HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS

Pablo Hernández Alonso

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DOCTORAL THESIS

Thesis supervisors:

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Unitat de Nutrició Humana
Departament de Bioquímica i Biotecnologia
UNIVERSITAT ROVIRA I VIRGILI
Reus, 2016

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Mònica Bulló Bonet, professora Agregada del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili,

CERTIFICO:

que aquest treball, titulat "***Health benefits of pistachio consumption in pre-diabetic subjects***", que presenta el Sr. Pablo Hernández Alonso per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica i Biotecnologia d'aquesta Universitat i que acompleix els requeriments per poder optar a Menció Internacional.

Reus, 26 d'agost de 2016.

La Directora de la Tesis Doctoral,

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ABSTRACT

ENGLISH:

Pre-diabetes and type 2 diabetes (T2D) are considered two major public health problems. Pre-diabetes is a metabolic reversible state between normoglycemia and T2D which is characterized by insulin resistance and it is associated to a higher risk of cardiovascular disease and T2D. As nuts are a rich and complex matrix of different macronutrients and micronutrients and other molecules such as antioxidants, they may have a modulatory role in insulin resistance via different targets. Among nuts, pistachios have a rich nutrient profile in antioxidants, fiber and fats which may lead to an improvement in glucose metabolism.

Our objective was to analyze the effects of pistachio consumption on parameters related to glucose metabolism and insulin resistance. Additionally, we evaluated the lipid profile, inflammatory, oxidative and other related markers, together with *in vitro* lymphocytes' glucose uptake and the expression of certain genes. We also evaluated the modulation of novel disease biomarkers related to glycemic and cardiovascular health such as microRNAs and lipoproteins (size and concentration).

Our results showed that chronic pistachio consumption, into the context of a healthy diet, reduced the insulin resistance status of the subjects with pre-diabetes. Importantly, pistachio consumption was also able to improve inflammatory and satiety markers and a set of genes linked to inflammation and glucose transport, together with *in vitro* lymphocytes' glucose intake. Finally, we reported a significant improvement in microRNAs related to T2D and pre-D and in lipoprotein profile which may reflect a reduced cardiovascular risk.

Overall, pistachio consumption seems effective to ameliorate the pre-diabetes state towards a healthier metabolic profile.

Resum

CATALÀ:

La prediabetis i la diabetis tipus 2 (DT2) són actualment dos problemes greus de salut pública. La prediabetis és un estat metabòlic reversible situat entre la normoglucèmia i la DT2, que es caracteritza per presentar resistència a la insulina i s'associa a un major risc de malaltia cardiovascular i de DT2. Degut a que els fruits secs són una matriu rica i complexa de diferents macronutrients, micronutrients i altres molècules com els antioxidants, poden tenir un paper important en la modulació de la resistència a la insulina mitjançant diferents mecanismes. Entre els fruits secs, els festucs són rics en antioxidants, fibra i lípids que poden conduir a una millora del metabolisme de la glucosa.

El nostre objectiu ha estat analitzar l'efecte del consum de festucs mitjançant paràmetres relacionats amb el metabolisme de la glucosa i la resistència a la insulina. Hem avaluat el perfil lipídic, inflamatori i oxidatiu, altres marcadors relacionats, el consum *in vitro* de glucosa en limfòcits i l'expressió de certs gens. Hem valorat la modulació de nous biomarcadors relacionats amb la glicèmia i amb la salut cardiovascular com ara els microRNAs i les lipoproteïnes (mida i concentració).

Els nostres resultats mostren que el consum crònic de festucs, en el context d'una dieta saludable, redueix l'estat de insulinoresistència en pacients amb prediabetis. A més, el consum de pistatxos és capaç de millorar marcadors d'inflamació i sacietat, l'expressió de gens relacionats amb la inflamació i amb el transport de glucosa, així com també el consum de glucosa en limfòcits *in vitro*. Finalment, s'ha mostrat una millora significativa en microRNAs relacionats amb la DT2 i prediabetis i en el perfil de lipoproteïnes que pot reflectir una disminució del risc cardiovascular.

En conjunt, el consum de festucs sembla efectiu per tal de millorar la prediabetis a un perfil metabòlic més saludable.

CASTELLANO:

La prediabetes y la diabetes tipo 2 (DT2) son actualmente dos importantes problemas de salud pública. La prediabetes es un estado metabólico reversible entre la normoglicemia y la DT2, que se caracteriza por presentar resistencia a la insulina y se asocia a un mayor riesgo cardiovascular y de DT2. Puesto que los frutos secos son una matriz rica y compleja de diferente macronutrientes y micronutrientes y otras moléculas antioxidantes, pueden tener un papel modulador de la resistencia a la insulina mediante diferentes dianas. Entre los frutos secos, los pistachos tienen un perfil de nutrientes rico en antioxidantes, fibra y lípidos que pueden originar una mejora en el metabolismo de la glucosa.

Nuestro objetivo fue analizar el efecto del consumo crónico de pistachos sobre parámetros relacionados con el metabolismo de la glucosa y la resistencia a la insulina. Se evaluó también el perfil lipídico, inflamatorio, oxidativo y de otros marcadores relacionados, además de la captación *in vitro* de glucosa en linfocitos y la expresión de ciertos genes. Evaluamos también la modulación de nuevos biomarcadores relacionados con la glicemia y de salud cardiovascular como microRNAs y lipoproteínas (tamaño y concentración).

Nuestros resultados mostraron que el consumo crónico de pistachos, en el contexto de una dieta saludable, reduce el estado de insulinorresistencia en pacientes con prediabetes. De forma importante, el consumo de pistachos fue capaz de mejorar marcadores de inflamación y saciedad y un conjunto de genes relacionados con la inflamación y el transporte de glucosa, así como con el consumo de glucosa en linfocitos *in vitro*. Finalmente, mostramos una mejora significativa en microRNAs relacionados con DT2 y prediabetes, y en el perfil de lipoproteínas que puede reflejar una disminución en el riesgo cardiovascular.

En conjunto, el consumo de pistachos parece efectivo para mejorar la pre-diabetes hacia un perfil metabólico más saludable.

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ABBREVIATIONS

-C, cholesterol
-P, particle
2-NBDG, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
ACTB, beta-actin
ADA, American Diabetes Association
AHA, American Heart Association
ANCOVA, analysis of covariance
ANOVA, analysis of variance
ARIC, Atherosclerosis Risk in Communities Study
ASC, apoptosis-associated speck-like protein containing caspase recruitment domain
BMI, body mass index
BW, body weight
CD, control diet
CGT, cellular glucose transport
CHD, coronary heart disease
CHO, carbohydrate
CI, confidence interval
Cq or **Ct**, cycle
CRP, c-reactive protein
CV, cardiovascular
CVD, cardiovascular disease
DBP, diastolic blood pressure
DOSY, diffusion-ordered ^{1}H NMR spectroscopy
DPP-4, dipeptidyl peptidase-4
DSTE, double stimulated echo
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay commercial kits
EPIC, European Prospective Investigation into Cancer and Nutrition Study
EPIRDEM, Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus
EVOO, extra-virgin olive oil
FFQ, food frequency questionnaire
FI, fasting insulin
FID, finite impulse decay
FPG, fasting plasma glucose
GD, gestational diabetes
GI, glycemic index
GIP, gastric inhibitory polypeptide
GL, glycemic load
GLP-1, glucagon-like peptide-1
GLP-1RA, GLP-1 receptor agonist
GLUT, glucose transporter
GWAS, genome-wide association study

Abbreviations

HbA_{1c}, glycated hemoglobin
HDL, high-density lipoprotein
HOMA-IR, homeostatic model assessment of insulin resistance
HPFS, Health Professionals' Follow-up Study
HPRT1, hypoxanthine phosphoribosyltransferase-1
HR, hazard ratio
IDF, International Diabetes Federation
IFG, impaired fasting glucose
IGT, impaired glucose tolerance
IHD, ischemic heart disease
IL, interleukin
IR, insulin resistance
IRAS, Insulin Resistance Atherosclerosis Study
IRS-1, insulin receptor substrate-1
ITT, intention-to-treat
JNK-1, c-Jun N-terminal kinase-1
LDL, low-density lipoprotein
LED, longitudinal eddy current delay
LP, lipoprotein
LPS, lipopolysaccharide
MAPK, mitogen-activated protein kinase
MESA, Multi-Ethnic Study of Atherosclerosis Study
MedDiet, Mediterranean Diet
miRNA or **miR**, microRNA
MUFA, monounsaturated fatty acid
NF-kB, nuclear factor kB
NGT, normal glucose tolerance
NHANES, National Health and Examination Nutrition Survey
NHS, Nurses' Health Study
NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases
NLR, nod-like receptors
NLRP3, nucleotide-binding domain and leucine-rich repeat containing NLR-pyrin domain containing 3
NMR, nuclear magnetic resonance
OGTT, oral glucose tolerance test
Ox-LDL, oxidized-LDL
PAI-1, plasminogen activator inhibitor-1
PBS, phosphate buffered saline
PD, pistachio diet
PF-4, platelet factor-4
PHS, Physicians' Health Study
PI3K, phosphoinositide-3-kinase
PLS, partial least-squares
PP, per protocol
Pre-D, pre-diabetes
PREDIMED, PREvención con Dieta MEDiterránea Study

Abbreviations

PRR, pattern-recognition receptors
PUFA, polyunsaturated fatty acids
RCT, randomized controlled trial
RETN, resistin
RPL30, ribosomal protein L30
SBP, systolic blood pressure
SFA, saturated fatty acids
SGLT-2, sodium-glucose cotransporter-2
SLC2A3, solute carrier family 2, facilitated glucose transporter member 3
SLC2A4, solute carrier family 2, facilitated glucose transporter member 4
SNP, single nucleotide polymorphism
sRAGE, soluble receptor for advanced glycation end-products
T1D, type 1 diabetes
T2D, type 2 diabetes
T2D-GENES, type 2 diabetes exploration by next-generation sequencing in multi-ethnic samples
TC, total cholesterol
TF, tissue factor
TLR, toll-like receptor
TNF-α, tumor necrosis factor-α
TE, tris ethylenediaminetetraacetic acid
TXB2, thromboxane B2
USDA, United States Department of Agriculture
VLDL, very low-density lipoprotein
vWF, Von Willebrand factor
YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
WHO, World Health Organization

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A. INTRODUCTION

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A. INTRODUCTION

1. Diabetes and pre-diabetes

1.1 Definition and subtypes

Diabetes is a chronic disease characterized by hyperglycemia, generated when the organism is no longer producing or efficacy using insulin. As a consequence of hyperglycemia, different tissues are damaged with short-, -mid and/or long-term fatal consequences[(1)].

There are different major subtypes of diabetes, namely: type 1 diabetes (T1D), type 2 diabetes (T2D), gestational diabetes (GD) and other types of diabetes including maturity onset diabetes of the young (MODY). T1D is a lifelong (chronic) disease characterized by a failure in β-cells' insulin production. This form of diabetes, which accounts for only 5-10% of total diabetes, previously encompassed by the terms insulin-dependent diabetes or juvenile-onset diabetes, results from a cellular-mediated autoimmune destruction of the β-cells of the pancreas[(2)]. T1D can occur at any age but it is most often diagnosed in children, adolescents, or young adults. MODY is a genetically and clinically heterogeneous group of disorders that results in β-cell dysfunction and which includes inheritable diabetes distinct from T1D and T2D. It is a rare form of diabetes as it accounts only for 1-2% of all diabetes and it is often difficult to distinguish and thus it is misdiagnosed as type 1 or type 2 diabetes[(3)]. On the other hand, GD develops during pregnancy and, like other types of diabetes, it affects how our cells use glucose. Gestational diabetes causes high blood glucose levels that can affect both mother's and fetus' health. However, glucose levels usually return to normal soon after delivery. Despite that, having gestational diabetes increases the risk of developing T2D in the following years[(2)]. Finally, T2D is the most common form of diabetes, and was previously referred as non-insulin-dependent diabetes or adult-onset diabetes. It is characterized by insulin resistance (IR) and usually a relative insulin deficiency and it is commonly associated to obesity or visceral fat accumulation. In these patients, hyperglycemia and hyperinsulinemia develop gradually from the normal glucose tolerance (NGT) situation to T2D, with an intermediate state of glucose intolerance known as pre-diabetes (pre-D), which length depends on different genetic and lifestyle factors. Therefore, pre-D is clinically defined as a non-chronic state of abnormal glucose homeostasis characterized by impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or both[(4,5)].

However, pre-D can be reverted to the NGT state, and for this reason it has received increasingly widespread attention as it is considered a potentially crucial indicator for preventing T2D and cardiovascular diseases (CVD)[6].

1.2 Worldwide epidemiology of diabetes and pre-diabetes

A dramatic increase in the prevalence and incidence of T2D have occurred in many parts of the world, especially in the newly industrialized and developing countries, where it represents from 85% to 95% of all diabetes[7]. Indeed, the majority of cases of T2D in the future will occur in developing countries with India and China having more cases than any other country in the world[7]. The last report from the International Diabetes Federation (IDF) published in 2015 showed a dramatic change in the prevalence of T2D and different glucose impairment conditions compared with 2013[8,9]. Currently, 415 million people have diabetes worldwide and by 2040 this is predicted to rise to 642 million people. In Europe, it will change from 59.8 to 71.1 million people (**Figure 1.A**). Almost half of the adults with diabetes are aged 40-59 and there are slightly gender differences in the global numbers of people with diabetes. Another alarming issue is that the number of people with undiagnosed diabetes has increased from 175 (in 2013) to 193 million people (in 2015), and are therefore at more risk of developing complications associated with the disease[9] (**Figure 1.B**). Despite IDF has no data regarding IFG, 318 million people worldwide (6.7% of adults) are estimated to have IGT thus with an increased risk of developing T2D[9]. Data from the National Health and Nutrition Examination Survey (NHANES) suggests that IFG is twice as prevalent as IGT[10], suggesting that the prevalence of pre-diabetes (IFG plus IGT) may actually affect more than 900 million people globally[1]. Moreover, by 2040, the number of people with IGT is projected to increase to 482 million people, or 7.8% of the global adult population.

Recently, several surveys' data indicate that previous modeled estimates have been generally underestimating the prevalence of both pre-D and T2D, especially based solely on fasting plasma glucose (FPG), thus many cases of diabetes are easily being missed[11,12]. Although the oral glucose tolerance test (OGTT) is a good tool for the diagnosis of diabetes, it is known to be poorly reproducible and is often not performed[13], which will cause a certain degree of misdiagnosis. Moreover, if we consider that approximately 5-10% of individuals with pre-diabetes will annually progress to T2D[14–17], the epidemia of T2D is increasing at alarming rates.

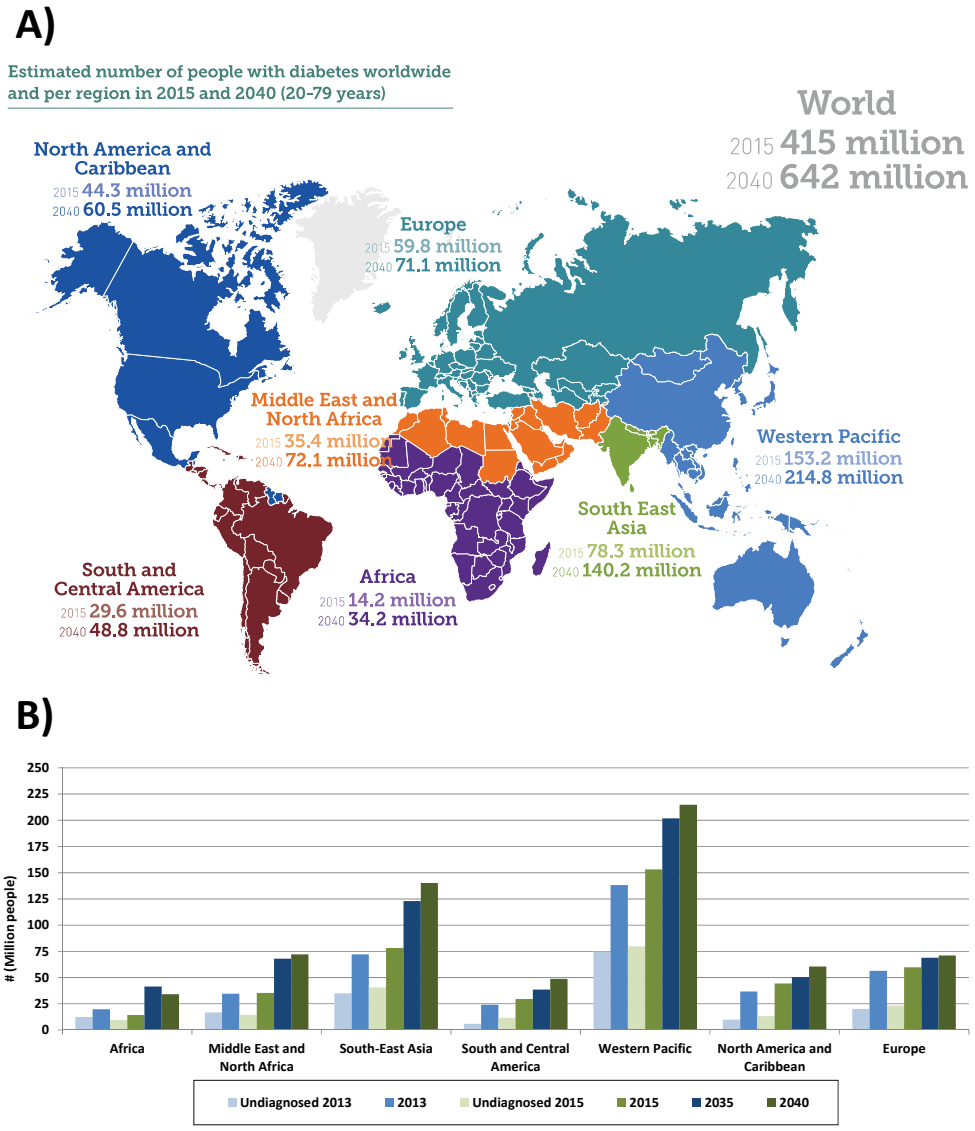

Figure 1. Estimated number of people with diabetes. (A) Worldwide data in 2015 and 2040 for an age range 20-79 years old. (B) Comparative results between International Diabetes Federation (*IDF*) Atlas from 2013 and 2015[8,9].

People with pre-D, even if they never progress to T2D, may also suffer from symptoms of fully developed diabetes[9], and they have an increased risk of different death outcomes and conditions related to their metabolic condition. In fact, there is a 40%, 87% and 33% higher risk of total death[18], cancer death[19] and

coronary heart disease (CHD)[20], respectively, compared with the NGT population. Moreover, pre-D subjects have a 7.9% higher risk of retinopathy[21]. In Spain, last data of prevalence of T2D and pre-D came from the Di@bet.es Study[22]. Soriguer et al. showed that almost 30% of the study population had some glucose disturbance with a 13.8% of T2D prevalence after adjusting by age and sex. Of these, almost half of the subjects did not know they had the disease (6.0%, 95% confidence interval (CI) (5.4-6.7%)). Contrary to other studies, they showed that isolated IFG was present in 3.4% (2.9-4.0%) and was less prevalent compared with isolated IGT, present in 9.2% (8.2-10.2%) of subjects. The prevalence of participants with combined IFG+IGT was 2.2% (1.7-2.7%), and the prevalence of T2D and both IFG/IGT have increased significantly with age[22].

1.3 Diagnosis criteria for type 2 diabetes and pre-diabetes

The diagnosis criteria for T2D may be based on different biochemical parameters even though FPG ≥126 mg/dL is the most common criteria[23]. Fortunately, since few years ago, a specific criteria for diagnosing pre-D has been also included[2,24]. The American Diabetes Association (ADA) defined in 1997 the criteria for IFG when FPG levels are between 110 and 125 mg/dL[25]. In 2003, the ADA reduced the lower FPG cut-off point for diagnosing IFG from the previous 110 mg/dL to 100 mg/dL[26,27] (**Table 1**). They justified it as subjects with a FPG levels between 100 and 109 exhibit a higher prevalence of T2D compared to subjects with a FPG level of <100 mg/dL[2]. As FPG levels are commonly used as an indicator in clinical tests[28], reducing the IFG threshold can facilitate the early identification of subjects at high risk of diabetes and the adoption of preventive measures that delay the development of T2D. Therefore, last diagnosis criteria from ADA is according to a positive result in one of the different tests[24] with different threshold values (**Table 1**). The different tests performed for the diagnosis of T2D and/or glucose impairments are:

A) Fasting plasma glucose. It measures glucose in a blood sample after an overnight fast of at least 8h.

B) 2-h plasma glucose after a 75g OGTT (WHO guidelines). Therefore, using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water, and after 2 hours, measuring plasma glucose.

C) Glycated hemoglobin (HbA_{1c}). It measures the percentage of hemoglobin which is glycated (glucose is attached to it). It may be useful both for the diagnosis of T2D and pre-D and for their control as it is an indicative of the average blood glucose levels for the past 2-3 months.

D) A random plasma glucose ≥200 mg/dL is indicative of T2D in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis.

Table 1. Summary of classification of glucose tolerance levels

Glucose tolerance levels	Fasting plasma glucose (mg/dL) *	2-hour plasma glucose after a 75g glucose load on OGTT (mg/dL) *	Glycated hemoglobin (HbA_{1C}, %) *
NGT	<100	<140	<5.7
IFG	100-125	<200	5.7-6.4
Isolated IFG	100-125	<140	5.7-6.4
IGT	<126	140-199	5.7-6.4
Isolated IGT	<100	140-199	5.7-6.4
Combined IFG+IGT	100-125	140-199	5.7-6.4
T2D	≥126	≥200	≥ 6.5

IFG, impaired fasting glucose; *IGT*, impaired glucose tolerance; *NGT*, normal glucose tolerance; *OGTT*, oral glucose tolerance test; *T2D*, type 2 diabetes. * In absence of unequivocal hyperglycemia, result to be confirmed by repeat testing. Data from Nathan et al.[5] and McLellan et al.[29] and following last criteria from American Diabetes Association (*ADA*)[2].

1.4 Pathophysiology of type 2 diabetes and pre-diabetes

The pathophysiology of T2D implies different cells, tissues, organs and systems of the body with a special focus on pancreas, liver, muscle tissue, gastrointestinal tract and circulatory system. Pancreas is a lobulated organ, which both has an exocrine (c.a. 85%) and endocrine action. The pancreatic islets of Langerhans are the regions of the pancreas that contain its endocrine cells responsible for the secretion of insulin and amilyn (β-cells), glucagon (α-cells), somatostatin (δ-cells) and pancreatic polypeptide (γ-cells). In the gut, different cells from the intestine produce incretins: gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Due to their important link with the pathophysiology of T2D, we will deepen the roles of insulin, glucagon and incretins. Importantly, changes that may begin in pre-D are established as chronic if the subject progress to T2D.

1.4.1 Glucose homeostasis and insulin resistance

Insulin, the most potent anabolizing hormone, is responsible for ensuring normal blood glucose levels and its use by target tissues and organs as an energy source[30]. It also suppresses postprandial glucagon secretion and promotes protein and fat synthesis. Insulin helps to control postprandial glucose in three ways. Initially, insulin increase the glucose uptake in the insulin-sensitive peripheral tissues, primarily skeletal muscle[31] (**Figure 2**). Secondly, insulin acts on the liver to promote glycogenesis. Finally, insulin

simultaneously inhibits glucagon secretion from pancreatic α-cells, thus signaling the liver to stop producing glucose via glucogenolysis and gluconeogenesis. All of these actions reduce blood glucose levels[32]. Postprandially, the secretion of insulin occurs in two phases: an initial rapid release of preformed insulin, followed by increased insulin synthesis and release in response to blood glucose. Long-term release of insulin occurs if glucose concentrations remains high[31,32]. Glucagon has the contrary effect of insulin, as it stimulates hepatic glucogenolysis and promotes both hepatic gluconeogenesis and ketogenesis. Hepatic glucose production, which is primarily regulated by glucagon, maintains basal blood glucose concentrations within a normal range during the fasting state. When plasma glucose falls below the normal range, glucagon secretion increases, resulting in hepatic glucose production and return of plasma glucose to the normal range[33] (**Figure 2**). This endogenous source of glucose is not needed during and immediately following a meal, thus glucagon secretion is suppressed. When coupled with insulin's direct effect on the liver, glucagon suppression results in a near-total suppression of hepatic glucose output. However, in the diabetic state, there is inadequate suppression of postprandial glucagon secretion (hyperglucagonemia)[34] resulting in elevated hepatic glucose production. Many pathophysiological studies have contributed to a better understanding of T2D (reviewed by Zaccardi et al.[35]). Available evidence clearly demonstrates that T2D is a spectrum of disorders characterized by variables degrees of IR and β-cell dysfunction[35]. In healthy individuals, blood glucose levels are strictly regulated to maintain health. With a progression towards T2D, abnormalities in glucose and insulin concentrations and dynamics occur continuously and insidiously over many years[36]. In fact, different results indicate that impairments in the glucose/insulin metabolism start many years before T2D development and the altered glucose homeostasis and decreased β-cell function is already present in the pre-D stage.

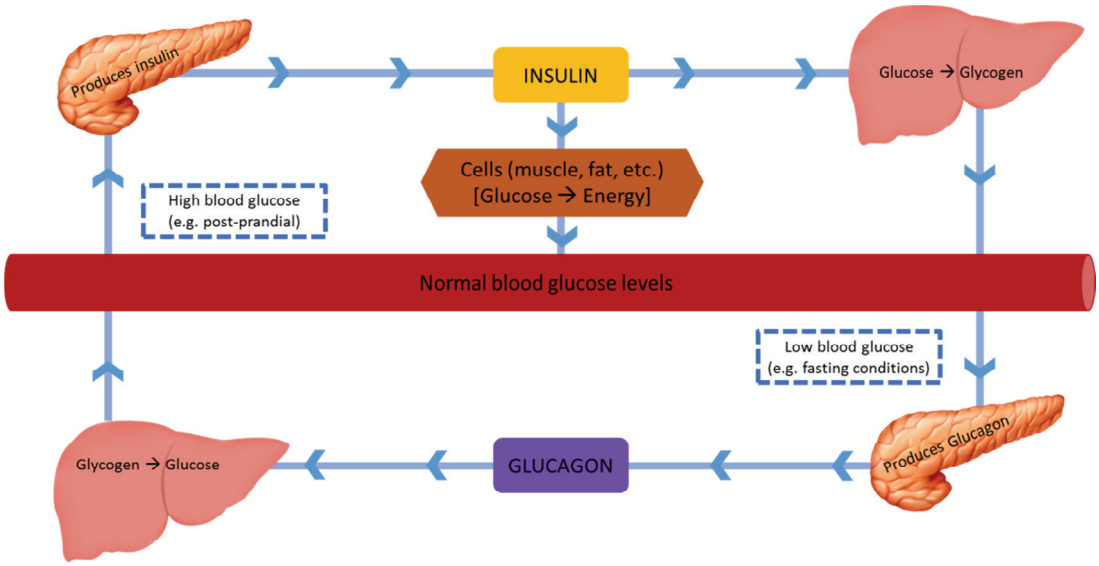

Figure 2. Systemic insulin and glucagon interplay. Pancreatic insulin, produced post-prandially in response to glucose from food, lead to the uptake of glucose from peripheral tissues and the production of hepatic glycogenesis. Contrarily, at low blood glucose levels, pancreas secretes glucagon to maintain normal blood glucose levels by stimulating hepatic glucogenolysis, gluconeogenesis and ketogenesis. Modified from the International Diabetes Federation (*IFD*)[9].

In fasting conditions, low glucose and insulin concentrations stimulates hepatic gluconeogenesis and glucogenolysis, reduces the synthesis of glycogen and the glucose uptake from target tissues and ensures adequate glucose levels in circulation[35,36] (**Figure 2**). On the other hand, in a post-prandial state, insulin stimulates the synthesis of glycogen from hepatic and muscle tissue, synthesis of protein and fatty acids in the liver, triglyceride synthesis in adipose tissue and the use of glucose as energy for their functions. However, apart from the direct action of insulin, glucose homeostasis is regulated by other two processes: production of glucose in the liver and use of glucose in peripheral tissues (mainly skeletal muscle). When the capacity of action of insulin over the insulin-sensitive tissues is diminished it is due to IR[37]. Peripheral resistance to insulin (mainly skeletal muscle, liver and adipose tissue) is described as a pathophysiological condition characterized by an impaired glucose uptake from target tissues at any blood insulin concentration. In this condition, adipocytes increase triglycerides hydrolysis and the release of circulating free fatty acids which migrates from skeletal muscle and liver; glucose uptake in muscle tissue is reduced and hepatic gluconeogenesis increases, overall contributing to hyperglycemia. To counteract this situation, β-pancreatic cells increase their cell mass thereby producing a starting higher amount of insulin

(hyperinsulinemia) which as time goes by generates a relative deficit of insulin and a chronic hyperglycemia state. This high blood glucose levels permanently, even in absence of symptoms, generates several injuries in tissues such as the small vessels of the retina, kidneys and peripheral nerves. Therefore, diabetes is considered one of the major causes of blindness, amputation, and end stage renal disease in developed societies[35,36].

