).

Dietary TAC was the major influential factor, as body weight ($p = 0.034$), BMI ($p = 0.026$) and fat mass ($p = 0.015$) were significantly improved by this variable (Table 4). Dietary TAC also seemed to have an effect on AST ($p = 0.004$) and a potential effect on ALT variations ($p = 0.062$). Concerning GI/GL, a trend towards an influence of insulin was found (Table 4). Since dietary TAC seemed to be the most influencing variable among the dietary analyzed elements, we categorized the sample considering the median value in: high- TAC (> 10.629 mmol day^{-1}) or low- TAC (<10.629 mmol day^{-1}) (Figure 2).

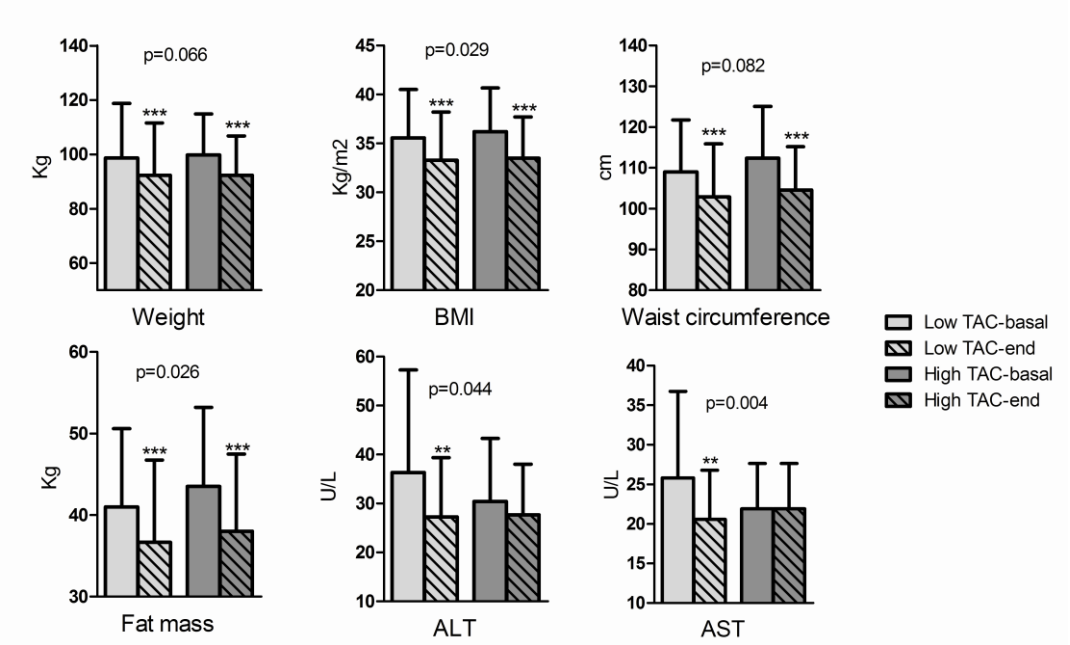

Figure 2. Changes on selected parameters categorized by low (<10.629 mmol day-1, n = 45) or high (>10.629 mmol day-1, n = 45) Total Antioxidant Capacity. BMI: Body Mass Index; ALT: Alanine aminotransferase; AST: Aspartate aminotranferase. p-values comparing the differences between low-TAC and high-TAC groups. ** $p < 0.01$; *** $p < 0.001$.

Table 4. Regression analysis, with changes in phenotypical measurements as dependent variable and dietary components as the independent

	Meal Frequency		Protein Intake		n-3 FA intake		TAC		GI		GL		HEI		Model	Corrected
Variable	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	**B**	***p***	***p***	**r^2**
Weight (Kg)	0.899	0.146	0.806	0.206	0.252	0.681	1.274	0.034	0.085	0.889	-0.519	0.399	1.140	0.059	0.024	0.122
BMI (kg/m²)	0.275	0.215	0.218	0.342	0.032	0.884	0.480	0.026	-0.025	0.910	-0.194	0.378	0.430	0.047	0.132	0.060
Waist circumference (cm)	0.731	0.446	1.392	0.157	-0.107	0.910	1.618	0.083	0.064	0.946	-0.722	0.447	0.624	0.508	0.559	-0.014
Bioimpedance																
Fat mass (kg)	0.613	0.394	-0.036	0.962	1.358	0.053	1.185	0.091	-0.215	0.760	-0.424	0.552	0.639	0.365	0.128	0.061
DXA																
Fat mass (kg)	0.949	0.063	0.819	0.120	0.434	0.392	1.211	0.015	0.050	0.921	0.154	0.763	0.806	0.109	0.133	0.059
Total Cholesterol (mg/dl)	3.886	0.670	0.444	0.962	-13.795	0.123	5.041	0.573	-2.462	0.783	-8.968	0.319	-16.091	0.071	0.381	0.010
HDL-c (mg/dl)	-0.786	0.612	-2.045	0.199	-2.055	0.178	0.558	0.714	-0.951	0.531	-1.558	0.308	-0.247	0.872	0.311	0.021
LDL-c (mg/dl)	5.174	0.522	1.930	0.817	-13.268	0.095	-0.164	0.984	-2.581	0.745	-4.266	0.594	-13.979	0.077	0.348	0.015
TG (mg/dl)	-2.511	0.865	2.792	0.855	7.639	0.601	23.238	0.107	5.349	0.712	-15.720	0.281	-9.324	0.522	0.471	-0.003
Glucose (mg/dl)	3.560	0.648	-8.493	0.290	6.908	0.370	-5.614	0.464	2.408	0.753	7.928	0.303	9.467	0217	0.781	-0.035
Insulin (µU/ml)	-1.662	0.233	-1.144	0.427	-1.814	0.188	-0.664	0.629	-2.546	0.061	-2.572	0.060	-1.597	0.246	0.198	-0.063
HOMA	-0.539	0.299	-0.487	0.364	-0.151	0.770	-0.371	0.468	-0.666	0.190	-0.465	0.365	-0.382	0.457	0.894	0.044
ALT (U/L)	-6.226	0.049	-6.852	0.035	-4.413	0.160	-5.795	0.062	3.721	0.232	3.708	0.238	5.855	0.060	0.066	0.089
AST (U/L)	-3.557	0.051	-4.539	0.015	0.084	0.963	-5.046	0.004	1.610	0.371	1.580	0.384	4.699	0.008	0.020	0.131

n-3 FA, n-3 Fatty Acids; TAC, Total Antioxidant Capacity; GI, Glycemic Index: GL, Glycemic Load; HEI, Healthy Eating Index; BMI, Body Mass Index; DXA, Dual-energy X-ray Absorptiometry; HDL-c, High Density Lipoprotein-cholesterol; LDL-c, Low Density Lipoprotein-cholesterol; TG, Triglycerides; HOMA, Homeostasis Model Assessment; ALT, Alanine minotransferase; AST, Aspartate aminotranferase. $p < 0.05$ was set-up as statistically significant.

As expected, the percentage of subjects from the RESMENA group (71%) included in the high-TAC group was significantly higher ($p < 0.001$) as compared with the Control group (29%). Body weight losses were marginally ($p = 0.066$) different when the subjects were categorized by the dietary TAC (Figure 2). Thus, the group with a higher dietary TAC showed a greater body weight reduction (-7.52 ± 0.64 kg) when compared with the low-TAC group (-6.35 ± 0.86 kg). Waist circumference decreased marginally towards significant differences between the two groups ($p = 0.082$) being greater in the high-TAC one (-7.77 ± 2.07 cm *vs.* -6.15 ± 0.31 cm). Fat mass was significantly reduced in both TAC groups and differences between them regarding densitometry fat mass kilograms ($p = 0.026$) were found. The low-TAC group significantly reduces ALT ($p = 0.001$) and AST ($p = 0.002$) transaminases levels, being statistically significant regarding the high-TAC group ($p = 0.044$ and $p = 0.004$, respectively) (Figure 2).

Gender variation influenced anthropometrical and biochemical parameters changes (Table 5). Body weight loss was significantly higher in males than in females ($p = 0.008$), as well as fat mass reduction ($p = 0.042$) (Figure 3). Interestingly, men showed a statistically significant decrease of insulin blood levels ($p = 0.020$). Concurrently, ALT values were significantly reduced in men, while we did not found any changes in this marker in females group (Figure 3). Regarding dietary TAC influence, differences concerning gender were found. Women with higher TAC levels, showed a significantly greater reduction of body weight ($p = 0.019$), BMI ($p = 0.028$) and fat mass ($p = 0.007$), while there were not any variable differences between high or low TAC in the male group.

Table 5. Analysis assessing diet and sex interactions concerning anthropometric and biochemical markers.

Variable	Groups				ANOVA 2x2		
	Control-men (n=27)	Control- women (n=21)	RESMENA-men (n=24)	RESMENA-women (n=24)	DIET	SEX	Diet * Sex
ΔWeight (Kg)	-7.60±3.29	-5.61±2.49	-7.73±2.58	-6.45±3.09	0.416	0.007	0.550
ΔBMI (kg/m²)	-2.56±1.12	-2.29±1.03	-2.55±0.90	-2.49±1.10	0.677	0.452	0.620
ΔWaist circumference (cm)	-6.57±4.02	-7.01±5.87	-8.15±2.88	-6.80±4.65	0.454	0.620	0.324
ΔBioimpedance Fat mass (kg)	-6.08±4.92	-4.28±1.84	-5.90±2.64	-4.89±3.50	0.764	0.054	0.582
ΔDXA Fat mass (kg)	-5.13±2.80	-3.99±1.49	-5.71±1.95	-4.78±2.75	0.156	0.034	0.835
ΔTotal Cholesterol (mg/dl)	-15.31±35.58	-19.05±30.86	-22.25±44.19	-10.42±49.55	0.921	0.635	0.362
ΔHDL-c (mg/dl)	-2.01±7.10	-6.67±6.98	-0.61±7.29	-3.32±6.03	0.098	0.011	0.495
ΔLDL-c (mg/dl)	-6.12±25.04	-7.48±27.41	-10.82±40.17	-0.51±47.30	0.880	0.553	0.440
ΔTG (mg/dl)	-35.88±78.11	-24.50±61.90	-54.08±88.48	-32.96±69.79	0.399	0.304	0.757
ΔGlucose (mg/dl)	-18.53±36.01	-5.91±19.86	-7.33±26.55	-19.84±46.17	0.847	0.994	0.079
ΔInsulin (IU/mg)	-7.68±9.02	-3.37±3.58	-6.26±5.02	-4.16±5.25	0.690	0.022	0.486
ΔHOMA	-2.64±2.65	-1.35±1.68	-1.74±1.63	-2.02±2.83	0.818	0.293	0.099
ΔALT (U/L)	-13.68±19.36	-6.52±12.77	-3.33±10.70	3.64±15.38	0.001	0.026	0.975
ΔAST (U/L)	-6.49±11.74	-3.37±6.16	-0.28±6.13	0.88±6.51	0.003	0.210	0.562

BMI, Body Mass Index; DXA, Dual-energy X-ray Absorptiometry; HDL-c, High Density Lipoprotein-cholesterol; LDL-c, Low Density Lipoprotein-cholesterol; TG, Triglycerides; HOMA, Homeostasis Model Assessment; ALT, Alanine minotransferase; AST, Aspartate aminotranferase. $p < 0.05$ was set-up as statistically significant.

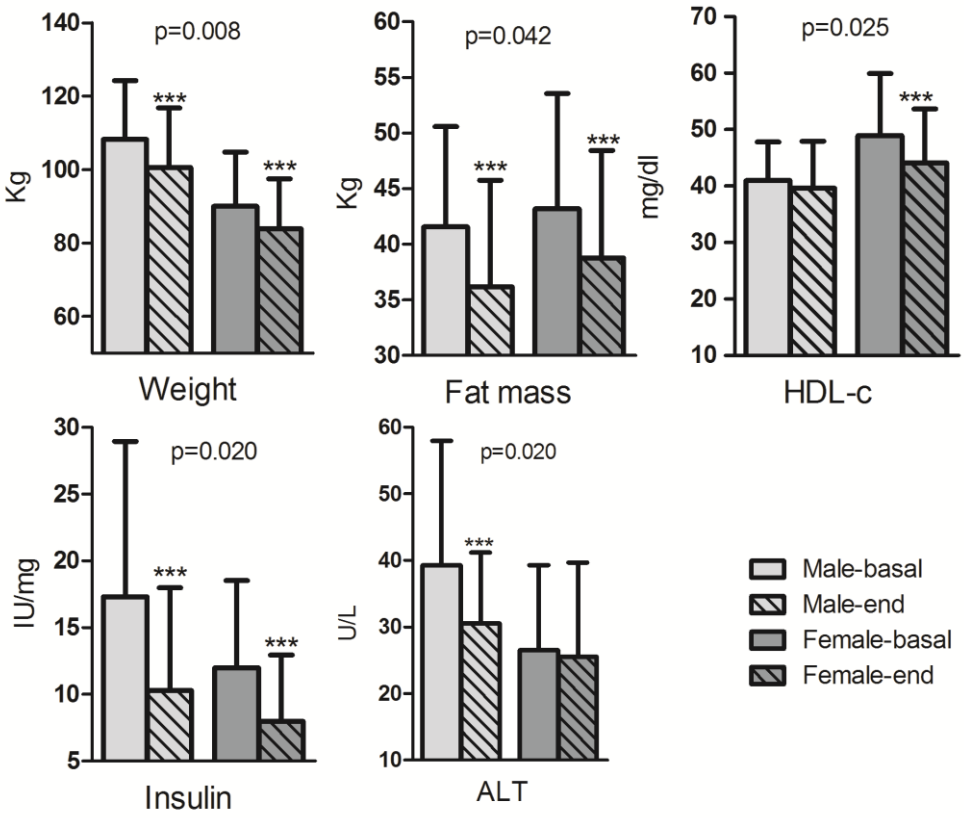

Figure 3. Changes on anthropometric and biochemical selected parameters regarding gender, male (n = 51) or female (n = 45). HDL-c: High Density Lipoprotein-cholesterol; ALT: Alanine aminotransferase. p-values comparing the differences between male and female groups. p-values comparing the differences between male and females.
*** $p < 0.001$.

Discussion

This study compared the effects of a novel dietary strategy with the AHA pattern (Krauss *et al.* 2000), considered to be effective to counteract obesity. To our knowledge, this is a pioneer intervention study in patients with MetS features evaluating the effects of an energy restricted diet based on food selection, including a modified macronutrient distribution, an increase in meal frequency, as well as the presence of bioactive ingredients, such as fiber and n-3 fatty acids, and controlling the GI/GL, dietary TAC and HEI score (Zulet *et al.* 2011).

Evaluating the two prescribed dietary strategies as a whole, both were effective for weight loss and improved anthropometric and biochemical markers, with minor differences between them. Waist circumference was reduced in all the participants. However, when considering the IDF criteria for abdominal obesity (> 90 cm. in men and > 80 cm. in women) 4 people reached lower values after the dietary intervention (1 man and 1 woman from the Control group, 1 man and 1 woman from the RESMENA group).

Specifically, the individual role of each diet component was analyzed in order to assess the dietary components with major influence on these measurements in a MetS sample. In this sense, several works have studied dietary components effects separately in humans, but available results are controversial (Aller *et al.* 2011; Cameron *et al.* 2009; Ghiselli *et al.* 2000).

Recent investigations have focused on the role of the macronutrient distribution in weight reduction treatments instead of considering only calorie restriction (Abete *et al.* 2010). In this context, increasing the dietary protein content has been a frequently-used strategy, due to the increased satiety with a reduction of energy intake in subsequent meals and the higher thermogenic effect attributed to them (Abete *et al.* 2010; Larsen *et al.* 2010). Thus, in the current work it was prescribed a dietary pattern including the 30% of total caloric value as protein at the expense of carbohydrates (40% total caloric value). This profile did not apparently induce changes on any anthropometrical parameters between the experimental groups. Different studies have shown high-protein intake effects in relation with body weight changes, specifically on weight regain. However, those effects were found in the long-term (Gogebakan *et al.* 2011) while the present work focused on the effects of an 8-week dietary treatment.

Interestingly, our study showed a positive association between protein intake and both ALT and AST transaminases levels. These enzymes are unspecific markers of hepatic function (Yang *et al.* 2012). Low-serum transaminases levels are found under normal conditions, indicating proper function of the liver, while increased serum values are related to hepatic dysfunction (Yang *et al.* 2012). They have shown a correlation with insulin resistance and later development of diabetes, liver lipid content and features of non-alcoholic fatty liver disease. The experimental data are consistent with those

other studies and suggest that moderately-high protein diets could influence negatively liver function. Concerning transaminases modification, it is important to notice that some differences were found before starting the intervention. For that reason we performed a percentage of change analyses in addition to the absolute values approach, in order to attenuate the possible bias. However, similar outcomes were obtained when applying this analysis, therefore, absolute values were maintained in Table 3.

Regarding the increased meal frequency, no differences in body weight loss in the context of iso-energetic energy-restricted diets were found, as also was reported by Cameron *et al.* (2009). Nevertheless, a direct relationship between increased meal frequency and the loss of fat mass was observed. With respect to biochemical parameters, no influences were found. However, Heden *et al.* (2012) observed that meal frequency differentially altered triglycerides and insulin postprandial concentrations.

The beneficial properties of n-3 fatty acids have been widely studied (Peairs *et al.* 2011; Ramel *et al.* 2010). In our study, a positive relationship between n-3 fatty acids intake and the reduction of fat mass was detected, being consistent with previous human studies (Ramel *et al.* 2010).

GI and GL are two concepts that refer to the absorption rate of carbohydrates (Foster-Powell *et al.* 2002). Increased values have been reported as potential type-2 Diabetes Mellitus risk factors (Aller *et al.* 2011). In this sense, an encouraging result was obtained in our study, since we found a trend towards a reduction in insulin levels related to lower values of GI and GL in diet, in agreement with (Salas-Salvado *et al.* 2011).

In order to assess the quality of the diet, numerous authors have designed and developed indexes or scores such as the Healthy Eating Index, the Alternate Healthy Eating Index or the Diet Quality Index and derivatives (Puchau *et al.* 2009). Most of them, take into consideration the Mediterranean Diet guidelines, widely recognized as a healthy pattern (Salas-Salvado *et al.* 2011). They consist on a single score that results from computing different component such as foods, food groups or a combination of foods and nutrients. In this context the HEI score was selected, obtained from the DIAL software. It takes into account macro and micronutrients intake, as well as food variety, to design the RESMENA diet. Considering the HEI score, a trend towards influencing

weight loss and total cholesterol, LDL-c, ALT and AST levels was found. Other works evaluating Mediterranean-based patterns reported similar results regarding lipid metabolism and hepatic function markers (Pitsavos *et al.* 2005; Psaltopoulou 2009).

The most relevant finding of this study is in relation to dietary TAC. This parameter has been recently considered as a useful tool to assess the effects of dietary antioxidants, since it has been positively associated with plasma total antioxidant capacity (Bahadoran *et al.* 2012). After 8 weeks of intervention, we evidenced positive associations between the dietary TAC and the reduction of weight, BMI, waist circumference and fat mass. Regarding these anthropometric measurements, our findings are in accordance with previous studies that also reported benefits of dietary TAC and antioxidants compounds on adiposity and obesity indicators (Psaltopoulou 2009; Zulet *et al.* 2008). This link may be associated with a reduction of cardiovascular risk, as previously described in other populations (Puchau *et al.* 2010). We also found an effect on ALT and AST transaminases suggesting an impact of the dietary TAC on hepatic metabolism. These data suggest that dietary TAC, as a measure of antioxidant intake, must be useful to assess the role of antioxidant intake as a single factor in the field of antioxidant consumption and disease prevention or therapy.

Contrary to most of the evaluated parameters, HDL-c values did not improve with none of the dietary treatments, which can be explained by the fact that the reduction on total cholesterol entails a reduction of this cholesterol fraction too, as previously reported by other researchers (Aicher *et al.* 2012). However, some other authors found higher HDL-c serum levels after similar dietary interventions (Belalcazar *et al.* 2012) especially when containing fish or fish derived products (Ramel *et al.* 2010; Smith *et al.* 2009). Despite our dietary strategy also focused on n-3 fatty acids intake, the participants did not reach a perfect adherence to this point, so that this can also contribute to the fact that HDL-c was found to decrease after the dietary intervention. Other important factor in relation to HDL-c is physical activity. In this sense, the participants were asked to maintain the normal activity and no exercise advice was given, which may allow discard differences due to physical activity changes.

