Universidad de Navarra

Facultad de Farmacia

INFLUENCE OF COCOA EXTRACT INTAKE ON OXIDATIVE, CARDIOMETABOLIC AND PSYCHOLOGICAL STATUS, INCLUDING A METABOLOMIC APPROACH IN MIDDLE-AGED OBESE SUBJECTS

INFLUENCIA DE LA INGESTA DE EXTRACTO DE CACAO EN EL ESTADO OXIDATIVO, CARDIOMETABÓLICO Y PSICOLÓGICO, INCLUYENDO UN ENFOQUE METABOLÓMICO EN SUJETOS OBESOS DE MEDIANA EDAD

Idoya Ibero Baraibar

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Memoria presentada por Dña. **Idoya Ibero Baraibar** para aspirar al grado de Doctor por la Universidad de Navarra.

Dña. Idoya Ibero Baraibar

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

Pamplona, 1 de Diciembre de 2015

Prof. J. Alfredo Martínez Hernández

Dr. M. Ángeles Zulet Alzórriz

A mis padres

"No basta dar pasos que un día puedan conducir hasta la meta, sino que cada paso ha de ser una meta, sin dejar de ser un paso"

(Johann P. Eckermann)

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¡Muchas gracias a todos!

LIST OF ABBREVIATIONS

-A-

ANCOVA: Analysis of covariance

ARIC: Atherosclerosis risk in communities cohort

-B-

BDI: Beck depression inventory

BMI: Body mass index

-C-

CHO: Carbohydrates

CNTA: National centre for food Safety and technology – Centro nacional de tecnología y seguridad alimentaria

CRP: C-reactive protein

CTNS: Centre tecnològic de nutrició i salut

CVD: Cardiovascular disease

-D-

DAPI: 4,6-diamidino-2-phenylindole

DBP: Diastolic blood pressure

DEXA: Dual-energy X-ray absorptiometry

DNA: Deoxyribonucleic acid

DORICA: Dislipidemia, obesidad y riesgo cardiovascular en España

DSM-V: Fifth edition of the diagnostic and statistical manual of mental disorders

-E-

EDTA: Ethylenediaminetetraacetic acid

EFSA: European food safety authority

eNOS: Endothelial nitric oxide synthase

ENRICA: Estudio de nutrición y riesgo cardiovascular en España - Study on nutrition and cardiovascular risk in Spain

EPIC-Norfolk: European prospective investigation into cancer-norfolk

ESI: Electrospray

ESMEeD: European study of the epidemiology of mental disorders

-F-

FFA: Free fatty acid

FFQ: Food frequency questionnaire

FMD: Flow mediated dilation

FPG: Formamidopyrimidine DNA glycosylase enzyme

-G-

GABAa: Adenosine and benzodiazepine receptor

-H-

HDL-c: High-density lipoprotein-cholesterol

HL: Hepatic lipase

HOMA-IR: Homeostasis model assessment of insulin resistance

HPA-axis: Hypothalamic pituitary adrenal axis

HPLC: High-performance liquid chromatography

-I-

ICAM-1: Intercellular cell adhesion molecule-1

IL-1: Interleukin-1

IL-6: Interleukin-6

IL-8: Interleukin-8

-L-

LDL-c: Low-density lipoprotein-cholesterol

LPL: Lipoprotein lipase

Lp-PLA2: Lipoprotein associated phospholipase A2

-M-

MAO: Monoamine oxidase

MCP-1: Monocyte chemoattractant protein-1

MPHG: 3-methoxy-4-hydroxyphenylglycol

MPO: Myeloperoxidase

MUFA: Monounsaturated fatty acid

-N-

NAFLD: Non-alcoholic fatty liver disease

NHANES: National health and nutrition examination survey

NO: Nitric oxide

-O-

oxLDL: Oxidised low-density lipoprotein-cholesterol

-P-

PCA: Principal component analysis

pHVA: Plasma homovanillic acid

PREDIMED: Prevención con dieta mediterránea

PUFA: Polyunsaturated fatty acids

-R-

RCT: Randomised controlled trial

ROS: Reactive oxygen species

RT: Retention time

-S-

SB: Strand break

SBP: Systolic blood pressure

SFA: Saturated fatty acid

STAI: State trait anxiety inventory

SULT: Sulfotransferase

-T-

TG: Triglyceride

TNF-α: Tumor necrosis factor-α

-U-

UDPGT: Uridine diphosphate glucuronyltransferase

-V-

VCAM-1: Vascular cell adhesion molecule-1

VitD: Vitamin D

VLDL: Very low-density lipoprotein-cholesterol

-W-

WHO: World health organization

25(OH)D: 25-hydroxivitamin D

8-oxodG: 8-oxo-deoxyguanosine

List of publications

I. **Ibero-Baraibar I**, Abete I, Navas-Carretero S, Massis-Zaid A, Martinez JA, Zulet MA. Oxidised LDL levels decreases after the consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet. *Nutr Metab Cardiovasc Dis* 2014, 24, 416-422.

II. **Ibero-Baraibar I,** Azqueta A, Lopez de Cerain, Martinez JA, Zulet MA. Assessment of DNA damage using comet assay in middle-aged overweight/obese subjects after following a hypocaloric diet supplemented with cocoa extract. *Mutagenesis* 2015, 30, 139-146.

III. **Ibero-Baraibar I**, Suarez M, Arola-Arnal A, Zulet MA, Martinez JA. Cocoa extract intake for 4 weeks reduces postprandial systolic blood pressure response of obese subjects, even after following an energy restricted diet. *Food & Nutrition Research* 2015 (Under review).

IV. **Ibero-Baraibar I**, Perez-Conago A, Ramirez MJ, Martinez JA, Zulet MA. An increase in plasma homovanillic acid with cocoa extract consumption is associated with the alleviation of depressive symptoms in overweight or obese adults on an energy restricted diet in a randomized controlled trial. *Journal of Nutrition* 2015 (Second revision).

V. **Ibero-Baraibar I**, Romo-Hualde A, Gonzalez-Navarro CJ, Zulet MA, Martinez JA. Urinary metabolomic profile following the intake of meals supplemented with cocoa extract in middle-aged obese subjects. *Food & Function* 2015 (First revision).

VI. **Ibero-Baraibar I,** Navas-Carretero S, Abete I, Martinez JA, Zulet MA. Increases in plasma 25(OH)D levels are related to improvements in body composition and blood pressure in middle-aged subjects after a weight loss intervention: Longitudinal study. *Clin Nutr* 2015, 34, 1010-1017.

Abstract

Obesity is considered the epidemic of the XXI century, which is associated with oxidative stress and inflammation. An excessive body weight contributes to increase the risk of suffering from other diseases, such as insulin resistance, dyslipidemia, cardiovascular disease (CVD), psychological disorders and cancer among others. The principal strategy to treat obesity is to improve dietary habits (increasing the consumption of vegetables and fruits and reducing fat intake) and increase physical activity. Interestingly, nowadays new approaches to combat obesity and associated comorbidities are being investigated.

Antioxidants and phytochemic compounds, such as polyphenols and vitamins are naturally occurring compounds with potential beneficial properties for human health. Cocoa is one of the richest sources of antioxidants, principally flavanols, a type of polyphenols. Cocoa flavanols can be found as monomeric (epicatechin and catechin) and polymeric flavanols (oligomers and procyanidins). According to previous studies cocoa consumption could be beneficial for blood pressure reduction, to control glucose and cholesterol levels and to improve endothelial dysfunction and oxidative stress impairments, among other disorders. Phytochemicals from cocoa are usually consumed within chocolate bars or cocoa beverages. However, taking into account the potential benefits of cocoa consumption, it could be used as a functional ingredient to be included in other foods for human consumption, such as ready-to-eat foods.

In this context, the principal aim of this study was to assess if the daily consumption of 1.4 g of cocoa extract (645 mg of polyphenols with 415 mg of flavanols) within ready-to-eat meals and consumed under an energy restricted diet (-15% E) of 4 weeks, could have beneficial effects on the nutritional and the metabolic status, as well as on depression and anxiety symptoms of middle-aged overweight/obese subjects. In this sense, the specific objectives were: **1)** to analyse the effect of the dietary intervention on general nutrition and metabolism; **2)** to assess the effects of cocoa extract consumption on anthropometry and body composition, blood pressure, routine blood biochemical determinations and biomarkers related to oxidative status, endothelial function and inflammation; **3)** to investigate the acute effects of cocoa consumption on blood pressure and blood biochemical markers (0, 60, 120 and 180 min), before and after 4 weeks of daily cocoa consumption within a diet for weight loss; **4)** to analyse the effect of cocoa intake within ready-to-eat meals on depressive and anxiety symptoms, as well as on the peripheral dopaminergic activity after 4 weeks of weight loss intervention; **5)** to evaluate plasma and urinary metabolomic profile in order to assess the presence of cocoa derived metabolites with the aim to obtain reliable information about the compliance of the volunteers concerning the intake of ready-to-eat meals, the availability of cocoa compounds as well as to investigate metabolomic changes with interest for human health.

In order to achieve the previously determined aims, a 4 week, double-blind, randomised, placebo-controlled parallel nutritional intervention with a simultaneous postprandial sub-study was carried out. Fifty subjects were recruited, 25 in each experimental group. The intervention

consisted of the daily intake of ready-to-eat meals, 1 dish and 1 dessert, integrated within a 15% energy restricted diet. Each dish and each dessert was supplemented with 0.7 g of cocoa extract in the case of cocoa group, representing a total consumption of 1.4 g of cocoa extract per day. At the end of the intervention, 24 subjects completed the study in the control group and 23 subjects in the cocoa group.

The principal results of this investigation showed that the prescription of a 4 week dietary strategy with a 15% of energy restriction and moderately high on protein content, lead to improvements in the general nutritional status, inflammatory, oxidative and endothelial dysfunction markers and it contributed to reduce depressive symptoms in middle-aged overweight/obese subjects.

Concerning the inclusion of 1.4 g of cocoa extract within the 15% energy restricted diet, a significantly higher reduction of oxidised low-density lipoprotein-cholesterol (oxLDL) levels were found in cocoa consumers. However, when the oxidative DNA damage was evaluated, no differences between cocoa consumers and the control group were found. Nevertheless, the cocoa group showed a negative correlation between oxidised bases and some cocoa metabolites in plasma. Moreover, subjects who started the intervention with higher levels of damage showed a greater reduction in oxidised bases when compared to those who had lower baseline levels, suggesting that the influence of cocoa intake over oxidative status at DNA level could be observed with a longer period of intervention or with higher levels of DNA damage at baseline.

Interestingly, the regular consumption of 1.4 g/day of cocoa extract within a hypocaloric diet during a period of 4 weeks resulted in a higher reduction of the acute systolic blood pressure (SBP) response independently of body weight loss.

The consumption of cocoa extract during 4 weeks did not show a direct effect on Beck Depression Inventory (BDI), but interestingly, pHVA, which is a marker reflecting dopaminergic activity in the brain, showed a greater increase in cocoa consumers in comparison with the control group. Importantly, pHVA was negatively associated with the reduction of depressive symptoms in cocoa consumers, suggesting the possible implication of cocoa on psychological behaviour.

On the other hand, metabolomic analyses revealed the presence of cocoa derived metabolites in plasma and urine on volunteers from cocoa group, who showed significantly higher amounts than in the control group. This outcome suggests adequate adherence of the volunteers to the intervention as well as the bioavailability of cocoa compounds within the ready-to-eat meals.

In summary, a weight loss dietary intervention with an energy restriction of 15% and moderately high in protein content, resulted in beneficial outcomes reducing anthropometric and body composition variables, cardiometabolic markers, inflammatory and oxidative markers, and contribute to the reduction of depressive symptoms. On the other hand, the consumption of 1.4 g of cocoa extract (645 mg of polyphenols with 415 mg of flavanols) resulted beneficial to improve the oxidative status by the reduction of oxLDL levels. Interestingly, the daily consumption of the cocoa extract during 4 weeks revealed a higher reduction of the postprandial SBP response compared to

the control group, suggesting an adaptive effect over time. Moreover, the intake of cocoa extract increased significantly the levels of pHVA, which was associated with a reduction on depressive symptoms. In addition, metabolomics analysis in plasma and urine suggested an adequate adherence to the nutritional intervention supported by the presence of metabolites derived from cocoa. Furthermore, the identification of metabolomic changes related with benefits attributed to cocoa consumption contributes to increase the current scientific knowledge in this area.

Resumen

La obesidad se asocia de forma positiva con un estado de estrés oxidativo y de inflamación, y es considerada la enfermedad del siglo XXI. El exceso de peso corporal contribuye a aumentar el riesgo de sufrir otras enfermedades como resistencia a la insulina, enfermedad cardiovascular, alteraciones psicológicas y cáncer entre otros. La principal estrategia para el tratamiento de la obesidad consiste en la mejora de los hábitos alimentarios (incrementando el consumo de frutas y verduras y disminuyendo la ingesta de grasas) y el aumento de la actividad física. En la actualidad se están investigando nuevas estrategias con el objetivo de combatir la obesidad y reducir las enfermedades asociadas al exceso de peso.

Los antioxidantes como los polifenoles y las vitaminas, son compuestos naturales que principalmente se encuentran en frutas y verduras, y que tienen potenciales propiedades beneficiosas para la salud. El cacao es uno de las fuentes naturales más ricas en antioxidantes y compuestos fitoquímicos, principalmente en flavanoles, una clase de polifenoles. Los flavanoles de cacao se pueden encontrar en forma monomérica (epicatequina y catequina) y polimérica (oligómeros y procianidinas). El consumo de flavanoles de cacao se asocia con diferentes beneficios para la salud, ya que estudios previos sugieren su implicación en la reducción de la presión arterial, los niveles de glucosa y el colesterol así como el estrés oxidativo y la inflamación entre otros. Los compuestos fitoquímicos del cacao son generalmente consumidos dentro de tabletas de chocolate o bebidas de cacao. Sin embargo, teniendo en cuenta los beneficios derivados de su consumo, los componentes del cacao podrían ser utilizados como ingrediente funcional, para ser incorporados en otros alimentos de consumo humano, como pueden ser los alimentos precocinados.

En este contexto, el objetivo general de este estudio fue evaluar si el consumo diario de 1,4g de extracto de cacao (645 mg de polifenoles de los cuales 415 mg flavanoles) incluido en platos precocinados y en el marco de una dieta para la pérdida de peso (-15% E y moderadamente alta en proteínas) durante 4 semanas, podría tener un efecto beneficioso sobre el estado nutricional y metabólico, así como en el estado anímico en sujetos sanos de mediana edad con sobrepeso/obesidad. En este sentido, los objetivos específicos fueron: **1)** analizar el efecto de la intervención nutricional sobre el estado nutricional general y metabólico; **2)** evaluar los efectos derivados de la ingesta de extracto de cacao sobre variables antropométricas y de composición corporal, la presión arterial, marcadores bioquímicos de rutina en sangre y marcadores vinculados al estado oxidativo, la función endotelial y la inflamación; **3)** investigar el efecto agudo (0, 60, 120 y 180 min) del consumo de extracto de cacao en variables cardiometabólicas y de presión arterial, antes y después de su consumo diario durante 4 semanas en el marco de una dieta para la pérdida de peso; **4)** evaluar si el consumo de platos precocinados suplementados con extracto de cacao muestra beneficios sobre síntomas depresivos y de ansiedad, así como en la actividad dopaminérgica del sistema periférico después de 4 semanas de intervención; **5)** Analizar metabolitos derivados del consumo de extracto de cacao en muestras de plasma y orina con el fin

de evaluar la adherencia de los voluntarios al estudio así como valorar la disponibilidad del extracto de cacao. Al mismo tiempo, se valoró en orina la posible presencia de cambios metabolómicos derivados del consumo de extracto de cacao que pudieran ser de interés para la salud humana.

Con el fin de alcanzar los objetivos previamente determinados se llevó a cabo un estudio de intervención nutricional, controlado, con una duración de 4 semanas, doble ciego, aleatorizado y paralelo. Además, también se diseñó un sub-estudio con objeto de evaluar el consumo agudo de cacao, el cual fue desarrollado simultáneamente junto al estudio principal. Se reclutaron 50 sujetos, 25 en cada grupo experimental. La intervención consistió en la ingesta diaria de platos precocinados, 1 plato y 1 postre diarios, integrados dentro de una dieta con un 15% de restricción energética. Cada plato y cada postre fueron suplementados con 0,7 g de extracto de cacao en el caso del grupo cacao, lo que supuso un consumo diario de 1,4 g de extracto de cacao, proporcionando 645 mg de polifenoles, de los cuales 415 mg fueron flavanoles. De los 50 voluntarios que iniciaron el estudio, 24 sujetos completaron la intervención en el grupo control y 23 sujetos en el grupo cacao.

Los principales resultados de esta investigación mostraron que la prescripción de una dieta con un 15% de restricción energética y moderadamente alta en proteínas durante un periodo de 4 semanas dio lugar a mejoras en el estado nutricional general, así como en los marcadores de inflamación, oxidación y disfunción endotelial y contribuyó a reducir los síntomas depresivos en sujetos de mediana edad con sobrepeso/obesidad.

En cuanto a los efectos de la inclusión de 1,4 g/día de extracto de cacao, cabe destacar que su consumo diario durante 4 semanas redujo significativamente en mayor magnitud los niveles de LDL oxidadas (oxLDL) en el grupo cacao. Sin embargo, cuando el daño oxidativo se evaluó a nivel de ADN, no se obtuvieron diferencias entre aquellos sujetos que consumieron el extracto de cacao y los sujetos del grupo control. No obstante, en el grupo cacao, el daño oxidativo en el ADN presentó una correlación negativa con algunos metabolitos del cacao presentes en plasma. Por otra parte, los sujetos que iniciaron la intervención con mayores niveles de daño oxidativo mostraron una mayor reducción de las bases oxidadas tras un periodo de 4 semanas, en comparación con aquellos que iniciaron el estudio con unos niveles basales más bajos. Estos resultados podrían indicar que con un mayor periodo de tiempo o con un mayor daño en el ADN a nivel basal, la ingesta de cacao podría tener algún beneficio adicional en cuanto al daño oxidativo en el ADN.

De forma interesante, el consumo regular de 1.4 g/día de extracto de cacao bajo una dieta para la pérdida de peso resultó en una reducción mayor de la respuesta de la presión arterial sistólica (PAS) independiente a la pérdida de peso.

La reducción de los síntomas depresivos así como el incremento del ácido homovaníllico (HVA) en plasma se observaron tras el seguimiento de la dieta para la pérdida de peso. Curiosamente, la ingesta de cacao mostró un incremento mayor de los niveles de HVA plasmático, encontrándose una asociación inversa entre este incremento y la reducción de los síntomas

depresivos solo en el grupo suplementado con cacao e independiente a la pérdida de peso, lo que sugiere que algunos componentes del cacao podrían estar involucrados en este hallazgo.

Por otra parte, los análisis metabolómicos revelaron la presencia de metabolitos derivados de cacao en plasma y orina de los voluntarios del grupo cacao, quienes mostraron mayor concentración en comparación con el grupo control. Este resultado sugiere una buena adherencia de los voluntarios al estudio así como que el cacao fue biodisponible dentro de los platos precocinados.

En conclusión, una estrategia nutricional con un 15% de restricción energética y ligeramente alta en proteínas, resultó beneficiosa en la reducción de variables antropométricas y de composición corporal, así como para mejorar los marcadores bioquímicos de rutina en sangre, la presión arterial, los marcadores de inflamación y estrés oxidativo y los síntomas de depresión, atribuyéndose estos efectos beneficiosos a la pérdida de peso. Por otro lado, la inclusión de 1,4 g de extracto de cacao al día (645 mg de polifenoles de los cuales 415 mg fueron flavanoles) durante 4 semanas resultó beneficiosa mejorando el estado oxidativo mediante la disminución de los niveles de oxLDL. Además la respuesta postprandial de la PAS se vio reducida en mayor magnitud tras el consumo diario de cacao durante 4 semanas. Por otra parte, el consumo de cacao aumentó los niveles de HVA en plasma, lo que se asoció con una reducción de los síntomas depresivos solo en el grupo cacao. Además, los análisis metabolómicos en plasma y en orina sugirieron una adherencia adecuada a la intervención nutricional apoyado por la presencia de metabolitos derivados del cacao. Además, la identificación de los cambios metabolómicos relacionados con los beneficios atribuidos al consumo de cacao contribuye a aumentar el conocimiento científico actual en esta área.

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I. INTRODUCTION

The importance of nutrition on health

According to World Health Organization (WHO) in 1946 health was defined as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (WHO, 2007). Health is determined by intrinsic (genetic, behaviour, culture, habits and lifestyle) and extrinsic factors (preventative, curative and promotional aspects, economical, social, environmental and technological factors)(WHO, 2007).

Obesity is today considered the epidemic of XXIst century (Flegal *et al.*, 2013). Genetic factors and principally habits and lifestyle factors are determinant aspects of obesity (Bhurosy and Jeewon, 2014; Chaput *et al.*, 2014). Poor lifestyle habits, such as the consumption of high-fat and high-sugar diets, increased alcohol consumption, low fruit and vegetable consumption and sedentary lifestyles are associated with the risk of suffering from obesity and comorbidities (Hill *et al.*, 2012). Nowadays, it is well known that nutritional status and health states are connected (Kostka *et al.*, 2014). In this context, some dietary patterns such as the Mediterranean diet, the increased consumption of fruits and vegetables as well as the increase of physical activity are considered healthy habits (Rautio *et al.*, 2015).

In the last years, the use of natural plants has been proposed as an alternative therapeutic approach to achieve an optimal health status (Liu, 2013). Plants are rich sources of antioxidants (polyphenols, vitamins, minerals etc.) with benefits recognised on disorders associated with oxidative stress and inflammation (Liu, 2013). Based on the scientific literature, cocoa has been postulated as a promising food source to prevent and control some health alterations such as cardiovascular diseases (CVD), insulin resistance, psychological disorders, oxidative stress and inflammation, among others (Hooper *et al.*, 2012; Kwok *et al.*, 2015; Mastroiacovo *et al.*, 2015).

1. Cocoa

Cocoa *(Theobroma cacao)* "Food of the Gods". This is the definition given by the nosologist Carl von Linné (Linnaeus) to the fatty seed of cocoa tree in 1753 (Dillinger *et al.*, 2000). However, the earliest cacao use dates from 1900 to 1500 BCE in Mesoamerica, where the Olmec, Maya and Aztec civilizations were the first who grow and consumed this seed (Coe and Coe, 1996; Verna, 2013). Christopher Columbus was the first European discovering chocolate in 1502 (Dillinger *et al.*, 2000). However, Hernán Cortés was the man who brought cocoa to King Charles of Spain in 1528 (Dillinger *et al.*, 2000). At that time, chocolate was very precious in Mesoamerica and Europe, and its consumption was reserved for few privileged groups such as governors, military officers, priests and warriors (Dillinger *et al.*, 2000).

From the ancient time, cocoa has been assigned with many beneficial properties for human health such as asthenia, digestion, anaemia, mental fatigue, fever, etc. and it came to be called "divine drink" by the Aztec Emperor Montezuma in the XVI century (Corti *et al.*, 2009; Lippi, 2013). Cocoa is a rich source of bioactive compounds with greater antioxidant capacity than other flavanol-rich foods (Lee *et al.*, 2003). Taking into consideration the benefits derived from antioxidant rich foods, the implication of cocoa consumption on health and disease has become a subject of interest.

1.1. Bioactive compounds in cocoa

Cocoa is a rich source of bioactive compounds known as phytochemicals (Ellam and Williamson, 2013; Kim *et al.*, 2014). Those phytochemicals are principally polyphenols (flavonoid and nonflavonoid polyphenols) and methylxanthines (theobromine) (Kim *et al.*, 2014).

1.1.1. Polyphenols

Polyphenols are the most extensive group of natural antioxidants in cocoa, comprising between 6-8% and 12-18% of dry cocoa bean (Wollgast and Anklam, 2000; Andres-Lacueva *et al.*, 2008). This group of compounds is synthesized by plants as secondary metabolites and are stored in the pigment cells of cotyledons, given colour to fruits and vegetables (Pandey and Rizvi, 2009). The main function of polyphenols in plants is to protect them against external stressors (Pandey and Rizvi, 2009). However, this group of compounds is also responsible of the sensory properties, stability and digestibility of plants (Serra Bonvehi and Ventura Coll, 1997; Misnawi *et al.*, 2004).

The chemical structure of polyphenols consists of an aromatic ring with one or more hydroxyl group attached (Tsao, 2010). According with the chemical structure, plant polyphenols are classified into four main categories: flavonoids, phenolic acids, stilbenes, lignans and others (Manach *et al.*, 2004; Perez-Jimenez *et al.*, 2010). Nowadays, more than 8000 phenolic structures are known and 4000 of them are flavonoids (Bravo, 1998; Harborne and Williams, 2000).

Regarding the polyphenol intake, the general population consumes around 1000 mg of total polyphenols per day (Scalbert and Williamson, 2000). In Spain, the mean daily intake of polyphenols was estimated between 2590 mg and 3016 mg (Saura-Calixto *et al.*, 2007) although recently the consumption of polyphenols in a Spanish population at high cardiovascular risk (PREDIMED study) was estimated at 820 mg per day (Tresserra-Rimbau *et al.*, 2013).

Flavonoids are the most extensive type of polyphenols in the plant kingdom and are normally classified as flavonoids and non-flavonoid phenols (Kim *et al.*, 2014; Oracz *et al.*, 2015). Flavanols, a subgroup of flavonoids, are the principal polyphenols in cocoa (Oracz *et al.*, 2015).

- *Flavonoid phenols: Flavanols*

The structure of flavonoids consists of two aromatic rings (A and B) and a heterocyclic ring (C) containing one oxygen atom (Manach *et al.*, 2004). Depending on the central heterocyclic ring and hydroxylation patterns, flavonoids can be divided into six different groups: flavones, isoflavones, flavanones, flavonols, flavanols or flavan-3- ols and anthocyanins, as is shown in **Figure 1** (Steinberg *et al.*, 2003; Yao *et al.*, 2004).

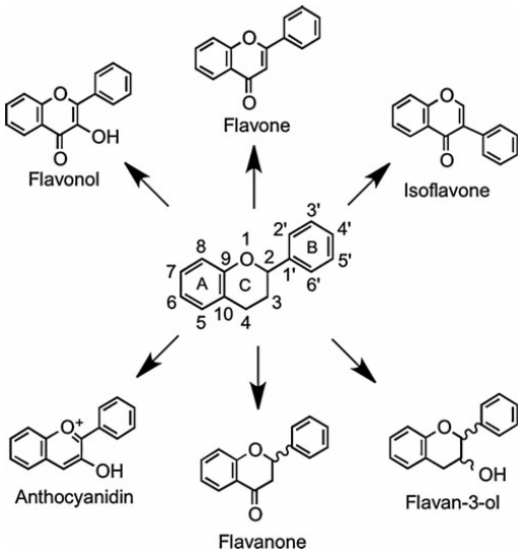

Figure 1. Flavonoid subclasses.

According with **Figure 2**, flavanols are the predominant flavonoids in cocoa (Wollgast and Anklam, 2000). Flavanols contain two aromatic rings linked by oxygenated heterocycle (Manach *et al.*, 2004). Monomers of flavanols are (−)-epicatechin and (+)-catechin. (-)-epicatechin is the major monomeric flavanol in cocoa, comprising the 35% of total phenolic content (Gu *et al.*, 2006; Andres-Lacueva *et al.*, 2008). Monomers can form links between C4 and C8 producing dimmers, oligomers and polymers (Manach *et al.*, 2004). Procyanidinis also known as condensed tannins, are polymers of epicatechin and catechin monomers, which are responsible of the astringency and bitterness of

cocoa due to the ability they have to form complexes with salivary proteins (Manach *et al.*, 2004; Del Rio *et al.*, 2013; Kim *et al.*, 2014). Oligomers (procyanidins B1, B2, B5 and C1) and polymers represent more than 90% of total polyphenols, while the monomers account for 5-10% (Andres-Lacueva *et al.*, 2008). Anthocyanins represent around the 4% of the total polyphenolic compounds in cocoa bean and are responsible of colour (Pettipher, 1986; Wollgast and Anklam, 2000).

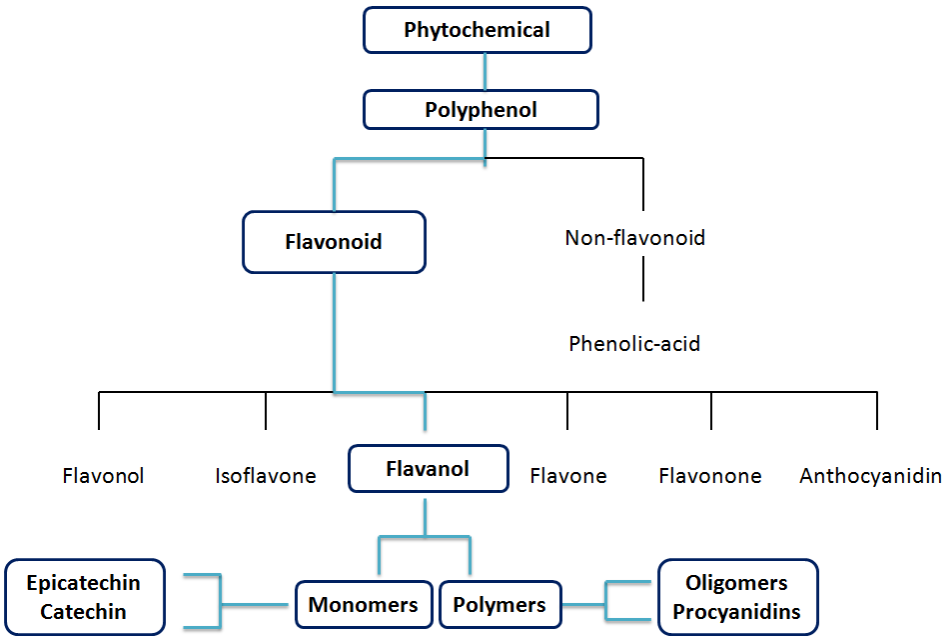

Figure 2. Classification of bioactive compounds in cocoa. Modified from : (Kim *et al.*, 2014)

Other types of flavonoids, such as flavonols, flavones and flavanones are present in cocoa, but in low amounts and often conjugated with sugars (Wollgast and Anklam, 2000; Andres-Lacueva *et al.*, 2008). Quercetin aglycone and its glycosides are the major representatives of flavonols (Oracz *et al.*, 2015). Concerning flavones, flavon-apigenin, luteolin and some glycosides are found (Jaganath and Crozier, 2010; Oracz *et al.*, 2015), while naringenin and its conjugates belong to flavanones (Oracz *et al.*, 2015).

The content of flavanols as well as the total amount of polyphenols is different in cocoa depending on the geographical origin, climate, growing region and manufacturing processes such as fermentation, roasting, high temperatures and storage (Ioannone *et al.*, 2015).

The dietary intake of flavanols (monomeric flavanols, polymeric flavanols and theaflavins) has been recently reported for Europe (Vogiatzoglou *et al.*, 2014). According with this investigation, the average intake of flavanols in the European Union is 369 mg/day. The consumption of total flavanols is higher in central regions (Belgium, Czech Republic, Germany, Hungary, Ireland, Latvia, The Netherlands and UK) reporting an intake of 449 mg/day, while the Northern (Denmark, Finland and Sweden) and the Southern (Italy, Spain and France) regions reported lower intakes, 283 mg/day and 241 mg/day respectively. Concerning the main sources of dietary flavanols in the

European Union, tea represents the 62%, pome fruits the 11%, berries the 3%, cocoa products the 3% and stone fruits the 3%. In the south of Europe, cocoa and cocoa derived products comprise the 12.6%, in the central regions the 2.3% and in the Northern regions the 2.4% (Vogiatzoglou *et al.*, 2014).

Focusing on Spain, the consumption of total flavanols is around 208 mg/day (24 mg of monomers, 174.7 mg of proanthocyanidins and 9.2 mg of theaflavins). Concerning food sources, fruits accounted the 44.8% of flavanols, vegetables the 31%, legume, nuts and oilseeds the 5.7% and grain products the 5.5%. Fruits and cocoa products are the major sources of flavanol monomers in Spain (Vogiatzoglou *et al.*, 2014).

- *Non-flavonoid phenols*

Among nonflavonoid phenols, derivatives of hydroxybenzoic acids (gallic acid, protocatechuic acid, syringic acid and vainillic acid) and hydroxycinnamic acids (coumaric acid, caffeic acid, ferulic acid and chlorogenic acid) have been identified in cocoa (Lafay and Gil-Izquierdo, 2008; Oracz *et al.*, 2015). Moreover, other non-flavonoids such as phloretic acid, phenylacetic acid, resveratrol, piceid, clovamide, deoxyclovamide and dideoxyclovamide are present in cocoa (Ramiro-Puig and Castell, 2009; Kim *et al.*, 2014).

1.1.2. Methylxanthines: theobromine

Methylxanthines are plant secondary metabolites derived from purine nucleotides (Franco *et al.*, 2013). Caffeine, theofylline and theobromine are the most popular methylxanthines appearing in coffee, tea and cocoa respectively (Franco *et al.*, 2013).

Focusing on cocoa, theobromine is the predominant methylxanthine, constituting the 2-3% of the dry weight (Risner, 2008). It is a 3,7-dimethylated xanthine alkaloid which is formed during caffeine metabolism (Risner, 2008; Katz *et al.*, 2011). Cocoa also contains caffeine representing the 0.2-0.25% of the dry weight and very low amounts of theophylline (Risner, 2008). As an example, in 50 g of dark chocolate there are 240-520 mg of theobromine and 17-36 mg of caffeine, while a cup of coffee contains 40-130 mg of caffeine (Smit and Blackburn, 2005).

Theobromine has been associated with cardiovascular protection, anti-tumoral and anti-inflammatory properties (Martinez-Pinilla *et al.*, 2015). Theobromine is not degraded during cocoa processing, whom main metabolites are 7-methylxanthine, 3-methylxanthine and 7-methyluric acid (Cornish and Christman, 1957; Ellam and Williamson, 2013). The half life of theobromine in plasma varies between 7.5 and 10 h with a maximum concentration at 2 h post consumption (Ellam and Williamson, 2013). Although the belief of the addictive effect of caffeine and theobromine is not longer true, theobromine seems to be toxic in some pets (Smith, 2011; Martinez-Pinilla *et al.*, 2015).

1.2. Bioavailability of cocoa flavanols

The consumption of cocoa flavanols has been associated with many cardioprotective benefits (Katz *et al.*, 2011; Hooper *et al.*, 2012). However, to establish a relationship between cocoa flavanols and its role on human health, it is necessary to assess the absorption and release into the circulation (Zheng *et al.*, 2015). For this reason, the bioavailability, which is defined as the fraction of a given food that is absorbed and secreted into the circulation and made available for tissue uptake and metabolism, needs to be evaluated (Rein *et al.*, 2013). Bioavailability comprises different phases such as the release from a food matrix, absorption, metabolism, distribution and elimination (Cifuentes-Gomez *et al.*, 2015). On the other hand, factors such as food matrix (physical state and macronutrient composition), processing, amounts of intake and genetic polymorphisms are factors affecting flavanol bioavailability (Neilson and Ferruzzi, 2011; Cifuentes-Gomez *et al.*, 2015).

The bioavailability of dietary compounds can be analysed by metabolomic tools, which is used to characterize small molecules within biological samples in order to assess the bioavailability of nutrients and identify new biomarkers of intake (Patti *et al.*, 2012; Astarita and Langridge, 2013; Zamboni *et al.*, 2015).

1.2.1. Digestion, absorption, metabolism and distribution

After ingestion of a food, the enzymes secreted from the intestinal mucosa break-down the food matrix liberating flavanols and making available for the intestinal absorption (Saura-Calixto *et al.*, 2007). The cocoa monomers and some oligomers are stable in the stomach and reach the jejunal part of the small intestine intact, where they are absorbed by enterocytes (**Figure 3**) (Rios *et al.*, 2002). Then, cocoa monomers are rapidly metabolized by phase-II metabolizing systems in the enterocyte and liver, appearing in plasma between 30 minutes and 1 hour after its consumption (Crozier *et al.*, 2010). The metabolization consists in glucoronidation by uridine diphosphate glucuronyltransferase (UDPGT), sulfation by sulfotransferase (SULT) and *O*-methylation by catechol-*O*-methyltransferase, giving *O*-glucoronidated, *O*-sulfated and *O*-methylated derivatives in plasma (**Figure 3**) (Hackman *et al.*, 2008). After that, those metabolites go into the bile through the enterohepatic circulation reaching the duodenum and travelling to the colon to be degraded by colonic microbiota, producing phenolic acids and other compounds (Hackman *et al.*, 2008; Neilson and Ferruzzi, 2011).

Procyanidins have higher molecular weight and for this reason are poorly absorbed in the small intestine (Urpi-Sarda *et al.*, 2009). To date, only flavanol dimmers such as dimer B2 and B5 are absorbed intact in the small intestine appearing in plasma in very low amounts and contributing to less than 1% of circulating flavanols (Holt *et al.*, 2002; Zhu *et al.*, 2005; Urpi-Sarda *et al.*, 2009). The remaining procyanidins together with some metabolized monomers are metabolized by colonic microflora in the large intestine producing derivatives of phenolic acids (**Figure 3**)

(Appeldoorn *et al.*, 2009). Such phenolic acids are absorbed into the bloodstream and metabolized in the liver producing potential bioactive compounds (Neilson and Ferruzzi, 2011; Rein *et al.*, 2013). Finally, flavanol derived metabolites are excreted in urine by kidneys (Rein *et al.*, 2013).

The distribution of flavanols in the organism is extensive and they have been found in the digestive tract, lung, liver, mammary gland, skin, pancreas, brain, kidney, ovaries and testes (Suganuma *et al.*, 1998; Ramiro-Puig *et al.*, 2007; Ramiro-Puig and Castell, 2009; Urpi-Sarda *et al.*, 2010b; Serra *et al.*, 2013). The tissue concentration of flavanol metabolites depends on the administered dose (Margalef *et al.*, 2015).

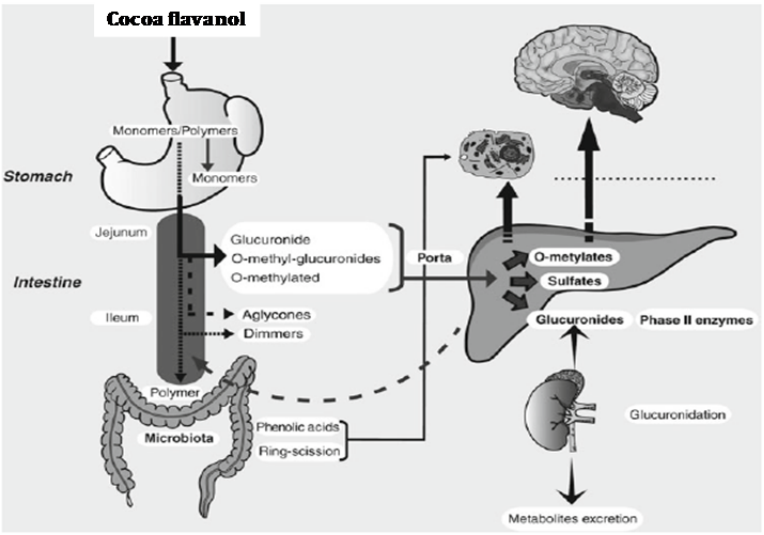

Figure 3. Bioavailability of cocoa flavanols in humans. Modified from (Watson *et al.*, 2013).

1.2.2. Factors affecting the bioavailability of cocoa flavanols

- *Food matrix*

In human diet, cocoa flavanols are usually consumed within food matrixes and not isolated or as pure substances, making difficult their bioavailability.

The *physical state* of the food (liquid or solid) may affect the availability of flavanols (Cifuentes-Gomez *et al.*, 2015). Liquid foods usually induce a faster absorption of flavanols and earlier appearance in plasma, probably due to a faster digestive release and faster stomach emptying compared with solids (Cifuentes-Gomez *et al.*, 2015).

The *macronutrient composition* (carbohydrate, lipid and protein profile) of the food matrix is also a determinant factor for flavanol absorption (Neilson *et al.*, 2009; Cifuentes-Gomez *et al.*, 2015).

Carbohydrates and specifically sugars, promote flavanol absorption (Schramm *et al.*, 2003; Neilson *et al.*, 2009; Rodriguez-Mateos *et al.*, 2012) by enhancing the solubility and intestinal uptake (Peters *et al.*, 2010).

Proteins do not have strong effect on flavanol availability except for the interaction with milk protein (Ferruzzi *et al.*, 2012). Some studies have reported a reduction of cocoa flavanols absorption when they are consumed mixed with milk (Serafini *et al.*, 2003). Indeed, in vitro studies have shown that β-globulin, which is the major milk protein, is able to bind epicatechin interfering in the bioavailability (Gallo *et al.*, 2013). However, other nutritional studies did not observe adverse effects (Keogh *et al.*, 2007). Interestingly, milk affects the profile of excreted metabolites in urine but not the amount of metabolites (Roura *et al.*, 2008; Mullen *et al.*, 2009; Urpi-Sarda *et al.*, 2010a).

Regarding the effect of lipids on flavanol absorption, there is no scientific evidence (Schramm *et al.*, 2003).

- *Food processing*

The flavanol composition of cocoa is conditioned by the environmental factors, climate, geographical growth, humidity and genetic variants, among others (Oracz *et al.*, 2015). However, there are other factors such as processing methods and storage condition, which may affect quantitative, and qualitatively the cocoa flavanol content, modulating also antioxidant activity and bioavailability (Andres-Lacueva *et al.*, 2008; Schinella *et al.*, 2010; Oracz *et al.*, 2015).

The fermentation process and high temperatures during the roasting, decrease the amount of flavanols in the cocoa bean (Ioannone *et al.*, 2015). The roasting process induces the characteristic brown colour, aroma and texture of cocoa beans and also reduces the astringency (Serra Bonvehi and Ventura Coll, 2002). The roasting temperature (120º-150º) and time (5-120 min) are important determinant factors of flavanol degradation.

Alkalization, also known as Dutch process, is the method used to reduce the bitterness of cocoa (Miller *et al.*, 2008). However, cocoa procyanidins are responsible of bitterness and therefore, when cocoa is subjected to this process the flavanol content is reduced (Andres-Lacueva *et al.*, 2008; Miller *et al.*, 2008; Hurst *et al.*, 2011).

The high temperatures during cocoa processing phases favours the epimerization of (-)-epicatechin to (-)-catechin, which is not naturally present in cocoa bean and is less bioavailable (Kofink *et al.*, 2007; Andres-Lacueva *et al.*, 2008; Hurst *et al.*, 2011; Ottaviani *et al.*, 2011). The absorption of cocoa flavanols has been determined as follows: (-)-epicatechin > (+)-catechin > (-)-catechin (Ottaviani *et al.*, 2011).

- *Intake and genetic polymorphisms*

The cocoa flavanol intake is associated in a dose-dependent manner with the concentration in plasma (Wang *et al.*, 2000).

On the other hand, genetic polymorphism is a determinant factor of the interindividual variability in the absorption and metabolism patterns (Borel, 2012). There are no studies assessing the absorption of cocoa flavanols depending on the genotype. However, in a study carried out with tea, a polymorphism in the gene encoding for catechol-*O*-methyl transferase enzymes produced an alteration of the metabolization process (Miller *et al.*, 2012). In this context, similar effect could be observed after cocoa consumption.

2. Obesity and associated comorbidities

2.1. Definition and prevalence

The WHO defines obesity as an abnormal or excessive fat accumulation that contributes to increase the risk of suffering from other diseases (WHO, 2015c). Due to its impact on morbid-mortality and healthcare cost obesity was declared the epidemic of XXI century (Flegal *et al.*, 2013).

The prevalence of obesity is high. More than 1.9 billion adults were overweight (39%) in 2014 and over 600 million were obese (13%) (WHO, 2015c). It has been estimated that more than 2.16 billion people will be overweight with a body mass index (BMI) ≥25 kg/m^2) and more than 1.12 billion people will be obese with a BMI≥30kg/m^2, by 2030 (Varemo *et al.*, 2013). Mexico, United States, Western Europe, Cook Islands and Middle East are the countries with major obesity rates (**Figure 4**).

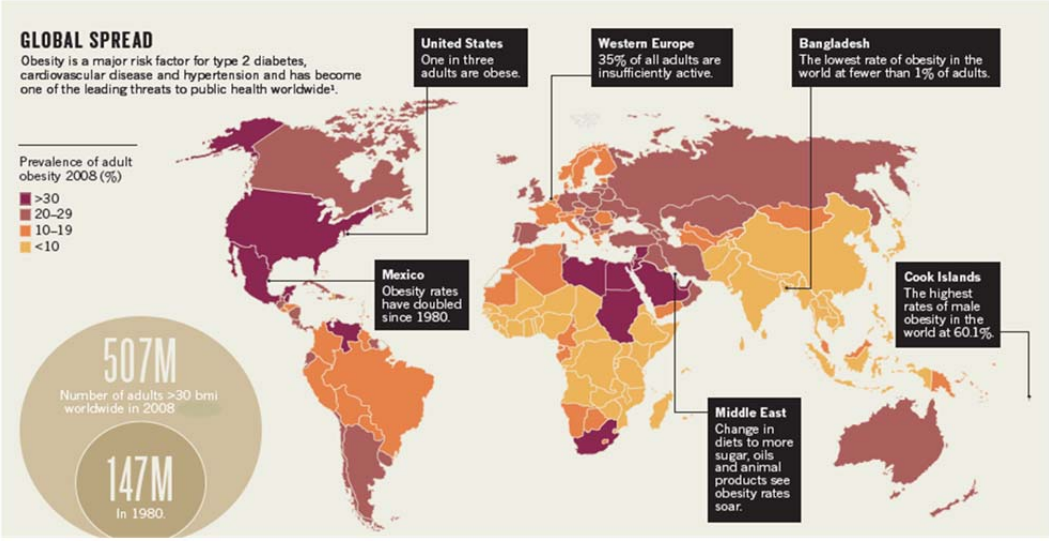

Figure 4. Prevalence of adult obesity worldwide. Adapted from: (Scully, 2014).

In Spain, the prevalence of overweight and obesity has strongly increased in the last years (Salcedo *et al.*, 2010; Gutierrez-Fisac *et al.*, 2012). The Spanish study on cardiovascular risk factors (DORICA study) conducted between 1990 and 2000 in subjects aged between 25–60 years reported a prevalence of obesity of 13% in men and of 18% in women (Aranceta-Bartrina *et al.*, 2005). The ENRICA study (Study on Nutrition and Cardiovascular Risk in Spain) is a cross-sectional study carried out between June 2008 and October 2010 in 12,883 Spanish subjects of 18 years and older (Gutierrez-Fisac *et al.*, 2012). The principal aim was to assess the frequency and distribution of the main components of CVD in Spain (Gutierrez-Fisac *et al.*, 2012). The prevalence of obesity was estimated at 22.9% (24.4% in men and 21.4% in women) with 36% of adults suffering from abdominal obesity (32% of men and 39% of women)(Gutierrez-Fisac *et al.*, 2012).

2.2. Causes of obesity

Obesity is principally the result of a positive imbalance between energy intake and expenditure, which contributes to increase body weight and fat mass (Galgani and Ravussin, 2008; Hill *et al.*, 2012). In addition, obesity is a multifactorial origin chronic disease (McAllister *et al.*, 2009) determined by lifestyle factors (diet, physical activity and socioeconomic status), environmental factors (infections, maternal age, ambient temperature, pharmacological iatrogenesis, etc.), genetic predisposition, behaviour and emerging factors such as gut microbiota (Bhurosy and Jeewon, 2014; Chaput *et al.*, 2014; Tai *et al.*, 2015). According to recent investigations, gene-nutrient and environment interactions strongly affect obesity (Doo and Kim, 2015; Huang and Hu, 2015).

2.2.1. Inflammation and oxidative stress the underlying mechanisms of obesity

Adipose tissue has been recognized as an active endocrine organ involved in numerous metabolic, hormonal, and immune processes by the secretion of molecules known as adipokines, leading to a pro-inflammatory and pro-oxidant environment (Mraz and Haluzik, 2014).

Adipose tissue is composed by adipocytes, the main energy storage of the organism (Kopecky *et al.*, 2004). Fat depots are increased in obesity due to hyperplasia and hypertrophy of the adipocytes (Jo *et al.*, 2009). Adipose tissue is categorized in brown and white adipose tissues. However, recently a beige adipose tissue has been found (Wu *et al.*, 2013). Brown adipose tissue is responsible of the thermogenic activity (Cannon and Nedergaard, 2004), while white adipose tissue is responsible of fat storage (Saely *et al.*, 2012). Beige adipose tissue is composed by inducible 'brown-like' adipocytes developed in white adipose tissue in response to various activators (Harms and Seale, 2013).

Obesity is characterized by a low-grade chronic inflammation, which is recognized as one of the most important pathogenic mechanisms involved in obesity and related disorders (Bondia-Pons *et al.*, 2012). Moreover, inflammation is associated with oxidative stress (Bondia-Pons *et al.*,

2012). Inflammation is a cellular and humoral reaction to defend the body from various insults and attacks, such as infections and tissue damage through inflammatory cytokine release like tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) (Cildir *et al.*, 2013; Lee and Lee, 2014). In the same manner, reactive oxygen species (ROS) and free radical production is a natural phenomenon derived from physiological processes, which are necessary to the normal function of the organism (Marseglia *et al.*, 2015).

The antioxidant system of the organism is composed by endogenous and exogenous antioxidants to neutralize the action of free radicals (Perez-Matute *et al.*, 2009). The endogenous antioxidants are enzymes such as superoxide dismutase, catalase and glutathione peroxidase, macromolecules such as albumin, ceruloplasmin and ferritin and small molecules such as uric acid and bilirubin (Wahlqvist, 2013). The exogenous antioxidants are molecules derived from foods, principally from fruits and vegetables such as vitamins, carotenoids, polyphenols and some minerals (Annuzzi *et al.*, 2014).

In obesity, the function of adipose tissue is disrupted and the production of circulating pro-inflammatory molecules increased (Karalis *et al.*, 2009). Those molecules act locally disturbing other organs and systems and affecting the whole-body homeostasis (Mraz and Haluzik, 2014). Simultaneously, the pro-inflammatory environment contributes to increase the ROS production breaking the balance between oxidants and antioxidants and establishing the oxidative stress (**Figure 5**)(Perez-Matute *et al.*, 2009). The imbalance is rapidly reversed in healthy subjects, while in obesity the imbalance is not restored (Marseglia *et al.*, 2015). Molecules, cells, tissues, systems and desoxirribonucleic acid (DNA) of the organism suffer from oxidative damage altering their function and increasing the risk of disease development and mortality (Lobo *et al.*, 2010). In this sense, oxidative stress is directly implicated in disease onset (Dai *et al.*, 2012; Hulsmans *et al.*, 2012).

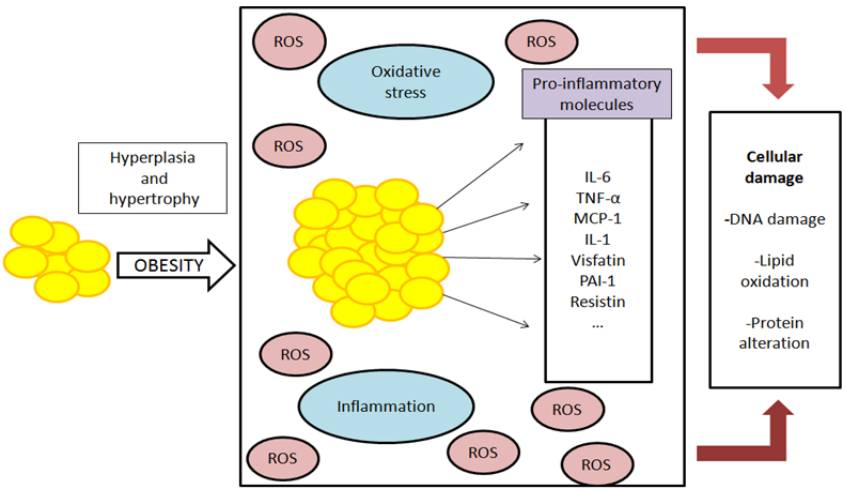

Figure 5. Consequences of oxidative stress and inflammation

Abbreviations: Il-6, interleukin-6; IL-1, interleukin-1; MCP-1, Monocyte Chemoattractant Protein-1; TNF-α, tumor necrosis factor-alpha; PAI-1, Plasminogen activator inhibitor-1; ROS, reactive oxygen species.

DNA stability and integrity are essential. However, DNA is subjected to environmental hazards such as free radicals (Kryston *et al.*, 2011). Damaged DNA leads to mutations and disease onset by the oxidative DNA damage, which induces the formation of single and double strand breaks (SBs) of oxidised bases in the DNA leading to genome destabilization (Shibutani *et al.*, 1991) and causing promutagenic lesions (e.g. 8-oxoguanine) (Shibutani *et al.*, 1991). This situation can play a significant role in the development of cancer and other diseases including atherosclerosis (Shibutani *et al.*, 1991; Loft *et al.*, 2008; Kryston *et al.*, 2011).

Dietary antioxidants can be in part responsible for disease prevention by acting directly as ROS scavengers, stimulating the cellular endogenous defences or even improving the DNA repair capacity (Azqueta and Collins, 2012; Collins *et al.*, 2012). Many nutritional intervention trials have successfully used the comet assay to monitor endogenous SBs and oxidised bases, antioxidant resistance and DNA repair capacity (Dusinska and Collins, 2008; Collins *et al.*, 2012).

On the other hand, endoplasmic reticulum is an active organelle which is involved in translocation and integration of proteins, lipid biosynthesis and calcium homeostasis (Chaudhari *et al.*, 2014). The stress of endoplasmic reticulum linked to inflammatory process is one of the major contributors for the development of pathologies (Chaudhari *et al.*, 2014).

2.3. Health consequences and comorbidities

Obesity has many health consequences such as medical comorbidities, psychological and social effects (Fan *et al.*, 2013). The interaction among genetics, environment and other factors influence weight gain and increases the development of metabolic disorders (**Figure 6**). Obesity and especially visceral obesity, increases the risk of morbidity and mortality (Britton *et al.*, 2013). The pro-inflammatory and pro-oxidant environment of obesity is an optimal situation for the development of insulin resistance, CVD, psychocogical disorders, sleep apenea, vitamin D (VitD) deficiency, cancer and osteoarthritis among other comorbidities of obesity (Katsareli and Dedoussis, 2014).

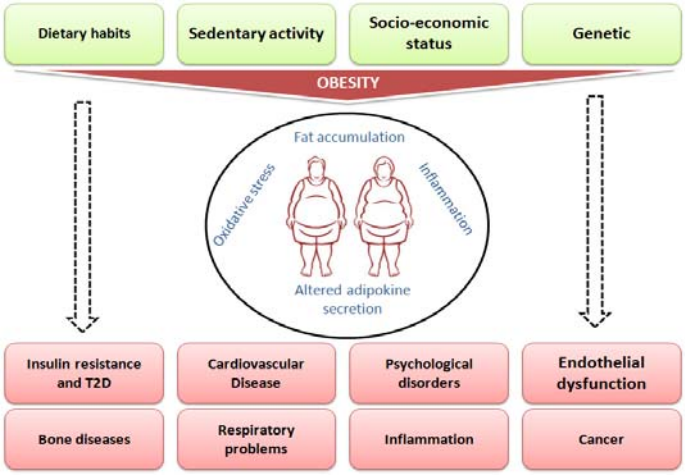

Figure 6. Risk factors and health consequences of obesity

The Atención Primaria de Navarra study (APNA), which was performed to evaluate the comorbidities associated with obesity in 40,010 subjects attending Primary Health Care Centres in Navarra reported that each unit of BMI increases in 12% the risk of suffering from hypertension (Martin-Rodriguez *et al.*, 2015). Moreover, they observed higher prevalence of type 2 diabetes in overweight (11.5%) and obese (25.2%) subjects (Martin-Rodriguez *et al.*, 2015).