Both IFG and IGT are characterized by IR of different tissue etiology[38–40]. While IFG is characterized by predominantly hepatic IR and normal muscle insulin sensitivity, IGT shows normal to slightly reduced hepatic insulin sensitivity and moderate to severe muscle IR[5]. However, both IFG and IGT are associated with impairment of insulin secretion[41]. Individuals with IFG have severe impaired early insulin responses to the OGTT and improvements in insulin secretion during the second phase of the test, while individuals with IGT have impaired early and late phase of insulin secretion[14,42–44]. Progressive β-cell loss characterizes the development of T2D. In the Insulin Resistance Atherosclerosis Study (IRAS), β-cell function was compromised by 40% in patients with IGT and 80% in patients with T2D[45]. Moreover, IGT and IFG have different implications for atherosclerotic CVD, in which IGT is associated with metabolic syndrome (MetS) and is a strong predictor of CVD[46]. Because pre-diabetic stages are asymptomatic, extended time periods may elapse before diagnosis of T2D, hindering early detection. IR is an early and important factor in the development of T2D and may be present for many years before the emergence of any changes in the glycemic control[47].

The hyperinsulinemic-euglycemic clamp technique is the gold standard for directly assessing IR in humans, however, it is time-consuming, labor-intensive, and overall expensive[48]. Therefore, the most common method for assessment of IR in clinical practice and epidemiologic studies is the homeostasis model assessment of insulin resistance (HOMA-IR) as it has been proved to be a robust tool for the surrogate assessment of IR[49,50]. In addition, HOMA-Beta-cell function (BCF) can be used as a surrogate measurement of β-cell function[51,52]. Therefore, a practical measure of IR would be valuable for early identification of individuals at risk for T2D and CVD in the general population and as a tool for monitoring progress in intervention strategies to prevent or delay these diseases. It should be noted that from a clinical point of view, the term “insulin resistance” implies that a greater than normal concentration of insulin is required to maintain the normoglycemia. In literature, the term “insulin resistance” implies the

resistance to the effects of insulin on glucose uptake, metabolism, and/or storage[53]. In obesity and T2D, IR is manifested by decreased insulin-stimulated glucose transport and metabolism in adipocytes and skeletal muscle and by impaired suppression of hepatic glucose output[53].

1.4.2 Role of incretins

Among the regulators of β-cells, a central role for gut hormones has also been described[54]. In fact, insulin secretion is greater after glucose ingestion than after an intravenous glucose infusion with superimposable glucose excursion (i.e. isoglycemic infusion)[35]. This observation suggests the existence of factors that overstimulate insulin secretion after glucose ingestion. These factors have been shown to be gut 'messengers' (termed incretins) able to stimulate insulin secretion[55]. GLP-1 (secreted by L cells located predominantly in the ileum and colon) and GIP (secreted by enteroendocrine K cells concentrated in the duodenum and proximal jejunum), have been identified as the central hormones responsible for this phenomenon. These polypeptides are secreted after food ingestion and are able to increase insulin (both GLP-1 and GIP) and reduce glucagon (only GLP-1) secretion[55]. While a major secretory defect in GIP does not seem to exist in T2D, significantly decreased secretion of GLP-1 has been consistently found in subjects with T2D[56], IR[57] and obesity[58]. Therefore, after food ingestion, subjects with T2D have a blunted GLP-1 response, resulting in lower postprandial GLP-1 and insulin concentrations and relative high glucagon levels. Furthermore, T2D subjects also show reduced sensitivity to both GIP and GLP-1, a condition that can be improved by restoring euglycemia[59], suggesting that the loss of incretins is secondary to the development of hyperglycemia and not a direct cause of it. While GIP is a more potent incretin hormone, GLP-1 is secreted in greater concentrations and is more physiologically relevant in humans[60]. Overall, beyond the action of GLP-1 promoting β-cell health, it enhances glucose-dependent insulin secretion, suppresses postprandial glucagon secretion, slows gastric emptying and reduces food intake (satiating effect) and body weight (BW)[61,62].

Overall, the pathophysiology of T2D is a complex process, which implicates the combination of different environmental factors (mainly sedentary lifestyle and unbalanced diet) and genetic factors. However, three impairments always coexist: peripheral IR, altered insulin secretion and increased glucose generation in the liver.

1.5 Cellular and molecular mechanisms: implications for type 2 diabetes and pre-diabetes

Beyond systemic, at cellular level, much of the variance in insulin sensitivity between non-diabetic humans may be explained by two mechanisms: differences in phospholipid profiles of skeletal muscle cell membranes, and in intramyocellular lipid stores[63]. High levels of lipids in the bloodstream result in an accumulation of triglycerides and their derivatives within muscle cells, which ultimately reduce the glucose uptake at any given level of insulin[64,65]. This mechanism is quite fast-acting and may induce IR within days or even hours in response to a large lipid influx[66]. However, draining the intracellular reserves is more challenging as moderate caloric restriction alone, even over a period of several months, appears to be ineffective[67,68], and it must be combined with physical exercise to have any effect. In the long term, diet has the potential to change the ratio of polyunsaturated to saturated phospholipids in cell membranes, correspondingly changing cell membrane fluidity. Despite full impact of such changes is not fully understood, it is known that the percentage of polyunsaturated phospholipids is strongly inversely-correlated with IR[69]. It is hypothesized that increasing cell membrane fluidity -by increasing PUFA concentration- might result in an enhanced number of insulin receptors, an increased affinity of insulin to its receptors, and a reduced IR. Moreover, many stressing factors may lead to increased cortisol levels in the bloodstream. Cortisol counteracts insulin, contributes to hyperglycemia (causing hepatic gluconeogenesis), and inhibits the peripheral utilization of glucose, which eventually leads to IR. It acts by decreasing the translocation of glucose transporters (GLUT) such as GLUT-4 to the cell membrane[70]. Although inflammation is often caused by cortisol, inflammation by itself also seems to be implicated in causing IR. In fact, mice without c-Jun N-terminal kinase-1 (JNK-1) signaling do not develop IR under dietary conditions that normally produce it[71,72]. There are several genes codifying proteins that are part of the T2D global pathway (**Figure 3**) in different routes which take place in cell types such as adipocytes, hepatocytes, skeletal muscle and pancreatic β-cells. The IR status and impaired insulin secretion are generated by an inadequate modulation of different molecules belonging to pathways involved not only in T2D. Both the impaired regulation of GLUT-4 and phosphoinositide-3-kinase (PI3K) are directly linked with the origin of the IR status. They are affected by the obese status which influences the levels of adiponectin, free fatty acids and tumor necrosis factor-α (TNF-α), and/or by hyperinsulinemia. However, in pancreatic β-cells, hyperglycemia and glucose levels *per se* could modulate apoptosis, synthesis of insulin and mitochondrial action that lead to impaired insulin secretion. Therefore, both IR and impaired insulin

secretion generates transient hyperglycemia and hyperinsulinemia that leads to T2D (**Figure 3**). These complex pathways are interrelated and are also part of other processes not directly linked with T2D or impaired glucose conditions such as IFG or IGT.

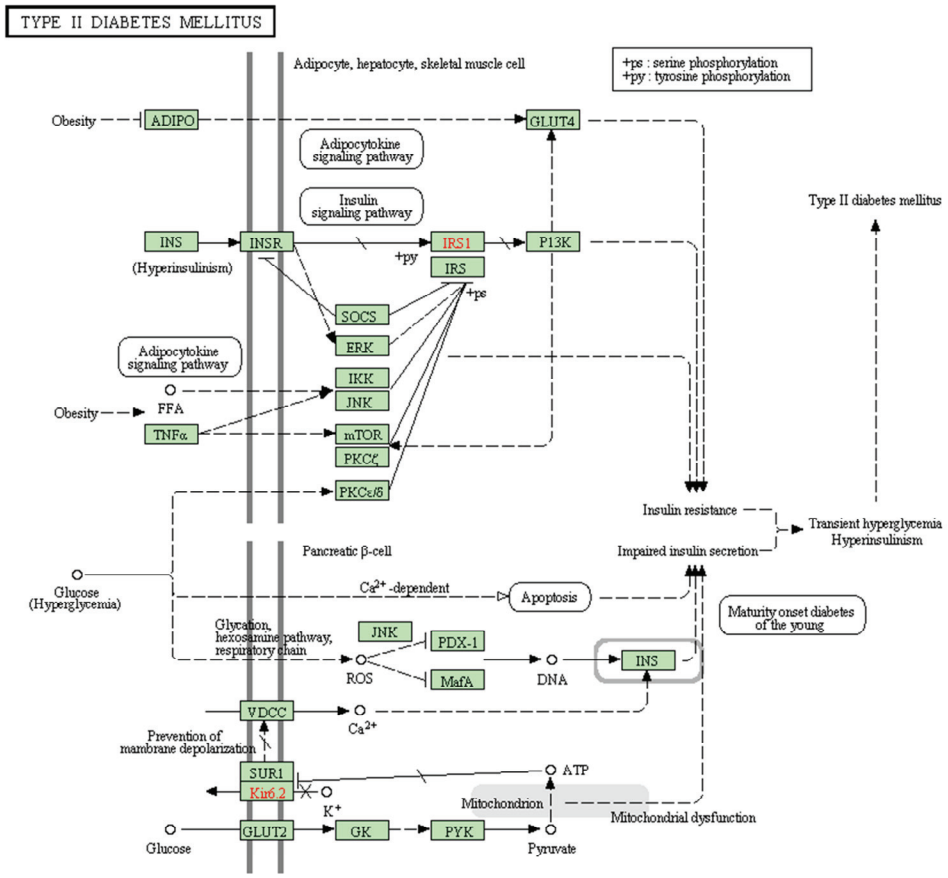

Figure 3. Pathways implicated in type 2 diabetes. *ADIPO*, adiponectin; *ERK*, extracellular signal-regulated kinase; *GK*, glucokinase; *GLUT*, glucose transporter; *IKK*, inhibitor of nuclear factor kappa-B kinase; *INS*, insulin; *IRS1*, insulin receptor substrate; *INSR*, insulin receptor; *JNK*, c-Jun N-terminal kinase; *Kir6.2*, type of potassium voltage-gated channel (*KCN*); *MafA*, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog A; *mTOR*, mechanistic target of rapamycin; *PDX-1*, pancreatic and duodenal homeobox 1; *PI3K*, phosphoinositide-3-kinase; *PKC*, protein kinase C; *PYK*, pyruvate kinase isozymes; *SOCS*, suppressor of cytokine signaling; *SUR1*, type of ATP binding cassette (*ABC*); *TNFα*, tumor necrosis factor-alpha; *VDCC*, voltage-dependent calcium channel. Obtained from the Kyoto Encyclopedia of Genes and Genomes (*KEGG*)[73,74]. Pathway hsa04930 ("Type II Diabetes Mellitus").

1.6 Classical risk factors for type 2 diabetes and pre-diabetes

Type 2 diabetes is a complex disorder resulting from an interaction between genes and environment. Several risk factors for T2D have been identified, including age, sex, obesity and central obesity, low physical activity, smoking, diet and ethnicity[75–78]. Due to the etiology of the disease, the same factors that increase the risk of developing T2D also increase the risk of developing pre-D. As there are modifiable and non-modifiable risk factors for pre-D and T2D, we will focus on each individual risk factor in this section.

1.6.1 Non-modifiable risk factors

These risk factors are based on inherent genetic or developmental factors, which are not modifiable by dietary and/or other lifestyle changes.

1.6.1.1 Race/ethnicity

Race has been related to a different risk for T2D and CVDs[7]. In this sense, African Americans, Mexican Americans, American Indians, Native Hawaiians, Pacific Islanders, Asian Americans and Gypsies have a higher risk for developing the aforementioned diseases. This is partly because these populations are more likely to be overweight, to have high blood pressure and to have T2D[7]. Singapore population had a frequency of T2D in 1992 of 7.7-8.5% in Chinese men and women aged 18-69 years compared with 13.3 and 12.3%, respectively, among the Asian Indians and Malays[79]. High prevalence rates of diabetes have also been found among Asian Indians compared with the indigenous populations in the United Kingdom, Fiji, South Africa and in the Caribbean[80–82]. In 2012, T2D Exploration by Next-generation sequencing in multi-Ethnic Samples (T2D-GENES) was founded[83]. It is a National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-funded research consortium which seeks to identify genetic variants for T2D through multiethnic sequencing studies. Therefore, while environmental factors undoubtedly account for some of this differential prevalence, they are likely also to reflect inherent ethnic differences in susceptibility to T2D.

1.6.1.2 Familiar aggregation and genetic risk factors

The empirical risk of having T2D is increased 2- to 6-fold if a parent or sibling has the disease[84,85]. Consequently, the familiar history of T2D is a practical way to estimate whether a subject is likely to have inherited susceptibility to the disease. On the other hand, familial aggregation may occur for non-genetic reasons. Family members often share a similar environment, particularly as children and in adolescence,

thus familial aggregation alone is not definitive evidence of genetic determinants. Both genetic and environmental factors, as well as their interactions, contribute to the pathogenesis of T2D[86]. In fact, the concordance of T2D in monozygotic twins is approximately 70% compared with 20-30% in dizygotic twins[87,88]. When both parents are affected with T2D, the lifetime risk of developing T2D is 70% in the offspring, and approximately 40% when only one parent has T2D. However, it is greater when the mother is the affected parent[89].

Family history of T2D has been associated in epidemiological studies with 2-fold increased risk of future T2D[76]. In the last decade, different genome-wide association study (GWAS)[90,91] have successfully identified numerous single-nucleotide polymorphisms (SNPs) located in and near genes that may be key in the development of T2D. Up to now, a total number of 88 genetic loci have been identified for T2D[92] in samples from populations of almost exclusively northern European ancestry. Therefore, novel genes (e.g. T*CF7L2, SLC30A8, IDE-KIF11-HHEX* and *FTO*) with uncertain functions affecting the risk of T2D have been identified[93]. Despite the increase in the number of genes, their known contribution to both the overall and the genetic risk remains small[94]. Although understanding the genetics of T2D has exhibited great progress in the past few years, a substantial amount of additional work will be required to identify causal variants/genes and molecular mechanisms conferring T2D risk.

1.6.1.3 Age

The prevalence of T2D increases with age (**Figure 4**). Even though the worldwide prevalence is higher in the oldest age categories, in certain populations a decrease in prevalence is seen in the oldest age groups (≥75 years) because of higher mortality rates in those with the disease. In fact, T2D usually develops in the middle to older age groups in relatively affluent societies. In developing countries, however, because of the younger age distribution of the population, many cases occur in young and middle aged adults. In Caucasian populations in the United States and Europe, the prevalence of T2D and pre-D increases with age at least into the seventies[95]. Even though T2D was formerly considered a disease of adults, in recent years, there have been many reports of its occurrence in childhood and adolescence[96–98]. As in adults, the disease in children is frequently asymptomatic and is detected mainly by screening. In Spain, adults aged 31-45 years have a prevalence of 2.15% (men) and 0.93% (women) whereas in the age group 46-60, it

increases to 11.9% (men) and 6.60% (women). Above 60 years, the prevalence is set between 18.7 and 24.8%[22].

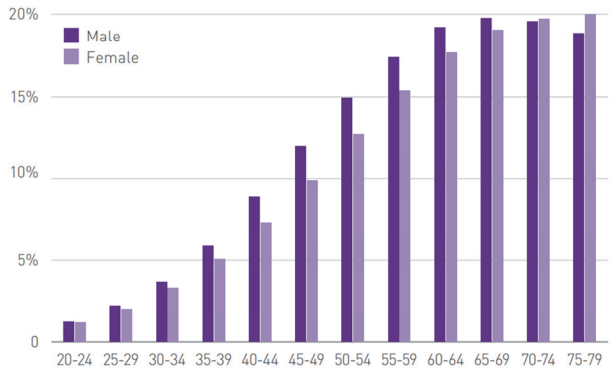

Figure 4. Worldwide prevalence of people with diabetes by age and stratified by sex. Obtained from International Diabetes Federation (*IDF*) 2015 report[9].

1.6.1.4 Gender

Recently, data from the IDF has showed that there are some differences in T2D prevalence according to gender even when we consider different age categories (**Figure 4**). They reported about 15.6 million more men than women with T2D (215.2 million men vs 199.5 million women) and estimated that this difference will slightly decrease by 2040 to about 15.1 million more men than women (328.4 million men versus 313.3 million women)[9]. Diabetes prevalence is higher in men, but there are more women with diabetes than men[99]. The combined effect of a greater number of elderly women than men in most populations and the increasing prevalence of diabetes with age is the most likely explanation for this observation [100,101]. Moreover, IFG is more common in men, whereas IGT is more prevalent among women, who instead have lower FPG[101,102]. Other studies have shown that T2D affects women disproportionately[103,104]. In fact, women with T2D generally have poorer glycemic control[105,106] and are less likely to reach the goals for HbA_{1c} compared with men[107,108]. Possible causes for varying outcome include differences in physiology, treatment response, and psychological factors[109]. However, some studies have found unlike rates considering age groups with higher rates in men in older but not in younger age groups in certain populations[110,111]. Moreover, researchers have found that one possible

explanation for this fact is due to men have to gain less BW (one of the main risk factor for T2D) to develop T2D than women, in part because men without T2D are generally more IR than women[112].

1.6.2 Modifiable risk factors

Beyond non-modifiable risk factors, there are different factors that we can vary in order to counteract the risk of developing pre-D and/or T2D. One of the main modifiable factors is related to diet, as food is the main activator/modulator of glucose and insulin metabolism.

1.6.2.1 Diet

Diet has a direct impact in the development and progression of T2D and many other metabolic diseases[113,114]. Many research has focused on macronutrient intake linked to T2D risk[115,116], mainly related to carbohydrate (CHO) and fat content. For several decades, the dominant approach used to test the association between diet and health indices was based on exploring the effects of single nutrients, individual food or dietary patterns. However, the fact that individuals consume complex combinations of foods consisting of several nutrients that interact with each other, highlighted the need for exploring the association between overall diet and disease[117]. Different trials have investigated the effects of lifestyle interventions (including diet and physical activity) on the development of T2D in people with IGT[118] showing a reduced risk in subjects following a diet rich in vegetables, fruits and moderate intake of alcohol compared to the non-intervention group. In all trials the effects of a lifestyle intervention, that included a combination of healthy eating and increased physical activity, was compared with no intervention or usual care. Dietary interventions in all studies were similar and overall contained the following recommendations: decreased intake of total energy, total fat, saturated fat, refined sugar and simple CHO and increased intake of unsaturated fat and fiber. In addition, increased intakes of vegetables, fruits and moderate intake of alcohol were recommended. Conclusively, all trials showed that, compared to no intervention or usual care, intensive lifestyle interventions reduced the development of T2D[118]. Importantly, the beneficial effects of diet on T2D could be even higher when considering dietary patterns such as MedDiet as a whole[119,120]. Undoubtedly, diet has a direct action on the risk of developing metabolic conditions such as pre-D and T2D even though more research is needed to determine the contribution of each food source to the development.

1.6.2.2 Physical activity

Several studies have stressed the importance of physical inactivity in the development of T2D[121–123]. However, in most studies its relative importance may be underestimated because of imprecision in measurement. The deleterious effect of low levels of physical activity was seen particularly among those subjects who have other risk factors such as high BMI, hypertension or family history of T2D. Investigations into the relationship between physical activity and insulin levels unequivocally demonstrate that high levels of sedentary time, low levels of daily movement, and little moderate to vigorous physical activity are associated with poor glycemic control[124–126]. Additionally, there is strong evidence that a dose-response relationship exists between insulin sensitivity and exercise "dose" (a combination of intensity, duration, and frequency)[127]. Individuals with pre-D have been shown to benefit significantly from intensive lifestyle intervention programs that include increased physical activity or a combination of physical activity and health dietary advices[128–130]. Specific recommendations of physical activity as a preventative strategy for the prevention of T2D are still unknown. Longitudinal studies have clearly indicated that increased physical activity reduces the risk of developing T2D regardless of the degree of adiposity[123,131,132]. Vigorous exercise (i.e. training to an intensity of 80-90% of age-predicted maximum heart rate for at least 20 minutes, at least 5 times/week) has the potential to substantially enhance insulin sensitivity[133]. However, the minimum intensity and duration of physical activity required to improve insulin sensitivity has not been established yet.

1.6.2.3 Obesity

Obesity is a frequent concomitant disorder of T2D, and in many longitudinal studies it has been shown to be one of the most powerful predictors of T2D development[134,135]. Obesity has increased rapidly in many populations in recent years[136–139] mainly due to an interaction between genetic and environmental factors[7]. This increase in obesity has been accompanied by an increasing prevalence of T2D[140]. Since obesity is such a strong predictor of T2D incidence, it appears that the rapid increases in the prevalence of T2D are almost certainly related to increasing obesity. However, only a limited number of studies have measured age and sex specific incidence rates for T2D in relation to obesity and the rates vary considerably according to other risk factors[141]. In 1990, data from the Nurses' Health Study (NHS) indicates that the relation between BMI and T2D risk is positive and continuous[135]. Moreover, last

prospective results from NHS I/II showed that both overweight and obesity duration are associated with a significantly higher risk of T2D[142], after adjusting by potential confounders. In men, the Danish cohort study showed that obesity was associated with more than 8-fold increased risk for T2D[143] compared to normoweight subjects.

Importantly, several studies indicate that waist circumference (WC) or waist-to-hip ratio are more powerful determinants of subsequent risk of T2D than BMI[7,144–150]. In fact, weight loss improves insulin sensitivity[133] and reduce the risk of progression from IGT to T2D[151,152]. However, central adiposity is also an important determinant of IR[149]. Given the importance of central adiposity as a determinant of T2D risk it is necessary to consider whether the usually quoted "normal range" for BMI (18.5-24.9 kg/m^2) is appropriate for all populations. It might be appropriate to also suggest an appropriate range for some measure of the distribution of body fat such as WC[153] as the influence of obesity on T2D risk is determined not only by the degree of obesity but also by where fat accumulates.

1.6.2.4 Tobacco smoking

Tobacco smoking is associated with an increased risk of T2D[154]. Different epidemiological studies have showed a positive association between cigarette consumption and risk of T2D[155–157]. More specifically, the Health Professionals' Follow-up Study (HPFS) showed that the risk for T2D among men who smoked ≥25 cigarettes/day was 1.94 (95% CI, 1.25-3.03) compared with non-smokers[157]. Several biological mechanisms have been proposed through which smoking may have an effect on the development of T2D, including inflammation and the effect of nicotine on glucose homeostasis and IR[158]. However, the exact molecular mechanisms connecting smoking to an increased risk of diabetes remain largely unknown. In healthy young men, acute smoking showed an increased IR[159,160]. In T2D subjects, insulin and c-peptide responses to oral glucose load were significantly higher in smokers than in non-smokers and the IR was positively correlated in a dose dependent manner[161]. Thus smoking induced IR in patients with T2D as well as in healthy subjects[161]. Beyond this, previous research has established that tobacco smoking has an important role in DNA methylation, the epigenetic mechanism of attachment of a methyl group to nucleotides[162–164]. In line with this, previous studies have suggested DNA methylation as a potential pathway in the association between smoking and an increased risk of T2D[165]. It has been recently showed

an effect of smoking on DNA methylation of some T2D-related genes (*ANPEP*, *KCNQ1* and *ZMIZ1*) giving further insight into potential mechanisms linking smoking to an excess risk of T2D[166].

1.6.2.5 Alcohol intake

Several studies have suggested that moderate alcohol intake is associated with a reduced incidence of T2D[167,168] and with modestly decreases in cardiometabolic risk of already T2D subjects[169]. Among women in the NHS, there was a reduced incidence of T2D in women who consumed alcohol compared with those who did not. There was a strong inverse relation between alcohol consumption and body weight, which could explain much of the apparent protective effect of alcohol consumption[170]. In males from the HPFS and Physicians' Health Study (PHS), they showed that moderate alcohol consumption among healthy people was associated with a reduced risk of T2D[157,171]. These was also examined among participants in the Atherosclerosis Risk in Communities Study (ARIC)[172]. After adjustment for other diabetes risk factors, men consuming >21 drinks/week had a significant increase in the incidence of diabetes, whereas no significant association with alcohol intake was found among the women. Therefore, like in many other diseases, there is a delicate balance between the harmful and beneficial effects of alcohol on the incidence of T2D. In moderate amounts, drinking is associated with a reduced risk of T2D, whereas in higher amounts with an increased risk.

1.7 Prevention and treatment of type 2 diabetes and pre-diabetes

Due to the worldwide epidemic of pre-D and T2D, many countries have prompted different prevention actions[103,173] and guidelines to manage already onset T2D[174]. Different clinical trials have demonstrated effective means of preventing or delaying diabetes onset comprising both pharmacological and/or lifestyle interventions[16,152,175,176]. However, as people with T2D and pre-D form a heterogeneous group, treatment regimens and therapeutic targets should be more personalized. Therefore, last efforts have been focused on delaying T2D by acting on subjects at risk and/or by acting on already established T2D to control its health complications.

1.7.1 Pharmacological management

As T2D is characterized by IR and ongoing decline in β-cell function, glucose levels likely will worsen over time. It means that treatment must be dynamic as therapeutic requirements increase with longer duration of disease. The desire to maintain HbA_{1c} levels that are less than 7% in many patients and the

progressive metabolic dysfunction of T2D predictably result in the need for increasingly complex medication regimens over time. A lifestyle intervention directed at weight loss and increased physical activity, similar to the one used successfully in the Diabetes Prevention Program[152], has been shown to ameliorate hyperglycemia and the need for medications in established T2D[177]. However, currently there is no medication specifically designed for pre-D and those subjects are usually prescribed the initial treatment that subjects with T2D follow.

Based on its efficacy in lowering glycemia, long history of use, demonstrated safety and tolerability, and other characteristics including the low reported level of hypoglycemia, associated weight loss, and low cost, metformin is the consensus choice as the first medicine that should be used to treat T2D[178–180]. Starting metformin at or near the time of diagnosis, together with lifestyle intervention aimed at weight loss is recommended[179,180]. Metformin is usually continued through the treatment course of T2D, assuming that contraindications or intolerance does not develop. However, in patients with adverse effects during metformin therapy or needing further treatment, clinicians are currently also considering the use of other drugs such as GLP-1 receptor agonists (GLP-1RA). In fact, GLP-1RAs are prescribed alone or in combination with metformin and/or other drugs depending on each subject. Therefore, GLP-1RAs represent a unique approach for the treatment of diabetes, with benefits extending outside glucose control, including positive effects on weight, blood pressure, cholesterol levels, and β-cell function[181]. They act by mimicking the effects of GLP-1 which includes increasing insulin secretion, decreasing glucagon release, increasing satiety, and slowing gastric emptying[182]. However, overall drug therapy has some potential adverse effects such as bowel movement alteration, diarrhea, weight gain, hypoglycemia, fluid retention, heart failure, bone loss, flatulence, nausea and vomiting. Therefore, a lifestyle modification, including nutritional therapy and physical activity, should continue to be emphasized while pharmacotherapy is being used as i) many drugs can cause weight gain as a side effect; and ii) the important role of lifestyle in the management of pre-D and T2D.

1.7.2 Lifestyle management: roles of physical activity and diet

As we have seen, both inadequate diet and physical inactivity have a direct impact on T2D and pre-D as they are well-established risk factors. Several landmark diabetes prevention trials have shown that lifestyle measures that incorporate healthy eating, physical activity, and weight control can successfully

delay and even prevent the onset of T2D in high-risk individuals to a greater extent than medical therapy[183]. Such studies showed reductions in risk from 29% up to 67%[183]. Most lifestyle recommendations for individuals with pre-D and/or at high risk of T2D focus on general strategies[29,152], such as the those listed in **Table 2**. However, lifestyle interventions are not only beneficial before the development of diabetes, as several studies have clearly demonstrated the benefits of control over diet, exercise, and weight loss in individuals already diagnosed with T2D. Despite much progress in the understanding of dietary macronutrient intake, there continues to be debate among diabetes and nutrition experts as to the relevance of each macronutrient to the development and management of pre-D and T2D is not fully understood.

Table 2. Strategies for preventing or delaying pre-diabetes and type 2 diabetes

Characteristics	Action plan
Subjects with IGT, IFG, or HbA_{1c} (5.7-6.4%)	Refer to intensive diet and physical activity behavior counseling: • Weight loss (5-10 kg of body weight) • Increased PA (≥ 150 min/week of PA) • Eliminate sugary drinks • Choose whole grains over refined CHO • Stop smoking • Drinking alcohol in moderation
In the presence of other characteristics: • BMI > 35 kg/m^2 • Age < 60 years • Women with prior GD	Consider metformin therapy for T2D prevention.
General considerations	a) Annual monitoring of individuals with pre-D b) Screening for and treatment of modifiable CVD risk factors (obesity, hypertension, and dyslipidemia) is suggested c) Diabetes self-management education and support are appropriate for pre-D to receive education and support to develop and maintain behaviors that can prevent and delay T2D

BMI, body mass index; *CHO*, carbohydrates; *CVD*, cardiovascular disease; *GD*, gestational diabetes; *HbA_{1C}*, glycated hemoglobin; *IFG*, impaired fasting glucose; *IGT*, impaired glucose tolerance; *PA*, physical activity; *pre-D*, pre-diabetes; *T2D*, type 2 diabetes. Modified from Knowler et al.[152], Portero et al.[29] and Lin et al.[184].