Differences between males and females regarding anthropometric and biochemical parameters have been widely investigated (Ejike and Ijeh 2012; Campbell

and Meckling 2012; Cameron *et al.* 2009). In this sense, we analyzed gender influence on the studied variable changes. Body weight loss was higher in men than women, due to the also higher restriction regarding absolute amount of calories. This greater reduction of weight was accompanied by an also higher decrease of fat mass, as previously reported (Cameron *et al.* 2009). Regarding biochemical parameters, insulin as well as ALT blood levels were significantly decreased within this group in accordance with previous studies (Ejike and Ijeh 2012). On the contrary, HDL-c reduction was greater among the women group, as other studies reported (Okuda *et al.* 2005), which confirms the influence of sex on this cholesterol fraction. Taken together, these outcomes indicate that gender may be taken into account in order to design specific dietary plans for males and females.

This work could benefit of increasing sample size. Additionally, although the adherence to the diet was acceptable, this kind of treatments could show higher benefits when reaching a stricter follow-up of the dietary pattern. On the other hand, we have analyzed the effects of this treatment on obese adults with MetS features. The effectiveness of this pattern should be also evaluated in a younger population since obesity and MetS rates have increased alarmingly among childhood (Haidar and Cosman 2011) and they represent a development problem (Giuca *et al.* 2012) leading to an increased morbimortality at the adult age (Park *et al.* 2012).

Conclusion

Taken together, the results of this study indicate that RESMENA diet could be considered as a new dietary strategy in order to improve anthropometrical and biochemical parameters in obese subjects presenting MetS manifestations. Furthermore, dietary TAC seems to be, among all the analyzed dietary aspects, the most relevant one in the obesity related markers.

Abbreviations

AHA: American Heart Association; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; DXA: Dual-energy X-ray Absorptiometry; GI: Glycemic index; GL: Glycemic load; HDL-c: High Density Lipoprotein-cholesterol; HEI: Healthy Eating Index; LDL-c: Low Density Lipoprotein-cholesterol; MetS: Metabolic syndrome; PQ: Protein Quality; PUFA: Poly Unsaturated Fatty Acids; TAC: Total antioxidant capacity; WHO: World Health Organisation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The authors contributions were as follows: PLL contributed to the design and the fieldwork, data collection, analysis and writing of the manuscript. RI and IA were involved in the design and the fieldwork. IBP contributed to the sample collection, interpretation and critical reading of the last version. SNC and LF were involved in recruitment and volunteers selection as well as in critical reading of the manuscript. MAZ was responsible for the general coordination, follow-up, design and financial management. JAM, project co-leader, was responsible of the follow-up, design, financial management and editing of the manuscript. All the authors actively participated in the manuscript preparation, as well as read and approved the final manuscript.

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4.2. CHAPTER 2

The protein type within a hypocaloric diet affects obesity-related inflammation: The RESMENA Project.

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Running head: Dietary protein and inflammation

Abstract

Objective

The aim of this study was to compare the effect of two energy-restricted diets differing on protein content on the inflammation state of obese subjects with MetS features.

Research Methods & Procedures

Ninety-six participants completed an 8-week randomized intervention trial comparing the RESMENA diet (-30% Energy, 30% Energy from protein) with a Control diet (-30% Energy, 15% Energy from protein) based on American Heart Association criteria.

Results

The mean body weight loss was 7.09±0.82 kg and 6.73±0.71 kg, respectively, without differences between groups. Endpoint inflammation score (hsCRP, IL-6, TNF-α, PAI-1) was significantly lower (p=0.012) in the low-protein group (6.81±2.32 vs. 7.94±1.94). The linear regression analyses revealed that total protein intake was positively associated with inflammation (p=0.007), as well as animal protein (p=0.025) and meat protein (p=0.015), but neither vegetable nor fish derived proteins were found to influence the inflammatory status.

Conclusion

Our results suggest that the type of protein more than the total protein intake within an energy-restricted diet influence the inflammation status associated to obesity-related comorbidities. www.clinicaltrials.gov; NCT01087086

Key words: cardiovascular diseases, inflammation markers, macronutrient distribution, weight loss, metabolic syndrome.

Introduction

Atherosclerotic cardiovascular disease is a chronic inflammatory disorder representing a major cause of morbid-mortality in many developed countries (Stranges and Guallar 2012). Furthermore, metabolic syndrome (MetS) encompasses important cardiovascular risk factors, including central obesity, insulin resistance, hypertension and serum lipid abnormalities (Mi *et al.* 2012). Effective strategies should be encouraged to reduce the burden of cardiovascular diseases, diet being a primary choice tool for both prevention and first-term treatment of such disorders (Abete *et al.* 2011).

Indeed, low-grade chronic inflammation has been proposed as a mechanism linking obesity and cardiovascular disturbances (Bondia-Pons *et al.* 2012), which is being currently redefined by including various markers indicative of inflammatory status, endothelial dysfunction and obesity-related vascular damage (Wijnstok *et al.* 2010). For that reason, appropriate dietary strategies must focus, in addition to inducing weight/fat loss, on inflammatory status improvement (Asemi *et al.* 2013; Richard *et al.* 2013).

Acute-phase proteins such as C-reactive protein (CRP) are relevant systemic inflammatory markers, which have been shown to be upregulated in obesity pre-clinical as well as in advanced stages of vascular damage (DeBoer 2013; Bugge *et al.* 2012). Some adipocytokines secreted by the adipose tissue such as tumor necrosis factor alpha (TNF-α) or interleukin 6 (IL-6) have been also assessed as useful markers of inflammation, since the adipose tissue is expanded in obesity. Furthermore, IL-6 seems to be a strong stimulator of CRP secretion in conditions of excessive fat accumulation (Hermsdorff *et al.* 2012). Plasminogen activator inhibitor-1 (PAI-1) levels are predictive of incident cardiovascular disease in the general population, whose secretion by adipose tissue is also increased in obese subjects associated to increased fat mass and pro-inflammatory status (Belalcazar *et al.* 2011). Given the association of these markers with cardiovascular risk, it is expected that any intervention focused on reducing inflammatory status may benefit cardiovascular health.

In this context, dietary nutrients have attracted attention in recent research due to their beneficial effects on cardiovascular disease by attenuating adverse lipid profiles, inflammation and/or oxidative stress (de Mello *et al.* 2011; Hermsdorff *et al.* 2009;

Tsitouras *et al.* 2008). Thus, recent nutritional investigations have considered the specific role of selected dietary components, since they may show additional benefits on top of weight loss (Lopez-Legarrea *et al.* 2013; Abete *et al.* 2011), as evidenced by the DiOGenes trial (Gogebakan *et al.* 2011; Goyenechea *et al.* 2011) or the Sysdimet study (de Mello *et al.* 2011; Lankinen *et al.* 2011), which revealed positive effects of glycemic index, omega-3 fatty acids, whole grain products or bilberries among others. Also, specific dietary patterns such as the Dietary Approaches to Stop Hypertension (DASH), based on reducing total and saturated fat, cholesterol and sugar and the American Heart Association (AHA) pattern have been widely evidenced as protective against cardiovascular diseases (Bhupathiraju *et al.* 2011; Salehi-Abargouei *et al.* 2013).

In this sense, the increase of protein content has been frequently assessed in weight lowering plans due to the increased satiety and a higher thermogenic effect (Westerterp-Plantenga *et al.* 2012). Nevertheless, the role of protein intake on obesity-related inflammation has shown controversial results, as well as the type of protein (Santesso *et al.* 2012; Hermsdorff *et al.* 2011, Azadbakht and Esmaillzadeh 2009).

In this context, this research aimed at evaluating the effects of two dietary strategies with different protein content on decreasing inflammation if an obese sample eliciting some metabolic syndrome features, through the RESMENA (MEtabolic Syndrome REduction in NAvarra) project.

Materials and Methods

Participants and study design

The study recruited 105 Caucasian adults presenting obesity and MetS features to follow an 8-week intervention trial. The participants were randomized to one of the dietary treatment groups: the AHA-diet (Control group) or the RESMENA-diet (RESMENA group). Presenting at least two of the International Diabetes Federation criteria for MetS was required for inclusion (Zimmet *et al.* 2005). On the other hand, psychiatric and eating disorders, chronic diseases related to nutrients metabolism, major body weight changes (≥3kg) in the last three months, as well as difficulties for changing food habits were considered exclusion criteria. The study was approved by the Ethics Committee of the University of Navarra (065/2009) and appropriately registered at

www.clinicaltrials.gov; NCT01087086. Consequently, all the participants gave written informed consent for participation in agreement with the Declaration of Helsinki. This work was performed following the CONSORT 2010 guidelines (Turner *et al.* 2012). Follow up visits were conducted fortnightly. Anthropometric and body composition measurements were assessed and blood samples for biochemical parameters and inflammation markers were taken at the beginning and at the endpoint of the 8-week period. There were 9 dropouts along the intervention, therefore, 96 volunteers were considered for the data reported *(see Figure 1 of chapter 1, page 124*). 47% were female, with an average age of 50±10 years-old and a mean body mass index of 35.85±4.67 kg/m². We could not get blood samples from two of the Control group participants, therefore, sample size in this group for biochemical analysis was n=46. Food record data were available from 90 of the 96 volunteers.

Diets

Two dietary strategies for reducing weight (-30% energy) with different protein intake (15% Total Caloric Value (TCV)- Control diet vs. 30% TCV- RESMENA diet) were evaluated and compared (Zulet *et al.* 2011). The rest of the macronutrients were distributed as following: 40% TCV from carbohydrates and 30% TCV from fats in the RESMENA diet and 55% TCV and 30% TCV in the Control diet, respectively. Diet composition including total, vegetable and animal protein intake, as well as meat and fish derived protein intake were determined using the DIAL software (Alce Ingenieria, Madrid, Spain) as described elsewhere (Lopez-Legarrea *et al.* 2011).

Anthropometric and biochemical assessments

Anthropometric measurements including body weight, waist and hip circumferences and body composition by Dual-energy X-ray Absorptiometry (Lunar Prodigy, software version 6.0, Madison, WI) were assessed at baseline and at the end of the intervention with the subjects in their underwear, following validated protocols (Zulet *et al.* 2011). Body mass index (BMI) was calculated as the body weight divided by the squared height (kg/m²).

Glucose, total cholesterol and high density lipoprotein-cholesterol (HDL-c) serum concentrations were measured by using an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits. Low-density lipoprotein-cholesterol (LDL-c)

levels were calculated following the Friedewald formula: LDL-c = TC - HDL-c - VLDL. Apolipoproteins AI and B were measured with specific kits (Tina-quant Apolipoprotein A-I ver.2 and Tina-quant Apolipoprotein B ver.2) using an autoanalyzer Roche/Hitachi 904/911/912/917/MODULAR.

Plasma concentrations of hsCRP (Demeditec), IL-6 (R&D Systems) TNF-α (R&D Systems) and PAI-1 (BioVendor) were measured with specific enzyme-linked immunosorbent assay kits from specified suppliers using an autoanalyzer system (Triturus, Grifols SA, Barcelona, Spain) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using the SPSS 15.1 software for Windows (SPSS Inc, Chicago, USA). Normality distributions of the measured variables were determined according to the Shapiro–Wilk test. Differences between the beginning and the end of the period were analyzed by a paired t-test of significance at an alpha level of 0.05. All between group analyses (RESMENA vs. Control) included two-tailed, unpaired t-tests. A linear regression analysis was fitted to examine the potential relationships and associations between the different dietary components included in the evaluated dietary strategies, as well as the type of protein and the inflammation score. The influence of each variable on the inflammation status was separately analyzed which avoided possible co-linearity bias. The arbitrarily defined inflammatory score was designed including the four measured markers (CRP, IL-6, TNF-α, PAI-1) in order to better evaluate each dietary strategy anti-inflammatory properties. First of all, each marker variation data were classified in three groups according to the Percentiles (P) values. Secondly, a punctuation of 1 (values <P33), 2 (values between P33-P66) or 3 (values >P66) was given to each participant taking into account their blood levels of each marker. Finally, we added the values obtaining an inflammation score indicating the inflammation status for each participant. This score therefore varied from 4 to 12 points (Richard *et al.* 2013). For this test, both groups were analyzed together as a whole following the model by per protocol analysis. A t-student test was also employed to compare the categorized sample according to total, vegetable and animal, and meat and fish protein intake. All results are expressed as mean ± SD.

Results

At the endpoint of the 8-weeks dietary intervention subjects in the Control group showed a significantly higher (p=0.013) intake of carbohydrates, whereas a greater (p<0.001) total protein intake was found in the RESMENA group (Table 1). Also according to the scheduled dietary patterns, there were no differences between dietary groups in relation to total fat intake. Both groups showed a similar intake (p>0.05) of vegetable and fish protein. In contrast, a significantly higher (p<0.001) intake of meat derived protein and consequently of animal protein (p<0.001) was found in the RESMENA group (Table 1).

Table 1. Comparison of the daily macronutrient intake of the two evaluated dietary groups at the end of the intervention.

Macronutrient	**Control group**	**RESMENA group**	***p***
Carbohydrates (g)	132.4±35.3	114.6±31.1	0.013
(% TCV)	(39.2)	(34.3)	
Total protein (g)	60.8±17.2	78.2±17.5	<0.001
(% TCV)	(18.0)	(23.4)	
Animal protein (g)	40.2±16.0	58.0±17.3	<0.001
Meat protein (g)	15.1±13.5	27.6±10.0	<0.001
Fish protein (g)	8.9±10.3	10.4±9.7	0.483
Vegetable protein (g)	18.8±7.4	19.8±9.4	0.906
Fats (g)	59.1±15.5	56.6±17.3	0.497
(% TCV)	(39.3)	(38.1)	

TCV: Total caloric value

The mean body weight loss was 6.73±0.71 kg and 7.09±0.82 kg in Control and RESMENA groups respectively, without differences between them. After 8 weeks of dietary intervention, participants in the both diets also showed a significant reduction in BMI, waist to hip ratio as well as in percentage of fat mass, with no statistical differences between groups (Table 2). Biochemical analyses revealed that at the end of the intervention period the two experimental groups improved glucose, total cholesterol and triglyceride serum levels without significant differences between them. Differences

between groups were found neither on apolipoproteins measurements nor when analyzing the atherogenic ratios (Table 2).

Four inflammatory markers were measured at baseline and month 2 in order to assess the obesity-related inflammatory status of the participants (Table 2). Control group significantly reduced PAI-1 plasma levels (p<0.001) after two months of intervention. In the RESMENA group, none of the inflammatory markers showed a significant decrease between baseline and endpoint values.

Table 2. Changes in selected anthropometric and biochemical variables of Control and RESMENA groups as well as the differences between them after the 8 weeks of intervention.

Variable	Control group			RESMENA group			P difference
	Baseline	Month 2	p	Baseline	Month 2	P	
Weight (kg)	99.45±19.21	92.72±18.50	<0.001	100.00±16.58	92.91±15.76	<0.001	0.555
BMI (kg/m²)	36.15±4.95	33.70±4.80	<0.001	35.55±4.40	33.03±4.24	<0.001	0.732
Waist/hip ratio	0.96±0.10	0.94±0.09	<0.001	0.96±0.10	0.93±0.10	<0.001	0.543
DXA Fat mass (%)	42.59±7.04	40.56±7.68	<0.001	42.76±6.01	40.37±6.63	<0.001	0.460
Glucose (mg/dl)	121.02±33.87	107.98±13.71	0.006	123.81±37.82	110.22±26.18	0.016	0.939
TC (mg/dl)	221±39	204±39	0.001	219±48	203±46	0.020	0.943
Triglycerides (mg/dl)	176±10	145±70	0.005	194±123	151±99	<0.001	0.421
TC/ HDL-c	4.94±1.02	4.96±0.94	0.856	5.18±1.24	5.04±1.24	0.398	0.419
LDL-c/HDL-c	3.09±0.77	3.22±0.78	0.178	3.20±0.83	3.23±0.92	0.803	0.623
Apo AI (mg/dl)	138.35±23.48	127.22±20.00	<0.001	129.36±19.03	117.85±18.75	<0.001	0.597
Apo B (mg/dl)	97.94±21.76	90.59±21.48	<0.001	95.33±25.26	84.00±22.16	<0.001	0.196
ApoB/ApoAI	0.72±0.17	0.72±0.16	0.988	0.73±0.18	0.72±0.18	0.313	0.441
CRP (µg/ml)	3.19±3.14	2.35±2.48	0.100	3.20±3.42	3.39±4.22	0.721	0.270
IL-6 (pg/ml)	2.61±1.39	2.56±1.40	0.838	2.71±1.83	2.79±1.56	0.714	0.710
TNF-α (pg/ml)	0.66±0.48	0.58±0.44	0.110	0.76±0.67	0.78±0.76	0.738	0.110
PAI –1 (ng/ml)	147.07±83.37	88.37±56.95	<0.001	151.19±122.77	124.95±113.23	0.052	0.068

BMI: Body mass index; DXA: Dual-energy X-ray absorptiometry; TC: Total Cholesterol; HDL-c: High density lipoprotein-cholesterol; LDL-c: Low density lipoprotein-cholesterol; Apo: Apolipoprotein, hsCRP: High Sensitive C-Reactive Protein: IL-6: Interleuquin-6: TNF-α: Tumor Necrosis Factor- α; PAI-1: Plaminogen Activator Inhibitor-1.

When comparing the inflammatory score which includes hsCRP, IL-6, TNF-α and PAI-1 markers at baseline there were no differences between groups (p=0.822). After 8 weeks of intervention, the Control group showed a significantly (*p*=0.012) lower value than the RESMENA dietary group (6.81±2.32 *vs.* 7.94±1.94), indicating that a greater benefit on the inflammatory state was achieved, in comparison with the RESMENA group (Figure 2).

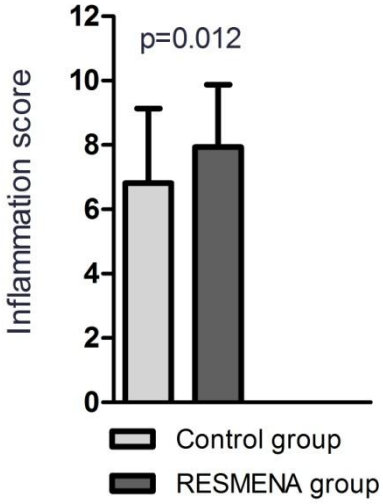

Figure 2. Comparison of the inflammation score values between Control and RESMENA groups at the endpoint of the 8-week intervention.

After adjusting for weight loss, the linear regression analysis evidenced that only protein intake showed an important positive (B=1.288±0.463; *p*=0.007) influence on inflammatory status. Consequently, the population was categorized by median daily total protein intake value (67.10 g) and the inflammatory score was compared between groups (Figure 3). Individuals consuming >67.10 g of proteins showed a higher value of inflammatory score than participants in the low-protein intake group (8.04±2.00 *vs.* 6.64±2.19), resulting in significant differences between groups (*p*=0.002). A subsequent linear regression analysis considering protein type was also performed. Animal protein showed an influence on the inflammatory score (B=0.822±0.323; *p*=0.025), whereas vegetable intake did not (*p*>0.05). The sample was categorized according to the animal protein intake median value in low-intake group (<45.13 g) and high-intake group (>45.13 g). The between groups comparison concerning animal protein intake showed a marginal trend towards signification (*p*=0.075), suggesting a positive relationship between high animal protein intake and inflammation (Figure 3). As expected, no differences were found when categorizing the sample by the median of the vegetable protein intake value. Specifically, among the animal group, meat and fish derived

protein was further explored. The linear regression analysis showed a positive association between meat protein intake and the inflammation score (B=0.256±0.017; *p*=0.015), but no statistical significant associations were found concerning fish intake. The sample was categorized considering the median intake value of both foods. Significant differences in the inflammatory score were found when comparing low vs. high meat protein intake (*p*=0.030). A higher meat intake was associated with a greater inflammation score after the 8-weeks intervention within an energy-restricted pattern. There were no differences when comparing low and high fish protein intake regarding inflammation.