2.3.1. Insulin resistance

Insulin resistance is the state in which the insulin-sensitive tissues (skeletal muscle, fat and heart) loss the response to insulin due to an impairment on insulin signalling pathway (**Figure 7**) (Lee and Lee, 2014).

Insulin is a hormone produced and secreted by pancreatic β-cells (Kahn *et al.*, 2006). The classical function of insulin is to regulate glucose homeostasis favouring glucose uptake by insulin-sensitive tissues, inhibiting hepatic glucose production and suppressing lypolisis in the adipose tissue (Ye, 2013). However, insulin is involved in the production of fatty acids and glycogen, in mitochondrial activity, microcirculation and cell proliferation (Maechler, 2013; Muris *et al.*, 2013; Ye, 2013).

In healthy condition, liver produces glucose via gluconeogenesis and glycogenolysis in order to keep fasting blood glucose in adequate levels (Boden, 2004). In contrast, during insulin resistance, the control over hepatic glucose production is altered (Kahn *et al.*, 2006). In consequence, glucose is produced by gluconeogenesis and glycogenolisis independently of blood glucose concentration (Lee and Lee, 2014). On the other hand, the glucose uptake by adipose tissue and muscle is not appropriate and as compensatory mechanism pancreatic β-cells produce more insulin leading to hyperinsulinemia (Kahn *et al.*, 2006). Nevertheless, pancreatic β-cells have a limit of insulin production and when that limit is overwhelmed, the β-cells fail decreasing insulin in peripheral tissues and leading to the development of type 2 diabetes (Kahn *et al.*, 2006).

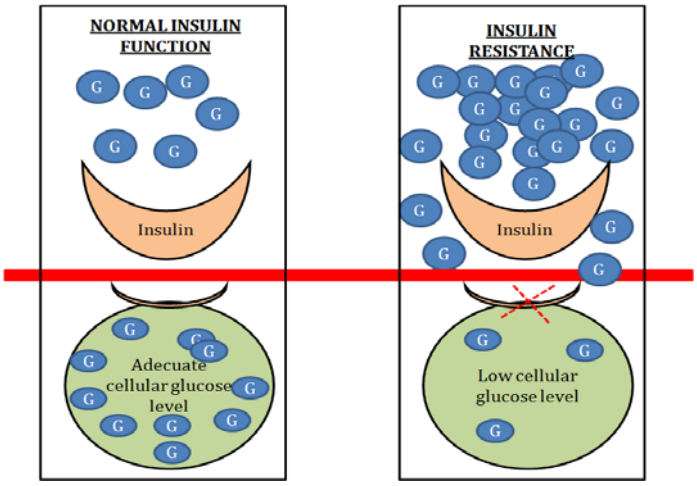

Figure 7. Normal insulin function and insulin resistance.

Type 2 diabetes is a complex metabolic disorder suffered principally by subjects living in low- and middle-income countries (Whiting *et al.*, 2011). It accounts for 90% of all diabetes cases and although until few years ago it was considered as a disease suffered by adult population, nowadays there are children developing it (Pulgaron and Delamater, 2014; WHO, 2015b). According to WHO, diabetes will be the seventh cause of death in 2030 (Mathers and Loncar, 2006; WHO, 2015b).

The principal treatment for insulin resistance and type 2 diabetes is based on lifestyle changes, mainly the control of dietary habits and the increase of physical activity (Torjesen *et al.*, 1997; WHO, 2015b). However, new alternatives such as the use of functional foods are being developed (Munir *et al.*, 2013; Dragan *et al.*, 2015).

2.3.2. Cardiovascular disease

According to WHO, CVD encloses heart and blood vessel disorders, including coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease as well as deep vein thrombosis and pulmonary embolism (WHO, 2015a).

The prevalence of CVD is high, 17.5 million people dead from CVD in 2012, representing the 31% of all global deaths (WHO, 2015a). Of those deaths, 7.4 million were due to coronary heart disease and 6.7 million were due to stroke (WHO, 2015a). By 2030 more than 23 million people will die annually due to CVDs (WHO, 2015a). Hypertension, smoking, diyslipidemia, endothelial dysfunction and increased blood glucose promote the risk for CVD (Cannon, 2007; Fan *et al.*, 2013). Lifestyle changes such as dietary control, no smoking and physical activity are the most important strategies to reduce the cardiovascular risk (Rautio *et al.*, 2015).

- *Lipid metabolism*

Obesity is linked to the development of dyslipidemia, which is defined as an abnormal amount of lipids in the blood and it is considered a risk factor for CVD (Jung and Choi, 2014).

Dyslipidemia is characterized by increased levels of plasma free fatty acids (FFA) and triglycerides (TG), decreased levels of high-density lipoprotein-cholesterol (HDL-c), and increased levels of low-density lipoprotein-cholesterol (LDL-c) (Jung and Choi, 2014).

Uncontrolled fatty acid release from adipose tissue, specifically visceral adipose tissue, via lipolysis, induces an increased delivery of fatty acids to the liver and synthesis of very-low density-lipoproteins (VLDL), becoming the mayor contributing factor for obesity-related dyslipidemia (Jensen, 2008). Increased levels of FFA, decrease the activity of lipoprotein lipase (LPL) in adipose tissue and skeletal muscle, while the increased synthesis of VLDL in the liver can inhibit lipolysis of chylomicrons promoting hypertriglyceridemia (Clemente-Postigo *et al.*, 2011; Klop *et al.*, 2012; Jung and Choi, 2014). In addition, hypertriglyceridemia alters the activity of the cholesterylester

transfer protein-mediated exchange of TG for cholesterol esters between TG-rich lipoproteins (VLDL) and lipoproteins that are relatively richer in cholesterol esters (LDL-c, HDL-c), leading to a decreased HDL-c concentration and a reduction of TG content in LDL-c (Jung and Choi, 2014). The increased TG content in LDL-c is hydrolyzed by hepatic lipase (HL), promoting the formation of LDL-c particles which are associated with a greater risk of CVD (Klop *et al.*, 2013; Trpkovic *et al.*, 2015).

The alteration of lipid metabolism and the increase of lipid peroxidation contribute to atherosclerotic plaque formation by the deposition of small cholesterol crystals in the intima and smooth muscle (Weber and Noels, 2011).

- *Blood pressure*

Hypertension is a risk factor for CVD and obesity is a risk factor for hypertension (Landsberg *et al.*, 2013). According to the data of National Health and Nutrition Examination Survey (NHANES), the prevalence of hypertension in individuals with a BMI greater than 30 kg/m^2 is 45%, for overweight subjects with a BMI between 25-29.9 kg/m^2 the prevalence is 27.8% and for subjects with a BMI less than 25 kg/m^2 is 15.3% (Wang and Wang, 2004). Factors such as elevated insulin and leptin levels, the activation of renin-angiotensin-aldosterone system as well as sodium excretion, pressure natriuresis and salt sensitivity are implicated in the pathophysiology of obesity related hypertension (Landsberg *et al.*, 2013).

The 50% of vascular events (47% of ischemic heart disease and 54% of stroke events) and the 13.5% of vascular death worldwide (37% of vascular death in the western populations) are associated with high blood pressure levels (Martiniuk *et al.*, 2007; Lawes *et al.*, 2008).

Lifestyle modifications (dietary management, increased physical activity, weight control and avoid of alcohol and tobacco) and pharmacological treatments are the conventional strategies to control blood pressure levels (Kokubo, 2014). A weight variation induces changes in blood pressure levels (Markus *et al.*, 2015). Weight gain increases blood pressure (Weisbrod *et al.*, 2013), while weight loss reduces blood pressure (De Ciuceis *et al.*, 2011). Nowadays, different strategies such as the use of plant derived products are being investigated to reduce blood pressure (Ried *et al.*, 2012; Ferri *et al.*, 2015; Mastroiacovo *et al.*, 2015).

- *Endothelial function and atheroma plaque formation*

The endothelium is a continuous, smooth, nonthrombogenic surface of all blood vessels that exhibits a highly selective permeability in healthy state (Deanfield *et al.*, 2007). The endothelial function is a measurement for cardiovascular risk prediction (Grassi *et al.*, 2013a). Hypertension, hypercholesterolemia, diabetes, obesity, poor lifestyle and smoking are risk factors for CVD and endothelial dysfunction (Yusuf *et al.*, 2004; Grassi *et al.*, 2011). The vascular homeostasis and tone

are mainly regulated by vasodilator and vasoconstrictor substances produced by the endothelium such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor, endothelin-1 and cell adhesion molecules (Aird, 2004, 2008).

NO is one of the most important endothelium derived products (Tousoulis *et al.*, 2012). This vasodilating substance is produced by the endothelial NO synthase (eNOS)(Lei *et al.*, 2013). NO maintains vascular homeostasis by the inhibition of inflammation, cellular proliferation and thrombosis (Lei *et al.*, 2013). Cardiovascular risk factors contribute to oxidative stress promoting a disruption in the balance between NO and ROS, with a relative decrease in NO bioavailability, which leads to endothelial dysfunction (Grassi *et al.*, 2011). This situation, promotes platelet and leucocyte activation as well as the activation of cytokines, which increases the permeability of the vessel wall to oxidised lipoproteins and inflammatory substances damaging structurally the arterial wall, promoting cell proliferation and plaque formation, becoming pro-atherosclerotic (**Figure 8**) (Grassi *et al.*, 2011). Endothelial dysfunction is considered the earliest step of atherosclerosis and is involved in the pathogenesis of hypertension and CVD (Grassi *et al.*, 2011; van Sloten *et al.*, 2014).

Flow mediated dilation (FMD) is the principal technique to measure the endothelial function (Deanfield *et al.*, 2007). It represents the endothelium-dependent relaxation of the artery mediated by NO release, in response to a hyperemic stimulus and reflecting the vascular activity (Deanfield *et al.*, 2007; Ghiadoni *et al.*, 2015). Interestingly, when FMD increases around 1%, the risk for cardiovascular events reduces in a 8-13% (Inaba *et al.*, 2010; Ras *et al.*, 2013) suggesting that FMD is inversely related to future cardiovascular events.

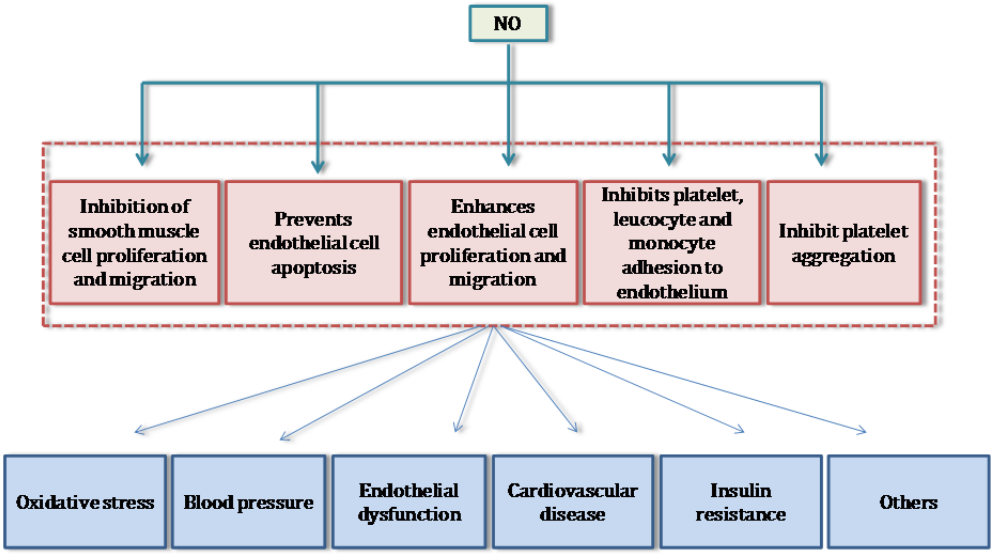

Figure 8. Vasoprotective effects of nitric oxide. Adapted from: (Lei *et al.*, 2013).
Abbreviations: NO, nitric oxide

Atherosclerosis is a chronic inflammatory disease of the arterial wall, which is the result of altered lipid metabolism, inflammation and lipid oxidation processes (Weber and Noels, 2011; Hajjar and Gotto, 2013). Oxidative modification of LDL-c particles is the early step of the atherosclerotic process, which occurs in a pro-inflammatory and pro-oxidant environment (Stocker and Keaney, 2004; Hajjar and Gotto, 2013). Circulating LDL-c particles are trapped in the subendothelial extracellular matrix to be oxidised, becoming susceptible to macrophages and contributing to foam cell production (Tabas *et al.*, 2007). This situation, leads to the production of adhesion molecules on the vascular surface such as intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) among others (Blankenberg *et al.*, 2003). Moreover, some immune cells are incorporated into the emerging atheroma plaque and ROS production contributing to tissue damage, lipid peroxidation and inflammation (Delporte *et al.*, 2013). Furthermore, smooth muscle cells move into the intima and proliferate, generating metalloproteinases that can digest extracellular elastin and collagen (Newby, 2006). Smooth muscle cells then encase foam cells with a fibrous cap, and the foam cell apoptosis gives rise to a lipid-rich necrotic core (Newby, 2006; Hajjar and Gotto, 2013). Pro-inflammatory mediators create the ideal environment for plaque rupture and subsequent thrombosis (Hajjar and Gotto, 2013; Rafieian-Kopaei *et al.*, 2014). The atherosclerotic process is shown in **Figure 9.**

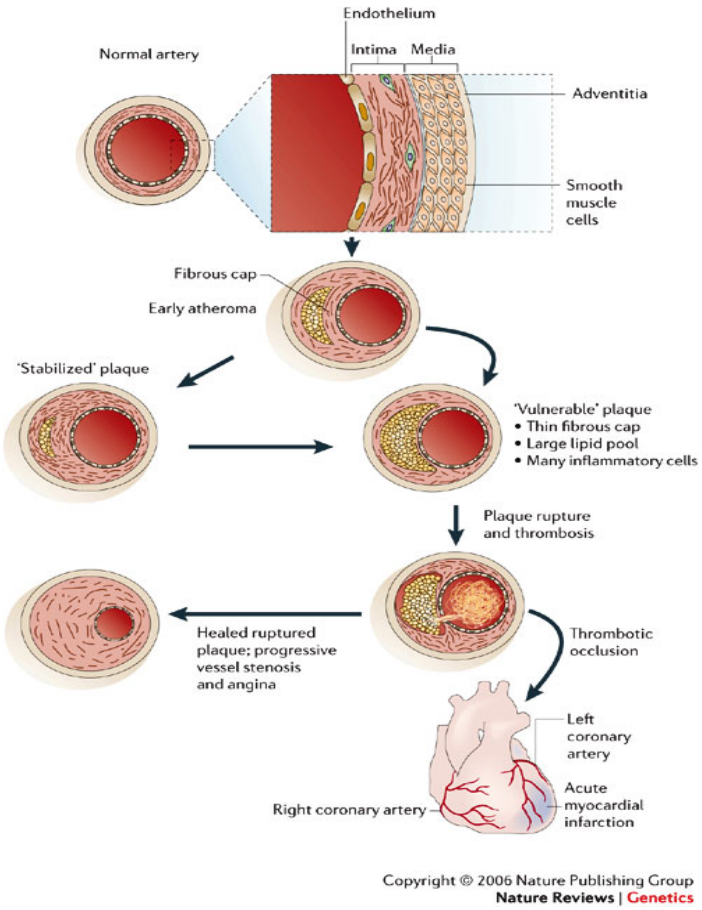

Figure 9. Atheromatous plaque progression. From: (Watkins and Farrall, 2006).

2.3.3. Mental and behavioural disorders

Mental disorders have a multifactorial aetiology and although there is no universal definition, generally are defined as those illnesses that have a disturbance in mood or affection (Belmaker and Agam, 2008; WHO, 2014; DSM-V, 2013). Mood disorders are characterized by abnormal thoughts, emotions, behaviour and relationships with others (WHO, 2014). Examples are schizophrenia, depression, intellectual disabilities, anxiety disorders and pathological conditions derived from drug abuse among others (WHO, 2014). Primary depression and generalized anxiety are the most prevalent mental disorders worldwide.

According to WHO, depression affects 350 million people worldwide and the prevalence is higher in women than in men (WHO, 2012). In Spain, the European Study of the Epidemiology of Mental Disorders project (ESEMeD-Spain) revealed that the lifetime prevalence of depression is 10.6% (Gabilondo *et al.*, 2010).

Depression is a multifactorial disease characterized by sadness, loss of interest or pleasure, feelings of guilt, disturbed sleep or appetite, tiredness, and poor concentration caused by biological, environmental, and psychological factors, as well as by the interaction of some of these elements (Belmaker and Agam, 2008). Obesity, inflammation, oxidative stress, genetic and epigenetic factors, neurotransmitter imbalance, unhealthy lifestyle, social factors and hypothalamic pituitary adrenal axis (HPA-axis) disturbance are factors affecting depression (Kunugi *et al.*, 2010; Soczynska *et al.*, 2011; Stegenga *et al.*, 2012; Hodgson and McGuffin, 2013; Lopresti *et al.*, 2013; Lopresti *et al.*, 2014).

The monoamine theory of depression has been accepted for several decades (Schildkraut, 1965). According to this, depression is caused by a depletion in the levels of serotonin, norepinephrine and/or dopamine in the central nervous system which could be restored with the use of antidepressants (Belmaker and Agam, 2008).

Depression can be categorized as mild, moderate or severe and it is diagnosed and treated (Turner *et al.*, 2014). In research, depressive symptoms are evaluated by rating questionnaires such as Beck Depression Inventory (BDI) (Beck *et al.*, 1961). Psychosocial treatments should be the first option for mild depression and pharmacological and psychological treatments should be used in cases of moderate and severe depression (Turner *et al.*, 2014).

On the other hand, anxiety disorder is a behavioural disturbance associated with tension and hypervigilance in preparation for future threat, when no real threat exists (DSM-V, 2013). Anxiety disorders are the most frequent mental disorders in Europe with a rate of 14.0% (Wittchen *et al.*, 2011). The prevalence of anxiety disorders is 8.7% in women and 4.3% in men (Steel *et al.*, 2014). In Spain, the ESEMeD evidenced that the lifetime prevalence of anxiety disorders is of the 6.2% and the incidence is twofold higher in women than in men (Haro *et al.*, 2006). Anxiety is

caused by genetic heritability, negative thought, health status, abnormalities in the HPA-axis and low grade inflammation and oxidative stress (DSM-V, 2013).

Generalised anxiety disorder is the most common anxiety disorder worldwide and is characterized by psychological symptoms such as excessive worry, fear, apprehension, and physical symptoms for instance fatigue, heart palpitation and muscular tension (DSM-V, 2013). It has a detrimental effect on health and social well-being (Sareen *et al.*, 2006), which is associated with gastrointestinal diseases, asthma, allergic conditions, migraine, thyroid diseases and arthritis among others (Roy-Byrne *et al.*, 2008).

Generalised anxiety disorder is often evaluated using rating instruments such as the State Trait Anxiety Inventory (STAI) (Spielberger, 1971) and the treatment includes psychological and pharmacological treatments (Stein *et al.*, 2010).

Obesity is associated with depression and anxiety disorders (de Wit *et al.*, 2010b). A meta-analysis of 17 community-based studies reported that obese subjects are 1.18 times more likely to suffer from depressive symptoms than those who are not obese, and this association is more clearly present among women (de Wit *et al.*, 2010a). Moreover, a reciprocal relationship between depression and excessive body-weight was found in a meta-analysis of longitudinal studies (Luppino *et al.*, 2010).

Subjects suffering from depression and anxiety follow unhealthy lifestyle patterns such as increased alcohol intake and unhealthy diets suffering also from sleep disturbances (Bonnet *et al.*, 2005; Gea *et al.*, 2013; van Mill *et al.*, 2013). On the other hand, obese individuals usually have poor healthy habits increasing the risk of suffering from depression and anxiety disorders (Pan *et al.*, 2012).

Oxidative stress and inflammation could be linked with obesity and mental disorders because pro-inflammatory and low antioxidant status have been observed in subjects suffering from depression and anxiety (Dantzer *et al.*, 2008; Miller *et al.*, 2009; Joshi and Pratico, 2014; Palta *et al.*, 2014).

In addition, monoamine imbalance in the central and peripheral nervous systems could be implicated in obesity and psychological disorders (Luppino *et al.*, 2010; Pan *et al.*, 2012). Although central nervous system has been extensively studied, the role of peripheral monoamines in mental disorders has been scarcely studied. Central monoaminergic system is responsible for cognition, the reward system and mood (Belmaker and Agam, 2008) and the peripheral monoaminergic system is implicated in physiological functions (Perez-Cornago *et al.*, 2015). Dopamine is a monoamine involved in mood, cognition, motor control, the reward system and well-being in the central nervous system (Schultz, 2007; Carlin *et al.*, 2013), while in the peripheral nervous systems it is implicated in gastrointestinal motility, heart function and blood pressure among others (Rubi

and Maechler, 2010). In relation with obesity, dopamine is inversely associated with BMI and low dopamine levels may increase the preference for consuming high-palatable and energetic foods, contributing to obesity condition (Wang *et al.*, 2001). However, central dopamine cannot cross the blood-brain barrier (Pardridge, 2007). Therefore, the determination of plasma homovanillic acid (pHVA), which is the main dopamine metabolite in body fluids, has been suggested as an appropriate indicator of central dopaminergic activity (Sternberg *et al.*, 1983; Amin *et al.*, 1992). The degradation of dopamine is caused by monoamine oxidase (MAO) enzyme which produces HVA as an end product (Lyles, 1996).

Feelings, mood and stress seem to play an important role in dietary choices increasing food intake and specifically the consumption of high fat and palatable foods (Wallis and Hetherington, 2009; Martin *et al.*, 2012). This activity produces comfort and distraction in subjects with negative emotions (Christensen and Pettijohn, 2001).

2.3.4. Vitamin D deficiency

VitD is implicated in multiple processes of human physiology, such as bone turnover and calcium homoeostasis, cardiovascular regulation, mental health, muscle and brain functions (Botella-Carretero *et al.*, 2007; Maddock *et al.*, 2013; Stocklin and Eggersdorfer, 2013). Two main sources of VitD are available: sunlight (exposure to solar UV-B radiation) and food (including dietary supplements). VitD exists in two forms: VitD2 (ergocalciferol) and VitD3 (cholecalciferol). VitD2 is found in plants and VitD3 is synthesized in the human epidermis through ultraviolet irradiation of 7-dehydrocholesterol. Moreover, it is found in animal derived foods such as oily fish and also in fortified foods and supplements. VitD is converted in the liver to 25-hydroxyvitamin D [25(OH)D], which is the major circulating metabolite of VitD and reflectsing the general VitD status (Hollis, 2008). In the kidney, 25(OH)D is converted by 1-hydroxylase to the active form, 1,25-dihydroxyvitamin D (Ferder *et al.*, 2013).

The scientific evidence shows that excesive BMI and adipose tissue are associated with low circulating VitD levels (Botella-Carretero *et al.*, 2007; Vimaleswaran *et al.*, 2013). However, the direction and causality remain unclear. Some researchers have suggested that obesity is the result of cold and dark environments, where VitD synthesis could be decreased due to the reduced sun exposure, promoting fat mass accumulation and increasing BMI (Foss, 2009). Other investigators have suggested the sequester capacity of VitD by adipose tissue, which has higher storage capacity (Blum *et al.*, 2008).

On the other hand, low 25(OH)D levels are associated with higher risk of suffering from cardiometabolic diseases (Gonzalez-Molero *et al.*, 2012; Mozos and Marginean, 2015). A recent meta-analysis involving 42,000 subjects reported that genetic variants associated with higher BMI were related to a lower 25(OH)D concentration, indicating that obesity involves low VitD circulating levels, but no vice versa (Vimaleswaran *et al.*, 2013). Moreover, middle-aged and elderly

populations are at risk of suffering from 25(OH)D deficiency due to a low dietary intake, sun exposure and synthesizing decrease (Perez-Lopez *et al.*, 2011; Samefors *et al.*, 2014). In addition, the risk of suffering from comorbidities, high body weight and excess of fat mass is higher in this collective (Chung *et al.*, 2013).

2.3.5. Other health alterations

Obesity is associated with a wide range of health disorders (Chopra *et al.*, 2013). The excessive caloric intake contributes to organ injury, for instance, promoting kidney disease, which is produced from the molecules secreted by the adipose tissue in obesity (Decleves and Sharma, 2015). Those molecules contribute to inflammatory processes and are related to insulin resistance and hypertension, which are the most important etiological factors of kidney disease (Wickman and Kramer, 2013). However, obesity directly impacts kidney disease inducing hyperfiltration, increasing glomerular capillary wall tension and the stress of podocytes (Wickman and Kramer, 2013). Moreover, obesity is associated with non-alcoholic fatty liver disease (NAFLD) with a prevalence higher than 80% (Williams *et al.*, 2011; Milic *et al.*, 2014). The excessive FFA release and the production of pro-inflammatory molecules from visceral adipose tissue are considered the most important factors contributing to liver injury progression in NAFLD (Milic *et al.*, 2014). Obstructive sleep apnea is also other consequence of obesity, which is characterized by the obstruction of the upper airway leading to sleep fragmentation and intermittent hypoxia during sleep (Drager *et al.*, 2013). In addition, obesity is associated with tooth loss, poor oral health (Ostberg *et al.*, 2012; Prpic *et al.*, 2013) and with different types of cancers such as mammary, renal, oesophageal, gastrointestinal and reproductive cancers (De Pergola and Silvestris, 2013). The pro-inflammatory and oxidative environment is optimal for tumours development (Park *et al.*, 2014). Obesity has also consequences in eating disorders and osteoarthritis, among others (Sim *et al.*, 2013; Bliddal *et al.*, 2014; Jasik, 2014).

2.4. Treatment of obesity

Considering the economical impact and health costs of obesity, different strategies and guidelines have been proposed to treat it (Abete *et al.*, 2011; de la Iglesia *et al.*, 2013; de la Iglesia *et al.*, 2014; Millen *et al.*, 2014). The principal aim is to change lifestyle factors through the reduction of energetic and fatty dietary patterns as well as by the increase of physical activity (Bales and Kraus, 2013). However, subjects with a BMI ≥ 30 kg/m^2 or those with a BMI ≥ 27 kg/m^2 and associated co-morbidities, such as type 2 diabetes mellitus or CVD, would be eligible for pharmacological treatment (Snow *et al.*, 2005; SEEDO, 2007). In addition, subjects with a BMI ≥ 40 kg/m^2 or a BMI ≥ 35 kg/m^2 together with serious co-morbidities would be eligible for surgery (Bray, 2007; SEEDO, 2007). Recently, new therapeutic approaches such as the use of plant bioactive components with adipose tissue lowering effects are investigating (Caimari *et al.*, 2013; Ali *et al.*, 2014; Wang *et al.*, 2014; de la Garza *et al.*, 2015).

3. Effect of cocoa flavanols on obesity and related comorbidities

Although the use of cocoa in health has been described since the XVII century, the interest concerning the benefits of cocoa flavanols on human health and specifically on cardiovascular health have increased in recent years (Dillinger *et al.*, 2000; Keen, 2001; McCullough *et al.*, 2006). Apart from the benefits on CVD, cocoa flavanols have shown positive effects on the immune-system, ageing, cancer, nervous-system, tooth health and other health alterations (Becker *et al.*, 2013; Badrie *et al.*, 2015; De Araujo et al., 2016).

3.1. Effect on body weight and body composition

In humans, the consumption of chocolate and cocoa derived products is often associated with body-weight gain due to sugar and fat content (Greenberg *et al.*, 2015), but the scientific evidence is inconclusive. Most intervention studies conducted in humans have not seen significant changes in BMI and adiposity markers after cocoa intake (Crews *et al.*, 2008; Njike *et al.*, 2011; Di Renzo *et al.*, 2013). However, some epidemiological studies have observed an inverse correlation between cocoa intake and BMI (Strandberg *et al.*, 2008; O'Neil *et al.*, 2011; Golomb *et al.*, 2012).

The anti-obesity properties of cocoa flavanols have been studied in animal models showing positive effects on body fat, blood lipids, adipose tissue, the expression of some enzymes involved in the regulation of lipid metabolism, the inhibition of key digestive enzymes and modification of satiating feelings (Matsui *et al.*, 2005; Gu *et al.*, 2011; Ali *et al.*, 2015). In this sense, Yamashita *et al* (2012) observed in high fat diet-fed mice that cocoa procyanidins promoted the activation of AMPK pathway inducing the inhibition of lipogenesis and fatty acid oxidation in liver and muscle (Yamashita *et al.*, 2012). In addition, the intake of a cocoa extract reduced fat mass by decreasing the adipogenesis in preadipocites and reducing insulin resistance kinase activity (Min *et al.*, 2013), whose activation induces a downstream signalling implicated in adipogenesis (Miki *et al.*, 2001; Bluher *et al.*, 2002). Moreover, the intake of cocoa polyphenols reduced the expression of genes encoding for lipogenic enzymes and promoted the expression of genes encoding for lipolytic enzymes in diet-induced obese rats (Ali *et al.*, 2015).

On the other hand, overweight and obesity may be prevented disturbing the hydrolysis and absorption of dietary carbohydrates and lipids. Thus, Gu *et al* (2011) noted that the major enzymes involved in the hydrolysis of dietary starch and fat (pancreatic α-amylase, pancreatic lipase and pancreatic phospholipase A2) were inhibited by the action of cocoa extract and cocoa procyanidins (Gu *et al.*, 2011). Moreover, mice supplemented with cocoa and fed on a high fat diet, reduced weight gain decreasing lipid absorption and increasing faecal excretion (Gu *et al.*, 2014).

Interestingly, the appetite feeling was found suppressed after chocolate smelling and negatively correlating with ghrelin levels in humans (Massolt *et al.*, 2010).

The most important randomized controlled trials (RCT) assessing the effect of cocoa flavanol intake on body-weight of subjects older than 50 years old is shown in **Table 1**. Five studies were found with these criteria (Taubert *et al.*, 2007; Monagas *et al.*, 2009; Sathyapalan *et al.*, 2010; Njike *et al.*, 2011; West *et al.*, 2014). The duration of the studies was between 4 and 18 weeks and cocoa flavonoids were provided through cocoa beverage or chocolate bar. All the studies reported no effect on body weight after cocoa consumption, except Monagas *et al* (2009) who observed an increase (Monagas *et al.*, 2009).

Table 1. Randomized controlled trials assessing the effect of cocoa on body-weight of subjects older than 50 years old.

Study	Design*	Population	Duration (week)	Treatment type	Dose (mg/d)	Control type	Dose (mg/d)	Outcome
(West *et al.*, 2014)	Crossover	n=30 Healthy Age: 53 Overweight	4	Chocolate bar + beverage	FL:814 EP:73.6	Chocolate bar + beverage	FL: 3 EP: 0.9	No effect
(Njike *et al.*, 2011)	Crossover	n= 37 Healthy Age: 52 Obese	6	Beverage (sugar free or sugar sweetened)	EP: 48	Cocoa-free beverage	0	No effect
(Sathyapalan *et al.*, 2010)	Crossover	n= 10 Chronic Fatigue syndrome Age: 52 Overweight	8	Chocolate bar	EP: 0.06	Chocolate bar	EP<0.05	No effect
(Monagas *et al.*, 2009)	Crossover	n= 42 Risk CVD Age: 70 Overweight	4	Cocoa beverage	PO: 495.2 EP: 46.08	Skimmed milk	0	Cocoa group increased body weight
(Taubert *et al.*, 2007)	Parallel	n= 44 Prehypertension Age: 63 Normoweight	18	Chocolate bar	PO: 30 EP: 5.1	White chocolate	0	No effect

*Double-blind, randomised controlled trial

PubMed search: cocoa + humans+ body weight+ randomised controlled trial

Abbreviations: EP, epicatechin,: FL, flavanol; PO, polyphenol

3.2. Effect on insulin resistance and type 2 diabetes

Several studies have demonstrated the benefits of consuming polyphenol rich foods on glucose metabolism (Hooper *et al.*, 2012; Grassi *et al.*, 2013b; Mastroiacovo *et al.*, 2015). During insulin resistance and type 2 diabetes, liver does not function properly and glucose levels are not controlled, leading to hyperglycemia which reduces the antioxidant defences (Evans *et al.*, 2002; Dey and Lakshmanan, 2013). Interestingly, cocoa flavanols are capable to improve antioxidant status of insulin resistance hepatic cells, suggesting the possible implication of cocoa flavanols against oxidative injury produced by hperglycemia (Cordero-Herrera *et al.*, 2015). Moreover, the consumption of cocoa improves glucose uptake and tolerance by translocation of GLUT4 to the plasma membrane in muscle cells (Yamashita *et al.*, 2012).

Shrime *et al* (2011) performed a meta-analysis including 24 short-term RCT studies and comprising 1106 subjects, with the aim to analyse the effect of flavonoid rich cocoa consumption on cardiovascular risk factors (Shrime *et al.*, 2011). A reduction of homeostasis model of insulin resistance (HOMA-IR) was found, but no effects were observed in fasting glucose concentrations (Shrime *et al.*, 2011).

In the same line, Hooper *et al* (2012) carried out a meta-analysis including 42 randomized trials and encompassing 1297 subjects in order to evaluate the effect of cocoa, chocolate and flavan-3-ols on major CVD risk factors (Hooper *et al.*, 2012). They reported a reduction of HOMA-IR due to a significant reduction in serum insulin (Hooper *et al.*, 2012) and suggested that the consumption of 50–100 mg epicatechin/day is necessary to improve fasting glucose levels (Hooper *et al.*, 2012).

Epidemiological studies observed also an inverse relationship between cocoa consumption and the incidence of insulin resistance or type 2 diabetes (Oba *et al.*, 2010; Greenberg, 2015; Matsumoto *et al.*, 2015). Interestingly, the consumption of chocolate up to 2-6 servings (1 oz) per week, decreases the risk to suffer from diabetes (Matsumoto *et al.*, 2015).

The RCT studies that evaluate the effect of cocoa consumption on HOMA-IR in subjects older than 50 years old are shown in **Table 2**. The design of the studies was crossover or parallel and double-blinded. The sample size was always less than 100 subjects per study and duration of the trials was between 5 days and 8 weeks. The food matrixes to supply cocoa polyphenols were beverages, chocolate bars or both, while control type consisted on flavanol free or low polyphenol variants. Flavanol dose ranged from 48 mg to almost 1000 mg per day. Concerning the results, some of the studies reported a reduction on insulin resistance (Desideri *et al.*, 2012; Mastroiacovo *et al.*, 2015), while others did not find any changes (Muniyappa *et al.*, 2008; Mellor *et al.*, 2010; West *et al.*, 2014).

Focusing on the studies with overweight and obese subjects, Mastroiacovo *et al* (2015) and Desideri *et al* (2012) assigned volunteers to consume a cocoa beverage among 3 different cocoa beverages with different flavanol content (~990 mg, 520 mg, 45 mg). After 8 weeks, they observed a reduction on insulin resistance with cocoa beverages containing 990 and 520 mg of flavanols and not with the beverage containing 45 mg of flavanols (Desideri *et al.*, 2012; Mastroiacovo *et al.*, 2015). West *et al* (2014) did not demosntrate a reduction of insulin resistance after 4 weeks consuming 814 mg of flavanols containing cocoa beverage and bar (West *et al.*, 2014), as well as Mellor *et al* (2010) or Muniyapa *et al* (2008), who did not reporte a reduction of insulin resistance after 8 weeks consuming 16.6 mg of epicatechin containing chocolate bar in type 2 diabetic overweigh/obese patients or after 2 weeks consuming 900 mg of flavanols containing beverage in obese hypertensive subjects respectively.

Table 2. Randomized controlled trials assessing the effect of cocoa flavanols on insulin resistance of human subjects older than 50 years

Study	Design*	Population	Duration (week)	Treatment type	Dose (mg/d)	Control type	Dose (mg/d)	Outcome
(Mastroiacovo *et al.*, 2015)	Parallel	n=90 Healthy Age: 70 y Overweight	8	Cocoa beverage	FL:993 EP:185, FL:520 EP:95, L:48 EP:5	—	—	Reduction with: 993 mg and 520 mg
(West *et al.*, 2014)	Crossover	n=30 Healthy Age: 53 Overweight	4	Chocolate bar + beverage	FL:814 EP:73.6	Chocolate bar + cocoa free beverage	FL:3 EP:0.9	No effect
(Desideri *et al.*, 2012)	Parallel	n=90 Mild cognitive impairment Age: 71 Overweight	8	Cocoa beverage	FL:990, FL:520, L:45	—	—	Reduction with: 990 mg and 520 mg
(Mellor *et al.*, 2010)	Crossover	n=12 Type 2 diabetes Age: 68 Overweight /obese	8	Chocolate bar	EP:16.6	Chocolate bar	EP:2	No effect
(Muniyappa *et al.*, 2008)	Crossover	n=20 Hypertension Age: 51 Obese	2	Cocoa beverage	FL:900 EP:174	Cocoa beverage	FL:28 EP:2	No effect

*Double-blind, randomised controlled trial

PubMed search: cocoa + humans+ insulin resistance+ randomised controlled trial

Abbreviations: EP, epicatechin,: FL, flavanol

3.3. Effect on cardiovascular diseases

Diet is an important lifestyle factor that strongly influences the incidence of CVD (Tourlouki *et al.*, 2009; Georgousopoulou *et al.*, 2014). Fruits, vegetables and dietary patterns such as Mediterranean diet reduce the incidence of CVD (Estruch *et al.*, 2013; Hartley *et al.*, 2013), while the increased consumption of saturated fats and alcohol increase the incidence of these diseases (Herieka and Erridge, 2014). Plant polyphenols such as flavanols, may decrease cardiovascular risk reducing oxidative damage, inflammation and improving vascular homeostasis regulation (Andriantsitohaina *et al.*, 2012; Khurana *et al.*, 2013; Quinones *et al.*, 2013). Cocoa is a rich source of flavanols with cardiovascular protection due to the involvement on blood lipid reduction, blood pressure and endothelial function among others (Hooper *et al.*, 2012; Arranz *et al.*, 2013; Ellam and Williamson, 2013).

3.3.1. Lipid metabolism

The scientific evidence has suggested the improvement of lipid profile after cocoa consumption, principally increasing HDL-c and decreasing TG levels (Flammer *et al.*, 2012; Khan *et al.*, 2012; Neufingerl *et al.*, 2013). However, other investigations did not reported changes on lipid profile (Heiss *et al.*, 2010; Njike *et al.*, 2011; Rull *et al.*, 2015).

Jia *et al* (2010) performed a meta-analysis to analyse the short term effects of cocoa product consumption on lipid profile (Jia *et al.*, 2010). They evaluated 8 RCT including 215 subjects, observing a reduction on LDL-c and a marginal decrease of total cholesterol (Jia *et al.*, 2010). Cholesterol lowering effect was only seen in those subjects who consumed low doses of cocoa and suffering from CVD risk. There were no changes in HDL-c and there was no a dose-dependent response (Jia *et al.*, 2010).

In the same way, Tokede *et al* (2011) performed a meta-analysis to evaluate the effect of chocolate and cocoa products on lipid metabolism, specifically on total cholesterol, LDL-c, HDL-c and TG (Tokede *et al.*, 2011). They analysed 10 short term RCT involving 320 subjects, finding beneficial effect of cocoa intake on LDL-c reduction and total cholesterol reduction. They did not observe any improvement on HDL-c ant TG levels. Moreover, they reported that cocoa bar was better food matrix compared with cocoa beverage (Tokede *et al.*, 2011).

The meta-analysis by Shrime *et al* (2011) observed that flavonoid rich chocolate consumption decreased LDL-c and increased HDL-c, whereas total cholesterol and TG levels remained unchanged (Shrime *et al.*, 2011).

Concerning the meta-analysis by Hooper *et al* (2012) a marginal significant effect on LDL-c and HDL-c was observed. Moreover, they found that only studies with less than 3 week of duration reduced fasting LDL-c and total cholesterol, and only those studies of more than 3-week of duration increased HDL-c. Concerning the TG levels, they observed a reduction at moderate doses (50–100 mg epicatechin/day) but no effect was detected with lower (<50 mg/day) or higher (>100 mg/day) doses (Hooper *et al.*, 2012).

The most important RCT studies assessing the effect of cocoa flavanols consumption on lipid profile of subjects older than 50 years old are shown in **Table 3**. Overall, in each study participated less than 155 subjects. The duration of the studies was between 1 and 12 weeks and food matrixes were chocolate bar or cocoa beverage.

Focusing on the studies with overweight obese subjects, 16 trials are reported (Taubert *et al.*, 2003; Wang-Polagruto *et al.*, 2006; Taubert *et al.*, 2007; Balzer *et al.*, 2008; Crews *et al.*, 2008; Muniyappa *et al.*, 2008; Monagas *et al.*, 2009; Heiss *et al.*, 2010; Mellor *et al.*, 2010; Njike *et al.*, 2011; Desideri *et al.*, 2012; Flammer *et al.*, 2012; Khan *et al.*, 2012; Neufingerl *et al.*, 2013; West *et al.*, 2014; Dower *et al.*, 2015; Grassi *et al.*, 2015; Mastroiacovo *et al.*, 2015; Rull *et al.*, 2015) 5 of them observing an increase of HDL-c (Wang-Polagruto *et al.*, 2006; Monagas *et al.*, 2009; Mellor *et al.*, 2010; Flammer *et al.*, 2012; Khan *et al.*, 2012). LDL-c was reduced in 2 studies (Balzer *et al.*, 2008; Mastroiacovo *et al.*, 2015) and total cholesterol and TG levels decreased in 1 study (Mastroiacovo *et al.*, 2015).

Table 3. Randomized controlled trials assessing the effect of cocoa flavanols on blood lipid profile of human subjects older than 50 years

Study	Design	Population	Duration (week)	Treatment type	Dose (mg/d)	Control type	Dose (mg/d)	Outcome
(Heiss *et al.*, 2015)	Parallel	n= 42 Healthy Age: 26 and 60 Overweight	2	Cocoa beverage	FL:900 EP:128	Flavanol free beverage	0	SBP reduction in elderly group
(Rull *et al.*, 2015)	Crossover	n= 26 Mild hypertension Age: 55.4 Overweight	6	Chocolate bar	FL:1064	Chocolate bar	FL:88	No effect
(Mastroiacovo *et al.*, 2015)	Parallel	n=90 Healthy Age: 70 y Overweight	8	Cocoa beverage	FL:993 EP:185, FL:520 EP:95, L:48 EP:5	—	—	SBP: reduced with 993 mg and 520 mg doses DBP: reduced with 993 mg, 520 mg and 48 mg doses
(Grassi *et al.*, 2015)	Crossover	n= 20 Healthy Age: 53.8 Overweight	1	Cocoa beverage	FD:80, 200, 500 , 800	Flavanol free beverage	0	SBP and DBP reduction compared to control
(West *et al.*, 2014)	Crossover	n=30 Healthy Age: 53 Overweight	4	Chocolate bar + beverage	FL:814 EP:73.6	Chocolate bar + beverage	FL:3 EP:0.9	No changes in fasting BP after intervention, but SBP increased at 2h of consumption compared to control
(Desideri *et al.*, 2012)	Parallel	n=90 Mild cognitive impairment Age: 71 Overweight	8	Cocoa beverage	FL:990 EP:185, FL:520 EP:95, FL:45 EP:5	—	—	SBP and DBP decreased with 990 and 520 mg doses
(Flammer *et al.*, 2012)	Parallel	n= 20 Congestive heart failure Age: 60 Overweight	2 h ; 4 w	Chocolate bar	PO:624 EP:36	Cocoa-liquor-free chocolate	0	No effect
(Njike *et al.*, 2011)	Crossover	n= 37 Healthy Age: 52 Obese	6	Beverage (sugar free or sugar sweetened)	EP:48	Cocoa-free beverage	0	No effect

(Heiss *et al.*, 2010)	Crossover	n=16 Coronary artery disease Age: 64 Overweight	4	Cocoa beverage	FL:375 EP:59	Cocoa beverage	FL:9 EP:1	SBP decreased with 375mg and was different to 9 mg. DBP no affected.
(Davison *et al.*, 2010)	Parallel	n=52 Mild hypertension Age: 55 Overweight /0bese	6	Cocoa beverage	FL:1052 EP:208, FL:712 EP:138, FL:372 EP:69, FL:33 EP:0	—	—	Reduction with 1052 mg
(Monagas *et al.*, 2009)	Crossover	n= 42 Risk CVD Age: 70 Overweight	4	Cocoa beverage	PO:495.2 EP:46.08	Skimmed milk	0	No effect
(Muniyappa *et al.*, 2008)	Crossover	n=20 Hypertension Age: 51 Obese	2	Cocoa beverage	FL:900 EP:174	Low flavanol cocoa beverage	FL:28 EP:2	No effect
(Balzer *et al.*, 2008)	Parallel	n= 41 Type 2 diabetes Age: 64 Obese	4	Cocoa beverage	FL:963	Low flavanol cocoa beverage	FL:75	No effect
(Crews *et al.*, 2008)	Parallel	n= 90 Healthy Age: 69 Overweight	6	Chocolate bar + cocoa beverage	PRO:5102	Low polyphenol placebo	PRO:452	No effect
(Taubert *et al.*, 2007)	Parallel	n= 44 Prehypertension Age: 63 Normoweight	18	Chocolate bar	PO:30 EP:5.1	White chocolate	0	SBP and DBP decreased with dark chocolate and compared to control.
(Wang-Polagruto *et al.*, 2006)	Parallel	n= 32 HC Age: 55 Overweight	6	Cocoa beverage	FL:446	Cocoa beverage	FL:43	446 mg dose did not change BP but 43 mg dose decreased SBP and DBP
(Taubert *et al.*, 2003)	Crossover	n= 13 Mild isolated systolic hypertension Age: 55-64 Normoweight	2	Chocolate bar	PO:500	White chocolate	0	SBP and DBP decreased in treatment arm

* Double-blind, randomised controlled trial
PubMed search: cocoa + humans+ HDL-c/LDL-c/total cholesterol/TG + randomised controlled trial
Abbreviations: CVD, cardiovascular disease, EP, epicatechin,: FL, flavanol; FD: Flavonoid; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; TC, total cholesterol; TG, trygliceride; PO: Polyphenol

3.3.2. Blood pressure

The consumption of cocoa flavanols is associated with blood pressure lowering properties due to the capacity to stimulate NO dependent vasodilation (Fraga *et al.*, 2011; Quinones *et al.*, 2011). Indeed, recently, a large prospective population study in the UK known as European Prospective Investigation into Cancer-Norfolk (EPIC-Norfolk), found a negative association between the consumption of chocolate and blood pressure levels (Kwok *et al.*, 2015).

The interest about the blood pressure lowering properties of cocoa flavanols started with the discovery of the low prevalence of hypertension in Kuna Indians in Central America who lived on the San Blas Island off Panama (McCullough *et al.*, 2006; N, 2006; Ried *et al.*, 2012). Some of the Kuna Indians migrated to the mainland and manifested higher incidences of hypertension and greater blood pressure levels linked to ageing comparing to the Kuna Indians in the Island. This difference between both populations suggested a strong influence of lifestyle and dietary factors (McCullough *et al.*, 2006; Ried *et al.*, 2012). While the Kuna Indians living in the island consumed daily about 3-4 cups of cocoa drinks, the Kuna Indians living in the mainland consumed around 10 times less cocoa, inferring cocoa as a blood pressure control agent (McCullough *et al.*, 2006).

An interesting meta-analysis by Ried *et al* (2012) assessed the effect of flavanol rich chocolate or cocoa products versus low flavanol products or placebo on blood pressure of adults with or without hypertension (Ried *et al.*, 2012). A total of 20 studies involving 856 participants were included in the meta-analysis. They concluded that blood pressure was significantly reduced by 2-3 mmHg after the consumption of flavanol rich cocoa products compared to control groups in 2-18 weeks short-term studies. Subjects consumed around 30-1080 mg of flavanols per day (mean of 545.5 mg) suggesting that 2 week duration studies were more effective than longer term trials. However, Ried *et al* (2012) pointed out that this result could be confounded by the flavanol dosage in control groups because most of the 2 week trials used no flavanol free controls and the unblinding of participants (Ried *et al.*, 2012).

The meta-analysis by Shrime *et al* (2011) concluded that the consumption of flavonoid rich chocolate for at least 2 weeks decreased systolic blood pressure (SBP) by 1.63 mmHg, while diastolic blood pressure (DBP) was unaffected (Shrime *et al.*, 2011).

Hooper *et al* (2012) reported a significant reduction of DBP and mean arterial pressure after the chronic intake of cocoa (Hooper *et al.*, 2012). Moreover, they suggested that 50 mg/day of epicatechin has greater effects for SBP and DBP control (Hooper *et al.*, 2012).

Table 4 shows the RCT trials carried out with cocoa in subjects older than 50 years old. Those studies were crossover or parallel and double-blinded. The sample size was less than 155 subjects per study and the duration of the trials was between 1 and 18 weeks. Selected food matrixes to provide cocoa polyphenols were beverages, chocolate bars or both, while control type

consisted on flavanol free or low polyphneol variants. Flavanol dose ranged from 30 mg to almost 1064 mg per day. Heterogeneous results have been reports, since some of the studies observed a reduction of blood pressure after cocoa intake (Taubert *et al.*, 2003; Wang-Polagruto *et al.*, 2006; Taubert *et al.*, 2007; Davison *et al.*, 2010; Heiss *et al.*, 2010; Desideri *et al.*, 2012; West *et al.*, 2014; Grassi *et al.*, 2015; Heiss *et al.*, 2015; Mastroiacovo *et al.*, 2015), while other studies did not (Balzer *et al.*, 2008; Crews *et al.*, 2008; Muniyappa *et al.*, 2008; Monagas *et al.*, 2009; Njike *et al.*, 2011; Flammer *et al.*, 2012; Rull *et al.*, 2015).

Focusing only on the studies carried out with overweight/obese subjects, 14 trials are reported (Wang-Polagruto *et al.*, 2006; Balzer *et al.*, 2008; Crews *et al.*, 2008; Muniyappa *et al.*, 2008; Monagas *et al.*, 2009; Davison *et al.*, 2010; Heiss *et al.*, 2010; Njike *et al.*, 2011; Desideri *et al.*, 2012; West *et al.*, 2014; Grassi *et al.*, 2015; Heiss *et al.*, 2015; Mastroiacovo *et al.*, 2015). From those studies, 9 trials did not improve or changed blood pressure levels after the intervention while 5 studies observed a reduction in blood pressure with doses of 993 mg and 520 mg of flavanols per day during 8 weeks (Desideri *et al.*, 2012; Mastroiacovo *et al.*, 2015), with 80, 200, 500 and 800 mg of flavonoids after 1 week (Grassi *et al.*, 2015), 375 mg of flavanols during 4 weeks (Heiss *et al.*, 2010) and after 1052 mg of flavanol intake during 6 weeks (Davison *et al.*, 2010).

Table 4. Randomized controlled trials assessing the effect of cocoa flavanols on blood pressure of human subjects older than 50 years

Study	Design	Population	Duration (week)	Treatment type	Dose (mg/d)	Control type	Dose (mg/d)	Outcome
(Heiss *et al.*, 2015)	Parallel	n= 42 Healthy Age: 26 and 60 Overweight	2	Cocoa beverage	FL:900 EP:128	Flavanol free beverage	0	SBP reduction in elderly group
(Rull *et al.*, 2015)	Crossover	n= 26 Mild hypertension Age: 55.4 Overweight	6	Chocolate bar	FL:1064	Chocolate bar	FL:88	No effect
(Mastroiacovo *et al.*, 2015)	Parallel	n=90 Healthy Age: 70 y Overweight	8	Cocoa beverage	FL:993 EP:185, FL:520 EP:95, L:48 EP:5	—	—	SBP: reduced with 993 mg and 520 mg doses DBP: reduced with 993 mg, 520 mg and 48 mg doses
(Grassi *et al.*, 2015)	Crossover	n= 20 Healthy Age: 53.8 Overweight	1	Cocoa beverage	FD:80, 200, 500 , 800	Flavanol free beverage	0	SBP and DBP reduction compared to control
(West *et al.*, 2014)	Crossover	n=30 Healthy Age: 53 Overweight	4	Chocolate bar + beverage	FL:814 EP:73.6	Chocolate bar + beverage	FL:3 EP:0.9	No changes in fasting BP after intervention, but SBP increased at 2h of consumption compared to control
(Desideri *et al.*, 2012)	Parallel	n=90 Mild cognitive impairment Age: 71 Overweight	8	Cocoa beverage	FL:990 EP:185, FL:520 EP:95, FL:45 EP:5	—	—	SBP and DBP decreased with 990 and 520 mg doses
(Flammer *et al.*, 2012)	Parallel	n= 20 Congestive heart failure Age: 60 Overweight	2 h ; 4 w	Chocolate bar	PO:624 EP:36	Cocoa-liquor-free chocolate	0	No effect
(Njike *et al.*, 2011)	Crossover	n= 37 Healthy Age: 52 Obese	6	Beverage (sugar free or sugar sweetened)	EP:48	Cocoa-free beverage	0	No effect

(Heiss *et al.*, 2010)	Crossover	n=16 Coronary artery disease Age: 64 Overweight	4	Cocoa beverage	FL:375 EP:59	Cocoa beverage	FL:9 EP:1	SBP decreased with 375mg and was different to 9 mg. DBP no affected.
(Davison *et al.*, 2010)	Parallel	n=52 Mild hypertension Age: 55 Overweight /0bese	6	Cocoa beverage	FL:1052 EP:208, FL:712 EP:138, FL:372 EP:69, FL:33 EP:0	—	—	Reduction with 1052 mg
(Monagas *et al.*, 2009)	Crossover	n= 42 Risk CVD Age: 70 Overweight	4	Cocoa beverage	PO:495.2 EP:46.08	Skimmed milk	0	No effect
(Muniyappa *et al.*, 2008)	Crossover	n=20 Hypertension Age: 51 Obese	2	Cocoa beverage	FL:900 EP:174	Low flavanol cocoa beverage	FL:28 EP:2	No effect
(Balzer *et al.*, 2008)	Parallel	n= 41 Type 2 diabetes Age: 64 Obese	4	Cocoa beverage	FL:963	Low flavanol cocoa beverage	FL:75	No effect
(Crews *et al.*, 2008)	Parallel	n= 90 Healthy Age: 69 Overweight	6	Chocolate bar + cocoa beverage	PRO:5102	Low polyphenol placebo	PRO:452	No effect
(Taubert *et al.*, 2007)	Parallel	n= 44 Prehypertension Age: 63 Normoweight	18	Chocolate bar	PO:30 EP:5.1	White chocolate	0	SBP and DBP decreased with dark chocolate and compared to control
(Wang-Polagruto *et al.*, 2006)	Parallel	n= 32 HC Age: 55, Overweight	6	Cocoa beverage	FL:446	Cocoa beverage	FL:43	446 mg dose did not change BP but 43 mg dose decreased SBP and DBP
(Taubert *et al.*, 2003)	Crossover	n= 13 Mild isolated systolic hypertension Age: 55-64 Normoweight	2	Chocolate bar	PO:500	White chocolate	0	SBP and DBP decreased in treatment arm

*Double-blind, randomised controlled trial
PubMed search: cocoa + humans+ blood pressure + randomised controlled trial
Abbreviations: BP, blood pressure; DBP, diastolic blood pressure; EP, epicatechin,: FL, flavanol; FD: Flavonoid; PO: Polypheno; SBP, systolic blood pressure

3.3.3. Endothelial function

The dysfunction of the vascular endothelium occurs before atherosclerotic changes and it is predictive of future coronary events (Grassi *et al.*, 2011). NO is the most important endothelium derived vasodilator molecule and it is involved in vascular relaxation, vasodilation, the prevention of leukocyte adhesion and migration as well as on the reduction of platelet adhesion and aggregation (Grassi *et al.*, 2011; Lei *et al.*, 2013). Under normal physiological conditions the endothelium maintains normal vascular tone and blood flow and the concentration of pro-inflammatory factors is low, whereas in presence of cardiovascular risk factors, pro-inflammatory processes increase vasoconstrictor and pro-inflammatory molecules (Grassi *et al.*, 2011). In this environment, eNOS expression and the bioavailability of NO are decreased, reducing normal blood-flow and leading to endothelial dysfunction increaseing the risk for an atherotrombotic event (Grassi *et al.*, 2011; Karbach *et al.*, 2014).

