UNIVERSITAT DE BARCELONA

Effects of Mediterranean Diet on Cardiometabolic Biomarkers in Randomized Clinical Trials: Molecular Mechanisms

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FACULTY OF PHARMACY AND FOOD SCIENCES
PhD PROGRAM: Food Sciences and Nutrition

Effects of Mediterranean Diet on Cardiometabolic Biomarkers in Randomized Clinical Trials: Molecular Mechanisms

Thesis submitted by Javier Hernando Redondo to obtain the degree of Doctor from the University of Barcelona.
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Barcelona, 2024

A mis padres, a mi hermana

A mi abuela

A Marina

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Abstract

Cardiovascular diseases remain the leading cause of death worldwide. This global burden continues to rise due to aging populations, unhealthy diets, sedentary lifestyles, and increasing rates of obesity and diabetes. This work explores the implementation of a Mediterranean diet intervention strategy on cardiovascular and metabolic health, focusing on elderly adults at high cardiovascular risk. Studying data from three subsamples of two randomized controlled studies, PREDIMED and PREDIMED-Plus, the work investigates the mid- and long-term outcomes of a hypocaloric Mediterranean diet (PREDIMED-Plus) promoted with physical activity, versus a traditional Mediterranean diet, on key metabolic markers, inflammatory processes, and gene expression. The first study demonstrates significant improvements in lipid profile, glucose metabolism, leptin levels, and pro-inflammatory markers. These results support the Mediterranean diet's role combined with moderate physical activity as a viable strategy for weight loss, low-grade inflammation and metabolic health improvement in populations with metabolic syndrome.

The second study, framed within the PREDIMED and PREDIMED-Plus trials, investigates gene expression linked to cholesterol efflux process, the first step of reverse cholesterol transport. The focus of the study lies on cholesterol transporters and the cholesterol efflux regulatory molecules involved. Mild upregulation was found in cholesterol-related genes after long-term adherence to an *ad libitum* Mediterranean diet enriched with extra-virgin olive oil or mixed nuts.
The third study also conducted within the PREDIMED trial, examines the relationship between Mediterranean diet supplementation (particularly with extra-virgin olive oil) and the modulation of gene expression related to both cardiovascular and neurodegenerative diseases. Significant gene expression changes were observed in pathways linked to neuroinflammation, suggesting that a cardioprotective diet like the Mediterranean diet may offer neuroprotective benefits. This modulation of inflammation-related genes may underlie the diet's protective effects against cognitive decline and neurodegenerative diseases, such as Alzheimer's disease, particularly in high-risk elderly populations.

In summary, these studies provide strong evidence for the Mediterranean diet's efficacy in improving cardiovascular health and regulating gene expression associated with reverse cholesterol transport and neuroinflammation. However, limitations include the specific focus on high-risk populations, limiting the generalizability of findings to broader populations. Future research should aim to explore these effects in more diverse cohorts and investigate the long-term neuroprotective effects of such dietary interventions.

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Abbreviations

ABCA1: ATP Binding Cassette Subfamily A Member 1
ABCG1: ATP Binding Cassette Subfamily G Member 1
ADA: American Diabetes Association
ASCVD: Atherosclerotic Cardiovascular Diseases
BBB: Blood-Brain Barrier
BMI: body mass index
CAD: Coronary Artery Disease
CHD: Coronary Heart Disease
CEC: Cholesterol Efflux Capacity
CRP: C-Reactive Protein
CVDs: Cardiovascular Diseases
DBP: Diastolic Blood Pressure
DM: Diabetes Mellitus
EC: Endothelial Cell
ESC: European Society of Cardiology
ESH: European Society of Hypertension
EVOO: Extra Virgin Olive Oil
Hba1c: Glycated Hemoglobin A1c
HDL-c: High-Density Lipoprotein Cholesterol
IL-6: Interleukin-6
IL-1b: Interleukin-1 Beta
LDL-c: Low-Density Lipoprotein Cholesterol
MedDiet: Mediterranean Diet
MetS: Metabolic Syndrome
MUFA: Monounsaturated Fatty Acids
NCDs: Non-Communicable Diseases
NDD: Neurodegenerative Disease
NO: Nitric Oxid
NR1H2/LXR-B (Liver X receptor β (LXR-β)): Nuclear Receptor Subfamily 1, Group H, Member 3 / Liver X receptor beta
NR1H3/LXR-A (Liver X receptor alpha (LXR-α)): Nuclear Receptor Subfamily 1 Group H Member 2 / Liver X receptor alpha
PPARs: Peroxisome Proliferator-Activated Receptor
PPARA (PPAR-α): Peroxisome Proliferator-Activated Receptor alpha
PPARD (PPAR-β/δ): Peroxisome Proliferator-Activated Receptor beta/delta
PPARG (PPAR-γ): Peroxisome Proliferator-Activated Receptor gamma
PUFA: Polyunsaturated Fatty Acids
RCT: Randomized Controlled Trial
RXRA: Retinoid X Receptor Alpha
RXRB: Retinoid X Receptor Beta
SBP: Systolic Blood Pressure
SCARB1/SR-B1: Scavenger Receptor Class B Type 1
SFA: Saturated Fatty Acids
TNF-α: Tumor Necrosis Factor-Alpha
T1DM: Type 1 Diabetes Mellitus
T2DM: Type 2 Diabetes Mellitus
VAT: Visceral Adipose Tissue
WHO: World Health Organization

INTRODUCTION

EPIDEMIOLOGY OF CARDIOVASCULAR DISEASE

Global burden of cardiovascular diseases (CVDs) still represents a significant number of deaths worldwide. Recent report providing updated data across the five continents, illustrated that ischemic heart disease remains the leading cause of global CVD mortality (Figure 1) (1). CVDs continue to be the leading cause of mortality in European countries (3.9 million per year), with rates strongly influenced by socioeconomic status (2). In Spain, according to National Statistics Institute, in 2022 diseases of the circulatory system ranked first as the leading cause of mortality (3). For the first time, in 2023, oncology diseases were the leading cause of mortality (4).

According to the World Health Organization (WHO), CVDs are defined as a group of disorders affecting heart and blood vessels, including: 1) coronary heart disease (CHD) – affecting the blood vessels supplying the heart muscle; 2) cerebrovascular disease – a disease involving the blood vessels supplying the brain; 3) peripheral arterial disease – a disease of blood vessels supplying the arms and legs; 4) congenital heart disease – birth defects that affect the normal development and functioning of the heart caused by malformations of the heart structure present from birth; and 5) deep vein thrombosis and pulmonary embolism – blood clots in the leg veins, which can dislodge and move to the heart and lungs (5).

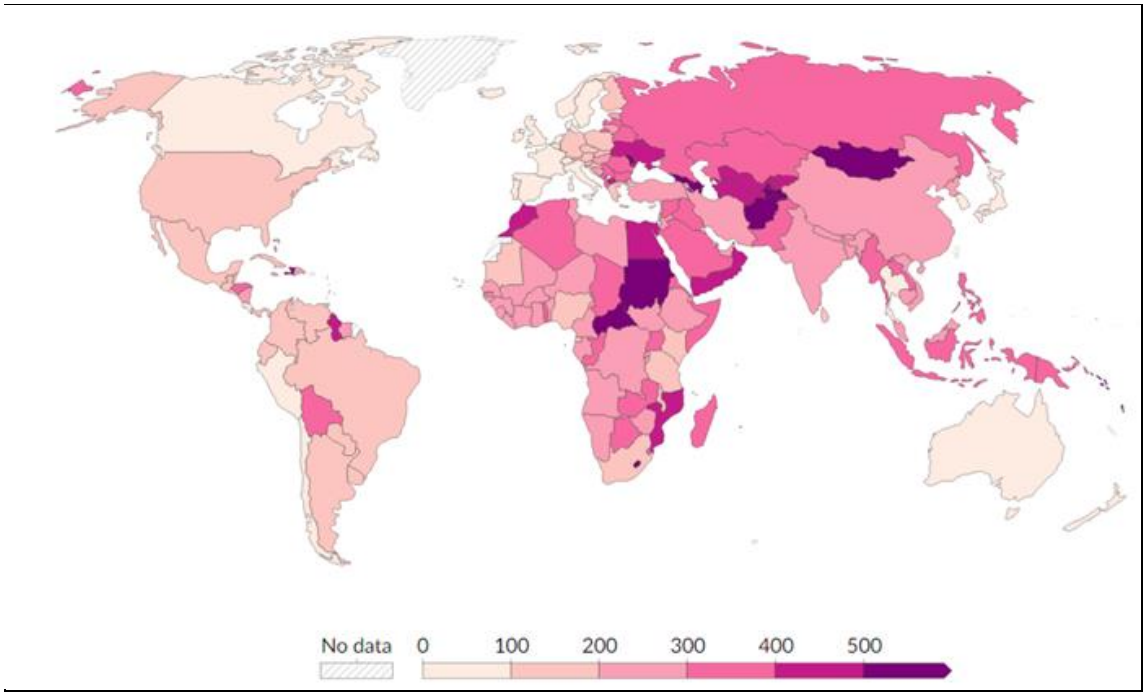

Figure 1 - Estimated death rate from cardiovascular diseases per 100,000 people in world population, 2019 (6)

Stronger preventive measures and investment in early diagnosis and treatment are essential. Population-level policies, including fiscal and regulatory measures targeting food policy, alcohol intake, physical activity (physical activity), and smoking, can yield significant health and economic benefits by reducing CVD mortality. Cost-effective policies that can quickly impact and alleviate pressures on the healthcare system are necessary. Reducing social inequalities in CVD requires targeted policies in deprived communities, along with broader structural policies to improve diets, increase physical activity, and reduce smoking and alcohol intake (7,8).

NON-COMMUNICABLE DISEASES

Noncommunicable diseases (NCDs) are non-transmissible diseases responsible for 41 million deaths annually, equivalent to 74% of all deaths globally. Of all NCD deaths, in 2021 77% were in low- and middle-income countries (Figure 2). CVDs account for most NCD deaths, around 17.9 million people annually (9). European data from 2016 estimated around two thirds of all deaths in the European region resulted from diabetes (E10-E14), cardiovascular diseases (ICD-10: I00-I99), chronic respiratory diseases (J40-J47), and cancers (C00-C97), the so-called NCDs (10).

There are significant disparities in life expectancy among different socioeconomic groups particularly concerning CVD, with low- and middle-income countries experiencing much higher premature mortality rates than others (2,9).

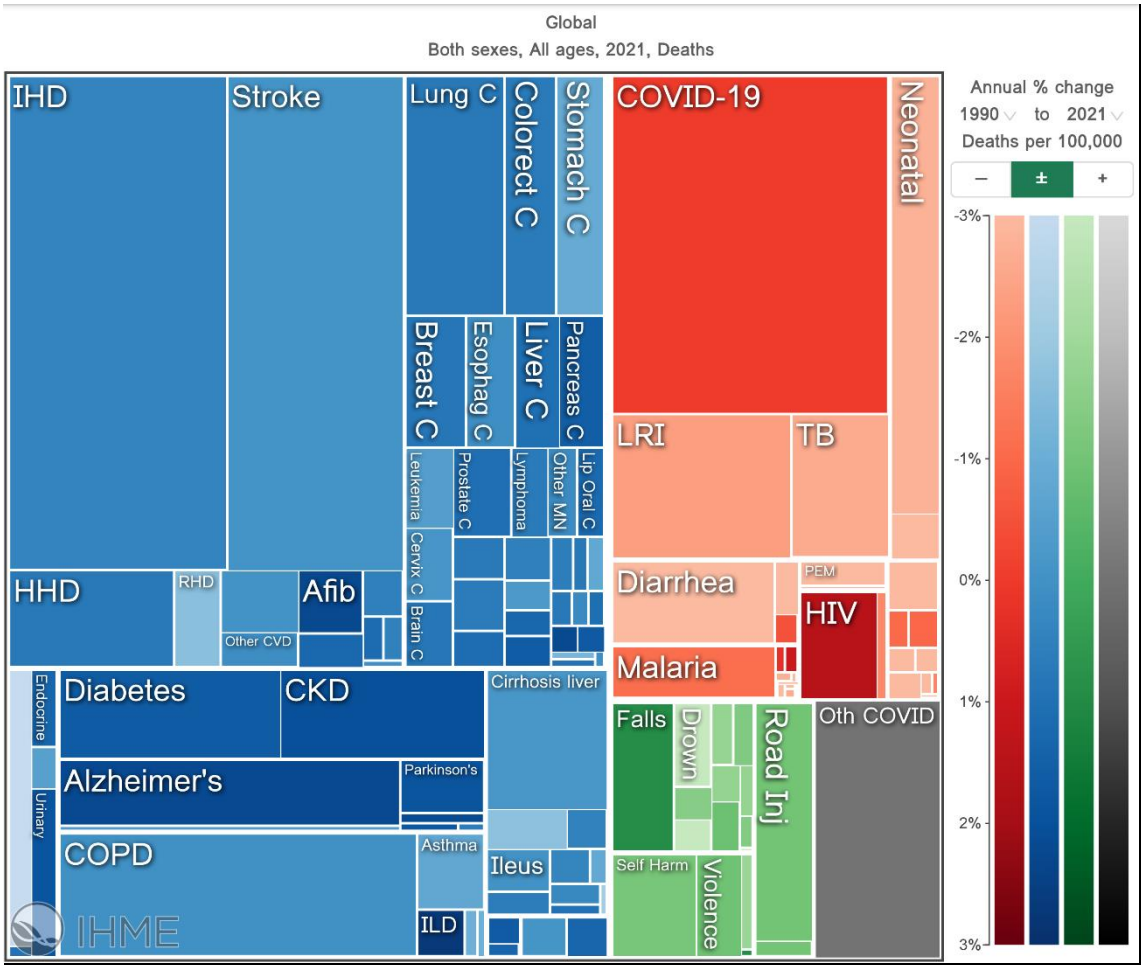

Figure 2 - Square pie chart of global mortality causes (11)

The four key behavioral factors affecting mortality are tobacco use, alcohol consumption, lack of physical activity and unhealthy diet. In this regard, overweight and obesity constitute the leading risk factor causing disability, even though morbidity rates may be underestimated. Multiple determinants have an influence on the environment contributing to the creation of the coined term “obesogenic environment”. Some of them are visible (diet or physical activity) however, others remain less tangible (urbanism, business or education) (12).

ATHEROSCLEROSIS

Atherosclerosis is a chronic, progressive, and lipid-driven inflammatory disease located in the arterial wall, characterized by the formation of fibrofatty plaques. As advanced plaques form, the artery wall becomes stiff, and its inner space narrows. Sometimes, these plaques can rupture or erode, leading to serious clinical outcomes (13,14).

The vessel wall is composed of three layers; tunica intima (inner layer), tunica media (middle layer), and tunica adventitia (outer layer). The tunica intima is a single layer of endothelial cells combined with collagen and elastic fibers facing the blood flow, directly exposed to the blood and shear forces (15). The initial step of atherosclerosis primarily begins with the accumulation of certain plasma lipoproteins, such as low-density lipoproteins (LDLs) and triglyceride-rich lipoprotein remnants, in the intimal region. This event tends to occur in arterial bifurcations, where blood flow is turbulent, increasing the permeability of endothelial cells, leading to the trapping of lipoproteins (Figure 3) (15–17). Activation of endothelial cells occurs by the mere flow disturbance (17,18), subsequently augmented by the oxidation of lipids and lipoproteins plus stacked debris in the nascent lesion. Endothelial dysfunction is also triggered by various factors, including age, diabetes, obesity, and hypertension (age, diabetes, obesity, hypertension), and is a key regulator of vascular tone, cellular adhesion, proliferation of smooth muscle cells, inflammation, and thrombosis (18–21). The inflammatory landscape enhances expression of chemotactic, chemoattractant and adhesion molecules, which facilitates monocytes recruitment to the atherosclerotic core (16,21). Once located in the lesion, monocytes enter the intima and differentiate into macrophages, highly-active tissue cells (13).

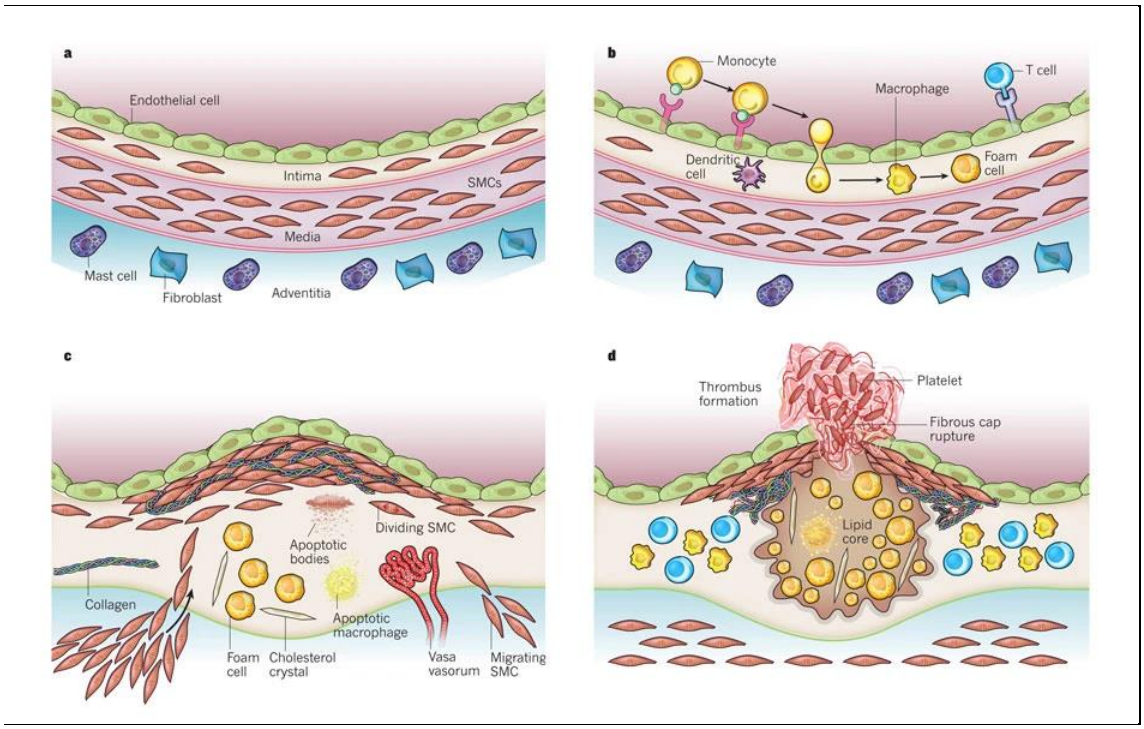

Figure 3 – Stages in the development of atherosclerotic lesions (14)

Once the initial stage of the lesion is established, T lymphocyte cells also infiltrate into the area and vascular smooth muscle cells transform and migrate into the intima (16). Advanced lesions undergo a transition from fatty streak to a fibrous state, characterized by a connective tissue matrix composition and lipid-free inner core loaded with apoptotic cells. This is partially caused by defective efferocytosis, which is the ability to remove apoptotic cells from injured location. In addition, cholesterol crystal deposition and calcification of the plaque stimulate enlarge the necrotic core (21–23).

The rupture of the atheroma plaque depends on the composition of the plaque more than the size itself: a thinner fibrous cap with larger lipid/necrotic core characterize the unstable plaques (24). Plaque rupture manifests in macrovascular complications, such as acute myocardial infarction or stroke (25). It usually occurs unpredictably, though inflammation has been associated with plaque instability. Foam cells tend to concentrate in the thinnest segment, which is regarded as the weakest point and the most likely to break. The thrombotic response occurs spontaneously after the rupture and is still not fully understood, including both the onset of the ischemia and the thrombogenic potential of the plaque material. However, it is well-established that different factors significantly increase the risk and severity of thrombosis (26).

It is speculated that differences in the composition of atheromatous plaques across European populations may contribute to the mortality rate gap from ischemic heart disease between southern and northern Europe. Research on the field suggests that plaques in northern European countries have a higher lipid content compared to those in southern countries (27).

CARDIOVASCULAR RISK FACTORS: TRADITIONAL AND EMERGENT

The Framingham Heart Study (FHS) was instrumental in identifying the "traditional cardiovascular risk factors" and has significantly influenced the estimation of developing CVD. The first significant findings included the association between hypertension, obesity, and hypercholesterolemia, with greater incidence of atherosclerotic heart disease. Later, age, male sex, diabetes, and left ventricular hypertrophy were also recognized as valuable variables for risk stratification (28,29). Over the years the FHS has collected multiple CVD phenotypes, such as blood biomarkers (including genetics, and 'omics'), urine biomarkers, imaging tests, vascular function tests, and adverse clinical outcomes (29). The “traditional” risk factors historically used in equations to predict cardiovascular events usually comprise overweight, diabetes, dyslipidemia, hypertension, and lifestyle habits (sedentarism/physical activity and tobacco consumption). These factors do not account for variability in CVD across all the populations (30). Emerging risk factors contribute to established paradigm from various aspects of physiopathology: inflammation, thrombosis, myocardial injury, HDL functionality, oxidative stress, adipogenesis, metabolic processes and lipoprotein metabolism such as LDL atherogenicity, HDL functionality, or Lp(a) levels (29,31,32). Besides the traditional and emergent factors, environmental factors evidence is growing due to the impact that gradually is being uncovered. These factors include extreme temperatures caused by climate change, air pollution, noise or urban environment (33–37).

Risk factors can also be classified as modifiable or non-modifiable, depending on their reversibility and whether they can be mitigated through lifestyle changes or drug therapy (38). Ongoing research for new markers in CVD pursues an accurate stratification of CVD risk in the new era of precision medicine. The FDA defines a biomarker as “a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions.” Several categories have been outlined to classify a biomarker’s usefulness, including diagnostic, prognostic, safety, or predictive (39).

Age

Age is a non-modifiable risk factor involved in the development of pathologies strongly associated with mortality rates (Figure 4). Age-associated physiological changes manifest in different organs and tissues. Vascular remodeling, such as increased vascular intima thickness or stiffness, usually precedes preclinical states, and can predict future cardiovascular events, in fact, these alterations are thought to occur before blood pressure increases and the development of hypertension (40). Cardiac structure is also affected by aging, thickness of left ventricular wall,

or increase the atrial size, are frequently observed transformations (41). The endothelium constitutes a highly heterogeneous monolayer (42) coating the luminal face of blood vessels. Numerous and complex functions has been attributed to endothelium, related to blood fluidity and coagulation, fluid filtration, promoting vascular tone, growth and integrity maintenance (19). In this regard, age-related changes has been observed, including the loss of competence, progression to a stiffer vascular tree, along with an impaired angiogenic and repairing processes, and a natural inclination to proinflammatory or prothrombotic status (19,21,40,43,44). Although multiple factors are known to damage the endothelium, dysfunction has been observed over the years even in healthy, normotensive individuals (40).

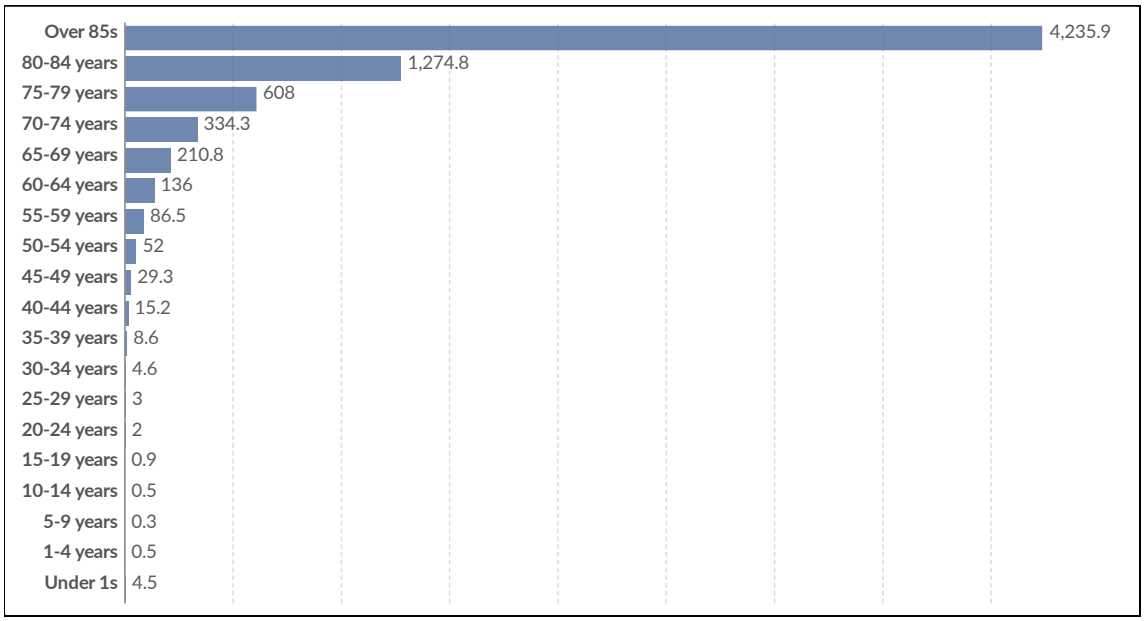

Figure 4 - Death rate from cardiovascular diseases by age group, Spain, 2021 (45)

The molecular mechanisms explaining aging are complex, and not completely elucidated; however, increasing evidence sheds light on the intricate hallmarks of aging: telomere attrition, genomic instability, stem cell exhaustion, epigenetic alterations, reactive oxygen species accumulation (ROS) or deregulated nutrient sensing among others (20,46). In particular, CVD development is characterized by a decline in the production of angiogenic cytokines, a drastic reduction in stem cells responsible for vascular homeostasis, and an unavoidable impairment of the endothelium; processes that cumulatively increase the overall risk of developing atherosclerotic disease (47,48). Even though aging is inevitable, biological processes can be tackled to decelerate the ongoing deleterious effects of time. Factors associated with lifestyle habits like physical activity, smoking habit or dietary patterns play a crucial role.

The homeostasis of cholesterol metabolism is regulated by multiple factors, including cholesterol synthesis, intestinal cholesterol absorption, hepatic cholesterol uptake, cholesterol excretion, bile acid production, and deconjugation by intestinal microflora and subsequent excretion (49,50). The disturbance of these factors can explain the dysregulation of cholesterol metabolism linked to aging (51). High-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) levels worsen with age, respectively decreasing and increasing. Several mechanisms have been proposed to explain these phenomena, such as the impairment of LDL particles removal, the increased catabolism of HDL, and aside from strict circulating levels, the HDL become inefficient transporting cholesterol over time (51,52).

Among the facts contributing to CVD advancement, loss of HDL functionalities is notably detrimental. Aging is characterized by alterations in high-density lipoprotein (HDL) composition and function (53), leading to decreased cholesterol efflux capacity (CEC) (54,55), accompanied

by a reduction of antioxidant activity (56) and an increased susceptibility to oxidation (57). These changes negatively affect cholesterol removal, favoring the accumulation of LDL-c. Susceptibility of LDL to oxidation is also affected in the elderly (58,59). This process has been partially attributed to Paraoxonase-1 activity effects over time (56,60,61).

Sex and gender

It's important to recognize the difference between sex and gender, as they carry distinct implications for an individual's experiences and identity. According to the WHO, sex refers to the biological and physiological characteristics that classify someone as female, male, or intersex, including chromosomes, hormones, and reproductive organs. Gender, on the other hand, refers to the socially constructed characteristics associated with being a woman, man, girl, or boy, such as behaviors, roles, expectations, and norms that vary across cultures and change over time (Figure 5) (62,63). Sex represents an inherent risk factor for CVD, with varying clinical manifestations between sexes, being cerebrovascular disease more prevalent in females (64) while ischemic heart disease is more prevalent among males (65).

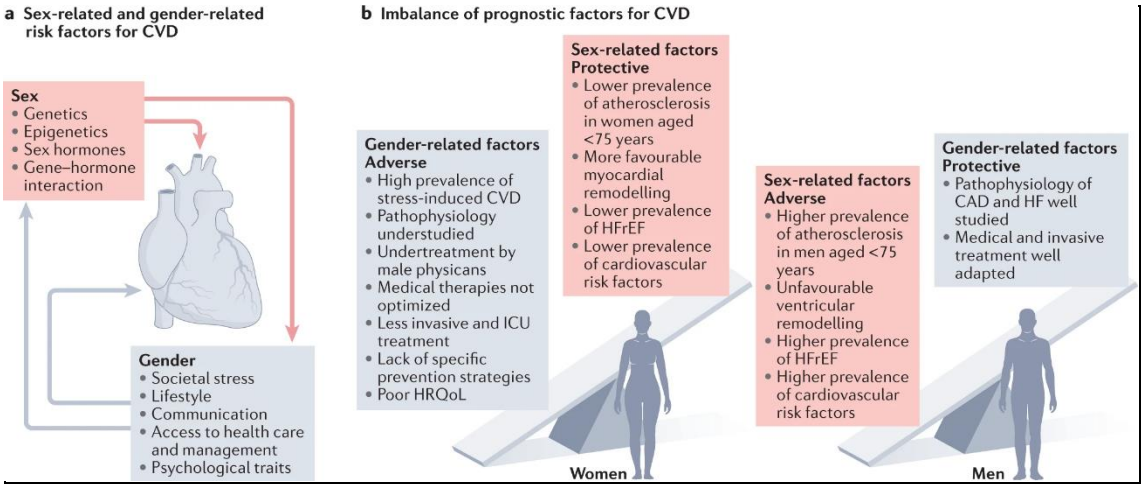

Figure 5 - Sex-related and gender-related disparities in CVD risk and outcomes (66)

Physiological sex disparities have been widely reported through years. Women experience unique situations related to menopause and pregnancy-related disorders (67,68). Both have been already demonstrated to involve particular cardiovascular complications that need to be accounted for (69). Data collected showed a lower incidence of coronary artery diseases (CAD) in women during the reproductive age (70), which increases during menopause (71). Overall, the onset of CAD disease tends to appear 6 to 8 years later in men (62), just as the development of atherosclerosis (70). Another differential biological factor is the role of hormones in lipid metabolism, with men showing higher pro-atherogenic lipid profile (72). Positive association between androgen levels and proatherogenic lipid profile has been previously reported (71). Blood pressure values are also highly influenced by sex. Blood pressure begins to rise earlier in men, however, analyzing trajectory courses by sex, the pattern displayed showed a faster increase in blood pressure in women (73).

Among gender-related disparities, psychosocial factors impact mortality and morbidity rates of cardiovascular diseases (74). Women more frequently experience disadvantages such as unemployment or lack of social support, which in turn result in higher levels of anxiety and depression. Women, especially belonging to minority ethnicities, also have unequal access to wealth and income, with poorer cardiovascular health and wellbeing status. In addition, women continue to be at a disadvantage because they remain severely under-represented in CVD clinical trials (75–77). The death rates per 100,000 population from 2000 to 2020 in Spain consistently show higher mortality rates from CVD in females compared to males (Figure 6).

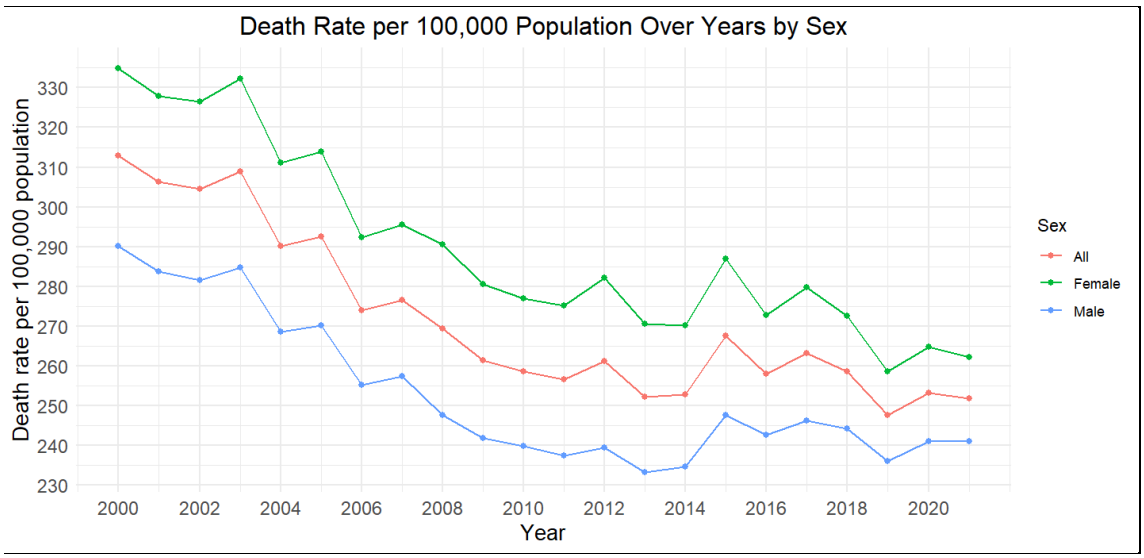

Figure 6 - Death rate sex stratified per 100,000 population caused per CVD (ICD-10 codes: I00-I99) in Spain (2000-2020). Adapted from https://platform.who.int/mortality/themes/theme-details/topics/topic-details/MDB/cardiovascular-diseases

Race and ethnicity

"The terms used for race, ethnicity, culture, and similar constructs often spark controversy due to issues with clear definitions and overlapping content" (78). Based on American Psychological Association race is a social construction and categorization of people based on perceived shared physical traits that result in the maintenance of a sociopolitical hierarchy. Ethnicity is defined as a characterization of people based on having a shared culture (e.g., language, food, music, dress, values, and beliefs) related to common ancestry and shared history. The meaning of these terms is continually under review and strong critical analysis (78).

The biological aspect of the socially defined concept of race is being abandoned by academics (biologists, anthropologists, and geneticists), claiming it is not a discrete variable that allows us to distinguish by physical characteristics. Racial groups are no longer considered to comprise biological variation as a whole (79,80). From an epidemiological standpoint data show that racially and ethnically minoritized patients experience higher rates of morbidity and mortality from CVD (81), probably due to the significant influence of factors such as socioeconomic status, income, education, neighborhood conditions, perceived racism, environmental exposures, healthcare access, and other social determinants of health (82,83). However, there is ongoing controversy regarding whether race or racism should be considered the risk factor, reflecting broader debates about the definition and interpretation of risk factors (84,85).

However, historical risk factors have been described in self-reported ethnic groups, therefore, evidence collected so far has been gathered in this manner. In this regard, different epidemiological studies have studied the prevalence of cardiovascular risk factors. Twenty-year trends in the United States population showed BMI, systolic blood pressure and Glycated Hemoglobin A_1c (HbA1c) to be increased in the black population compared to white population. Adjustments for income, home ownership, employment, health insurance, and access to health care were considered. Obesity, hypertension, and diabetes were more prevalent among Black participants compared to White participants, while hypercholesterolemia was less prevalent (86,87). Diabetes is also more present among Hispanics (88). Regarding hypertension, Black people have shown higher blood pressure values than White population (88). Mortality rates from all CVDs were significantly higher in African Americans compared to the White population (87).

The vast majority of research has been performed on White population, although some efforts towards validating prediction algorithms on different ethnic groups have been made (89). In this line, calibration of FHS risk models for specific racial/ethnic cohorts (non-Hispanic White (NHW), non-Hispanic black (NHB), and Mexican American (MA)) have yielded optimal results predicting mortality rates. However, risk factors displayed varying degrees of association with CVD mortality, and the prevalence of the risk factors varied among different racial and ethnic groups (90). Further investigations have demonstrated in different cohorts that the FHS prediction functions perform reasonably well for both White and Black men and women. However, when applied to Japanese American and Hispanic men, as well as Native American women, recalibration was necessary (91).

Hypertension

Hypertension is a well-recognized risk factor for CVD. Adult population presenting with systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 80 mmHg is the cutoff values presented by the American College of Cardiology (ACC)/American Heart Association (AHA). For elevated blood pressure (SBP between 120-129 mmHg and DBP < 80 mmHg) or stage 1 hypertension (blood pressure 130-139/80-89 mmHg) nonpharmacological recommendations are proven to be beneficial for prevention and treatment of hypertension, including weight loss, healthy diet, reduced sodium intake, physical activity and moderated alcohol intake. Guidelines uniformly recommend pharmacological therapy when blood pressure ≥ 140/90 mmHg (92), although threshold for hypertension varies between ACC/AHA (>130/80 mm Hg) and the European Society of Cardiology (ESC)/European Society of Hypertension (ESH) (93). Mediterranean diet (MedDiet) has consistently shown an improvement in blood pressure associating higher adherence with lower blood pressure (94–96).

Smoking

Certain chemicals in cigarette smoke induce oxidative stress, which activates endothelial cells, macrophages, and platelets. This activation prompts the release of proinflammatory cytokines, C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), leading to endothelial dysfunction. Cigarette smoking also impairs the vasodilatory function of the endothelium, largely due to a reduction in nitric oxide (NO) availability. NO, produced by endothelial cells, is crucial for vasodilation and regulation of vascular tone. In addition, tobacco consumption introduces a large number of free radicals, particularly from its gas and tar phases. These radicals initiate oxidative stress, a key factor in the progression of atherosclerosis because LDL, which are highly susceptible to oxidation, accumulate in the arterial wall, and are more easily absorbed by macrophages (97,98).

Epidemiological studies have demonstrated that smoking significantly impacts long-term cardiovascular health by increasing the risk of various CVD subtypes (99). Smokers experience an earlier onset of CVD and a greater risk of fatal CVD events as their first presentation of the disease. Additionally, smoking is associated with a higher long-term risk of heart failure, particularly pronounced in younger men and older women. Overall, smoking accelerates the onset of CVD, reduces overall survival, and contributes to a higher burden of CVD across all age and sex groups (100). Not only active smoking is harmful for health, but secondhand smoking has also been observed in epidemiological studies to increase CHD, with the estimated detrimental effects being much larger than expected (101).

From an economic standpoint, reducing smoking prevalence yields substantial short-term benefits, particularly in terms of reducing hospitalizations for acute myocardial infarctions (AMIs) and strokes, as well as saving significant amounts of money in direct medical costs (102).

Overweight and obesity

Defined as accumulation of excessive fat deposits, overweight is the pre-stage of obesity. Obesity is defined as a chronic relapsing disease caused by the excess of adipose tissue, with a detrimental impact in the development or prognosis of different conditions (Figure 7) (62). Currently regarded as a pandemic, different transformations in Western societies have driven to favor this "obesogenic" landscape: economic growth, industrialization, mechanized transportation or urbanization. From the behavioral standpoint, the dietary pattern has shifted towards a higher consumption of sweetened, high-density and nutrient-poor food than recommended. This is probably caused by accessibility and inexpensiveness that characterize ultra-processed food. To complete the picture, sedentary lifestyle has become a widespread reality that adds up to the current scenario (62,103,104).

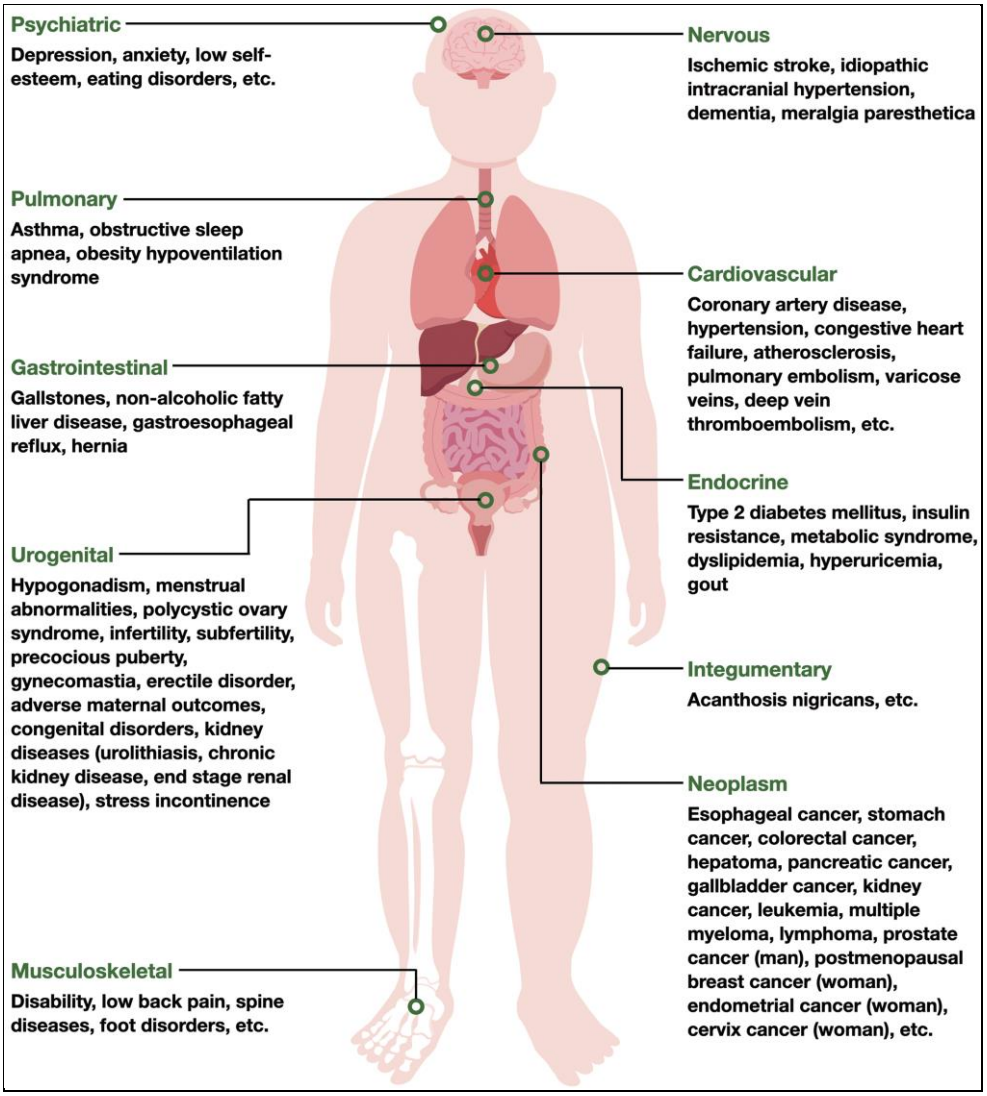

Figure 7 - Main and additional conditions of the MetS as the consequences of obesity (105)

The most accessible approach to measure is body mass index (BMI), which is calculated as weight (kg)divided by height squared (m^2). BMI is usually categorized by establishing thresholds:

	Age	Indicator	Normal weight	Overweight	Obese
Adults	> 20 years	BMI (kg/m^2)	18.50 to 24.99	25 to 29.99	≥30.00 Class1: 30 to 34.99 Class2: 35 to 39.99 Class3: ≥ 40.00

Table 1 Adapted from (103) – Classification of overweight/obesity grades based on BMI values

Abdominal adiposity has been strongly correlated with total body fat. Waist circumference, a measure of abdominal adiposity, is widely recognized as an indicator of cardiovascular risk worldwide (106,107).

Population	Organization	Normal Waist Circumference Threshold for Abdominal Obesity	
		Men	Women
Europid	IDF	≥ 94 cm	≥ 80 cm
Caucasian	WHO*	≥ 94 cm ≥ 102 cm	≥ 80 cm ≥ 88 cm
United States	AHA/NHLBI (ATP III)*	≥ 102 cm	≥ 88 cm
Canada	Health Canada	≥ 102 cm	≥ 88 cm
European	European Cardiovascular Societies	≥ 102 cm	≥ 88 cm
Asian (including Japanese)	IDF	≥ 90 cm	≥ 80 cm
Asian	WHO	≥ 90 cm	≥ 80 cm
Japanese	Japanese Obesity Society	≥ 85 cm	≥ 90 cm
China	Cooperative Task Force	≥ 85 cm	≥ 80 cm
Middle East, Mediterranean	IDF	≥ 94 cm	≥ 80 cm
Sub-Saharan African	IDF	≥ 94 cm	≥ 80 cm
Ethnic Central and South American	IDF	≥ 90 cm	≥ 80 cm

Table 2 (Adapted from (106)) - Current Recommended Waist Circumference Thresholds for Abdominal Obesity

Differences can also be encountered in the fat type proportion, with a higher risk for individuals who have greater visceral adipose tissue compared to subcutaneous adipose tissue (62,107). Visceral adipocytes are metabolically more active and exhibit a pro-inflammatory profile. They usually comprise the highest proportion of adipose tissue in the abdominal cavity (108). The expansion of adipose tissue resulting from a positive energy balance consists of an increase in the number of adipocytes, referred to as hyperplasia, and the enlargement of adipocyte size, a process known as hypertrophy (109).