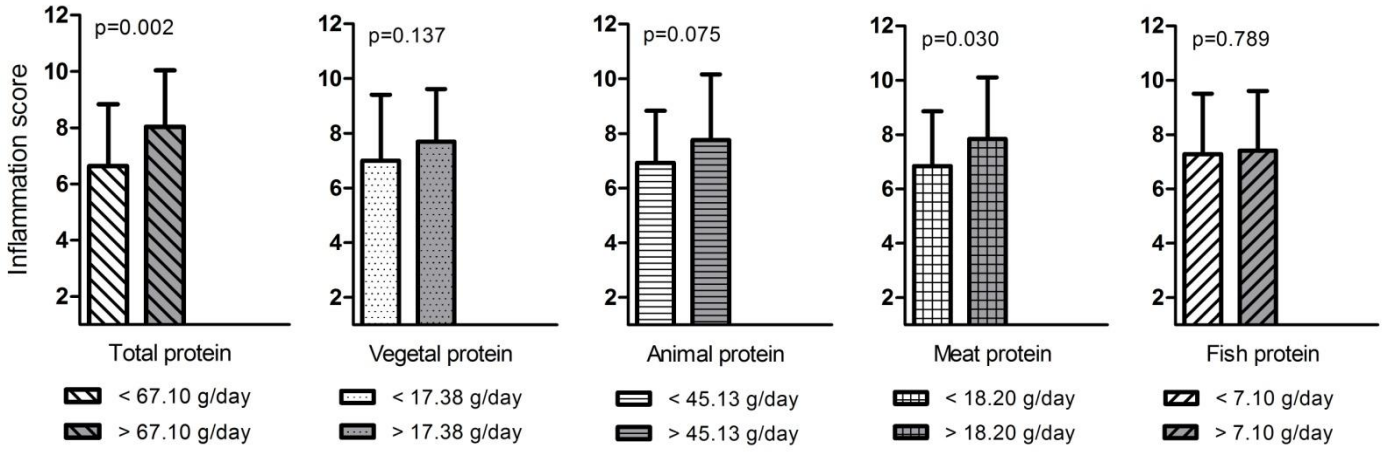

Figure 3. Comparison of the inflammatory score at the end of the study, regarding total protein intake, animal and vegetable protein, as well as fish and meat intake.

Discussion

This work compared the effects of two energy-restricted diets with different protein content on anthropometric, biochemical and inflammatory markers over an 8-weeks intervention period on obese subjects presenting MetS features. Cardiovascular disease is an important social burden, therefore, designing new dietary strategies for its prevention and combating causal disorders is needed. Obesity is one of the main causative problems so weight loss strategies have been widely studied (Jebb *et al.* 2011; Tapsell *et al.* 2010).

Considering just weight and fat mass loss both evaluated diets showed to be equally effective, in agreement with previous works (Clifton *et al.* 2009). Obesity and

MetS features represent important risk factors for cardiovascular disease, so that, improvement of biochemical parameters related to lipid and glucose metabolism is another requirement of any appropriate dietary strategy. In this sense, both Control and RESMENA plans were also effective. Thus, these positive outcomes regarding MetS indicators may allow making an approach of the effectiveness of the evaluated strategies on cardiovascular diseases, showing both to be beneficial for improving cardiovascular risk factors. However, as inflammation has been proposed as the link between obesity and related vascular comorbidities, improving the inflammatory status must be also considered a goal. In this sense, only the Control diet significantly reduced PAI-1, and the rest of the markers showed a similar trend within this group. The Control group followed the guidelines of the AHA and the obtained outcomes are in agreement with previous studies that reported an association of this pattern as a whole with lower fasting values of inflammatory markers such as CRP (Bhupathiraju *et al.* 2011).

Calorie restriction is known to be an activator of some protective metabolic pathways, accompanying a reduction on inflammatory markers (Calder *et al.* 2011; Clement *et al.* 2004). Also some dietary components, such as omega-3 fatty acids, have been widely investigated in relation to inflammation (Tsitouras *et al.* 2008). Nevertheless, one of the most commonly used dietary modifications consists of increasing the protein content of the diet (Wycherley *et al.* 2012) and its role on inflammation is controversial (Santesso *et al.* 2012). There is a lack of literature reporting protein intake influence on inflammation and if varying the protein content of the diet may have some beneficial effects in that respect remains elusive. In this sense, the DiOGenes project reported apparently for first time that dietary protein content influences inflammation, specifically, hsCRP concentrations. This pan-European controlled dietary intervention study compared a high *vs.* a low protein diet in overweight and obese adults and found that the lower protein content appeared to be associated with a further decrease of hsCRP compared with the high-protein diet. A small effect although significant in all analyses was found indicating the robustness of the result and it was concluded that low-protein intake may specifically reduce low-grade inflammation in overweight/obese adults (Gogebakan *et al.* 2011). In the present work, we compared two diets with different protein content: 15% of the TCV of the diet, classically considered as a healthy amount (Grundy *et al.* 2004) *vs.* a moderately-increased input of the 30%, suggested as positive for greater weight loss and better

weight maintenance (Westerterp-Plantenga *et al.* 2012; Larsen *et al.* 2010). In turn, we evaluated the specific role of protein on inflammation in addition to energy-restriction effects. Our results indicate that lower protein intake levels within a hypocaloric diet are associated with a better inflammation status, represented by the inflammatory score, after 8 weeks of dietary intervention, which is in agreement with the DiOGenes project findings (Gogebakan *et al.* 2011). Therefore, the Control diet appears more beneficial than the RESMENA strategy. In any case, more studies are needed in order to confirm these results and elucidate the potential mechanisms underlying this effect. Controversial results have been found in relation to this outcome, but a recent meta-analysis concerning the most relevant investigations in last years concluded, although only as a trend, that a higher protein intake is associated with deterioration on inflammation (Santesso *et al.* 2012). Nevertheless, it has been reported that depending on the source, animal or vegetable, protein intake may have different influences on health (Azadbakht *et al.* 2007). Based on these outcomes, separate evaluation of animal and vegetable protein effects was carried out within this research work. Our results showed that after 8 weeks of dietary intervention, a higher animal protein intake was positively associated with inflammation, whereas no association was evidenced between vegetable protein and the inflammatory score in obese individuals with MetS symptoms. The first finding is in agreement with previous research, which found that a high consumption of red meat was associated with elevated plasma CRP concentrations (Azadbakht and Esmaillzadeh 2009).

Concerning vegetable origin protein, our outcomes suggest that vegetable protein does not have beneficial effects, but neither detrimental ones, as other authors have reported for soy protein, for example (Azadbakht *et al.* 2007). In contrast, there are some previous studies that have evidenced positive effects on inflammation of vegetable protein consumption (Hermsdorff *et al.* 2011).

When comparing protein intake between groups, RESMENA showed a higher intake of animal protein, whereas both groups showed similar vegetable protein intake. This situation means that the higher total protein amount scheduled for the RESMENA group was reached by increasing the animal type, although this does not agree with the study prescription. In order to further explore the role of animal protein, we separately analyzed meat and fish protein consumption. As previously reported, meat intake was positive associated with inflammation (Azadbakht and Esmaillzadeh 2009), whereas

fish did not. The observed higher inflammation related to meat and consequently to animal protein could be attributed to components occurring in meat such as advanced glycation end products (AGEs), iron or saturated fat. The AGEs occur naturally in meat and are formed through heat processing (Uribarri *et al.* 2010) and may have proinflammatory actions (Uribarri *et al.* 2005). It has been shown that a high-AGE diet increased concentration of plasma CRP in participants with diabetes, compared with a low-AGE diet (Vlassara *et al.* 2002). In line with these findings, an increase of circulating inflammation markers has been demonstrated to occur after eating a high-AGE diet for 6 weeks compared with eating a low-AGE diet (Peppa *et al.* 2002). On the other hand, free iron can increase oxidative stress, thereby acting as a proinflammatory agent (Fernandez-Real *et al.* 2002). The higher inflammatory status linked to meat may also be explained by the higher content of saturated fat (Benatar *et al.* 2011) since a detrimental role of these components is suggested by their association with elevated levels of the inflammatory marker CRP (Santos *et al.* 2013). Therefore, the higher animal protein intake showed by the RESMENA group was due to meat intake rather than to fish consumption. Although a protective role of fish intake could be expected due to the anti-inflammatory properties of omega-3 fatty acids (Tsitouras *et al.* 2008; Guebre-Egziabher *et al.* 2013) we failed to find any association between fish and inflammation. In any case, we have not separately studied oily and lean fish intake, so this can be a confusing factor. This fact can be also explained because the adherence was not as good as expected and participants did not completely reach the scheduled omega-3 fatty acids amounts. Indeed, the obtained outcomes could benefit from a higher compliance to the dietary strategies. This study has some other limitations to be considered. Sample size could be larger and the intervention period longer in order to confirm the outcomes. Nevertheless they have an important translational value. Moreover, the Spanish food composition tables may have gaps concerning some items. In addition, the use of the AHA diet as a control instead of a "typical diet" for evaluating the new RESMENA strategy may have hampered to find differences between groups, because both dietary patterns are healthy.

To our knowledge this is the first intervention study that separately evaluates the effects of vegetable and animal protein on the inflammatory status associated to obesity and MetS, when following a weight reducing diet.

In summary, a higher intake of animal origin protein, specifically meat, is associated with higher plasma levels of inflammatory markers in obese adults with MetS features, at least for the short term. For that reason, guidelines regarding macronutrient distribution in relation to inflammation, must take into consideration the type of protein consumed. Additionally, some studies even suggest considering the specific protein-food included (Murphy *et al.* 2012).

Conclusion

The RESMENA diet could be considered a more convenient dietary approach if more vegetable protein consumption is encouraged since the type of protein consumed is apparently more influential than the total protein amount intake on inflammatory status.

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The authors contributions were as follows: PLL contributed to the design and the fieldwork, data collection, analysis and writing of the manuscript. RI and IA were involved in the design and the fieldwork. SNC was involved in recruitment and volunteers selection as well as in critical reading of the manuscript. MAZ was responsible for the general coordination, follow-up, design and financial management. JAM, project co-leader, was responsible of the follow-up, design, financial management and editing of the manuscript. All the authors actively participated in the manuscript preparation, as well as read and approved the final manuscript.

Conflict of interest

None declared.

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4.3. CHAPTER 3

Higher baseline irisin concentrations are associated with greater reductions in glycemia and insulinemia after weight loss in obese subjects

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Abbreviated title: Irisin and glucose homeostasis

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Abstract

Irisin is assumed to be a relevant link between muscle and weight maintenance as well as in mediating the exercise benefits on health. The aim of this study was to assess the possible associations between irisin levels and glucose homeostasis in obese subjects suffering metabolic syndrome under an energy-restricted treatment. Ninety-six adults with excessive body weight and metabolic syndrome in accordance to the International Diabetes Federation criteria underwent hypocaloric dietary pattern for 8 weeks, within the RESMENA randomized controlled trial (www.clinicaltrials.gov; NCT01087086). After the intervention, dietary restriction significantly reduced body weight and evidenced a dietary-induced decrease in irisin circulating levels in parallel with improvements in glucose homeostasis markers. Interestingly, participants with higher irisin values at baseline (above median) showed a greater reduction on glucose ($p=0.022$) and insulin ($p=0.021$) concentrations as well as on the HOMA index ($p=0.008$) and triglycerides ($p=0.006$) after the dietary intervention, compared with those with low irisin baseline values (below median). Interestingly, a positive correlation between irisin and carbohydrate intake was found at the end of the experimental period. In conclusion, irisin appears to be involved in glucose metabolism regulation after dietary induced weight loss.

Key words: glucose homeostasis, hormone, myokine

Introduction

Obesity is a worldwide health burden, accompanied by a number of comorbidities including glucose intolerance, insulin resistance and type 2 diabetes (Yang *et al.* 2013). Irisin (Bostrom *et al.* 2012), which is a cleavage product of the type I membrane protein fibronectin type III domain-containing 5 (FNDC5) has been hypothesized as a target to counteract obesity and type 2 diabetes (Stengel *et al.* 2013; Liu *et al.* 2013). Irisin is expressed in the muscle and the adipose tissue and has been associated with adiposity and body weight in animals (Roberts *et al.* 2013; Roca-Rivada *et al.* 2013) and humans (Moreno-Navarrete *et al.* 2012; Huh *et al.* 2012). However, the precise role and underlying mechanisms concerning irisin actions and signaling pathways remain incompletely understood.

The aim of this research was to assess changes in irisin circulating concentrations after a hypocaloric treatment to lose weight in obese subjects presenting metabolic syndrome (MetS) features and to analyze the potential irisin relationships with glucose homeostasis after dieting.

Methods

Study protocol

This research reports the findings of a secondary analysis of the 8-week intervention period of the RESMENA randomized intervention trial conducted following CONSORT guidelines and registered on the ClinicalTrials.gov database (www.clinicaltrials.gov;NCT01087086). A full list of inclusion criteria, as well as a complete description of the study methodology can be found in earlier publications (Lopez-Legarrea *et al.* 2013; Zulet *et al.* 2011). Briefly, participants were randomized into two intervention groups, with the same energy restriction (-30% E), but differing mainly in carbohydrate/protein ratio and meal frequency: Control group- 55% E CHO, 15% E Proteins and 3-5 meals/day, and RESMENA group- 40% E CHO, 30% E Proteins and 7 meals/day.

Subjects

Ninety-six adults (50±9 years old) with excessive body weight (mean BMI=35.85±4.67 kg/m^2) suffering MetS according to the IDF criteria completed the intervention period. All the participants gave written informed consent to participate as approved by the Ethics Committee of the University of Navarra (065/2009) and in accordance with the Declaration of Helsinki.

Participant's dietary intake was assessed by 48-hour weighed records at baseline and at the end of the intervention, which were analyzed using the DIAL software (Alce ingenieria, Madrid, Spain). Participants were asked to maintain their usual activity levels during the study, which was evaluated at the beginning and at the endpoint by a 24-h physical activity questionnaire (Lopez-Legarrea *et al.* 2013).

Anthropometric measurements and body composition determinations were performed, as described elsewhere (Zulet *et al.* 2011). Overnight fasting plasma levels of glucose and triglycerides were measured in an autoanalyser Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits from this company. Insulin concentrations were determined by an ELISA kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). The homeostasis model (HOMA-IR) was applied to estimate insulin resistance.

Irisin concentrations were determined in human plasma using a commercial ELISA kit following the manufacturer's instructions (Irisin ELISA kit EK-067-52; Phoenix Pharmaceuticals, INC, CA), on a spectrophotometric reader at wavelength of 450 nm (Versamax Microplate Reader, East Falmouth, MA). This test provided a range of detection of 0.066-1024ng/mL and exhibited a coefficient of variation of 6-10% inter-and intra-assay. The samples were kept at -80ºC and were analyzed immediately after the experiment was ended.

Statistical analysis

The sample size of this secondary analysis was calculated for an α=0.05 and a power of 80% based on the waist circumference reduction, as described elsewhere (Lopez-Legarrea *et al.* 2013). Normality distributions of the measured variables were determined according to the Shapiro Wilk test. Irisin plasma levels were not normally

distributed, but based on the sample size (n>60) a parametric test was performed. Indeed, after analysis with a log transformation of irisin values the statistical outcomes were maintained. Differences between baseline and endpoint values within groups were analyzed by a paired *t*-test. Between group analyses included unpaired *t*-tests. A multiple linear regression analysis was applied in order to assess the potential relationship between irisin and anthropometric and biochemical parameters (CI 95%). To analyze the effect of high or low irisin levels on glucose regulatory factors, the median (above and below the 50th percentile) cutoff values in the irisin were considered in the assayed population as previously applied (Crujeiras *et al.* 2010) and based on assigning the studied population into two groups of disease risk (Martinez-Gonzalez *et al.* 2005). The association between irisin levels and carbohydrate intake was analyzed using the parametric Pearson correlation. Data are reported as mean±SE. Statistical analysis was performed using the SPSS15.1 software (SPSS Inc, Chicago, USA). Significance was set at an alpha level of 0.05.

Table 1. Changes in selected anthropometric and biochemical parameters within each dietary group (Control and RESMENA) after the 8-week intervention and comparison between groups.

	Control group			**RESMENA group**			**P**
	Baseline	**Endpoint**	**p**	**Baseline**	**Endpoint**	**p**	**difference**
Body weight (kg)	99.45±2.80	92.72±2.71	<0.001	100.00±2.39	92.91±2.28	<0.001	0.555
BMI (kg/m²)	36.15±0.71	33.70±0.69	<0.001	35.55±0.64	33.03±0.61	<0.001	0.732
Fat mass (%)	39.13±1.10	36.19±1.10	<0.001	39.21±0.93	36.38±1.02	<0.001	0.854
Fat mass (kg)	38.97±1.57	33.68±1.48	<0.001	39.23±1.37	33.84±1.31	<0.001	0.886
Glucose (mg/dL)	121.02±4.99	107.98±2.02	0.006	123.81±5.46	110.22±3.78	0.016	0.939
Insulin (μU/mL)	15.30±1.69	9.32±1.06	<0.001	14.36±1.20	9.14±0.88	<0.001	0.557
HOMA	4.69±0.56	2.61±0.34	<0.001	4.48±0.44	2.60±0.29	<0.001	0.686
Triglycerides (mg/dL)	176±13	145±10	0.005	194±18	151±14	<0.001	0.421

BMI: Body Mass Index; HOMA: Homeostasis Model Assessment.

Results

Before starting the intervention there were not between-group differences in any of the anthropometric and routine biochemical markers ($p>0.05$). After the intervention, an improvement was observed on such measurements with apparently equal effectiveness between the two dietary treatments ($p>0.05$, Table 1). Therefore, both groups were merged for subsequent analyses. Mean body weight loss of the participants was -6.91±2.99kg. Irisin plasma levels were diminished after following the dietary treatment (Figure 1A) in association with changes in baseline body weight ($r=0.21$; $p=0.046$) and fat mass ($r=0.22$; $p=0.037$).

There were no differences in the physical activity from the beginning to the end of the intervention in both groups. Moreover, the regression analysis showed no association between physical activity factor and irisin changes ($p=0.736$). An association of glucose (B= -0.134, 95%CI: -0.245 to -0.024; $p=0.018$) and irisin changes, irrespective of confounding factors: gender, age, diet, body weight loss and irisin baseline values was found.

Interestingly, after adjusting for gender, age and weight loss, participants belonging to the high-irisin group at baseline (>307.99ng/ml) evidenced significantly greater reductions on glucose ($p=0.022$), insulin ($p=0.021$), HOMA index ($p=0.008$) and triglycerides ($p=0.006$; Figure 1B). Furthermore, irisin decreased more in the high-baseline irisin group (-126.62±15.93 ng/ml vs. -18.22±9.14 ng/ml; $p<0.001$) compared with the low-irisin (<307.99 ng/ml) group. Relevantly, after 8 weeks of nutritional intervention irisin levels positively correlated with carbohydrate intake ($r=0.234$, $p=0.023$; Figure 1C).

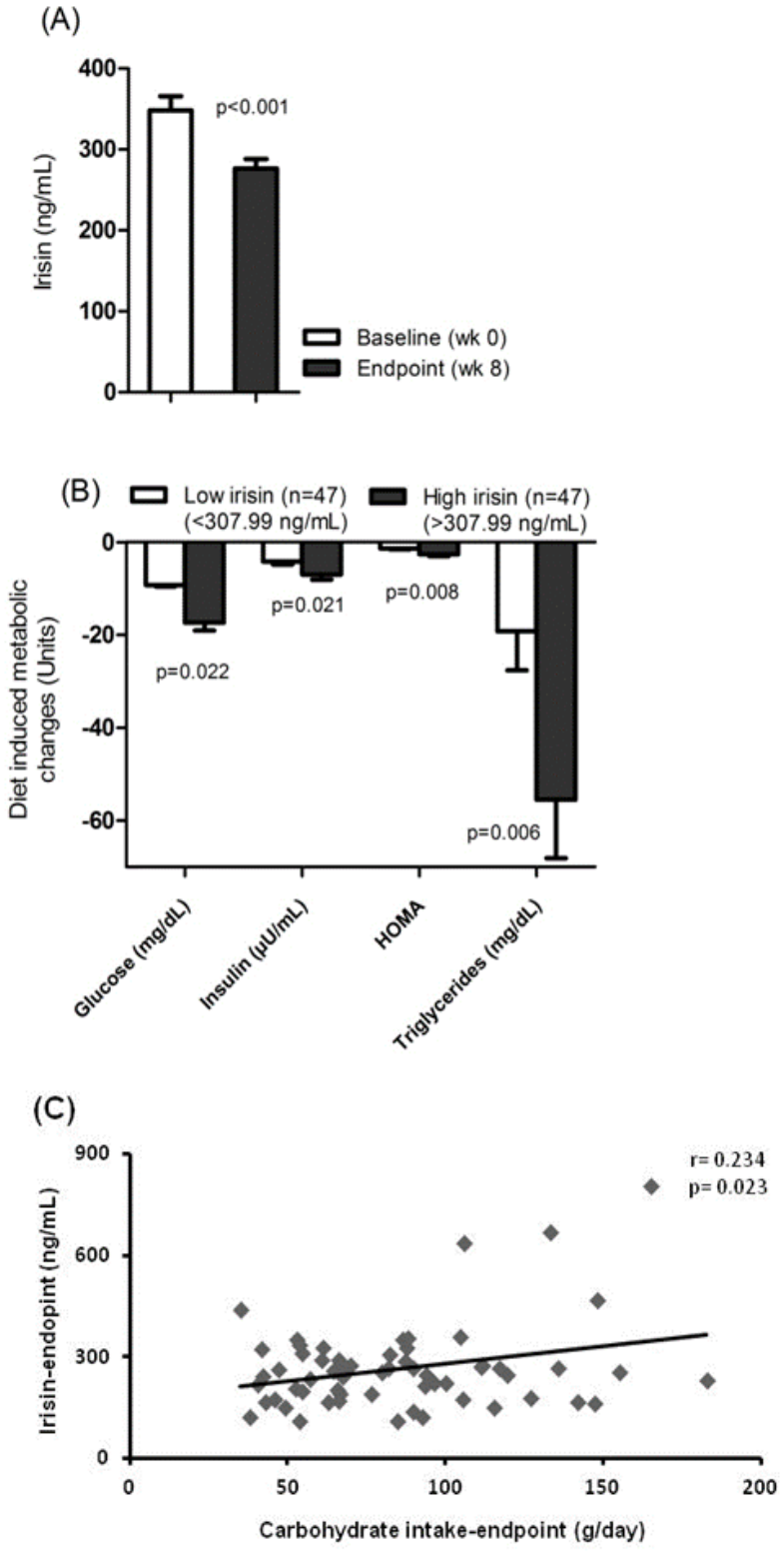

Figure 1. Irisin changes from baseline (wk 0) to the end of the intervention (wk 8) (A); changes in glucose, insulin, HOMA index and triglycerides, according to irisin baseline levels after the intervention of 8 weeks duration (B) and irisin correlation with carbohydrate intake at the endpoint of the intervention (C).