Scientific evidence shows that cocoa flavanols as well as the consumption of plant flavonoids improve endothelial function by increasing the NO dependent FMD and decreasing vasoconstrictor molecules such as endothelin-1 levels and sVCAM-1 and sICAM-1 adhesion molecules (Monahan, 2012; Grassi *et al.*, 2013b). In this context, the European Food Safety Authority (EFSA) supported a cause and effect relationship between the consumption of cocoa flavanols and endothelium health (EFSA, 2012). They stated that "'In order to obtain the claimed effect, 200 mg of cocoa flavanols should be consumed daily. This amount could be provided by 2.5 g of high-flavanol cocoa powder or 10 g of high-flavanol dark chocolate. These amounts of cocoa powder or dark chocolate can be consumed in the context of a balanced diet. The target population is the general population" (EFSA, 2012).

3.4. Effect on mental and behavioural disorders: depression and anxiety

In the last years, the use of some nutrients, dietary patterns (Sanchez-Villegas *et al.*, 2013) and antioxidants (Payne *et al.*, 2012) have been used in neurological health, mood and behavioural health (Vauzour *et al.*, 2010; Kennedy, 2014).

Plant polyphenols such as cocoa flavanols, have been evaluated in mental disorders as promising therapy (Dias *et al.*, 2012; Pathak *et al.*, 2013). Scientific studies suggest that cocoa flavanols may display several neurocognitive properties, such as the improvement of memory, learn and cognitive functions as well as the reduction of mental fatigue and the increase of neuronal protection (Scholey *et al.*, 2010; Messerli, 2012; Pase *et al.*, 2013). Curiously, significant linear correlation between chocolate consumption per capita and the number of Nobel laureates was observed in 23 countries (Messerli, 2012).

In animal studies have shown that flavanols and their metabolites can cross the blood-brain barrier, inducing beneficial effects on brain tissue and function implicated in learning,

memory, and cognition especially in the hippocampus (Vauzour *et al.*, 2008; Sokolov *et al.*, 2013). The neurobiological actions of flavanols occur mainly by direct interactions with cellular signalling pathways or by enhancing brain blood-flow by the central and peripheral nervous system through the stimulation of NO production (Fisher *et al.*, 2006; Francis *et al.*, 2006; Hollenberg *et al.*, 2009). In this context, Scholey *et al* (2010) observed that the consumption of drinks containing 520 mg or 994 mg cocoa flavonoids improved cognitive performance and several studies using brain imaging techniques reported a correlation between cerebrovascular blood flow and cognitive function in humans (Scholey *et al.*, 2010).

On the other hand, it has been suggested that eating chocolate improves mood states and psychological disorders such as anxiety and depression, making people feel good (Pase *et al.*, 2013). However, few studies have been conducted to demonstrate the hypothesis that cocoa is associated with pleasure and well-being (Parker *et al.*, 2006; Kuijer and Boyce, 2014). The affinity of polyphenols for adenosine and benzodiazepine receptors (GABAa), suggests a calming effect after cocoa consumption (Medina *et al.*, 1997; Jager and Saaby, 2011). It has been suggested that chocolate craving is produced fundamentally by the palatability and sweet taste of chocolate (Michener and Rozin, 1994). The opioid system appears to play a role in the palatability of preferred food and it seems that endogenous opioid peptides enhance dopaminergic activity in the mesolimbic pathways altering the reward value of food (Si *et al.*, 1986; Scholey and Owen, 2013). Focusing on specific components of cocoa, theobromine and flavanols have been proposed as components implicated in mood enhancing effects. In this context, Sathylapalan *et al* (2010) reported that polyphenol rich versus polyphenol poor chocolate reduced symptoms of anxiety in humans with chronic fatigue (Sathyapalan *et al.*, 2010) and Scholey *et al* (2010) observed that the acute consumption of 520 and 994 mg of cocoa flavanols improved mood and cognitive performance in healthy young subjects (Scholey *et al.*, 2010). However, it seems that the ingestion of whole chocolate is more effective than the influence of specific components (Scholey and Owen, 2013). In general, there are scarce data answering how cocoa affects mood.

Moreover, cocoa may interact with some neurotransmitter systems such as dopaminergic system (chocolate contains the dopamine precursor tyrosine), serotonin and endorphins (contained in cocoa and chocolate) regulating appetite, reward and mood regulation (Jenny *et al.*, 2009).

On the other hand and considering that obesity is associated with depression and anxiety (Kim *et al.*, 2014; Konttinen *et al.*, 2014), weight loss has been suggested to be useful for the improvement of mental disorders in obese subjects (Perez-Cornago *et al.*, 2015). However, while some researchers suggest that a weight loss is positively associated with a reduction in anxiety and depression symptomatology (Stapleton *et al.*, 2013; Perez-Cornago *et al.*, 2015), other investigators did not find this effect (Eyres *et al.*, 2014; Jackson *et al.*, 2014).

Two RCT assessing anxiety and depressive symptoms in subjects older than 50 years old were found (Sathyapalan *et al.*, 2010; Pase *et al.*, 2013). Both studies reported beneficial effects of cocoa flavanol consumption on anxiety and depressive symptoms. Moreover, Scholey *et al* (2010) observed a decrease in mental fatigue after acute consumption of cocoa flavanols by young subjects (Scholey *et al.*, 2010).

3.5. Other possible health benefits of cocoa flavanols

In the scientific literature, cocoa flavanols have been mainly investigated according with their effect on CVD (Khan *et al.*, 2012; Arranz *et al.*, 2013; Mastroiacovo *et al.*, 2015). However, cocoa flavanols has more beneficial properties on health. In this sense and taking into account the antioxidant capacity and the free radical scavenging ability of cocoa, it interferes in **cancer** initiation, promotion and progression, protecting cells against ROS (D'Archivio *et al.*, 2008; Martin *et al.*, 2013). Moreover, cocoa flavanols may have antiproliferative effects by inhibiting cell growth as well as antimutagenic and chemoprotective properties to inhibit the metabolic activation of carcinogens (Martin *et al.*, 2013). In animal studies have been demonstrated that cocoa phenol components can prevent and slow down the initiation or progression of different types of cancers such as prostate, colon, leukemia, mammary, pancreatic, hepatic and lung among others (Martin *et al.*, 2013). In humans, cocoa flavanols consumption has been associated with a reduction on oxidative and inflammatory markers, which are closely related with carcinogenesis (Gu and Lambert, 2013). Indeed, some epidemiological studies have found the inverse association between cocoa flavanol consumption and the incidence of cancer (Garcia-Closas *et al.*, 1999; Arts *et al.*, 2002). However, other studies have failed to report this relation (Rouillier *et al.*, 2005).

The negative connotation of chocolate in tooth health is attributed to components such as sugar (Varoni *et al.*, 2012). However, cocoa polyphenols have anti-cariogenic effects and also reduce periodontal induced oxidative stress suggesting a protective action against **tooth damage** (Ferrazzano *et al.*, 2009; Tomofuji *et al.*, 2009).

The **antimalarial** effect of cocoa polyphenols has been proposed due to the reduced incidence of malaria in people from Ghana who regularly drank cocoa beverage (Addai, 2010). The mechanisms involved may be related to the increased availability of antioxidants in plasma which strength the immune system (Addai, 2010).

Other healthy function of cocoa polyphenols has been found on **skin health** (Scapagnini *et al.*, 2014). Cocoa polyphenols has been used in skin alterations such as skin cancer, psoriasis, acne and wound healing (Scapagnini *et al.*, 2014). The beneficial effect is associated with the antioxidant properties of cocoa to neutralize ROS and confers also anti-wrinkle, skin whitening and sunscreen effects (Karim *et al.*, 2014).

Immune system, particularly the innate inflammatory response and the systemic and intestinal adaptive immunity are influenced by cocoa polyphenols (Becker *et al.*, 2013; Perez-Cano *et al.*, 2013). Cocoa is able to induce changes in T cells and cytokine secretion (Becker *et al.*, 2013). Moreover, cocoa intake induces changes in the functionality of gut-associated lymphoid tissue modulating IgA secretion and intestinal microbiota (Perez-Cano *et al.*, 2013). Thus, long-term cocoa intake can even modify microbiota composition and immune response at intestinal level (Tzounis *et al.*, 2011). On the other hand, cocoa has also been investigated in allergy (Abril-Gil *et al.*, 2012).

II. HYPOTHESIS AND AIMS

1. Hypothesis

Based on the scientific evidence, antioxidants and phytochemical compounds may contribute to improve health status reducing cardiovascular risks, metabolic alterations, oxidative stress impairment and inflammatory disturbances among others. Therefore, the hypothesis of this study was that the intake of ready-to-eat dishes and desserts containing a cocoa extract as bioactive compound, confers benefits to improve the health status of middle-aged overweight/obese subjects, mainly mediated by the content of flavanols in the cocoa extract.

2. Aims

General aim

The general aim of this research is to assess if the consumption of a cocoa extract integrated on ready-to-eat meals and consumed within a 15% energy restricted diet for 4 weeks could have beneficial effects on the nutritional and metabolic status, as well as on depression and anxiety symptoms of healthy middle-aged overweight/obese subjects.

Specific aims

1. To evaluate the effect of a weight loss dietary intervention with an energy restriction of 15% and moderate high protein content during 4 weeks, on metabolism and general nutritional status ***(Chapters 1, 2, 3, 4 and 6)***.
2. To assess the effects derived from the daily inclusion of a cocoa extract through the consumption of ready-to-eat meals and within a weight loss dietary intervention of 4 weeks, on anthropometry and body composition, blood pressure, routine blood biochemical determinations and biomarkers linked to oxidative status, endothelial function and inflammation ***(Chapters 1 and 2).***
3. To investigate the acute effects of cocoa intake during 3 h (0, 60, 120 and 180 min) on blood biochemical and blood pressure determinations before and after 4 weeks of its daily consumption within a weight loss diet ***(Chapter 3).***
4. To analyse the effect of cocoa intake within ready-to-eat meals and under a weight loss diet on depressive and anxiety symptoms, as well as on the peripheral dopaminergic activity after 4 weeks of intervention ***(Chapter 4).***
5. To evaluate the compliance of the volunteers to the intervention assessing the presence of cocoa derived metabolites in plasma and urine through a metabolomic approach , as well as to explore metabolomic changes of interest for human health ***(Chapters 3 and 5).***

III. SUBJECTS AND METHODS

1. The overview of the study

The present study belongs to SENIFOOD project, about research of diets and foods with specific characteristics for elderly people (http://www.senifood.com/), supported by The Centre for Industrial Technological Development within the National Strategic Consortia (http://www.cdti.es/). From the scientific point of view the aim of the project was to investigate the effect of some food components over common diseases in elderly population, while from the technological point of view, the objective was to combine functional properties with physicochemical and organoleptic characteristics of the products, to make simple, palatable and attractive for consumers.

The intervention was performed in the Metabolic Unit of the University of Navarra in Pamplona (Spain), between March and July of 2012. The research group was a multidisciplinary team integrated by Medical Doctors, a nurse, a dietitian, two PhD in Pharmacy and a Professor in Nutrition. The research group had adequate facilities and methodological tools for the implementation of the investigation.

The protocol of the study was supervised and approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and it was registered at www.clinicaltrials.gov (NCT01596309) on 9th May 2012. The study followed the Helsinki Declaration guidelines and it was performed following the CONSORT guidelines (2010). The information provided by the volunteers was treated according to the law 15/1999 of 13th of December about protection of personal data. A numerical code was assigned to each volunteer in order to preserve the confidentiality and the anonymity.

2. Study design

The research study was designed as a 4 week, double-blind, randomised, placebo-controlled parallel nutritional intervention with a simultaneous postprandial sub-study in order to assess the effects of cocoa extract on cardiometabolic, oxidative, inflammatory markers and psychological behaviour, as well as to evaluate the availability of cocoa flavanols within the ready to eat meals to investigate the compliance of the volunteers and to identify metabolomic changes with interest for human health. The studied population was overweight/obese men and women aged between 50 and 80 years old.

The intervention consisted on the daily intake of ready-to-eat meals, 1 dish and 1 dessert, integrated within a weight loss controlled diet. Volunteers were randomly assigned to control or cocoa groups using the “random between 1 and 2” function in the Microsoft Office Excel (Microsoft Iberica, Spain). Cocoa group received ready-to-eat meals supplemented with cocoa extract and the control group received the same meals but without cocoa extract supplementation. Test meals consisted of a variety of ready-to-eat dishes and desserts. Each dish and each dessert was

supplemented with 0.7 g of cocoa extract in the case of cocoa group. From those meals, volunteers had to consume one dish and one dessert per day, consuming 1.4 g of cocoa extract daily. The amount of cocoa extract (1.4 g/day) was selected by the researchers and the Tutti-Pasta company, which provided the ready-to-eat meals. The meals had the same appearance and differed only on the code label, ensuring the double-blind model of the study. At the same time and considering the overweight/obese condition of the volunteers, a 15% energy restricted diet was prescribed to improve the health status of the participants and to encourage them to follow the intervention protocols. Diet, which was designed including the study products, provided 45% of total energy value from carbohydrates, 30-33% of energy from lipids and 22–25% of energy from proteins (**appendix 1**).

Participants were asked not to consume cocoa and cocoa derived products from one week before the beginning of the study and during the intervention period. In addition, three days prior to the start of the trial, participants were required to consume a low-polyphenol diet without energy restriction (**appendix 2**). On the other hand, three days prior to end of the study, subjects were asked to consume predetermined types of ready-to-eat meals. Thus, all the volunteers started and finished the intervention in comparable conditions.

Sample and data collection were taken at baseline and after 4 weeks of intervention in overnight fasting condition. Apart from the baseline and the endpoint visits, volunteers had to attend the Metabolic Unit 3 more times for the follow-up visits. Those visits were weekly programmed to provide volunteers with ready-to-eat meals, to supervise that volunteers were following the intervention properly, to check the weight loss and to encourage them to follow the intervention.

On the other hand, a postprandial sub-study was performed within the 4 week intervention to evaluate the acute effects of cocoa extract before and after 4 weeks of its daily consumption. This sub-study was carried out in the first and the last day of intervention in half of the volunteers, who also participated in the intervention study.

This part of the trial consisted on the consumption of a ready-to-eat dish and a dessert in the Metabolic Unit of the University of Navarra, supplemented or not with cocoa extract according with the group allocation. The test was performed in the morning at overnight fast condition. Samples and data collection were taken just before consuming the test meal and at 60, 120 and 180 minutes post-consumption. Between both postprandial test days, volunteers followed the 4 week intervention study under 15% energy restricted diet and consuming ready-to-eat meals daily (one meal and one dessert per day) with or without 1.4 g of cocoa supplementation, depending on the group they were assigned (**appendix 1**). **Figure 10** shows study procedures.

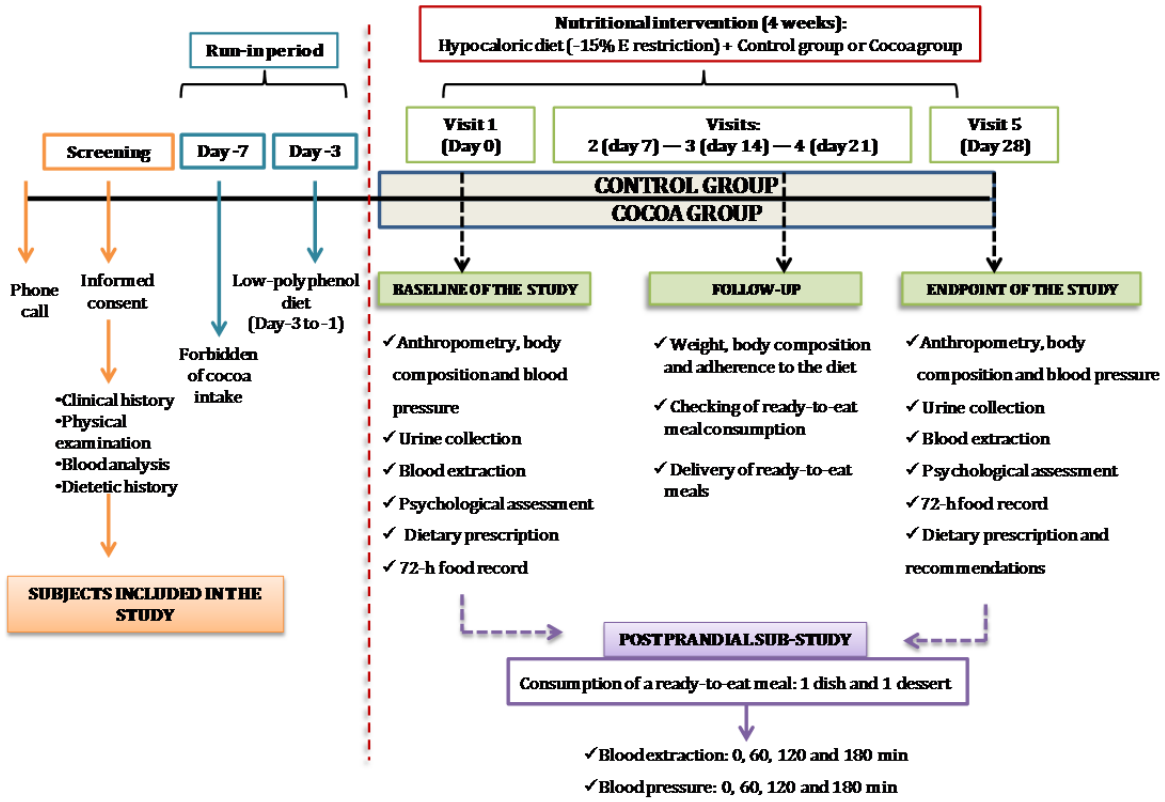

Figure 10. Chronogram of scheduled procedures of the study

3. Subjects

3.1. Sample size calculation

The sample size of this study was calculated considering oxLDL as the main variable. The sample size was estimated taking into account a reduction of 14.1 U/L and an interquartile range of 16.3 U/L, according to the study carried out by (Khan *et al.*, 2012). With a bilateral confidence index of 95% (α=0.05) and a statistical power of 80% (β=0.80) the sample size was estimated to be of 22 subjects in each group. Considering a possible drop-out rate of 15%, the final sample size was established in 50 subjects, 25 subjects in each group.

Volunteers were recruited between March and May of 2012 through advertisements in the local newspapers. A phone number and email address of the Metabolic Unit were provided in the advertisements. Thus, those subjects who were interested in participating in the trial contacted the research group and arranged an appointment for the screening process. Before beginning the study, all participants were provided with the required information and if they agree, signed the informed consent in duplicate (**appendix 3**).

3.2. Inclusion, exclusion and retirement criteria

In the screening process, inclusion and exclusion criteria were assessed by a physician, a nurse, and a dietician by means of medical history, blood biochemical analysis, physical examination and anthropometrical measurements in the Metabolic Unit of the University of Navarra. Below are the inclusion, exclusion and retirement criteria:

Inclusion criteria:

- Age: 50-80 years.
- BMI: 27.0-35.5 kg/m^2.
- Maintain a stable weight (<5% of variation) during the previous three months to the intervention.

Exclusion criteria:

- History of metabolic disorders.
- Gastrointestinal diseases.
- Diabetes.
- Cancer.
- Inflammatory diseases (such as rheumatoid arthritis).
- Food allergies.
- Cognitive alterations.
- Current sliming, hormone replacement, anti-inflammatory or BP lowering treatments.
- Medication that could influence appetite or nutrient absorption.
- Inability to perform the follow-up.
- Being smoker.
- Intake of antioxidant supplements.

Retirement criteria

Volunteers could leave the study at any time-point. The researchers had also the criteria to exclude any volunteer according with the following situations:

- Intolerable adverse events.
- Violation of the protocol.
- Lost the follow-up.
- Diagnosis of a new disease or aggravation of previously existing one requiring continue therapeutic or pharmacological treatment.
- Not to follow the prescribed diet or not to consume the test products. In the case of the study products, no consumption of test meal during 3 consecutive days was established as retirement criteria.

3.3. Flow-chart of the volunteers

- *Four week intervention study*

From 488 subjects interested in participating in the trial 113 met the inclusion criteria, but according with the sample size calculation only 50 subjects were selected to participate. Those 50 subjects were randomly assigned to follow control or cocoa interventions, 25 volunteers in each group. During the follow-up, 3 dropouts were detected, one in control group (no adherence to the diet) and the remaining 2 in cocoa group (no adherence to the diet and personal reasons). Therefore, 24 subjects completed the intervention under control group and 23 subjects under cocoa group. **Figure 11** shows the flow-chart of the 4-week intervention study.

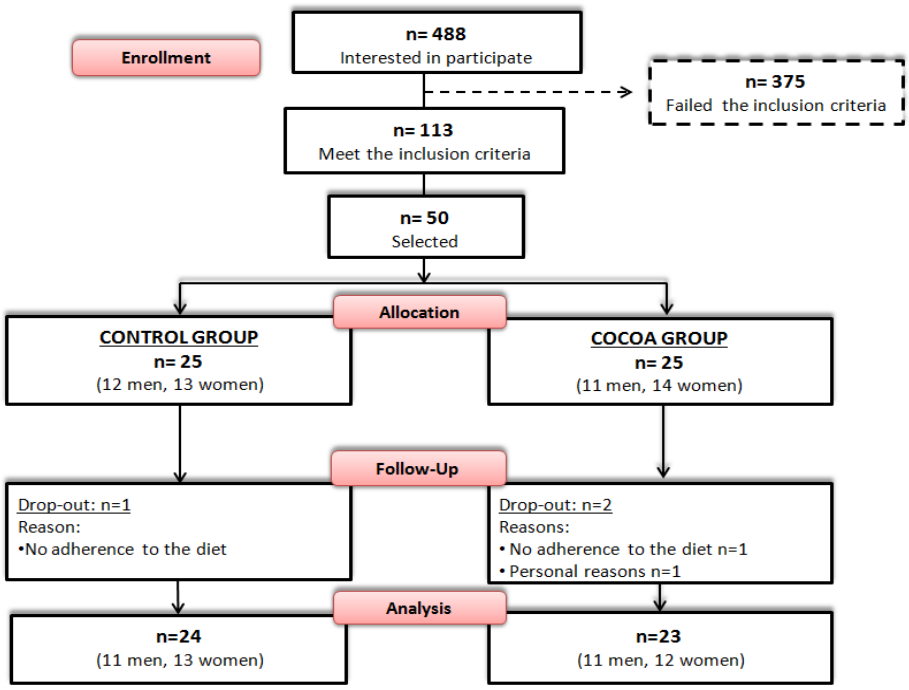

Figure 11. Flow-chart of the volunteers for 4 week intervention sub-study

- *Postprandial study*

The postprandial trial was a sub-study performed within the main study. From the 50 volunteers who participated in the main study, 24 volunteers took part on it, 12 subjects were allocated in control group and the remaining 12 in cocoa group. One subject was excluded due to non-compliance of the diet. Thus, 12 subjects completed the postprandial study under the control treatment and 11 subjects under cocoa group. Flow-chart of the postprandial sub-study is shown in **Figure 12.**

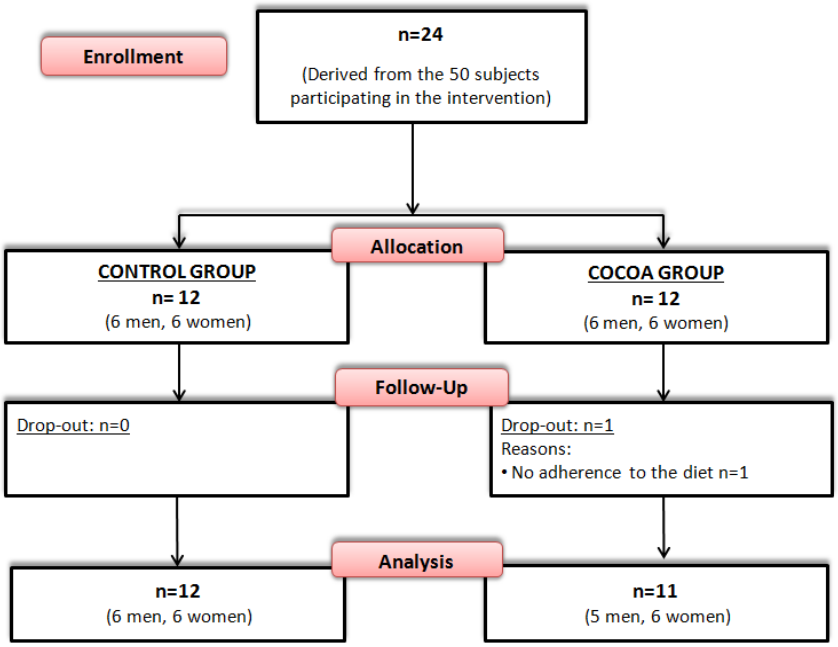

Figure 12. Flow-chart of the volunteers participating in the postprandial study

4. Nutritional strategy

4.1. Ready-to-eat meals

The Tutti Pasta® Company (http://www.tuttipasta.com/) provided the dishes and desserts used in the study. Ready-to-eat meals were designed considering likes and dislikes of middle-aged/elderly subjects, improving the nutritional profile of meals and facilitating the chewing and swallowing process. The consumption of ready-to-eat meals was established to consume one dish and one dessert per day for both intervention groups (7 dishes and 7 desserts per week). However, volunteers were weekly provided with a box containing 10 varieties of dishes (300 g each) and 8 desserts of 4 different varieties (150 g each). Ready-to-eat dishes consisted of chicken, seafood paella, zucchini cream, gazpacho, bolognese pasta, risotto funghi, chickpeas with spinach, creamed spinach, hake with tomato and gnocchi with vegetables (**Figure 13**). Desserts were coffee flavored custard, yogurt cream, nougat flavored cream and cocoa flavored cream (**Figure 14**).

Figure 13. Ready-to-eat dishes (10 different varieties)

Figure 14. Ready-to-eat desserts (4 different varieties)

The composition of each dish and dessert was analysed by the National Center for Food Safety and Technology (CNTA, http://www.cnta.es/) of Navarra, Spain. The ready-to-eat meals containing cocoa extract and the control meals had the same macronutrient and micronutrient composition, the unique difference between them was the content of cocoa extract or not. In **Table 5** is reported the composition of ready-to-eat dishes and in **Table 6** the composition of ready-to-eat desserts.

Table 5. Composition of ready-to-eat dishes used in the study (g/300g)

Composition*	Chicken	Seafood paella	Zucchini cream	Gazpacho	Bolognese pasta	Risotto funghi	Chickpeas with spinach	Creamed spinach	Hake with tomato	Gnocchi with vegetables
Energy (kcal)	363.9	359.3	246.7	233.4	400.3	376.6	418.7	215.6	210.9	339.2
CHO (g)	10.7	55.6	10.3	13.6	57.4	53.9	29.1	16.3	12.1	53.1
Protein (g)	34.3	12.1	4.6	2.8	17.6	8.4	9.1	9.4	22.7	7.9
Lipids (g)	18.2	9	20.1	18	9.8	13.7	27.5	11	7	9.1
Fiber (g)	9.9	3.8	3	2.8	6	2.2	9	6.9	4.5	6.8
SFA (g)	88.4	76	82.5	76.7	94.6	110.8	43.8	76.8	89	95.7
MUFA (g)	163	182	194.6	198	130.4	165.1	81.1	191	154	173.6
PUFA (g)	48.6	42.2	22.8	25.3	75	24.1	175.1	32.2	57	30.7
Omega 3 (g)	4.1	10.7	2.6	2.2	3.3	2.1	2.3	12.7	32.4	3.5
Omega 6 (g)	44.4	31.6	20.3	23.1	71.8	21.7	172.8	19.5	24.6	27.1
Sodium (g)	1.4	0.9	0.9	0.7	1	0.8	0.4	1	1	0.8

* The nutritional information is reported per 300 g, which was the established portion to consume per day.
Abbreviations: CHO: carbohydrates; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

Table 6. Composition of ready-to-eat desserts used in the study (g/150g)

Composition*	Coffee flavored custard	Yogurt cream	Nougat flavored cream	Cocoa flavored cream
Energy (kcal)	117	114.8	134	116.8
CHO (g)	24.5	24.2	17.4	23.9
Proteins (g)	4.2	3.5	4	4.3
Lipids (g)	<0.5	<0.5	4.6	<0.5
Fiber (g)	1.3	2.1	3.3	1.9
Sodium (g)	0.2	0.2	0.2	0.2

*The nutritional information is reported per 150 g, which was the established portion to consume per day.

Abbreviations: CHO: carbohydrates.

In order to ensure the double-blind design of the study, Tutti Pasta Company provided the ready-to-eat meals in boxes with the same external appearance, but differently codified (**Figure 15**).

Figure 15. The appearance of the boxes where ready-to-eat meals were provided.

4.2. Cocoa extract

Cocoa extract was the bioactive compound added to the ready-to-eat meals, whose composition is detailed in **Table 7**. The cocoa extract and the analytical characterization were provided by Nutrafur S.A (Murcia, Spain). The total polyphenol content was determined by the Folin-Ciocalteu colorimetric method (Singleton and Rossi, 1965; Song *et al.*, 2010) and High-performance liquid chromatography (HPLC) was used to quantify flavonoids and theobromine in the cocoa extract (Cai *et al.*, 2003). Each dish and dessert contained 0.7 g of cocoa extract. Thus, each volunteer assigned to cocoa group consumed 1.4 g of cocoa extract per day.

Table 7. The composition of cocoa extract in 1.4 g

Component	**Amount** mean (SD)
Total polyphenols as catechin (mg)[a]	645.30 (32.27)
Total flavanols as catechin (mg) [b]	414.26 (20.71)
- Epicatechin (mg)[b]	153.44 (7.67)
- Catechin (mg)[b]	14.56 (0.73)
- Dimer B2 (mg)[b]	99.40 (4.97)
- Dimer B1 (mg)[b]	13.44 (0.67)
- Oligomeric procyanidins (mg)[b]	133.53 (6.68)
Theobromine (mg)[b]	140.42 (7.02)

[a] Determined by the Folin-Ciocalteu reagent (colorimetric assay) [b] Determined by HPLC (High-performance liquid chromatography) assay (chromatographic technique)

4.3. Prescribed diet

Taking into account the overweight/obese condition of the participants and in order to encourage them to follow the intervention, a controlled energy restricted diet (15% energy restriction from the total energy requirements) was prescribed to all the volunteers (control and cocoa). The total energy requirements were estimated by means of the Harris-Benedict equation and individualised physical activity factor, which was calculated as average daily exercise (The National Academy Press, 1989).

The macronutrient distribution of the diet was 45% of total caloric value from carbohydrates, 30-33% from lipids and 22-25% from proteins. In order to comply with the macronutrient distribution a diet was designed for each dish. Diets were analysed and quantified using the DIAL software (Engineering, Madrid, Spain). An example of the diet is shown in **appendix 1.**

Moreover, volunteers were provided with a list of forbidden polyphenol rich foods (cocoa and derived products, coffee, tea, spices, wine, antioxidant supplements, fruits and vegetables rich in flavanoids) and some nutritional recommendations for the study (**appendix 1**).

5. Clinical and biochemical measurements

5.1. Anthropometry and body composition

Anthropometric and body composition measurements were assessed following validated procedures. Body weight was determined using Tanita bioelectrical impedance (SC-330, Tanita, Tokyo, Japan) accurate to 0.1 kg and height was measured by a wall-mounted stadiometer (Seca 220, Vogel & Halke, Germany) accurate to 1cm. BMI was calculated by the Quetelet equation, dividing the body weight (kg) by the squared height (m) (Perez S, 2005). The waist circumference was measured at the narrowest point between the rib cage and the iliac crest and the hip circumference at the widest point over the buttocks. Body composition was determined using DEXA Lunar Prodigy, GE Medical Systems, Madison, WI, USA (DEXA) with validated procedures (Perez S, 2005; Sun *et al.*, 2005).

5.2. Blood pressure

Blood pressure was measured three times after a five minute resting period with an automatic monitor device (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands) following WHO criteria (Whitworth and Chalmers, 2004). The average value obtained from the last two measurements was used. The measures were taken with the subject in seat position with hand resting on a table and the cuff at the level of heart, always in the same arm. The volunteers were maintained in resting conditions between measurements, free of any alterations in a quiet and

temperature-controlled room. The same protocol was used to measure the blood pressure in the postprandial sub-study, but it was taken also at 60, 120 and 180 minutes after meal consumption.

5.3. Blood collection and blood biochemical analysis

5.3.1. Blood collection and storage

Fasting (10 h) blood samples were collected between 8:00-9:30 a.m at baseline and at the end of the intervention using 7 ethylenediaminetetraacetic (EDTA) and 2 serum (CLOT) tubes. In the postprandial test, blood samples were collected also at 60, 120 and 180 minutes after meal consumption, 1 EDTA and 1 CLOT tubes at each time. Then, samples were left for 10-15 minutes at room temperature and were centrifuged to obtain plasma and serum aliquots (15 minutes, 1 500g, 4ºC). Afterwards, samples were stored at -80ºC until analysis (de la Iglesia *et al.*, 2014).

5.3.2. Glycemic, lipid, oxidative and inflammatory markers

Plasma glucose, total cholesterol, HDL-c, triglycerides, FFA, proteins and homocysteine were measured by colorimetric methods in an auto-analyser Pentra C200 (Horiba Medical, Montpellier, France) following the supplier instructions. Plasma insulin, oxLDL, MPO (Mercodia, Upssala, Sweden), TNF-α, IL-6, lipoprotein associated phospholipase A2 (Lp-PLA2) (R&D Systems, Minneapolis, USA) sVCAM-1, sICAM-1(R&D Systems, Minneapolis, USA) and C-reactive protein (Demeditec Diagnostic) were quantified with specific ELISA kits in a Triturus auto-analyser (Grifols, Barcelona, Spain) (de la Iglesia *et al.*, 2013; Lopez-Legarrea *et al.*, 2014).

The 25(OH)D levels were analysed by an external certified laboratory (Megalab S.A, Madrid, Spain) using a radioimmunoassay technique (Glendenning *et al.*, 2003; Wallace *et al.*, 2010).

Friedewald equation [LDL-c = TC-HDL-c – VLDL(TG/5)] was used to calculate LDL-c concentration (Friedewald *et al.*, 1972) and HOMA-IR index was used to estimate insulin resistance [HOMA-IR = [glucose (mmol/L) × insulin (μU/mL)]/22.5] (Matthews *et al.*, 1985). Atherogenic index was calculated as follows: total cholesterol/ HDL-c.

5.3.3. Peripheral monoamines

Peripheral concentrations of dopamine, dopac and HVA were analysed in plasma samples using HPLC technique as described elswhere (Perez-Cornago *et al.*, 2014c). MAO activity was measured in plasma samples using a commercial kit (Amplite Fluorometric Monoamine Oxidase Assay Kit, AAT Bioquest, ref 11303) and following the manufacturer's instructions.

5.3.4. Comet assay

The comet assay (single-cell gel electrophoresis) is a method used to measure DNA strand breaks in eukaryotic cells (Azqueta and Collins, 2013). It is simple, sensitive and economic and it is applied in genotoxicity testing, ecological monitoring, human studies, biomonitoring and in nutritional studies (Azqueta and Collins, 2013). Focusing on nutrition, it is used to assess the effect of antioxidants at DNA level (Collins and Azqueta, 2012).

Comet assay was carried out in lymphocytes isolated from the volunteer's blood samples which were collected in EDTA tubes. Lymphoprep sedimentation method was used to lymphocyte isolation. The comet assay format of 12 minigels per slide was used as previously described (Shaposhnikov *et al.*, 2010).

Briefly, cells were embedded in agarose on a microscope slide and then were lysed in a solution containing detergent and NaCl (Dusinska and Collins, 2008). Membranes and soluble cell constituents, as well as histones, were removed, leaving the DNA still supercoiled, known as nucleoid, and attached to the nuclear matrix (Azqueta *et al.*, 2013). Then, alkaline incubation and electrophoresis caused DNA loops containing breaks to move towards the anode forming a 'comet tail' that was visualised by fluorescence microscopy after stained with 4,6-diamidino-2-phenylindole (DAPI)(Dusinska and Collins, 2008). The % of DNA in the tail reflected the frequency of DNA breaks (Azqueta *et al.*, 2013).

In order to detect oxidative damage, the enzyme-modified version of the comet assay was used. This variant measured 8-oxo-deoxyguanosine (8-oxodG) lesions (Moller and Loft, 2006). For that a DNA digestion step with DNA formamidopyrimidine DNA glycosylase enzyme (FPG) was included after the lysis (Collins *et al.*, 1993).

In addition, with the aim of measure the antioxidant capacity of the cell, a new step was included. In this step cells were exposed to a DNA breaking agent (hydrogen peroxide) producing DNA damage and then the repair capacity of the cell was measured *(*Azqueta *et al.,* 2013*).*

The principal steps of the comet assay procedure are shown in **Figure 16** (more details in chapter 2).

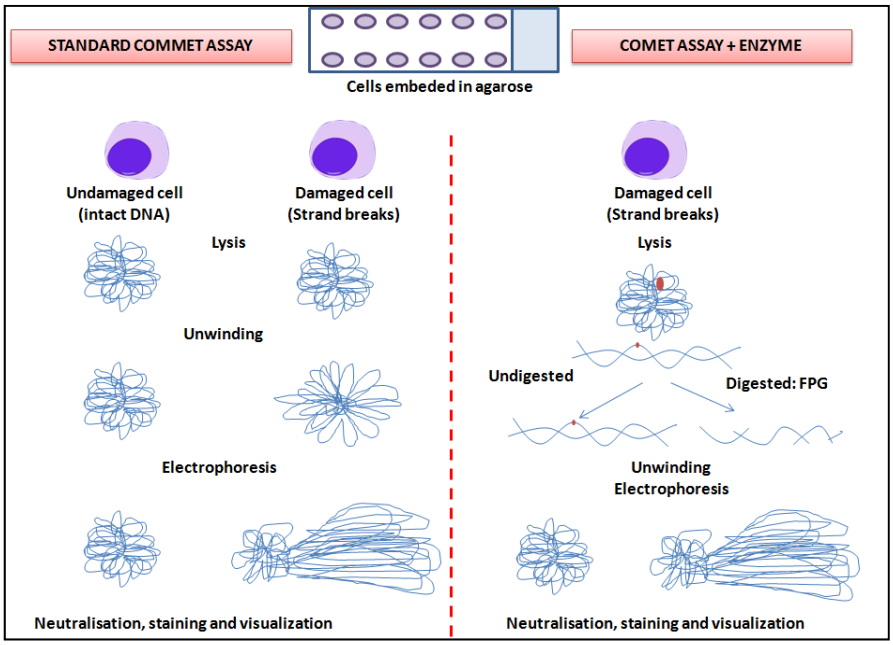

Figure 16. Diagram of the standard comet and the modified assay including digestion by FPG enzyme (Azqueta *et al.*, 2011)

5.3.5. Metabolite determination: targeted and untargeted metabolomics

Metabolomics belongs to the new field of "omics" research concerned with the comprehensive characterization of the small molecule metabolites in biological samples (Fiehn, 2002; Patti *et al.*, 2012). Metabolome is the characterization of all the metabolites and reflects genetic and environmental components such as drugs, diet, gut microflora activity etc (Zamboni *et al.*, 2015). It can provide an overview of the metabolic status and global biochemical processes associated with a cellular or biological system (Nicholson *et al.*, 2012).

Targeted metabolomics is focused on analysing selected metabolites, often related to a specific metabolic pathway. In nutrition it is usually used to determine the concentration, bioavailability, turnover, or metabolism of dietary compounds (Astarita and Langridge, 2013). On the other hand, untargeted metabolomics consists in the screening of metabolites with the intention to compare profiles of metabolites among different groups of subjects and to identify new biomarkers (Astarita and Langridge, 2013).

- *Targeted metabolomics in plasma*

In this research, the assessment of cocoa derived metabolites in plasma was used to evaluate the bioavailability of cocoa extract within the food matrix, to assess how cocoa flavanols may interact with the biochemical network of the organism and to check the adherence of the

volunteers to the intervention (Jones *et al.*, 2012; Odriozola and Corrales, 2015). Fourteen cocoa derived metabolites were analysed in plasma by targeted metabolomic in the postprandial sub-study: catechin, epicatechin, methyl-epicatechin-glucoronide, procyanidin B2, methyl-catechin-glucoronide, catechin-sulphate, epicatechin-glucoronide, epicatechin-sulphate, 3-*O*-methyl-epicatechin, methyl-epicatechin-*O*-sulphate, 3,7-dimethyluric acid, 1-methylxanthine, 3-methylxanthine and theophylline. These analysis were performed by external certified research centre (Centre Tecnològic de Nutrició i Salut (CTNS), Reus, Spain).

Briefly, the first step was the extraction of phenolic metabolites and further analysis by HPLC-MS/MS following the method described by Serra *et al* (2009) with minor modifications (Serra *et al.*, 2009), while theophylline metabolites were extracted following the method described by Ogawa *et al* (2012) with some minor modifications (Ogawa, 2012). Then, two chromatographic methods were used to analyse the whole range of metabolites. Ionisation was performed by electrospray (ESI) in the negative mode (detailed in chapter 3).

- *Untargeted metabolomics in urine*

In this investigation, untargeted metabolomic analysis was carried out in 24 h urine samples. The aim was to assess the presence of metabolites related to cocoa extract intake and the identification of metabolomic changes with interest for human health. The laboratory procedures were as follows:

Urine collection

Twenty-four hours urine specimen was collected the previous day to the beginning of the study and the day before to the last day of the intervention. Urine samples were always maintained at 4ºC and then were stored in vials of 1 mL at -80ºC until the analysis like in other studies (Llorach *et al.*, 2013).

Sample preparation and HPLC-TOF-MS analysis

The urine samples were thawed and centrifuged for 10 min at 10.000 rpm. A 100 µL aliquot of the supernatant was diluted with 100 µL of Milli-Q water and vigorously vortexed. The solution was transferred to a vial for the subsequent analyses.

Agilent Technologies 1200 liquid chromatographic system equipped with a 6220 Accurate-Mass TOF LC/MS, operated in positive electrospray ionization mode (ESI+) or negative electrospray ionization mode (ESI-), controlled by MassHunter Workstation 06.00 software (Agilent Technologies, Barcelona, Spain) was used for the analysis. The column used was a Zorbax SB-C18 (15 cm × 0.46 cm; 5 µm) from Agilent Technologies with a SB-C18 precolumn from Teknokroma (Barcelona, Spain).

The mobile phase consisted of A (formic acid 0.1%) and B (acetonitrile with formic acid 0.1%). The gradient elution, 1-20% B, 0-4 min, 20-95% B 4-6 min, 95% B 9-7.5 min, 95-1% B 7.5-8 min, 1% B 8-14 min. After the analyses, the column was re-equilibrated during 5 min at 1% B. The injection volume was 15 µL and the flow rate was 0.6 mL min−1. Chromatography was performed at 40 ºC. ESI conditions were as follows: gas temperature, 350 ºC; drying gas, 10 L min−1 ; nebulizer, 45 psig; capillary voltage, 3500 V; fragmentor, 175 V; and skimmer, 65 V. The instrument was set out to acquire over the m/z range 100 –2000 with an acquisition rate of 1.03 spectra s-1.

To evaluate the quality in this metabolomic study, a procedure from Gika *et al* (2007) and Llorach *et al* (2009) with some modifications was used (Gika *et al.*, 2007; Llorach *et al.*, 2009). Two types of quality control sample (QCs) were used: i) standard mixture solution implemented of cytosine, L-carnitine hydrochloride, betaine, leucine, deoxyadenosine and deoxyguanosine at concentration of 1 mg/L. ii) pool urine was prepared by mixing equal volumes from each of the 94 samples. These samples were injected 3 times at the beginning of the run to ensure system equilibration, and then every 5 samples to further monitor stability of the analysis. Finally, samples were randomized to reduce the systematic error associated with instrumental variability. Samples were analysed in sets of 10-15 samples/day.

Data Processing and metabolite identification

Liquid Chromatography–Mass Spectrometry (LC-MS) data was analyzed using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain) to detect and align features. Alignment used 0.15 min retention time tolerance window, and 0.002 Da mass tolerance window. For the screening of metabolites, the following filters were specified: the m/z of metabolites should appear in at least one of 94 samples. Subsequently, the detected m/z should be present in 50% of samples in only experimental group. Metabolites contributing to the discrimination among groups were then identified on the basis of their exact mass, which was compared to the registered in METLIN (https://metlin.scripps.edu/index.php) within a mass accuracy below 5 mDa, as well as based on the score given by the software , the scientific literature and the metabolic pathways reported in Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and Lipidmaps (http://www.lipidmaps.org/).

A one-way ANOVA was conducted followed by a Tukey range test, and a Benjamini–Hochberg multiple correction procedure was used to statistically compare and define statistically significant metabolites ($p < 0.01$). Finally, metabolites that satisfy a fold change cut-off of 2.0 were selected. Then, the resulting data were analyzed by principal components analysis (PCA), using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain).

5.4. Psychological assessment

Psychological assessment was performed using validated questionnaires at baseline and at 4 week of intervention.

5.4.1. Depressive symptoms

Symptoms of depression were evaluated using the Spanish version of the Beck Depression Inventory, BDI (Conde and Useros, 1975), which is a validated and reliable measure of depressive symptoms (Beck *et al.*, 1961) (**appendix 4**). The BDI is a 21-item test answered on a 4-point scale. This questionnaire measures the presence and degree of depressive symptoms. Scores can range from 0 to 63, with a score of 10 or higher indicating moderate depressive symptoms (Conde and Useros, 1975). Question number 19 of the test, relating to weight loss, was discarded from the analyses given that losing weight is considered a manifestation of depression (Perez-Cornago *et al.*, 2014b). However, in the volunteers participating in this study, it was considered a positive manifestation because they were enrolled in a weight loss program (Perez-Cornago *et al.*, 2014b).

5.4.2. Anxiety symptoms

Symptoms of anxiety were assessed using the validated Spanish translation of the STAI (**appendix 5**) (Spielberger, 1971). This questionnaire consists of 20 items answered on a 4-point Likert-type scale. The total score was obtained by summing all items, higher values indicating greater anxiety (Spielberger, 1971).

5.5. Dietary assessment

At the beginning and at 4 week of the study, a 3-day validated food-recall questionnaire was used to assess nutrient intake (**appendix 6**). Two days had to be working days and the third day weekend day. According with other studies, the questionnaire was analysed using the DIAL software (Alce Ingenieria S.L, Madrid, Spain) (Perez-Cornago *et al.*, 2014b). On the other hand, in order to evaluate the adherence to meal consumption, volunteers had to fill a notebook with the name of the dish and dessert they consumed daily (**appendix 7**).

6. Statistical analysis

Normality of the variables was assessed using the Shapiro–Wilk test. According to whether variables were normally distributed or not, comparisons between baseline and the endpoint (4 week) were analysed by paired Student's *t*-test or Wilcoxon signed-rank test. Independent sample t-test or Mann–Whitney *U* test were used to compare between groups the differences of measures taken at baseline and at 4 weeks (Δ= 4 week-baseline).

Analysis of covariance (ANCOVA) was performed to compare variables between groups adjusted for predetermined covariates in each case.

Correlation analyses were performed by Pearson correlation test (normally distributed variables) or Spearman correlation test (non-normally distributed variables) with the aim to assesses the potential relationships between metabolites, biochemical, anthropometrical and body composition variables. Data derived from the postprandial test were analysed by repeated measures analysis of variance (ANOVA) with multiple comparisons by Bonferroni correction to analyse differences between test meals at different time points.

SPSS 15.1 software for Windows (SPSS Inc, Chicago, USA) and STATA version 12.0 (StataCorp, College Station, TX, USA) were used to perform the statistical analyses. Principal component analysis was carried out using Mass Proffesional 12.6.1. software (Agilent Technologies; Barcelona, Spain). Specific statistical analyses are explained in more detail in each corresponding chapter.

IV. RESULTS

CHAPTER 1

Oxidised LDL levels decreases after the consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet

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Oxidised LDL levels decreases after the consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet

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KEYWORDS
Cocoa extract;
Oxidative stress;
oxLDL;
Obesity;
Middle-aged

Abstract *Background and aims:* Cocoa flavanols are recognised by their favourable antioxidant and vascular effects. This study investigates the influence on health of the daily consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet, on middle-aged overweight/obese subjects.
Methods and results: Fifty healthy male and female middle-aged volunteers [57.26 ± 5.24 years and body mass index (BMI) 30.59 ± 2.33 kg/m^2] were recruited to participate in a 4 week randomised, parallel and double-blind study. After following 3 days on a low-polyphenol diet, 25 volunteers received meals supplemented with 1.4 g of cocoa extract (645.3 mg of polyphenols) and the other 25 participants received control meals, within a 15% energy restriction diet. On the 4th week of intervention individuals in both dietary groups improved ($p < 0.05$) anthropometric, body composition, blood pressure and blood biochemical measurements. Oxidised LDL cholesterol (oxLDL), showed a higher reduction ($p = 0.030$) in the cocoa group. Moreover, myeloperoxidase (MPO) levels decreased only in the cocoa supplemented group ($p = 0.007$). Intercellular Adhesion Molecule-1 (sICAM-1) decreased significantly in both groups, while Vascular Cell Adhesion Molecule-1 (sVCAM-1) did not present differences after the 4 weeks of intervention. Interestingly, cocoa intake showed a different effect by gender, presenting more beneficial effects in men.
Conclusions: The consumption of cocoa extract as part of ready-to-eat meals and within a hypocaloric diet improved oxidative status (oxLDL) in middle-aged subjects, being most remarkable in males.
Registration number: Registered at www.clinicaltrials.gov (NCT01596309).

Introduction

Obesity and overweight status are associated with the risk of suffering from chronic diseases such as cardiovascular disease or atherosclerosis [1]. Middle-aged and elderly population is growing up in developed countries due to the increase of life expectancy. However, the prevalence of obesity and chronic diseases is high in these age groups [2]. Moreover, changes in cardiovascular physiology with ageing and the presence of comorbidities, make

Abbreviations: BMI, body mass index; BP, blood pressure; DBP, diastolic blood pressure; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; MPO, myeloperoxidase; NO, nitric oxide; oxLDL, oxidised low-density lipoprotein-cholesterol; SBP, systolic blood pressure; sICAM-1, soluble intercellular adhesion molecule-1; sVCAm-1, soluble vascular cell adhesion molecule-1; TG, triglyceride.

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atherosclerotic complications the leading cause of death in these countries [2].

Oxidative stress is a process directly implicated in atherosclerosis, and therefore associated with the development of cardiovascular diseases [3]. Despite the presence of an antioxidant system in the human organism, the imbalance created when antioxidant system is weak and the production of reactive oxygen species is increased, favours the establishment of a harmful oxidative stress situation [4]. Moreover, antioxidant status tends to be unfavourable with age [5], and it seems that the antioxidant protection is lower in men than in women [6].

Some foods consumed in human diet, contain natural bioactive compounds with antioxidant properties, such as plant derived polyphenols whose consumption provides protection against oxidative stress and apparently prevents the incidence of cardiovascular events [7].

In this context, cocoa bean is one of the richest dietary source of flavanols, a type of polyphenols with recognised potential health effects [8]. Several studies have observed a strong link between their consumption and a decrease of blood pressure (BP) levels [9]. Moreover, cocoa flavanols can influence blood lipid levels by decreasing low-density lipoprotein-cholesterol (LDL-c), increasing high-density lipoprotein-cholesterol (HDL-c) and inhibiting oxidised LDL-c production (oxLDL), which is one of the most important triggers implicated in atherosclerosis [10,11]. Furthermore, cocoa polyphenol intake has a positive effect on inflammation, insulin resistance and endothelial function [12,13].

Given the increasing availability of ready-to-eat meals and the potential beneficial effects of cocoa flavanols on human health, the objective of this trial was to assess the influence on oxidative status, blood glucose and lipid profile of the daily consumption of ready-to-eat meals supplemented with cocoa extract within a hypocaloric diet on middle-aged overweight/obese subjects. Moreover, we analysed whether the effect of cocoa consumption on oxidative status depends on gender.

Methods

Study population

The volunteers, 50 healthy Caucasian (23 men and 27 women) with 57.26 ± 5.24 years and with BMI of 30.59 ± 2.33 kg/m^2 were recruited between March and May of 2012, by advertisements in local newspapers. The study was carried out in the Metabolic Unit of the University of Navarra (Spain). The participants gave written informed consent to participate in the trial. The inclusion criteria were age (50–80 years), BMI (27.0–35.5 kg/m^2) and to maintain a stable weight (<5% of variation) the previous three months to the intervention. The exclusion criteria were history of metabolic disorders, gastrointestinal diseases, diabetes, cancer, inflammatory diseases (such as rheumatoid arthritis), food allergies, cognitive alterations, current sliming, hormone replacement, anti-inflammatory or BP lowering treatments, medication that could influence appetite or nutrient absorption, inability to perform the follow-up and being smoker. The study was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and followed the Helsinki Declaration guidelines. The trial was registered at www.clinicaltrials.gov (NCT01596309) on 9th may 2012.

Study design and diet monitoring

The study was designed as a 4 week double-blind, randomised, placebo-controlled parallel nutritional intervention. One week before the beginning of the study the volunteers had to exclude cocoa and cocoa containing products from their habitual diet and three days prior to the start of the trial were asked to consume a low-polyphenol diet without energy restriction. After these previous preparatory days, volunteers were provided every week with ready-to-eat meals, which were weekly supplied by Tutti Pasta S.A (Navarra, Spain). From 50 allocated volunteers, 25 subjects received meals supplemented with 1.4 g/d cocoa extract and 25 volunteers received control meals (the composition of cocoa extract is shown in Table 1). The randomisation was performed using the "random between 1 and 2" function in the Microsoft Office Excel (Microsoft Iberica, Spain). Boxes in which the meals were provided had the same appearance and differed only on the code label, ensuring the double-blind. Those meals were consumed within a hypocaloric diet with an energy restriction of 15%. Resting metabolic rate was calculated by the Harris–Benedict equation applying the corresponding individualised physical activity factor, which was calculated as average daily exercise [14]. The macronutrient distribution of the diet was 45% of total caloric value from carbohydrates, <30% from lipids and 22–25% from proteins. The volunteers were asked to exclude cocoa containing foods and polyphenol rich foods maintaining their habitual physical activity during the intervention. At the beginning and end of the study, a 3-day validated food-recall questionnaire was used to assess nutrient intake, which was analysed using the DIAL software (Alce Ingenieria S.L, Madrid, Spain). To evaluate the adherence to meal consumption, volunteers had to fill a notebook with the name of the dish and dessert that they consumed daily.