The endocrine role of adipose tissue has recently been established within the scientific community. Currently, the paradigm of adipose tissue is now considered a potent organ with capacity to secrete hormones, cytokines and adipokines, which contributes to an increased inflammatory status (110–114). In this regard, research on MedDiet has revealed an attenuating effect on the inflammatory biomarkers (115–117). One of the most frequent comorbidities of obesity is type 2 diabetes mellitus (T2DM), where fat mass proportion and distribution correlate with glucose metabolism impairment (impaired insulin sensitivity and β-cell secretion, hyperinsulinemia, elevated fasting glucose levels) (107,118,119). In this line, adipokines, cytokines produced by adipose tissue, are elevated in T2DM patients with impaired glucose-related metabolic tests (120).

Diabetes

Diabetes mellitus (DM) constitutes a chronic metabolic condition characterized by the presence of hyperglycemia in the absence of treatment. It affects an estimated 537 million individuals around the world, with a prevalence rate of 10.5 %, based on data from 2021 (121). The diagnosis is mainly established from biochemical tests assessing plasma glucose levels; either the fasting concentration, 2-hour levels during the glucose tolerance test, random glucose, and HbA1c concentrations (Table 3) (121,122). HbA1c has shown strong evidence associating circulating levels with clinical outcomes. In fact, clinical trials have elucidated that improvements in HbA1c levels reduce the risk of microvascular complications (123).

Glycemic biochemical criteria	**ADA**	**ESC**
FPG	FPG ≥126 mg/dL (≥7.0 mmol/L)	
2-hours PG during OGTT*	≥200 mg/dL (≥11.1 mmol/L)	
HbA1c	HbA1c ≥ 6.5% (≥ 48 mmol/mol)	
RPG	≥200 mg/dL (≥11.1 mmol/L)	
* FPG: fasting plasma glucose, OGTT: oral glucose tolerance test, HbA1c: glycated hemoglobin, RPG: random plasma glucose		

Table 3 (adapted from ESC (European Society of Cardiology) and ADA (American Diabetes Association) guidelines (121,122)) - Diagnostic Criteria for Diabetes: Comparison Between ESC and ADA Guidelines

DM is usually classified according to the etiology causing the hyperglycemic state. Type 1 diabetes mellitus (T1DM) is caused by destruction of pancreatic islets due to an autoimmune process, leading to deficiency in insulin synthesis. This type accounts for 5 - 10 % of all cases of diabetes and is most commonly diagnosed in individuals under the age of 35 presenting with diabetes symptoms. T2DM encompasses 90 – 95% of all-type diabetes, and is defined by insulin resistance and, a relative inadequacy of insulin secretion. In absolute terms, plasma insulin levels (both fasting and after food intake) are typically elevated; however, they are insufficient to maintain normal glucose homeostasis. Concerning this matter, the Homeostasis Model Assessment (HOMA) is a parameter that estimates insulin resistance (HOMA-IR) and β-cell function (HOMA-B) from fasting glucose and insulin levels. It correlates well with established techniques like the euglycemic clamp and hyperglycemic clamp (124). Despite moderate precision, HOMA effectively quantifies insulin resistance and β-cell dysfunction, supporting the role of a feedback loop in glucose-insulin regulation (125), although some caveats have been reported regarding reproducibility and statistical distribution, requiring careful application (126).

In the field of carbohydrate metabolism, GLP-1 (glucagon-like peptide-1) has become a significant biomarker. This peptide hormone, which is released from the intestines in response to

food intake, plays a crucial role in regulating blood glucose levels by enhancing insulin secretion and inhibiting glucagon release (127). Additionally, GLP-1 slows gastric emptying and contributes to satiety, promoting fullness and reducing food intake, thereby further supporting glucose control and weight management (Figure 8) (128). Monitoring GLP-1 levels provides valuable insights into an individual's metabolic status and the effectiveness of therapeutic interventions for managing conditions such as T2DM (129), and also for lowering the risk of cardiovascular death, heart attack, and stroke in adults who have cardiovascular disease and either obesity or overweight (130).

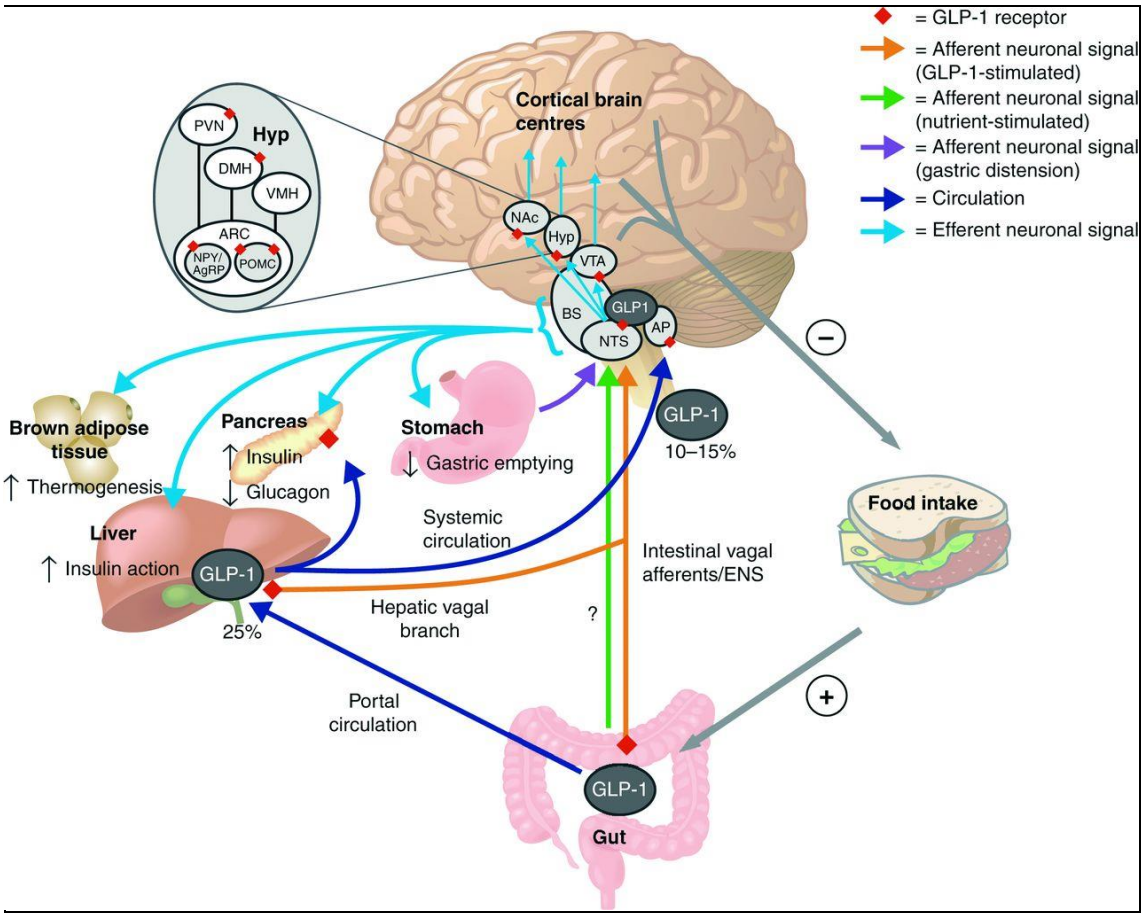

Figure 8 - Proposed routes of action of GLP-1 in the central regulation of feeding and glucose metabolism (128)

There are other entities of DM, also known as hybrid forms: the slowly evolving immune-mediated diabetes and ketosis-prone diabetes. Additionally, gestational diabetes mellitus is a diabetes subtype, which constitutes one of the most frequent medical complications during pregnancy, which can cause problems at delivery and postpartum, and may lead to an increase in cardiovascular risk in the future. Lastly, genetic advances in the last decades have encouraged the unraveling of molecular mechanisms behind diseases (defects in β-cells or insulin receptors) (121,122,131–133).

Among the already mentioned NCDs, T2DM has an enormous negative impact in both health indicators (morbidity and premature mortality), and economic costs (at individual and collective level), especially in developing countries (134,135). However, Western countries have also been experiencing a disproportionate increase of T2DM, probably driven by the sedentary lifestyles, and consumption of ultra-processed and high-calorie foods (2,136). In this regard, European countries have committed to collaborate in reducing DM burden, working on priority points such as prevention, or care and management of T2DM (137,138). The effect of emerging dietary patterns in Western societies contributes to the early development of prediabetic states or the onset

of T2DM. On this subject, Mediterranean dietary pattern consisting of several pieces of fruit, minimally processed wholegrains, complex carbohydrates and vegetables has proven to reduce the risk of diabetes (139–143).

Structural microvascular damage precedes the development of cardiovascular events in patients with T2DM, whereas changes in microvascular function occur before microangiopathy. Mechanisms through which hyperglycemia causes vascular damage include activation of nuclear factor κ-B (NFκB), which increases gene expression in endothelial cells, monocyte activation, and vascular smooth muscle cell proliferation. T2DM patients often present abnormalities in lipoprotein particles, reduction in HDL-c levels, and an increase in triglyceride-rich lipoproteins, along with an elevated oxidative and inflammatory status that accelerate atherosclerosis (144). At the genetic and epigenetic level, both T2DM and CVD have been found to share common traits that predispose individuals to either condition, which may lead to potentially useful biomarkers in the future (145).

Dyslipidemia

Dyslipidemia is a condition characterized by abnormal lipid and/or lipoprotein levels, grouping elevated triglycerides (hypertriglyceridemia) and LDL cholesterol (LDL-c), along with reduced HDL-c levels (146). Different classifications have been proposed over time for dyslipidemia. In 2001, the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III, or ATP III) established the following classification (in mg/dL):

Variable	Level (mg/dL)	Classification
LDL-c	<100	Optimal
	100-129	Near or above optimal
	130-159	Borderline high
	160-189	High
	≥190	Very high
Total cholesterol	<200	Desirable
	200-239	Borderline high
	≥240	High
HDL-c	<40	Low
	≥60	High
Triglycerides	<150	Normal
	150-199	Borderline high
	200-499	High
	≥500	Very high

Table 4 - ATP III Dyslipidemia Classification Criteria (mg/dL)

Dyslipidemia is generally described as the total cholesterol, LDL-c, triglycerides, apo B or lipoprotein (a) levels above the 90th percentile or HDL and apo A levels below the 10th percentile of the general adult population (147). Optimal lipid levels differ based on a person's age, sex, and various risk factors . Primary dyslipidemia is caused by genetic mutations affecting specific points in pathways of lipid metabolism. Secondary dyslipidemia results from various causes related to lifestyle habits (lack of physical activity, unhealthy nutrition patterns, alcohol abuse) or concomitant comorbidities (diabetes mellitus, kidney disease, hypothyroidism). Epidemiologically, Western countries have seen a marked decrease, while developing countries

have experienced a general increase (148). The impact of both the quantity and type of dietary fat has been extensively researched over recent decades, yet further controlled studies are needed to address unsolved questions (149).

Hypertriglyceridemia

Hypertriglyceridemia is defined as plasma triglyceride levels > 150 mg/dL and constitutes a criterion of metabolic syndrome (MetS). The causal relationship between triglycerides levels and the future of CVD events has been controversial due to different methodological grounds (quality of the studies, sample size or distribution of the variable across populations) (150,151). It has even failed to demonstrate an overall improvement in CVD events with triglyceride-lowering therapy (152). Additionally, Mendelian randomization studies support plasma triglycerides constitutes an independent risk factor for CVD (151,153–155), although this statement must be taken cautiously, because most variants associated with triglycerides are also associated with HDL-c, LDL-c or Lp(a) (156,157). Weight reduction has led to an overall improvement in triglyceride levels (158), which is usually achieved through diet intervention and physical activity (159–161). In this regard, MedDiet has demonstrated consistent effect in reducing triglycerides levels (62,162,163).

Hypercholesterolemia

Hypercholesterolemia is defined as total cholesterol plasma concentrations above 200 mg/dL. Traditionally, the atherogenic LDL fraction is represented by the LDL-c, which is a primary risk factor for atherosclerosis. Different estimation formulas to calculate LDL-c have been published over the years with varying degrees of accuracy (165). The most popular is the Friedewald formula, which need to meet certain conditions (triglycerides ≤ 400 mg/dL) along with the assumption of 1:5 ratio of VLDL to triglycerides (62). However, updated evidence has demonstrated undertreatment due to unreliable results when LDL-c < 70 mg/dL, especially if triglycerides exceeds 150 mg/dL (164). Alternative estimation methods have already been proposed to address limitations of the Friedewald formula, and direct methods to quantify have been developed, although they have certain limitations (166–169).

Risk assessment for atherosclerotic cardiovascular disease (ASCVD) has been addressed by the American Heart Association, whose guidelines provide multiple recommendations classified by the strength of the evidence. Healthy lifestyle habits are still emphasized as the primary choice for maintaining optimal LDL-c values, including healthy dietary options (Table 5). Depending on the ASCVD risk, pharmacological therapy should be started at different intensities (170). New cholesterol-lowering agents have been introduced (PCSK9 inhibitors and ezetimibe) and tested in randomized controlled trials (RCT) for prescription in treating various indications (164). In this regard, MedDiet is known to improve lipid metabolism, reducing total cholesterol and LDL-c circulating levels (94,117).

Recent findings have pointed out remnant cholesterol contributes to atherosclerosis, and therefore cardiovascular risk stratification (171). Multiple definitions have recently emerged, but remnant cholesterol is mostly characterized by cholesterol contained in triglyceride-rich lipoproteins: chylomicron residues in the non-fasting state, intermediate-density lipoprotein (IDL) in the fasting state, and very low-density lipoprotein (VLDL) (172). Adverse cardiovascular events and cardiovascular mortality have been found to be negatively influenced by remnant cholesterol concentration (171,173).

	To be preferred	To be used in moderation	To be chosen occasionally in limited amounts
Cereals	Wholegrains	Refined bread, rice, and pasta, biscuits, corn flakes	Pastries, muffins, pies, croissants
Vegetables	Raw and cooked vegetables	Potatoes	Vegetables prepared in butter or cream
Legumes	Lentils, beans, fava beans, peas, chickpeas, soybean		
Fruit	Fresh or frozen fruit	Dried fruit, jelly, jam, canned fruit, sorbets, ice lollies/popsicles, fruit juice	
Sweets and sweeteners	Non-caloric sweeteners	Sucrose, honey, chocolate, sweets/candies	Cakes, ice creams, fructose, soft drinks
Meat and fish	Lean and oily fish, poultry without skin	Lean cuts of beef, lamb, pork, and veal, seafood, shellfish	Sausages, salami, bacon, spare ribs, hot dogs, organ meats
Dairy food and eggs	Skimmed milk and yoghurt	Low-fat milk, low-fat cheese and other milk products, eggs	Regular cheese, cream, whole milk and yoghurt
Cooking fat and dressings	Vinegar, mustard, fat-free dressings	Olive oil, non-tropical vegetable oils, soft margarines, salad dressing, mayonnaise, ketchup	Trans fats and hard margarines (better to avoid them), palm and coconut oils, butter, lard, bacon fat
Nuts/seeds		All, unsalted (except coconut)	Coconut
Cooking procedures	Grilling, boiling, steaming	Stir-frying, roasting	Frying

Table 5 - Food choices to lower low-density lipoprotein cholesterol and improve the overall lipoprotein profile. Adapted from (156)

Metabolic syndrome

MetS is a condition characterized by the presence of a cluster of risk factors for CVD and T2DM (174). There is controversy about whether MetS is a distinct syndrome or just a collection of unrelated phenotypes. The harmonization of MetS has yielded the following conditions to meet (106,175):

- Elevated waist circumference: Population- and country-specific definitions are defined in Table 2
- Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator): ≥ 150 mg/dL (1.7 mmol/L)
- Reduced HDL-c (drug treatment for reduced HDL-c is an alternate indicator): < 40 mg/dL (1.0 mmol/L) in males; < 50 mg/dL (1.3 mmol/L) in females
- Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator): Systolic ≥ 130 mm Hg and/or diastolic ≥ 85 mm Hg
- Elevated fasting glucose (drug treatment of elevated glucose is an alternate indicator): ≥ 100 mg/dL

The key risk factors for the syndrome are abdominal obesity and insulin resistance. Research suggests that excess visceral fat is more closely linked to insulin resistance than other types of adipose tissue. In general, abdominal (or upper-body) obesity is more strongly associated with insulin resistance and MetS compared to lower-body obesity (174). Other relevant characteristics include a proinflammatory and prothrombotic state which aggravate the atherosclerotic process

leading to cardiovascular events. Several biomarkers have been postulated as useful tools to assess the status of individuals (176,177).

HDL functions and cholesterol efflux capacity

A wide range of functionalities are attributed to HDL lipoproteins, and CEC is possibly one of the most extensively studied. CEC is defined as the output of cholesterol removal from different donors to the subsequent acceptors, mainly composed by HDL lipoproteins (Figure 9) (178,179). On the other hand, from an epidemiological standpoint HDL-c negatively correlates with CVD events (180,181), although accumulated evidence has questioned whether HDL-c levels constitute a causal factor for CVD event. Mendelian randomization studies have challenged the assertion of HDL-c levels as a predictive variable of CVD events (62,182). The intersection of both scientific statements has led to explore HDL functionalities as a plausible scientific bridge to explain the CVD events reduction and HDL particles (179,180,183,184).

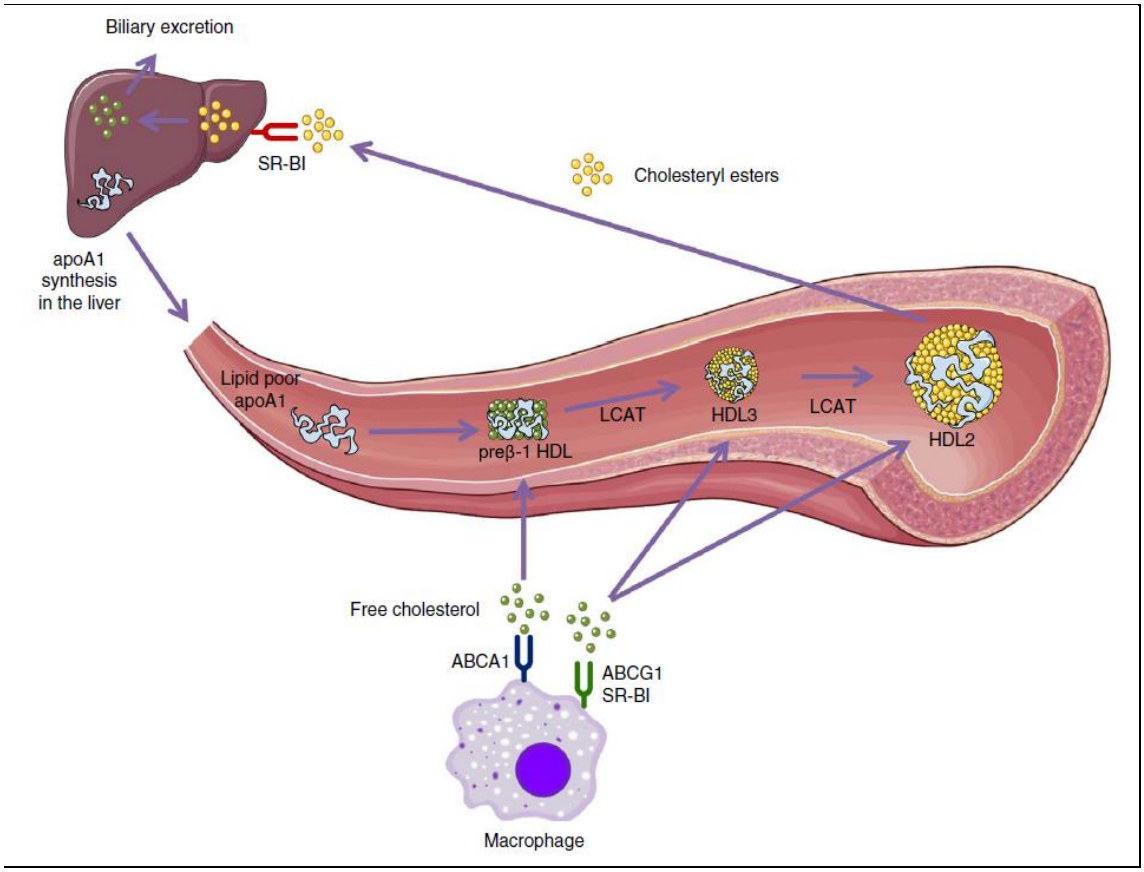

Figure 9 - HDL metabolism and reverse cholesterol transport pathway (185)

Cholesterol efflux has been observed as an adjustable functional activity, which inversely correlates with overweight and BMI (186). Diverse metabolic conditions impair HDL functionality, including obesity, hyperglycemia, and inflammation (187,188). However, certain factors have a positive influence, such as adherence to MedDiet pattern which has been shown to improve HDL functionalities (189), including CEC (32,190). Based on our previous experience and functional studies with PREDIMED participants, we have demonstrated that the MedDiet enhances CEC. Using *in vitro* techniques with cell cultures, we observed that isolated HDL from MedDiet intervention participants extracted cholesterol from macrophage THP-1 more effectively than HDL from the control group (32). To fully understand the underlying regulation of cholesterol efflux processes, the study of transcriptomic signature may be a prolific approach to picture a mechanistic profile. Prior PREDIMED substudies examined MedDiet transcriptomic response modulation, proving MedDiet enriched with extra-virgin olive oil (EVOO) pro-

inflammatory genes attenuation at midterm(191,192). On the contrary, 3-year follow-up did not observe transcriptomic amelioration in MedDiet groups; however enhancement in systemic inflammatory biomarkers was reported (193). The hydroxytyrosol a phenolic compound characteristic of olive oil intake has been proven to interact with regulatory pathways involved in the expression of the transporters in cholesterol efflux. *In vitro* experiments described stimulation of the PPAR-γ/LXR-α/ABCA1 pathway, where PPAR-γ (peroxisome proliferator activated receptor gamma) and LXR-α (liver X receptor alpha) act as enhancers of cholesterol efflux, upregulating *ABCA1* (ATP binding cassette subfamily A member 1) (194). In this regard, human studies have also described modulation of key players (*ABCA1*, *SR-B1*, *PPARA*, *PPARG*, *PPARD* and *CD36*) after 2 weeks of high-phenolic olive oil intake (195). Cholesterol efflux is a complex process with plenty of molecules interacting with each other. ABCA1 and ABCG1 (ATP binding cassette subfamily A or G member 1) are transporters, pumping cholesterol to different acceptors (nascent lipid-free HDL or more mature HDL already containing cholesterol, respectively). Both membrane proteins are expressed in peripheral blood cells, macrophages and foam cells shuttling cholesterol in the first step of reverse cholesterol transport (179). There is also an alternative ATP-free mechanism by passive diffusion performed by SR-B1 (Scavenger Receptor Class B Type 1), interplaying with mature HDL. SR-B1 seems to particularly be present and promote cholesterol uptake in steroidogenesis (196).

Regulation is executed by various nuclear receptors, acting as cholesterol sensors, and also working as signal transducers involved in a wide range of activities. First, the liver X receptors (LXR-α and LXR-β), encoded by *NR1H2* and *NR1H3* respectively, form obligatory heterodimerization with different receptors, such as the retinoid X receptors (RXRs) or PPAR-γ. Among lipid-related functions, nuts play a crucial role in reverse cholesterol (Figure 10) transport by enhancing cholesterol efflux, which is achieved through the upregulation of *ABCA1* and *ABCG1* gene expression. The regulatory control is primarily exerted at transcriptional level (179,197,198).

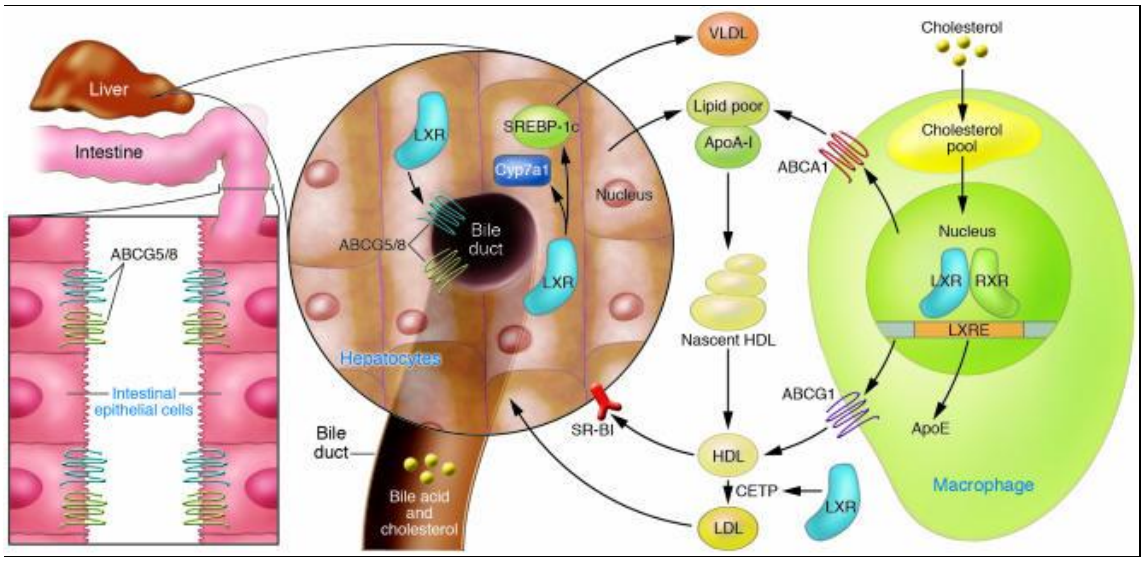

Figure 10 - Role of LXRs in reverse cholesterol transport from macrophages (199)

The RXRs are versatile receptors that can form obligate heterodimers to control cholesterol efflux transcription (200). RXR form obligate heterodimers with LXR, creating LXR-RXR, which can be activated by different ligands that can potentially upregulate ABCA1 (201). They can also form permissive partnerships with PPARs (peroxisome proliferator-activated receptors), being susceptible of activation for ligands of both receptors. PPARs are nuclear receptors highly expressed in active metabolic tissues, known as fatty acid sensors (202). The PPAR-γ isoform, encoded by the *PPARG* gene, plays a role in glucose metabolism as an insulin-sensitizing agent,

increases adipogenesis, and activates cholesterol efflux primarily through the PPAR-γ/LXR-α/ABCA1 pathway (202,203).

The natural ligands of these nuclear receptors include cholesterol intermediate metabolites, unsaturated fatty acids and synthetic molecules with varying degrees of therapeutic potential (203,204). In this regard, the hypothesis is that nuts, due to their high content of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), influence gene expression involved in lipid metabolism. PUFAs, in particular, mediate the expression of *PPARG* and *NR1H2* (190). Nuts are known for their ability to enhance CEC (32,205), among other antiatherogenic actions related to shifting lipoprotein composition towards a less atherogenic profile (size, density and number) (206). In this line, MedDiet food consumption (virgin olive oil, nuts, legumes, whole grains and fish) improves HDL CEC but also paraoxonase-1 activity (PON1) antioxidant activity and cholesteryl ester transfer protein (CETP) function (207).

The purported health benefits of EVOO, which also include CEC, are attributed to its high MUFA, polyphenols and several antioxidants constituents (208,209). The MUFA content in EVOO can induce physicochemical changes in the lipid composition of HDL particles favoring the efflux of cholesterol from macrophages to HDL (210–212). In addition, phenolic compounds especially abundant in EVOO, can induce cholesterol efflux (211,213–215). Certain constituents of EVOO have been found to indirectly enhance cholesterol efflux. Olive oil, specially EVOO, contains phenolic compounds could induce the gene expression of cholesterol efflux-related processes (211,213–215). These compounds protect against oxidative damage, which can otherwise impair the cholesterol efflux process. (213,216–218).

Physical activity

Physical activity constitutes a preventive measure in the reduction of CVD. A minimum recommendation of 150 minutes per week of moderate intensity physical activity, or 75 minutes per week of vigorous-intensity physical activity, plus two strength training sessions per week (219,220). One-third of the adult population in Europe accomplish the minimum recommended (2). Certain recommendations have been specified for adults older than 65 years, combining different types of training, intensity-varying activities, meeting a minimum of time and frequency (221).

A combination of caloric restriction and increased energy expenditure through physical activity is crucial for weight control, with the health benefits of physical activity being well-established (222,223). Both diet-induced weight loss and aerobic and resistance exercises improve cardiometabolic risk factors. Physical activity enhances cardiometabolic processes such as blood pressure, lipid profile, carbohydrate metabolism, and inflammation, while sedentarism is closely linked to obesity (224). Several RCTs have demonstrated exercise-induced weight loss generally achieve lower results than combined with caloric restriction. Conversely, following a diet combined with physical activity can achieve almost a 20% greater reduction than a diet-only program (225). Strong evidence (category A) states physical activity plays an influential role in weight maintenance, especially when the amount of time devoted to physical activity reach approximately 200 minutes per week (226).

Regardless of weight loss, physical activity has been shown to provide significant health benefits for risk factors including visceral fat, glucose and insulin metabolism, cardiorespiratory fitness or improvements associated with HDL cholesterol (227–229). Besides, older adults benefit by mitigating age-related impairments such as cognitive decline or frailty (230,231), plus the benefits of regular physical activity extend to psychological (satisfaction), physical, and social aspects.

The role of exercise influence on HDL functionality has been observed to improve cellular CEC promoted by HDL, reduction in endothelial vascular cell adhesion molecule expression, and the overall anti-inflammatory activity (232,233). Physical activity has demonstrated a synergistic

effect in CEC, the habit of moderate- or high-intensity exercise has been associated with an improvement in cholesterol efflux (234,235). Additionally, upregulation of key regulatory receptors and the cholesterol transporters *ABCA1* and *ABCG1* genes (236,237).

Mediterranean dietary pattern

The MedDiet composition has long been recognized as a healthy nutritional pattern (238,239), associated improved all-cause mortality rates, less incidence of cardiovascular outcomes, and a reduction in metabolic disorders (139,240,241). MedDiet is characterized by abundant intake of plant foods, including fruits, vegetables, but also includes frequent consumption of legumes and nuts (Figure 11). MedDiet typically consists of a high content of vegetal fat where the main source of lipids is based on MUFA, with olive oil as the primary source of lipids, added to vegetables and legumes to make them palatable. Additionally, traditional MedDiet is also characterized by a high intake of raw nuts. Substitution of saturated fatty acids (SFA) by PUFA is recommended by consumption of nuts, walnuts or peanuts. The sources of carbohydrate are typically low-glycemic index forms, rich in fiber and cereals as whole-grain. Moderate consumption of fish, poultry and dairy products, and a significantly reduced amount of red meat are another of the MedDiet features. Low to none consumption of ultra-processed and high-density foods, sweetened beverages (238,242–244).

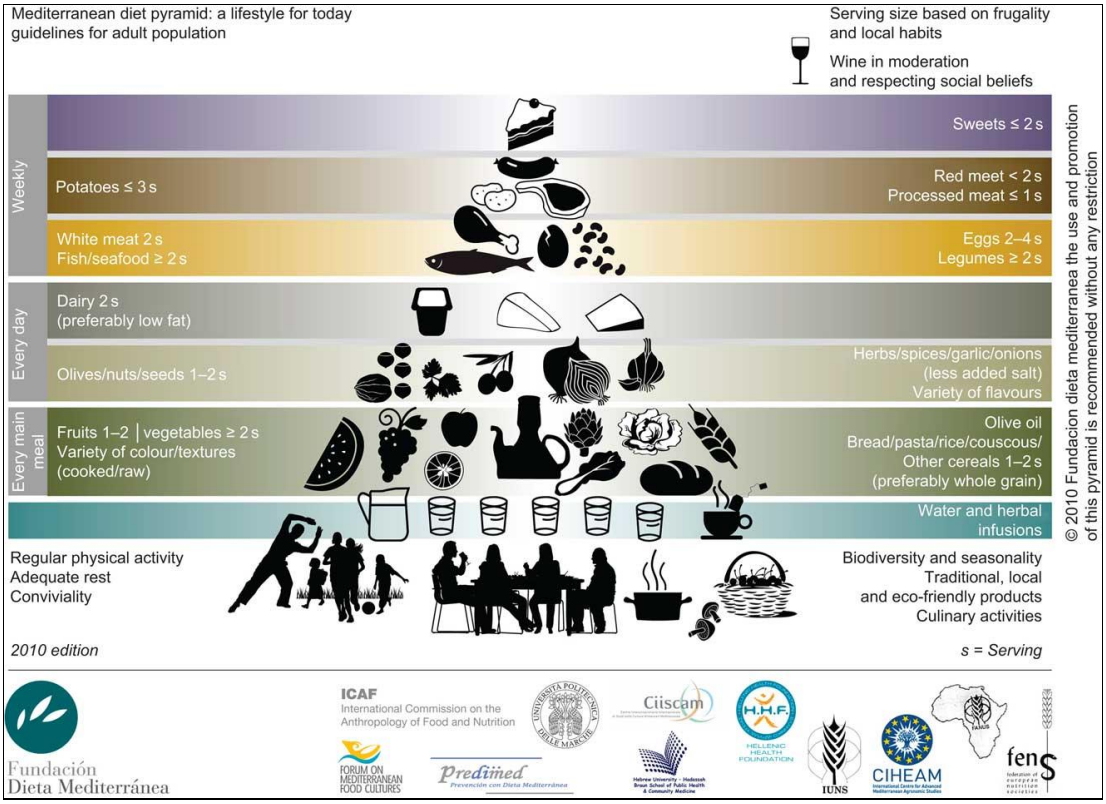

Figure 11 - Mediterranean diet pyramid: a lifestyle for today (245)

Olive oil, abundant in oleic acid, is acknowledged as the main fat in MedDiet pattern (238,246–248). Mediterranean inhabitants consume around 25-50 mL of olive oil per day, either raw for dressing salads and vegetables, or for culinary purposes as cooking oil. The chemical composition of olive oil differs according to olive variety, environmental conditions, ripening, and processing methods. Based on production methods, olive oil is classified into four types: EVOO, virgin olive oil, common olive oil, and refined olive oil (ROO). Extra-virgin olive oil criteria are: 1) maximum 0.8 % oleic acidity; 2) mechanical extraction methods without chemicals and hot water; 3) first cold pressing; and 4) adequate taste. Virgin olive oil has a maximum free acidity of 2%. Those with acidity >3.3 % are submitted to a refining process in which mainly phenolic compounds and,

to a lesser extent squalene, are lost (EU Regulation). Ordinary or common olive oil is produced by mixing virgin and ROO, which undergoes chemical extraction that removes most of its minor components (248,249). Olive oil is known to exert a wide range of protective actions (209), related to vascular integrity, LDL antioxidation (250) , blood pressure (251), cardioprotection (248,252), age-related cognitive decline or neurodegeneration (209,253,254).

Nuts are typically energy-dense food, enriched with unsaturated fats, vitamins, phytosterols, minerals, and fiber. A substantial body of evidence has associated nut consumption with numerous health benefits, particularly addressing cardiometabolic disorders (190). Lipid profile ameliorates, with a decrease in total cholesterol, triglycerides and LDL-c, while raising HDL-c concentration. Extensive research has examined the connection between nut consumption and cardiovascular health. Nuts are nutrient-dense foods rich in MUFA (linoleic acid) and PUFA (α-linolenic acid), with low SFA content, but also plant-based protein, fiber, and essential vitamins. They also contain antioxidants, such as polyphenols, which are fundamental to the cardioprotective actions attributed to them (255).

Clinical trials and long-term studies have clarified the protective role of nuts, decreasing the risk of T2DM, obesity and therefore MetS (256,257). The primary endpoint of PREDIMED was to determine if following a MedDiet may reduce the incidence of CHD and stroke, which was successfully demonstrated (258). In this line, several cohorts have assessed different types of nut supplementation, and its association with incidence and mortality. The high degree of heterogeneity among studies hinders comparability, however it can be stated that nuts perform a protective effect against CVD outcomes (259).

To answer these questions, RCTs with sufficient sample size, multicentric design and long-term follow-up are essential to draw robust conclusions. The PREDIMED study aimed to determine whether two traditional MedDiets, one enriched with virgin olive oil and another enriched with nuts, would exert superior beneficial effects on combined cardiovascular endpoints (myocardial infarction, stroke, and cardiovascular mortality) compared to a low-fat control diet. PREDIMED Study was a large-scale observational and multicentric RCT with adult elderly participants at high-cardiovascular risk. The PREDIMED trial provided with strong evidence and for the first time that the traditional MedDiet provides protection against CVD in individuals at high cardiovascular risk (260).

The available evidence suggests a general improvement in MetS, although the published evidence remains limited (261). The PREDIMED study confirmed MedDiet is a potential alternative for managing MetS (243,262). The PREDIMED-Plus study, encouraged by previous results dealing with weight loss following a restricted-calorie MedDiet (263,264), was conceived to design an optimal approach for body weight reduction, cardiovascular prevention and MetS amelioration in patients suffering from overweight/obesity (265). Results from one-year effect showed a median weight loss of 3.4% of baseline value, improvement in metabolic profile (fasting glucose, HbA1c, insulin, and HOMA of insulin resistance, HDL-c or triglycerides) and blood pressure measurements, along with reduction in several inflammatory markers (CRP, IL-1, IL-6, tumor necrosis factor-α, MCP-1) (266). Also, a substudy in PREDIMED-Plus has described how MetS severity correlates with higher inflammatory profile, depression risk or lower adherence to MedDiet (267). An interim substudy observed the evolution at 1 and 3-year follow-ups of several anthropometric measurements, including body fat distribution and composition. They found a significant fat mass reduction (visceral fat and total fat mass) in the first year, that is partially regained at 3-year follow-up, but still below baseline levels (268).

Neuroinflammation

Neuroinflammation is an inflammatory response within the brain primarily mediated by the secretion of cytokines (interleukin-1b (IL-1b), interferon gamma (IFN-γ), and TNF-α, chemokines and other proinflammatory molecules. This response can be triggered by different

types of insults, including metabolic disorders, traumatic injuries or infectious agents (269). Systemic inflammation triggers neuroinflammatory responses, potentially compromising the integrity of the blood-brain barrier (BBB), which increases its permeability, and allows cytokines to enter the brain. Subsequent reaction to cytokines primes resident glia cells which in turn amplifies the inflammatory reaction and indirectly promotes the recruitment of leukocytes. Long-term exposure drives neuronal damage, disrupting synaptic connections and causing neuronal death. The aforementioned pathological processes constitute a primary force in the development of neurodegenerative diseases (NDDs) (270).

The common comorbidities shared by NDDs, and CVD suggest that causes behind both types of conditions may be driven similarly. Individuals with overweight, diabetes or dyslipidemia are conditions more prevalent in individuals with NDDs and CVD. These conditions are known to induce chronic low-grade inflammation. Obesity effects on BBB inflammation are largely mediated by upregulation of adhesion molecules and pro-inflammatory cytokines, astrocytic response and extravasation of macrophages. Similarly, T2DM cause effects related to cytokine increase, but also deleterious mechanisms characterized by generation of advanced glycated end products and abnormal angiogenesis process (271).

Dietary composition also plays a significant role influencing chronic low-grade inflammation. In this regard, high-fat diet consumption has demonstrated to increase circulating levels of cytokines and proinflammatory molecules. Various molecules can serve as surrogate markers for neuroinflammation, which are linked to cardiometabolic diseases, cerebrovascular disorders, arteriosclerosis, and dementia.

On the other hand, Mediterranean diet research have shown an inverse correlation between adherence to the MedDiet and inflammatory markers levels (266,272). Polyphenols, a large family of naturally occurring compounds found in the Mediterranean diet, have been studied for their neuroprotective effects (which may reduce oxidative stress, neuroinflammation, and improve synaptic plasticity, Figure 12). Preclinical studies have shown promising results attenuating neuroinflammation, but human studies have produced inconsistent results regarding memory and cognitive function, indicating that further investigation is needed (273).

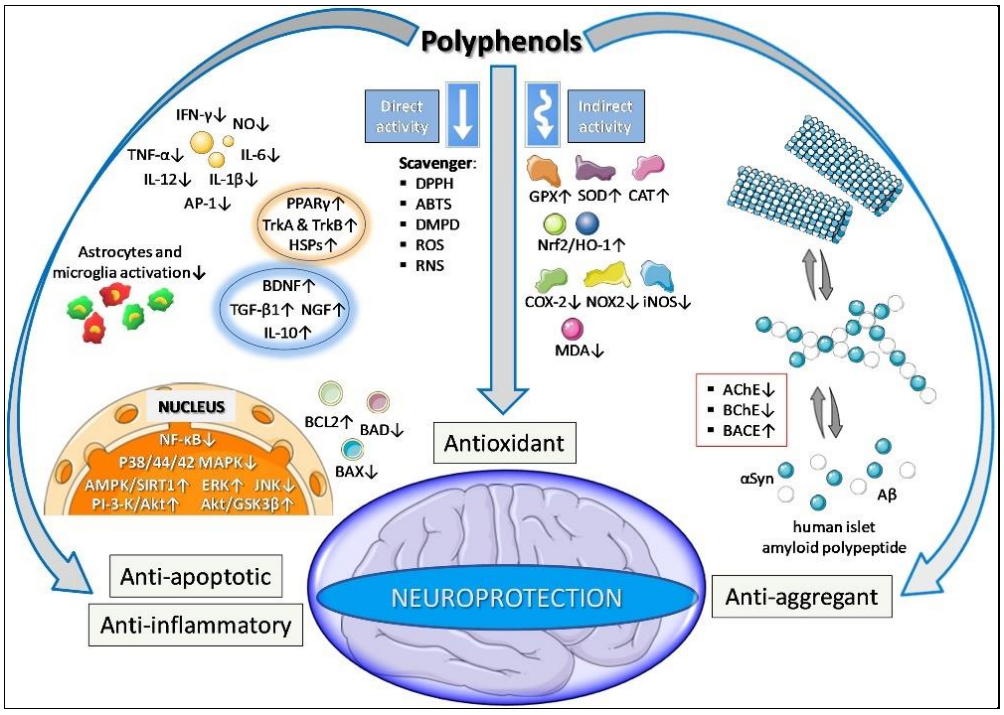

Figure 12 - Multimodal mechanism of action of phenolic acids in exerting neuroprotection (273)

Cardiometabolic risk, omic biomarkers and mechanisms

Lately, there have been advancements in the discovery of markers in the healthcare field. New parameters have arisen to challenge the well-established paradigm providing new insights and, hopefully, more accurate diagnosis and prognosis processes.

The recent incorporated molecules comprise metabolites, genotype and epigenetic markers that play a role in processes related to atherosclerosis, cardiac ischemia and fibrosis, thrombosis, nutrition, tissue repair, hemodynamics, inflammation, lipid- and glucose-related metabolism. Pro-inflammatory markers have been broadly used to follow inflammatory processes regardless of the cause (tumoral, metabolic, infectious or autoimmune). The production of certain cytokines may contribute to the low-grade acute-phase response, linking obesity and its related comorbidities. Obesity is characterized by chronic low-grade inflammation, due to secretion of adipokines that increase this dysfunctional state. C-reactive protein (CRP), primarily produced in hepatocytes, constitutes an independent risk factor for cardiovascular events (274–276). CRP concentrations in the blood increase in response to circulating cytokines, reflecting downstream inflammation though no direct causative role has been associated with CVD (31). CRP circulating levels function as a proxy of chronic low-grade inflammation (277) frequently observed in patients with overweight ,T2DM (278,279) but also in NDDs and dementia-related diseases (277).

PAI-1 is a prothrombotic factor produced by multiple cell types, including endothelial cells, mononuclear cells, and adipocytes. Biologically related to thrombosis, the plasminogen activator inhibitor 1 (PAI-1) restrain fibrinolytic process, facilitating a prothrombotic environment. Pro-thrombotic state enhances the chronic low-grade inflammation that progressively increases atherosclerosis. In fact, PAI-1 expression has been shown to be upregulated in atherothrombotic lesions (38,280).

PAI-1 contributes to insulin resistance in the development and progression of T2DM (281) , which can be partially explained by the modifiers that influence PAI-1: obesity, high-fat diet or alcohol (38), while a Mediterranean dietary pattern can be instrumental to maintain low circulating levels (282). In this context, PAI-1 has been shown to individually correlate with several components of MetS, including BMI, HDL-c, triglycerides, and glucose, highlighting its strong association with CVD (283). Epidemiological studies have supported the solid connection between PAI-1 and CVD, partially explained by the role exerted in hemostasis, where PAI-1 inhibits the fibrinolytic system causing a prothrombotic state (284–286). It is commonly accepted that PAI-1 is an enhancing factor for atherosclerosis, however, some controversy remains (287).