Discussion

Irisin levels decreased after following an energy-restricted treatment in obese subjects presenting MetS features and higher baseline circulating irisin levels are associated with a greater improvement of glucose related markers. These results suggest that irisin may modulate the relationship between excess body weight and glucose homeostasis, altered in the MetS (Stengel *et al.* 2013).

Because irisin is induced by exercise and it is able to increase energy expenditure, may play an important role in obesity and diabetes (Bostrom *et al.* 2012; Castillo-Quan 2012; Kelly 2012; Sanchis-Gomar *et al.* 2012). Interestingly, individuals with higher irisin levels at the beginning of the intervention achieved higher beneficial effects on glucose homeostasis markers such as insulin and HOMA index, but carefully examined. Our results do not contradict previous studies that reported an association of low irisin concentrations with type 2 diabetes situations (Liu *et al.* 2013; Choi *et al.* 2013; Hojlund and Bostrom 2013). The potential role of irisin on metabolism regulation was reflected also in the association between baseline irisin levels and a decrease in triglycerides, as others reported (Zhang *et al.* 2013). This hypothesis agrees with previous preclinical and clinical studies (Stengel *et al.* 2013; Roca-Rivada *et al.* 2013; Huh *et al.* 2012) suggesting that irisin production may accompany fat changes, but also that could contribute to fight against excessive fat content (Bostrom *et al.* 2012). This behavior was also observed for leptin and insulin (Marti *et al.* 1999). Thus, irisin could mean a physiological feedback to counteract potential glucose metabolism-related disturbances associated to an excessive body weight state, which may diminish as a consequence of the weight loss, since irisin is then "less" needed to restore glucose tolerance. Despite that irisin was speculated to have an effect on weight lowering (Bostrom *et al.* 2012) and that it seems that more weight is related with high irisin levels, a decrease in body weight may accompany a reduction in this myokine because the regulatory requirements of irisin are lower (Stengel *et al.* 2013). In any case, it should be noted that irisin has been also reported to be decreased within obese subjects (Moreno-Navarrete *et al.* 2012).

The association between irisin concentrations and carbohydrate intake was related to the consumption of some sources of carbohydrates (cereals, pulse, fruits and vegetables). This outcome may be explained because the dietary modifications during

the hypocaloric intervention evolved with shifts in carbohydrate consumption within the energy restriction. Thus, irisin could be increased in response to inappropriate dietary pattern in order to prevent/improve the elevation of parameters such as glucose, insulin or HOMA index values, linked to latter damage on multiple organs (Nolan *et al.* 2011). This finding is interesting given that different macronutrient distribution is a recurrent approach for treating obese and MetS patients (Lopez-Legarrea *et al.* 2013). These results appear to be irrespective to the physical activity level, since the patients in this study maintained physical activity.

The statistical adjustments for sex did not revealed specific differences between males and females concerning the analyzed irisin outcomes. A limitation of this study is that it demonstrated an association but not evidenced causation. Moreover, the methods to assess the dietary intake and physical activity were based on questionnaires, which could bias the results interpretation. However, the design of the current trial based on a nutritional intervention is an important strength allowing within-subject pre- and post-test comparisons.

This investigation concerns a potential role of irisin on impaired glucose homeostasis associated to obesity and, consequently, suggesting a metabolic interplay on glucose metabolism and insulin secretion control.

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Conflict of interest

None declared

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4.4. CHAPTER 4

SERPINE1, PAI-1 protein coding gene, methylation levels and epigenetic relationships with adiposity changes in obese subjects with metabolic syndrome features under dietary restriction

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5. GENERAL DISCUSSION

5.1. Background

The hypothesis for this research holds that the RESMENA program, a novel dietary strategy, based on increasing protein content and meal frequency, enhancing bioactive compounds and antioxidant-rich food intake and promoting low GI/GI meals is associated with a higher effectiveness on improving anthropometric, biochemical and inflammatory markers on obese subjects with MetS, as compared with a pattern based on the AHA guidelines (Krauss *et al.* 2000).

The WHO together with other international organisms has laid down that obesity has evolved into a worldwide 21st century epidemic due to the huge dimensions acquired along the last decades (WHO 2012). The MetS concept not only refers to obesity, but also encompasses interrelated alterations that share action mechanisms and risk factors such as insulin resistance and CVD (Grundy *et al.* 2005). Despite the use of different definitions for MetS has lead to a wide range of prevalence data, there is no doubt that it represents a leading health burden worldwide (Bhanushali *et al.* 2013; Di Daniele *et al.* 2012; Anagnostis 2012). In Spain, despite these criteria-dependent and some geographic variations MetS mean rates range between the 20% and the 30% of the adult population, according to several investigations carried out such as DARIOS (Fernandez-Berges *et al.* 2012), MADRIC (Martinez *et al.* 2008), CLYDIA (Palma-Gamiz *et al.* 2007), ESOPOH (de la Sierra 2006) and MESYAS (Alegria *et al.* 2005) projects.

Nevertheless, mechanisms accompanying obesity and clinical related diseases remain under investigation. Recent studies have proposed low-grade chronic inflammation as a related etiological factor in the onset and development of these disturbances (Bondia-Pons *et al.* 2012; Calder *et al.* 2011). Consequently, treatments for excessive body weight must target not only on weight and fat mass loss, but also on improving comorbidities such as the low-grade inflammation. To prevent and counteract excessive body fat accumulation and associated features dietary strategies have been the most frequently employed tool (Papadaki *et al.* 2013; Gargallo Fernandez *et al.* 2012). Since obesity has been traditionally defined as an excessive body fat accumulation as a consequence of a chronic positive energy balance (Salas-Salvado *et al.* 2007) dietary approaches have mainly focused on energy restriction (Crujeiras *et al.* 2008). Nonetheless, in the last years several dietary elements have separately been

studied to potentially exert additional benefits to calorie restriction. Indeed, alternative macronutrient distributions (Westerterp-Plantenga *et al.* 2012; Clifton 2012), meal frequency variations (Allirot *et al.* 2013) or rising the content on bioactive compounds such fiber or omega-3 fatty acids (Grube *et al.* 2013; Peairs *et al.* 2011) have shown diverse positive effects on controlling hunger/satiety and reducing/maintaining weight or improving obesity-related inflammation (Chaves *et al.* 2013). On the other hand, carbohydrates selection based on the GI/GL (Mirrahimi *et al.* 2012; Gogebakan *et al.* 2011), promoting the intake of antioxidant-rich food (Tresserra-Rimbau *et al.* 2013) or encouraging the adherence to specific dietary patterns evidenced as beneficial such as the Mediterranean standard have been also revealed to help in the prevention of glucose metabolism alterations and cardiovascular disease, among others (Zamora-Ros *et al.* 2013; Estruch *et al.* 2013).

Therefore, the current situation results in the need to enclose all available knowledge in order to reach greater effectiveness on treating obesity and related disturbances as well as for curbing the growth of affected population (Marchetti *et al.* 2012). Thus, the RESMENA project aimed at integrating all the aforementioned elements reported in the literature as beneficial within a dietary plan based on traditional food intake for a greater improvement in anthropometric and biochemical parameters, as well as the related inflammation status of obese subjects presenting MetS.

In this sense, scientific literature has reported nutritional intervention studies which have been carried out with the aim of reducing MetS criteria, being those focusing on the supplementation with a specific nutrient or food the most highlighted investigations. Among them, the PREDIMED (Ibarrola-Jurado *et al.* 2013) or the SUVIMAX (SUpplementation on VItamines et Mineraux AntioXydants) projects (Vergnaud *et al.* 2007) are some of the main endeavous. Briefly, the first one compared the effect of a MedDiet pattern supplemented on virgin olive oil with another supplemented on nuts and with a control diet reduced in fat, in people free of CVD at baseline but presenting major cardiovascular risk factors (Martinez-Gonzalez *et al.* 2012). Regarding the latest, it was designed to test the efficacy of daily supplementation with antioxidant vitamins and minerals at nutrition-level doses in reducing several major health problems in industrialized countries such as cancers and CVD (Hercberg *et al.* 1998). Notwithstanding, some research groups have focused on dietary patterns involving several nutritional aspects such as the DASH strategy (Dietary Approaches to

Stop Hypertension) with a reduced content on total and saturated fats, cholesterol and sugar (Shirani *et al.* 2013) or the OmniHeart (Optimal Macronutrient Intake Trial to Prevent Heart Disease) project in which a carbohydrate-rich, a protein-rich and an unsaturated-rich diet were compared (Gadgil *et al.* 2012); and the DiOGenes (DIet Obesity and Genes) trial, which assessed the effect of GI and protein consumption modifications on body weight loss and maintenance (Larsen *et al.* 2010).

Moreover, observational studies based on validated questionnaires have related food intake with several biochemical and body composition markers obtaining important conclusions in the understanding of the role of food patterns followed by a specific population and the specific nutrients/food associated with a healthy status. Some of them are the SUN (*Seguimiento Universidad of Navarra*) prospective dynamic cohort among Spanish university alumni with the aim of identifying the dietary determinants of stroke, coronary disease and other disorders (Barrio-Lopez *et al.* 2013) or the EPIC (European Prospective Investigation of Cancer-Norfolk) project, which included eight Europeans cohorts (Vergnaud *et al.* 2013).

In this context, the present study was designed as a randomized controlled intervention trial for comparing the RESMENA diet with a pattern considered a reference for losing weight and maintaining a healthy status such us the AHA approach (Krauss *et al.* 2000). The AHA guidelines were designed with the aim of preventing the development of recurrent heart and circulatory diseases and for promoting overall well-being, by enhancing the intake of a variety of food, limiting saturated fats, cholesterol, salt and alcohol (Krauss *et al.* 2000). Participants were allocated into one of the two groups and the project was divided in two sequential periods for both evaluating the effectiveness of the proposed strategy in the short and in the medium-term (Zulet *et al.* 2011). This thesis reports the findings of the first period in which participants were expected to learn and embrace the novel dietary habits achieving a high adherence in order to better evaluate the potential benefits of the dietary plans. During this period, a close fortnightly follow-up was performed by qualified professionals who controlled anthropometric and body composition by bioimpedance measurements as well as the adherence to the prescribed dietary treatment. Additionally, at the beginning and at the endpoint of the intervention body composition assessment by DXA was performed, blood and urine samples collected and 48-h weighted food dietary records, previously validated for Spanish population (Martinez-Gonzalez *et al.* 2005), required (Zulet *et al.*

2011). Thus, this experimental design allowed us to separately evaluate each dietary strategy compliance and effects along the 8 weeks of intervention and also to compare between-diet effectiveness and to assess the possible causes of different behaviors. Additionally, each specific component action could also be evaluated.

The dietary approaches were based on an energy restriction of the 30% to the total energy requirements of each patient after calculating resting metabolic rate by Harris-Benedict equation (Frankenfield 2013), where the Wilkenss adjusted weight was applied (de Oliveira *et al.* 2013), and considering the physical activity factor according to WHO classification (WHO 2012). Available literature evidences that low-calorie nutritional interventions for inducing weight loss which apply an energy restriction of the 30% to the required calorie intake per day lead to weight reductions between 0.5 kg and 1 kg per week (Papadaki *et al.* 2013).

5.1.1. Strengths and limitations of the study

The sample size of the present study was calculated considering the waist circumference reduction as the main variable in which a difference of 4.3±6.8 cm between the Control and the RESMENA groups was expected to be observed, taking into account inclusion criteria (Alberti *et al.* 2009) and previously reported outcomes (Katcher *et al.* 2008; Konig *et al.* 2008). The confidence index was set up at the 95% (α=0.05) and the statistics power at the 80% (β=0.8). For this purpose, one hundred people were estimated to be required 50 in each group, considering an expected withdrew rate of the 25%. One of the main problems of nutritional interventions is the high withdrew rate along the programs (Gonzalez-Cabrera *et al.* 2013). In this sense, during the RESMENA project intervention period 9 people dropped out, 3 participants from the Control group and 6 from the RESMENA group, which represents around the 8% of the participants. Therefore, 96 volunteers (48 for each group) finished the intervention and the final reached sample size as well as the follow up of the plan can be considered acceptable.

Another important issue to be considered is the adherence to the dietary treatment. Regarding this aspect, the 48 h weighed records allowed to evidence that the participants did not completely reach the scheduled amount of all of the dietary

components included. Thus, the obtained outcomes could benefit from a higher compliance of the prescribed treatments. It is also appropriate to notice the variability concerning the Spanish food composition tables employed for analyzing dietary records (DIAL software, Alce Ingenieria, Madrid), which may have gaps concerning some items, and that might influence the values regarding food intake and adherence to the prescribed patterns. In relation to the duration of the intervention, available literature reports many intervention studies developing 8-week duration programs which showed significant reductions in anthropometric parameters (Papadaki *et al.* 2013; Alves *et al.* 2013; Gogebakan *et al.* 2011). Nevertheless, sample size could be larger and the intervention period longer in order to confirm the outcomes.

Another strong point of the study was that both groups resulted homogeneous regarding mean age (50±9 years old in the Control group *vs.* 49±10 years old in the RESMENA group) and gender distribution (21 women and 27 men in the Control group *vs.* 24 women and 24 men in the RESMENA group), without baseline differences between them. Contrariwise, the fact the AHA diet, widely accepted as a healthy a beneficial pattern, was employed as the control for evaluating the new RESMENA strategy effectiveness instead of using a "typical diet" may have hampered to find more differences between groups, but give support to the differential findings. Finally the employed inflammatory score represents a good marker of inflammation and although not validated is a "trending" tool employed for assessing the inflammation status (Richard *et al.* 2012), and has been traditionally used for the evaluation of other health aspects.

In summary, the experimental design of this research allowed to show the relevance of focusing on different dietary components in addition to calorie content within intervention programs for improving anthropometric, biochemical and inflammation markers of obese subjects presenting MetS features.

5.2. Dietary effectiveness on anthropometric and biochemical parameters

The main objective of the RESMENA trial was to evaluate and compare two different dietary patterns in order to determine the effectiveness on improving MetS features, as well as the associated inflammation status. Both strategies applied the same energy restriction in order to better discriminate the potential additional benefit of the RESMENA-contained dietary elements, as are the increased protein consumption, the higher meal frequency, the greater intake of omega-3 PUFA, the enhanced total antioxidant capacity and the reduced GI/GL (Zulet *et al.* 2011). The two groups adjusted to the calorie content guideline and subsequently, the mean expected body weight reduction was achieved, as also reported by other authors employing this caloric restriction (Cameron *et al.* 2010). There were no differences between dietary groups, which suggested that none of the patterns as a whole is better than the other for inducing body weight reductions.

Initially, the adherence to the prescribed treatments was evaluated. RESMENA and control diets were designed maintaining the same fat intake but differing on protein and consequently on carbohydrate content. A moderately-high protein intake effects seeked to be evaluated, since this macronutrient has been related to greater weight reductions and better weight maintenance because of the satiety power and the thermogenic effect attributed to them (Westerterp-Plantenga *et al.* 2012; Abete *et al.* 2009). The RESMENA group did not completely achieve the protein prescribed intake, although it was high enough to significantly differentiate from the Control group. Simultaneously, differences on carbohydrate intake were also found, as scheduled. Concerning carbohydrates, the emphasis was focused on GI and GL, reported to be good indicators for carbohydrate-quality assessment (Goto *et al.* 2012). As prescribed, the RESMENA group evidenced a significantly higher GL, compared with the Control group, although we failed on reaching differences concerning GI, as well as fiber. Diverse results have been found in the literature in relation to fat content of the diet (Summerbell *et al.* 2008; Due *et al.* 2008, Petersen *et al.* 2006) but overall, the evidence support that low-fat diets are a good choice for the prevention of weight gain and obesity (Abete *et al.* 2010). In addition, recent reports enhanced the role of specific fat-

types intake because of have been revealed as beneficial (Urpi-Sarda *et al.* 2012; Aarsetoey *et al.* 2012). Therefore, the RESMENA diet maintained a 30% of total daily energy intake from fats enhancing the consumption of monounsaturated, as extra virgin olive oil, and polyunsaturated fatty acids, mainly from fatty fish. Dietary TAC indicates the global antioxidant content of the diet (Carlsen *et al.* 2010). Given that food rich in those components have been associated with a lowered incidence of various chronic diseases (Del Rio *et al.* 2011) the RESMENA strategy was designed as a high TAC pattern, achieving significant differences in consumption when compared with the Control group at the end of the nutritional intervention. Finally, the last aspect considered in the RESMENA diet was meal frequency. There is not much consistent evidence about this issue but some studies report that increasing the number of meals per day helps in controlling satiety and consequently favors weight loss (Allirot *et al.* 2013). Seven meals per day were included within the RESMENA pattern, higher that the 3-5 meals prescribed for the Control group, as recommended by the AHA (Krauss *et al.* 2000).

Regarding the role of the RESMENA and Control dietary strategies on improving anthropometric and biochemical determinations both resulted equally effective for reducing weight and the rest of the evaluated anthropometric markers, as well as body composition indicators. Such manner, although they were significantly reduced by each dietary treatment along the 8 weeks of intervention, there were no between-diet differences neither on lipid, non on glucose metabolism. Only transaminases reduction was significantly higher in the Control group, as compared with the RESMENA group, which might be explained by the fact that the RESMENA group was prescribed moderately-higher protein content than the Control group and we found an association between protein intake and transaminases levels. HDL-c was the only parameter that did not improve with none of the dietary treatments, which can be probably explained because of the reduction on total cholesterol which entails a reduction of this fraction too, as other authors found (Aicher *et al.* 2012). On the contrary, some researchers have evidenced higher HDL-c levels after comparable dietary interventions (Aicher *et al.* 2012; Belalcazar *et al.* 2012) specially related to the consumption omega-3 fatty acids intake (Ramel *et al.* 2010; Smith *et al.* 2009). The adherence to this guideline, however, was not as stricter as expected in our intervention. Therefore, these outcomes could benefit a better omega-3 PUFA adscription. Other

authors have also reported widespread low compliance with omega-3 fatty acids recommendations (Neville *et al.* 2012). Additionally, it is important to consider a relevant factor in relation to HDL-c values such as physical activity (Henson *et al.* 2013), where participants were asked to maintain along the intervention, and no specific exercise advice was provided. Therefore, it could not apparently contribute to increase the levels of this cholesterol fraction. Furthermore, HDL-c is also influenced by gender, as this study confirm revealing a significantly greater HDL-c among the women participants. In these line, differences between males and females regarding anthropometric and biochemical parameters have been widely investigated (Neville *et al.* 2012; Campbell and Meckling 2012; Alhazmi *et al.* 2012). We analyzed gender influence on the studied variable changes and it was found that body weight reduction was higher in men than in women, explained by the also higher restriction regarding absolute amount of calories. This weight reduction was accompained by an also higher decrease of fat mass, which agrees with previous reports (Neville *et al.* 2012). Insulin as well as ALT blood levels were significantly decreased within the group of men in accordance with other authors (van den Beld *et al.* 2000). These outcomes suggest that gender may be considered in order to design appropriated dietary plans.