Total polyphenol content and characterisation of the cocoa extract

Cocoa extract and analytical characterisation were provided by Nutrafur S.A (Murcia, Spain). Total polyphenol content was determined by the Folin–Ciocalteu colourimetric method. Briefly, 50 mg of cocoa extract was mixed with 100 mL of water for soluble extracts or 4 mL of ethanol + 96 ml of water for insoluble extracts. Then they were shaken during 15 min and filtered. Absorbance was measured at 765 nm. The total polyphenol content was calculated from the calibration curve (1 ppm–8 ppm) using catechin hydrate (Sigma–Aldrich C1788) as a standard value and expressed as milligrams of catechin per 1.4 g.

HPLC was used to quantify the flavonoids and theobromine in the cocoa extract. For that, the extract was

Table 1 Polyphenol composition of the cocoa extract (1.4 g) used in the study.

Component	Value
Theobromine (mg)[b]	140.42 (7.02)
Total polyphenols as catechin (mg)[a]	645.30 (32.27)
Total flavanols as catechin (mg)[b]	414.26 (20.71)
- Epicatechin (mg)[b]	153.44 (7.67)
- Catechin (mg)[b]	14.56 (0.73)
- Dimer B2 (mg)[b]	99.40 (4.97)
- Dimer B1 (mg)[b]	13.44 (0.67)
- Oligomeric procyanidins[b]	133.53 (6.68)

[a] Determined by the Folin–Ciocalteu reagent (colourimetric assay).
[b] Determined by HPLC (High-performance liquid chromatography) assay (chromatographic technique).

dissolved in dimethylsulfoxide (DMSO) 5 mg/mL. This solution was filtered through a 0.45 mm nylon membrane. The HPLC equipment used was a Hewlett–Packard Series HP 1100 equipped with a diode array detector. The stationary phase was a C18 LiChrospher 100 analytical column (250 × 4 mm i.d.) with a particle size of 5 mm (Merck, Darmstadt, Germany) thermostated at 30 °C. The flow rate was 1 mL/min and the absorbance changes were monitored at 280 nm. The mobile phases for chromatographic analysis were: (A) acetic acid/water (1:99) and (B) acetonitrile. A linear gradient was run from 96% (A) and 4% (B) to 90% (A) during 25 min; changed to 87% (A) in 5 min (30 min, total time); in 5 min changed to 50% (A) (35 min, total time), after equilibrate in for 10 min. Phenolic compounds in Cocoa extract were identified by comparison of their retention time with the correspondence standard (Sigma–Aldrich) and by their UV spectra obtained with the diode array detector.

Anthropometric, body composition and blood pressure

At baseline and at the end of the dietary intervention, anthropometric, body composition and blood pressure measurements were performed. The measurements of weight, height, waist circumference and body composition were taken in underwear after an overnight fast as described elsewhere [15]. BP was taken 3 times with automatic monitor (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands), to use the average value obtained from the last two measurements.

Blood biochemical analysis

Fasting (10 h) blood samples were collected between 8:00–9:30 a.m at baseline and at the end of the intervention using EDTA and CLOT tubes. After that, samples were left for 10–15 min at room temperature. Then, samples were centrifuged to obtain plasma and serum aliquots (15 min, 1500 g, 4 °C), which were stored at −80 °C until analysis. Plasma glucose, total cholesterol, HDL-c, triglycerides (TG) and proteins were measured by colourimetry in an autoanalyser Pentra C200 (Horiba Medical, Montpellier, France). LDL-c was calculated using Friedewald equation. Plasma insulin, oxLDL, MPO (Mercodia, Uppsala, Sweden), sVCAM-1 and sICAM-1 (R&D Systems, Minneapolis, USA) were quantified with specific ELISA kits in a Triturus auto-analyser (Grifols, Barcelona, Spain).

Statistical analysis

Considering oxLDL as the main variable, the sample size was estimated taking into account a reduction of 14.1 U/L and an interquartile range of 16.3 U/L, according to the study carried out by Khan et al. [11].With a bilateral confidence index of 95% ($\alpha = 0.05$) and a statistical power of 80% ($\beta = 0.80$) the sample size was estimated in 44 subjects. Considering a possible drop-out rate of 15%, the final sample size was established in 50 subjects. Normality of the variables was assessed using Shapiro–Wilk test. Data are expressed as mean (SD) for normally distributed variables or as median and interquartile range for non-normally distributed. Comparisons between baseline and end point, and between studies groups were accordingly analysed by Student paired *t*-test, Wilcoxon test, independent *t*-test or *U* Mann–Whitney, depending on the normality of the variables. Spearman correlation tests were applied to evaluate the relationship between changes (Δ) on oxLDL, total cholesterol, and glucose levels. Multiple linear regression analysis was used to assess the effect of cocoa supplementation over %ΔoxLDL adjusting for different models, in which the independent variables were weight, total cholesterol and LDL-c, differently combined. $p < 0.05$ was considered significant. The software used was SPSS 15.0 for Windows (SPSS Inc, Chicago, USA).

Results

Participants and adherence to diet and meal consumption

From the initial 488 subjects who contacted with the Metabolic Unit, 113 met the inclusion criteria and 50 subjects were randomised. Finally 47 volunteers completed the intervention, 24 subjects in control group and 23 in cocoa group (Fig. 1). After 4 weeks of intervention, both groups reported similar adherence to meal consumption (98.4 (2.2) % control and 98.5 (3.3) % cocoa group) and the dietary records showed that there were no significant differences between groups in macronutrient and calorie intake (data not shown). None of the volunteers reported side effects, taste dislike or changes in physical activity during the study. Moreover, there were no statistical differences at baseline in any of the assessed variables between groups.

Anthropometric, body composition, blood pressure and blood biochemical parameters after 4 weeks of intervention

Subjects in both dietary interventions significantly improved BP, anthropometric and body composition variables. Interestingly, lean mass percentage increased

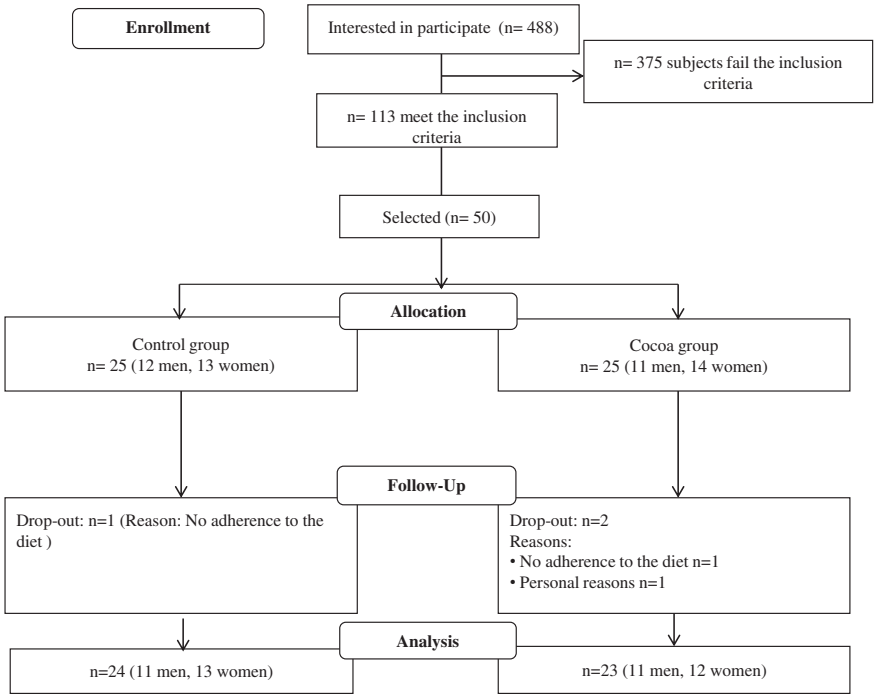

Figure 1 Flow-chart of the intervention.

significantly in both groups (Table 2). Also, concentrations of total cholesterol, LDL-c, HDL-c, TG, total proteins and insulin significantly decreased (Table 2). However, no changes were observed in blood glucose levels (Table 2).

oxLDL, MPO, sVCAM-1 and sICAM-1 after 4 weeks of intervention

After 4 weeks of intervention, oxLDL concentration decreased significantly ($p < 0.001$) in both groups, showing higher reduction in cocoa group ($p = 0.030$) as it is shown in Fig. 2. Moreover, ΔoxLDL was positively correlated with Δtotal cholesterol (rho = 0.574; $p < 0.001$) and Δglucose (rho = 0.297; $p = 0.043$). MPO levels decreased significantly only in cocoa supplemented subjects ($p = 0.007$), without observing differences with control group. Concerning sICAM-1, a significant decrease in both arms was observed, while no changes were found in sVCAM-1 (Table 2).

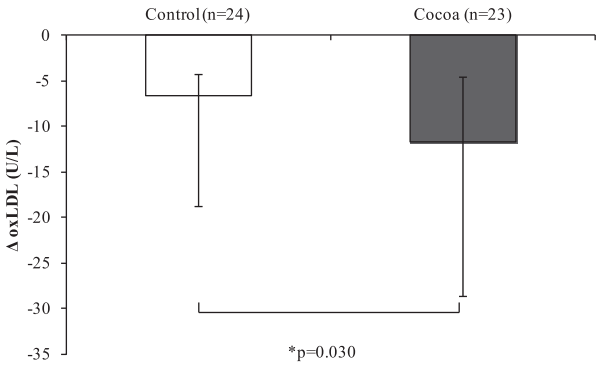

Figure 2 Oxidised LDL (oxLDL) reduction in control and cocoa group.

Considering that the literature reports poorer antioxidant status in men than in women, Table 3 shows the effect of cocoa consumption by gender on %ΔoxLDL. Different multiple linear regression models (model 1, 2 and 3) were obtained. %Δweight was included as independent variable because it could have an effect on %ΔoxLDL, and %Δtotal cholesterol and %ΔLDL-c were included due to the correlations found between them. The lineal regression analysis showed a significant effect of dietary group on %ΔoxLDL with model 2 and 3 in men. Moreover, model 1, 2 and 3 were significant only in men. The 2nd model was the best to predict the variability of %ΔoxLDL, which was 50.3% ($p = 0.001$).

Discussion

In this study, weight, waist circumference, total fat mass and truncal fat mass of the participants improved after following the intervention. The improvements were observed in both groups, indicating that cocoa supplementation did not have additional effect on those variables and attributing the observed changes to the hypocaloric diet followed by the volunteers. Our results are in agreement with two previous studies and one meta-analysis, in which no changes were observed in body size and composition of overweight/obese subjects after the cocoa supplementation [9,16,17]. Golomb et al. observed a negative correlation between the frequency of dark chocolate consumption and BMI [18]. However that is an epidemiological study in which the population

Table 2 Changes in blood biochemical parameters in both intervention groups (control and cocoa).

Variables	Control group ($n = 24$)		Cocoa group ($n = 23$)		
	Baseline	4 week	Baseline	4 week	Δ p
Anthropometric, body composition and blood pressure					
Weight (kg)	81.51 (11.41)	79.03 (11.36)***	83.25 (10.45)	80.63 (10.15)***	0.700
Waist (cm)	102.58 (8.72)	98.28 (8.99)***	105.54 (7.18)	100.37 (7.69)***	0.482
Total fat mass (%)	40.50 (6.61)	39.22 (6.61)***	41.80 (7.76)	40.30 (7.71)***	0.812
Truncal fat mass (%)	45.21 (5.69)	43.33 (5.81)***	46.08 (6.60)	44.06 (6.74)***	0.725
Lean mass (%)	57.42 (6.55)	58.62 (6.40)***	56.21 (7.41)	57.58 (7.30)***	0.583
SBP (mmHg)	116 (13.02)	108 (10.81)***	123 (14.29)	116 (15.12)***	0.477
DBP (mmHg)	79 (8.24)	71 (6.96)***	82 (8.45)	77 (8.51)***	0.302
Blood biochemical parameters					
Glucose (mg/dL)	98.35 (9.99)	95.67 (9.48)	98.85 (10.03)	96.73 (10.96)	0.868
Insulin (μU/mL)	6.51 (3.08; 11.10)	4.93 (2.24; 7.21)**	6.19 (3.83; 9.45)	3.9 (1.25; 5.89)***	0.941
Total-c (mg/dL)	234 (217.25; 259.50)	194.5 (171; 212.5)***	252 (221; 285)	213 (185; 235)***	0.509
LDL-c (mg/dL)	162.09 (32.27)	130.40 (25.45)***	179.18 (50.98)	147.40 (35.31)***	0.992
HDL-c (mg/dL)	48.92 (39.23; 60.48)	43.92 (38.96; 50.70)**	45.16 (40.29; 57.43)	44.84 (33.18; 50.74)*	0.808
TG (mg/dL)	108 (86; 138.75)	80 (63; 103)***	103 (79; 119)	71 (56; 93)**	0.887
Total proteins (g/L)	70.58 (3.19)	68.11 (2.76)***	71.36 (4.54)	68.78 (3.39)**	0.917
Biomarkers of oxidative stress and endothelial dysfunction					
oxLDL (U/L)	42.75 (34.95; 49.67)	35.82 (28.62; 38.41)***	52.62 (42.71; 60.36)	36.35 (31.56; 45.21)***	0.030
MPO (μg/L)	68 (46.26; 83.38)	46.88 (37.43; 71.13)	83.50 (61.5; 100)	43.10 (31.95; 53.0)**	0.171
sVCAM-1 (ng/mL)	681.04 (287.63)	729.48 (232.18)	655.22 (177.04)	699.48 (215.60)	0.955
sICAM-1 (ng/mL)	252.42 (68.65)	232.92 (57.30)***	254.33 (48.92)	236.05 (44.01)**	0.874

Data are expressed as mean (SD) for normally distributed variables or as median and interquartile range for non-normally distributed. Comparisons between baseline and end of the study were analysed by paired student t-test or Wilcoxon test (***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Comparisons between both groups were performed with an independent t-test or U Mann–Whitney test (Δ p). $n = 23$ in sVCAM-1 control group and $n = 22$ in sICAM-1 cocoa group. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; Total-c, total cholesterol; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high-density lipoprotein-cholesterol; TG, triglycerides; oxLDL, oxidised low-density lipoprotein-cholesterol; MPO, myeloperoxidase; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular cell adhesion molecule-1; Δ p (final-initial).

studied had different characteristics. The increase observed on lean mass is interesting, as it is generally reduced after following a hypocaloric diet, being more notable with ageing [19]. Based on previous observations, the 22–25% of protein instead of 15% can be pointed out as the responsible of this positive outcome [19].

On the other hand, no additional benefits on blood lipid and glucose profile after cocoa supplementation were found. Some studies have reported that cocoa flavanols may increase HDL-c and decrease LDL-c levels [10,11], although these outcomes are controversial and there is not a consensus on these effects [17]. A meta-analysis carried out by Shrime et al. [17], suggests that HDL-c levels increase in longer term trials with low fat consumption, whereas LDL-c and total cholesterol decrease in short-term studies in patients younger than 50 years-old [17]. This finding is in agreement with our results. On the other hand, Neufingerl et al. [20] have recently observed that the frequent consumption of 850 mg of pure theobromine significantly increased HDL-c levels. They explained that theobromine increases the concentration of apolipoprotein A-I, the major apolipoprotein of HDL-c. However, to consume 850 mg of theobromine, 100 g of dark chocolate are needed, which additionally contains saturated fats and calories. Moreover they did not completely control the

Table 3 Multiple regression analyses to assess the effect of cocoa extract supplementation on %ΔoxLDL as dependent variable, divided by sex.

%ΔoxLDL						
		B	95% CI	p	R^2	p model
Men ($n = 22$)	Unadjusted	−0.100	−0.240; 0.040	0.154	0.054	0.154
	Model 1	−0.100	−0.227; 0.028	0.119	0.196	0.049
	Model 2	−0.108	−0.209; −0.008	0.036	0.503	0.001
	Model 3	−0.123	−0.226; −0.020	0.022	0.484	0.001
Women ($n = 25$)	Unadjusted	−0.007	−0.158; 0.144	0.924	−0.043	0.924
	Model 1	−0.006	−0.161; 0.148	0.932	−0.088	0.970
	Model 2	−0.008	−0.164; 0.147	0.912	−0.089	0.981
	Model 3	−0.008	−0.163; 0.147	0.913	−0.089	0.978

Model 1: Adjusted for %Δweight (kg).
Model 2: Adjusted for %Δtotal cholesterol (mg/dL).
Model 3: Adjusted for %ΔLDL-c (mg/dL).

physical activity and diet during the intervention, factors that may influence HDL-c levels [20].

No significant changes of TG, total cholesterol and glucose were observed in the meta-analysis by Shrime et al. [17]. However, Hooper et al. [13] suggest a possible beneficial effect on TG and HOMA after the daily consumption of 50–100 mg of epicatechins [13], although no effects were evident at higher or lower doses. Our subjects consumed 153.44 mg/d of epicatechins in the cocoa group, which could explain the absence of effects. Moreover, a limitation is the length of the study, which no provides information about long-term effects.

Although BP decreased, no additional effect due to cocoa consumption was noted, which is in agreement with the meta-analysis carried out by Ried et al. [21] where it is suggested that flavanol-rich chocolate does not reduce blood pressure below 140/80 mmHg. However, some studies have found a reduction in BP probably associated with the increase of nitric oxide (NO) bioavailability after cocoa flavanols consumption [12].

MPO and oxLDL are molecules involved in the development of oxidative stress and consequently in atherosclerosis [22,23]. Particles of oxLDL are generated during lipid peroxidation, and are able to damage endothelial cells. MPO is a leukocyte-derived enzyme that produces reactive intermediate compounds, which plays an oxidative role over LDL-c, contributing to the transformation into oxLDL. Nitrite, the major oxidation product of NO, is the substrate of MPO and favours the production of nitrogen reactive species, which contribute to oxidise LDL-c [23]. In our study, the decrease on oxLDL concentration was significantly higher in the cocoa group. This finding could be linked to the significant decrease of MPO levels only in cocoa group, an effect probably related to the capacity of cocoa flavanols to modulate oxidative reactions catalysed by MPO [24]. On the other hand, sICAM-1 and sVCAM-1, indicators of endothelial dysfunction, are usually overexpressed in atherosclerotic plaque. Although they are up-regulated by oxLDL [25,26], we have not observed a higher reduction in cocoa group as a consequence of the oxLDL decrease. In this sense, Farouque et al. [27] reported that consumption of flavanol-rich chocolate bar and beverage during 6 week did not alter the concentrations of ICAM-1 and VCAM-1. However, Jung-Suk et al. [28], suggested that flavonoids are able to inhibit the expression of sVCAM-1 and s-ICAM-1.

The antioxidant status becomes weak with age [5], with apparent poorer antioxidant status in men than in women [6]. In agreement with this, we have found that cocoa consumption, adjusted for different models, has an effect on %ΔoxLDL of men, while in women this result was not observed. This antioxidant effect is probably related with the radical scavenging capacity of cocoa flavanols, which protects cells against oxidative stress and endothelial dysfunction [11]. Other hypothesis could be that men were more oxidised than women, thus, the effect of cocoa flavanols is more effective, given that some studies have not observed additional benefits after intake of antioxidants [29].

Conclusion

This study supports that the consumption of cocoa extract as part of a ready-to-eat meals and within a hypocaloric diet, improve oxidative status (oxLDL) in middle-aged subjects, and particularly in men, supporting the antioxidant effect of cocoa flavanols. However, additional research is needed to better understand the role of cocoa flavanols in cardiovascular diseases. Thus, a long-term trial including a larger sample of men, could contribute to clarify these promising findings.

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CHAPTER 2

Assessment of DNA damage using comet assay in middle-aged overweight/obese subjects after following a hypocaloric diet supplemented with cocoa extract

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CHAPTER 3

Cocoa extract intake for 4 weeks reduces postprandial systolic blood pressure response of obese subjects, even after following an energy restricted diet

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ABSTRACT

Background: Postprandial cardiometabolic profile is usually altered in obesity. Interestingly, the consumption of flavanol-rich foods might be protective against those metabolic alterations.

Objective: To evaluate the postprandial cardiometabolic effect after the acute cocoa extract consumption before and after 4 weeks of its daily intake. Moreover, the bioavailability of cocoa extract was investigated.

Design: Twenty-four overweight/obese middle-aged subjects participated in a 4 week intervention study. Half of the volunteers consumed a test meal enriched with 1.4 g of cocoa extract (415 mg flavanols), while the rest of the volunteers consumed the same meal without the cocoa extract (control group). Glucose and lipid profile, as well as blood pressure and cocoa metabolites in plasma were assessed before and at 60, 120 and 180 min post consumption at the beginning of the study (Postprandial 1) and after following a 4 week -15% energy restricted diet including foods containing or not the cocoa extract (Postprandial 2).

Results: In the Postprandial 1 test, the area under the curve (AUC) of systolic blood pressure (SBP) was higher in the cocoa group compared to the control ($p=0.007$), showing significant differences after 120 min of intake. However, no differences between groups were observed at Postprandial 2. Interestingly, the reduction of postprandial AUC of SBP (AUC_Postprandial 2-AUC_Postprandial 1) was higher in the cocoa group ($p=0.016$). Moreover, cocoa derived metabolites were detected in plasma of cocoa group, while the absence or significantly lower amounts of metabolites were found in the control group.

Conclusions: The daily consumption of cocoa extract within a hypocaloric diet resulted in a greater reduction of postprandial AUC of SBP compared to the effect of control group and independently of body weight loss. The present study suggests the implication of cocoa flavanols on postprandial blood pressure homeostasis and the availability of cocoa flavanols.

Trial registration: www.clinicaltrials.gov (NCT01596309).

Keywords: Blood pressure, Cocoa, Diet, Bioavailability, Polyphenols, Weight loss.

INTRODUCTION

Obesity is considered one of the major public health problems associated with cardiovascular mortality (Mitchell *et al.*, 2011). In that situation, different fasting and postprandial metabolic markers are altered contributing to the development of the obesity associated comorbidities such as diabetes, insulin resistance, hypertension, atherosclerosis and dyslipidemia, among others (Andersson *et al.*, 2008; Katsareli and Dedoussis, 2014). Interestingly, the prescription of nutritional strategies as well as lifestyle changes, such as the reduction of energy intake and adherence to healthy dietary patterns reduces the risk of suffering from cardiometabolic disorders (Fayh *et al.*, 2013; Stradling *et al.*, 2014). On the other hand, the intake of plant extracts, which are rich on polyphenos is receiving especial attention in the protection against obesity associated comorbidities (Etxeberria *et al.*, 2012). In this context, cocoa is one of the richest sources of polyphenols with claimed benefits on blood pressure (Desideri *et al.*, 2012; Sarria *et al.*, 2012), insulin resistance (Desideri *et al.*, 2012; Sarria *et al.*, 2014), lipid profile (Khan *et al.*, 2012; Martinez-Lopez *et al.*, 2014; Sarria *et al.*, 2014), endothelial dysfunction or oxidative stress (Rodriguez-Ramiro *et al.*, 2011; Khan *et al.*, 2012; Martin *et al.*, 2013; Ibero-Baraibar *et al.*, 2014) and inflammation (Gu and Lambert, 2013; Ali *et al.*, 2014; Gu *et al.*, 2014; Khan *et al.*, 2014; Sarria *et al.*, 2014). Such therapeutic effects have been attributed to some of the bioactive compounds occurring in cocoa, mainly flavanols, which are the most abundant polyphenols in this seed (Heiss *et al.*, 2010). Flavanols in cocoa are found as monomers ((-)-epicatechin and (+)-catechin) and procyanidins (Rusconi and Conti, 2010). Moreover, cocoa contains also other bioactive compounds such as methylxanthines (caffeine and theobromine) and minerals (magnesium, cupper, iron, etc.) with potential healthy properties (Franco *et al.*, 2013).

In order to establish a relationship between cocoa consumption and healthy benefits through physiological mechanisms, flavanols from cocoa need to be absorbed into the circulation (Rein *et al.*, 2013). Bioavailability depends on different factors such as food matrix, physical state and the degree of flavanol polymerisation (Neilson and Ferruzzi, 2011). Cocoa monomeric and some oligomeric flavanols are stable in the stomach and small intestine (Holt *et al.*, 2002; Urpi-Sarda *et al.*, 2009a) and are rapidly absorbed appearing in plasma between 30 and 60 minutes post-consumption (Richelle *et al.*, 1999; Rusconi and Conti, 2010). Afterwards, glucoronidation, sulphation and methylation of the monomeric flavanols in the liver and in the small intestine produces *O*-glucoronidated, *O*-sulphated and *O*-methylated flavanol derivates in plasma (Neilson and Ferruzzi, 2011). Once in the bloodstream, those metabolites undergo additional conjugations in the liver and returned back to the small intestine by enterohepathic circulation (Neilson and Ferruzzi, 2011). Procyanidins are poorly absorbed, only procyanidin dimer B2 has been detected in human plasma (Holt *et al.*, 2002; Urpi-Sarda *et al.*, 2009a). Unabsorbed flavanols reach the colon and after the degradation by the intestinal microbiota and the transformation to phenolic acids are then absorbed into the circulation (Appeldoorn *et al.*, 2009). In addition, phase-II metabolites are excreted into the bile, where after the bacterial enzyme activities are reabsorbed into the

circulation. Finally, metabolites are transferred from bloodstream to kidneys and are excreted in urine (Neilson and Ferruzzi, 2011).

On the other hand, the intake of a food component could have a different effect when is consumed as a first time or when is consumed regularly during a determined period of time (Crujeiras *et al.*, 2009). Surprisingly, it has not yet well documented if the acute response of cocoa extract consumption on cardiometabolic markers could be persistent with time or it could be influenced by its regular consumption along time showing an adaptive or tachyphylactic effect.

The present research is a sub-study carried out within a clinical trial (NCT01596309), whose principal purpose was to evaluate the effect of consuming ready-to-eat meals containing cocoa extract under a moderate energy restricted diet for 4 weeks on the general nutritional status as well as on cardiometabolic and oxidative status of middle-aged obese subjects. The obtained results evidenced the improvement of oxidized low-density lipoprotein-cholesterol levels (Ibero-Baraibar *et al.*, 2014). In addition, the prescribed energy restricted diet reduced the adiposity as well as improved blood pressure, routine blood biochemical profile and 25-hydroxivitamin D levels (Ibero-Baraibar *et al.*, 2015a; Ibero-Baraibar *et al.*, 2015b).

The aim of the present study was specifically to analyse the postprandial response of the acute cocoa extract consumption during the immediate 3h of intake on routine blood biochemical and blood pressure markers before and after 4-weeks of its daily consumption. Moreover, the bioavailability of the cocoa extract within the ready-to-eat meals was studied by measuring cocoa derived metabolites in plasma.

SUBJECTS AND METHODS

Subjects

Twenty-four volunteers [58.2 (5.2) years] from the main study (n=50) took part in the present sub-study, 12 allocated in the control group (6 men and 6 women) and the remaining 12 in the cocoa group (5 men and 7 women) as shown in Figure 1-A.

All the participants gave written informed consent to participate in the trial. Enrolled subjects needed to be between 50 and 80 years old, with a body mass index (BMI) of 27.0-35.5 kg/m^2 and maintaining a stable weight (<5% of variation) within the previous three months to the intervention. Subjects under the following conditions were not included in the trial: suffering from gastrointestinal disease, hepatic diseases, diabetes, cancer or inflammatory disease, food allergies or cognitive and psychiatric alterations. Also, individuals following a weight loss treatment or hormone replacement or being presented anti-inflammatory, hypocholesterolemic or blood pressure lowering treatments were not allowed to participate. Subjects taking antidepressant

drugs, antioxidant rich supplements, medication that could influence appetite or nutrient absorption, inability to follow-up the intervention and smokers were also exclusion criteria.

Study design

The study was carried out in the Metabolic Unit of the University of Navarra (Pamplona, Spain). The trial was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and followed the Helsinki Declaration guidelines. Moreover, it was registered at www.clinicaltrials.gov (NCT01596309). CONSORT guidelines 2010 were considered.

One week before the start of the intervention volunteers were asked to remove cocoa and cocoa containing products from their habitual diet. In addition, three days prior to the first day of the study all of the volunteers were required to follow a low-polyphenolic diet, restricted in fruits and vegetables and excluding cocoa, coffee, tea, infusions, botanical or antioxidant rich supplements as well as juices and alcoholic drinks.

The postprandial study was performed on the first (Postprandial 1) and the last day (Postprandial 2) of a 4-week nutritional intervention (Figure 1-B). The procedure consisted in the consumption of a test meal (a ready-to-eat dish and a dessert) which provided 1.4 g of cocoa extract, or the same meal without cocoa extract, depending on the group allocation. The experiment was carried out after a 10 h overnight fast. Blood samples and blood pressure were taken just before consuming the test meal (time 0) and at 60, 120 and 180 minutes post-consumption. The postprandial test was repeated after 4 weeks (day 28) of following an energy restricted diet (-15%E) including or not the daily consumption of ready-to-eat meals containing 1.4 g/day of cocoa extract, depending of the experimental group (Figure 1-B). The energy restricted diet was composed of 45% of the total caloric value from carbohydrates, <30% from lipids and 22-25% from proteins as explained elsewhere (Ibero-Baraibar *et al.*, 2014).

The randomisation to participate in the main study was performed using the ''random between 1 and 2'' function in the Microsoft Office Excel (Microsoft Iberica, Spain). Boxes in which the meals were provided had the same appearance and differed only on the code label, ensuring the double-blind protocol. The restriction of cocoa containing foods and polyphenol rich foods was maintained during the 4-weeks of the study and volunteers were also asked not to change their physical activity patterns. Three days prior to the second postprandial test volunteers were prescribed to consume pre-determined types of test meals and desserts (with or without the inclusion of cocoa), in order to all volunteers reach in comparable nutritional conditions to the Postprandial 2 test.

Test meals

The postprandial test included a meal containing 1.4 g of cocoa extract or the same meal without cocoa extract, which was based on a ready-to-eat dish and dessert: courgette cream (300) and chocolate custard (150 g). The meal provided 365 kcal and was composed in grams and proportions (%) by 34.2 g (7.6%) of carbohydrates, 20.9 g (4.6%) of lipids, 8.9 g (2.0%) of proteins, 4.9 g (1.1%) of fibre, 377.7 g (83.9%) of water and 4.1 g (0.9%) of ashes.

Characterisation of cocoa extract

The cocoa extract and the analytical characterisation were supplied by Nutrafur S.A. (Murcia, Spain). The composition of 1.4 g of cocoa extract as mean (SD) was the following: 140.4 (7.1) mg of theobromine, 645.3 (32.3) mg of total polyphenols as catechin, 414.3 (20.7) mg of flavanols as catechin, 153.4 (7.7) mg of epicatechin, 14.6 (0.7) mg of catechin, 99.4 (5.0) of procyanidin B2, 13.4 (0.7) mg of procyanidin B1 and 133.5 (6.7) mg of oligomeric procyanidins. Total polyphenol content was determined by Folin-Ciocalteu method and flavanoids and theobromine were analysed by high-performance liquid chromatography (HPLC), whose analytical procedures have been described in detail elsewhere (Ibero-Baraibar *et al.*, 2014).

Body weight and blood biochemical analysis

Body weight was measured underwear and overnight fasting conditions using Dual-energy X-ray absorptiometry following manufacturer´s instructions (Lunar Prodigy, software version 6.0, Madison, WI). Blood samples were drawn before (time 0) and after tested meal consumption (60, 120 and 180 min) through an intravenous catheter inserted into an antecubital vein using ethylenediaminetetraacetic acid (EDTA) and CLOT tubes (BD Vacutainer®). After each extraction, samples were centrifuged in order to obtain plasma and serum aliquots (15 min, 1500 g, 4ºC), which were then stored at -80 ºC until analysis. Plasma glucose, total cholesterol (Total-c) and high-density lipoprotein-cholesterol (HDL-c) were measured by colorimetric procedures in a Pentra C200 autoanalyser (Horiba Medical, Montpellier, France).

Blood pressure determination

Blood pressure was determined immediately before the test meal consumption (time 0) and at 60, 120 and 180 minutes post-consumption with an automatic monitor device (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands). Measures were carried out on subjects in the seat position with hand resting on a table and the cuff at the level of heart, always in the same arm. Between measurements volunteers were maintained in resting conditions, free of any alterations in a quiet and temperature-controlled room.

Analysis of cocoa extract derived metabolites

The assessment of cocoa derived metabolites in plasma was used to evaluate the bioavailability of cocoa extract and the adherence of the volunteers to the intervention, as well as to compare the metabolite concentration in plasma before and after 4 weeks of daily cocoa consumption. Fourteen cocoa derived metabolites were targeted in plasma of cocoa group in the Postprandial 1 test and the same metabolites were measured in the Postprandial 2 in cocoa and control groups. The analysed metabolites were: catechin, epicatechin, methyl-epicatechin-glucoronide, procyanidin B2, methyl-catechin-glucoronide, catechin-sulphate, epicatechin-glucoronide, epicatechin-sulphate, 3-*O*-methyl-epicatechin, methyl-epicatechin-*O*-sulphate, 3,7-dimethyluric acid, 1-methylxanthine, 3-methylxanthine and theophylline.

Chemicals and reagents

Procyanidin B2 was purchased from Extrasynthese (Genay, France). (+)-catechin and (-)-epicatechin were purchased from Fluka Co. (Buchs, Switzerland) while 3-methylxanthine and 3,7-dimethyluric acid were from Sigma Aldrich (St. Louis, MO). Cathecol was used as internal standard (IS). Methanol (HPLC grade), acetonitrile (HPLC grade) and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). Ortho-phosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Water was of MilliQ quality (Millipore Corp, Bedford, MA, USA).

Phenolic metabolites' extraction

Phenolic metabolites were extracted by using microelution plates (Waters, Milford, USA) packed with 2 mg of OASIS HLB sorbent (Waters, Milford, USA) following the method described by Serra et al (Serra *et al.*, 2009) with minor modifications. Briefly, the wells were sequentially conditioned by using 250 µL of methanol and 250 µL of milliQ water:acetic acid (99.8:0.2, v/v). Then, 200 µL of plasma mixed with 350 µL of phosphoric acid 4% and 50 µL of catechol (IS) at 0.5 mg/L were loaded into the wells. After that, the clean-up of the plates was sequentially done with 200 µL of milli-Q water and 200 µL of milli-Q water:acetic acid (99.2:0.2, v/v) to eliminate any interference that the sample might contain. Finally, the elution of the retained metabolites was performed with 2x50 µL of acetone:milli-Q water:acetic acid (70:29.5:0.5, v/v/v). 5 µL of the eluted was directly injected into the HPLC–MS/MS (Agilent Technologies, Palo Alto, U.S.A.).

Theophylline metabolites extraction

Theophylline metabolites were extracted following the method described by Ogawa et al (Ogawa *et al.*, 2012) with some minor modifications. Thus, 50 µL of plasma were mixed with 100 µL of acetonitrile, vortexed 1 minute and centrifuged 5 min at 1000 rpm. Pellets were removed and solvent was evaporated to dryness under nitrogen flow rate. Finally, samples were reconstituted with 50 µL of milli-Q water:acetic acid (99.9:0.1, v/v) and were analysed by HPLC-MS/MS.

HPLC–ESI-MS/MS measurements

The HPLC-MS/MS system consisted of an Agilent 1200Series instrument (Agilent Technologies, Palo Alto, U.S.A.) using a Zorbax SB-Aq column (3.5 μm, 150 mm x 2.1 mm i.d.) equipped with a Pre-Column Zorbax SB-C18 (3.5 μm, 15 mm x 2.1 mm i.d.) and coupled to a triple quadrupole 6410 also from Agilent.

Two chromatographic methods were used to analyse the whole range of metabolites. To carry out the analysis of phenolic metabolites the column was kept at 25ºC and the flow rate was 0.4 mL/min. The composition for solvent was A: milli-Q water:acetic acid (99.8:0.2 v/v) while solvent B: acetonitrile. The elution gradient was 0-10 min, 5-55% B; 10-11 min, 55-80% B; 11-12 min, 80% B; 12-13 min, 80-5% B and 5 min post-time. On the other hand, to analyse theophylline metabolites milli-Q water:acetic acid (99.8:0.2 v/v) was used as solvent A and acetonitrile as solvent B. The elution gradient was 0-2 min, 3-10% B; 3-4 min, 10-50% B; 4-5 min, 50-95% B; 5-6 min, 95% B; 6-6.5 min, 95-3% and 2 min post-time. The flow rate was held at 0.5 mL/min throughout all the run time.

Ionisation was performed by electrospray (ESI) in the negative mode and the source parameters were: drying gas temperature 350 ºC, flow rate 12 L/min, gas nebulizer pressure 45 psi and the capillary voltage 4000 V. The selected reaction monitoring (SRM) transitions and the instrumental parameters for each compound are reported (Supplemental Table 1). Due to the lack of standards of some metabolites, they were tentatively quantified by using the calibration curves corresponding to their phenolic precursors as is specified in Supplemental Table 2. Quality parameters are reported (Supplemental Table 2). Results are expressed as nmol/L of each metabolite/compound in the plasma sample. Values under the limit of quantification (LOQ) are expressed as “n.q.” (no quantified) while values under the limit of detection (LOD) are showed as “n.d.” (no detected).

Statistical analyses

Data were analysed using the SPSS 15.0 for Windows statistical program (SPSS, Inc., Chicago, IL, USA) considering results with p values less than 0.05 as statistically significant. Normality of the variables was assessed by the Shapiro–Wilk test. Data are expressed as mean (SD). Comparisons between baseline and the endpoint of the intervention were analysed by the Student paired t-test or the Wilcoxon test. The comparisons between both groups were performed by parametric t-test or Mann-Whitney U test, depending on the normality of the variables. Area under the curve (AUC) was calculated for blood biochemical markers, blood pressure and cocoa extract derived metabolites in plasma according to the trapezoid rule (Tai, 1994) in GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California USA). Thus, the AUC was determined per hour and then the sum of the 3 AUC-s was performed. For cocoa metabolites, the AUC was calculated when at least the metabolites were quantifiable at one time-point. However, to

perform the statistical analysis and to calculate the p-value derived from the comparison of AUC of control and cocoa groups, the original data of each participant was used, even if it was under the LOQ or LOD. Analysis of covariance (ANCOVA) was performed to compare groups in the Postprandial 1and Posprandial 2 adjusted for the fasting value of each variable in each test and weight at baseline or weight change (Δ=endpoint-baseline) when appropriate. The comparisons of the AUC changes (Postprandial 2-Postprandial 1) between groups were adjusted for the changes of each assessed variable at time 0 and Δweight. Covariates were included in the ANCOVA analysis to avoid results bias. The comparisons between Postprandial 1 and Postprandial 2 at different time points were evaluated by repeated measures analysis of variance (ANOVA) for multiple comparisons by Bonferroni correction. Although some metabolites were under the LOQ or LOD, repeated measures ANOVA were performed with those values where appropriate.

RESULTS

Subjects

Twenty-three subjects of the initial twenty-four [58.2 (5.2) years] completed the study. A woman from the cocoa group was removed due to dietary non-compliance (self-reported), and hence 11 subjects finished the study under cocoa treatment (Figure 1-A). This volunteer was also excluded from the statistical analyses following per protocol criteria. On the other hand, no differences were observed at baseline in any of the assessed variables, confirming that all of the participants started the intervention in the same condition (Table 1). As expected, body weight was significantly reduced in both intervention groups, control: -2.8 (1.3) and cocoa -2.7 (0.9), after the 4 week period, without statistical differences between them.

Postprandial glucose and lipid metabolism response

Differences between groups were not found neither in the AUC of glucose nor in the lipid metabolism variables during Postprandial 1 and Postprandial 2 tests. Both groups reported lower AUC of total cholesterol and HDL-c at Postprandial 2 (Table 2), but no differences were observed when the AUC changes (Postprandial 2-Postprandial 1) were compared between groups (Table 2).

Postprandial blood pressure response

The AUC concerning SBP was significantly (p=0.007) higher in the cocoa group compared to the control group during the Postprandial 1 (Table 2). Specifically, repeated measures analysis revealed that cocoa supplemented meal resulted in a significantly higher (p=0.018) SBP levels at 120 min post-consumption when compared to control group (Figure 2). However, no differences were found along time in the SBP within the cocoa or control groups in the Postprandial 1. In the Postprandial 2, no differences concerning AUC of SBP were found between groups (Table 2). When the AUC of Postprandial 1 was compared with the AUC of Postprandial 2 within each group, AUC of

SBP decreased significantly in both groups, (control p=0.015 and cocoa p=0.001), but interestingly, the reduction of AUC (Postprandial 2-Postprandial 1) was significantly higher in cocoa group when compared to control group (p=0.016) (Figure 3).

Concerning the AUC of DBP, no differences were noted between groups neither in Postprandial 1 nor in Postprandial 2 (Table 2). When the AUC of DBP was compared within the same group between Postprandial 1 and 2, significant reduction was observed in both groups (control p <0.001, cocoa p= 0.001). However, when the AUC changes (Postprandial 2-Postprandial 1) for DBP were compared between groups no differences were found (Table 2).

Cocoa derived metabolites in plasma

Fourteen metabolites from cocoa intake were measured in plasma of cocoa group at Postprandial 1 and in plasma of control and cocoa groups at Postprandial 2. Data are presented when at least in one of the groups the metabolite was quantifiable (Table 3). Metabolites were monomeric and dimeric flavanols (catechin, epicatechin and procyanidin B2), alkaloid metabolites (3-methylxanthine, 1-methylxanthine, 3,7-dimethyluric acid and theophylline) and flavanol phase II metabolites (methyl-epicatechin-glucoronide, methyl-catechin-glucoronide, catechin-sulphate, epicatechin-glucoronide, epicatechin-sulphate, methyl-epicatechin-*O*-sulphate and 3-*O*-methyl-epicatechin).

Except theophylline, cocoa derived metabolites were no detected in fasting condition (time 0) at the beginning of the study. Interestingly, cocoa derived metabolites were detected in plasma samples of cocoa consumers after the meal consumption in both postprandial trials, while in the control group the absence or significantly lower concentration of those metabolites was found at Postprandial 2 (Table 3).

As expected, a significant variation was noted in the cocoa derived metabolites along the 180 min post-consumption, where the highest concentration of metabolites was found at 60 and 90 min post-consumption (Table 3). The repeated measures ANOVA revealed no differences in the amount of metabolites between Postprandial 1 and 2 except for 3-methylxanthine and theophylline, which presented higher amounts in the Postprandial 2 (Table 3). Similarly, when the AUC of Postprandial 1 and Postprandial 2 were compared between groups significantly higher AUC of 3-methylxanthine (p<0.001) and theophylline (p=0.001) were detected in the second postprandial trial (Table 3).

DISCUSSION

In this study the postprandial effect of consuming ready-to-eat meals containing cocoa extract was studied before and after 4 weeks of following a moderate hypocaloric diet including cocoa. One of the major findings of this trial was the different postprandial response of SBP to the acute intake of cocoa extract before and after the daily consumption for 4 weeks. In the Postprandial 1, the intake of cocoa extract resulted in a significantly higher acute AUC of SBP, showing significant differences at 120 minutes post-consumption when compared to the control group. However, when the postprandial test was repeated after 4 weeks of daily cocoa extract consumption, that effect was not maintained, suggesting an adaptive process of the postprandial SBP when cocoa is consumed after its regular intake during a short period of time. Nevertheless, the present results should be viewed with caution because although significant differences were observed when compared with the control group, no differences between baseline and the 120 min of consuming the cocoa extract were found. However, some studies observed an increment of blood pressure after the cocoa intake or respect to the treated group, such as De Gottardi et al., (2012) who found an increment of blood pressure in cirrhotic patients after 30 min of consuming a liquid meal containing 0.55 g of dark chocolate per kg of body weight (De Gottardi *et al.*, 2012). Similarly, West et al (2014) reported that the acute consumption of 22 g of cocoa (814 mg flavanol) after 4 weeks of regular consumption resulted in a higher SBP at 2 hours post-consumption in comparison with a flavanol free group, not observing differences in blood pressure after the regular cocoa intake (West *et al.*, 2014). The increment of SBP after the cocoa intake could be attributed to the theobromine content, which is the most important methylxanthine in cocoa, and although it does not have the same stimulating effect as caffeine, it is able to stimulate the heart rate (Smit, 2011; Baggott *et al.*, 2013).

More important is the result obtained when the difference between both postprandial (AUC_Postprandial 2-AUC_Postprandial 1) tests was compared concerning SBP between both groups. Interestingly, a greater reduction of postprandial SBP was observed in cocoa consumers after 4 weeks of daily cocoa consumption independently of body weight loss, suggesting that postprandial reduction of SBP could be due to the daily consumption of cocoa extract. In our previously reported data, the reduction of SBP after the 4 week intervention in the fasting period was the result of a weight loss and not additional effect due to cocoa consumption was noted (Ibero-Baraibar *et al.*, 2014). However, in this research, the postprandial SBP was assessed, revealing that it could be affected by the regular consumption of the cocoa extract. This result may be related to the blood pressure lowering effects that cocoa has (Desideri *et al.*, 2012; Sarria *et al.*, 2012).

As expected, a reduction was observed in the AUC of routine blood biochemical markers which should be attributed to the weight loss of following the 15% of energy restricted diet because no differences were found between groups (Gutierrez-Salmean *et al.*, 2014).

Importantly, the measurement of metabolites in plasma is an useful method to assess the bioavailability of nutrients (Neilson and Ferruzzi, 2011) and the compliance of food intake (Khan *et al.*, 2012; Llorach *et al.*, 2013; O'Callaghan and Noakes, 2014). In this study, fourteen cocoa derived metabolites where analysed in plasma. Ten metabolites were detected and quantified in cocoa consumers and 5 in the control group. As expected, cocoa metabolites in the plasma samples of cocoa consumers were detected during both postprandial test and the absence or significantly lower concentration of those metabolites were detected in control group. This outcome suggests the bioavailability of cocoa flavanols and theobromine within the ready-to-eat meals as well as the compliance of the volunteers during the intervention. In accordance with other studies, most of the metabolites where phase II metabolites, as catechin and epicatechin-sulphates, methylates and glucoronidates (Actis-Goretta *et al.*, 2012), but also alkaloid metabolites were detected in both groups. Epicatechin and catechin were not quantifiable intact since one hour after cocoa extract consumption, probably due to a rapid metabolization in sulphates, methylates and glucoronidates. Interestingly, the procyanidin B2 was detected in plasma of cocoa consumers (Holt *et al.*, 2002).

Focusing on the cocoa group, 3-methylxanthine and theophylline levels were significantly higher in the second postprandial period. This phenomenon could be due to the half life of theobromine and derivatives in the circulation. While the half-life of flavanols in plasma varies between 6-8h with a maximum concentration at 1-2h post-consumption (Holt *et al.*, 2002), the half-life of theobromine and derivatives varies between 7.5 and 10h with a maximum concentration at 2h post-consumption (Ellam and Williamson, 2013). The presence of some cocoa derived metabolites in the control group could be explained because of the presence of other flavanol sources in the diet such as apples, pome fruits etc. The concentrations of those flavanols in subjects from control group were in significantly lower amounts in accordance with the results obtained by other studies (Llorach *et al.*, 2013). The metabolites reported in this study have been already identified in other cocoa related studies (Urpi-Sarda *et al.*, 2009a; Urpi-Sarda *et al.*, 2009b; Llorach *et al.*, 2013).

Finally, it is important to highlight the strengths and limitations of the present investigation. First, a cross-over design would have been appropriate, since each cross-over patient serves as his/her own control reducing the effect of confounding covariates. Nevertheless, the cross-over design was declined because the volunteers would present a weight loss due to the prescribed energy restricted diet. In this sense, weight change would have been a confounding factor since weight loss would not have been the same in the first phase comparing to the second phase of the cross-over. For this reason, it was decided to perform a parallel study. Second, the assessment of cocoa derived metabolites in plasma to evaluate the nutritional compliance of the volunteers could be considered as a reliable method. Nevertheless, not to consider the effect of cocoa extract beyond the 180 minutes post-consumption is a limitation of this study.

CONCLUSION

In conclusion, the intake of cocoa extract during 4 weeks revealed a higher reduction of postprandial AUC of SBP respect to the first time that cocoa was consumed (Postprandial 1) when compared to control group. In addition, cocoa derived metabolites were detected in plasma of cocoa consumers suggesting the bioavailability of cocoa extract within the ready-to-eat meals. Overall, this research provides a new evidence to understand the implication of cocoa flavanols on postprandial cardiometabolic markers.

Authors' contribution

I.I-B. contributed to the design of the study, was involved in the fieldwork, data collection, analysis and writing of the manuscript; M.S. and A.A-A contributed to the analyses of cocoa derived metabolites and editing the manuscript; J.A.M and M.A.Z were responsible for the general coordination, design, interpretation of the data, 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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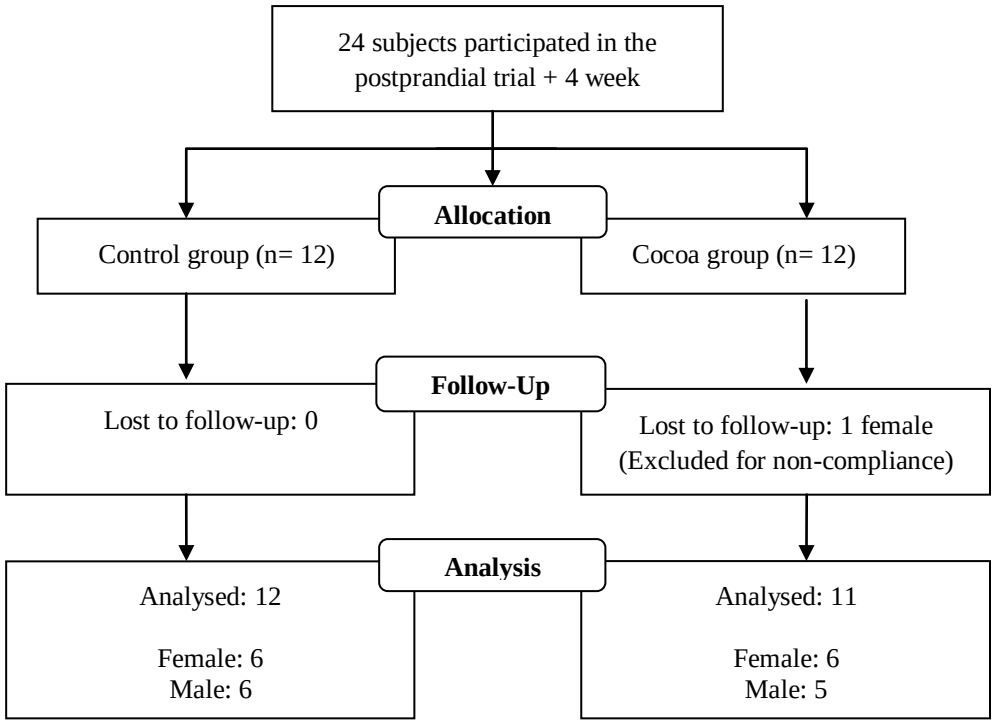
A



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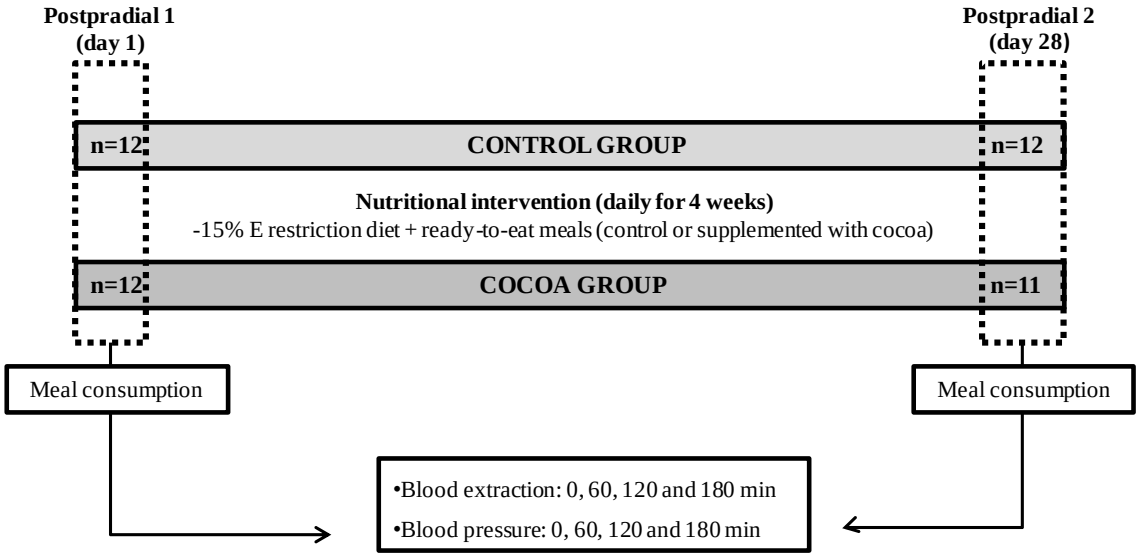

Figure 1. A) Flow-chart of the participants. B) Study diagram

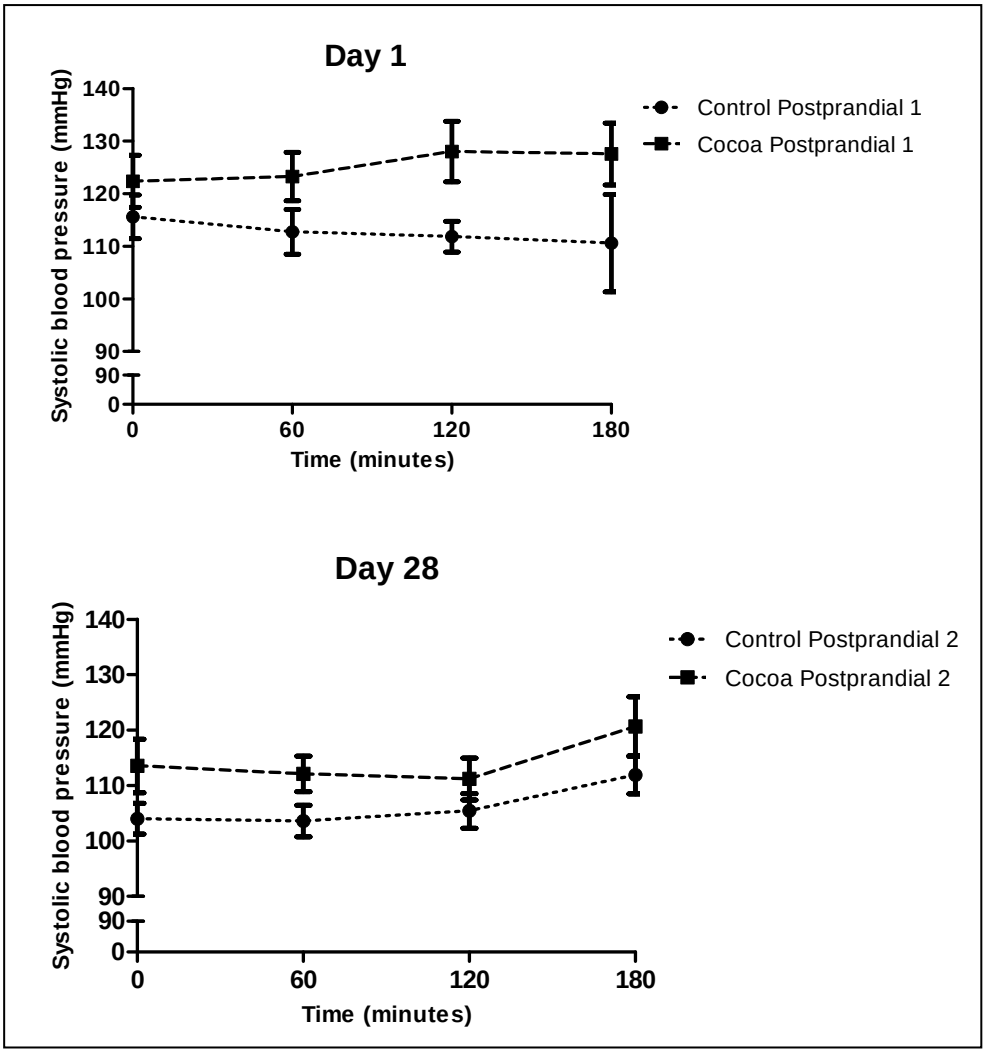

Figure 2. Systolic blood pressure before (time 0) and after meal consumption (60,120 and 180 min) in control (n=12) and cocoa (n=11) groups

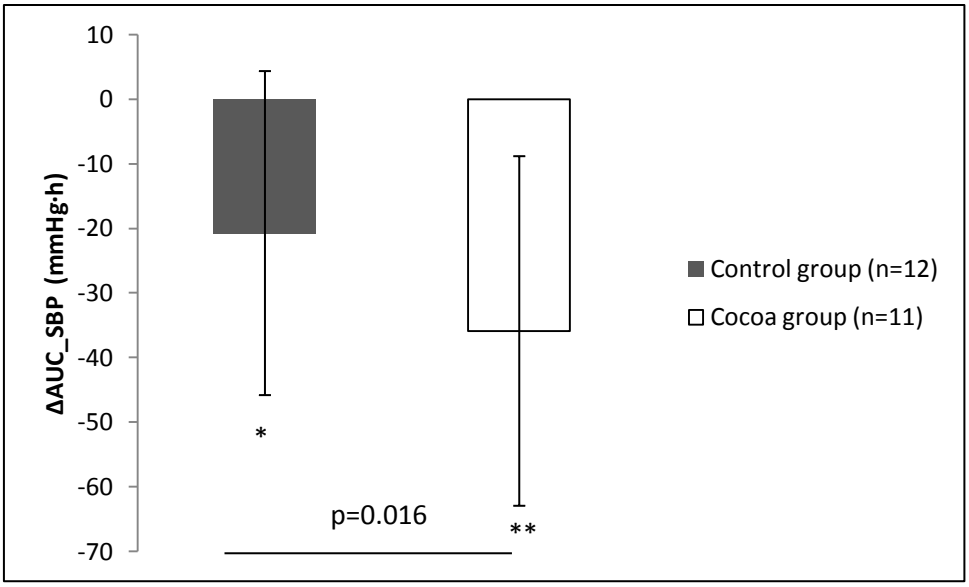

Figure 3. Comparison of ΔAUC_SBP (AUC_SBP Postprandial 2 – AUC_SBP Postprandial 1) between groups

Abbreviations: AUC, area under the curve; SBP, systolic blood pressure.