Protein

In industrialized countries, dietary protein intake has increased substantially during the last few decades, exceeding 50% of the recommended dietary allowance[185]. Diets with increased protein and reduced CHO have been shown to improve glycemic regulations in healthy subjects and people with T2D[186,187], with caution regarding renal problems and the source of the protein (animal versus vegetable) as stated by the ADA[188,189]. ADA has also established that there is no ideal intake to improve glycemic control or CVD risk but CHOs' sources high in protein should not be used to treat or prevent hypoglycemia[189]. However, it is known that suggested beneficial acute effects of dietary protein on insulin secretion and glycemic control[190] do not seem to persist mid- and long-term[191,192]. Some epidemiological studies addressing the association between protein intake and T2D have found an increased risk of T2D with high protein and/or meat protein intake[193–195]. However, Sluijs et al.[196] did not observe a significant association for total protein after adjustment for BMI and WC in the Dutch cohort of European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct. Based on sub-analyses, the authors concluded that high protein intake increases T2D risk only for participants with a BMI <25 kg/m^2[196]. In a recent analysis of the global EPIC consortium, they found that high total and animal protein intake was associated with a modest elevated risk of T2D[197]. Recent results from the NHS I/II and HPFS showed that higher intake of animal protein was associated with an increased risk of T2D, while higher intake of vegetable protein was associated with a modestly reduced risk. However, besides total and animal protein, prior research suggests that the protein source could be of relevance. T2D risk is associated with higher meat consumption, particularly red and processed meat[198–200], whereas is reported to be lower in subjects with high dairy consumption[201–204] and/or high plant product consumption, especially of legumes[205] and nuts[206].

Fat

Both the amount and quality of dietary fat may modify glucose tolerance and insulin sensitivity[207–209]. However, subtypes of fat have a different role on glucose homeostasis[210]. Overall, a high fat content in the diet may result in deterioration of glucose tolerance by several mechanisms including decreased binding of insulin to its receptors, impaired glucose transport, reduced proportion of glycogen synthase and accumulation of stored triglycerides in skeletal muscle[211–215]. As we have previously seen, the fatty

acid composition of the diet, in turn, affects tissue phospholipid composition, which may relate to insulin action by altering membrane fluidity and insulin signaling[207]. General guidelines for T2D and pre-D show that they need to reduce SFA (<10% total caloric intake) and keep a intake of monounsaturated fatty acids (MUFA) as following Mediterranean-style (i.e. nuts, EVOO) as alternative to low-fat high CHO diets to improve glycemic control and reduce CVD risk[189]. However, in which regards prevention of T2D and pre-D, data from epidemiological and human intervention studies is inconsistent. In two cross-sectional studies, total fat intake was higher in glucose intolerant and T2D subjects[216] and in subjects with recurrent GD[217] compared with normo-glycemic controls. Furthermore, a high fat intake has been shown to predict development of IGT in a group of healthy subjects[218] and progression from IGT to T2D in a group of subjects with IGT[219]. In fact, high total fat intake has also been associated with higher fasting insulin concentrations[220] and a lower insulin sensitivity index. On the other hand, there are several studies which show no association between diabetes risk and total fat intake[221–229]. Additionally, a recent meta-analysis have shown that saturated fats are not associated with the development of T2D, but the evidence is still heterogeneous in the literature[230].

Carbohydrate, fiber and glycemic index

The ideal CHO intake in subjects with T2D is not established but some recommendations have been related to improvements in glycemic control of T2D subjects[189]. ADA recommends CHO intake from vegetables, fruits, whole grains, legumes and dairy, avoiding other CHO sources (e.g. added fats, sugar and sodium). It is also important to take into account the glycemic index (GI) and glycemic load (GL) of the foods, and the overall diet, and try to consume foods with low-moderate GI as it produce a modest improvement in glycemic control[231]. Moreover, dietary fiber and whole grains should be also considered. However, some controversy surrounds the optimal ratio of CHO-to-fat in the diet with respect to the prevention of chronic diseases including T2D[232–234]. It is known that a high CHO intake increases the requirement for insulin secretion in order to maintain glucose homeostasis[235]. However, there is some evidence that a high CHO intake decreases the prevalence of T2D[235]. Moreover, numerous studies have reported that an increased intake of CHO can reduce high-density lipoprotein (HDL) levels and raise fasting plasma triglycerides concentrations[233]. Last results from systematic review and meta-analysis have shown protective effects of low dietary GI and GL but non-significant associations of total CHO intake and

T2D[236]. Therefore, evidence points to a major role of CHO quality rather than quantity in the diet[235,237]. Dietary fiber is one of the factors that influences post-prandial glucose and insulin responses[238]. The effects of the various components of dietary fiber have been implicated in the prevention and management of a range of diseases, including T2D[235]. According to the last systematic review and meta-analysis of epidemiological studies, a relatively low intake of dietary fiber significantly increases the risk of T2D[239]. Moreover, last meta-analysis of RCT showed that increased fiber intake improved glycemic control in subjects with T2D[240]. Cross-sectional studies suggest that lack of dietary fiber may be related to T2D and have shown an inverse relationship between fiber intake and blood insulin levels[235], implying that fiber improves insulin sensitivity.

Mediterranean diet

In addition to the isolated macronutrients, the evaluation of dietary patterns is important to determine the whole effect of the diet into T2D and pre-D. Mediterranean diet has emerged as an overall healthy diet to prevent and manage different conditions[241]. Although the MedDiet is a dietary pattern with plenty, well-established health benefits, a progressive shift towards "westernized" dietary habits has lately occurred in the Mediterranean regions[242,243]. As a result, the term "Mediterranean-style diet" is currently used in the literature in order to describe not a specific diet, but rather a collection of dietary habits traditionally followed by the populations of countries bordering the Mediterranean Sea[244]. In brief, a Mediterranean-style diet is characterized by high consumption of olive oil, vegetables, legumes, whole grains, fruits and nuts, moderate consumption of poultry and fish, low consumption of full fat dairy products and red meat, and low-to-moderate consumption of wine as the main source of alcohol accompanying meals[244]. All available cohort studies[245–252] as reviewed by Georgoulis et al.[253] and Salas-Salvadó et al.[254] support the protective role of the MedDiet against T2D development with overall reductions in risk ranging from 12% to 83% for subjects closely adhering to the MedDiet, compared to those reporting the lowest adherence, after adjusting for several confounding factors. Apart from cohort studies, there is also a sub-analysis of a multicenter, randomized trial (the PREvención con Dieta MEDiterránea (PREDIMED) study) which so far provides the strongest evidence on the association between MedDiet and T2D development[120]. In this interventional study 7,447 high cardiovascular risk subjects were randomly assigned to education on either a low-fat diet or to one of two MedDiets,

supplemented with either free virgin olive oil (1 L/week) or nuts (30 g/day). After a mean follow-up of 4 years, participants (without T2D at baseline) allocated in the MedDiet supplemented with olive oil and nuts groups exhibited a 40% and 18% reduced risk of T2D, respectively, compared with the low-fat diet group, after adjusting for several confounders[120].

Beyond the best mix of carbohydrates (with a special emphasis on fiber intake and GI), proteins, and fats, the adjustments for total caloric intake must be appropriate to weight management goals. Overall, as we have seen, different clinical trials have been performed to measure how dietary interventions can manage and prevent T2D from a normo-glycemic state. However, there is a lack of information in which regards medium- and long-term nutritional interventional studies designed to test the effect of specific food on ameliorating cardiometabolic alterations of pre-D in the context of a Mediterranean diet.

2. Nutrition: from nuts to pistachios

Nuts constitute an independent group among food and is part of the cornerstone of the MedDiet[244]. From a botanical definition, a nut is simple a dry fruit with one seed (rarely two) in which the ovary walls becomes very hard (stony or woody) at maturity, and where the seed remains unattached or free within the ovary wall. However, common use of the word "nut" implies only any large, oily kernels found within a shell and used as food. In the present thesis, we will refer to the term "nuts" as a group composed by almonds, walnuts, hazelnuts, pinions, peanuts, pistachios, Brazil nuts, cashews, Macadamia nuts and pecans. Although peanuts are part of the leguminous group, due to their similar nutrient composition and their proved beneficial CV health benefits, we have also included them in the nuts group. Nuts have a wide variety of cultural connections to the areas where they grow and to the people who live there or eat them. History, symbolism and legends reveal the ancient tradition of nuts and how they are related to the lives of our ancestors[255]. In fact, archaeological excavations in eastern Turkey have uncovered the existence of a non-migratory society whose economy centered on harvesting nuts. This shows that nuts have been a feedstock in the human diet since the beginnings of history[255,256]. Moreover, since ancient times nuts have been used for their medicinal properties and they also play a role in many old legends and traditions. This thesis will focus on pistachio as a model of nut due to their balanced nutrient composition, and to the lack of evidences concerning some health-promoting properties of this specific green nut.

2.1 A focus on pistachios' history

Pistachio (Pistacia vera L.) is a member of the *Anacardiaceae* family, which is native to arid zones of Central and West Asia, and distributed throughout the Mediterranean basin[257]. From the Greek word *pistákion* [πιστάκιον], pistachio is widely cultivated in the Mediterranean region, even though it probably originated in central and southwest Asia. Evidence of its consumption has been found in archaeological excavations, which proves that it has long been associated with human activities[256]. Remains of pistachio nuts dating from the sixth millennium BC have been found in both Afghanistan and southeastern Iran where pistachio (*Pistacia vera L*) was probably first cultivated in regions close to where it grew wild. It was widely cultivated in the ancient Persian Empire, from where it gradually expanded to the west. For example, legends suggest that the Queen of Sheba (Assyria, c.a. tenth century BC) monopolized a limited crop of pistachios for her exclusive use[258]. However, the Assyrians and the Greeks knew that pistachios

could be used as medicines, aphrodisiacs and antidotes. By the end of his reign, Emperor Tiberius, the Roman consul of the province, introduced pistachios into Italy. From Italy, they had spread into Mediterranean regions in southern Europe and North Africa. Around the tenth century, pistachios were also cultivated in China, and more recently in Australia, New Mexico and California[256]. Nowadays, pistachio is widely commercialized and it is an important part of different food dishes all over the globe.

2.2 Nutritional composition of nuts

From a nutritional point of view, nuts are a complex matrix of different macro- and micronutrients (**Figure 5.A**). Nuts are naturally low in sodium and high in unsaturated fatty acids, CHOs with low glycemic index, dietary fiber, plant protein and phytochemicals, some of which may act synergistically to produce a wide range of health benefits[259–262]. Moreover, their composition regarding micronutrients and other bioactive molecules is also extensive. Nuts contain many vitamins (vitamins E, B_6, niacin and folic acid), minerals (magnesium, potassium, calcium and phosphorus) and other phytochemical constituents (stigmasterol, campesterol, resveratrol and catechins)[263]. Compared to other nuts, pistachios have lower amount of fat (mostly from poly- and monounsaturated fatty acids) and energy content (**Figure 5.B**), and higher levels of fiber (both soluble and insoluble), potassium, phytosterols, γ-tocopherol, vitamin K, xanthophyll and carotenoids[263]. Importantly, pistachios are among the top 50 foods with a high antioxidant potential[264] and are as well a known source of bioactive compounds, including plant sterols[265]. Particularly, pistachios are rich in β-carotenes which have been widely associated with a protective T2D role[266,267]. In addition, pistachios are the only nut that contains significant amounts of lutein and zeaxanthin[263]. Polyphenols, xanthophylls and tocopherols from pistachios have been demonstrated to be rapidly accessible in the stomach, thus maximizing the possibility of absorption in the upper small intestine, and thereby contributing to the beneficial relation between pistachio consumption and health-related outcomes[268].

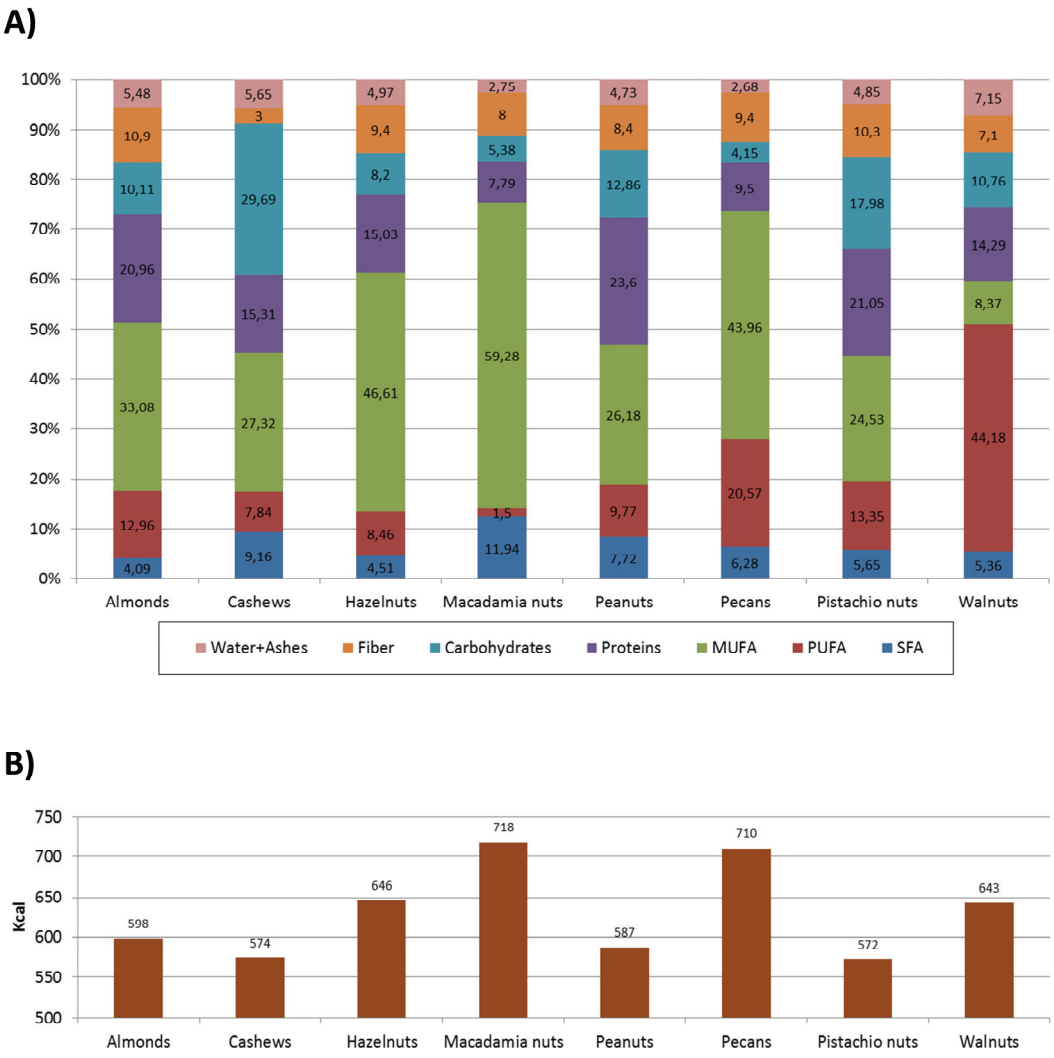

Figure 5. Nutrient and caloric content of selected nuts per 100 g (dry roasted). A) Percentage of macronutrient composition of selected nuts. *MUFA*, monounsaturated fatty acids; *PUFA*, polyunsaturated fatty acids; *SFA*, saturated fatty acids. B) Caloric content (*Kcal*) of selected nuts. Walnuts' data is from raw nuts as data from dry roasted walnuts is not available. Data obtained from the United States Department of Agriculture (*USDA*), Nutrient Database for Standard Reference, Release 28, 2015[269].

2.3 Health benefits of nuts

Research on nuts' contribution to health outcomes is not recent. The CV health benefits associated with consuming different nuts such as almonds[270], pistachios[271] and walnuts[272] have been extensively investigated. Since the last more than 20 years, a huge amount of research has been focused in analyzing the beneficial properties of nut consumption (**Figure 6**). Many epidemiological studies have succeeded in their prospective analyses of the good properties of nuts and many clinical trials have also studied their potential role on different conditions. Beyond this, research regarding nuts has also been evaluated in *in vivo* or *in vitro* models to test their contribution for the amelioration of different parameters and diseases such as oxidative processes, inflammation and cancer[273–279].

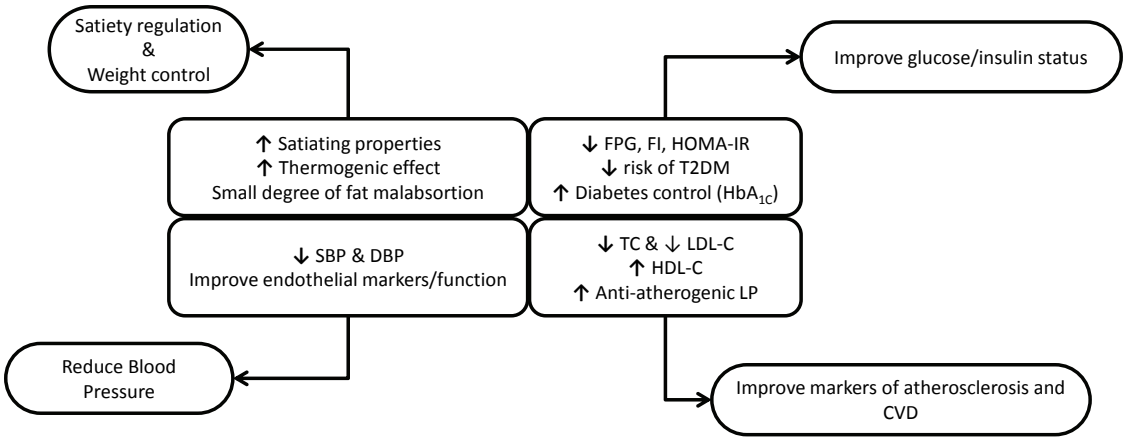

Figure 6. Beneficial properties of nut consumption for different outcomes. ↑, increase; ↓, decrease; *CVD*, cardiovascular disease; *DBP*, diastolic blood pressure; *FI*, fasting insulin; *FPG*, fasting plasma glucose; *HbA_{1c}*, glycated hemoglobin; *HDL-C*, high-density lipoprotein cholesterol; *HOMA-IR*, homeostatic model assessment of insulin resistance; *LDL-C*, low-density lipoprotein cholesterol; *LP*, lipoprotein; *SBP*, systolic blood pressure; *T2D*, type 2 diabetes; *TC*, total cholesterol.

Undoubtedly, nuts are one of the most studied food categories mainly due to their particular healthy matrix-based nutritional profile. First investigations pointed to a beneficial protective role of habitual nut consumption over CHD[280]. However, these beneficial properties could be spread to many other conditions such as T2D, obesity and hypertension. Therefore, their potential role on weight control could be mainly attributed to their satiating properties (i.e. fiber, protein) and low GI. Nuts have an effect on blood pressure modulating SBP and DBP, mainly because nuts improve endothelial function and markers.

Moreover, some markers of atherosclerosis and CVD are also regulated by nut intake in terms of total cholesterol (TC), low-density lipoprotein (LDL-C) and HDL-C along with anti-atherogenic lipoprotein subfractions. Additionally, their modulation of glucose metabolism is by affecting both glycemia and insulinemia (**Figure 6**). Overall, many clinical trials have analyzed the potential beneficial effect of nuts (individual or mixed nuts) on different health outcomes. However, due to this thesis is focused on the beneficial effects of pistachio consumption in pre-D, we will report pistachios' contribution to different diseases and/or conditions with a main focus on glucose and insulin metabolism.

2.4 Diabetes- and pre-diabetes-nuts axes

The wide health benefits of nuts consumption are based on different mechanisms that may interact synergically to generate their health-promoting properties. Therefore, as we have seen, several molecular pathways and targets could be modulated by the incorporation of nuts into our healthy diet. However, a huge amount of data is currently pointing to a global beneficial role of nuts on T2D and other glucose-related derangements in both epidemiological and RCT.

2.4.1 Epidemiological studies

Apart from T2D, different epidemiological studies have evaluated the effect of nut intake on CHD. In fact, last pooled analysis of epidemiological studies stated that for each increase in one serving/day of nut consumption (28 g/d), there is a reduction of 28%, 29% and 17% in ischemic heart disease (IHD), CVD and all-cause death, respectively[281]. As T2D is considered a major risk factor for CHD, this last systematic review and meta-analysis performed by Luo et al. also assessed the relation between nut intake and incidence of T2D (from 4 prospective studies). The evaluation of the association between nut consumption and T2D[281] showed that for each increment in one serving/day of nut intake, the risk of T2D is reduced by 12% in case of the model not adjusted by BMI. However, when adjusting for BMI the association was not significant. Zhou et al. and Guo K. et al. also found no significant associations in their respective meta-analysis[282,283]. However, another meta-analysis (from 5 prospective studies and 1 RCT) also published in 2014, reported different results as they reported a significant 13% less risk of T2D in subjects consuming 4 servings/week[284].

These meta-analyses came from epidemiological research that has been conducted in different studies with cohorts of men or women. In 2002, the NHS ascertained the incidence of T2D by frequency of nut consumption (16 years of follow-up)[285]. Nut consumption was inversely associated with risk of T2D after multivariate adjustment for traditional risk factors. Compared with the category of "never/almost never", consuming 1 serving/d in the category >4 times/week was associated with a reduction of 27% in the risk of T2D (95% CI, 0.60-0.89). However, in the Iowa Women's Health Study[286] the association between nut consumption and T2D risk was less clear. In the 11 years of follow-up, postmenopausal women who ate nuts often had no reduced risk of diabetes compared to those who ate nuts occasionally after adjusting for multiple confounders (hazard ratio (HR): 1.26; 95% CI, 0.95-1.66). A subsequent report of a Chinese cohort from the Shanghai women health study of nearly 64,000 women followed up for 4.6 years also suggests a protective effect of nuts on diabetes risk[205]. This study showed an adjusted 20% risk reduction (95% CI; 0.68-0.93) between the lowest quintile (0.1 g) and upper quintile (3.1 g) of daily peanut consumption. At odds with the findings in women, results from the PHS[287] suggests no protective effect of nut consumption on diabetes risk in men. In this study 20,224 male participants were followed for an average of 19 years. Adjusted risks for development of diabetes were 0.87 (95% CI, 0.61-1.24) for those eating at least a daily serving of nuts, and the results were similar in lean or overweight/obese participants. Finally, last results came from the NHS I/II[288] which found significant results for walnut consumption. Pan et al. showed that walnut consumption was associated with a lower risk of T2D, thus participants consuming ≥2 servings/week of walnuts had a 24% less risk (95% CI, 0.62-0.94) of developing T2D compared with women who never/rarely consumed walnuts. **Figure 7** illustrates the findings of the main prospective studies relating nut consumption to the risk of developing T2D. In summary, regular consumption of nuts is clearly beneficial for CHD risk, but is less consistent and any protective role on diabetes risk must await further prospective and clinical studies.

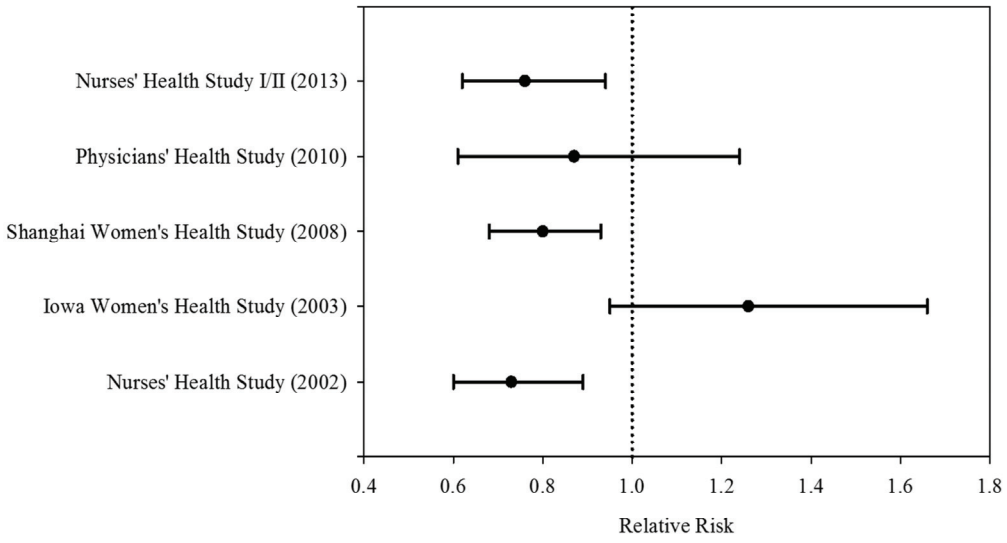

Figure 7. Relative risk for type 2 diabetes by nut consumption in different prospective studies. All data is relative risk and 95% confidence interval when comparing highest versus lowest nut consumption categories assessed with the full-adjusted model. Data obtained from [205,285–288].

2.4.2 Clinical trials

Even though epidemiological studies imply a huge amount of subjects, RCT are considered the gold standard for clinical trials when testing the effectiveness or efficacy of an intervention and for reporting causal inference. One of the most important clinical trials in the field of nuts in the context of a Mediterranean diet (MedDiet) is the PREDIMED trial. As we have previously commented, in the PREDIMED trial both MedDiets showed beneficial effects in terms of CV health and T2D prevention[120,289]. Subjects which followed MedDiet+nuts had a significant 28% and 18% lower incidence of major CV events and T2D, respectively. However, as we have seen in epidemiological studies, regular nut consumption and incidence of T2D is not fully conclusive. For this reason, few studies have evaluated the effect of nuts on glucose and insulin metabolism. There is a consensus in acute feeding studies as all of them reported beneficial effects on glucose and insulin parameters after nut consumption (reviewed by Ros[280]). However, inconsistent results have been found in medium and long-term clinical trials as some RCT showed positive benefits and others non-significant modulations. In fact, last results from a systematic review and meta-analysis after evaluating the effect of nuts on glycemic control in diabetes showed

significant reductions in HbA_{1c} and fasting glucose, whereas no significant effects considering fasting insulin and HOMA-IR[290]. Therefore, supporting their inclusion in a healthy diet and reinforcing the need for longer, higher quality RCT evaluating the nut-T2D axis. As it is the main topic of this thesis, we have recently reviewed the health properties of pistachios[271] (**Annex I & II**) exploring their beneficial effects on lipid profile, blood pressure and body weight modulation (through satiety), among others. Trials evaluating different parameters after pistachio consumption are shown in **Table 3**. These clinical trials were conducted to evaluate glucose/insulin control, lipid profile, weight changes and/or blood pressure. These studies have been conducted in different subjects: healthy, MetS or T2D subjects; in a parallel or crossover way, and the reference diet - when it exists – was a control diet, regular diet or an energy restricted diet. The main outcomes refer to glucose metabolism, with pistachio reducing fasting plasma glucose (and HbA_{1C})[291–293], but with no effect on insulin. Pistachio consumption modulates lipid profile by reducing TC, LDL-C and/or TG[291,292,294–299], and increasing HDL-C[295,297,300]. In which regards BW changes, pistachio diet have shown no increase in BW, and two studies have reported a decrease of BMI following PD compared with CD[293,301]. Blood pressure has been improved in three studies[293,302,303] and oxidative stress status or associated-markers have been ameliorated following pistachio diets[291,295,296].

However, beyond short-, medium- and long-term clinical trials, investigation of pistachio consumption has also been focused in acute studies. In fact, four acute intervention studies with pistachio consumption have been performed to evaluate different parameters. Two published studies have evaluated the satiating properties of pistachio nuts. The impact of consuming in-shell pistachios or pistachios kernels on fullness and caloric intake was evaluated in a randomized, cross-over, controlled feeding trial including 140 university students aged 18-24 years. Consumption of in-shell pistachios resulted in a lower calorie intake than consumption of kernels[304]. The same authors, in a second crossover feeding trial with 118 healthy individuals, demonstrated that the visual cue of the empty pistachios shells may have helped the participants to consume fewer pistachios and about 18% fewer calories[305]. In addition, among all nuts pistachios have a low GI, suggesting a possible effect on reducing postprandial glycemia and insulinemia, thereby potentially decreasing the risk of T2D. The effect of pistachios, consumed alone or combined with meals, on postprandial glycemia has been also evaluated[306,307]. Thus, whereas pistachios consumed alone had a minimal effect on postprandial glycemia, the addition of pistachios (56 g) to foods with a high GI

(pasta, parboiled rice and instant mashed potatoes) reduced, in a dose-dependent manner, the total postprandial glycemic response by 20 to 30%[307]. In a recent randomized, crossover study conducted on 20 subjects with MetS, 85.04g of pistachios consumed with bread reduced postprandial glycemia and increased GLP-1 levels compared with bread alone[306].

Table 3. Human clinical trials aimed to assess different outcomes through pistachio consumption.

