Universidad de Navarra

Facultad de Farmacia

EFFECTS OF A NEW DIETARY STRATEGY IN THE TREATMENT OF THE METABOLIC SYNDROME: THE RESMENA DIET

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THE RESMENA DIET

Memoria presentada por Dª **Rocío de la Iglesia González** para aspirar al grado de Doctor por la Universidad de Navarra

Rocío de la Iglesia González

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

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Dr. M. Ángeles Zulet Alzórriz   Dr. J. Alfredo Martínez Hernández

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ABBREVIATIONS

8-OHdG	8-Hydroxydeoxyguanosine
AHA	American Heart Association
ALA	α- Linolenic Acid
ALT	Alanine Aminotransferase
ANOVA	Analysis of Variance
Apo A-I	Apolipoprotein A-I
Apo B	Apolipoprotein B
ARE	Arylesterase
AST	Aspartate Aminotransferase
BIA	Bioelectric Impedance Analysis
BMI	Body Mass Index
CAT	Catalase
CVD	Cardiovascular Disease
CHO	Carbohydrates
CKD-EPI	Chronic Kidney Disease-Epidemiology Collaboration
CONSORT	Consolidated Standards of Reporting Trials
CpG	Cytosine-phosphate-Guanine
DBP	Diastolic Blood Pressure
DHA	Docosahexaenoic Acid
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
DXA	Dual-energy X-ray Absorptiometry
EDTA	Ethylenediaminetetraacetic Acid
EFSA	European Food Safety Authority
eGFRs	Estimated Glomerular Filtration Rates

Abbreviations

EGIR	European Group of Insulin Resistance
ELISA	Enzyme-linked Immunosorbent Assay
ENRICA	*Estudio de Nutrición y Riesgo Cardiovascular en España/* Nutrition and Cardiovascular Risk Study in Navarra
EPA	Eicosapentaenoic Acid
FESNAD	*Federación Española de Sociedades de Nutrición, Alimentación y Dietética/* The Spanish Federation of Nutrition and Dietetics Societies
FFA	Free Fatty Acids
FNDC5	Fibronectin Type 3-domain containing Protein 5
GI	Glycaemic Index
GL	Glycaemic Load
GPX	Glutathione Peroxidase
HCIS	Homocysteine
HDL-c	High Density Lipoprotein-cholesterol
HOCL	Hypochlorous Acid
HOMA-IR	Homeostasis Model Assessment Insulin Resistance Index
IAS	International Atherosclerosis Society
ICO	Index of Central Obesity
IDF	International Diabetes Federation
LDL-c	Low Density Lipoprotein-cholesterol
MBP	Median Blood Pressure
MCP-1	Monocyte Chemoattractant Protein-1
MDA	Malondialdehyde
MedDiet	Mediterranean Diet
MetS	Metabolic Syndrome

MPO	Myeloperoxidase
NCEP ATP III	National Cholesterol Education Program Adult Treatment Panel III
NHANES	National Health and Nutrition Examination Survey
NHLBI	National Heart, Lung, and Blood Institute
oxLDL	Oxidized Low Density Lipoproteins
PGC1α	Peroxisome Proliferator-activated Receptor-coactivator 1α
PON	Paraoxonase
PUFAs	Polyunsaturated Fatty Acids
RESMENA	*Reducción del Síndrome Metabólico en Navarra/* Reduction of the Metabolic Syndrome in Navarra
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SBF	Simulated Body Fluid
SBP	Systolic Blood Pressure
Scr	Serum Creatinine
SD	Standard Deviations
SE	Standard Errors
SEEDO	*Sociedad Española para el estudio de la Obesidad/* The Spanish Society for the Study of Obesity
SOD	Superoxide Dismutase
TAC	Dietary Total Antioxidant Capacity
TC	Total Cholesterol
TG	Triglycerides
UCP1	Uncoupling Protein 1
WBC	White Blood Cells

Abbreviations

WHO   World Health Organization

WHR   Waist to Hip Ratio

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INTRODUCTION

1. METABOLIC SYNDROME

1. 1. Definition

The metabolic syndrome (MetS) is a clinical entity of substantial heterogeneity, commonly represented by the combination of related physiopathological derangements such as obesity (especially abdominal obesity), hyperglycaemia, dyslipidaemia (high levels of triglycerides (TG) and low levels of high density lipoprotein-cholesterol (HDL-c)) and hypertension (Eckel et al., 2010).

It was during the period of 1910-20 when it was suggested for the first time that a cluster of associated metabolic disturbances tend to coexist together (Kylin, 1923, Sarafidis et al., 2006). Later on, in 1947, Vague suggested the term "android obesity" to define an association of different metabolic impairments related to diabetes and cardiovascular diseases (Vague, 1947).

The concept of syndrome X was coined by Reaven in 1988, when he reported that several cardiovascular risk factors tend to coexist together at the same time (Reaven, 1988). Another name attributed to this heterogenic syndrome is the insulin resistance syndrome, as some authors consider the insulin resistance as the main clinical outcome of the MetS (DeFronzo et al., 1991).

Nowadays, the name most used to refer to this syndrome is the MetS; however, its precise definition has not been well-established yet. During the past fifteen years, different organisms have suggested diverse definitions. Among them, the most common ones are hereafter described and summarized in Table 1.

In 1999, the **World Health Organization (WHO)** was the first organism to suggest a definition for the MetS (Alberti et al., 1998), considering the insulin resistance as the main factor for the MetS development.

Soon after, the **European Group of Insulin Resistance (EGIR)** proposed a new definition for nondiabetic individuals (Balkau et al., 1999).

Lately, in 2001 the **National Cholesterol Education Program Adult Treatment Panel (NCEP ATP) III** gave the same importance to all the MetS components (ATPIII, 2001) suggesting that when 3 of 5 of the listed characteristics in Table 1 are present, a diagnosis of MetS can be made.

Table 1. Criteria to define the MetS depending on different organisms.

WHO
One of this:
- Type 2 diabetes, insulin resistance or impaired glucose tolerance.
Plus at least two:
- TG ≥ 1.7 mmol/L and/or HDL-c < 0.9 mmol/L (men) and < 1.0 mmol/L (women).
- Urine albumin excretion > 20 µg/min or albumin:creatinine ratio > 30 mg/g.
- SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or treatment for hypertension.
- Central obesity: BMI ≥ 30 kg/m² or waist:hip ratio > 0.90 (men), > 0.85 (women).

EGIR
- Insulin resistance defined as the top 25 % of the fasting insulin values among nondiabetic individuals.
Plus at least two:
- Central obesity: waist circumference ≥ 94 cm (men), ≥ 80 (women).
- TG ≥ 2.0 mmol/L and/or HDL-c < 1.0 mmol/L or specific treatment.
- SBP ≥ 140 mmHg or DBP ≥ 90 mmHg or treatment for hypertension.
- Fasting glucose ≥ 6.1 mmol/L.

NCEP ATP III
At least three:
- Abdominal obesity: waist circumference > 102 cm (men) > 88 cm (women).
- TG ≥ 1.7 mmol/L.
- HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women.
- SBP ≥ 130 mmHg or DBP ≥ 85 mmHg.
- Fasting plasma glucose ≥ 6.1 mmol/L.

AHA/NHLBI
At least three:
- Waist circumference ≥ 102 cm (men), ≥ 88 cm (women) or diagnosed type 2 diabetes.
- TG ≥ 1.7 mmol/L or specific treatment for hypertriglyceridemia.
- HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women or specific treatment.
- SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or drug treatment for hypertension.
- Fasting plasma glucose ≥ 5.6 mmol/L.

IDF
- Central obesity: waist circumference ≥ 94 cm (Europids men), ≥ 80 (Europids women).
Plus at least two:
- TG ≥ 1.7 mmol/L or specific treatment for hypertriglyceridemia.
- HDL-c < 1.03 mmol/L in men, < 1.3 mmol/L in women or specific treatment.
- SBP ≥ 130 mmHg or DBP ≥ 85 mmHg or drug treatment for hypertension.
- Fasting plasma glucose ≥ 5.6 mmol/L or type 2 diabetes previously diagnosed.

AHA/NHLBI, American Heart Association/National Heart, Lung, and Blood Institute; BMI, body mass index; DBP, diastolic blood pressure; EGIR, European Group of Insulin Resistance; HDL-c, high density lipoprotein-cholesterol; IDF, International Diabetes Federation; NCEP ATP, National Cholesterol Education Program Adult Treatment Panel; SBP, systolic blood pressure; TG, triglycerides; WHO, World Health Organization.

In 2005, both the **International Diabetes Federation** or **IDF** (Alberti et al., 2005) and the **American Heart Association/National Heart, Lung, and Blood Institute or AHA/NHLBI** (Grundy et al., 2005) designed their own definitions. The **IDF** dropped the WHO requirement for insulin resistance, but recognized the central obesity as a determinant for the MetS development.

The **AHA/NHLBI** slightly modified the NCEP ATP III criteria but did not mandate abdominal obesity as a required risk factor. The remaining 4 risk factors were identical in definition to those of the IDF (Grundy et al., 2005).

Finally, in 2009 it was published a scientific statement in order to facilitate the creation of a new definition for the MetS unifying the diverse criteria of the major organizations (Alberti et al., 2009). In this work it was stated that waist circumference should not be an compulsory component, but that it would continue to be a main preliminary screening tool; that three out of five abnormal findings would define the presence of the MetS; and finally, that a single set of cut points would be used for all components except waist measurement, for which further research is needed.

1. 2. Epidemiology

The prevalence of the MetS varies broadly around the world and between different populations from 10 % to 50 % (Pistrosch et al., 2013). It is increased in developed countries, sedentary people, smokers, low socioeconomic status population, as well as individuals with unhealthy dietary habits. It is also positively associated with age, although whether this association is direct or rather correlated to the variation in body composition that occurs as people age, is not well-known (Borch-Johnsen, 2013). According to sex, the association with the MetS is not entirely clear, as some researchers report that the MetS is positively associated with women (Gundogan et al., 2013, Soares et al., 2009) while others reported that it is more frequent in men (Grundy, 2011). Those who support that the prevalence is more likely in male believe that androgens may have a role in its development, since in disorders where these hormones are elevated, such as polycystic ovary syndrome, the MetS prevalence is more frequent (Grundy, 2011). Moreover there are authors who reported that there is no difference between sexes regarding the MetS prevalence (Grundy, 2011).

However, due to the different definitions of the MetS, the prevalence varies depending on the source used. Nonetheless, what is clear is that the number of people suffering the MetS is increasing in epidemic proportions around the world over the last 40-50 years,

mainly due to the obesogenic environment of most of the developed regions (Oresic et al., 2014).

A systematic review published in 2013 reported that the general prevalence of MetS in Brazil was 29.6 % (de Carvalho Vidigal et al., 2013). Data obtained from the China Health and Nutrition Survey conducted in 2009 indicated an overage of age-standardized prevalence of 16.7 % among the Chinese population (Xi et al., 2013). In Turkish adults, the MetS prevalence was found as high as 40.3 % (Gundogan et al., 2013). Regarding the United States, the prevalence of the MetS based on the National Health and Nutrition Examination Survey (NHANES 2009-10) was estimated at 22.9 % (Beltran-Sanchez et al., 2013).

Concerning Spain, the di@bet.es study reported an age-standardized MetS prevalence of 38.37 % in men and 29.62 % in women (Marcuello et al., 2013). Moreover, it has been just published the data from the ENRICA Study (*Estudio de Nutrición y RIesgo CArdiovascular en España*), a cross-sectional study performed from 2008 to 2010 which indicates a prevalence of 22.7 % of MetS adults among the Spanish population (Guallar-Castillon et al., 2014).

On specific populations, the prevalence of the MetS in Europe among obese people ranges from 43 % in Italy to 78 % in Finland among men, and from 24 % in Italian women to 65 % in Finnish women (van Vliet-Ostaptchouk et al., 2014). In hospitalized elderly patients, the prevalence of MetS is 66 % (Castro Vilela et al., 2014). And, regarding children and adolescents, a systematic review of all the relevant literature published since 2003, estimated a median prevalence of MetS of 3.3 % around the world (Friend et al., 2013).

1. 3. Aetiology/Causes

The specific causes of the MetS triggering are still unclear. However, it is known that both genetics and environmental factors, as well as their interactions, have a role in the aetiology of this clinical entity (Rodriguez, 2009). Thus, the MetS is related with a general unhealthy lifestyle (Figure 1) such as the lack of physical activity (Lee et al., 2014), smoking habits, alcoholism (Kong et al., 2013) or an unhealthy dietary pattern (Bernabe Garcia et al., 2014). Moreover, genetics and epigenetics also have a role in the development of the MetS as many susceptible genes and different genetic factors are implicated in the phenotypic variation of this syndrome (Rodriguez, 2009).
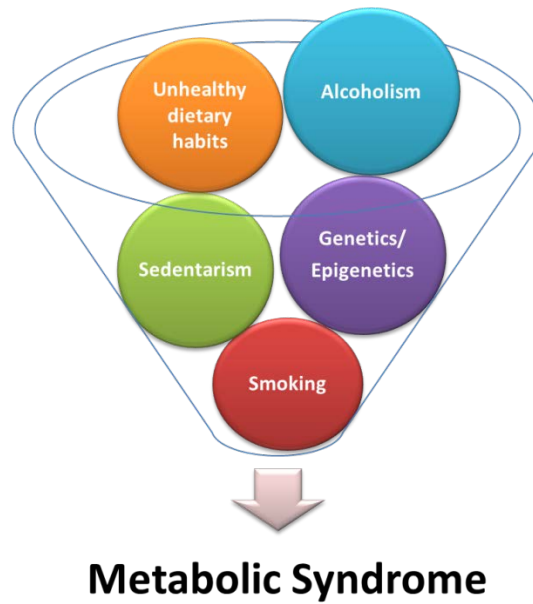
Figure 1. Causes of the metabolic syndrome.

1. 4. Physiopathology

It is common to find obesity, glucose metabolism impairments, high blood pressures and/or dyslipidaemias at the same time in an individual (Figure 2). That is the MetS, a cluster of related metabolic disturbances (Eckel et al., 2010).
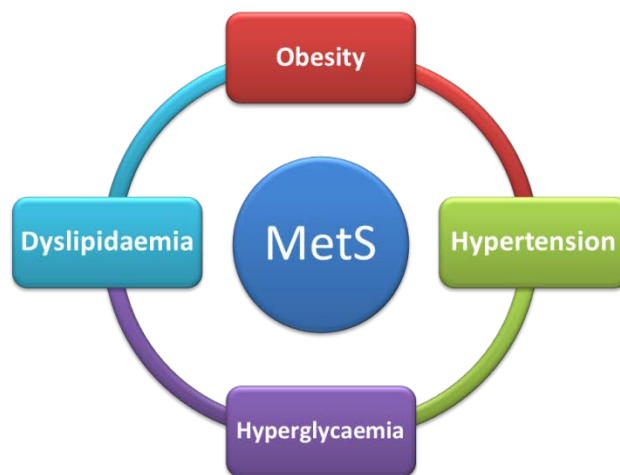
Figure 2. Pathophysiology of the metabolic syndrome. MetS, metabolic syndrome.

Therefore, the design of strategies for general hypertension, hyperglycaemia and dyslipidaemia management in MetS population is needed, where changes in lifestyle, such as diet or exercise, should be an essential first step (Gomez-Huelgas et al., 2014, IAS, 2014).

1. 4. 1. Obesity

The word "obese" comes from the Latin "obedere", which means "one who eats too much" (Zhang et al., 2005). The first use of this term is attributed to the medical practitioner and social reformer Noha Biggs in 1951. Today, the WHO defines overweight and obesity as "an abnormal or excessive fat accumulation that may be harmful to health"(WHO, 2012).

Percentage of adults with obesity

0   6   12   18   24
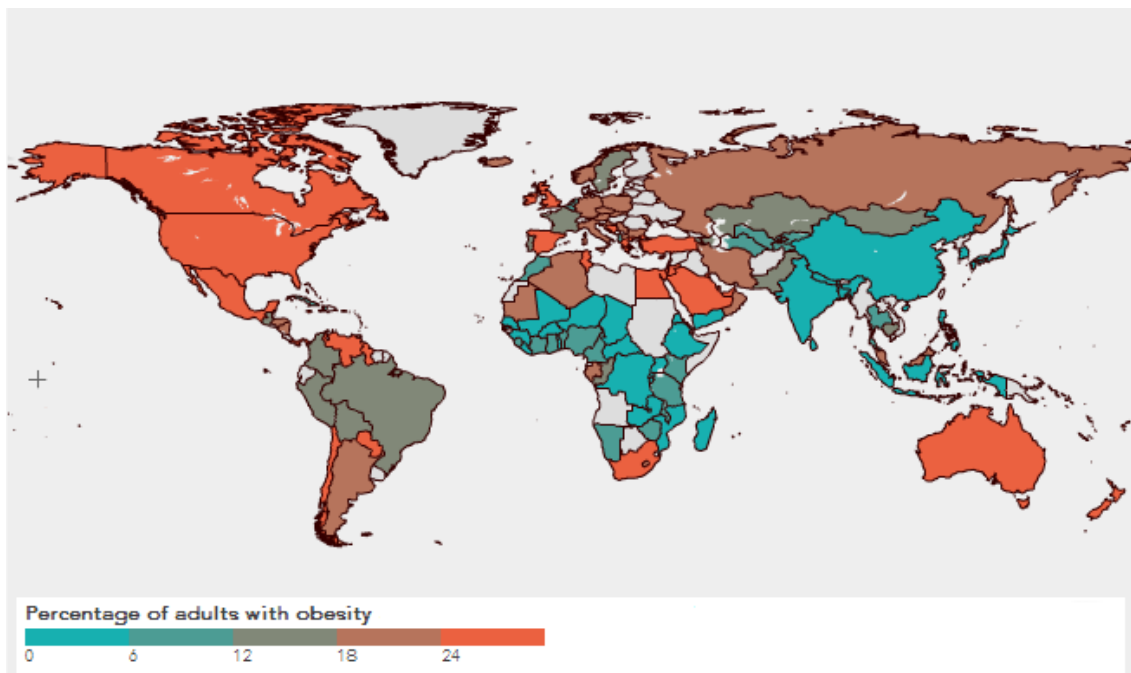
Figure 3. Prevalence of obesity around the world. Source: World Obesity Federation, 2014.

The main cause of obesity is a chronic imbalance between energy intake and energy expenditure (Selassie et al., 2011). The excess of energy consumed is primarily deposited in the adipose tissue as TG (Shimano, 2012). The purpose of this fat accumulation is to be used by other cells in the body in future periods of external energy deficiency (lack of

food) because, while food intake is carried out discontinuously, the energy requirements of the cells in our body are constant (Abdel-Hamid, 2009).

The prevalence of obesity varies geographically (Figure 3), but overall it follows an upward trend, reaching epidemic proportions in many societies. In fact, nowadays the number of people with overweight or obesity has equalled the number of individuals underweight not only in the developed countries but also in some developing areas, and this difference is estimated to increase over the coming years (WHO, 2013).

The WHO last update indicates that in 2008, 35 % of adults aged 20 and over were overweight (more than 1.4 billion) and 11 % were obese (200 million men and nearly 300 million women) around the world (WHO, 2013). Similarly, the World Obesity Federation estimated in 475 million the number of obese adults and around 1.5 billion of people with overweight worldwide (WOF, 2012).

Regarding the Spanish population (Table 2), between 2008 and 2010, the ENRICA study was carried out. Data from this study states that almost 23 % (24. 4 % men, 21.4 % women) from the Spanish adult population is obese and about 39 % (46.4% male, 32.5 % female) is in ranges of overweight (Gutierrez-Fisac et al., 2012).

Table 2. Prevalence of obesity and overweight in Spanish population (ENRICA Study, 2011).

	Overweight (%)	Obesity (%)	Central Obesity (%)
Total	39.4	22.9	35.5
18-44 y.o	33.4	15.0	20.4
45-64 y.o	44.9	27.8	43.0
≥ 65 y.o	46.0	35.0	61.6
Men	46.4	24.4	31.7
18-44 y.o	41.5	18.6	19.8
45-64 y.o	51.9	30.9	41.4
≥ 65 y.o	51.7	30.6	50.9
Women	32.5	21.4	39.2
18-44 y.o	24.6	11.1	21.1
45-64 y.o	38.0	24.7	44.6
≥ 65 y.o	41.7	38.3	69.7

Concerning central obesity (waist circumference > 102 cm in male, > 88 cm in female), the prevalence in the Spanish population is 35.5 % (31.7 % male, 39.2 % female) (Gutierrez-Fisac et al., 2012). Moreover, 1.2 % (0.6 % male, 1.8 % female) individuals from Spain has a BMI ≥ 40, which is considered severe or extreme obesity (Gutierrez-Fisac et al., 2012).

1. 4. 2. Dyslipidaemia

Dyslipidaemia encompasses elevated serum TG levels, increased low density lipoprotein-cholesterol (LDL-c) particles, and reduced levels of HDL-c (Bosomworth, 2013).

This disorder is associated with hepatic steatosis (Vidal-Puig, 2014), dysfunction of β-cells (Poitout et al., 2008) and elevated risk of atherosclerosis, among others (Rizza et al., 2014).

The atherosclerosis development (Figure 4) can be defined as a chronic inflammatory process that leads to endothelial dysfunction and narrowing of medium and large-sized arteries (Weber et al., 2011). In this process, lipoproteins, leucocytes and macrophages penetration, in addition to oxidative processes, lead to foam cells formation which finally end in atherosclerotic plaque development (Luque et al., 2013, Stocker et al., 2004).
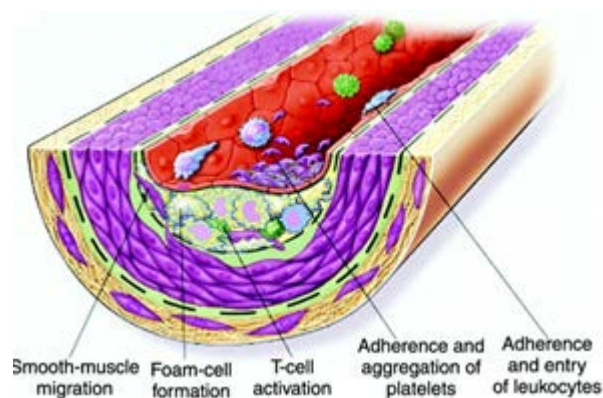
Figure 4. Atherosclerosis process. Source: modified from Stocker et al., 2004.

Atherosclerosis is considered the main cause of cardiovascular diseases such as coronary heart disease, myocardial infarction or stroke, among others (Musunuru, 2010, Rizza et al., 2014).

Cardiovascular diseases constitute the leading cause of death in the world, therefore treating dyslipidaemias has become a matter of main importance (IAS, 2014). In fact, lowering cholesterol levels has demonstrated to reduce cardiovascular events and mortality (Khatana et al., 2014). In this context, a reduction of 1.0 mmol/L of plasma LDL-c lowers over a fifth the possibilities of suffering a cardiovascular event and about 40-50 % if the reduction reaches 2-3 mmol/L (Baigent et al., 2010).

1. 4. 3. Hypertension

Another main modifiable MetS manifestation is the hypertension, which is mainly defined as a resting systolic blood pressure (SBP) ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg or drug prescription to lower hypertension (Lloyd-Jones et al., 2013).

The exhibition of this clinical entity that usually involves narrowed arteries, is conditioned by genetics, environmental and behavioural factors and affects a large number of adults as well as an important proportion of children (Lloyd-Jones et al., 2013). The last statistical update from the AHA estimates a hypertension population of 77.9 million ≥ 20 years old around the world (Go et al., 2014).

Hypertension is identified as a major reversible cardiovascular and renal risk factor, related to heart disease, vascular disease, stroke and myocardial infarction (James et al., 2014, Rizza et al., 2014, Thomas et al., 2014, Zanchetti, 2014). Furthermore, it has been shown that every additional 20 mmHg in SBP and 10 mmHg in DBP double the risk of cardiovascular disease (Lewington et al., 2002). For all of this, hypertension is considered a public health problem (Lloyd-Jones et al., 2013). In fact, in Spain, together with the control of plasma cholesterol levels, the maintenance of a healthy blood pressure is considered the most important achievement contributing to cardiovascular mortality reduction (Gomez-Huelgas et al., 2014). Thus, public health and clinical efforts to detect, treat and control the hypertension are essential for lowering the prevalence of this clinical condition.

1. 4. 4. Hyperglycaemia

Hyperglycaemia and related insulin resistance and type 2 diabetes mellitus, are other metabolic abnormalities encompassing the MetS that involves a damaged glucose metabolism.

The stages of impaired glucose tolerance and impaired fasting glycaemia are previous conditions to the development of type 2 diabetes, a disease known since ancient times which first written reference dates from 1500 before Christ, in Egypt (Klandorf, 2013).

This illness is characterized by an impaired uptake of glucose by the cells which leads to hyperglycaemia and consequently glycosuria and ketoacidosis (Klandorf, 2013). This statement leads to different tissue damage that shorten the life expectancy of diabetics, involving cardiovascular diseases, atherosclerosis, hypertension (Ballard et al., 2013), β-cells dysfunction (Poitout et al., 2008), kidney disease (Pugliese et al., 2014) or blindness (Asif, 2014). Among them, cardiovascular lesions are reported to be the most common cause of premature death in diabetic persons (Klandorf, 2013).

In 2011 it was estimated that 347 million people around the world presented diabetes (Danaei et al., 2011) and the prevalence is growing each year, being hypothesized that in 2030, 552 million people will suffer this disease becoming the seventh leading cause of death (Whiting et al., 2011, WHO, 2011). Although type 2 diabetes is a disease that usually occurs in the adulthood, in the last decades its prevalence is also increasing among young people (Klandorf, 2013).

The most important factors contributing to the development of type 2 diabetes are an unhealthy lifestyle together with genetic susceptibility (Asif, 2014). Among the environmental factors related to type 2 diabetes, obesity and the lack of exercise are considered main contributors. In fact, even small amounts of weight loss can result in an increased insulin sensitivity (Klandorf, 2013).

Moreover, some dietary compounds such as vitamin C are thought to improve the hyperglycaemic-related complications (Klandorf, 2013). Therefore, in the treatment of this disease both antidiabetic drugs and insulin, depending on the degree of development of the illness, can be used, but always a healthy dietary pattern together with exercise should be established (Asif, 2014, Klandorf, 2013).

2. OXIDATIVE STRESS IN THE METABOLIC SYNDROME

Oxidative stress is defined as an imbalance between the prooxidants and antioxidants in the body (Rahal et al., 2014). Reactive oxygen species (ROS) are chemically reactive molecules derived from oxygen formed by different endogenous systems, exposure to various physicochemical conditions or pathophysiological states (Devasagayam et al., 2004). At low concentrations, they have beneficial effects participating in different processes such as in the energy production, the defence against infectious agents, the control of the vascular tone or the cell apoptosis (Ho et al., 2013). However, when there is an imbalance between ROS and the antioxidant systems, whether through an increase in ROS levels or a decrease in the cellular antioxidant defence, the accumulation of ROS occurs, leading to an oxidative stress status (Jialal et al., 2012).
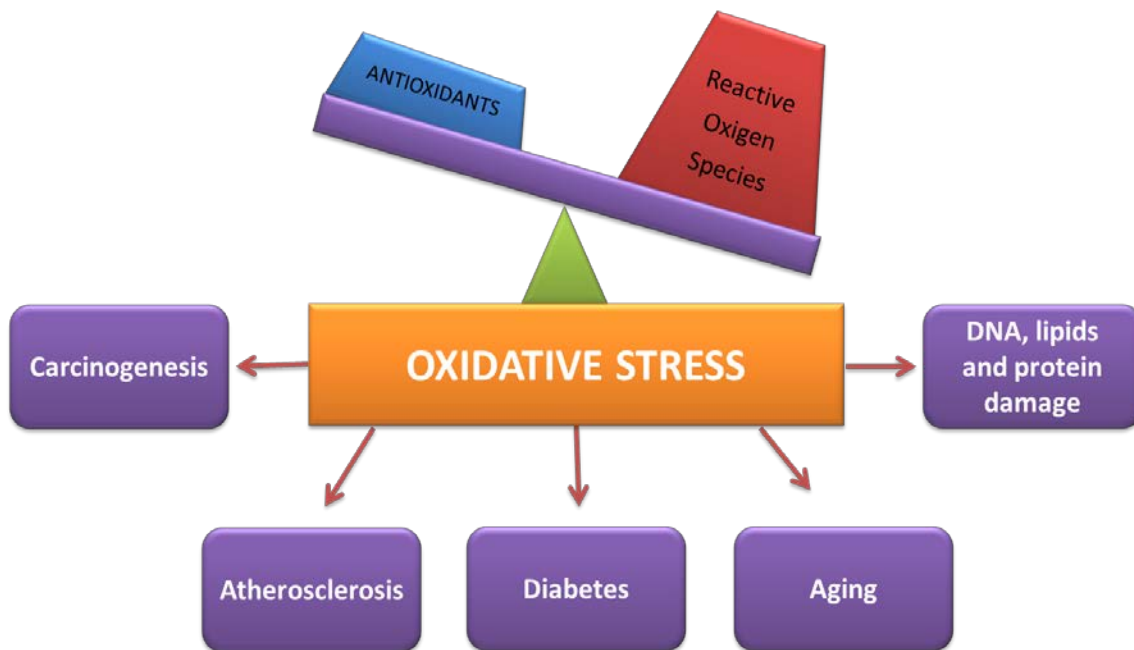
Figure 5. Oxidative stress status and associated comorbidities. DNA, deoxyribonucleic acid.

The oxidative stress (Figure 5) is associated with carcinogenesis, atherosclerosis, diabetes, aging and MetS by causing injury of nucleic acids, proteins and lipids (Jialal et al., 2012, Ray et al., 2012). Concerning the MetS, there are several studies that confirm a higher oxidative stress in individuals suffering this clinical entity than in healthy people (Cardona et al., 2008). In this context, it is suggested that the oxidative stress is involved in the aetiology, pathogenesis, and development of the MetS (Soares et al., 2009).

Moreover, there are different studies that have reported a positive correlation between fat mass accumulation and a systemic oxidative stress (Cervellati et al., 2014, Matsuda et al., 2013).

Furthermore, the impairment of the glucose uptake by the cells of the organism, the dysfunction of the pancreas β-cells and the insulin resistance in diabetics, is thought to be partly caused by free radicals as a consequence of their ability to activate stress-sensitive signalling pathways (Monickaraj et al., 2013).