Table 1. Anthropometric and clinical characteristics of subjects from control and cocoa groups at baseline

	Baseline		
Variables	**Control (n=12)**	**Cocoa (n=11)**	**p[a]**
Age	57 (4.9)	59 (5.4)	ns
Weight (kg)	83.5 (9.8)	83.3 (10.9)	ns
Waist (cm)	103.7 (4.8)	105.2 (6.8)	ns
SBP (mmHg)	116 (14.4)	122 (16.5)	ns
DBP (mmHg)	76 (8.9)	80 (7.1)	ns
Glucose (mg/dL)	96.2 (8.6)	99.1 (7.0)	ns
Insulin (μU/mL)	7.6 (5.1)	9.2 (7.4)	ns
Total-c (mg/dL)	220.6 (37.4)	237.0 (64.8)	ns
HDL-c (mg/dL)	55.5 (15.2)	52.2 (8.2)	ns

Data presented as mean (SD)

[a] Comparison between control and cocoa groups.

$p<0.05$ was considered significant.

Abbreviations: DBP: Diastolic blood pressure; HDL-c: High-density lipoprotein-cholesterol; ns: no significant; SBP: Systolic blood pressure; Total-c: Total cholesterol

Table 2. Area under the curve of biochemical and blood pressure variables at Postprandial 1 and 2

AUC	Postprandial 1 (day 1)			Postprandial 2 (day 28)					
	Control (n=12)	Cocoa (n=11)	p[a]	Control (n=12)	Cocoa (n=11)	p[b]	p[c]	p[d]	p[e]
Glucose (mg·h/dL)	286.9 (43.5)	314.8 (29.1)	ns	292.9 (37.3)	314.4 (33.6)	ns	ns	ns	ns
Insulin (μU·h/dL) [f]	75.9 (60.4)	97.6 (73.8)	ns	68.6 (50.3)	77.0 (48.5)	ns	ns	ns	ns
Total-c (mg·h/dL)	664.5 (120.2)	714.1 (194.7)	ns	551.0 (88.1)	577.2 (138.8)	ns	<0.001	0.001	ns
HDL-c (mg·h/dL)	161.7 (44.0)	154.0 (25.4)	ns	141.0 (25.6)	134.3 (23.8)	ns	0.011	0.001	ns
SBP (mmHg·h)	338.0 (31.0)	376.0 (46.6)	0.007	317.0 (28.8)	340.0 (36.6)	ns	0.015	0.001	0.016
DBP (mmHg·h)	226.0 (19.8)	240.0 (22.8)	ns	205.0 (17.9)	220.0 (21.7)	ns	<0.001	0.001	ns

Data presented as unadjusted mean (SD). Comparisons between both groups were performed with an independent t-test or Mann-Whitney U test and comparisons between Postprandial 1 and 2 were analysed by paired student t-test or Wilcoxon test, according with the normality of the variables. ANCOVA univariate analysis adjusted for fasting value of each assessed variable, weight at baseline and Δweight differently combined were determined.

$p<0.05$ was considered as significant

[a] AUC control vs. AUC cocoa group in Postprandial 1, adjusted for fasting value at Postprandial 1 and baseline weight

[b] AUC control vs. AUC cocoa group in Postprandial 2, adjusted for fasting value at Postprandial 2 and Δweight

[c] AUC control group: Postprandial 1vs. Postprandial 2

[d] AUC cocoa group: Postprandial 1 vs. Postprandial 2

[e] ΔAUCcontrol vs. ΔAUCcocoa: adjusted for Δfasting value of the variable (fasting value at Postprandial 2-fasting value at Postprandial 1) and Δweight

[f] No-normally distributed variables.

Abbreviations: AUC: Area under the curve; DBP: Diastolic blood pressure; HDL-c: High-density lipoprotein cholesterol; ns: no significant; SBP: Systolic blood pressure; Total-c: Total cholesterol; Δweight: weight change.

Table 3. Cocoa derived metabolites in plasma before (time 0) and after meal consumption (60, 120, 180 min)

Cocoa metabolites (nmol/L)	Time (min)				p^a			AUC (nmol·h/L)	$p_{AUC}{}^b$
	0	60	120	180	$p_{0\text{-}180'}$	$p_{P1\text{-}P2}$	$p_{0\text{-}180' \times P1\text{-}P2}$		
[e] **Procyanidin B2**									
Cocoa-P1	n.d.Z	3.2 (1.8)	3.1 (1.1)	n.q.	<0.001	ns	ns	7.6 (3.1)	ns
Cocoa-P2	n.d.	3.1 (1.3)	n.q.	n.q.				7.3 (3.7)	
Control-P2	n.d.	n.d.	n.d.	n.d.				—	
Methyl-catechin-glucoronide									
Cocoa-P1	n.d.	42.6 (23.9)	43.2 (12.8)	39.2 (11.9)	<0.001	ns	ns	105.9 (38.2)	ns
Cocoa-P2	n.q.	55.2 (18.0)	62.6 (45.1)	41.4 (12.8)				143.7 (58.87)	
Control-P2	n.d.	n.q.	n.q.	n.q.				—	
Epicatechin-glucoronide									
Cocoa-P1	n.d.	1346.8 (677.8)	1267.6 (499.7)	968.1 (360.7)	<0.001	ns	ns	3098.4 (1234.3)	ns
Cocoa-P2	104.4 (63.9)	1576.5 (478.9)	1371.1 (366.4)	998.6 (388.1)				3499.2 (955.0)	
Control-P2	14.4 (29.9)	40.6 (22.3)	44.4 (21.9)	63.9 (101.3)				124.2 (82.4)	
[e] **Catechin-sulphate**									
Cocoa-P1	n.d.	133.7 (53.3)	106.0(40.1)	65.8 (24.2)	<0.001	ns	ns	272.6 (85.6)	ns
Cocoa-P2	12.1 (17.0)	138.1 (51.0)	106.8 (48.7)	62.6 (45.9)				282.2 (110.2)	
Control-P2	n.d.	n.d.	n.d.	n.d.				—	
Methyl-epicatechin-*O*-sulphate									
Cocoa-P1	n.d.	353.7 (138.5)	353.3 (111.7)	276.9 (59.7)	<0.001	ns	ns	845.4 (249.0)	ns
Cocoa-P2	24.2 (12.1)	397.5 (108.4)	385.6 (79.8)	284.6 (80.9)				937.5 (185.9)	
Control-P2	n.q.	n.d.	13.9 (4.0)	16.5 (26.1)				36.3 (19.8)	

Epicatechin-sulphate									
Cocoa-P1	n.d.	2847.6 (1034.2)	2970.6 (958.2)	2074.4 (524.2)	<0.001	ns	ns	6734.1 (2164.3)	ns
Cocoa-P2	152.2 (98.8)	3311.2 (1029.2)	3374.3 (996.0)	2201.2 (885.0)				7862.1 (1862.2)	
Control-P2	n.q.	92.2 (42.6)	80.0 (20.8)	107.0 (175.7)				229.7 (135.1)	
3-*O*-methyl-epicatechin									
Cocoa-P1	n.d.	137.8 (51.8)	136.6 (47.0)	92.2 (37.9)	<0.001	ns	ns	320.5 (104.2)	ns
Cocoa-P2	n.q.	151.1 (40.7)	147.8 (33.3)	110.1 (36.6)				358.2 (76.6)	
Control-P2	n.d.	n.q.	n.q.	n.q.				—	
3,7-dimethyluric acid									
Cocoa-P1	n.d.	66.1 (138.3)	n.q.	n.q.	0.002	ns	ns	135.4 (171.8)	ns
Cocoa-P2	n.q.	68.0 (43.4)	61.9 (32.1)	77.6 (82.7)				176.8 (117.4)	
Control-P2	n.d.	n.q.	n.q.	n.q.				—	
3-methylxanthine									
Cocoa-P1	n.d.[c]	458.3 (190.8)[c]	634.0 (146.8)[c]	705.5 (158.3)[c]	<0.001	<0.001	ns	1445.0 (328.1)	<0.001
Cocoa-P2	881.1 (310.9)[d]	1301.1 (394.5)[d]	1386.4 (427.7)[d]	1560.9 (528.1)[d]				3908.5 (1146.7)	
Control-P2	335.2 (303.5)	542.2 (315.7)	592.7 (331.4)	680.0 (412.8)				1642.5 (983.8)	
[e] **Theophylline**									
Cocoa-P1	121.3 (212.3)[c]	285.5 (190.2)[c]	360.7 (143.3)[c]	391.0 (164.9)[c]	<0.001	0.013	ns	902.3 (512.3)	0.001
Cocoa-P2	726.2 (742.8)[d]	858.7 (563.9)[d]	854.4 (689.4)[d]	867.4 (555.7)[d]				2509.8 (1876.9)	
Control-P2	339.7 (341.3)	398.7 (328.7)	407.9 (291.1)	415.5 (329.8)				1184.2 (949.0)	

Data presented as unadjusted mean (SD). When a mean value was not quantifiable or no detectable in control group at 0, 60, 120 or 180 minutes, the real values were used, although been under the limit of quantification or detection, to perform statistical analysis where appropriate. AUC was calculated when at least the metabolite was above the limit of quantification in one time-point. In those cases also the corresponding p_{AUC} is reported. p_{AUC} was calculated using the original values of each participant where appropriate. Comparisons between P1 and P2were analysed by paired student t-test or Wilcoxon test, according with the normality of the variables. $p<0.05$ was considered as significant.

— Not possible to calculate AUC

[a] p values of the repeated measures ANOVA.

[b] AUC of cocoa group at Postprandial 1 vs. AUC of cocoa group at Postprandial 2.

[c,d] Cocoa group at Postprandial 1 vs. Cocoa groups at Postprandial 2 at each time-point by multiple comparison by Bonferroni correction.

[e] Non-normally distributed variables.

Abbreviations: AUC: Area under the curve; n.d: no detected; ns: no significant; n.q: no quantified; P1: Postprandial 1; P2: Postprandial 2.

Supplementary tables

- CHAPTER 3-

Supplemental Table 1. Optimized selected reaction monitoring conditions for the analyses of cocoa derived metabolites by HPLC–MS/MS.

Compound	MW	Quantification			Confirmation		
		SRM $_1$	Fragmentor (V)	Collision energy (V)	SRM $_2$	Fragmentor (V)	Collision energy (V)
Catechin	290	289 > 203	120	20	289 > 245	120	25
Epicatechin	290	289 > 245	130	10	289 > 203	130	20
Procyanidin B2	578	577 > 425	130	10	577 > 407	130	30
Methyl-catechin-glucuronide	480	479 > 303	160	20	479 > 289	160	20
Methyl-epicatechin-glucuronide	480	479 > 303	160	20	479 > 289	160	20
Epicatechin-glucuronide	466	465 > 289	140	20	465 > 203	140	40
Catechin-sulphate	370	369 > 289	140	20	369 > 245	140	20
Methyl epicatechin-*O*-sulphate	384	383 > 303	140	20	383 > 245	140	10
Epicatechin-sulphate	370	369 > 289	140	20	369 > 245	140	20
3-*O*-methyl-epicatechin	304	303 > 137	180	20	303 > 245	180	10
3,7-dimethyluric acid	196	195 > 124	110	20	195 > 180	110	20
3-methylxanthine	166	165 > 122	110	20	165 > 150	110	20
1-methylxanthine	166	165 > 108	40	20	165 > 122	40	20
Theophylline	180	179 > 164	40	20	179 > 122	40	20

Abbreviations: MW: Molecular weight; SRM : Selected reaction monitoring

Supplemental Table 2. Retention time, calibration curves, linearity, process efficiency, limits of detection and limits of quantification

Compound	RT (min)	Calibration curve	Linearity (nM)	R^2 (%)	Process efficiency (%)	LOD (nM)	LOQ (nM)
Catechin	6.3	y = 0.1493x	11.1-3100.6	0.998	102	3.3	11.1
Epicatechin	6.8	y = 0.1459x	13.5-3100.6	0.999	80	4.0	13.5
Procyanidin B2	6.6	y = 0.2267x	2.9-864.3	0.981	76	0.9	2.9
3,7-dimethyluric acid	2.4	y = 0.753x	53.6-112170.2	0.981	71	16.0	53.6
3-methylxanthine	2.8	y = 0.932x	211.9-99192.4	0.945	76	63.6	211.9

Specifications for the determination of phenolic compounds in spiked plasma samples.

Methyl-catechin-glucoronide and catechin-sulphate were quantified using the calibration curve of catechin. Methyl epicatechin-glucoronide, epicatechin-glucoronide, methyl-epicatechin–*O*–sulphate, epicatechin-sulphate and 3-*O*-methil-epicatechin were quantified using the calibration curve of epicatechin. 1-methylxantine and theophylline quantified using the calibration curve of 3-methylxanthine.

Abbreviations: Retention time (RT, min), linearity, process efficiency (%R^2), LOD: Limit of detection; LOQ: Limit of quantification.

CHAPTER 4

An increase in plasma homovanillic acid with cocoa extract consumption is associated with the alleviation of depressive symptoms in overweight or obese adults on an energy restricted diet in a randomized controlled trial

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ABSTRACT

Background: Obesity has been associated with various health disorders such as psychological alterations. Cocoa consumption and weight management may produce a beneficial effect on them.

Objective: The aim of this study was to investigate the effect of a cocoa extract supplementation within an energy restriction diet on psychological status as well as on the peripheral dopaminergic activity of overweight or obese middle-aged subjects.

Methods: A 4 week, double-blind, randomized, placebo-controlled parallel nutritional intervention, encompassing 22 men and 25 women (57(5) years, 30.6(2.3) kg/m2) was designed. After a week of run-in period, half of the volunteers randomly received ready-to-eat meals supplemented with 1.4 g/d of cocoa extract (645 mg total polyphenols), while the rest of the volunteers received the same meals without cocoa supplementation, all of them under the same 15% energy restriction diet. Plasma monoamines [dopamine, dopac and homovanillic acid (pHVA)], monoamine oxidase (MAO) and psychological status (anxiety and depressive symptoms) were analyzed at fasting at baseline and at endpoint. Variables were analyzed along time and comparisons between groups were performed. Moreover, regression and correlation analyses were carried out. Registered at www.clinicaltrials.gov (NCT01596309).

Results: Both groups significantly decreased depressive symptoms after the intervention (control: -9.4%, $p<0.001$ and cocoa: -6.3%, $p=0.008$), but not anxiety symptoms. pHVA presented a greater increase (11.5%) in the cocoa group ($p=0.016$), while dopamine, dopac and MAO did not evidence differences between groups. Interestingly, multiple regression analysis showed a negative relationship between changes in depressive symptoms and changes in pHVA only in the cocoa supplemented group ($\beta=-0.39$; $p=0.029$). Importantly, Δdopamine was positively associated with Δmethyl-catehchin-*O*- glucoronide cocoa metabolite ($r= 0.69$; $p=0.019$).

Conclusion: The intake of cocoa extract within a weight loss diet contributes to increase plasma HVA levels, which were associated with the reduction of depressive symptoms, suggesting the implication of cocoa intake on psychological behavior.

Keywords: cocoa, depression, dopamine, homovanillic acid, weight-loss

INTRODUCTION

Obesity is a multifactorial disease mainly caused by genetic and environmental factors, or the interaction among them (Rey-Lopez *et al.*, 2011; Milagro *et al.*, 2013; Goni *et al.*, 2014). Mental disorders such as anxiety and depression are also associated with obesity and weight gain in a bidirectional manner (Pan *et al.*, 2012; Preiss *et al.*, 2013; Kim *et al.*, 2014).

Interestingly, central and peripheral monoamine systems are related with obesity and mental disorders (Luppino *et al.*, 2010; Pan *et al.*, 2012). Central monoaminergic system is responsible of cognition, the reward system and mood (Belmaker and Agam, 2008), while the peripheral monoaminergic system is mainly implicated in physiological functions (Perez-Cornago *et al.*, 2014b). Dopamine is a monoamine produced from the hydroxylation of the amino acid tyrosine and it is present in central and peripheral monoaminergic systems. While the central dopamine is involved in mood, cognition, motor control, the reward system and well-being (Schultz, 2007; Carlin *et al.*, 2013), the peripheral dopamine is involved in gastrointestinal motility, heart function and blood pressure among others (Rubi and Maechler, 2010). In relation with the incidence of obesity, dopamine is inversely associated with body mass index (BMI) and low dopamine levels may increase the preference for consuming high-palatable and energetic foods, contributing also to obesity condition (Wang *et al.*, 2001; Wang *et al.*, 2009). Plasma homovanillic acid (pHVA) is the main metabolite produced from the dopamine degradation by the action of monoamine oxidase (MAO), which is an amine oxide enzyme (Lyles, 1996). Taking into account that dopamine cannot cross the blood brain barrier (Pardridge, 2007), the determination of pHVA has been suggested as an appropriate indicator of central dopaminergic activity, because the HVA changes in the brain are positively correlated with the HVA changes in plasma (Sternberg *et al.*, 1983; Amin *et al.*, 1992).

In the last years, some nutrients (Sanchez-Villegas and Martinez-Gonzalez, 2013; Perez-Cornago *et al.*, 2014b; Perez-Cornago *et al.*, 2015), dietary patters (Sanchez-Villegas *et al.*, 2013) and antioxidants (Payne *et al.*, 2012) have been used as new approaches to treat obesity and mental disorders, finding plant polyphenols as a promising therapy (Dias *et al.*, 2012; Pathak *et al.*, 2013). Cocoa is a rich source of polyphenols, mainly flavanols, which have been recognised of having positive effects on disturbances such as cardiovascular disease, cancer, diabetes, inflammation, oxidative stress and blood pressure, among others (Shrime *et al.*, 2011; Arranz *et al.*, 2013; Latif, 2013). In addition, chocolate consumption has the capacity to make people feel good and some studies have suggested that cocoa and cocoa polyphenols may have positive psychological and neuroprotective benefits (Messerli, 2012; Sokolov *et al.*, 2013). Indeed, flavanols have the capacity to cross the brain blood barrier enhancing brain blood flow through the increased production of nitric oxide, which has been proposed as a mechanism involved in brain health (Vauzour *et al.*, 2008; Nehlig, 2013). On the other hand and considering that obesity is associated with depression and anxiety, it seems that weight loss is a useful strategy to improve mental disorders in obese subjects (Perez-Cornago *et al.*, 2014b). However, there is a controversy concerning this hypothesis.

While some researchers suggest that weight loss is positively associated with a reduction in anxiety and depressive symptoms (Stapleton *et al.*, 2013; Perez-Cornago *et al.*, 2014b), other investigators did not find this effect (Eyres *et al.*, 2014; Jackson *et al.*, 2014).

In this context, the results presented in this work aimed to answer questions related to the effect of cocoa consumption on psychological behavior and peripheral monoamine system under a weight loss nutritional strategy. The results reported in this research are secondary outcomes derived from a clinical trial (NCT01596309), whose principal aim was to assess the effect of consuming ready-to-eat meals containing 1.4 g/day of cocoa extract under a moderate energy restriction diet on the general nutritional status as well as on cardiometabolic and oxidative status of middle-aged overweight or obese subjects. Some results derived from that investigation evidenced the improvement of oxidative status through the reduction of oxidized low-density lipoprotein-cholesterol (oxLDL-C) levels (Ibero-Baraibar *et al.*, 2014a). In addition, the prescribed dietary strategy resulted beneficial in improving the general status of the volunteers through the reduction of anthropometric and body composition variables as well as by the improvement of blood pressure, routine blood biochemical profile and 25-hydroxivitamin D levels (Ibero-Baraibar *et al.*, 2014b; Ibero-Baraibar *et al.*, 2015).

Taking into account such background, the objective of this study was focused on answering questions based on the effects of consuming 1.4 g/day of cocoa extract during 4 weeks and within a moderate energy restriction diet on depressive and anxiety symptoms as well as on the peripheral dopaminergic activity of overweight or obese middle-aged subjects.

SUBJECTS AND METHODS

Subjects

Fifty subjects (23 men and 27 women) participated in the study. The trial was performed in the Metabolic Unit of the University of Navarra (Spain) following the CONSORT 2010 guidelines. This study was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012), followed the Helsinki Declaration guidelines and it was registered at www.clinicaltrials.gov with the number NCT01596309 and the name “Cocoa Extract-enriched Meals and Cardiovascular Risk in Older Population”.

Participants were recruited between March and May of 2012 by advertisements in local newspapers. After the screening process, those subjects who met the inclusion criteria were included in the study. All the participants gave written informed consent before starting the intervention. The inclusion criteria were subjects with an age between 50 and 80 years old, BMI between 27.0 and 35.5 kg/m^2 and to have maintained a stable weight (<5% of variation) three months prior to the intervention. The information concerning the exclusion criteria have been reported elsewhere (Ibero-Baraibar *et al.*, 2014a).

Study design

The trial was designed as a 4 weeks double-blind, randomized, placebo-controlled parallel nutritional intervention. As shown in Figure 1, a run-in period was carried out one week before the beginning of the intervention. In that period, volunteers were encouraged to exclude cocoa and cocoa containing products from their habitual diet and three days before starting the trial they were asked to consume a low-polyphenol diet without energy restriction (Ibero-Baraibar *et al.*, 2014a; Ibero-Baraibar *et al.*, 2015). Then, volunteers were randomized using the "random between 1 and 2" function in the Microsoft Office Excel (Microsoft Iberica, Spain) in control or cocoa group.

Volunteers were provided with a variety of ready-to-eat meals. Each dish and each dessert was supplemented with 0.7 g of cocoa extract in the case of cocoa group, while no extract was included in the ready-to-eat meals of control group. Taking into account the overweight or obese condition of the participants, subjects from both groups were prescribed with a 15% energy restriction diet as described elsewhere (Ibero-Baraibar *et al.*, 2014a). Once per week volunteers attended the Metabolic Unit to carry out the follow-up visits, where volunteers received the test products and were encouraged to carefully follow the intervention. Three days before the last day of the trial, volunteers were prescribed to consume pre-determined types of test meals in order to make all the volunteers were in the same nutritional conditions at the last day of the study, avoiding putative nutritional interferences. The first day and the last day of the intervention the corresponding measurements and data collection were performed.

Adherence to the intervention was assessed by a notebook where volunteers self-reported the name of the test dish and dessert they consumed daily. Nutrient intake was analyzed by a 3-day validated food-recall questionnaire which was processed using the DIAL software (Alce Ingenieria SL, Madrid, Spain) according to other studies (Perez-Cornago *et al.*, 2014a). Moreover, plasma metabolites derived from cocoa consumption (methyl-catechin-glucoronide, catechin-sulphate and 3-*O*-methyl-epicatechin) were analyzed at baseline and the endpoint of the study by an external certified research center (Centre Tecnòlogic de Nutició i Salut (CTNS), Reus, Spain). Phenolic metabolites were extracted following the method described by Serra et al (2009). Then HPLC-MS/MS analysis was performed in ESI negative mode (Serra *et al.*, 2009).

Cocoa extract used in the study

Cocoa extract and its analytical characterization were supplied by Nutrafur S.A. (Murcia, Spain). The composition of 1.4 g of cocoa-extract provided as mean ± SD was as follows: 140.4 ± 7.1 mg of theobromine, 645.3 ± 32.3 mg of total polyphenols as catechin, 414.3 ± 20.7 mg of flavanols as catechin, 153.4 ± 7.7 mg of epicatechin, 14.6 ± 0.7 mg of catechin, 99.4 ± 5.0 of procyanidin B2, 13.4 ± 0.7 mg of procyanidin B1 and 133.5 ± 6.7 mg of oligomeric procyanidins. Total polyphenol content was determined by Folin-Ciocalteu method and flavanoids and theobromine were analyzed by HPLC. The analyses have been described in detail elsewhere (Ibero-Baraibar *et al.*, 2014a).

Anthropometric and blood biochemical measurements

Anthropometric, body composition and routine blood biochemical markers were collected at baseline and at the endpoint of the study in fasting conditions following validated procedures as previously described (de la Iglesia *et al.*, 2013; Ibero-Baraibar *et al.*, 2014a). These data are reported and discussed in more detail elsewhere (Ibero-Baraibar *et al.*, 2014a). In this study these data have been only presented to describe the sample.

Assessment of anxiety and depressive symptoms

The validated Spanish translation of the State-Trait Anxiety Inventory (STAI) was used to evaluate the degree of anxiety symptoms (Spielberger, 1971). This questionnaire consisted of 20 items answered on a 4-point scale. Total score was obtained by summing all items, higher values indicating greater anxiety symptoms. Depressive symptoms were evaluated with the Spanish translation of the Beck Depression Inventory (BDI). The questionnaire consisted of 21-items answered on a 4-point scale that after summing all items provided a score of depressive symptoms (Conde, 1975). Taking into account that losing weight has been related to depression, item number 19 of the test, relating to weight loss, was discarded because losing weight was considered as a positive aspect in this study, as described elsewhere (Perez-Cornago *et al.*, 2014a). Questionnaires assessing the psychological status of the participants were completed at the beginning and at the end of the study.

Monoamine analyses

Peripheral concentrations of dopamine, dopac and HVA were analyzed in plasma samples using HPLC technique as described elsewhere (Perez-Cornago *et al.*, 2014b). MAO activity was measured in plasma samples using a commercial kit (Amplite Fluorometric Monoamine Oxidase Assay Kit, AAT Bioquest, ref 11303) and following the manufacturer's instructions.

Statistical analyses

The sample size was calculated *a priori* for the main study, considering oxLDL-C marker as the main variable (Ibero-Baraibar *et al.*, 2014a). The estimation was made taking into account a reduction in oxLDL-C levels of 14.1 U/L and an interquartile range of 16.3 U/L, according to the study carried out by Khan et al (Khan *et al.*, 2012). With a bilateral confidence index of 95% (α=0.05) and a statistical power of 80% (β= 0.20) the sample size was estimated to be 44 subjects (22 subjects in each arm). A possible drop-out rate of 15% was considered, establishing the final sample size in 50 subjects (25 subjects in each arm). Data are expressed as mean (SD). The distribution of the variables was assessed using the Shapiro-Wilk test. According to the normality of the variables, comparisons between baseline and the endpoint of the intervention were studied by paired Student´s t-test or Wilcoxon signed-rank test. Analyses between both groups were performed by independent sample t-test or Mann-Whitney U test. ANCOVA analyses were carried

out adjusting for age, sex and weight change (Δ= 4 weeks – baseline) when appropriate. The analytical results concerning specific variables have been reported as p-value as well as the 95% CI, which expresses also the power of the trial. Regression analyses were performed to evaluate the relationship between changes in BDI (ΔBDI) as dependent variable and changes in dopac, dopamine and HVA levels (Δdopac, Δdopamine and ΔpHVA) as independent variables, separated by groups and adjusted for age, sex and Δweight. In addition, a regression analysis was carried out to assess the effect of group and Δweight on ΔpHVA as well as a correlation analysis between ΔMAO and ΔpHVA. Spearman and Pearson correlation analyses were performed in cocoa group to evaluate any relationships between changes of cocoa derived metabolites and routine blood biochemical variables and monoamine changes in plasma. Data were analyzed using SPSS 15.0 software for Windows (SPSS Inc, Chicago, USA) considering $p<0.05$ as statistically significant.

RESULTS

Subjects

Of the 50 subjects who were enrolled in the study, 47 subjects with a mean age of 57.5 ± 5.3 and BMI of 30.5 ± 2.2 completed the trial and were included in the data analyses (Figure 2). In the control group, one man dropped out because of poor adherence to the diet, while in the cocoa group two women were excluded, one for no adherence to the diet and the other one for personal reasons. Regarding the STAI questionnaire, 43 subjects (21 subjects in the control and 22 subjects in the cocoa group) were assessed because 4 subjects did not answer the whole questionnaire. Concerning the BDI questionnaire, 37 subjects completed it (19 subjects from the control and 18 subjects from the cocoa group). Subjects from the control and the cocoa groups started the intervention in the same conditions given that no significant differences were found at baseline in the assessed variables (Table 1). Moreover, after 4 weeks intervention, both groups significantly reduced most of the anthropometric, body composition and routine blood biochemical markers (Table 1). The comparison of changes did not demonstrate significant differences in the unadjusted as well as in the adjusted models (Table 1).

These data have been previously reported elsewhere (Ibero-Baraibar *et al.*, 2014a; Ibero-Baraibar *et al.*, 2014b; Ibero-Baraibar *et al.*, 2015). Moreover, no differences between groups were found at baseline in dietary macronutrient and energy intake [energy (kcal/day): 1780 ± 375 vs. 1845 ± 560; carbohydrate (g/day): 173 ± 56; 176 ± 50; proteins (g/day): 80 ± 25 vs. 82 ± 19; lipids (g/day):76 ± 18 vs. 79 ± 35].

Both groups reported similar adherence to meal consumption (98.4 ± 2.2 % control and 98.5 ± 3.3 % cocoa group) and dietary records showed no significant differences between groups in macronutrient and calorie intake during the study (Ibero-Baraibar *et al.*, 2014b). In addition, the analytical determination of cocoa extract derived metabolites in plasma revealed the occurrence of methyl-catechin-glucoronide, catechin-sulphate and 3-*O*-methyl-epicatechin only in the cocoa

group, which confirmed the compliance of the intervention. No volunteers reported adverse events during the intervention.

Depressive and anxiety symptoms after 4 weeks of intervention

A significant reduction in depressive symptoms was observed after 4 weeks of intervention in both experimental groups, while anxiety symptoms did not show significant changes (Table 1). No differences were found between groups in depressive and anxiety symptoms when changes were compared unadjusted or adjusted for age, sex and Δweight (Table 1).

Peripheral monoamine levels and monoamine oxidase concentration after 4 weeks of intervention

Circulating dopamine presented a tendency towards significant decrease in the control group (p= 0.05) at the end of the intervention (**Table 2**), while no changes concerning dopamine were observed in the cocoa group. Dopac did not change in the control group, whereas it presented a significant raise in the cocoa group. Moreover, pHVA and MAO levels significantly increased in both groups. The ratio dopac + HVA/dopamine significantly decreased after 4 weeks. When changes between the 4th week and baseline were compared between groups, significantly a major rise of pHVA levels were observed in cocoa group comparing to control group, unadjusted as well as adjusted for age, sex and Δweight. Dopamine, dopac, ratio and MAO did not show differences between groups.

Regression and association analyses between monoamines and BDI score

Interestingly, the multiple regression analyses between ΔHVA levels and ΔBDI score adjusted for age, sex and Δweight showed a negative relationship in cocoa supplemented group (β=-0.29; p=0.039), but not in control group. Concerning the relationship of ΔBDI with Δdopamine (control: β= 0.07; p= 0.73; cocoa: β= 0.22, p= 0.25) and Δdopac (control: β= 0.20, p= 0.46; cocoa: β= -0.11; p= 0.58) no associations were found neither in cocoa nor in control group. The regression analysis to assess the effect of Δweight and group on ΔHVA, resulted significant for group (β= 2.62; p= 0.045) and not significant for Δweight (β= -0.17; p= 0.70). Moreover, no association was found between ΔMAO and ΔHVA neither in control (rho= 0.35; p= 0.15) nor in cocoa group (rho= 0.06, p= 0.83) after the 4 weeks nutritional intervention.

Association analyses between cocoa derived metabolites in plasma and blood biochemical and monoamine markers

No statistical association between cocoa derived metabolites (Δmethyl-catechin-glucoronide, Δcatechin-sulphate and Δ3-*O*-methyl-epicatechin) and plasma biochemical and monoamine markers were found except for Δcatechin-sulphate, which significantly correlated with

Δglucose (rho= -0.75; p=0.008) and Δmethyl-catechin-glucoronide which significantly correlated with Δdopamine (r = 0.69; p= 0.019).

DISCUSSION

This study demonstrated that depressive symptoms measured by BDI index decreased significantly in both experimental groups after the 4 weeks intervention, demonstrating that weight loss contributed to reduce depressive symptoms (Swencionis *et al.*, 2013; Perez-Cornago *et al.*, 2014b). On the other hand, pHVA levels significantly increased in both groups after the 4 weeks, suggesting that weight loss also may produce an increase in the metabolite derived from dopamine metabolism. Brain dopamine, which is related with mood, cannot cross the blood-brain barrier (Pardridge, 2007) and for this reason, the dopaminergic activity in the brain was determined by the measurement of pHVA, which is positively correlated with the central dopaminergic activity (Sternberg *et al.*, 1983). Taking into account these results, it could be postulated that weight loss may be related with the improvement of psychocological behavior (Perez-Cornago *et al.*, 2014b; Perez-Cornago *et al.*, 2015). However, the increase of pHVA was significantly higher in cocoa consumers compared to control group indicating that the intake of cocoa extract produces an additional increment of pHVA levels. More importantly, a statistically significant relationship was observed between the changes of pHVA and the changes of BDI only in the cocoa group. Moreover, the assessment of cocoa derived metabolites in plasma revealed that Δmethyl-catechin-glucoronide was positively associated with plasma Δdopamine, the neurotransmitter which according to the scientific literature is inversely associated with BMI and whose low levels may increase the preference for consuming high-palatable and energetic foods, contributing also to obesity condition (Wang *et al.*, 2001; Wang *et al.*, 2009).

The results from this study suggest that the intake of cocoa extract within a nutritional strategy for weight loss could be associated with a specific improvement of psychological status. The lack of differences between groups in BDI index could be due to the variability of data reported because BDI is a subjective questionnaire, whereas pHVA is an objective determination. The scientific evidence concerning cocoa supports our results since Fernandez-Fernandez et al (2015) observed that the consumption of a diet with a high proportion of cocoa enhanced the dopaminergic system and catecholamine production in the striatum of mice. Moreover, cells treated with theobromine increased the production of dopamine and HVA among other neurotransmitters (Fernandez-Fernandez *et al.*, 2015). Similarly, the administration of cocoa rich in procyanidins, resulted protective against dopaminergic loss and the reduction of HVA in neurons from a rat model with Parkinson disease (Datla *et al.*, 2007). Likewise, previous investigations have suggested the positive effects of cocoa consumption and weight loss on well-being and mood of obese subjects (Sokolov *et al.*, 2013; Swencionis *et al.*, 2013; Perez-Cornago *et al.*, 2014b).

In connection with this, cocoa flavanols are able to cross the blood brain barrier inducing an increment of brain blood flow through the increase on nitric oxide production, which is

negatively associated with depressive symptoms (Fisher *et al.*, 2006). In addition, cocoa flavanols may interact with a number of neurotransmitters in the brain, which are implicated in the regulation of the food reward system, mood (Parker *et al.*, 2006), cognitive performance (Scholey *et al.*, 2010), reduction of fatigue (Sathyapalan *et al.*, 2010) and improvement of calmness and contentedness (Pase *et al.*, 2013). Indeed, there are studies suggesting the antidepressant effect of cocoa (Messaoudi *et al.*, 2008; Pase *et al.*, 2013), which is in agreement with some of our findings.

A controversy exists concerning pHVA levels. For this reason it is important to be cautious with the interpretation. The increment of pHVA level has been postulated as beneficial for cognitive functions in healthy subjects (Sumiyoshi *et al.*, 1998). However, schizophrenic subjects show elevated levels of pHVA associated to the hyperactivity in the dopaminergic system which is reduced by antipsychotics treatment (Baeza *et al.*, 2009; Zumarraga *et al.*, 2011). Contrary, Parkinson disease is characterised by the loss of dopaminergic neurons and a decrease of pHVA concentration (Herbert *et al.*, 2013). Concerning depressed subjects, mixed results have been observed. The pHVA levels in psychotic depression might be high (Goto *et al.*, 2006) while low levels of pHVA have been observed in major depression (Mitani *et al.*, 2006).

MAO, which is the enzyme responsible of degrading dopamine to HVA (Lyles, 1996) increased significantly after the intervention, but it was not apparently related with the rise of pHVA at the end of the study. Interestingly, it has been suggested that amine oxidases are not simple amine scavengers and they have also a role on insulin function (Carpene *et al.*, 2006) because MAO substrates induce MAO activation exhibiting insulin-mimicking properties, at least in adipocytes (Carpene *et al.*, 2006).

On the other hand, excessive body weight and the incidence of depression are associated (de Wit *et al.*, 2010b). According with a meta-analysis, obese people have higher risk to suffer from depression compared to those who are not obese (de Wit *et al.*, 2010a). Moreover, a bidirectional relationship between depression and excessive body weight was found in a meta-analysis of longitudinal studies (Luppino *et al.*, 2010). Subjects suffering from depression and anxiety seem to follow unhealthy lifestyle patterns contributing to weight gain (Gea *et al.*, 2013; van Mill *et al.*, 2013). In the same manner, obese individuals or those with dopaminergic hypofunction usually follow poor health habits, increasing the consumption of high-palatable and energetic foods which increase the risk of suffering from depression and anxiety disorders (Reinholz *et al.*, 2008; Pan *et al.*, 2012). In this context, in order to improve depressive symptoms in obese subjects, a body weight reduction has been suggested by other investigators (Perez-Cornago *et al.*, 2014b; Perez-Cornago *et al.*, 2015). According to this, the weight loss diet followed by the volunteers in this trial caused a significant reduction of weight and consequently may have improved depressive symptoms in both intervention groups.

Furthermore, depression is associated with a great oxidative stress and poor antioxidant status, which may be seen improved by the consumption of antioxidants such as cocoa flavanols

(Pandya *et al.*, 2013). According to Lopresti et al 2013 (Lopresti *et al.*, 2014), the potential oxidative stress biomarkers to diagnose depression are malondialdehyde, 8-Hydroxy-2-deoxyguanosine, isoprostanes, superoxide dismutase and glutathione, and the potential biomarkers to measure the treatment response are C-reactive protein, cytokines and neopterin (Lopresti *et al.*, 2014). Nevertheless, more studies should be conducted to determine if the reduction of oxidative stress could be one of the mechanisms involved in the improvement of depressive symptoms.

Concerning anxiety symptoms, no significant changes were observed after the intervention, suggesting that neither weight loss nor cocoa extract intake had any effects on anxiety after the 4 weeks intervention. In this sense, while Scholey et al (2010) did not observe an improvement in STAI score after the acute consumption of 520 mg or 994 mg of cocoa flavanols (Scholey *et al.*, 2010), Sathyapalan et al 2010 (Sathyapalan *et al.*, 2010) reported a reduction in anxiety after polyphenol rich chocolate consumption in humans with chronic fatigue. On the other hand, although a positive association has been proposed between weight loss and anxiety symptoms, a recent review concluded that diet induced weight loss did not always reduce anxiety (Eyres *et al.*, 2014). Maybe, the period of the study was not enough to observe significant effects.

As far as we know, this is the first time that peripheral monoamine levels have been assessed in relation to depressive and anxiety symptoms after cocoa extract consumption within an energy restriction diet. Results of the present study contribute to shed light about the implication of cocoa flavanols and energy restriction diet on psychological symptoms during obesity. The design of this study, which is a randomized controlled trial, could be considered as an strength because this type of trials are recognized on clinical research, due to the reduction of allocation bias equally distributing the participants. However, some bias could not be discarded due to the selection of participants prior to the randomization. Some limitations could be addressed in this investigation. First, the missing data of some volunteers reduced the sample size of the study in some analysis, even if significant differences were found. Second, type I and type II errors cannot be discarded due to the low number of volunteers. Third, the lack of differences between groups in BDI questionnaire could be due to the lack of power as we can see in the width of the confidence intervals for this variable, while for the pHVA is enough. Fourth, although psychological questionnaires used in this study are validated and have been recently used in weight loss studies, participants could hide their real feelings because questionnaires were self-reported (Perez-Cornago *et al.*, 2014a; Perez-Cornago *et al.*, 2014b). Fifth, although it is well described that pHVA levels represents dopaminergic activity within the central nervous system, pHVA also comes from noradrenergic neurons.

In conclusion, this study revealed that the consumption of cocoa within a weight loss diet increased pHVA levels more than the dietary strategy alone. In addition, the increase of pHVA was negatively associated with the reduction of depressive symptoms only in the cocoa group. Considering that pHVA reflects HVA levels in the brain, it is suggested the implication of cocoa on the improvement of psychological behavior.

Conflict of interest and financial disclosure

Idoia Ibero-Baraibar, Aurora Perez-Cornago, Maria J Ramirez, J. Alfredo Martinez and M. Angeles Zulet declare no conflicts of interest.

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Abbreviations

BDI, Beck depression inventory; BMI, Body mass index; HOMA-IR, Homeostatic model assessment-insulin resistance; HPLC, High-performance liquid chromatography; MAO, Monoamine oxidase; oxidised LDL, oxidised low-density lipoprotein-cholesterol; pHVA, Plasma homovanillic acid; STAI, State-Trait Anxiety Inventory.

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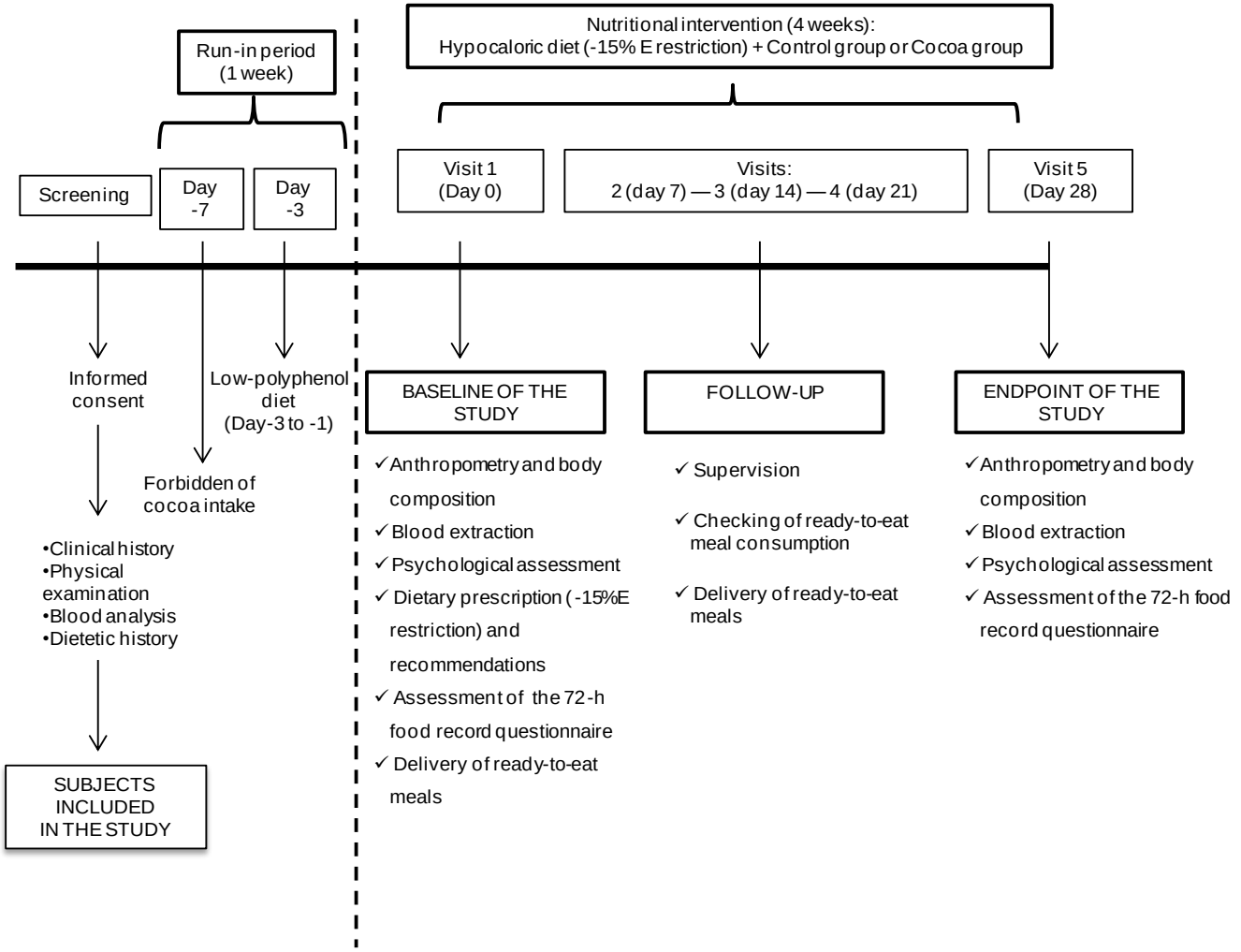

Figure 1. Chronological sequence of events during the intervention period.

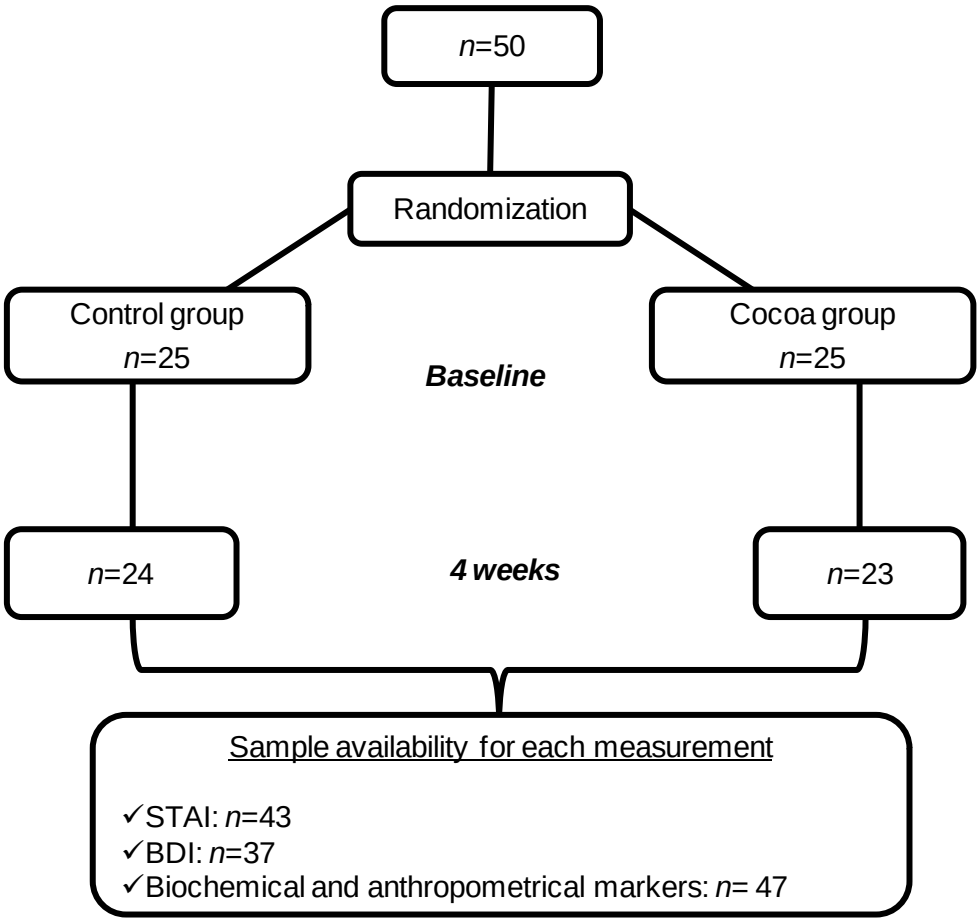

Figure 2. Flow-chart of participants during the study period including control and cocoa groups.

Abbreviations: BDI, Beck Depression Inventory; HVA, Homovanillic acid; STAI, State-Trait Anxiety Inventory

Table 1. Phenotypic variables, plasma biochemical markers and psychological symptoms of control and cocoa groups at baseline and after 4 weeks of intervention [1]

Variables	Control group (*n*=24)		Cocoa group (*n*=23)		p [3]	95%CI	Δp [4]	95%CI	Δp [5]	95%CI
	Baseline	4 week[2]	Baseline	4 week[2]						
Age, years	57 ± 5.0		58 ± 5.6		0.45	—	—	—	—	—
Sex ,♂ or ♀	11 ♂, 13♀	11♂, 13♀	11 ♂, 12 ♀	11♂, 12 ♀	—	—	—	—	—	—
Weight, kg	81.5 ± 11.4	79.0 ± 11.4	83.3 (10.5)	80.6 ± 10.2	0.62	(-4.9, 8.1)	0.70	(-1.0, 0.7)	Colineality	Colineality
BMI, kg/m^2	30.3 ± 1.9	29.4 ± 2.1***	30.7 ± 2.5	29.7 ± 2.3***	0.45	(-0.9, 1.7)	0.74	(-0.3, 0.3)	Colineality	Colineality
Waist/hip ratio	0.95 ± 0.09	0.93 ± 0.09*	0.96 ± 0.06	0.93 ± 0.07***	0.55	(-0.03, 0.1)	0.32	(-0.03, 0.01)	0.23	(-0.03, 0.01)
Glucose, mg/dL	98 ± 10.0	96 ± 9.5	99 ± 10.0	97 ± 11.0	0.54	(-5.4, 6.4)	0.87	(-6.2, 7.3)	0.78	(-5.7, 7.6)
Insulin, µU/mL [6]	7.1 ± 4.8	5.4 ± 4.2**	7.1 ± 5.9	4.7 ± 4.4***	0.86	(.3.2, 3.1)	0.94	(-3.3, 1.9)	0.69	(-3.2, 2.1)
HOMA-IR	1.7 ± 1.2	1.3± 1.0**	1.8 ± 1.5	1.2 ± 1.2**	0.99	(-0.8, 0.8)	0.99	(-0.9, 0.5)	0.69	(-0.9, 0.6)
Total-c, mg/dL [6]	234 ± 33.5	192 ± 30.4***	248 ± 53.4	206 ± 41.4***	0.93	(-11.7, 40.4)	0.51	(-19.0, 19.1)	0.89	(-20.8, 18.2)
STAI, points [6, 7]	11.3 ± 7.7	12.5 ± 6.3	10.4 ± 6.0	9.6 ± 6.3	0.17	(-6.1, 2.4)	0.47	(-5.4, 1.5)	0.33	(-5.4, 1.8)
BDI, points [6, 8]	11.8 ± 7.4	6.1 ± 6.1***	9.4 ± 8.5	5.7 ± 6.2**	0.49	(-4.6, 1.8)	0.56	(-1.0, 3.1)	0.21	(-0.8, 3.4)

[1] Values are means ± SDs. BDI, beck depression inventory; BMI, body mass index; HOMA-IR, homeostasis model assessment insulin resistance; Total-c, total cholesterol; STAI, state-trait anxiety inventory; Δ, change (4 week-baseline); ♂, male; ♀, female

[2] ***p≤0.001; **p≤0.01; *p≤0.05. Comparison between baseline and 4week

[3] p-value and 95%IC of comparing control and cocoa at baseline adjusted for age and sex

[4] unadjusted p-value and 95% CI of comparing control and cocoa changes

[5] p-value and 95% CIof comparing control and cocoa changes adjusted for age, sex and Δweight

[6] Non-normally distributed variables

[7] STAI : control *n*=21 and cocoa *n*=22

[8] BDI: control *n*=19 and cocoa *n*=18

Table 2. Plasma monoamine and MAO levels of control and cocoa groups at baseline and after 4 weeks of intervention [1]

Variables	Control (*n*=24)			Cocoa (*n*=23)			Δp[3]	95%CI	Δp[4]	95%CI
	Baseline	4 week	P [2]	Baseline	4 week	P [2]				
Dopamine, pg /µL	16.8 ± 5.0	14.7 ± 4.2	0.05	17.3 ± 5.9	16.1 ± 7.7	0.39	0.60	(-2.6, 4.4)	0.51	(-2.4, 4.7)
Dopac, pg /µL [5,6]	3.1 ± 3.9	4.4 ± 2.8	0.05	5.9 ± 5.9	8.3 ± 6.2	0.021	0.11	(-1.1, 4.6)	0.24	(-1.2, 4.5)
Homovanillic acid, pg /µL	24.4 ± 3.5	26.8 ± 4.0	<0.001	25.3 ± 4.9	30.5 ± 6.6	<0.001	0.014	(0.6, 5.1)	0.016	(0.6, 5.3)
Ratio dopac + HVA/dopamine [7]	1.8 ± 0.7	2.3 ± 0.9	0.005	2.2 ± 1.3	3.1 ± 1.6	0.002	0.18	(-0.2, 1.1)	0.19	(-0.2, 1.1)
MAO, mU/mL [5,8]	35.4 ± 21.0	46.3 ± 21.4	0.019	34.9 ± 16.9	48.3 ± 28.8	0.018	0.92	(-11.6, 16.4)	0.45	(-8.5, 18.7)

[1] Values are means ± SDs. HVA, homovanillic acid; MAO, monoamine oxidase

[2] Comparison between baseline and 4 week of the study

[3] Unadjusted p-value and 95% CI of comparing control and cocoa changes

[4] p-value and 95% CI of comparing control and cocoa changes adjusted for age, sex and Δweight

[5] Non-normally distributed variables

[6] Dopac: control *n*= 24 and cocoa *n*= 21

[7] Ratio dopac + HVA/dopamine: control *n*= 24 and cocoa *n*= 21

[8] MAO: control *n*=21 and cocoa *n*=19

CHAPTER 5

Urinary metabolomic profile following the intake of meals supplemented with cocoa extract in middle-aged obese subjects

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ABSTRACT

Background: Metabolomics is a useful tool to assess the compliance and bioavailability of food components, as well as to evaluate metabolic changes associated with food consumption.