Given that there were no major differences in relevant anthropometrical and biochemical measures, both dietary groups were considered together for specifically analyze the potential role of each single included dietary element. In this context, increasing protein intake in modifying the macronutrient distribution in place of just controlling energy content has been thoroughly investigated (Alves *et al.* 2013; Soenen *et al.* 2013; Gogebakan *et al.* 2011). This macronutrient has been associated to several benefits in weight reduction and maintenance (Westerterp-Plantenga *et al.* 2012; Larsen *et al.* 2010; Muzio *et al.* 2007), but they report on long-term findings (Gogebakan *et al.* 2011), which could explain the fact that in our research, despite an increased protein prescription in the RESMENA group no relationships with any anthropometric or body composition parameter was found. Nonetheless, an association protein consumption-transaminases was evidenced. ALT and AST are considered hepatic function markers and low levels are found under normal conditions. Thus, our outcomes might suggest that a moderately-high consumption of protein could not be beneficial for hepatic metabolism, in consistence with other studies which have previously reported a relationship of protein consumption with hepatic function (Yang *et al.* 2012). In

addition to the increase of total protein, the RESMENA pattern focused on enhancing the intake of fish derived protein, and specifically, fatty fish, in order to achieve a greater consumption of omega-3 fatty acids, widely reported as presenting beneficial properties (Raatz *et al.* 2013; Alhazmi *et al.* 2012). Our study evidenced a positive effect of the intake of fatty acids on fat mass reduction, being in agreement with previously trials (Ramel *et al.* 2010). This result could be more obvious improving the compliance of this nutrient because the RESMENA group did not completely reach the scheduled amounts.

Overweight and obesity are defined as a body fat accumulation subsequent to a chronic imbalance between energy intake and energy expenditure and several factors may influence energy intake (Martinez 2000). Behavioral aspects involving the number of eating episodes in a day are a fundamental one. However, there is no much recent literature concerning the optimal number of meals per day in the context of obesity and MetS and there is no scientific consensus because available data are controversial (Allirot *et al.* 2013; Cameron *et al.* 2010; Ohkawara *et al.* 2012). It has been traditionally postulated that increased meal frequency may confers benefits on body weight, adiposity and energy intake (Metzner *et al.* 1977; Kant *et al.* 1995; Hejda and Fabry 1964) by modulating appetite control (maximising sensation of fullness and minimising sensation of hunger) (Allirot *et al.* 2013) and possible gut peptides as well (Speechly *et al.* 1999). However, some other investigations did not find any relevant effects (Cameron *et al.* 2010; Palmer *et al.* 2009) or even some negatives ones (Allirot *et al.* 2013). In addition to excessive body weight, one of the disturbances entailed by the MetS is glucose metabolism impairment. In this sense, an increased feeding could be associated with the maintenance of constant glycemia levels. Therefore, taking into account lack of agreement about decreased, normal or increased feeding role in obesity and the trend to positive effects in glucose homeostasis we designed a pattern including 7 meals per day, with two snacks in the morning and other two in the afternoon in addition to breakfast, lunch and dinner. After 8 weeks of intervention there were no associations between the number of meals per day and the reduction in body weight, in accordance with a previous study from Cameron *et al.* (2010) that did not observe differences on body weight after an intervention comparing 3 *vs.* 6 meals per day. However, our study found a direct association of increased feeding and fat mass loss, that they did not notice (Cameron *et al.* 2010).

The RESMENA strategy also focused on GI and GL, which refer to the absorption rate of carbohydrates and measure the ability of a carbohydrate-containing food to raise the blood glucose level (Goto *et al.* 2012; Brand-Miller *et al.* 2002) . Low GI food provide a slower more consistent source of glucose to the bloodstream, thereby stimulating less insulin release than high GI food. The glycemic index and load of the included food were assessed in base on the database from the University of Sydney by Foster-Powel *et al.* (2002) researches and based on previous studies (Moore *et al.* 2010). A trend to a reduction in insulin levels according to lower GI/GL values was demonstrated after the intervention in agreement with other authors (Bao *et al.* 2011). This finding is an encouraging result since increased GI/GL values have been reported as potential risk factors for type 2 DM (Aller *et al.* 2011).

After analyzing every component isolated function on anthropometric and biochemical values on obese subjects with MetS features in the context of an energy restricted pattern, dietary TAC turned an important influencing factor, since it showed a positive association with body weight, BMI, waist circumference and fat mass reductions. Dietary TAC has been established in recent years as useful for assessing the global antioxidant capacity of a diet, based on its correlation with plasma total antioxidant capacity (Bahadoran *et al.* 2012). This index has been positively associated with improvements in several health conditions. Indeed, dietary TAC values have been reported to be inversely associated with central adiposity measurements (Hermsdorff *et al.* 2011) being the outcomes obtained in this research in agreement with the reported findings. Additionally, the TAC of the diet has been inversely related to BMI (Puchau *et al.* 2010), as we also reported; to glucose and lipid biomarkers (Hermsdorff *et al.* 2011, Puchau *et al.* 2010) and to systolic blood pressure (Puchau *et al.* 2010). Moreover, dietary TAC has been associated with lower risk of heart failure, indicating that a healthy diet, high in antioxidants may help to prevent this adverse feature (Rautiainen *et al.* 2013). Surprisingly, there was a higher reduction in the serum transaminases concentrations among the low-TAC group. A plausible explanation for this issue could be related to the protein intake, and not specifically to the dietary TAC, because the majority of participants presenting low dietary TAC values were also those with a lower protein intake as belonging to the Control group.

Another objective when designing the RESMENA pattern including all the reported dietary elements was to reach a high adherence to the MedDiet. For better carry out and evaluate the compliance of this purpose we took into consideration the Healthy Eating Index (Ye *et al.* 2013) obtained through the DIAL software, which computed different dietary aspects in order to assess the quality of the diet (Puchau *et al.* 2009). When evaluating the role within the intervention period a trend on influencing weight loss, as well as total cholesterol, LDL-c and transaminases levels was found, in agreement with previous studies that reported a cluster of several health benefits by the consumption of the MedDiet pattern (Abiemo *et al.* 2012; Perez-Guisado and Munoz-Serrano 2011; Pitsavos *et al.* 2005).

In summary, the reported outcomes indicate that the RESMENA novel dietary strategy resulted as effective as the AHA pattern for reducing weight and improving MetS features and therefore, could be considered a good alternative to the AHA treatment.

5.3. Dietary effects on the inflammation status associated to obesity

Once known each dietary strategy effects on anthropometric and biochemical and evidenced that there were no major differences between them, the other part laid out in this research concerned the assessment of dietary patterns role and possible discriminated impact on the inflammation status linked to obesity.

Inflammation is nowadays accepted as a mechanism mediating the association between obesity and related disturbances (Bondia-Pons *et al.* 2012). The concept of systemic, chronic but low-grade inflammation as a risk factor for the MetS is based on the observation of elevated blood levels of inflammation-associated markers in people with the syndrome (Calder *et al.* 2011). Therefore, it was raised that an appropriate approach for MetS should be effective on decreasing inflammation markers concentrations (Hermsdorff *et al.* 2012). A mechanistic link between obesity and low-grade inflammation was first proposed by Hotamisgil *et al.* (1993) who showed that white adipose synthesizes and releases the pro-inflammatory cytokine TNF-α. Thus,

TNF-α was the first pro-inflammatory cytokine associated to obesity and related insulin resistance (Hotamisligil *et al.* 1993) and subsequently several markers have been proposed as representative of the inflammation status. TNF-α has been shown to correlate with BMI and to be higher among obese with MetS as compared with those without complications (Hermsdorff *et al.* 2012; Xydakis *et al.* 2004). TNF-α together with IL-6 are considered to be useful markers of the inflammation linked to obesity since both are secreted by the adipose tissue, which is expanded in the obesity condition. CRP as a representative marker of acute reactants phase protein from the liver has been also linked to inflammatory processes (Zulet *et al.* 2007). CRP levels are increased in patients with MetS (Ridker *et al.* 2008) and it is considered an important independent predictor of cardiovascular events and coronary heart diseases (Jialal and Devaraj 2003). Finally, PAI-1 which is the principal inhibitor of tissue plasminogen activator and urokinase, and hence inhibits fibrinolysis (Declerck and Gils 2013), has been also found to be increased among obese subjects and it has been recently proposed as a possible constituent of the MetS definition (Belalcazar *et al.* 2011).

In this investigation, these four markers were assessed at the beginning and at the endpoint of the nutritional intervention in order to determine the two dietary approaches action on inflammation. When comparing each single marker after the 8 weeks no significant differences were noted between diets, except for PAI-1, significantly reduced within the Control group, but not within the RESMENA one. This finding is in accordance with previous studies that reported an association of the AHA pattern, in which the Control diet was based, with lower fasting values of inflammatory markers, such as CRP (Bhupathiraju *et al.* 2011). We failed to find significant differences concerning the other markers despite weight losses have been related in the literature with decreases in inflammation indicators. Indeed, when looking the rest of markers we observed the same trend although without reaching statistical signification, considering a two-tailed model, which can be due to the duration of the intervention. The outcomes could be more evident if the intervention time is prolonged and also by increasing the sample size. In any case, to further explore this issue an arbitrary inflammatory score was designed, not validated but "trendy", similar to previously employed by other authors (Richard *et al.* 2012), which integrated the four inflammatory indicators.

When comparing the final inflammatory scores between Control and RESMENA groups significant differences were evidenced showing the Control group lower values after 8 weeks of following its corresponding pattern, as compared with the RESMENA one. Subsequently, we aimed at discriminating whose of the dietary elements were involved in this observation and no associations with the inflammatory score were found for any of them, except for protein consumption. Aforementioned, increased protein of the diet has been one of the most evaluated approaches and several benefits have been attributed to them (Clifton *et al.* 2009). However, its role on inflammation remains under controversy. Whereas calorie restriction is known to be an activator of some metabolic pathways, which lead to a reduction on inflammatory markers, there is a lack of literature reporting if varying the protein content of the diet may have some beneficial effects in that issue. In this sense, one of the most important European controlled dietary intervention, as is the DiOGenes trial, reported a further decrease on hsCRP concentrations and low-grade inflammation after a low-protein diet, compared with the high-protein pattern (Gogebakan *et al.* 2011). The reported finding is consistent therefore with the outcomes obtained in this work, since here the Control group was prescribed a 15% TCV of proteins in contrast with the 30% TCV designed for the RESMENA group. Additionally, a recent meta-analysis evaluating the role of protein intake on diverse metabolic aspects revealed although without statistical signification that a higher protein intake has a detrimental effect for inflammation (Santesso *et al.* 2012). Therefore, just considering this issue, Control diet based on AHA criteria seems to be more favorable for inflammation than the RESMENA pattern. In any case, more studies are needed to confirm this information and elucidate potential underlying mechanisms.

Here vegetable and animal protein influences were separately explored. In the literature some discriminated behaviors for both kinds of protein have been reported regarding several health aspects (Takata *et al.* 2013; Martinez-Augustin *et al.* 2012; Abete *et al.* 2009). Therefore, we wondered if this outcome could also be influencing the protein-inflammation relationship. Thus, animal origin protein showed a positive association with inflammation while vegetable did not in obese subjects presenting MetS features after 8-weeks of nutritional intervention. The first result is in accordance with previous studies that report associations between red meat intake and CRP inflammation marker (Azadbakht and Esmaillzadeh 2009) or others evidencing

detrimental effects of meat intake (Takata *et al.* 2013). With respect to vegetable protein, there is some research that have evidenced a protective role against the inflammatory status (Hermsdorff *et al.* 2011; Crujeiras *et al.* 2007), whereas some others did not found any effect (Azadbakht *et al.* 2007) agreeing with data reported here. Subsequently, meat and fish derived protein was specifically explored. We found a positive association between meat intake and the inflammation score after 8 weeks of nutritional intervention, in agreement with aforementioned reports (Azadbakht and Esmaillzadeh 2009). We failed however on finding associations that concern fish intake, despite a protective role because of the high content on omega-3 PUFA could be expected (Tsitouras *et al.* 2008). This result might be explained by the failure to follow omega-3 fatty acids recommendations and also to the fact that we did not differentiate between white and fatty fish. The positive association observed between meat and inflammation can be attributed to several components occurring in meat. Thus, advanced glycation end products have been shown to increased plasma hsCRP and other inflammatory markers concentrations (Uribarri *et al.* 2005; Peppa *et al.* 2002). Moreover, free iron can increase oxidative stress acting as a proinflammatory agent (Fernandez-Real *et al.* 2002) and finally, the high content on saturated fat in meat could also contribute to the noted effects, given that it has been associated with elevated levels of CRP (Santos *et al.* 2013).

Indeed, it is appropriate to discuss on the protein profile of each dietary strategy. The RESMENA diet was designed as moderately-high protein (30% TCV) enhancing fatty fish and vegetable protein intake. However endpoint data from the 48 h weighted-records showed that the RESMENA group did not achieve those prescriptions since data of vegetable and fish protein intake were similar for both groups. In contrast, the RESMENA group evidenced a significantly higher intake of meat when compared with the Control group. Therefore, this information revealed that the higher protein intake within the RESMENA group was probably reached by increasing meat intake, which is not in accordance with prescriptions.

In summary, a moderately-high protein intake might be associated with a worse inflammatory status in obese subjects presenting MetS symptoms following an energy-restricted pattern if the protein source is animal and specifically meat. Contrariwise, no adverse effects have been evidenced at least in the short-term for fish or vegetable origin protein. This observation indicates that protein source must be considered when

designing a dietary pattern. Moreover, it can be suggested that dietary strategies moderately-high in protein could be a good alternative for achieving the benefits on weight loss/maintenance attributed to this macronutrient without negatively affect inflammation if the enhanced protein sources are from vegetable and fish.

5.4. Irisin role in the context of obesity and metabolic syndrome

The recently discovered myokine is named irisin to honor the Greek Goddess Iris, who served as a messenger among the Gods in the ancient Greek, given that it is probably palying a role as mediator in the metabolism (Bostrom *et al.* 2012). Irisin is a newly exercise-induced hormone produced by skeletal muscle that has drawn much attention as a novel preventive and therapeutic target to treat obesity and metabolic diseases like type 2 diabetes (Raschke and Eckel 2013). The scientific community has placed high hopes on it since it has been involved in the regulation of human energy metabolism (Wen *et al.* 2013; Swick *et al.* 2013; Moreno-Navarrete *et al.* 2012).

This hormone was characterized by Boström and collaborators and it was shown to be expressed in muscle especially stimulated with exercise (Bostrom *et al.* 2012). Boström *et al.* observed in mice that overexpression of PGC1-alpha in muscle increased the expression of the membrane protein FNDC5, further proteolytically processed, being the main source of circulating irisin. Subsequently, irisin produces profound changes in the subcutaneous adipose tissue stimulating browning and UCP1 expression (Bostrom *et al.* 2012). After this finding, several studies have further confirmed muscle FNDC5 gene expression (Lecker *et al.* 2012; Timmons *et al.* 2012) and increased circulating irisin levels in humans (Huh *et al.* 2012) in response to acute exercise. Moreover, lower expression levels of FNDC5 have been also detected in other major organs, such as kidney, liver, lung and adipose tissue as compared to muscle (Huh *et al.* 2012). Interestingly, Roca-Rivada *et al.* (2013) have recently identified FNDC5/irisin as a potential adipokine since demonstrated to be expressed in white adipose tissue and especially by subcutaneous adipose tissue.

Given the potential role of irisin against obesity, the relationship between this myokine and BMI has been widely investigated. Thus, Huh *et al.* (2012) observed a positive association, although not significant, between irisin and BMI, being in concordance with another experiment were the association achieved statistical significance ($p<0.001$), in which they compared anorexic, normal, overweight and obese participants (Stengel *et al.* 2013). Irisin circulation levels were significantly greater among the subjects with more fat content, suggesting a physiological protective role. Thus, it could be speculated that irisin may be released as a compensatory mechanism for the appearance of obesity, somehow acting to protect the body against increase fat accumulation. However, these results differ from those of Moreno-Navarrete *et al.* (2012), were an inverse correlation between circulating irisin and BMI was observed. Interestingly, both muscle FNDC5 mRNA levels and circulating irisin levels were significanlty decreased 6 months after bariatric surgery (Huh *et al.* 2012), suggesting again the relationship of irisin to body weight and adiposity.

In the present study the potential relationship of irisin and glucose homeostasis-related parameters in volunteers with MetS participating in the RESMENA weight-loss programme for 8 weeks was evaluated. Firstly, irisin levels were significantly decreased after the hypocaloric intervention. Interestingly, this reduction was higher within the group of participants presenting greater baseline values, in the same way that happens with other conditions such as body weight, which lost is higher among subjects presenting a more pronounced overweight (Thorsdottir *et al.* 2007). This irisin behavior could indicate a similar pattern as leptin or insulin, which are elevated under obesity and reduced after following a hypocaloric diet (Cordero *et al.* 2011). In other words, irisin may diminish subsequently to weight loss, since it is less needed due to the metabolic condition improvement after a body weight reduction. Secondly, high baseline circulating irisin levels were associated with a greater reduction of glucose, insulin and HOMA index at the end of the interventional period, indicating that irisin may constitute a physiological attempt to restore glucose metabolism altered in the MetS. In this regard, elevated irisin levels have been associated with reduced new onset of type-2 DM (Choi *et al.* 2013) and may give rise to a physiological function on improving glucose tolerance as suggested in a rodent model (Bostrom *et al.* 2012). Triglycerides levels were also significantly associated to irisin baseline concentrations and the participants who showed a greater improvement were those allocated in the high-irisin

baseline values group. Ttriglycerides correlate with glucose and therefore, the same response must be expected for them (Karpe *et al.* 2011). Another possible explanation for this could be that the increased energy expenditure induced by irisin entails a higher utilization of these compounds as energy substrate. Also, Zhang *et al.* (2013) reported a relationship between irisin and triglycerides. The investigations found that higher serum irisin levels were associated with preferable systemic triglycerides levels. Moreover their study provided for first time evidence that serum irisin concentrations were inversely associated with the triglycerides content in the liver. Based on these inverse associations of irisin with liver fat and triglycerides in obese adults, they suggested that irisin might be a protective factor for fatty liver disease. On the other hand, a study performed in patients with chronic kidney disease also evidenced a role for irisin in energy expenditure, since they found that plasma irisin levels were significantly decreased in these patients and suggested that this feature is associated to the abnormal energy expenditure and metabolism found among chronic kidney disease patients (Wen *et al.* 2013).

On the other hand, since a nutritional intervention was performed, we wanted to evaluate the potential influence of dietary components on irisin levels. Interestingly, a positive correlation with carbohydrates was found at the endpoint of the period. Since carbohydrates are a macronutrient, whose excessive consumption lead to the elevation of some markers (glucose, insulin, HOMA index) associated to glucose metabolism impairements (Nolan *et al.* 2011). These results may indicate again that irisin could act somehow as a compensatory mechanisms. Barely works exist focusing on irisin role on carbohydrate metabolism, but other myokines, mainly IL-6 (Pedersen and Febbraio 2012), as well as other hormones such as leptin, have been revealed to positively influence glucose homeostasis (Zuo *et al.* 2013; Coppari and Bjorbaek 2012; German *et al.* 2010).

The main outcome of these anlyses was that the variation of these indicators along the nutritional intervention program was associated with irisin baseline concentrations. In this context, it can be speculated that, as is it a metabolism-activator, the more the glucose homeostasis parameters levels decrease, the less the irisin action and vice-versa.

As a conclusion, the outcomes of this area of research suggest an involvement of irisin on glucose metabolism probably acting as a compensatory mechanism in metabolically altered situations and may play an important role in the pathology of insulin-resistance related disorders. In other words, irisin may constitute a physiological attempt to restore glucose metabolism, altered in the MetS In turn, the correlation found with carbohydrate consumption represent a very interesting finding considering that dietary strategies and specially, macronutrient modification are one the most recurrent approaches for the treatment of obesity and associated comorbidities. Although more research is needed in order to confirm these outcomes and deepen on underlying acting mechanisms pathways, they will open new insights concerning the role of the recently discovered myokine irisin.