MetS is a composite of different disorders, being overweight and obesity an influential risk factor among the meeting criteria. Adipokines could serve as a valuable biomarker for assessing the impact of cardiometabolic disorders in patients. On this matter, visfatin an adipokine principally secreted by visceral adipose tissue, with both intracellular and extracellular activity, along with pro-inflammatory effects (288–290). The carbohydrate-related metabolic role is not well elucidated, some report insulin-mimetic effects while others have not found out correlation (291–293). Visfatin has been reported to correlate with subclinical atherosclerosis showing an association with carotid intima-media thickness (290). Published evidence suggests that dietary patterns, along with other lifestyle factors such as physical activity, can influence visfatin levels (111).

Another adipokine of interest is resistin, which is ubiquitously expressed but particularly high in adipose tissue whose attributed functions are proinflammatory actions, lipid accumulation, foam cell formation, and a contradictory relationship with insulin functions (120,280,294–296). Resistin is an adipocyte-secreted hormone known for impairing insulin sensitivity. It can be the bridge between obesity and diabetes due to the correlation between serum resistin and, both BMI and insulin resistance (120,280). Weight loss has been observed to concurrently decrease with resistin levels.

Complex mechanisms modulate energy homeostasis, including lipid metabolism, hunger or satiety. Regarding intake and satiety mechanisms, leptin and ghrelin binomial account for great regulatory responsibility. Leptin is a polypeptide predominantly secreted by adipose tissue, although not exclusively, in direct proportion to fat mass, whose most well-known actions are exerted at hypothalamic level regulating the satiety circuit. Leptin exerts different actions across the body, from hypothalamus where the hunger-satiety regulation occurs, to inflammatory immune response. As an anorexigenic hormone, leptin modulates appetite impulses and indirectly regulating energy expenditure and body weight (Figure 13) (280,297).

Resistance to leptin and excess circulating levels are typical findings in obesity, although mechanisms remain unclear. Hypothetical explanations comprise a reduced number of receptors and impaired transport across the BBB caused by saturation of transport mechanisms (298–300). Although the physiological role of leptin with weight maintenance is solid (300) several aspects remain controversial. No clear association between leptin exogenous administration, weight loss and maintenance has been established (298,301,302). Different functions have been attributed to leptin, such as enhancing macrophagic response, proinflammatory cytokine secretion, acute-phase proteins (303–306) or immune regulation (298,300). Research on circulating levels, combined with other biomarkers, has been conducted to gather and predict information on cardiovascular risk (300,304,305,307).

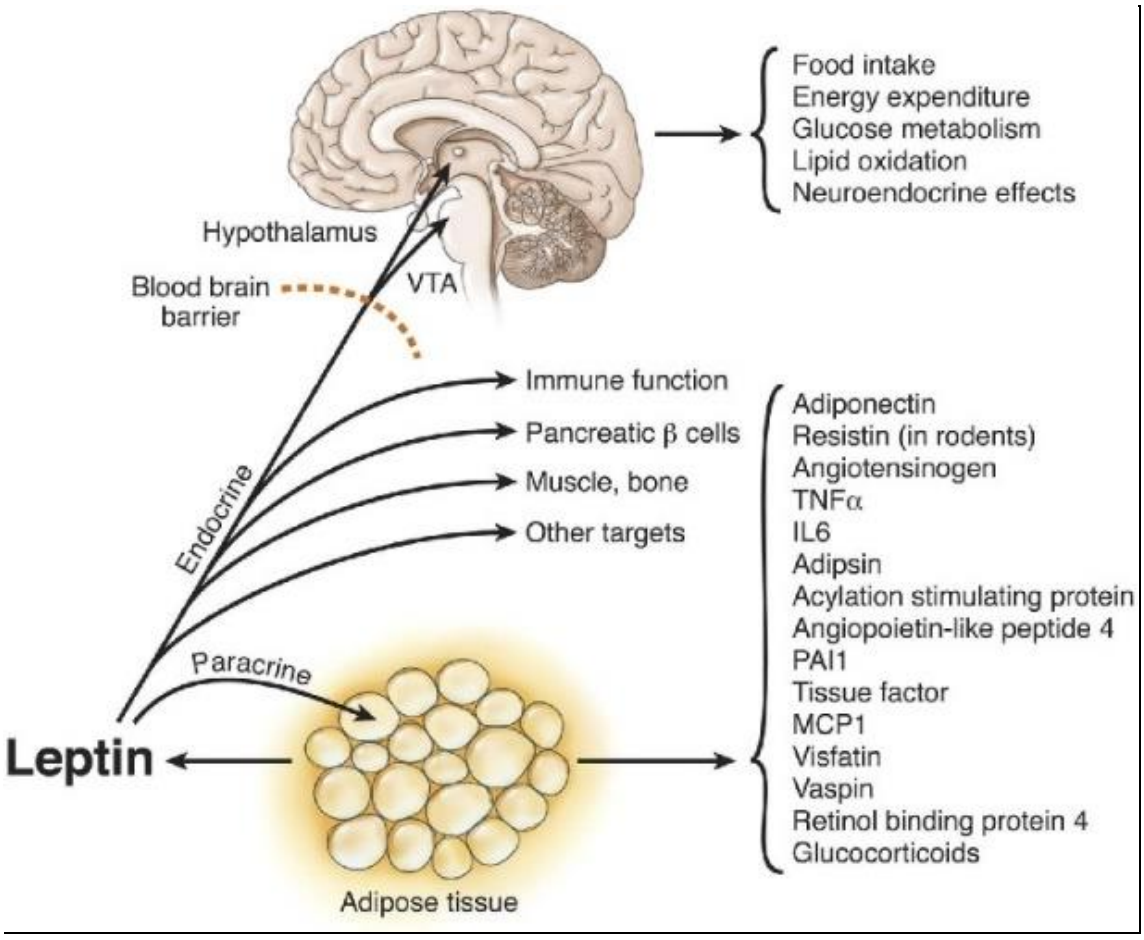

Figure 13 – Leptin's Endocrine and Paracrine Effects on Brain and Peripheral Tissues

On the other hand, ghrelin, an endogenous peptide predominantly secreted by the stomach and intestine, plays a key role in stimulating appetite in response to orexigenic signals (308,309). Ghrelin regulates short-term hunger and long-term body weight, but also decreases energy expenditure (310). Ghrelin levels have been found to be reduced in obese individuals, partially

due to a counterregulatory mechanism involving insulin or leptin. Decreased levels can also be observed in weight maintenance period, after weight-loss program (311).

In the short term, ghrelin administration raises plasma glucose levels (312), reducing insulin secretion and insulin sensitivity (312,313). Related to fat metabolism, ghrelin promotes adiposity and lipogenesis (314–316). Beyond its metabolic functions, ghrelin also plays a role in various physiological functions (Figure 14), including learning and memory, psychological stress, mood and anxiety, sleep/wake rhythm, and aging (316). Circulating ghrelin levels are characterized by pre-prandial rise and a postprandial fall, and they are also influenced by circadian rhythm (317). Ghrelin decreases inflammation by inhibiting the nuclear factor kB (NF-kB) pathway and mitigates fibrosis (309). Research on animal models has shed light on its participation in CVD processes such as cardiac output, ischemia or peripheral resistance (317,318).

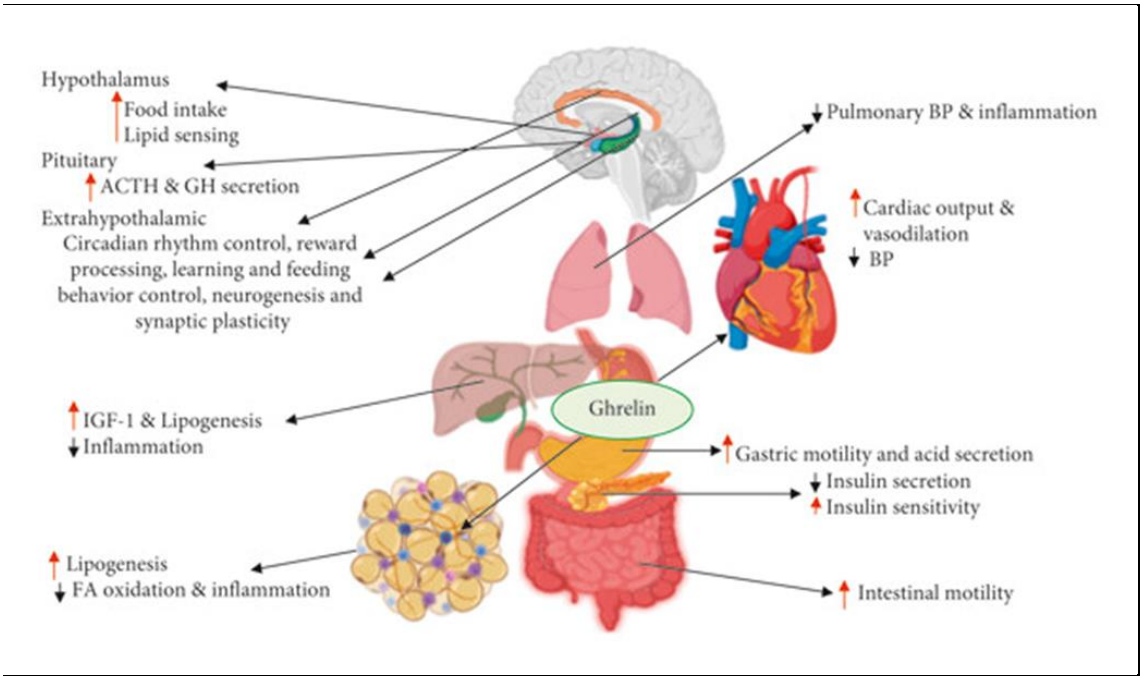

Figure 14 – Physiological Effect of Ghrelin on Body Systems

New strategies have been proposed using circulating leptin and ghrelin levels, particularly in overweight and obese patients, to predict weight loss outcomes and the likelihood of weight regain during and after energy-restriction programs (319,320). There is some controversy whether circulating levels of these hormones can reliably predict anthropometric changes during and after interventions (321).

HYPOTHESES

Hypothesis 1

To examine whether an intensive lifestyle intervention with a calorie-restricted traditional Mediterranean diet and physical activity, versus a non-intensive *ad libitum* traditional Mediterranean dietary pattern intervention, in a cohort of old participants meeting metabolic syndrome criteria, is associated with less cardiometabolic risk. The expectation that inflammation will improve particularly in the group following the intensive calorie-restricted Mediterranean diet combined with physical activity. The specific hypotheses for category:

Anthropometric measurements

- Participants following an intensive lifestyle intervention will exhibit a greater reduction in body weight and waist circumference than those on a traditional Mediterranean diet. These changes in lifestyle habits are hypothesized to be sustained over time, given the traditional Mediterranean diet's palatability and adherence, driven by its plant-based content and use of olive oil.

Carbohydrate and lipid metabolism

- Intensive lifestyle intervention will lead to a more significant improvement in glycemic and lipid profiles. However, the control group following the traditional Mediterranean diet is also expected to show some improvement in both glycemic and lipid metabolism.

Inflammatory status

- Participants allocated in the intensive lifestyle intervention group will demonstrate a more pronounced reduction in inflammatory markers. Mild amelioration of biomarkers is expected to occur in the control group.

Hunger-satiety circuit

- Participants following the intensive lifestyle intervention are expected to show a more significant modulation of the hunger-satiety circuit, with a greater reduction in leptin levels and an increase in ghrelin levels, compared to the traditional Mediterranean diet group. The control group is expected to experience some degree of regulatory changes.

Hypothesis 2

The Mediterranean diet improves HDL function in high cardiovascular risk patients as published in the PREDIMED studies (PREDIMED and PREDIMED-Plus). We expect the modulation of gene expression to be a key mechanism underlying the improvement of HDL functionality. Specifically, we expect to observe:

- An upregulation of cholesterol transporter gene expression in response to a targeted dietary pattern.
- A positive correlation between gene expression and anthropometric measurements.

- Greater upregulation of genes involved in cholesterol efflux in in the group with promoted physical activity.

Hypothesis 3

The Mediterranean diet has been associated with a risk reduction of dementia and Alzheimer's disease. The study hypothesizes that the traditional Mediterranean diet has the capacity to modulate neuroinflammation, a cornerstone of neurodegenerative diseases. In this regard, attenuation of systemic inflammation can mitigate the neuroinflammatory response, resulting in beneficial effects for both neurodegenerative diseases and cardiovascular disease. In this line Mediterranean dietary pattern may modify multiple inflammatory and oxidative pathways, with transcriptomic analysis serving as a surrogate marker of neuroinflammation, studied in a population of adults at high-cardiovascular risk.

The hypothesis is:

- The traditional Mediterranean diet will alter the expression of genes related to neuroinflammation in adults at high cardiovascular risk compared to the control low-fat dietary pattern.

OBJECTIVES

Manuscript 1

To assess whether an intensive lifestyle intervention with a calorie-restricted traditional Mediterranean diet and physical activity, versus a non-restrictive traditional Mediterranean diet pattern, in subjects with obesity/overweight meeting criteria for metabolic syndrome improves overall cardiometabolic profile. The objectives can be summarized in:

Anthropometric measurements

- Assess changes in body weight, waist circumference, and BMI from baseline to 6 and 12 months, and compare these changes between the calorie-restricted and non-restrictive traditional Mediterranean diet groups.

Carbohydrate and lipid metabolism

- Assess changes in glucose, insulin, HOMA-IR (Homeostatic Model Assessment for Insulin Resistance), total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides from baseline to 6 and 12 months, and compare differences between the calorie-restricted and non-restrictive traditional Mediterranean diet groups.

Inflammatory status

- Measure changes in inflammatory markers such as C-reactive protein (CRP), plasminogen activator inhibitor-1 (PAI-1), resistin, and visfatin from baseline to 6 and 12 months, and evaluate differences between the calorie-restricted and non-restrictive traditional Mediterranean diet groups.

Hunger-satiety circuit

- Measure changes in leptin and ghrelin levels from baseline to 6 and 12 months and assess the differences between the calorie-restricted and non-restrictive Mediterranean diet groups.

Trend assessment

- Evaluate variable trends, linear or quadratic, in the adjusted models for the different biomarkers previously mentioned.

Manuscript 2

To examine the expression of cholesterol efflux-related genes involved in regulatory functions in patients at high cardiovascular risk subjected to two non-restrictive traditional Mediterranean diets, supplemented with extra-virgin olive oil or nuts, compared to a control low-fat diet.

In this regard we aimed to:

- Investigate the regulatory molecules involved in cholesterol efflux gene expression within and between-groups.

- Examine the gene expression of cholesterol transporters in response to a dietary pattern, and analyze the correlation with anthropometric measurements.
- Examine the relationship among the regulated genes involved in cholesterol efflux.

Manuscript 3

To assess evaluate the impact of a long-term traditional Mediterranean diet interventions supplemented with extra-virgin olive oil or nuts, versus a low-fat control diet, on the expression of genes related to neuroinflammation and cardiovascular risk in an elderly population at high cardiovascular risk.

RESULTS

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Mid- and long-term changes in satiety-related hormones, lipid and glucose metabolism, and inflammation after a Mediterranean diet intervention with the goal of losing weight: A randomized, clinical trial

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Background: Obesity is produced by the enlargement of the adipose tissue. Functioning as an endocrine organ, it releases and receives information through a complex network of cytokines, hormones, and substrates contributing to a low-chronic inflammation environment. Diet and healthy habits play key roles in the prevention of obesity and its related pathologies. In this regard, there is a need to switch to healthier and more appetizing diets, such as the Mediterranean one.

Objective: To compare the mid-and long-term effects of two Mediterranean diet (MedDiet) interventions, one energy-reduced plus physical activity promotion versus a non-restrictive diet, on peripheral satiety-related hormones, weight loss, glucose/lipid metabolism, and pro-inflammatory markers in subjects with obesity/overweight and metabolic syndrome.

Materials and methods: A randomized, lifestyle intervention was conducted in 23 Spanish centers, with a large cohort of patients presenting metabolic syndrome. Our study is a subproject set in IMIM (Hospital del Mar Research

Institute). Participants were men and women, aged 55–75 and 60–75, respectively, who at baseline met at least three metabolic syndrome components. Subjects were assigned to two intervention groups: (1) an intensive lifestyle intervention with an energy-reduced MedDiet and physical activity promotion (intervention group) with the aim of weight loss; and (2) a normocaloric MedDiet (control). We quantified in a subsample of 300 volunteers from Hospital del Mar Research Institute (Barcelona), following analytes at baseline, 6 months, and 1 year: glucose, HbA1c, triglycerides, total cholesterol, high-density lipoprotein cholesterol, LDL cholesterol, C-peptide, ghrelin, GLP-1, glucagon, insulin, leptin, PAI-1, resistin, and visfatin. Anthropometric and classical cardiovascular risk factors were also determined. A multivariate statistical model was employed to compare the two groups. Linear mixed-effect models were performed to compare changes in risk factors and biomarkers between intervention groups and over time.

Results: Compared to participants in the control group, those in intervention one showed greater improvements in weight, waist circumference, insulin ($P < 0.001$), glucose metabolism-related compounds ($P < 0.05$), triglyceride-related lipid profile ($P < 0.05$), leptin, blood pressure, and pro-inflammatory markers such as PAI-1 ($P < 0.001$) at mid-and/or long-term. High-sensitivity C-reactive protein, resistin, and vifastin also decreased in both groups.

Conclusion: A weight loss intervention employing a hypocaloric MedDiet and physical activity promotion has beneficial effects on adiposity, glucose metabolism, lipid profile, leptin, and pro-inflammatory markers, such as PAI-1 in both mid-and long-term.

KEYWORDS

metabolic syndrome, Mediterranean diet (MedDiet), leptin, PAI-1, inflammation

Introduction

Over the past 40 years, obesity has come to be considered an emerging global pandemic. Described by the World Federation of Obesity "as a chronic relapsing disease process," it has proven influence on the development of hypertension, diabetes mellitus, and cardiovascular events (1). Current nutrition habits, which include the excessive consumption of sweetened beverages and high-density energy food, have notably increased the prevalence of overweight and obesity in both child and adult populations. Moreover, western society has embraced sedentary routines which further contribute to an augmented positive energy balance, thus worsening insulin resistance and perpetuating unhealthy behavioral patterns (2, 3).

Abbreviations: MedDiet, Mediterranean diet; HbA1c, glycated hemoglobin; HDL cholesterol, high-density lipoprotein cholesterol; LDL cholesterol, low-density lipoprotein cholesterol; hs-CRP, highly sensitivity C-reactive protein; K2-EDTA, dipotassium ethylenediaminetetraacetic acid; GLP-1, glucagon-like peptide-1; PAI-1, plasminogen activator inhibitor-1; CV, coefficient of variation.

Metabolic syndrome, characterized by high cardiovascular risk due to prediabetes/diabetes, hypertension, dyslipidemia, and overweight/obesity, is associated with several comorbidities, including cardiovascular conditions, diabetes, cancer, and liver disease. Specific pharmacological agents apart, there is a need to switch to healthier diets, such as the Mediterranean one (MedDiet), given that diet is a key factor in the prevention of such comorbidities (4).

The traditional MedDiet, largely based on plant-derived products, is characterized by seasonal and proximity products. It includes: (a) olive oil as the main source of fat; (b) high consumption of cereals, vegetables, legumes, fruit, and nuts; (c) moderate intake of poultry, fish, eggs, milk, and dairy products; (d) regular, but moderate, consumption of red wine at meals; and (e) low intake of red meat, processed meat, and industrial confectionery (5). The protective effect of the traditional MedDiet against cardiovascular disease in primary prevention has been demonstrated with the PREDIMED Study. This randomized, controlled, multicenter clinical trial had three intervention groups: two with a traditional MedDiet supplemented with extra virgin olive oil and nuts, respectively,

and low-fat diet control (6). In addition, a meta-analysis of 50 epidemiological and clinical trials (534,906 participants) determined that adherence to the MedDiet was associated with a reduced risk of metabolic syndrome (7).

Obesity is characterized by an increase of adipose tissue which, due to its involvement in metabolic regulation functions, has been acknowledged as an endocrine tissue organ. A maze of cytokines, hormones, substrates, and products, with both pro-and anti-inflammatory effects, regulate feelings of hunger and satiety through signals from the gastrointestinal tract and adipose tissue. Dietary interventions accompanied by weight loss have been shown in mid-and long-term programs to substantially influence satiety hormones. Feelings of hunger and satiety involve complex interactions between ghrelin and leptin in the hypothalamus which integrates both signals to regulate the body's energy homeostasis (8–10). Leptin, an adipose tissue-specific adipokine, is crucial in the control of appetite, energy expenditure, behavior, and glucose metabolism. It crosses the blood–brain barrier and acts on specific receptors to decrease appetite and increase energy expenditure. Reduction in leptin levels has been observed short and mid-term (11, 12), while fewer studies have demonstrated MedDiet effectivity beyond a 12-month intervention (13). Physical activity and a caloric-restricted diet have jointly been reported to augment leptin decrease (14). Ghrelin, an endogenous peptide mainly secreted by the gut, contributes to orexigenic stimulus thus increasing appetite (15, 16). Higher circulating levels have been observed in short-term, with lesser evidence after 1 year of initial weight loss (13).

By interacting with different cell lineages (17–20), leptin acts as a pro-inflammatory adipokine and increases C-reactive protein levels in primary hepatocytes and human coronary endothelial cells (21, 22). Low-grade chronic inflammation is associated with adiposity, advanced age, dyslipidemia, and hyperglycemia. Inflammatory status can be counteracted by modifying diet patterns, including moderate physical activity (23–25). Several biomarkers engage in the complex process of inflammation, such as C-reactive protein, considered to reflect inflammatory reactions in atherosclerotic vessels, as well as circulating cytokines and necrosis in acute myocardial infarction (26). Plasminogen activator inhibitor-1 (PAI-1), a physiological inhibitor of plasminogen, acts as a biomarker of a pro-thrombotic state. MedDiet interventions have been reported to ameliorate pro-thrombotic status decreasing PAI-1 serum levels (27, 28). Smoking, alcohol consumption, and age are positively correlated with PAI-1 levels (29).

White adipose tissue has been broadly accepted as a metabolic active organ. However, some of its peptides are unclear, for instance, resistin, an antagonist polypeptide of insulin action that may play a role in obesity (30). Controversial results have been obtained regarding the identification of changes in its levels in both obesity and diabetes mellitus (31, 32). Regarding visfatin, an adipokine with arguably insulin-mimetic effects (33) and which is highly expressed in visceral fat (34, 35) appears to be upregulated in patients with obesity (36) and type 2 diabetes mellitus (37). Results, however, are inconsistent with respect to insulin sensitivity, waist circumference, body mass index (BMI), and HbA1c (38–40).

Our objective is to assess whether an intervention with a restrictive MedDiet plus physical activity promotion, versus a non-restrictive MedDiet, is associated with an improvement in satiety-related hormones, weight loss, pro-inflammatory biomarkers, and glucose/lipid metabolism at mid-and long-term (6-and 12-month follow-ups). In addition, we will establish the association of these markers with weight loss irrespective of the intervention group.

Materials and methods

Study design and population recruitment

The PREDIMED-PLUS is a multicenter lifestyle intervention with 6,874 eligible participants. It is a 6-year randomized trial conducted in 23 Spanish centers with a large cohort presenting metabolic syndrome recruited from primary healthcare centers. Inclusion criteria were: men aged 55–75 years and women 60–75 years, with overweight/obesity (BMI: 27–40), and meeting at least three metabolic syndrome components at baseline (41, 42).

Patients were randomly allocated either to the intervention group or control (41). Those in the former followed an energy-reduced MedDiet with physical activity promotion and behavioral support so as to meet specific weight loss objectives. The participants received recommendations based on a 17-item energy-restricted score. In addition, physical activity counseling to gradually increase exercise intensity to 150 min/week, and attitudinal lifestyle advice through frequent sessions with dietitians (both individual and collective), were provided. Participants in the control group received educational sessions on an *ad libitum* MedDiet based on a 14-item non-energy-restricted score. No specific advice for increasing physical activity or losing weight was provided.

Regarding the individual sessions, participants in both groups received periodical group sessions and telephone calls (once a month in the intensive intervention group and two times a year in the control one).

Adherence to diet was assessed with a previously validated 14-item questionnaire employed in the PREDIMED Study for the control group (43, 44), which was adapted to the 17-item energy-restricted diet questionnaire for the intervention

group. According to the score obtained, the scale was estimated as approximate tertiles: low (≤ 7), medium (8–10), and high (11–17) (45). Physical activity practice was evaluated at the beginning of the study and during follow-up. Participants reported activities through the Regicor Short Physical Activity Questionnaire, a validated version adapted from the Minnesota leisure time physical activity questionnaire (46, 47). Physical activity was measured in MET-min/week.

Hormone and inflammation-related determinations were performed in a subsample of 300 patients at baseline, with measurements at 6-and 12-month follow-ups of 298 and 266 subjects, respectively. The sample size of glycosylated A1c hemoglobin (HbA1c) was made up of 300, 353, and 369 individuals at the three visits, respectively. Due to sample availability, high sensitivity C-reactive protein (hs-CRP) was analyzed in 228 individuals.

Laboratory, anthropometric, and clinical data

The following information was gathered before and after the intervention: (i) the participants' general clinical status (sex, age, BMI, waist circumference, systolic/diastolic blood pressure); (ii) adherence to the energy-reduced MedDiet (with a 17-point questionnaire); and (iii) levels of physical activity. Sample collection was performed after an overnight fasting period at baseline, 6-months, and 12-months of follow-up. Venous blood samples were collected in vacuum tubes with a silica clot activator and K_2-EDTA anticoagulant (Becton Dickinson, Plymouth, United Kingdom) to yield serum and plasma, respectively. Serum tubes were centrifuged after the completion of the coagulation process, and plasma tubes immediately after collection, both for 15 min at 1.700 g room temperature. With the exception of HbA1c which was analyzed with K_2-EDTA anticoagulated whole blood, the following analytes were quantified in serum with an ABX Pentra-400 auto-analyzer (Horiba-ABX, Montpellier, France): glucose, HbA1c, triglycerides, high-density lipoprotein (HDL) cholesterol, and total cholesterol. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula whenever triglycerides were < 300 mg/dL. Remnant-C was estimated as total cholesterol minus LDL cholesterol minus HDL cholesterol. Finally, leptin, ghrelin, glucagon-like peptide-1 (GLP-1), C-peptide, glucagon, insulin, PAI-1, resistin, and visfatin were simultaneously analyzed in plasma by Bio-Plex Pro methodology, a bead-based multiplexing technology with specific capture antibodies coupled with magnetic beads to discriminate analytes using an XMAG-Luminex assay (Bio-Rad, Hercules, CA, USA). The fluorescence signal was read on a Bio-Plex 200 equipment (Bio-Rad) (14). After several washes to remove unbound protein, a biotinylated detection antibody conjugated with fluorescent dye reporter. Homeostatic model assessment for insulin resistance (HOMA) was calculated as fasting plasma glucose (mg/dL) x fasting serum insulin (μ units/mL)/405. The inter-assay coefficients of variation (CVs) of these determinations were between 4.92 and 12.43%, except for GLP-1 (24.11%) and vifastin (32.42%). Values under the methodological limit of detection were reported with the limit of detection itself. Leptin measurements from six individuals were removed from the database due to analytical sampling error, and two hs-CRP values were considered outliers.

Statistical analysis

The assessment of the normality distribution of the variables was performed based on normality probability plots and boxplots. Continuous variables were normally shaped, except for triglycerides which were normalized by Napierian logarithm, and median and interquartile ranges were displayed. Lifestyle categorical variables were compared between groups with the Chi-square test.

A descriptive statistic table stratified by intervention and control group was summarized including mean values (or median if non-normally shaped), and mean differences between 6-and 12-month intervals. In addition, multivariate linear regression models adjusted for sex, age, energy intake baseline value, and baseline value of the variable under study were fitted. Mean differences between groups were estimated and 95% confidence intervals were reported. To identify possible statistical differences across time, we performed the paired *t*-test among baseline, 6 months, and 12 months in each group (Mann–Whitney *U* test was carried out for non-normal variables).

Weight loss and waist circumference changes were stratified according to the tertiles of the population at the different time points (baseline, 6 months, and 12 months). To estimate the extent of variation among the first, second, and third tertiles, the analysis of variance was calculated by adjusting for baseline value and baseline weight. Major weight and waist circumference losses corresponded to the first tertile. The linear mixed-effect models were constructed considering potential significant covariates with age, sex, time, weight, and adherence to MedDiet as fixed effects. Given that time affects individuals differently, it was contemplated as a varying covariate and a random slope constructed. The model contains both linear and quadratic time components so as to determine which trend better fits the model. We also included possible interaction between sex and weight, using the latter to correct the model in all variables (except for weight itself). Linear mixed-effect estimation was carried out with the use of restricted maximum likelihood. Graphical representation of variables that showed significant results for the and/or group: time interaction (linear and/or quadratic component) was performed. In addition,

analysis of 1-year weight loss correlation with these variables was calculated with Pearson's correlation formula. A p-value of < 0.05 was considered significant.

Sample size

Accepting an alpha risk of 0.05 and a beta risk of < 0.2 in a bilateral contrast, 116 subjects in both groups allow the detection of a difference $\geq 1{,}2$ pg/mL for leptin circulating levels, when the standard deviation is assumed to be 3.26 pg/mL.

Results

Our study population was a sample of 407 (215 women) participants from the IMIM (Hospital del Mar Research Institute) site within the framework of the PREDIMED PLUS Study. The mean age was 65.44 years (± 4.62 years). With respect to participants' lifestyles at baseline, the diet and physical activity questionnaire scores did not show significant differences between groups, and they met the minimal physical activity requirements suggested by the American Heart Association (450–750 MET·min·week^{-1}) (48). Diabetes, dyslipidemia, hypertension, and smoking conditions were equally distributed between the two groups without significant differences.

Baseline, 6-and 12-month follow-ups, characteristics of continuous variables regarding clinical features, lifestyle, lipid/glucose metabolism, satiety-related hormones, and studied pro-inflammatory markers are shown in Table 1. The main food items and nutritional parameters are shown in Table 2. In comparison to the control group, the adjusted multivariate of MedDiet adherence, physical activity, weight, waist circumference, remnant cholesterol, triglyceride levels, and HDL cholesterol showed an improvement at 6-month follow-up which was maintained at 12 months. Systolic and diastolic blood pressure presented significant improvements at 6-month follow-up but did not reach significance at 12 months. Regarding carbohydrate metabolism, we found differences between the two groups at 6-and 12-month follow-ups in HOMA, insulin, and C-peptide. Borderline inter-group P-value these explanations were aimed to clarify the meaning of borderline to reviewer 2. Borderline inter-group was observed for glucose at 6 and 12 months [a tendency to ameliorate results over time: $\beta_{6m} = -3.58$ (−7.39, 0.23) and $\beta_{12m} = -4.22$ (−9.16, 0.72)] and a significant decrease for HbA1c only at 12 months. Changes in leptin and PAI-1 levels were reported at 12 months, with a 6-month P-value close to significance in the case of PAI-1. Mean multivariate-adjusted differences (95% CI) for 6-and 12-month follow-ups were estimated and are depicted in common units of baseline standard deviations in Supplementary Figure 1.

As expected, the weight loss tertiles showed improvements at mid-and long-term follow-up for MedDiet adherence and physical activity practice regardless of the group. In particular, we observed changes in the triglyceride-related measurements (total cholesterol, HDL cholesterol, triglycerides, and remnant cholesterol), systolic/diastolic blood pressure, and carbohydrate metabolism (HOMA, HbA1c, insulin, glucagon, C-peptide, GLP-1). In addition, changes in leptin, PAI-1, and visfatin levels were observed at 6-and 12-month follow-ups (Table 3). Waist circumference change tertiles showed similar results to body weight tertiles (Table 4).

Changes were graphically examined through linear mixed-effect models of cardiovascular risk factors at 6-and 12-month follow-ups to observe the behavior of the repeated measures in both groups. The time:group (linear and quadratic) interaction as a potential predictor of the outcome variable was significant in weight, waist circumference, HDL, and remnant cholesterol, systolic/diastolic blood pressure, triglycerides, and PAI-1 levels (Supplementary Figure 2). Pearson's correlation at 1 year yielded a moderately positive correlation ($r > 0.20$) between weight loss and reduction of leptin, glucagon, PAI-1, HbA1c, and insulin levels. Comparably, moderately positive correlations ($r > 0.20$) between waist circumference changes and reduction of leptin, PAI-1, and insulin levels were observed. Weight change with moderate positive correlation was reported (Supplementary Figure 3).

Discussion

The intervention with an energy-reduced MedDiet and physical activity, versus a non-reduced one, was associated with an improvement in weight, waist circumference, glucose metabolism, triglyceride-related lipid profile, satiety-related hormones (leptin), and pro-inflammatory markers (PAI-1) at mid-and long-term in subjects with metabolic syndrome.

Such changes being maintained over time have been previously reported. Moreover, it has been hypothesized that MedDiet pattern interventions lead to greater compliance and adherence rates, in fact, the number of dropouts registered in trials has been reported to be larger in the control groups (7, 49–52). The MedDiet fat component is of vegetable origin (olive oil and nuts) and includes an abundance of plant foods (vegetables, fruit, whole grains, and legumes), limited fish consumption, and red wine in moderation (usually during meals). The intake of red and processed meats, refined grains, potatoes, dairy products, and ultra-processed foods (ice cream, sweets, creamy desserts, industrial confectionery, and sugar-sweetened beverages) (41, 53).

The hypothesis that the MedDiet is an eating pattern that can be maintained in mid-and long-term with a high degree of acceptance has been reflected in several studies introducing behavioral and nutritional patterns into small population groups (52, 54, 55). During other interventions,

TABLE 1 Baseline and 6-and 12-month changes (mean and standard deviation) stratified in the control and intervention groups of the participants on the 17-item questionnaire, physical activity, biomarkers, and anthropometric measurements, lipid profile, carbohydrate metabolism, and hormones. Adjusted for sex and age.

	Control group			Intervention group			Control group vs. Intervention group		
	Baseline	6 month-change	12 month-change	Baseline	6 month-change	12 month-change	Baseline	6 month-adjusted model	12 month-adjusted model
Diet adherence and physical activity									
Mediterranean diet adherence (17-point item score)	7.18 (2.36)	3.03* (3.03)	2.55 + ^ (2.94)	7.52 (2.60)	4.13* (3.27)	3.89^ (3.38)	0.30 (−0.17, 0.77)	1.37 (0.88, 1.86)	1.62 (1.13, 2.12)
Physical activity (MET·min/week)	2477 (2132.37)	275.77 (2311.75)	367.91^ (2272.46)	2648 (2216.67)	838.78* (2430.42)	872.23 ^ (2207.52)	150.64 (−269.65, 570.93)	649.28 (233.83, 1064.72)	591.67 (206.63, 976.71)
Lipid profile									
Total cholesterol (mg/dL)	218.30 (41.22)	−3.69 (31.71)	−0.16 (33.93)	221.88 (41.65)	−4.50* (32.54)	−1.70 (32.66)	3.62 (−4.06, 11.30)	0.69 (−4.95, 6.34)	−0.34 (−6.53, 5.85)
HDL cholesterol (mg/dL)	54.29 (11.06)	0.91 (6.89)	−0.13 + (7.06)	52.73 (11.21)	2.53* (7.79)	2.62 ^ (7.83)	−1.74 (−3.69, 0.22)	1.35 (−0.10, 2.79)	2.29 (0.84, 3.74)
LDL cholesterol (mg/dL)	133.67 (35.19)	−4.17* (25.22)	2.03 + (28.77)	139.22 (38.03)	−3.03 (27.71)	−0.36 (27.70)	5.67 (−1.45, 12.80)	3.44 (−1.38, 8.26)	−0.41 (−5.74, 4.92)
Triglycerides (mg/dL)	144 [107: 186]	−5.93 (55.03)	−13.48^ (55.65)	134 [104:182]	−13.48* [−38:6]	−16^ [−42:10]	0.00 (−0.09, 0.08)	−0.12 (−0.18, −0.07)	−0.08 (−0.14, −0.02)
Remnant cholesterol (mg/dL)	29.13 (10.60)	−1.08 (8.66)	−1.52^ (8.05)	28.52 (10.88)	−3.72* (7.82)	−3.03^ (9.49)	−0.48 (−2.62, 1.67)	−2.81 (−4.30, −1.33)	−1.78 (−3.43, −0.13)
Blood pressure and anthropometric measurements									
Systolic pressure (mmHg)	139.25 (13.46)	−2.04* (14.30)	−3.64^ (14.61)	140.01 (12.90)	−6.38* (13.90)	−5.18^ (15.43)	0.63 (−1.92, 3.18)	−3.90 (−6.46, −1.35)	−1.09 (−3.79, 1.61)
Diastolic pressure (mmHg)	74.59 (10.74)	−0.81 (11.22)	−2.10^ (11.11)	75.59 (9.77)	−4.99* (10.20)	−4.06^ (10.87)	1.13 (−0.80, 3.05)	−3.39 (−5.24, −1.53)	−1.21 (−3.13, 0.71)
Weight (kg)	88.98 (13.71)	−2.66* (3.47)	−2.67^ (3.99)	87.54 (13.87)	−6.31* (4.09)	−7.41 + ^ (4.07)	−1.12 (−3.46, 1.21)	−3.74 (−4.46, −3.03)	−4.84 (−5.60, −4.08)
Waist circumference (cm)	111.47 (9.56)	−2.81* (3.70)	−2.83 ^ (4.48)	110.25 (9.74)	−6.21* (4.73)	−7.28 + ^ (4.37)	−1.10 (−2.84, 0.64)	−3.52 (−4.33, −2.70)	−4.57 (−5.44, −3.70)
Carbohydrate metabolism									
HOMA	3.14 (3.18)	−0.30* (1.82)	−0.34 ^ (1.52)	2.97 (2.17)	−0.75* (1.24)	−0.70^ (1.38)	−0.17 (−0.79, 0.45)	−0.49 (−0.83, −0.14)	−0.38 (−0.70, −0.06)
Glucose (mg/dL)	119.90 (33.19)	−2.92 (22.11)	−0.53 (27.27)	120.86 (31.32)	−6.67* (17.69)	−4.95^ (21.75)	1.04 (−5.25, 7.33)	−3.58 (−7.39, 0.23)	−4.22 (−9.16, 0.72)

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TABLE 1 (Continued)

	Control group			Intervention group			Control group vs. Intervention group		
	Baseline	6 month-change	12 month-change	Baseline	6 month-change	12 month-change	Baseline	6 month-adjusted model	12 month-adjusted model
HbA1c (%)	6.40 (1.11)	−0.27* (0.72)	−0.06 + (0.55)	6.33 (0.84)	−0.33* (0.45)	−0.21 + ^ (0.50)	−0.07 (−0.30, 0.15)	−0.06 (−0.19, 0.07)	−0.14 (−0.27, −0.02)
Insulin (pg/mL)	351.81 (230.53)	−24.19 (160.44)	−33.22^ (142.44)	336.79 (189.70)	−77.56* (129.63)	−79.59^ (131.15)	−14.65 (−62.49, 33.19)	−57.09 (−90.06, −24.12)	−49.39 (−79.44, −19.33)
Glucagon (pg/mL)	455.03 (163.32)	−36.43* (114.51)	−39.24^ (115.95)	446.44 (154.60)	−47.00* (128.95)	−48.99^ (125.23)	−6.35 (−41.12, 28.43)	−13.46 (−39.90, 12.98)	−11.33 (−38.03, 15.37)
C-peptide (pg/mL)	1054.99 (522.52)	−68.82* (303.26)	−89.74^ (313.09)	1066.02 (432.07)	−155.66+ (327.24)	−170.98^ (316.98)	11.15 (−97.34, 119.65)	−83.93 (−149.45, −18.41)	−82.90 (−149.68, −16.12)
GLP_1 (pg/mL)	172.39 (134.29)	−8.33 (81.05)	−12.46 (98.24)	166.78 (120.69)	−9.71 (91.22)	−23.20^ (82.12)	−5.75 (−34.43, 22.93)	−4.45 (−22.92, 14.01)	−13.74 −33.43, 5.95)
Hormones and inflammation biomarkers									
Ghrelin (pg/mL)	807.38 (415.02)	−30.86 (245.98)	−31.21 (236.83)	831.31 (412.41)	−11.05 (207.24)	5.69 (227.47)	26.33 (−67.05, 119.72)	26.96 (−24.03, 77.95)	40.68 (−12.90, 94.25)
Leptin (pg/mL)	7746.99 (4152.80)	−730.11 (2486.83)	−776.03 (2727.56)	7246.06 (3850.42)	−1020.43 (3141.41)	−1310.85 (2524.41)	−385.77 (−1145.57, 374.04)	−425.74 (−1036.51, 185.04)	−698.56 (−1295.48, −101.64)
PAI_1 (pg/mL)	2631.62 (838.39)	−250.14* (715.26)	−129.02 + ^ (729.33)	2652.39 (828.08)	−425.63+ (837.60)	−371.54^ (692.88)	29.66 (−158.92, 218.24)	−153.42 (−312.00, 5.16)	−252.41 (−403.90, −100.92)
Resistin (pg/mL)	4625.87 (2138.00)	−286.75* (1670.16)	−362.79^ (1815.51)	4254.35 (1635.32)	−74.65* (1343.08)	−12.26 + ^ (1176.19)	−378.91 (−812.86, 55.05)	55.85 (−247.20, 358.89)	151.36 (−160.26, 462.98)
Visfatin (pg/mL)	1309.09 (1620.44)	−302.97* (1314.76)	−300.98^ (1375.44)	1194.63 (1093.55)	−270.33+ (668.66)	−258.90^ (613.18)	−93.64 (−387.15, 199.87)	−52.22 (−205.86, 101.41)	−47.97 (−214.70, 118.76)
hs-PCR (mg/dL)	0.45 (0.61)	−0.07 (0.81)	−0.13^ (0.47)	0.45 (0.61)	−0.13* (0.63)	−0.11 (0.81)	0.00 (−0.15, 0.16)	−0.01 (−0.09, 0.08)	0.02 (−0.11, 0.15)

#Median and interquartile range were displayed in non-normal distributed variables *: significant *P-value* between baseline and 6-month follow-up; + : significant *P-value* between 6-month follow-up and 12-month follow-up; ^: significant *P-value* between baseline and 12-month follow-up.

TABLE 2 Baseline and differences at 6-and 12-month follow-ups (mean and standard deviation) stratified in the control and intervention groups in the consumption of key food items and dietary parameters between the control and intensive group adjusted for the baseline value.