Objective: The aim of this study was to analyse the urinary metabolomic profile to evaluate the metabolomic changes related to the intake of ready-to-eat meals containing a cocoa extract.

Methods: Fifty middle-aged volunteers [30.6 (2.3) kg/m^2] participated in a 4 week randomised, parallel and double-blind study. Half of them consumed meals supplemented with 1.4 g of cocoa extract (645 mg polyphenols) while the remaining subjects received the same meals without cocoa extract supplementation. Ready-to-eat meals were included within a 15% energy restriction diet. Urine samples (24 h) were collected at baseline and after 4 weeks and were analyzed by High Performance Liquid Chromatography-Time-Of-Flight Mass Spectrometry (HPLC-TOF-MS) in negative and positive ionization modes followed by multivariate analysis. Spearman correlation test was used to evaluate the relation between urinary metabolites

Results: The principal component analysis revealed separation among baseline and both groups at 4th week, principally in the negative ionization mode ($p<0.01$). Putative metabolites included those related to theobromine (3-methylxanthine and 3-methyluric acid), food processing (L-beta-asparyl-L-phenylalanine), flavonoids (2,5,7,3',4'-pentahydroxyflavanone 5-*O*-glucoside and 7,4'-dimethoxy-6-c-methylflavanone), catecholamine metabolism (3-mthoxy-4-hydroxyphenylglycol sulphate) and endogenous ones (uridin monophosphate). These metabolites were present in significantly ($p<0.001$) higher amounts in the cocoa consumers.

Conclusion: In conclusion, the metabolomics approach supported the compliance of the study and indirectly suggests the availability of cocoa compounds within the ready-to-eat meals. On the other hand, the identified metabolomic changes contribute to provide more information concerning the beneficial effects of cocoa intake.

INTRODUCTION

Metabolomics is an emerging *omics* tool applied to evaluate metabolic modifications in body fluids (Patti *et al.*, 2012). The interest for metabolomics has recently increased in nutritional science, but it is also used in clinical, pharmaceutical and toxicological fields in order to explain biological functions and biochemical responses of the organism (Zamboni *et al.*, 2015).

In nutrition science, metabolomic analysis is used to detect biomarkers of intake, which are metabolites produced from the absorption, digestion and metabolism of dietary components (Manach *et al.*, 2009; McGhie and Rowan, 2012). Moreover, metabolomics is used for identifying biomarkers of nutritional exposure related to endogenous changes and alteration of metabolic pathways induced by dietary components (Herrero *et al.*, 2012). There are two types of metabolomic analyses: targeted metabolomic is used to analyse predetermined metabolites in body fluids in order to assess the nutritional compliance or endogenous changes associated with dietary consumption (Astarita and Langridge, 2013), while untargeted metabolomic is focused on investigate changes in metabolites on plasma, urine or faeces to identify new biomarkers of dietary exposure after the intake of a specific food or dietary pattern (Astarita and Langridge, 2013; Andersen *et al.*, 2014; Etxeberria *et al.*, 2015).

The consumption of fruits and vegetables, which are rich sources of antioxidants, are related with the reduction of some diseases (Boeing *et al.*, 2012). Those benefits are principally linked to the polyphenol content of plants (Khurana *et al.*, 2013). However, in order to establish a relationship between the consumption of plants and the effects on health, polyphenols need to be bioavailable for the organism (Rein *et al.*, 2013; Zheng *et al.*, 2015).

Cocoa is a rich source of antioxidants due to the content on flavanols (Oracz *et al.*, 2015), whose consumption has been associated with the prevention or improvement of some health disorders such as hypertension, endothelial dysfunction, inflammation, oxidative stress and psychological disorders, among others (Ellam and Williamson, 2013; Kim *et al.*, 2014; Peluso *et al.*, 2015). Indeed, European Food Safety Authority (EFSA) approved a claim about the benefits of cocoa consumption on endothelial function (EFSA, 2014). Nevertheless, it seems that the bioavailability of cocoa flavanols is poor. Cocoa monomers and some dimmers are directly absorbed into the circulation and are sulphated, methylated and glucoronidated by phase-II enzymes in the entherocyte and liver (Rein *et al.*, 2013). Whereas, oligomers and procyanidins reach the colon, where are degraded by colonic microflora producing phenolic acids that then are absorbed into the circulation and metabolised in the liver (Neilson *et al.*, 2009; Rein *et al.*, 2013), finally to be excreted in bile and urine (Rein *et al.*, 2013). On the other hand, manufacturing processes, such as high temperatures and the storage conditions, are able to modify the polyphenolic content in cocoa (Andres-Lacueva *et al.*, 2008).

The common consumption of cocoa is as cocoa bar or as a powder mixed with milk (Roura *et al.*, 2008). However, the inclusion of cocoa flavanols within other food matrixes has not been sufficiently studied before. In this context, the aim of the present study was to assess the effect of consuming ready-to-eat meals containing 1.4 g of cocoa extract within a weight loss diet, on urinary metabolome in order to investigate the compliance of intake in overweight/obese middle-aged subjects, as well as to evaluate the presence of metabolomic changes with interest for human health.

SUBJECTS AND METHODS

Subjects

The inclusion criteria were an age between 50 and 80 years old and a body mass index (BMI) between 27.0 and 35.5 kg/m^2. The exclusion criteria to participate in the study have been previously reported elsewhere (Ibero-Baraibar *et al.*, 2014). Fifty subjects were recruited to participate in the study, but 47 of them completed it; 24 in control and 23 in cocoa groups (Ibero-Baraibar *et al.*, 2014). The study was carried out in the Metabolic Unit of the University of Navarra (Spain) from April to July 2012 and all participants signed a written informed consent before starting the intervention. The trial was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012). Moreover, Helsinki Declaration guidelines and further updates were followed. The trial was registered in www.clinicaltrials.gov with the number NCT01596309.

Study design

The study was a 4 week, randomised, placebo-controlled double-blind parallel nutritional intervention. One week before the beginning of the study volunteers were asked to exclude cocoa and cocoa containing products from their habitual diet and three days before starting the trial they were required to consume a low-polyphenol diet without energy restriction. After that, subjects were randomised to control or cocoa groups through "random between 1 and 2" function in the Microsoft Office Excel (Microsoft Iberica, Spain). Cocoa and control groups were provided with a variety of ready-to-eat meals (dishes and desserts), supplemented with cocoa extract in the case of cocoa consumers and without cocoa extract supplementation in the case of control group. Meals were supplied by Tutti Pasta S.A. Company (Navarra, Spain). Volunteers had to choose daily a dish and a dessert from the variety of dishes and desserts they had available for each week. All the volunteers were provided with the same variety of dishes and desserts, but they were not instructed to consume them in a predetermined order. On the other hand and taking into account that participants were overweight/obese, both groups were prescribed with the same hypocaloric diet (-15% energy restriction), which provided 45% of total energy value from carbohydrates, 30-33% of energy from lipids and 22-25% of energy from proteins. Moreover, volunteers were

provided with a list of forbidden polyphenol rich foods and were requested not to change their physical activity patterns during the intervention. In addition, three days prior to the end of the intervention, volunteers were prescribed to consume pre-determined types of test meals from the variety of meals provided, in order to make all the volunteers reach the endpoint of the study in comparable nutritional conditions. The day before the intervention (at the beginning and at the endpoint of the study) volunteers collected a 24 h urine specimen. Moreover, the first day and the last day of the intervention clinical and biochemical measures were taken in fasting conditions early in the morning, which showed an improvement of anthropometric and body composition measures as well as of the routine blood markers, blood pressure and inflammatory markers at the end of intervention (Ibero-Baraibar *et al.*, 2014; Ibero-Baraibar *et al.*, 2015a; Ibero-Baraibar *et al.*, 2015b). Interestingly, cocoa supplemented group reported a greater reduction of oxidized-low-density lipoprotein-cholesterol (oxLDL) levels after the intervention (Ibero-Baraibar *et al.*, 2014). Some of those results are reported in Table 1, where the principal characteristics of volunteers are presented.

The consumption of ready-to-eat meals was assessed by a notebook, where volunteers self-reported the name of the test dish and dessert they consumed daily. Volunteers reported a high adherence, as reported before (Ibero-Baraibar *et al.*, 2014). In addition, nutritional intake was analysed by a 3-day validated food-recall questionnaire, which was completed at the beginning and the end of the study. According to other studies, the data were analysed using the DIAL software (Alce Ingenieria SL, Madrid, Spain)(Perez-Cornago *et al.*, 2014) and no differences between groups concerning the macronutrient consumption were found (Ibero-Baraibar *et al.*, 2015b).

Ready-to-eat meals

Each ready-to-eat dish and dessert was supplemented with 0.7 g of cocoa extract in the case of cocoa group, while control group received the same meals without cocoa extract supplementation. Considering that the daily consumption consisted in a ready-to-eat dish and a dessert, volunteers under cocoa treatment consumed 1.4 g/d of cocoa extract, which was composed of [mean (SD)]: 140.4 (7.1) mg of theobromine, 645.3 (32.3) mg of total polyphenols as catechin, 414.3 (20.7) mg of flavanols as catechin, 153.4 (7.7) mg of epicatechin, 14.6 (0.7) mg of catechin, 99.4 (5.0) of procyanidin B2, 13.4 (0.7) mg of procyanidin B1 and 133.5 (6.7) mg of oligomeric procyanidins. Total polyphenol content was determined by Folin-Ciocalteu method and flavanoids and theobromine were analysed by HPLC (Ibero-Baraibar *et al.*, 2014). The cocoa extract was supplied by Nutrafur S.A (Murcia, Spain). Cocoa extract was added within the ready-to-eat meals before a pasteurization process (95ºC during 15-20 min). Then, the temperature was decreased to 10ºC and afterwards, ready-to-eat meals were ultra-frozen during 90 min. The store temperature was at -20ºC. In the moment of consumption, volunteers had to heat it using the microwave, where meals were heated reaching the 65ºC in the core of the meal.

Urine collection

Twenty-four hours urine specimen was collected the previous day to the beginning of the study and the day before to the endpoint of the intervention. Urine samples were collected by all the volunteers in a urine container and chilled at 4ºC. In accordance with other studies, urine samples were stored in vials of 1 mL at -80ºC until analysis (Llorach *et al.*, 2013). Considering the number of baseline and endpoint samples, a total of 94 samples were collected, 2 samples from each subject.

Sample preparation and HPLC-TOF-MS analysis

The urine samples were thawed and centrifuged for 10 min at 10.000 rpm. A 100 µL aliquot of the supernatant was diluted with 100 µL of Milli-Q water and vigorously vortexed. The solution was transferred to a vial for the subsequent analyses. Agilent Technologies 1200 liquid chromatographic system equipped with a 6220 Accurate-Mass TOF LC/MS, operated in positive electrospray ionization mode (ESI+) or negative electrospray ionization mode (ESI-), controlled by MassHunter Workstation 06.00 software (Agilent Technologies, Barcelona, Spain) was used for the analysis. The column used was a Zorbax SB-C18 (15 cm × 0.46 cm; 5 µm) from Agilent Technologies with a SB-C18 precolumn from Teknokroma (Barcelona, Spain). The mobile phase consisted of A (formic acid 0.1%) and B (acetonitrile with formic acid 0.1%). The gradient elution, 1-20% B, 0-4 min, 20-95% B 4-6 min, 95% B 9-7.5 min, 95-1% B 7.5-8 min, 1% B 8-14 min. After the analyses, the column was re-equilibrated during 5 min at 1% B. The injection volume was 15 µL and the flow rate was 0.6 mL min−1. Chromatography was performed at 40 ºC. ESI conditions were as follows: gas temperature, 350 ºC; drying gas, 10 L min−1 ; nebulizer, 45 psig; capillary voltage, 3500 V; fragmentor, 175 V; and skimmer, 65 V. The instrument was set out to acquire over the m/z range 100 –2000 with an acquisition rate of 1.03 spectra s-1.

To evaluate the quality in this metabolomic study, a procedure from Gika *et al* (2007) and Llorach *et al* (2009) with some modifications was used (Gika *et al.*, 2007; Llorach *et al.*, 2009). Two types of quality control sample (QCs) were used: i) standard mixture solution implemented of cytosine, L-carnitine hydrochloride, betaine, leucine, deoxyadenosine and deoxyguanosine at concentration of 1 mg/L. ii) pool urine was prepared by mixing equal volumes from each of the 94 samples. These samples were injected 3 times at the beginning of the run to ensure system equilibration, and then every 5 samples to further monitor stability of the analysis. Finally, samples were randomized to reduce the systematic error associated with instrumental variability. Samples were analysed in sets of 10-15 samples/day.

Data Processing and metabolite identification

Liquid Chromatography–Mass Spectrometry (LC-MS) data was analyzed using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain) to detect and align

features. Alignment used 0.15 min retention time tolerance window, and 0.002 Da mass tolerance window. For the screening of metabolites, the following filters were specified: the m/z of metabolites should appear in at least one of 94 samples. Subsequently, the detected m/z should be present in 50% of samples in only experimental group. Metabolites contributing to the discrimination among groups were then identified on the basis of their exact mass, which was compared to the registered in METLIN (https://metlin.scripps.edu/index.php) within a mass accuracy below 5 mDa, as well as based on the score given by the software , the scientific literature and the metabolic pathways reported in Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/), Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and Lipidmaps (http://www.lipidmaps.org/).

Statistical analyses

The sample size was primarily calculated for the main study considering oxLDL as the main variable (Ibero-Baraibar *et al.*, 2014). It was calculated taking into account the study by Khan *et al.* with a reduction of 14.1 U/L and an interquartile range of 16.3 U/L (Khan *et al.*, 2012).With a bilateral confidence index of 95% (α=0.05) and a statistical power of 80% (β=0.80) the sample size was estimated in 44 subjects. A possible drop-out rate of 15% was considered, establishing the final sample size in 50 subjects (Ibero-Baraibar *et al.*, 2014). Data are expressed as mean (SD) and normality of the variables was assessed using Shapiro-Wilk test. According to whether variables were normally distributed or not, comparisons between baseline and the endpoint were analysed by paired Student's t-test or Wilcoxon signed-rank test, while comparisons between both groups were performed with and independent sample t-test or Mann–Whitney U test. Spearman correlation tests were carried out to evaluate the relationship between metabolite changes when appropriate. $p < 0.05$ was considered significant. STATA version 12.0 (Stata- Corp, College Station, TX, USA) software was used to carry out statistical analysis.

Concerning metabolites, a one-way ANOVA was conducted followed by a Tukey range test, and a Benjamini–Hochberg multiple correction procedure was used to statistically compare and define statistically significant metabolites ($p < 0.01$). Finally, metabolites that satisfy a fold change cut-off of 2.0 were selected. Then, the resulting data were analyzed by principal components analysis (PCA), using Mass Profiler Professional 12.6.1 software (Agilent Technologies; Barcelona, Spain).

RESULTS AND DISCUSSION

Subjects

The principal characteristics of volunteers at baseline and the changes after the 4 weeks intervention are reported in Table 1. Control and cocoa groups were homogeneous at baseline since

no differences were found at the beginning of the intervention in any of the assessed variables (data not shown). From the 50 subjects enrolled in the intervention 47 volunteers finalized it. Overall, volunteers were men and women with a BMI of 30.3 (2.0) kg/m^2 and 57.5 (5.3) years old.

Urinary metabolomic profile

The PCA of urine samples was able to discriminate three groups: the baseline group (all the volunteers before initiate the study), the control group at endpoint and the cocoa group at endpoint in the negative ionization mode, while the PCA in the positive ionization mode shows clear discrimination between control and cocoa groups at the endpoint, while baseline and control group at the endpoint were not so clearly distinguished (Figure 1). Therefore, it seems that both, cocoa consumption and to follow a weight-loss diet influenced the excretion of metabolites in urine. This result suggest the compliance of the volunteers to the intervention and indirectly indicates the bioavailability of cocoa compounds within the ready-to-eat meals because cocoa specific putative metabolites were identified (Worley and Powers, 2013). In general, the most used food matrixes to cocoa phytochemical consumption are chocolate bars and cocoa beverages (Actis-Goretta *et al.*, 2012; Khan *et al.*, 2012; Llorach *et al.*, 2013). In addition, when cocoa beverages or chocolate bars are consumed, they usually do not suffer additional heating process by the consumers, with the exception of hot chocolate, which is added to boiling water or milk before consumption. In the present study, test products suffered an additional heating process before being consumed by the volunteers and still metabolites from cocoa consumption were detected.

Identification of putative molecules and the implication on human metabolism

In the global urine metabolite profile 1095 features in the positive ionization mode and 1154 features in the negative ionization mode were detected. Then, predetermined cut off values were applied in order to select the metabolites that were different ($p<0.01$) among the 3 groups. Thus, 26 putative metabolites were identified in positive ionization mode and 20 putative metabolites in negative ionization mode. From those, we focused on those metabolites that showed a highly significant difference between groups ($p< 0.001$) in the change from baseline to endpoint. In this situation, 8 metabolites were detected, 5 in negative ionization mode and 3 in positive mode. These metabolites are listed and grouped according their origin (Table 2). The remaining metabolites are reported in Supplemental Table 1 (negative ionization mode) and in Supplemental Table 2 (positive ionization mode). Tables contain information concerning mean intensity of each metabolite at baseline as well as in control and cocoa groups at the endpoint, retention time, detected mass, putative metabolite identification, assignation and mass difference.

Cocoa is a rich source of phytochemicals such as polyphenols and purine alkaloids (Ellam and Williamson, 2013). Moreover, manufacturing procedures as well as metabolization processes may influence the content of phenolic compounds (Andres-Lacueva *et al.*, 2008). Focusing on the

putative identification, discriminating metabolites could be grouped in metabolites derived from theobromine metabolism, food processing, flavonoid metabolism, catecholamine metabolism and endogenous metabolism (Table 2).

Cocoa and control groups significantly increased the excretion of theobromine metabolites such as 3-methylxanthine and 3-methyluric acid after 4 weeks intervention, not finding those metabolites at baseline and showing significantly higher excretion in the cocoa group (Table 2). This finding is in accordance with the results obtained by Llorach *et al* (2013) and Garcia-Aloy *et al* (2015), who found similar metabolites in human urine after cocoa intake (Llorach *et al.*, 2013; Garcia-Aloy *et al.*, 2015). Theobromine is a 3,7-dimethylated xanthine alkaloid that is also formed during caffeine metabolism (Risner, 2008). Its acute consumption stimulates heart rate and specific benefits in lipoprotein levels have been observed, specifically in high-density lipoprotein-cholesterol (HDL-c) (Neufingerl *et al.*, 2013; West *et al.*, 2014). Taking into account that theobromine is not degraded during cocoa processing and that it is highly bioavailable and well absorbed in the small intestine, it can be used as an indicator of cocoa intake (Risner, 2008; Ellam and Williamson, 2013). 3-methylxanthine metabolite is produced from theobromine degradation by P450 enzyme. Then, 3-methylxanthine is degraded by xanthine oxidase producing 3-methyluric acid (Ellam and Williamson, 2013). These metabolites demonstrates the compliance of the volunteers to the intervention, since according with scientific literature those are metabolites derived from cocoa intake (Garcia-Aloy *et al.*, 2015).

Concerning food processing compounds, L-beta-aspartyl-L-phenylalanine putative metabolite was detected in urine. The excretion of L-beta-aspartyl-L-phenylalanine was detected in both groups after 4 week intervention, but the excretion was significantly higher in cocoa consumers, not finding this metabolite at baseline. L-beta-aspartyl-L-phenylalanine is an isomer of aspartyl-phenylalanine, which was reported by García-Aloy *et al* (2015) in human urine after cocoa consumption (Garcia-Aloy *et al.*, 2015). This metabolite is a food processing derived compound and it is produced from aspartame degradation, a sweetening agent that could present in the ready-to-eat meals (Garcia-Aloy *et al.*, 2015). Garcia-Aloy *et al* (2015) also reported the presence of a similar metabolite named cyclo(aspartyl-phenylalanil) which has been identified as a constituent of roasted cocoa nibs.

Although the principal cocoa flavonoids are flavanols, cocoa also contains other types of flavonoids in minor contents (Sanchez-Rabaneda *et al.*, 2003). In this study, two putative metabolites derived from flavanones were detected: 2,5,7,3',4'-pentahydroxyflavanone-5-*O*-glucoside and 7,4'-dimethoxy-6-C-methylflavanone. Both metabolites were not detected at baseline. Thus, 2,5,7,3',4'-pentahydroxyflavanone-5-*O*-glucoside was identified only in the urine samples of cocoa group at the endpoint, while 7,4'-dimethoxy-6-C-methylflavanone was found in both groups, showing a significantly higher excretion in cocoa supplemented group (Table 2). To our knowledge,

those metabolites are derived from flavanones that could be present in cocoa extract. However, they still have not been apparently described in the scientific literature related to cocoa intake.

Interestingly, a metabolite related with catecholamine metabolism was identified, 3-methoxy-4-hydroxyphenylglycol sulphate (MHPG-sulphate). MHPG-sulphate excretion increased after the 4 weeks intervention in both intervention groups, appearing in higher amounts in the cocoa group. MHPG can be found in sulphated or in glucoronidated forms, being the sulphated form more common in humans (Peyrin *et al.*, 1985). Interestingly, there are previous studies reporting that 3-methoxy-4-hydroxyphenylglycol (MHPG) levels are decreased in brain and urine of depressive subjects and it is an indicator of brain norepinephrine metabolism (Peyrin *et al.*, 1985; Dabidy Roshan *et al.*, 2011). Interestingly, scientific literature supports that central MHPG excretion is best represented by the urinary MHPG-sulphate fraction than the total MHPG levels (Peyrin, 1990). On the other hand, there are scientific studies suggesting that weight loss and the consumption of cocoa are beneficial for psychological disorders (Sokolov *et al.*, 2013; Stapleton *et al.*, 2013). Taking into account that the excretion of MHPG-sulphate metabolite increased in both groups showing a significantly higher excretion in the cocoa group, it could be speculated that cocoa extract supplementation could has a possible additional benefit in terms of improving depressive symptoms. Indeed, ΔMHPG-sulphate was positively correlated in the whole sample with Δ3-methylxanthine and Δ 7,4'-dimethoxy-6-C-methylflavanone (Table 3). As previously described, 3-methylxanthine is a metabolite related with cocoa intake. Therefore, this outcome shows that higher excretion of MHPG-sulphate is associated with higher excretion of 3-methylxantine (Figure 2). Concerning this result, a recent investigation in mouse model has reported that the consumption of a diet rich in theobromine, polyphenols and polyunsaturated fatty acids which is composed by cocoa, hazelnuts, polyphenols, vegetable oils and flours rich in soluble fibre and known as LMN cream, modulated the catecholaminergic and cholinergic systems of mouse brain (Fernandez-Fernandez *et al.*, 2015). However, this hypothesis should be viewed with caution because MHPG-sulphate in the present study is a putative metabolite and authentic standards should have been run to confirm the retention time with the matching compound, assuring that it is MHPG-sulphate. In addition, the relation of MHPG-sulphate with other depressive markers would be necessary.

Finally, uridin monophosphate was identified as a putative endogenous molecule involved in nucleotide metabolism (Macdonald *et al.*, 1987). This metabolite was not excreted at baseline or in control group after the 4 weeks intervention (Table 2). However, it was detected after the intervention in cocoa group. To our knowledge, uridine monophosphate has not previously found in urine after cocoa consumption.

Some limitations can be mentioned. First, targeted metabolomic analysis would be interesting to confirm the putative compounds and second, a group without weight-loss diet consuming only the cocoa extract would be interesting to discriminate possible interactions between weight loss metabolite changes and the changes derived from cocoa intake.

CONCLUSION

The untargeted metabolomics analysis was able to discriminate the intervention groups supporting the compliance of the cocoa intake and indirectly suggesting the availability of cocoa compounds within the ready-to-eat meals. Furthermore, the described metabolomic changes contribute to provide more information concerning the beneficial effects of cocoa intake.

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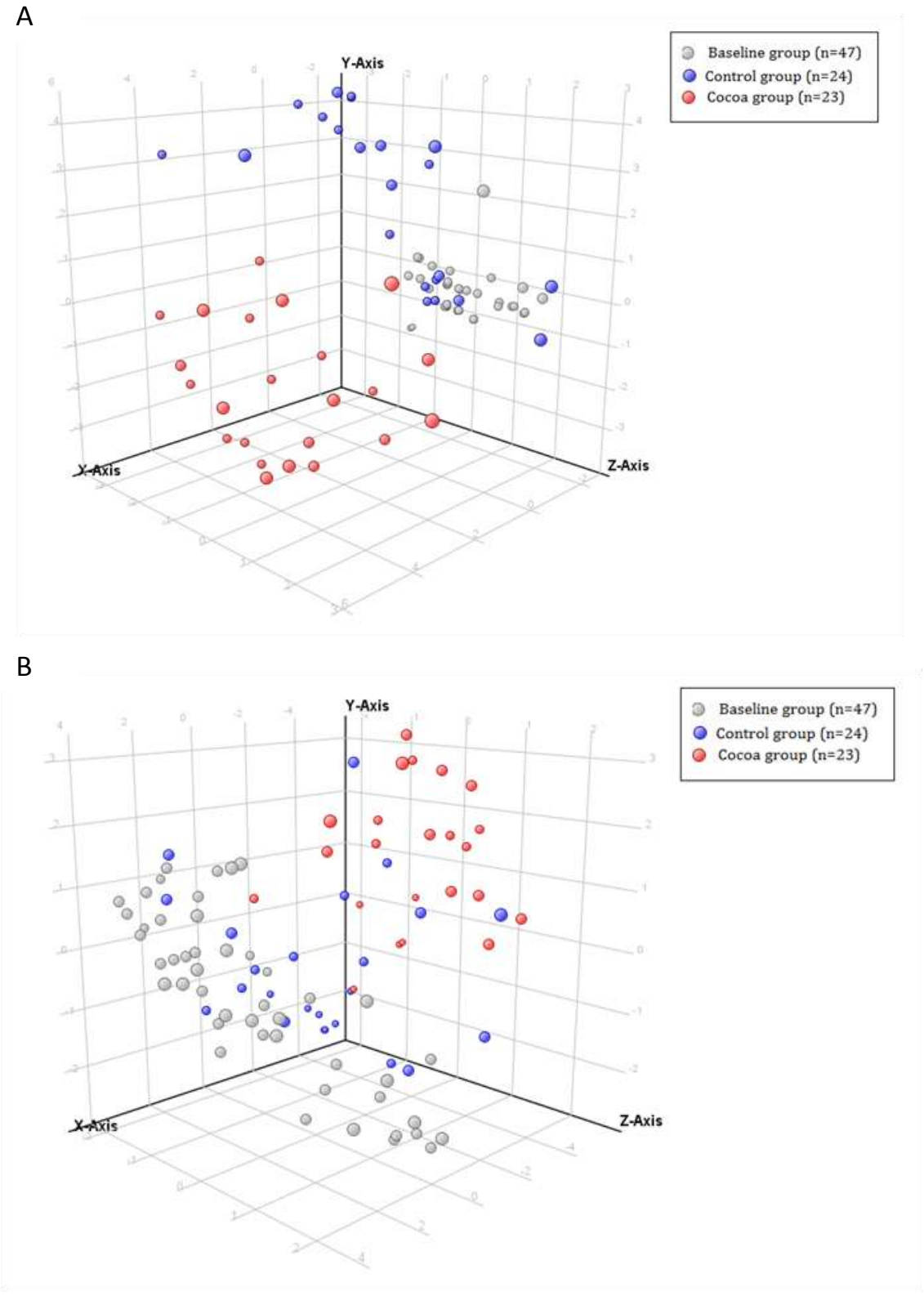

Figure 1. Principal component analysis (PCA) including baseline group (n=47), control group at the endpoint (n=24) and cocoa group at the endpoint (n=23).

A: PCA in negative ionization mode (ESI-)

B: PCA in positive ionization mode (ESI+)

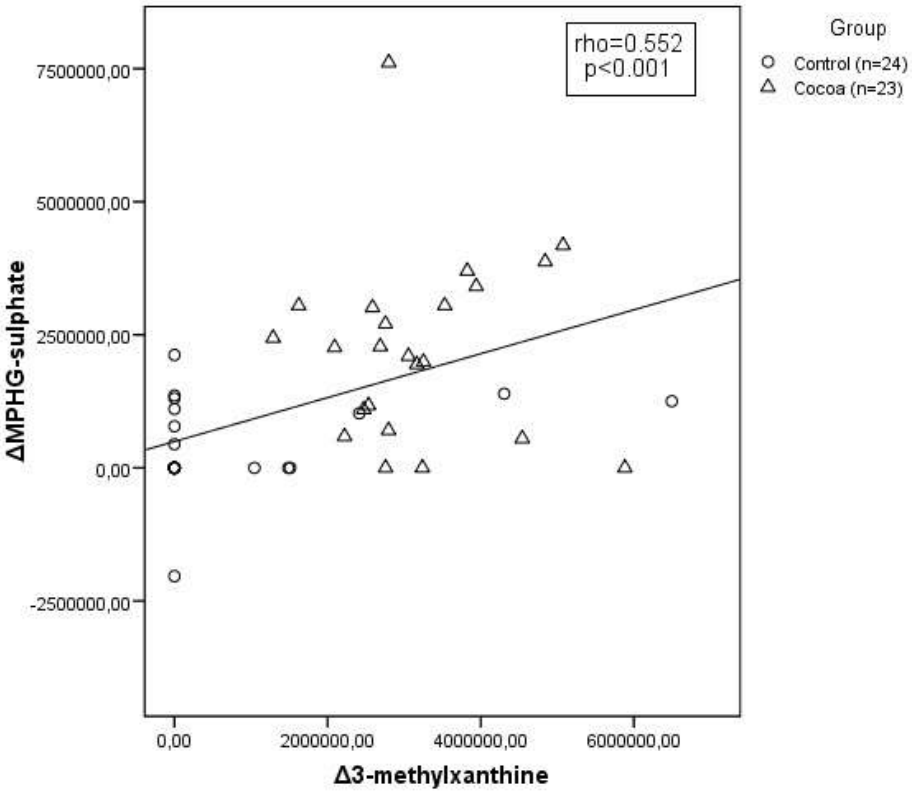

Figure 2. Association between ΔMHP-sulphate and Δ3-Methylxanthine (n=47)

Table 1. General characteristics of volunteers at baseline and changes after 4 week nutritional intervention

Variables	Baseline (n=47)	ΔControl (n=24)	ΔCocoa (n=23)	Δp
Age (years)	57.5 (5.3)	—	—	—
BMI (kg/m^2)	30.3 (2.0)	-0.9 (0.5)***	-1. (0.5)***	0.744
SBP (mmHg)	120 (19.93)	-8.7 (10.8)***	-6.7 (8.5)***	0.477
DBP (mmHg)	81 (8.43)	-7.63 (6.88)***	-5.74 (5.38)***	0.302
Total-c (mg/dL)†	240.55 (44.45)	-41.50 (24.22)***	-41.43 (34.26)***	0.509
HOMA-IR	1.75 (1.35)	-0.43 (1.38)**	-0.61 (0.90)**	0.992

Data are expressed as mean (SD). Comparisons between baseline and end of the study were analysed by paired student t-test or Wilcoxon test (***p≤0.001; **p≤0.01; *p≤0.05).

Comparisons between both groups were performed with an independent t-test or Mann-Whitney U test (Δp).

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment insulin resistance; SBP, systolic blood pressure, Total-c, total cholesterol.

†: no-normally distributed variables

Δ: endpoint– baseline

Table 2. Putative metabolites different between baseline, control and cocoa groups.

Baseline	Control 4 weeks	Cocoa 4 weeks	Δp (Δcontrol vs. Δcocoa)	RT (min)	Detected mass (m/z)	Putative metabolites	Assignation	Mass difference (mDa)
Theobromine metabolism								
nd	5.29	21.51	<0.001	6.740	166.0508	3-Methylxanthine	[M+H]+	-1.7
nd	1.72	13.10	<0.001	6.334	182.0450	3-Methyluric acid	[M+H]+	-1.0
Food processing								
nd	1.72	11.04	<0.001	7.969	280.1045	L-beta-aspartyl-L-phenylalanine	$[M+H]^+$	1.4
Flavonoid metabolism								
nd	nd	10.09	<0.001	8.594	466.1151	2,5,7,3',4'-Pentahydroxyflavanone 5-*O*-glucoside	[M-H]-	-4.0
nd	3.30	14.99	<0.001	7.995	298.1186	7,4'-Dimethoxy-6-C-methylflavanone	$[M-H]^{\cdot}[-H_2O]$	1.9
Catecholamine metabolism								
0.45	7.53	18.28	<0.001	8.694	264.0332	MHPG-sulphate	$[M-H]^{\cdot}[-H_2O]$	-2.8
Endogenous metabolism								
nd	nd	13.21	<0.001	8.666	324.0355	Uridine monophosphate	[M+HCOO]-	0.4
nd	nd	16.85	<0.001	8.696	324.0347		[M+CH3COO]-	1.2

p<0.05 was considered as significant. Δ= endopoint– baseline. Comparisons between both groups were performed with by U Mann–Whitney test.
The data in baseline, control and cocoa columns refers to mean intensity of metabolites and are presented as log 2.
Abbreviations: nd, no detected; RT, retention time; MHPG-sulphate, 3-Methoxy-4-hydroxyphenylglycol sulphate. The following comparisons were p<0.05: baseline vs. control, baseline vs. cocoa and control vs. cocoa

Table 3. Correlation analysis between changes in MHPG-sulphate and metabolites related with cocoa intake

Metabolites	Δ MHPG-sulphate	
	rho	p
Δ 3-Methylxanthine	0.552	<0.001
Δ 3-Methyluric acid	0.265	0.072
Δ 2,5,7,3',4'-Pentahydroxyflavanone 5-*O*-glucoside	0.202	0.173
Δ 7,4'-Dimethoxy-6-C-methylflavanone	0.447	0.002

rho: Spearman correlation coefficient
p<0.05 was considered significant
Δ: change (endpoint– baseline)
Abbreviations: MHPG-sulphate, 3-Methoxy-4-hydroxyphenylglycol-sulphate

Supplementary tables

-CHAPTER 5-

Supplemental table 1. Other putative metabolites in negative ionization mode (ESI-)

Baseline	Control 4 weeks	Cocoa 4 weeks	RT (min)	Detected mass (m/z)	Metabolite putative identification	Assignation	Mass difference (mDa)
nd	10.15	7.75	4.086	247.0536	Phenylephrine 3-O-sulfate	$[M-H]^-$	-2.2
0.87	5.00	10.68	6.131	198.077	5-Acetylamino-6-amino-3-methyluracil	$[M-H]^-$	-1.7
13.25	5.71	8.89	6.367	233.0369	Dopamine 4-O-sulfate	$[M-H]^-$	-1.1
			6.367	233.0369	Dopamine 3-O-sulfate	$[M-H]^-$	-1.1
3.96	6.90	13.06	6.944	166.0511	7-Methylxanthine	$[M-H]^-$	-2.0
			6.944	166.0511	3-Methylxanthine	$[M-H]^-$	-2.0
1.21	11.55	8.55	7.971	262.0166	2,4-dichlorophenoxybutyric acid. methyl ester	$[M-H]^-$	-0.2
			7.971	234.0221	phenylglycol 3-O-sulfate	$[M+HCOO]^-[-H_2O]$	-2.3
			7.971	201.9959	4-Sulfobenzoate	$[M+CH_3COO]^-$	-2.3
2.20	10.66	6.96	8.267	376.1393	Asp Trp Gly	$[M-H]^-$	-1.0
			8.267	376.1393	Riboflavin	$[M-H]^-$	-1.0
			8.267	376.1393	Asp Ile Trp	$[M-H]^-$	-1.0

			8.267	376.1393	Trp Asp Gly	$[M-H]^-$	-1.0
0.93	1.64	14.92	8.312	156.0441	(2S,3S)-2,3-Dihydro-2,3-dihydroxybenzoate	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	3-Methyl-cis.cis-hexadienedioate	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	3-Methylmuconolactone	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	4-Methylmuconolactone	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	1,6-Dihydroxycyclohexa-2,4-diene-1-carboxylate	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	2-Hydroxy-6-keto-2,4-heptadienoate	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	(1R,6S)-1.6-Di,hydroxycyclohexa-2,4-diene-1-carboxylate	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	2-Hydroxy-5-methyl-cis,cis-muconic semialdehyde	$[M-H]^-[-H_2O]$	-1.8
			8.312	156.0441	4-Methyl-3-oxoadipate-enol-lactone	$[M-H]^-[-H_2O]$	-1.8
nd	2.50	16.16	8.317	217.9911	5-Sulfosalicylate	$[M-H]^-$	-2.6
2.09	6.59	11.50	8.318	390.1084	Ser-Tyr-OH	$[M-H]^-$	-2.1
			8.318	390.1084	Tyr-Ser-OH	$[M-H]^-$	-2.1
			8.318	390.1084	Dopaxanthin	$[M-H]^-$	-2.1
			8.318	390.1084	3,5-Pyridinedicarboxylic acid. 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-carboxymethyl methyl est	$[M-H]^-$	-2.1

			8.318	408.1189	Glabrescin	$[M-H]^-[-H_2O]$	2.0
			8.318	408.1189	Dehydroamorphigenin	$[M-H]^-[-H_2O]$	2.0
			8.318	408.1189	Ferrugone	$[M-H]^-[-H_2O]$	2.0
			8.318	408.1189	5-Methoxydurmillone	$[M-H]^-[-H_2O]$	2.0
6.02	15.16	8.45	8.352	331.0383	C13H9N5O4S	$[M-H]^-$	-0.8
			8.6	144.0433	(E)-2-Methylglutaconic acid	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	3-hexenedioic acid	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	2,3-Dimethylmaleate	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	(E)-3-methylglutaconic acid	$[M-H]^-[-H_2O]$	-1.06
4.58	11.76	5.08	8.6	144.0433	(E)-hex-2-enedioic acid	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	1.5-Anhydro-4-deoxy-D-glycero-hex-1-en-3-ulose	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	1.5-Anhydro-4-deoxy-D-glycero-hex-3-en-2-ulos	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	Allylmalonic acid	$[M-H]^-[-H_2O]$	-1.0
			8.6	144.0433	Methylitaconate	$[M-H]^-[-H_2O]$	-1.0
0.40	9.94	3.36	8.681	274.0168	C6H10O12	$[M-H]^-$	0.4

0.41	10.09	4.26	8.685	194.0588	Scytalone	$[M-H]^-$	-0.9
			8.685	194.0588	Isoferulic acid	$[M-H]^-$	-0.9
			8.685	194.0588	Monoethyl phthalate	$[M-H]^-$	-0.9
			8.685	194.0588	5-Hydroxyconiferaldehyde	$[M-H]^-$	-0.9
13.74	6.69	6.07	8.723	477.1227	Isorhamnetin 7-alpha-D-Glucosamine	$[M-H]^-$	4.4
nd	0.84	11.43	8.107	434.1462	Asp Asp Trp	$[M-H]^-[-H_2O]$	-2.4
			8.107	434.1462	Trp Asp Asp	$[M-H]^-[-H_2O]$	-2.4
			8.107	434.1462	Asp Trp Asp	$[M-H]^-[-H_2O]$	-2.4
			8.107	388.1407	N5-Dinitrophenyl-L-ornithine methyl ester	$[M+HCOO]^-[-H_2O]$	-2.4
			8.107	434.1461	HoPhe-Phe-OH	$[M-H]^-[-H_2O]$	1.7
			8.107	434.1461	Phe-HoPhe-OH	$[M-H]^-[-H_2O]$	1.7

Abbreviations: nd, no detected; RT, retention time.
The following comparisons were $p<0.01$: baseline vs. control, baseline vs. cocoa and control vs. cocoa.
The data in baseline, control and cocoa columns refers to mean intensity of metabolites and are presented as log 2.

Supplemental table 2. Other putative metabolites in positive ionization mode (ESI+)

Baseline	Control 4 weeks	Cocoa 4 weeks	RT (min)	Detected mass (m/z)	Metabolite putative identification	Assignation	Mass difference (mDa)
18.04	9.78	7.23	2.178	141.9919	Bis(2-chloroethyl)ether	$[M+H]^+$	3.3
11.38	3.31	2.53	2.466	141.0906	L-Histidinol	$[M+H]^+[-H_2O]$	-0.4
16.57	7.74	5.29	2.651	128.0594	4-Amino-4-cyanobutanoic acid	$[M+H]^+$	-0.8
			2.651	128.0594	L-γ-Cyano-γ-aminobutyric acid	$[M+H]^+$	-0.8
			2.651	128.0594	2-Amino-4-cyanobutanoic acid	$[M+H]^+$	-0.8
			2.651	128.0594	Dihydrothymine	$[M+H]^+$	-0.8
20.01	9.80	10.10	2.855	170.0706	2,3,4-Trihydroxybenzylhydrazide	$[M+H]^+$	-1.5
			2.855	170.0706	N-Nitrosoguvacoline	$[M+H]^+$	-1.5
			2.855	170.0706	3-Hydroxybiphenyl	$[M+H]^+$	2.6
			2.855	170.0706	4-Hydroxybiphenyl	$[M+H]^+$	2.6
			2.855	188.0812	N-Acetylglutamine	$[M+H]^+[-H_2O]$	-1.5
			2.855	188.0812	L-glycyl-L-hydroxyproline	$[M+H]^+[-H_2O]$	-1.5

			2.855	188.0811	5,8,11-Dodecatriynoic acid	$[M+H]^+[-H_2O]$	2.6
17.11	8.95	6.37	3.954	139.0278	6-Hydroxynicotinic acid	$[M+NH_4]^+[-H_2O]$	-0.9
13.79	6.36	3.74	4.199	183.1255	Acetyltropine	$[M+Na]^+[-H_2O]$	0.4
			4.199	183.1255	Acetylpseudotropine	$[M+Na]^+[-H_2O]$	0.4
			4.199	165.1149	Hordenine	$[M+Na]^+$	-0.5
			4.199	165.1149	Anatoxin a	$[M+Na]^+$	-0.5
			4.199	165.1149	Ephedrine	$[M+Na]^+$	-0.5
			4.199	165.1149	Pseudoephedrine	$[M+Na]^+$	-0.5
			4.199	165.1149	Racephedrine	$[M+Na]^+$	-0.5
			4.199	188.0810	N-Acetylglutamine	$[M+NH_4]^+[-H_2O]$	-1.3
			4.199	188.0810	L-glycyl-L-hydroxyproline	$[M+NH_4]^+[-H_2O]$	-1.3
			4.199	170.0704	2,3,4-Trihydroxybenzylhydrazide	$[M+NH_4]^+$	-1.3
12.68	6.14	3.69	5.881	201.1125	C8H15N3O3	$[M+H]^+$	-1.2
			5.881	219.1230	Meperidinic acid	$[M+H]^+[-H_2O]$	2.9
			5.881	219.1230	Ritalinic acid	$[M+H]^+[-H_2O]$	2.9

			5.881	219.1230	Darlingine	$[M+H]^+[-H_2O]$	2.9
12.33	5.09	6.91	5.904	203.1280	Lys Gly	$[M+H]^+$	-1.0
			5.904	203.1280	Gly Lys	$[M+H]^+$	-1.0
13.40	8.83	5.25	5.915	120.0569	4-Hydroxystyrene	$[M+H]^+$	0.6
			5.915	120.0569	Phenylacetaldehyde	$[M+H]^+$	0.6
			5.915	120.0569	Acetophenone	$[M+H]^+$	0.6
			5.915	138.0675	3-Ethylcatechol	$[M+H]^+[-H_2O]$	0.6
12.53	4.43	4.41	5.934	169.0860	Nalpha-Methylhistidine	$[M+H]^+$	-0.9
			5.934	169.0860	1-Methylhistidine	$[M+H]^+$	-0.9
11.39	2.60	3.54	6.170	522.0639	C14H22N2O17S	$[M+H]^+$	0.0
13.46	6.99	2.63	7.538	232.1459	Alantolactone	$[M+Na]^+[-H_2O]$	0.4
			7.538	232.1459	Isoalantolactone	$[M+Na]^+[-H_2O]$	0.4
			7.538	232.1459	Costunolide	$[M+Na]^+[-H_2O]$	0.4
			7.538	232.1459	Frullanolide	$[M+Na]^+[-H_2O]$	0.4
			7.538	232.1459	Pinguisone	$[M+Na]^+[-H_2O]$	0.4

			7.818	295.1441	C18H25NO5S	$[M+H]^+$	1.2
			7.818	295.1439	C15H21NO5	$[M+H]^+$	-1.9
13.29	6.83	1.78	7.944	298.1066	2-Phenylethanol glucuronide	$[M+H]^+[-H_2O]$	-1.3
			8.075	159.0686	N-Hydroxy-1-aminonaphthalene	$[M+NH_4]^+[-H_2O]$	-0.2
			8.075	159.0686	Echinopsine	$[M+NH_4]^+[-H_2O]$	-0.2
			8.075	159.0686	3-Methyl-quinolin-2-ol	$[M+NH_4]^+[-H_2O]$	-0.2
16.61	6.61	3.90	8.423	137.0488	p-Aminobenzoic acid	$[M+H]^+[-H_2O]$	-1.1
17.72	9.82	8.39	8.738	135.0696	2-Phenylacetamide	$[M+H]^+$	-1.2
			8.738	135.0696	Vanillylamine	$[M+H]^+$	-1.2
15.10	9.03	5.54	8.993	325.1922	C14H31NO5S	$[M+H]^+[-H_2O]$	0.1
11.49	4.49	4.70	2.162	157.0136	Potassium oxonate	$[M+K]^+$	-1.2
12.70	4.51	7.27	5.174	147.0540	L-Glutamate	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	N-Acetylserine	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	O-Acetylserine	$[M+H]^+[-H_2O]$	-0.8

			5.174	147.0540	Glutamate	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	N-Methyl-D-aspartic acid	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	2-Oxo-4-hydroxy-5-aminovalerate	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	L-4-Hydroxyglutamate semialdehyde	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	Isoglutamate	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	D-Glutamate	$[M+H]^+[-H_2O]$	-0.8
			5.174	147.0540	N-(Carboxymethyl)-D-alanine	$[M+H]^+[-H_2O]$	-0.8
2.56	8.79	14.90	8.126	278.1181	3-(7'-Methylthio)heptylmalic acid	$[M+NH_4]^+$	0.7
			8.126	278.1181	2-(7'-Methylthio)heptylmalic acid	$[M+NH_4]^+$	0.7
nd	11.88	7.59	2.626	159.0907	Valerylglycine	$[M+H]^+$	-1.2
			2.626	159.0907	Isovalerylglycine	$[M+H]^+$	-1.2
			2.626	159.0907	2-Methylbutyrylglycine	$[M+H]^+$	-1.2
			2.626	159.0907	Calystegin A3	$[M+H]^+$	-1.2
			2.626	159.0907	Acetyl-DL-Valine	$[M+H]^+$	-1.2
			2.626	159.0907	N-Acetyl-DL-Valine	$[M+H]^+$	-1.2

			2.626	159.0907	5-Acetamidopentanoate	$[M+H]^+$	-1.2
			2.626	159.0907	5-Acetamidovalerate	$[M+H]^+$	-1.2
10.35	4.31	nd	6.690	165.0660	7-Methylguanine	$[M+H]^+$	-0.9
			6.690	165.0660	1-Methylguanine	$[M+H]^+$	-0.9
			6.690	165.0660	3-Methylguanine	$[M+H]^+$	-0.9
			6.690	165.0660	6-O-Methylguanine	$[M+H]^+$	-0.9
			6.690	165.0660	N2-Methylguanine	$[M+H]^+$	-0.9
12.16	1.77	2.76	2.161	257.9680	C10H10S4	$[M+H]^+$	-1.5
			2.161	257.9680	C6H10O5S3	$[M+H]^+$	-1.0
10.74	0.89	nd	5.778	133.0748	1,4-Dideoxy-1,4-Imino-D-Arabinitol	$[M+H]^+[-H_2O]$	-0.9
			5.778	133.0748	3-Hydroxynorvaline	$[M+H]^+[-H_2O]$	-0.9
			5.778	133.0748	L-O-Methylthreonine	$[M+H]^+[-H_2O]$	-0.9
			5.778	116.0482	Alpha-ketoisovaleric acid	$[M+NH_4]^+[-H_2O]$	-0.9
			5.778	116.0482	2-Methyl-3-ketovaleric acid	$[M+NH_4]^+[-H_2O]$	-0.9
			5.778	116.0482	Methyl acetoacetate	$[M+NH_4]^+[-H_2O]$	-0.9

5.778	116.0482	5-Oxopentanoate	$[M+NH_4]^+[-H_2O]$	-0.9
5.778	116.0482	2-Ketovaleric acid	$[M+NH_4]^+[-H_2O]$	-0.9
5.778	116.0482	Levulinic acid	$[M+NH_4]^+[-H_2O]$	-0.9
5.778	116.0482	3-Ketovaleric acid	$[M+NH_4]^+[-H_2O]$	-0.9

Abbreviations: nd, no detected; RT, retention time.
The following comparisons were $p<0.01$: baseline vs. control, baseline vs. cocoa and control vs. cocoa.
The data in baseline, control and cocoa columns refers to mean intensity of metabolites and are presented as log 2.

CHAPTER 6

Increases in plasma 25(OH)D levels are related to improvements in body composition and blood pressure in middle-aged subjects after a weight loss intervention: Longitudinal study

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Original article

Increases in plasma 25(OH)D levels are related to improvements in body composition and blood pressure in middle-aged subjects after a weight loss intervention: Longitudinal study

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SUMMARY

Background & aims: The aim of this study is to further clarify the role of plasma 25(OH)D concentration after a weight-lowering nutritional intervention on body composition, blood pressure and inflammatory biomarkers in overweight/obese middle-aged subjects.
Methods: This longitudinal research encompassed a total of 50 subjects [57.26 (5.24) year], who were under a 15% energy restricted diet for 4 weeks. Anthropometric and body composition variables, blood routine, inflammatory markers as well as 25(OH)D were analysed.
Results: Circulating 25(OH)D levels [12.13(±17.61%)] increased while anthropometric, body composition, routine blood markers as well as the concentration of TNF-α, C-reactive protein and Lp-PLA2 were significantly reduced after the intervention. Multiple linear regression analyses evidenced that Δ25(OH)D increase was linked to the decrease in weight, adiposity, SBP and IL-6 levels. Moreover, a relationship was found between Δ25(OH)D, Δfat mass ($r = -0.405$; $p = 0.007$), ΔSBP ($r = -0.355$; $p = 0.021$) and ΔIL-6 ($r = -0.386$; $p = 0.014$). On the other hand, a higher increase in 25(OH)D was accompanied by reductions in weight, BMI, SBP, IL-6 and an increase in bone mineral concentration ($p < 0.05$). Interestingly, higher levels of 25(OH)D at the endpoint, showed a significantly higher decrease in weight, BMI and total fat mass.
Conclusions: The increase in plasma 25(OH)D level is linked with the decrease in SBP and adiposity in middle-aged subjects after a weight-loss intervention. Therefore, 25(OH)D assessment is a potential marker to be accounted in metabolic measures related to blood pressure, adiposity and inflammation in obesity management.
Trial registration: www.clinicaltrials.gov (NCT01596309).

1. Introduction

Obesity is characterised by an excess body fat mass accumulation and usually this abnormal adipose tissue deposition is linked with different comorbidities and metabolic alterations [1]. Furthermore, adipose tissue is an active endocrine organ through which pro-inflammatory molecules, known as adipokines, are released [2]. Among those compounds, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as C-reactive protein and Lipoprotein-associated phospholipase A2 (Lp-PLA2), are responsible of triggering processes and diseases related to inflammation [3].

Abbreviations: AI, Atherogenic index; BMC, Bone mineral concentration; BMI, Body mass index; C-RP, C-reactive protein; DBP, Diastolic blood pressure; HDL-c, High-density lipoprotein-cholesterol; HOMA-IR, HomoeostasisHomoeostasis model assessment insulin resistance; IL-6, Interleukin-6; LDL-c, Low-density lipoprotein-cholesterol; Lp-PLA2, Lipoprotein-associated phospholipase A2; SBP, Systolic blood pressure; TFM1, Truncal fat mass; TFM2, Total fat mass; TG, Triglyceride; TNF-α, Tumor necrosis factor-alpha; 25(OH)D, 25-hydroxyvitamin-D.

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Vitamin D is found in circulating blood as 25-hydroxyvitamind D [25(OH)D], and it is considered as an estimate of vitamin D status [4]. Vitamin D is involved in multiple processes of human physiology, such as bone turnover and calcium homoeostasis, cardiovascular regulation, mental health, muscle and brain functions [5–7]. Epidemiological studies have shown that a high body mass index (BMI), increased weight and adipose tissue enhancement, are associated with low circulating vitamin D levels [5,8]. However, the direction and causality are unclear. Some researchers have suggested that obesity is the result of cold and dark environments, where vitamin D synthesis could be seen decreased due to the reduced sun exposure, promoting fat mass accumulation and increasing BMI [9]. Other investigators have suggested that vitamin D could be sequestered by adipose tissue, which has a higher storage capacity in obesity [10] Furthermore, there is evidence that low 25(OH)D levels are associated with higher risk of suffering from cardiometabolic diseases [11]. A recent meta-analysis involving 42,000 subjects performed by Vimaleswaran et al. (2013) reported that genetic variants associated with higher BMI were related to a lower 25(OH)D concentration, indicating that obesity involves a low vitamin D circulating levels, but no vice versa [8]. In contrast, Ortega et al. (2008) reported that overweight/obese women who started a 20% energy restriction diet with higher levels than 50 nmol/L of 25(OH)D, presented a higher reduction of body fat mass 2 weeks after the intervention [12].

Middle-aged and elderly populations are at risk of suffering from 25(OH)D deficiency due to a low dietary intake, sun exposure and synthesizing decrease [13]. Moreover, within these age groups the risk of suffering from commorbidities, high body weight and excess of fat mass is higher [14].

The actual controversy in the causality direction and the scarcity of studies about the effects of 25(OH)D changes [Δ25(OH)D] over anthropometric, body composition, blood pressure, blood parameters and inflammatory status after weight-loss suggests that more investigations to explain the relation between vitamin D and those aspects are needed. In this sense, the objective of this study was to analyse the involvement of 25(OH)D changes on body composition, blood pressure, routine blood parameters and inflammation markers, in overweight/obese middle-aged subjects after a weight-loss intervention.