5.5. *SERPINE1* methylation levels associated with obesity indicators

In the last years, epigenetic research has risen as a new tool that allows the study of the influence of several environmental factors, such as diet, on some multifactorial-origin diseases, among which obesity is found (Hermsdorff *et al.* 2013). Indeed, it has been observed that epigenetic changes and consequently, the regulation of specific genes expression (Niculescu and Lupu 2011; Lomba *et al.* 2010) can be modulated by interindividual differences due to inheritance transmission, dietary components, metabolic features and also by physical activity modifications (Lim and Song 2012; Slomko *et al.* 2012). Moreover, it has been recently proposed that each individual methylation pattern could condition the response capacity to an intervention. In this sense, epigenetics marks have been suggested as predictive tools for prognosis individual responses to an energy restriction (Milagro *et al.* 2011; Kussmann *et al.* 2010) and have been also related to the adipogenesis process (Zhu *et al.* 2012) and to age-related inflammatory diseases (Gowers *et al.* 2011). Nowadays, there are three major objectives in epigenetics research in relation to obesity, including the search of epigenetics biomarkers to predict future health problems or detect the individuals at most risk; the understanding of obesity-related environmental factors that could modulate gene expression by affecting epigenetics mechanisms; and the study of novel

therapeutic strategies based on nutritional or pharmacological agents that can modify epigenetic marks (Milagro *et al.* 2013)

One of the most common epigenetics mechanisms is DNA methylation, which is mainly of cytosine nucleotide at the carbon 5 position and often found in sequence context CpG (cytosine followed by a guanine) (Mansego *et al.* 2013). Promoter methylation is typically associated with repression, whereas genic methylation is correlated with transcriptional activity (Ball *et al.* 2009). In this context, there are multiple methods to study the epigenetic DNA methylation pattern, such as bisulfite sequencing PCR-BSP (Frommer *et al.* 1992), pyrosequencing (Ahmadian *et al.* 2006), combined bisulfate restriction analysis (Xiong and Laird 1997), methylation microarrays (Bouchard *et al.* 2010), methylation specific PCR-MSP (Herman *et al.* 1996) or matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Lomba *et al.* 2010). An array-based specific DNA methylation analysis was performed in this study (Mansego *et al.* 2013).

In this research *SERPINE1* gene DNA methylation levels were specifically investigated. *SERPINE1* codes for PAI-1 which is the main inhibitor of tissue-type (t-PA) and urokinase-type plasminogen activator and therefore plays an important role in the plasminogen-plasmin system (Declerck and Gils 2013). PAI-1 is involved in a variety of cardiovascular diseases as well as in cell migration and tumor development (Fortenberry 2013). This serine protease is widely accepted as a biological marker of inflammation and cardiovascular disease risk, both issues closely associated to obesity. Indeed, several findings suggest that there are multiple mechanisms through which inhibition of PAI-1 may promote cardiovascular health (Simone and Higgins 2012). Therefore, the main objective of this study was to deepen in PAI-1 behavior by establishing PAI-1 signaling pathway mechanisms, thought epigenetic analysis.

In the adult population, it has been observed that some gene methylation processes before an energy-restriction can predict a better response to the treatment (Crujeiras *et al.* 2013; Moleres *et al.* 2013). Not many investigations have been performed concerning PAI-1 and *SERPINE1*, but several authors have observed how methylation levels of other proinflammatory genes, such as, TNF-α are associated with the response to an intervention program (Cordero *et al.* 2011; Milagro *et al.* 2011) and even that methylation levels vary from the beginning to the end of an intervention

depending on the response magnitude (Bouchard *et al.* 2010). On the other hand, Wang and colleagues (Wang *et al.* 2010) showed that the methylation pattern determined in peripheral blood leukocytes in obese adolescents differ from those no obese. Moreover, previous studies have also highlighted that DNA methylation levels of several CpG sites of TNF-α may play a relevant role in inflammation-mediated diseases (Nimmo *et al.* 2012; Gowers *et al.* 2011; Campion *et al.* 2009; El Gazzar *et al.* 2009).

In the present study the sample was categorized accorded to *SERPINE1* CpG 10 median methylation level before starting the dietary program to better known the influence of epigenetic marks on the response to the intervention. Such site was selected for subsequent assessments given that in the preliminary analysis it evidenced potential associations with several adiposity indicators. Thus, it was found that anthropometric and biochemical modifications differentially varied depending on the methylation levels. Briefly, it was found that higher methylation levels at baseline, known to lead to lower mRNA (Gao *et al.* 2005), were associated with major reductions in body weight, total and android fat mass, triglycerides and total cholesterol, in accordance with previously reported studies that evidenced differences for changes in body weight, fat mass percentage and lipid profile (Cordero *et al.* 2011) and truncal fat (Hermsdorff *et al.* 2013) depending on the methylation degree of specific genes. However, a recent study (Zhang *et al.* 2012) did not find any relationship between IL-6 promoter methylation and age, gender, race/ethnicity, body mass index, physical activity or diet. In any case, the relationship between *SERPINE1* methylation and obesity indicators reported here could contribute to understand the link between PAI-1, an important pro-inflammatory marker and anthropometric and biochemical changes related to obesity. In turn, this leads to suggest that *SERPINE1* methylation levels were related to a better response to the 8-week intervention under energy-restriction and, consequently, to a major improvement of obesity indicators in obese individuals presenting MetS features.

These results are encouraging, since it was found that *SERPINE1* initial methylation levels could be used as a good marker for predicting the individual response to a dietary treatment based on energy restriction and can contribute on the way toward personalized nutrition based on epigenetic criteria. This term has risen as a new concept in the line with the fields of nutrigenomics, proteomics and metabolomics and the advances are expected to lead to genome-customized diets for obesity and related comorbidities prevention and treatment based on personalized approaches.

5.6. References

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6. CONCLUSIONS / CONCLUSIONES

1. A dietary strategy including a moderately-increased amount of protein, a higher meal frequency, enhancing antioxidant-rich and low-GI/GL food, promoting the intake of bioactive compounds and encouraging the adherence to the MedDiet pattern (RESMENA) was found to be as effective as the AHA-based pattern for reducing adiposity and biochemical parameters of obese subjects presenting MetS features along an 8-week intervention.

2. Dietary total antioxidant capacity within both diets turned apparently the most influential among the analyzed dietary elements on improving obesity indicators, such as body weight, BMI and fat mass, at least in the short-term, in the context of a hypocaloric dietary treatment.

3. Protein consumption was the only dietary element included within the dietary patterns that evidenced an association, which was positive, with the inflammatory status linked to obesity and MetS within a hypocaloric diet. Moreover, the type of protein showed to influence inflammation, since animal derived protein and specifically meat protein evidenced a detrimental effect on the inflammatory status of obese subjects presenting MetS traits, whereas fish and vegetable protein did not show any association, at least in the short-term.

4. The recently discovered myokine irisin was associated with the reduction of some glucose metabolism markers (glucose, insulin and HOMA) on obese subjects presenting MetS symptoms following an energy-restricted dietary treatment for 8 weeks, suggesting a role for irisin in glucose metabolism regulation after dietary induced weight loss.

5. Methylation levels of *SERPINE1* gene, which codes for PAI-1, were found to be associated to the reduction of body weight, fat mass, total cholesterol and triglycerides in obese subjects with MetS features under an 8-week energy restriction, which suggest that *SERPINE1* epigenetic modifications could have a predictive role to the response concerning a nutritional energy-restricted intervention.

General conclusion:

Overall, the outcomes obtained in this research enabled to suggest that the novel dietary strategy RESMENA could be considered a good alternative for improving adiposity and biochemical markers of obese subjects presenting MetS features in the short-term. Moreover, this dietary plan could be also beneficial for reducing inflammation if vegetable protein consumption is encouraged. Finally, the role of hormones, such as irisin, and epigenetics modifications must be taken into account for better understand the response to an energy-restricted dietary treatment of obese subjects with MetS traces.

1. Un régimen dietético con una cantidad de proteínas moderadamente elevada, una alta frecuencia de tomas diarias, que promueve el consumo de alimentos con bajo índice/carga glucémica y alto contenido en antioxidantes, así como una elevada adherencia a la Dieta Mediterránea (RESMENA) resultó tan efectiva como el patrón basado en las recomendaciones de la Asociación Americana del Corazón (AHA) en la reducción de parámetros bioquímicos y de adiposidad de sujetos obesos, que presentaban manifestaciones de síndrome metabólico, tras una intervención de 8 semanas.

2. La capacidad antioxidante total en ambas dietas resultó aparentemente el elemento más influyente de cara a la mejora de los indicadores de obesidad analizados, como el índice de masa corporal o la masa grasa, en el contexto de una dieta hipocalórica, al menos a corto plazo.

3. El consumo de proteína fue el único elemento incluido en los patrones dietéticos que mostró una relación, que fue positiva, con el estado inflamatorio asociado a obesidad y síndrome metabólico, en el contexto de una dieta hipocalórica. El tipo de proteína consumido influyó de manera diferente en los indicadores inflamatorios, de forma que la proteína de origen animal, y específicamente la proteína de la carne, mostró un efecto desfavorable en el estado inflamatorio de sujetos obesos con signos de síndrome metabólico, mientras que la proteína del pescado, así como la de origen vegetal no evindenciaron ningún tipo de asociación con marcadores inflamatorios, al menos a corto plazo.

4. La mioquina irisina, recientemente descubierta, se asoció con una reducción en algunos marcadores del metabolismo de la glucosa (glucosa, insulina e índice HOMA) en sujetos obesos con síndrome metabólico, tras una pérdida de peso como consecuencia de un tratamiento dietético de restricción energética. Este hallazgo podría sugerir un papel de la irisina como mecanismo compensatorio

ante situaciones de alteración en la homeostasis de la glucosa, como ocurre en la obesidad.

5. Los niveles de metilación del gen S*ERPINE1*, que codifica para PAI-1, se asociaron con la reducción del peso corporal, la masa grasa, el colesterol y los triglicéridos de sujetos obesos con síndrome metabólico bajo restricción energética de 8 semanas, sugiriendo que las modificaciones epigenéticas del gen *SERPINE1* podrían ser un buena herramienta predictora de la respuesta a una intervención nutricional con dieta hipocalórica.

Conclusión general:

Los resultados obtenidos en esta investigación permiten sugerir que una estrategia dietética incluyendo una cantidad de proteína moderadamente aumentada, una mayor frecuencia de tomas, bajo índice/carga glucémica, alta capacidad antioxidante y elevada adherencia a la Dieta Mediterránea puede considerarse una buena alternativa de cara a mejorar marcadores antropométricos y bioquímicos de sujetos obesos con manifestaciones de síndrome metabólico, a corto plazo. Además, este plan dietético podría ser también beneficioso de cara a reducir la inflamación asociada a obesidad si se fomenta el consumo de proteína de origen vegetal. Finalmente, el papel de las hormonas, como irisina, y la modulación epigenética deben tenerse en cuenta para comprender mejor la respuesta a un tratamiento de restricción energética de sujetos obesos con síndrome metabólico.

7. SUMMARY / RESUMEN

SUMMARY

This PhD dissertation report ecompasses the effect of a new energy-restricted dietary strategy (RESMENA) involving different dietary aspects such us a modified macronutrient profile including a moderately increased amount of proteins, an augmented meal frequency, an enhancement of low GI/GL and high antioxidant content food and with a high adherence to the Mediterranean Diet, on anthropometric, biochemical and inflammatory markers in obese subjects presenting metabolic syndrome (MetS) features after a nutritional intervention of 8 weeks duration. Additionally, this research compared the effectiveness of the RESMENA diet with the one of a pattern based on the American Heart Association (AHA) guidelines and studied potential underlying mechanisms such us hormonal influences and epigenetic modifications.

In the first chapter the effects of the two dietary patterns on anthropometric and biochemical indicators from baseline to the end of the intervention were compared. Moreover, the potential influence of each specific dietary component included in the pattern was separately analyzed. Firstly, it was observed that the two dietary regimens were equally effective on improving the obesity indicators since there were not statistical differences between them. Secondly, dietary total antioxidant capacity was evidenced as the major influential element given that several anthropometric and biochemical indicators such us body weight, BMI and fat mass, as well as AST and ALT were changed.

The second chapter reports the findings after analyzing the dietary patterns influence on the inflammatory markers CRP, PAI-1, TNF-α and IL-6 separately, and also considering all together by computing a score. The AHA-based pattern showed to be more beneficial in order to improve the inflammation status associated to obesity, at least in the short-term. Again, the potential role of each specific component was evaluated and it was observed an association between protein intake and the inflammation condition. Subsequently, vegetable and animal derived protein, and specifically among the latter, meat and fish proteins were evaluated. Interestingly, animal origin protein as a whole, and meat derived protein showed to negatively

influence the inflammatory status. However associations were found neither for vegetable origin protein, nor for fish.

In the third research aim, the recently discovered myokine irisin was studied. First of all, it was observed that irisin plasma concentrations decreased after following the energy-restricted dietary patterns, without differences between them, in association with body weight reduction. Then, this substudy evidenced that irisin levels were associated with changes in glucose homeostasis parameters, being greater the reductions on glucose, insulin and HOMA index among the participants with higher irisin baseline levels.

Finally, the fourth chapter analyzed epigenetic modifications, specifically DNA methylation, of *SERPINE1* gene, which codes for PAI-1, and its potential influences on the observed outcomes after the energy-restriction. The higher methylation levels at baseline, the greater reductions were observed in some obesity indicators such us body weight, total and android fat mass, as well as triglycerides and cholesterol, which suggests a potential role of *SERPINE1* methylation levels as a predictor of the response to a hypocaloric dietary treatment.

In summary, data from the current Doctoral Thesis report enabled to provide evidence about the effectiveness of a new dietary pattern and about the influence of specific dietary components (total antioxidant capacity and proteins) on anthropometrical, biochemical and inflammatory markers of obese subjects with MetS after a nutritional intervention of 8-weeks duration. Furthermore, the influence of the recently discovered irisin and epigenetics modifications has been found as influential factors in mediating the observed outcomes.

RESUMEN

La presente memoria de Tesis Doctoral recoge la investigación sobre el efecto de una nueva estrategia dietética hipocalórica (RESMENA) basada en la modificación de diferentes aspectos dietéticos como el contenido en macronutrientes, aumento del aporte de proteínas en detrimento de los hidratos de carbono, una mayor frecuencia de tomas diarias, favoreciendo el consumo de alimentos con bajo índice/carga glucémica y con una elevada capacidad antioxidante y adherencia a la Dieta Mediterránea sobre marcadores antropométricos, bioquímicos e inflamatorios de sujetos obesos con síndrome metabólico, tras una intervención nutricional de 8 semanas de duración. Además, este trabajo comparó dichos efectos con los de un patrón basado en las recomendaciones de la Asociación Americana del Corazón (AHA) y estudió posibles mecanismos hormonales y epigenéticos involucrados.

En el primer capítulo se comparó el efecto de los dos patrones estudiados sobre las variables antropométricas y bioquímicas, desde el comienzo hasta el final de la intervención. Además, se analizó de manera individual el potencial papel de cada uno de los componentes dietéticos incluidos. Primeramente, se observó que los dos planes resultaron igualmente efectivos, sin encontrarse diferencias estadísticamente significativas para ninguna de las variables estudiadas. A continuación, la capacidad antioxidante total de la dieta resultó ser el elemento más influyente, ya que mostró una asociación negativa tanto con el peso corporal, como con el índice de masa corporal, la masa grasa.

El segundo capítulo recoge la resultados obtenidos al analizar la influencia de ambos patrones dietéticos en los marcadores de inflamación CRP, PAI-1, TNF-α e IL-6, considerándolos de manera individual, pero también conjuntamente en forma de "score". El patrón basado en las recomendaciones de la AHA resultó más beneficioso para estado inflamatorio asociado a obesidad, al menos a corto plazo. Además, se evaluó el papel específico de cada componente dietético y se observó una asociación entre el consumo de proteína y los niveles de marcadores inflamatorios. A continuación se analizó por separado la proteína de origen vegetal y animal, y dentro de esta última, la proteína de la carne y del pescado. Tanto la proteína de origen animal en su conjunto, como específicamente la de la carne, mostraron un efecto negativo en el estado

inflamatorio. Por el contrario, no se observó ninguna asociación ni para la proteína vegetal ni para la del pescado.

En el tercer artículo se estudió la nueva mioquina irisina. En primer lugar, se observó que las concentraciones plasmáticas de irisina disminuyeron tras la intervención con dieta hipocalórica, sin diferencias entre los dos planes analizados, y en asociación con la pérdida de peso. Posteriormente, se encontró una asociación de los niveles de irisina con los cambios en parámetros relacionados con el metabolismo de la glucosa, siendo mayor la reducción de los niveles de glucosa, insulina e índice HOMA entre los individuos con mayores concentraciones plasmáticas de irisina al inicio de la intervención.

Por último, en el cuarto capítulo se analizaron modificaciones epigenéticas, específicamente metilación del DNA, en el gen *SEPRINE1* que codifica para PAI-1, así como su posible influencia en los resultados obtenidos tras la intervención nutricional. Se observó que cuanto más altos eran los niveles de metilación al inicio del estudio, mayores fueron las reducciones observadas en algunos indicadores de obesidad, como el peso corporal, la masa grasa total y androide, así como en los niveles de triglicéridos y colesterol, lo cual sugiere un papel potencial de los niveles de metilación de *SERPINE1*, como herramienta predictora de la respuesta a un tratamiento dietético de restricción energética.

En resumen, los resultados de la presente Tesis Doctoral aportan evidencias sobre la efectividad de un nuevo patrón dietético en el tratamiento de la obesidad y el síndrome metabólico, así como sobre la influencia de componentes dietéticos específicos (capacidad antioxidante total y proteínas), principalmente sobre marcadores antropométricos, bioquímicos e inflamatorios tras una intervención nutricional de 8 semanas. Además, se ha evidenciado la importancia de la nueva mioquina irisina en la regulación de aspectos metabólicos, como la homeostasis de la glucose. Por último, esta investigación ha puesto de manifiesto la utilidad del análisis de modificaciones epigéneticas de cara a la optimización de posibles tratamientos de la obesidad, contribuyendo a una nutrición personalizada basada en criterios epigenéticos.

8. APPENDIX: OTHER PUBLICATIONS

APPENDIX 1

The reduction of the metabolyc syndrome in Navarra-Spain (RESMENA-S) study; a multidisciplinary strategy based on chrononutrition and nutritional education, together with dietetic and psychological control

El estudio RESMENA-S: reducción del síndrome metabólico; una estrategia multidisciplinar basada en la crononutrición y la educación nutricional, junto con control dietético y psicológico

Zulet, M.A. (María Ángeles), Bondia-Pons, I. (I.), Abete, I. (Itziar), Iglesia, R. (Rocío) de la, Lopez-Legarrea, P. (Patricia), Forga, L. (Luis), Navas-Carretero, S. (Santiago)

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Zulet MA, Bondia-Pons I, Abete I, de la Iglesia R, Lopez-Legarrea P, Forga L, et al. The reduction of the metabolyc syndrome in Navarra-Spain (RESMENA-S) study: a multidisciplinary strategy based on chrononutrition and nutritional education, together with dietetic and psychological control. Nutr Hosp 2011 Feb;26(1):16-26.

Abstract: The high prevalence of metabolic syndrome (MS) in Spain requires additional efforts for prevention and treatment. Objective: The study RESMENA-S aims to improve clinical criteria and biomarkers associated with MS though an integral therapy approach. Methods: The study is a randomized prospective parallel design in which is expected to participate a total of 100 individuals. The RESMENA-S group (n = 50) is a personalized weight loss (30% energy restriction) diet, with a macronutrient distribution (carbohydrate / fat / protein) of 40/30/30, high meal frequency (7 / day), low glycemic index/load and high antioxidant capacity as well as a high adherence to the Mediterranean diet. The control group (n = 50) is assigned to a diet with the same energy restriction and based on the American Heart Association pattern. Both experimental groups are under dietary and psychological control during 8 weeks. Likewise, for an additional period of 16 weeks of self-control, is expected that volunteers will follow the same pattern but with no dietary advice. Results: Anthropometrical data and body composition determinations as well as blood and urine samples are being collected at the beginning and end of each phase. This project is registered at www.clinicaltrials.gov with the number NCT01087086 and count with the Research Ethics Committee of the University of Navarra approval (065/2009). Conclusions: Intervention trials to promote the adoption of dietary patterns and healthy lifestyle are of great importance to identify the outcomes and nutritional mechanisms that might explain the link between obesity, metabolic syndrome and associated complications.