Reference	Study	Glucose metabolism		Lipid profile							Body weight			Blood pressure		Oxidative stress	
First author (year)[reference]	Subjects, Design, Reference Diet	Glucose	Insulin	TC	TC/HDL-C	HDL-C	LDL-C	VLDL-C	LDL-C/HDL-C	TG	BMI	BW	WC	SBP	DBP	Markers	AC
Edwards (1999)[294]	MH, C, RD	-	-	↓	↓	NS	NS	-	↓	NS	-	-	-	-		-	-
Kocyigit (2006)[295]	HS, P, RD	-	-	↓	↓	↑	NS	-	↓	NS	NS	NS	-	-	-	↓ MDA	↑ AOP
Sheridan (2007)[300]	MH, C, RD	-	-	NS	↓	↑	NS	NS	↓	NS	NS	NS	-	NS	NS	-	-
Gebauer (2008)[296] **Kay (2010)**[308]**a** **Holligan (2014)**[309]**b**	HL, C, CD	-	-	↓	-	-	↓ (sLDL-P)[b]	NS	NS	NS	-	NS	-	NS	NS	↓ (ox-LDL)[a]	↑
Li (2010)[301]	OB, P, CD	NS	NS	NS	-	NS	NS	NS	NS	↓	↓	↓	-	-	-	-	-
Sari (2010)[291]	HS, S, NCD	↓	-	↓	↓	NS	↓	-	↓	↓	-	NS	-	NS	NS	↓ IL-6	↑
Aldemir (2011)[297]	ED, S, NCD	NS	-	↓	↓	↑	↓	-	↓	NS	-	-	-	NS	NS	-	-
Baer (2012)[299]	HS, C, CD	-	-	NS	-	NS	↓	-	-	NS	-	-	-	-	-	-	-
Wang (2012)[310]	MS, P, CD	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	-	-
West (2012)[302]	HL, C, CD	-	-	-	-	-	-	-	-	-	-	-	-	↓	NS	-	-
Nieman (2014)[311]	HC, C, CD	NS	-	-	-	-	-	-	-	-	-	NS	-	-	-	↓ CRP	-
Gulati (2014)[292]	MS, P, CD	↓	NS	↓	-	NS	↓	-	-	NS	-	NS	↓	-	-	↓ TNF-α	-
Parham (2014)[293]	DM, C, CD	↓ HbA_{1c}	-	-	-	-	-	-	-	-	↓	-	-	↓	NS	↓ CRP	-
Sauder (2014)[303] **Sauder (2015)**[298]**c**	DM, C, CD	NS[c]	NS[c]	↓[c]	↓[c]	NS[c]	NS[c]	NS[c]	NS[c]	↓[c]	-	NS[c]	-	↓	NS	-	-
Kasliwal (2015)[312]	MD, P, CD	NS	NS	NS	NS	NS	NS	-	-	NS	NS	NS	NS	NS	NS	-	-

Major findings in different clinical trials in pistachio supplemented diet versus control or regular diets, when applicable. Full information about each clinical trial could be found in their referenced paper or in Bulló's et al. review[271].

Abbreviations: ↓, decrease; ↑, increase; -, not reported; *NS*, non-significant. **Subjects:** *DM*, type 2 diabetes; *ED*, erectile dysfunction; *HC*, healthy cyclist; *HL*, high LDL-C (≥ 2.8 mmol/L); *HS*, healthy subjects; *MD*, mild dyslipidemia; *MH*, mild hypercholesterolemic; *MS*, metabolic syndrome; *OB*, obese. **Design:** *C*, crossover; *P*, parallel; *S*, sequential feeding trial. **Reference Diet:** *CD*, control diet; *NCD*, no control diet; *RD*, regular diet. **Other:** *AC*, antioxidant capacity; *AOP*, antioxidant potential; *BMI*, body mass index; *BW*, body weight; *CRP*, c-reactive protein; *DBP*, diastolic blood pressure; *HbA_{1C}*, glycated hemoglobin; *HDL-C*, high-density lipoprotein cholesterol; *IL-6*, interleukin-6; *LDL-C*, low-density lipoprotein cholesterol; *MDA*, malondialdehyde; *Ox-LDL*, oxidized-LDL; *SBP*, systolic blood pressure; *sLDL-P*, small-LDL-particle; *TC*, total cholesterol; *TG*, triglycerides; *TNF-α*, tumor necrosis factor-α; *VLDL-C*, very-low density lipoprotein cholesterol; *WC*, waist circumference. [a,b,c] refer to specific findings published in other manuscripts with the same subjects of study.

3. Metabolic changes in type 2 diabetes and pre-diabetes

The immune system (e.g. inflammatory responses) influences metabolic parameters such as IR and *vice versa*. Because of this cross-talk, the immune system plays a central role in metabolic changes associated with different diseases such as pre-D and T2D. In fact, systemic and local activation of the immune system accompanies obesity, and contributes to the development of IR, T2D and CVD[313,314]. However, it is not entirely understood which mechanisms trigger the onset of T2D in subjects at risk, but inflammation is a critical candidate. The consequence of acute hyperglycemia (accompanying T2D and pre-D) on circulating immune cells is largely unknown, but a pro-inflammatory role for hyperglycemia has been observed, as an OGTT increases transcript levels for TNF-α and IL-6 in peripheral white blood cells from MetS subjects, but not from healthy controls without MetS[315]. Therefore, blocking inflammation improves glucose tolerance in T2D patients, but whether *in vivo* hyperglycemia is able to initiate the activation of the immune system, without the influence of obesity, dyslipidemia, and high blood pressure, is not known. Many metabolic changes take place once T2D appears. However, there are also some parameters that vary before the onset of T2D. These molecules (e.g. inflammatory/oxidation markers, lipoprotein profile, gene expression, microRNAs) are of important relevance because they can monitor pre-D and T2D at the same time that they can be modulated by treatments to counteract these diseases.

3.1 Current biomarkers of type 2 diabetes and pre-diabetes

As we have seen, the routine diagnosis of T2D is generally based on the levels of fasting plasma glucose (**Table 1**)[316]. In 2009, an international expert panel also recommended the use of glycated hemoglobin (HbA_{1C}) levels for the diagnosis of T2D and pre-D (**Table 1**)[317]. These, in combination with measurements of lipid metabolites (i.e. triglycerides, cholesterol and lipoproteins), small molecule intermediates (e.g. α-hydroxybutyrate) and other metabolites (e.g. creatinine) help predict T2D with a probability of about 0.65-0.75[318]. Supplemental information about lifestyle factors as we have commented before (including physical inactivity, inadequate diet, tobacco smoking, and genetic risk factors) further increase the probability values to 0.85-0.90[318]. Research clinical studies also support the use of novel biomarkers like c-reactive protein (CRP), adipokines, incretins, cytokines, among others, in combination but not alone, for predicting T2D with similar probabilities as the traditional biomarkers[319–323] (**Table 4**). However, these biomarkers, both traditional and novel, are not entirely specific for T2D and are also predictive of other

metabolic disorders[324]. In addition to this, the detection of all these biomarkers is generally late and occurs in individuals already displaying metabolic imbalance[325]. Therefore, these biomarkers can predict T2D susceptibility only a few years before actual disease manifestation and hence cannot be used to assess disease susceptibility in the general population[321]. This calls for a need to identify new biomarkers (emerging biomarkers) that help to identify the individuals who are at risk of developing T2D - or associated diseases - much before the metabolic imbalance sets in, and thus help to a better assessment of therapeutics and drug targets.

Table 4. Association of different classical and novel biomarkers for pre-diabetes, type 2 diabetes and diseases associated with them

Association	Biomarkers	References
Classical biomarkers		
Higher levels in T2D subjects	Glycemia and HbA_{1C}	(326)
	Cholesterol, triglycerides	(327)
	Creatinine	(328)
	α-hidroxybutyrate	(329)
Lower levels in T2D subjects	High-density lipoprotein (HDL)	(327)
Novel biomarkers		
Higher levels in T2D subjects	C-reactive protein	(320,322)
	Ferritin	(320)
	Cytokines (e.g. IL-6, IL-18, TGF-β1, TNF-α)	(320,322)
	Chemokines (e.g. MCP-1)	(322)
	1-Deixysphingolipids	(319)
Lower levels in T2D subjects	Leptin and adipokines	(320,322,323)
Anti-diabetic	Incretins (e.g. GLP-1)	(320)
Emerging biomarkers		
Some miRNAs related to IFG/IGT and T2D	Tissue or circulating miRNAs	(330–333)
LP mean size and abundance related to CV risk in IFG/IGT and T2D	Lipoprotein subclases (size, concentration)	(334,335)

CV, cardiovascular; *GLP-1*, glucagon-like peptide-1; *HbA_{1C}*, glycated hemoglobin; *IL*, interleukin; *LP*, lipoprotein; *MCP*, monocyte chemoattractant protein; *miRNA*, microRNA; *TGF*, transforming growth factor; *TNF-α*, tumor necrosis factor-α. Modified from Guay et al. [324] and Herder et al.[321].

3.2 Inflammation and other related molecules

Different inflammatory pathways were suggested as the underlying mediators of T2D. In fact, chronic inflammation may lead to IR via insulin signaling[336]. The interaction of insulin with its receptor initiates insulin action. This activation and recruitment processes (also of the insulin receptor substrate (IRS) family proteins) lead to the activation of multiple downstream effectors that ultimately transmit the insulin signal to a branching series of intracellular pathways that regulate cell differentiation, growth, survival, and metabolism. The narrow link between inflammation processes and T2D have generated terms such as "inflammasome", an aggrupation of different proteins constituting a multiproteic complex, which process inactive pro-IL-1β and IL-18 into their active forms, regulating chronic inflammation and metabolic processes[337]. Inflammasome are composed by: i) pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) and intracellular nod-like receptors (NLR) that trigger downstream signaling cascades; ii) the adaptor molecule ASC (apoptosis-associated speck-like protein containing caspase recruitment domain); and iii) caspase-1. Several inflammasomes have been identified, and NLRP3 (nucleotide-binding domain and leucine-rich repeat containing NLR-pyrin domain containing 3) is the most extensively studied and is linked to T2D and β-cell destruction, obesity, and atherosclerosis[337]. TLRs are activated by a variety of dietary factors and endogenous signals in response to metabolic alterations induced by obesity. Free fatty acids bind to two different toll-like receptors, TLR2 and TLR4. Other inflammatory stimuli, such as ceramides and oxidized-LDL (ox-LDL), bind to and activate TLR4. The downstream pathways of both TLRs activate nuclear factor kB (NF-kB) and mitogen-activated protein kinases (MAPK) to inhibit insulin signaling via serine phosphorylation of the IRS-1 and induce transcription of the proinflammatory cytokines TNF-α and IL-6, as well as the biologically inactive pro-IL-1β and pro-IL-18, which require posterior processing to become active[338]. Some of the most important molecules implicated in inflammation and their link with IR and glucose metabolism processes are CRP, IL-6, leptin, adiponectin, resistin and ox-LDL.

3.2.1 C-reactive protein

C-reactive protein is a well-established component of the inflammatory response[339]. As the synthesis of CRP by the liver is induced by pro-inflammatory cytokines[340,341], plasma CRP levels provide a sensitive and quantitative assessment of overall inflammatory activity. Huge evidence has suggested that high CRP

levels are also a powerful predictor of CVD[342,343] as well as that chronic inflammation is a key mechanism underlying the development and progression of diseases such as atherosclerosis[344,345]. Importantly, CRP levels have also been shown to correlate strongly with IR and fasting insulin in non-diabetic subjects without clinical coronary artery disease[346]. Moreover, prospective studies have found high CRP levels to be predictive of the development of IR, MetS and T2D[347–349].

3.2.2 Interleukin-6

IL-6 is a pluripotent cytokine secreted by several tissues and cells, such as immune cells, endothelial cells, myocytes, and adipocytes[350] and involved in multiple physiological processes, including inflammation, tissue injury and host defense[351]. Adipose tissue is responsible for originating almost one third of circulating IL-6. Increased IL-6 levels are positively correlated with obesity, IR and T2D and are also predictive for the development of MetS and CVD. Inflammatory stimuli (e.g. TNF-α) and adipocyte hypertrophy favor increased IL-6 production. The mechanisms associated with IR and IL-6 are similar to those reported for TNF-α occurring via serine residue phosphorylation of IRS-1 and inhibition of lipoprotein lipase[350].

3.2.3 Leptin

Leptin was first described in the *Ob/Ob* mouse, which has spontaneous mutation in the leptin gene, and these mice become obese, hyperphagic, intolerant to cold, and unfertile[352]. Leptin is a non-glycosylated polypeptide which can circulate freely or as a stable complex associated with α2-macroglobulin[353]. It is secreted by adipose tissue usually 2 to 3 h after a meal. The circulating levels of leptin directly reflect the amount of energy stored in the adipose tissue; therefore, its production increases proportionally to the amount of body fat, and women have greater amounts than men[353]. Additionally, leptin is expressed at other sites, such as brown adipose tissue, the gastrointestinal tract, bone, lung, intestine, kidney, liver, skin, stomach, heart, placenta, and spleen, suggesting a pleiotropic effect[354]. The primary biological effect of leptin is the control of food intake and increased energy expenditure by activating its receptor, which is highly expressed in the hypothalamus. Leptin induces the expression of pro-inflammatory cytokines in macrophages and T cells and activates the same receptors activated by pro-inflammatory cytokines[354]. Therefore, in addition to its role in metabolism, leptin is important during the adipose tissue inflammation[353,354].

3.2.4 Adiponectin

Adiponectin is a plasma protein that circulates as a multimeric complex[355]. Adiponectin is highly expressed in the subcutaneous adipose tissue, and its levels decrease with increasing BW and adiposity[356]. The metabolic effects of adiponectin occur in several organs. In the liver, adiponectin improves insulin sensitivity, inhibits free fatty acid uptake and oxidation, and reduces the secretion of glucose. In adipose tissue, adiponectin increases glucose uptake and adipogenesis and in the muscles, stimulates glucose metabolism and accelerates the oxidation of free fatty acids[356]. The anti-inflammatory and anti-atherogenic effects of adiponectin are mediated through the inhibition of monocyte adhesion, macrophage growth, differentiation into foam cells, and remodeling of the vascular wall muscle[357].

3.2.5 Resistin

Human resistin is induced in response to various inflammatory stimuli such as lipopolysaccharide (LPS), TNF-α, or IL-6, and resistin itself induces pro-inflammatory cytokines, suggesting a role for resistin in inflammation in humans[358,359]. Although its expression was initially defined in adipocytes, significant levels of resistin expression in humans are mainly found in mononuclear leukocytes, macrophages, spleen and bone marrow cells[360]. Circulating resistin levels correlate with inflammatory markers in subjects with T2D, coronary atherosclerosis and chronic kidney disease, among others[359,361]. However, there is still debate regarding resistin's metabolic role in different diseases[362]. Resistin, originally described as an adipocyte-specific hormone, could be an important link between obesity, IR and T2D.

3.2.6 Oxidized-LDL

Ox-LDL has multiple pro-atherogenic properties, which include induction of cholesterol accumulation in macrophages as well as potent pro-inflammatory, immunogenic, apoptotic and cytotoxic activities[363]. Most of the pro-inflammatory properties of ox-LDL arise from bioactive products of LDL lipid peroxidation. As a result, LDL oxidation further propagates the inflammatory process in the arterial wall, thereby accelerating atherogenesis[363]. Atherosclerosis is regarded, therefore, as a chronic inflammatory disease of the arterial wall, mediated in part by ox-LDL and other pro-inflammatory agents, such as cytokines, produced by inflammatory macrophages and macrophage-derived foam cells. Moreover, systemic oxidative stress, as measured by ox-LDL, has been suggested to be a novel risk factor for T2D and

obesity[364], and the association between dyslipidemia and oxidation of LDL has been demonstrated in individuals with pre-D[365].

3.3 Other biomarkers linked to type 2 diabetes, pre-diabetes and cardiovascular diseases

Beyond established parameters (i.e. glucose, HOMA-IR), other molecules are currently being investigated as new "emerging" biomarkers for an early detection of pre-D, T2D and other associated CVD. Within them, lipoprotein size and composition together with microRNAs are postulated as good candidates[330–335].

3.3.1 Lipoprotein subclasses: particle concentration and size

Conventional CVD prevention strategies focus on decreasing LDL-C concentrations in order to ameliorate the CV risk. Therefore, classical lipid profile assessment (i.e. LDL-C, HDL-C, total cholesterol, triglycerides) has been used in order to monitor the CV risk of the population including subjects with pre-D and T2D. However, increasing data suggests that preventive and therapeutic strategies should be focusing on monitor abnormalities on lipoprotein subfractions (small, medium, large; **Figure 8**) beyond classical lipid profile[366]. Previous investigations of lipoprotein profile have been based most often on results regarding lipoprotein size and subclass quantification from gradient gel electrophoresis (GGE)[367]. Recently, a new procedure for quantifying plasma levels of lipoprotein subclass particles by proton nuclear magnetic resonance (NMR) spectroscopy has been developed[368,369]. NMR spectroscopy provides concentrations of VLDL, LDL, and HDL subspecies simultaneously, and NMR-determined subclasses correspond well with those obtained with established methods[369].

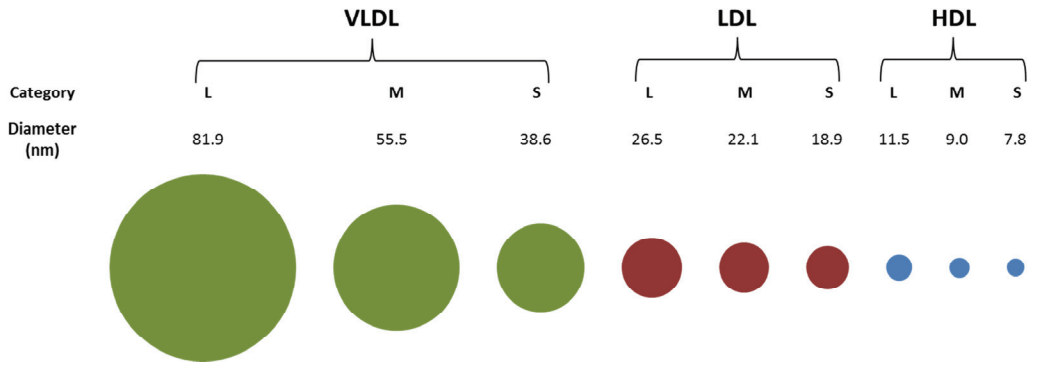

Figure 8. Lipoprotein subclasses. Lipoprotein subclasses and size, namely, large, medium, and small VLDL, LDL, and HDL as determined and classified according to the protocol of Mallol et al.[370]. **Lipoprotein:** *HDL*, high-density lipoprotein; *LDL*, low-density lipoprotein; *VLDL*, very low-density lipoprotein. **Size:** *L*, large; *M*, medium; *S*, small.

Several metabolic disorders as IR, adiposity, and T2D have been widely associated with alterations in the classical lipid profile[75,371–375]. Even so, the lipid and lipoprotein profile often displays other abnormalities in the pre-D stage, which may contribute to the subsequent increased risk of developing T2D and CVD. In this regard, large VLDL and small LDL particles have been related to a higher severity and incidence of coronary artery disease and T2D[334,376–379]. However, results on HDL-P subfractions are more controversial. Thus, whereas some studies showed an association between small HDL-P and coronary risk[380], others found that small and medium-sized HDL-P were associated with a lower risk of total stroke[381], and the implications on pre-D and T2D is not fully understood.

Currently, there is a lack of information on the potential modulatory effects of diet on lipoprotein subfractions and its effects on health and disease. As far as nutritional factors are concerned, both epidemiological and clinical studies have provided a body of scientific evidence on the cardioprotective effects of nuts and their lipid-lowering properties. A pooled analysis of 25 clinical trials including different types of nuts have showed a significant dose-related reduction in total cholesterol and LDL-C, but no effect on HDL-C or triglycerides (except in participants with hypertriglyceridemia) after nut consumption[382]. However, only two studies have assessed the effect of nut consumption on the composition and particle size of lipoprotein subfractions. These studies showed beneficial changes in lipid distribution in lipoprotein subfractions after walnut or pistachio consumption, and no changes in plasma lipid composition[309,383] along with the amelioration of other metabolic parameters. Overall, this reinforces the importance of evaluating classical and novel lipid profile to fully elucidate the CV risk associated with diseases such as pre-D and T2D.

3.3.2 Circulating microRNAs

Among RNA subtypes, microRNAs (miRNAs) are a family of endogenous, highly conserved, functional, single stranded RNA molecules of 22-24 nucleotides that can act by decreasing target mRNA levels or inhibiting their translation[324,384]. Although miRNAs originate in the cell nucleus (**Figure 9**), they have been detected in biological fluids such as blood, urine, saliva, sperm or breast milk[385]. miRNAs are excreted into the circulation and can regulate gene expression in cells/tissues far from where they were produced[386], suggesting an endocrine or paracrine signaling[387]. Interestingly, a single miRNA can

potentially bind and regulate the expression of hundreds of targets, whereas a specific mRNA can be targeted by different miRNAs.

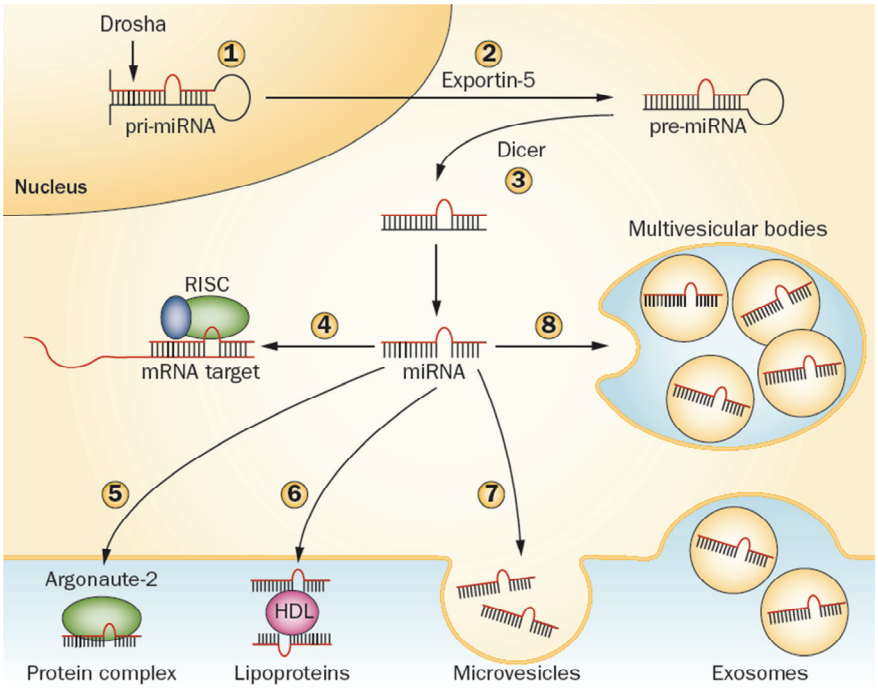

Figure 9. The biogenesis of microRNAs (miRNAs). Biogenesis of miRNAs begins with transcription of pri-miRNA (1). Pri-miRNAs are processed by Drosha to produce pre-miRNA hairpins which are exported into the cytosol (2). They are processed into 19-24 nucleotide mature miRNA duplexes by Dicer (3). One strand of the mature miRNA duplex is incorporated into the RISC (RNA-induced silencing complex) where it can regulate expression of target mRNAs (4) or either be degraded, or possibly prepared for export from the cell: in the presence of RNA-binding proteins (5); lipoproteins (6); microvesicles shed during membrane blebbing (7); or in exosomes derived from multivesicular bodies (8). Once in the extracellular space, these miRNAs could be taken up by other cells, degraded by RNases, or excreted. Obtained from Guay and Regazzi[324].

Their high stability makes peripheral circulating miRNAs potentially novel sources of noninvasive biomarkers of cancers and other diseases[388–392]. In fact, in body fluids, miRNAs are stable and resistant to treatment with ribonucleases, pH fluctuations (very high or low), extended storage at -20°C, boiling temperatures and up to ten freeze-thaw cycles[392]. This kind of stability in miRNA is observed because of the formation of complexes of circulating miRNAs with specific proteins (like Argonaute-2 and HDLs)[393,394]. Other reports have demonstrated that miRNA are packaged inside specialized microvesicles (exosomes)[395] (**Figure 9**).

The molecular mechanisms underlying T2D and pre-D are not totally understood. However, miRNAs emerge as powerful regulators not only for specific cell processes such as apoptosis, proliferation and differentiation[391], but also for several metabolic pathways including insulin secretion, glucose homeostasis and CHO and lipid metabolism[387,396–398]. For a silent disease like T2D, which develops slowly as a result of metabolic imbalance in the body and goes unnoticed for several years, alterations in miRNA expression profiles can be easily detected 5-10 years before the actual manifestation of T2D[324,399]. The first study that highlighted the usefulness of circulating miRNAs as early predictors of T2D and its vascular complications was conducted by Zampetaki et al. in 2010, using blood samples collected from randomly selected individuals from the Bruneck population from Italy[399]. Deregulated expression of five miRNAs, namely miR-15a, miR-28-3p, miR-29b, miR-126 and miR-223 was observed in pre-D or T2D subjects. Importantly, the determination of expression levels for these miRNAs was sufficient enough to identify 70% of T2D subjects, thereby suggesting a unique miRNA signature from plasma that could help with other factors distinguish between individuals with T2D from healthy controls at least in the Bruneck population[399]. Despite some methodological limitations, results regarding the use of miRNAs as biomarkers for assessing the risk of T2D and detecting and monitoring its associated complications are promising. However, few studies have evaluated miRNA modulation after a nutritional intervention in an attempt to determine the effect of particular nutrients and/or supplements rather than food or dietary patterns[400]. In humans, consumption of grape extract rich in resveratrol, and vitamin supplementation modulated specific miRNAs[401]. Similarly, a PUFA-enriched diet including almonds and walnuts was effective at modifying several miRNAs[402]. Therefore, there is a current lack of information regarding nutrition-miRNAs axis.

Overall, the presence of miRNAs in different human biofluids has resulted in the pursuit of miRNA-based biomarkers (miRNA signatures) for multiple diseases including cardiometabolic diseases. Undoubtedly, miRNAs are potent regulators of different disease-related pathways and they have important targets on cell metabolism that could be of relevant importance to fully determine the molecular mechanisms of several diseases.

B. JUSTIFICATION

UNIVERSITAT ROVIRA I VIRGILI
HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS
Pablo Hernández Alonso

B. JUSTIFICATION

Pre-diabetes and T2D are considered two major worldwide health problems. Pre-diabetes is a metabolic reversible state between normoglycemia and T2D which is characterized by insulin resistance and it is associated to a higher risk of cardiovascular disease and T2D. Therefore, we have the opportunity of acting on pre-D in order to stop its progression to T2D. Traditionally, subjects with T2D are prescribed with oral antidiabetic drugs and advised to increase physical activity and follow a healthy diet. However, it was not until few years ago when pre-D was managed as a condition or state that the patient needs to handle to stop its evolution to T2D. Nowadays, depending on the type (IFG or IGT) and presence of other metabolic diseases, pre-D subjects are advised to change their lifestyle and in some cases, they are prescribed antidiabetic drugs. The main problem is still the low amount of glucose control in the overall population which leads to many cases of undiagnosed T2D and pre-D.

Among the different modifiable factors that can affect the high worldwide prevalence of T2D, nutritional interventions and drug therapy are the major focus of research. However, there is still scarce information aiming to study the pre-D state. Despite of that, strong evidence from epidemiological studies supports the beneficial effects of certain food such as chronic nut consumption on T2D prevention. However, long-term clinical studies have found inconsistent results as both protective and non-significant results have been obtained. Moreover, as far as we are concerned, only one study in almonds has focused on the putative role of nuts on pre-diabetes stage.

Nuts are a rich and complex matrix of different macro- and micronutrients and other molecules such as antioxidants, thus they may have a role improving glucose metabolism via different targets. We decided to investigate the putative protective role of pistachio nut on pre-D as it has a healthy composition of fat (rich unsaturated fatty acids), fiber and protein, with a healthy profile of micronutrients (e.g. magnesium and potassium) and antioxidants, together with a moderate-to-low GI. In fact, due to their content in antioxidants, its intake may also affect some inflammatory and immune systemic factors, reducing also the risk of CVD. Particularly, they are rich in beta-carotene, which seems to be inversely related to T2D.

Therefore, pistachio consumption incorporated into a balanced diet could be a great nutritional tool to ameliorate the IR associated with pre-D state.

Most studies have evaluated the beneficial effects of nut consumption on few peripheral or systemic effects. In addition to this, our study assessed the beneficial effects of pistachio consumption using a multilevel approach at systemic, cellular and molecular levels.

Importantly, alterations in lipid profile are one of the main CV risk factors. Traditionally, the lipid profile consists in detecting and measuring the concentration of total cholesterol, HDL, LDL, VLDL and triglycerides. However, recent investigations have confirmed that conventional lipid profile should be integrated with the measurement of subtypes and concentration of lipoproteins in order to fully determine CVD risk. This analysis includes the determination of the size and subtypes (large, medium and small) of the different lipoproteins. Beyond lipid parameters, gene expression evaluation is a powerful procedure for determining how different factors may alter the expression of specific genes related to a wide range of outcomes. In fact, certain macro-, micronutrients, and whole foods may modify our expression profile related to diseases or pathological conditions (e.g. obesity, diabetes, cancer). Moreover, several investigations are being focused in the role of miRNAs over gene regulation. Different *in vivo* and *in vitro* research has showed circulating modulation of certain miRNAs in specific diseases or following particular treatments.