	Baseline	6 month-change	12 month-change	Baseline	6 month-change	12 month-change	Baseline	6 month-adjusted model	12 month-adjusted model
Energy intake (kcal/day)	2464.42 (548.70)	−135.32 (559.65)	−160.20 (576.76)	2357.21 (528.53)	−111.46 (585.36)	−110.20 (581.21)	< 0.05	0.054	0.368
Carbohydrates (g/day)	227.24 (66.12)	−20.31 (73.14)	−20.84 (64.67)	218.22 (69.56)	−22.88 (72.54)	−18.93 (75.03)	0.181	< 0.05	0.241
Protein (g/day)	105.80 (19.94)	2.07 (20.69)	−1.74 (22.29)	102.25 (19.77)	6.84 (23.13)	6.23 (22.15)	0.072	0.288	< 0.05
Total fat (g/day)	118.22 (28.89)	−4.14 (30.72)	−5.15 (33.56)	113.22 (28.08)	−2.12 (33.58)	−3.45 (33.79)	0.077	0.272	0.255
Saturated fatty acids (g/day)	30.80 (9.50)	−5.47 (9.11)	−5.84 (9.62)	29.25 (9.03)	−6.18 (9.58)	−6.19 (9.89)	0.093	< 0.001	< 0.05
Monounsaturated fatty acids (g/day)	61.05 (15.04)	1.83 (19.54)	2.11 (20.07)	58.08 (14.90)	5.59 (21.29)	4.85 (19.85)	< 0.05	0.451	0.968
Polyunsaturated fatty acids (g/day)	19.01 (5.83)	3.12 (6.90)	2.29 (7.32)	18.82 (6.84)	3.64 (7.78)	2.62 (8.19)	0.761	0.528	0.800
Cholesterol (mg/day)	426.16 (105.26)	−40.19 (106.38)	−48.23 (121.61)	418.58 (118.16)	−35.06 (124.82)	−41.95 (132.46)	0.495	0.934	0.733
Trans-fatty acids (g/day)	0.72 (0.41)	−0.27 (0.39)	−0.28 (0.43)	0.70 (0.44)	−0.37 (0.45)	−0.37 (0.46)	0.579	< 0.001	< 0.001
Linolenic acid	1.74 (0.67)	0.52 (0.84)	0.37 (0.95)	1.72 (0.78)	0.63 (0.98)	0.44 (0.92)	0.775	0.248	0.538
Carbohydrate percentage (%)	36.72 (5.44)	−1.34 (6.41)	−1.01 (5.90)	36.72 (5.89)	−2.06 (6.67)	−1.35 (6.71)	1	0.076	0.462
Protein percentage (%)	17.44 (2.46)	1.28 (2.98)	0.84 (2.79)	17.62 (2.68)	1.88 (3.02)	1.83 (3.27)	0.481	< 0.001	< 0.001
Total fat percentage (%)	43.22 (5.36)	0.90 (6.52)	0.97 (6.14)	43.33 (5.70)	1.27 (6.70)	0.64 (6.93)	0.837	0.203	0.526
Saturated fatty acid percentage (%)	11.18 (1.97)	−1.44 (2.16)	−1.48 (1.96)	11.09 (1.82)	−1.86 (1.95)	−1.91 (1.99)	0.635	< 0.05	< 0.001
Monounsaturated fatty acid percentage (%)	22.41 (3.58)	1.91 (5.33)	2.27 (4.86)	22.34 (4.10)	3.16 (5.55)	2.89 (5.49)	0.856	0.004	0.219

(Continued)

TABLE 2 (Continued)

	Baseline	6 month-change	12 month-change	Baseline	6 month-change	12 month-change	Baseline	6 month-adjusted model	12 month-adjusted model
Polyunsaturated fatty acid percentage (%)	6.95 (1.65)	1.64 (2.25)	1.37 (2.15)	7.21 (2.17)	1.82 (2.48)	1.38 (2.51)	0.185	0.014	0.198
Meat and meat products (g/day)	174.48 (59.31)	−17.35 (60.32)	−24.14 (61.51)	166.54 (52.82)	−7.08 (60.80)	−9.02 (63.08)	0.155	0.279	< 0.05
Fish (g/day)	120.91 (42.98)	17.11 (49.01)	6.91 (47.91)	126.49 (46.18)	15.53 (60.00)	14.27 (57.86)	0.207	0.523	0.005
Vegetables (g/day)	343.34 (149.73)	42.53 (186.18)	33.25 (171.62)	354.26 (122.21)	49.97 (176.83)	57.45 (143.06)	0.422	0.226	< 0.05
Total cereals (g/day)	129.44 (58.75)	−5.29 (69.71)	−7.00 (61.00)	119.38 (63.50)	−0.15 (63.61)	6.69 (78.60)	0.098	0.248	0.450
Dairy products (g/day)	370.65 (181.52)	14.88 (209.43)	−10.69 (208.67)	339.42 (168.83)	27.83 (214.99)	35.79 (189.12)	0.073	0.629	0.105
Nuts (g/day)	15.69 (15.92)	21.24 (26.01)	19.97 (25.91)	15.83 (16.73)	28.66 (25.76)	25.12 (25.57)	0.933	< 0.05	< 0.05
Fruit (g/day)	351.48 (174.25)	0.63 (224.44)	35.68 (223.37)	351.14 (174.26)	19.97 (221.11)	22.88 (209.81)	0.984	0.255	0.479
Legumes (g/day)	20.53 (10.15)	3.99 (12.26)	3.35 (13.21)	19.73 (9.02)	7.26 (12.01)	5.32 (11.78)	0.399	0.007	0.145
Olive oil (g/day)	47.77 (13.78)	−0.11 (17.34)	1.35 (16.49)	45.47 (14.44)	1.89 (17.33)	2.17 (16.41)	0.100	0.906	0.244
Virgin olive oil (g/day)	30.39 (20.44)	13.40 (22.33)	13.94 (21.70)	31.61 (20.07)	12.29 (21.66)	13.61 (21.43)	0.544	0.963	0.580
Sunflower oil (g/day)	0.74 (2.81)	−0.65 (2.77)	−0.40 (2.56)	1.35 (6.44)	−1.31 (6.65)	−1.19 (6.24)	0.214	0.755	0.327
Dietary fiber (g/day)	24.93 (7.18)	5.80 (8.72)	5.08 (8.75)	25.25 (6.93)	7.29 (8.83)	6.93 (8.16)	0.645	< 0.05	< 0.001
Alcohol (g/day)	9.76 (12.52)	−3.59 (10.38)	−3.36 (8.86)	8.05 (9.84)	−4.02 (9.45)	−4.04 (7.81)	0.128	< 0.05	< 0.05

TABLE 3 Tertiles of weight loss change (mean, standard deviation, and their comparison) adjusted for weight and baseline value of the participants on the 17-item questionnaire, physical activity, biomarkers and anthropometric measurements, lipid profile, carbohydrate metabolism, and hormones.

	First tertile of 6-month weight-loss change	Second tertile of 6-month weight-loss change	Third tertile of 6-month weight-loss change	Global *P-value*	First tertile of 12-month weight-loss change	Second tertile of 12-month weight-loss change	Third tertile of 12-month weight-loss change	Global *P-value*
Diet adherence and physical activity								
Mediterranean diet adherence (17-point item score)	4.93 (3.12)	3.31* (2.91)	2.42 + ^ (3.04)	< 0.001	4.56 (3.10)	2.70* (3.18)	2.34 + ^ (2.98)	< 0.001
Physical activity (MET·min/week)	1084.68 (2503.52)	390.80* (2197.49)	167.02^ (2356.66)	< 0.001	933.72 (2398.89)	596.32* (1969.18)	311.94^ (2346.88)	< 0.05
Lipid profile								
Total cholesterol (mg/dL)	−8.99 (30.57)	−4.73 (36.49)	1.64^ (28.05)	< 0.05	−2.52 (32.39)	−3.51 (36.13)	3.76 (30.54)	0.414
HDL cholesterol (mg/dL)	3.34 (8.04)	0.71* (6.96)	0.96^ (6.80)	< 0.05	3.54* (7.87)	0.71* (7.12)	−0.73^ (7.11)	< 0.001
LDL cholesterol (mg/dL)	−8.00 (25.82)	−1.21 (30.34)	−1.29^ (22.28)	0.113	−1.17 (27.43)	0.03 (30.99)	4.05 (25.70)	0.370
Triglycerides (mg/dL)	−33.70 (61.93)	−14.25* (53.21)	1.22^ (53.76)	< 0.001	−27.33 (53.10)	−21.30* (57.28)	−2.96 + ^ (60.50)	< 0.001
Remnant cholesterol (mg/dL)	−5.35 (8.44)	−1.90* (7.54)	0.33^ (8.05)	< 0.001	−4.72 (9.12)	−2.59* (8.30)	0.90 + ^ (8.12)	< 0.001
Blood pressure and anthropometric measurements								
Systolic pressure (mmHg)	−7.71 (13.62)	−4.48* (14.46)	−0.14 + ^ (13.74)	< 0.05	−7.86 (13.49)	−3.96* (15.40)	−1.18^ (15.50)	< 0.05
Diastolic pressure (mmHg)	−5.41 (10.11)	−2.84 (11.21)	−0.21 + ^ (10.89)	< 0.001	−4.71 (10.98)	−3.39 (11.29)	−0.95 + ^ (10.51)	< 0.05
Waist circumference (cm)	−8.20 (4.24)	−3.88* (3.28)	−1.19 + ^ (2.87)	< 0.001	−9.32 (4.64)	−4.45* (2.83)	−1.05 + ^ (3.21)	< 0.001
Carbohydrate metabolism								
HOMA	−0.94 (1.25)	−0.38* (2.08)	−0.02^ (1.01)	< 0.001	−1.02 (1.18)	−0.49* (1.61)	0.13 + ^ (1.36)	< 0.001
Glucose (mg/dL)	−10.73 (19.68)	−4.47* (21.84)	1.20 + ^ (16.96)	< 0.001	−8.16 (18.06)	−2.87 (31.30)	3.46^ (21.29)	< 0.001
HbA1c (%)	−0.44 (0.54)	−0.30 (0.77)	−0.07 + ^ (0.33)	< 0.001	−0.35 (0.47)	−0.05* (0.48)	0.06^ (0.57)	< 0.05
Insulin (pg/mL)	−93.90 (134.85)	−30.79* (177.62)	−5.45^ (102.70)	< 0.001	−108.89 (124.15)	−43.90* (143.14)	1.47^ (126.86)	< 0.001
Glucagon (pg/mL)	−72.75 (131.53)	−29.64* (106.92)	−7.82^ (112.95)	< 0.001	−83.56 (126.80)	−30.87* (102.37)	−5.49^ (116.94)	< 0.001

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TABLE 3 (Continued)

	First tertile of 6-month weight-loss change	Second tertile of 6-month weight-loss change	Third tertile of 6-month weight-loss change	Global *P-value*	First tertile of 12-month weight-loss change	Second tertile of 12-month weight-loss change	Third tertile of 12-month weight-loss change	Global *P-value*
C-peptide (pg/mL)	−207.60 (322.66)	−71.29* (299.50)	−9.15^ (292.38)	< 0.001	−227.06 (305.38)	−104.96* (303.46)	−26.49^ (313.63)	< 0.001
GLP_1 (pg/mL)	−14.42 (84.28)	−3.39* (99.23)	−7.89 (68.07)	0.105	−21.13 (75.58)	−18.21 (88.02)	−12.87^ (111.02)	0.109
Hormones and inflammation biomarkers								
Ghrelin pg/mL)	−32.28 (205.27)	−21.59 (195.07)	−3.61 (295.65)	0.653	−8.91 (225.22)	−20.47 (224.18)	−9.35 (253.85)	0.966
Leptin (pg/mL)	−1549.05 (3235.36)	−724.65* (2355.01)	22.19^ (2400.70)	< 0.001	−1789.81 (2299.54)	−1194.54+ (2562.08)	156.79 +^ (2762.06)	< 0.001
PAI_1 pg/mL)	−484.66 (811.69)	−261.77* (786.25)	−193.61^ (684.39)	< 0.05	−464.06 (640.85)	−239.00* (718.15)	31.85 +^ (735.85)	< 0.001
Resistin (pg/mL)	−142.90 (1542.54)	−183.42 (1322.29)	−−253.10 (1745.68)	0.588	−106.24 (1215.95)	−129.09 (1457.42)	−369.27 (1971.13)	0.710
Visfatin (pg/mL)	−352.27 (702.77)	−227.70 (568.43)	−263.99^ (1780.08)	< 0.001	−388.93 (627.74)	−226.48* (553.56)	−192.99^ (1765.19)	< 0.001
hs-PCR (mg/dL)	−0.21 (0.72)	−0.42 (2.94)	−0.04 (0.33)	0.297	−0.23 (0.65)	−0.01 (0.86)	−0.54 (3.35)	0.141

*: significant *P-value* between first and second tertile; +: significant *P-value* between second and third tertile; ^: significant *P-value* between first and third tertile.

TABLE 4 Tertiles of waist circumference change (mean, standard deviation, and their comparison) adjusted for weight and baseline value of the participants on the 17-item questionnaire, physical activity, biomarkers and anthropometric measurements, lipid profile, carbohydrate metabolism, and hormones.

	First tertile of 6-month waist circumference change	Second tertile of 6-month waist circumference change	Third tertile of 6-month waist circumference change	Global *P-value*	First tertile of 1-year waist circumference change	Second tertile of 1-year waist circumference change	Third tertile of 1-year waist circumference change	Global *P-value*
Diet adherence and physical activity								
Mediterranean diet adherence (17-point item score)	4.76 (3.12)	3.38* (3.04)	2.55 + ^ (3.04)	< 0.001	4.73 (3.34)	2.92* (2.58)	2.48 + ^ (2.87)	< 0.001
Physical activity (MET·min/week)	932.61 (2453.74)	617.23 (2262.68)	122.75 + ^ (2462.50)	< 0.05	1202.29 (2242.48)	531.83* (2238.96)	263.94 + ^ (2051.53)	< 0.001
Lipid profile								
Total cholesterol (mg/dL)	−5.33 (31.52)	−4.50 (33.51)	−2.09 (31.08)	0.796	−3.66 (30.10)	2.11 (34.04)	−1.36 (35.69)	0.168
HDL cholesterol (mg/dL)	2.89 (8.22)	1.63 (6.61)	0.36^ (7.05)	< 0.05	2.01 (7.33)	2.60 (7.65)	−1.19 + ^ (7.24)	< 0.001
LDL cholesterol (mg/dL)	−3.70 (27.74)	−2.60 (26.89)	−4.74 (24.42)	0.567	0.74 (25.35)	1.77 (29.73)	−0.13 (29.73)	0.231
Triglycerides (mg/dL)	−31.64 (64.98)	−17.41* (53.28)	5.09 + ^ (48.90)	< 0.001	−33.86 (54.90)	−12.75* (50.83)	−5.30^ (64.12)	< 0.001
Remnant cholesterol (mg/dL)	−4.41 (8.71)	−2.81* (7.26)	0.60 + ^ (8.39)	< 0.001	−5.65 (8.85)	−1.48* (7.86)	0.61 + ^ (8.65)	< 0.001
Blood pressure and anthropometric measurements								
Systolic pressure (mmHg)	−7.86 (14.34)	−4.32* (13.46)	0.51 + ^ (13.90)	< 0.001	−7.00 (13.94)	−4.02 (15.69)	−1.99^ (15.07)	< 0.05
Diastolic pressure (mmHg)	−5.02 (10.19)	−3.57 (10.68)	0.68 + ^ (11.31)	< 0.001	−3.72 (11.06)	−4.64 (10.58)	−0.56 + (11.14)	< 0.05
Weight (kg)	−8.00 (4.49)	−4.07* (1.97)	−0.81 + ^ (2.24)	< 0.001	−9.16 (4.60)	−4.83* (2.59)	−1.15 + ^ (2.58)	< 0.001

(Continued)

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TABLE 4 (Continued)

	First tertile of 6-month waist circumference change	Second tertile of 6-month waist circumference change	Third tertile of 6-month waist circumference change	Global *P-value*	First tertile of 1-year waist circumference change	Second tertile of 1-year waist circumference change	Third tertile of 1-year waist circumference change	Global *P-value*
Carbohydrate metabolism								
HOMA	−0.83 (1.30)	−0.68 (1.54)	0.12 + ^ (1.78)	< 0.001	−0.95 (1.22)	−0.57* (1.29)	−0.05 + ^ (1.71)	< 0.001
Glucose (mg/dL)	−9.30 (19.90)	−5.65 (20.32)	1.84 + ^ (18.58)	< 0.001	−5.92 (28.74)	−2.98 (20.17)	1.12^ (24.32)	0.051
HbA1c (%)	−0.45 (0.51)	−0.30* (0.71)	−0.09 + ^ (0.49)	< 0.001	−0.36 (0.49)	−0.14* (0.43)	0.10 + ^ (0.58)	< 0.001
Insulin (pg/mL)	−82.04 (132.49)	−59.17* (130.24)	5.55 + ^ (176.87)	< 0.001	−95.44 (123.25)	−66.84* (128.61)	−5.78 + ^ (149.81)	< 0.001
Glucagon (pg/mL)	−78.51 (122.47)	−31.70* (118.85)	−6.87^ (112.42)	< 0.001	−69.39 (139.30)	−47.78* (96.20)	−14.95 + ^ (120.76)	< 0.001
C-peptide (pg/mL)	−184.51 (354.04)	−105.02* (277.13)	−21.46^ (299.70)	< 0.001	−216.09 (296.89)	−139.44* (295.42)	−34.91 + ^ (336.88)	< 0.001
GLP_1 (pg/mL)	−7.86 (86.89)	−11.69 (93.79)	−6.64 (73.08)	0.620	−12.84 (73.14)	−25.92 (88.71)	−13.53 (107.03)	0.239
Hormones and inflammation biomarkers								
Ghrelin (pg/mL)	−36.13 (212.62)	−4.74 (183.25)	−25.43 (296.16)	0.657	−23.74 (259.87)	8.61 (182.88)	−26.26 (254.03)	0.541
Leptin (pg/mL)	−1654.22 (3115.84)	−545.42* (2525.77)	−281.55^ (2583.37)	< 0.001	−1741.04 (2490.24)	−924.76* (2237.87)	−468.22^ (3030.65)	< 0.001
PAI_1 (pg/mL)	−561.69 (620.62)	−295.04* (949.65)	−92.44^ (609.46)	< 0.001	−456.89 (603.42)	−243.57* (710.70)	−52.24 + ^ (785.81)	< 0.001
Resistin (pg/mL)	−166.84 (1586.23)	−137.04 (1494.89)	−275.84 (1492.57)	0.668	−225.48 (1522.45)	−40.72 (1367.82)	−317.87 (1727.57)	0.935
Visfatin (pg/mL)	−379.89 (695.73)	−308.23* (1494.60)	−136.14 ^ (571.59)	< 0.001	−355.01 (579.81)	−375.96* (1571.81)	−96.18^ (599.17)	< 0.001
hs-PCR (mg/dL)	−0.22 (0.77)	−0.39* (2.77)	−0.06^ (0.42)	< 0.05	−0.13 (0.87)	−0.17 (0.62)	−0.43 (3.01)	0.413

*: significant *P-value* between first and second tertile; + : significant *P-value* between second and third tertile; ^: significant *P-value* between first and third tertile.

several participants reported freshness and palatability of food, with variance across the studies regarding taste (56–58). Meal plans resulted in hedonic appreciation and satisfaction by most participants (58), although this differed according to age and dishes (57). There were, however, a number of barriers, such as dislike of some foods (including olive oil) and/or reduction of red meat. In addition to diet acceptability, various limitations have been reported such as the perception of expense, expectation of time commitment, perceived impact on body weight, and cultural differences (56, 58–60). Among a group of schoolchildren, a study found that food neophobia correlated negatively with certain healthy dietary habits, such as fruit and vegetable consumption.

The intervention group was based on a hypocaloric diet with moderate fat consumption of vegetable origin: olive oil, tree nuts, and peanuts. Furthermore, it was designed to augment complex carbohydrates and fiber-rich products. Moderate intake of monounsaturated fat in the form of olive oil is one of the cornerstones of MedDiet due to its culinary versatility. Its beneficial effects on the reduction of cardiovascular disease include cardioprotective characteristics, improvement in lipid profile (decrease in total and LDL cholesterol and an increase of HDL cholesterol) and blood pressure decrease, amelioration of LDL cholesterol oxidation and low-chronic inflammation, and anti-atherogenic properties (61–67).

Weight and waist circumference

While short-term changes are relatively easy to accomplish, successfully maintaining them over time is considerably more difficult. The combination of diet-induced weight loss with exercise training has demonstrated greater improvement in cardiovascular risk factors than diet alone (68, 69). Our findings from the intervention group showed a decrease in waist circumference and weight at both 6-and 12-month follow-ups, and the comparison with the control was significant for both periods. The weight loss experienced by the control group, despite following a non-reduced diet, can be explained by their motivation to participate in a clinical trial for subjects with overweight/obesity. In the intervention group, the maximum weight loss was at 1 year. Such a finding is particularly relevant since in most studies on the effects of restrictive diets this occurs at 6 months followed by a reward effect. Interventions with hypocaloric diets which can be sustainable over time could, therefore, provide a better approach to weight loss. In this regard, a MedDiet is appropriate as its better palatability, due to its mainly vegetal content and use of olive oil leads to greater adherence.

Leptin–Ghrelin binomial

Hyperleptinemia is a characteristic manifestation of obesity in humans. Resistance to leptin action in obesity has been suggested, and elevated circulating concentrations may be necessary to maintain sensitivity to hormone and energy homeostasis (70, 71). Leptin, as a polypeptide secreted by adipocytes, might be decreased as a result of fat mass reduction (72, 73). We observed a significant reduction in its levels after both the intervention and control groups. The former displayed an overall stronger decrease probably caused by the further reduction of anthropometric measurements. In fact, a significant reduction was reported comparing the intervention arm to the control at 12-month follow-up.

Individuals with overweight/obesity have typically lower circulating ghrelin levels. This adipogenic hormone seems to indicate downregulation in human obesity, supposedly as an adaptive mechanism in response to positive energy balance (74, 75). Diet-induced effects usually show an increase in circulating levels, although reversion to baseline levels at 12 months after a 6-month peak has been reported (76). Our cohort reflected an initial reduction followed by a minor increase in circulating levels in the intensive group, with no statistical significance.

Carbohydrate metabolism-related hormones

Weight loss interventions lead to changes in carbohydrate homeostasis, and increased insulin sensitivity has been observed following dietary interventions, physical activity, and bariatric surgery (77, 78). Nevertheless, in contrast to isolated interventions, the combined effects of a restricted diet and physical exercise have been reported to improve to a greater extent such sensitivity and variables related to the cardiometabolic syndrome. In our intervention group, insulin levels decreased during the first 6 months and were maintained up to the 12-month follow-up. The control group also experienced a steady reduction although it presented higher levels at 6-and 12-month follow-ups. HOMA, C-peptide, HbA1c, and glucose levels followed a similar pattern.

Glucagon improvement caused by diet and exercise training has been reported in the literature. A meta-analysis made up of 29 interventions assessed body weight change, glucagon, insulin, and glucose fasting concentrations after two different weight reduction methods (bariatric surgery versus low-caloric diet intervention). More than half the diet interventions resulted in a decrease from 17 to 27%. The mean decrease in fasting glucagon, however, was not significantly different between both weight reduction approaches (77). Although no inter-group differences in the present study were obtained, a linear time component proved to be a predictor of weight loss regardless of the intervention.

Lipid profile

Triglyceride reduction is crucial in the management of dyslipidemia, particularly atherogenic dyslipidemia which is highly prevalent in metabolic syndrome subjects. Atherogenic dyslipidemia is characterized by high circulating triglyceride levels and low levels of HDL cholesterol, and even optimal concentrations of LDL cholesterol. We have recently reported in subjects with overweight/obesity at high cardiovascular risk, that triglycerides and remnant cholesterol levels, but not LDL cholesterol, were associated with cardiovascular outcomes irrespective of other risk factors (79, 80). Triglyceride concentration is an independent risk factor for cardiovascular disease and is strongly associated with subcutaneous abdominal adipose tissue. In fact, it has been suggested that triglycerides could be a predictor of cardiovascular disease (79). The MedDiet has been previously studied as a dietary tool to improve metabolic syndrome and subsequent events (6, 79, 81). In this respect, our results show an overall triglyceride reduction in both groups, with a greater reduction in the intervention group than in the control. In concordance, we have recently reported that an energy-reduced MedDiet plus physical activity improves HDL-related triglyceride metabolism versus a non-reduced MedDiet without physical activity (82). Regarding remnant cholesterol, its levels follow a similar pattern to that of triglycerides. Although we did not observe changes after the intervention in total cholesterol, remnant cholesterol decreased in mid-and long-term versus the control group. Such a finding could be a good indicator that the intensive intervention shifted toward protection against cardiovascular risk.

High-density lipoprotein (HDL) cholesterol lipoproteins are known for their atheroprotective effects through a number of anti-inflammatory, anti-oxidative, anti-thrombotic, and anti-apoptotic properties (83, 84). An inverse association between triglycerides and HDL cholesterol concentrations usually occurs. In fact, HDL lipoproteins are catabolized faster in the presence of hypertriglyceridemia in non-pathological states. In our study, while the intervention group experienced an increase in the first 6 months and kept a steady concentration at 12 months, the control group had increased HDL cholesterol in the first 6 months which was slightly decreased at 12 months.

Pro-inflammatory markers

High sensitivity C reactive protein (hs-CRP) is broadly used to monitor inflammatory processes, including autoimmune, infectious, tumoral, and metabolic diseases. Prospective epidemiological studies have reported elevated hs-CRP as an independent factor associated with cardiovascular events (26, 85). Dietary interventions usually lead to inflammatory profile improvement (86), we observed a reduction in hs-CRP levels across time in both groups, with no significant inter-group results.

Plasminogen activator inhibitor-1 plasma levels are positively associated with cardiovascular disease, thrombosis, fibrosis, and the progression of coronary syndromes (87). They are also positively correlated with individual risk factors (BMI, triglycerides, glucose, and mean arterial pressure) which may be indicative of their relevance in metabolic syndrome events (88). Diet composition has been demonstrated to affect circulating levels of PAI-1 and the fibrinolytic system as much as alcohol intake and smoking. High-fat diet consumption increases PAI-1 levels impairing clot lysis (29, 89). In our study, both groups produced a marked change in PAI-1 levels, although decreases were higher in the intensive group, mainly at the 12-month follow-up.

Cross-sectional studies have demonstrated that, compared to lean individuals, those with obesity have higher resistin levels (90–92). Some weight loss programs, however, have not always resulted in a decrease in circulating levels (31, 93, 94), while others reflect parallel reduction (95, 96). Regarding visfatin, weight loss programs have achieved a decrease in their levels, with no significant difference between them (94, 97). Nevertheless, there is evidence that a MedDiet has not always demonstrated an improvement in visfatin concentrations (98). In our study, resistin and visfatin levels displayed parallel behavior in both groups with an initial reduction at 6 months followed by steady maintenance at 12 months.

Strengths and limitations

Our large sample size and randomized design provide high-quality evidence that minimizes confounding and bias influences. We have comprehensively assessed diverse cardiovascular risk biomarkers and satiety-related hormones. There are, however, some limitations. First, results were obtained in adult/elderly participants with metabolic syndrome and excess body weight; therefore, our findings cannot be extrapolated to other populations. Second, we observed only moderate differences between the two intervention arms. Such a finding was to be expected as the control group was an active comparator following a healthy traditional MedDiet. Moreover, due to the physiological regulation of ghrelin, among other hormones, the measurement of post-prandial levels would have been inestimable contribution, further research is warranted. Nevertheless, this randomized trial provides high-level evidence of the benefits of an intervention with a restrictive MedDiet and physical activity, especially on weight, waist circumference, leptin levels, lipid/glucose metabolism, blood pressure, and the pro-inflammatory marker PAI-1 at mid-and long-term intervention in subjects with metabolic syndrome. Given that

such changes were maintained over time, and the marked palatability and acceptability of the MedDiet on the part of the consumers, MedDiet pattern interventions with hypocaloric diets could be a pertinent approach to weight loss.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committees of all centers approved the study protocol during 2013 and 2014. The trial was registered in 2014 at (www.isrctn.com/ISRCTN89898870). The patients/participants provided their written informed consent to participate in this study.

Author contributions

MF, JS-S, MM-G, DC, ER, FT, and RE designed the clinical trial. OC and MF designed the conceptualization sub-study. JH-R performed the formal and laboratory analysis. AT and JH-R carried out the statistical analysis. OC, MF, and JH-R drafted the manuscript. AT, DB, JS-S, MM-G, DC, RE, AG, OC, and MF revised and approved the final version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2022.950900/full#supplementary- material

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2nd Manuscript

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Mediterranean Diet Modulates Gene Expression of Cholesterol Efflux Receptors in High-Risk Cardiovascular Patients

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Abstract (300 words maximum)

In this study, we investigated gene expression related to cholesterol efflux receptors in individuals at high cardiovascular risk undergoing Mediterranean dietary interventions. Through transcriptomic analysis, we examined samples from two trials: PREDIMED and PREDIMED-Plus, with 151 and 89 elderly adults, respectively. Blood cells were isolated at baseline and after a 12-month intervention. In the PREDIMED trial, participants followed different Mediterranean diets: one supplemented with extra-virgin olive oil (MedDiet-EVOO), another with nuts (MedDiet-Nuts), and a low-fat control diet. The PREDIMED-Plus trial compared an energy-reduced Mediterranean diet (Er-MedDiet) with physical activity to an ad libitum Mediterranean diet. Over time, mild but significant upregulation of genes like ABCA1, RXRA, RXRB, and NR1H3 was observed in response to MedDiet-EVOO, MedDiet-Nuts, and Er-MedDiet. Notably, RXRA expression was higher in both MedDiet-EVOO and MedDiet-Nuts compared to the control diet. Differences in gene expression, particularly RXRA, ABCG1, NR1H3, and PPARD, were evident between MedDiet-Nuts and the control diet. In the PREDIMED-Plus trial, no significant differences in gene expression were found between dietary groups. Principal component analysis (PCA) and linear discriminant analysis (LDA) showed overlapping gene expression profiles across different Mediterranean diet interventions. In conclusion, our study highlights mild upregulation of cholesterol efflux-related genes—specifically involving RXRA, RXRB, ABCA1, ABCG1, NR1H3, and PPARD—in response to long-term adherence to Mediterranean diets among elderly adults at high cardiovascular risk. This suggests a potential mechanism by which these diets may exert cardiovascular protective effects.

Background: Atherosclerosis marks the initial stage of most cardiovascular diseases. Omics tools might help uncover the mechanisms underlying atherosclerosis complexity.

Objective: In individuals at high cardiovascular risk, we examined the gene expression of receptors involved in HDL-mediated cholesterol efflux across different Mediterranean dietary interventions.

Methods: Transcriptomic expression analysis was conducted on samples obtained from two distinct clinical trials, PREDIMED and PREDIMED-Plus, involving 151 and 89 elderly adults, respectively. Peripheral nucleated blood cells were isolated at baseline and after a 12-month intervention for, in three PREDIMED groups [Mediterranean diet (MedDiet) supplemented with extra-virgin olive oil (EVOO) (MedDiet-EVOO), MedDiet enriched with nuts (MedDiet-Nuts) and low-fat control diet] in the PREDIMED study; and in two PREDIMED-Plus groups [energy-reduced MedDiet (Er-MedDiet) with physical activity compared to an ad libitum MedDiet]. Gene expression analysis was studied along time, between groups (adjusting by multiple covariates) and applying supervised and unsupervised learning methods, linear discriminant (LDA) and principal component analysis (PCA), respectively.

Results: Mild but significant upregulation was observed along time in ABCA1 in the MedDiet-EVOO (fold-change (FC)=1.18), MedDiet-Nuts (FC = 1.22) and Er-MedDiet (FC = 1.23); RXRA was upregulated in both non-restrictive MedDiets, the MedDiet-EVOO (FC=1.11) and MedDiet-Nuts (FC=1.13). In addition, RXRB expression increased after the MedDiet-EVOO (FC=1.13) while NR1H3 increased after the MedDiet-Nuts (FC = 1.18). Intergroup differences were observed especially in RXRA between both MedDiet-EVOO and MedDiet-Nuts versus the control diet. In the comparison between MedDiet-Nuts and control, we observed significant results also in ABCG1, NR1H3, and PPARD. No statistically significant differences were observed between groups in PREDIMED-Plus trial. PCA and LDA methods displayed an overlapping profile of gene expression among different MedDiet interventions.

Conclusions: Mild upregulation of cholesterol efflux-related genes involving retinoid X (RXRA, RXRB), ATP-binding cassette family (ABCG1, ABCA1), liver X (LXRa/NR1H3), and

peroxisome proliferator activated (PPARD) receptors, occurred as long-term responses to different Mediterranean diets in elderly adults at high cardiovascular risk.

Abbreviations:

CVDs: cardiovascular diseases
MedDiet: Traditional Mediterranean diet
HDL: High-density lipoprotein
MedDiet-EVOO: traditional Mediterranean diet enriched with extra-virgin olive oil
MedDiet-Nuts: MedDiet enriched with nuts MedDiet-Nuts
ABCA1: ATP binding cassette subfamily A member 1
ABCG1: ATP binding cassette subfamily G member 1
NR1H2/LXR-α: nuclear receptor subfamily 1 group H member 2
NR1H3/LXR-β: nuclear receptor subfamily 1 group H member 3
RXRA: retinoid X receptor alpha
RXRB: retinoid X receptor beta
CEC: cholesterol efflux capacity
SCARB1: Scavenger Receptor Class B Type 1
CAV1: Caveolin-1
PPARs: peroxisome proliferator activated receptors
BP: blood pressure
LDL-c: low-density lipoprotein cholesterol
HDL-c: High-density lipoprotein cholesterol
Er-MedDiet: energy-reduced MedDiet
FC: fold-change
Ct: cycle threshold
PCA: principal component analysis
SFA: saturated fatty acid
PUFA: polyunsaturated fatty acid

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Keywords: Mediterranean diet, cardiovascular risk, transcriptomic, nutrigenomic, randomized controlled trial, omics

Introduction

Cardiovascular diseases (CVDs) are the leading cause of death globally, representing an estimation of 17.9 million of deaths from CVDs (1). Lifestyle and dietary patterns are key factors in the development of metabolic syndrome or its components (2,3). Traditional Mediterranean diet (MedDiet) is characterized by a high intake of extra-virgin olive oil, cereals, legumes, fish, vegetables, and fruit (4), along with a lower consumption of red and processed meat. This diet has demonstrated beneficial effects on cardiovascular risk factors by reducing inflammatory biomarkers levels and improving the lipid profile, in particular, HDL (high-density lipoprotein) functionality (5–8). HDL function enhancement has been reported under different MedDiet scenarios, such as a 12-month longitudinal clinical trial with a MedDiet supplemented with EVOO and nuts (8).

In the era of precision medicine, blood transcriptome has been put forward as a surrogate and accessible tissue that allows to infer or predict disease-related data for different purposes (9,10), including CVDs and nutrients interaction (11,12). Previous PREDIMED sub-studies have examined blood transcriptome response to dietary interventions supplemented with EVOO or nuts. The effects of a three-month MedDiet intervention on the expression of cardiovascular risk-related genes were reported using whole transcriptome microarray analyses in elderly subjects at high cardiovascular risk (MedDiet-EVOO through IL1b, IL1RN, TNF-α, and ICAM1). The effects of a three-month MedDiet intervention on the expression of cardiovascular risk-related genes were reported using whole transcriptome microarray analyses in elderly subjects at high cardiovascular risk (MedDiet-EVOO through IL1b, IL1RN, TNF-α, and ICAM1) (13). Furthermore, following a three-month intervention with the PREDIMED MedDiet enriched with mixed nuts or EVOO, a downregulation of transcriptomic pathways related to neuroinflammation (MedDiet-Nuts, with

downregulation levels of TNF-α, CCL3, IL-8, and IL10) was observed (14). Additionally, after a three-month intervention with a traditional MedDiet, particularly when supplemented with virgin olive oil, decreased gene expression linked to inflammation (INF-γ, ARHGAP15, IL7R) and oxidative stress (ADRB2, POLK) was observed in healthy subjects (15). Within the framework of the PREDIMED study, following a long-term MedDiet intervention (3 years), no statistically significant changes were observed between the MedDiet groups and the control group, whereas the control group showed a tendency to increase the gene expression of two inflammatory receptors involved in the pathogenesis of atherosclerosis (CXCR2, CXCR3) (16).

Based on the association between MedDiet and the overall cardiovascular benefit, along with HDL functionality we selected a subset of candidate genes involved at different stages of cholesterol efflux, to study the transcriptional landscape. First, ATP binding cassette subfamily A and G member 1 (ABCA1 and ABCG1) are membrane-bound proteins involved in cholesterol and phospholipid transport that are expressed in multiple tissues, where they play a role in reverse cholesterol transport, HDL lipoprotein formation, and pumping cholesterol to HDL particles at different stages (17). At the regulation stage, nuclear receptor subfamily 1 group H member 2 and 3 (*NR1H2* and *NR1H3*, also known as LXRB and LXRA) belong to a superfamily involved in modulation of reverse cholesterol transport through the translated protein's ability to form partnerships with functionally related molecules to regulate *ABCA1* and *ABCG1* expression (18–20). In a similar way, Retinoid X Receptors (RXRs) can operate as lipid sensors and partner with a variety of molecules to exert a wide range of functions including cholesterol efflux capacity (CEC) promotion (21). ABCA1, ABCG1, and Scavenger Receptor Class B Type 1 (*SCARB1*, also called *SR-BI*) cholesterol transporters are involved in cholesterol efflux from macrophages to lipid-free apoA-I and HDL as a first stage of reverse cholesterol transport (22). Caveolin (CAV1) is a

structural protein of caveolae (or plasma membrane invaginations) involved in cholesterol transport and signaling (23). Finally, peroxisome proliferator activated receptors (PPARs) are ligand-transcription factors with upregulating activity in several proteins involved in CEC and reverse cholesterol transport, fat storage, and oxidation (24,25).

To understand these molecules behavior in well-established interventions with solid evidence of ameliorating HDL functionality, the aim of the present study was to examine the transcriptomic response of cholesterol efflux-related genes and assess the long-term effects after 12 months of different Mediterranean diet-interventions in older adult subsamples at high cardiovascular risk.

Methods

Study design and subject recruitment

Our study population came from two clinical trial samples: PREDIMED (PREvención con DIeta MEDiterránea) and PREDIMED-Plus. Both studies are randomized, parallel, controlled, nutritional trials (Flow chart in **Image S1**, **Supplementary material**). Baseline characteristics of the subsample volunteers compared to general population in both studies, PREDIMED and PREDIMED-Plus, are described in **Supplementary table 1**.

PREDIMED Study

The PREDIMED study was a large-scale multicenter trial of 7.447 participants, that assessed the effect of a supplemented MedDiet on the primary prevention of CVD (26). The PREDIMED population for our study, was a random subsample of volunteers (n = 151, 77 women, and 74 men). The subsample included individuals from seven different recruiting sites, with similar baseline main characteristics (age, sex, hypertension, weight, body mass index (BMI), smoking status, cholesterol, triglycerides, and glucose levels, with the exception of diabetes status proportion). The

hypothesis was based on the comparison of two traditional MedDiets, one supplemented with extra-virgin olive oil (MedDiet-EVOO), another with nuts (MedDiet-Nuts), plus a third one as low-fat diet advice (control group). Participants in the MedDiet group received educational sessions on an ad libitum MedDiet based on a 14-item non-energy restricted score (27). No specific advice for increasing physical activity or losing weight was provided.

Eligible participants were women aged 60 – 80 y and men between 55 – 80 y who met at least one of the following criteria: 1) type 2 diabetes or 2) ⩾ 3 major cardiovascular risk factors, out of the following: current smoking (> 1 cig/day during the last month); hypertension (systolic BP (Blood Pressure) ⩾ 140 mmHg or diastolic BP ⩾ 90 mmHg or antihypertensive medication); LDL-c (low-density lipoprotein cholesterol) ⩾ 160 mg/dl or lipid-lowering therapy; HDL-c (high-density lipoprotein cholesterol) ⩽ 40 mg/dl in men or ⩽ 50 mg/dl in women; BMI ⩾ 25 kg/m2; or family history of premature coronary heart disease (28). Exclusion criteria included: prior history of CVD, severe chronic illness, drug or alcohol addiction, history of allergy or intolerance to olive oil or nuts, a low predicted likelihood of changing dietary habits according to the stages of change model (29) or any condition that could impair study participation (26). Main subsample characteristics, age, sex, glucose and lipid metabolism, and lifestyle habits including diet and physical activity questionnaire scores are described in **Table 1**.

PREDIMED-Plus Study

The PREDIMED-Plus is a multicenter lifestyle intervention with 6.874 eligible participants. It is a randomized trial conducted in 23 Spanish centers with a large cohort presenting metabolic syndrome recruited from primary healthcare centers (30). In the PREDIMED-Plus trial, the study population was a subgroup of 89 participants randomly selected (39 women and 40 men) from the IMIM (Hospital del Mar Research Institute) recruiting site. Inclusion criteria were men aged 55–

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75 years and women 60–75 years, with overweight/obesity (BMI: 27 – 40 kg/m2) and meeting at least three metabolic syndrome components at baseline: 1) triglycerides ⩾150 mg/dL or triglyceride-lowering medication; 2) fasting glucose ⩾100 mg/dL or glucose-lowering medication; 3) systolic/diastolic blood pressure ⩾130/85 mmHg or antihypertensive medication; 4) low HDL-c levels <50 mg/dL in women and <40 mg/dL in men or medication; and/or 5) waist circumference in women and ⩾102 cm in men (31,32).

Participants were randomly to either an energy-reduced MedDiet (Er-MedDiet) intervention) or an ad libitum MedDiet control group. Those in the active intervention group, followed an Er-MedDiet with physical activity promotion and behavioral support, to meet specific weight loss objectives. The participants received recommendations based on a 17-item MedDiet score (33). In addition, physical activity counseling to gradually increase exercise intensity to 150 min/week, and attitudinal lifestyle advice through frequent sessions with dietitians (both individual and collective), were provided. As used in the PREDIMED study, participants in the control group received educational sessions on an ad libitum MedDiet based on a 14-item non-energy restricted score, and no specific advice for increasing physical activity or losing weight was provided. Main subsample characteristics, age, sex, glucose and lipid metabolism, and lifestyle habits including diet and physical activity questionnaire scores are described in **Table 2**.

Cardiovascular and lifestyle factors in PREDIMED and PREDIMED-Plus studies

Dyslipidemia was defined as meeting any of the following criteria: HDL-c < 40 mg/dL or 50 mg/dL (for men and women respectively), LDL-c > 100 mg/dL, triglycerides > 150 mg/dL or taking any lipid-lowering drugs.

Adherence to diet was assessed with a previously validated 14-item questionnaire used in the PREDIMED Study (27,34), which was adapted to the 17-item energy-restricted diet questionnaire

for the intervention. PREDIMED-Plus participants reported activities through the Regicor Short Physical Activity Questionnaire (35), a validated version adapted from the Minnesota leisure time physical activity questionnaire (36) which was employed for PREDIMED participants.

Blood chemistry analysis

Sample collection was performed after an overnight fasting period at baseline, and 12-month follow-up. Venous blood samples were respectively collected in K3-EDTA anticoagulant to yield plasma in PREDIMED, and vacuum tubes with a silica clot activator for serum in PREDIMED-Plus (Becton Dickinson, Plymouth, United Kingdom). Serum tubes were centrifuged after the completion of the coagulation process, and plasma tubes immediately after collection, both for 15 min at 1.700 g room temperature. The following analytes were quantified in serum with an ABX Pentra-400 auto-analyzer (Horiba-ABX, Montpellier, France): glucose (mg/dL), triglycerides (mg/dL), HDL-c (mg/dL), and total cholesterol (mg/dL). LDL-c was calculated according to the Friedewald formula whenever triglycerides were < 300 mg/dL.

RNA extraction, reverse transcription, and gene expression quantification

Gene expression related to receptors involved in cholesterol efflux including nuclear receptors (*RXRA, RXRB, NR1H2, NR1H3, PPARA, PPARD, PPARG*), membrane transporters (*ABCA1, ABCG1, SR-B1*), and structural receptors (*CAV1*) were established.

Blood samples were collected, at baseline and one-year post-intervention, and stored at -80ºC until further analysis. Nuclear cells were isolated from peripheral blood by using tubes for purification of intracellular RNA from human whole blood (range of white blood cells 4.8 x 106 – 1.1 x 107 leukocytes/ml) for in vitro diagnostics applications (PAXgene Blood RNA Tube, BRT). The RNA concentration (A260) and purity were calculated spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies). RNA integrity was assessed by using microcapillary gel electrophoresis

(Bioanalyzer, NanoChip; Agilent Technologies) and the RNA integrity number value was calculated with Agilent 2100 Expert Software (Agilent Technologies). Samples were selected with RNA integrity number above 7.

Preamplification step was intended to increase low-input samples, whose concentration lied between 50-200 ng/µl. Recommended target levels were above 200 ng/µl and were obtained using TaqMan® PreAmp Master Mix (Applied Biosystems). Reverse transcription to cDNA was carried out with High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies). Microarray RT- PCR step was performed using QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies) and TaqMan® OpenArray™ Real-Time PCR Master Mix (Applied Biosystems). Subsequently, yielded results were analyzed with QuantStudio™ 12K Flex Software.

Normalization, relative quantification, repeated measurements, and gene expression change

We employed relative quantification approach to present the analysis of gene expression data. We tested multiple candidates as possible control genes, allegedly being unaffected by the treatment conditions (known as reference genes). Reference genes used to normalization were selected following the geNorm algorithm running a preliminary analysis to discriminate among 21 candidates, selecting *gapdh* due to the higher stability displayed. To study differences between baseline value and 12-month follow-up we compared the difference between cycle threshold (Ct), 12-month follow-up minus baseline values (ΔCt). In compliance with the premise that efficiency of target and reference genes are approximately equal (100% ±10%) (37–39), we applied the 2-ΔΔCt method to quantify change in gene expression. Therefore, the data is presented as a fold-change (FC) value normalized to the reference gene and relative to baseline value. Each pair of patient samples were allocated in the same plate to remove potential run-to-run variation (40–46).

Statistics

The assessment of the normality distribution of the variables was performed based on normality probability and box plots. To examine temporal changes and group differences analysis, we performed paired Student-T test to assess temporal changes along the intervention and independent samples Student-T test, respectively on ΔCt values. Linear mixed-effect models were fitted to estimate whether the evolution of ΔCt values differs among groups. These models were further adjusted for possible confounding variables such as age, sex, time, and weight, while individuals were included as random effect factor to consider repeated measurements. Finally, the interaction term between time and intervention group was formulated to assess inter-group variability across the trial. Inter-individual variability was contemplated through random intercept. Linear mixed-effect estimation was carried out with the use of restricted maximum likelihood. Analysis was executed using the lme function from nlme R package (47).