APPENDIX 2

A NEW DIETARY STRATEGY FOR LONG-TERM TREATMENT OF THE METABOLIC SYNDROME IS COMPARED WITH THE AMERICAN HEART ASSOCIATION (AHA) GUIDELINES: THE METABOLIC SYNDROME REDUCTION IN NAVARRA (RESMENA) PROJECT

Iglesia, R. (Rocío) de la, Lopez-Legarrea, P. (Patricia), Abete, I. (Itziar), Bondia-Pons, I. (I.), Navas-Carretero, S. (Santiago), Forga, L. (Luis), Martinez, J.A. (José Alfredo), Zulet, M.A. (María Ángeles)

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de la Iglesia R, Lopez-Legarrea P, Abete I, Bondia-Pons I, Navas-Carretero S, Forga L, et al. A new dietary strategy for long-term treatment of the metabolic syndrome is compared with the American Heart Association (AHA) guidelines: the MEtabolic Syndrome REduction in NAvarra (RESMENA) project. Br J Nutr 2014 2014 Feb (Epub 2013 Aug 23);111(4):643-652

Abstract: The long-term effects of dietary strategies designed to combat the metabolic syndrome (MetS) remain unknown. The present study evaluated the effectiveness of a new dietary strategy based on macronutrient distribution, antioxidant capacity and meal frequency (MEtabolic Syndrome REduction in NAvarra (RESMENA) diet) for the treatment of the MetS when compared with the American Heart Association guidelines, used as Control. Subjects with the MetS (fifty-two men and forty-one women, age 49 (se 1) years, BMI 36·11 (se 0·5) kg/m) were randomly assigned to one of two dietary groups. After a 2-month nutritional-learning intervention period, during which a nutritional assessment was made for the participants every 15 d, a 4-month self-control period began. No significant differences were found between the groups concerning anthropometry, but only the RESMENA group exhibited a significant decrease in body weight (- 1·7%; P= 0·018), BMI (- 1·7%; P= 0·019), waist circumference (- 1·8%; P= 0·021), waist:hip ratio (- 1·4%; P= 0·035) and android fat mass (- 6·9%; P= 0·008). The RESMENA group exhibited a significant decrease in alanine aminotransferase and aspartate aminotransferase (AST) concentrations (- 26·8%; P= 0·008 and - 14·0%; P= 0·018, respectively), while the Control group exhibited a significant increase in glucose (7·9%; P= 0·011), AST (11·3%; P= 0·045) and uric acid (9·0%; P< 0·001) concentrations. LDL-cholesterol (LDL-C) concentrations were increased (Control group: 34·4%; P< 0·001 and RESMENA group: 33·8%; P< 0·001), but interestingly so were the LDL-C:apoB ratio (Control group: 28·7%; P< 0·001, RESMENA group: 17·1%; P= 0·009) and HDL-cholesterol concentrations (Control group: 21·1%; P< 0·001, RESMENA group: 8·7; P= 0·001). Fibre was the dietary component that most contributed to the improvement of anthropometry, while body-weight loss explained changes in some biochemical markers. In conclusion, the RESMENA diet is a good long-term dietary treatment for the MetS.

APPENDIX 3

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Article

Beneficial Effects of the RESMENA Dietary Pattern on Oxidative Stress in Patients Suffering from Metabolic Syndrome with Hyperglycemia Are Associated to Dietary TAC and Fruit Consumption

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Abstract: Hyperglycemia and oxidative stress are conditions directly related to the metabolic syndrome (MetS), whose prevalence is increasing worldwide. This study aimed to evaluate the effectiveness of a new weight-loss dietary pattern on improving the oxidative stress status on patients suffering MetS with hyperglycemia. Seventy-nine volunteers were randomly assigned to two low-calorie diets (−30% Energy): the control diet based on the American Health Association criteria and the RESMENA diet based on a different macronutrient distribution (30% proteins, 30% lipids, 40% carbohydrates), which was characterized by an increase of the meal frequency (seven-times/day), low glycemic load, high antioxidant capacity (TAC) and high *n*-3 fatty acids content. Dietary records, anthropometrical measurements, biochemical parameters and oxidative stress biomarkers were analyzed before and after the six-month-long study. The RESMENA (Metabolic Syndrome Reduction in Navarra) diet specifically reduced the android fat mass and demonstrated more effectiveness on improving general oxidative stress through a greater

decrease of oxidized LDL (oxLDL) values and protection against arylesterase depletion. Interestingly, oxLDL values were associated with dietary TAC and fruit consumption and with changes on body mass index (BMI), waist circumference, fat mass and triacilglyceride (TG) levels. In conclusion, the antioxidant properties of the RESMENA diet provide further benefits to those attributable to weight loss on patients suffering Mets with hyperglycemia.

Keywords: metabolic syndrome; hyperglycemia; oxidative stress; TAC; fruit

1. Introduction

The prevalence of metabolic syndrome (MetS), established as the combination of central obesity and different metabolic disturbances, such as insulin resistance, hypertension and dyslipidemia, is increasing worldwide [1,2]. Among the different metabolic abnormalities encompassing MetS, insulin resistance has been considered a common manifestation of the MetS, which leads to tissue damage and health features, involving cardiovascular diseases (CVD), atherosclerosis and hypertension [3–5]. Moreover, oxidative stress has been investigated as a potential contributor to the etiology of different pathophysiological complications, including MetS and type 2 diabetes [4,6]. Therefore, many scientific efforts are under way to detect, treat and prevent MetS, focusing on lowering the risk of type 2 diabetes and oxidative stress development [7,8]. Thus, several studies have been designed and implemented to reduce these oxidative stress-related diseases based on different lifestyle modification strategies, such as giving up smoking, increasing physical activity, controlling alcohol intake, implementing healthy sleep habits, controlling anxiety and depression, losing weight and modifying unhealthy dietary patterns [7–9]. Since it has been demonstrated that central obesity is associated with increased risks of type 2 diabetes, hypertension, CVD [10,11], oxidative stress [12] and MetS manifestations in general [11], android fat mass reduction should be a main target in order to improve MetS related diseases. Concerning nutritional strategies, most of the studies have examined the effects of single dietary factors, such as the hypotriglyceridemic effect of *n*-3 fatty acids consumption [13], the protection against oxidative damage of the dietary total antioxidant capacity (TAC) [14,15], the control of blood glucose levels of low glycemic load (GL) diets [16] or the meal frequency related appetite control [17]. However, the role of a complete dietary pattern on oxidative stress and its related diseases remains unclear [18]. Thus, it was hypothesized that the combination of all these components (*n*-3 fatty acids, TAC, GL, meal frequency) may be effective when included in an integrated adequate dietary pattern. Therefore, in the present work, the effectiveness of a new dietary strategy involving different nutritional elements is studied in order to improve oxidative stress markers, as well as biochemical and body composition measurements on a population suffering MetS with hyperglycemia. The RESMENA-S (Metabolic Syndrome Reduction in Navarra-Spain) project [19,20].

2. Results and Discussion

2.1. Anthropometrical, Body Composition and Blood Pressure Parameters

After the six-month trial, both control and RESMENA dietary strategies proved to be effective on improving anthropometric, body composition and blood pressure parameters (Table 1). Both groups significantly reduced the body weight, body mass index (BMI), waist circumference, waist to hip ratio (WHR), total fat mass, lean mass, fat-free mass, systolic blood pressure (SBP) and diastolic blood pressure (DBP). However, regarding the android fat mass and related waist circumference measurement, the RESMENA diet demonstrated more benefits than the control, as volunteers of the RESMENA group presented a bigger waist circumference decrease, leading to a trend towards a marginally significance between groups ($p = 0.060$). Indeed, the RESMENA subjects were the only group that significantly reduced android fat mass values ($p < 0.001$), which resulted in significant differences between groups ($p < 0.044$). As it has been previously described, central obesity is associated with increased risks of type 2 diabetes mellitus [21], hypertension, cardiovascular diseases and MetS manifestations in general [10,11]. Moreover, only the individuals belonging to RESMENA group showed a significantly decrease in their heart rate ($p < 0.001$). Therefore, although both strategies were effective on improving general anthropometric and body composition measurements, the RESMENA diet showed additional benefits that should be taken into account in future nutritional intervention research.

Table 1. Changes in anthropometric parameters, body composition, blood pressure and activity level in both experimental groups (control and Metabolic Syndrome Reduction in Navarra (RESMENA)).

	Control		RESMENA		$P^{\dagger}$
	Day 0	Day 180	Day 0	Day 180	Difference
Weight (kg)	103.1 ± 2.9	95.35 ± 2.9 ***	106.0 ± 3.2	96.7 ± 3.0 ***	0.281
BMI (kg/m^2)	36.4 ± 0.7	33.7 ± 0.8 ***	37.41 ± 0.8	34.12 ± 0.8 ***	0.206
Waist circumference (cm)	114.6 ± 2.0	107.4 ± 2.0 ***	117.2 ± 2.1	107.1 ± 2.0 ***	0.060
WHR	1.00 ± 0.02	0.97 ± 0.02 ***	0.99 ± 0.02	0.95 ± 0.02 ***	0.098
Total fat Mass (kg)	42.3 ± 1.5	36.4 ± 1.6 ***	45.4 ± 1.9	37.9 ± 1.8 ***	0.139
Android Fat Mass (kg)	4.7 ± 0.2	4.3 ± 0.3	5.3 ± 0.2	4.0 ± 0.2 ***	0.044
Lean mass (kg)	58.0 ± 2.2	55.6 ± 2.1 ***	57.1 ± 2.1	55.5 ± 2.0 **	0.197
Fat-free mass (kg)	60.9 ± 2.3	58.6 ± 2.2 ***	60.0 ± 2.1	58.4 ± 2.1 **	0.220
SBP (mmHg)	152.9 ± 3.3	138.7 ± 2.2 **	154.2 ± 4.4	137.1 ± 3.1 **	0.637
DBP (mmHg)	86.3 ± 1.6	79.2 ± 1.8 **	85.8 ± 1.8	79.5 ± 2.0 *	0.766
Heart rate (bpm)	75 ± 3	72 ± 3	82.3 ± 2.6	72.1 ± 2.5 ***	0.587
Activity level [1]	1.59 ± 0.04	1.54 ± 0.04	1.54 ± 0.03	1.55 ± 0.03	0.191

Abbreviations: BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; Symbols: ** $p < 0.005$; *** $p < 0.001$ (comparison between day 0 and day 180 in each group); $P^{\dagger}$, comparison between dietary group differences. [1] Average daily exercise calculated by twenty forth physical activity questionnaire.

Regarding physical activity, as designed, volunteers of both dietary patterns maintained their activity levels along the study, with no significant differences between groups (Table 1). Therefore, the

effects on anthropometric and biochemical parameters cannot be related to changes in physical activity, but to the different dietary patterns.

2.2. General Biochemical Parameters

Regarding biochemical values (Table 2), both, control and RESMENA diets, proved to be effective on ameliorating the plasma biochemical profile. As it was mentioned before, insulin resistance has been postulated as a major risk condition for the MetS development [3]. Volunteers of both groups significantly reduced their insulin and Homeostasis Model Assessment Index (HOMA-IR) values, although only those under RESMENA dietary patterns ended with significantly lower glucose levels. These results agree with the review and meta-analysis carried out by Santos *et al*. [22], where it was described that caloric restriction, despite the type of diet, leads to an improvement on insulin, HOMA-IR and plasma glucose levels, but the intake of a low-carbohydrate diet demonstrated a markedly bigger effect on decreasing fasting plasma glucose levels. Since volunteers included in this study presented hyperglycemia, the fact that the RESMENA group were the only that significantly decreased the glucose values has to be highlighted and might be considered in future dietary treatments of hyperglycemic patients.

Table 2. Changes in biochemical parameters in both experimental groups (control and RESMENA).

	Control		RESMENA		$P^{\dagger}$
	Day 0	Day 180	Day 0	Day 180	Difference
Total Cholesterol (mmol/L)	5.56 ± 0.19	5.66 ± 0.19	5.44 ± 0.21	5.44 ± 0.20	0.397
HDL-c (mmol/L)	1.14 ± 0.05	1.28 ± 0.06 ***	1.11 ± 0.04	1.15 ± 0.04	0.057
LDL-c (mmol/L)	3.47 ± 0.18	4.38 ± 0.17 ***	3.34 ± 0.17	4.29 ± 0.19 ***	0.884
LDL-c/ApoB	1.43 ± 0.04	1.91 ± 0.04 ***	1.50 ± 0.11	1.92 ± 0.03 **	0.593
TG (mmol/L)	2.06 ± 0.21	1.67 ± 0.21 *	2.17 ± 0.21	1.72 ± 0.20 **	0.574
Apo A-I (mg/dL)	134.3 ± 4.3	139.2 ± 4.1	126.3 ± 3.5	131.2 ± 4.3	0.978
Apo B (mg/dL)	93.4 ± 3.7	88.7 ± 3.4	90.3 ± 4.6	86.9 ± 4.1	0.737
FFA (mmol/L)	0.55 ± 0.04	0.48 ± 0.04	0.60 ± 0.18	0.50 ± 0.23 *	0.349
Glucose (mmol/L)	7.14 ± 0.36	6.68 ± 0.28	7.59 ± 0.43	6.49 ± 0.35 **	0.118
Insulin (μU/mL)	15.22 ± 1.56	10.01 ± 1.54 ***	15.36 ± 1.53	9.41 ± 1.21 ***	0.685
HOMA-IR	4.92 ± 0.55	3.25 ± 0.61 **	5.24 ± 0.56	2.80 ± 0.37 ***	0.475
Uric Acid (mg/dL)	6.08 ± 0.21	6.29 ± 0.22	6.19 ± 0.28	6.23 ± 0.22	0.310
Total Proteins (mg/dL)	73.01 ± 0.94	76.30 ± 1.19 ***	71.48 ± 0.79	73.51 ± 0.97 *	0.186
eGFRs (mL/min/1.73 m^2)	83.97 ± 2.92	79.85 ± 2.60	79.07 ± 2.72	81.46 ± 3.08	0.080
ALT (U/L)	41.59 ± 4.29	27.16 ± 1.56 **	28.90 ± 2.13	22.54 ± 1.60 **	0.172
AST (U/L)	27.73 ± 2.26	22.86 ± 1.15*	22.68 ± 1.08	20.38 ± 1.00	0.685

Abbreviations: HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triacilglycerides; Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; FFA, free fatty acids; HOMA-IR, homeostasis model assessment of insulin resistance; eGFRs, estimated glomerular filtration rates; ALT, alanine aminotranferase; AST, aspartate aminotransferase. Symbols: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ (comparison between day zero and day 180 in each group); $P^{\dagger}$, comparison between dietary group differences.

Furthermore, both dietary groups significantly reduced triglyceride (TG) values, a feature that has been associated with an amelioration of coronary heart disease risks [23]. However, concerning low

density lipoprotein-cholesterol (LDL-c), unexpectedly, the two groups increased their values, results that agree with Clifton *et al*. [24], who described that in some cases, LDL-c may raise despite weight loss. However, this significant increase was not observed on apolipoprotein B (Apo B) concentrations, which has been considered a better predictor of cardiovascular disease than any other lipid measurement [25]. Moreover, according to the LDL/Apo B ratio that predicts the LDL-particle size, the values being significantly raised in both groups, it indicates an increase in LDL-particle size and a lower risk of ischemic cardiac events [26,27]. With regards to high density lipoprotein-cholesterol (HDL-c) concentrations, they rose in both groups, but this increase was statistically significant only in the control group, although apolipoprotein A-I (Apo A-I), a major protein component of HDL-c [28], did not show any changes in any of the dietary groups.

Some studies associate the rise of uric acid with gout, uric acid kidney stones, diabetes and hypertension, among other diseases [29], but it also has been proposed to have a protective role and to be able to function as an antioxidant [30]. In the present study, uric acid levels slightly raised in both groups; however, no significant differences were found, neither between day zero and 180, nor between dietary groups.

Interestingly, free fatty acids (FFA), which are known to impair aortic elastic function [31], were only significantly decreased in the RESMENA group.

Concerning renal function, low levels of estimated glomerular filtration rates (eGFRs) have been positively correlated to cardiovascular disease [32]. In the present study, the control group slightly decreased these values, whereas the RESMENA group mildly increased them, leading to a trend towards significance between groups. Although decreases in protein intake has been associated to increases of eGFRs [33], our results agree with other studies where protein intake was not associated with renal function [34,35].

Transaminases, mainly alanine aminotransferase (ALT), are markers of hepatocyte injury that have shown a correlation with insulin resistance and later development of diabetes [36]. Dietary weight loss has been associated with a depletion of this liver enzyme [37] irrespective of the type of diet [38], which agrees with the present study, where both control and RESMENA group volunteers significantly decreased their ALT levels. The control group lowered aspartate aminotransferase (AST) values, as well.

2.3. Oxidative Stress Biomarkers

Oxidative stress, defined as an imbalance between production and degradation of reactive oxygen species, is a potential biochemical mechanism involved in the pathogenesis of MetS and diabetes [39–41]. Therefore, the study of oxidative stress-related markers on people suffering MetS and/or diabetes is important to be approached in their treatment.

High levels of plasma malondialdehyde (MDA), a biomarker of lipid peroxidation [42], have been associated with type 2 diabetes [43]. Moreover, energy-restricted dietary strategies have demonstrated to be able to decrease MDA levels [44]. At the end of the study, both dietary treatments had reduced these biomarker levels; the control group showed statistically significant changes ($p = 0.007$), and the RESMENA group showed a trend towards significance ($p = 0.079$). When comparing both groups, no statistically significant differences were found (Table 3).

Table 3. Changes in oxidative stress parameters in both experimental groups (control and RESMENA).

	Control		RESMENA		$P^{\dagger}$
	Day 0	Day 180	Day 0	Day 180	Difference
MDA (µM)	0.86 ± 0.07	0.75 ± 0.07 *	0.83 ± 0.07	0.76 ± 0.05	0.449
MPO (µg/L)	71.69 ± 7.36	65.39 ± 7.65	69.53 ± 8.39	66.48 ± 7.42	0.723
ARE (U/L)	458 ± 44	442 ± 43	370 ± 31	361 ± 28	0.778
ARE:HDL-c (U/mmol)	413.6 ± 0.1	366.8 ± 0.1 *	343.8 ± 0.1	327.1 ± 0.1	0.227
ARE:Apo A-I (U/mg)	0.347 ± 0.030	0.319 ± 0.027 *	0.295 ± 0.024	0.281 ± 0.022	0.424
oxLDL (U/L)	35.36 ± 1.80	36.39 ± 2.60	46.53 ± 4.46	41.03 ± 3.22 *	0.025
oxLDL:LDL-c (U/mmol)	10.34 ± 0.52	8.25 ± 0.62 **	14.88 ± 1.80	9.52 ± 0.58 **	0.046
oxLDL:HDL-c (U/mmol)	30.89 ± 1.52	28.46 ± 1.76	42.78 ± 4.19	4.19 ± 2.64 *	0.186
oxLDL:Apo B (U/mg)	0.038 ± 0.002	0.043 ± 0.004	0.051 ± 0.004	0.048 ± 0.003	0.040

Abbreviations: MDA, malondialdehyde; MPO, myeloperoxidase; ARE, arylesterase; HDL-c, high density lipoprotein-cholesterol; ApoA1, apolipoprotein A1; oxLDL, oxidized low density lipoprotein; LDL-c, low density lipoprotein-cholesterol; ApoB, apolipoprotein B. Symbols: * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ (comparison between day zero and day 180 in each group); $P^{\dagger}$, comparison between dietary group differences.

Regarding myeloperoxidase (MPO), a leucocyte-derived enzyme that catalyzes the formation of a number of reactive oxidant species and that is known to oxidize the HDL-c [45], it has been described that energy restriction diets let to depletions on its levels [46]. In the present study, both diets slightly decreased their MPO values, but no significant differences were found, neither between day zero and day 180 in each group, nor between both dietary groups (Table 3).

Arylesterase (ARE) activity, one of the three functions of the paraoxonase enzyme (PON1), is associated with HDL-c and has been shown to protect LDL-c and HDL-c against oxidation [47]. In diabetic patients, PON1 ARE activity dissociates from HDL-c [48]. Studies focusing on the effect of the diet on the ARE activity are scarce, but it has been reported that flavonoids, fish oil, nori algae and pomegranate-rich based diets are positively associated with PON1 ARE activity in diabetic patients [49–52]. In the present study, volunteers of the control diet decreased ARE:HDL-c ($p = 0.006$) and ARE:Apo A-I ($p = 0.029$) ratio values, while they remained almost unchanged in the RESMENA group. Therefore, the RESMENA diet showed a specific protection effect against ARE depletion (Table 3).

Oxidation of LDL-c is considered an important cardiovascular risk factor, since it lets to foam cell formation induction, alongside propagation of atherosclerosis [53]. Moreover, oxidized-LDL (oxLDL) has been found to be a biomarker increased in type 2 diabetic patients [54]. Our results evidenced that between both dietary patterns, RESMENA is significantly more effective on reducing oxLDL ($p = 0.025$), oxLDL:LDL-c, ($p = 0.046$) and oxLDL:Apo B ($p = 0.040$) than the control diet. Moreover, the RESMENA group was the only that significantly reduced oxLDL:HDL-c values ($p = 0.025$) (Table 3). These results agree with previous studies, where an inverse relationship between high TAC dietary patterns and MetS related-oxidative stress was established [15]. Moreover, when the correlation between TAC and changes on oxLDL was studied, taking into account the entire sample, that is volunteers of both control and RESMENA groups, a significant positive relationship between oxLDL reduction and TAC values was found (Figure 1). Furthermore, the same association was

observed when studying the relationship between oxLDL and consumed energy (kcal) from fruits (Figure 2). Finally, BMI, waist circumference, fat mass and TG value reductions are associated with decreases of oxLDL circulating concentration levels, taking again into account the entire sample (Figure 2). These results correlate with other studies, where a diet-induced weight loss resulted in significant reductions of oxLDL levels [46,55].