Additionally, increased oxidative stress also motivates the pathophysiology of hypertension via up-regulating the renin-angiotensin system (Matsuda et al., 2013).
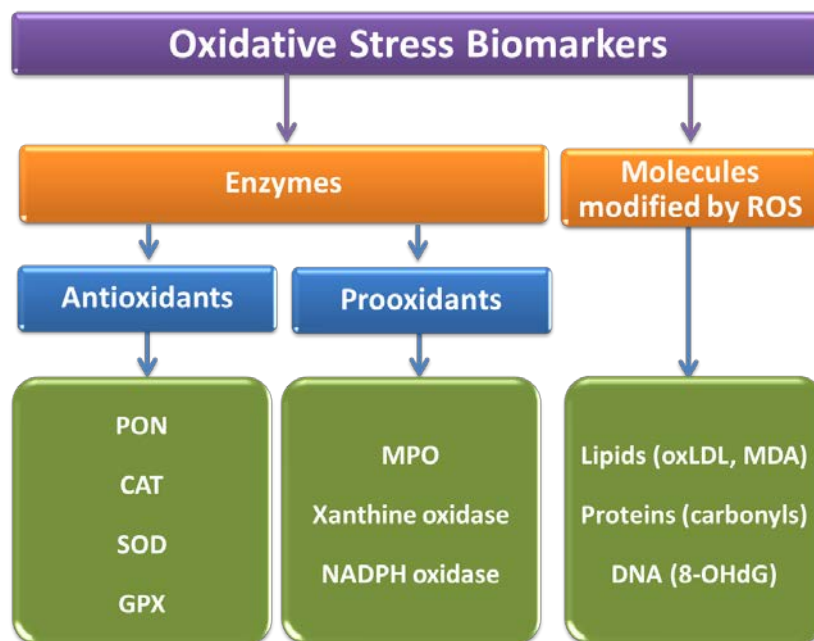
Figure 6. Some oxidative stress biomarkers. 8-OHdG, 8-Hydroxydeoxy-guanosine; CAT, catalase; DNA, deoxyribonucleic acid; GPX, glutathione peroxidase; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; oxLDL, oxidized low density lipoproteins; PON, paraoxonase; ROS, reactive oxygen species; SOD, superoxide dismutase.

Concerning the MetS-related disease atherosclerosis, it is well-known that the oxidative stress plays a key role in its development by different mechanisms such as the oxidation of LDL-c particles (Parthasarathy et al., 2008) or the impairment of HDL-c functions (McGrowder et al., 2011).

Introduction

The measurement of parameters indicative of the antioxidant/oxidant status (Figure 6) in the population, especially in individuals suffering the MetS, is important in order to define whether the subjects present oxidative stress, as well as to determine if the choice, dosage, and duration of an antioxidant treatment achieve the proposed biochemical or physiological endpoint (Rahal et al., 2014).

The identification and measurement of biomarkers of lipid peroxidation like malondialdehyde or oxidized low density lipoproteins, prooxidant enzymes such as the myeloperoxidase, enzymes with antioxidant properties such as the paraoxonase or dietary intake antioxidants as vitamin C or tocopherols, has been one of the main topics among the biological researchers in the last two decades (Mansego et al., 2011, Rahal et al., 2014).

2. 1. Oxidized Low Density Lipoproteins

Oxidized low density lipoproteins (oxLDL) consist of LDL-c particles which lipids and apolipoprotein B content has been modified by oxidation (Soares et al., 2009). This complex process takes place mainly within vascular cells and can occur in two different ways, non-enzymatically or catalysed by enzymes such as 12/15-lipoxygenase (Ho et al., 2013).
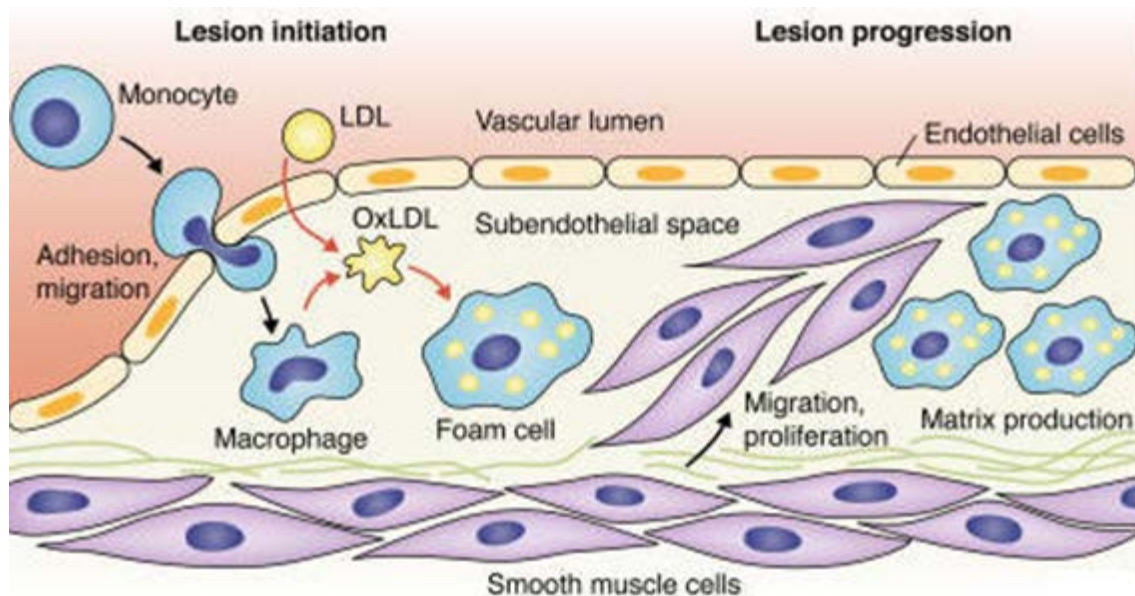
Figure 7. Initiation and progression of atherosclerotic lesions. LDL, low density lipoprotein; oxLDL, oxidized low density lipoprotein. Source: Heinecke JW, 2006.

OxLDL induce the migration of monocytes into the tunica intima (Figure 7) of the blood vessels where they differentiate into macrophages (Chavez-Sanchez et al., 2014, Heinecke, 2006). These macrophages interact with the oxLDL via scavenger receptor pathways and form foam cells (Farooqui, 2013). This situation is a clear hallmark of the development of atherosclerosis since foam cells form fatty streak lesions that finally result in atherosclerotic plaques (Luque et al., 2013).

Plasma oxLDL levels depend on LDL-c particles size and concentration. Thus, the higher the LDL-c levels and the prevalence of small, dense LDL-c particles, the greater the oxLDL concentrations (Camont et al., 2013).

It has been reported that high levels of oxLDL are associated with the MetS in general (Tumova et al., 2013) and with each of its components such as diabetes mellitus (Tousoulis et al., 2013), obesity (Njajou et al., 2009), dyslipidaemia (Camont et al., 2013) or cardiovascular diseases (Holvoet et al., 2008).

Finally, oxLDL are considered one of the main biomarkers of oxidative stress, in fact, it is the parameter used by the European Food Safety Authority (EFSA) to study the approval of a health claim which refers to have an effect against oxidative damage (EFSA, 2011).

2. 2. Malondialdehyde

Malondialdehyde (MDA) is a 3-carbon, low molecular weight aldehyde produced as an end-product of the lipid peroxidation, especially of polyunsaturated fatty acids. In fact, it is one of the most studied aldehydes formed when lipid hydroperoxides break down in biological systems, being in many instances the most abundant (Uchida, 2000).

MDA occurs in various covalently bound forms (Esterbauer et al., 1991) and it is widely employed as an oxidative stress biomarker (Spirlandeli et al., 2014).

The manner in which MDA is produced is not clear. There are different hypothesis (Lykkesfeldt, 2007), all of them postulated some time ago: some authors were based on the non-volatile nature of the MDA precursor when suggested that the mechanism by MDA is produced involves the formation of prostaglandin-like endoperoxides from polyunsaturated fatty acids (PUFAs) with at least two methylene-interrupted double bonds (Pryor et al., 1975), while another hypothesis is based on sequential hydroperoxide formation and β-cleavage of PUFAs where MDA is formed by β-scission of a 3-hydroperoxyaldehyde or by reaction between acrolein and hydroxyl radicals (Esterbauer et al., 1991).

It has been described that MDA can interact with proteins and aminoacids such as lysine residues, generating products which impair the interaction between oxLDL and macrophages and thereby promote atherosclerosis (Del Rio et al., 2005).

Moreover, it has been reported that serum MDA levels are increased in patients with atherosclerosis (Lykkesfeldt, 2007), in obese people (Sankhla et al., 2012), in individuals with type 2 diabetes (Kaefer et al., 2012), in patients with obstructive apnea, in hypertensives (Ahmad et al., 2013) and in people suffering MetS (Yardim-Akaydin et al., 2013).

2. 3. Myeloperoxidase

Myeloperoxidase (MPO) is a leukocyte-derived enzyme discovered in 1994, which is crucial in immune system due to its distinctive capacity to produce hypochlorous acid (HOCL). HOCL is a weak acid with a potent antimicrobial activity (Ho et al., 2013) but also an oxidizing agent (Maitra et al., 2011).

MPO also generates other intermediates particles, such as $\cdot OH$, $ONOO^-$ and $NO2$, in the process of oxidizing chloride ions, Cl^-, in the presence of H_2O_2. These are ROS particles involved as well in oxidative stress development (Karakas et al., 2012).

These MPO-derived ROS can produce endothelial dysfunction via several pathways, such as: (1) reducing the bioavailability of nitric oxide, a molecule with well-known atheroprotective and vascular relaxation effects (Eiserich et al., 2002); (2) modifying LDL-c particles in the subendothelial space of the vessels wall leading to oxLDL (Carr et al., 2000) or (3) impairing the HDL-c function by causing oxidative damage to apolipoprotein A1 (Nicholls et al., 2005) or generating HDL-c receptor inhibitors (Binder et al., 2013). All these actions contribute to atherogenic processes and cardiovascular diseases development (Anatoliotakis et al., 2013). Thus, several studies support that high levels of MPO are associated with increased risk of myocardial infarction (Kaya et al., 2012), coronary artery disease (Schuhmann et al., 2014), accelerated progression of coronary atherosclerosis (Kataoka et al., 2014), acute coronary syndrome (Graner et al., 2013) or poor cardiovascular outcomes in chronic obstructive pulmonary disease patients (Park et al., 2013).

2. 4. Paraoxonase-1

Paraoxonase-1 (PON1) is a calcium-dependent glycoprotein, hepatically synthesized, that belongs to the paraoxonase family (Precourt et al., 2011). Since it is a hydrophobic enzyme, it requires HDL-c as a serum transport vector (Reddy, 2010). In fact, many of the antiatherogenic properties of HDL-c are attributed to PON1 functions such as the protection against oxidative stress or its anti-inflammatory capacity (Aharoni et al., 2013, Cohen et al., 2012).

The precise mechanisms by which the PON1 acts remain still unclear, but several in vitro activities have been attributed to this enzyme (Nus M, 2008): paraoxonase (hydrolysis of organophosphates), lactonase (hydrolysis of lipophilic lactones) and arylesterase or ARE (hydrolysis of aromatic carboxylic acid esters).
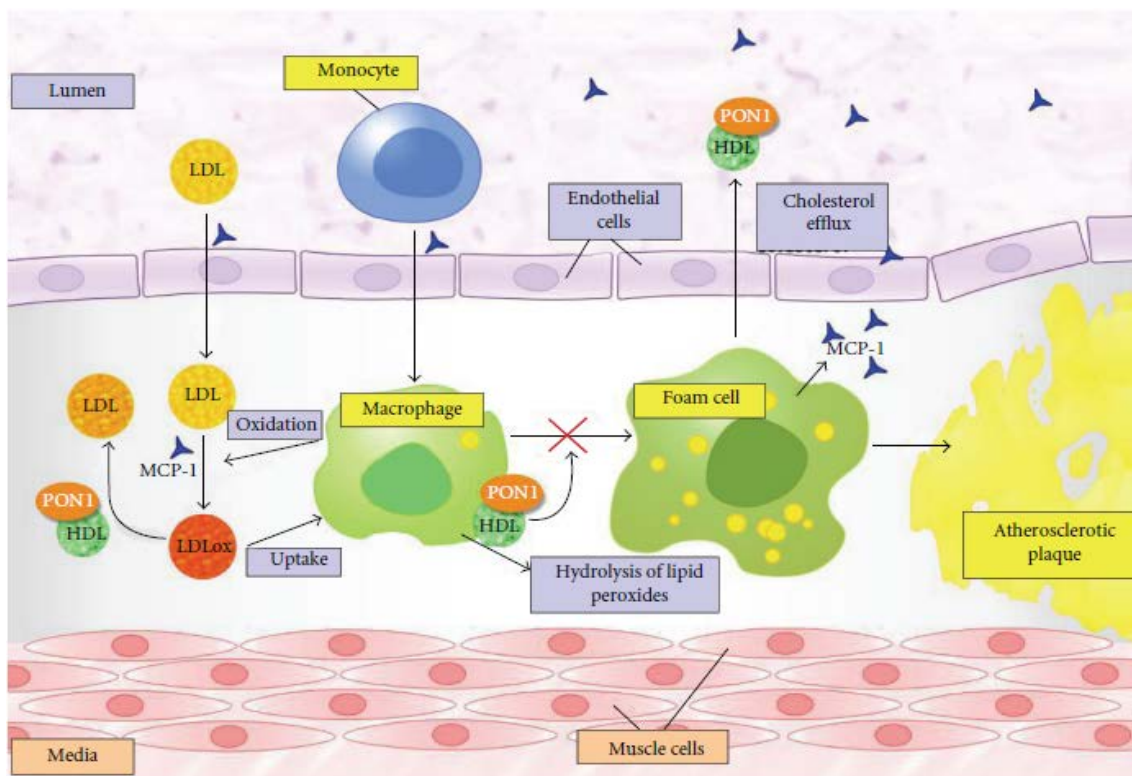

Figure 8. The protective role of paraoxonase 1 (PON1) in atherosclerosis. HDL, high density lipoprotein; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein-1. Source: Camps et al., 2012.

Moreover, it has been described that PON1 has the ability to inhibit the oxidation of lipoproteins such as HDL-c or LDL-c (Kim et al., 2013, Kumar et al., 2013). Additionally,

PON1 can diminish the adhesion of monocytes to the endothelial cells and their differentiation to macrophages (Rosenblat et al., 2011). It has also been shown that PON1 prevents cholesterol biosynthesis and uptake by macrophages (Rozenberg et al., 2005), has the ability to lower lipid peroxide levels and increase the cholesterol efflux from macrophages (Berrougui et al., 2012). All these actions (Figure 8) lead to an inhibition of atherosclerosis development (Aharoni et al., 2013, Camps et al., 2012, Reddy, 2010).

Furthermore, a positive association between the PON1 activity and the plasma levels of HDL-c has been described. Besides, a negative correlation between lipid peroxidation markers and PON1 activity was suggested (Reddy, 2010).

Interestingly, HDL-c, especially the associated apolipoprotein A1, has been shown to contribute to PON1 function, activity and stability (Reddy, 2010). In fact, it has been reported that when the binding of PON1 to HDL-c is blocked, the antiatherogenic properties of the enzyme decrease significantly (Mackness et al., 2006). In this context, reduced levels of PON1 are associated with atherosclerosis as well as with other related diseases such as hypercholesterolemia, diabetes, hypertension, coronary heart disease or other cardiovascular diseases (Efrat et al., 2009, Rosenblat et al., 2006).

3. A NEW POTENTIAL MARKER OF THE METABOLIC SYNDROME: IRISIN

As a heterogenic clinical entity, many biomarkers can be used in the identification of the MetS. Thus, we can find inflammatory biomarkers, oxidative stress parameters, energy metabolism indicators and genetic and epigenetics marks as indirect measurements that can contribute to the diagnosis and control of the MetS development (Rao et al., 2014).

Irisin, which name comes from the Greek messenger goddess Iris (Bostrom et al., 2012), is a recently discovered peptide expressed and produced especially by the skeletal muscle but also by the adipose tissue (Moreno-Navarrete et al., 2013) and others such as saliva or breast milk (Aydin et al., 2013). It is a 112 amino acid peptide secreted into the circulation following proteolytic cleavage from its cellular form, fibronectin type 3-domain containing protein 5 (FNDC5). The FNDC5 expression is induced by the peroxisome proliferator-activated receptor-coactivator 1α (PGC1α) (Bostrom et al., 2012).

Research in rodents and humans has associated circulating plasma irisin levels with increased energy expenditure, suggesting a possible role of this hormone in the regulation of energy metabolism (Polyzos et al., 2013, Swick et al., 2013). But, as a novel hormone,

several studies are being carried out in order to find and understand its potential different functions (Lopez-Legarrea et al., 2014). In fact, as irisin has been found in several tissues in addition to the skeletal muscle or the adipose tissue, such as liver, lung or kidney as well as in breast milk and saliva; further functions to that of thermoregulation are being hypothesized (Crujeiras et al., 2014, Hofmann et al., 2014).
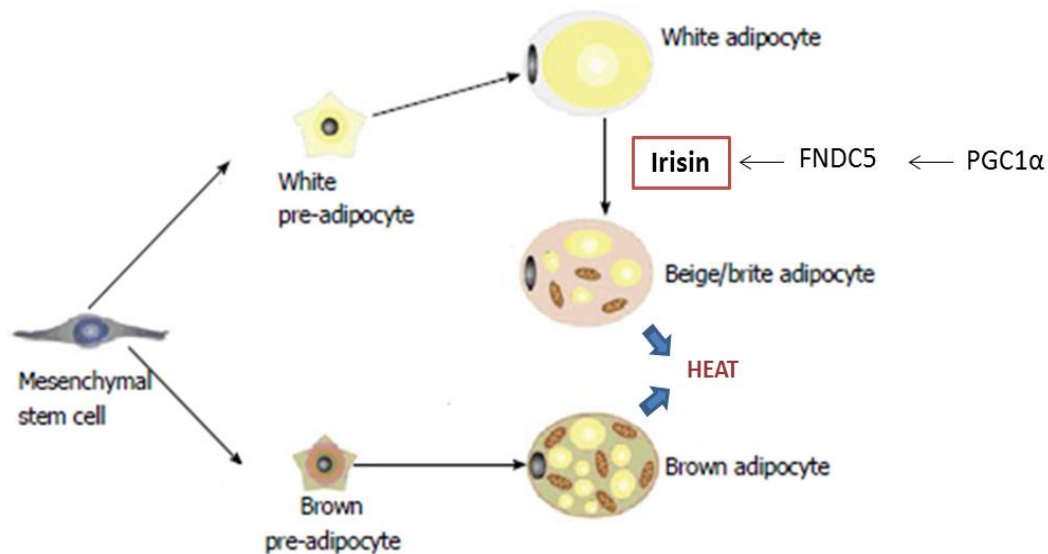
Figure 9. Classes of adipocytes and their differentiation. Source: modified from Park et al., 2014.

Fat browning (Figure 9) is a process that consists in the formation of the so-called beige or brite adipocytes from white adipose tissue (Park et al., 2014). These kinds of adipocytes have the characteristics of both, brown and white fat cells (Petrovic et al., 2010). Therefore, they can dissipate energy as heat to maintain the body temperature via uncoupling protein 1 (UCP1), as brown adipocytes do (Aydin et al., 2013, Spiegelman, 2013). In murine models, irisin, has been reported to participate in white fat browning (Bostrom et al., 2012). However, the role of irisin in white fat browning of humans cells remains unclear. Actually, it has been reported that in human cell models this hormone has no effect on browning of the mayor white adipose tissue depots, being likely to selectively target a small population of adipocytes (Elsen et al., 2014).

It was initially reported that exercise induced rising irisin levels (Bostrom et al., 2012, Kraemer et al., 2014, Spiegelman, 2013) as well as it was shown a relation of irisin with follistatin, a peptide that regulates muscle growth (Vamvini et al., 2013). However, later research came across controversial results and recent reviews have stated that more investigation is needed in order to conclude if irisin is directly influenced by exercise and if this relationship happens in all populations equally (Hofmann et al., 2014, Irving et al., 2014).

Moreover, it has been shown lower levels of plasma irisin in type 2 diabetes mellitus patients compared to controls with normal glucose tolerance (Liu et al., 2013), as well as it has been reported a possible antidiabetic role of this hormone by improving glucose homeostasis (Choi et al., 2013, Hojlund et al., 2013, Liu et al., 2013). Moreover, a recent study has associated high levels of irisin with healthy centenary people and low levels with young patients with myocardial infarction (Aydin et al., 2014, Emanuele et al., 2014). However, at the same time, positive association between irisin and obesity or MetS has been described, suggesting an irisin resistance similar to leptin or insulin resistance in these populations (de la Iglesia et al., 2013b, Hee Park et al., 2013). Furthermore, other studies reported no association between irisin levels and BMI, diabetes or other related parameters (Sanchis-Gomar et al., 2014).

This disagreement between different studies may be due to the fact that they have been carried out in different populations and under different conditions.

4. EPIGENETICS IN THE METABOLIC SYNDROME

The deoxyribonucleic acid (DNA) is a double-stranded helix, formed by nucleotides (Figure 10). Each nucleotide contains a monosaccharide, a phosphate group and a nitrogen base (guanine, adenine, thymine or cytosine), recorded using the letters G, A, T, and C, respectively (Pray, 2008, Shapiro et al., 2014).

The DNA encodes the genetic instructions used in the development and functioning of the living organisms. However, the information of the DNA sequence does not completely explain human development, physiology and disease (Jang et al., 2014). Hence, there are inheritable reversible changes in gene expression that cannot be explained by changes in DNA sequence but involve changes in phenotype, which is called epigenetics (Goossens et al., 2009, Morgan et al., 2008). The science which studies the epigenetics modifications is called epigenomics (Martinez et al., 2014).
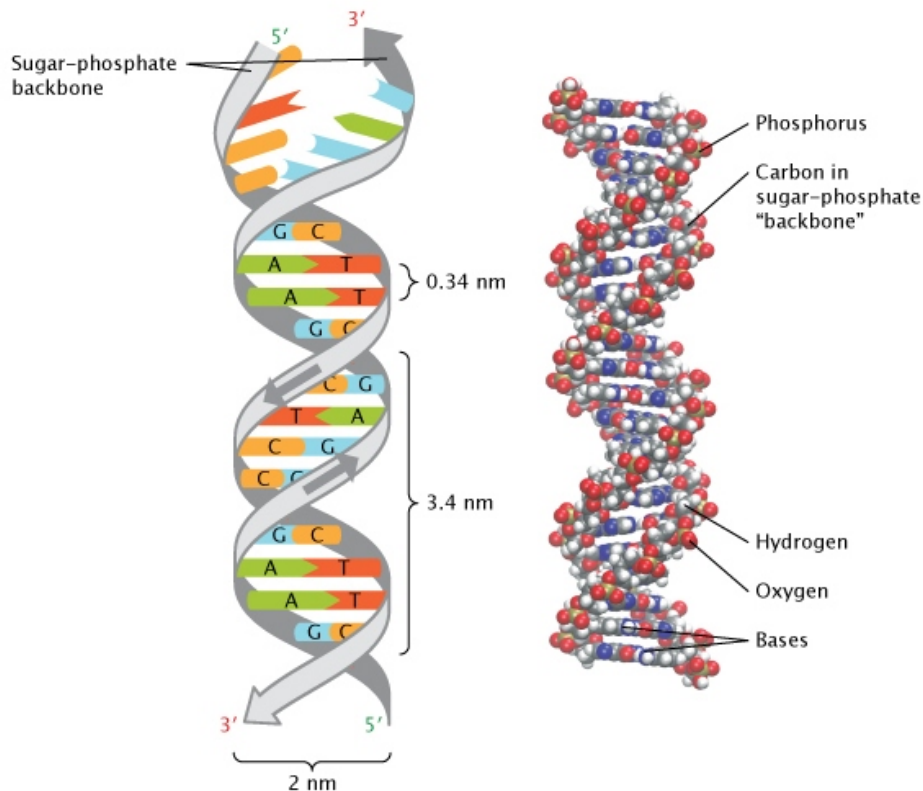
Figure 10. The double-helical structure of DNA. Source: Pray, 2008.

The prefix epi- comes from the Greek, meaning "above", "on" or "over". Thus, epigenetics means "above/on/over genetics". Therefore, genes can be silenced or activated by different "above" molecules that modify its expression, but not the DNA sequence (Christensen et al., 2011).

The DNA is packaged as structural units called nucleosomes by proteins named histones, forming the chromatin, in order to store the large mass of genetic material within the cell nucleus. DNA unpacking occurs for gene expression, a process that can be altered by epigenetic processes (Figure 11) such as DNA methylation, histone modifications, critical nuclear proteins for epigenetic gene regulation, genomic imprinting, non-coding ribonucleic acids or non-covalent mechanisms as physical alterations in nucleosomal positioning (Mansego et al., 2013, Milagro et al., 2013).

Among the different possible epigenetic modifications, microRNAs, DNA methylation and histone modifications (Figure 12) are probably the most widely studied (Lavebratt et al., 2012, Milagro et al., 2013).
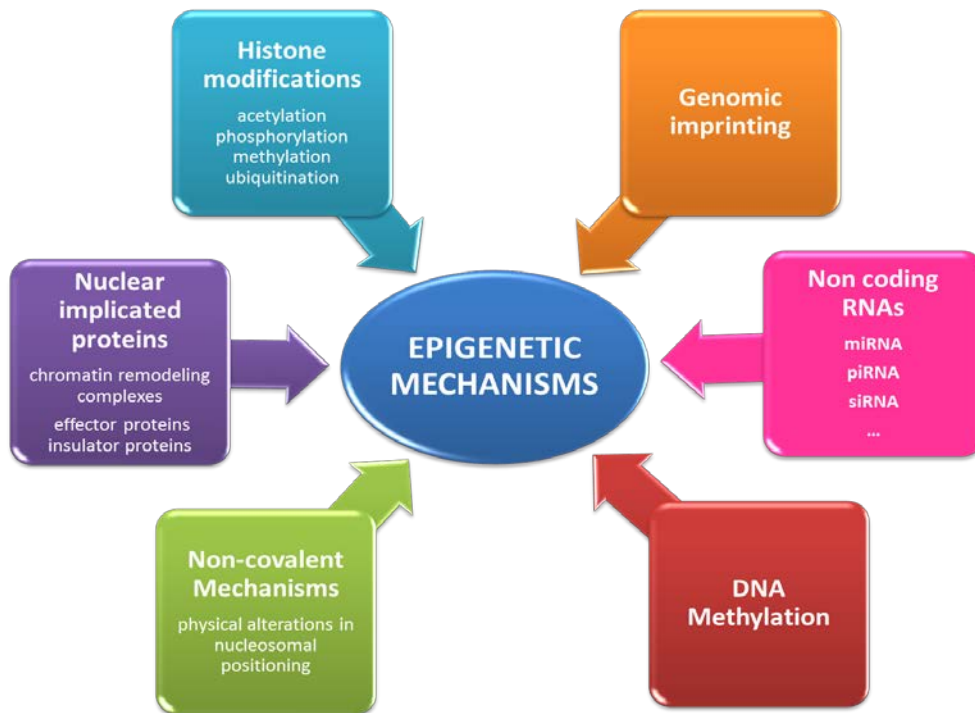
Figure 11. Different epigenetic mechanisms that regulate gene expression.
DNA, deoxyribonucleic acid; miRNA, microRNA; piRNAs, Piwi-interacting RNAs; RNA, ribonucleic acid; siRNAs, small interfering RNAs.

DNA methylation is a biochemical process where a one-carbon methyl group is covalently adhered to the cytosine base. This process typically occurs in Cytosine-phosphate-Guanine (CpG) dinucleotides, naturally associated with the 5 position of gene sequences, where CpG dinucleotides islands are particularly dense (Campion et al., 2009).

Hypermethylation of the CpG islands is usually associated with suppression of the expression of surrounding genes, while hypomethylation is generally associated with their transcriptional activation (Bird, 1986).

Moreover, the histones proteins are susceptible of a wide range of covalent modifications including acetylation, phosphorylation, methylation, an ubiquitination (Martinez et al., 2012). These changes are thought to have and influence on chromatin structure and gene function (Cedar et al., 2009). Additionally, it seems that there is a relationship between DNA methylation and histone modifications in both directions, as it is hypothesized that the DNA methylation may be a template for histone modifications and, in the other way round, histone methylation can encourage DNA methylation (Cedar et al., 2009).
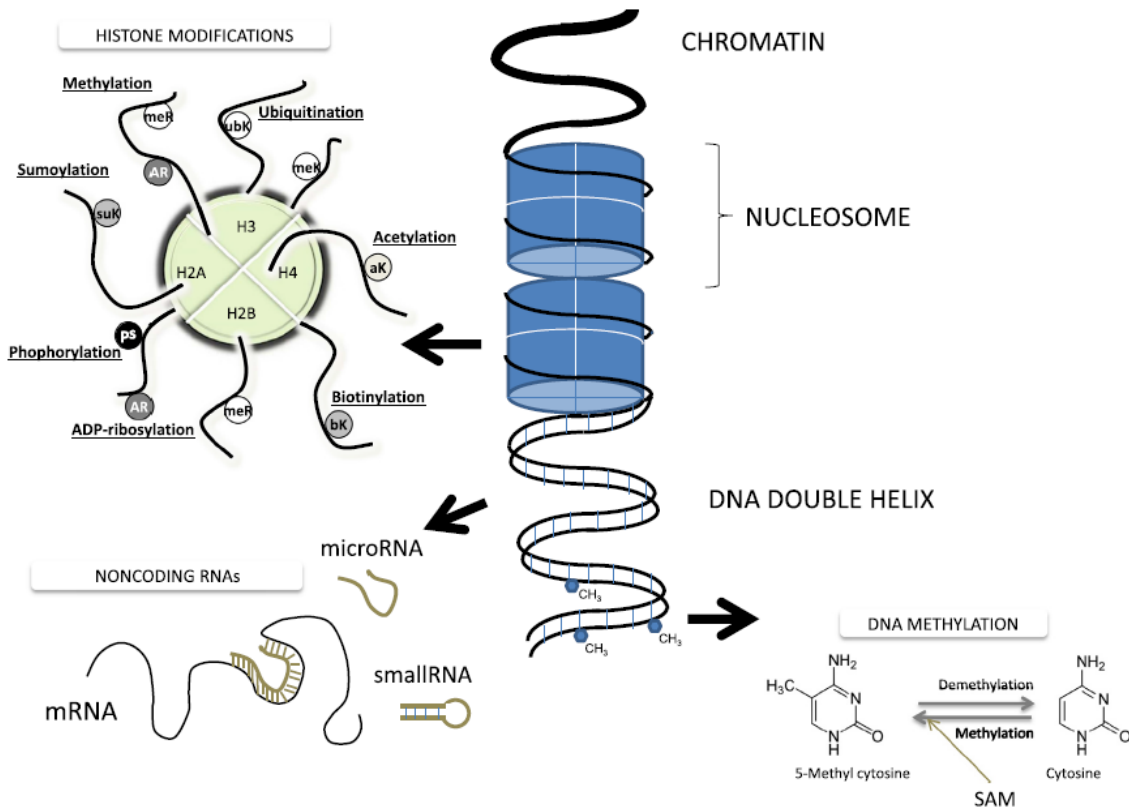
Figure 12. Major epigenetic mechanisms involved in gene expression regulation. Source: Milagro et al., 2013.