Descriptive statistics (mean and standard values) and comparison were calculated at baseline, post-intervention and 12-month change to display nutritional parameters, energy intake and key food components. In the PREDIMED population, both MedDiets were compared using independent Student T test with control diet, while Er-MedDiet was compared with MedDiet in the PREDIMED-Plus population. Mean of differences between groups at baseline, post-intervention and 12-month change (MedDiet-EVOO – control and MedDiet-Nuts – control in PREDIMED; and Er-MedDiet – MedDiet in PREDIMED-Plus) were also computed and displayed next to confidence intervals from Student T test comparison (**Supplementary table 2 and 3**). Pearson correlation was calculated between BMI, 12-month weight change and the genes (ABCA1, ABCG1).

Principal component analysis (PCA) was conducted to represent the individuals according to their expression along all genes (*ABCA1, ABCG1, CAV1, NR1H2, NR1H3, PPARA, PPARD, PPARG, RXRA, RXRB, SRB1*). This analysis aimed to visualize outlier individuals or to depict the three PREDIMED groups (**Image S2, Supplementary material**). Principal component analysis was executed with FactoMineR package. Along with PCA, linear discriminant analysis was executed to classify individuals in predefined groups, maximizing between-class variance and minimizing within-class (**Image S3, Supplementary material**). Complete cases were selected for all analysis. The level of confidence established for statistical procedures was 0.95. All analysis were executed using R Statistical software.

Statistical power and sample size

The sample size of 31 and 23 participants allowed at least 80% power to detect a statistically significant difference in *ABCA1* gene expression, among the 3 and 2 groups, within the PREDIMED and PREDIMED-Plus respectively, of 0.5 units of the relative quantification log_2FC, assuming a 2-sided type error of 0.05. A common standard deviation of 0.6 is estimated.

Untargeted functional analysis

Canonical pathways modulated by dietary interventions were defined using Ingenuity (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity), a web-based software application that identifies biological pathways and functions relevant to biomolecules of interest. Functional analysis was executed using log_2FC (**Image 3**).

Results

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The flow-chart of the study is depicted in the **Image S1 (Supplementary material)**. Finally, the present study employed samples from 151 and 89 participants of the PREDIMED and PREDIMED-Plus studies, respectively. Samples from 3 and 2 participants from the PREDIMED and PREDIMED-Plus trials, were respectively omitted due to atypical gene expression values. Additionally, 14 and 5 values, from the PREDIMED and PREDIMED-Plus trials, respectively, were not included due to lack of amplification of either the target or control gene.

The mean age was 66.3(± 6.4 years) and 65.5 years (± 4.7 years) for the PREDIMED and PREDIMED-Plus participants, respectively. With respect to participants' lifestyles at baseline, in both studies the diet and physical activity questionnaire scores did not show statistically significant differences among groups, and they met the minimal physical activity requirements suggested by the American Heart Association (450–750 METminweek-1).

Table 1.- Mean and standard deviation of key lipid and glucose metabolism variables, anthropometric measurements, adherence to diet and physical activity questionnaire scores of PREDIMED at baseline and 12-month follow-up. Median and 1st-3rd quartile range are displayed for triglycerides (non-normal distributed variable). Mean of differences and confidence intervals from Student-T test comparison between groups at baseline and 12-month follow-up are presented.

	MedDiet-EVOO			MedDiet-Nuts			Control			Baseline comparison to control		12 months comparison to control		12-month change comparison to control	
	Baseline	12 months	12-month change	Baseline	12 months	12-month change	Baseline	12 months	12-month change	MedDiet-EVOO	MedDiet-Nuts	MedDiet-EVOO	MedDiet-Nuts	MedDiet-Nuts	MedDiet-EVOO
Weight1 (kg)	75.61 (10.31)	75.03 (10.28)	-0.58 (2.47)	81.06 (13.81)	80.74 (13.92)	-0.33 (3.24)	84.23 (12.51)	83.52 (13.88)	-0.71 (4.18)	**-8.55(-13.31 - (-3.93))**	-5.31(-8.90 - 2.57)	**-8.29(-13.50 - (-3.48))**	-4.77(-8.80 - 3.24)	0.26(-1.28 - 1.55)	0.54(-1.22 - 1.99)
Waist circumference (cm)	99.94 (9.20)	100.27 (8.48)	0.17 (5.56)	101.68 (8.91)	101.79 (10.80)	-0.57 (5.18)	104.96 (9.71)	105.16 (10.78)	0.04 (5.21)	**-5.35(-8.90 - (-1.14))**	-4.51(-7.32 - 0.78)	**-4.71(-8.89 - (-0.89))**	-5.1(-8.10 - 1.37)	0.02(-2.10 - 2.34)	-0.8(-2.91 - 1.68)
BMI (kg/m^2)	29.84 (3.65)	29.62 (3.73)	-0.22 (1.02)	29.28 (3.62)	29.18 (3.77)	-0.10 (1.14)	31.37 (3.51)	31.06 (3.87)	-0.30 (1.62)	**-1.57(-2.99 - (-0.07))**	**-2.53(-3.63 - (-0.53))**	-1.43(-2.99 - 0.11)	**-2.26(-3.53 - (-0.23))**	0.14(-0.47 - 0.65)	0.27(-0.40 - 0.80)
Total cholesterol (mg/dL)	209.21 (43.77)	201.13 (40.55)	-4.63 (38.13)	198.86 (39.08)	195.35 (31.15)	-3.10 (32.51)	195.39 (34.49)	191.44 (37.69)	-2.96 (42.58)	11.33(-2.33 - 29.98)	2.94(-12.73 - 19.68)	8.8(-7.01 - 26.38)	1.05(-11.94 - 19.76)	0.73(-19.05 - 15.71)	-0.7(-17.46 - 17.19)
LDL-c (mg/dL)	129.15 (37.30)	130.10 (38.33)	3.69 (37.64)	120.06 (31.94)	116.03 (27.75)	-2.57 (20.70)	120.38 (31.15)	115.00 (26.38)	-7.18 (27.02)	8.81(-5.34 - 22.88)	2.73(-14.36 - 13.71)	**17.57(1.02 - 29.18)**	1.15(-12.60 - 14.65)	12.78(-3.17 - 24.93)	-0.56(-7.42 - 16.65)
HDL-c (mg/dL)	47.92 (7.45)	45.13 (10.26)	-2.45 (6.82)	48.63 (10.64)	50.97 (12.08)	0.62 (6.57)	48.44 (9.96)	47.74 (11.00)	-0.64 (7.39)	-1.74(-4.18 - 3.15)	0.74(-4.36 - 4.75)	-1.87(-7.18 - 1.95)	1.52(-2.27 - 8.73)	-1.47(-4.86 - 1.25)	1.49(-2.06 - 4.57)
Triglycerides (mg/dL)	136.68 [106.03:172.55]	125.60 [105.46:168.53]	-21.66 (148.82)	125.26 [83.22:178.54]	114.22 [81.43:138.65]	-23.84 (61.27)	112.27 [91.51:159.66]	129.39 [98.07:162.99]	7.96 (52.60)	34.06(-14.49 - 81.22)	1.44(-21.56 - 40.70)	-1.49(-18.94 - 32.40)	-14.55(-44.44 - 2.71)	-12.08(-76.14 - 16.89)	**-26.07(-58.57 - (-5.05))**
Triglycerides/ HDL-c	3.65 (4.45)	3.41 (2.07)	-0.28 (4.31)	3.13 (2.25)	2.42 (1.31)	-0.54 (1.64)	2.87 (1.56)	3.10 (1.68)	0.30 (1.36)	0.82(-0.57 - 2.14)	0.07(-0.60 - 1.13)	-0.01(-0.49 - 1.12)	-0.21(-1.38 - 0.03)	-0.16(-1.91 - 0.75)	**-0.59(-1.58 - (-0.12))**
Glucose (mg/dL)	125.53 (37.83)	119.79 (39.04)	-7.08 (36.65)	125.84 (41.82)	124.90 (37.26)	-3.85 (36.70)	131.64 (55.12)	117.28 (26.70)	-8.87 (46.24)	-3.76(-25.86 - 13.64)	-9.46(-27.04 - 15.44)	1.69(-11.60 - 16.61)	6.93(-7.54 - 22.78)	-2.72(-16.26 - 19.84)	7.95(-14.10 - 24.12)
Adherence to MedDiet (14-point item score)	8.57 (2.02)	10.24 (1.41)	1.67 (2.15)	8.62 (2.01)	10.72 (1.57)	2.10 (2.57)	8.98 (1.72)	9.07 (1.68)	0.09 (1.87)	-0.41(-1.17 - 0.36)	-0.41(-1.18 - 0.45)	**1.16(0.55 - 1.81)**	**1.63(0.95 - 2.35)**	**1.57(0.77 - 2.41)**	**2.04(1.03 - 3.01)**
Physical activity (MET-min/ week)	1854 (1372)	1623 (959)	-230 (1263)	2232 (1995)	2573 (2233)	341 (1352)	1684 (1650)	2033 (2151)	348 (1601)	208.26(-450.70 - 791.56)	441.37(-252.35 - 1348.14)	-357.72(-1099.83 - 281.78)	487.9(-410.41 - 1492.30)	-565.97(-1170.48 - 11.57)	46.52(-644.32 - 630.42)

Bold letters indicate confidence intervals excluding zero. MedDiet-EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet-Nuts, Mediterranean diet supplemented with nuts; BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein

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Table 2. Mean and standard deviation of key lipid and glucose metabolism variables, anthropometric measurements, adherence to diet and physical activity questionnaire scores of PREDIMED-Plus at baseline and 12-month follow-up. Median and 1st-3rd quartile range are displayed for triglycerides (non-normal distributed variable). Mean of differences and confidence intervals from Student-T test comparison between groups at baseline and 12-month follow-up are presented.

	Er-MedDiet baseline	**Er-MedDiet 12m**	**Er-MedDiet 12m-change**	**MedDiet baseline**	**MedDiet 12m**	**MedDiet 12m-change**	**Baseline**	**12 months**	**12month-change**
Weight (kg)	88.38 (14.87)	80.30 (14.54)	-8.09 (3.31)	88.99 (9.83)	86.14 (9.86)	-2.84 (4.14)	-0.6(-6.14 - 4.94)	**-5.85(-6.90 - (-3.65))**	**-5.24(-6.89 - (-3.60))**
Waist circumference (cm)	109.74 (9.97)	106.13 (9.94)	-3.61 (2.15)	112.46 (8.21)	111.26 (7.94)	-1.20 (2.11)	-2.67(-6.76 - 1.32)	**-5.07(-3.47 - (-1.58))**	**-2.4(-3.36 - (-1.47))**
BMI (kg/m^2)	32.28 (3.02)	29.33 (3.31)	-2.95 (1.11)	33.74 (3.43)	32.66 (3.36)	-1.09 (1.58)	**-1.46(-2.88 - (-0.04))**	**-3.32(-2.56 - (-1.33))**	**-1.86(-2.47 - (-1.26))**
Total cholesterol (mg/dL)	232.80 (49.35)	220.41 (42.23)	-12.39 (35.35)	217.12 (35.94)	217.37 (38.80)	0.24 (38.10)	15.68(-3.29 - 34.66)	3.05(-20.39 - 8.34)	-12.63(-28.79 - 3.52)
LDL-c (mg/dL)	148.63 (45.05)	139.65 (35.68)	-9.74 (32.15)	133.74 (31.78)	133.59 (31.74)	0.49 (32.09)	10.67(-2.93 - 32.72)	5.34(-15.98 - 9.48)	-10.57(-25.01 - 4.56)
HDL-c (mg/dL)	48.93 (10.01)	51.78 (10.12)	2.85 (8.00)	52.27 (11.92)	52.05 (10.76)	-0.22 (8.28)	-3.34(-8.18 - 1.50)	-0.27(-1.29 - 5.27)	3.07(-0.51 - 6.65)
Triglycerides (mg/dL)	143 [109:213]	131 [99 - 183]	-26.29 (81.23)	150 [102:180]	144 [99.2-202]	-3.16 (78.94)	13.93(-25.70 - 53.55)	-14.71(-45.44 - 5.29)	-28.63(-63.63 - 6.36)
Triglycerides/HDL-c	3.90 (2.48)	2.98 (1.73)	-0.92 (2)	3.42 (2.46)	3.24 (1.71)	-0.18 (2.13)	0,48 (-0.60 – 1.56)	-0,25 (-1.08 – 0.19)	-0,74 (-1.08 – 0.19)
Glucose (mg/dL)	125.30 (38.63)	115.50 (32.44)	-9.80 (25.13)	116.37 (20.88)	110.79 (16.62)	-5.58 (16.22)	8.17(-5.90 - 22.24)	3.85(-8.94 - 6.52)	-4.32(-13.63 - 4.99)
Adherence to MedDiet (17-point item score)	7.82 (2.85)	11.14 (2.39)	3.32 (3.24)	7.07 (2.59)	9.91 (2.34)	2.84 (2.91)	0.46(-0.71 - 1.63)	1.15(0.00 - 2.08)	0.68(-0.68 - 2.05)
Physical activity (MET·min/week)	2842 (2745)	3593 (2535)	751 (2287)	2646 (2418)	3041 (2298)	395 (2121)	281.03(-871.61 - 1433.66)	640.42(-389.26 - 1349.64)	359.39(-635.35 - 1354.13)

Bold letters indicate confidence intervals excluding zero. MedDiet, Mediterranean diet; Er-MedDiet, energy-reduced Mediterranean diet. BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol.

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Energy intake, nutritional parameters, and key food components are summarized in **Supplementary tables 2 and 3** for the PREDIMED and PREDIMED-Plus studies, respectively. Cardiovascular risk factors stratified per group of intervention of PREDIMED and PREDIMED-Plus are represented in **Supplementary table 4**. Percentage of participants with hypertension, dyslipidemia, and prevalent diabetes are slightly higher in the PREDIMED-Plus study than in PREDIMED.

Gene expression

$2^{-\Delta\Delta Ct}$ values correspondent to gene expression patterns (upregulation or downregulation) were depicted through divergent bars chart of both study populations (**Image 1 and 2**, PREDIMED and PREDIMED-Plus respectively).

PREDIMED Study: Significant changes occurred along time for baseline to endpoint comparison between baseline and post-intervention using Student-T test. These changes occurred in *ABCA1* and *RXRA* in MedDiet-EVOO and MedDiet-Nuts, plus *RXRB* in MedDiet-EVOO and *NR1H3* in MedDiet-Nuts group.

On the other hand, when conducting Student-T test comparison between independent groups yielded statistically significant differences between *RXRA* gene between MedDiet-EVOO and control groups. In the case of the MedDiet-Nuts and control groups, our analysis revealed differences in *RXRA* and *PPARD*. Linear mixed-effects models adjusted for sex, age and weight resulted in statistically significant differences for the interaction term time-group of intervention in *RXRA* between MedDiet-EVOO and control. In the comparison between MedDiet-Nuts and control, we observed statistically significant results in *RXRA, ABCG1, NR1H3*, and *PPARD*. In concordance, in the analysis joining both MedDiet groups, *ABCG1, PPARD,* and *RXRA* were differently expressed versus the control group **(Supplementary table 4)**. BMI change and weight

loss displayed moderate negative Pearson correlation with *ABCA1* in MedDiet-Nuts (r = -0.38 and r = -0.39, data not shown).

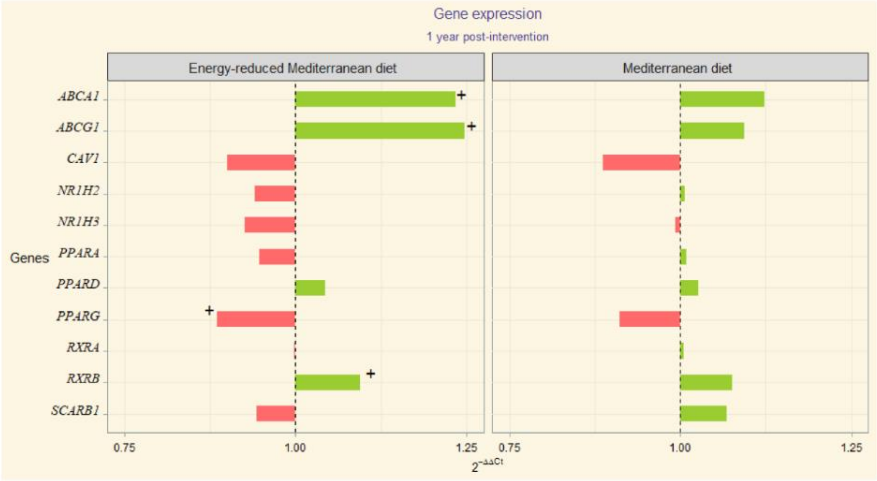
Image 1.- Divergent bar plot depicting the fold-change mean values per group of PREDIMED (Green color: upregulation and red color: downregulation of genes)

Statistically significant (p-value < 0.05): baseline to post-intervention change +; time:group interaction (p-value) from mixed- effects model compared to control #. Numerical p-values are presented in supplementary table 3.

PREDIMED-Plus Study: Comparison between baseline and endpoint results yielded statistically significant changes when performing independent Student-T test. These changes occurred after Er-MedDiet in *ABCA1* and *ABCG1, PPARG* and *RXRB*. Also, borderline p-values were observed in NR1H2. No statistically significant values resulted from linear model or independent Student-T test between arms **(Supplementary table 5)**. No differences were observed in gene expression when comparing subjects with the most extreme outcomes regarding atherogenic dyslipidemia in both groups in the PREDIMED-Plus study. *ABCA1* and *ABCG1* correlated moderately with weight

loss and BMI change in the MedDiet group (r = -0,39, r = -0,38, r = -0,38, r= 0,378) (data not presented).

Image 2.- Divergent bar plot depicting the FC mean values per group of PREDIMED-Plus (Green color: upregulation and red color: downregulation of genes)



Statistically significant (p-value < 0.05): baseline to post-intervention change +

Linear discriminant analysis model applied to PREDIMED classified correctly 45.34% of individuals using the selected genes and their relative expression values as predictor variables. Cross-validated table for classification of PREDIMED groups using MedDiets combined shown in **Supplementary table 6**. When applied to PREDIMED-Plus trial the estimated classification accuracy was 32.2% for MedDiet and Er-MedDiet groups. Cross-validated table shown in **Supplementary table 7**.

PCA and linear discriminant analysis performed in PREDIMED and PREDIMED-Plus are depicted in the **Images S2 and S3 (Supplementary material)**. PCA method displayed an overlapping profile of gene expression among the individuals in the different groups. Probability density function plots were depicted for MedDiets combined versus control in PREDIMED, and MedDiet versus Er-MedDiet in PREDIMED-Plus, according to the selected genes. The density probability functions overlapped in a broad area of both curves.

Pathway analysis illustrated the relationships between biological functions efflux and transport of cholesterol with the selected gene set: *ABCA1, ABCG1, NR1H2, NR1H3, PPARA, PPARD, RXRA, RXRB, CAV1* and *SCARB1*. In accordance with our findings, an upregulation pattern in cholesterol transporters and key regulators was displayed when overlaying the correspondent pathways to the provided nodes. Inconsistent predictions were found for *PPPARG, PPARA* and *CAV1* interaction with cholesterol efflux and transport, as far as the selected genes. A proposed scheme with expression quantification, relationship and signaling among the different nodes is displayed below in **Image 3**. The network depiction represents the predicted interactions between the selected molecules using MedDiets combined dataset of PREDIMED. Gene expression values determine the type of interaction (activation, inhibition) among molecules and functions.

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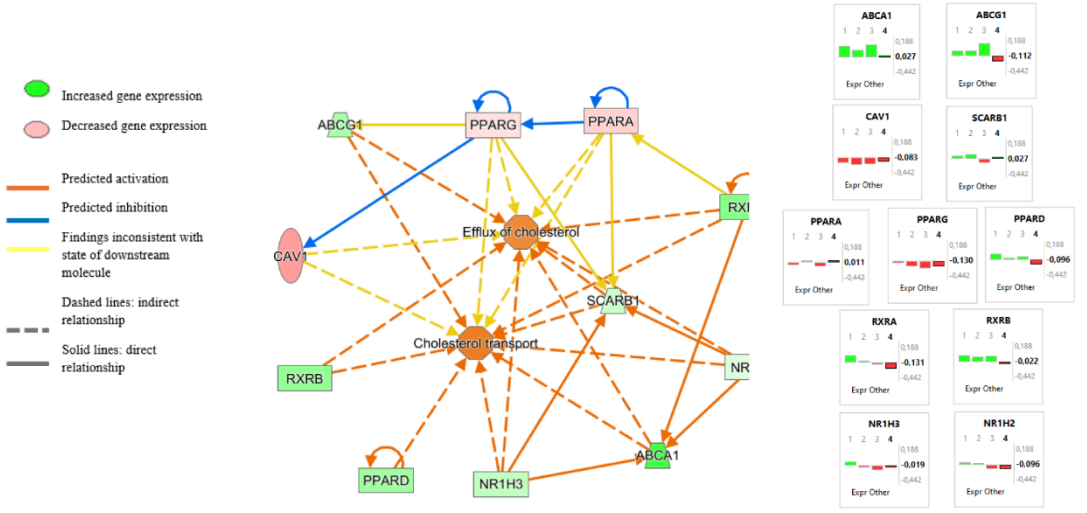

Image 3. Ingenuity Pathway Analysis regulatory network prediction using log_2FC. Legend illustrates relations of molecules in functional processes related to cholesterol homeostasis.

Legend 1: MedDiets combined (PREDIMED), 2: MedDiet (PREDIMED-Plus), 3: ErMedDiet (PREDIMED-Plus), 4: Control diet (PREDIMED);

Discussion

We observed an up-regulation of the gene expression related to receptors involved in cholesterol efflux function including nuclear receptors (*RXRA, RXRB, NR1H3, PPARD*), and membrane transporters (*ABCA1, ABCG1*). Long-term response was determined within the frame of two MedDiet-intervention trials after 1 year follow-up in elderly adults at high cardiovascular risk. *ABCA1* and *ABCG1* proteins actively participate in the cholesterol removal, the first step of the reverse cholesterol transport. While *ABCA1* promotes cholesterol movement to nascent HDL particles (lipid-poor apoA-I), probably induced by cholesterol-loaded cells, *ABCG1* and scavenger receptor B1 (*SR-B1*) perform a similar task to larger HDL lipoproteins (17,48,49). Our experiment disclosed baseline to post-intervention mild changes in *ABCA1* expression after MedDiet-EVOO

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and MedDiet-Nuts. Weight-adjusted model attested also significant *ABCA1* upregulation comparing the MedDiet-Nuts intervention and control. In concordance, within the frame of the PREDIMED-Plus trial, both groups experienced an upregulation of *ABCA1* and *ABCG1* comparing baseline to endpoint measurements ($p < 0.01$).

Individual lifestyle habits play an influential role in the overall regulation of reverse cholesterol transport, with special interest focused on cholesterol efflux. Dietary pattern's composition has been hypothesized to be a crucial factor affecting CEC gene-related expression, even though controversial results have been encountered (50), and studies mainly reflect the effects of certain components of the diet, such as fatty acids. The short-term effects of a high saturated fatty acid (SFA) diet during a 5-week diet intervention, resulted in a downregulation of *ABCA1* and *ABCG1* blood expression, without an increase of plasma inflammatory markers (51). On the other hand, there was an upregulation of *ABCG1* blood expression after an 8-week diet intervention where a specific quantity of SFA was replaced with n-6 polyunsaturated fatty acids (PUFA), while maintaining the same monounsaturated fatty acid (MUFA) content in a double-arm randomized trial (52). In this regard, our findings indicated a common decrease in the consumption of SFA in every group of PREDIMED and PREDIMED-Plus. However, a rise in the consumption of MUFA occurred in MedDiet pattern groups (MedDiet-EVOO and MedDiet-Nuts in PREDIMED; and MedDiet and Er-MedDiet in PREDIMED-Plus), along with a PUFA increase (in MedDiet-Nuts), which also displayed an upregulated gene expression of *ABCA1* and *ABCG1* in both studies.

Poor dietary pattern standards are usually associated with increased overweight and obesity, therefore changes in anthropometric measurements may indirectly correlate with diet quality. In this line, the association of weight and BMI with cholesterol efflux activity is widely considered to relate in an opposite direction, being overweight and obesity known to impair of CEC (53).

Previous research has demonstrated in different manners the BMI is inversely associated with CEC (53,54). We did not observe correlation between baseline values of anthropometric measurements and *ABCA1* and *ABCG1* expression levels. However, in the PREDIMED study we observed a statistically significant moderate correlation between *ABCA1*, BMI and weight change respectively, in the MedDiet-Nuts group. Similar results were found in the MedDiet group of PREDIMED-Plus, where *ABCA1* and *ABCG1* correlated moderately with weight loss and BMI change. Conversely, in the Er-MedDiet group, which underwent physical activity promotion and experienced weight loss, along a larger *ABCA1* and *ABCG1* upregulation, we observed no such correlations probably due to inter-subject variability and adherence to the intervention.

Regarding the impact of physical activity on CEC, the evidence points to an enhanced functionality under moderate or high exercise at midterm (55,56). Mid-term effects have been also evaluated on blood mononuclear cells, yielding an increase in gene expression of *ABCA1* and *ABCG1* during an 8-week longitudinal study involving low-intensity exercise (57). In PREDIMED-Plus we observed a larger upregulation in the group engaging in physical activity, Er-MedDiet, than in the MedDiet traditional group. However, no statistically significant differences were found from baseline to endpoint.

The regulation of *ABCA1* and *ABCG1* constitutes a critical point due to the impact in the overall CEC. This process operates at multiple levels (transcriptional, post-transcriptional and post-translational), with a plethora of interrelated molecules participating, forming partnerships, stimulating, and inhibiting each other. Among the most significant we can highlight *NR1H3* also known as *LXRα* (liver-X-receptor alpha) and *NR1H2*, also known as *LXRβ* (liver-X-receptor beta), homeostasis cholesterol sensors, that regulate and actively participate in reverse cholesterol transport (58). Through obligatory heterodimerization with retinoid X receptors (RXRs) LXRα

and LXRβ form a multifunctional partnership susceptible to stimulation by ligands (cholesterol and its metabolites). One of them is the heterodimer LXRα-RXR working as a major transcriptional regulator of transporters *ABCA1* and *ABCG1*, enhancing cholesterol efflux (20,49,59,60). With regard to our studies, gene expression patterns varied across groups, from non-significant downregulation (Med-EVOO, MedDiet, or Er-MedDiet) to mild, but statistically significant upregulation (MedDiet-Nuts). The inconsistence behind downregulation effect cannot be fully ascertained, it could be as a result of negative feedback mechanism after transcriptional stimulation; however, it might also be attributed to the concentration decrease of cholesterol or its derivative compounds (61). The lack of correlation between *ABCG1, ABCA1* and *NR1H2, NR1H3* has already been studied in peripheral blood mononuclear cells (PBMC) samples of hypercholesterolemic patients, under different lipid-lowering treatments and in controls (62). In vitro experiments involving human PBMC have led to consider first a differential regulation mechanism between *ABCA1* and *ABCG1*, and secondly, the short span of time of these transcripts upon agonist stimulation (59).

As previously described, RXRs work as transcription factor in various biological processes (24,59,63,64). Among the natural ligands, different unsaturated fatty acids (docosahexaenoic, linoleic, oleic, and arachidonic acids) and phenolic compounds contained in olive oil have demonstrated activity on RXRs (63–66). Previous research has provided insight of how VOO enriched with phenolic compounds, enhanced proteomic expression of LXR/RXR among the top signaling pathways (67). We observed a mild, but statistically significant upregulation of *RXRA* and *RXRB* in the MedDiet-EVOO participants. Meanwhile, previous studies have pointed out that PUFA contained in nuts mediate the expression liver X-receptors (68,69). Our results revealed a

slight but significant upregulation of *RXRA* in MedDiet-NUTS participants. In fact, both MedDiets displayed a *RXRA* upregulation trend in comparison to control.

One of the multiple partners that typically collaborates with RXRs are PPARs, a family of nuclear receptors involved in multiple metabolic pathways related to glucose and lipid regulation, even serving as therapeutic targets (fibrates in PPARα or thiazolidinediones in PPARγ). Differently represented in tissues, the PPARs family is known to intervene in biological processes such as CEC, clearance of oxidized LDL fraction and reverse cholesterol transport (20,25,70,71). Based on previous research (72–74), the initial speculation leant towards an upregulation over time, however the tendency in both trials showed mild downregulation, without meaningful results for *PPARA* gene expression, apart from a downregulation over time for combined MedDiets in PREDIMED. Unexpected findings have been reported earlier in referral to *PPARA* expression, after long-term of single PUFA supplementation (75).

PPARG, is a well-known insulin sensitizing agent, with already proven activity on lipid metabolism (24,76). The PPARγ-LXRα partnership has been shown to trigger a signaling cascade improving cholesterol efflux (77,78). However, we did not observe statistically significant temporal changes nor even upregulation in MedDiet-EVOO. In fact, the results indicated a slight tendency to downregulation only significant in the Er-MedDiet group. It has been reported though, that the modulation produced by physical activity would exert upregulation (79,80). Within our study, the Er-MedDiet arm, which provided physical activity promotion, there was a significant downregulation in *PPARG* and non-significant downregulation in *NR1H3*. This finding might be due to alternative transient downregulation mechanisms in the pathway. It should be pointed out *PPARG* has been found to control *ABCA1* by inducing the expression of LXRα, and in an independent manner (76,81,82). Meanwhile, *ABCG1* has also been proved to be influenced by

PPARγ, in LXR-independent manner (84). The alternative mechanism could influence regulation pattern across the different MedDiet patterns with or without physical activity.

Strengths and limitations

The first strength of our study lies in its randomized and controlled design, conducted among free-living individuals. This approach enabled us to generate foundational scientific evidence regarding the effects of the dietary interventions under investigation in the target population. Second, the assembled cohort constitutes a specific group of participants meeting age and risk factors criteria, allowing conclusions to be transferred to analogous population at high-cardiovascular risk. On the other hand, the same reasoning hinders the possibility to extrapolate to different populations.

Third, peripheral blood cell analysis has been reported as prolific tissue to study cardiovascular diseases, inflammation, and cholesterol efflux biomarkers. However, it must be taken in account the fact that simultaneous protein analysis has not been performed and could contribute to understanding biological mechanisms. Special attention should be given to the fact that active diet components were not supplemented or provided individually but were incorporated into various sources within a whole diet.

Conclusion

Mild upregulation of cholesterol efflux-related genes involving retinoid X (RXRA, RXRB), ATP-binding cassette family (ABCG1, ABCA1), liver X (LXRa/NR1H3), and peroxisome proliferator activated (PPARD) receptors, occurred as long-term responses to different Mediterranean diets in elderly adults at high cardiovascular risk.

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MF, JS-S, MM-G, DC, ER, FT, and RE designed the clinical trial. OC and MF designed the conceptualization sub-study. IS and JH-R performed the formal analysis, DM and JH-R performed the laboratory analysis. OC, MF, and JH-R drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Data Availability:

PREDIMED

The dataset analyzed during the current study cannot be made publicly available due to national data regulations and ethical considerations, including the absence of explicit written consent from study participants to make their deidentified data available upon study completion. However, data described in the manuscript will be provided to bona fide investigators upon request. Requests can be made by sending a letter to the PREDIMED Steering Committee (predimed-steering-committee@googlegroups.com).

PREDIMED PLUS

The generation and analysis of the data sets within this study are not projected to be open to access beyond the core research group. This is because the participants' consent forms and ethical approval did not include provisions for public accessibility. However, we follow a controlled data-sharing collaboration model, as the informed consent documents signed by the participants allowed for regulated collaboration with other researchers for study-related research. Following an application and approval process by the PREDIMED-Plus Steering Committee, the data described in the manuscript, alongside the codebook and analytic code, will be available upon request. Researchers interested in this study can reach out to the Committee by sending a request letter to predimed_plus_scommittee@googlegroups.com. For those proposals that gain approval, a data-sharing agreement, outlining the specifics of the collaboration and data management, will be prepared and finalized.

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Mediterranean Diet Modulation of Neuroinflammation-Related Genes in Elderly Adults at High Cardiovascular Risk

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Abstract: Individuals with dementia and neurodegenerative diseases (NDDs) often suffer from cardiovascular diseases (CVDs). Neuroinflammation driven by conditions involved in CVDs is linked to disruptions in the central nervous system triggering immune reactions, perpetuating an "inflammatory-like" environment. The Mediterranean diet (MedDiet), known for its anti-inflammatory and antioxidant properties, has been proposed as a key factor to attenuate these risks. Blood nuclear cell samples were collected from 134 participants of the PREDIMED trial, which randomized participants to three diets: one supplemented with extra-virgin olive oil (MedDiet-EVOO), another with nuts (MedDiet-Nuts), and a low-fat control diet. These samples were analyzed at baseline and 12-month follow-up to assess the impact of these dietary interventions on gene expression markers. We first selected target genes by analyzing intersections between NDD and CVD associations. Significant gene expression changes from baseline to 12 months were observed in the participants allocated to the MedDiet-EVOO, particularly in CDKN2A, IFNG, NLRP3, PIK3CB, and TGFB2. Additionally, TGFB2 expression changed over time in the MedDiet-Nuts group. Comparative analyses showed significant differences in TGFB2 between MedDiet-EVOO and control, and in NAMPT between MedDiet-Nuts and control. Longitudinal models adjusted for different covariates also revealed significant effects

for TGFB2 and NAMPT. In conclusion, our results suggest that one year of traditional MedDiet, especially MedDiet-EVOO, modulates gene expression associated with CVD risk and NDDs in older adults at high CV risk.

Keywords: Mediterranean diet; neuroinflammation; cardiovascular disease; nutrigenomics

1. Introduction

A significant proportion of patients affected by dementia have common comorbidities such as cardiovascular diseases (CVDs), type 2 diabetes, hypertension, dyslipidemia or excess body weight. CVDs and neurodegenerative diseases (NDDs) share risk factors that predispose and accelerate both pathologies [1]. One critical non-modifiable pro-inflammatory factor that must be considered is inflammaging [2], a term referring to the chronic progressive stress caused by aging, which increases inflammatory status. Inflammaging added to cardio metabolic risk factors first triggers immune responses in peripheral organs, initiating a systemic inflammatory state that later affects the central nervous system (CNS) and eventually disrupts homeostasis in both areas [3,4]. Once inflammatory cells, molecules, or cytokines infiltrate the CNS, resident glial cells are activated, perpetuating neuroinflammation [3,5–7].

Neuroinflammation is characterized by a primary immune reaction to brain injury mediated by key pro-inflammatory cytokines, particularly interleukin (IL)-1b, IL-6, and tumor necrosis factor (TNFα). The neuroinflammatory process involves the activation and priming of glial cells, during which microglia exert macrophage-like functions such as vital surveillance, scavenging, antigen presentation, and cell repair [8]. The released cytokines increase blood–brain barrier (BBB) permeability [9], thereby increasing their own concentrations in the brain, which intensify microglia's pro-inflammatory responses [10]. The type, degree, and duration of the stimulus determine the neuroinflammatory damage responsible for the destruction of brain tissues characteristic of NDDs [10–12]. NDDs are influenced by a wide range of factors such as age, sex, nutrition, socio-economic, and genetic determinants, in addition to lifestyle habits (physical activity, metal health, and wellbeing) that interplay in triggering or accelerating the disease [12–14].

It is known that there are components in Mediterranean dietary patterns that exert a protective effect, contributing to attenuate the neuroinflammatory state [12,15]. Resveratrol, tyrosol, anthocyanins, and isoflavones, found in common sources of the Mediterranean diet (such as olive oil, fruits, and legumes), have been associated with varying degrees of consistency in improving cognitive function. From a mechanistic point of view, they exert anti-inflammatory, anti-apoptotic, and neuroprotective effects by modulating various pathways involved in oxidative stress, inflammatory mediators, and promoting cell survival mechanisms [16]. Further, the gut–brain axis has recently been proved as a key entity influencing multiple states or diseases, being susceptible to modification by a variety of factors including diet, drug intake, or medical procedures [17].

Epidemiological studies have tried to bridge the gap between CVDs and Alzheimer's disease (AD) through association studies, identifying common variants of both diseases [18–20]. Additionally, research on the transcriptional profile has inferred genetic information from blood cell analysis, reflecting primary alterations occurring in tissue. Cell-free RNA has been demonstrated to distinguish AD patients versus age-matched controls by comparing transcript levels of AD-related genes [21]. In this regard, advancements in this field have revealed correlations between disease severity and the characteristics of circulating transcriptomes [22].

Thus, systemic inflammation and neuroinflammation play pivotal roles in the progression of NDDs, wherein both established chronic inflammatory processes contribute to brain damage and cognitive impairment. MedDiets may benefit brain function by attenuating

both neuroinflammation and systemic inflammation, measured through different gene biomarkers linked to these pathological processes.

The aim of this sub-study of the PREDIMED randomized trial [21] was to determine the effect of a long-term Mediterranean diet (MedDiet) intervention on the gene expression of transcriptomic biomarkers related to neuroinflammation and cardiovascular risk in an older population at high cardiovascular risk.

2. Materials and Methods

2.1. Study Design and Population Recruitment

The study population was a random subsample of volunteers (n = 134, 67 men and 67 women) recruited in different sites of the large-scale multicenter randomized, controlled trial PREDIMED (flow chart in Figure S1 Supplementary Material). PREDIMED assessed the effect of MedDiets on the primary prevention of CVD [23]. In this trial, two traditional MedDiets were tested for long-term effects on CVD risk, one enriched with extra-virgin olive oil (MedDiet-EVOO) and another enriched with raw nuts (MedDiet-Nuts) vs. a control diet based on advice to reduce the fat content of the diet.

Eligible participants were women and men, aged 60–80 years and 55–80 years, respectively, who met at least one of the following criteria: (1) type 2 diabetes or (2) $\geq$3 major cardiovascular risk factors: current smoking (>1 cig/day during the last month); hypertension (systolic BP $\geq$ 140 mmHg or diastolic BP $\geq$ 90 mmHg or antihypertensive medication); LDL cholesterol $\geq$ 160 mg/dL or lipid-lowering therapy; HDL cholesterol $\leq$ 40 mg/dL in men or $\leq$50 mg/dL in women; body mass index $\geq$ 25 kg/m^2; and family history of early-onset coronary heart disease [23]. Exclusion criteria were a prior history of CVD, severe chronic illnesses, substance abuse, allergies or intolerance to olive oil or nuts, a low predicted likelihood of changing dietary habits based on the stages of change model [24], or any condition that might hinder study participation.

2.2. Blood Chemistry Analysis

Sample collection was performed after an overnight fast at baseline and after a 12-month follow-up. Collected samples were centrifuged immediately after extraction, both for 15 min at 1.700$\times$ *g* room temperature. The following analytes were quantified in serum with an ABX Pentra-400 auto-analyzer (Horiba-ABX, Montpellier, France): glucose (mg/dL), triglycerides (mg/dL), HDL-cholesterol (mg/dL), and total cholesterol (mg/dL). LDL-cholesterol was calculated according to the Friedewald formula when triglycerides were <300 mg/dL.

2.3. Cardiovascular and Lifestyle Factors

Dyslipidemia was defined as meeting any of the following criteria: HDL-cholesterol < 40 mg/dL or 50 mg/dL (for men and women respectively), LDL-cholesterol > 200 mg/dL, triglycerides > 150 mg/dL or taking any lipid-lowering drugs. Adherence to the MedDiet was assessed by a validated 14-item questionnaire [25]. Physical activity was recorded via the Minnesota leisure time physical activity questionnaire [26,27].

2.4. RNA Extraction, Reverse Transcription, and Gene Expression Analysis

Nuclear cells were extracted from peripheral blood by using tubes for purification of intracellular RNA from human whole blood (range of white blood cells 4.8 $\times$ 10^6–1.1 $\times$ 10^7 leukocytes/mL) for in vitro diagnostics applications (PAXgene Blood RNA Tube, BRT, Hombrechtikon, Switzerland). The RNA concentration (A260) and purity were calculated spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, v3.5, Wilmington, NC, USA). RNA integrity was assessed by using microcapillary gel electrophoresis (Bioanalyzer, NanoChip; Agilent Technologies, version 2.6, Waldbronn, Germany) and the RNA integrity number value (RIN) was calculated with Agilent 2100 Expert Software (Agilent Technologies).

Low-input samples (50–200 ng/µL) underwent preamplification because recommendations pointed target levels should be above 200 ng/µL. Amplification was performed using TaqMan® PreAmp Master Mix (Applied Biosystems, Vilnius, Lithuania). Reverse transcription to cDNA was carried out with a High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, Vilnius, Lithuania). Microarray RT-PCR step was performed using a QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies) and TaqMan® OpenArray™ Real-Time PCR Master Mix (Aplied Biosystems, Vilnius, Lithuania). Finally, results were analyzed with QuantStudio™ 12K Flex Software version 1.3.

2.5. Selection of Gene Targets

To identify genes related to both neurodegenerative disease and CVD, we performed a disease enrichment analysis to search human gene–disease interactions. The search was executed including the following terms: "atherosclerosis", "cognitive", "cholesterol", "lipid", "metabolism", "cerebrovascular", "dementia", and "arteriosclerosis". From the intersection of the selected diseases, "Arteriosclerosis" and "Cerebrovascular disease" in DisGeNET (Database of Gene-Disease Associations) v6 and v7 [28] and "Hyperlipidemia" and "Cerebrovascular disorders" in the Human Disease Ontology [29] (Figure 1), a total of 46 genes were identified. For the present study, the nine genes selected from the intersection according to their biological functions were (a) IFNG (Interferon Gamma); IL10 (Interleukin 10); NFE2L2 (Nuclear Factor, Erythroid 2 Like 2); NLRP3 (NLR Family Pyrin Domain Containing 3); TGFB2 (Transforming Growth Factor Beta 2); (b) Regulators of the senescence and cell cycle: CDKN2A (Cyclin Dependent Kinase Inhibitor 2A); (c) Metabolic and Cell Signaling Regulators: PIK3CB (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta); and NAMPT (Nicotinamide Phosphoribosyltransferase).

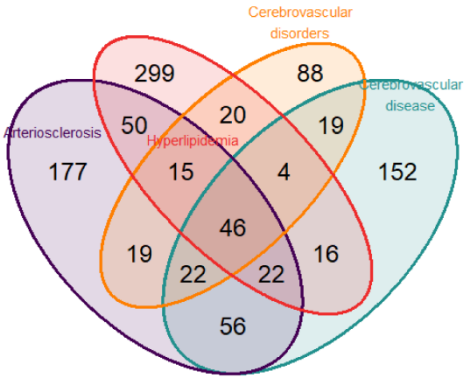

Figure 1. Venn diagram displaying the number of genes found from the overlap of the 4 selected pathologies (atherosclerosis, hyperlipidemia, cerebrovascular disorders, and cerebrovascular disease) employing the public databases DisGeNET and Disease Ontology.

2.6. Reference Genes and Relative Quantification

We employed the relative quantification approach to present the analysis of gene expression data. We tested 3 potential candidates as control genes (also known as reference or housekeeping genes). The reference genes were chosen using the geNorm algorithm, which conducted a preliminary analysis to distinguish among 21 candidates. The algorithm tested gene expression variation across different samples, resulting in genes with lower M values, or those that were more stably expressed and, therefore, better candidates: we selected GAPDH as a reference. To study differences between baseline value and 12-month follow-up we compared ΔCt.

The efficiency of the target and reference genes was 100% ± 10% [30–32], we applied the $2^{-\Delta\Delta Ct}$ method to quantify changes in gene expression. Therefore, the data are presented as a fold change value normalized to the reference gene and relative to the baseline value. Each pair of patient samples was allocated in the same plate to remove potential run-to-run variation [33–36].

2.7. Statistical Analyses

The sample size of 36 participants allowed at least 80% power to detect statistically significant differences in PIK3CB gene expression among the 3 groups, within the PREDIMED study of 0.4 units of the relative quantification log2FC, assuming a 2-sided type error of 0.05. A common standard deviation (SD) of 0.6 was estimated.