Overall, the matrix-based composition of nuts makes them an optimal food to counteract the derangements of preventable metabolic conditions such as pre-D and T2D. Therefore, the incorporation of nuts into a balanced and varied diet may be a proper nutritional lifestyle intervention to fight against stationary states such as pre-D or related diseases. This thesis is based in the huge need for nutritional interventions aiming to counteract the progression of this high prevalent and silent condition: pre-diabetes.

C. HYPOTHESIS

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Pablo Hernández Alonso

C. HYPOTHESIS

A chronic pistachio consumption, in the context of a balanced diet, may ameliorate the insulin resistance status of the pre-diabetes stage and improve the associated metabolic risk profile.

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D. OBJECTIVES

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D. OBJECTIVES

The main objective of this thesis was to analyze the effectiveness of a chronic pistachio diet in ameliorating pre-diabetes state by altering the insulin resistance state.

Specific objectives

To analyze the effect of chronic pistachio intake on:

1. Changes of plasma glucose, insulin levels and HOMA-IR.

2. Changes of inflammatory, satiety and other related metabolic risk markers.

3. Modulation of gene expression (glucose transport and inflammation) along with *in vitro* cellular glucose uptake at lymphocytes' level.

4. Changes in the classical (i.e. cholesterol level) and novel lipid profile (lipoprotein particle composition and size).

5. Modulation of circulating concentration of different microRNAs widely related to glucose and insulin metabolism.

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E. METHODOLOGY

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E. METHODOLOGY

1. The EPIRDEM study

The EPIRDEM (Effects of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus) study is a randomized, controlled, crossover trial with a 4-month dietary intervention in subjects with pre-D that was designed to assess the effect of a pistachio-rich diet on glucose and insulin metabolism and other metabolic-related risk factors.

1.1 Study population

Eligible participants were community-living men and women between 25 and 65 years of age with a BMI below 35 kg/m^2 and FPG levels between 100 and 125 mg/dL (IFG-pre-diabetes criteria following last ADA guidelines). Subjects were excluded if they met one of the following criteria:

a) Diabetes mellitus or using oral anti-diabetic drugs
b) Alcohol, tobacco or drug abuse
c) Frequent consumption of nuts or known history of allergy to them
d) Use of plant sterols, psyllium, fish oil supplements and multivitamins, vitamin E or other antioxidant supplements
e) Bad dentures, involving difficulty to chew pistachios
f) Following a vegetarian or a hypocaloric diet to lose weight
g) Being pregnant or wishing to become pregnant 9 months before or during the study, lactating 6 weeks before or during the study
h) Significant liver, kidney, thyroid or other endocrine diseases
i) Medical, dietary or social conditions that hinder compliance to the intervention

Participants were recruited from primary care centers affiliated to the University Hospital of Sant Joan de Reus. We obtained executed informed consent from all study participants. Moreover, the Institutional Review Board of the University Hospital of Sant Joan de Reus (Spain) approved the study protocol in September 2011. The trial was registered in Clinical Trials (National Institutes of Health) with identifier NCT01441921.

1.2 Interventions

After the participants fulfilled the inclusion criteria, they were randomly assigned to two different intervention periods of 4 months each (**Figure 10**). A 15-days run in period preceded the four-month treatment period and a 2-week wash-out period separated the two crossover periods. At baseline, data on medical history, physical examination and fasting blood for biochemical analysis were collected. Subjects who met the inclusion criteria were randomly assigned to one of the two different intervention periods using a computer-generated random-number table. They were instructed to follow a normo-caloric diet that provided 50% of energy as carbohydrates, 15% as protein, and 35% as total fat during the 2 weeks proceeding each study period. The isocaloric diet was individually calculated using WHO equations adjusted by the estimated energy expenditure in physical-activity leisure-time.

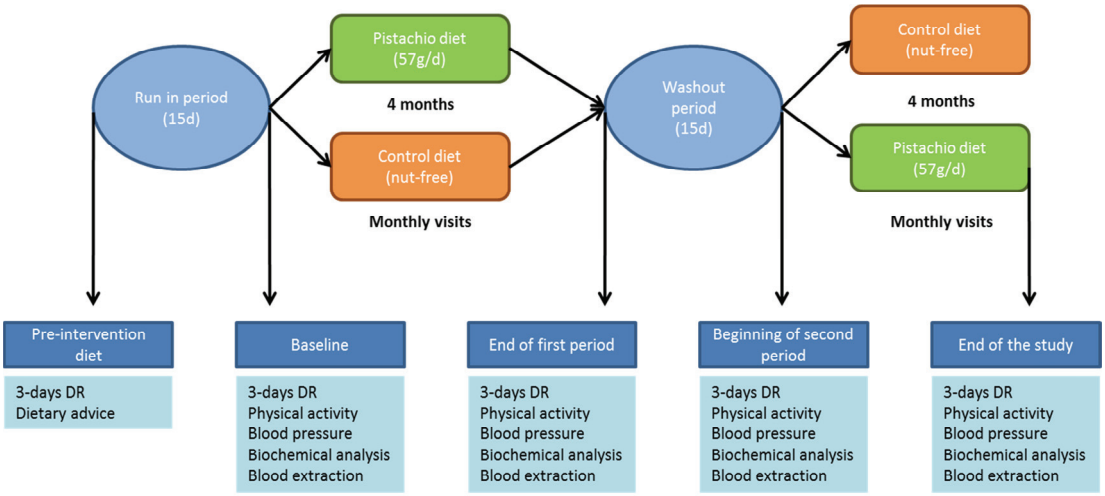

Figure 10. Design of the EPIRDEM study. Intervention period and scheduled visits. *DR*, dietary record; *EPIRDEM*, Effects of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus.

After the 2-week run-in period, subjects were randomized to the control diet (CD) or the pistachio supplemented diet (PD). Detailed nutrient composition is shown in **Table 5**. Participants allocated to the pistachio diet were supplemented with 2 ounces of pistachio (57 g/day) that were provided free to the subjects. The nuts were half roasted, and half roasted and salted. In the control diet, the energy intake of other fatty foods, mostly olive oil, was adjusted to compensate for the energy from pistachios included in

the PD. Participants were provided with detailed dietary instructions, including biweekly menus and seasonal recipes according to the type of diet - pistachio or control.

Table 5. Nutrient composition of each intervention diet

Nutrient	Control diet	Pistachio diet
Carbohydrate (% of energy)	55	50
Protein (% of energy)	15	15
Total fat (% of energy)	30	35
Saturated fatty acids (% of energy)	7	7
Monounsaturated fatty acids (% of energy)	16	20
Polyunsaturated fatty acids (% of energy)	3	5
Fibre (g in a 2000 Kcal diet)	35.8	41.0
Cholesterol (mg in a 2000 Kcal diet)	191	166
Na (mg in a 2000 Kcal diet)	2,100	2,100

Nutrient composition of research diets using Spanish[403] and United States Department of Agriculture (*USDA*) (Release 28)[269] databases.

1.3 Specific measurements

Individual examinations were scheduled at baseline, after the run-in period - consisting in two weeks - and then monthly until the end of each intervention period.

1.3.1 Anthropometry, body composition and blood pressure

Weight, height and waist circumference were determined at each visit with subjects wearing light clothes and no shoes. BMI was then calculated. At the beginning and end of each four-month intervention period, body composition was measured by bio-electrical impedance analysis (Human-Im-Scan, Dietosystem, Spain). Blood pressure was measured in the non-dominant arm, using a validated semiautomatic oscillometer (Omron HEM-705CP, Hoofddorp, Netherlands) in duplicate with a five-minute interval between each measurement.

1.3.2 Dietary and physical activity assessment

At the beginning of each intervention period, and every two months subsequently, dietary intake was estimated using the mean of 3-day dietary records including two workdays and a weekend day. Energy and nutrient intake were calculated using Spanish food composition tables[403]. Adherence to the intervention period was assessed by counting the empty sachets of pistachio administered and by measuring plasma lutein-zeaxanthin and γ-tocopherol levels with liquid chromatography coupled to a 6490 triple quadrupole mass spectrometer (QqQ/MS) (Agilent Technologies, Palo Alto, USA). Physical activity was evaluated using the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire[404,405]. All participants received instructions to maintain constant physical activity during the study. A questionnaire was used to assess gastrointestinal side effects such as mouth symptoms, bloating, fullness, or indigestion, altered bowel habit, and any other diet-related symptoms.

1.3.3 Biological samples: collection and storage

Fasting blood samples were collected at baseline and at the end of each four-month intervention period. Therefore, each participant was submitted to four visits with blood extraction. We collected blood samples to obtain EDTAK2-plasma, heparin-plasma, citrate-plasma and serum.

1.4 Analyses

1.4.1 Routinely biochemistry, inflammation and other related parameters

Fasting plasma glucose and serum lipid profile were determined using standard enzymatic automated methods. LDL-C was estimated using the Friedewald formula in those subjects whose triglyceride levels were less than 400 mg/dL. Plasma tissue factor (TF) (AssayPro, MO, USA), thromboxane B2 (TXB2) (Cayman Chemical Company, MI, USA) and oxidized LDL (Ox-LDL) (Qayee Bio-Technology, Shanghai, China) were measured using enzyme-linked immunosorbent assay commercial kits (ELISA). Soluble receptor for advanced glycation end-products (sRAGE) (Sigma-Aldrich, MO, USA), interleukin-18 (IL-18) (Boster Biological Technology, CA, USA) and interleukin-6 (IL-6) (R&D Systems, MN, USA) were determined in serum using commercial ELISAs according to their specific procedures. Plasma fibrinogen, Von Willebrand factor (vWF), platelet factor-4 (PF-4), GIP, GLP-1, insulin, leptin, C-peptide, adiponectin, plasminogen activator inhibitor-1 (PAI-1) and resistin, were determined using a MILLIPLEX® MAP Plex Kit (Merck

Millipore, MA, USA). Insulin resistance and insulin secretion were estimated by the HOMA-IR and HOMA-BCF methods, respectively[51].

$$HOMA - IR = \frac{Glucose\left(\frac{mg}{dL}\right) * Insulin\left(\frac{mU}{mL}\right)}{405} \qquad HOMA - BCF = \frac{360 * Insulin\left(\frac{mU}{mL}\right)}{Glucose\left(\frac{mg}{dL}\right) - 63}$$

1.4.2 Cellular glucose transport

In order to perform the cellular glucose transport (CGT) assessment, we evaluated the *in vitro* uptake of glucose by lymphocytes collected from the study subjects. Lymphocytes were obtained from blood, collected in heparin tubs and incubated in a final concentration of $4 \cdot 10^6$ cells/well with 10µM of a fluorescent glucose analog, the 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) (Molecular Probes, Life Technologies, OR, USA) in RPMI 1640 (Gibco, Life Technologies, MD, USA), as previously described[406]. After 30 minutes of incubation (37°C under 95% humidity, 5% CO_2, and atmospheric O_2 levels) reaction was stopped by adding cold 1X phosphate buffered saline (PBS), and fluorescence was read (λ_{ex}= 485 nm; λ_{em}= 538nm) (Fluoroskan Ascent, Thermo Fisher S., Madrid, Spain).

1.4.3 Gene expression analysis

Total RNA was extracted from blood samples using the Tempus Spin RNA Isolation Kit (Ambion, Madrid, Spain). The purity and quantification of the RNA were determined by spectrophotometry (NanoDrop; Thermo Fisher Scientific, Madrid, Spain). The retrotranscription step was performed using the high-capacity cDNA reverse transcription kit (Invitrogen, Madrid, Spain). Gene expression was analyzed using Taqman gene expression assays (Applied Biosystems, Madrid, Spain) in a 7900HT Fast Real-Time PCR System (AB, Madrid, Spain). Primers and probes for the genes were selected as follows: *TLR2, TLR4, SLC2A3* (solute carrier family 2, facilitated glucose transporter member 3; also known as *GLUT-3*), *SLC2A4* (solute carrier family 2, facilitated glucose transporter member 4; also known as *GLUT-4*), *IL-6* and *RETN* (Resistin) were analyzed. Genes tested as endogenous controls were selected from a pool of genes that Genevestigator (http://www.genevestigator.com/gv)[407] gave as potential reference genes based on previous experiments on T2D, and glucose or insulin impairment. Hence, *ACTB* (beta-actin), *HPRT1* (hypoxanthine phosphoribosyltransferase 1), *RPL30* (Ribosomal protein L30) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta) were initially tested as putative reference genes. *HPRT1* and *YWHAZ* were finally selected as the best endogenous for our samples, so they

were both used as reference genes for target gene normalization. Each qPCR reaction contained 2 µL of 1:7 fold diluted cDNA, 0.5 µL of the validated TaqMan assay for target or reference gene, 5 µL of Universal TaqMan PCR master mix (Applied Biosystems, Madrid, Spain) and 2.5 µL RNAse-free water (Ambion, Madrid, Spain). All measurements were performed in duplicate and qPCR data were acquired using sequence detector software (SDS version 2.4, Applied Biosystems, Madrid, Spain). Mean quantification cycle (Cq) values (also known as threshold cycle, Ct, values) were calculated as the average of two replicates if the SD was less than 0.5; otherwise the sample was repeated in the qPCR experiment. Normalized expression was calculated for individual samples using the $2^{-\Delta Cq}$ method (Expression Suite Software v1.0.3). Changes in expression were shown as the ratio between final and baseline values.

1.4.4 Plasma lipoprotein analysis

Lipoprotein analysis of plasma samples by 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY) was performed using a previous protocol[370]. This protocol measures lipid concentrations (i.e., triglycerides and cholesterol), sizes and particle numbers for VLDL (38.6 to 81.9 nm), LDL (14.7 to 26.6 nm) and HDL (6.0 to 10.9 nm) classes, as well as the particle numbers of nine subclasses (namely large, medium and small VLDL, LDL and HDL, respectively). To determine lipoprotein size, the methyl signal was surface fitted with the numbers of functions so that the nine lipoprotein subclasses could be determined. The mean particle size of every main fraction was derived by averaging the NMR area of each fraction by its associated size. To obtain particle-weighted lipoprotein sizes, each NMR area was divided by its associated volume. The particle numbers of each lipoprotein main fraction were calculated by dividing the lipid volume by the particle volume of a given class. The lipid volumes were determined by using common conversion factors to convert concentration units obtained from the partial least-squares (PLS) models into volume units. The relative areas of the lipoprotein components used to decompose the 2D spectra were used to derive the particle numbers of the nine lipoprotein subclasses.

1.4.5 Circulating microRNAs

Plasma was obtained by centrifugation at 1,800 x g for 15 min at 4ºC, using K_2EDTA-coated vacutainer tubes, which were aliquoted and stored at -80ºC until use. We centrifuged thawed plasma samples for 10 min at 2,000 x g at RT, and then transferred the upper supernatant (3/4) to a fresh tube to proceed with total RNA isolation. Total RNA (including miRNA) was isolated from plasma samples using the mirVana

PARIS Isolation Kit (Applied Biosystems, Darmstadt, Germany) according to manufacturer's protocol. As there is no appropriate endogenous miRNA for plasma samples we decided to use a spike-in exogenous control. *Caenorhabditis elegans* miR-39 (cel-miR-39, Qiagen, Madrid, Spain), which lacks sequence homology to human miRNAs, was selected to control for extraction procedures and to normalize the experimental quantitative-RT-PCR (qRT-PCR). Previous results in serum and plasma samples have shown that the use of spike-in is one of the most stable normalizing methodologies[408,409]. The final recovery of these synthetic oligonucleotides was measured for each sample using TaqMan qRT-PCR miRNA assays (Applied Biosystems, Darmstadt, Germany). To validate the success of each extraction, we also assessed Cq values obtained for a serial dilution (10^{-1}) of cel-miR-39. We selected seven human circulating miRNAs (hsa-miR-15a-5p, -miR-21-5p, -miR-29b-3p, -miR-126-3p, -miR-192-5p, -miR-223-3p, -miR-375) widely related to glucose metabolism, IR status, pre-D status and biomarkers of T2D, using updated reviews[324,387,398,410] and databases[411–413] together with the spike-in cel-miR-39. A fixed 3 µL volume of RNA eluate was the input for the RT. cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) with the following conditions per sample reaction: 6 µL of RT primer pool of miRNAs (Applied Biosystems, Darmstadt, Germany), 0.3 µL of dNTPs (100 mM), 1.5 µL of 10X RT Buffer, 3.01 µL of nuclease-free water, 0.19 µL of RNase Inhibitor (20 U/µL) and 1 µL of MultiScribe Reverse Transcriptase (50 U/µL) up to a final volume of 15 µL. Primer pool was obtained using each 5X RT miRNA-specific primers (250 nM) which were pooled and diluted in 1× Tris-EDTA (TE) buffer to obtain a final dilution of 0.05x each. The reaction mixtures were incubated at 16ºC for 30 min, 42ºC for 30 min and 85ºC for 5 min before being held at 4ºC in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Darmstadt, Germany). Various qPCR reactions were carried out using 2 µL of pre-diluted cDNA (1:5 in nuclease-free water), 5µL of TaqMan® Universal Master Mix II, no UNG (Applied Biosystems, Darmstadt, Germany), 0.5 µL of gene-specific primer/probe (TaqMan® MicroRNA Assay) and 2.5 µL of nuclease-free water in a final volume of 10 µL. The reaction mixtures were incubated at 50ºC for 2 min, 95ºC for 10 min and 45 cycles of 95ºC for 15s, ending with 60ºC for 1 min in an ABI 7900HT real-time PCR system (Applied Biosystems). We ran a negative control (nuclease-free water) and an inter-plate control (same cDNA sample) in each plate and several negative RT controls (nuclease-free water instead of RNA). All measurements were performed in duplicate, and qPCR data were acquired using sequence detector software (SDS version 2.4, Applied Biosystems, Darmstadt, Germany). Mean

quantification Cq values were calculated as the average of two replicates if the SD was 0.5; otherwise, the sample was repeated in the qPCR experiment. Normalized expression was calculated for individual samples using the $2^{-\Delta Cq}$ method (Expression Suite Software v1.0.3) using cel-miR-39 as normalizer. Changes in expression were shown as the ratio between final and baseline values.

1.5 Statistical analysis

We used standard methods (Shapiro-Wilk) to test the normal distribution of the analyzed variables before statistical analysis. Normalized relative $\log_{10}$ ratios were used for statistical tests, when necessary. The anti-log-transformed values were after reported. The descriptive data of participants at baseline and differences during the intervention periods are shown as means and 95% CI for continuous variables, and number (%) for categorical variables. Differences in all variables were evaluated by analysis of variance (ANOVA), with intervention diet as the independent and repeated measures factor. A paired t-test was used to evaluate differences in each outcome from baseline to the end of the period. The differences in variable changes between dietary intervention periods were analyzed by an analysis of covariance (ANCOVA) test using baseline values as a covariate. When we compute the percentage of change from baseline, baseline values were not used as a covariate. Diet sequence (order of diet treatments) was analyzed as an independent factor in order to evaluate the carry-over effect. As it was not significant, we were able to analyze data as a cross-over study. All statistical analyses were conducted using intention-to-treat (ITT) and *per protocol* (PP) approaches. ITT analysis included all randomized participants with at least fulfilling all baseline measurements. The PP analysis excluded participants who did not attend the last visit. The sample size was calculated considering changes in HOMA-IR as a primary endpoint. Assuming an α error of 0.05 and 90% power, we required a sample size of 40 subjects to identify significant differences in HOMA-IR similar to those observed in a previous study by our group[414]. We finally selected a sample of 54 individuals to compensate for an expected 17% loss in participants. All analyses were done using SPSS v20.0 and v22.0 (SPSS Inc, Chicago, IL). All tests were 2-sided, and significance was defined as $P<0.05$.

F. RESULTS

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F. RESULTS

Publication 1.

Title: Beneficial effect of pistachio consumption on glucose metabolism, insulin resistance, inflammation, and related metabolic risk markers: a randomized clinical trial.

Authors: Pablo Hernández-Alonso; Jordi Salas-Salvadó; Mònica Baldrich-Mora; Martí Juanola-Falgarona and Mònica Bulló.

Year and Journal information: 2014. *Diabetes care*. 37(11): 3098 – 3105.

IF: 8.420; 7/128; Q1; Endocrinology & Metabolism.

Abstract:

OBJECTIVE: To examine whether a pistachio-rich diet reduces the prediabetes stage and improves its metabolic risk profile.

RESEARCH DESIGN AND METHODS: Prediabetic subjects were recruited to participate in this Spanish randomized clinical trial between 20 September 2011 and 4 February 2013. In a crossover manner, 54 subjects consumed two diets, each for 4 months: a pistachio-supplemented diet (PD) and a control diet (CD). A 2-week washout period separated study periods. Diets were isocaloric and matched for protein, fiber, and saturated fatty acids. A total of 55% of the CD calories came from carbohydrates and 30% from fat, whereas for the PD, these percentages were 50 and 35%, respectively (including 57 g/day of pistachios).

RESULTS: Fasting glucose, insulin, and HOMA of insulin resistance decreased significantly after the PD compared with the CD. Other cardiometabolic risk markers such as fibrinogen, oxidized LDL, and platelet factor 4 significantly decreased under the PD compared with the CD ($P < 0.05$), whereas glucagon-like peptide-1 increased. Interleukin-6 mRNA and resistin gene expression decreased by 9 and 6%, respectively, in lymphocytes after the pistachio intervention ($P < 0.05$, for PD vs. CD). SLC2A4 expression increased by 69% in CD ($P = 0.03$, for PD vs. CD). Cellular glucose uptake by lymphocytes decreased by 78.78% during the PD ($P = 0.01$, PD vs. CD).

CONCLUSIONS: Chronic pistachio consumption is emerging as a useful nutritional strategy for the prediabetic state. Data suggest that pistachios have a glucose- and insulin-lowering effect, promote a healthier metabolic profile, and reverse certain metabolic deleterious consequences of prediabetes.

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Pablo Hernández Alonso

Beneficial Effect of Pistachio Consumption on Glucose Metabolism, Insulin Resistance, Inflammation, and Related Metabolic Risk Markers: a Randomized Clinical Trial

Pablo Hernández-Alonso,[1] *Jordi Salas-Salvadó,*[1,2] *Mònica Baldrich-Mora,*[1,2] *Martí Juanola-Falgarona,*[1,2] *and Mònica Bulló*[1,2]

Diabetes Care 2014;37:1–8 | DOI: 10.2337/dc14-1431

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RESULTS

Fasting glucose, insulin, and HOMA of insulin resistance decreased significantly after the PD compared with the CD. Other cardiometabolic risk markers such as fibrinogen, oxidized LDL, and platelet factor 4 significantly decreased under the PD compared with the CD ($P < 0.05$), whereas glucagon-like peptide-1 increased. Interleukin-6 mRNA and resistin gene expression decreased by 9 and 6%, respectively, in lymphocytes after the pistachio intervention ($P < 0.05$, for PD vs. CD). SLC2A4 expression increased by 69% in CD ($P = 0.03$, for PD vs. CD). Cellular glucose uptake by lymphocytes decreased by 78.78% during the PD ($P = 0.01$, PD vs. CD).

CONCLUSIONS

Chronic pistachio consumption is emerging as a useful nutritional strategy for the prediabetic state. Data suggest that pistachios have a glucose- and insulin-lowering effect, promote a healthier metabolic profile, and reverse certain metabolic deleterious consequences of prediabetes.

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The global prevalence of impaired glucose tolerance (IGT) is estimated at 316 million people and is expected to increase to 471 million by 2035 (1). Data from the National Health and Nutrition Examination Survey (NHANES) suggest that impaired fasting glucose is twice as prevalent as IGT (2), suggesting that the prevalence of prediabetes (impaired fasting glucose plus IGT) may actually affect more than 900 million people globally (3). This metabolic condition is preventable and treatable if recognized early. If left untreated, however, the yearly progression rate to diabetes is estimated to be between 3.5 and 7.0%. This has far-reaching implications for morbidity and mortality (4). Reflecting the global burden of prediabetes, its high rate of progression to type 2 diabetes mellitus (T2DM), and the increased risk of micro- and macrovascular complications and death (5), its prevention and treatment is one of the major goals of public health strategies. This goal goes beyond just T2DM; it looks to prevent the risk of cardiovascular disease (CVD). Therefore, healthy lifestyles, including diet, stand out as effective strategies for reversing the prediabetic stage and its related complications (6). In fact, lifestyle interventions and the use of antidiabetic drugs in prediabetic subjects have been associated with a lower progression to diabetes (7,8) and a greater reduction in CVD risk (9).

Since the publication of the results of the Adventist Health Study, which showed that nut consumption was associated with a lower risk of coronary heart disease (10), a large body of evidence from epidemiological studies and controlled clinical trials has demonstrated the beneficial impact of nut consumption on health outcomes and total mortality (reviewed in Ros [11]) (12,13). The data consistently show nut consumption's cholesterol-lowering effect, and there is emerging evidence that it also benefits other cardioprotective mechanisms, such as endothelial function (14), and susceptibility to LDL cholesterol (LDL-c) oxidation and inflammatory processes (15). Nevertheless, the results of epidemiological studies on the impact of nut consumption on T2DM incidence, one of the major risk factors for CVD, are less conclusive (11,16,17). The results of several interventional studies examining the effects of nut-enriched diets on glycemic control and insulin sensitivity have also been inconsistent (reviewed by Bulló et al. [18]). Thus, whereas acute feeding studies reported reduced postprandial glucose and insulin excursions after nut consumption in healthy or T2DM subjects (19,20), medium- and long-term changes in fasting glucose or insulin sensitivity in response to nut diets are still controversial (21–23). Recently, it has been reported that acute consumption of pistachios can attenuate postprandial glucose levels when they are consumed with carbohydrates (24,25). It has also been demonstrated that chronic consumption of pistachios can improve blood glucose levels, LDL-c, and some inflammatory markers, but not fasting insulin, in healthy subjects and subjects with metabolic syndrome (14,26,27).

To the best of our knowledge, to date, no studies have evaluated the chronic effect of nut intake on glucose metabolism and insulin resistance in the prediabetic state. The EPIRDEM (Effects of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus) study is a randomized, controlled, crossover trial with a 4-month dietary intervention in prediabetic subjects that aimed to assess the effect of a pistachio-rich diet on glucose and insulin metabolism and other metabolic-related risk factors.

RESEARCH DESIGN AND METHODS

Participants

Eligible participants were community-living men and women between 25 and 65 years of age in Reus (Spain) with a BMI <35 kg/m^2 and fasting plasma glucose levels between 100 and 125 mg/dL. Subjects were excluded if they met one of the following criteria: *1*) diabetes or using oral antidiabetic drugs; *2*) alcohol, tobacco, or drug abuse; *3*) frequent consumption of nuts or known history of allergy to them; *4*) use of plant sterols, psyllium, fish oil supplements and multivitamins, vitamin E, or other antioxidant supplements; *5*) bad dentures, involving difficulty to chew pistachios; *6*) following a vegetarian or a hypocaloric diet to lose weight; *7*) being pregnant or wishing to become pregnant 9 months before or during the study or lactating 6 weeks before or during the study; *8*) significant liver, kidney, thyroid, or other endocrine diseases; or *9*) medical, dietary, or social conditions that hinder compliance to the intervention.

Participants were recruited from primary care centers affiliated with the Universitari Hospital of Sant Joan de Reus. Executed informed consent was obtained from all study participants. The institutional review board of the Universitari Hospital of Sant Joan de Reus approved the study protocol in September 2011. The trial was registered in Clinical Trials (National Institutes of Health) with identifier NCT01441921.

Study Design

The design of the crossover clinical trial is illustrated in Supplementary Fig. 1. A 15-day run-in period preceded the 4-month treatment period. A 2-week washout period separated the two crossover periods. At baseline, data on medical history, physical examination, and fasting blood for biochemical analysis were collected. Subjects who met the inclusion criteria were randomly assigned to one of the two different intervention periods using a computer-generated random-number table. They were instructed to follow a normocaloric diet that provided 50% of energy as carbohydrates, 15% as protein, and 35% as total fat during the 2 weeks preceding each study period. The isocaloric diet was individually calculated using World Health Organization equations adjusted by the estimated energy expenditure in physical-activity leisure time. After the 2-week run-in period, subjects were randomized to the control diet (CD) or the pistachio-supplemented diet (PD). The main characteristics of both intervention diets are shown in Supplementary Table 1. Participants allocated to the PD were supplemented with 2 ounces of pistachio (57 g/day) that was provided free to the subjects. The nuts were half roasted, and half roasted and salted. In the CD, the energy intake of other fatty foods, mostly olive oil, was adjusted to compensate for the energy from pistachios included in the PD. Participants were provided with detailed dietary instructions, including biweekly menus and seasonal recipes according to the type of diet, pistachio or control. A qualitative example of a daily menu is shown in Supplementary Table 2.

Measurements

Individual examinations were scheduled at baseline, after a 2-week run-in, and then monthly until the end of each intervention period.