Finally, RNA based mechanisms like microRNAs have also been described to be involved in modifying gene expression of target genes (Chaturvedi et al., 2014).

Concerning the MetS, although there are still scarce studies investigating the epigenetics in this type of population, it is hypothesized that there is a link between aberrant DNA methylation pattern and obesity, atherosclerosis or type 2 diabetes, which makes patients more predisposed to developing MetS (Misiak et al., 2013).

On the other way round, there are different conditions that can affect DNA indirectly by modifying epigenetics factors such as oxidative stress, inflammation, physical activity, aging or nutrition (do Amaral et al., 2014, Santos et al., 2006).

Regarding nutrition, the study of the individuals' epigenetic patterns can be an effective tool to evaluate the response to a dietary treatment or to design a personalized diet, in order to assess the risk of developing obesity or related comorbidities such as MetS, (Cordero et al., 2010, San-Cristobal et al., 2013).
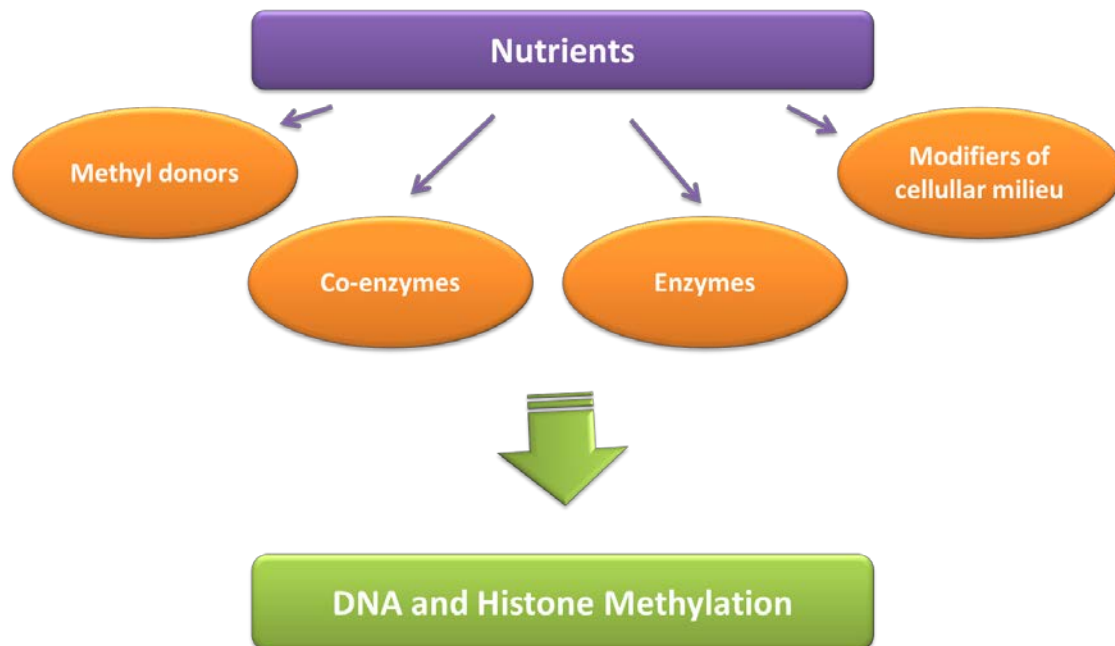
Figure 13. **Nutrients' influence on DNA and histone methylation.** DNA; deoxyribonucleic acid.

Dietary factors can influence phenotypic changes by different pathways (Figure 13). Among the possible mechanisms by which dietary components exert their effects, modulation of gene expression through regulation of DNA and histone methylation is one of the main studied (Bartels et al., 2007, Malireddy et al., 2012, Milagro et al., 2009). They can act as methyl donors (such as folate or vitamins B2, B6 or B12) or as co-enzymes (as methionine, choline, betaine or serine) promoting the transfer of methyl groups for DNA and histone methylation (Kim et al., 2009, Martinez et al., 2014). Moreover, nutrients can also stimulate/depress enzymes that participate in methylation epigenetic changes (Choi et al., 2010). Furthermore, the diet can modify the cellular milieu promoting changes in gene expression by methylation of DNA or histones (Cyr et al., 2011).

Among the different dietary factors related to epigenetics, antioxidants have become agents of strong interest in the field due to its high capacity to alter gene expression and thereby affect phenotype (Malireddy et al., 2012, Milagro et al., 2009).

In this context, lycopene, among other dietary factors (apigenin, curcumin, sulforaphane) is known to decrease DNA methyltransferase activity (Milagro et al., 2013).

Studies in vitro have shown that ascorbate (vitamin C) has the capacity of demethylate the genome, an action related not to its antioxidant activity but to its role as a cofactor and

electron donor for various hydroxylases involved in the regulation of gene transcription and epigenetics (Carr et al., 2013, Pera, 2013).

Moreover, different forms of tocopherols have also been reported to participate in epigenetics by inhibiting CpG methylation (Huang et al., 2012).

To sum up, the field of nutritional epigenetics, emerged as a novel mechanism, is of main importance due to the support that provides for understanding the role of nutrition in determining phenotype from genotype.

5. DIETARY APPROACHES FOR THE METABOLIC SYNDROME

The dietary treatment of the MetS, as a clinical entity of substantial heterogeneity, is a complex challenge. Thus, the different cornerstones presented in this illness should be addressed: obesity, dyslipidaemias, hypertension or impaired glucose metabolism, and, as not all individuals with the MetS have the same symptoms and pathologies, it must be personalized (Zulet et al., 2011).

5. 1. Energy-Restricted Diets

Since most individuals with the MetS are overweight, dietary treatment of this clinical entity should be primarily focused on weight reduction. Thus, it is well-known that in order to achieve this goal, a negative energy balance is needed. Therefore, it is necessary either to increase the energy expenditure or to decrease the caloric intake.

A hypocaloric diet is defined as a regime that supplies less energy than the amount of calories that would be consumed ad libitum (Bales et al., 2013). A reduction of 10–50 % calorie intake below usual ad libitum has been shown to cause a proportionate increase in maximum life span (Bales et al., 2013, Fontana et al., 2010).

In this context, hypocaloric diets have demonstrated to be effective in managing obesity promoting weight reduction as well as improving obesity-related disorders (Rossmeislova et al., 2013). It has been described that individuals under a long-term calorie restriction with an adequate nutrient intake are not at risk of developing abdominal obesity, type 2 diabetes, hypertension, dyslipidaemia, inflammation or atherosclerosis (Fontana et al., 2004). Moreover, accumulating data showed that even a weight loss of 2-5 % of the total body weight led to an improvement of cardiometabolic risk profile and reduced the risk of type 2 diabetes and cardiovascular diseases development (Golay et al., 2013, Turk et al.,

2009). In addition, different intervention trials have reported a relationship between weight loss and decreases of SBP and DBP and plasma TG levels, improvements of cholesterol profile (Wing et al., 2011) or reductions in hepatic fat (Lazo et al., 2010).

Among the different nutritional intervention trials, a reduction of 30 % of the energy requirements is a well-established hypocaloric dietary strategy which has demonstrated to be effective in weight reduction (Abete et al., 2008, Abete et al., 2009). However, the challenge lies in maintaining the weight reduction over time, as many subjects can follow a prescribed diet for a few months, but most people have difficulty in maintaining the acquired habits over the long term (Ebbeling et al., 2012).

5. 2. Diets Rich in *Omega*-3 Polyunsaturated Fatty Acids

The *n*-3 PUFAs are essential fatty acids with a double bond at the third carbon atom from the end of the carbon chain. Among them, α- linolenic acid (ALA) and the very long-chain eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the three most important *n*-3 PUFAs for the human physiology. We can find ALA mainly in vegetables oils such as soybean and flaxseed oils, while fish oils and fatty fish are the major dietary sources of EPA and DHA (Calder et al., 2009).

There is a moderate body of evidence from epidemiologic and clinical studies suggesting that *n*-3 PUFAs, mainly EPA and DHA, have a positive role in the prevention and treatment of the pathologies associated to the MetS (Abete et al., 2011, Lopez-Huertas, 2012). In this context, it has been described that EPA and DHA have the ability to reduce the risk of developing cardiovascular diseases and cardiometabolic abnormalities as well as cardiovascular disease-related mortality (Rizza et al., 2014). These beneficial effects are partly due to the anti-inflammatory and anti-thrombotic properties of these long-chain fatty acids as they have been described to have the ability to reduce the blood pressure and plasma TG levels, to decrease the thrombotic tendency and to improve the vascular endothelial function, among others (De Caterina, 2011, Lopez-Huertas, 2012, Rizza et al., 2014). An increment of paraoxonase levels as well as an improvement of insulin sensitivity in individuals with a high consumption of EPA and DHA has also been described (Lopez-Huertas, 2012).

Concerning ALA dietary intake-benefits on human health, it has been stated that a consumption of 1.5-3 g of ALA per day is cardioprotective (Albert et al., 2005, Mozaffarian et al., 2005). However, it is thought that these health benefits are due to the conversion of ALA into EPA and DHA and that this conversion is very low in humans (Calder et al., 2009,

Holligan et al., 2012). Therefore it is considered that an increased consumption of ALA might be of little benefit in improving health outcomes compared with increased intake of EPA and DHA (Calder et al., 2009, Holligan et al., 2012). However, concerning the last ones, different health authorities such as the European Food Safety Authority (EFSA) or the WHO had established dietary recommendations (Rangel-Huerta et al., 2012) of 250 mg of EPA plus DHA/day for the general adult population (males and non-pregnant/non-lactating females). Nevertheless, there is a need to carry out more studies with MetS patients in order to establish dietary recommendations for this specific population (Lopez-Huertas, 2012).

5. 3. Diets based on Low Glycaemic Index/Load

The glycaemic index (GI) is a ranking on a scale from 0 to 100 based on the area of blood glucose traced on a graph (Figure 14) generated during a 2-h period time following the ingestion of a food containing 50 g of CHO compared with the area plotted after giving a similar quantity of glucose or white bread which are giving a rating of 100 (Zulet et al., 2012). The higher the index, the more promptly the postprandial serum glucose rises and the more rapid the insulin response (Codairo, 2011, Sydeny, 2011). The glycaemic load (GL) is equal to the GI multiplied by the number of grams of CHO in a serving (Codairo, 2011).
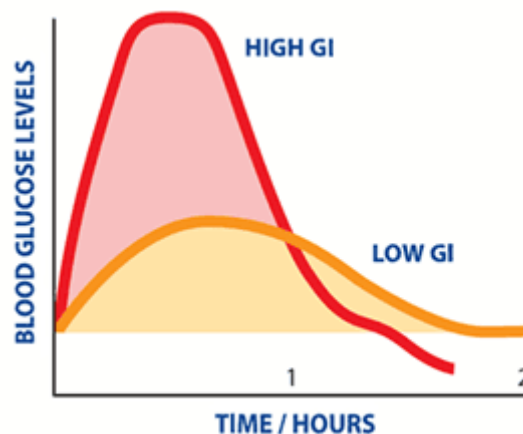
Figure 14. Difference between low-GI and high-GI foods. GI, glycaemic index. Source: University of Sydney, 2011.

The quick insulin response leads to a rapid hypoglycaemia associated to a consequent caloric ingestion increment which has been reported to be implicated in an increased cardiometabolic risk in patients suffering the MetS (Codairo, 2011). Actually, there is a hypothesis which states that the MetS is a consequence of an elevated intake of high GI foods, among others unhealthy dietary habits (Nakagawa et al., 2006). Moreover, following a diet rich in high GI CHO has been associated with hyperglycaemia, insulin resistance, type 2 diabetes, hypertriglyceridemia and obesity (Codairo, 2011, Farooqui, 2013), abnormalities directly related to the MetS.

For all of this, it is not uncommon to find the limitation of CHO at high GI among the advices for the MetS treatment (D'Amore et al., 2014).

5. 3. 1. Dietary Fibre

Several health benefits of dietary fibre have been described, including the prevention and mitigation of type 2 diabetes mellitus, cardiovascular diseases and colon cancer by reducing the risk of hyperlipidaemia, hypercholesterolemia, hyperglycaemia or oxidative stress (Hermsdorff et al., 2012, Straznicky et al., 2012). Moreover, diverse clinical studies have examined the role of this dietary component in body-weight reduction, and a strong relationship has been established (Du et al., 2010, Kristensen et al., 2012). Different mechanisms by which dietary fibre intake can influence body weight have been proposed. Recently, the role of dietary fibre in gut microbiota in the development of obesity and its associated co-morbidities has come to the forefront (Parnell et al., 2012). Data suggest that fibre can reduce the risk of obesity by promoting satiety and reducing energy intake (Brownawell et al., 2012, Burton-Freeman, 2000), and numerous studies have been carried out to determine the effects of dietary fibre on satiety (Howarth et al., 2001, Pereira et al., 2001, Wanders et al., 2011). Many different mechanisms have been suggested, such as a lower metabolisable energy content of fibre than of other nutrients (Livesey, 1992), a relatively constant meal intake volume (Poppitt et al., 1996), a decreased total energy intake by consuming foods rich in fibre, and the increased chewing activity or oral exposure time to foods after a high dietary fibre intake, which may result in earlier satiation (Zijlstra et al., 2009). Furthermore, fibre can slow down gastric emptying and consequently increase stomach distension, which also leads to satiation (de Graaf et al., 2004).

5. 4. Diets with High Total Antioxidant Capacity

Dietary total antioxidant capacity (TAC) is defined as the sum of antioxidant activities of the pool of antioxidants present in a food (Bartosz, 2003).

Taking into account that oxidative stress is one of the remarkable unfortunate physiological states of the MetS, dietary antioxidants are of main interest in the prevention and treatment of this multifactorial disorder (Zulet et al., 2012). Accordingly, it is well-accepted that a diet rich in fruits, vegetables and other plant-based foods may decrease the risk of oxidative stress-related diseases (Carlsen et al., 2010).

In this context, several studies analysing the effects of dietary TAC in individuals suffering the MetS or related diseases have been carried out. Thus, the Tehran Lipid and Glucose Study demonstrated that a high TAC has beneficial effects on metabolic disorders and especially prevents weight and abdominal fat gain (Bahadoran et al., 2012). In the same line, our team came across similar results as TAC was the most contributing factor involved in body weight and obesity after an eight-week hypocaloric intervention trial in obese adults with MetS symptoms (Lopez-Legarrea et al., 2013). Shortly beforehand, it was suggested that dietary TAC could be a good tool to estimate the risk of developing MetS features (Puchau et al., 2010).

Moreover, research conducted in our department also evidenced that beneficial effects on oxidative stress biomarkers were positively related with higher TAC consumption in patients suffering from MetS with hyperglycaemia (de la Iglesia et al., 2013a).

For all of this, diets with a high content of spices, herbs, fruits (including berries), vegetables, nuts and chocolate, the most antioxidant rich food categories (Carlsen et al., 2010), may be effective in the prevention and treatment of the MetS and related diseases.

5. 4. 1. Vitamin C

Vitamin C or ascorbate is an essential water-soluble antioxidant (Fain, 2013) mainly found in fruits and vegetables (Gallie, 2013). This dietary component produces its antioxidant effect primarily by quenching damaging free radicals, although it can also regenerate other oxidized antioxidants such as tocopherol (Chambial et al., 2013).

Moreover, several physiological functions have been attributed to ascorbate such as the biosynthesis of collagen or catecholamines as well as the prevention of cancer and cardiovascular diseases (Grosso et al., 2013).

Vitamin C deficiency provokes scurvy which is mainly caused by a lack of daily dietary amount (Fain, 2013). On the other hand, an excess of vitamin C ingestion leads to an oxidative process where oxidative particles are formed (Mamede et al., 2011).

5. 4. 2. Tocopherols

Tocopherols (Figure 15), also known as vitamin E, are a family of eight fat-soluble phenolic compounds (Mamede et al., 2011, Traber et al., 2012) which main dietary sources are vegetable oils (Yang et al., 2013).

These compounds are considered essential for human health since they act as antioxidants by quenching oxidative free radicals (Yang et al., 2013). In this context, it has been described that tocopherols inhibit the oxidation of LDL-c as well as exert anti-inflammatory and antiatherogenic effects *in vitro*. Nevertheless, results from *in vivo* studies are still controversial (Wallert et al., 2014).
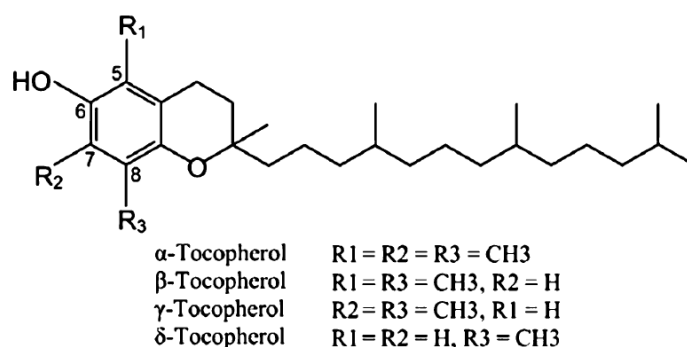
Figure 15. **Structures of tocopherols.** Source: Yang et al, 2013.

5. 4. 3. Carotenoids

Dietary carotenoids are a wide group of lipid-soluble micronutrients found mainly in fruits and vegetables, which are readily accumulated by vegetable consuming animals (Sommer et al., 2012, Stahl et al., 2012).

Different roles have been attributed to these dietary components, such as photoprotection, natural coloration or oxidative protection. Indeed, their capacity to act as scavengers of oxidant free radicals have received much attention (Britton et al., 2008).

There has been isolated more than 700 different carotenoids from natural sources (Britton et al., 2008). Among them, some of the most important, such as lycopene, lutein or

zeaxanthin, have been described to be related with prevention of a number of chronic diseases such as cancer, cardiovascular disease, cataract or macular degeneration (Sommer et al., 2012).

5. 5. Changes in Macronutrient Distribution

The most common macronutrient distribution recommendation for the general population as well as for weight loss dietary treatments has been set for a long time as 15 % protein, < 30 % lipids and 50-55 % CHO (Abete et al., 2010, Zulet et al., 2012).

A hypocaloric diet based on this macronutrient distribution has demonstrated to be effective in weight reduction over the short term (Abete et al., 2010). However, most people have difficulty in maintaining these dietary habits over time (Abete et al., 2011, Ebbeling et al., 2012).

For this reason, research on different macronutrient distribution, looking for a "formula" to increase dietary adherence based on promoting satiety and decreasing hunger, is being carried out (Arciero et al., 2013, Bray et al., 2012, de Jonge et al., 2012, Koppes et al., 2009, Stocks et al., 2013, Westerterp-Plantenga et al., 2009, Wikarek et al., 2014).

In this context, a moderate increment of protein intake at the expense of CHO has been seen to enhance weight-loss and weight-loss maintenance (Larsen et al., 2010, Petzke et al., 2014, Stocks et al., 2013). Two mechanisms have been proposed to explain these beneficial effects: the increment of diet-induced thermogenesis (Bray et al., 2012) and the increase of satiety (Westerterp-Plantenga et al., 2009). The increment of the thermogenesis is explained by the fact that the synthesis of peptide bonds, production of urea and gluconeogenesis are processes with a higher energy requirement than the metabolism of lipids or CHO (Koppes et al., 2009). The satiety effect may be clarified by an increment of cholecystokinin production or other appetite-control related hormones (Bendtsen et al., 2013).

Other beneficial effects attributed to moderate-high protein diets in the literature are the improvement of glucose homeostasis (Heer et al., 2014), the possibility of lower blood lipids (Layman et al., 2009), the reduction of blood pressure (Pedersen et al., 2013), the preservation of lean body mass (Daly et al., 2014) or the lower of cardiometabolic disease risk (Arciero et al., 2008, Gregory et al., 2011).

Conversely, there are some studies that have not found all these benefits attributed to a moderate-high protein diet (de Jonge et al., 2012). This fact can be explained by the different kind of proteins and their amino acid composition investigated in each study (Heer et al., 2014) as well as by the different type of populations (Gregory et al., 2011). Therefore, more research in the field is needed in order to make these results consistent.

In any case, when a hypocaloric diet is implemented, it is necessary to slightly increase the amount of proteins. Otherwise it would be difficult to reach the protein energy requirements, established as 0.83 g/kg/day for isocaloric diets and which should probably be at least 1 g/kg/day for energy-restricted diets (FESNAD-SEEDO, 2011).

5. 6. Meal Frequency Pattern

Lately, the term chrononutrition, referring to mealtimes and type of food consumed at each mealtime, has arisen together with meal frequency studies.

Research in chrononutrition is focused on the study of which time of the day is better to ingest specific foods for improving biorhythms, physical performance or health status in general (Bravo et al., 2013). In this context, a very recent review article has concluded that timing of food intake is related to obesity and to the success of weight loss therapy (Garaulet et al., 2014)

The pattern of increasing meal frequency in weight loss and weight control interventions has currently begun popular among professionals (Jakubowicz et al., 2012, Schwarz et al., 2011). The idea is to distribute the total daily energy intake into more frequently and smaller meals. However, there is not yet high evidence about the efficacy of this habit (Ohkawara et al., 2013). While some investigations have found an inverse association between the increment of meals per day and body weight, BMI, fat mass percentage or metabolic diseases such as coronary heart disease or type 2 diabetes (Arciero et al., 2013, Bhutani et al., 2009, Ekmekcioglu et al., 2011, Lioret et al., 2008, Schwarz et al., 2011), others have found no association (Cameron et al., 2010, Leidy et al., 2011, Mills et al., 2011).

Different mechanisms by which high meal frequency can have a positive effect on weight and metabolism management have been proposed. An increment of energy expenditure was hypothesized; however the studies carried out in this line have concluded that total energy expenditure does not differ among different meal frequencies (Smeets et al., 2009, Taylor et al., 2001). Another postulated hypothesis is that the more the number of meals a day, the higher the fat oxidation, but again no consensus has been

achieved (Ohkawara et al., 2013, Smeets et al., 2008). An additional suggested mechanism is that increasing meal frequency leads to plasma glucose levels with lower oscillations and reduced insulin secretion which is thought to contribute to a better appetite control. However, this associations have been found in population with overweight or high glucose levels but in normal-weight or normoglycaemic individuals the results are still inconsistent (Bachman et al., 2012, Heden et al., 2012, Leidy et al., 2011).

5. 7. Mediterranean Diet

The concept of the Mediterranean Diet (MedDiet) was for the first time defined by the scientific Ancel Keys after the Seven Countries Study. Keys studied the coronary events and dietary patterns of seven countries (Finland, Greece, Italy, Japan, the Netherlands, the United States, and Yugoslavia) and concluded that those around the Mediterranean Sea, which had a characteristic diet, had less risk of suffering coronary heart diseases (Keys, 1997, Keys et al., 1984).

The traditional MedDiet is rich in plant foods (fruits, vegetables, cereals, whole grains, legumes, tree nuts, seeds and olives), with extra-virgin olive oil as the main lipid source. It is also characterized by a low to moderate consumption of dairy products and a low intake of red meat as well as by a moderate consumption of red wine during the meals (Serra-Majem et al., 2006).

There is a lot of literature supporting the general health benefits of the MedDiet. One of the latest meta-analysis carried out in this field corroborates that a high adherence to this dietary pattern protects against mortality and morbidity from several causes (Sofi et al., 2013). Other studies have found a positive correlation between the adherence of a MedDiet pattern and reduced risk of developing cardiovascular diseases (Estruch et al., 2013, Fito et al., 2014, Martinez-Gonzalez et al., 2011, Salas-Salvado et al., 2008), diabetes (Salas-Salvado et al., 2014), MetS or its related comorbidities (Babio et al., 2009) or even depression (Sanchez-Villegas et al., 2013).

Furthermore, a MedDiet pattern follow-up has been suggested not only as a preventive tool but also as a treatment for the MetS (Mayneris-Perxachs et al., 2014) and cardiovascular diseases (Ortega-Azorin et al., 2014). A former meta-analysis concluded that the MedDiet is associated with significant reductions in body weight and waist circumference (Kastorini et al., 2011).

In addition, high plasma antioxidant capacity and reduced body weight gain (Razquin et al., 2009) are other of the beneficial effects attributed to the MedDiet.

The high amount of fibre which, among other beneficial effects, helps to weight control providing satiety; and the high antioxidants and anti-inflammatory nutrients such as *n*-3 fatty acids, oleic acid or phenolic compounds, are thought to be the main contributors to the positive effects attributed to the MedDiet (Bertoli et al., 2014).

5. 8. American Heart Association Guidelines

In 1989, nine health organizations and governmental bodies met under the aegis of the American Heart Association (AHA) in order to consolidate and establish nutritional dietary guidelines to improve the overall health of the general population. Later, in 2000, revised guidelines were published (Krauss et al., 2000), establishing the following principles:

1. **Include foods from all major food groups** in order to maintain a healthy eating pattern: consume fish, legumes, poultry, fat-free and low fat dairy products, lean meats and a variety of vegetables, fruits and grain products including whole grain.

2. **Maintain a healthy body weight** by doing exercise and controlling the calories intake (limitation of high caloric density foods).

3. **Preserve a desirable blood cholesterol and lipoprotein profile** limiting the consumption of food rich in saturated fatty acids and cholesterol and emphasizing the intake of unsaturated fatty acids from vegetables, fish, legumes and nuts.

4. **Maintain a desirable blood pressure** by preserving a healthy body weight and limiting salt and alcohol consumption.

The AHA also recommends a macronutrient distribution of ≤ 30 % total energy value from lipids, 55-60 % from carbohydrates (CHO) and the rest from proteins, as well as a cholesterol content of less than 300 mg/day (Krauss et al., 1996).

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AIM AND OBJECTIVES

1. GENERAL AIM

This current experimental trial was designed to evaluate and compare the effectiveness of the American Heart Association (AHA) guidelines with a new dietary strategy, the RESMENA (MEtabolic Syndrome REduction in Navarra) diet, based on the MedDiet pattern and characterized by a specific macronutrient distribution (40 % total energy value from carbohydrates, 30 % from proteins and 30 % from lipids), elevated meal frequency (7 meals/day), high *n*-3 PUFAs and low GI CHO consumption, which promotes also a high natural antioxidant food intake.

2. SPECIFIC OBJECTIVES

1. To evaluate changes on anthropometric and biochemical biomarkers throughout the nutritional intervention and to assess the differences on effectiveness between the two dietary hypocaloric treatments.

2. To examine changes on the MetS-related oxidative stress status throughout the nutritional trial and to assess the potential differences between both dietary strategies.

3. To determine the potential influence of specific dietary components included in the dietary patterns on the modification of anthropometric, biochemical, oxidative stress and epigenetics markers.

4. To investigate the influence of the recently discovered hormone irisin on the phenotype of MetS patients under an energy-restricted programme.

5. To research the potential role of methylation levels of oxidative stress-related genes on oxidative stress markers to better understand the influence of epigenetic mechanisms on the MetS.

SUBJECTS AND METHODS

1. STUDY DESIGN

The study was designed as a randomized, longitudinal and controlled trial to compare the effects of two dietary strategies on improving the comorbidities of the MetS such as body composition, biochemical, hormonal, oxidative stress or epigenetic parameters. Participants were randomly assigned to the control or the experimental diet, Control and RESMENA (*REducción del Síndrome MEtabólico en Navarra*) groups, respectively (Zulet et al., 2011).

The study lasted for 6 months encompassing two sequential stages (Figure 16): an initial 8-week nutritional-learning intervention period, during which nutritional assessment was carried out for the participants every 15 days (Lopez-Legarrea et al., 2013), and a 4-month self-control or autonomy period, during which the volunteers applied on their own the previously acquired dietary habits (de la Iglesia et al., 2014). The participants were asked to maintain their normal physical activity throughout the study (Lopez-Legarrea et al., 2014b).

During the 2-month nutritional-learning intervention period, the volunteers visited the Metabolic Unit at the University of Navarra every 2 weeks for anthropometric measurements, body composition analysis by bioelectric impedance, blood pressure measurements and psychological control, carried out by trained nutritionists following validated protocols (de la Iglesia et al., 2013b). Moreover, the nutritionists asked the participants about the feelings and sensations that they were experiencing with the new diet to determine their well-being. Finally, different advice was given to the participants in each situation as well as recipes, general information about food and the importance of dietary adherence (Zulet et al., 2011).

At the first visit as well as before and after the 4-month-long self-control period, body composition was measured using Dual-energy X-ray Absorptiometry (DXA), fasting blood and 24 h urine samples were collected and a 24 h physical activity questionnaire (Food and Nutrition Board, 1989, Navas-Carretero et al., 2009) and 48 h food record were implemented, in addition to the anthropometric measurements, blood pressure determinations, bioimpedance assessments and psychological tests (Perez-Cornago et al., 2013).