The assessment of the normality distribution was conducted using normality probability plots and boxplots. Descriptive statistics (mean and standard values) and comparison were calculated at baseline, post-intervention, and 12-month change to display nutritional parameters, energy intake, and key food components. The MedDiets were compared with the control diet using independent Student *T*-tests. We compared individuals' changes along the intervention. To assess inter-group differences, independent Student *T*-tests were calculated employing ΔCt values. To account for the effect of relevant covariates, we performed two models: the first one was adjusted for age, sex, time, and education level; and the second one was additionally adjusted for diabetes, BMI, physical activity, smoking status, hypertension, and dyslipidemia. Diabetes and hypertension were treated as binary qualitative variables, categorized as either 'Yes' or 'No'. Smoking status was stratified as a qualitative variable with three categories: current smoker, former smoker, and never smoker. Dyslipidemia was previously defined as a composite variable based on HDL-cholesterol, LDL-cholesterol, triglyceride levels, or lipid-lowering drugs. Educational level was a qualitative variable comprising 3 categories: higher education or equivalent, secondary education, or primary education. Physical activity was measured as a continuous variable in MET-minutes per week (METmin/week). BMI was also treated as a continuous quantitative variable, expressed in kg/m^2. The interaction term time:group of intervention to explain inter-group variability across the trial. Inter-individual variability was assessed through random intercept. The linear mixed-effect model was estimated using restricted maximum likelihood. We used the lme function from the package nlme, using R software version 4.3.2 [37].

3. Results

The mean age and standard deviation of the study population was 66.1 (±6.34) years. Table 1 displays cardiovascular risk factors stratified per group of intervention. Among the subsample, 53% of participants had diabetes, 57% had dyslipidemia, 79% had hypertension, and 15% were smokers. Baseline characteristics of the groups were similar to those of the entire PREDIMED cohort (Supplementary Table S1). We discarded a total of 17 samples due to technical issues during the experimental phase of RT-qPCR.

Energy intake, nutritional parameters, and key food components are summarized in Supplementary Table S2. No between-group differences in 12-month change in energy intake were found. Between-group 12-month differences in changes were observed for total fat intake, mainly due to monounsaturated fatty acids (MUFA), between MedDiet-EVOO and MedDiet-Nuts compared to the control diet. We also observed differences in polyunsaturated fatty acids (PUFA) changes between the MedDiet-Nuts and control diet groups. The overall consumption of virgin olive oil and nuts were respectively increased in the MedDiet-EVOO and MedDiet-Nuts interventions compared to the control diet.

Table 1. General characteristics of the study population. Values are expressed as a percentage (for categorical variables) and mean (and standard deviation) for quantitative continuous variables. MedDiet-EVOO, Mediterranean diet supplemented extra virgin olive oil; MedDiet-Nuts, Mediterranean diet supplemented with nuts.

	All Participants (134)	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**
Age (years. mean ± SD)	65.82 ± 6.29	65.61 ± 5.49	66.10 ± 6.93	64.73 ± 6.50
Sex (% women)	67 (50%)	29 (59.2%)	13 (33.3%)	25 (54.3%)
Hypertension	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**
No	28 (20.9%)	10 (20.48%)	10 (25.64%)	8 (17.39%)
Yes	106 (79.1%)	39 (79.59%)	29 (74.35%)	38 (82.6%)
Diabetes	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**
No	63 (47.01%)	20 (40.81%)	18 (46.15%)	25 (54.34%)
Yes	71 (52.99%)	29 (59.18%)	21 (53.84%)	21 (45.65%)
Dyslipidemia *	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**
No	56 (42.75%)	20 (40.82%)	18 (47.37%)	18 (40.91%)
Yes	75 (57.25%)	29 (59.18%)	20 (52.63%)	26 (59.09%)
Tobacco use	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**
Current smoker	20 (14.93%)	9 (18.37%%)	6 (15.38%)	5 (10.87%)
Former smoker	38 (28.36%)	9 (18.37%%)	14 (35.90%)	15 (32.61%)
Never smoker	76 (56.72%)	31 (63.27%)	19 (48.72%)	26 (56.52%)
Adherence to diet (14-point item score)	8.72 ± 1.91	8.57 ± 2.02	8.62 ± 2.01	8.98 ± 1.72
Physical activity (MET·min/week)	1906 ± 1670	1855 ± 1373	2232 ± 1995	1684 ± 1651

* Dyslipidemia is defined: HDL-cholesterol < 40 mg/dL or 50 mg/dL (for men and women respectively), LDL-cholesterol > 200 mg/dL, triglycerides > 150 mg/dL or taking any lipid-lowering drugs.

Gene Expression

The $2^{-\Delta\Delta Ct}$ values correspondent to gene expression quantification (upregulation or downregulation) are depicted through divergent bars in a visual chart (Figure 1). In the MedDiet-EVOO intervention group, we observed significant 12-month changes in the following gene expression values: CDKN2A, IFNG, NLRP3, PIK3CB, and TGFB2. In this line, we observed temporal change in TGB2 in MedDiet-Nuts. Significant between-group differences in TGFB2 expression were found between the MedDiet-EVOO and control diet. Additionally, significant differences in NAMPT expression were observed in between-group comparisons of MedDiet-Nuts and the control diet group. Model 1 (adjusted for sex, age, and education level) resulted in statistically significant differences for the interaction term time-group of intervention in TGFB2 between the MedDiet-EVOO and control diet. The comparison between the MedDiet-Nuts and control diet disclosed significant results in NAMPT. Meanwhile, model 2 only showed significant differences by statistical criterion in

NAMPT in MedDiet-Nuts. The results of the statistical analyses, including within-group, between-group comparisons, and model outputs, are presented in Supplementary Table S3.

4. Discussion

In the search of the molecular mechanisms by which a cardioprotective diet like the Mediterranean one benefits brain health, we analyzed neuroinflammation- and systemic inflammation-related genes' behavior after a Mediterranean diet intervention. Specifically, the MedDiet-EVOO modulated the expression of the CDKN2A, IFNG, NLRP3, PIK3CB, and TGFB2 genes in the peripheral blood mononuclear cells of older adults at high cardiovascular risk, whereas the MedDiet-Nuts resulted in a different expression of the NAMPT gene compared to the control diet (Figure 2).

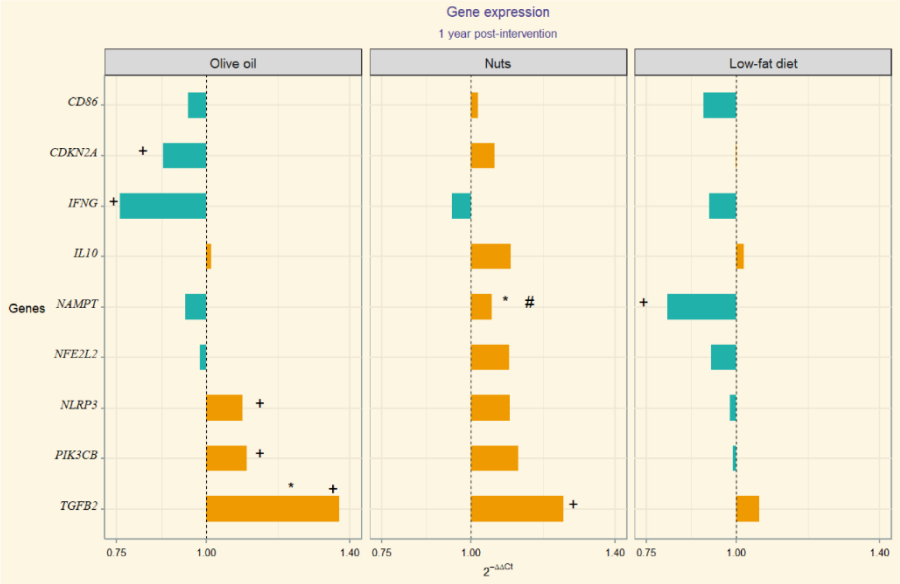

Figure 2. Divergent bar showing $2^{-\Delta\Delta Ct}$ per group (Orange bars represent upregulation of gene expression, while blue bars indicate downregulation). Statistically significant (p-value < 0.05): + baseline to post-intervention change; * individual comparison between MedDiet-EVOO and MedDiet-Nuts versus control; # time:group interaction (p-value) from mixed-effects of the fully adjusted model compared to control. Numerical p-values are presented in Supplementary Table S3.

There is evidence that AD patients display high plasma levels of numerous pro-inflammatory markers [38,39], including IFN-γ protein levels [40]. In this line, it is known that TGFβ interacts with IFN-γ, indicating a crosstalk between these pleiotropic cytokines, functioning as reciprocal regulation [41]. TGF-β's signaling is involved in multiple neurological pathways, regulating synaptic growth, neurotrophic functions, and cell survival, although their role is not completely understood, especially in AD [42–44]. Research on the role of TGF-β1 in dementia [41,42,44] has shown reduced plasma concentrations in patients with AD [45].

Despite that Aβ42 levels rise at the onset of AD, as the disease progresses, they typically decrease [46,47]. In the present study, we observed an increased expression of TGF-β2 (Figure 2), in particular after the MedDiet interventions, suggesting a possible protective role in this older population. This is aligned with some limited evidence that points at a neurotrophic effect [48], although TGF-β2 functions remain unclear. However, prior research has shown that in AD patients, neurons bearing neurofibrillary tangles exhibit

increased expression of TGF-β2 compared to cognitive age-matched controls [49–51]. There is limited evidence, based on small studies, that TGF-β2 may play a deleterious role in AD, justified by the higher protein levels found in the brains of AD patients [50,52], but on the other hand, there seems to be a reduced presence of receptors in the neurons of AD patients [51,53]. Our results may be in favor of the effect of the Mediterranean diet through the TGFβ2 anti-apoptotic effect described in other cell types [54].

The inflammasome is a multiprotein complex of the innate immune system that detects danger signals such as tissue damage, cellular stress, or infection. Cytokines become active by inflammasome mediation, triggering local and systemic inflammatory responses essential for immune defense but also involved in chronic inflammatory diseases when deregulated [55]. The NLRP3 inflammasome plays a pivotal role in the onset and progression of Aβ in mice, and may participate in protein tau pathology as well [56]. Prior research conducted to assess the effect of MedDiet adherence counteracting neuroinflammation and age-related diseases revealed that a 3-month intervention with a MedDiet supplemented with different olive oils was associated with downregulation of IFNG transcriptomic levels [57,58], among other inflammatory and proatherogenic biomarkers [59]. In a long-term (3 years) transcriptomic PREDIMED sub-study, a downregulation of both IFN-γ and NLRP3 was reported [60].

Hormesis is based on exposure to a substance exerting a biphasic response depending on the dose [61,62]. This may explain possible differences regarding the gene expression modulation by diet, specifically the upregulation pattern observed in MedDiet-EVOO (Figure 2). The inter-species hormesis theory postulates that the stress-induced synthesis of plant polyphenols, among other phytochemicals, can produce an environmental chemical signature that leads to stress resistance in other species [63–65]. In this regard, an hormetic dose-response behavior regarding NLRP3 has been described considering polyphenol intake [66–68] that aligns with this hypothesis, suggesting that hormesis may explain these findings.

NAMPT is the gene encoding visfatin, a dual-form ubiquitously expressed, whose functionality encompasses multiple processes related to insulin sensitivity, NDDs, lipid metabolism, atherosclerosis, and pro-inflammatory effects [69–71], including NLRP3 inflammasome activation [72,73]. It has also been described that NAMPT promotes IFN-γ secretion in CD4+ T lymphocytes, but paradoxically the IFNG transcript was upregulated when NAMPT was inhibited, suggesting a posttranslational level regulation [74]. An influence of diet on NAMPT expression has been described [75,76]. With regard to visfatin circulating levels, a decrease after a hypocaloric diet with significant weight loss has been described [77]. It has also been reported that the fat type can influence the visfatin response [78,79]. In this regard, no significant weight change was apparent with the MedDiet interventions in the full PREDIMED cohort [80]. Nevertheless, we have observed a significant downregulation of NAMPT in the control diet group compared to the MedDiet supplemented with nuts (Figure 2).

In the present study, the MedDiet-EVOO was the unique group in which an upregulation of PIK3CB and downregulation of CDKN2A versus baseline was observed (Figure 2). In one RNA-seq data study, the mean expression of PIK3CB in AD patients was lower than that of controls, suggesting a role in AD pathogenesis through apoptosis [81]. Similarly, altered blood expression of CDKN2A, measured by qRT-PCR, was reported in preclinical AD patients compared to controls [82]. The CDKN2A protein has previously been described to be upregulated in the brains of patients with AD [83]. These findings suggest that whole blood could be an emerging valuable tissue for unraveling the pathophysiology of AD and differentiating it from normal aging [84].

Several meta-analyses conducted in older adults (60–80 y) have concluded that greater MedDiet adherence usually correlates with an overall better cognitive performance [85–87]. Nevertheless, the evidence for causality is weak. The Three-City cohort is a French longitudinal (4-year follow-up) study designed to assess the risk of dementia, with almost 10,000 older

participants (mean age, 74 years) [88,89], which did not demonstrate an association between adherence to the MedDiet and the risk of developing dementia [90].

The disbalance between oxidative stress and antioxidant systems has been suggested to play a role in the pathophysiology of NDDs. Further evidence is required to demonstrate if MedDiet can exert a protective effect on dementia or delay its onset [91,92] and explore the underlying molecular mechanisms of these benefits.

Strengths and Limitations

The first limitation is the relatively small sample size, which may result in limited statistical power. More repeated measurements are required to study the dynamic behavior of molecular mechanisms, and therefore improve the strength of the conclusions. As a consequence, the study may not have sufficient sensitivity to identify relevant associations between the variables of interest. The second limitation is that cognitive impairment and risk of dementia were not primary endpoints in the PREDIMED study. No cognitive tests were performed, even though alternative subcohorts within the PREDIMED study have evaluated such outcomes. The third limitation is the biological specimen employed in the transcriptomic study, taking into account that gene expression varies depending on the tissue analyzed. In this regard, PBMCs from blood are useful for studying cardiovascular biomarkers such as inflammation and peripheral cholesterol efflux. However, blood is not a good indicator for selective gene expression in other tissues. Although neurons and cerebral microvasculature cells are closely related to neurodegenerative risk, collecting these cells in population-based research is impractical. Finally, our cohort comprised participants at high cardiovascular risk. Thus, results may not be generalized to the average elderly population.

4.1. Conclusions

Our results suggested that the expression of inflammatory pathways-related genes linked to both CVD and NDDs is moderately modulated by a traditional MedDiet, especially when supplemented with extra-virgin olive oil, in circulating peripheral blood nuclear cells of older adults at high cardiovascular risk. In particular, the MedDiet-EVOO modulated the expression of CDKN2A, IFNG, NLRP3, PIK3CB, and TGFB2 genes, whereas the MedDiet-Nuts differently expressed NAMPT compared to the control diet. The underlying molecular mechanisms could explain the brain benefits of a cardioprotective diet, such as the Mediterranean diet, although more evidence on large-sized and long-term lifestyle interventions is needed. The research on biomarkers may discover specific molecules able to assess neuroinflammatory process in an accessible way. The combination with imaging techniques may enhance the detection and monitoring of these processes, offering a more comprehensive approach to diagnosis and treatment strategies for neuroinflammatory conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nu16183147/s1, Figure S1: Flowchart of participant enrollment, randomization, and analysis in the PREDIMED; Table S1: Baseline characteristics of the subsample participants of PREDIMED (N = 134) and comparison with the correspondent baseline characteristics of the complete PREDIMED Study (N = 7237). Values are expressed as a percentage (for categorical variables), mean (standard deviation). Chi-Square test was performed for categorical variables and Student-T test for quantitative continuous variables. Table S2: Mean Consumption and Dietary Changes at Baseline, 12 Months, and Follow-up Comparison. Table S3: Student-T Test and Mixed-Effects Model Comparison of Gene Expression Changes Over 12 Months.

Author Contributions: M.F., J.S.-S., M.Á.M.-G., D.C., E.R. and R.E. were responsible for the development and planning of the clinical trial. O.C. and M.F. conceptualized the sub-study. J.H.-R. carried out the formal analysis, data curation, and software management, while also contributing to the visualization and methodology. O.C., M.F. and J.H.-R. prepared the initial draft of the manuscript. M.M., K.A.P.-V., I.P.-G. and I.B.-C. contributed to the manuscript by reviewing and editing the content. M.Á.M.-G., D.C. and R.E. were also involved in the investigation and conceptualization of the study. X.P., F.A., J.L. and E.R. contributed to the investigation and writing, focusing on reviewing and editing the article. D.R. and R.C.-G. assisted in the writing, review, and editing processes. All authors

contributed to the content review and editing of the article and approved the final submission. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Review Board of Hospital del Mar d'Investigacions Mèdiques: protocol code 2017/7482/I in Barcelona, 7 March 2017.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The dataset analyzed during the current study cannot be made publicly available due to national data regulations and ethical considerations, including the absence of explicit written consent from study participants to make their deidentified data available upon study completion. However, data described in the manuscript will be shared with bona fide investigators for collaboration upon request. Requests for collaboration can be made by sending a letter to the PREDIMED Steering Committee (predimed-steering-committee@googlegroups.com).

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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DISCUSSION

General overview

This thesis presents three original publications focusing on two different scopes. The first manuscript is framed within the PREDIMED-Plus trial. We assessed the effect of a hypocaloric MedDiet intervention, combined with physical activity promotion, versus an *ad libitum* MedDiet in subjects with MetS. We compared weight loss and long-term maintenance, carbohydrate and lipid metabolism, leptin and ghrelin behavior, and attenuation of inflammatory response measuring several biomarkers.

In the second and third publications, we investigated the underlying molecular mechanisms of the MedDiet effects on cardiovascular risk by determining the gene expression of circulating nuclear peripheral cells in subjects at high cardiovascular risk. Firstly, we explored how different MedDiet interventions regulate the cholesterol efflux process in two different clinical trials (PREDIMED and PREDIMED-Plus). In the PREDIMED, we compared two traditional MedDiets supplemented with EVOO and nuts respectively, versus a control following a low-fat restricted diet. Then, we also analyzed gene expression within the frame of PREDIMED-Plus, comparing a hypocaloric MedDiet intervention with physical activity routine versus an *ad libitum* MedDiet.

Finally, in the third paper, we investigated the molecular mechanisms regulating neuroinflammation within the context of the PREDIMED trial in subjects at high cardiovascular risk. This study compared the impact of two traditional Mediterranean diets, one supplemented with EVOO and the other with nuts against a low-fat control diet. Our analysis focused on gene expression related to neuroinflammatory and inflammatory markers.

1st Manuscript

Mid- and long-term changes in satiety-related hormones, lipid and glucose metabolism, and inflammation after a Mediterranean diet intervention with the goal of losing weight: A randomized, clinical trial

Association hypocaloric MedDiet weight loss maintenance

Short-term weight change is relatively straightforward, but maintaining these improvements over time is considerably more challenging, especially once the intervention has ended (322). Researchers have suggested various time frames for the onset of the weight regain phenomenon, also depending on the intervention (pharmacotherapy, lifestyle changes (323,324) or bariatric surgery) (322,325).

Several predictors of weight-loss maintenance are those related to: a) lifestyle such as physical activity, low-calorie or low-fat diet, b) genetic factors such as metabolism, and c) psychological traits, environmental factors, socio-economic status, and social cohesion, and d) clock-related factors regarding eating habits, physical activity, and sleep (326–329). In this regard, given its unique position at the intersection of multiple domains of science, nutrition science has the potential to lead initiatives and common policies for delivering impactful research (330). Treatment for obesity prioritizes weight loss through lifestyle recommendations aimed at achieving an energy deficit. Nutritional therapy should be individualized to address personal causes of obesity, culture, and individual preferences, ensuring long-term adherence to treatment (331).

After a follow-up of almost 5 years and 7447 individuals following non-energy restricted MedDiets (enriched with EVOO or nuts) in the PREDIMED trial, 90% of whom were overweight or obese at baseline, significant differences in body weight evolution were observed, although only a slight reduction was noted when compared to the control group. (332). Consequently, an intensive energy-restricted MedDiet together with physical activity should intensify the weight

loss and its maintenance over time, as evidenced in a posterior study, the PREDIMED-Plus trial (266,268).

Aligned with the PREDIMED-Plus trial, our results in a subset of participants, the intensive lifestyle intervention group revealed reductions in both waist circumference and weight at the 6-month and 12-month follow-ups, with significant differences compared to the control group at both intervals. The weight loss observed in the control group, despite following a non-restricted energy diet, may be attributed to their motivation to actively engage in a clinical trial targeting individuals with overweight or obesity.

Combining diet-induced weight loss with exercise training has shown greater improvements in cardiovascular risk factors compared to diet by itself (333,334). The combination of physical activity and calorie-restricted diet has strongly demonstrated to prevent weight regain after a substantial initial loss (335). The difficulty in maintaining weight loss is often attributed to lack of adherence to the diet that led the initial weight loss, highlighting the need to focus on behavioral interventions (336). In our study, the weight loss tertiles showed improvements at mid-and long-term follow-ups, correlating with MedDiet adherence and the amount of physical activity, regardless of the group.

The key challenge for restrictive diets is the difficulty of adhering to long-term interventions, partially due to the unappetizing nature of the diet. In our study, the intervention group achieved maximum weight loss at 1 year, with no rebound effect observed from six- to twelve-month period. In this regard, a Mediterranean diet is characterized by strong palatability, due to its high intake of vegetal fat, such as olive oil and nuts, encourages participants to long-term commitment (337–340).

Leptin and Ghrelin: Balancing Hunger and Satiety in Weight Regulation

The physiological mechanisms governing weight stability or regain are complex and not completely understood, resulting from a combination of factors (socioeconomic, genetic, environmental, etc.) (341). The flexibility of adipose tissue allows for the enlargement of adipocyte size, to be able to maintain lipid homeostasis in the postprandial state. During long-term processes such as weight loss, maintenance or regain, adipocyte balance functions similarly, but the regulatory mechanisms become more complex (Figure 15). Individuals suffering from obesity experience a disruption in homeostatic mechanisms, where adipocytes fail to respond to physiological stimuli (342).

The energy expenditure and storage circuit is regulated among others through leptin effects, primarily exerted on hypothalamus and adipose tissue (300,341). A significant decrease in peripheral levels of leptin in energy-restricted interventions is caused by fat mass reduction (343–345). Leptin levels can be modulated by certain dietary components (300), and prior research has reported systemic leptin concentration decrease after following a MedDiet for up to 24 weeks (116,346,347), although limited evidence exists for diet interventions beyond 24 weeks (348). In this regard, we observed a reduction in leptin levels in both the intervention and control groups. The intervention group showed a more pronounced decrease, likely due to greater reductions in anthropometric measurements. Notably, significant difference was found between the intervention and control groups at the 12-month follow-up.

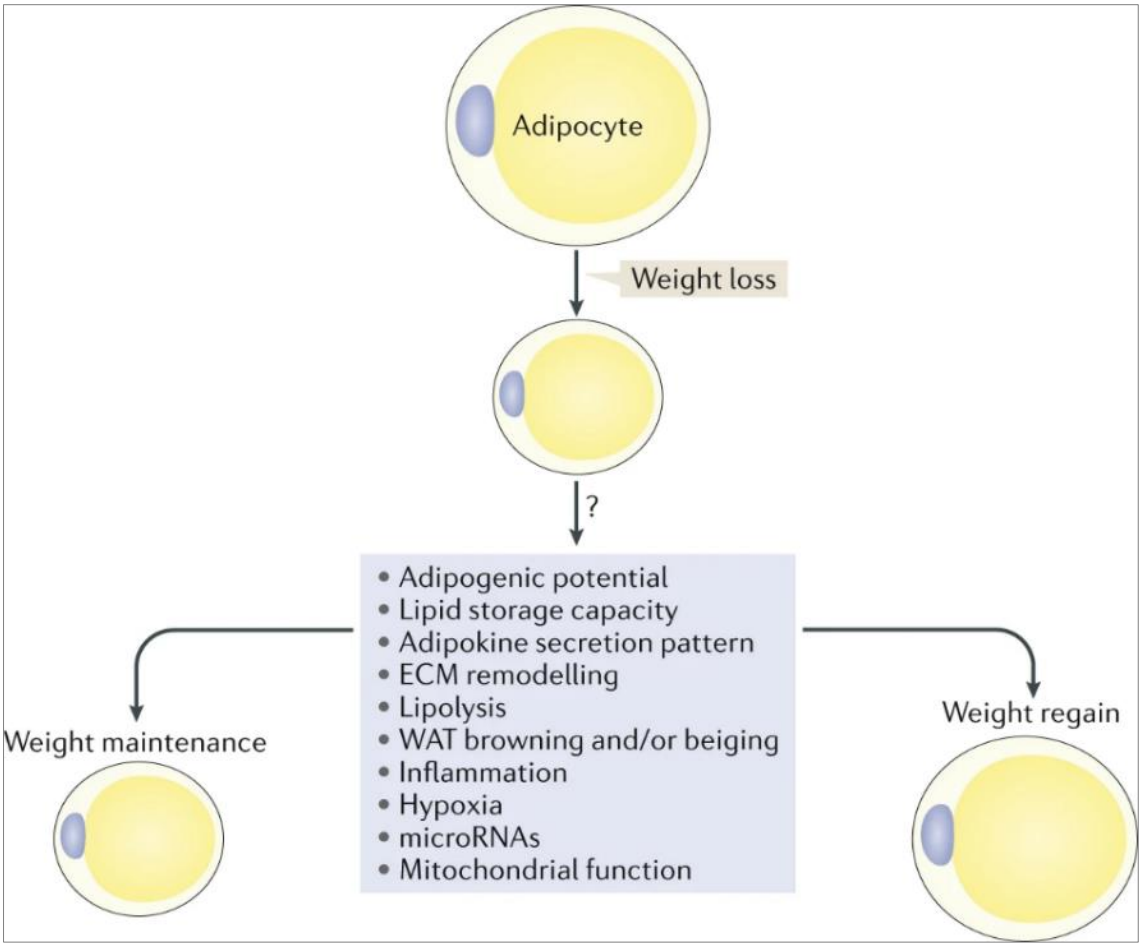

Figure 15 - Adipocyte Changes in Weight Loss, Maintenance, and Regain (341). ECM: Extracellular matrix; WAT: White Adipose Tissue; miRNAs: micro ribonucleic acids

The counterpart to leptin is partially exerted by ghrelin, an orexigenic, lipogenic and adipogenic hormone, whose deficiency is associated with lower weight (349–351). Obese individuals tend to have lower fasting plasma levels of ghrelin, which negatively correlate with body fat percentage, fasting insulin, and leptin concentrations. Weight loss is typically associated with an increase in ghrelin levels, regardless of the underlying factors contributing to weight reduction (352–354). In the same vein, ghrelin levels can be influenced by specific dietary components (314–316).

Previous dietary interventions based on the Mediterranean dietary pattern have produced consistent results regarding ghrelin behavior, though varying outcomes have been observed when examining the relationship between ghrelin and body composition. A significant increase in ghrelin levels was observed among participants with MetS who gained visceral adipose tissue (VAT) after one year. Additionally, ghrelin changes based on VAT tertiles revealed that those who lost the most VAT had the highest baseline ghrelin levels and experienced the smallest increase in ghrelin over time (355). On the other hand, an 18-month MedDiet intervention showed a significant increase in ghrelin among men, suggesting that ghrelin plays an independent role in improving insulin sensitivity. The 18-month change in fasting ghrelin levels inversely correlates with VAT and weight. Additionally, higher ghrelin levels were significantly associated with decreases in HbA1c and HOMA-IR, as well as an increase in HDL cholesterol. These associations remained significant after adjusting for age, baseline measurements, and intervention group (356).

Our results showed a pattern previously observed, in which ghrelin levels initially decreased to slightly increase over time, eventually returning to baseline values (357). However, these changes were not statistically significant when compared to the control group.

Carbohydrate metabolism

Compelling evidence has clarified that diabetes can be tackled through lifestyle interventions, including various dietary strategies. Concurrent weight loss is likely a driving force leading to beneficial effects in the management and prevention of diabetes. The abundant scientific literature presents several approaches, although conflicting results exist around the optimal dietary composition to achieve these outcomes (358,359). The European and American Institutions recommend lifestyle as the primary approach for diabetes mellitus management (331). In this regard, Mediterranean dietary pattern supports the consumption of carbohydrate mostly based on whole grains, cereals and fruit (245,265,360). Evidence on this matter suggests that higher-quality carbohydrates improves CVDs risk factors (360,361) including favorable adipose tissue distribution changes (362).

With regard to high-fat content, particularly MUFA, which are reported to promote positive effects on regulating the glycemic response and enhancing insulin sensitivity (363–365). In fact, clinical trials have consistently demonstrated such improvements, especially in individuals predisposed to insulin resistance, strengthening the positive impact of this dietary pattern (364). Previous findings from the PREDIMED study also reported improvements in carbohydrate-related variables after a non-calorie-restricted MedDiet intervention enriched with EVOO or nuts (143).

In contrast to isolated interventions, the combined effects of a calorie-restricted diet and physical activity have been shown to more significantly improve insulin sensitivity and cardiometabolic syndrome-related variables (333,334) . Analogously, we observed insulin level reduction in the intervention group during the first 6 months, that remained stable up to 12-month follow-up. The control group also showed a consistent reduction, though insulin levels were higher at 6- and 12-month follow-ups. HOMA, C-peptide, HbA1c, and glucose levels displayed a comparable trend.

Lipid metabolism

Dyslipidemia is characterized by abnormal lipid and/or lipoprotein levels, comprising elevated triglycerides (hypertriglyceridemia) and LDL-c, along with reduced HDL-c levels. Hypertriglyceridemia is influenced by several factors, including body weight, fat distribution or diet composition. Reducing triglycerides often requires multiple interventions with additive effect. One key strategy is eliminating trans fatty acids from the diet, as these fats raise triglyceride levels and harmful lipoproteins like LDL-c, increasing cardiovascular risk.

In contrast, a low-fat and high-carbohydrate diet has been linked to a rise in triglycerides and a decrease of HDL-c, which can be detrimental, since elevated triglycerides levels often coexist with T2DM (150). The MedDiet replaces animal fat with vegetable, namely SFA with MUFA and PUFA (366). The evidence has demonstrated MedDiet improves lipid profile over the long term, ameliorating triglycerides, LDL-c and HDL-c (263,367). Regarding our results, the data reflects a more substantial reduction in triglycerides in the intervention group at both the 6- and 12-month time points, after adjusting for sex and age.

Changes in LDL particles may contribute to increase atherogenicity, especially when LDL particles are small and dense (146). MedDiet has been associated with positive changes regarding LDL particle size, the degree of resistance to oxidation, and LDL cytotoxicity in macrophages (368,369). At the same time, the functionality of the HDL particle is gaining increasing clinical relevance. We have previously observed in a substudy of PREDIMED-Plus, that a hypocaloric diet combined with physical activity promotes the reduction of triglycerides in HDL lipoproteins, suggesting an improvement in HDL's role in triglyceride metabolism. We also reported a decrease in apo-CIII levels after the intervention, which contributes to hepatic clearance of plasma triglycerides. These effects were shown to be mediated by BMI reduction (370).

Pro-inflammatory biomarkers

CRP circulating levels are routinely measured to monitor multiple inflammatory processes regardless of the underlying cause (infectious, autoimmune, tumoral or metabolic). It is a widely used acute-phase reaction marker that helps to assess low-grade inflammation characteristic in patients with cardiovascular risk factors. The MedDiet has been demonstrated as an effective strategy for reducing inflammation and mitigating the inflammatory response, manifested with decreased CRP or IL-6 levels (371). The anti-inflammatory effect of the traditional MedDiet has also been reported in the PREDIMED study, with a significant reduction of pro-inflammatory markers observed at the 3- and 5-year follow-ups (95). Concretely, we observed a reduction in CRP levels over time in both groups, though without significant differences between them.

Meanwhile, PAI-1 function is an indirect thrombotic enhancer by acting as an antifibrinolytic agent (287). Previous studies have shown that high adherence to MedDiet correlates with an improved inflammatory profile, which includes low PAI-1levels (282). In our experience, we observed significant changes in PAI-1 levels in both groups, with greater decreases occurring in the intensive intervention group, particularly at the 12-month follow-up. Adipose tissue constitutes a major source of PAI-1, linking obesity directly to elevated PAI-1 levels (283). The weight loss experienced by the energy-restricted group may have contributed to the steeper reduction observed.

Visfatin levels decreased in both groups in a similar way, without significant inter-group differences. This suggests that both MedDiet types were effective in lowering visfatin independently of calorie restriction or promoted physical activity. In contrast, it has been previously reported no significant effects of MedDiet in visfatin levels in adult population with low cardiovascular risk according to ESC (116).

Resistin levels decreased in both groups, with a steeper and significant reduction in the control group, but without significant inter-arm difference. Research on the field has sometimes shown a limited impact on resistin levels, which may be influenced by the designs, durations, and inclusion criteria for the population (age, sex, health status). However, both studies involved calorie restriction and physical activity, and achieved weight loss and inflammatory markers improvements (372,373)

2nd Manuscript:

Mediterranean diet transcriptomic modulation of cholesterol efflux molecular mechanisms in elderly adults at high cardiovascular risk

CEC is probably the most well-known function of HDL. It is the process of cholesterol removal from various donors, typically peripheral cells in the blood or macrophages in the intima, to subsequent acceptors, which are primarily HDL lipoproteins (185). A wide range of molecules are involved in the cholesterol efflux process, making it challenging to quantify the individual contribution of each compound influence. In the context of a Mediterranean dietary pattern, these bioactive compounds, such as polyphenols, fatty acids, and other nutrients may work synergistically, likely through molecular mechanisms, to exert their effects.

Rather than measuring the impact of isolated compounds, we assessed the intervention as a whole dietary pattern. This approach allows us to capture the combined effect of the various components of the MedDiet on cholesterol efflux gene expression regulators, providing a more realistic and comprehensive understanding of how this dietary pattern supports cardiovascular health. We have previously reported improvements in CEC as a result of a traditional MedDiet intervention, especially enriched with EVOO (32).

We observed mild upregulation of cholesterol efflux-related genes involving nuclear receptors such as RXRs (*RXRA*, *RXRB*), LXR-β, encoded by *NR1H2*, and peroxisome proliferator activated (*PPARD*), *ABCG1* and *ABCA1*, occurred as long-term responses to different MedDiets in elderly adults at high cardiovascular risk. In the analysis joining both MedDiet groups, *ABCG1*, *PPARD*, and *RXRA* were differently expressed versus the control group.

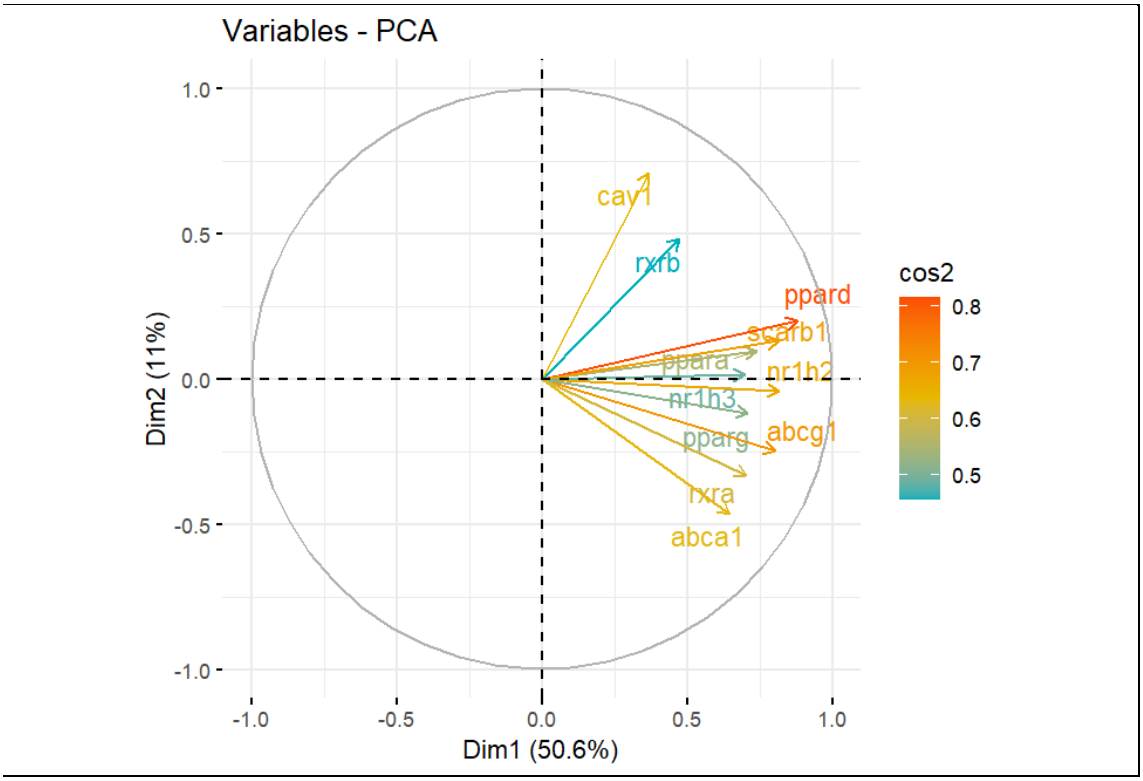

Figure 16 - Gene Contribution to Principal Components in Mediterranean Diets
Dim 1: dimension 1; Dim 2: dimension 2.
In parentheses, the total percentage of variance explained by the component

We can observe from Figure 16 (Gene Contribution to Principal Components in Mediterranean Diets) that *ABCA1* and *ABCG1* display a similar contribution to overall variability. Both show a moderate-high positive contribution to Dim1, and slightly and negative contribution to Dim2. These genes are primarily associated with variance captured by Dim 1, which accounts for most of the variance (50.6 %), likely due to the fact that the studied genes are related with cholesterol efflux. In this regard, most of the cholesterol efflux-related enhancers such as *NR1H2*, *NR1H3*, *RXRA* and *PPARs,* also display high contribution to Dim1, being *NR1H2*, *NR1H3*, *RXRA* and *PPARD* upregulated in response to MedDiet. *RXRB* and *PPARG*, on the other hand, contribute moderately to Dim2, although *RXRB* is also upregulated by MedDiet, and *PPARG* slightly downregulated. This contribution to Dim2 may be due to these genes could be involved in additional biological processes captured by Dim2 (11 %), or due to alternative regulatory mechanisms that we cannot explain. A similar reasoning may justify *CAV1* and *SCARB1* which are also involved in the cholesterol efflux process but have not been modulated by MedDiets. A previous study found downregulation of *RXRB* and upregulation of *CAV1* following functional virgin olive oil (FVOO) consumption, which contrasts with our findings. However, it should be noted that the study was based on three intervention periods of three weeks each, with washout periods between interventions, and focused solely on FVOO consumption, which may explain the difference (374).

ABCA1 is tightly regulated by a network of transcriptional factors that can form partnerships that activate the promoter regions. In this role, LXR-α activity stands out due to the capacity to directly

enhance *ABCA1* expression in response to cholesterol derivatives, as well as through dimerization with various regulatory molecules, such as RXRα (Figure 17) or PPAR-γ.(375). RXR amplify transcriptional activity o working synergistically with LXR (376), thus it is interesting to observe the contrast in the differing gene expression.

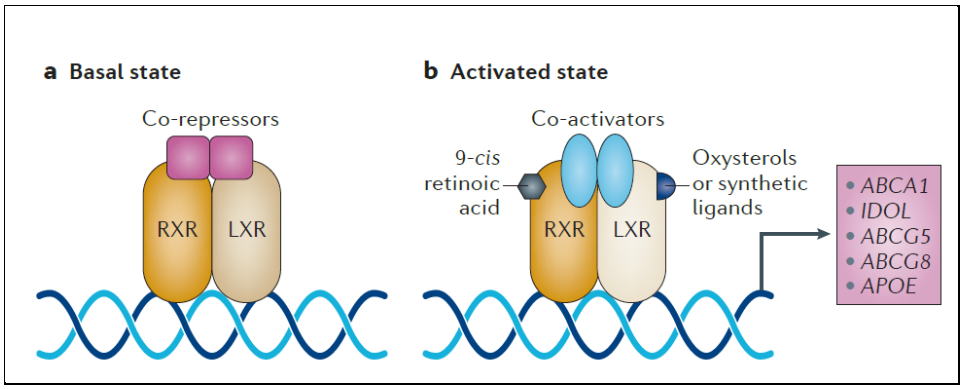

Figure 17 - LXR–RXR heterodimers are ligand-activated transcription factors (377).

In the PREDIMED-Plus, we found a similar pattern involving *ABCA1* and *ABCG1*, which experienced a significant upregulation from baseline to 12-month follow-up- The larger upregulation may be consequence of the physical activity, which positively increases *ABCA1* and *ABCG1*(236). However, we reported *PPARG* downregulation despite previous research found that physical activity enhances its expression (378). We hypothesized the concurrence of the different regulatory pathways that modulate its expression, may explain the unexpected results (379–381). Figure 18 illustrates the regulatory network of cholesterol efflux, involving participant gene encoding proteins. The graphic shows the interactions among the molecules with each other and their influence on cholesterol efflux and transport processes. The diagram shows gene expression values, along with predicted effect (activation, inhibition or inconsistent) and the nature of this one (direct or indirect)

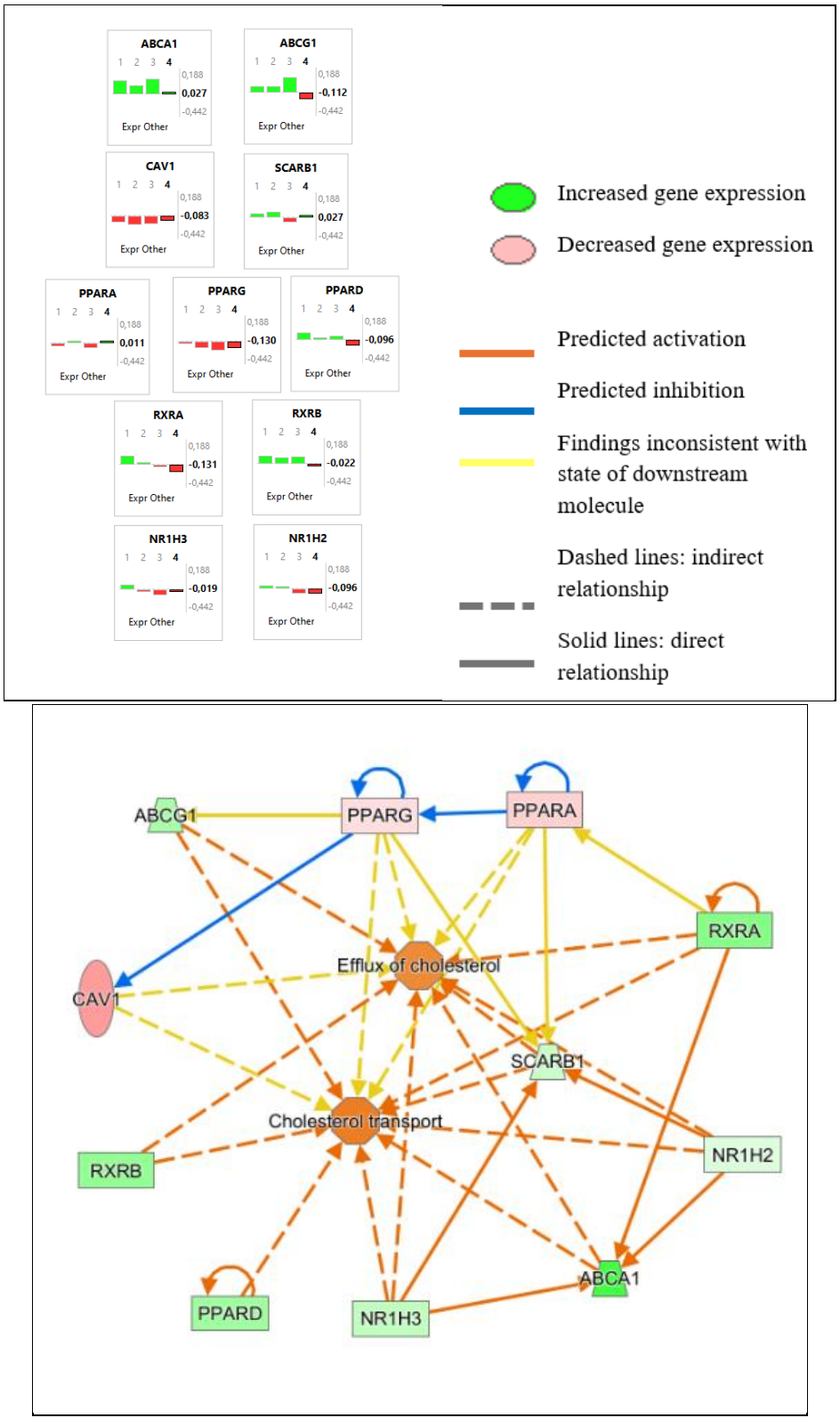

Figure 18 - Ingenuity Pathway Analysis regulatory network prediction using log_2FC. Legend illustrates relations of molecules in functional processes related to cholesterol homeostasis.