Anthropometry, Body Composition, and Blood Pressure

Weight and waist circumference were determined at each visit with subjects wearing light clothes and no shoes. BMI was calculated. At the beginning and end of each 4-month intervention period, body composition was measured by bioelectrical impedance analysis (Human-Im-Scan; Dietosystem, Barcelona, Spain). Blood pressure was measured in the nondominant arm, using a validated semiautomatic oscillometer (HEM-705CP; OMRON, Hoofddorp, the Netherlands) in duplicate with a 5-min interval between each measurement.

Dietary and Physical Activity Assessment

At the beginning of each intervention period, and every 2 months subsequently, dietary intake was estimated using the mean of 3-day dietary records including two workdays and a weekend day. Energy and nutrient intake were calculated using Spanish food composition tables (28). Adherence to the intervention period was assessed by counting the empty sachets of pistachio administered and measuring plasma lutein-zeaxanthin and γ-tocopherol levels with a liquid chromatograph coupled to a 6490 QqQ/MS (Agilent Technologies, Palo Alto, CA) as previously described. The detailed protocol is provided in Supplementary Methods 1.

Physical activity was evaluated using the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire (29). All participants received instructions to maintain constant physical activity during the study. A questionnaire was used to assess gastrointestinal side effects such as mouth symptoms; bloating, fullness, or indigestion; altered bowel habit; and any other diet-related symptoms.

Biological Samples, Collection, and Storage

Fasting blood samples were collected at baseline and at the end of each 4-month intervention period. Plasma fasting glucose and serum lipid profile were determined using standard enzymatic automated methods. LDL-c was estimated using the Friedewald formula in those subjects whose triglyceride levels were $<$400 mg/dL. Plasma tissue factor (TF) (AssayPro, St. Charles, MO), thromboxane B_2 (TXB2) (Cayman Chemical Company, Ann Arbor, MI), and oxidized LDL (ox-LDL) (Qayee Bio-Technology, Shanghai, China) were measured using ELISA commercial kits. Soluble receptor for advanced glycation end products (sRAGE) (Sigma-Aldrich, St. Louis, MO), interleukin-18 (IL-18) (Boster Biological Technology, Fremont, CA), and IL-6 (R&D Systems, Minneapolis, MN) were determined in serum using commercial ELISAs. Plasma fibrinogen, von Willebrand factor (vWF), platelet factor 4 (PF4), gastric inhibitory polypeptide (GIP), GLP-1, insulin, leptin, C-peptide, adiponectin, plasminogen activator inhibitor-1 (PAI-1), and resistin (RETN) were determined using a MILLIPLEX MAP Plex Kit (Merck Millipore, Billerica, MA). Both insulin resistance and insulin secretion were estimated by the HOMA of insulin resistance (HOMA-IR) and HOMA of β-cell function (HOMA-BCF) methods.

Cellular Glucose Uptake

Lymphocytes were obtained from blood, collected in heparin tubs, and incubated in a final concentration of 4×10^6 cells/well with 10 μmol/L of deoxy-D-glucose-2^{3}H(G) (2-NBDG) (Molecular Probes, Life Technologies, Eugene, OR) in RPMI 1640 (Gibco, Life Technologies, Frederick, MD), as previously described (30). After 30 min of incubation (37°C under 95% humidity, 5% CO_2, and atmospheric O_2 levels), the reaction was stopped by adding cold 1× PBS, and fluorescence was read (λ_{ex} = 485 nm; λ_{em} = 538 nm) (Fluoroskan Ascent; Thermo Fisher Scientific, Madrid, Spain).

Gene Expression Analysis

Total RNA was extracted from blood samples using the Tempus Spin RNA Isolation Kit (Ambion, Madrid, Spain). The purity and quantification of the RNA were determined by spectrophotometry (NanoDrop; Thermo Fisher Scientific, Madrid, Spain). The retrotranscription step was performed using the high-capacity cDNA reverse transcription kit (Invitrogen, Madrid, Spain). Gene expression was analyzed using Taqman gene expression assays (AB, Madrid, Spain) in a 7900HT Fast Real-Time PCR System (AB). Primers-probes for the genes were selected as follows: TLR2 (Toll-like receptor 2) (Hs00152932_m1), TLR4 (Hs00152939_m1), SLC2A3 (solute carrier family 2, facilitated glucose transporter member 3) (Hs00359840_m1), SLC2A4 (Hs00168966_m1), IL-6 (Hs00985639_m1), and RETN (Hs00220767_m1) were analyzed. Genes tested as endogenous controls were selected from a pool of genes that Genevestigator (http://www.genevestigator.com/gv) gave as potential reference genes based on previous experiments on T2DM, and glucose or insulin impairment. Hence, ACTB (β-actin) (Hs99999903_m1), HPRT1 (hypoxanthine phosphoribosyltransferase 1) (Hs99999909_m1), RPL30 (ribosomal protein L30) (Hs00265497_m1), and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ) (Hs00237047_m1) were initially tested as putative reference genes. HPRT1 and YWHAZ were finally selected as the best endogenous for our samples, so they were both used as reference genes for target gene normalization. Each quantitative PCR (qPCR) reaction contained 2 μL of 1:7 fold diluted cDNA, 0.5 μL of the validated TaqMan assay for target or reference gene, 5 μL of Universal TaqMan PCR master mix (Applied Biosystems, Madrid, Spain), and 2.5 μL RNase-free water (Ambion). All measurements were performed in duplicate, and qPCR data were acquired using Sequence detector software (SDS version 2.4; Applied Biosystems). Mean quantification cycle (Cq) values (also known as threshold cycle, Ct, values) were calculated as the average of two replicates if the SD was $<$0.5; otherwise the sample was repeated in the qPCR experiment. Normalized expression was calculated for individual samples using the $2^{-\Delta Cq}$ method (ExpressionSuite Software v1.0.3). Changes in expression were shown as the ratio between final and baseline values. The nontemplate sample did not produce a signal in any assay, and internal control showed a mean variation between independent plates of $<$8% for all the genes tested.

Statistical Analysis

Descriptive data of participants at baseline and differences during the intervention periods are shown as means and 95% CIs for continuous variables and number (%) for categorical variables. Differences in all variables were evaluated by ANOVA, with intervention diet as the independent and repeated measures factor. Diet sequence (order of diet treatments) was analyzed as independent factor, but as it was not significant, it was not further considered. The differences in

variable changes between dietary intervention periods were analyzed by an ANCOVA test using baseline values as a covariate. All statistical analyses were conducted using intent-to-treat (ITT) and per protocol (PP) approaches. ITT analysis included all randomized participants at least fulfilling all baseline measurements. The PP analysis excluded participants who did not attend the last visit, and results are shown in the Supplementary Data. The sample size was calculated considering changes in HOMA-IR as a primary end point. Assuming an α error of 0.05 and 90% power, we required a sample size of 40 subjects to identify significant differences in HOMA-IR similar to those observed in a previous study by our group (31). We finally decided on a sample of 54 individuals to compensate for an expected 17% loss in participants. All analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL). All tests were two sided, and significance was defined as $P < 0.05$.

RESULTS

Study Participants

The study flowchart is shown in Supplementary Fig. 2. A total of 108 participants were assessed for eligibility. Of these, 30 declined to participate and 24 did not meet the inclusion criteria. Fifty-four participants were randomly assigned to one of the two intervention sequences. During the pistachio period, 5 of the 54 randomized participants (9.25%) dropped out of the study for personal reasons. No gastrointestinal side effects were observed during the trial. The baseline characteristics of the study participants are shown in Table 1. No changes in medication were reported during the study. No significant differences were observed between dietary interventions at baseline in any of the analyzed parameters.

Dietary Compliance

As expected, plasma lutein-zeaxanthin levels significantly increased (Table 2) during the PD compared with the CD (mean 222.53 nmol/L [95% CI 150.89, 294.16] and −16.65 nmol/L [−63.46, 30.17], respectively; $P < 0.001$) (Table 2). The results were similar for γ-tocopherol (684.53 nmol/L [469.97, 899.09] and −99.76 nmol/L [−264.84, 65.33], respectively; $P < 0.001$).

Glucose Metabolism and Lipid Profile

In the ITT analysis, glucose and insulin circulating levels decreased significantly ($P < 0.001$) in the PD intervention (Table 2) compared with the CD. Subsequently, HOMA-IR decreased during the PD intervention compared with the CD (mean −0.69 [95% CI −1.07, −0.31] and 0.97 [0.49, 1.44], respectively; $P < 0.001$). No significant changes, however, were observed in HOMA-BCF and glycated hemoglobin (HbA_{1c}) between PD and CD. Although lipid profile did not change significantly between groups, LDL-c showed a nonsignificant reduction after pistachio intervention compared with the increase observed in the CD period (mean −4.00 mg/dL [95% CI −9.03, 1.03] and 1.20 mg/dL [−4.35, 6.74], respectively; $P = 0.16$). The PP results were similar to those analyzed by the ITT approach (Supplementary Table 3).

Lymphocyte Glucose Uptake

A significant decrease in cellular glucose transport was observed in lymphocytes after a PD compared with the CD (−78.78% [95% CI 127.46, −30.10] and 15.86% [−34.55, 66.27], respectively; $P = 0.01$) (Fig. 1*A*). Results by the PP approach provided equivalent results (Supplementary Fig. 3*A*).

Inflammation and Metabolic Risk Markers

Table 3 depicts baseline inflammatory and metabolic risk markers and changes during the intervention. No significant differences were observed in these parameters at baseline according to the sequence of randomization. After the PD, fibrinogen and PF-4 significantly decreased in comparison with the CD. In contrast, GLP-1 increased during the PD intervention compared with the CD. There were no different effects between diets for the other inflammatory and metabolic biomarkers analyzed, although all changes were in the expected direction. Although nonsignificant, ox-LDL decreased during the PD but increased during the CD ($P = 0.10$). In the PP approach, ox-LDL decreased significantly during the PD intervention but increased during the CD (−7.22 ng/mL [95% CI −21.28, 6.83] and 11.24 ng/mL [2.82, 19.66], respectively; $P = 0.03$) (Supplementary Table 4).

Gene Expression in Peripheral Lymphocytes

The gene expression data (Fig. 1*B*) show that IL-6 mRNA decreased by a significant 9% in the PD in comparison with

Table 1—Baseline characteristics of the study population

Variable	Whole population	Male	Female
Subjects, *n* (%)	54	29 (53.7)	25 (46.3)
Age (years)	55 (53.4, 56.8)	54.5 (52.1, 56.9)	55.7 (53.1, 58.3)
Weight (kg)	77.6 (74.8, 80.3)	82.7 (79.0, 86.4)	71.6 (68.7, 74.5)*
BMI (kg/m^2)	28.9 (28.2, 29.6)	28.4 (27.4, 29.5)	29.4 (28.4, 30.3)
Waist circumference (cm)	94.7 (92.8, 96.6)	96.9 (94.2, 99.6)	92.1 (89.7, 94.5)*
Systolic blood pressure (mmHg)	134 (130, 137)	137 (132, 142)	130 (125, 136)
Diastolic blood pressure (mmHg)	81 (79, 83)	82 (78, 85)	81 (78, 83)
Dyslipidemia, *n* (%)	27 (50)	15 (51.7)	12 (48.0)
Hypertension, *n* (%)	23 (42.6)	11 (37.9)	12 (48.0)
Statins, *n* (%)	5 (9.3)	2 (6.9)	3 (12.0)
Fibrates, *n* (%)	2 (3.7)	2 (6.9)	0 (0.0)
ACE inhibitors, *n* (%)	6 (11.1)	2 (6.9)	4 (16.0)
Beta-blockers and other antihypertensive drugs, *n* (%)	13 (24.1)	7 (24.1)	6 (24.0)
Leisure-time physical activity (kcal/day)	347 (307, 387)	328 (267, 387)	370 (317, 423)

Data are given as mean (95% CI) or number (%). *Significant differences between sexes.

Table 2—Baseline and changes after intervention period in anthropometric and biochemical parameters

	PD		CD		Treatment effect
Characteristics	Baseline	Change	Baseline	Change	*P* value
Waist circumference (cm)	94.19 (92.27, 96.11)	0.63 (−0.02, 1.29)	94.80 (92.88, 96.72)	−0.44 (−1.30, 0.43)	0.08
Weight (kg)	77.29 (74.46, 80.14)	0.40 (−0.08, 0.08)	77.74 (74.93, 80.54)	0.21 (−0.74, 0.32)	0.07
BMI (kg/m^2)	28.76 (28.03, 29.49)	0.12 (−0.06, 0.30)	28.90 (28.21, 29.59)	−0.07 (−0.28, 0.12)	0.12
Systolic blood pressure (mmHg)	133.89 (129.75, 138.03)	−3.64 (−6.23, −1.06)	132.17 (128.70, 135.64)	−1.47 (−4.40, 1.46)	0.22
Diastolic blood pressure (mmHg)	80.48 (78.33, 82.63)	0.19 (−1.25, 1.61)	79.94 (77.80, 82.07)	−0.25 (−1.59, 1.10)	0.75
Fasting plasma glucose (mg/dL)	116.24 (112.37, 120.11)	−5.17 (−8.14, −2.19)*	108.06 (104.27, 111.84)	6.72 (4.38, 9.07)	<0.001
HbA_{1c} (%)	5.92 (5.82, 6.02)	−0.03 (−0.12, 0.05)	5.87 (5.75, 5.99)	0.03 (−0.03, 0.10)	0.13
HbA_{1c} (mmol/mol)	41.18 (40.10, 42.27)	−0.46 (−1.38, 0.46)	40.66 (39.36, 41.96)	0.32 (−0.47, 1.11)	0.14
Fasting plasma insulin (mU/mL)	14.36 (12.65, 16.07)	−2.04 (−3.17, −0.92)*	11.44 (9.81, 13.07)	2.51 (1.02, 4.00)	<0.001
HOMA-IR	4.22 (3.66, 4.77)	−0.69 (−1.07, −0.31)*	3.10 (2.64, 3.56)	0.97 (0.49, 1.44)	<0.001
HOMA-BCF	98.22 (86.35, 110.09)	−3.46 (−11.45, 4.53)	96.76 (78.45, 115.07)	−0.25 (−9.65, 9.16)	0.62
Total cholesterol (mg/dL)	217.44 (208.10, 226.79)	−3.74 (−9.20, 1.72)	213.83 (205.48, 222.19)	2.11 (−4.41, 8.63)	0.15
HDL-c (mg/dL)	54.28 (50.43, 58.13)	1.33 (−1.65, 4.32)	54.42 (51.00, 57.83)	1.34 (−0.71, 3.39)	0.96
LDL-c (mg/dL)	137.93 (128.18, 147.67)	−4.00 (−9.03, 1.03)	136.77 (128.85, 144.70)	1.20 (−4.35, 6.74)	0.16
VLDL-c (mg/dL)	25.19 (22.23, 28.14)	−1.04 (−3.20, 1.13)	22.74 (20.32, 25.15)	0.36 (−1.65, 2.38)	0.28
Total cholesterol/ HDL-c ratio	4.29 (3.87, 4.72)	−0.19 (−0.38, −0.01)	4.14 (3.80, 4.47)	−0.05 (−0.25, 0.15)	0.31
LDL-c/HDL-c ratio	2.78 (2.39, 3.17)	−0.15 (−0.32, 0.02)	2.68 (2.40, 2.96)	−0.04 (−0.20, 0.11)	0.33
Triglycerides (mg/dL)	125.81 (111.07, 140.56)	−4.96 (−15.72, 5.79)	113.89 (101.70, 126.07)	7.47 (−7.87, 22.81)	0.15
Lutein-zeaxanthin (nmol/L)	452.90 (399.86, 505.95)*	222.53 (150.89, 294.16)	465.01 (408.27, 521.75)	−16.65 (−63.46, 30.17)	<0.001
γ-Tocopherol (nmol/L)	625.49 (464.20, 786.78)*	684.53 (469.97, 899.09)	755.06 (603.04, 907.09)	−99.76 (−264.84, 65.33)	<0.001

ITT analysis, *n* = 54. All values are means (95% CI). Intragroup analysis was assessed by the paired Student *t* test. Basal-adjusted changes between groups were analyzed using adjusted ANOVA of repeated measurements. HDL-c, HDL cholesterol. *Significant difference ($P < 0.05$) between baseline and end of a particular intervention period.

the CD ($P = 0.004$). After the PD, RETN gene expression was also lower than after the PD (6%, $P = 0.04$). Facilitated glucose transporter gene expression assessed by SLC2A3 and SLC2A4 showed different patterns. SLC2A3 expression showed no significant changes between groups, whereas SLC2A4 showed a significant 69% increase during the CD compared with the PD ($P = 0.03$, PD vs. CD). Neither of the TLR genes analyzed, TLR2 and TLR4, differed statistically between treatments. The PP analysis (Supplementary Fig. 3*B*) showed similar results.

CONCLUSIONS

Several studies have evaluated the effect of nut intake on glycemia and insulin levels (21–23), but few have focused on pistachio intake (14,27) and none have been conducted in the prediabetic stage. This is the first study to evaluate the chronic effect of nut intake on glucose metabolism, insulin resistance, inflammation, and related-metabolic risk markers, at both the systemic and molecular levels in prediabetic subjects. The results of this crossover, randomized, controlled clinical trial provide evidence that chronic consumption of pistachio decreases glucose and insulin levels, thus improving insulin resistance and other inflammatory and metabolic risk markers.

A beneficial dose-response effect of pistachios has been observed on postprandial glycemia and insulinemia when pistachios are consumed with carbohydrate foods (24,25). Likewise, significant improvements have been reported in fasting blood glucose after healthy young men consumed 20% of their daily energy intake as pistachios for 4 weeks (14). Moreover, a 24-week randomized clinical trial conducted on subjects with metabolic syndrome demonstrated a significant improvement in fasting glucose but not insulin levels (27). In contrast, a 12-week weight reduction diet that included pistachios had no effect on glucose or insulin when compared with an isocaloric diet containing pretzels (32). The results of our study demonstrate that pistachio intake has a chronic lowering effect on fasting glucose and a beneficial effect on insulin resistance measured indirectly by HOMA-IR. The effect of pistachios on insulin metabolism could be partly explained by an increase in GLP-1 levels during pistachio consumption. GLP-1 and GIP are gastric hormones that stimulate pancreatic insulin secretion and suppress glucagon secretion in a

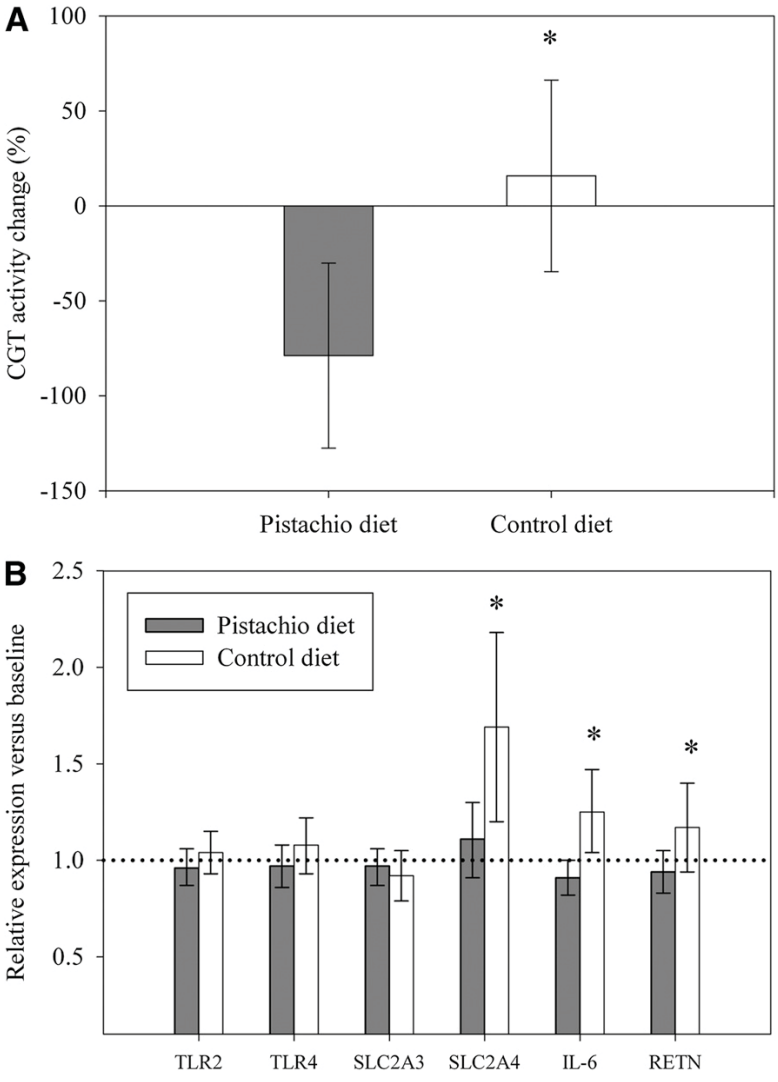

Figure 1—Changes in peripheral leukocyte gene expression and cellular glucose uptake. *A*: Percentage of change in cellular glucose transport (CGT) activity through both intervention diets. *B*: Expression changes across intervention diets. *$P < 0.05$, significant differences in changes between dietary interventions.

glucose-dependent manner (33). The acute upregulatory effect of pistachio intake on the secretion of GLP-1 was reported by Kendall et al. (25) in individuals with metabolic syndrome. In the current study, we extend their results and show that pistachios have a long-term stimulating effect on GLP-1 and insulin-sparing effects in patients with prediabetes.

In contrast to previous epidemiological and clinical studies reporting a cholesterol-lowering effect of nut consumption, including pistachios, mainly in hypercholesterolemic subjects but also in normolipidemic and healthy subjects (18), we failed to find any significant differences between the changes in total cholesterol and LDL-c between intervention diets. Our results are in agreement with those previously published on overweight and metabolic syndrome individuals (31,34,35) and support the hypothesis that the lipid profile of metabolic syndrome subjects is less likely to undergo changes because alteration in the cholesterol homeostasis inherent to the obese state makes them resistant to downregulating cholesterol (36).

Because they are richer in lutein, β-carotene, and γ-tocopherol than other nuts, pistachios tend to have a beneficial effect on the inflammatory and oxidative state. An improvement in the circulating levels of IL-6, CRP, TNF, and adiponectin has previously been reported in both healthy men and individuals with metabolic syndrome (14,27). A dose-dependent reduction in oxidized LDL by a 4-week pistachio consumption diet has also been demonstrated (26). This trial found a significant reduction in ox-LDL during the PD in the PP approach. Although no change in circulating IL-6 levels was observed, other inflammatory and metabolic risk markers underwent a significant reduction. Reductions in fibrinogen and PF4 were measured during the PD.

Unlike circulating measures, however, lymphocyte expression of IL-6 and RETN was significantly lowered by pistachio consumption. These data clearly suggest a pistachio-mediated impact on classical inflammatory markers of glucose and insulin metabolism. Because lymphocytes play an important role in inflammatory responses, they need to be tightly regulated if health is to be maintained. Correct fuel requirements of these cells are vitally important for their growth and effector function (37). Therefore, whereas hypoglycemia decreases the viability of peripheral blood cells, hyperglycemia leads to an excessive glucose uptake, thus promoting immune hyperactivity and compromising health (38). Further, a significantly increased SLC2A4 protein expression on the surface of lymphocytes has been described in both diabetic subjects (39) and subjects with impaired glucose metabolism (40). We have demonstrated that pistachio consumption leads to a significant decrease in SLC2A4 mRNA (in parallel with a lower increase in cellular glucose uptake). This decrease can be explained by circulating insulin levels after dietary treatment. Therefore, these results suggest a potential mechanism by which pistachios could lead to a healthier systemic inflammatory profile. Taken together, our results reinforce the potential anti-inflammatory effect of pistachio nuts and their role in chronic inflammatory diseases.

Several strengths and limitations of our study deserve comment. Among its strengths are the crossover randomized

Table 3—Baseline and changes after intervention period in inflammatory, satiety, and other related markers

	PD		CD		Treatment effect
Characteristics	Baseline	Change	Baseline	Change	*P* value
Platelets ($\times 10^3/\mu L$)	225.70 (214.39, 237.02)	−3.90 (−8.07, 0.27)	224.00 (209.63, 238.37)	−5.68 (−12.93, 1.57)	0.89
Lymphocytes ($\times 10^3/\mu L$)	1.87 (1.74, 2.00)	−0.02 (−0.08, 0.04)	1.87 (1.72, 2.02)	−0.01 (−0.08, 0.06)	0.67
Fibrinogen (ng/mL)	71.18 (65.62, 76.75)	−2.24 (−5.94, 1.46)	65.13 (60.45, 69.81)	3.24 (−0.19, 6.67)	0.02
Tissue factor (pg/mL)	195.71 (143.16, 248.26)	16.33 (−10.60, 43.27)	225.57 (169.88, 281.26)	−14.46 (−40.35, 11.43)	0.16
PAI-1 (pg/mL)	158.37 (134.65, 182.10)	13.26 (−13.81, 40.33)	177.42 (136.40, 218.43)	−12.91 (−42.41, 16.59)	0.15
vWF (ng/mL)	0.61 (0.47, 0.75)	0.27 (0.00, 0.55)	0.99 (0.59, 1.39)	−0.04 (−0.53, 0.45)	0.15
PF4 (ng/mL)	0.20 (0.07, 0.32)	−0.07 (−0.13, −0.02)	0.12 (0.09, 0.15)	0.00 (−0.02, 0.02)	0.01
TXB2 (ng/mL)	2.20 (1.60, 2.80)	−0.18 (−0.55, 0.19)	2.20 (1.69, 2.71)	0.13 (−0.33, 0.58)	0.31
C-peptide (ng/mL)	1.83 (1.68, 1.98)	−0.06 (−0.18, 0.06)	1.75 (1.60, 1.91)	0.01 (−0.11, 0.14)	0.34
GIP (pg/mL)	32.55 (26.99, 38.11)	−0.04 (−4.17, 4.09)	34.19 (29.17, 39.21)	−1.31 (−5.08, 2.46)	0.61
GLP-1 (pg/mL)	46.62 (37.24, 56.00)	4.09 (1.25, 6.94)*	47.40 (37.77, 57.04)	−0.59 (−2.98, 1.80)	0.01
RETN (pg/mL)	105.70 (89.89, 121.50)	2.29 (−7.14, 11.73)	108.63 (89.55, 127.71)	4.19 (−21.01, 29.39)	0.67
IL-6 (pg/mL)	1.48 (1.18, 1.77)	−0.13 (−0.32, 0.06)	1.39 (1.08, 1.71)	0.01 (−0.27, 0.29)	0.27
IL-18 (pg/mL)	104.60 (87.06, 122.13)	−8.99 (−16.04, −1.94)*	115.88 (92.54, 139.22)	−12.14 (−23.50, −0.77)	0.64
Leptin (ng/mL)	10.83 (8.40, 13.27)	−0.40 (−1.49, 0.68)	10.60 (8.41, 12.79)	0.09 (−0.70, 0.88)	0.34
Adiponectin (ng/mL)	69.16 (54.33, 84.00)	−3.12 (−8.49, 2.25)	66.89 (52.42, 81.35)	−0.64 (−6.59, 5.31)	0.56
Ox-LDL (ng/mL)	279.07 (253.29, 304.85)	−2.64 (−16.14, 10.86)	267.45 (247.87, 287.03)	10.20 (2.51, 17.89)*	0.10
sRAGE (pg/mL)	319.12 (242.22, 396.01)	20.16 (−17.60, 57.92)	312.01 (237.75, 386.27)	10.93 (−15.37, 37.22)	0.72

ITT analysis, $n = 54$. All values are means (95% CI). Intragroup analysis was assessed by the paired Student *t* test. Basal-adjusted changes between groups were analyzed using basal-adjusted ANOVA of repeated measurements. PAI-1, plasminogen activator inhibitor-1; sRAGE, soluble receptor of advanced glycation end products. *Significant difference ($P < 0.05$) between baseline and end of a particular intervention period.

design, its medium-term duration, the presence of dietary compliance markers, and the two-level approach (systemic and cellular). However, by design, an important limitation of this trial is that it focuses on the prediabetic patient. Results may only be extrapolated to healthy subjects or subjects with T2DM with caution pending further studies.

This study's findings build on the literature by describing the health-enhancing properties of pistachios along with nuts in general, and in particular the benefits for glucose metabolism and cardiovascular health that could be attributed to their higher amount of polyunsaturated fatty acid and other bioactive compounds such as procyanidins and carotenoids (11). In the context of a healthy diet, pistachios have important glucose- and insulin-lowering effects and improve the inflammatory profile by downregulating both the expression and the circulating levels of several metabolic risk markers. Overall, the integration of pistachios into a balanced diet is proving to be a safe nutritional strategy that can help reverse the risks associated with prediabetes. These benefits may well provide novel dietary tools for managing other prevalent chronic inflammatory diseases as well. Future studies should be designed to investigate the impact of regular pistachio consumption on the development and management of T2DM and other chronic diseases.

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Publication 2.

Title: Effect of pistachio consumption on plasma lipoprotein subclasses in pre-diabetic subjects.