The CONSORT (CONsolidated Standards Of Reporting Trials) 2010 guidelines (Moher et al., 2012) were followed by taking into account the design of the present study, two-group longitudinal intervention, except for blinding.
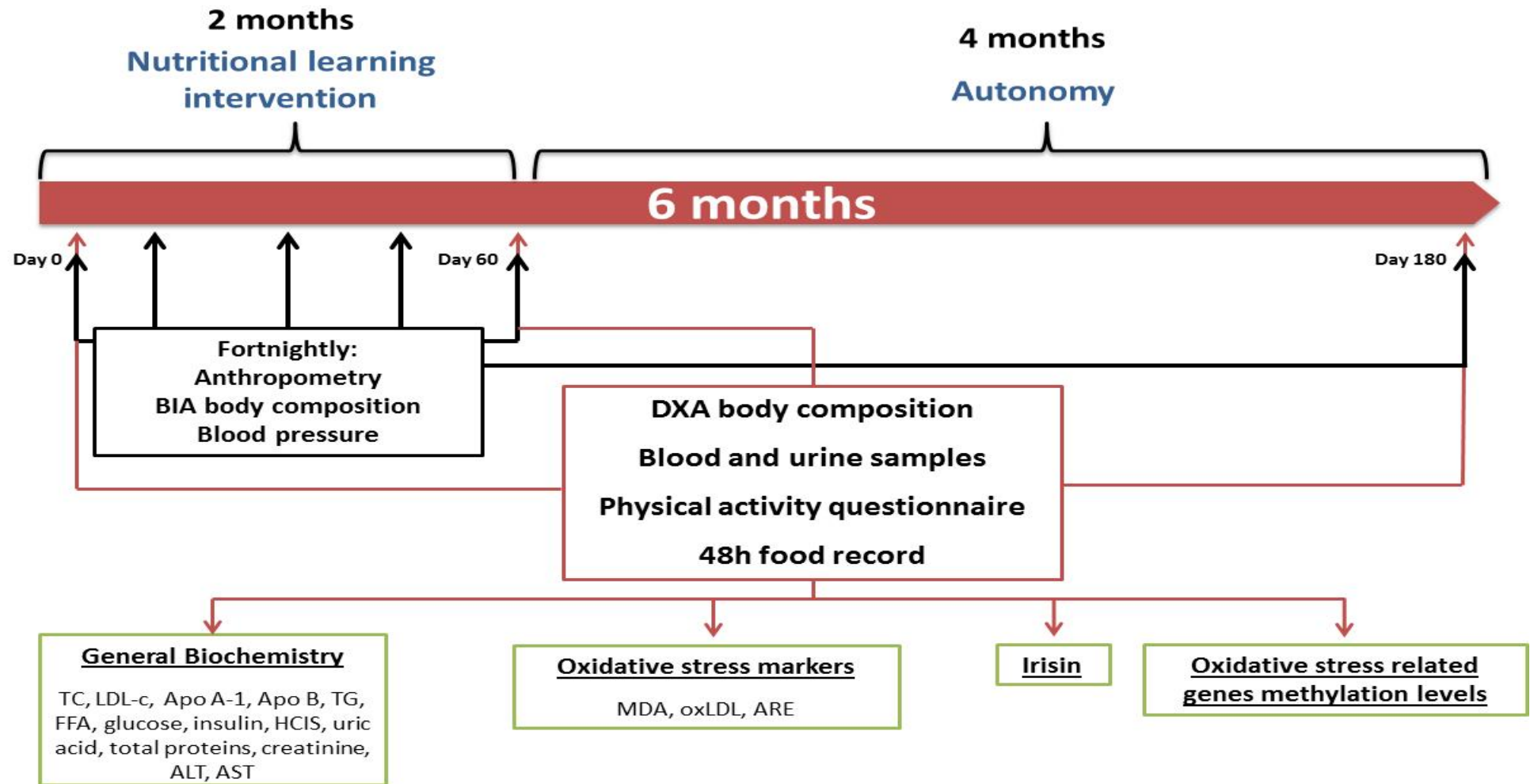
Figure 16. Study design. ALT, alanine aminotransferase; Apo A-1, apolipoprotein 1; Apo B, apolipoprotein B; ARE, arylesterase; AST, aspartate aminotransferase; BIA, bioelectric impedance analysis; DXA, dual-energy X-ray absorptiometry; FFA, free fatty acids; HCIS; homocysteine; LDL-c, low density lipoprotein-cholesterol; MDA, malondialdehyde; oxLDL, oxidized low density lipoproteins; TC, total cholesterol; TG, tryglicerides.

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Written informed consent was obtained from all the volunteers before starting the intervention trial (www.clinicaltrials.gov; NCT01087086) and the study was approved by the Research Ethics Committee of the University of Navarra (ref. 065/2009).

2. SUBJECTS

A total of 109 volunteers with MetS symptoms were enrolled to participate in the study. Among them, a total of ninety-three Caucasian adults (52 men/ 41 women) were diagnosed with the MetS according to the International Diabetes Federation criteria (Grundy et al., 2005). Eighty-four of them completed the 8-weeks intervention, while sixty seven finished the whole study (Figure 17).
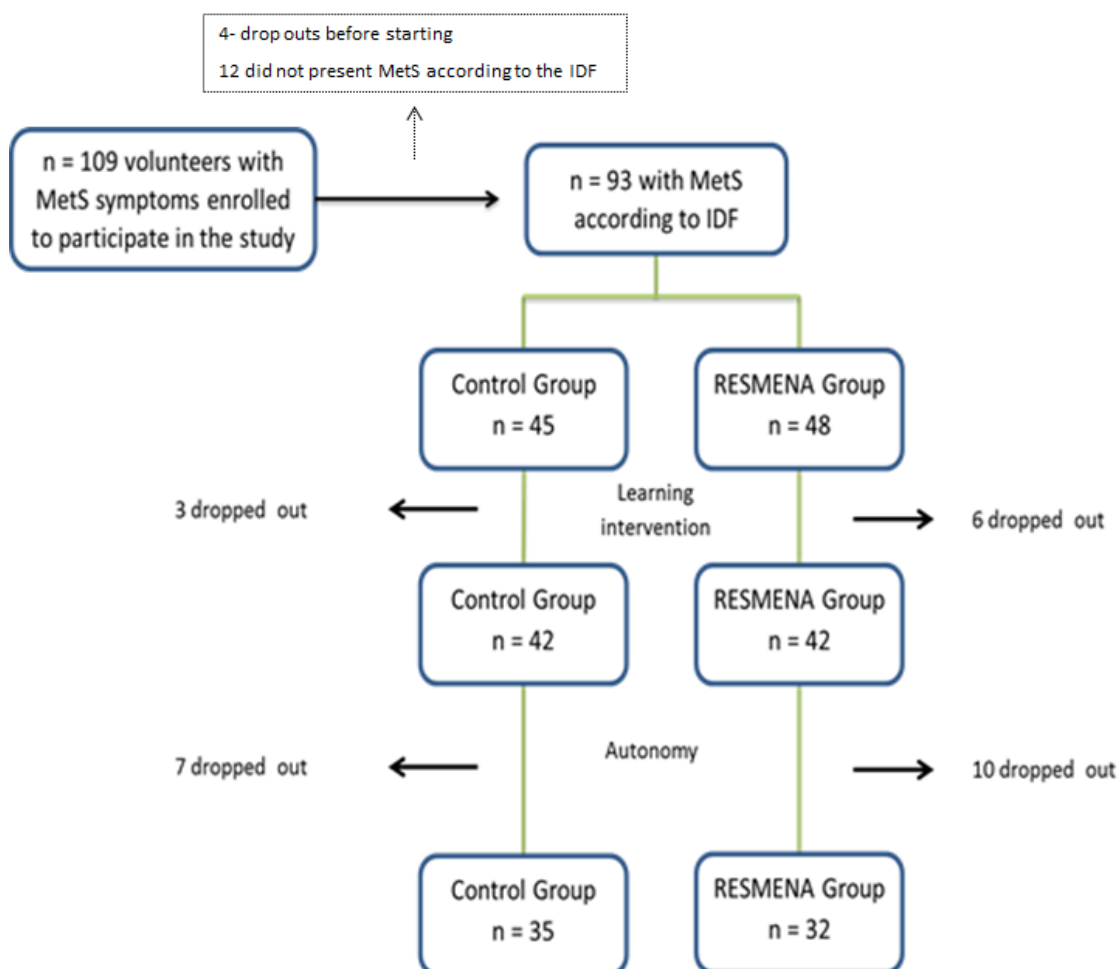
Figure 17. Flow diagram of the participants of the study. IDF, International Diabetes Association; MetS, metabolic syndrome.

Subjects presenting the following criteria were excluded (Perez-Cornago et al., 2014):

1. Subjects with difficulty for changing dietary habits.

2. Subjects with psychiatric or psychological disorders.

3. Subjects with eating disorders (bulimia; test of Edinburgh).

4. Subjects with weight instable for 3 months before the beginning of the study.

5. Subjects under any pharmacological treatment (except those drugs included in the IDF diagnostic criteria for the MetS).

6. Subjects with chronic diseases related to the metabolism of energy and nutrients (gastric ulcer, disorders of the digestive system, hyperthyroidism or hypothyroidism).

7. Subjects on special diets.

8. Subjects with food allergies or intolerances.

3. DIETARY STRATEGIES

Two energy-restricted diets (-30 % energy of the studied requirements) were prescribed and compared (Figure 18). The Control diet was based on the AHA guidelines (Grundy et al., 2005), including 3-5 meals per day, a macronutrient distribution of 55 % total energy value from CHO, 15 % from proteins and 30 % from lipids, a healthy fatty acid profile and a cholesterol content of less than 300 mg/day.

The RESMENA diet was characterized by a higher meal frequency, consisting of seven meals per day (including breakfast, lunch, dinner and two snacks in the morning and two snacks in the afternoon), and by a different macronutrient distribution, 40 % total energy value from CHO, 30 % from proteins and 30 % from lipids. Furthermore, this pattern tried to reinforce a MedDiet pattern, high *n*-3 polyunsaturated fatty acids and high natural antioxidant food consumption and promoted low glycaemic load CHO intake. It also maintained a healthy fatty acid profile and a cholesterol content of less than 300 mg/day as the Control diet (Zulet et al., 2011).

The RESMENA participants were prescribed a 7-day menu plan, while the Control group was prescribed a food exchange system plan. A 48-hour weighed food record was collected at the beginning and at the end of both the nutritional-learning and autonomous periods in order to assess the participants' adherence to the prescribed nutritional

patterns. The composition of the designed diets, as well as the different dietary records, were analysed using the DIAL software (Alce-Ingenieria, 2011).

	AHA	RESMENA
Energy (Kcal)	-30%	-30%
Meal Frequency (meals/day)	3-5	7
Carbohydrates (% E)	55%	40%
Proteins (% E)	15%	30%
Lipids (% E)	30%	30%

MedDiet pattern
High *n*-3 PUFAs
High natural antioxidant foods
Low glycemic load carbohydrates

Figure 18. Characteristics of the two dietary strategies compared in the study. % E, percentage of the total dietary energy; AHA, America Heart Association; MedDiet, Mediterranean diet; PUFAs, polyunsaturated fatty acids; RESMENA, MEtabolic Syndrome REduction in NAvarra-Spain.

4. CLINICAL AND BIOCHEMICAL ASSESSMENTS

4. 1. Anthropometric, Body Composition and Blood Pressure Measurements

Anthropometric and body composition measurements were taken in fasting conditions and with the volunteers in their underwear (Perez et al., 2005). Body weight was assessed to the nearest 0.1 kg using bioelectric impedance (TANITA SC-330; Tanita Corporation) equipment. BMI was calculated as the body weight divided by height squared (kg/m^2).

Waist and hip circumferences were measured using a commercial measuring tape following validated protocols. Total body fat mass, android fat mass, lean mass and fat-free mass were determined using DXA (Lunar iDXAe, software version 6.0; GE Healthcare). SBP, DBP and heart rate were assessed using a digital monitor (Medisana AG, 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 WHO criteria (Whitworth et al., 2004). Median blood pressure (MPB) was calculated as: [(DPB x 2) + SBP]/3 as advised elsewhere (Shapiro et al., 2010).

4. 2. Sample Collection

Blood samples (Figure 19) were taken at baseline and at the endpoint of each period in fasting conditions, as described elsewhere (Abete et al., 2008). In each visit, 3 serum tubes and 6 ethylenediaminetetraacetic acid (EDTA) tubes per volunteer were collected. The EDTA–plasma and serum samples as well as white blood cells (WBC) were separated from whole blood by centrifugation at 3,500 rpm, 5 ºC, 15 min (Model 5804R, Eppendorf, Germany), and were frozen immediately at -80 ºC until assay (WBC in buffy-coat).
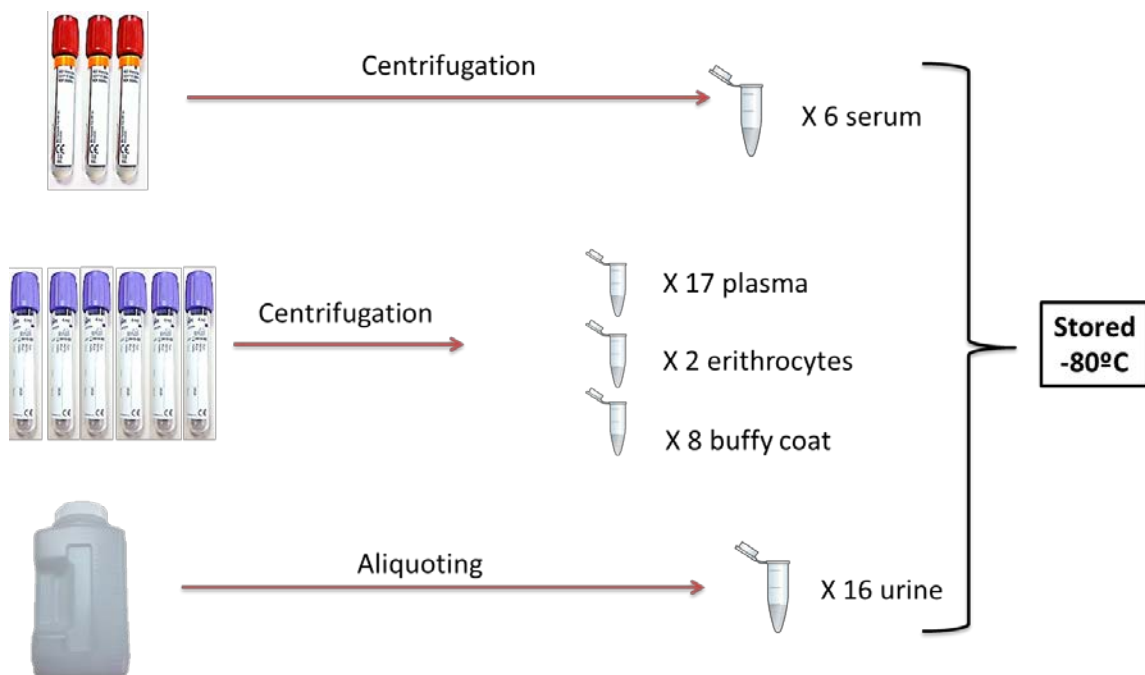
Figure 19. **Blood and urine samples collection, processing and storage.**

Twenty-four hour-urine samples for the day before to the corresponding visit, including the last micturition in the morning before coming, were collected at baseline and at the endpoint of each period. Samples were storage at -80 ºC (de la Iglesia et al., 2013a).

4. 3. Biochemical Measurements

Serum total cholesterol (TC), HDL-c, TG, free fatty acids (FFA), glucose, homocysteine, uric acid, total protein, creatinine, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations were measured using an autoanalyser Pentra C-200 (Horiba ABX) with specific kits (Lopez-Legarrea et al., 2014a).

LDL-c levels were calculated using the Friedewald formula (Friedewald et al., 1972): LDL-c = TC - HDL-c – TG/5.

Apolipoprotein A-I (Apo A-I) and apolipoprotein B (Apo B) values were measured with a specific kits (Tina-quant Apolipoprotein A-I ver.2 and Tina-quant Apolipoprotein B ver.2, Mannheim, Germany) using a Roche/Hitachi autoanalyser (Mod.904 Modular, Tokio, Japan).

Insulin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyser (Grifols SA, Barcelona, Spain). Insulin resistance was estimated using the homeostasis model assessment insulin resistance index (HOMA-IR), which was calculated using the following formula (Aller et al., 2011): HOMA-IR = (glucose (mmol/L) x insulin (μIU/mL))/22.5.

Estimated glomerular filtration rates (eGFRs) were calculated from serum creatinine values using the CKD-EPI (Chronic Kidney Disease-Epidemiology Collaboration) equation, which takes into account sex, age and race (Levey et al., 2009):

eGFRs = 141 x min $(Scr/k, 1)^{\alpha}$ x max$(Scr/k, 1)^{-1.209}$ x 0.993^{Age} x 1.018 [if female] x 1.159 [if black], where Scr is serum creatinine, k is 0.7 for females and 0.9 for males, α is - 0.329 for females and -0.411 for males, min indicates the minimum of Scr/k or 1, and max indicates the maximum of Scr/k or 1.

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 (de la Iglesia et al., 2013a).

Plasma oxLDL and MPO were measured using capture ELISA assay kits (Hermsdorff et al., 2012, Ibero-Baraibar et al., 2014) 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 (Nus et al., 2006). 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).

The quantitative measurement of irisin in human plasma samples was performed using a commercial ELISA kit directed against amino acids 31-143 of the FNDC5 protein according to the manufacturer's instructions (Irisin ELISA Kit EK-067-52; Phoenix Pharmaceuticals, INC, CA). 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). This test provided a range of detection of 0.066-1024 ng/mL and exhibited a coefficient of variation of 6-10 % inter and intra-assay. The samples were kept at -80 ºC and were analysed within the month following the end of the study (Lopez-Legarrea et al., 2014b).

4. 4. Methylation Measurements

Genomic DNA from WBC was extracted using the Master Pure kit (Epicenter, Madison, WI, USA), and its quality was assessed with PicoGreen double-stranded DNA (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.

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 (Mansego et al., 2013). The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, USA). Eight CpG sites of the PON1 gene that codes for the ARE enzyme were selected. We included CpG sites located in the transcriptional regulatory region (promoter, 5'-untranslated region and exon 1).

5. STATISTICAL ANALYSES

Statistical analyses were carried out using SPSS 15.1 software for Windows (SPSS Inc, Chicago, USA). Based upon previous studies (Katcher et al., 2008, Konig et al., 2008), the required sample size was calculated to detect a difference of 4.3 cm, with a variation of ± 6.8 cm between the dietary groups in the reduction of waist circumference with a $p < 0.05$ and a statistical power of 80 %. Mean values and standard errors (SE) or standard deviations (SD) are reported for the measured variables (Martinez-Gonzalez et al., 2010).

To compare the changes due to the treatment before and after each period a paired *t*-test was used. The between group analyses were performed applying an unpaired *t*-test or a multivariate analysis of variance (ANOVA) adjusted for sex and age or for baseline values of each variable, as appropriate.

In order to evaluate potential relationships and associations between variables, linear regression and Pearson correlation analyses were performed, including as co-variables those estimated necessary for adjusting the model (Natale et al., 2014).

Moreover, multiple testing correction (Benjamini–Hochberg) analyses were performed when appropriate. Globally, values of $p < 0.05$ were considered as statistically significant (Abbas et al., 2013).

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RESULTS

CHAPTER 1

Plasma irisin depletion under energy restriction is associated with

improvements in lipid profile in metabolic syndrome patients

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CHAPTER 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

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CHAPTER 3

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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Article

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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.

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		$P^{†}$
	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); $P^{†}$, 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.
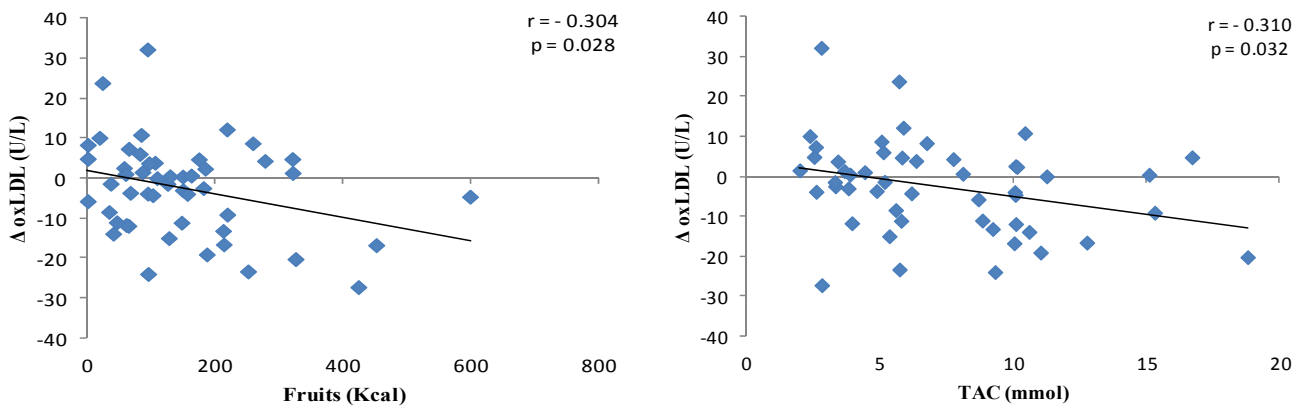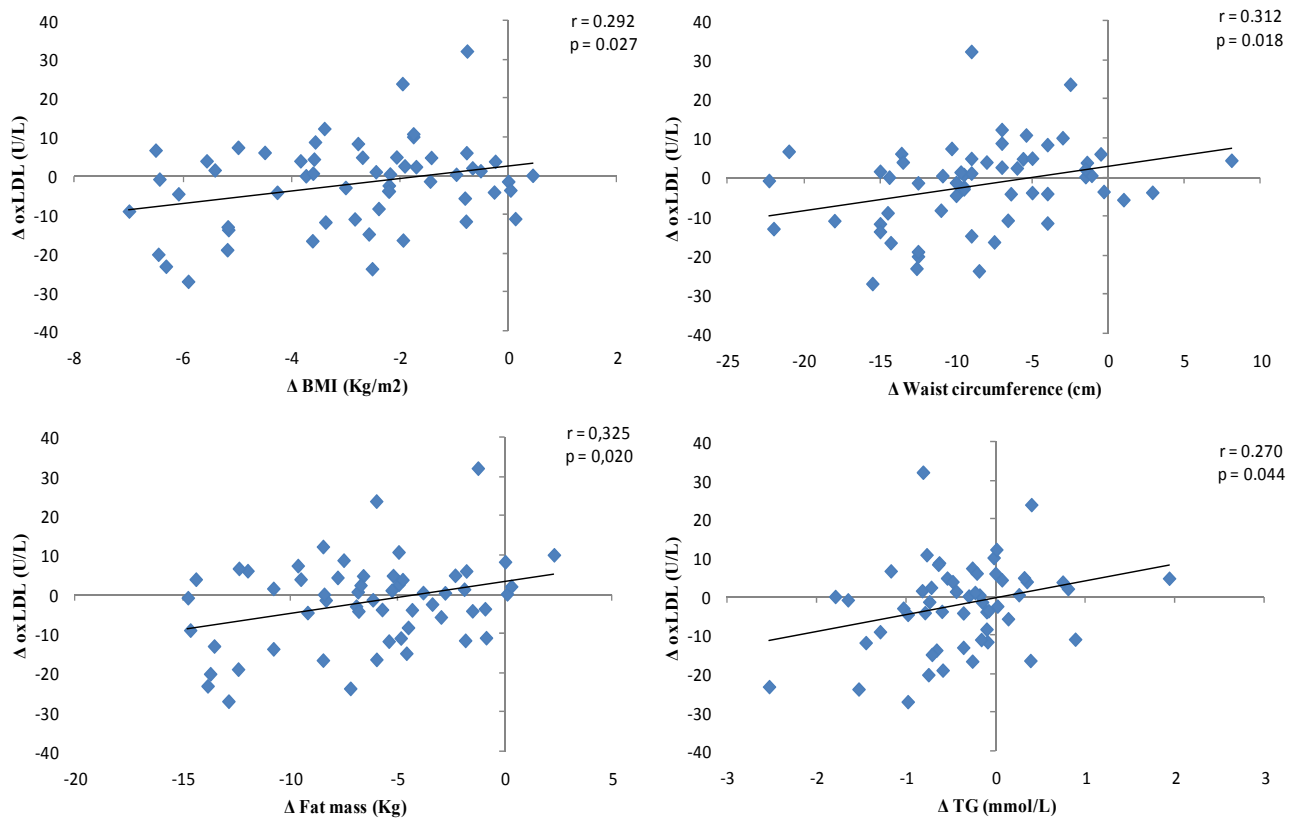

Figure 2. Correlations between changes on oxLDL and changes on adiposity parameters. Abbreviations: BMI, body mass index; TG, triglycerides.

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)
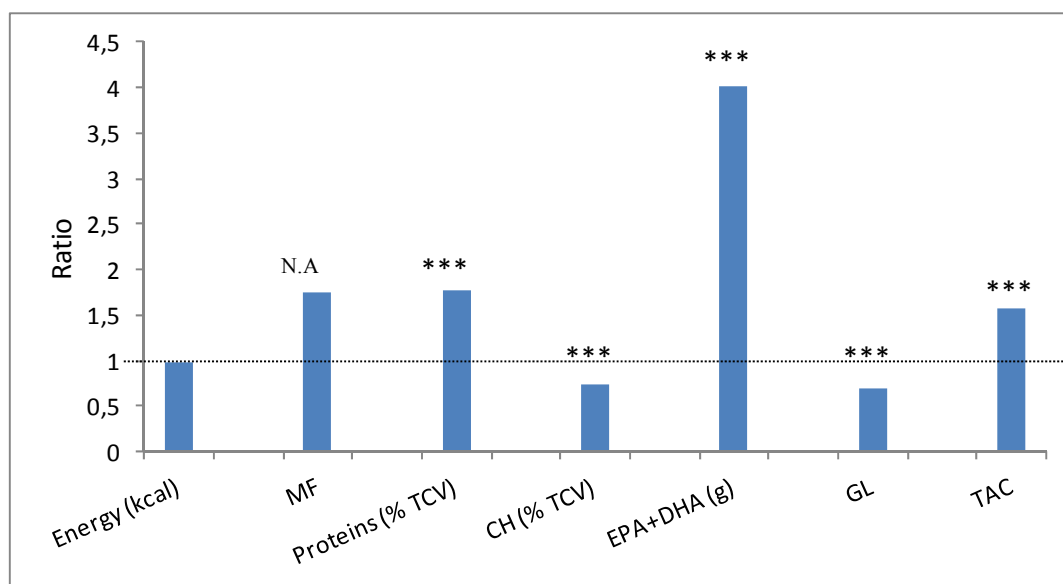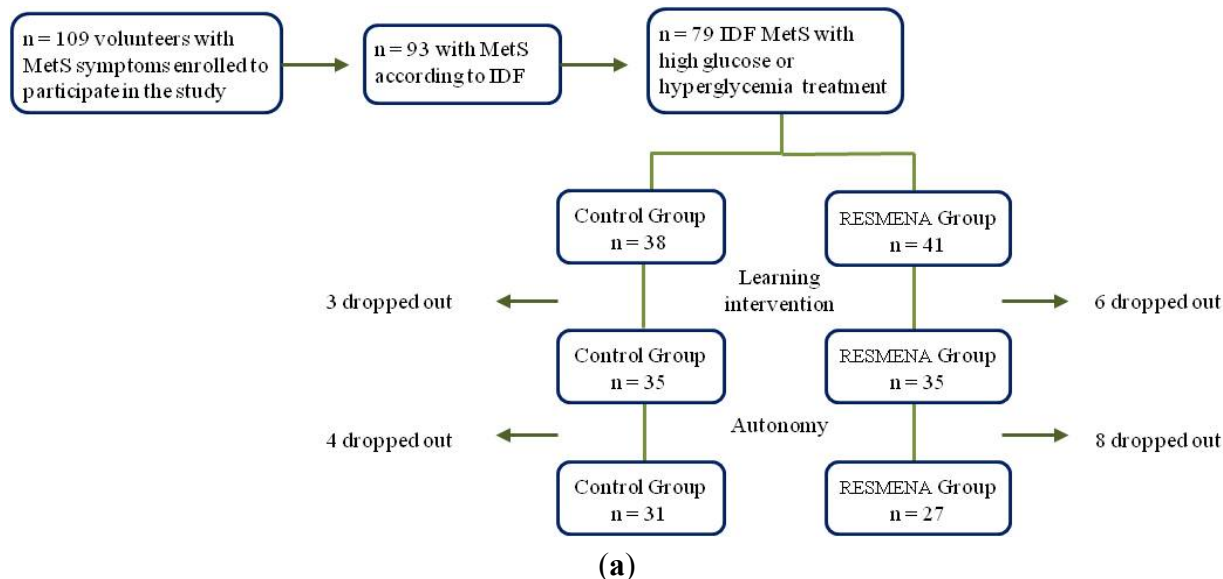
(b)

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/m²). 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.

Conflict of Interest

The authors declare no conflict of interest.

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CHAPTER 4

Arylesterase activity is associated with antioxidant intake and paraoxonase 1 (PON1) gene methylation in metabolic syndrome patients following an energy restricted diet

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Original article:

ARYLESTERASE ACTIVITY IS ASSOCIATED WITH ANTIOXIDANT INTAKE AND *PARAOXONASE-1* (*PON1*) GENE METHYLATION IN METABOLIC SYNDROME PATIENTS FOLLOWING AN ENERGY RESTRICTED DIET

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ABSTRACT

The arylesterase (ARE) activity linked to the *paraoxonase-1* (*PON1*) gene is known to protect lipoproteins from oxidation and provide defense against metabolic syndrome (MetS) and cardiovascular diseases. The epigenetic regulation of enzymatic activities is gaining importance nowadays. This research aimed to assess the potential relationships between the ARE activity with the methylation levels of the *PON1* gene transcriptional regulatory region, anthropometrics, biochemical markers and antioxidant dietary components. Forty-seven subjects (47 ± 10 y.o; BMI 36.2 ± 3.8 kg/m^2; 46.8 % female) with MetS features, who followed a six-month energy-restricted dietary weight-loss intervention, were included in this study (*www.clinicaltrials.gov; NCT01087086)*. Anthropometric, biochemical, enzymatic and dietary data were assessed using validated procedures. *PON1* transcriptional regulatory region methylation was analyzed by a microarray technical approach. Volunteers reduced ARE activity in parallel with body weight ($p = 0.005$), BMI ($p = 0.006$), total fat mass ($p = 0.020$), diastolic blood pressure ($p = 0.018$), mean blood pressure ($p = 0.022$) and triglycerides ($p = 0.014$). Methylation levels of some CpG sites of the *PON1* gene correlated negatively with ARE activity ($p < 0.05$). Interestingly, dietary vitamin C ($p = 0.001$), tocopherols ($p = 0.009$) and lycopene ($p = 0.038$) were positively associated with ARE activity and showed an inverse correlation ($p = 0.004$, $p = 0.029$ and $p = 0.021$, respectively) with the methylation of some selected CpG sites of the *PON1* gene. In conclusion, ARE activity decreased in parallel with MetS-related markers associated to the energy restriction, while dietary antioxidants might enhance the ARE activity by lowering the PON1 gene methylation in patients with MetS features.

Keywords: DNA methylation, ARE, PON1 gene, obesity, metabolic syndrome, energy restriction, antioxidants

INTRODUCTION

Paraoxonase-1 (PON1) is a calcium-dependent glycoprotein hepatically synthesized that belongs to the paraoxonase family (Precourt et al., 2011). This enzyme is related to high density lipoprotein-cholesterol (HDL-c) and has been described to protect lipoproteins, particularly low density lipoprotein-cholesterol (LDL-c), from oxidation (Kim et al., 2013; Kumar et al., 2013). Thus, some of the antiatherogenic properties of HDL-c are attributed to PON1 functions (Cohen et al., 2012). The precise mechanisms by which the PON1 acts remains unclear, but several *in vitro* activities have been attributed to this enzyme (Nus et al., 2008): paraoxonase (hydrolysis of organophosphates), lactonase (hydrolysis of lactones), and arylesterase or ARE (hydrolysis of aromatic carboxylic acid esters).