Figure 1. Relationship between changes on oxLDL and fruits and TAC dietary records. Abbreviations: oxLDL, oxidized low density lipoprotein; TAC, total antioxidant capacity.

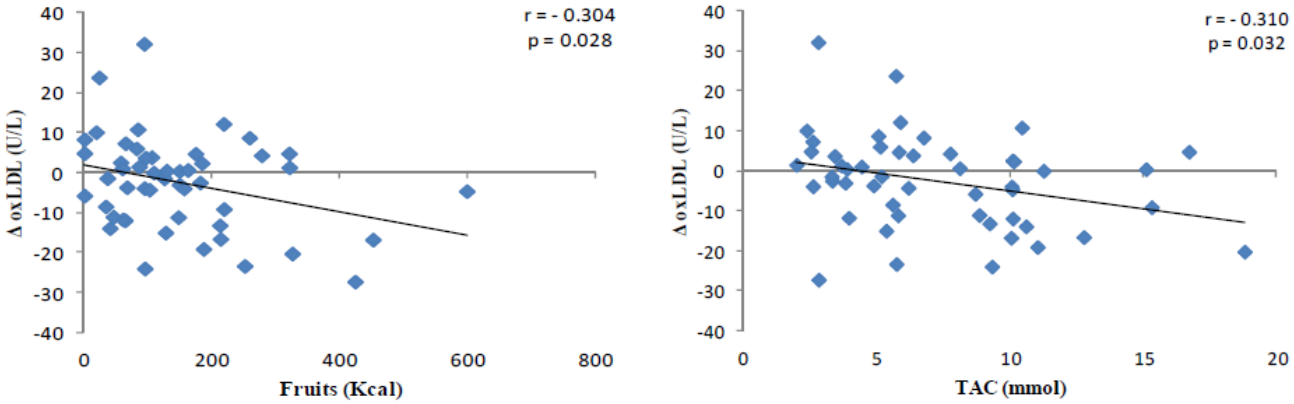
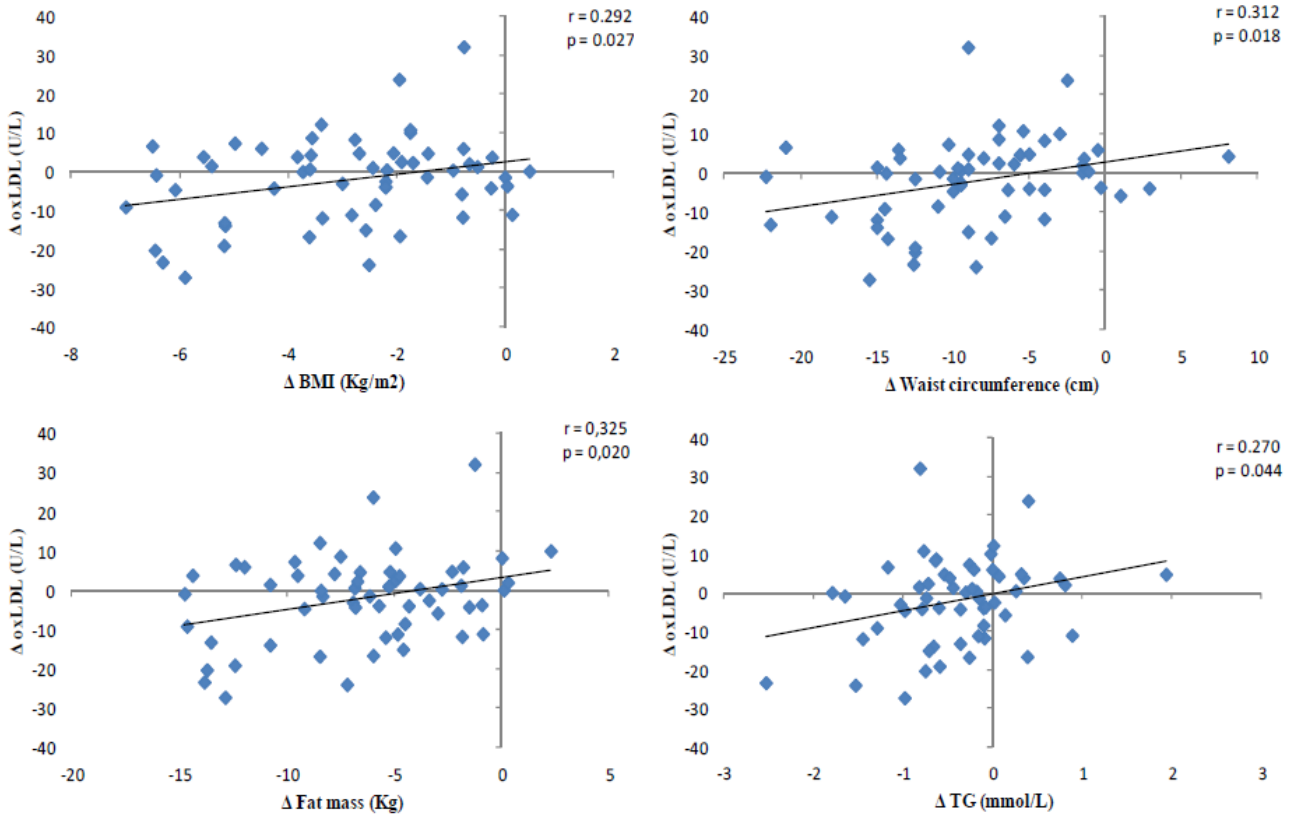


Figure 2. Correlations between changes on oxLDL and changes on adiposity parameters. Abbreviations: BMI, body mass index; TG, triglycerides.



2.4. Dietary Records

The dietary records at the end of the study showed that the designed differences between the two dietary patterns composition were met, although no statistically significant differences were found for fiber, GL or EPA + DHA (Table 4). This outcome could be explained by the fact that the dietary records analyzed in this study were collected at the endpoint, once volunteers had completed four months of autonomy and after the six months that lasted the study. Therefore, volunteers may not complete them with the thoroughness required or might not followed the diet as strictly as at the beginning of the study. However, it was achieved that the RESMENA individuals had a higher meal frequency ($p < 0.001$), protein ($p = 0.001$) and TAC ($p = 0.031$) intake than the control group ones. Furthermore, the fruit consumption was also higher in the RESMENA group ($p = 0.049$). Moreover, both groups declared to consume the same amount of energy (Table 4), as designed. In the RESMENA group, a higher number of drop-outs than in the control group appeared, which may be a limitation of the study, although the difference was not statistically significant ($p > 0.10$).

Table 4. Comparison of control and RESMENA dietary records at the endpoint.

	Control	RESMENA	*p*
Energy (kcal/day)	1513 ± 54	1569 ± 77	0.542
Meal Frequency (meals/day)	4.3 ± 0.2	5.8 ± 0.2	<0.001
Proteins (% TCV/day)	16.9 ± 0.4	20.4 ± 0.9	0.001
Lipids (% TCV/day)	40.8 ± 1.5	37.7 ± 1.0	0.108
CHO (% TCV/day)	37.1 ± 1.5	36.9 ± 1.1	0.940
Fiber (% TCHO/day)	11.4 ± 0.8	12.0 ± 0.6	0.573
GL (U/day)	73.4 ± 5.9	70.0 ± 5.5	0.682
EPA+DHA (g/day)	0.30 ± 0.08	0.39 ± 0.17	0.617
TAC (mmol/day)	6.1 ± 0.6	8.5 ± 0.9	0.031
Fruits (kcal/day)	117 ± 21	185 ± 27	0.049

Abbreviations: TCV, total caloric value; CHO, carbohydrates (without fiber); TCHO, total carbohydrates (included fiber); GL, glycemic load; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TAC, total antioxidant capacity.

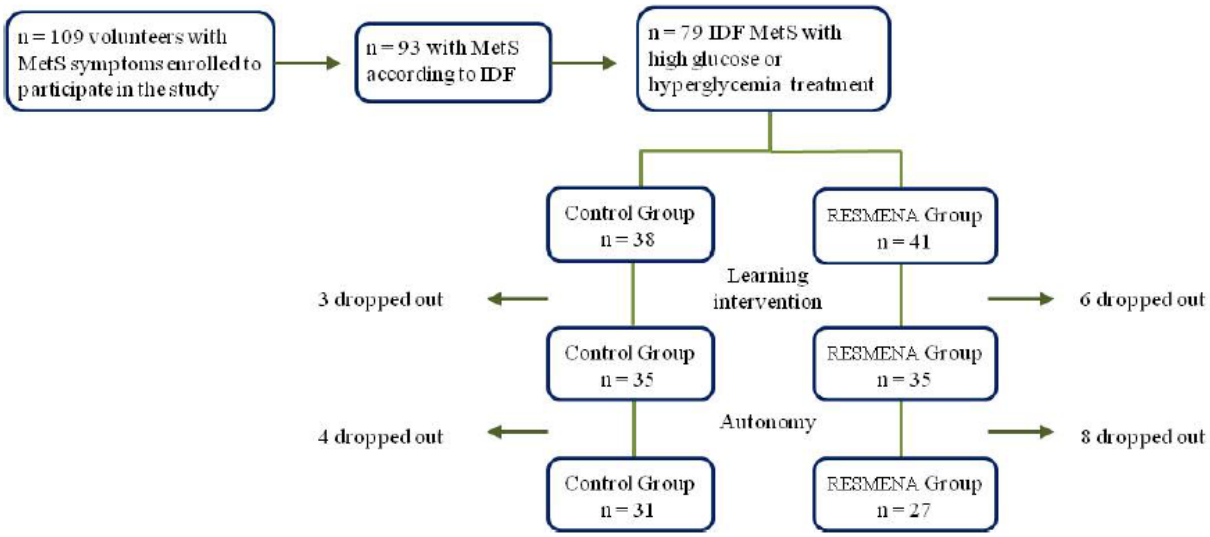
3. Experimental Section

3.1. Subjects

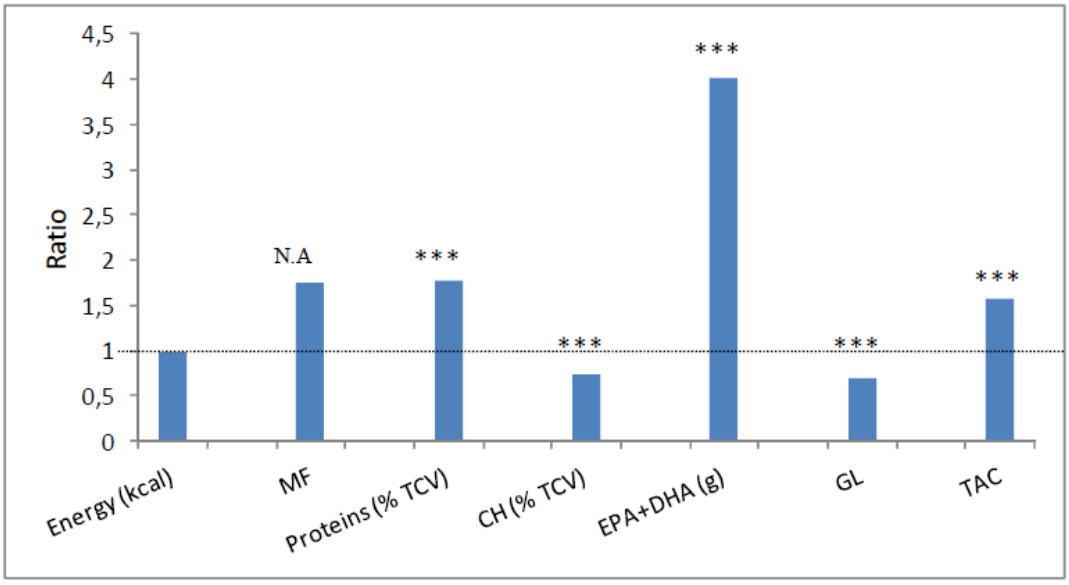
A subsample of 79 hyperglycemic adults diagnosed of MetS according to the IDF criteria [56] were selected from the 109 volunteers with Mets symptoms enrolled to participate in the RESMENA-S project. During the 6-month-study, 21 volunteers dropped out. Therefore, 58 individuals of the subsample completed the study and were included in the final statistical analysis (Figure 3a).

This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of the University of Navarra (065/2009). Written informed consent to participate in the intervention trial [20] was obtained from all subjects.

Figure 3. Flow diagram of participants during the study (**a**) and ratio RESMENA/control of energy and specific dietary components of the scheduled diet (**b**). Abbreviations: MetS, metabolic syndrome; IDF, International Diabetes Association; MF, meal frequency; TCV, total caloric value; CH, carbohydrates; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; GL, glycemic load; TAC, total antioxidant capacity. Symbols: *** $p < 0.001$ differences between control and RESMENA scheduled diets; N.A, not applicable.



(**a**)



(**b**)

3.2. Study Protocol

The study was designed as a randomized, controlled trial to compare the effects of two dietary strategies (Figure 3b) on improving body composition, biochemical and oxidative stress parameters in a MetS population with hyperglycemia. Participants were randomly assigned to the control or the experimental diet (control and RESMENA groups, respectively). The study lasted a total of six months implemented in two sequential stages: an initial 8-week nutritional learning intervention period, during which the study participants received nutritional assessment every fifteen days, and a follow-up

4-month self-control period, in which they applied on their own the previously acquired nutritional habits. The CONSORT 2010 guidelines [57] were followed by taking into account the design of the present study as two-groups longitudinal intervention, except for blinding.

Participants were asked to maintain their normal physical activity during the study, which was checked by a 24-h physical activity questionnaire [58] at the beginning and at the end of the study. For assessing physical activity, all participants were asked about their occupation, sleeping hours and additional activities at work and during the rest of the day. The physical activity questionnaire included representative values expressed as multiples of Resting Energy Expenditure. Average daily physical activity level was calculated taking into account the intensity and time spent on each activity. Activities were divided in 5 categories (resting, very light, light, moderate and heavy) [58].

At baseline and at the end point of the 6-month study, trained nutritionists performed anthropometrical measurements and body composition analyses by Dual-energy X-ray Absorptiometry (DXA) following validated protocols [19]. Moreover, fasting blood samples for biochemical analyses were collected.

3.3. Diets

Two energy-restricted diets (−30% energy of the studied requirements) were prescribed and compared (Figure 3b). Thus, the control diet was based on the AHA guidelines [59], including 3–5 meals per day, a macronutrient distribution of 55% total caloric value (TCV) from carbohydrates, 15% proteins and 30% lipids, a healthy fatty acids (FA) profile and a cholesterol consumption lower than 300 mg/day. The RESMENA diet was characterized by a higher meal frequency, consisting of seven meals per day and by a different macronutrient distribution, 40% TCV from carbohydrates, 30% proteins and 30% lipids [19]. Furthermore, this pattern tried to reinforce the high *n*-3 polyunsaturated FA (*n*-3 PUFAs) and high natural antioxidant foods consumption and promoted low GL carbohydrates intake. It also maintained a healthy FA profile and a cholesterol content of less than 300 mg/day as the control diet.

RESMENA participants were prescribed a 7-day menu plan, while in the control group, a previously described [60] food exchange system plan was provided to volunteers. A 48-hour weighed food record was collected at the beginning and at the end of both the nutritional-learning and the autonomous periods, in order to assess the volunteer's adherence to the prescribed nutritional patterns. The designed diets composition, as well as the different dietary records, were analyzed by the DIAL software (Alce Ingenieria, Madrid, Spain) [61]. The sum of eicosapentaenoic and docosahexaenoic fatty acid (EPA+DHA) obtained by the DIAL program [61] was used to estimate *n*-3 PUFAs consumption. TAC was calculated using the validated data, considering raw or cooked preparations [62]. Finally, the GL was obtained from the international updated website database based in the Human Nutrition Unit, School of Molecular Biosciences from the University of Sydney [63].

3.4. Clinical and Biochemical Assessments

Anthropometric measurements were performed in fasting conditions, as previously described [64]. Body weight was assessed to the nearest 0.1 kg by using a bioimpedance (TANITA SC-330, Tanita, Corporation, Tokyo, Japan). BMI was calculated as the body weight divided by the squared height

(kg/m^2). Waist and hip circumferences were measured with a commercial tap following validated protocols, as previously described [19]. Total body fat mass android fat mass, lean mass and fat-free mass were evaluated by DXA (Lunar iDXA™, software version 6.0, Madison, WI, USA). Measurements of SBP, DBP and heart rate were assessed using a digital monitor (Medisana, MTC, Düsseldorf, Germany) in the right arm, with the patient seated and relaxed, with an appropriate cuff for the arm size of each patient. Measurements were taken three times after a five-minute resting period, following World Health Organization (WHO) criteria [65].

Total cholesterol, HDL-c, TG, FFA, glucose, uric acid, total proteins, creatinine, ALT and AST serum concentrations were measured in an autoanalyzer 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) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain). Insulin resistance was estimated by the Homeostasis Model Assessment Index (HOMA-IR), which was calculated as stated in the following formula: HOMA-IR = [glucose (mmol/L) × insulin (μU/mL)]/22.5, as described elsewhere [66]. LDL-c levels were calculated following the Friedewald formula: LDL-c = Total cholesterol − HDL-c − TG/5 [67]. Apo A-I and Apo B were measured with specific kits (Tina-quant Apolipoprotein A-I ver.2 and Tina-quant Apolipoprotein B ver.2, Mannheim, Germany) using a Roche/Hitachi autoanalyzer (Mod.904 Modular, Tokio, Japan). Estimated glomerular filtration rates (eGFRs) were calculated from serum creatinine values using the equation CKD-EPI, which takes into account sex, age and race [68].

Plasma MDA was colorimetrically determined with a commercial kit (BIOXYTECH® LPO-586™, Oxis Research™, Portland, OR, USA). Each sample (200 μL of serum) was mixed with 650 μL of *N*-methyl-2-phenylindole in acetonitrile and 150 μL of 37% (12 N) HCl. Tubes were capped, mixed and incubated at 45 °C for 60 min. Samples were centrifuged at 15,000 × *g* for 10 min, and the supernatant was read on a spectrophotometer at 586 nm (Multiskan Spectrum, Thermo Electron Corporation, Vantaa, Finland). The assay included a six-point standard curve, the measurement was performed in replicate and the mean value was computed.

Plasma ox-LDL and MPO were measured using capture ELISA assay kits from Mercodia (Uppsala, Sweden). ARE activity was measured with simulated body fluid (SBF) as buffer and phenylacetate as substrate at pH 7.34–7.4 and 37 °C, as described elsewhere [48]. Reaction rates of ARE were followed at 270 nm in thermostatically controlled 10-mm Lightpath quartz cuvettes using a Shimadzu UV-2401PC spectrophotometer (Tokio, Japan). The final reaction volume in the cuvettes was 2.0 mL, and the total time was 3 min. One unit of ARE activity is equal to 1 mol of phenylacetate hydrolyzed/(L min)

3.5. Statistical Analyses

Mean values and standard errors were reported for the measured variables. Differences between the beginning and the end of the complete study were analyzed by a paired *t*-test. The analysis between both groups (RESMENA *vs*. Control) was performed through an independent measures *t*-test. Correlation analyses were applied to assess the potential relationships and associations, between some components of the diet and anthropometrical and biochemical parameters variation. For drop-out analysis, the χ^2 test was applied. The SPSS 15.1 software for Windows (SPSS Inc., Chicago, USA) was used for all statistical analyses. Values of $p < 0.05$ were considered as statistically significant.

4. Conclusions

Both energy-restricted dietary patterns, AHA guidelines-based diet and the RESMENA diet were successful on improving anthropometrical measurements, body composition, blood pressure levels and biochemical markers on patients suffering MetS with hyperglycemia. However, the RESMENA diet showed greater benefits regarding android fat mass reduction and improvement of the general oxidative stress status, specifically oxLDL related markers. Interestingly, dietary TAC and fruit consumption were apparently the nutritional components that potentially contributed most to the oxLDL depletion. Moreover, the decrease on BMI, waist circumference, fat mass and TG levels were also directly associated with the oxLDL decrease levels. For all of this, the prescription of the RESMENA diet is a good antioxidant dietary treatment for people suffering MetS with hyperglycemia to further improve the benefits associated to weight loss.

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Conflict of Interest

The authors declare no conflict of interest.

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