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Abstract:

BACKGROUND AND AIMS: Nuts have been demonstrated to improve several cardiovascular risk factors and the lipid profile in diabetic and pre-diabetic subjects. However, analysis of conventional serum lipid profiles does not completely explain the atherogenic risk associated with pre-diabetes. We therefore investigated whether chronic consumption of pistachio modifies the lipoprotein subclasses to a healthier profile in pre-diabetic subjects.

METHODS AND RESULTS: Randomized cross-over clinical trial in 54 subjects with pre-diabetes. Subjects consumed a pistachio-supplemented diet (PD, 50% carbohydrates, 33% fat, including 57 g/d of pistachios daily) and a control diet (CD, 55% carbohydrates, 30% fat) for 4 months each, separated by a 2-week wash-out. Diets were isocaloric and matched for protein, fiber and saturated fatty acids. Nuclear magnetic resonance (NMR) was performed to determine changes in plasma lipoprotein subclasses. Small low-density lipoprotein particles (sLDL-P) significantly decreased after pistachio consumption compared to the nut-free diet (P = 0.023). The non-high-density lipoprotein particles (non-HDL-P i.e. VLDL-P plus LDL-P) significantly decreased under the PD compared to CD (P = 0.041). The percentage of sHDL-P increased by 2.23% after the PD compared with a reduction of 0.08% after the CD (P = 0.014). Consequently, the overall size of HDL-P significantly decreased in the PD (P = 0.007).

CONCLUSION: Chronic pistachio consumption could modify the lipoprotein particle size and subclass concentrations independently of changes in total plasma lipid profile, which may help to explain the decreased risk of cardiovascular disease and mortality associated with those individuals who frequently consumed nuts.

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HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS
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Effect of pistachio consumption on plasma lipoprotein subclasses in pre-diabetic subjects

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Abstract *Background and aims:* Nuts have been demonstrated to improve several cardiovascular risk factors and the lipid profile in diabetic and pre-diabetic subjects. However, analysis of conventional serum lipid profiles does not completely explain the atherogenic risk associated with pre-diabetes. We therefore investigated whether chronic consumption of pistachio modifies the lipoprotein subclasses to a healthier profile in pre-diabetic subjects.
Methods and results: Randomized cross-over clinical trial in 54 subjects with pre-diabetes. Subjects consumed a pistachio-supplemented diet (PD, 50% carbohydrates, 33% fat, including 57 g/d of pistachios daily) and a control diet (CD, 55% carbohydrates, 30% fat) for 4 months each, separated by a 2-week wash-out. Diets were isocaloric and matched for protein, fiber and saturated fatty acids. Nuclear magnetic resonance (NMR) was performed to determine changes in plasma lipoprotein subclasses. Small low-density lipoprotein particles (sLDL-P) significantly decreased after pistachio consumption compared to the nut-free diet ($P = 0.023$). The non-high-density lipoprotein particles (non-HDL-P i.e. VLDL-P plus LDL-P) significantly decreased under the PD compared to CD ($P = 0.041$). The percentage of sHDL-P increased by 2.23% after the PD compared with a reduction of 0.08% after the CD ($P = 0.014$). Consequently, the overall size of HDL-P significantly decreased in the PD ($P = 0.007$).
Conclusion: Chronic pistachio consumption could modify the lipoprotein particle size and subclass concentrations independently of changes in total plasma lipid profile, which may help to explain the decreased risk of cardiovascular disease and mortality associated with those individuals who frequently consumed nuts.
Registration number: This study is registered at www.clinicaltrials.gov as NCT01441921.

Abbreviations: CV, cardiovascular; CD, control diet; DOSY, diffusion-ordered ^{1}H NMR spectroscopy; DSTE, double stimulated echo; EPIRDEM, Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus; FID, finite impulse decay; HDL, high-density lipoprotein; IRAS, Insulin Resistance Atherosclerosis Study; ITT, intention-to-treat; LDL, low-density lipoprotein; LED, longitudinal eddy current delay; NMR, nuclear magnetic resonance; -P, particle; PD, pistachio diet; PLS, partial least-squares; PP, per protocol; T2DM, type 2 diabetes mellitus; VLDL, very low-density lipoprotein.

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Introduction

Atherogenic dyslipidemia is a common feature of type 2 diabetes (T2DM) characterized by high levels of serum triglycerides, low HDL-cholesterol (HDL-C) concentrations and a relative increase in the number of small dense LDL particles (sLDL-P). Nonetheless, the lipid and lipoprotein profile often displays other abnormalities in the pre-diabetic stage, which may contribute to the subsequent increased risk of developing T2DM and cardiovascular (CV) disease. In this regard, large VLDL and small LDL particles have been related with a higher severity and incidence of coronary artery disease and type 2 diabetes [1]. However, results on HDL subfractions are more controversial. Thus, whereas some studies showed an association between small HDL-P and coronary risk [2], others found that small and medium-sized HDL particles were associated with a lower risk of total stroke [3].

While conventional cardiovascular prevention strategies focus on decreasing LDL-C concentrations, increasing data suggests that preventive and therapeutic strategies could be focusing on lipoprotein subfractions abnormalities [4]. However, there is a lack of information on the potential modulatory effects of diet on lipoprotein subfractions and its effects on health and disease.

As far as nutritional factors are concerned, both epidemiological and clinical studies have provided a body of scientific evidence on the cardioprotective effects of nuts and their lipid-lowering properties. A pooled analysis of 25 clinical trials including different types of nuts showed a significant dose-related reduction in total cholesterol and LDL-C, but no effect on HDL-C or triglycerides (except in participants with hypertriglyceridemia) after nut consumption [5]. However, only one study has assessed the effect of nut consumption on the composition and particle size of lipoprotein subfractions. This study showed beneficial changes in lipid distribution in lipoprotein subfractions after walnut consumption, and no changes in plasma lipid composition [6]. Compared to other nuts, pistachios have lower fat (mostly from poly- and monounsaturated fatty acids) and energy content, and higher levels of fiber (both soluble and insoluble), potassium, phytosterols, γ-tocopherol, vitamin K, xanthophyll and carotenoids thereby contributing to explain the beneficial relation between pistachio consumption and health-related outcomes [7].

The aim of the present study is to evaluate the effect of chronic intake of pistachios on lipoprotein size and subclass concentration in pre-diabetic subjects as a potential mechanism for decreasing the cardiovascular risk associated with pre-diabetes.

Methods

Study characteristics

The EPIRDEM (Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus) study is a randomized, controlled, cross-over trial with a four-month dietary intervention in each period conducted in pre-diabetic subjects. The institutional review board of the Universitary Hospital of Sant Joan de Reus approved the study protocol in September 2011. Executed informed consent was obtained from all study participants. The trial was registered in Clinical Trials (identifier NCT01441921).

Study population

Eligible participants were community-living men and women aged between 25 and 65 years, body mass index $\leq$ 35 kg/m^2 and pre-diabetes was considered when fasting plasma glucose levels were between 100 and 125 mg/dL. Subjects were excluded if they met one of the following criteria: a) diabetes mellitus or using oral antidiabetic drugs; b) alcohol, tobacco or drug abuse; c) frequent consumption of nuts or known history of allergy to them; d) use of plant sterols, psyllium, fish oil supplements and multivitamins, vitamin E or other antioxidant supplements, e) bad dentition, involving difficulty to chew pistachios; f) following a vegetarian or a hypocaloric diet to lose weight; g) being pregnant or wishing to become pregnant 9 months before or during the study, lactating 6 weeks before or during the study; h) significant liver, kidney, thyroid or other endocrine diseases; i) medical, dietary or social conditions that hinder compliance with the intervention.

Study design

A 15-days run-in period proceeded the four-month treatment period. A 2-week wash-out period separated the 2 crossover sequences. At baseline, data on medical history, physical examination and fasting blood for biochemical analysis were collected. Subjects who met the inclusion criteria were randomly assigned to one of the two different intervention sequences, before the 15-days run-in period, using a computer-generated random-number table. They were instructed to follow a normocaloric diet that provided 50% of energy as carbohydrates, 15% as protein, and 35% as total fat during the 2 weeks proceeding each study period.

The isocaloric diet was individually calculated using WHO equations adjusted by the estimated energy expenditure in physical-activity leisure-time. After the 2-week run-in period, subjects were randomized to one of the two sequences: starting with a control diet (CD) followed by the pistachio supplemented diet (PD), or starting with the pistachio diet followed by the control diet. The main characteristics of both intervention diets have already been published [8]. Participants allocated to the pistachio diet (PD) were supplemented with 2 ounces of pistachio (57 g/day, half roasted and half roasted and salted). In the control diet (CD), the energy intake of other fatty foods, mostly olive oil, was adjusted to compensate for the energy from pistachios included in the PD. Adherence to the intervention period was assessed by counting the empty sachets of pistachio administered and by measuring

plasma lutein-zeaxanthin and γ-tocopherol levels as previously described [8].

Data collection

Medical and anthropometric measurements were collected at the beginning and at the end of each dietary period. Blood samples were also collected during the same visits after 12 h of fasting.

Lipoprotein analysis by NMR spectroscopy of plasma samples

Lipoprotein analysis of plasma samples by 2D diffusion-ordered ^{1}H NMR spectroscopy (DOSY) was performed using a previous protocol [9]. This protocol measures lipid concentrations (i.e., triglycerides and cholesterol), sizes and particle numbers for VLDL (38.6–81.9 nm), LDL (14.7–26.6 nm) and HDL (6.0–10.9 nm) classes, as well as the particle numbers of nine subclasses (namely large, medium and small VLDL, LDL and HDL, respectively). Briefly, 2D ^{1}H NMR spectra were recorded on a Bruker-Avance III 600 spectrometer at 310 K (Bruker BioSpin, Rheinstetten, Germany). We used the double stimulated echo (DSTE) pulse program with bipolar gradient pulses and a longitudinal eddy-current delay (LED). The relaxation delay was 2 s, the finite impulse decays (FIDs) were collected into 64K complex data points and 32 scans were acquired for each sample. The gradient pulse strength was increased from 5 to 95% of the maximum strength of 53.5 Gauss cm^{-1} in 32 steps. The squared gradient pulse strength was linearly distributed.

To determine lipoprotein size, the methyl signal was surface fitted with the numbers of functions so that the nine lipoprotein subclasses could be determined. The mean particle size of every main fraction was derived by averaging the NMR area of each fraction by its associated size. To obtain particle-weighted lipoprotein sizes, each NMR area was divided by its associated volume. The particle numbers of each lipoprotein main fraction were calculated by dividing the lipid volume by the particle volume of a given class. The lipid volumes were determined by using common conversion factors to convert concentration units obtained from the partial least-squares (PLS) models into volume units. The relative areas of the lipoprotein components used to decompose the 2D spectra were used to derive the particle numbers of the nine lipoprotein subclasses.

Statistical analysis

The descriptive data of participants at baseline and differences during the intervention periods are shown as means and 95% confidence intervals (95% CI) for continuous variables, and number (%) for categorical variables. Differences in all variables were evaluated by analysis of variance (ANOVA), with intervention diet as the independent and repeated measures factor. Diet sequence (order of diet treatments) was analyzed as an independent factor but as it was not significant it was not further considered. The differences in variable changes between dietary intervention periods were analyzed by an ANCOVA test using baseline values as a covariate. All statistical analyses were conducted using intention-to-treat (ITT) and *per protocol* (PP) approaches. ITT analysis included all randomized participants and data was analyzed following the assumption that the drop outs (all of them attended only to the baseline visit) did not change their variables' values through the whole study. The PP analysis excluded participants who did not attend the last visit, and the results are shown in Supplement. The sample size estimation has been previously described [8]. All analyses were done using SPSS 20.0 (SPSS Inc, Chicago, IL). All tests were 2-sided, and significance was defined as $P < 0.05$.

Results

A total of 108 participants were assessed for eligibility, of whom 30 declined to participate and 24 did not meet the inclusion criteria. Fifty-four participants were randomly assigned to one of the two intervention sequences. During the pistachio period, 5 of the 54 randomized participants (9.25%) dropped out of the study for personal reasons (Fig. S1, Supporting information). No gastrointestinal side effects were observed during the trial and no changes in medication were reported during the study. The baseline characteristics of the study participants are shown in Table 1. No significant differences were observed between dietary interventions at baseline in any of the analyzed parameters. As expected, plasma lutein-zeaxanthin and γ-tocopherol levels were significantly higher in the PD compared with the CD (already published data, [8]).

Total LDL-P was non-significantly lowered in the PD diet than in the CD diet (mean (95% CI): −46.67 nM (−88.22, −5.12) and 20.66 nM (−23.62, 64.94), respectively, P = 0.10) (Table 2). However, when the concentration of each LDL-P subclass (i.e. small, medium and large) was analyzed, the concentration of small LDL-P was significantly lowered in the PD diet than in the CD diet (mean (95% CI): −28.07 nM (−60.43, 4.29) and 16.49 nM (−14.19, 47.18) respectively, P = 0.02). However, results for total VLDL-P, total HDL-P concentrations and their relative subclasses, showed non-significant changes between intervention periods.

The mean size of HDL particles was significantly lowered in the PD diet than in the CD diet (mean (95% CI): −0.13 Å (−0.22, −0.03) and 0.02 Å (−0.07, 0.10), respectively, P = 0.01), whereas no significant changes were observed for mean VLDL or LDL particle size.

Moreover, there were significant differences in non-HDL-P concentrations (i.e. sum of total VLDL-P and LDL-P) between the two periods: they were reduced in the PD period (mean (95% CI): −36.02 nM (−77.56, 5.20)) and increased in the CD period (21.11 nM (−23.85, 66.06)); P = 0.04. The proportion (expressed as percentage) of small-HDL particle concentrations from total HDL particles, was significantly higher after PD than after the control period (mean (95% CI): 2.23% (0.57, 3.89) and −0.08%

Table 1 Baseline characteristics of the study population.

Variable	Subjects (n = 54)
Female sex, n (%)	25 (46)
Age (years)	55 (53.4, 56.8)
Weight (kg)	77.6 (74.8, 80.3)
Body mass index (kg/m^2)	28.9 (28.2, 29.6)
Waist circumference (cm)	94.7 (92.8, 96.6)
Systolic blood pressure (mmHg)	134 (130, 137)
Diastolic blood pressure (mmHg)	81 (79, 83)
Total cholesterol (mg/dL)	213.13 (205.00, 221.26)
LDL-C (mg/dL)	135.66 (127.65, 143.67)
HDL-C (mg/dL)	54.47 (50.83, 58.11)
Triglycerides (mg/dL)	115.34 (102.68, 128.00)
VLDL-P (nM)	41.62 (36.07, 47.18)
LDL-P (nM)	1210.40 (1147.26, 1273.54)
HDL-P (μM)	30.34 (29.13, 31.56)
Fasting plasma glucose (mg/dL)	112.80 (108.51, 117.08)
Fasting plasma insulin (mU/mL)	12.19 (10.59, 13.79)
HOMA-IR	3.50 (2.97, 4.02)
Glycated HbA_{1c} (%)	5.92 (5.81, 6.03)
Glycated HbA_{1c} (mmol/mol)	41.20 (40.01, 42.38)
Dyslipidemia, n (%)	27 (50)
Hypertension, n (%)	23 (42.6)
Statins, n (%)	5 (9.3)
Fibrates, n (%)	2 (3.7)
Angiotensin converter enzyme inhibitors, n (%)	6 (11.1)
Beta-blockers and other antihypertensive drugs, n (%)	13 (24.1)
Leisure-time physical activity (Kcal/day)	347 (307, 387)

Data are given as mean (95% CI) or number (%). VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; -C, cholesterol; -P, particle; HOMA-IR, homeostatic model assessment of insulin resistance; HbA_{1c}, glycated hemoglobin.

(−1.66, 1.49), respectively, P = 0.01), whereas the percentage of medium- and large-HDL particles was significantly lower. No significant differences in the proportion of small LDL-P were observed between intervention periods (Fig. 1). The *Per Protocol* analysis showed similar results (Table S1 and Fig. S2, Supporting information).

Discussion

In the present study, we have demonstrated that the chronic consumption of pistachios shifts the lipoprotein size and particle profile to a less atherogenic pattern, thus suggesting that pistachios may play a beneficial role in cardiovascular disease, even though they have no effect on the classic lipid factors of cardiovascular risk.

In hypercholesterolemic, normolipidemic and healthy subjects, it has been consistently reported that the regular intake of nuts has the beneficial effect on lipid profile of lowering serum LDL-C, without significantly affecting triglycerides or HDL-C [10]. However, these lipid-lowering properties attributed to nuts are more controversial in obese subjects or subjects who are resistant to insulin. In these subjects, whereas some authors have reported significant reductions in LDL-C and increases in HDL-C after nut consumption [11], others have failed to find significant changes in lipid profile [12]. In particular, consumption of pistachio has been reported to induce a significant reduction in total cholesterol (TC), TC/HDL-C ratio and LDL-C/HDL-C ratio [13–15] and a significant increase in plasma HDL-C [13,14] in healthy and hypercholesterolemic subjects. The effect of pistachio consumption on LDL-C concentrations is less consistent: they tend to decrease [13,15] but not always significantly [13]. In a previous publication conducted in the same population as the present study, we found no significant changes in either total cholesterol, LDL-C or HDL-C after pistachio consumption [8], supporting the hypothesis that obese or insulin-resistant subjects are less likely to have changes in their lipid profile [16].

In recent years, interest in the concentrations of lipoprotein subclass particles (small, medium, large) has been increasingly focused not on the total amount of cholesterol within these particles but on their potential effect on atherosclerosis and cardiovascular risk [4]. A decade ago, Liu et al. found that non-HDL-cholesterol (LDL-C plus VLDL-C) was strongly associated with an increased risk of coronary heart disease [17], which was even greater than that attributed to LDL-C [18]. Unlike large LDL particles, small, dense LDL-P confers greater atherogenic risk because of its interaction with the arterial wall (e.g. increased residence time in the circulation, easy penetration into the sub-endothelial space and greater susceptibility to oxidative modification) [4]. Additionally, high levels of small, dense LDL-P have been positively correlated with microalbuminuria and negatively with glomerular filtration rate as predictors of diabetic nephropathy [19] and as emerging CV risk factors [20]. Otherwise, small HDL-P have been associated both with more non-calcified plaque or higher coronary risk but also with an atheroprotective role [21], whereas larger HDL-P have been associated with less non-calcified plaques and lower coronary risk [22]. Lipoprotein subclass abnormalities have also been related to insulin resistance and T2DM. Concentrations of small, dense atherogenic LDL particles are commonly higher in insulin-resistant subjects [23]. A study conducted on subjects with or without T2DM demonstrated that progressive insulin resistance was associated with an atherogenic profile characterized by an increase in VLDL size (mainly in large-VLDL particle concentrations), a decrease in LDL size (reflected by an increase in small- and a reduction in large-LDL), and a decrease in HDL size (by reduction in large- and an increase in small-HDL particles) [24]. The results found after 1,371 T2DM, impaired glucose-tolerant and normoglycemic participants were analyzed in the Insulin Resistance Atherosclerosis Study (IRAS) [25], were similar and suggested that dyslipidemia associated with insulin resistance or type 2 diabetes is not detected when the traditional lipid profile is evaluated. The IRAS study has also demonstrated for the first time that, independently of triglyceride and HDL-C concentrations, lipoprotein subclasses were positively associated with the incidence of T2DM at 5-year follow-up [1].

Table 2 Baseline and changes in lipoprotein concentration and size after the intervention period.

Characteristics	Pistachio diet		Control diet		Treatment effect
	Baseline	Change	Baseline	Change	P-value
Total VLDL-P (nM)	46.24 (39.74, 52.73)	−0.58 (−5.12, 3.95)	42.12 (36.61, 47.64)	2.27 (−3.36, 7.90)	0.33
Large VLDL-P (nM)	1.42 (1.06, 1.78)	−0.06 (−0.33, 0.21)	1.10 (0.87, 1.34)	0.22 (−0.20, 0.65)	0.23
Medium VLDL-P (nM)	7.09 (5.64, 8.54)	−0.11 (−1.09, 0.87)	6.04 (4.89, 7.18)	0.75 (−0.47, 1.97)	0.25
Small VLDL-P (nM)	37.72 (32.93, 42.51)	−0.41 (−3.81, 2.98)	34.98 (30.73, 39.22)	1.35 (−2.73, 5.43)	0.39
Total LDL-P (nM)	1236.37 (1160.70, 1312.04)	−46.67 (−88.22, −5.12)	1219.11 (1154.56, 1283.67)	20.66 (−23.62, 64.94)	0.10
Large LDL-P (nM)	131.36 (113.49, 149.23)	−8.15 (−18.99, 2.69)	133.98 (120.49, 147.47)	1.80 (−8.13, 11.73)	0.30
Medium LDL-P (nM)	463.62 (421.63, 505.62)	−10.45 (−31.39, 10.49)	462.91 (431.15, 494.68)	2.37 (−21.26, 26.01)	0.49
Small LDL-P (nM)	647.68 (604.74, 690.61)	−28.07 (−60.43, 4.29)	620.60 (581.13, 660.06)	16.49 (−14.19, 47.18)	0.02
Total HDL-P (μM)	30.32 (29.02, 31.62)	1.18 (−0.33, 2.70)	30.72 (29.46, 31.98)	0.70 (−0.28, 1.68)	0.55
Large HDL-P (μM)	1.14 (0.83, 1.44)	−0.08 (−0.18, 0.03)	1.04 (0.79, 1.29)	0.05 (−0.06, 0.17)	0.09
Medium HDL-P (μM)	6.69 (5.77, 7.61)	−0.28 (−0.76, 0.20)	6.44 (5.73, 7.15)	0.13 (−0.25, 0.51)	0.13
Small HDL-P (μM)	22.58 (21.24, 23.91)	1.57 (0.22, 2.91)	23.34 (22.19, 24.49)	0.56 (−0.53, 1.65)	0.18
Particle size (Å)					
VLDL	200.59 (199.15, 202.04)	0.27 (−0.77, 1.31)	199.58 (198.38, 200.78)	0.41 (−0.58, 1.39)	0.83
LDL	99.20 (98.85, 99.54)	0.09 (−0.16, 0.35)	99.46 (99.20, 99.71)	−0.15 (−0.40, 0.11)	0.17
HDL	40.30 (40.09, 40.51)*	−0.13 (−0.22, −0.03)	40.21 (40.05, 40.37)	0.02 (−0.07, 0.10)	0.01
Non-HDL-P (nM)	1282.61 (1206.53, 1358.69)	−36.02 (−77.56, 5.52)	1261.35 (1195.00, 1327.70)	21.11 (−23.85, 66.06)	0.04
Total-P/HDL-P ratio	1.04 (1.04, 1.05)	−0.003 (−0.005, −0.0006)	1.04 (1.04, 1.05)	−0.0004 (−0.002, −0.002)	0.09
LDL-P/HDL-P ratio	0.04 (0.04, 0.05)	−0.003 (−0.005, −0.0005)	0.04 (0.04, 0.04)	−0.003 (−0.005, −0.0007)	0.86

Intention-to-treat analysis, n = 54. All values are means (95% CI). Intra-group analysis was assessed by the paired t-test. Basal-adjusted changes between groups were analyzed using adjusted ANOVA of repeated measurements. * Significant difference ($P < 0.05$) between baseline and end of a particular intervention period. -P, particle; -C, cholesterol; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

Although a considerable amount of research has been carried out on the conventional lipid profile, the modulatory effect of dietary fatty acids on the size and concentration of lipoprotein subclasses has been poorly analyzed. A lower consumption of saturated fatty acids (15%, 9% or 6.1%) for 4 weeks significantly decreased large HDL and increased small HDL subclasses [26]. A reduction in large, medium and small, dense LDL-P was observed after a portfolio diet including almonds (15 g/day) for 4 weeks [27]. However, a significant decrease in the proportion of small LDL-P has been reported in normolipidemic subjects after 3 days on a high-fat diet (37% energy from fat) [28] and also 6 h after a high-fat meal (83% energy from fat) [29]. To the best of our knowledge, only one study has evaluated the effect of nut consumption on the distribution and particle size of lipoprotein subclasses. The study was conducted in a small number of adults (n = 18) with combined hyperlipidemia who consumed 48 g of walnuts for 6 weeks. Cholesterol decreased, particularly in small-LDL-P and large-HDL particles, which suggested that the beneficial properties of nut intake on cardiovascular risk may be underlying by an additional mechanism [6]. In agreement with these results, our study demonstrates that chronic consumption of pistachios induces a significant decrease of small-LDL concentrations and HDL-P size, despite the absence of change in TC, LDL-C or HDL-C. The mean decrease observed in HDL-P size could be explained by the significant increase in the proportion of small HDL-P and the significant decrease in the proportion of medium and large HDL particle concentrations. Similarly, we found a significant decrease in non-HDL-particle concentrations, which are also strongly associated with cardiovascular risk [30].

Several strengths and limitations of our study deserve comment. Among the strengths are its cross-over randomized design, its medium-term duration and the biochemical dietary compliance markers. We have also used NMR to analyze lipoprotein subfractions, which it has been suggested as a better coronary heart disease estimator than non-denaturing polyacrylamide gradient gel electrophoresis [31]. Because data on HDL function was not measured, the implications of changes in HDL subtype distribution need to be further investigated. However, because the study focused on pre-diabetic subjects, the results cannot be extrapolated to healthy subjects or subjects with T2DM. Despite the cross-over design, other limitations could be related to the relatively heterogeneity of the subjects studied as the presence of dyslipidemia or the use of lipid-lowering treatments. Whether other nuts can modify lipoprotein subclasses in a similar way deserves further research.

In conclusion, the results of the present study suggest that the chronic consumption of pistachios has a beneficial effect on emergent cardiovascular risk factors, even though it has little or no effect on classical lipid risk markers. Further research is necessary to corroborate our results and to extend them in the knowledge of which subclasses are reliable markers of cardiovascular diseases and potential targets for effective therapies.

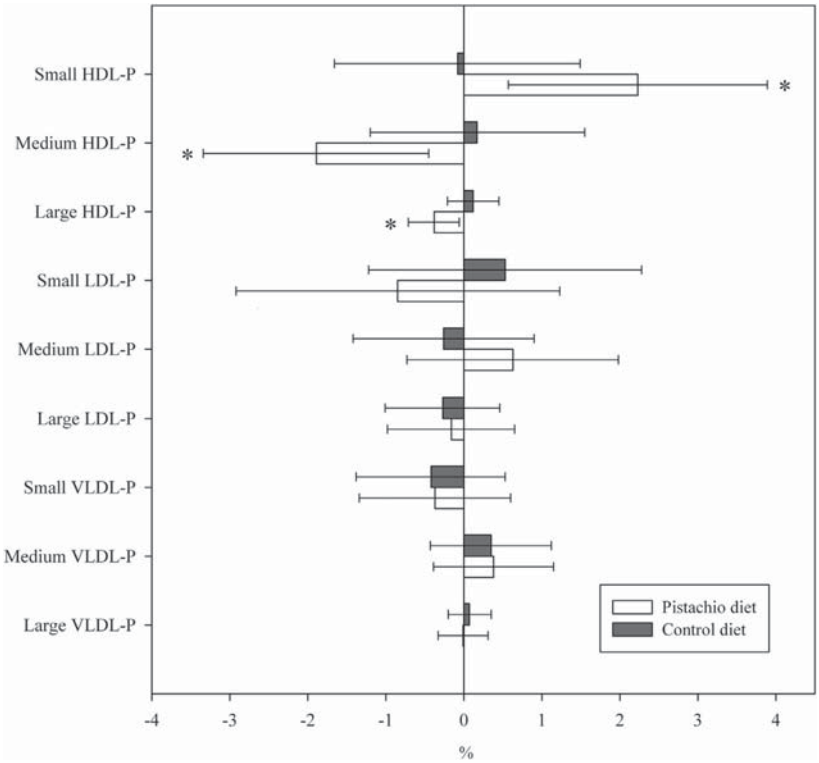

Figure 1 Changes (expressed as percentages) in the proportion of lipoprotein subclasses (small, medium or large) related to the total lipoprotein particle concentration (HDL-P, LDL-P or VLDL-P) according to intervention period. Intention-to-treat analysis, n = 54. Values are means (95% CI). Changes between groups were analyzed using ANOVA of repeated measurements.* stands for significant differences ($P < 0.05$) in changes between pistachio diet and control diet. HDL-P, high-density lipoprotein particle; LDL-P, low-density lipoprotein particle; VLDL-P, very low-density lipoprotein particle.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.numecd.2015.01.013.

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UNIVERSITAT ROVIRA I VIRGILI
HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS
Pablo Hernández Alonso

Publication 3.

Title: Chronic pistachio intake modulates circulating microRNAs related to glucose metabolism and insulin resistance in prediabetic subjects.

Authors: Pablo Hernández-Alonso; Simona Giardina; Jordi Salas-Salvadó; Pierre Arcelin and Mònica Bulló.

Year and Journal information: 2016. *European Journal of Nutrition*. Epub ahead of print.

IF: 3.239; 27/78; Q2; Nutrition & Dietetics.

Abstract:

PURPOSE: To assess the effects of a pistachio-enriched diet on the profile of circulating microRNAs (miRNAs) related to glucose metabolism and insulin resistance (IR).