Obesity and metabolic syndrome (MetS) features are main risk factors for the development of atherosclerosis and cardiovascular diseases (CVD) onset (Garg et al., 2014). Due to the increasing prevalence of obese people, these accompanying diseases are considered a major concern for public health authorities and many scientific efforts are being carried out to detect, treat and prevent them. Hypocaloric diets are one of the most common treatments employed to combat MetS and related diseases (Straznicky et al., 2010). However, more investigation is needed to understand the PON1 activity role as part of these nutritional strategies. In this context, some research have been performed focusing on the relationship between the PON1 activity levels and the MetS/ obesity states (Ferretti et al., 2012; Koncsos et al., 2011; Kota et al., 2013; Tabur et al., 2010) while other authors have investigated the influence of some specific dietary factors on the activity levels of this enzyme (Canales et al., 2011; Jarvik et al., 2002; Vazquez-Velasco et al., 2011). But the studies carried out are scarce and reported controversial results.

Epigenetics is defined as heritable changes in gene expression that cannot be explained by changes in DNA sequence (Christensen et al., 2011). Among the different possible epigenetic modifications, DNA methylation is probably the most widely studied (Milagro et al., 2011). In this context, dietary factors, specially antioxidants, have become agents of strong interest in the field of epigenetics due to their prominent role as potent modulators of epigenome-regulated gene expression through regulation of DNA methylation (Bartels, 2007; Malireddy et al., 2012; Milagro et al., 2009).

Thus, within this scenario, the current study aimed to assess the potential relationships between the PON1 ARE activity and anthropometric and biochemical markers, dietary antioxidants intake, and the cytosine methylation levels of *PON1* gene transcriptional regulatory region, in volunteers with MetS symptoms after following an energy-restricted dietary program.

MATERIALS AND METHODS

Subjects and study protocol

The current analysis was an ancillary study conducted within the RESMENA (Metabolic Syndrome Reduction in Navarra) project, a randomized controlled trial (Zulet et al., 2011) where a subsample of 47 obese adults (47 ± 10 y.o; BMI 36.2 ± 3.8 kg/m^2; 46.8 % female) who presented MetS features was selected. The study lasted a total of 6 months divided in two sequential stages: an initial 8-week nutritional-learning intervention period, during which nutritional assessment was carried out for the participants every 15 days (Lopez-Legarrea et al., 2013) and a 4-month self-control period, during which the participants followed the previously acquired dietary habits (de la Iglesia et al., 2013).

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 Hel-

sinki. More details about the procedures and protocols have been previously reported (Zulet et al., 2011).

Anthropometric, body composition, blood pressure and dietary intake assessment

Anthropometric measurements were conducted in fasting conditions according to previously described procedures (Zulet et al., 2011). Body mass index (BMI) was calculated as the body weight divided by height squared (kg/m^2). Body composition analyses were carried out by Dual Energy X-ray Absorptiometry (DXA) following validated protocols as reported elsewhere (Zulet et al., 2011). Systolic (SBP) and diastolic (DBP) blood pressures were measured following standardized World Health Organization criteria (Whitworth et al., 2004). Mean blood pressure (MBP) was calculated as: [(DPB x 2) + SBP]/3 as advised elsewhere (Shapiro et al., 2010).

Information about dietary intake was collected using a 48 h weighed food record and analysed using the DIAL (Alce Ingeniería) software (http://www.alceingenieria.net/nutricion) as previously reported (Perez-Cornago et al., 2013).

Biochemical assessments

Venous blood samples were drawn after a 12 h overnight fast by venipuncture. The EDTA–plasma and serum samples as well as WBC were separated from whole blood by centrifugation at 3,500 rpm, 5 °C, 15 min (Model 5804R, Eppendorf, Germany), and were frozen immediately at -80 °C until assay (WBC in buffy-coat).

Plasma concentrations of triglycerides (TG), total cholesterol (TC), HDL-c (Wako Chemicals, GmbH, Nuiss, Germany) and glucose (Horiba ABX Diagnostics, Montpellier, France) were measured by specific colorimetric assays, using an automated analyzer system Pentra C-200 (HORIBA ABX, Madrid, Spain). LDL-c levels were calculated using the Friedewald formula: LDL-c = TC - HDL-c – TG/5 (Friedewald et al., 1972). Apolipoprotein B (Apo B) was measured with a specific kit (Tina-quant Apolipoprotein B ver.2, Mannheim, Germany) using a Model 904 Modular Roche/ Hitachi autoanalyser (Roche Diagnostics, Tokio, Japan).

Plasma concentrations of ARE activity were measured with simulated body fluid (SBF) as buffer and phenylacetate as substrate at pH 7.34–7.4 and 37 °C, as published elsewhere (Nus et al., 2006). 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 was defined as the mmol phenol formed from phenyl acetate per min.

DNA isolation and DNA methylation study

Genomic DNA from 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. 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 (Mansego et al., 2013). The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina, USA). Eight Cytosine-phosphate-guanine (CpG) sites of the *PON1* gene that codes for the PON1 enzyme were selected. CpG sites located in the transcriptional regulatory region (promoter, 5'-untranslated region and exon 1) were included (Figure 1). Refer-

ence names and characteristics of the selected CpG sites are shown in Table 1.

Statistical analyses

Results are shown as mean value ± standard deviation. Variable distribution was determined by the Shapiro-Wilk test and no normal variables (glucose, HDL-c, LDL-c, TG, ARE and ARE/HDL-c) were logarithmically transformed for statistical purposes. Differences between the beginning and the end of the complete study were analyzed by paired Student *t*-test. Pearson correlations adjusted for age and sex were fitted to evaluate the potential associations of PON1 ARE activity and anthropometric, body composition, blood pressure and biochemical variables, and also to assess the relationships between *PON1* transcriptional regulatory region methylation and ARE activity and specific dietary factors. Pearson correlations adjusted for sex and age were also used to study the association between the ARE activity

and the dietary factors intake at the end of the study. Moreover, multiple testing correction (Benjamini–Hochberg) analyses were performed when appropriate. The SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) for Windows XP (Microsoft, USA) was used for statistical analyses. Globally, p < 0.050 was considered as statistically significant.

RESULTS

Information about selected anthropometric and biochemical measurements was recorded at baseline and at day 180 (Table 2). After the 6-month-long trial, participants presented significantly lower (p < 0.001) values concerning anthropometric and body composition variables, such as body weight, BMI, waist circumference, WHR, total fat mass and android fat mass. Volunteers also significantly reduced the SBP, DBP and MBP values as well as the plasma glucose
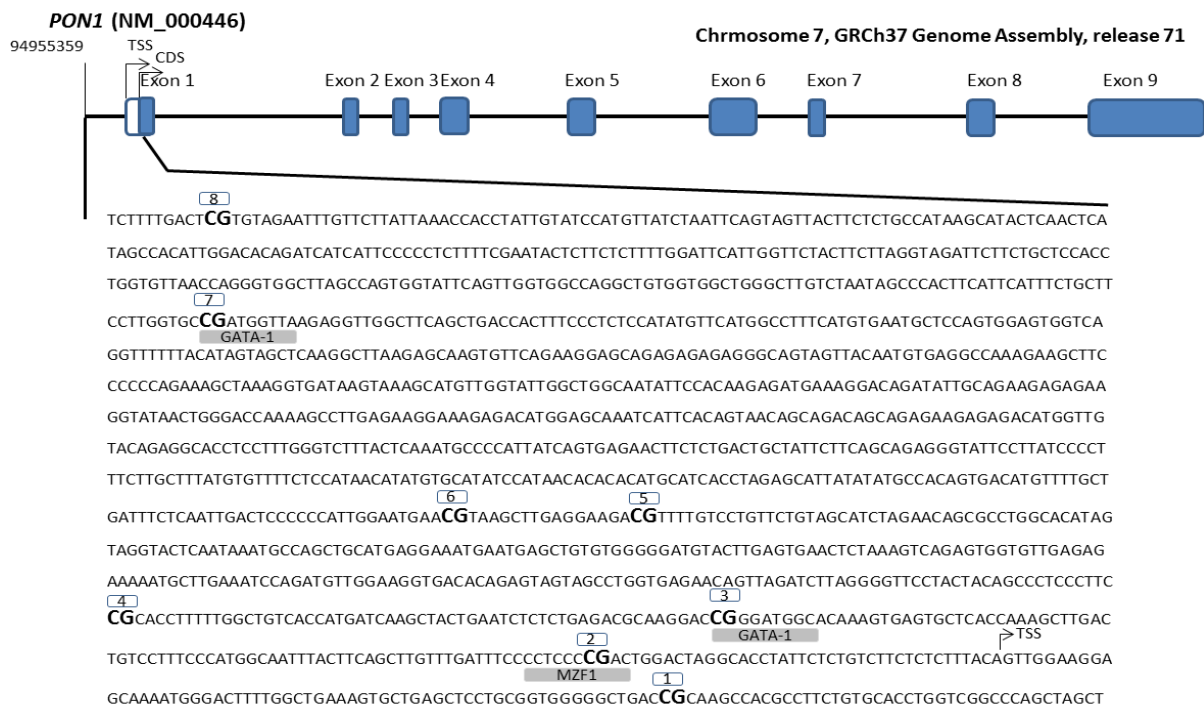
Figure 1: Genomic localization and nucleotide sequence of 8 CpG sites covered by the Illumina probe for the study of DNA methylation levels of *PON1* promoter (from - 1330 to +104 pb). Transcription Start Site (TSS).

Table 1: Information of the selected CpG sites for each *PON1* gene

CpG ID[1]	Illumina ID	CHR position[2]	Reference[3]
1	cg17330251	7: 94953956	c.+63
2	cg01874867	7: 94954059	c.-41
3	cg20119798	7: 94954144	c.-126
4	cg04871131	7: 94954202	c.-184
5	cg23055772	7: 94954438	c.-420
6	cg07809369	7: 94954455	c.-437
7	cg17020263	7: 94955053	c.-1035
8	cg15887283	7: 94955348	c.-1330

1: Studied CpG identifier

2: Genome assembly: GRCh37, Ensemble release 73.37

3: It begins in the first nucleotide of exon 1

Table 2: Changes in anthropometric, body composition, blood pressure, biochemical parameters and arylesterase (ARE) activity after 6 month-study (N = 47)

Variable	Baseline	Day 180	p
Body weight (kg)	103.5±18.7	94.8±20.1	< 0.001
BMI (kg/m^2)	36.0±4.1	32.9±4.6	< 0.001
Waist circumference (cm)	112.7±12.9	104.7±14.8	< 0.001
WHR	0.97±0.10	0.94±0.10	< 0.001
Total fat mass (kg)	43.0±9.6	36.0±10.8	< 0.001
Android fat mass (kg)	4.75±1.37	3.75±1.31	< 0.001
SBP (mmHg)	151.2±17.2	134.4±13.2	< 0.001
DBP (mmHg)	85.6±8.9	77.7±10.5	< 0.001
MBP (mmHg)	107.4±11.1	96.6±10.5	< 0.001
Glucose[#] (mmol/L)	6.77±1.90	6.16±1.41	< 0.001
TC (mmol/L)	5.61±1.34	5.50±1.14	0.584
HDL-c[#] (mmol/L)	1.13±0.28	1.22±0.32	0.011
LDL-c[#] (mmol/L)	3.50±1.19	4.27±0.98	< 0.001
ApoB (g/L)	0.94±0.27	0.88±0.21	<0.001
TG[#] (mmol/L)	2.12±1.12	1.75±1.19	0.001*
ARE[#] (IU/L)	483.4±241.0	461.5±217.1	0.222
ARE/HDL-c[#] (IU/mmol)	436.2±189.1	393.1±174.4	0.003

Data are mean ± SD. p values from Student t test. [#]Log transformed variables. BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MDP, mean blood pressure; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triglycerides; Apo B, apolipoprotein B; ARE, arylesterase.

(p < 0.001), Apo B (p < 0.001), TG (p = 0.001). On the other hand, LDL-c (p < 0.001) and HDL-c (p = 0.011) values were significantly higher at the end of the study.

Plasma TC and ARE activity tended to be reduced, although did not reach significance, while the ARE/HDL-c ratio was significantly decreased (p = 0.003).

Pearson correlation analyses adjusted for sex and age were performed to assess the possible associations between the PON1 ARE activity and the anthropometric, body composition, blood pressure and biochemical variables. The PON1 ARE activity levels significantly correlated with TC (r = 0.362, p = 0.016), HDL-c (r = 0.346, p = 0.021) and Apo B (r = 0.446, p = 0.002) concentrations, at baseline. Moreover, significant positive associations were found between changes in ARE activity and variation of body weight, BMI, total fat mass, DBP, MBP and plasma TG levels (Table 3). The association with waist circumference and SBP resulted in a trend towards significance. Some of these relationships should be considered as explorative since after applying a multiple comparison correction, the statistical significance was toned down. Interestingly, in the case of BMI variation, which is one of the main variables, remained statistically associated with the change of PON1 ARE activity.

At the end of the intervention, the PON1 ARE activity measurements showed positive correlations with the intake of the selected antioxidants vitamin C (p = 0.001), total tocopherols (p = 0.009) and lycopene (p = 0.038) recorded by the 48h weighed food record (Figure 2). Interestingly, when assessing the association between the selected CpG sites methylation of the *PON1* gene and the related ARE activity at baseline (Table 4), a significant inverse correlation was found concerning the *PON1* CpG 1, CpG 2, CpG 3 and CpG 4 sites with the enzymatic ARE activity. Moreover, these associations remained significant after ap-

plying the Benjamini-Hochberg test for multiple comparisons.

Table 3: Pearson correlation analyses between arylesterase (ARE) activity variation and changes in selected anthropometric, body composition, blood pressure and biochemical variables.

Variable	r	p
Δ **Body weight (kg)**	0.473	0.005
Δ **BMI (kg/m²)**	0.459	0.006*
Δ **Waist circumference (cm)**	0.336	0.052
Δ **WHR**	0.131	0.459
Δ **Total fat mass (kg)**	0.396	0.020
Δ **Android fat mass (kg)**	0.020	0.910
Δ **SBP (mmHg)**	0.323	0.062
Δ **DBP (mmHg)**	0.402	0.018
Δ **MBP (mmHg)**	0.392	0.022
Δ **Glucose# (mmol/L)**	0.123	0.487
Δ **TC (mmol/L)**	0.216	0.221
Δ **HDL-c# (mmol/L)**	0.201	0.254
Δ **LDL-c# (mmol/L)**	0.000	1.000
Δ **Apo B (g/L)**	0.200	0.249
Δ **TG# (mmol/L)**	0.419	0.014

r and p values from Pearson correlations adjusted for sex and age. #Log transformed variables. BMI, body mass index; WHR, waist to hip ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; Apo B, apolipoprotein B; TG, triacilglycerides. *P-value < 0.05 after correcting for Benjamini–Hochberg multiple comparisons.

Table 4: Pearson correlation analyses between arylesterase (ARE) activity and the selected CpG sites methylation (%) of PON1 gene at baseline

CpG site	r	p
CpG 1	-0.490	0.003*
CpG 2	-0.606	< 0.001*
CpG 3	-0.503	0.002*
CpG 4	-0.434	0.009*
CpG 5	-0.013	0.939
CpG 6	-0.326	0.056
CpG 7	-0.235	0.174
CpG 8	-0.159	0.361

*P-value < 0.05 after correcting for Benjamini–Hochberg multiple comparisons.
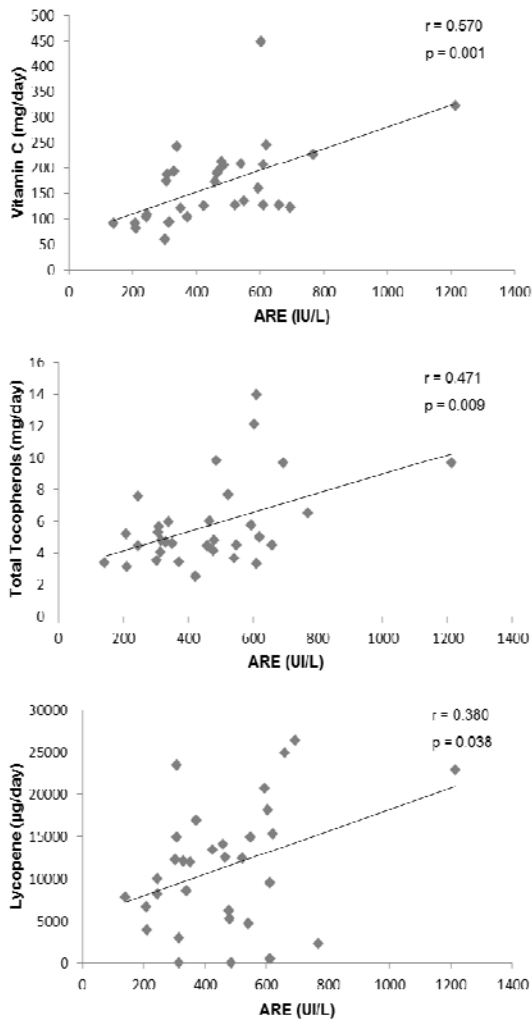
Figure 2: Associations between antioxidant dietary components intake and arylesterase (ARE) activity

Figure 3: Statistically significant correlations between antioxidant dietary components intake and methylation (%) of *PON1* gene CpG sites at baseline

Finally, the analysis of the possible relationships between the antioxidant dietary intake and the percentage of methylation of the different *PON1* gene CpG sites, revealed a correlation between the selected antioxidants: vitamin C, total tocopherols and lycopene and the selected CpG sites at baseline (Figure 3).

DISCUSSION

The assessment of processes associated to weight loss may contribute to a better understanding of the metabolic and genetic machinery, which will benefit the implementation of personalized dietary treatments.

Indeed, as reported in other studies concerning hypocaloric diets (Straznicky et al., 2010), the energy-restricted intervention proved to be effective in improving MetS abnormalities except for LDL-c, which was higher at the end of the study. This outcome agrees with Clifton et al. who described that in some cases, LDL-c may increase despite weight loss (Clifton et al., 2007). Nevertheless, Apo B, which has been considered a better predictor of CVD onset than any other lipid measurement (McQueen et al., 2008), evidenced statistically significant decreased levels.

Concerning PON1 ARE activity, it seems logical the positive correlation found with the HDL-c levels at baseline since it is an HDL-c related enzyme, as other studies have previously reported (Aksoy et al., 2009; Mascarenhas-Melo et al., 2013; Tabur et al., 2010). The positive correlation with LDL-c and TC also agree with previous studies (Aksoy et al., 2009; Younis et al., 2013).

The PON1 ARE activity was reduced in parallel to some anthropometric (body weight, BMI), body composition (total fat mass), blood pressure and biochemical biomarkers (TG) related to the MetS. Although there is scarce research about the relationship of PON1 activity and obesity or MetS, the studies carried out point to relate obese/MetS status to lower levels of ARE activity (Ferretti et al., 2012; Kota et al., 2013). Nevertheless, when it comes to clinical trials, in accordance to our results, a significant decrease of ARE activity and serum PON1 protein levels after a weight loss intervention, as well as a significant association between reduced PON1 and reductions in body fat has been reported (Rector et al., 2007). Similarly, it has been shown that significant depletions of all metabolic outcomes in addition to a significant reduction of PON1 activity correlated with BMI reduction, after a low calorie diet intervention (Kotani et al., 2009). In this last study, a significant association of PON1 activity changes and LDL-c depletion was also observed; however, no significant relationships were found in the present experimental trial. On the other hand, to our knowledge, this is the first research that has found an association of ARE activity and TG changes after a dietary intervention. With regards to the significant association of PON1 activity depletion and reduction of blood pressure levels, it has been reported significantly higher ARE levels in hypertensive than in normotensive children (Akis et al., 2009), while in other studies carried out in different adult populations no association was found (Tabur et al., 2010; Usta et

al., 2011). However, the studies mentioned above were cross-sectional studies, while the present work is a clinical intervention. Taking into account the previous issues and the lack of research in the area, the findings observed in the present study may indicate that reductions on PON1 general activity might be indicative of the improvement in the overall MetS/obese related features, in patients with MetS manifestations under an energy-restricted programme.

The activity of PON1 is under genetic and environmental regulation (Jarvik et al., 2002). Within the environmental factors that may alter PON1 activity, dietary antioxidants are of major importance, since PON1 is an oxidative stress related enzyme (de la Iglesia et al., 2014; Kheir-Eldin et al., 2008). Among the different antioxidants that can be incorporated in the diet, the antioxidant capacity of vitamins C and E and lycopene is well-established (Chen et al., 2013; Mustacich et al., 2007; Story et al., 2010). The positive relationships between the ARE activity and the selected antioxidant components reported in the present work may be explained by the capacity of the dietary antioxidants to scavenge free-oxygen radical products that may depress PON1 activity, as previous studies have suggested (Jarvik et al., 2002; Tsakiris et al., 2009).

DNA methylation is one of the major epigenetic mechanisms considered to regulate gene expression, together with histone modifications and noncoding RNA activity (Portela et al., 2010). Methylation of the CpG-rich region (CpG island) overlapping a gene's promoter is a generally accepted mechanism for silencing expression (Vanderkraats et al., 2013). Our findings confirm this outcome since four CpG sites and the average methylation of all the CpG sites studied inversely correlated with the ARE activity.

About the possible mechanisms by which the dietary antioxidants exert their effects, modulation of gene expression through regulation of DNA methylation is

one of the main studied mechanisms (Bartels, 2007; Malireddy et al., 2012; Milagro et al., 2009). In the present work, inverse correlations between dietary vitamin C, total tocopherols and lycopene with different CpG sites methylation was observed, which may influence in the ARE activity as other studies have suggested (Jarvik et al., 2002; Tsakiris et al., 2009).

This work has the limitations that we have not determined *PON1* methylation levels at the end of the intervention and we have not measured expression levels. However, we found that CpG2 and CpG7 sites match a core-binding consensus motif for the GATA binding protein 1 (globin transcription factor 1) and CpG2 site matches for myeloid zinc finger 1 (MZF1), which are known transcriptional regulators for several pathways (Gaboli et al., 2001; Zheng et al., 2010). Furthermore, some statistical associations concerning changes in PON1 ARE activity and anthropometric and biochemical measurements variations were lost after the application of a multiple comparison correction test.

In conclusion, the present study reports that ARE activity decreased in parallel with MetS-related markers, while dietary antioxidants intake may enhance the ARE activity by lowering the *PON1* gene methylation in patients with MetS features, under an energy-restricted intervention. These results suggest that further investigations into PON1 activity might be a good target to better understand the metabolic effects of the dietary intervention studies, in which epigenetic processes may be involved.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DISCUSSION

1. JUSTIFICATION OF THE STUDY

As the prevalence of the MetS is reaching epidemic rates (Oresic et al., 2014), and the maintenance of acquired healthy dietary habits is still a pending subject for clinical nutrition (Abete et al., 2011), there is a need to find effective and easy-to-follow dietary strategies to combat excessive body weight and the MetS comorbidities.

There are several dietary factors/strategies that have been reported to show positive effects on dietary adherence and treatment of the MetS. Among them we can find the MeBDiet pattern (Grosso et al., 2013, Mayneris-Perxachs et al., 2014), a high *n*-3 fatty acids diets (Fan et al., 2013, Puchau et al., 2010, Robinson et al., 2013), an increased dietary TAC consumption (Bahadoran et al., 2012, Puchau et al., 2010) high meal frequency patterns (Alves et al., 2014, Jaaskelainen et al., 2013, Zizza, 2014), low GI diets (Melanson et al., 2012, Misra et al., 2011) or moderate increases of protein intake (Petzke et al., 2014, Westerterp-Plantenga et al., 2012).

Moreover, it is usual to find people highly motivated during the first few months after having begun to follow a hypocaloric diet, especially when they are in hands of nutritionists and health professionals, who frequently assess their progress (Dubnov-Raz et al., 2010). Difficulties appear when they stop attending the nutritionist and have to apply on their own the acquired dietary habits (Ebbeling et al., 2012). Therefore, it is important to find a dietetic plan reliable and efficient when followed by volunteers without a continuous monitoring.

For all of this, the present study hypothesized that considering all these demonstrated-efficient dietary patterns integrated within a unique dietetic plan, would result in a good strategy to improve the MetS, being feasible to follow-up over the 6-months whole study (2-months of nutritional learning period followed by 4-months of autonomy).

Taking into account that a diet based on the AHA guidelines (Krauss et al., 2000), a well-recognized healthy dietary pattern, was used as control, the stake of the present work was reasonably high.

2. MAIN DIFFERENCES BETWEEN THE RESMENA DIET vs AHA GUIDELINES-BASED DIET

2. 1. Effects on Body Composition after the Period of Autonomy (from day 60 to day 180)

Among the different anthropometric measurements used to analyse the state of health of the population, it has been demonstrated that central obesity is of key importance since it is associated with increased risks of diabetes mellitus, cardiovascular disease (CVD) hypertension, (Goh et al., 2014, Khalili et al., 2012) and MetS manifestations in general (Rosety-Rodriguez et al., 2013).

Lately, there is a debate about which abdominal obesity measurement is most appropriate in order to prevent metabolic disorder risks. In this context, the last scientific statement about the MetS definition (Alberti et al., 2009) considers the waist circumference as a main preliminary screening tool, but not an obligatory component to define the MetS, which is in accordance with other scientific opinions (Lee et al., 2012). Another recent study showed that waist circumference and WHR were useful measurements to predict the presence of MetS (Ferreira-Hermosillo et al., 2014). Moreover, the BMI not always has been well considered as a health risk determinant (Allison et al., 2002). However, a recent study stated the opposite, considering the BMI a predictive measurement as strong as waist circumference, WHR or fat mass percentage assessed by bioimpedance (Mooney et al., 2013). Also, the index of central obesity (ICO), defined as the ratio of waist circumference and height, was proposed as a better parameter to define central obesity among different ethnicities (Parikh et al., 2012).

In the present research, under the same energy restriction (- 30% total energy value), the RESMENA diet showed more effectiveness to maintain/improve some anthropometric measurements than the diet based on the AHA guidelines during a 4 month period of autonomy (chapter 1). The positive effects of the RESMENA diet were specifically highlighted by the significant loss of body weight at the expense of android fat mass and reduction of waist circumference, WHR and BMI, altogether, while these parameters remained unaltered in the volunteers under the AHA diet. These results are consistent with those of other studies where diets moderately high in protein at the expense of carbohydrates, were seen to enhance weight-loss and weight-loss maintenance (Larsen et al., 2010, Petzke et al., 2014, Stocks et al., 2013). The increment of diet-induced

thermogenesis (Bray et al., 2012) and the increase in satiety (Westerterp-Plantenga et al., 2009) are two mechanisms proposed to explain these beneficial effects.

2. 2. Effects on Biochemical Variables after the Period of Autonomy (from day 60 to day 180)

In treating the MetS features, it is not only important to improve the anthropometric measurements, but also the plasma biochemical determinations representative of the metabolism status. In this context, transaminases, mainly ALT, are markers of hepatocyte injury that have been reported to show a correlation with insulin resistance and later development of diabetes, liver lipid content and histological features of non-alcoholic fatty liver disease, which is increasingly being regarded as the main hepatic manifestation of the MetS (Hamaguchi et al., 2005, Monteiro et al., 2014). ALT transaminase concentration has also been reported to be correlated with the levels of C-reactive protein, a marker of low-grade inflammation associated with the MetS (Kerner et al., 2005). Dietary weight loss has been reported to be associated with a depletion of these liver enzymes (Straznicky et al., 2012), but irrespective of the type of diet (Rodriguez-Hernandez et al., 2011). However, in the present study (chapter 1), the volunteers following the RESMENA diet, in addition to losing more weight, they decreased the levels of both ALT and AST transaminases, while AST concentrations were significantly increased in the AHA guidelines-based diet group.

Although uric acid has been proposed to be able to function as an antioxidant quenching reactive species (Kamogawa et al., 2014, Yu et al., 2013), some studies have reported an association between increased levels of this purine end-product and gout and uric acid kidney stones (Kamogawa et al., 2014) and, more importantly, with adverse effects in obesity (Yin et al., 2014), diabetes (Zhang et al., 2014) hypertension (Bombelli et al., 2014), CVD (Jeemon et al., 2012), fatty liver (Sertoglu et al., 2014) and with the prevalence of the MetS in general (Viazzi et al., 2014). Based on the results of the present study (chapter 1), the RESMENA diet can be considered as a better option for patients with high uric acid concentrations than the diet based on the AHA guidelines, as uric acid levels were significantly increased in the AHA guidelines based diet group, while they remained almost unchanged in the RESMENA group, during the 4-months of autonomy.

Since insulin resistance has been proposed to be related to the development of the MetS (Samson et al., 2014), one of the main aims of the dietary treatment of the MetS is the amelioration of related markers such as serum glucose concentrations and HOMA-IR. In

the present study (chapter 1), in those participants following the AHA guidelines-based diet, serum glucose values significantly increased and HOMA-IR also rose, while they remained unaltered in the RESMENA group. This finding could be explained by the fact that central obesity usually precedes insulin resistance, being a risk factor for the development of type 2 diabetes (Roberts et al., 2014, Shao et al., 2014), and the RESMENA group was the only dietary group that exhibited a significant decrease in android fat mass. Furthermore, these results are in accordance with those from other investigations that have shown a positive role of low carbohydrates/low GL diets in preventing insulin resistance complications (Abete et al., 2010).

2. 3. Effects on Oxidative Stress Markers and Hyperglycaemia after the Whole Study (from day 0 to day 180)

Taking into account the importance of hyperglycaemia and related insulin resistance and type 2 diabetes in the MetS development (Roberts et al., 2014, Samson et al., 2014, Shao et al., 2014), and considering the better results obtained concerning the RESMENA diet on glucose metabolism as shown during the autonomy period, we sought to deep in the study of the potential beneficial effects of this new nutritional pattern on a hyperglycaemic population suffering MetS (chapter 2). Thus, among the whole sample of volunteers suffering MetS enrolled in the study, we selected those that had hyperglycaemia or type 2 diabetes previously diagnosed and compared the effects of the two different diets.

Regarding the android fat mass and related waist circumference measurements, the RESMENA diet demonstrated more benefits than the AHA guidelines-based diet after the 6-month long study. This outcome should be taken into account in future studies since central obesity is considered to be the main cause of insulin resistance and type 2 diabetes (Radzeviciene et al., 2013) and it is associated with increased risks of hypertension, CVD and MetS manifestations in general (Goh et al., 2014, Khalili et al., 2012, Rosety-Rodriguez et al., 2013).