Legend - 1: MedDiets combined (PREDIMED), 2: MedDiet (PREDIMED-Plus), 3: ErMedDiet (PREDIMED-Plus), 4: Control diet (PREDIMED)
ABCA1: ATP binding cassette subfamily A member 1; ABCG1: ATP binding cassette subfamily G member 1; NR1H2/LXR-α: nuclear receptor subfamily 1 group H member 2; NR1H3/LXR-β: nuclear receptor subfamily 1 group H member 3; RXRA: retinoid X receptor alpha; RXRB: retinoid X receptor beta; SCARB1: Scavenger Receptor Class B Type 1; CAV1: Caveolin-1 PPARs: peroxisome proliferator activated receptors (A (alpha), D (delta), G (gamma))

3rd Manuscript:

Mediterranean diet transcriptomic modulation of neuroinflammation-related genes in elderly adults at high cardiovascular risk

Systemic inflammation is triggered by different conditions, including atherosclerosis, dyslipidemia, obesity or hypertension, leading to an increase in proinflammatory cytokines, endothelial damage and oxidative stress. The inflammation may cause the impairment of the BBB, becoming permeable and allowing the entrance of pro-inflammatory molecules which activate the microglia. Neuroinflammatory states can be transient or chronic, with NDDs typically associated with a chronic neuroinflammatory response (266, 366). Both chronic low-grade inflammation and neuroinflammation involve similar genes and pathways (JAK-STAT, NLRP3 inflammasome), establishing a direct link between systemic inflammation and brain inflammation (271,383,384).

In this regard, we selected several genes from the overlapping pathways identified in the functional analysis using keywords related to cardiovascular and neurodegenerative conditions (Figure 19).

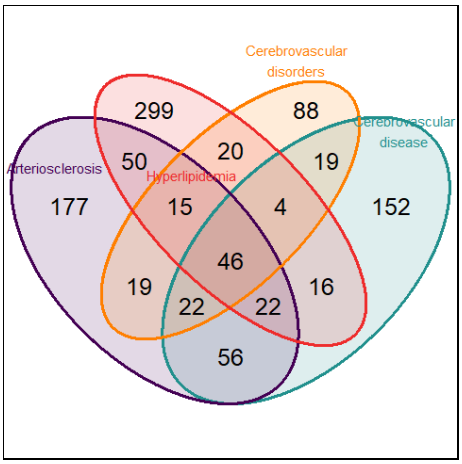

Figure 19 - Venn diagram displaying the number of genes found from the overlap of the 4 selected pathologies (atherosclerosis, hyperlipidaemia, cerebrovascular disorders, and cerebrovascular disease) employing the public databases DisGeNET and Disease Ontology.

Neuroinflammatory response is a direct consequence of various conditions that promote this state (270,279). These conditions are closely associated with the modern lifestyle, particularly the sedentary lifestyle and the Western dietary pattern (271,272). Adopting a healthy dietary pattern may help attenuate the detrimental effects caused by systemic inflammation (385,386).

Peripheral blood transcriptomic profiling has proven to be a valuable tool for measuring neuropathological biomarkers in a convenient and accessible manner (387), although it does not precisely reflect alterations in primary tissue (388,389). In this regard, prior research conducted in the PREDIMED trial assessed whether a 3-month MedDiet enriched with either EVOO or nuts on pathways related to neuroinflammation, reported a potential delay on the risk of neurodegenerative diseases. The study found a downregulation of the *TREM1* signaling pathway with the MedDiet enriched with EVOO, while MedDiet enriched with nuts downregulated the neuroinflammation signaling pathway in a whole transcriptome microarray analyses (390). In the Manuscript 3, we assessed the MedDiet effect on neuroinflammatory response. In this regard, related to MedDiet enriched with EVOO intervention group, we observed significant 12-month changes in the Real Time PCR (RT-PCR) expression of the following genes: *IFNG*, *CDKN2A*, *NLRP3*, *PIK3CB*, and *TGFB2*. Evidence has reported MedDiet supplemented with various types of olive oil cause a reduction in *IFNG* after 3 months (218, 360).

We also observed changes in *TGFB2* expression from baseline to one-year follow-up in the MedDiet enriched with nuts group. Significant between-group differences in *TGFB2* expression were found between the MedDiet enriched with EVOO and the control groups. Furthermore, significant differences in *NAMPT* expression were observed in the comparison between the MedDiet enriched with nuts and control groups. Model 1, adjusted for sex, age, and education level, revealed statistically significant differences in the interaction term (time-group intervention) for *TGFB2* between the MedDiet enriched with EVOO and control groups, as well as significant results for *NAMPT* in the comparison between MedDiet enriched with nuts and control. However, Model 2 only showed statistically significant differences for *NAMPT* in the MedDiet enriched with nuts group versus the control group. In this regard, we were also unable to detect changes in systemic vifastin levels between groups following an energy-restricted diet (with physical activity) an *ad libitum* MedDiet in manuscript 1. However, we observed changes from baseline to the 6- and 12-month follow-ups, with a steady trajectory between 6 and 12 months.

Although, an increase in the *TGFB2* expression has been observed in the three PREDIMED groups, including the control group, significant between-group differences in *TGFB2* expression were found between the MedDiet enriched with EVOO and control groups. Although a direct correlation between TGF-β2 levels and soluble neurofibrillary tangles and Aβ42 (key form of Aβ aggregation) has been described (392), neurotrophic effects (393) have also been reported. In addition, whereas Aβ42 levels initially rise at the onset of AD, they typically decrease as the disease progresses (394,395). Interestingly, Aβ42 in cerebrospinal fluid has been associated with better cognitive outcomes and brain preservation in patients with amyloidosis (396,397). Furthermore, significant differences in *NAMPT* expression were observed in comparison between the MedDiet enriched with nuts and control groups. Model 1, adjusted for sex, age, and education level, revealed statistically significant differences in the interaction term (time-group intervention) for *TGFB2* between the MedDiet enriched with EVOO and control groups, as well as significant results for *NAMPT* in the comparison between MedDiet enriched with nuts and control. However, Model 2 only showed statistically significant differences for *NAMPT* in the MedDiet enriched with nuts group.

The dose-response phenomenon known as hormesis may explain the differences observed in gene expression modulation by diet, in particular when olive oil is supplemented. Hormesis refers to the biphasic response triggered by exposure to a substance, where the effect varies depending on the dose (398,399). The inter-species hormesis theory suggests that the stress-induced production of plant polyphenols and other phytochemicals creates an environmental chemical signature that can promote stress resistance in other species (400–402). In this context, a hormetic dose-response behavior related to *NLRP3* has been observed in relation to polyphenol intake (366–368).

STRENGTHS AND LIMITATIONS

The overall strengths of our research across the three studies lie in the randomized, and controlled designs, which minimize bias and confounding factors enhancing the reliability of our results. The long-term longitudinal design and the focus on populations at high cardiovascular risk allow for in-depth analysis of metabolic and cardiovascular health outcomes. This approach provides foundational evidence on the effects of dietary interventions in target population. In terms of general limitations, generalizability is constrained by the focus on older adults and individuals with MetS or high cardiovascular risk, limiting the applicability of the findings to broader populations. The self-reported dietary intake, although assessed using a validated questionnaire, may provide inaccurate data, potentially undermining the reliability of the results.

In the case of the first manuscript, the lack of post-prandial hormone measurements, such as ghrelin and leptin, prevents a full understanding of how the interventions affect short-term hormonal responses to meals. Given that leptin secretion is proportional to fat depots the leptin measurements were partially biased by the inability to estimate the overall adipose mass, as we did not utilize measurement direct techniques (Dual-Energy X-ray Absorptiometry or Bioelectrical Impedance Analysis). Due to the PREDIMED-Plus design based on a hypocaloric MedDiet versus a normocaloric MedDiet we cannot infer whether the Mediterranean dietary pattern may trigger a specific response in hunger-satiety circuit beyond evidence reported so far. On the other hand, longitudinal analysis allowed us to observe weight loss maintenance over time, along with reduction in proinflammatory biomarkers in a one-year period.

In the case of transcriptomic studies in both the second and third manuscripts, the small sample size reduces statistical power, potentially missing key associations between variables. Variations in the preanalytical phase (blood collection, storage, pipetting and processing) may carry significant changes in the results and difficult comparability among transcriptomic studies. A potential limitation may be the lack of technical replicates in our study. In the second manuscript, the cholesterol efflux-related study, simultaneous protein investigation could provide valuable insights into regulatory mechanisms and enable predictions of the relationship between transcriptomic and proteomic results. Post-transcriptional modifications, transcripts stability, and activity are crucial for understanding the impact of dietary interventions on cholesterol efflux pathways. Transcriptomic analysis alone might not capture the overall influence of the intervention. Additionally, individual responses to dietary interventions can vary greatly due to genetic, environmental, and lifestyle factors. This variability can make it difficult to distinguish diet-specific effects from other factors, such as physical activity in the PREDIMED-Plus trial. Furthermore, lipid-lowering treatments, commonly prescribed to participants of PREDIMED and PREDIMED-Plus, can influence the expression of genes related to cholesterol efflux and lipid metabolism. On the other hand, studying multiple genes involved in the key regulatory mechanisms of cholesterol efflux simultaneously allows us to observe expression patterns and attempt to infer the dynamic landscape of this biological process. However, some of the selected genes are involved in multiple metabolic processes, which can sometimes mask the specific effects of dietary intervention.

Regarding the third manuscript, the reliance on blood as a biological specimen limits the understanding of gene expression in tissues that are anatomically more directly related to neurodegenerative risk. Neuroinflammation occurs in the central nervous system, a region with strict immune regulation. Blood samples may not always accurately reflect the inflammatory processes occurring in the brain due to differences in gene expression and regulation between the CNS and peripheral blood. It must be noted that many participants in the study were taking statins, whose pleiotropic effects have been reported to affect inflammatory pathways such as NFE2L2. Additionally, neuroinflammatory processes in peripheral mononuclear cells may not reflect brain cells like microglia and astrocytes. The studies did not include cognitive assessments, limiting conclusions about how dietary interventions might influence cognitive function or dementia risk.

CONCLUSIONS

1. An intensive lifestyle intervention with a calorie-restricted traditional Mediterranean diet combined with physical activity leads to several beneficial outcomes on adiposity, blood pressure, glucose metabolism, lipid profiles, leptin levels, and pro-inflammatory markers at both mid-term and/or long-term.
   - An intensive lifestyle intervention has demonstrated to achieve greater weight loss goals in comparison to an *ad libitum* Mediterranean diet, and can be sustained over a 6 and 12-month period.
   - Higher adherence to the intensive lifestyle intervention was achieved in the intervention group compared to the control group at 6 and 12 months.
   - Reduction in triglyceride and remnant cholesterol levels was significantly greater in the intensive lifestyle intervention than in the *ad libitum* Mediterranean diet at 6 and 12 months whereas HDL-c increased at 12 months.
   - Reduction in insulin, C-peptide, and insulin resistance was significantly greater in the intensive lifestyle intervention than in the *ad libitum* Mediterranean diet at 6 and 12 months while HbA1c, leptin, and PAI-1 levels improved at 12 months.
   - Improvements in diastolic and systolic blood pressure at 6 months in the intensive lifestyle intervention than with the *ad libitum* Mediterranean diet.
2. An increase of the expression of *ABCG1*, *PPARD*, and *RXRA* after 1 year-intervention considering the combined traditional Mediterranean diet groups versus the low-fat diet control group were observed.
3. The gene expression patterns of cholesterol-efflux genes overlap between the Mediterranean diet enriched with extra-virgin olive oil and Mediterranean diet enriched with nuts.
4. Both intensive lifestyle intervention and the traditional Mediterranean diet promote upregulation versus baseline in genes encoding transporters of cholesterol, *ABCA1* and *ABCG1*, in older participants with metabolic syndrome.
5. Mediterranean diet, specially enriched with extra-virgin olive oil has demonstrated to modulate pathways linked to neurodegenerative disorders, highlighting the potential brain benefits of a cardioprotective diet, in high cardiovascular elderly population Specifically, the traditional Mediterranean diet plus nuts resulted in a different expression of the *NAMPT* gene compared to the control diet.
6. Gene expression changes from baseline to 12 months were observed mainly in the participants allocated to the traditional Mediterranean diet enriched with extra-virgin olive oil, particularly in *CDKN2A*, *IFNG*, *NLRP3*, *PIK3CB*, and *TGFB2*.

FUTURE RESEARCH

Weight loss, carbohydrate and lipid metabolic changes, and inflammatory response

Future research may address the objectives of this manuscript simultaneously analyzing and integrating multi-omics data. Weight loss and weight loss maintenance comprise different phases that share the same objective, requiring different approaches to achieve sustained results. On one hand, the biological standpoint is currently questioning the impact of the microbiota on the long-term treatment of obesity. Unlike genetic traits, microbiota is highly modifiable and dynamic (403), emerging as a potential target for weight loss and prevention of rebound effect (404). Several interventions, such as diet, physical activity, medication, and bariatric surgery, capable of modifying the microbiota (405).

The Mediterranean diet has been shown to modulate microbiota composition, although the strength of the evidence is limited and heterogeneous (406). Some positive results have been reported (407,408) regarding phyla's relative abundance has been associated with weight loss. However the dynamism in the microbiota composition and the complex interaction with host make it difficult to draw firm conclusions (404). A potential limitation is that Mediterranean dietary pattern includes multiple sources; therefore, each component may exert a unique influence on microbiota that cannot be inferred. On the other hand, adherence to diet is crucial for achieving goals, therefore identifying the behavioral and psychological barriers is a cornerstone of successful weight loss interventions (409,410).

New drugs targeting GLP-1 have demonstrated optimal results regarding DM2 and obesity treatment. It may be interesting research on lifestyle and dietary habits interaction with GLP-1 receptor, which is known to participate in reward, motivation and mood regulation. Moreover, GLP-1 analogues have proven to shift microbiota towards a healthier profile (404). However, regulation is bidirectional, as microbiota changes in response to a Western diet can trigger hypothalamic diet-induced inflammation and leptin sensitivity via GLP-1 receptor (411). This complex interaction may also be worthwhile to explore.

Regarding the low-grade inflammation, novel biomarkers with improved overall predictive power are emerging in certain contexts. For example, glycoprotein acetyls have shown potential as a biomarker with several advantages, though larger and more diverse population studies are needed to confirm its effectiveness in predicting cardiovascular risk (412). New dietary approaches, such as intermittent fasting or paleo diet, have recently gained interest due to promising results in improving cardiometabolic markers. However, long-term randomized clinical trials are required to report solid evidence (413–415).

Cholesterol efflux

The removal of peripheral cholesterol is a crucial step to avoid or delay atherosclerosis, as it plays a key role in the accumulation and formation of plaque within arterial walls. The first unsolved question is to elucidate regulatory mechanisms capturing genomic, transcriptomic, proteomic, and epigenomic, in response to multiple stimuli regarding cholesterol efflux (diet intervention, physical activity, bariatric surgery or drug treatments). The complexity of such achievement would require multiple studies to determine molecules involved in the process (416). In this line, not only the effects of exogenous factors need to be elucidated, but also those of endogenous hormones and cytokines that participate in the process (417), with the additional challenge of distinguishing their effects between in healthy and diseased states. In this regard, mediation analysis could provide evidence of overall molecular mechanisms, for example to study the mediation effect of different Mediterranean diet nutrients on HDL functionality, both at transcriptomic and systemic level.

A significant challenge lies in understanding the regulation of ABC transporters, such as ABCA1 and ABCG1. Cellular and structural studies should also gain insight in conformational and biochemical mechanisms (418). Future projects should consider look into alternative pathways of cholesterol transport (419), that may help reduce atherosclerosis.

Deficiency in ABCA1 and ABCG1 transporters leads to cholesterol accumulation, which activates the inflammasome, resulting in systemic inflammation and neuroinflammatory responses. Targeting ABCA1, ABCG1 or NLRP3 proteins may be potential approaches to counteract inflammatory response (419) . This complexity becomes even more pronounced when considering their roles in diseases like atherosclerosis, Alzheimer's or metabolic syndrome. Future research may look into enhancing the efficiency of cholesterol efflux transporters to treat associated diseases.

Neuroinflammatory response

The inflammatory response plays a central role in neurodegenerative diseases, making the discovery of novel biomarkers or identifying those with the strongest prognostic potential key targets for future treatments. Various signals are responsible for triggering different responses in glial cells; thus, selectively neutralizing or enhancing specific signaling pathways may improve inflammatory regulation and promote metabolic health. Microglial intervention in response to pathological insults can assume either neuroprotective or neurotoxic roles, partly mediated by gut metabolites generated from diet (420). Research on metabolites, the microbiota, and factors capable of modulating microglial status may provide valuable information.

Lifestyle habits, particularly dietary patterns, can influence these specific pathways; however, further research is needed to determine how nutrients affect these processes, and which dietary composition is most beneficial. The gut-brain axis is a complex communication system currently investigated, influenced by microbiota composition. As previously mentioned, gut microbiota regulates different inflammatory pathways, such as GLP-1, which has been identified as a potential target with neuroprotective effects (421). In this context, the integrity of the BBB also depends on these pathways, as well as metabolites, which can either impair or help to restore it.

Integrating this knowledge may be beneficial, or even essential, for assessing information from multiple sources, such as transcriptomic data, while simultaneously analyzing proteomic and epigenetic profiles alongside imaging techniques. The comprehensive perspective provided by combining these diverse data sources could further aid in the identification of pathological mechanisms. Potential precision nutrition tailored according to genotype and phenotype, presents a future challenge in the prevention based on individual traits.

However, a key challenge remains the limited accessibility of brain samples, hindering the evaluation of how systemic inflammation affects central nervous system and contributes to the development or worsening of NDDs. In this context, longitudinal studies are particularly valuable, as they enable researchers to monitor changes in inflammatory markers, glial cell activity, and disease progression over time. These studies can provide insight into the temporal relationships between systemic inflammation and the onset and progression of NDDs.

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SUPPLEMENTARY MATERIAL

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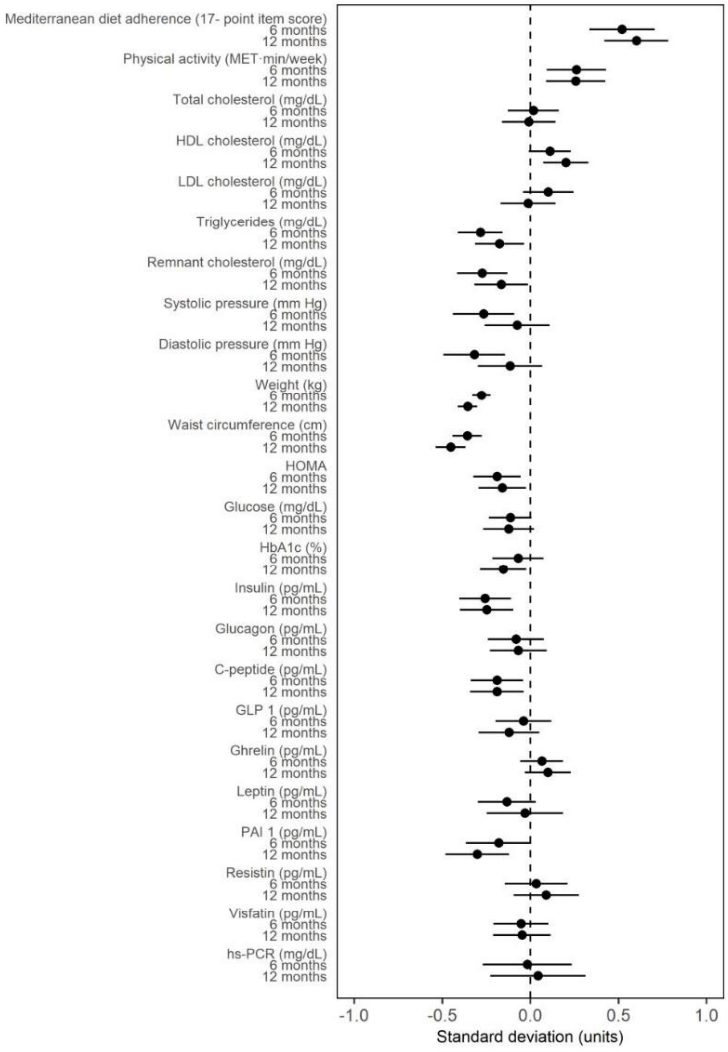
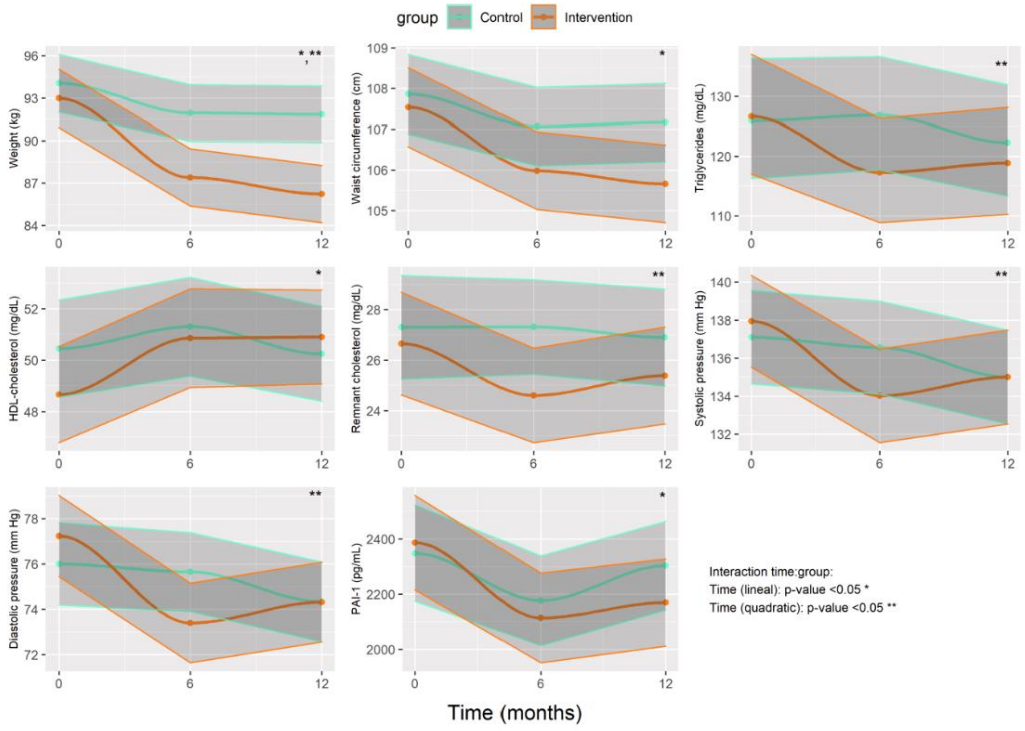
Supplementary Material

1 Supplementary Figures

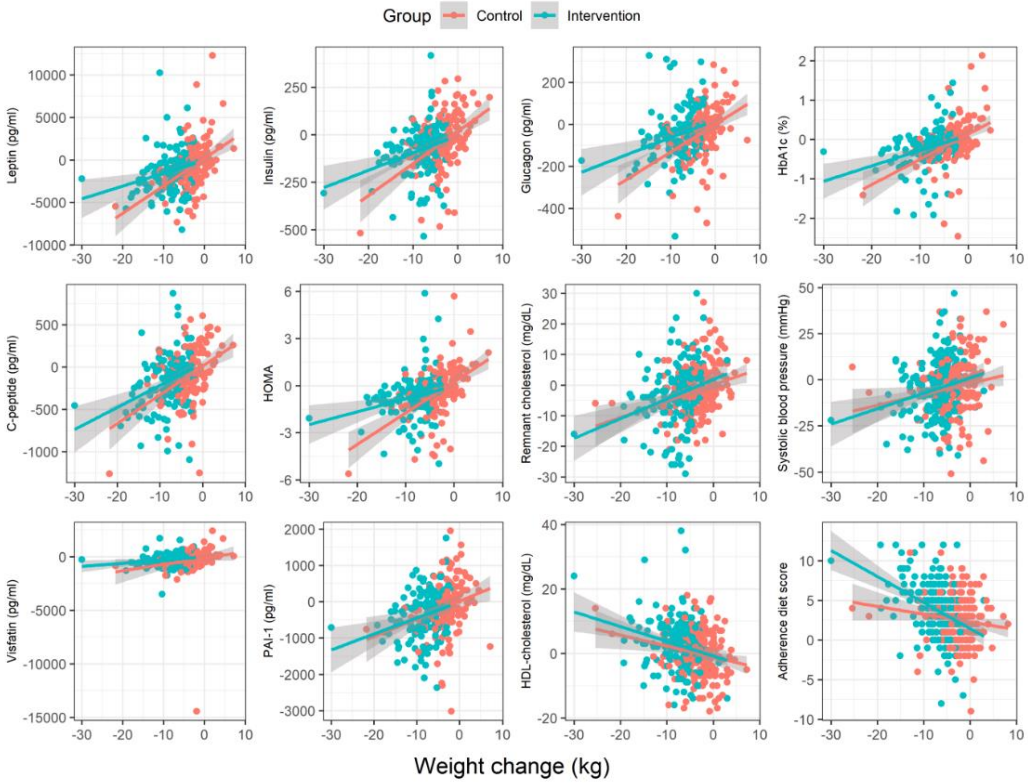


1.1 Supplementary Figure 1. Mean multivariable-adjusted differences (95% CI) for changes at 6- and 12-month follow-ups in 17-item questionnaire, physical activity, biomarkers and anthropometric measurements, lipid profile, carbohydrate metabolism, and hormones.

Supplementary Material



1.2 Supplementary Figure 2. Graphics of linear mixed-effect models in cardiovascular risk factors over time: weight, waist circumference, triglycerides, HDL-cholesterol, remnant cholesterol, systolic and diastolic blood pressure, and PAI-1.



1.3 Supplementary Figure 3. Graphics of Pearson's correlation of weight loss with 12-months change of: leptin, insulin, glucagon, HbA1c, C-peptide, HOMA, remnant cholesterol, systolic blood pressure, visfatin and PAI-1, HDL cholesterol and adherence diet score.

Supplementary material

Supplementary Table 1. Baseline characteristics of the subsample participants of PREDIMED (*N* = 134), and PREDIMED-Plus respectively (*N* = 82), and comparison with the correspondent baseline characteristics of the complete PREDIMED Study (*N*=7237) and PREDIMED-Plus (*N* = 9595). Values are expressed as a percentage (for categorical variables), mean (standard deviation). Chi-Square test was performed for categorical variables and Student-T test for quantitative continuous variables.

	Sample (N = 134)	**PREDIMED (N = 7313)**	**p-value**	**Sample (N = 82)**	**PREDIMED (N = 9595)**	**p-value**
Sex (%women)	67 (50.0%)	4215 (57.6%)	0.092	27 (32.9%)	2651 (27.6%)	0.229
Age	65.8 (6.29)	67.0 (6.20)	0.033	65.5 (4.66)	65.2 (4.98)	0.592
Weight (kg)	80.2 (12.6)	76.7 (11.9)	0.002	88.7 (12.5)	86.2 (13.3)	0.084
BMI (kg/m^2)	30.2 (3.67)	30.0 (3.85)	0.468	33.0 (3.30)	32.6 (3.71)	0.277
Waist circumference (cm)	102 (9.48)	100 (10.3)	0.035	111 (9.18)	108 (10.0)	0.001
Hypertension (% hypertensive)	106 (79.1%)	6057 (82.8%)	0.310	71 (86.6%)	7819 (81.5%)	0.661
Diabetes (% diabetic)	71 (53.0%)	3561 (48.7%)	0.369	27 (32.9%)	2651 (27.6%)	0.51
Dyslipidemia (%dyslipidemic)	75 (57.3%)	3316 (48.0%)	0.044	68 (82.9%)	7379 (90.1%)	0.044
Adherence to MedDiet (14-point item score)	8.72 (1.91)	5.67 (4.40)	<0.001	7.50 (2.65)	8.51 (2.67)	<0.001
Physical activity (MET·min/week)	1906 (1670)	1608 (1675)	0.042	2759 (2610)	2452 (2267)	0.292
Smoking status			0.525			0.619
Former smoker	20 (14.92%)	1027 (14.04%)		7 (8.54%)	1222 (12.7%)	
Current smoker	38 (28.35%)	1799 (24.6%)		38 (46.3%)	3942 (41.1%)	
Never smoker	76 (56.71%)	4487 (61.35%)		37 (45.1%)	4355 (45.4%)	

Supplementary table 2. Mean and standard deviation in the consumption of key food items and dietary parameters of PREDIMED at baseline, 12 months and 12-month change. Mean of differences and confidence intervals from Student-T test comparison between groups at baseline, 12 months and 12-month change are presented. Mean of differences and confidence intervals from Student-T test

	MedDiet-EVOO			MedDiet-Nuts			Control			Baseline comparison to control		12 months comparison to control		12-month change comparison to control	
	Baseline	12 months	12-month change	Baseline	12 months	12-month change	Baseline	12 months	12-month change	MedDiet-EVOO	MedDiet-Nuts	MedDiet-EVOO	MedDiet-Nuts	MedDiet-EVOO	MedDiet-Nuts
Energy intake (kcal/day)	2208.62 (524.79)	2152.81 (412.71)	-55.81 (570.03)	2211.03 (525.85)	2190.95 (476.61)	-20.08 (596.64)	2258.26 (501.67)	2078.13 (586.84)	-180.13 (560.66)	-74.62(-258.78 - 159.50)	-82.97(-270.30 - 175.83)	85.5(-133.67 - 283.04)	86.3(-116.66 - 342.30)	160.12(-106.12 - 354.75)	169.27(-91.42 - 411.53)
Carbohydrates (g/day)	235.64 (62.64)	219.39 (59.46)	-16.25 (77.39)	241.93 (74.84)	219.12 (68.17)	-22.81 (90.36)	232.95 (70.87)	222.66 (88.03)	-10.29 (79.13)	-3.83(-24.64 - 30.03)	5.39(-22.66 - 40.63)	-5.26(-34.14 - 27.62)	-4.44(-37.28 - 30.20)	-1.43(-37.88 - 25.97)	-9.83(-49.54 - 24.50)
Protein (g/day)	94.02 (26.20)	92.44 (18.17)	-1.58 (24.06)	89.73 (17.24)	87.97 (17.01)	-1.76 (19.35)	94.42 (17.72)	87.24 (17.89)	-7.18 (21.15)	-1.2(-9.48 - 8.68)	-5.43(-12.26 - 2.87)	5.7(-2.15 - 12.55)	0.06(-6.81 - 8.27)	6.9(-3.62 - 14.82)	5.49(-3.32 - 14.17)
Total fat (g/day)	93.47 (27.83)	95.34 (19.04)	1.87 (27.73)	91.41 (25.82)	100.51 (23.24)	9.10 (26.52)	98.63 (27.77)	85.52 (27.46)	-13.12 (26.41)	-4.93(-16.50 - 6.17)	-8.72(-18.80 - 4.35)	**11.39(0.12 - 19.53)**	**13.35(4.06 - 25.93)**	**16.32(3.96 - 26.02)**	**22.07(10.75 - 33.68)**
Saturated fatty acids (g/day)	23.64 (9.16)	21.75 (6.15)	-1.89 (7.31)	23.56 (7.79)	21.78 (7.29)	-1.78 (7.35)	25.63 (9.96)	20.43 (7.25)	-5.19 (9.54)	-1.83(-5.89 - 1.92)	-2.23(-5.90 - 1.77)	1.66(-1.44 - 4.06)	0.8(-1.80 - 4.50)	3.49(-0.18 - 6.78)	3.03(-0.23 - 7.06)
Monounsaturated fatty acids (g/day)	46.50 (14.14)	51.09 (10.25)	4.59 (14.04)	45.05 (12.77)	51.01 (11.97)	5.96 (13.57)	49.92 (15.02)	43.60 (15.42)	-6.32 (15.01)	-3.48(-9.37 - 2.54)	-5.82(-10.86 - 1.13)	**8.37(2.10 - 12.87)**	**6.8(1.49 - 13.32)**	**11.85(4.97 - 16.84)**	**12.62(6.10 - 18.44)**
Polyunsaturated fatty acids (g/day)	15.06 (6.23)	14.10 (4.00)	-0.95 (6.53)	14.42 (5.77)	18.26 (5.41)	3.84 (6.82)	14.43 (4.43)	13.39 (6.12)	-1.03 (5.90)	0.81(-1.56 - 2.82)	-0.43(-2.26 - 2.25)	0.96(-1.42 - 2.84)	**4.53(2.38 - 7.36)**	0.15(-2.45 - 2.61)	**4.96(2.10 - 7.66)**
Meat and meat products (g/day)	133.79 (55.41)	131.78 (51.14)	-2.01 (52.96)	119.91 (42.71)	108.36 (49.94)	-11.55 (43.42)	141.02 (51.90)	123.57 (47.50)	-17.45 (58.71)	-7.91(-29.09 - 14.63)	-19.49(-41.53 - (-0.70))	9.28(-11.88 - 28.31)	-16.28(-36.37 - 5.95)	17.18(-7.40 - 38.28)	3.21(-16.19 - 27.99)
Fish (g/day)	107.68 (54.42)	115.20 (47.25)	7.52 (53.25)	94.00 (41.86)	104.78 (41.22)	10.77 (51.73)	109.67 (41.01)	116.32 (40.21)	6.64 (38.75)	-1.38(-21.57 - 17.57)	-16.83(-33.63 - 2.29)	1.19(-18.96 - 16.72)	-10.38(-29.20 - 6.11)	2.56(-18.03 - 19.78)	6.46(-15.94 - 24.20)
Vegetables (g/day)	385.05 (168.52)	373.10 (165.82)	-11.95 (157.29)	329.74 (151.87)	315.91 (123.08)	-13.83 (146.35)	372.41 (127.72)	374.83 (150.55)	2.42 (152.86)	14.99(-48.09 - 73.37)	-58.4(-103.94 - 18.61)	-2.87(-66.20 - 62.73)	-64.55(-117.96 - 0.13)	-17.86(-77.57 - 48.83)	-6.14(-80.93 - 48.43)
Cereals without potato (g/day)	139.23 (79.12)	129.76 (81.64)	-9.47 (107.09)	141.27 (83.67)	138.12 (94.28)	-3.14 (109.34)	124.97 (57.18)	139.13 (91.98)	14.16 (97.45)	8.47(-13.77 - 42.28)	13.06(-15.32 - 47.90)	-8.72(-44.91 - 26.16)	0.14(-41.39 - 39.38)	-17.19(-65.31 - 18.05)	-12.92(-62.40 - 27.80)
Dairy products (g/day)	369.50 (250.63)	361.32 (233.26)	-8.18 (179.79)	395.69 (228.52)	324.56 (212.02)	-71.13 (199.05)	383.07 (237.47)	280.97 (154.20)	-102.10 (163.04)	-20.11(-113.01 - 85.88)	6.5(-88.13 - 113.38)	**81.23(0.13 - 160.58)**	37.15(-37.94 - 125.13)	**101.34(24.06 - 163.78)**	30.65(-48.59 - 110.53)
Nuts (g/day)	8.63 (10.82)	6.85 (8.26)	-1.78 (11.74)	10.23 (11.45)	27.42 (9.98)	17.19 (15.80)	7.74 (9.67)	5.71 (9.62)	-2.02 (12.20)	1.36(-3.28 - 5.07)	2.23(-2.14 - 7.12)	0.96(-2.53 - 4.80)	**21.07(17.45 - 25.96)**	-0.4(-4.64 - 5.13)	**18.84(13.02 - 25.40)**
Fruit (g/day)	390.19 (163.96)	429.95 (233.09)	39.76 (242.56)	374.75 (174.70)	407.48 (190.65)	32.73 (182.42)	464.25 (228.36)	435.95 (296.14)	-28.30 (282.25)	-88.95(-155.66 - 7.55)	**-90.41(-176.58 - (-2.42))**	-35.97(-115.17 - 103.17)	-33.09(-134.54 - 77.61)	52.98(-39.54 - 175.66)	57.32(-40.19 - 162.26)
Legumes (g/day)	24.54 (13.75)	26.13 (12.89)	1.60 (18.07)	22.99 (11.51)	24.94 (10.47)	1.95 (14.03)	21.66 (9.18)	22.20 (14.48)	0.53 (13.91)	1.94(-1.87 - 7.61)	1.13(-3.23 - 5.88)	3.46(-1.67 - 9.54)	2.83(-2.66 - 8.14)	1.53(-5.49 - 7.61)	1.7(-4.64 - 7.47)
Virgin olive oil (g/day)	18.06 (18.95)	51.84 (13.22)	33.78 (21.59)	16.23 (19.00)	28.17 (22.01)	11.94 (19.55)	22.78 (24.50)	23.87 (21.46)	1.09 (20.73)	-3.41(-13.69 - 4.26)	-6.85(-15.95 - 2.84)	**29.14(20.63 - 35.31)**	4.53(-5.12 - 13.73)	**32.55(24.06 - 41.31)**	**11.38(2.15 - 19.56)**
Sunflower oil (g/day)	1.98 (5.25)	0.00 (0.00)	-1.98 (5.25)	0.95 (4.07)	1.03 (4.19)	0.08 (5.86)	0.99 (3.82)	2.09 (6.45)	1.10 (4.75)	1.05(-0.88 - 2.85)	-0.19(-1.76 - 1.67)	**-1.97(-4.01 - (-0.18))**	-1.22(-3.39 - 1.25)	**-3.01(-5.12 - (-1.04))**	-1.04(-3.36 - 1.31)
Fiber (g/day)	27.21 (8.71)	26.85 (7.66)	-0.36 (10.68)	26.56 (8.45)	27.44 (8.58)	0.89 (9.72)	27.10 (9.10)	26.28 (9.86)	-0.82 (10.23)	-0.76(-3.52 - 3.74)	-1.19(-4.34 - 3.25)	-0.33(-3.05 - 4.19)	0.68(-2.82 - 5.14)	0.43(-3.80 - 4.72)	1.87(-2.61 - 6.02)
Alcohol (g/day)	6.96 (11.43)	6.77 (11.15)	-0.19 (9.61)	8.81 (10.68)	8.28 (11.24)	-0.53 (11.49)	8.73 (15.58)	9.84 (14.31)	1.11 (11.52)	-1.44(-7.37 - 3.84)	-0.62(-5.61 - 5.79)	-2.68(-8.32 - 2.18)	-2.34(-7.07 - 3.96)	-1.24(-5.65 - 3.04)	-1.72(-6.63 - 3.34)

Bold letters indicate confidence intervals excluding zero.
MedDiet-EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet-Nuts, Mediterranean diet supplemented with nuts; Control. BMI, body mass index; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; MedDiet, Mediterranean diet.

Supplementary table 3.- Mean and standard deviation in the consumption of key food items and dietary parameters of PREDIMED-Plus at baseline, 12 months and 12-month change. Mean of differences and confidence intervals from Student-T test comparison between groups at baseline, 12 months and 12-month change are presented.

Component	Er-MedDiet baseline	Er-MedDiet 12 months	Er-MedDiet 12 month-change	MedDiet control group baseline	MedDiet control group 12 months	MedDiet control group 12 month-change	Baseline Er-MedDiet - MedDiet control	12 months Er-MedDiet - MedDiet control	12 month - change Er-MedDiet - MedDiet control
Energy intake (kcal/day)	2377.07 (498.48)	2252.18 (344.96)	-124.89 (434.63)	2382.13 (506.34)	2280.29 (481.16)	-101.84 (469.87)	-5.06(-225.89 - 215.78)	-28.11(-182.96 - 131.20)	-23.05(-221.98 - 175.88)
Carbohydrates (g/day)	222.64 (67.08)	204.87 (39.67)	-17.77 (58.32)	225.32 (65.59)	206.53 (57.43)	-18.79 (61.52)	-2.68(-31.84 - 26.48)	-1.66(-19.64 - 18.30)	1.03(-25.32 - 27.37)
Protein (g/day)	98.61 (18.26)	107.81 (15.14)	9.20 (18.22)	99.05 (17.59)	102.65 (16.31)	3.59 (19.67)	-0.45(-8.33 - 7.43)	5.16(-1.16 - 11.77)	5.6(-2.73 - 13.94)
Total fat (g/day)	111.32 (23.00)	106.75 (19.97)	-4.58 (25.10)	112.20 (24.30)	110.92 (25.04)	-1.28 (23.14)	-0.88(-11.27 - 9.52)	-4.17(-12.70 - 5.13)	-3.29(-13.90 - 7.32)
Saturated fatty acids (g/day)	29.18 (8.68)	23.11 (5.95)	-6.06 (8.39)	28.25 (8.73)	24.50 (6.98)	-3.75 (7.80)	0.92(-2.90 - 4.75)	-1.39(-4.26 - 0.84)	-2.31(-5.88 - 1.25)
Monounsaturated fatty acids (g/day)	57.94 (10.38)	60.09 (11.23)	2.15 (12.94)	58.96 (12.00)	63.31 (17.15)	4.35 (14.00)	-1.02(-5.95 - 3.91)	-3.22(-8.24 - 3.06)	-2.19(-8.12 - 3.73)
Polyunsaturated fatty acids (g/day)	17.65 (4.92)	19.92 (5.02)	2.27 (6.40)	17.97 (5.06)	20.97 (5.73)	3.00 (5.60)	-0.32(-2.51 - 1.87)	-1.05(-3.19 - 1.31)	-0.73(-3.37 - 1.91)
Meat and meat products (g/day)	151.47 (47.75)	161.99 (31.79)	10.52 (51.34)	163.97 (46.65)	148.01 (41.42)	-15.96 (51.30)	-12.5(-33.25 - 8.24)	13.98(0.82 - 32.51)	26.48(3.92 - 49.04)
Fish (g/day)	120.67 (44.40)	131.64 (40.09)	10.97 (51.65)	109.25 (41.91)	120.09 (43.16)	10.84 (39.66)	11.42(-7.56 - 30.39)	11.56(-9.92 - 23.98)	0.14(-20.10 - 20.38)
Vegetables (g/day)	370.23 (119.78)	400.16 (123.66)	29.93 (145.50)	329.00 (123.08)	374.78 (95.77)	45.78 (130.57)	41.23(-12.15 - 94.60)	25.38(-33.09 - 61.84)	-15.85(-76.61 - 44.91)
Cereals (g/day)	112.49 (50.62)	126.97 (42.87)	14.48 (61.18)	131.02 (66.36)	126.52 (64.03)	-4.50 (58.37)	-18.53(-44.47 - 7.41)	0.45(-13.73 - 29.93)	18.98(-7.30 - 45.26)
Dairy products (g/day)	351.19 (183.73)	392.46 (171.21)	41.27 (226.91)	316.77 (174.38)	358.16 (202.35)	41.39 (258.16)	34.42(-44.31 - 113.15)	34.3(-52.76 - 112.62)	-0.12(-106.94 - 106.70)
Nuts (g/day)	13.51 (12.38)	32.30 (17.13)	18.79 (20.75)	15.41 (16.58)	38.71 (23.06)	23.30 (22.46)	-1.9(-8.33 - 4.53)	-6.41(-14.38 - 2.97)	-4.51(-14.01 - 4.99)
Fruit (g/day)	355.11 (170.72)	322.43 (123.42)	-32.68 (173.39)	401.37 (149.57)	386.87 (119.98)	-14.50 (163.34)	-46.27(-116.81 - 24.28)	-64.45(-105.18 - (-1.80))	-18.18(-92.22 - 55.85)
Legumes (g/day)	19.42 (8.15)	24.93 (9.63)	5.52 (11.57)	23.92 (9.96)	24.85 (8.65)	0.93 (13.42)	-4.5(-8.50 - (-0.50))	0.08(-3.82 - 4.52)	4.59(-0.92 - 10.09)
Virgin olive oil (g/day)	33.73 (18.74)	43.54 (13.00)	9.81 (16.72)	33.52 (20.40)	44.15 (11.40)	10.63 (19.67)	0.21(-8.40 - 8.82)	-0.61(-5.58 - 4.25)	-0.82(-8.84 - 7.20)
Fiber (g/day)	48.20 (9.20)	47.37 (9.21)	-0.83 (13.39)	49.46 (8.23)	47.65 (8.10)	-1.81 (11.71)	-1.25(-5.09 - 2.58)	-0.28(-4.18 - 3.51)	0.98(-4.55 - 6.50)
Alcohol (g/day)	25.59 (6.40)	31.35 (6.34)	5.76 (7.53)	26.41 (5.94)	30.79 (5.34)	4.38 (6.68)	-0.83(-3.54 - 1.89)	0.56(-1.69 - 3.27)	1.38(-1.75 - 4.51)

Bold letters indicate confidence intervals excluding zero. MedDiet, Mediterranean diet; Er-MedDiet, energy-reduced Mediterranean diet.