2. Materials and methods

2.1. Study design

This study is based on an intervention trial of 4 weeks, designed as a double-blinded, randomised controlled trial to evaluate the effect of the daily consumption of ready-to-eat meals supplemented with 1.4 g of cocoa extract, within a hypocaloric diet, in middle-aged overweight/obese subjects [15]. Half of the sample was randomly assigned to control group and the other half to cocoa group (1.4 g of cocoa extract) following both groups a 15% energy restricted diet. Energy intake was adjusted for resting metabolic rate. Harris–Benedict equation was used to calculate the corresponding individualised physical activity factor [16]. The macronutrient distribution of the diet in both groups was 45% of total caloric value (TCV) from carbohydrates, <30% TCV from lipids and 22–25% TCV from proteins and it was controlled by a trained dietician. Volunteers were instructed to keep up their habitual physical activity during the intervention. The DIAL software (Alce Ingenieria S.L, Madrid, Spain) was used to assess the nutrient intake by the three day validated food record questionnaire, at the beginning and at the end of the study period, including vitamin D intake. The intervention was carried out in the spring, being all the participants assessed during the same season. The study was performed in the Metabolic Unit of the University of Navarra (Spain), following the CONSORT 2010 guidelines. The study was approved by the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and followed the Helsinki Declaration guidelines. It was registered in www.clinicaltrials.gov: (NCT01596309). The design and more details about this study have been reported elsewhere [15].

2.2. Subjects

The study population encompassed 23 men and 27 women [57.26 (5.24) year old and body mass index (BMI) of 30.59 (2.33 kg/m^2)]. Written informed consent was obtained from all the enrolled participants before the beginning of the study. Volunteers were screened according to the inclusion and exclusion criteria previously detailed [15]. Thus, subjects should be between 50 and 80 years, BMI between 27.0 and 35.5 kg/m^2 and maintaining a stable weight (<5% of weight variation) three months prior to the intervention. Subjects suffering from diseases, consuming antioxidants, dietary supplements, following some pharmacological treatment, reporting inability to perform the follow-up or being smokers were excluded.

2.3. Anthropometry, body composition and blood pressure

Anthropometric variables (weight, waist and hip circumference), body composition (total fat mass, truncal fat mass and bone mineral concentration-BMC) and blood pressure (systolic and diastolic blood pressure) measurements were carried out at baseline and at the end of the dietary intervention following validated procedures as described elsewhere [17]. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were taken in triplicate with an automatic device (Intelli Sense. M6, OMRON Healthcare, Hoofddorp, Netherlands) to use the average value obtained from the last two measurements.

2.4. Blood biochemical analysis

Blood samples were collected between 8:00 and 9:30 am, after a 10 h overnight fasting period at baseline and at the end of the intervention with EDTA and CLOT prepared tubes (Vacutainer). After the extraction, samples were centrifuged to obtain plasma and serum aliquots, and stored at −80 °C until running the analysis. Plasma glucose, triglycerides (TG) and homocysteine were measured by colourimetry in an auto-analyser Pentra C200 (Horiba Medical, Montpellier, France). Plasma insulin (Mercodia, Upssala, Sweden), TNF-α, IL-6, Lp-PLA2 (R&D Systems, Minneapolis, USA) and C-reactive protein (Demeditec Diagnostic) were quantified with specific ELISA kits in a Triturus auto-analyser (Grifols, Barcelona, Spain). 25(OH)D levels were analysed by an external certified laboratory (Megalab S.A, Madrid, Spain) using a radioimmunoassay technique. LDL-c levels were calculated applying the Friedewald equation: LDL-c = TC-HDL-c − VLDL and HOMA index was used to estimate insulin resistance: HOMA-IR = [glucose (mmol/L) × insulin (μU/ml)]/22.5. Atherogenic index was calculated as follows: total cholesterol/HDL-c.

2.5. Statistical analysis

The sample size was estimated in 50 subjects as was previously reported [15], and was based on the expected differences in means and dispersion criteria with a p value < 0.05 and a statistical power of 80%. The distribution of the variables was assessed using the Shapiro–Wilk test and data are expressed as mean (SD). According to the normality of the variables, comparisons between baseline and the endpoint of the intervention were studied by paired

Student's *t*-test or Wilcoxon signed-rank test and the analysis between both groups was performed by independent sample *t*-test or Mann–Whitney *U* test.

Considering that we did not observe differences between dietary groups either in the dietary intake (Table 1) nor for the rest of the variables reported in this work (Table 2), we have analysed the data merging control and cocoa groups, considering that this approach is valid as has been demonstrated by Concato J (2000) [18]. Moreover, we performed a multiple regression analysis adjusted for sex, age, baseline value of each dependent variable and Δtotal fat mass (%) to be sure that the intervention group (control/cocoa) did not have an effect on the variables presented in this work (Table 3). The group variable was also included in the multiple adjusted models and in the ANCOVA analysis. Therefore, we treated the participants as a whole group, analysing as a unique longitudinal observational design.

Weight, BMI, total fat mass, truncal fat mass, SBP and ΔIL-6 were selected as representative of anthropometry, body composition, blood pressure and inflammatory status. Multiple linear regression analyses were fitted to assess the effect of Δ25(OH)D, as an independent variable, over changes on weight, total fat mass, truncal fat mass, SBP and blood IL-6 levels, as dependent variables, adjusting for age, sex, Δtotal fat mass (kg), the baseline value of each dependent variable and the intervention group (control/cocoa) differently combined. Adjusted Pearson correlation tests were applied to evaluate the relationship between ΔSBP, Δtotal fat mass and ΔIL-6 with Δ25(OH)D, adjusted for age, sex, Δtotal fat mass and baseline values of each measurement. Finally, ANCOVA analyses were performed considering the median of the increase (1.6 ng/mL), as well as the median of baseline (17.30 ng/mL) and endpoint (19.19 ng/mL) 25(OH)D plasma levels as cut off points including sex, age, intervention group (control/cocoa), baseline value of each measurement and Δtotal fat mass (%) as a covariates. $p < 0.05$ was considered as significant and data were statistically analysed using SPSS 15.0 software for Windows (SPSS Inc, Chicago, USA). Correlation figure was performed using STATA 12.0 (StatacCorp) software.

3. Results

3.1. Participants and adherence to the diet

From 50 subjects who participated in the study 47 completed the trial, 24 subjects in the control group and 23 subjects in the cocoa group. Dropouts during the study were due to loss to follow-up or personal reasons as were shown in the flow-chart previously reported [15]. The dietary records reveals no differences observed in macronutrient and energy consumption between groups after 4 weeks of intervention (Table 1). Concerning the vitamin D intake, the collective sample ($n = 47$) consumed a mean of 3.23 (4.74) μg/day prior to the beginning of the study and 4.16 (4.10) μg/day at the endpoint, finding no significant differences ($p = 0.119$) between the baseline and the endpoint of the intervention. Moreover, no significant differences were observed between control and cocoa groups (Table 1).

Table 1
Comparison of real dietary intake between control ($n = 24$) and cocoa ($n = 23$) groups.

Variables	Control group ($n = 24$)	Cocoa group ($n = 23$)	p
Energy (kcal/day)	1512.5 (305.3)	1535.6 (237.8)	0.775
Carbohydrates (g/day)	160.9 (34.6)	167.4 (27.8)	0.484
Protein (g/day)	82.6 (17.7)	83.1 (12.7)	0.923
Total fats (g/day)	58.5 (13.5)	57.9 (13.3)	0.893
Vitamin D (μg/day)	3.56 (4.15)	4.79 (4.04)	0.260

Data are expressed as mean (SD). Data were assessed by independent *t* test. $p < 0.05$ was considered as significant.

3.2. Anthropometry, body composition and blood pressure

Anthropometry (weight, BMI, waist/hip circumference), body composition (total fat mass, truncal fat mass and BMC) and blood pressure (SBP and DBP) significantly decreased after 4 weeks of intervention in control and cocoa groups, no observing significant differences between groups (Table 2). More details are reported in our previous publication, where was demonstrated that 15% energy restriction diet is effective at improving anthropometric, body composition and blood pressure variables in both groups [15].

3.3. Blood markers: routine blood and inflammatory variables

After 4 weeks of the intervention, HOMA index, LDL-c, atherogenic index and TG significantly decreased in both groups, while glucose and homocysteine levels remained unchanged (Table 2). Concerning inflammatory biomarkers, TNF-α decreased only in cocoa supplemented group ($p = 0.034$) while C-reactive protein decreased in the control group ($p = 0.028$). Lp-PLA2 significantly decreased in control and cocoa groups, whereas IL-6 did not present any changes. On the other hand, 25(OH)D significantly increased after the nutritional intervention in the control ($p < 0.001$) and cocoa ($p = 0.014$) groups (Table 2).

As no significant differences were observed between interventions groups in any of the assessed variables (Table 2), both groups were merged and analysed together as a unique observational sample in the rest of the analyses. However, to avoid confounding effects, regression analyses were carried out to assess the effect of group (control/cocoa) over the change of anthropometry, body composition, blood pressure, blood biochemical parameters, biomarkers of inflammation and 25(OH)D, adjusted for sex, age, baseline value of each measurement and Δfat mass% (Table 3). Thus, anthropometric, body composition and blood pressure decreased significantly in the collective sample (Table 4). In addition, except glucose, homocysteine and IL-6, the rest of the blood markers also decreased and 25(OH)D increased (Table 4). The rest of the analyses were adjusted for the intervention group to avoid confounding effects.

3.4. Multiple linear regression and correlation analysis

Two models of multiple regression analyses were performed to assess the relationship of Δ25(OH)D levels with Δweight, Δtotal fat mass, Δtruncal fat mass, ΔSBP and ΔIL-6 markers. As model 1 shows in Table 5, Δ25(OH)D evidenced a significant influence on Δweight ($p = 0.010$), Δtotal fat mass (kg) ($p = 0.007$), Δtruncal fat mass (kg) ($p = 0.019$), ΔSBP ($p = 0.032$) and ΔIL-6 ($p = 0.010$). In model 2, Δ25(OH)D evidenced a relationship only with ΔSBP ($p = 0.025$) and ΔIL-6 ($p = 0.015$). Moreover, as is shown in Fig. 1, adjusted Pearson correlation models showed that Δ25(OH)D was negatively associated with Δtotal fat mass ($r = -0.405$; $p = 0.007$), ΔIL-6 ($r = -0.386$; $p = 0.014$) and ΔSBP ($r = -0.355$; $p = 0.021$).

3.5. Comparison according to the increase, baseline and endpoint 25(OH)D level categorisation

Significant differences were observed in weight, BMI, total fat mass (kg), BMC, SBP and IL-6 between subjects below and above the median of the increment in 25(OH)D. Subjects whose 25(OH)D levels increased by more than 1.6 ng/mL presented a higher decrease in weight ($p = 0.026$), BMI ($p = 0.045$), total fat mass

Table 2
Comparison of anthropometric, body composition, blood pressure, routine blood biochemical parameters, inflammatory biomarkers and 25(OH)D in control ($n = 24$) and cocoa ($n = 23$) groups.

Variables	Control group ($n = 24$)			Cocoa group ($n = 23$)			
	Baseline	4 week	p	Baseline	4 week	p	Δp
Anthropometry, body composition and blood pressure							
Weight (kg)	81.51 (11.41)	79.03 (11.36)	<0.001	83.25 (10.45)	80.63 (10.15)	<0.001	0.700
BMI (kg/m^2)	30.34 (1.97)	29.40 (2.09)	<0.001	30.73 (2.46)	29.74 (2.34)	<0.001	0.744
Waist/Hip ratio	0.95 (0.09)	0.93 (0.09)	0.048	0.96 (0.06)	0.93 (0.07)	0.001	0.324
Total fat mass (kg)	31.44 (5.05)	29.50 (4.90)	<0.001	33.46 (6.75)	31.15 (6.27)	<0.001	0.273
Truncal fat mass (kg)	18.11 (3.45)	16.69 (3.08)	<0.001	18.88 (3.42)	17.29 (3.15)	<0.001	0.477
BMC (g)	2.68 (0.57)	2.60 (0.58)	0.275	2.59 (0.55)	2.40 (0.62)	0.091	0.376
SBP (mmHg)	116 (13.02)	108 (10.81)	0.001	123 (14.29)	116 (15.12)	0.001	0.477
DBP (mmHg)	79 (8.24)	71 (6.96)	<0.001	82 (8.45)	77 (8.81)	<0.001	0.302
Blood routine variables							
Glucose (mg/dL)	98.35 (9.99)	95.67 (9.48)	0.132	98.85 (10.03)	96.73 (10.96)	0.474	0.868
HOMA[a]	1.73 (1.17)	1.31 (1.03)	0.007	1.77 (1.54)	1.15 (1.18)	0.002	0.992
LDL-c (mg/dL)	162.09 (32.27)	130.40 (25.45)	<0.001	179.18 (50.98)	147.40 (35.31)	<0.001	0.992
Atherogenic index[a]	5.39 (2.25)	4.65 (1.46)	0.001	5.82 (2.45)	5.18 (1.84)	0.007	0.650
TG (mg/dL)[a]	114.75 (38.60)	87.00 (33.18)	<0.001	109.13 (49.14)	79.87 (35.12)	0.002	0.887
Homocystein (μmol/L)	12.94 (4.15)	13.16 (4.43)	0.765	13.45 (3.80)	13.52 (3.23)	0.926	0.885
Inflammation biomarkers							
TNF-α (pg/mL)	1.28 (0.63)	1.09 (0.49)	0.059	1.31 (0.60)	1.13 (0.50)	0.034	0.977
IL-6 (pg/mL)	2.65 (2.44)	2.72 (2.42)	0.831	2.51 (1.56)	2.75 (1.63)	0.549	0.737
C-reactive protein (μg/mL)[a]	2.04 (1.74)	1.47 (1.48)	0.028	2.63 (2.26)	1.96 (1.90)	0.105	0.903
Lp-PLA2 (ng/mL)	188.58 (76.41)	157.58 (53.30)	0.001	204.34 (44.51)	169.43 (37.68)	<0.001	0.704
Plasma vitamins							
25(OH)D (ng/mL)	16.68 (4.04)	18.72 (4.69)	<0.001	18.97 (6.69)	20.75 (7.00)	0.014	0.737

Data are expressed as mean (SD). Comparisons between baseline and endpoint (p) of the study within the same group were analysed by paired student t-test or Wilcoxon test, and comparison between groups was performed by independent t-test or U Mann–Whitney, according with the normality of the variables (Δp).
$P < 0.05$ was considered as significant.
Abbreviations: *BMI* body mass index; *BMC* bone mineral concentration; *SBP* systolic blood pressure; *DBP* diastolic blood pressure; *HOMA* Homoeostasis model assessment; *LDL-c* low-density lipoprotein-cholesterol; *TG* triglycerides; *TNF-α* tumor necrosis factor alpha; *IL-6* interleukin 6; *Lp-PLA2* Lipoprotein-associated phospholipase A2; Δ = 4 week − Beginning.
[a] Non-normally distributed variables.

Table 3
Regression analysis to assess the effect of group (control/cocoa) over the change of anthropometry, body composition, blood pressure, blood biochemical parameters, biomarkers of inflammation and 25(OH)D, adjusted for sex, age, baseline value of each measurement and Δfat mass% ($n = 47$).

Dependent variables	Independent variables				
	Group (control/cocoa)	Sex (male/female)	Age (years)	Baseline value	ΔFat mass (%)
Anthropometry, body composition and blood pressure					
ΔWeight (kg)	−0.08 (0.861)	−0.21 (0.702)	−0.03 (0.493)	−0.04 (0.138)	−0.01 (0.978)
ΔBMI (kg/m^2)	−0.02 (0.884)	−0.20 (0.888)	−0.01 (0.424)	−0.04 (0.369)	−0.01 (0.927)
ΔWaist/Hip ratio	−0.01 (0.208)	−0.02 (0.201)	0.002 (0.061)	−0.17 (0.095)	−0.01 (0.127)
ΔTotal fat mass (kg)	−0.09 (0.733)	0.07 (0.885)	−0.02 (0.541)	−0.08 (0.007)	0.41 (<0.001)
ΔTruncal fat mass (kg)	0.01 (0.933)	0.23 (0.112)	−0.02 (0.162)	−0.06 (0.012)	0.50 (<0.001)
ΔBMC (g)	−0.14 (0.275)	−0.09 (0.714)	−0.004 (0.757)	−0.02 (0.718)	−0.31 (0.188)
ΔSBP (mmHg)	4.13 (0.127)	−5.04 (0.074)	0.03 (0.909)	−0.38 (0.001)	−1.49 (0.222)
ΔDBP (mmHg)	2.82 (0.096)	−2.41 (0.152)	0.26 (0.100)	−0.33 (0.002)	0.78 (0.308)
Blood biochemical parameters					
ΔGlucose (mg/dL)	0.76 (0.778)	1.94 (0.483)	1.94 (0.090)	−0.74 (<0.001)	−3.28 (0.017)
ΔHOMA	−0.15 (0.594)	0.08 (0.782)	−0.05 (0.081)	−0.56 (<0.001)	−0.15 (0.238)
ΔLDL-c (mg/dL)	8.91 (0.137)	6.44 (0.275)	0.84 (0.139)	−0.48 (<0.001)	7.16 (0.010)
ΔAtherogenic index	0.26 (0.201)	0.15 (0.452)	0.001 (0.948)	−0.34 (<0.001)	0.09 (0.367)
ΔTG (mg/dL)	−3.85 (0.669)	8.95 (0.346)	−1.18 (0.180)	−0.61 (<0.01)	−2.23 (0.603)
ΔHomocysteine (μmol/L)	−0.12 (0.894)	1.44 (0.166)	0.07 (0.463)	−0.34 (0.011)	−0.44 (0.310)
Biomarkers of inflammation					
ΔTNF-α (pg/mL)	0.03 (0.748)	−0.02 (0.890)	−0.003 (0.782)	−0.40 (<0.001)	0.06 (0.215)
ΔIL-6 (pg/mL)	0.13 (0.788)	−0.08 (0.877)	−0.04 (0.437)	−0.32 (0.010)	−0.30 (0.185)
ΔC-RP (μg/mL)	0.45 (0.379)	0.06 (0.911)	−0.07 (0.148)	−0.80 (<0.001)	−0.01 (0.982)
ΔLp-PLA2 (ng/mL)	0.04 (0.996)	1.84 (0.797)	2.03 (0.007)	−0.32 (<0.001)	5.82 (0.085)
Plasma vitamins					
Δ25(OH)D (ng/mL)	−0.25 (0.755)	−0.14 (0.862)	−0.10 (0.223)	−0.02 (0.792)	−0.63 (0.089)

Data are expressed as B (p-value). Abbreviations: *BMI* body mass index; *BMC* bone mineral concentration; *SBP* systolic blood pressure; *DBP* diastolic blood pressure; *HOMA* Homoeostasis model assessment; *LDL-c* low-density lipoprotein-cholesterol; *TG* triglycerides; *TNF-α* tumor necrosis factor alpha; *IL-6* interleukin 6; C-RP C-reactive protein; *Lp-PLA2* Lipoprotein-associated phospholipase A2; Δ = 4 week − Beginning.
$P < 0.05$ was considered as significant.

Table 4
Comparison of anthropometric, body composition, blood pressure, routine blood biochemical parameters and inflammatory biomarkers between baseline and the endpoint of the intervention of the collective sample ($n = 47$).

Variables	Collective sample		p
	Baseline ($n = 47$)	4 week ($n = 47$)	
Anthropometry, body composition and blood pressure			
Weight (kg)	82.18 (10.96)	79.60 (10.70)	<0.001
BMI (kg/m^2)	30.53 (2.21)	29.57 (2.20)	<0.001
Waist/Hip ratio	0.96 (0.01)	0.93 (0.01)	<0.001
Total fat mass (kg)	32.43 (5.97)	30.31 (5.61)	<0.001
Truncal fat mass (kg)	18.49 (3.42)	16.98 (3.10)	<0.001
BMC (g)	2.64 (0.56)	2.51 (0.60)	0.042
SBP (mmHg)	120.00 (13.93)	112.00 (13.69)	<0.001
DBP (mmHg)	81.00 (8.43)	74.00 (8.28)	<0.001
Blood routine variables			
Glucose (mg/dL)	98.60 (9.91)	96.19 (10.13)	0.153
HOMA[a]	1.75 (1.35)	1.23 (1.11)	<0.001
LDL-c (mg/dL)	170.45 (42.87)	138.72 (31.53)	<0.001
Atherogenic indexa	5.60 (2.33)	4.91 (1.66)	<0.001
TG (mg/dL)[a]	112 (43.68)	83.51 (33.96)	<0.001
Homocystein (μmol/L)	13.19 (3.95)	13.33 (3.85)	0.777
Inflammation biomarkers			
TNF-α (pg/mL)	1.30 (0.61)	1.11 (0.49)	0.004
IL-6 (pg/mL)	2.59 (2.05)	2.74 (2.07)	0.553
C-reactive protein (μg/mL)	2.33 (2.01)	1.71 (1.70)	0.010
Lp-PLA2 (ng/mL)	196.29 (62.69)	163.38 (46.21)	<0.001
Plasma vitamins			
25(OH)D (ng/mL)	17.80 (5.56)	19.71 (5.96)	<0.001

Data are expressed as mean (SD). Comparisons between baseline and endpoint (p) of the study were analysed by paired student t-test or Wilcoxon test, according with the normality of the variables.
$P < 0.05$ was considered as significant.
Abbreviations: *BMI* body mass index; *BMC* bone mineral concentration; *SBP* systolic blood pressure; *DBP* diastolic blood pressure; *HOMA* Homeostasis model assessment; *LDL-c* low-density lipoprotein-cholesterol; *TG* triglycerides; *TNF-α* tumor necrosis factor alpha; *IL-6* interleukin 6; *Lp-PLA2* Lipoprotein-associated phospholipase A2.
[a] Non-normally distributed variables.

($p = 0.021$), SBP ($p = 0.011$), IL-6 ($p = 0.044$) and an increase in BCM ($p = 0.043$). When the analysis was performed considering the median of baseline 25(OH)D plasma concentration, no significant differences were observed between groups (Table 6). However, statistically significant differences were found in weight, BMI and total fat mass when category groups were defined by the median of the endpoint 25(OH)D plasma concentration. Interestingly, subjects who finished the intervention with a 25(OH)D concentration higher than 19.10 ng/mL presented lower body weight ($p = 0.024$), BMI ($p = 0.025$) and total fat mass ($p = 0.017$) as is shown in Table 6.

4. Discussion

Obesity and ageing are negatively associated with 25(OH)D levels [5,8] and 25(OH)D deficiency is involved in the development of various adverse cardiometabolic conditions [11]. Therefore, the aim of this study was to evaluate the relationship of 25(OH)D changes and levels with anthropometric, body composition, blood pressure, blood biochemical parameters and inflammatory markers, after a weight-lowering nutritional intervention in overweight/obese middle-aged subjects. In our study, 25(OH)D levels significantly increased after 4 weeks of weight-loss nutritional intervention, as occurred in the study carried out by Tzotzas et al. (2010), where after a 10% of weight-loss, 25(OH)D levels increased in middle-aged women [19]. Likewise, Mason et al. (2011) reported a greater increase of 25(OH)D levels in overweight/obese postmenopausal women who after have followed a reduced-calorie diet for 12 months presented a greater decrease of body weight [20]. Thus, when the collective sample was analysed together, weight and fat loss were seen negatively associated with 25(OH)D increase. Moreover, 25(OH)D increase was higher in those women who reduced more than 15% of body-weight [20]. On the other hand, Ortega et al. (2008) observed that overweight/obese women who were allocated in a 20% energy restricted diet for 2 weeks and started the intervention with more than 50 nmol/L of 25(OH)D, were those who reduced more body fat mass after the 2 weeks of intervention [12]. Consequently, it was suggested that subjects with more vitamin D reserves at baseline respond better to an energy restriction, losing more body fat mass. Valuable results have been obtained after a supplementation with 200 IU of vitamin D3 during 12 months [21]. Those researchers did not find an additional effect on weight-loss, but subjects who reached more than 32 ng/mL of 25(OH)D at the end of that period presented greater improvements in weight, waist circumference and body fat mass [21]. However, in the study of Piccolo et al. (2013), who measured vitamin D in serum and adipose tissue, no significant changes were found during a weight-loss intervention for 12 weeks [22]. They assessed if the concentration of 25(OH)D from the subcutaneous white adipose tissue of overweight and obese subjects could be altered by a weight-loss. Moreover, they studied if the 25(OH)D from the adipose tissue was correlated with blood 25(OH) levels. Actually, they did not find changes either in adipose 25(OH)D levels nor in serum levels, suggesting that weight-loss is not sufficient to increase serum 25(OH)D levels. Nevertheless, according to other studies [12,20], an inverse relationship between fat mass and 25(OH)D levels is suggested. We can mention that in our study the fat mass percentage change was of 3.38% after one month, whereas in the study of Piccolo et al. was of 6% after 3 months. It is known that the fat and weight-loss is higher in the first stages of a weight-loss treatment. Therefore, the rate of fat mass loss was higher in our study and for this reason the release of vitamin D could be also higher. Moreover, the absolute amount of body fat was more reduced after 3 months as compared to 1 month, which could also contribute to explain the lack of differences in the circulating vitamin D levels.

Table 5
Multiple regression analysis to assess the effect of Δ25(OH)D (ng/mL) (independent variable) over the change of Δ weight, Δ fat mass, Δ truncal fat mass, Δ SBP and Δ IL-6 ($n = 47$) (dependent variables).

Dependent variables															
Models	Δ Weight (kg)			Δ Fat mass (kg)			Δ Truncal fat mass (kg)			Δ SBP (mmHg)			Δ IL-6 (pg/mL)		
	B	*p*	*R2*	*B*	*p*	*R2*	*B*	*p*	*R2*	*B*	*p*	*R2*	*B*	*p*	*R2*
Unadjusted	−0.20	0.009	0.12	−0.18	0.005	0.14	−0.10	0.038	0.07	−0.95	0.077	0.05	−0.19	0.052	0.06
Model 1	−2.04	0.010	0.12	−0.16	0.007	0.29	−0.09	0.019	0.34	−1.07	0.032	0.24	−0.30	0.010	0.19
Model 2	−0.12	0.139	0.22	Colineality			0.01	0.693	0.82	−1.24	0.025	0.24	−0.25	0.015	0.19

Δ = 4 week − Beginning.
Unadjusted.
Model 1: Adjusted for age (years), sex, baseline value of each dependent variable and intervention group (control and cocoa).
Model 2: Adjusted for age (years), sex, baseline value of each dependent variable, Δ fat mass (kg) and intervention group (control and cocoa).
Abbreviations: *25(OH)D* 25-hydroxyvitamin D, *SBP* systolic blood pressure, *IL-6* interleukin-6.

The association between the increase of 25(OH)D and the reduction of SBP is one of the most interesting finding of our study. This outcome is in agreement with other studies carried out in humans, where an inverse correlation between 25(OH)D and blood pressure levels were found [23]. Thus, Bhandari et al. (2011) suggested that polymorphisms in the genes related to vitamin D receptors such as BsmI and FokI, are associated with the risk of suffering from hypertension [24]. However, other investigations have not found this association [25]. On the other hand, Li et al. [26] proposed that vitamin D is involved in the renin-angiotensin system as a negative regulator. These researchers reported that vitamin D receptor knockout mice increased the activity of the renin-angiotensin system, and in consequence, those animals developed hypertension [26].

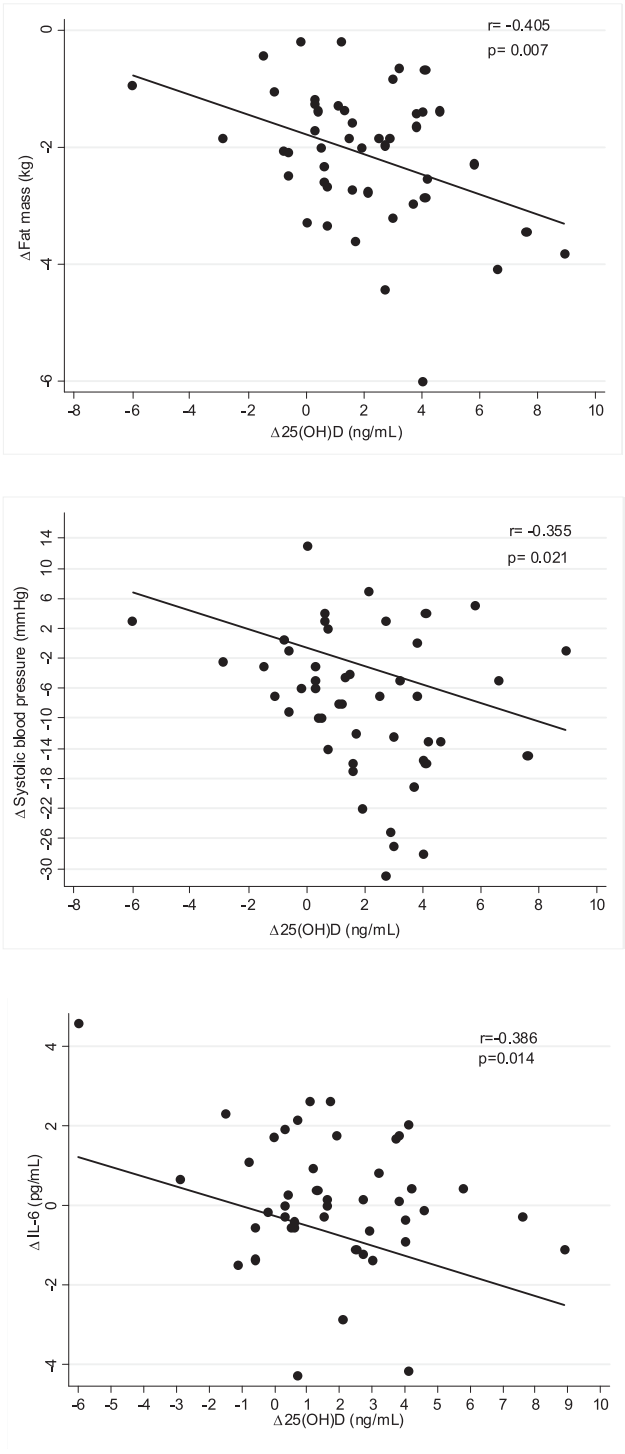

Fig. 1. Correlation analyses between Δ25(OH)D (ng/mL), Δfat mass (kg), ΔSBP (mmHg) and Δ IL-6 (pg/mL), adjusted for sex, age, Δfat mass (kg) and baseline value of each measurement. The correlation between Δ25(OH)D (ng/mL) and Δfat mass (kg) was not adjusted for Δfat mass (kg). Abbreviations: *25(OH)D* 25-hydroxyvitamin D; *SBP* systolic blood pressure, *IL-6* interleukin 6.

On the other hand, 25(OH)D deficiency is negatively correlated with obesity and it is suggested that adipose tissue sequesters 25(OH)D, reducing its availability in the plasma of obese subjects [10]. Also, recent investigations have shown a potential modulating role of vitamin D on adipose tissue formation and function, becoming a direct target [27]. The expression of vitamin D related genes is occurred in human adipocytes, suggesting that vitamin D may inhibit adipogenesis by suppressing the expression of adipogenic transcription factors and decreasing the lipid addition in adipocytes, avoiding preadipocyte differentiation to adipocites [28].

Adipose tissue is an endocrine organ that produces adipokines such as IL-6, which is involved in inflammatory processes [3]. To date, there are few studies that have investigated how vitamin D could participate in human adipokine production, but some of them have shown a negative influence of vitamin D over inflammation [29,30]. In our study, a negative association between Δ25(OH)D and ΔIL-6 levels was found, as in the study of Laird et al., where a negative association between 25(OH)D and IL-6 levels was observed in older Irish adults [30]. Similarly, in the study of Kim et al. [29], a negative relationship between 25(OH)D and IL-6 in overweight/obese Koreans was reported [29].

The effect between 25(OH)D and weight, fat mass, SBP and IL-6 was confirmed when subjects were categorised according to their change or endpoint 25(OH)D levels. If subjects are categorised according to their change on 25(OH)D levels, individuals who had a higher increase in 25(OH)D levels showed a higher decrease in weight, BMI, total fat mass, SBP and IL-6. This suggests that a relationship between 25(OH)D and those variables exist, which is improved after the weight-loss. The same phenomena was observed in the study of Ortega et al. (2008), where who started the weight-loss intervention with more than 50 nmol/L of 25(OH)D were who presented a higher reduction of body fat [12]. On the other hand, if the subjects are categorised according to their endpoint 25(OH)D level, individuals who finalised the study with high levels of 25(OH)D had a higher reduction in anthropometric (weight and BMI) and body composition (total fat mass) variables. Since after weight-loss treatment, 25(OH)D increased and anthropometric and body composition variables decreased, it can be suggested that 25(OH)D levels and weight-loss treatment are related to those changes.

Our data reveal that 25(OH)D has a role in blood pressure and fat mass, giving additional light to this issue. However, the following limitations should be mentioned: first, as both intervention groups (control and cocoa) were merged, there was not a control group to compare with. A control group no losing weight would be interesting to be sure that the increase of 25(OH)D was produced as a consequence of a weight-loss. Second, the sample size was relatively small to allow us to obtain reliable conclusions. Third, even if all the volunteers were assessed in the same season, it would have been useful to control them for the sun exposure to avoid the confounding factor of vitamin D synthesis from the sun. Finally, the food table composition of micronutrients may be inaccurate for some foods.

Table 6
Effect of baseline, endpoint (4 weeek) and Δ25(OH)D levels on anthropometric, body composition, blood pressure, blood routine and inflammation variables.

Variables	Baseline 25(OH)D ≤17.30 ng/mL ($n = 24$)	Baseline 25(OH)D >17.30 ng/mL ($n = 23$)	Δ*p*	End 25(OH)D ≤19.10 ng/mL ($n = 24$)	End 25(OH)D >19.10 ng/mL ($n = 23$)	Δ*p*	Δ 25(OH)D ≤1.6 ng/mL ($n = 23$)	Δ 25(OH)D >1.6 ng/mL ($n = 24$)	Δ*p*
Anthropometric and body composition variables									
ΔWeight (kg)	−2.24 (2.06)***	−2.92 (2.06)***	0.124	−2.07 (1.99)***	−3.10 (2.06)***	0.024	−2.07 (2.06)***	−3.06 (2.06)***	0.026
ΔBMI (kg/m^2)	−0.84 (0.75)***	−1.09 (0.75)***	0.112	−0.78 (0.69)***	−1.15 (0.75)***	0.025	−0.80 (0.75)***	−1.12 (0.75)***	0.045
ΔWaist/hip	−0.01 (0.07)*	−0.03 (0.07)**	0.112	−0.02 (0.07)**	−0.03 (0.07)*	0.256	−0.03 (0.07)***	−0.02 (0.07)*	0.187
ΔTFM^1 (kg)	−1.29 (0.69)***	−1.62 (0.69)***	0.117	−1.41 (0.69)***	−1.61 (0.75)***	0.206	−1.40 (0.69)***	−1.61 (0.69)***	0.178
ΔTFM^2 (kg)	−2.01 (0.89)***	−2.01 (0.89)***	0.239	−1.90 (0.82)***	−2.35 (0.82)***	0.017	−1.90 (0.82)***	−2.33 (0.82)***	0.021
ΔBMC (g)	−0.17 (0.62)	−0.08 (0.62)	0.503	−0.19 (0.62)	−0.07 (0.62)	0.409	−0.27 (0.62)	0.01 (0.62)	0.043
ΔSBP (mmHg)	−7.38 (12.75)***	−8.10 (13.03)***	0.793	−5.95 (12.68)**	−9.59 (12.96)***	0.193	−4.16 (12.27)**	−11.16 (12.00)***	0.011
ΔDBP(mmHg)	−5.90 (7.95)***	−7.54 (8.16)***	0.344	−6.19 (8.09)***	−7.24 (8.30)***	0.553	−5.56 (8.09)***	−7.80 (7.95)***	0.196
Blood routine variables									
ΔGlucose (mg/dL)	−0.56 (12.96)	−4.34 (13.23)	0.182	−3.92 (13.44)	−0.83 (13.71)	0.300	−4.48 (13.51)**	−0.42 (13.23)	0.167
ΔHOMA	−0.47 (1.37)**	−0.56 (1.37)***	0.761	−0.50 (1.37)*	−0.58 (1.37)	0.688	−0.51 (1.37)***	−0.53 (1.37)***	0.956
ΔLDL-c (mg/dL)	−28.82 (27.97)***	−34.77 (28.59)***	0.326	−26.18 (27.63)***	−37.53 (28.25)***	0.066	−25.83 (29.00)***	−37.39 (28.31)***	0.073
ΔAI	−0.60 (0.60)***	−0.79 (1.03)***	0.369	−0.53 (0.96)**	−0.87 (1.03)	0.117	−0.52 (1.03)***	−0.86 (1.03)***	0.127
ΔTG (mg/dL)	−23.88 (45.18)***	−33.30 (46.28)***	0.351	−23.57 (46.41)***	−33.62 (47.58)	0.343	−19.25 (43.74)***	−37.35 (42.71)***	0.058
ΔHomocysteine (μmol/L)	−0.29 (4.59)	0.61 (4.66)	0.364	0.13 (4.66)	0.16 (4.80)	0.977	−0.04 (4.73)	0.33 (4.66)	0.714
Inflammation biomarkers									
ΔTNF-α (pg/mL)	−0.28 (4.94)*	−0.09 (0.48)	0.075	−0.23 (0.55)*	−0.14 (0.55)	0.453	−0.29 (0.51)***	−0.09 (0.48)	0.070
ΔIL-6 (pg/mL)	0.09 (2.40)***	0.21 (2.47)	0.831	0.57 (2.19)	−0.33 (2.40)	0.076	0.66 (2.26)***	−0.39 (2.33)***	0.044
ΔC-RP (μg/mL)	−0.87 (2.40)***	−0.35 (2.47)**	0.318	−0.54 (2.47)	−0.70 (2.54)	0.762	−0.62 (2.74)**	−0.61 (2.45)**	0.982
ΔLp-PLA2 (ng/mL)	−29.70 (34.48)***	−36.26 (35.31)***	0.382	−27.13 (34.48)***	−38.95 (35.31)***	0.125	−33.45 (37.30)***	−32.40 (36.40)***	0.897

Values are expressed as mean (SD). Data and *p*-values are analysed by ANCOVA using sex, age, intervention group, baseline value of each dependent variable and Δtotal fat mass (%) as covariates. Comparisons between baseline and end of the study were analysed by paired student *t*-test or Wilcoxon test
(***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$), according with the normality of the variables. *Δ p* of the difference between each categorized group adjusted for sex, age, intervention group, baseline value of each dependent variable and total fat mass (%).
Abbreviations: *25(OH)D* 25-hydroxyvitamin D; *BMI* body mass index; *BMC* bone mineral concentration; *SBP* systolic blood pressure; *DBP* diastolic blood pressure;; *TFM^1* truncal fat mass; *TFM^2* total fat mass *HOMA* Homeostasis model assessment; *LDL-c* low-density lipoprotein-cholesterol; *AI* atherogenic index; *TG* triglycerides; *TNF-α* tumor necrosis factor alpha; *C-RP* C-reactive protein; *Lp-PLA2* Lipoprotein-associated phospholipase A2 *IL-6*, interleukin-6; *Δ* = 4 week − Beginning.

5. Conclusions

The rise in plasma 25(OH)D level is linked to the decrease of fat mass and SBP in middle-aged individuals after a nutritional weight-lowering intervention. Moreover, a rise in vitamin D is closely associated with adiposity and inflammatory processes. In this sense, 25(OH)D is a variable to be accounted for metabolic alterations related to blood pressure, adiposity and inflammation. This research, within a nutritional intervention study provides a new evidence to help understand and to clarify the role of 25(OH)D in blood pressure and lipid metabolism management.

Ethical approval

The investigation was carried out under the approval of the Research Ethics Committee of the University of Navarra (ref. no 006/2012) and followed the Helsinki Declaration guidelines.

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Contributors

The authors' contributed as follows: I.I.B. contributed to the design of the study, was involved in the fieldwork, data collection, analysis and writing of the manuscript; S.N.C. and I.A. contributed to the scientific content and editing the manuscript; J.A.M and M.A.Z were responsible for the general coordination, design, interpretation of the data, financial management and editing of the manuscript.

Conflict of interests

The authors declare that they have no conflict of interest.

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V. GENERAL DISCUSSION

1. Justification of the Study

Obesity is considered one of the most important public health problems of the XXI century (Flegal *et al.*, 2013; WHO, 2015c), where poor lifestyle habits (inadequate dietary patterns and reduced physical activity) are the principal factors affecting excessive weight gain (WHO, 2015c). The imbalance between energy intake and expenditure, the increased consumption of fat, simple sugars and alcohol, as well as to follow sedentary lifestyle patterns contribute to overweight and obesity condition (Hill *et al.*, 2012; Bhurosy and Jeewon, 2014; Lopez-Legarrea *et al.*, 2014). On the other hand, obesity is associated with the onset of different comorbidities and clinical complications such as CVD, insulin resistance, type 2 diabetes, sleep apnea, psychological disorders, reproductive problems, cancer and VitD deficiency, among other disorders (Fan *et al.*, 2013; Martin-Rodriguez *et al.*, 2015).

Different dietary strategies have been proposed in order to combat obesity and accompanying comorbidities (Abete *et al.*, 2011; Millen *et al.*, 2014). However, many of them have a detrimental result on muscular mass (Villareal *et al.*, 2012). For this reason, the design of effective dietary strategies to preserve muscular mass and reduce adipose tissue is needed. In this context, higher protein intake and increased physical activity have been suggested as approach to manage excessive weight gain (Moize *et al.*, 2013; DeLany *et al.*, 2014; Hector *et al.*, 2015). On the other hand, the use of plant components with anti-obesity properties has increased in the last years (Wang *et al.*, 2014). Vegetables are rich sources of antioxidants with many health benefits, such as reduction of blood pressure, glycemic control or blood lipid reduction as well as neutralization of ROS, free radicals and pro-inflammatory molecules, which contribute to generate oxidative stress and inflammatory condition (Slavin and Lloyd, 2012; Rodriguez-Casado, 2014).

Cocoa is a rich source of antioxidants and phytochemical compounds whose consumption has been proposed to treat different health disorders (Lee *et al.*, 2003; Andujar *et al.*, 2012; Ellam and Williamson, 2013). Cocoa intake has been associated with the prevention or treatment of CVD through the modification of blood pressure, lipid profile and endothelial dysfunction (Ferri *et al.*, 2015). Moreover, cocoa has been associated with cognitive performance, as well as with well-being and pleasure (Scholey *et al.*, 2010; Pase *et al.*, 2013; Scholey and Owen, 2013). The implication of cocoa on peripheral monoaminergic system, which could be related with psychological alterations, has been scarcely studied. On the other hand, cocoa flavanols are usually consumed as chocolate bar or cocoa beverage, but considering its beneficial properties for health, the inclusion of cocoa within different food matrixes could be interesting.

Taking into account the positive effects of cocoa consumption and body weight loss, the present study was focused on the assessment of metabolic (inflammatory and oxidative stress status) and nutritional status as well as the evaluation of depressive and anxiety symptoms on middle-aged obese subjects, after daily consumption of ready-to-eat meals containing 1.4 g of cocoa extract (645 mg polyphenols with 415 mg of flavanols) within a weight loss diet.

2. General discussion

2.1. Effect of cocoa extract intake and moderate energy restricted diet on anthropometry, body composition and blood biochemical determinations

In the present study, the effect of a nutritional strategy with a 15% of energy restriction and moderately high in protein content, as well as other additional benefits derived from the supplementation with cocoa extract were assessed on body weight and body composition.

The prescribed energy restricted diet resulted beneficial in the reduction of body weight and the improvement of body composition in both intervention groups as reported elsewhere (Hermsdorff *et al.*, 2011; de la Iglesia *et al.*, 2014). Interestingly, volunteers preserved the lean mass after the intervention, suggesting that a greater intake of proteins together with a moderate energy restriction could be beneficial to obtain a weight loss, but avoiding the loss of lean mass (Leidy *et al.*, 2007).

On the contrary, the addition of cocoa extract to the energy restricted diet did not suppose additional benefit concerning body weight and body composition changes, since no differences were observed between groups. In agreement with our results, most of the RCT studies did not find any changes in body weight and body composition after consumption of cocoa or chocolate during a short period of time (Grassi *et al.*, 2005a; Taubert *et al.*, 2007; Muniyappa *et al.*, 2008; Heiss *et al.*, 2010; Njike *et al.*, 2011; Khan *et al.*, 2012). Exceptionally, Monagas *et al* (2009) and Desch *et al* (2010) reported an increment of 0.5 and 1 kg of body weight respectively, which was attributed to increased energy intake from the inclusion of cocoa in the diet (Monagas *et al.*, 2009; Desch *et al.*, 2010).

On the other hand, cross-sectional epidemiological studies have reported that the habitual consumption of chocolate is associated with lower body weight and BMI (Strandberg *et al.*, 2008; O'Neil *et al.*, 2011; Golomb *et al.*, 2012; Cuenca-Garcia *et al.*, 2014). Interestingly, based on the Atherosclerosis Risk in Communities cohort (ARIC) study, Greenberg and Buijsse (2013) reported that subjects with greater BMI are the individuals suffering from obesity related comorbidities and in order to improve their health, those subjects tend to decrease the consumption of energetic foods, such as chocolate. This fact could explain the inverse causality found between chocolate consumption and BMI (Greenberg and Buijsse, 2013). On the contrary, the prospective analysis revealed that more frequent consumption of chocolate was significantly associated with a long-term greater weight gain in a dose-response manner (Greenberg and Buijsse, 2013).

Nevertheless, chocolate and cocoa extract have no the same composition. Chocolate, apart from cocoa flavanols contains sugar and fat in high proportions and for this reason chocolate consumption has been usually associated with body weight gain (Greenberg *et al.*, 2015). Contrariwise, the cocoa extract is mainly composed by cocoa phytochemicals (Ortega *et al.*, 2008a).

Therefore, it cannot be strictly compared the results observed after chocolate intake with those observed after cocoa extract intake.

With the aim of elucidate the anti-obesity mechanisms of flavanols, the effect of cocoa intake has been specifically assessed in animal model (Ali *et al.*, 2014), where cocoa polyphenols appear to regulate lipid utilisation through the activation of the expression of several genes or transcription factors involved in energy metabolism (Ali *et al.*, 2014; Ali *et al.*, 2015). Interestingly, Dorenkott *et al* (2014) observed that oligomeric cocoa procyanidins were the most effective type of cocoa flavanols to prevent the development of obesity (Dorenkott *et al.*, 2014).

However, obesity is not only reflected in body image and appearance, it is also associated with the alteration of relevant blood biochemical measurements (Jung and Choi, 2014). Indeed, obesity is related with hyperglycaemia and insulin resistance, which is the previous state of type 2 diabetes (Kahn *et al.*, 2006). In this context, weight loss and the consumption of cocoa phytochemicals could be beneficial for the control of glucose metabolism (Torjesen *et al.*, 1997; Grassi *et al.*, 2013b).

In our study, glucose levels did not change after 4 weeks of intervention, while insulin and HOMA-IR levels reduced in both intervention groups, without finding any differences between them. This outcome suggested that the inclusion of 1.4 g of cocoa extract did not suppose additional benefits in the glycemic profile and that the insulin and HOMA-IR reductions were probably due to weight loss (Pedersen *et al.*, 2015). According to these results, other studies about cocoa flavanols intake did not report changes on glucose, insulin or HOMA-IR levels (Muniyappa *et al.*, 2008; Mellor *et al.*, 2010; West *et al.*, 2014). However, other RCT studies have observed the effectiveness of cocoa intake reducing blood glucose and insulin levels and consequently improving insulin resistance (Grassi *et al.*, 2005b; Mastroiacovo *et al.*, 2015; Matsumoto *et al.*, 2015). In this sense, Masctroiacovo *et al* (2015) and Desideri *et al* (2012) found a reduction of HOMA-IR after consuming a beverage containing around 990 and 520 mg of flavanols during 8 weeks in overweight elderly subjects (Desideri *et al.*, 2012; Mastroiacovo *et al.*, 2015).

The meta-analysis by Shrime *et al* (2011) who investigated 24 RCT studies encompassing 1106 subjects, reported a significant reduction of HOMA-IR without finding a reduction on fasting glucose after the cocoa intake (Shrime *et al.*, 2011). Moreover, Hooper *et al* (2012) observed a reduction of HOMA-IR in a meta-analysis including 42 RCT studies and comprising 1297 subjects (Hooper *et al.*, 2012). They found a reduction of glucose concentration after the consumption of 50-100 mg of epicatechin (Hooper *et al.*, 2012). Our volunteers consumed 153 mg/day of epicatechin, which is over 50-100 mg previously reported. However, Mastroiacovo *et al* (2015) observed a reduction of glucose levels with epicatechin concentration higher than 100 mg (Mastroiacovo *et al.*, 2015).

The alteration of lipid profile is associated with obesity and the development of CVD (Karthikeyan *et al.*, 2009). In accordance with the INTERHEART study, subjects with altered lipid profile have 3 times higher risk of suffering from a heart attack compared to subjects with normal lipid concentrations (Karthikeyan *et al.*, 2009).

In this study, lipid profile (total cholesterol, LDL-c, TG, HDL-c and FFA), significantly decreased in both intervention groups, but no differences were found between them, suggesting that the inclusion of 1.4 g of cocoa extract did not represent an additional benefit in blood lipid profile at least after 4 weeks of consumption. Thus, blood lipid reduction was attributed to the prescription of 15% energy restricted diet (Melanson *et al.*, 2003). In agreement with these results, other investigators did not find benefits on lipid profile after the consumption of cocoa (Taubert *et al.*, 2003; Taubert *et al.*, 2007; Crews *et al.*, 2008; Muniyappa *et al.*, 2008; Heiss *et al.*, 2010; Njike *et al.*, 2011; West *et al.*, 2014; Grassi *et al.*, 2015; Rull *et al.*, 2015).

The meta-analysis by Hooper *et al* (2012) concluded that studies with less than 3 week of duration reduced LDL-c and total cholesterol, while those with more than 3 week of duration increased HDL-c (Hooper *et al.*, 2012). Moreover, the consumption of 50 - 100 mg of epicatechin per day reduced TG levels, but no effect was found with lower or higher levels (Hooper *et al.*, 2012). Our volunteers consumed 153 mg/day of epicatechin during 4 weeks. In this context, it could be hypothesized that for this reason we did not find a significant reduction on LDL-c and TG levels in comparison to control group. Concerning HDL-c, although the duration of our study was over 3 weeks, we did not observe an increment in HDL-c because it is usually reduced after weight loss diets without physical activity prescription (Ribeiro *et al.*, 2008; Aicher *et al.*, 2012).

Blood pressure and endothelial dysfunctions are also characteristic features of obesity and both are risk factors of CVD (Grassi *et al.*, 2013b; Landsberg *et al.*, 2013). The current scientific evidence has showed that the intake of cocoa flavanols increases NO production, a vasodilatatory molecule that is negatively related with blood pressure and endothelial function (Fraga *et al.*, 2011). Although in our study the systolic and diastolic blood pressure significantly reduced after the intervention, no differences were observed between groups, suggesting that the supplementation of the diet with 1.4 g of cocoa extract did not represent additional benefits on these measures. In accordance with these observations, other investigations did not find benefits on blood pressure after cocoa flavanol consumption (Njike *et al.*, 2011; Flammer *et al.*, 2012; Rull *et al.*, 2015), while other studies have reported a reduction on blood pressure due to the involvement of cocoa flavanols on NO production (Taubert *et al.*, 2007; Heiss *et al.*, 2010; Grassi *et al.*, 2015; Heiss *et al.*, 2015).

Endothelial function is usually evaluated by FMD (Ghiadoni *et al.*, 2015). However, we measured sICAM-1 and sVCAM-1 adhesion molecules, observing a reduction on sICAM-1 in both groups without differences between groups, while sVCAM-1 did not change during the intervention.

Therefore, the reduction of sICAM-1 could be attributed to the prescription of a 15% energy restricted diet and not to the inclusion of 1.4 g/d of cocoa extract. In agreement with our results, Farouque *et al* (2007) and Muniyappa *et al* (2008) did not find any changes after cocoa intake (Farouque *et al.*, 2006; Muniyappa *et al.*, 2008), while Wan-Polagruto *et al* (2006) and Monagas *et al* (2009) reported an improvement of endothelial function (Wang-Polagruto *et al.*, 2006; Monagas *et al.*, 2009).

In connection with this, VitD is a molecule implicated in multiple processes of human physiology (Botella-Carretero *et al.*, 2007; Maddock *et al.*, 2013; Stocklin and Eggersdorfer, 2013) and the scientific evidence has shown that low VitD levels are associated with greater BMI and adipose tissue (Botella-Carretero *et al.*, 2007; Vimaleswaran *et al.*, 2013) as well as with higher risk of suffering from cardiometabolic diseases (Gonzalez-Molero *et al.*, 2012; Mozos and Marginean, 2015).

In this study, no differences were observed in 25(OH)D after cocoa extract consumption. For this reason, and considering the importance of VitD in obesity and CVD, we merged both intervention groups and evaluated the role of plasma 25(OH)D concentration after a weight loss nutritional intervention on body composition, blood pressure and inflammatory markers (Concato *et al.*, 2000). Thus, 25(OH)D levels showed a significant increase after the 4 week intervention which was linked to the reduction of weight, adiposity, SBP and IL-6 (Tzotzas *et al.*, 2010; Bhandari *et al.*, 2011; Mason *et al.*, 2011; Kim *et al.*, 2013; Laird *et al.*, 2014). Concerning the relation with SBP, other studies suggest that polymorphisms in the genes related to VitD receptors are associated with the risk of suffering from hypertension (Wang *et al.*, 2013). In addition, VitD might be involved in the renin-angiotensin system as a negative regulator (Li *et al.*, 2004). Interestingly, individuals who reported a higher increase of 25(OH)D showed a greater decrease of body weight, BMI, SBP and IL-6 and an increase in bone mineral concentration suggesting a relationship with 25(OH)D (Ortega *et al.*, 2008b). On the other hand, subjects who finalised the study with greater levels of 25(OH)D showed higher reduction in anthropometric (weight and BMI) and total fat mass. Considering that after weight loss treatment 25(OH)D concentration increased and anthropometric and body composition variables decreased, it could be suggested that 25(OH)D levels and weight loss treatment are related to those changes (Vimaleswaran *et al.*, 2013).

To sum up, a diet with a 15% of energy restriction and with moderately high protein content improved the anthropometric variables, body composition, insulin levels, HOMA-IR, lipid profile, blood pressure, markers of endothelial dysfunction and 25(OH)D levels. However, the intake of 1.4 g/day of cocoa did not show additional benefits in those experimental variables, since no differences were found between both intervention groups. The flavanol dose, potential interactions with other nutrients, study period or interindividual variability could be possible explanations for the absence of benefits derived from cocoa intake.

2.2. Effect of cocoa extract intake and moderate energy restricted diet on oxidative stress and inflammation

Oxidative stress and inflammation are the main underlying mechanisms of obesity (Bondia-Pons *et al.*, 2012). Adipose tissue releases a wide range of molecules contributing to a pro-inflammatory and pro-oxidant environment (Mraz and Haluzik, 2014). This situation is optimal for the onset of endothelial dysfunction and consequently the formation of atherosclerotic plaque contributing to the development of comorbidities associated to obesity (Martinez, 2006; Hajjar and Gotto, 2013).