Furthermore, oxidative stress is a potential biochemical mechanism involved in the pathogenesis of MetS and diabetes (Jialal et al., 2012, Kaneto et al., 2012, Matsuda et al., 2013). Therefore, the study of oxidative stress-related markers on people suffering MetS and/or diabetes is important to be approached in their treatments.

In this context, ARE activity, one of the three functions of the PON1 enzyme, has been shown to protect LDL-c and HDL-c against oxidation (Kim et al., 2013, Kumar et al., 2013). Moreover, in patients diagnosed of type 2 diabetes, low levels of PON1 ARE are directly associated with development of related complications (Nair et al., 2011). Studies focusing on the effect of the diet on the ARE activity are scarce, but it has been reported that flavonoids, fish oil, algae, dates and pomegranate-rich based diets are positively associated with PON1 ARE activity (Ghorbanihaghjo et al., 2012, Sanchez-Muniz, 2012, Schultz Moreira et al., 2014, Takaeidi et al., 2014), some of them carried out specifically in diabetic populations (Lixandru et al., 2010, Rock et al., 2008). In the present study, volunteers of the AHA guidelines-based diet decreased ARE:HDL-c and ARE:Apo A-I ratio values, while they remained almost unchanged in the RESMENA group. Therefore, the RESMENA diet apparently showed a specific protection effect against PON1 ARE activity depletion.

Oxidation of LDL-c is estimated as an important cardiovascular risk factor since it lets to foam cell formation induction, alongside propagation of atherosclerosis (Farooqui, 2013). Moreover, it has been reported that high levels of oxLDL are associated with the MetS in general (Tumova et al., 2013) and with obesity (Njajou et al., 2009), dyslipidaemia (Camont et al., 2013), CVD (Holvoet et al., 2008) and type 2 diabetes mellitus (Parfentyeva et al., 2014, Tousoulis et al., 2013). Our results evidenced that between both dietary patterns, RESMENA was significantly more effective on reducing oxLDL, oxLDL:LDL-c, and oxLDL:Apo B and oxLDL:HDL-c than the AHA guidelines-based diet. These results agree with previous studies, where an inverse relationship between high TAC dietary patterns and MetS related-oxidative stress was established (Puchau et al., 2010).

Globally, the additional benefits shown by the RESMENA diet should be taken into account in future nutritional intervention research, especially as a good antioxidant dietary treatment for people suffering MetS with hyperglycaemia and as an efficient dietary pattern to be followed over time during self-control.

3. CONJOINT ANALYSES IN SUBJECTS BELONGING TO BOTH NUTRITIONAL INTERVENTIONS

3. 1. Relationship between Irisin and Lipid Profile Variables after the Nutritional Learning Period (from day 0 to day 60)

The recently discovered myokine, irisin, has received much attention because it has been involved in the regulation of human energy metabolism (Lopez-Legarrea et al., 2014, Polyzos et al., 2013, Swick et al., 2013). Therefore, it can be interesting to study if irisin could also be involved in fat metabolism homeostasis or lipid disorders (chapter 3). Thus, the main objective was to evaluate the potential relationships between changes in atherogenic markers and the variation in irisin levels in patients suffering MetS after a weight-loss programme.

Associations between this myokine levels and plasma cholesterol have been previously described in other studies, but there is no general agreement among investigations concerning obese subjects and weight changes. While some researchers showed a positive correlation between this myokine and TC (Liu et al., 2013), others reported a negative association (Huh et al., 2012). Moreover, in another study a positive correlation between irisin and HDL-c was observed, but not with TC or LDL-c (Wen et al., 2013). Furthermore, to our knowledge, this is the first report that shows a positive relationship between irisin and the atherogenic index TC/HDL-c or Apo B levels. Additionally, it should be mentioned that all the above-cited studies were cross-sectional studies, while our findings belong to a weight-loss intervention trial where an association between irisin changes and TC, LDL-c, TC/HDL-c and Apo B variations is reported. Although the exact mechanisms of irisin action remain unclear, these findings suggest that irisin may participate in the regulation of lipid metabolism. In this context, it can be speculated that given irisin's putative role as a "metabolism-activator", changes in irisin concentration might reflect a response to metabolic (or atherogenic) burdens. This outcome might therefore explain our observation of a positive correlation between irisin and atherogenic factors such as total cholesterol and Apo B. Moreover, a resistance phenomenon could not be excluded. Indeed, irisin could follow a similar pattern as leptin or insulin, which are elevated in obese subjects and reduced after subjects undergo a hypocaloric diet as other authors have also suggested (Cordero et al., 2011, Hee Park et al., 2013, Moreno-Navarrete et al., 2013).

3. 2. Effects of Fibre after the Period of Autonomy (from day 60 to day 180)

Several health benefits of dietary fibre have been described, including the prevention and mitigation of type 2 diabetes mellitus, CVD and colon cancer by reducing the risk of hyperlipidaemia, hypercholesterolemia and hyperglycaemia (Kaczmarczyk et al., 2012, Leeds, 2014, Threapleton et al., 2013). Moreover, diverse clinical studies have examined the role of this dietary component in body-weight reduction, and a strong protective relationship has been established (Kristensen et al., 2012, Turner et al., 2013). Different mechanisms by which dietary fibre intake can influence body weight have been proposed including satiety and nutrient availability (Monteiro et al., 2014). Recently, the role of dietary fibre in gut microbiota in the development of obesity and associated co-morbidities has come to the forefront (Zeng et al., 2014). Data suggest that fibre can reduce the risk of obesity by promoting satiety and reducing energy intake (Monteiro et al., 2014). Many different mechanisms have been suggested to explain these effects, such as a lower metabolisable energy content of fibre than of other nutrients, a relatively constant meal intake volume, a decreased total energy intake and the increased chewing activity or oral exposure time to foods, which may result in earlier satiation (Wanders et al., 2011). Furthermore, fibre can slow down gastric emptying and consequently increase stomach distension, which also leads to satiation (Juvonen et al., 2009). Finally, a positive association between dietary fibre and hormones that induce satiation has also been found (Barone Lumaga et al., 2012, Juvonen et al., 2009). In the present study, when the impact of this dietary component on anthropometric and body composition measurements during a 4-month period of autonomy was studied, the results obtained are in agreement with those of the studies mentioned above, as fibre consumption showed positive effects on the improvement of body weight, BMI, waist circumference, WHR, total fat mass and android fat mass in individuals affected by the MetS (chapter 1).

3. 3. Effects of Antioxidants after the Whole Study (from day 0 to day 180)

Taking into account that exacerbated oxidative stress is a potential biochemical mechanism involved in the pathogenesis of MetS and diabetes, dietary antioxidants are of main interest in the prevention and treatment of these disorders (Zulet et al., 2012). In this context, it is well-accepted that a diet rich in fruits, vegetables and other plant-based foods reach in antioxidants such as vitamins C and E or lycopene, may decrease the risk of

oxidative stress-related diseases (Carlsen et al., 2010, Chen et al., 2013, Mustacich et al., 2007, Story et al., 2010).

When the correlation between TAC and changes on oxLDL in the population suffering MetS with hyperglycaemia was studied, a significant positive relationship between oxLDL reduction and TAC values was found. Furthermore, the same association was evidenced when studying the relationship between oxLDL and consumed energy (kcal) from fruits (chapter 2). Moreover, a positive relationship between PON1 ARE activity and dietary intake of vitamin C, tocopherols and lycopene was found in people presenting MetS symptoms (chapter 4).

These results agree with previous studies, where an inverse relationship between high TAC dietary patterns and MetS related-oxidative stress was established (Lopez-Legarrea et al., 2013). The positive relationships between the ARE activity and the selected antioxidant components may be explained by the capacity of the dietary antioxidants to scavenge free-oxygen radical products that may depress PON1 activity, as previous studies have suggested (Jarvik et al., 2002, Tsakiris et al., 2009).

Moreover, DNA methylation is one of the major epigenetic mechanisms considered to regulate gene expression, together with histone modifications and noncoding RNA activity (Portela et al., 2010). Methylation of the CpG-rich region (CpG island) overlapping a gene's promoter is a generally accepted mechanism for silencing expression (Vanderkraats et al., 2013). Modulation of gene expression through regulation of DNA methylation is one of the main studied mechanisms by which the dietary antioxidants exert their effects (Bartels M, 2007, Malireddy et al., 2012, Milagro et al., 2009). Our findings (chapter 4) confirm these outcomes since four of the studied CpG sites of the *PON1* gene were inversely correlated with the PON1 ARE activity. Moreover, inverse correlations between dietary vitamin C, total tocopherols and lycopene with different CpG sites methylation levels of the *PON1* gene were observed, which may influence the ARE activity as other studies have suggested (Jarvik et al., 2002, Tsakiris et al., 2009).

4. STRENGTHS AND LIMITATIONS OF THE STUDY

One of the main strengths of the present research is that it is a randomized controlled trial, considered the gold standard in the hierarchy of research designs for evaluating the efficacy and safety of a treatment intervention (Silverman, 2009). In addition, the CONSORT guidelines, used to improve the reporting of randomized controlled trials, were also followed (Schulz et al., 2011).

Moreover, the design of the RESMENA diet was based on the last findings in terms of nutritional interventions (Alves et al., 2014, Fan et al., 2013, Mayneris-Perxachs et al., 2014, Petzke et al., 2014), a fact that provides strength to the study. Besides, what makes this work unique is that all those demonstrated-efficient dietary patterns have been integrated in a single dietetic plan, presenting a new dietary strategy.

Moreover, the fact that every dietary pattern has been personally designed for each patient taking into account the sex, height, initial body weight, physical activity and working timetables should also be highlighted (Zulet et al., 2011). Furthermore, nutritionists asked to each participant throughout the first nutritional-learning period about the feelings and sensations that they were experiencing with the new diet to determine their well-being. In addition, different advice was given to the volunteers in each situation as well as recipes and general information about food and the importance of dietary adherence (de la Iglesia et al., 2014).

Taking into account that a diet based on the AHA guidelines (Krauss et al., 2000), a well-recognized healthy dietary pattern, was used as control, the positive results attributed to the RESMENA diet should be considered of reasonably importance.

Besides, the variables analysed can be considered fairly complete as they range since well-known and well-recognized measurements (Dubnov-Raz et al., 2010) in the assessment of obesity and the MetS (body weight, waist circumference, plasma glucose levels, plasma cholesterol concentrations...) to very novel molecules such as irisin (Crujeiras et al., 2014), or processes such as gene methylation levels measure (do Amaral et al., 2014).

However, this study also presents some limitations that should be mentioned. The volunteers' food consumption was compiled using dietary records which can vary depending on the thoroughness which each participant completed them, the nutritionist responsible for the analysis, the food composition tables used or the program to evaluate them employed (De Keyzer et al., 2011). Nevertheless, the dietary records are considered

good methods to estimate dietary intake and a scale and precise explanations about how to fulfil them were given to each volunteer (Zulet et al., 2011).

Moreover, although well-recognized high quality devices such as DXA or Triturus autoanalyser (Hangartner et al., 2013) have been used along the study, it is probably that not always the most accurate technique was used. For instance, to quantify the MDA there are different possible assays (Spirlandeli et al., 2014), the probability that we would have not chosen the best technique may explain that we did not achieve very relevant results regarding these biomarker as it did happen with the PON1 or the oxLDL.

Furthermore, chapter 3 consist of a subanalysis of the first 8-weeks of the nutritional intervention, therefore the limitations of a post hoc analysis should also be taking into account and the study considered as observational.

Finally, although the relevant CpG sites described in the fourth chapter match a core-binding consensus motif for known transcriptional regulators for several pathways (Gaboli et al., 2001; Zheng et al., 2010), the measurement of expression levels would have strengthened the outcomes obtained.

5. COROLLARY

This study presents a new dietary strategy, the RESMENA diet, to combat the comorbidities of the MetS and compares it with following a diet based on the AHA guidelines. The RESMENA diet proved to be more effective throughout a self-control period, where the participants had to apply on their own previous acquired dietary habits, regarding body composition, especially central obesity, and by reducing transaminase levels and maintaining uric acid and serum glucose levels in patients with MetS. Among people suffering MetS with hyperglycaemia, the RESMENA diet showed more benefits than the AHA diet concerning android fat mass reduction and improvement of the general oxidative stress status.

Moreover, this investigation evidenced how fibre, TAC, vitamin C, tocopherols, lycopene and fruits are the dietary components that most contributed to improve the MetS related parameters. Furthermore, a link between the recently discovered myokine irisin and several lipid profile biomarkers was reported, suggesting a response of this myokine to atherogenic burdens or a resistance phenomenon. Finally, different dietary antioxidants were proposed to enhance the PON1 ARE activity by epigenetic regulation, lowering the *PON1* gene methylation levels.

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CONCLUSIONS

Conclusions regarding patients with MetS after the 2-month long nutritional-learning intervention period (Chapter 1: from day 0 to day 60)

1. The RESMENA diet proved to be as effective as the AHA guidelines-based diet in improving adiposity and biochemical markers in patients with MetS.

2. Decreases in plasma irisin levels were associated with reductions in lipid profile markers (total cholesterol, TC/HDL-c ratio, LDL-c and Apolipoprotein B) when considering the whole sample.

Conclusions concerning patients with MetS after the 4-months period of autonomy (Chapter 2: from day 60 to day 180)

3. The patients under the RESMENA diet showed more beneficial effects than the AHA diet concerning body composition, involving a significant loss of body weight, android fat mass, waist circumference, WHR and BMI, since these variables remained unaltered in the subjects following the AHA diet.

4. The RESMENA diet presented more favorable effects than the AHA guidelines-based diet regarding biochemical profile. Levels of uric acid, glucose, HOMA-IR and AST increased in the AHA group, while they remained unaltered (uric acid, serum glucose, HOMA-IR) or even decreased (ALT and AST) in the RESMENA group.

5. Fibre consumption was the dietary component that apparently most contributed to the improvement of anthropometric variables (body weight, BMI, waist circumference, WHR, total fat mass and android fat mass) in the whole sample.

6. Body weight loss partly explained the improvements in some biochemical parameters (TG, glucose, insulin, HOMA-IR, ALT and AST) in the total population.

Conclusions regarding patients with MetS and hyperglycaemia after the 6-month long study (Chapter 3: from day 0 to day 180)

7. The RESMENA diet showed greater benefits than the AHA guidelines-based diet regarding android fat mass reduction and improvement of the general oxidative stress status, specifically on oxLDL related markers in volunteers suffering from MetS with hyperglycaemia.

8. Dietary TAC and fruit consumption were the nutritional components that potentially contributed most to the oxLDL depletion, in the total population.

9. The decrease on BMI, waist circumference, fat mass and TG levels were directly associated with the fall on oxLDL levels when considering the total sample.

Relationships of PON1 activity with *PON1* gene methylation, MetS related markers and dietary antioxidants intake after the 6-month long study (Chapter 4: from day 0 to day 180)

10. Vitamin C, tocopherols and lycopene intake within an energy-restricted diet may enhance the ARE activity by lowering the PON1 gene methylation levels in patients with MetS symptoms under a hypocaloric treatment, suggesting a potential epigenetic regulation.

General Conclusion

The RESMENA diet is a new dietary strategy designed to combat the comorbidities of the MetS, which has proved to be especially effective during autonomy and among people suffering MetS with hyperglycemia, as compared with a diet based on the AHA guidelines. Moreover, fibre and antioxidants are the dietary components that must be specially considered in future nutritional intervention in populations with MetS. Potential biomarkers and epigenetic processes related to the MetS (irisin and DNA methylation) should be deeply studied to draw any definitive conclusions.

APPENDICES

Appendix 1: *Participants Written Informed Consent*

TÍTULO: Proyecto de intervención nutricional para pacientes con síndrome metabólico
RESPONSABLE DEL ESTUDIO: Marian Zulet Alzórriz
HOJA INFORMATIVA PARA EL PARTICIPANTE

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

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

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

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

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

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

En la cita del día 0 se le volverá a recordar en qué consiste su participación y se le tomarán medidas de peso, talla, perímetro cintura y de composición corporal. Al mismo tiempo se le realizará una serie de preguntas relacionadas con el estado anímico y el grado de ansiedad. A continuación se le tomará la tensión arterial y la enfermera le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina (colesterol, glucosa, etc) y otros más específicos de relacionados con síndrome metabólico, entre ellos un análisis de la expresión de determinados genes. La dietista le proporcionará su dieta personalizada para comenzar el estudio de intervención nutricional. Deberá ajustarse a las pautas que se le establezcan, como la ingesta de alimentos, forma de preparación y a las recomendaciones de estilo de vida.

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

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

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

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

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

SU PARTICIPACIÓN EN EL ESTUDIO NO ESTÁ REMUNERADA.

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

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

Entiendo que mi participación es voluntaria.
    Entiendo que puedo retirarme del estudio:
        1º   Cuando quiera.
        2º   Sin tener que dar explicaciones.
        3º   Sin que esto repercuta en mis cuidados médicos.

Presto libremente mi conformidad para participar en el estudio.

Fecha   Firma del participante

Fecha   Firma del investigador

Appendix 2: *An Example of the RESMENA Diet*

LUNES

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

DESAYUNO

- 175g de naranja (unidad mediana).

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

MEDIA MAÑANA I

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

MEDIA MAÑANA II

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

COMIDA

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

2º plato elegir entre:
- 200g de pescado en crudo (170g cocinado): bacaladilla, bacalao, besugo, congrio, dorada, gallo, lenguado, lubina, merluza, mero, pescadilla, pez espada, rape, rodaballo, trucha, calamares, sepia, gambas o langostinos, pulpo, bonito, atún, sardinas, salmón, anchoas frescas…
- 150g en crudo de carne magra de cerdo (120g cocinada), conejo, pavo, pollo, ternera, jamón serrano, jamón york.

O elegir entre:

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

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

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

Postre: 125g kiwi (1 unidad pequeña).

MERIENDA I

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

MERIENDA II

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

CENA

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

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

VERDURAS DE CONSUMO LIBRE:

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

RECOMENDACIONES GENERALES:

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

Appendix 3: *An Example of the AHA Diet*

DIETA

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

DESAYUNO:

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

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

COMIDA:

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

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

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

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

CENA:

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

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

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

VERDURAS DE CONSUMO LIBRE:

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

RECOMENDACIONES GENERALES:

✓ Realizar todas las tomas a lo largo del día.

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

✓ Respetar las cantidades especificadas en la dieta, en especial frutas y grasas. Durante los primeros días conviene pesar los alimentos para hacerse una idea de lo que puede comer.

✓ Consuma como bebida, principalmente, agua. De modo ocasional puede elegir también cerveza sin alcohol, gaseosa y refrescos lights.

✓ Evite el consumo de alcohol. Vino tinto (250cc) los fines de semana.

✓ Puede tomar infusiones sin azúcar (con sacarina) siempre que quiera.

Appendix 4: *Congress Communications*

IUNS 20th International Congress of Nutrition (IUNS)

15th to 20th of September, 2013. Granada (Spain)

Oral Communication

The beneficial effects of the RESMENA dietary pattern on oxLDL in patients with Mets

de la Iglesia R[1], Lopez-Legarrea P[1], Abete I[3], Navas-Carretero S[1, 2], Martínez JA[1, 2], Zulet MA[1, 2]

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Background and objectives: The prevalence of metabolic syndrome (MetS) is increasing worldwide. Insulin resistance, hyperglycemia and type 2 diabetes have been considered major traits of the MetS. Moreover, oxidative stress is considered an important contributor to these pathophysiological complications. Therefore, the aim of the present study was to evaluate the interactions and effectiveness of a weight loss dietary pattern on improving the oxidative stress status on patients suffering MetS with hyperglycemia.

 Methods: seventy-nine volunteers recruited according to specific inclusion criteria were randomly assigned to two low-calorie dietary treatments (-30% Energy): the Control diet based on the American Heart Association criteria and the RESMENA diet based on a different macronutrient distribution (30% proteins, 30% lipids, 40% carbohydrates), which was characterized by a high adherence to the Mediterranean diet, increased meal frequency (7times/day), low glycemic load (mainly in the afternoon and at night) as well as high total antioxidant capacity and omega-3 fatty acids content. Anthropometrical measurements and biochemical analyses were performed before and after 6-months of intervention. Plasma ox-LDL were measured using a capture ELISA assay kit.

Results: Both dietary groups decreased body weight, BMI, waist circumference and total fat ($p < 0.001$ both groups) as well as plasma TG ($p < 0.05$ Control, $p < 0.005$ RESMENA), insulin ($p < 0.001$ both groups) and HOMA-IR ($p < 0.005$ Control, $p < 0.001$ RESMENA) levels, associated to the energy restriction. Interestingly, after the intervention, only subjects of the RESMENA group significantly reduced plasma oxLDL, the main variable of the study, resulting in significant differences between groups ($p < 0.025$).

 Conclusions: the RESMENA dietary pattern might be a good option for patients specifically suffering Mets and hyperglycemia not only due to the beneficial effects of weight loss, but also on oxidative stress status.

Key words: metabolic syndrome, hyperglycemia, oxidative stress, oxLDL.

XIV Spanish Nutrition Society Congress (SEN)

27th-29th of September, 2012. Zaragoza (Spain)

Oral Communication

Efecto de dos estrategias dietéticas con diferente contenido en macronutrientes sobre marcadores inflamatorios en pacientes con Síndrome Metabólico: Proyecto RESMENA.

de la Iglesia R[1], López-Legarrea P[1], Abete I[1], Rosa FT[2], Navas-Carretero S[1], Forga L[3], Zulet MA[1], Martínez JA[1]

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Introducción: La inflamación crónica de bajo grado es un mecanismo vinculado a síndrome metabólico (SM). Actualmente, numerosas investigaciones se centran en encontrar estrategias dietéticas eficaces que mejoren la sintomatología y reduzcan dicho proceso inflamatorio. En este sentido, el objetivo de este trabajo fue comparar el efecto de dos estrategias dietéticas de pérdida de peso (-30% Valor Calórico Requerido Total), una de ellas basada en las directrices de la Asociación Americana del Corazón (AHA) frente a otra con mayor aporte proteico, en detrimento del contenido en hidratos de carbono (HC).

Material y Métodos: Participaron 96 (48H/48M) sujetos adultos (49±9 años) con criterios de SM según la Federación Internacional de Diabetes, que fueron asignados aleatoriamente al grupo Control-AHA (55% HC, 15% Proteínas y 30% Lípidos) y al grupo en estudio-Resmena (40% HC, 30% Proteínas, 30% Lípidos), durante 8 semanas bajo seguimiento nutricional quincenal. Los marcadores de inflamación IL 6, TNFalfa, hsCRP y PAI I fueron analizados en plasma al inicio y al final de la intervención y se computaron conjuntamente a través de un sumatorio de variaciones, permitiendo calcular un índice del estado inflamatorio.

Resultados: Ambas estrategias nutricionales mostraron una reducción significativa de los parámetros antropométricos, de composición corporal (DXA) y bioquímicos (metabolismo lipídico y glucídico), sin diferencias entre dietas. El análisis estadístico del índice inflamatorio (PCR, IL-6, PAI-I, TNF-alfa) reveló una mejora significativamente mayor (p=0.012) en el grupo de dieta basada en las recomendaciones de la AHA, tras 2 meses de intervención.

Conclusiones: Ambos regímenes dietéticos resultaron efectivos en la reducción de variables antropométricas, de composición corporal y bioquímicas asociadas a SM. La dieta hipocalórica con la distribución de macronutrientes recomendada por la AHA presentó unos beneficios mayores en cuanto a su comportamiento antiinflamatorio, vinculado a SM, al menos a corto plazo.

21st European Congress of Obesity (ECO)

28th-31st of May, 2014. Sofia (Bulgaria)

Poster

Plasma irisin depletion under energy restriction is associated with improvements in lipid profile in metabolic syndrome patients.

de la Iglesia R[1], Lopez-Legarrea P[1], Crujeiras AB[2,3], Pardo M[2,3], Zulet MA[1,2], Martinez JA[1,2], Casanueva FF[2,3].

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Introduction: Irisin is a novel myokine that has received much attention because it has been hypothesized to have an important role in fuel metabolism. The aim of the current research was to study the relationships between the lipid profile of patients with Metabolic Syndrome (MetS) under an energy-restricted programme and plasma levels of this hormone.

Methods: MetS subjects (n = 84, 49±10 y.o, BMI 36.1±4.6 kg/m^2, 56% men) who followed an 8-week hypocaloric regimen (-30% of the energy requirements), were enrolled in the present study. Anthropometric, biochemical and plasma irisin data were assessed using validated procedures at the beginning and at the end of the dietary intervention.

Results: Most anthropometric and biochemical parameters were improved at the end of the study. Plasma irisin levels were significantly lowered (-20.0%, $p < 0.001$) paralleling the weight loss (-7.0±3.0 kg) after the nutritional intervention. The irisin reduction positively correlated with the variation of some lipid profile variables, such as total cholesterol ($p = 0.018$), total cholesterol/high density lipoprotein-cholesterol ratio ($p = 0.036$), low density lipoprotein-cholesterol ($p = 0.037$) and apolipoprotein B ($p = 0.002$), regardless the weight loss.

Conclusion: This study revealed important relationships between the decrease in irisin levels and the reductions in atherogenic-related variables in patients with MetS following a controlled hypocaloric diet.

8th Congress International Society of Nutrigenetics/Nutrigenomics (ISNN)

2nd-3rd of May, 2014. Gold Coast (Australia)

Poster

Associations between arylesterase (ARE) activity, methylation of *paraoxonase 1* (*PON1*) gene and dietary antioxidants in patients with metabolic syndrome features under a hypocaloric diet

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Objectives: Understanding the epigenetic regulation of enzymatic activities involved in body metabolism is gaining interest. The arylesterase (ARE) activity attributed to the paraoxonase-1 (PON1) enzyme is known to protect against lipoproteins oxidation and against the development of metabolic syndrome (MetS) manifestations. The aim of the current research was to study the potential relationships between the PON1 ARE activity and anthropometrics, biochemical markers, dietary antioxidants and the *PON1* gene methylation levels, in volunteers with MetS symptoms after following a hypocaloric diet.

Methods: Adults with MetS features (n=47, 47±10 y.o; BMI 36.2±3.8 kg/m2; 46.8% female), who followed a six-month energy-restricted dietary trial, were enrolled in the intervention. *PON1* transcriptional regulatory region methylation was analysed using validated protocols (microarray) at the beginning of the study. Anthropometric, biochemical, enzymatic and dietary data were also assessed before and after the dietary implementation.

Results: Participating subjects decreased body weight, BMI, total fat mass, diastolic blood pressure, mean blood pressure and triglycerides accordingly to the ARE activity reduction. Methylation levels of *PON1* gene were positively associated with ARE activity at baseline. Noteworthy, dietary tocopherols, lycopene and vitamin C intakes, were positively correlated with ARE activity at the end of the study and showed a negative association with the methylation of some CpG sites of the *PON1* gene at baseline.

Conclusions: Volunteers with MetS symptoms following a hypocaloric diet decreased ARE activity in parallel with MetS-related markers. Interestingly, dietary antioxidants might have a role in the enhancement of the ARE activity by lowering the *PON1* gene methylation levels.

Poster

El seguimiento de una dieta hipocalórica durante dos meses mejora el estrés oxidativo en pacientes con síndrome metabólico.

de la Iglesia R[1], López-Legarrea P[1], Abete I[1], Rosa FT[2], Navas-Carretero S[1], Forga L[3], Zulet MA[1], Martínez JA[1]

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Introducción: El síndrome metabólico (SM) se asocia con un desequilibrio en el estado oxidativo, favoreciendo de este modo el desarrollo de alteraciones cardiovasculares y disfunción endotelial, entre otras complicaciones. En este contexto, el objetivo del presente estudio ha sido comprobar si una dieta efectiva frente a la pérdida de peso y grasa corporal, produce un beneficio adicional sobre el estado oxidativo en pacientes con SM.

Material y métodos: Un total de 96 voluntarios con SM, según los criterios de la Federación Internacional de Diabetes (IDF, 2005), siguieron durante 2 meses una dieta hipocalórica personalizada con una restricción del 30% sobre el valor calórico total requerido (VCT). Al principio y al final de la intervención se tomaron medidas antropométricas y de composición corporal (DXA) y se analizaron marcadores del perfil lipídico en suero. Para analizar los cambios en el estado oxidativo, se determinaron las concentraciones plasmáticas de malondialdehído (MDA) y LDL-oxidadas (ox-LDL) mediante técnicas espectrofotométricas.

Resultados: Al final de la intervención, los voluntarios redujeron significativamente el peso, el IMC, el perímetro de la cintura, el porcentaje de masa grasa total y el porcentaje de masa grasa androide (p < 0,001). Además, presentaron niveles significativamente menores de colesterol total, LDL-colesterol, HDL-colesterol, triglicéridos y ratio TG:HDL (p < 0,01). Adicionalmente, los niveles de MDA y de ox-LDL se redujeron significativamente (p < 0,01) tras los dos meses de estudio.

Conclusiones: El seguimiento de una dieta hipocalórica, además de producir un efecto beneficioso sobre variables antropométricas y del metabolismo lipídico, mejoró el estado oxidativo evaluado a través de los niveles de MDA y de ox-LDL, en personas con SM.

19th European Congress of Obesity (ECO)

9th-12th of May, 2012. Lyon, (France)

Poster

The RESMENA Diet: a new long-term strategy to reduce metabolic syndrome comorbidities

de la Iglesia R[1], López-Legarrea P[1], Abete I[1], Ibáñez-Melo A[1], Navas-Carretero S[1], Bondia-Pons I[1], Forga L[2], Zulet MA[1], Martínez JA[1]

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Introduction: There are different dietary strategies to reduce metabolic syndrome symptoms, but studies researching the long-term effects are scarce. In this context, this study compared the long-term effects of two low-calorie diets (-30% Energy): a Control diet based on the American Heart Association criteria; and the RESMENA diet based on a different macronutrient distribution (30%proteins, 30% lipids, 40% carbohydrates) and characterized by a high adherence to the Mediterranean diet, increased meal frequency (7times/day), low glycemic load (mainly in the afternoon and at night) as well as high total antioxidant capacity and omega-3 fatty acids content. The specific aim of this study was to analyze changes in volunteers during an autonomous free-living period after a two-month personally advised nutritional intervention.

Methods: Seventy-eight volunteers presenting metabolic syndrome were randomly assigned to the Control and RESMENA diets. After the nutritional intervention a four-month autonomous free-living period began, during which participant must apply the advice received. Anthropometrical measurements and biochemical analyses were performed before and after the free-living period.

Results: Only the RESMENA group individuals significantly continued losing body weight, body mass index and waist circumference, while in both groups a significant reduction of fat mass was found. Total cholesterol and glucose concentrations significantly increased in the control group, but no changes occurred in the RESMENA group. Additionally, an improvement in transaminases levels (AST and ALT) was observed in the RESMENA group.