Supplementary table 4. Cardiovascular risk factors stratified per group of intervention of PREDIMED and PREDIMED-Plus

	PREDIMED				PREDIMED-Plus		
	All participants	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**	**All participants**	**Er-MedDiet**	**MedDiet control group**
Age (years, mean ± SD)	65.82 ± 6.29	65.61 ± 5.49	66.10 ± 6.93	64.73 ± 6.50	65.51 ± 4.66	65.46±5.19	65.56±4.12
Hypertension (n)	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**	**All participants**	**Er-MedDiet**	**MedDiet control group**
No	28	10 (35,71%)	10 (35,71%)	8 (28,57%)	11	6 (54.55%)	5 (45.45%)
Yes	106	39 (36,79%)	29 (27,36%)	38 (35,85%)	71	35 (49.30%)	36 (50.70%)
Diabetes (n)	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**	**All participants**	**Er-MedDiet**	**MedDiet control group**
No	63	20 (31,75%)	18 (28,57%)	25 (39,68%)	55	30 (54.55%)	25 (45.45%)
Si	71	29 (40,85%)	21 (29,58%)	21 (29,58%)	27	11 (40.74%)	16 (59.26%)
Dyslipidemia* (n)	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**	**All participants**	**Er-MedDiet**	**MedDiet control group**
No	56	20 (40.82%)	18 (47.37%)	18 (40.91%)	14	8 (57,14%)	6 (42,86%)
Si	75	29 (59.18%)	20 (52.63%)	26 (59.09%)	68	33 (48,53%)	35 (51,47%)
Tobacco use (n)	**All participants**	**MedDiet-EVOO**	**MedDiet-Nuts**	**Control**	**All participants**	**Er-MedDiet**	**MedDiet control group**
Current smoker	20	9 (45,00%)	6 (30,00%)	5 (25,00%)	7	1 (14.29%)	6 (85.71%)
Former smoker	38	9 (23,68%)	14 (36,84%)	15 (39,47%)	38	20 (52.63%)	18 (47.36%)
Never smoker	76	31 (40,79%)	19 (25,00%)	26 (34,21%)	37	20 (45.95%)	17 (54.05%)

SD, standard deviation; MedDiet, Mediterranean diet; EVOO, extra-virgin olive oil; Er-MedDiet, energy-reduced Mediterranean diet. Dyslipidemia is defined by meeting any of the following criteria: HDL-c < 40 mg/dL or 50 mg/dL (for men and women respectively), LDL-c > 200 mg/dL, triglycerides > 150 mg/dL. * Three individuals were discarded for missing values

Supplementary table 4. Student-T test comparison between baseline and 12 months gene expression (ΔCt) values (p-values) of PREDIMED.
Student-T test comparison of log_2FC values (p-values) between groups of PREDIMED.
Interaction term group:time (p-value) from the mixed-effects model adjusted for sex, age, and weight

Gene	Baseline to 12 months comparison				MedDiet vs Control comparison			Linear mixed-effects model time:group term (p-value)		
	MedDiet-EVOO	MedDiet-Nuts	Control	MedDiets combined	MedDiet-EVOO - Control	MedDiet-Nuts - Control	MedDiets combined vs Control	MedDiet-EVOO vs Control	MedDiet-Nuts vs Control	MedDiets combined vs Control
ABCA1	**0,016**	**0,026**	0,826	**<0.001**	0,167	0,139	0,461	0,179	0,125	0,097
ABCG1	0,323	0,120	0,299	0,065	0,156	0,064	0,641	0,163	**0,038**	**0,048**
CAV1	0,477	0,313	0,578	0,217	0,982	0,740	0,811	0,981	0,724	0,862
NR1H2	0,396	0,186	0,302	**0,017**	0,729	0,093	0,437	0,753	0,056	0,227
NR1H3	0,565	**0,017**	0,838	0,548	0,846	0,056	0,152	0,841	**0,036**	0,319
PPARA	0,560	0,870	0,936	**0,003**	0,655	0,863	0,662	0,811	0,761	0,754
PPARD	0,209	0,052	0,313	0,568	0,123	**0,034**	0,674	0,154	**0,023**	0,036
PPARG	0,497	0,893	0,231	0,643	0,699	0,335	0,947	0,831	0,386	0,555
RXRA	**0,011**	**0,048**	0,113	**0,002**	**0,006**	**0,011**	0,108	**0,009**	**0,006**	**0,002**
RXRB	**0,006**	0,356	0,810	**0,016**	0,067	0,385	0,120	0,088	0,323	0,110
SRB1	0,809	0,064	0,804	0,219	0,734	0,278	0,249	0,786	0,219	0,636

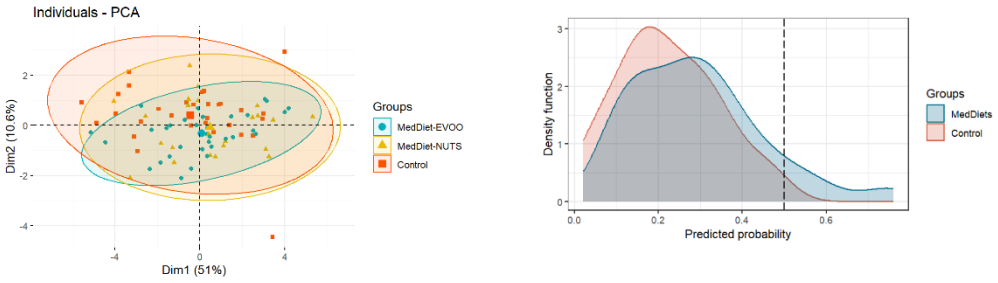
Bold letters indicate statistically significant
MedDiet-EVOO, Mediterranean diet supplemented with extra-virgin olive oil; MedDiet-Nuts, Mediterranean diet supplemented with nuts; Control; MedDiet, Mediterranean diet.

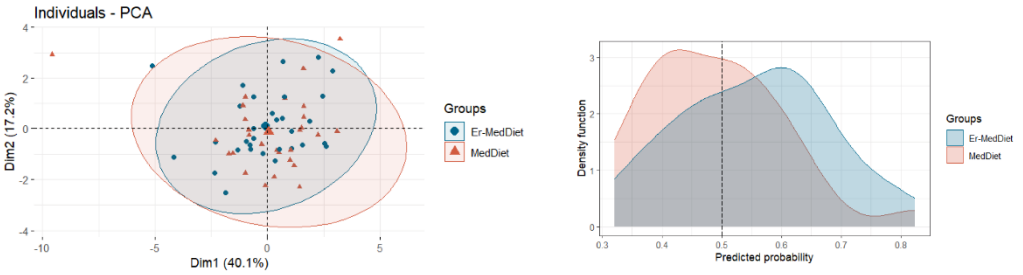
Supplementary table 6 and 7. Cross-validated table for classification of PREDIMED groups using MedDiets combined, and PREDIMED-PLUS. The rows of the table show the actual groups to which individuals belong, while the columns display the predicted groups.

PREDIMED	Control	MedDiets
Control	11	19
MedDiets	12	44

PREDIMED-Plus	MedDiet	Er-MedDiet
MedDiet	7	21
Er-MedDiet	19	12

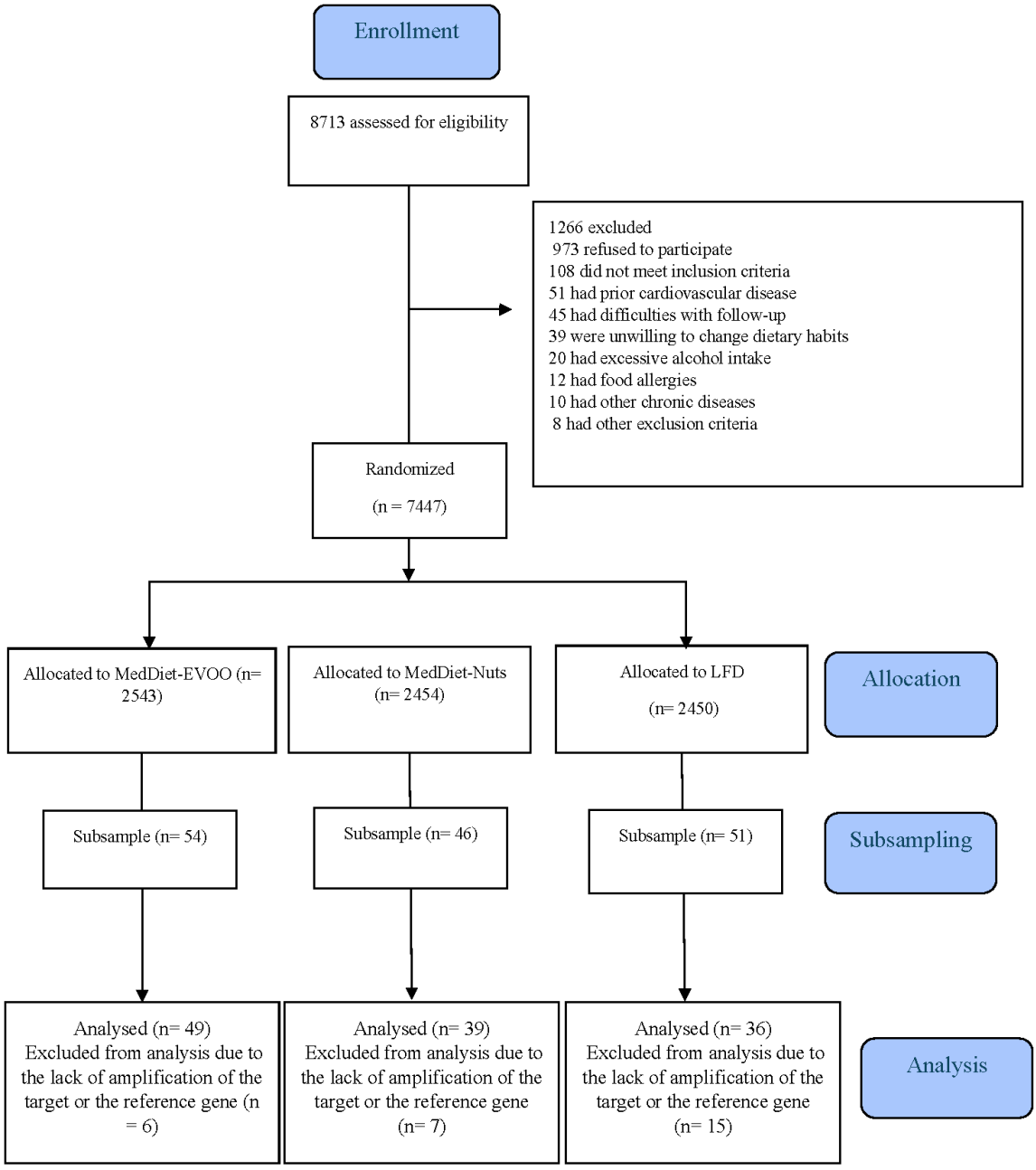
Image S2 and S3. Principal component analysis and linear discriminant analysis of the mean ΔΔCt values per group of PREDIMED. Principal components analysis of the mean ΔΔCt values per group of PREDIMED-PLUS along the density function plot correspondent to PREDIMED-PLUS





Supplementary material

Supplementary Figure 1. Flowchart of participant enrollment, randomization, and analysis in the PREDIMED



Supplementary Table S1. Baseline characteristics of the subsample participants of PREDIMED (N = 134) and comparison with the correspondent baseline characteristics of the complete PREDIMED Study (N=7237). Values are expressed as a percentage (for categorical variables), mean (standard deviation). Chi-Square test was performed for categorical variables and Student-T test for quantitative continuous variables.

	Sample (N = 134)	PREDIMED (N = 7313)	Confidence intervals
Sex (%women)	67 (50.0%)	4215 (57.6%)	(-0.001-0.012)
Age	65.8 (6.29)	67.0 (6.20)	(-2.291-(-0.117))
Weight (kg)	80.2 (12.6)	76.7 (11.9)	(1.280-5.628)
BMI (kg/m^2)	30.2 (3.67)	30.0 (3.85)	(-0.400-0.867)
Waist circumference (cm)	102 (9.48)	100 (10.3)	(0.125-3.424)
Hypertension (% hypertensive)	106 (79.1%)	6057 (82.8%)	(-0.004-0.013)
Diabetes (% diabetic)	71 (53.0%)	3561 (48.7%)	(-0.009-0.003)
Dyslipidemia (%dyslipidemic)	75 (57.3%)	3316 (48.0%)	(-0.013-0.000)
Adherence to MedDiet (14-point item score)	8.72 (1.91)	5.67 (4.40)	(2.713-3.397)
Physical activity (MET·min/week)	1906 (1670)	1608 (1675)	(10.777-586.495)
Smoking status			(0.095-0.223)
Former smoker	20 (14.92%)	1027 (14.04%)	
Current smoker	38 (28.35%)	1799 (24.6%)	
Never smoker	76 (56.71%)	4487 (61.35%)	

SD, standard deviation; MedDiet, Mediterranean diet; EVOO, extra-virgin olive oil; Dyslipidemia is defined by meeting any of the following criteria: HDL-c < 40 mg/dL or 50 mg/dL (for men and women respectively), LDL-c > 200 mg/dL, triglycerides > 150 mg/dL. * Three individuals were discarded for missing values

Supplementary table S2. Mean and standard deviation in the consumption of key food items and dietary parameters of PREDIMED at baseline, 12 months and 12-month change. Mean of differences and confidence intervals from Student-T test comparison between groups at baseline, 12 months and 12-month change are presented. Mean of differences and confidence intervals from Student-T test comparison between groups at baseline and 12-month follow-up are presented.

	MedDiet-EVOO			MedDiet-Nuts			Control			Baseline comparison to control		12 months comparison to control		12-month change comparison to control	
	Baseline	12 months	12-month change	Baseline	12 months	12-month change	Baseline	12 months	12-month change	MedDiet-EVOO	MedDiet-Nuts	MedDiet-EVOO	MedDiet-Nuts	MedDiet-EVOO	MedDiet-Nuts
Energy intake (kcal/day)	2208.62 (524.79)	2152.81 (412.71)	-55.81 (570.03)	2211.03 (525.85)	2190.95 (476.61)	-20.08 (596.64)	2258.26 (501.67)	2078.13 (586.84)	-180.13 (560.66)	-74.62 (-258.78 - 159.50)	-82.97 (-270.30 - 175.83)	85.5 (-133.67 - 283.04)	86.3(-116.66 - 342.30)	160.12 (-106.12 - 354.75)	169.27 (-91.42 - 411.53)
Carbohydrates (g/day)	235.64 (62.64)	219.39 (59.46)	-16.25 (77.39)	241.93 (74.84)	219.12 (68.17)	-22.81 (90.36)	232.95 (70.87)	222.66 (88.03)	-10.29 (79.13)	-3.83 (-24.64 - 30.03)	5.39 (-22.66 - 40.63)	-5.26 (-34.14 - 27.62)	-4.44 (-37.28 - 30.20)	-1.43 (-37.88 - 25.97)	-9.83 (-49.54 - 24.50)
Protein (g/day)	94.02 (26.20)	92.44 (18.17)	-1.58 (24.06)	89.73 (17.24)	87.97 (17.01)	-1.76 (19.35)	94.42 (17.72)	87.24 (17.89)	-7.18 (21.15)	-1.2 (-9.48 - 8.68)	-5.43 (-12.26 - 2.87)	5.7 (-2.15 - 12.55)	0.06 (-6.81 - 8.27)	6.9 (-3.62 - 14.82)	5.49 (-3.32 - 14.17)
Total fat (g/day)	93.47 (27.83)	95.34 (19.04)	1.87 (27.73)	91.41 (25.82)	100.51 (23.24)	9.10 (26.52)	98.63 (27.77)	85.52 (27.46)	-13.12 (26.41)	-4.93 (-16.50 - 6.17)	-8.72 (-18.80 - 4.35)	**11.39 (0.12 - 19.53)**	**13.35 (4.06 - 25.93)**	**16.32 (3.96 - 26.02)**	**22.07 (10.75 - 33.68)**
Saturated fatty acids (g/day)	23.64 (9.16)	21.75 (6.15)	-1.89 (7.31)	23.56 (7.79)	21.78 (7.29)	-1.78 (7.35)	25.63 (9.96)	20.43 (7.25)	-5.19 (9.54)	-1.83 (-5.89 - 1.92)	-2.23 (-5.90 - 1.77)	1.66 (-1.44 - 4.06)	0.8 (-1.80 - 4.50)	3.49 (-0.18 - 6.78)	3.03 (-0.23 - 7.06)
Moncounsaturated fatty acids (g/day)	46.50 (14.14)	51.09 (10.25)	4.59 (14.04)	45.05 (12.77)	51.01 (11.97)	5.96 (13.57)	49.92 (15.02)	43.60 (15.42)	-6.32 (15.01)	-3.48(-9.37 - 2.54)	-5.82 (-10.86 - 1.13)	**8.37 (2.10 - 12.87)**	**6.8 (1.49 - 13.32)**	**11.85 (4.97 - 16.84)**	**12.62 (6.10 - 18.44)**
Polyunsaturated fatty acids (g/day)	15.06 (6.23)	14.10 (4.00)	-0.95 (6.53)	14.42 (5.77)	18.26 (5.41)	3.84 (6.82)	14.43 (4.43)	13.39 (6.12)	-1.03 (5.90)	0.81 (-1.56 - 2.82)	-0.43 (-2.26 - 2.25)	0.96 (-1.42 - 2.84)	**4.53 (2.38 - 7.36)**	0.15 (-2.45 - 2.61)	**4.96 (2.10 - 7.66)**
Meat and meat products (g/day)	133.79 (55.41)	131.78 (51.14)	-2.01 (52.96)	119.91 (42.71)	108.36 (49.94)	-11.55 (43.42)	141.02 (51.90)	123.57 (47.50)	-17.45 (58.71)	-7.91 (-29.09 - 14.63)	-19.49 (-41.53 - (-0.70))	9.28 (-11.88 - 28.31)	-16.28 (-36.37 - 5.95)	17.18 (-7.40 - 38.28)	3.21 (-16.19 - 27.99)
Fish (g/day)	107.68 (54.42)	115.20 (47.25)	7.52 (53.25)	94.00 (41.86)	104.78 (41.22)	10.77 (51.73)	109.67 (41.01)	116.32 (40.21)	6.64 (38.75)	-1.38 (-21.57 - 17.57)	-16.83 (-33.63 - 2.29)	1.19 (-18.96 - 16.72)	-10.38 (-29.20 - 6.11)	2.56 (-18.03 - 19.78)	6.46 (-15.94 - 24.20)
Vegetables (g/day)	385.05 (168.52)	373.10 (165.82)	-11.95 (157.29)	329.74 (151.87)	315.91 (123.08)	-13.83 (146.35)	372.41 (127.72)	374.83 (150.55)	2.42 (152.86)	14.99 (-48.09 - 73.37)	-58.4 (-103.94 - 18.61)	-2.87 (-66.20 - 62.73)	-64.55 (-117.96 - 0.13)	-17.86 (-77.57 - 48.83)	-6.14 (-80.93 - 48.43)
Cereals without potato (g/day)	139.23 (79.12)	129.76 (81.64)	-9.47 (107.09)	141.27 (83.67)	138.12 (94.28)	-3.14 (109.34)	124.97 (57.18)	139.13 (91.98)	14.16 (97.45)	8.47 (-13.77 - 42.28)	13.06 (-15.32 - 47.90)	-8.72 (-44.91 - 26.16)	0.14 (-41.39 - 39.38)	-17.19 (-65.31 - 18.05)	-12.92 (-62.40 - 27.80)
Dairy products (g/day)	369.50 (250.63)	361.32 (233.26)	-8.18 (179.79)	395.69 (228.52)	324.56 (212.02)	-71.13 (199.05)	383.07 (237.47)	280.97 (154.20)	-102.10 (163.04)	-20.11 (-113.01 - 85.88)	6.5 (-88.13 - 113.38)	**81.23 (0.13 - 160.58)**	37.15 (-37.94 - 125.13)	**101.34 (24.06 - 163.78)**	30.65 (-48.59 - 110.53)
Nuts (g/day)	8.63 (10.82)	6.85 (8.26)	-1.78 (11.74)	10.23 (11.45)	27.42 (9.98)	17.19 (15.80)	7.74 (9.67)	5.71 (9.62)	-2.02 (12.20)	1.36 (-3.28 - 5.07)	2.23 (-2.14 - 7.12)	0.96 (-2.53 - 4.80)	**21.07 (17.45 - 25.96)**	-0.4 (-4.64 - 5.13)	**18.84 (13.02 - 25.40)**
Fruit (g/day)	390.19 (163.96)	429.95 (233.09)	39.76 (242.56)	374.75 (174.70)	407.48 (190.65)	32.73 (182.42)	464.25 (228.36)	435.95 (296.14)	-28.30 (282.25)	-88.95 (-155.66 - 7.55)	**-90.41 (-176.58 - (-2.42))**	-35.97 (-115.17 - 103.17)	-33.09 (-134.54 - 77.61)	52.98 (-39.54 - 175.66)	57.32 (-40.19 - 162.26)
Legumes (g/day)	24.54 (13.75)	26.13 (12.89)	1.60 (18.07)	22.99 (11.51)	24.94 (10.47)	1.95 (14.03)	21.66 (9.18)	22.20 (14.48)	0.53 (13.91)	1.94 (-1.87 - 7.61)	1.13 (-3.23 - 5.88)	3.46 (-1.67 - 9.54)	2.83 (-2.66 - 8.14)	1.53 (-5.49 - 7.61)	1.7 (-4.64 - 7.47)
Virgin olive oil (g/day)	18.06 (18.95)	51.84 (13.22)	33.78 (21.59)	16.23 (19.00)	28.17 (22.01)	11.94 (19.55)	22.78 (24.50)	23.87 (21.46)	1.09 (20.73)	-3.41 (-13.69 - 4.26)	-6.85 (-15.95 - 2.84)	**29.14 (20.63 - 35.31)**	4.53 (-5.12 - 13.73)	**32.55 (24.06 - 41.31)**	**11.38 (2.15 - 19.56)**
Sunflower oil (g/day)	1.98 (5.25)	0.00 (0.00)	-1.98 (5.25)	0.95 (4.07)	1.03 (4.19)	0.08 (5.86)	0.99 (3.82)	2.09 (6.45)	1.10 (4.75)	1.05 (-0.88 - 2.85)	-0.19 (-1.76 - 1.67)	**-1.97 (-4.01 - (-0.18))**	-1.22 (-3.39 - 1.25)	**-3.01 (-5.12 - (-1.04))**	-1.04 (-3.36 - 1.31)
Fiber (g/day)	27.21 (8.71)	26.85 (7.66)	-0.36 (10.68)	26.56 (8.45)	27.44 (8.58)	0.89 (9.72)	27.10 (9.10)	26.28 (9.86)	-0.82 (10.23)	-0.76 (-3.52 - 3.74)	-1.19 (-4.34 - 3.25)	-0.33 (-3.05 - 4.19)	0.68 (-2.82 - 5.14)	0.43 (-3.80 - 4.72)	1.87 (-2.61 - 6.02)
Alcohol (g/day)	6.96 (11.43)	6.77 (11.15)	-0.19 (9.61)	8.81 (10.68)	8.28 (11.24)	-0.53 (11.49)	8.73 (15.58)	9.84 (14.31)	1.11 (11.52)	-1.44 (-7.37 - 3.84)	-0.62 (-5.61 - 5.79)	-2.68 (-8.32 - 2.18)	-2.34 (-7.07 - 3.96)	-1.24 (-5.65 - 3.04)	-1.72 (-6.63 - 3.34)

Supplementary table 3. Student-T test comparison between baseline and 12 months gene expression (ΔCt) values (95% confidence interval).
Student-T test comparison of log_2FC values (95% confidence interval) between MedDiet-EVOO, MedDiet-Nuts and both combined against control.
Interaction term group:time (p-value) from the mixed-effects models:

- Model 1: adjusted for time, group, age, sex and education level
- Model 2: adjusted for time, group, age, sex, bmi, diabetes, dyslipidemia, physical activity, hypertension, smoking status, education level

Gene	Baseline to 12 months comparison			MedDiet vs Control comparison			Linear mixed-effects model time:group term (p-value)			
							Model 1		Model 2	
	MedDiet-EVOO	MedDiet-Nuts	Control	MedDiet-EVOO - Control	MedDiet-Nuts - Control	MedDiets combined vs Control	MedDiet-EVOO vs Control	MedDiet-Nuts vs Control	MedDiet-EVOO vs Control	MedDiet-Nuts vs Control
cd86	(-0.2019 - 0.0505)	(-0.1814 - 0.2317)	(-0.3318 - 0.0585)	(-0.169 - 0.291)	(-0.118 - 0.442)	(-0.118 - 0.33)	0,578	0,199	0,428	0,162
cdkn2a	**(-0.3591 – (-0.0118))**	(-0.0903 - 0.2726)	(-0.1866 - 0.1887)	(-0.439 - 0.066)	(-0.167 - 0.347)	(-0.287 - 0.162)	0,106	0,584	0,235	0,325
ifng	**(-0.6584 – (-0.1341))**	(-0.3522 - 0.1949)	(-0.3996 - 0.1718)	(-0.665 - 0.1)	(-0.354 - 0.425)	(-0.483 - 0.196)	0,114	0,98	0,139	0,920
il10	(-0.1633 - 0.2023)	(-0.088 - 0.389)	(-0.1864 - 0.2482)	(-0.292 - 0.269)	(-0.198 - 0.437)	(-0.212 - 0.305)	0,901	0,360	0,875	0,256
nampt	(-0.276 - 0.0999)	(-0.1961 - 0.3548)	**(-0.5094 – (-0.1026))**	(-0.055 - 0.491)	**(0.048 - 0.723)**	**(0.037 - 0.547)**	0,167	**0,018**	0,203	**0,023**
nfe2l2	(-0.1543 - 0.1044)	(-0.0503 - 0.3409)	(-0.3083 - 0.1018)	(-0.162 - 0.318)	(-0.031 - 0.528)	(-0.078 - 0.385)	0,534	0,065	0,678	0,132
nlrp3	**(0.0109 - 0.2649)**	(-0.0687 - 0.3644)	(-0.2366 - 0.1865)	(-0.081 - 0.407)	(-0.125 - 0.471)	(-0.072 - 0.407)	0,08	0,083	0,081	0,077
pik3cb	**(0.0368 - 0.2683)**	(-0.015 - 0.3666)	(-0.1943 - 0.1669)	(-0.046 - 0.378)	(-0.069 - 0.448)	(-0.03 - 0.383)	0,152	0,137	0,155	0,171
tgfb2	**(0.2268 - 0.681)**	**(0.0534 - 0.6055)**	(-0.1947 - 0.3727)	**(0.006 - 0.724)**	(-0.149 - 0.63)	(-0.02 - 0.639)	**0,035**	0,213	0,086	0,377

Review: Atherogenic LDL and cardiovascular risk

Current Opinion in Lipidology

Atherogenic LDL and cardiovascular risk

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review

Atherogenic LDL and cardiovascular risk

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Abbreviations: ACS: acute coronary syndrome, CAD: coronary artery disease, CVD: cardiovascular disease, HDL-c: high-density lipoprotein cholesterol, LDL-c: low-density lipoprotein cholesterol, LDL-Ps: LDL particles, MedD: Mediterranean diet, oxLDL: oxidized LDL, RCT: randomized controlled trial, OxPL: oxidized phospholipids, SMC: smooth muscle cell, T2DM: type 2 diabetes mellitus

Keywords: atherogenesis, LDLc, oxidized LDL, cardiovascular disease,

Abstract

LDL cholesterol (LDLc) is a well-established therapeutic target for managing cardiovascular disease (CVD). Despite the benefits of reducing LDLc concentration, cardiovascular risk may persist. Beyond lipoprotein composition, particle count, diameter, electronegativity, and structural modifications, such as oxidation, can contribute to this residual risk. The direct relationship between LDL oxidation and CVDs has been extensively studied over the past few decades, especially in subjects with diabetes and oxidative stress. Specifically, small dense LDL (sdLDL) has been linked to the risk of atherosclerotic CVD across various analytic methods. Nowadays, parameters providing data regarding all lipoprotein subtypes have been increasingly assessed as they are more feasible and comparable. Several short- to long-term follow-up studies have reported that circulating oxidized LDL (oxLDL) could be a predictor of acute coronary syndrome in both the general population and patients with coronary artery disease. Research on biomarkers could potentially yield useful tools for predicting cardiovascular risk .
A complementary panel of different biomarkers related to LDL atherogenicity could potentially enhance global cardiovascular risk prediction.
An atheroprotective diet combined with the promotion of physical activity are appropriate recommendations for improving LDL oxidation status. OxLDL has become a target for immune-modulatory anti-atherosclerosis therapy. Finally, delivering LDL-based nanocarriers holds promise for both imaging atherosclerosis and therapeutics.

Cholesterol LDL, cardiovascular risk and residual risk

Increased cholesterol accumulated in ApoB100 containing lipoproteins (VLDL, IDL, LDL), and low HDL cholesterol (HDLc) levels have been described as cardiovascular risk factors in epidemiological studies. Meanwhile, controversial results exist referring cardioprotective effect of increased HDLc concentration (1).
LDL cholesterol (LDLc) is a well-established therapeutic target for managing CVD. Current guidelines include different statin intensity therapy combined with a non-statin add-on drugs (ezetimibe or PCSK9) under certain circumstances (2). Statins, an inhibitor of hydroxymethyl glutaryl coenzyme A reductase, play a solid role in primary and secondary prevention of

cardiovascular events and mortality. Meta-analyses have shown a 22% reduction in cardiovascular events with significant lowering of LDLc (3). Despite these benefits, residual cardiovascular risk persists, underscoring the need of optimizing lipid management strategies (4) .

Modifications of LDL structure can play a role in residual cardiovascular risk. A systematic review and meta-analysis of 20 randomized controlled trials (RCTs) involving 1,874 patients found that high-dose statins or combinations with ezetimibe reduced circulating oxLDL levels more effectively than low-to-moderate intensity statin therapy alone (4). Interestingly, in the MIRACL study, a placebo-controlled trial involving a large cohort of acute coronary syndrome (ACS) patients, an increase in oxidized phospholipids (OxPL)/apoB levels was observed in response to atorvastatin across subgroups of CVD risk factors and lipoproteins. Previous studies in smaller patient cohorts and experimental animal models have suggested that this increase in OxPL/apoB levels following statin intervention may indicate plaque regression and/or stabilization(5).

Among 10,497 patients with type 2 diabetes mellitus (T2DM) (66.9% with prior CVD) who had mild-to-moderate hypertriglyceridemia and low levels of HDLc and LDLc, those treated with pemafibrate did not experience a lower incidence of cardiovascular events versus those receiving placebo (6). Thus, despite pemafibrate effectively reduces triglyceride, VLDL cholesterol, remnant cholesterol, and Apo C-III levels, a residual risk could still be explained by the LDL atherogenicity. Furthermore, the levels of oxLDL did not differ significantly across concentration categories of cLDL in patients with type 2 diabetes (T2DM) under statin treatment, indicating that the behavior of both parameters is not strictly parallel (7). Furthermore, despite attainment of LDLc <50 mg/dl or non-HDL cholesterol <80 mg/dl, patients with T2DM exhibited an increase in LDL particle levels (>500 nmol/L), suggesting a potential residual coronary heart disease (CHD) risk (8). Hyperglycemia in T2DM exacerbates enteral cholesterol absorption and suppresses LDL receptor (LDLR) expression in the liver. This metabolic environment fosters the formation of small dense LDL (sdLDL) particles, which are particularly atherogenic and contribute to endothelial dysfunction in the microcirculation, independent of oxLDL levels (9).

Atherogenicity of LDL-related biomarkers and cardiovascular risk

Beyond lipoprotein composition (such as LDLc), additional characteristics of lipoproteins could contribute to this residual risk, including protein nature and activity, particle count, diameter, electronegativity (electric net charge), and structural modifications (measured as oxidation, inflammation, resistance against oxidation, glycation, acetylation, immune complexes, aggregation, antibodies against oxLDL) (10).

OxLDL has been related to endothelial dysfunction, monocyte chemotaxis, macrophage polarization, smooth muscle cell (SMC) proliferation and migration, and platelet activation, ultimately leading to plaque instability and rupture (11). Furthermore, oxidatively modified LDL promotes platelet activation and arterial thrombosis through constitutively expressed scavenger receptors (12) .

A systematic review and meta-analysis of 3 observational studies involving 1,060 participants dealing with chronic inflammatory diseases showed increased levels of circulating oxLDL in individuals with CVD (13). Another systematic review highlighted that oxLDL (n=3,271), along with other inflammation-related biomarkers, such as sCD40L (n=2,660) and IL-6 (n=6,196), are linked to an increased relative risk of developing CVD. Additionally, these biomarkers combined can be used to estimate and have prognostic value for cardiovascular disease (14). Recent research underscores the feasibility of using a subset of biomarkers to provide a more accurate assessment of LDL oxidation. Incorporating markers such as ApoB100, non-HDL cholesterol, ultrasensitive C reactive protein (hsCRP), or the number and size of LDL postulate to better determine residual cardiovascular risk (15).

LDL particles (LDL-Ps) can be categorized into various subclasses based on density, size, electric charge, and protein composition. Laboratory methods such as ultracentrifugation, gradient gel

electrophoresis (GGE), high-performance liquid chromatography (HPLC), and nuclear magnetic resonance (NMR) are employed for lipoprotein separation. The density of LDL-Ps ranges from 1.019 to 1.060 g/ml, and ultracentrifugation is a commonly used method for density-based separation. Despite its high resolution, ultracentrifugation has low throughput and may lead to overlapping of LDL subclasses and destructive alterations due to centrifugal forces. In contrast, the vertical auto profile technique allows for the simultaneous identification of different LDL subclasses (16).

In the ^{1}H-NMR spectrum, the terminal methyl protons of lipids in different chemical environments exhibit distinct chemical shifts, which are used to determine LDL subclasses (particle count, size, lipid, and protein mass). Compared to ultracentrifugation and GGE-related methodologies, NMR can analyse samples quickly without inducing destructive alterations and with minor variation among laboratories (17). Therefore, the NMR method, which is time-saving, reproducible, and entail a high throughput application, could be useful for explaining residual risk. It has been observed that patients with a premature cardiovascular event, versus a control group, presented LDL and HDL with smaller and larger diameters (featured by NMR), respectively. Patients with cardiovascular events also have more triglycerides in HDL and LDL, which is an indicator of particle instability (18).

LDL particles exist in various forms, with L1 being the most electropositive and L5 the most electronegative. L1 is recognized and internalized via LDLR through receptor-mediated endocytosis, whereas L5 is internalized through lectin-like oxidized LDL receptor 1 (LOX-1) and the platelet-activating factor receptor, leading to endothelial cell apoptosis. LOX-1, a scavenger receptor abundantly expressed on endothelial cells, plays a crucial role in endothelial dysfunction by facilitating the transcytosis of oxLDL and electronegative LDL to subendothelial space. Given that LOX-1 is a membrane-bound protein with a single C-terminal domain can be cleaved and released as a soluble form in the circulation, thus allowing its measurement (19). In this regard, the development of immunoassays for measuring soluble LOX-1 (sLOX-1) levels has allowed to dig into its potential as a biomarker for CVD. Additionally, myeloperoxidase-oxidized LDL (Mox-LDL) has been shown to upregulate LOX-1 expression in human aortic endothelial cells (20). Electronegative LDL [LDL(-)] exhibits *in vitro* effects including inflammation, apoptosis, cell proliferation, low affinity for LDL receptors, and high affinity for arterial proteoglycans (21). Patients with early atherosclerosis often present with LDL(-) (22).

Autoantibodies against oxidized LDL (oxLDL-Ab) bind to oxidative epitopes, forming immune complexes blocking any interaction with macrophage receptors, thereby they could attenuate atherogenesis. The measurement of oxLDL-Ab indirectly reflects the immunological response to lipid oxidation, which has an inherent inter-individual variability.

The limitations of current measurement methods for quantifying LDL oxidative status include the lack of automated analyser support, time-consuming processes, and large-volume blood sample-processing requirements. Emerging techniques are facilitating the isolation of LDL and the fractionation of its protein and lipid components, including its antioxidant arsenal like carotenoids and tocopherols (23).

Predictive value for cardiovascular diseases of oxidized LDL

Several short- to long-term follow-up studies have reported that circulating oxLDL could be a predictor of ACS in both, the general population (24,25) and CAD patients (26,27). OxLDL measurement by ELISA in 88 men with incident CHD and 258 age- and survey-matched controls predicted acute CHD events (follow-up around 5 years) in apparently healthy, middle-aged men from the general population (24). Similarly, oxLDL was independently associated with 10-year CAD events, but not subclinical atherosclerosis in the general population. It also proved to enhance the reclassification capacity of Framingham-derived CAD risk functions (25). In secondary prevention, in 433 patients with unstable CAD included in the FRISC-II Study, and in 233 of these subjects at follow-up 4-7 weeks later, high levels of oxLDL identified patients at increased risk for future myocardial infarction (26). Furthermore, both oxLDL and Lp(a)

concentrations were independently associated with a poorer prognosis at 6-month follow-up in the multicenter cohort FORTIAM study, which included 1,371 acute myocardial infarction patients admitted within 24 hours of symptom onset (27). In contrast, a recent prospective study involving 105 participants over a follow-up period of approximately 15 years found that 27 individuals (25.8%) experienced 30 cardiovascular outcomes. However, there was no significant difference in the incidence of the primary composite outcome among groups with varying circulating oxLDL levels (28).

In patients with T2DM and CAD (n= 96), elevated circulating oxLDL levels were established as an independent predictor of future cardiac events in a 52 month-follow up, underscoring its role in atherosclerotic progression (29). Modified LDL forms, such as LDL (-), oxLDL, and glycated LDL, are more prevalent in the plasma of individuals with T2DM and accelerate the development of atherosclerotic lesions (30). In general, antidiabetic drugs decrease the uptake of oxLDL by vascular cells, reduce subsequent inflammatory signaling, prevent macrophage infiltration and suppress the oxLDL-induced transformation of macrophages. These properties help to attenuate atherosclerosis in diabetic patients (31).

Levels of sdLDL have been linked to the risk of atherosclerotic CVD across various analytic methods. A direct association between plasma sdLDL cholesterol and incident CHD events in a follow-up of approximately 11 years, was observed in 11,419 participants from the ARIC study using a newly developed homogeneous assay. In addition, genome-wide association analyses identified genetic variants in 8 loci, including PCSK7, which were associated with sdLDL-C levels, providing new insights into the role of this gene in lipid metabolism (32). In a separate case-control study involving Chinese individuals, sdLDL alone was found to efficiently predict CHD risk, similar to the combination of triglycerides, HDLc, and sdLDL(33). Additionally, in a longitudinal study involving 386 patients from China with T2DM followed for 48 months, sdLDL-C levels effectively predicted future CV events (34).

A systematic review and meta-analysis compiled data from prospective studies, including techniques such as GGE, NMR, and the sdLDL-cholesterol assay. This meta-analysis encompassed a total of 30,628 subjects and identified 5,693 incident CHD events associating higher sdLDL and sdLDL cholesterol levels with higher risk of CHD (35).

Regarding the susceptibility of LDL to oxidation, a 5-year case-control study (n=394) reported strong correlation between higher susceptibility with an increase of carotid artery atherosclerosis (36).

Elevated levels of remnant cholesterol, Lp(a), and hs-CRP together exert a synergistic adverse effect on major cardiovascular events among statin-treated patients with chronic coronary syndrome, suggesting that comprehensive assessment of multiple cardiovascular risk factors could enhance risk stratification in the very-high-risk population (37). The Framingham risk score remains the most widely used system for cardiovascular risk assessment. In the European population, the Systematic Coronary Risk Evaluation (SCORE) and the Framingham-REGICOR risk charts are recommended. However, these charts may underestimate risk, particularly in women and young populations (38). Among individuals with low-to-moderate risk algorithms do not accurately predict the risk (39), reflecting gaps in our understanding of pathophysiological mechanisms. Therefore, exploring novel and predictive biomarkers related to LDL oxidation status could potentially enhance global cardiovascular risk prediction.

Effect of lifestyle on LDL oxidation

The Mediterranean Diet (MedD) has demonstrated its ability to decrease LDL atherogenic features, as evidenced by several studies (40). Adherence to this diet, particularly when enriched with virgin olive oil, has been shown to mitigate LDL atherogenicity, improving traits related to oxidation, estimated size, and composition, as well as reducing LDL *ex vivo* cytotoxicity (41). Olive oil, the primary source of fat in the MedD, has been extensively studied for its health benefits. The EUROLIVE Study, a European project, investigated the effects of three types of olive oil varying in phenolic content on blood lipids and oxidative/antioxidative status in healthy

volunteers. This RCT found that olive oils with higher phenolic content effectively reduced *in vivo* lipid oxidative damage, including oxidized LDL, uninduced conjugated dienes, and hydroxy-fatty acids (42). In 2011, the European Food Safety Authority approved a health claim related to phenolic compounds and LDL oxidation, suggesting that daily consumption of 5 mg of hydroxy-tyrosol and its derivatives in olive oil can confer these benefits, easily achievable within a balanced diet (43). Studies comparing dietary effects on LDL oxidation resistance have shown that MUFA-rich diets promote higher resistance to oxidation compared to PUFA-rich diets (44) . Additionally, a meta-analysis of RCT reported a decrease in oxidized LDL levels following consumption of high-phenolic olive oil (45).
Physical activity practice contributes to improve triglyceride and HDLc concentration and promotes a shift from sdLDL particles to larger LDL particles. Finally, recent research also highlights an inverse and linear relationship between vigorous physical activity and circulating levels of oxidized LDL, particularly among men in the general population (46). Future studies should focus on determining the clinical benefits of improving these biomarkers.

Lipoprotein as targeted delivery of drugs and imaging agents and target for drug development
Native nanostructured lipoproteins such as LDL and HDL represent novel platforms for targeted drug delivery and imaging agents. The predominant mode of LDL-based delivery involves the interaction between apolipoprotein B (Apo-B), the primary lipoprotein of LDL, and the LDLR. Recent studies have also highlighted that delivering LDL-based nanocarriers to macrophages via fluid-phase pinocytosis are promising for imaging atherosclerosis (47).
Furthermore, recent advances in synthetic Lox-1 inhibitors and neutralizing antibodies have shown promising results in the reduction of noncalcified atherosclerotic plaque (48). Given that oxLDL serves as a critical autoantigen in atherosclerosis, there is growing interest in immune-modulatory therapies that promote the activity of apoB peptide-specific and regulatory T cells (Tregs) as an approach to prevent or stabilize atherosclerosis process. In this regard, several experimental oxLDL tolerance vaccines have demonstrated promising outcomes in animal models of atherosclerosis (49).

Conclusion
The limitations of current measurement methods for quantifying LDL oxidative status include the lack of automated analyzer, laborious protocols and large-volume samples. Therefore, there is a need for simpler, more accurate, faster, and standardized assays.
Evidence suggests a relationship between detrimental modifications in LDL structure and lipoprotein's atherogenic potential role in the residual cardiovascular risk, especially in pathological conditions like T2DM. However, future studies should focus on determining the clinical benefits of improving these biomarkers. A complementary panel of different biomarkers related to LDL atherogenicity would be relevant, as exploring novel and predictive biomarkers could potentially enhance global cardiovascular risk prediction through current risk equations.
An atheroprotective diet, such as the MedD where olive oil is the primary fat source, is an appropriate recommendation for improving LDL oxidation status. Physical activity should also be recommended as part of lifestyle changes.
Since oxidized LDL is a key autoantigen in atherosclerosis, it has become an interesting target for immune-modulatory therapy aimed at the tailored prevention and stabilization of atherosclerosis. Delivering LDL-based nanocarriers holds promise for both imaging atherosclerosis and therapeutics.

Summary:

- LDL cholesterol (LDLc) is a crucial therapeutic target for managing cardiovascular disease, but residual risk remains despite LDLc reduction.
- Elevated oxidized LDL (oxLDL) levels have demonstrated to predict acute coronary syndromes.
- LDL particles are prone to modifications, which exacerbate residual cardiovascular risk.
- The MedDiet and physical activity are effective approaches to improve LDL oxidation status.
- Emerging research targeting oxLDL receptor and LDL-based nanocarriers delivery can be promising approaches to both treatment and diagnosis of atherosclerosis.

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Conflict of interest

We declare no conflict of interest

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