Plant polyphenols could be effective components to reduce or prevent oxidative stress and inflammation due to their high antioxidant capacity (Wang *et al.*, 2014). These compounds can be at least in part responsible by acting directly on ROS, stimulating the cellular endogenous defences or repairing DNA damage (Azqueta and Collins, 2012; Collins *et al.*, 2012). Specifically, cocoa is a rich source of polyphenols with high antioxidant capacity (Lee *et al.*, 2003).

In our study, a significant reduction of oxLDL concentration was observed in both intervention groups after the 4 week intervention, suggesting the implication of body weight loss in this outcome (de la Iglesia *et al.*, 2013; Tumova *et al.*, 2013). Interestingly, according with other studies, a higher oxLDL reduction was found in cocoa supplemented group (Khan *et al.*, 2012; Sola *et al.*, 2012). This finding could be linked to the significant reduction of MPO in cocoa group, a molecule that catalyses the oxidative reactions favoring oxLDL production (Steffen *et al.*, 2006).

Apart from lipid damage, oxidative stress is able to produce an oxidative DNA damage that could be repaired by the consumption of antioxidants (Collins, 2013). In our trial, the DNA damage was not reduced neither after weight loss nor after cocoa intake. According to our results, Spadafranca *et al* (2010) did not observe differences in DNA damage after consuming 45 g of dark chocolate *vs.* white chocolate during 2 weeks (Spadafranca *et al.*, 2010). However, when both groups were merged and analysed as a collective group, a marginal decrease of oxidised DNA bases was observed, attributing the effect to body weight loss (Kouda *et al.*, 2000). Moreover, volunteers who started the intervention with greater oxidative damage showed a greater reduction in oxidised bases after 4 weeks compared to those who had lower levels at baseline (Lettieri-Barbato *et al.*, 2013; de la Iglesia *et al.*, 2014).

Metabolomics is an interesting tool to show if the biochemical processes of the organism are related to the ingested nutrients (Nicholson *et al.*, 2012). In this context, the oxidised bases of DNA negatively correlated with methyl-epicatechin-*O*-sulphate and epicatechin-sulphate, metabolites derived from cocoa consumption in plasma. This finding suggests that a protective effect of cocoa consumption might be seen in a longer period of time or in subjects with greater baseline DNA damage suggesting that a protective effect of cocoa consumption might be observed

after a longer period of time or in subjects with higher DNA damage at baseline. (Lettieri-Barbato *et al.*, 2013).

On the other hand, obesity is associated with a low-grade chronic inflammatory status, which is also involved in the pathogenesis of other health disorders (Mraz and Haluzik, 2014). The scientific evidence suggests that cocoa intake regulates cytokine secretion and expression and modulates eicosanoid metabolism, as well as the effects mediated by NO (Gu and Lambert, 2013).

In this study, the group supplemented with cocoa extract significantly reduced TNF-α and Lp-PLA2 inflammatory markers, while the control group significantly reduced C-reactive protein and Lp-PLA2. According to other studies, the consumption of cocoa did not induce differences between groups on inflammatory markers (Mathur *et al.*, 2002; Balzer *et al.*, 2008; Muniyappa *et al.*, 2008; Mellor *et al.*, 2010; Njike *et al.*, 2011). Indeed, the improvement of inflammatory markers could be associated with the reduction of body weight and fat mass (Richard *et al.*, 2013; Petelin *et al.*, 2014; Ryan *et al.*, 2014). Thus, the inclusion of 1.4 g per day of cocoa extract within an energy restricted diet did not represent additional benefits on inflammatory makers compared to the energy restricted diet alone. Nevertheless, some studies showed benefits on inflammation after cocoa consumption (Gu and Lambert, 2013).

To sum up, the dietary strategy proposed in this study resulted effective to improve the antioxidant status by the reduction of oxLDL levels and oxidised DNA bases. Moreover, the reduction of some inflammatory markers is attributed to body weight and fat mass loss due to the energy restricted diet. Interestingly, the consumption of 1.4 g per day of cocoa extract induced a greater oxLDL reduction, improving the oxidative status.

2.3. Postprandial cardiometabolic effects of cocoa extract consumption before and after a 4 week intervention

Considering that humans spend more than three-quarters of their lives in the postprandial state, the postprandial effect of consuming ready-to-eat meals containing cocoa extract was studied before and after 4 weeks of following a moderate hypocaloric diet including cocoa (Laville *et al.*, 2013). Interestingly, a different acute response of cocoa extract intake was observed on postprandial SBP before and after the 4 week intervention. At the beginning of the study, the intake of cocoa extract resulted in a significantly higher acute SBP response compared to the control group, which could be attributed to theobromine content in cocoa, a methylxanthine from the same family of caffeine, which has been related to the increase of heart rate (Baggott *et al.*, 2013; West *et al.*, 2014). However, after a regular consumption of cocoa extract during 4 weeks no differences were observed between groups concerning the acute SBP levels, suggesting some kind of tolerance after the daily intake of cocoa (Monahan *et al.*, 2011; De Gottardi *et al.*, 2012; West *et al.*, 2014). It is important to highlight that a greater reduction of postprandial SBP was obtained in cocoa consumers after 4 weeks of daily cocoa consumption independently of body weight loss, suggesting

that postprandial reduction of SBP could be due to the regular consumption of cocoa extract. This effect could be explained by the blood pressure lowering effects attributed to cocoa (Desideri *et al.*, 2012; Sarria *et al.*, 2012).

2.4. Effect of cocoa extract intake and moderate energy restricted diet on psychological behaviour and peripheral monoamine levels

Obesity is associated with mental disorders in a bidirectional manner (Pan *et al.*, 2012). Obese individuals usually follow unhealthy dietary habits, based on the consumption of high palatable and energetic foods leading to increase the risk of suffering from depression and anxiety disorders (Reinholz *et al.*, 2008; Pan *et al.*, 2012; Rahe *et al.*, 2014), while subjects suffering from depression and anxiety symptoms seem to follow unhealthy lifestyle patterns contributing to obesity (Savolainen *et al.*, 2014).

Nowadays, nutritional strategies based on balanced dietary patterns, the increased intake of some nutrients and the use of plant components is increasing to combat, prevent or reduce the incidence of those disorders (Sanchez-Villegas and Martinez-Gonzalez, 2013; Sanchez-Villegas *et al.*, 2013; Perez-Cornago *et al.*, 2014a; Xu *et al.*, 2014), while unhealthy dietary patterns tend to cause detrimental effects (Le Port *et al.*, 2012; Hiles *et al.*, 2015).

On the other hand, circulating monoamines could be involved on obesity and psychiatric diseases (Williams *et al.*, 2006; Wang *et al.*, 2009). Few studies have analysed the effect of weight loss diets and the effect of cocoa consumption on peripheral monoamine and psychological features (Pase *et al.*, 2013), (Perez-Cornago *et al.*, 2014c). Indeed, to our knowledge, this is the first time that the effect of cocoa extract intake has been investigated on peripheral monoamine levels, especially on dopaminergic system, in relation with depression and anxiety symptoms.

In this study, self-reported depressive symptoms, which were measured by BDI index (Beck *et al.*, 1961), were reduced after the 4 weeks intervention in both intervention groups, suggesting the contribution of body weight loss to the reduction of depressive symptoms (Swencionis *et al.*, 2013; Perez-Cornago *et al.*, 2014c). Indeed, Fabricatore *et al* (2011) reported that obese subjects who lose weight may improve body image, physical functioning and quality of life, and consequently decrease psychological stress (Fabricatore *et al.*, 2011). However, anxiety symptoms measured by STAI questionnaire (Spielberger, 1971) did not show any changes after the intervention. Although the reduction of anxiety symptoms has been associated with body weight reduction (Swencionis *et al.*, 2013; Perez-Cornago *et al.*, 2015), a recent review by Eyres *et al* (2014) did not find clear evidence to confirm that energy-restricted diets are effective to reduce anxiety symptoms (Eyres *et al.*, 2014).

Concerning the peripheral dopaminergic system, pHVA significantly increased in both groups after the 4 weeks intervention, suggesting that weight loss may also produce an increase in

pHVA levels. HVA is the main metabolite produced from the dopamine degradation and taking into account that brain dopamine, which is related with mood, cannot cross the blood-brain barrier (Pardridge, 2007) the dopaminergic activity in the brain was determined by the measurement of pHVA, which is positively correlated with the central dopaminergic activity (Sternberg *et al.*, 1983; Amin *et al.*, 1992). Interestingly, the increment of pHVA was higher in cocoa consumers indicating that the intake of cocoa extract produced an additional increment of pHVA levels. More importantly, the increment of pHVA showed a negative association with depressive symptoms only in the group supplemented with cocoa extract.

These results suggest that the intake of cocoa extract within a nutritional strategy for weight loss could be associated with a specific improvement of psychological status. Indeed, cocoa flavanols are able to cross the blood brain barrier and increase the brain blood flow by the rise of nitric oxide production (Fisher *et al.*, 2006). In addition, cocoa flavanols may interact with a number of neurotransmitters in the brain which are implicated in the regulation of the food reward system, mood, cognitive performance and improvement of calmness and contentedness (Parker *et al.*, 2006; Scholey *et al.*, 2010; Pase *et al.*, 2013). The lack of differences between groups in BDI index could be due to the variability of data reported because BDI is a subjective questionnaire, whereas pHVA is an objective determination.

To sum up, a weight loss diet with a 15% of energy restriction contributed to significantly decrease depressive symptoms, but not anxiety symptoms. Concerning the inclusion of 1.4 g/day of cocoa extract no direct effect was observed on psychological behaviour, but interestingly, pHVA, which is a marker that reflects dopaminergic activity in the brain, showed a greater increase in cocoa consumers in comparison with the control group, and was also negatively associated with the reduction of depressive symptoms in cocoa consumers, suggesting the possible implication of cocoa on psychological behaviour.

2.5. Effect of cocoa extract intake and weight loss diet on plasma and urinary metabolomic profiles

Metabolomics is an important “omic” discipline that enables the identification and quantification of small molecules in biological samples (Fiehn, 2002; Weckwerth and Morgenthal, 2005; Patti *et al.*, 2012). Metabolomic studies provide valuable information about the metabolic status and global biochemical processes associated with a cellular or biological system (Nicholson *et al.*, 2012). Hence, metabolomics is able to display alterations of metabolic pathways linked to phenotypic perturbations (Patti *et al.*, 2012).

Targeted metabolomics is focused on analysing selected metabolites, often related to a specific metabolic pathway. In nutrition, it is usually used to determine the concentration, bioavailability, turnover, or metabolism of dietary compounds (Astarita and Langridge, 2013). On the other hand, untargeted metabolomics consists in the assessment of global metabolome with the

intention to compare profiles of metabolites among different groups of subjects and to identify new biomarkers (Astarita and Langridge, 2013).

In order to establish a relationship between the consumption of cocoa flavanols and the observed results, it is necessary to assess the absorption and release of cocoa flavanols into the circulation (Zheng *et al.*, 2015). For this reason, cocoa derived metabolites had to be present in plasma and urine of cocoa consumers (Tomas-Barberan *et al.*, 2007; Zheng *et al.*, 2015).

In this research, the effect of cocoa consumption on plasma and urine metabolomic profile was investigated. On one hand, targeted metabolomics approach was applied to assess the cocoa derived metabolites in plasma during the postprandial period (0-180 min) after the consumption of ready-to-eat meals supplemented or not with cocoa extract. This procedure was carried out before and after 4 weeks of intervention. On the other hand, untargeted metabolomics approach was used to evaluate the 24h urinary metabolomic profile before and after 4 weeks of intervention.

Plasma and urine metabolomic profiles revealed the presence of cocoa derived metabolites in plasma and urine, suggesting that cocoa flavanols and theobromine were absorbed and went into the circulation (Zheng *et al.*, 2015). In addition, the present results demonstrated the compliance of the volunteers to the intervention due to the higher plasma concentration of cocoa derived metabolites in the cocoa consumers compared to the absence or lower amounts of those metabolites in the control group (Bondia-Pons *et al.*, 2013). Similarly, the principal component analysis (PCA) of urine samples was able to discriminate the baseline group (no cocoa consumption, no diet), the control group at endpoint (-15%E diet) and the cocoa group at endpoint (1.4 g of cocoa extract and -15%E diet) corroborating the compliance of the volunteers to the intervention (Worley and Powers, 2013; Andersen *et al.*, 2014). The presence of some cocoa derived metabolites in the urine and plasma of the control group could be due to the presence of other flavanol sources in the diet. However, the concentration of those metabolites in subjects from control group was found in significantly lower amounts. According to other studies, the assessment of metabolites is a reliable method to assess the dietary compliance (Bondia-Pons *et al.*, 2013; Garcia-Aloy *et al.*, 2015; Zheng *et al.*, 2015).In cocoa consumers, theobromine derived metabolites in plasma were present in higher amounts at the end of intervention, which could be related to the half life of those metabolites in the circulation (Ellam and Williamson, 2013). Concerning the urinary putative metabolites, it included those related to theobromine, cocoa polyphenols, food processing, catecholamine and endogenous metabolism. Theobromine and polyphenol derived metabolites supported the adherence to the intervention (Garcia-Aloy *et al.*, 2015).

It is important to note the presence of MHPG-sulphate in urine, a metabolite from catecholamine metabolism. Previous studies have reported that MHPG-sulphate levels are decreased in brain and urine of depressive subjects (Peyrin *et al.*, 1985). Taking into account that the urinary excretion of MHPG-sulphate increased in both groups after the 4 week intervention, the

weight loss might be related with psychological behaviour, as was shown by BDI index. However, the cocoa supplemented group showed higher excretion of MHPG-sulphate at the endpoint, indicating a possible additional benefit of cocoa consumption in terms of improving psychological behaviour, as was revealed by pHVA increase. Nevertheless, these results should be viewed with caution because MHPG-sulphate was a putative metabolite and authentic standards should be run to confirm the retention time with the matching compound. Moreover, the relation of MHPG-sulphate with monoamine levels as well as with psychological questionnaires would be necessary.

3. Strengths and limitations

There are various strengths to mention in this research:

First, this is a pioneer trial, including cocoa extract within a variety of ready-to-eat meals, since usually cocoa flavanols are consumed within chocolate bars or as cocoa powder mixed with milk or water. Second, the energy restricted diet prescribed to all the volunteers was closely controlled. Volunteers received the same energy restriction according to their energy requirements. In this context, the differences found between groups after the intervention would be attributed to cocoa extract supplementation. Third, no differences between intervention groups were observed in the variables assessed at the beginning of the study. Therefore, the control and cocoa groups were homogeneous. Fourth, the regular and the acute effect of cocoa consumption were simultaneously evaluated. Fifth, the compliance of meal consumption was self-reported, but also new methodologies such as metabolomic analysis were used to corroborate the obtained results (Odriozola and Corrales, 2015). Indeed, the adherence to the intervention was high, suggesting that ready-to-eat meals were palatable and attractive to consume. Sixth, the cocoa extract used in the study seems to be bioavailable within the ready-to-eat meals.

On the other hand, some limitations were detected during the intervention:

First, the duration of the study could be considered short to find changes in some of the assessed variables. Second, the BDI (Beck *et al.*, 1961), the STAI (Spielberger, 1971) and the 3 days-validated food record questionnaires are self-reported validated questionnaires used in the clinical practice and have been previously used to assess depressive and anxiety symptoms (Fabricatore *et al.*, 2011) and dietary consumption respectively (Perez-Cornago *et al.*, 2015). However, these types of questionnaires are subjective and volunteers could hide their real feelings or food consumption. Third, the sample size of this study was small, which have increased the type II errors (failing to detect real differences) if several adjustments are performed (Smith, 2002). Thus, limit number of covariates was used accepting type I errors (asserting something that is absent) in order to avoid type II errors (Cohen *et al.*, 2013). Fourth, the cocoa derived metabolites in plasma were presented at the end of the intervention for control group, but it would be interesting to evaluate these metabolites also at the beginning of the study. Fifth, NO determination would have been interesting to assess any relation with oxLDL reduction or blood pressure. Six, targeted metabolomic analysis

or the use of authentic standards to confirm the retention time with the matching compound would be interesting procedures to confirm the putative compounds that were found in urine.

4. Corollary

The results presented in this dissertation reveal that the prescription of an energy restricted diet (-15% E) with moderately high protein content was an effective strategy to improve anthropometric and body composition variables, routine blood biochemical markers, blood pressure, VitD levels, oxidative and inflammatory markers as well as depressive symptoms. On the other hand, cocoa extract intake during a short period of time (4 weeks) resulted beneficial to improve the oxidative status by the reduction of oxLDL levels as well as the psychological status by the influence on peripheral dopaminergic system increasing pHVA levels. Moreover, the daily consumption of cocoa extract within ready-to-eat meals and under a weight loss diet reduced the postprandial SBP response and also showed an adaptive effect of the acute SBP. In addition, the presence of cocoa derived metabolites in plasma and urine after 4 weeks of the study revealed the adequate compliance of the volunteers to the intervention and the bioavailability of cocoa within the ready-to-eat meals. Furthermore, the identification of metabolic changes related with benefits attributed to cocoa consumption contributes to increase the current scientific knowledge.

In recent years, the general population has changed eating habits, mainly due to the modification of lifestyle features such as shift work, social habits and accelerated rhythm of life among others. On the other hand, ageing processes increase the occurrence of health problems. In this context, food companies are investigating how to facilitate society in the eating process in a healthy and nutritive manner. Although the consumption of ready-to-eat foods is not well considered, the nutritional profile and the outward appearance of meals is improving, including also functional ingredients in order to solve nutritional deficiencies and to improve the health condition of consumers.

In this context, we have evidenced that the inclusion of a cocoa extract within ready-to-eat meals, as well as the inclusion of ready-to-eat meals within an energy restricted diet was able to improve the nutritional and metabolic status. Nevertheless, further studies are needed to confirm the finding of this investigation as well as in order to shed more light about the duration of the treatment, the doses of flavanols and the target population which would be more benefited.

VI. CONCLUSIONS

Conclusions derived from aim 1:

1. The prescription of a 4 week dietary strategy with a 15% of energy restriction and moderately high in protein content improved the general nutritional status as well as specific inflammatory and oxidative markers and depressive symptoms in middle-aged overweight/obese subjects.
2. A weight loss diet with a 15% of energy restriction resulted beneficial to increase plasma Vit D levels in overweight/obese subjects after an intervention of 4 weeks. Interestingly, the increase of VitD levels was inversely associated with a reduction of body weight, SBP and IL-6, conferring a relationship of Vit D circulating levels with markers related to CVD.

Conclusions derived from aim 2:

3. The inclusion of cocoa as a functional compound within ready-to-eat meals could be a healthy approach to improve oxidative status of overweight/obese subjects under a -15% energy restricted diet, which was ascertained by a reduction of oxLDL levels after 1.4 g cocoa extract (645 mg of total polyphenols with 415 mg flavanols) consumption during 4 weeks.
4. The inverse association of some cocoa derived metabolites in plasma with oxidative DNA damage suggested that the intake of cocoa could be useful to improve oxidative status in middle-aged overweight/obese individuals after 4 weeks under a moderate energy restricted diet.

Conclusion derived from aim 3:

5. The daily consumption of cocoa extract within a hypocaloric diet during a period of 4 weeks resulted in a reduction of postprandial SBP response independent of body weight loss, which was achieved after following of a weight loss diet. This result suggested the implication of cocoa on the regulation of postprandial blood pressure response.

Conclusion derived from aim 4:

6. The intake of cocoa extract within a weight loss diet contributed to increase peripheral monoamine HVA levels, which was associated with a reduction of depressive symptoms, suggesting a putative involvement of cocoa components on psychological behaviour of overweight/obese subjects.

Conclusion derived from aim 5:

7. The presence of cocoa derived metabolites in plasma and urine samples evidenced the compliance to the dietary intervention and contributed to identify metabolomic changes supporting information to the current scientific knowledge about metabolites of interest for human health.

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VIII. APPENDICES

Appendix 1: Example of the diet (1500 kcal)

NOTA IMPORTANTE

EL PARTICIPANTE DEBERÁ EXCLUIR DE SU DIETA:

- Cacao y productos derivados del cacao: chocolate, crema de cacao, cacao en polvo, bombones y productos que contengan como ingrediente al cacao.
- Suplementos nutricionales con vitaminas y/o minerales.
- Productos de herboristería, naturales y/o fitoquímicos, a base de compuestos ricos en polifenoles o antioxidantes.
- Condimentos y especias como mostaza, canela, azafrán, perejil, tomillo, orégano, hinojo, etc., así como bayas de goji.
- Infusiones como las de rooibos, frutas rojas, té verde, té rojo, menta poleo, manzanilla, etc.
- Todos los productos (leche, galletas, huevos, harinas, cafés solubles, margarinas, cereales, etc.) enriquecidos con algún antioxidante.

RECOMENDACIONES GENERALES

- Debe consumir diariamente **el plato precocinado del estudio** que desee entre las 10 opciones con la dieta que corresponde para cada plato.
- Debe consumir **1 postre diario del estudio**, como postre en la comida o en la cena.
- Usted deberá realizar **las 5 tomas al día**.
- Los postres de la comida y la cena, como las medias mañanas y meriendas, son intercambiables, dentro del mismo día.
- Las **VERDURAS DE CONSUMO LIBRE** las puede utilizar para acompañar a carnes, pescados, legumbres, elaborar ensaladas o añadir a las verduras de pesar. Las puede consumir cuando quiera y en la cantidad que desee.
- Pesar los alimentos en crudo y en limpio, siempre que sea posible. Ej: acelga, después de limpiar pero antes de cocinar.
- Respetar las cantidades especificadas en la dieta.
- **Utilizar pan, galletas, tostadas y cereales de desayuno integrales.**
- Carne magra: **retirar la grasa visible** antes de cocinar, ya que es mucho más fácil e higiénico. En el caso del jamón serrano, antes de comer.
- Si consume nata, margarina, mantequilla o mayonesa (5 g – 1cucharilla), restar 5 g de aceite de oliva.
- Si consume nueces, almendras o avellanas (8 g), restar 5 g de aceite de oliva.
- Se puede sustituir 30 g de pan por: 1) una ración de sopa de fideos o 2) 20 g de pasta en crudo o 70 g cocida para una ensalada de pasta como opciones para la cena.
- El consumo de embutidos, charcutería, carnes grasas (costillas, patas de cerdo, morcilla, vísceras…), así como de fritos, rebozados y productos conservados en aceite **no está permitido**. Desgrasar los caldos. Hacer los guisos y estofados sin aceite adicional.
- El consumo de azúcares y golosinas (como azúcar, miel, chocolates, helados) **está prohibido.**

- Utilizar los siguientes modos de cocción: se aconseja el **hervido, asado, plancha, parrilla, papillote, cocina al vapor, microondas y estofados sin grasa añadida.**
- **Evite el consumo de alcohol**. Consuma como bebida, principalmente, agua y de forma esporádica gaseosa, cerveza sin alcohol y refrescos *light*.

GARBANZOS CON ESPINACAS

Toma	Alimentos	Cantidad	Ración
Desayuno	Lácteo	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
	Cereal	*Elegir entre:* -45 g pan integral (barra/molde) -45 g biscotes integrales -20 g galletas tipo María integrales -40 g cereales integrales	1 ½ rebanada pequeña 4 unidades pequeñas 4-5 unidades 4 cucharadas soperas
	Proteico	45 g de jamón cocido	1 ½ loncha
Media Mañana	Lácteo	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 vaso 2 unidades
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Comida	Primer plato	***300g DE GARBANZOS CON ESPINACAS***	***1 ENVASE ENTERO***
	Segundo plato	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	Lácteo	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 vaso 2 unidades
Cena	Primer plato	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Segundo plato	-280 g crudo ó 240 g cocinado de pescado blanco ó azul - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete mediano/grande Acompañamiento
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 15 g	1 ½ cuchara sopera

CREMA DE CALABACÍN

Toma	Alimentos	Cantidad	Ración
Desayuno	Lácteo	*Elegir entre:* -250 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
	Cereales	*Elegir entre:* -30 g pan integral (barra/molde) -30 g biscotes integrales -20 g galletas tipo María integrales -30 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
Media Mañana	Lácteo	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela, 75 g plátano	1 unidad pequeña/ mediana
Comida	Primer plato	-200 g de verdura en crudo o 240 g en cocido (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…)	1 plato mediano
	Segundo plato	-180g crudo ó 160 g cocinado de pescado blanco ó azul - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 ración mediana Acompañamiento
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela, 75 g plátano	1 unidad pequeña/ mediana
Merienda	Lácteo	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 vaso 2 unidades
Cena	Primer plato	***300 g CREMA DE CALABACÍN***	***1 ENVASE ENTERO***
	Segundo plato	-140g de clara de huevo - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento -60 g Jamón cocido	2-3 claras Acompañamiento 2 lonchas
	Postre	***-150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 20 g	2 cucharas soperas
Pan integral	-TOTAL DÍA: 50 g	5 biscotes ó 2 rebanadas medianas de pan

ESPINACAS A LA CREMA

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -30 g pan integral (barra/molde) -30 g biscotes integrales -20 g galletas tipo María integrales -30 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
	Fruta	*Elegir entre:* -245 g naranja, melocotón, fresas -210 g albaricoque, mandarina, pera -175 g manzana, piña, ciruela -105 g plátano	1 unidad mediana
Comida	**Primer plato**	***300 g ESPINACAS A LA CREMA***	***1 ENVASE ENTERO***
	Segundo plato	-80 g crudo ó 240 g cocido de alubias, garbanzos, lentejas - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano Acompañamiento
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	-30 g crudo ó 50 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Segundo plato	-155g crudo ó 135 g en cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete pequeño Acompañamiento
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 25 g	2 1/2 cucharas soperas

GNOCHI CON VERDURAS

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -35 g pan integral (barra/molde) -35 g biscotes integrales -20 g galletas tipo María integrales -35 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Comida	**Primer plato**	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento -100 g crudo o 80 g cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…)	1 plato 1 filete pequeño
	Segundo plato	***300 g GNOCHI CON VERDURAS***	***1 ENVASE ENTERO***
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	-250 g de verdura en crudo o 290 g en cocido (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…)	1 plato mediano/grande
	Segundo plato	-170 g crudo ó 140 g en cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete pequeño/mediano Acompañamiento
	POSTRE	***-150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA:20 g	2 cucharas soperas

GAZPACHO

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -250 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
	Cereales	*Elegir entre:* -30 g pan integral (barra/molde) -30 g biscotes integrales -20 g galletas tipo María integrales -30 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Comida	**Primer plato**	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano
	Segundo plato	-75 g crudo ó 225 g cocido de alubias, garbanzos, lentejas - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano Acompañamiento
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	***300 g GAZPACHO***	***1 ENVASE ENTERO***
	Segundo plato	-170 g crudo ó 150 g en cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…) -CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete mediano Acompañamiento
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 20 g	2 cucharas soperas

MERLUZA CON TOMATE

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereal	*Elegir entre:* -45 g pan integral (barra/molde) -45 g biscotes integrales -20 g galletas tipo María integrales -40 g cereales integrales	1 ½ rebanada pequeña 4 unidades pequeñas 4-5 unidades 4 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Comida	**Primer plato**	-200 g crudo ó 240 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano
	Segundo plato	***300 g MERLUZA CON TOMATE***	***1 ENVASE ENTERO***
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Segundo plato	-130 g de jamón cocido -60 g de queso fresco 0% MG	4 ½ lonchas
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 30 g	3 cucharas soperas
Pan integral ó patata	*Elegir entre:* -TOTAL DÍA: 55 g de pan -70 g de patata cruda ó 80 g cocida	2 rebanadas medianas 1 unidad pequeña

PAELLA DE MARISCO

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -35 g pan integral (barra/molde) -35 g biscotes integrales -20 g galletas tipo María integrales -35 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Comida	**Primer plato**	-200 g crudo ó 240 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano
	Segundo plato	-170 g crudo ó 150 g cocido de pescado blanco ó azul - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 porción pequeña/mediana Acompañamiento
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	***300 g PAELLA DE MARISCO***	***1 ENVASE ENTERO***
	Segundo plato	-80 g jamón serrano -90 g de queso fresco 0%MG	2 ½ lonchas
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 25 g	2 ½ cucharas soperas

PENNE BOLOÑESA

Toma	Alimentos	Cantidad	Ración
Desayuno	Lácteo	*Elegir entre:* -250 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -20 g pan integral (barra/molde) -20 g biscotes integrales -15 g galletas tipo María integrales -20 g cereales integrales	1 rebanada pequeña 2 unidades pequeñas 2 unidades 2 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	Lácteo	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Comida	Primer plato	- CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento -40 g de jamón serrano -40 g de queso fresco 0%MG	2 lonchas finas
	Segundo plato	***300 g PENNE BOLOÑESA***	***1 ENVASE ENTERO***
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	Lácteo	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	Primer plato	-200 g de verdura en crudo o 240 g en cocido (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Segundo plato	-240 g crudo ó 210 g cocinado de pescado blanco ó azul -20 g de queso fresco 0% MG - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete mediano/grande 1 loncha Acompañamiento
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 25 g	2 ½ cucharas soperas

RISOTTO AL FUNGHI

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -30 g pan integral (barra/molde) -30 g biscotes integrales -20 g galletas tipo María integrales -30 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Comida	**Primer plato**	***300 g RISOTTO AL FUNGHI***	***1 ENVASE ENTERO***
	Segundo plato	-120 g crudo ó 100 g en cocinado de carne magra (pollo, pavo, ternera, conejo, potro, lomo cerdo…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 filete pequeño Acompañamiento
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -120 g leche desnatada -125 g yogur desnatado	1 vaso pequeño 1 unidad
Cena	**Primer plato**	-150 g de verdura en crudo o 170 g en cocido (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…)	1 plato pequeño
	Segundo plato	-125 g de clara de huevo -45 g de atún sin aceite	2 claras ¾ de una lata sin aceite
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 15 g	1 ½ cucharas soperas

POLLO SANFAINA

Toma	Alimentos	Cantidad	Ración
Desayuno	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 tazón 2 unidades
	Cereales	*Elegir entre:* -30 g pan integral (barra/molde) -30 g biscotes integrales -20 g galletas tipo María integrales -30 g cereales integrales	1 rebanada pequeña 3 unidades pequeñas 4 unidades 3 cucharadas soperas
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Media Mañana	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 vaso 2 unidades
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Comida	**Primer plato**	-200 g crudo ó 240 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato mediano
	Segundo plato	***300 g POLLO SANFAINA***	***1 ENVASE ENTERO***
	Fruta	*Elegir entre:* -175 g naranja, melocotón, fresas -150 g albaricoque, mandarina, pera -125 g manzana, piña, ciruela -75 g plátano	1 unidad pequeña/ mediana
Merienda	**Lácteo**	*Elegir entre:* -240 g leche desnatada -250 g yogur desnatado	1 vaso 2 unidades
Cena	**Primer plato**	-100 g crudo ó 120 g cocido de verdura (judía verde, coliflor, puerro, tomate, acelga, cardo, borraja, berenjena, zanahoria, champiñón…) - CONSUMO LIBRE: achicoria, calabacín, lechuga, endibia, canónigos, rúcula, escarola, apio, cebolla, pepino y pimiento	1 plato
	Segundo plato	-60 g de huevo	1 unidad pequeña
	Postre	***150 g de POSTRE LÁCTEO***	***1 ENVASE ENTERO***

REPARTIR ENTRE COMIDA Y CENA:		
Aceite de oliva	-TOTAL DÍA: 20 g	2 cucharas soperas
Pan integral	-TOTAL DÍA: 30 g	1 rebanada mediana

Appendix 2: Low-polyphenol diet and dietary recommendations

RECOMENDACIONES

- Una semana antes de comenzar el estudio, es decir, a partir del día ___/____/___ tiene prohibido consumir cacao o productos derivados del cacao (cacao en polvo, chocolate, crema de cacao, bombones, batidos de chocolate etc.)
- 3 días antes de acudir a la primera visita tendrá que seguir la dieta que se le ha indicado, respetando cantidades y alimentos.

Los días: ___/___/___, ___/____/___ y ___/____/___

- Consumir todo lo que indica la dieta
- Realizar las 5 tomas al día
- Minimice la actividad física durante los 3 días previos a la visita 1

PROHIBIDO DURANTE LOS 3 DÍAS ANTERIORES:

- Consumir alcohol (vino, cerveza, sidra, etc.)
- Productos derivados del cacao (chocolate, cacao en polvo, crema de cacao)
- Cereales integrales (enriquecidos y con contenido de fibra)
- Aceite de girasol.
- Consumir cualquier tipo de té, infusiones y café
- Zumos de frutas y conservas
- Suplementos de vitaminas y minerals
- Especias: tomillo, romero, laurel, canela, pimienta, perejil, hierbabuena, pimentón, comino, menta y orégano…

- **DÍA 1 __/__/__ y Día 3 __/__/__**

Toma	Alimentos	Cantidad
Desayuno	**Lácteo**	Leche (puede añadir azúcar o edulcorante)
	Cereal	Galletas tipo María sin dorar (integrales no)
Media Mañana	**Lácteo**	Yogur natural (puede añadir azúcar o edulcorante)
Comida	**Primer plato: pasta con tomate y jamón**	Pasta no integral Tomate frito Jamón serrano magro
	Segundo plato: Merluza	Merluza sin rebozar
	Postre	100g de Platano (pesado con la cáscara)
	Pan	Pan blanco
Merienda	**Lácteo**	Yogur natural (puede añadir azúcar o edulcorante)
Cena	**Lomo de cerdo con arroz blanco**	Lomo de cerdo con arroz blanco
	Pan	Pan blanco
	Postre	Leche (puede añadir azúcar o edulcorante)

COMIDA Y CENA PARA COCINAR:
ACEITE DE OLIVA (no tiene porque ser extra virgen)

DÍA 2 __/__/__

Toma	Alimentos	Cantidad
Desayuno	**Lácteo**	Leche (puede añadir azúcar o edulcorante)
	Cereal	Galletas tipo María sin dorar (integrales no)
Media Mañana	**Lácteo**	Yogur natural (puede añadir azúcar o edulcorante)
Comida	**Primer plato: Puré de patata y zanahoria**	Patata Zanahoria (30g)
	Segundo plato: Pechuga pollo a la plancha con patatas	Pechuga de pollo Patata
	Postre	Queso
	Pan	Pan blanco
Merienda	**Lácteo**	Yogur natural (puede añadir azúcar o edulcorante)
Cena	**Bocadillo de tortilla de jamón cocido**	Huevo Jamón cocido
	Pan	Pan blanco
	Postre	Yogur natural (puede añadir azúca r/edulcorante)

COMIDA Y CENA PARA COCINAR:
ACEITE DE OLIVA (no tiene porque ser extra virgen)

- Cantidades libres excepto en los alimentos indicados.
- Por favor, no consuma más fruta y más verdura de la indicada.
- Por favor, no consuma más fruta y más verdura de la indicada.

Appendix 3: Participant's written informed consent

HOJA DE INFORMACIÓN AL VOLUNTARIO

Esta hoja informativa le invita a participar en el estudio: "**Efecto de la ingesta de platos preparados incluyendo cacao, como potencial ingrediente funcional, sobre marcadores de riesgo cardiovascular en personas mayores".**

Se trata de un estudio de intervención nutricional que se llevará a cabo en la Unidad Metabólica del Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra. En este estudio se incluirán 50 voluntarios como usted. Este estudio forma parte de un proyecto de investigación en alimentación muy amplio llamado CENIT (Investigación industrial de dietas y alimentos con características específicas – Acrónimo: SENIFOOD). El estudio está financiado por la empresa TUTTI PASTA S.A como promotor.

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

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

¿Cuál es el objetivo del estudio?

Investigar el posible efecto beneficioso de la ingesta de platos preparados conteniendo extracto de cacao, dentro de una dieta saludable, sobre marcadores de riesgo cardiovascular en personas mayores.

¿Cuáles son los procedimientos del estudio?

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

En la primera cita se le hace entrega de la presente hoja informativa para que usted la lea y pregunte dudas sobre el estudio. A continuación se le hace entrega de la hoja de consentimiento informado, por duplicado y aprobado por el Comité de Ética de la Investigación de la Universidad de Navarra, para que muestre su conformidad. Si usted acepta participar en el estudio, la médico del equipo le realizará una breve entrevista y una historia clínica con exploración física. En caso de que usted no haya presentado una analítica reciente (últimos 3 meses), la enfermera procederá a la extracción de una

muestra de sangre. Para ello, será necesario recoger la sangre en 1 tubo de 4 ml. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio.

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

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

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

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

3) Se le hará entrega de un bote de orina para que el día anterior a su primera visita, recoja la orina de 24 horas.

Este estudio tiene una duración total de 4 semanas consecutivas durante las cuales deberá seguir la dieta pautada y consumir 1 plato preparado y 1 postre al día. Cada plato y cada postre contendrán el extracto vegetal en estudio (0,7g/plato) o un placebo, que le será asignado de manera aleatoria.

En el caso de que usted cumpla los criterios de inclusión, se le seleccionará para la primera visita del estudio, *Visita 1* (Día 0) y se le tomarán medidas de peso, talla, pliegues cutáneos, circunferencia de la cintura y cadera y de composición corporal. A continuación la enfermera le tomará la presión arterial y le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina relacionados con el metabolismo glucídico, lipídico y proteico y otros análisis relacionados al estudio. Para ello será necesario recoger la sangre en 9 tubos de bioquímica, 4 ml en cada tubo. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio.

Se le hará entrega de los 10 platos y 10 postres preparados junto a la dieta (que le será explicada) para que los consuma de la forma indicada. También se le entregará el cuestionario VAS para que lo complete cuando se le indique. Finalmente tendrá que

cumplimentar algunos cuestionarios psicológicos durante la visita. Esta visita durará unos 60 minutos.

Pasada esta primera visita tendrá que acudir a la Unidad Metabólica una vez cada semana ─ Visita 2 (Día 7), Visita 3 (Día 14), Visita 4 (Día 21) ─. En las *Visitas 2, 3 y 4* solo acudirá a entregar y recoger los platos preparados, también servirá como visita de seguimiento, en la que se realizará una breve entrevista y se le pesará. Estas visitas le llevará no más de 45 minutos. Asimismo, durante la visita 3 de seguimiento, se le entregarán el registro de 72 h, el cuestionario de hambre-saciedad, VAS, que tendrá que rellenar los días indicados por nosotros y un bote de orina (para proceder de la misma manera que el día anterior a la *Visita 1* y entregarlos en la última visita. En esta visita también tendrá que responder a algunos cuestionarios psicológicos.

La última visita (Visita 5) se realizará el día 28, en la que le realizaremos las mismas medidas y pruebas que en la primera visita (Día 0). También completará los cuestionarios psicológicos. Al finalizar la visita se le hará entrega de una dieta de mantenimiento o pérdida de peso, según su situación. Esta visita durará aproximadamente 50 minutos.

¿Qué debe hacer si quiere participar en este estudio?

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

Para participar en este estudio deberá usted firmar el consentimiento informado y una vez evaluemos su analítica y confirmemos que no existe ningún impedimento para su participación, le llamaremos al teléfono que nos haya facilitado para citarle y comenzar las 4 semanas de estudio.

Posibles problemas para su salud

El producto en estudio ha pasado absolutamente todos los controles sanitarios oportunos. Durante la extracción de sangre puede sentir alguna molestia y pueden aparecer hematomas en la zona del pinchazo, y excepcionalmente puede sufrir mareo. Ninguna otra prueba de las que le realizaremos implica un riesgo para su salud.

Seguro

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

Confidencialidad de los datos

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

HOJA DE INFORMACIÓN AL VOLUNTARIO QUE TAMBIÉN PARTICIPA EN EL ESTUDIO DE BIODISPONIBILIDAD

Esta hoja informativa le invita a participar en el estudio: "**Efecto de la ingesta de platos preparados incluyendo cacao, como potencial ingrediente funcional, sobre marcadores de riesgo cardiovascular en personas mayores".**

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

¿Cuál es el objetivo del estudio?

Investigar el posible efecto beneficioso de la ingesta de platos preparados conteniendo un extracto de cacao, dentro de una dieta saludable, sobre marcadores de riesgo cardiovascular en personas mayores.

¿Cuáles son los procedimientos del estudio?

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

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analítica reciente (últimos 3 meses), la enfermera procederá a la extracción de una muestra de sangre. Para ello, será necesario recoger la sangre en 1 tubo de 4 ml. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio.

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

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

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

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

3) Se le hará entrega de un bote de orina para que el día anterior a su primera visita, recoja la orina de 24 horas.

Este estudio tiene una duración total de 4 semanas consecutivas durante las cuales deberá seguir la dieta pautada y consumir 1 plato preparado y 1 postre al día. Cada plato y cada postre contendrán el extracto vegetal en estudio (0,7g/plato) o un placebo, que le será asignado de manera aleatoria.

En el caso de que usted cumpla los criterios de inclusión, se le seleccionará para la primera visita del estudio, *Visita 1* (Día 0) y se le tomarán medidas de peso, talla, pliegues cutáneos, circunferencia de la cintura y cadera y de composición corporal. A continuación la enfermera le tomará la presión arterial y le extraerá una muestra de sangre para llevar a cabo análisis bioquímicos de rutina relacionados con el metabolismo glucídico, lipídico, proteico y otros análisis relacionados al estudio. Para ello será necesario recoger la sangre en 9 tubos de bioquímica, 4 ml en cada tubo. En ningún caso se utilizará la sangre obtenida para otros fines que no sean propiamente los del estudio. En esta primera visita tendrá que consumir también uno de los platos del estudio. Tras la ingesta se llevarán a cabo una extracciones de sangre (9 tubos - 4 ml/tubo) a los 60, 120 y 180 minutos y se recogerá la

orina antes y durante las 24 h posteriores a la ingesta (0-6, 6-12, 12-24). En los tiempos 0, 60, 120 y 180 también tendrá que rellenar los cuestionarios VAS. Se le hará entrega de los 10 platos y 10 postres preparados junto a la dieta (que le será explicada) para que los consuma de la forma indicada. Se le hará entrega de un cuestionario VAS para que lo complete una vez por semana y lo entregue en la V*isita 3.* Finalmente tendrá que cumplimentar algunos cuestionarios psicológicos durante la visita. Esta visita durará unas 3,5 horas.

Pasada esta primera visita tendrá que acudir a la Unidad Metabólica una vez cada semana ─ Visita 2 (Día 7), Visita 3 (Día 14), Visita 4 (Día 21) ─. En las *Visitas 2 y 4* solo acudirá a entregar y recoger los platos. Sin embargo, la *Visita 3* será una visita de seguimiento, en la que se realizará una breve entrevista y se le pesará. Esta visita le llevará no más de 45 minutos. Asimismo, durante esta visita de seguimiento, se le entregarán el registro de 72 h, el cuestionario de hambre-saciedad, VAS, que tendrá que rellenar los días indicados por nosotros y un bote de orina (para proceder de la misma manera que el día anterior a la *Visita 1*) y entregarlos en la última visita. En esta visita también tendrá que responder a algunos cuestionarios psicológicos.

La última visita (Visita 5) se realizará el día 28, en la que le realizaremos las mismas medidas y pruebas que en la primera visita (Día 0). También completará los cuestionarios psicológicos y hará entrega de los cuestionarios VAS cumplimentados las dos últimas semanas. Al finalizar la visita se le hará entrega de una dieta de mantenimiento o pérdida de peso, según su situación. Esta visita durará aproximadamente 3,5 horas.

¿Qué debe hacer si quiere participar en este estudio?

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

Para participar en este estudio deberá usted firmar el consentimiento informado y una vez evaluemos su analítica y confirmemos que no existe ningún impedimento para su participación, le llamaremos al teléfono que nos haya facilitado para citarle y comenzar las 4 semanas de estudio.

Posibles problemas para su salud

El producto en estudio ha pasado absolutamente todos los controles sanitarios oportunos. Durante la extracción de sangre puede sentir alguna molestia y pueden aparecer

hematomas en la zona del pinchazo, y excepcionalmente puede sufrir mareo. Ninguna otra prueba de las que le realizaremos implica un riesgo para su salud.

Seguro

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

Confidencialidad de los datos

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

Formulario de consentimiento

Efecto de la ingesta de platos preparados incluyendo cacao, como potencial ingrediente funcional, sobre marcadores de riesgo cardiovascular en personas mayores.

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.

Presto libremente mi conformidad para participar en el estudio.

Fecha Firma del participante

Fecha Firma del investigado

Appendix 4: Beck Depression Inventory

BDI. INVENTARIO DE BECK

1.

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

2.

_____0. No me siento especialmente desanimado de cara al futuro.
_____1. Me siento desanimado de cara al futuro.
_____2. siento que no hay nada por lo que luchar.
_____3. El futuro es desesperanzador y las cosas no mejorarán.

3.

_____0. No me siento fracasado.
_____1. he fracasado más que la mayoría de las personas.
_____2. Cuando miro hacia atrás lo único que veo es un fracaso tras otro.
_____3. Soy un fracaso total como persona.

4.

_____0. Las cosas me satisfacen tanto como antes.
_____1. No disfruto de las cosas tanto como antes.
_____2. Ya no obtengo ninguna satisfacción de las cosas.
_____3. Estoy insatisfecho o aburrido con respecto a todo.

5.

_____0. No me siento especialmente culpable.
_____1. Me siento culpable en bastantes ocasiones.
_____2. Me siento culpable en la mayoría de las ocasiones.
_____3. Me siento culpable constantemente.

6.

_____0. No creo que esté siendo castigado.
_____1. siento que quizás esté siendo castigado.
_____2. Espero ser castigado.
_____3. Siento que estoy siendo castigado.

7.

_____0. No estoy descontento de mí mismo.
_____1. Estoy descontento de mí mismo.
_____2. Estoy a disgusto conmigo mismo.
_____3. Me detesto.

8.

_____0. No me considero peor que cualquier otro.
_____1. me autocritico por mi debilidad o por mis errores.
_____2. Continuamente me culpo por mis faltas.
_____3. Me culpo por todo lo malo que sucede.

9.

_____0. No tengo ningún pensamiento de suicidio.
_____1. A veces pienso en suicidarme, pero no lo haré.
_____2. Desearía poner fin a mi vida.
_____3. me suicidaría si tuviese oportunidad.

10.

_____0. No lloro más de lo normal.
_____1. ahora lloro más que antes.
_____2. Lloro continuamente.
_____3. No puedo dejar de llorar aunque me lo proponga.

11.

_____0. No estoy especialmente irritado.
_____1. me molesto o irrito más fácilmente que antes.
_____2. me siento irritado continuamente.
_____3. Ahora no me irritan en absoluto cosas que antes me molestaban.

12.

_____0. No he perdido el interés por los demás.
_____1. Estoy menos interesado en los demás que antes.
_____2. He perdido gran parte del interés por los demás.
_____3. He perdido todo interés por los demás.

13.

_____0. tomo mis propias decisiones igual que antes.
_____1. Evito tomar decisiones más que antes.
_____2. Tomar decisiones me resulta mucho más difícil que antes.
_____3. Me es imposible tomar decisiones.

14.

_____0. No creo tener peor aspecto que antes
_____1. Estoy preocupado porque parezco envejecido y poco atractivo.
_____2. Noto cambios constantes en mi aspecto físico que me hacen parecer poco atractivo.
_____3. Creo que tengo un aspecto horrible.

15.

_____0. Trabajo igual que antes.
_____1. Me cuesta más esfuerzo de lo habitual comenzar a hacer algo.
_____2. Tengo que obligarme a mí mismo para hacer algo.
_____3. Soy incapaz de llevar a cabo ninguna tarea.

16.

_____0. Duermo tan bien como siempre.
_____1. No duermo tan bien como antes.
_____2. Me despierto una o dos horas antes de lo habitual y ya no puedo volver a dormirme.
_____3. Me despierto varias horas antes de lo habitual y ya no puedo volver a dormirme.

17.

_____0. No me siento más cansado de lo normal.
_____1. Me canso más que antes.
_____2. Me canso en cuanto hago cualquier cosa.
_____3. Estoy demasiado cansado para hacer nada.

18.

_____0. Mi apetito no ha disminuido.
_____1. No tengo tan buen apetito como antes.
_____2. Ahora tengo mucho menos apetito.
_____3. he perdido completamente el apetito.

19.

_____0. No he perdido peso últimamente.
_____1. He perdido más de 2 kilos.
_____2. He perdido más de 4 kilos.
_____3. He perdido más de 7 kilos.

20.

_____0. No estoy preocupado por mi salud
_____1. Me preocupan los problemas físicos como dolores, malestar de estómago, catarros, etc.
_____2. Me preocupan las enfermedades y me resulta difícil pensar en otras cosas.
_____3. Estoy tan preocupado por las enfermedades que soy incapaz de pensar en otras cosas.

21.

_____0. No he observado ningún cambio en mi interés por el sexo.
_____1. La relación sexual me atrae menos que antes.
_____2. Estoy mucho menos interesado por el sexo que antes.
_____3. He perdido totalmente el interés sexual.

Appendix 5: State Trait Anxiety Inventory

N.º 124

STAI

A/E	PD= 30	+	—	=	
A/R	PD= 21	+	—	=	

AUTOEVALUACION A (E/R)

Apellidos y nombre ..... *Edad* ..... *Sexo* ..... V ó M

Centro ..... *Curso/Puesto* ..... *Estado civil* .....

Otros datos ..... *Fecha* .....

A-E

INSTRUCCIONES

A continuación encontrará unas frases que se utilizan corrientemente para describirse uno a sí mismo. Lea cada frase y señale la puntuación 0 a 3 que indique mejor cómo se *SIENTE* Vd. *AHORA MISMO*, en este momento. No hay respuestas buenas ni malas. No emplee demasiado tiempo en cada frase y conteste señalando la respuesta que mejor describa su situación presente.

	Nada	Algo	Bastante	Mucho
1. Me siento calmado	0	1	2	3
2. Me siento seguro	0	1	2	3
3. Estoy tenso	0	1	2	3
4. Estoy contrariado	0	1	2	3
5. Me siento cómodo (estoy a gusto)	0	1	2	3
6. Me siento alterado	0	1	2	3
7. Estoy preocupado ahora por posibles desgracias futuras	0	1	2	3
8. Me siento descansado	0	1	2	3
9. Me siento angustiado	0	1	2	3
10. Me siento confortable	0	1	2	3
11. Tengo confianza en mí mismo	0	1	2	3
12. Me siento nervioso	0	1	2	3
13. Estoy desasosegado	0	1	2	3
14. Me siento muy «atado» (como oprimido)	0	1	2	3
15. Estoy relajado	0	1	2	3
16. Me siento satisfecho	0	1	2	3
17. Estoy preocupado	0	1	2	3
18. Me siento aturdido y sobreexcitado	0	1	2	3
19. Me siento alegre	0	1	2	3
20. En este momento me siento bien	0	1	2	3

COMPRUEBE SI HA CONTESTADO A TODAS LAS FRASES CON UNA SOLA RESPUESTA

Ahora, vuelva la hoja y lea las Instrucciones antes de comenzar a contestar a las frases.

Appendix 6: 72-h food recall questionnaire

REGISTRO DE PESADA PRECISA DE 72 HORAS

INSTRUCCIONES PARA LA CUMPLIMENTACIÓN DEL REGISTRO DE PESADA PRECISA DE ALIMENTOS DE 72 HORAS

Este cuestionario consta de un total de 4 páginas, aunque usted puede añadir más si lo considera necesario.

Las tres primeras páginas corresponden cada una a un día, recuerde anotar el día de la semana y la fecha correspondiente.

Usted deberá pesar todos los alimentos que consuma cada día que rellene el cuestionario, intentando no olvidar el aceite que usa para cocinar y para aliñar, así como todas las bebidas que ingiera, incluido el agua de grifo, y los alimentos consumidos entre horas.

Para evitar que se le olvide algún alimento o plato, intente anotarlos nada más comerlos siempre que sea posible.

Recuerde también, en el caso de alimentos envasados, anotar la marca y si es posible adjuntarnos la etiqueta del producto.

Si alguna de las comidas le es imposible pesar (por comer fuera de casa, por ejemplo), intente anotar con medidas caseras, es decir, si el plato era llano u hondo, si estaba lleno, a la mitad, etc., tamaño de la fruta (pequeña, mediana o grande), usar medidas como cucharadas, tazas o puñados, etc..

En el caso de que el plato que ha tomado sea un plato elaborado con varios ingredientes (por ejemplo, paella), utilice la hoja adjuntada al final para describir con toda la precisión posible dicho plato. Si lo necesita puede utilizar más hojas como la que le hemos proporcionado.

Elegir 3 días en total:

- **2 días entre semana:**
- **1 día fin de semana:**

REGISTRO PESADO 72 HORAS
ID PACIENTE:
Fecha:

DIA 1

Hora am/pm	Lugar	Nombre comercial, excepto para los alimentos frescos	Descripción completa de cada uno de los alimentos incluyendo: - Si es fresco, congelado, en conserva… - Como se ha cocinado (plancha, asado, etc) - Tipo de grasa o aceite utilizado	Peso del recipiente (g)	Peso de la comida (g)	Peso de sobras (g)	Peso (g)

DIA 2

Hora am/pm	Lugar	Nombre comercial, excepto para los alimentos frescos	Descripción completa de cada uno de los alimentos incluyendo: - Si es fresco, congelado, en conserva… - Como se ha cocinado (plancha, asado, etc) - Tipo de grasa o aceite utilizado	Peso del recipiente (g)	Peso de la comida (g)	Peso de sobras (g)	Peso (g)

DIA 3

Hora am/pm	Lugar	Nombre comercial, excepto para los alimentos frescos	Descripción completa de cada uno de los alimentos incluyendo: - Si es fresco, congelado, en conserva… - Como se ha cocinado (plancha, asado, etc) - Tipo de grasa o aceite utilizado	Peso del recipiente (g)	Peso de la comida (g)	Peso de sobras (g)	Peso (g)

Utilice esta hoja para las comidas elaboradas en casa a partir de varios ingredientes, ampliando la información de lo que refleja en el registro.

Escriba todos los detalles de los platos cocinados en casa a partir de los ingredientes. Pese todos los ingredientes en crudo y anote debajo sus pesos.

Cuándo se comió: Hora: ……. Día: ……. Fecha: …….

INGREDIENTES	PESO	INGREDIENTES	PESO

Appendix 7: Notebook to register the ready-to-eat meal consumption

REGISTRO DEL CONSUMO DE PLATOS Y POSTRES

Traer este cuaderno en cada visita

- Se le entregará una bolsa con 10 platos y 10 postres preparados (suficiente para 10 días) en cada visita, salvo en la última visita, ya que finalizará el estudio.
- Debe tomar 1 plato y 1 postre diarios.
- En el apartado de observaciones anote los aspectos que crea importantes de nuestro conocimiento (dificultades …)
- En el caso de no consumo de algún plato o postre o parte del mismo indicar en el apartado de observaciones el motivo, la toma en la que no se tomó, así como si la tomó en otro momento.

REGISTRO DEL CONSUMO DE PLATOS Y POSTRES

_____ semana de estudio. Del día ___ al ___ de _____

	_/_/_	_/_/_	_/_/_	_/_/_	_/_/_	_/_/_	_/_/_
¿Ha consumido el plato?	si/no	si/no	si/no	si/no	si/no	si/no	si/no
¿Ha consumido el postre?	si/no	si/no	si/no	si/no	si/no	si/no	si/no
¿Los consumió en la toma indicada?	si/no	si/no	si/no	si/no	si/no	si/no	si/no
¿Los consumió en su totalidad?	si/no	si/no	si/no	si/no	si/no	si/no	si/no
Indique el nombre del plato y del postre que ha consumido	- -	- -	- -	- -	- -	- -	- -
Observaciones							