Conclusion: The adherence to the RESMENA diet could be a good option as a long-term dietary treatment for combating metabolic syndrome comorbidities.

V Experimental Science and Health Research Journey

27th of May, 2012. Pamplona (Spain)

Poster

Different macronutrient distribution in the dietary treatment of metabolic syndrome

de la Iglesia R[1], López-Legarrea P[1], Abete I[1], Ibáñez-Melo A[1], Navas-Carretero S[1], Bondia-Pons I[1], Forga L[2], Zulet MA[1], Martínez JA[1]

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Introduction: Long-term outcomes of two low-calorie diets (-30% Energy) are compared in this research: a Control diet based on the American Heart Association criteria; and the RESMENA diet based on different macronutrient distribution (30%proteins, 30% lipids, 40% carbohydrates) and characterized by high adherence to the Mediterranean diet, increased meal frequency (7times/day), low glycemic load as well as high total antioxidant capacity and omega-3 fatty acids content. The specific aim was to determine changes in volunteers during an autonomous free-living period after two-month personally advised nutritional intervention.

Methods: Seventy-eight volunteers presenting metabolic syndrome were randomly assigned to Control and RESMENA groups. After the nutritional intervention a four-month autonomous free-living period began. Anthropometrical measurements and biochemical analyses were performed before and after the free-living period.

Results: Only individuals assigned to the RESMENA diet significantly lost body weight, BMI and waist circumference, while in both groups a significant reduction of fat mass was found. Total cholesterol and glucose levels significantly increased in control group, but no changes were found in RESMENA group. Moreover, an improvement in transaminases concentrations was observed in the individuals that followed RESMENA diet.

Conclusion: The adherence to RESMENA diet could be a good option as a long-term dietary treatment for combating metabolic syndrome.

Appendix 5: *Additional RESMENA Project Articles*

Nutrition&Metabolism

RESEARCH　　　　　　　　　　　　　　　　　　　　　　**Open Access**

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

Patricia Lopez-Legarrea[1], Rocio de la Iglesia[1], Itziar Abete[2,4], Isabel Bondia-Pons[1], Santiago Navas-Carretero[1,4], Lluis Forga[3], J Alfredo Martinez[1,4]* and M Angeles Zulet[1,4]

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 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: 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 [1]. 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) [2-4]. Also according to the WHO estimations, obesity prevalence rates will tend to increase in the next years. So that new effective proposals are needed 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 [5]. While dietetic programs have traditionally focused on calorie restriction, new nutritional alternatives are nowadays being investigated. They entail macronutrient distribution [6], meal frequency [7], consumption of

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bioactive ingredients, such as fiber [8] and n-3 fatty acids [9], glycemic index (GI)/glycemic load (GL) [10] or the dietary total antioxidant capacity (TAC) [11]. Dietary TAC is considered an appropriate approach to measure the cumulative antioxidant properties of food [12], despite its controversial use at evaluating the role of antioxidants *in vivo* [13]. 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 [11]. In this context, dietary TAC is gaining importance as a valuable tool to investigate the relationship between diet and oxidative stress-related diseases [14]. 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 [15], meal frequency [7], fiber [8], n-3 fatty acids [16], GI/GL [17] or dietary TAC [18]. 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 (www.clinicaltrials.gov; NCT01087086) [19] 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 [20].

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 [21] 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 (www.clinicaltrials.gov;
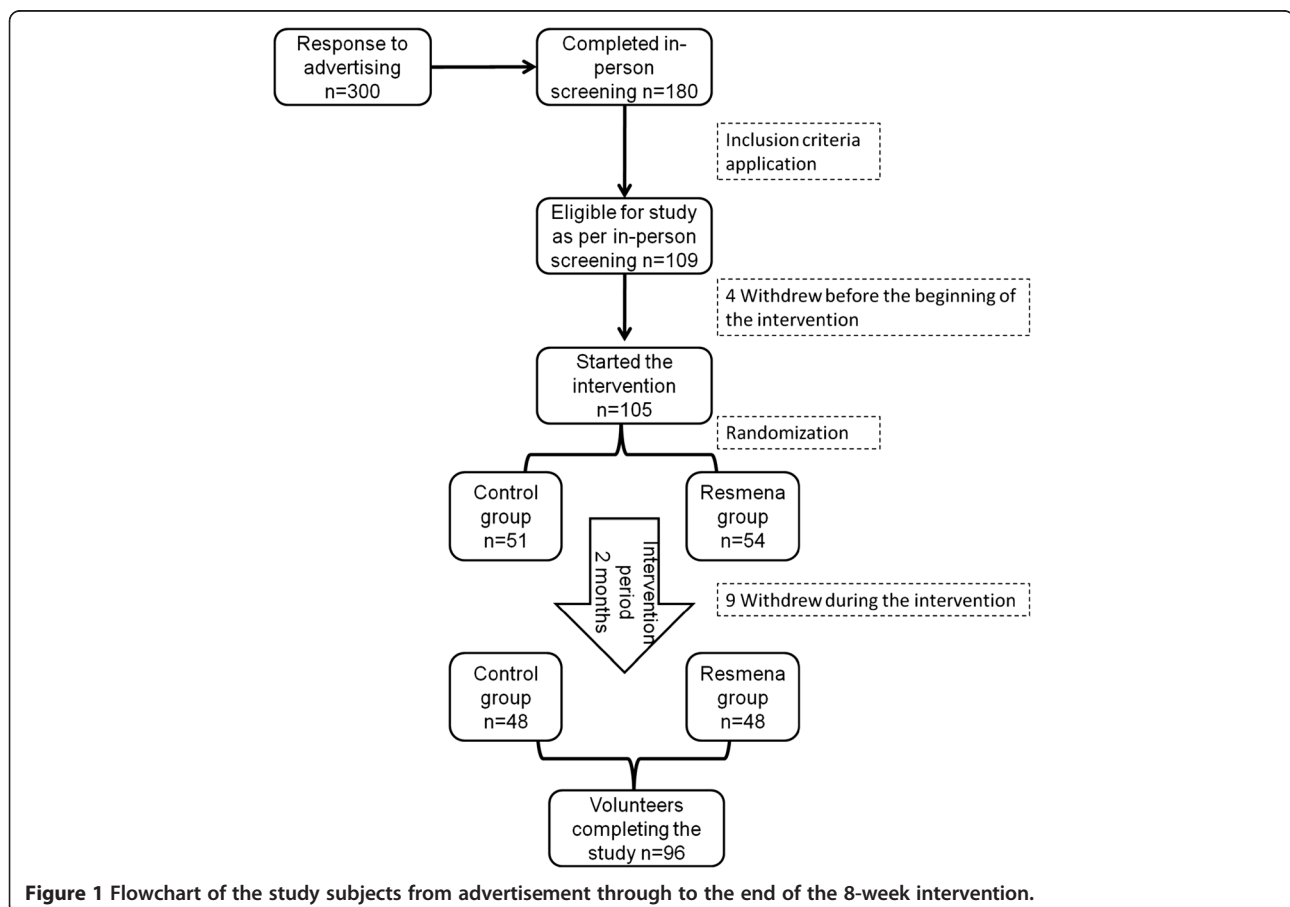

Figure 1 Flowchart of the study subjects from advertisement through to the end of the 8-week intervention.

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.

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." [22]. The Control diet was based on the AHA guidelines [20], 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).

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 [2]. Usual diet was assessed with a semiquantitative 136-item food frequency questionnaire previously validated in Spain for energy and nutrient intake [14]. 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 [23]. 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 [24]. Dietary TAC was calculated using the list

Table 1 Selected anthropometric characteristics of the whole sample and categorized 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.

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

Variable	Control group (n = 48)			RESMENA group (n = 48)					
	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 (points)	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
Meal Frequency (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. $ $p < 0.05$; $$ $p < 0.01$; $$$ $p < 0.001$.

from Carlsen et al. 2010, which takes into consideration raw or cooked food preparations [25]. 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. [26] (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 [19]. 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 [19].

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} [5]. Low-density lipoprotein-cholesterol (LDL-c) levels were calculated following the Friedewald formula: LDL-c = TC-HDL-c - VLDL [10].

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 completely 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). 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 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), fat mass (*p* = 0.015) and AST (*p* = 0.004) were significantly improved by this variable (Table 4). Dietary TAC also seemed to have a potential effect in 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). 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

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 0)	Visit 2 (day 60)	p^a	Visit 1 (day 0)	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/m^2)	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 (IU/mg)	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.

regarding the high-TAC group ($p = 0.044$ and $p = 0.004$, respectively) (Figure 2).

As expected, 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.

Discussion

This study compared the effects of a novel dietary strategy with the AHA pattern [20], 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 [19].

Evaluating the two prescribed dietary strategies as a whole, both were 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 [5,7,13].

Recent investigations have focused on the role of the macronutrient distribution in weight reduction treatments instead of considering only calorie restriction [27]. 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 [6,27]. 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

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

Variable	Meal Frequency		Protein Intake		n-3 FA intake		TAC		GI		GL		HEI		Model p	Corrected r²
	B	p	B	p	B	p	B	p	B	p	B	p	B	p		
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 (IU/mg)	−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.
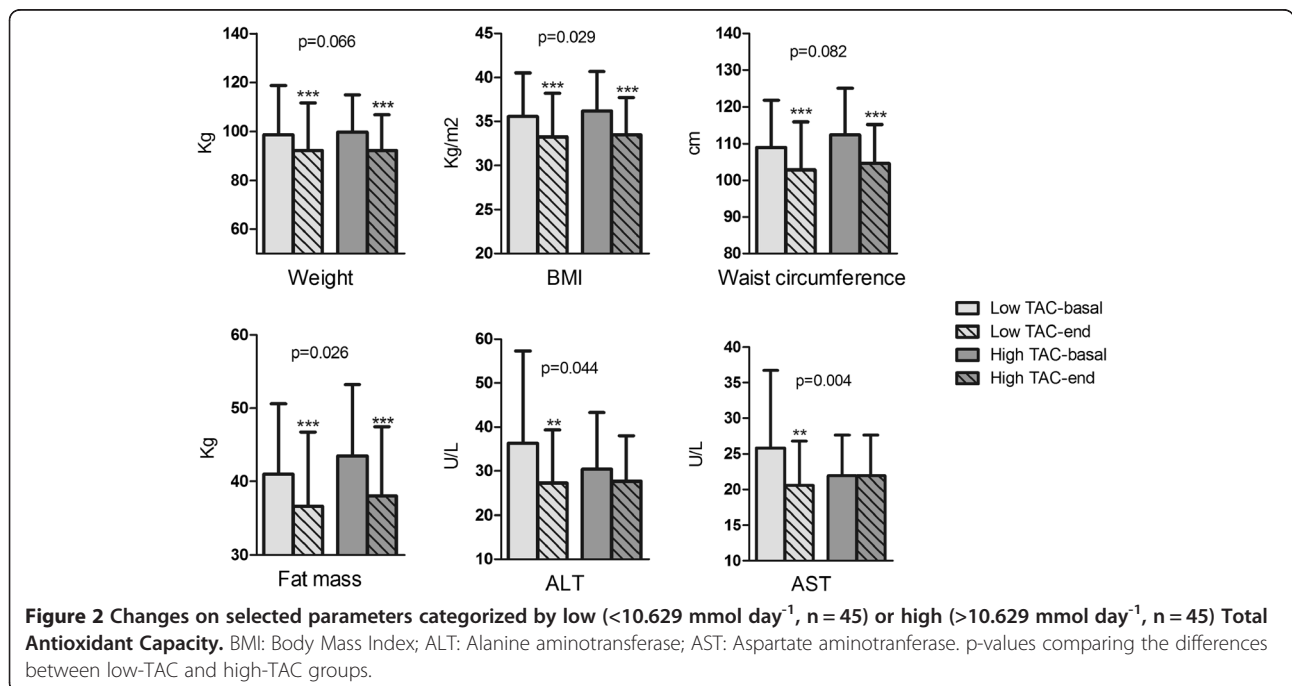

Figure 2 Changes on selected parameters categorized by low (<10.629 mmol day⁻¹, n = 45) or high (>10.629 mmol day⁻¹, n = 45) Total Antioxidant Capacity. BMI: Body Mass Index; ALT: Alanine aminotransferase; AST: Aspartate aminotranferase. p-values comparing the differences between low-TAC and high-TAC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

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

Variable	Groups				ANOVA 2×2		
	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/m^2)	−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 aminotransferase; *AST*, Aspartate aminotranferase. $p < 0.05$ was set-up as statistically significant.

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 [10] while the present work focused on the effects of an 8-week dietary treatment.

Interestingly, our study showed an inverse association between protein intake and both ALT and AST transaminases levels. These enzymes are unspecific markers of hepatic
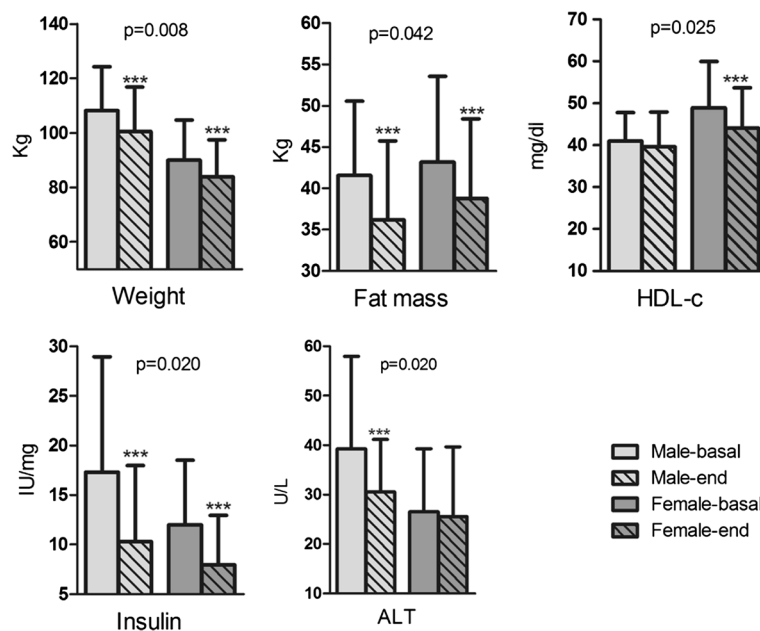

Figure 3 Changes on anthropometric and biochemical selected parameters regarding gender, male (n = 51) or female (n = 45).
HDL-c: High Density Lipoprotein-cholesterol; ALT: Alanine aminotransferase. *p* -values comparing the differences between male and female groups.

function [28]. Low-serum transaminases levels are found under normal conditions, indicating proper function of the liver, while increased serum values are related to hepatic dysfunction [28]. 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 [7]. 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 [29].

The beneficial properties of n-3 fatty acids have been widely studied [9,16]. 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 [16].

GI and GL are two concepts that refer to the absorption rate of carbohydrates [26]. Increased values have been reported as potential type-2 Diabetes Mellitus risk factors [5]. 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 Bao et al. 2010 [30].

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 [14]. Most of them, take into consideration the Mediterranean Diet guidelines, widely recognized as a healthy pattern [30]. 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 [31,32].

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 [33]. 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 [31,34]. This link may be associated with a reduction of cardiovascular risk, as previously described in other populations [35]. 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 [36]. However, some other authors found higher HDL-c serum levels after similar dietary interventions [37] especially when containing fish or fish derived products [16,38]. 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 [1,7,39]. 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 [7]. Regarding biochemical parameters, insulin as well as ALT blood levels were significantly decreased within this group in accordance with previous studies [1]. On the contrary, HDL-c reduction was greater among the women group, as other studies reported [40], 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 [41] and they represent a development problem [42] leading to an increased morbimortality at the adult age [43].

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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SHORT COMMUNICATION

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

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Irisin is assumed to be a relevant link between muscle and weight maintenance as well as to mediate exercise benefits on health. The aim of this study was to assess the possible associations between irisin levels and glucose homeostasis in obese subjects with metabolic syndrome (MetS) following an energy-restricted treatment. Ninety-six adults with excessive body weight and MetS features underwent a 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 circulating levels of irisin in parallel with improvements on glucose homeostasis markers. Interestingly, participants with higher irisin values at baseline (above the median) showed a greater reduction on glucose ($P = 0.022$) and insulin ($P = 0.021$) concentrations as well as on the homeostasis model assessment index ($P = 0.008$) and triglycerides ($P = 0.006$) after the dietary intervention, compared with those presenting low-irisin baseline values (below the 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 a dietary-induced weight loss.

INTRODUCTION

Obesity is a worldwide health burden, accompanied by a number of comorbidities including glucose intolerance, insulin resistance and type 2 diabetes.[1] In this context, the myokine irisin,[2] which is a cleavage product of the type I membrane protein fibronectin type III domain-containing 5, has been hypothesized as a target to counteract obesity and type 2 diabetes.[3,4] Irisin is expressed in the muscle and the adipose tissue and has been associated with adiposity and body weight in animals[5,6] and humans.[7,8] 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 on circulating irisin concentrations in obese subjects presenting metabolic syndrome (MetS) features after a treatment designed to lose weight and to analyze the potential relationships of this myokine with glucose homeostasis after dieting.

MATERIALS AND METHODS

Study protocol

This research reports the findings of the 8-week intervention period of the RESMENA randomized intervention trial (www.clinicaltrials.gov; NCT01087086), which was conducted following the CONSORT 2010 criteria. A full list of inclusion criteria, as well as a complete description of the study methodology can be found in earlier publications.[9,10] Briefly, participants were randomized into two intervention groups, with the same energy restriction (−30% E), but differing mainly in the carbohydrate/protein ratio and meal frequency: control group supplying 55% E from CHO and 15% E from proteins within a 3–5 meals per day pattern, and RESMENA group providing 40% E from CHO and 30% E from proteins within a 7 meals per day plan.

Subjects

Ninety-six adults (mean age = 50 years old; range 21–70 years old) with excessive body weight (mean body mass index = 35.9 kg m^{-2}; range 26.9–49.4 kg m^{-2}) suffering MetS according to the International Diabetes Federation criteria completed the intervention period. All the participants gave a 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 means of 48-h weighed records at baseline and at the end of the intervention and further analyzed using the DIAL software (Alce Ingenieria, Madrid, Spain). Subjects were asked to maintain their usual activity levels during the study, which was monitored at the beginning and endpoint with a validated 24-h physical activity questionnaire.[9]

Anthropometric measurements and body composition determinations were performed, as described elsewhere.[9] Overnight fasting plasma levels of glucose and triglycerides were measured in an autoanalyzer Pentra C-200 (HORIBA ABX, Madrid, Spain) with specific kits from this company. Insulin concentrations were determined with an enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden) in a Triturus autoanalyzer (Grifols SA, Barcelona, Spain) and the homeostasis model (homeostatic model assessment-insulin resistance (HOMA-IR)) was applied to estimate insulin resistance.

Irisin plasma levels were determined using a commercial enzyme-linked immunosorbent assay kit following the manufacturer's instructions (Irisin ELISA kit EK-067–52; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA),

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on a spectrophotometric reader at a wavelength of 450 nm (Versamax Microplate Reader, East Falmouth, MA, USA). This test provided a range of detection of 0.066–1024 ng ml^{-1} and exhibited a coefficient of variation of 6–10% inter- and intra-assay. The samples were kept at $-80\,^{\circ}$C and were analyzed immediately after the experiment was ended.

Statistical analysis

The sample size of this secondary analysis was calculated for an $\alpha = 0.05$ and a power of 80% based on the waist circumference reduction, as described elsewhere.[9] 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. Analyses between dietary groups were performed with unpaired t-tests. A multiple linear regression analysis was applied in order to assess the potential relationships among irisin with anthropometric and biochemical measurements (95% confidence interval). The median value of irisin baseline concentrations was considered as the cutoff for analyzing the effect of high- or low-irisin levels on glucose regulatory factors, as previously applied.[11] This tool is based on the assignment of the studied population into two groups of disease risk. The association between irisin levels and carbohydrate intake was assessed using the parametric Pearson correlation. Specific statistical analyses (analysis of covariance) were performed after excluding outlier values in order to control the regression to the mean phenomenon. Statistical analysis was performed using SPSS15.1 software (SPSS Inc., Chicago, IL, USA). An alpha level of 0.05 was set up for determining statistically significant differences. Data are reported as mean ± s.e.

RESULTS

At the beginning of the intervention, there were no differences between groups in any of the anthropometric and routine biochemical markers ($P > 0.05$). After the intervention, an improvement (reduction) was observed on these measurements with apparently equal effectiveness between the two dietary treatments ($P > 0.05$, Table 1), except for adiponectin, which was increased in both groups, but without reaching statistical significance. Changes in irisin concentrations were similar ($P > 0.05$) in the control group (-87.3 ± 18.4 ng ml^{-1}) as compared with the RESMENA group (-59.8 ± 11.8 ng ml^{-1}), after following the energy-restricted treatment. Therefore, both groups were merged for subsequent analyses. Considering the whole sample, participant's mean body weight loss was -6.9 ± 3.0 kg and irisin plasma concentrations diminished (Figure 1a) in association with changes in body weight ($r = 0.21$; $P = 0.046$) and fat mass ($r = 0.22$; $P = 0.037$). As the main objective of this study was to evaluate the

potential role of irisin on glucose homeostasis and given that some of the participants were diabetic, a preliminary analysis separating non-diabetic and diabetic participants was also performed. Differences were found for glucose concentrations and HOMA index between both groups after the nutritional intervention with energy restriction, but similar outcomes were found concerning irisin concentrations (data not shown).

Similar values were found concerning physical activity assessments at the beginning and at the end of the intervention in both dietary groups. Moreover, the regression analysis showed no relationships between physical activity factor and irisin levels changes ($P = 0.736$). An association of circulating glucose ($B = -0.134$, 95% confidence interval: -0.245 to -0.024; $P = 0.018$) and irisin concentrations changes was found, irrespective of confounding factors: gender, age, diet, body weight loss and irisin baseline values.

Interestingly, after adjusting for gender, age and weight loss, participants belonging to the high-irisin group at baseline (>308.0 ng ml^{-1}) evidenced significantly greater reductions (Figure 1b) on glucose ($P = 0.022$), insulin ($P = 0.021$), HOMA index ($P = 0.008$) and triglycerides ($P = 0.006$), compared with those belonging to the low-irisin group at baseline (<308.0 ng ml^{-1}). Furthermore, the decrease in irisin concentrations was significantly greater ($P < 0.001$) within the group with high-irisin values at baseline (-126.6 ± 15.9 ng ml^{-1}) than within the lower irisenemia group (-18.2 ± 9.1 ng ml^{-1}). After 8 weeks of nutritional intervention, irisin concentrations were positively correlated with carbohydrate intake (cereals, pulse, fruits and vegetables; $r = 0.234$, $P = 0.023$; Figure 1c).

DISCUSSION

This study evidenced that irisin *per se* may exert an effect on the reduction of glucose, insulin and triglycerides concentrations after prescribing an 8-week nutritional intervention to obese subjects with MetS traits.

Irisin is a recently discovered muscle-derived hormone, whose secretion is induced by exercise.[2] This myokine has been shown to be able to increase energy expenditure, and therefore, it has been proposed to have a potential role in obesity and diabetes treatments.[2,12–14] Since discovery, a number of original studies have addressed various aspects of the biology of irisin.[15] However, the regulation and specific role of irisin in human's glucose metabolism remain still unclear. Thus, the main objective of the current research was to investigate the potential relationships between irisin concentrations and glucose homeostasis, after dieting.

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			Difference between diet groups (P-value)
	Baseline	Endpoint	P-value	Baseline	Endpoint	P-value	
Body weight (kg)	99.5 ± 2.8	92.7 ± 2.7	<0.001	100.0 ± 2.4	92.9 ± 2.3	<0.001	0.555
BMI (kg m^{-2})	36.2 ± 0.7	33.7 ± 0.7	<0.001	35.6 ± 0.6	33.0 ± 0.6	<0.001	0.732
Fat mass (%)	39.1 ± 1.1	36.2 ± 1.1	<0.001	39.2 ± 0.9	36.4 ± 1.0	<0.001	0.854
Fat mass (kg)	39.0 ± 1.6	33.7 ± 1.5	<0.001	39.2 ± 1.4	33.8 ± 1.3	<0.001	0.886
Glucose (mg dl^{-1})	121.0 ± 5.0	108.0 ± 2.0	0.006	123.8 ± 5.5	110.2 ± 3.8	0.016	0.939
Insulin (µU ml^{-1})	15.3 ± 1.7	9.3 ± 1.1	<0.001	14.4 ± 1.2	9.1 ± 0.9	<0.001	0.557
HOMA	4.7 ± 0.6	2.6 ± 0.3	<0.001	4.5 ± 0.4	2.6 ± 0.3	<0.001	0.686
Triglycerides (mg dl^{-1})	176 ± 13	145 ± 10	0.005	194 ± 18	151 ± 14	<0.001	0.421
Irisin (ng ml^{-1})	412.3 ± 31.6	326.7 ± 22.6	<0.001	299.4 ± 16.3	239.6 ± 8.8	<0.001	0.234
Leptin (ng ml^{-1})	22.4 ± 2.3	14.8 ± 1.8	<0.001	20.2 ± 2.1	12.8 ± 1.6	<0.001	0.883
Adiponectin (ng ml^{-1})	13.6 ± 1.5	13.8 ± 1.3	0.863	12.1 ± 1.3	17.6 ± 3.3	0.127	0.152

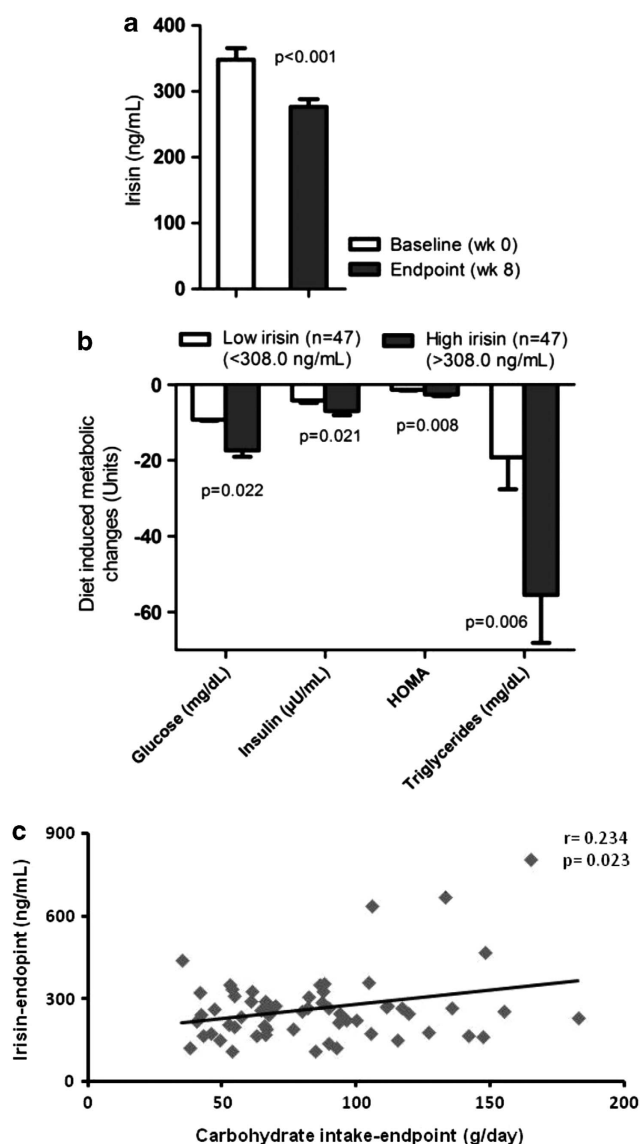Abbreviations: BMI, body mass index; HOMA, homeostasis model assessment.

Figure 1. Irisin changes from baseline (week 0) to the end (week 8) of the intervention (**a**); changes in glucose, insulin, HOMA index and triglycerides, depending on irisin baseline levels after the intervention of 8 weeks duration (**b**); and irisin correlation with carbohydrate intake (cereals, pulse, fruits and vegetables) at the endpoint of the intervention (**c**).

The study was designed as a randomized controlled nutritional intervention comparing two energy-restricted dietary treatments.[9] Both control and RESMENA dietary strategies proved to be effective for improving MetS disturbances by lowering anthropometric and biochemical markers, being these outcomes in agreement with other studies concerning hypocaloric diets.[16] However, no differences between treatments were observed for any of the studied variables including irisin. For that reason, the sample was merged and considered as a whole for the subsequent analyses regarding irisin concentrations and its potential associations with glucose metabolism.

First, changes on irisin concentrations after the 8 weeks of nutritional intervention were evaluated. This study evidenced that irisin plasma concentrations decreased after the energy restriction program and the subsequent weight loss, independently of the dietary group. This finding is in agreement with a previous study that reported a reduction in irisin levels after surgically induced weight markdown.[8]

Then, the potential role of irisin on glucose homeostasis-related parameters was analyzed in order to reach the main objective of the research. The prime finding of the current investigation was that higher irisin concentrations at the beginning of the intervention were associated with greater reductions on glucose and insulin concentrations as well as on the HOMA index, independently of body weight loss. Although this outcome should be carefully examined, similar results have been reported in children by Al-Daghri et al.[17] where a crucial role for irisin in glucose homeostasis was suggested. On the other hand, those individuals with higher irisin concentrations at the beginning of the intervention also achieved higher beneficial effects regarding the lowering of triglycerides concentrations. This effect could be explained by the fact that triglycerides levels have been revealed to positively correlate with glucose levels.[18] Thus, the effects of irisin on the changes of glucose concentrations may have been subsequently reflected on triglycerides. In addition, taking into account that irisin has been evidenced to increase energy expenditure,[19] the greater reduction observed in triglycerides according to the high-irisin levels at baseline may be also due to a higher utilization of triglycerides as energy substrate. Previous studies have also evidenced an inverse association of irisin levels with triglycerides concentrations.[20] Taking together these outcomes, it can be suggested that irisin may be involved in the regulation of glucose homeostasis in obese subjects presenting MetS features. Thus, irisin could mean a physiological feedback to counteract potential glucose metabolism-related disturbances associated to an excessive body weight state. Irisin would seem to be increased in unfavorable metabolic situations acting as a compensatory triggering mechanism. Other authors have likewise claimed that the increase in irisin under obesity conditions may indicate a physiological adaptation to improve glucose tolerance, which is often impaired in obese subjects.[3] Indeed, this behavior has been observed predominantly in individuals with metabolic disease[21] as it is the condition of our study population. However, other studies reported associations between plasma irisin levels and important metabolic factors in non-diabetic subjects, but not in individuals with type 2 diabetes.[4,22] Our suggested corollary would be that irisin is increased in metabolically altered situations and may diminish as a consequence of the weight loss, as irisin is then 'less' needed to restore the altered metabolic state. Thus, the theory about a possible irisin resistance appears similar to the well-known leptin insensitivity in obesity and cannot be discarded[21] as has been reported for leptinemia and insulinemia after dieting.[11]

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 the dietary pattern, depending on the carbohydrate content, in order to prevent/improve the rise on glucose, insulin or HOMA index values, linked to latter damage on multiple organs.[23] This finding is interesting given that modifying the macronutrient distribution is a recurrent approach for treating obese and MetS patients.[24]

The observed results appear to be irrespective to the physical activity, as patients in this study maintained the same physical activity level along the intervention. 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. Also, some other relevant measurements in relation to glucose metabolism, such as OGTT or Clamp-test would be appropriate. However, the design of the current trial based on a

nutritional intervention involving a quite large human sample is indeed a valuable feature enabling pre- and post-test comparisons within subjects. An effect of regression to the mean could not be attributed since pertinent statistical procedures were performed in order to control this phenomenon.

This research concerns the investigation of a potential role of irisin on impaired glucose homeostasis associated to obesity and, consequently, the metabolic interplay on glucose metabolism and insulin secretion control. Indeed, the search of predictive laboratory markers is of value for clinical practice.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Nutrición Hospitalaria

Artículo especial

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

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Abstract

Introduction: 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.

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Key words: *Metabolic syndrome. Weight loss. Inflammation. Oxidative stress. Mediterranean diet.*

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

Resumen

Introducción: La alta prevalencia del síndrome metabólico (SM) en España requiere de esfuerzos adicionales para su prevención y tratamiento.

Objetivo: El estudio RESMENA-S tiene como objetivo mejorar criterios clínicos de SM y biomarcadores asociados a través de un tratamiento integral.

Métodos: El estudio consiste en un ensayo aleatorizado de diseño paralelo y prospectivo en el que está previsto participen un total de 100 individuos. El grupo RESMENA-S (n = 50) sigue una dieta personalizada de pérdida de peso (restricción energética 30%), con una distribución en macronutrientes (hidratos de carbono/grasas/ proteínas) de 40/30/30, elevada frecuencia de ingestas (7/día), bajo índice/ carga glucémica y elevada capacidad antioxidante y adherencia a la dieta Mediterránea. El grupo control (n = 50) sigue una dieta con la misma restricción energética y basada en la Asociación Americana del Corazón. El estudio tiene una duración de 8 semanas bajo control dietético y psicológico en ambos grupos. Durante un periodo adicional de 16 semanas de auto-control, los voluntarios siguen el mismo patrón dietético pero sin ningún asesoramiento específico.

Resultados: Datos antropométricos y de composición corporal, así como muestras sanguíneas y de orina están siendo recogidas al inicio y al final de cada fase. Este proyecto está registrado en www.clinicaltrials.gov con el número NCT01087086 y cuenta con la aprobación del Comité de Ética de Investigación de la Universidad de Navarra (065/2009).

Conclusiones: Las intervenciones que favorezcan la adopción de patrones dietéticos y de estilo de vida más saludables, son de elevada importancia para identificar los mecanismos que podrían explicar el nexo de unión entre obesidad, SM y complicaciones asociadas.

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Palabras clave: *Síndrome metabólico. Pérdida de peso. Inflamación. Estrés oxidativo. Dieta mediterránea.*

Tumor necrosis factor alpha (TNF-alpha); interleukin (IL); plasminogen activator inhibitor protein-1 (PAI-1); protein C reactive (PCR); complement factor 3 (C3); retinol binding protein 4 (RBP4); malondialdehyde (MDA); cardiovascular disease (CVD); calorie restriction (CR); dual-energy X-ray absorptiometry (DEXA); State-Trait Anxiety Inventory (STAI); homeostasis model assessment-estimated insulin resistance (HOMA-IR),

Introduction

Obesity rates are currently achieving epidemic proportions worldwide.[1,2] The metabolic syndrome describes a clustering of metabolic abnormalities that increase the cardiovascular and diabetes risk, which not only includes the obesity as a process characterized by an excess of body fat conditioned by genetic and environmental determinants, but also other related diseases sharing risk factors and most likely mechanisms of action.[3-6] Inflammatory markers, such as tumor necrosis factor alpha (TNF-alpha), interleukin 6 and 18 (IL-6; IL-18), plasminogen activator inhibitor protein-1 (PAI-1), protein C reactive (PCR), complement factor 3 (C3), retinol binding protein 4 (RBP4), intercellular adhesion molecules (ICAMs), vascular cell adhesion molecules (vCAMs), asymmetric dimethylarginine (ADMA), ceruloplasmin and leptin have been negatively correlated to clinical features of the metabolic syndrome.[7-11] In addition, the evaluation of several markers of the oxidant/antioxidant status such as malondialdehyde (MDA), oxidized LDL and the total antioxidant capacity of the plasma might be also relevant to understand the mechanisms behind the development of clinical metabolic syndrome features.[12] Further research in both, the role of inflammation and oxidative stress in the metabolic syndrome is therefore needed to elucidate those mechanisms.[13] For this purpose, new nutritional interventions and complementary approaches for effective prevention and treatment of metabolic syndrome must be designed.

The Mediterranean diet, a dietary pattern that has attracted considerable interest because of its potential advantages in the prevention of chronic diseases[14,15] contains many food compounds with putative anti-inflammatory and/or antioxidant effects.[16] A Mediterranean diet supplemented with virgin olive oil or nuts has for example recently shown an anti-inflammatory effect reducing serum CRP, IL-6 and endothelial and monocytary adhesion molecules and chemokines in comparison to a low-fat diet in subjects at a high cardiovascular risk in the PREvención con DIeta MEDiterránea (PREDIMED) Study.[17] A regular weekly consumption of legumes within a hypocaloric diet also resulted in a specific reduction in CRP and C3 and a clinically significant improvement of the lipid profile and blood pressure in overweight and obese subjects.[13] The application of nutrigenomics techniques

for large-scale profiling of genes, proteins and metabolites recently showed that a short-term double-blind, crossover study with a dietary mix containing resveratrol, alpha-tocopherol, vitamin C, n-3 polyunsaturated fatty acids and tomato extract, all of them naturally present in the Mediterranean diet, was able to modulate inflammation of adipose tissue, improve endothelial function, and increase liver fatty acid oxidation in overweight men with mildly increased C-reactive protein concentrations.[18]

Other dietary factors have been shown to be effective in metabolic syndrome and related pathologies.[19] For example, high protein diets might guarantee a satiety effect and a lower recovery of the lost weight according to the results derived from The DIet, Obesity and GENES (DIOGENES) study due to the higher thermogenic effect of this nutrient.[20-22] Low glycemic load diets have proved to positively affect gene expression in subcutaneous adipose tissue in persons with metabolic syndrome[11,23] and a daily consumption of 3 portions of whole-grain foods has recently demonstrated to significantly reduce CVD risk in middle-aged people mainly through blood pressure-lowering mechanisms.[24]

In addition to the quantitative and qualitative composition of the diet, other factors related to eating behavior habits might significantly influence the success of a weight-loss nutritional intervention.[24-28] Factors such as the meal frequency, the size of the eating portions, the distribution of the portions along the day, the subjective feelings of hunger and the quality of life and mood of the subject might be taken into account in current and future interventions.[29]

A decrease in energy intake by means of dietary restriction has also shown to lower the risk of CVD in obese populations.[30] The most common form of dietary restriction implemented is daily calorie restriction (CR), which requires individuals to decrease their energy intake by 15-40% of baseline needs each day.[19,31] Preliminary results from the Comprehensive Assessment of the Long-term Effect of Reducing Intake of Energy (CALERIE) Study, aimed to test the effects of 25% CR in 150 non-obese healthy subjects aged 25-45 years, have pointed out significant alterations in energy metabolism, oxidative damage, insulin sensitivity, and functional changes in both the neuroendocrine and sympathetic nervous systems.[32,33] Interestingly, the 6-month CR intervention not only caused favorable physiological responses in body composition, diabetes risk factors, CVD risk, biomarkers of longevity, energy expenditure, endocrinology and physical activity, but also psychological and behavioral responses.[34,35] However, it is still a challenge for most individuals to practice CR in an obesogenic environment so conducive to overfeeding. Other dietary and non-dietary factors must be also considered in the design of new nutritional strategies involving psychological, health-care and social support to deal with the problem of obesity and overweight.[36,37] Behavioural

therapy based on the Mediterranean diet has been recently reported as a useful tool for obesity treatment.[38] Behavioural therapy in relation to body weight management is based on the principles of "conditioning", which indicate that eating is frequently associated with external events closely linked to ingestion.[39] There are different techniques used in behavioural therapy, such as stimulus control, self-monitoring, positive reinforcement, or cognitive restructuring.[38] A healthy lifestyle needs planning, skill in the choice of alternatives and in estimating portion sizes, and compliance in recording food intake and energy expenditure. All this needs time to be learnt and maintained, which is one of the objectives of applying behavioural therapy techniques in nutritional studies dealing with weight loss and maintenance. Subjects can then develop skills in order to adopt proper habits and attain their healthiest weight, learning to establish realistic goals and evaluating their progressin modifying eating and physical activity habits.

Objective

The main aim of the present study is to reduce body weight and to manage the oxidative and inflammatory impaired status of Spanish obese adults with metabolic syndrome features by means of a controlled parallel nutritional intervention based on caloric restriction personalized diets accompanied by dietary counseling and psychological control.

The specific objectives of the study were the following:

1. To diagnose subjects with clinical features of metabolic syndrome based on medical history and measurements of anthropometry and biochemistry.
2. To evaluate the dietary intake and non dietary habits (alcohol intake, smoking habits, and physical activity) of the study participants through validated questionnaires.
3. To gather psychosocial information that might influence the selection of foods (socio-economical factors) and meal habits (meals outside home per week, eating rate, etc.) of the subjects.
4. To estimate the resting energy expenditure of each study participant to design a personalized diet adjusted to his own intake real needs.
5. To design a personalized diet characterized by variety, a high adherence to the Mediterranean Diet, a high intake of dietary antioxidants from natural sources, a low glycemic index and a macronutrient distribution by energy of 40/30/30 (carbohydrate/fat/protein) distributed with a high meal frequency (7 meals a day) that compete to conventional AHA-recommendation based diets.
6. To promote nutritional education, firstly by means of an informative session for the study participants and their families at the beginning of the study, emphasizing in selecting and distributing foods thorough the day; and secondly by attending dietary counseling with dietitians every 15 days during the intervention period.
7. To evaluate the body composition through bioimpedance and dual-energy X-ray absorptiometry (DEXA), at the beginning of the study, after two months of intervention and at the end of the study.
8. To measure representative markers of the oxidative and inflammatory status related to adiposity, CVD and insulin resistance, at the beginning and after two months of the intervention.
9. To capture changes in the lipid, glucose and hormone metabolism of the subjects due to the nutritional intervention by applying non-targeted metabolic profiling approaches.
10. To elucidate gene-diet interactions due to the nutritional intervention.
11. To evaluate the effects of the nutritional intervention on anxiety and psychological traits related metabolism.

Subjects and methods

Inclusion and exclusion criteria

The inclusion and exclusion criteria for the study are shown in table I. The criteria for metabolic syndrome were based on those established by the International Diabetes Federation.[40]

The study 065/2009 was approved by the local ethical committee (Research Ethics Committee of the University of Navarra). All study participants signed an informed consent document after verbal and written instructions and according to local legislation (see document). This trial is registered at www.clinicaltrials.gov as NCT01087086.

Recruitment of participants

The recruitment of the participants is being carried out with the help of the Department of Endocrinology of the Health Department of Navarra and the Department of General Medicine of the University Hospital of Navarra. Advertisements (poster approved by the Ethical Committee), internet, interviews to local press and to the University of Navarra information office, and databases from previous studies in the Department have been used for recruitment.

Sample size estimations

Calculations were based on findings of previous studies.[41,42] A group size of 40 was estimated to be necessary

Table I
Inclusion and exclusion criteria

Inclusion		Exclusion
Age: 35-65 years old		Subjects with difficulty for changing dietary habits
Central obesity (WC[1] > 94 cm males and > 80 cm females)		Subjects with psychiatric or psychological disorders
plus any two of the following four factors:		Subjects with eating disorders (bulimia; test of Edinburgh)
Raised triglycerides	≥ 150 mg/dL or specific treatment for this lipid abnormality	Subjects with weight instable for 3 months before the beginning of the study
Reduced HDL cholesterol	< 40 mg/dL in males < 50 mg/dL in females or specific treatment for this lipid abnormality	Subjects under any pharmacological treatment
Raised blood pressure	Systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg or treatment of previously diagnosed hypertension	Subjects with chronic diseases related to the metabolism of energy and nutrients (gastric ulcer, disorders of the digestive system, hyperthyroidism, hypothyroidism)
Raised fasting plasma glucose	≥ 100 mg/dL or previously diagnosed type 2 diabetes	Peri- and postmenopausal women
		Subjects on special diets
	If above 100 mg/dL , OGTT is strongly recommended but is not necessary to define presence of the syndrome	Subjects with food allergies or intolerances

[1]WC: waist circumference; BP: blood pressure; OGTT: oral glucose tolerance test.

in order to obtain a significant (P < 0.05) difference in the reduction of the waist circumference of 4.3 ± 6.8 cm with a power of 80%. Given an estimated dropout rate of 25%, the sample size was fixed to 50 subjects in each group (intervention and control group).

Study design

The study was designed as a 6-month weight-loss caloric restriction trial divided in 2 consecutive phases. The first phase consisted of an 8-week controlled parallel intervention period, which was followed by a 16-week self-control second phase. At the beginning of the study, subjects were randomized either to a moderately high protein weight-loss diet (group A, n = 50) or to a weight-loss diet based on the American Heart Association recommendations (Group B, n = 50). Diets in both groups (table II) were designed on a daily caloric restriction of 30% of the subjects total energy baseline needs. Group A diets were characterized by a macronutrient distribution of 40/30/30 (carbohydrate/lipid/protein), a high adherence to the Mediterranean dietary pattern, an intake of low glycemic index carbohydrates, a higher supply of energy from protein at the end of the day, a high total antioxidant capacity, and a meal frequency of 7 meals/day. A weekly intake of at least 3 portions of wholegrain pasta, 3-4 portions of legumes, 3 portions of fatty fish and 6 fruits/vegetables portions was mandatory. Group B diets were characterized by a macronutrient content of 55/30/15 (carbohydrate/lipid/protein) distributed in 3- 5 meals/day.

Table II
*Examples of 1-day diet for each dietary group
(1,300 kcal diet)*

	Group A	Group B
Breakfast	Orange (175 g) 2 low-fat yogurts (2 x 125 g)	Orange (175 g) 2 low-fat yogurts (2 x 125 g) 1 slice of refined white bread (15 g)
Morning snack I	1 low-fat yogurt (125 g)	Apple (125 g) 1 low-fat yogurt with sugar (125 g)
Morning snack II	2 thin slices of ham (45 g) 2 slices of whole-grain bread (20 g)	
Lunch	Vegetables (cooked; 250 g) Whole-grain pasta (cooked; 45 g) Lean fish (cooked; 140 g) Apple (125 g)	Vegetables (cooked; 240g) Pasta (cooked; 90 g), 1 slice of refined white bread (15 g) Lean fish (40 g) Melon (250 g) 1 low-fat yogurt (125 g)
Afternoon snack I	1 low-fat yogurt (125 g)	Banana (75 g)
Afternoon snack II	Walnuts (10 g) Low-fat cheese (60 g)	
Dinner	Salad (200 g) 1 slice of whole-grain bread (30 g) Lean meat (cooked, 80 g) Pear (150 g)	Salad (200 g) 1 slice of refined white bread (30 g) 2 slices of ham (60 g) Pear (150 g)

During the first phase of the study, subjects received dietary counseling by qualified nutritionists every 15 days, while during the second phase of the study sub-
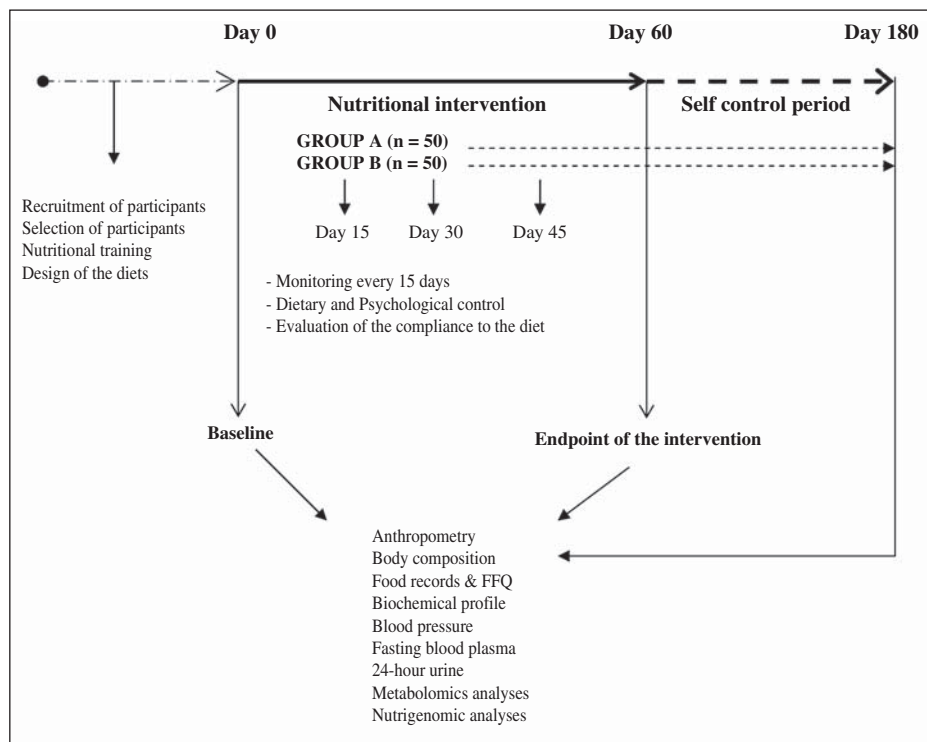
Fig. 1.—Experimental design of the RESMENA-S study.

jects were asked to follow the diets without any dietary advice. In addition, subjects were asked to fill in a validated personality test (NEO-PIR) at the beginning of the study. Validated psychological tests (Beck depression Inventory;[43] State-Trait Anxiety Inventory (STAI);[44] and Anxiety thermometer)[45] to evaluate their levels of anxiety during the weight-loss diet intervention were filled at the beginning of the study, every 15 days during the first phase, and at the end of the study. Figure 1 shows the experimental design of the study.

Screening visit

Recruited participants will attend a screening visit in which they will receive a written document with information about the study together with the informed consent to be signed (Appendix). Both documents were approved by the Research Ethics Committee of the University of Navarra. During this visit, any doubt concerning the participation of the subject in the study will be solved by qualified staff. After a medical examination by a physician, anthropometric parameters and blood pressure will be measured and a fasting blood sample will be drawn by a nurse for the biochemical determination of metabolic syndrome clinical features. The subject will be asked to fill in a validated questionnaire concerning food frequency and dietary habits (SUN questionnaire) and a 72-hour food record, data that together with the calculation of the basal metabolic rate of the subject will be used by the dietitian to design their personalized diets.[46] A val-

idated test of personality (NEO-PI-R) will be also filled in by the participant. Those subjects who meet the inclusion criteria will be given an appointment for the first visit of the intervention and for an informative group session.

Study visits

Six visits were planned for each subject in the course of the study at days 0, 15, 30, 45, 60 and 180. Details of the determinations and measurements for each of them are given in table III. The following metabolic determinations and markers will be also calculated at the beginning and end of the study: body mass index, waist-to-hip index, homeostasis model assessment-estimated insulin resistance (HOMA-IR) and atherogenic index.

In addition to the intervention visits, participants from group A were requested to attend an informative group session to reinforce psychological attitude at the beginning of the study. In this session, two qualified nutritionists are explaining the benefits of the dietary pattern to follow in the study, the options to create their own personalized menus with the established personalized diets, as well as emphasized the importance of eating habits and compliance during both the controlled and self-control phases of the study. At the end of the study, participants are receiving a report about the evaluation of their nutritional status in a last dietary counseling visit with the dietitian.

HOJA INFORMATIVA PARA EL PARTICIPANTE

TÍTULO: PROYECTO DE INTERVENCIÓN NUTRICIONAL PARA PACIENTES CON SÍNDROME METABÓLICO

Responsable del estudio: Marian Zulet Alzórriz

(Informative document about the study for the participants)

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

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

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

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

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

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

En la cita del día 0 se le volverá a recordar en qué consiste su participación y se le tomarán medidas de peso, talla, perímetro cintura y de composición corporal. Al mismo tiempo se le realizará una serie de preguntas relacionadas con el estado anímico y el grado de ansiedad. A continuación se le tomará la tensión arterial y la enfermera le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina (colesterol, glucosa, etc) y otros más específicos de relacionados con síndrome metabólico, entre ellos un análisis de la expresión de determinados genes. La dietista le proporcionará su dieta personalizada para comenzar el estudio de intervención nutricional. Deberá ajustarse a las pautas que se le establezcan, como la ingesta de alimentos, forma de preparación y a las recomendaciones de estilo de vida.

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

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

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

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

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

SU PARTICIPACIÓN EN EL ESTUDIO NO ESTÁ REMUNERADA

Application of "omics" tools in the study

In addition to traditional determinations, the RESMENA-S study will also apply metabolomics and transcriptomics approaches.

Metabolites are the cellular endpoints of gene expression and of any physiological regulatory process. Measuring such compounds might therefore offer deeper insights into mechanisms of health and disease as well as might provide a greater understanding the role of lifestyle and dietary factors[47] in relation to specific diseases such as metabolic syndrome. Currently metabolomics is driven by the breathtaking advancements of analytical techniques providing constantly improved sensitivity and larger metabolite panels, but also by advances in chemometrics and bioinformatics. Among the metabolomics platforms, those based on mass spectrometry are increasingly used to characterize the complex meta-

Visit day	0	15	30	45	60	180
Body weight	x	x	x	x	x	x
Blood pressure (SBP, DBP)	x				x	x
Waist and hip circumference	x				x	x
Skin-folds[(1)]	x				x	x
Body composition by bioimpedance	x	x	x	x	x	x
Body composition by DEXA	x				x	x
Collection of fasting blood[(2)]	x				x	x
24-h urine collection	x				x	x
Fasting plasma glucose	x				x	x
Fasting plasma insulin	x				x	x
Free fatty acids	x				x	x
Cholesterol, HDL-Chol, LDL-Chol	x				x	x
Total proteins	x				x	x
Inflammatory markers[(3)]	x				x	x
Oxidative-stress markers[(4)]	x				x	x
Metabolomics analyses[(5)]	x				x	x
Gene expression analyses[(6)]	x				x	x
Dietary counseling	x	x	x	x	x	x
72 hour-weight food record	x	x	x	x	x	x
VAS questionnaire					x	
State-Trait Anxiety Inventory (STAI)	x				x	x
Beck depression Inventory	x				x	x
Anxiety thermometer	x	x	x	x	x	x

[(1)]Bicipital, tricipital, subescapular suprailiac skin-folds.
[(2)]Plasma, erythrocytes and peripherial blood mononuclear cells sampling.
[(3)]Homocistein, C3, Ceruloplasmin (Turbidimetry COBAS MiraS); Retinol binding protein 4 (RBP4), asymmetric dimethylarginine (ADMA), Protein C reactive (PCR), Interleukins IL-6 and IL-18, TNF-alpha, PAI-1, fibrinogen.
[(4)]Selenium, Oxidized LDL Malondialdehyde, Total antioxidant capacity of plasma.
[(5)]Metabolomics analyses will be performed by non targeted metabolic profiling based on LC/MS.
[(6)]Nutrigenomics gene expression analyses will be performed by Real time PCR.

bolic effects of nutrients and foods.[48] In this sense, in the RESMENA-S study we aim to monitor metabolic changes associated with the 6-month weight loss nutritional intervention by applying non-targeted metabolic profiling approaches to blood and urine samples of the participants at the beginning and end of each phase of the study.[49,50] The application of metabolomics might help to elucidate the effect of the personalized caloric restriction diets on low-grade inflammation, oxidative stress and whole body metabolism with a special interest in the lipid and glucose metabolism. In addition, the metabolomic analyses will be used to investigate the effects that the different diets in the RESMENA-S intervention can have on stress and anxiety trait-related metabolism. In fact, the identification of different metabolic phenotypes and its combination with other phenotypic data to give dietary advice could be envisaged as a potential role of metabolomics in personalized nutrition,[51,52] with a special interest in obesity related diseases such as the metabolic syndrome.

Nutrigenomic profiling and epigenetics studies are envisaged in both dietary groups in order to establish predictive patterns concerning the response to the nutritional intervention as well as prognostic markers of the outcomes.[53] These tasks will be performed using molecular and genetic tools such as microarrays, RT-PCR and sequencing in selected genes related to inflammation, energy homeostasis, and lipid metabolism in order to elucidate the role of gene expression in metabolic syndrome related phenotypes.

Facilities

The Department of Nutrition, Food Sciences, Physiology and Toxicology at the University of Navarra is provided with the facilities and equipment needed for the evaluation and monitoring of the nutritional status of the study participants. This is mainly based in medical data collection, anthropometry and body composi-
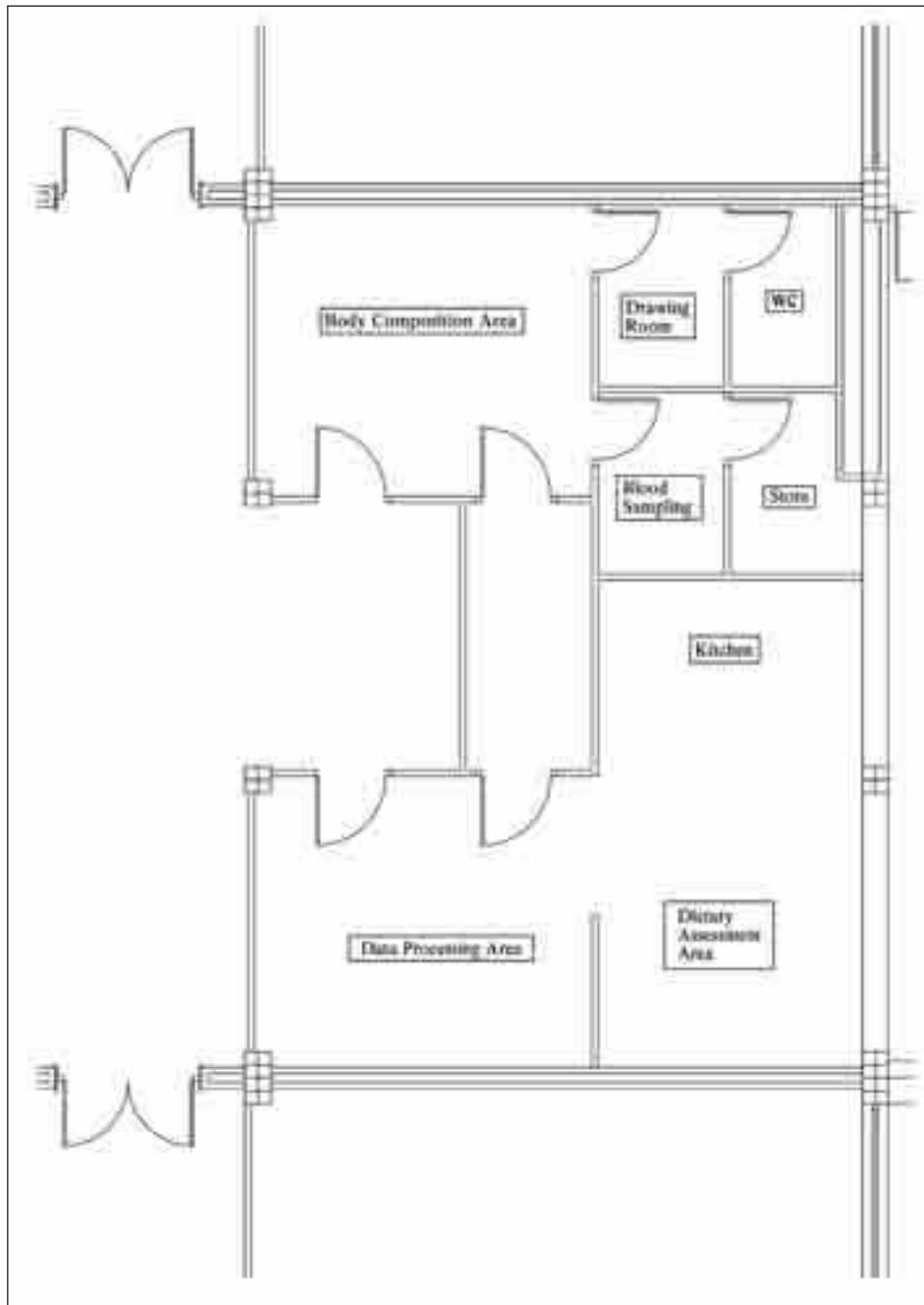
Fig. 2.—Schematic distribution of the metabolic unit.

tion equipments (tapes, scales, tensiometers, skin fold calipers, devices for bioelectrical impedance analysis and DEXA) and dietary measurements (calorimeter, validated food questionnaires, nutrition softwares such as Medisystem, DIAL, Nutriciun). The availability of a recently renovated Metabolic Unit for individual attention to the patient, monitoring and dietary counseling along the intervention study has been of great help (fig. 2). The unit is divided in different areas: an office for medical visiting hours equipped with DEXA and bioimpedance devices; a changing room and a toilet; an area for blood collection; an area for calorimeter measurements;

an room for dietary counseling, equipped with a dining room table, microwave, a cooktop, a fridge, and a sink; and an area for participant recruitment formalities, control of medical histories and writing of patient reports.

Novelty of the study and conclusions

The present initiative is based on our traditional diet but from a more personalized point of view. RES-MENA-S Study aims to integrate the main results obtained from the latest observational epidemiological

studies and interventional studies in the dietary pattern designed for obese subjects with metabolic syndrome features.[23,54-58] The study will apply the concept of selecting and distributing the foods thorough the day according the physiological needs of each individual, and will not only consider the quantitative and qualitative composition of the diets, but other important factors related to dietary habits. The importance of regular dietary support and psychological control will be also evaluated for a successful loss of weight and improvement of clinical features of metabolic syndrome.

Furthermore, the combination of varied tools generating detailed clinical chemistry, anthropometry, and metabolite and gene data will allow to perform a functional analysis for biological interpretation of the impact of personalized diets based on the Mediterranean dietary pattern and energy restriction in the treatment of metabolic syndrome features.

Acknowledgements

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Appendix 6: *Other Articles*

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