METHODS: Randomized crossover clinical trial in 49 subjects with prediabetes was performed. Subjects consumed a pistachio-supplemented diet (PD, 50 % carbohydrates, 33 % fat, including 57 g/day of pistachios) and an isocaloric control diet (CD, 55 % carbohydrates and 30 % fat) for 4 months each, separated by a 2-week washout period. The plasma profile of a set of seven predefined miRNAs related to glucose and insulin metabolism was analyzed by quantitative RT-PCR.

RESULTS: After the PD period, subjects have shown significant lower circulating levels of miR-192 and miR-375 compared to CD period, whereas miR-21 nonsignificantly increased after PD compared with CD (47 vs. 2 %, P = 0.092). Interestingly, changes in circulating miR-192 and miR-375 were positively correlated with plasma glucose, insulin and HOMA-IR.

CONCLUSION: Chronic pistachio consumption positively modulates the expression of some miRNA previously implicated on insulin sensitivity.

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HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS
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ORIGINAL CONTRIBUTION

Chronic pistachio intake modulates circulating microRNAs related to glucose metabolism and insulin resistance in prediabetic subjects

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Abstract
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Methods Randomized crossover clinical trial in 49 subjects with prediabetes was performed. Subjects consumed a pistachio-supplemented diet (PD, 50 % carbohydrates, 33 % fat, including 57 g/day of pistachios) and an isocaloric control diet (CD, 55 % carbohydrates and 30 % fat) for 4 months each, separated by a 2-week washout period. The plasma profile of a set of seven predefined miRNAs related to glucose and insulin metabolism was analyzed by quantitative RT-PCR.
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Conclusion Chronic pistachio consumption positively modulates the expression of some miRNA previously implicated on insulin sensitivity.

Keywords Prediabetes · Pistachio · microRNA · Glucose

Introduction

Type 2 diabetes (T2D) is a worldwide chronic disease characterized by hyperglycemia [1]. However, before the onset of the disease, prediabetes (pre-D) occurs, a preventable and treatable condition if recognized early enough [2]. Although the molecular mechanisms underlying these metabolic conditions are not totally understood, microRNAs (miRNAs) emerge as powerful regulators not only for specific cell processes such as apoptosis, proliferation and differentiation [3], but also for several metabolic pathways including insulin secretion, glucose homeostasis and carbohydrate and lipid metabolism [4]. MicroRNAs are a family of endogenous, highly conserved, functional, single-stranded RNA molecules of 22–24 nucleotides that can act by decreasing target mRNA levels or inhibiting their translation [5]. Although miRNAs originate in the cell nucleus, they have been detected in such biological fluids as blood, urine, saliva or breast milk [6]. MiRNAs are excreted into the circulation and can regulate gene expression in cells/tissues far from where they were produced [7], suggesting an endocrine or paracrine signaling [8]. Their high stability in plasma and other fluids [9] makes circulating miRNA potentially novel sources of both noninvasive biomarkers and targets in different diseases.

Electronic supplementary material The online version of this article (doi:10.1007/s00394-016-1262-5) contains supplementary material, which is available to authorized users.

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Despite some methodological limitations, results on the use of miRNAs as biomarkers for T2D risk and detecting and monitoring its associated complications have been promising. However, few studies have evaluated miRNA modulation after a nutritional intervention in an attempt to determine the effect of particular nutrients and/or supplements rather than food or dietary patterns [10]. In humans, consumption of grape extract rich in resveratrol, and vitamin supplementation modulated specific miRNAs [11]. Similarly, a polyunsaturated fatty acid-enriched diet including almonds and walnuts was effective at modifying several miRNAs [12].

We have previously demonstrated that chronic pistachio consumption significantly improves glucose and insulin metabolism as well as other cardiovascular risk markers in the prediabetic state. The current study attempts to explore changes in circulating glucose- and insulin-related miRNAs in response to the regular consumption of pistachios.

Subjects and methods

Study characteristics

The EPIRDEM (Effect of Pistachio Intake on Insulin Resistance and Type 2 Diabetes Mellitus) study is a randomized, controlled, crossover trial with two 4-month dietary intervention sequences separated by a 2-week washout period which was conducted in prediabetic subjects. The trial was registered in clinical trials (identifier NCT01441921), and the institutional review board of the University Hospital of Sant Joan de Reus approved the study protocol in September 2011. Written informed consent was obtained from all study subjects.

Study population

Eligible participants were community-living men and women of 25–65 years of age, body mass index (BMI) $\leq$35 kg/m^2 and fasting plasma glucose levels between 100 and 125 mg/dL (prediabetes state). Subjects were excluded if they had T2D or met one of the exclusion criteria defined in the protocol [13].

Study design

Subjects were screened to determine inclusion/exclusion criteria. Those who met the inclusion criteria were included in a 15-days run-in period and were advised to follow a normocaloric diet (50 % energy (E %) as carbohydrates, 15 E % as protein and 35 E % as total fat). After the 2-week run-in period, subjects were randomized to one of the two sequences using a computer-generated random table: a preliminary control diet (CD) followed by the pistachio-supplemented diet (PD) or a preliminary PD followed by the CD (Fig. 1a). The main characteristics of both intervention diets have already been published [13]. Participants allocated to the pistachio diet (PD) were supplemented with 2 oz of pistachios per day (57 grams/day, half roasted and half roasted and salted). Control diet was free of nuts. Data on medical history, physical examination and blood sampling were collected at the beginning and at the end of each intervention period.

Biological samples, biochemical and molecular parameters

Blood samples were obtained after a 12-h fast at baseline and at the end of each intervention period. Plasma fasting glucose was determined using standard enzymatic automated methods. Plasma thromboxane B2 (TXB2) (Cayman Chemical Company, MI, USA) was measured using enzyme-linked immunosorbent assay commercial kits (ELISA). Plasma von Willebrand factor (vWF), platelet factor 4 (PF4), insulin, C-peptide and resistin were determined using a MILLIPLEX® MAP Plex Kit (Merck Millipore, MA, USA). Insulin resistance was estimated by the homeostatic model assessment of insulin resistance (HOMA-IR). Gene expression data were obtained as previously described [13]. Briefly, total RNA was extracted from whole blood samples using the Tempus Spin RNA Isolation Kit (Ambion, Madrid, Spain), and we analyzed the expression of the following set of genes: TLR2 (Toll-like receptor 2), TLR4, SLC2A3 (solute carrier family 2, facilitated glucose transporter member 3), SLC2A4, IL-6 (interleukin 6) and RETN (resistin).

Analysis of circulating plasma miRNAs

Plasma was obtained by centrifugation at 1800×g for 15 min at 4 °C, using K_2EDTA-coated Vacutainer tubes, which were aliquoted and stored at −80 °C until use. We centrifuged thawed plasma samples for 10 min at 2000×g at RT and then transferred the upper supernatant (3/4) to a fresh tube to proceed with total RNA isolation.

Total RNA (including miRNA) was isolated from plasma samples using the mirVana PARIS Isolation Kit (Applied Biosystems, Darmstadt, Germany) according to manufacturer's protocol. Briefly, 400 µL of plasma was diluted with the same volume of 2X Denaturing Solution. As there is no appropriate endogenous miRNA for plasma samples, we decided to use a spike-in exogenous control. *Caenorhabditis elegans* miR-39 (cel-miR-39, Qiagen, Madrid, Spain), which lacks sequence homology to human miRNAs, was selected to control for extraction procedures and to normalize the experimental quantitative RT-PCR

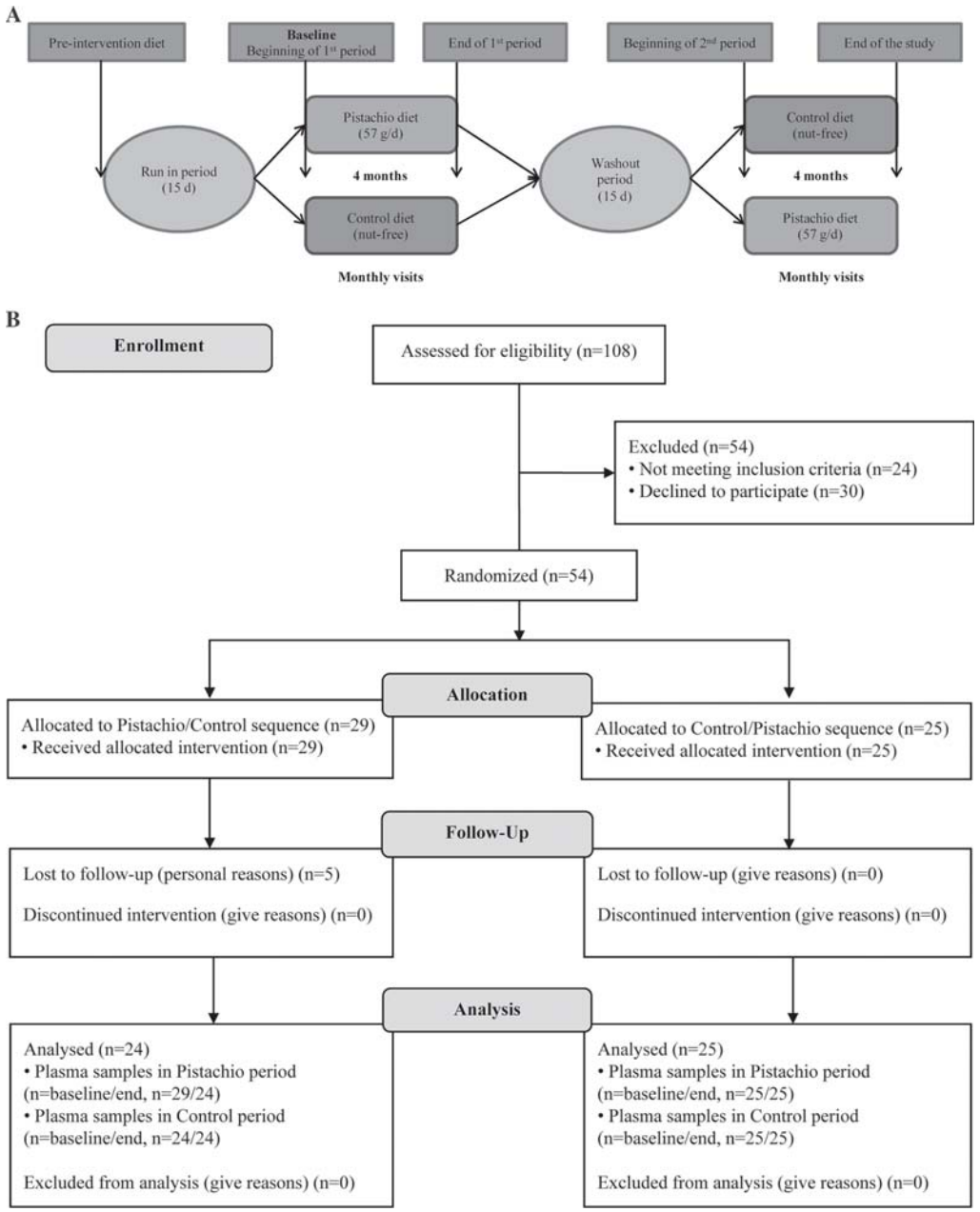

Fig. 1 Study design and flow diagram of the study **a** study design. **b** Consort 2010 flow diagram for EPIRDEM study

(qRT-PCR). We empirically added 5 µL of 5 nM synthetic *C. elegans* miR-39 and then 800 µL of acid–phenol: chloroform. After of the various centrifugation steps and washing with 100 % ethanol, elution was done with 50 µL of preheated (95 °C) nuclease-free water, aliquoted and stored at −80 °C until reverse transcription (RT) was performed. Plasma RNA was extracted from the four samples of each participant in the same batch to reduce further variability. Previous results in serum and plasma samples have shown that the use of spike-in is one of the most stable normalizing methodologies [14, 15]. The final recovery of these synthetic oligonucleotides was measured for each sample using TaqMan qRT-PCR miRNA assays (Applied Biosystems, Darmstadt, Germany). To validate the success of each

extraction, we also assessed cycle (Cq) values obtained for a serial dilution (10^{-1}) of cel-miR-39. Briefly, known quantities of synthetic cel-miR-39 were reverse transcribed in the same conditions as plasma samples. The qPCR was performed for each tenfold dilution of cel-miR-39 in triplicate, and a regression curve was generated in order to calculate the extraction efficiency by comparing predicted and each sample-specific value of cel-miR-39. Individual samples with recovery values less than approximately 60 % and/or differences in recovery rates between intra-subject's samples ≥3 % were excluded or remade.

We selected seven human circulating miRNAs (hsa-miR-15a-5p, hsa-miR-21-5p, hsa-miR-29b-3p, hsa-miR-126-3p, hsa-miR-192-5p, hsa-miR-223-3p, hsa-miR-375) widely related to glucose metabolism, insulin resistance status, pre-D status and biomarkers of T2D, using updated reviews [4, 8, 16, 17] and databases [18–20] together with the spike-in cel-miR-39 (Supplementary Table 1).

A fixed 3 µL volume of RNA eluate was the input for the RT. Then, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) with the following conditions per sample reaction: 6 µL of RT primer pool of miRNAs (Applied Biosystems, Darmstadt, Germany), 0.3 µL of dNTPs (100 mM), 1.5 µL of 10X RT buffer, 3.01 µL of nuclease-free water, 0.19 µL of RNase inhibitor (20 U/µL) and 1 µL of MultiScribe Reverse Transcriptase (50 U/µL) up to a final volume of 15 µL. A primer pool was obtained using a 5X solution of each RT miRNA-specific primer (250 nM) which was pooled and diluted in 1 × Tris–EDTA (TE) buffer to obtain a final dilution of 0.05× for each primer.

The reaction mixtures were incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min before being held at 4 °C in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Darmstadt, Germany).

Various qPCR reactions were carried out using 2 µL of pre-diluted cDNA (1:5 in nuclease-free water), 5 µL of TaqMan® Universal Master Mix II, no UNG (Applied Biosystems, Darmstadt, Germany), 0.5 µL of gene-specific primer/probe (TaqMan® MicroRNA Assay) and 2.5 µL of nuclease-free water in a final volume of 10 µL. The reaction mixtures were incubated at 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s, ending with 60 °C for 1 min in an ABI 7900HT real-time PCR system (Applied Biosystems). We ran a negative control (nuclease-free water) and an inter-plate control (same cDNA sample) in each plate and several negative RT controls (nuclease-free water instead of RNA).

All measurements were performed in duplicate, and qPCR data were acquired using Sequence Detector software (SDS version 2.4, Applied Biosystems, Darmstadt, Germany). Mean quantification Cq values were calculated as the average of two replicates if the SD was 0.5; otherwise, the sample was repeated in the qPCR experiment. Normalized expression was calculated for individual samples using the $2^{-\Delta Cq}$ method (ExpressionSuite software version 1.0.3) using cel-miR-39 as normalizer. Changes in expression were shown as the ratio between final and baseline values. The non-template sample did not produce a signal in any assay.

Statistical analysis

The descriptive data for the participants during the intervention periods are shown as means, with 95 % confidence intervals (95 % CI) for continuous variables and numbers (%) for categorical variables. Before statistical analysis, normal distribution and homogeneity of the variances were evaluated using Levene's test. Normalized relative log10 ratios were used for statistical tests, when necessary. The antilog-transformed values are reported. Differences in all variables were evaluated by analysis of variance (ANOVA), with intervention diet as the independent and repeated measures factor. Diet sequence (order of diet treatments) was analyzed as the independent factor, but as it was not significant, it was not considered any further. Paired *t* tests were performed to evaluate changes within each specific intervention period. Ratio of expression relative to the baseline of each miRNAs is presented. Therefore, values higher than 1 mean up-regulation throughout the intervention period and lower than 1 down-regulation.

Pearson's correlation coefficients were used to evaluate whether the changes (end/beginning of each intervention period) in plasma levels of different miRNAs correlated with the clinical parameters for glucose metabolism, insulin resistance and metabolic markers associated with T2D (end/beginning) in the whole population, regardless of the intervention period. We also computed Pearson's correlation coefficients between miRNAs (divided by up- or down-regulation) and the expression of a set of genes related to glucose metabolism and inflammation.

We also showed percentages of total cases showing up- or down-regulation in each miRNA according to the intervention period. All analyses were carried out using SPSS 22.0 (SPSS Inc, Chicago, IL). All the tests were two-sided, and significance was set as $P < 0.05$. The potential target genes and pathways were identified in silico according to the intersection of miRNAs with significant changes between treatments using DIANA-mirPath, a free Web-based computational tool that identifies potentially altered molecular pathways by the expression of a single or multiple significantly modulated miRNAs [21] (Supplementary Table 3).

Results

A total of 108 subjects were assessed for eligibility. Of these, 30 subjects declined to participate and 24 did not meet the inclusion criteria (Fig. 1b). Finally, 54 participants were randomly assigned to one of the two intervention sequences (i.e., pistachio–control or control–pistachio). Five participants dropped out for personal reasons and 49 successfully completed the study. The baseline characteristics of the study participants are shown in Table 1. No significant differences were observed between dietary interventions at baseline in any of the analyzed parameters.

Recovery efficiency and inter-plate control

Supplementary Table 1 shows basic information about the miRNA studied in the sample set. The standard curve of cel-miR-39 shows linearity within the concentrations assayed ($y = -3.2698x + 20.7671$; $R^2 = 0.9983$) (Supplementary Fig. 1). Using this standard curve, we calculated the efficiency of recovery in all the plasma samples which ranged from 66.2 to 71.3 %. We also evaluated the variability of cel-miR-39 recovery within the four plasma samples analyzed for each subject. The CVs of all subjects ranged from 0.86 to 1.03 %. Assessment of the inter-plate control showed that cel-miR-39 had the lowest coefficient of variation (CV) of all the miRNAs (0.86 %) (Supplementary Table 2). Although we did not perform the standard curve (Supplementary Fig. 1) in a background of plasma, we diluted cDNA (empirically) before qPCR analysis to minimize the possibility of contamination with PCR inhibitors. Furthermore, we show that the efficiency of recovery in all the plasma samples was appropriate and not dispersed

Table 1 Baseline characteristics of the study population before the start of the study

Variable	Subjects ($n = 49$)
Female sex, *n* (%)	23 (46.9)
Age (years)	55.7 (53.9, 57.4)
Weight (kg)	77.0 (74.2, 79.9)
Body mass index (kg/m^2)	28.9 (28.2, 29.6)
Waist circumference (cm)	94.5 (92.6, 96.4)
Systolic blood pressure (mmHg)	134 (130, 138)
Diastolic blood pressure (mmHg)	81 (79, 83)
Total cholesterol (mg/dL)	212.73 (204.01, 221.46)
LDL-C (mg/dL)	135.04 (126.44, 143.64)
HDL-C (mg/dL)	54.42 (50.42, 58.42)
Triglycerides (mg/dL)	116.75 (102.83, 130.67)
Fasting plasma glucose (mg/dL)	114.31(109.91, 118.70)
Fasting plasma insulin (mU/mL)	12.45 (10.77, 14.12)
HOMA-IR	3.59 (3.04, 4.14)
HbA_{1c} (%)	5.93 (5.81, 6.05)
Dyslipidemia, *n* (%)	26 (53.1)
Hypertension, *n* (%)	22 (44.9)

Data are given as means (95 % CI) or numbers (%)

LDL low-density lipoprotein, *HDL* high-density lipoprotein, *C* cholesterol, *HOMA-IR* homeostatic model assessment of insulin resistance, *HbA_{1c}* glycated hemoglobin

miRNA modulation by the intervention diets

The miRNA expression data (Fig. 2a) showed that of the seven miRNAs studied, miR-15a, miR-21, miR-29b, miR-126 and miR-223 had a nonsignificantly higher expression after the PD than the CD although it was at the limit of statistical significance in the case of miR-21 [mean 1.47 (95 % CI 1.03, 1.92) and 1.02 (0.82, 1.23), respectively, $P = 0.092$]. Interestingly, the changes in expression of miR-15a, miR-21, miR-29b and miR-126 were significantly increased after the PD period ($P < 0.05$) (Fig. 2a) although a nonsignificant trend was observed in the case of miR-223 ($P = 0.054$). As expected, after PD, circulating miR-192 expression was significantly lower than in CD [mean 1.00 (95 % CI 0.80, 1.20) and 1.34 (1.14, 1.55), respectively, $P = 0.018$]. This same down-regulation following PD compared with CD was also exhibited by plasma miR-375 [mean 0.91 (95 % CI 0.62, 1.19) and 1.64 (1.29, 1.99), respectively, $P = 0.004$, Fig. 2a]. Moreover, we found a nonsignificant effect of the diet sequence order (Supplementary Table 4) together with a significant recovery in almost all miRNAs evaluated when changing from the first intervention period (CD or PD) to the second intervention period (PD or CD). We additionally plotted the paired mean (95 % CI) values of miR-192 and miR-375 for each subject sorted by increasing ratio of expression values in the pistachio period (Fig. 3). Percentages of subjects having up- or down-regulation in each miRNA, comparing the end value with the baseline value according to the intervention period, are shown in Fig. 2b, c.

Correlations between miRNAs, biochemical parameters and gene expression

Changes in circulating miR-192 and miR-375 were positively correlated with plasma glucose, insulin (at the limit of statistical significance in the case of miR-192, $P = 0.064$) and HOMA-IR, indicating that increase in miR-192 and miR-375 levels mirror an increase in insulin resistance (Table 2). Interestingly, we found a significant inverse association between circulating levels of miR-29b and BMI, miR-21 and serum resistin concentrations, and miR-15a and von Willebrand factor levels (Table 2). Moreover, thromboxane B2 showed a significant association with

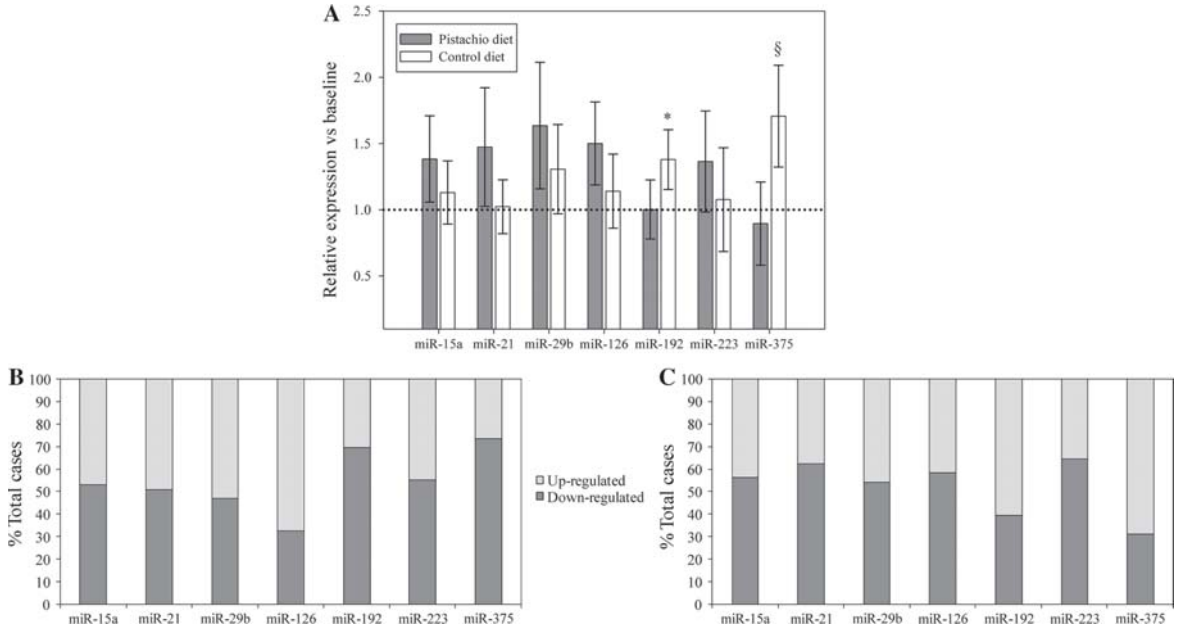

Fig. 2 Expression of the different miRNAs **a** expression relative to the baseline of the microRNAs (miRNAs) in plasma samples across intervention diets. Data are given as mean (95 % CI). Values equal to 1 mean the same expression at baseline and at the end of a certain period, whereas values higher than 1 mean up-regulation throughout the intervention period and lower than 1 down-regulation. $^{*}P < 0.05$ and $^{\S}P < 0.01$, significant differences in changes between dietary interventions. Intra-group changes showed that miR-15a, miR-21, miR-29b and miR-126 were significantly up-regulated in pistachio diet, whereas miR-192 and miR-375 in control diet. Normalization was performed using spike-in cel-miR-39. **b**, **c** Distribution (% of total cases) of subjects exhibiting down-regulation or up-regulation in PD (**b**) and in CD (**c**) for the different miRNAs. Expression relative to the baseline of each miRNA with values higher than 1 is categorized here as up-regulation throughout the intervention period and lower than 1 as down-regulation. miR, microRNA; *PD* pistachio diet, *CD* control diet and *CI* confidence interval

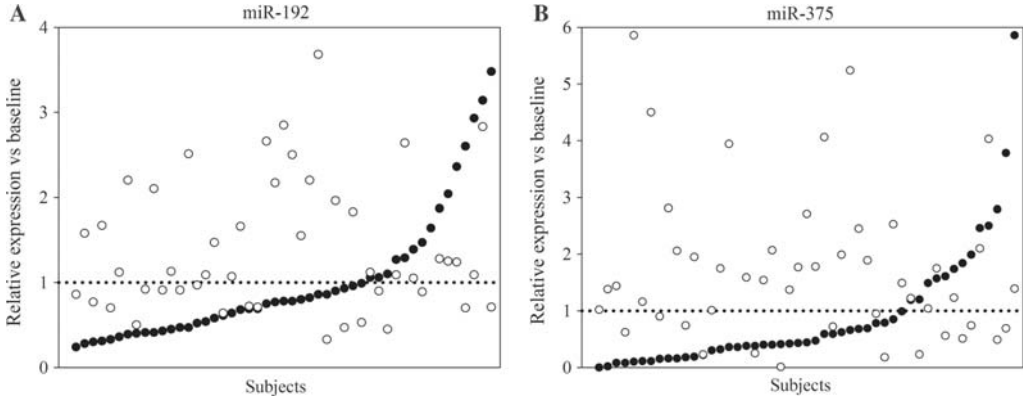

Fig. 3 Plot of the relative expression versus baseline for the study subjects. Paired values of the study subjects ($n = 49$) were sorted according to increasing relative expression versus baseline in pistachio period. *Black dots* refer to pistachio period and *white dots* to the control period. **a** miR-192 and **b** miR-375

most of the circulating miRNAs studied. Significant inverse associations were shown by miR-21, miR-29b, miR-126 and miR-223 with TXB2, whereas circulating miR-192 showed a significant direct association with TXB2. Table 3 shows the correlation between changes in each miRNA (grouped by up- and down-regulated) and changes in

Table 2 Correlations between the miRNAs and biochemical parameters related to glucose metabolism, insulin resistance and metabolic derangements associated with T2D

	miR-15a	miR-21	miR-29b	miR-126	miR-192	miR-223	miR-375
Glucose	0.056 (0.584)	−0.034 (0.743)	−0.019 (0.853)	−0.007 (0.942)	**0.207 (0.042)**	0.064 (0.535)	**0.331 (0.001)**
Insulin	0.063 (0.540)	0.087 (0.395)	0.066 (0.521)	−0.018 (0.864)	0.189 (0.064)	0.002 (0.985)	**0.312 (0.002)**
HOMA−IR	0.075 (0.465)	0.071 (0.490)	−0.054 (0.599)	−0.011 (0.911)	**0.218 (0.032)**	0.031 (0.764)	**0.353 (< 0.001)**
BMI	0.136 (0.183)	0.105 (0.304)	**−0.208 (0.041)**	0.101 (0.324)	0.112 (0.275)	0.096 (0.350)	−0.049 (0.631)
Resistin	0.082 (0.422)	**−0.241 (0.017)**	−0.170 (0.096)	0.041 (0.693)	−0.030 (0.770)	0.075 (0.467)	0.021 (0.839)
C-peptide	0.097 (0.342)	0.177 (0.083)	0.172 (0.093)	0.139 (0.174)	0.092 (0.368)	0.101 (0.324)	0.051 (0.617)
von Willebrand factor	**−0.229 (0.025)**	−0.026 (0.800)	0.126 (0.223)	−0.022 (0.834)	0.097 (0.346)	0.024 (0.819)	0.012 (0.910)
Platelet factor 4	0.182 (0.085)	0.198 (0.061)	0.115 (0.280)	0.210 (0.072)	0.043 (0.685)	**0.227 (0.032)**	0.157 (0.140)
Thromboxane B2	0.190 (0.062)	**−0.313 (0.002)**	**−0.338 (0.001)**	**−0.319 (0.001)**	**0.257 (0.011)**	**−0.264 (0.009)**	0.160 (0.117)

Single associations were tested between the end/baseline ratio by Pearson's correlation coefficient for the whole cohort (49 subjects per intervention group, $n = 98$). Results represent the coefficient of correlation (*P* value)

Significant associations ($P < 0.05$) are shown in bold

miRNA microRNA, *T2D* type 2 diabetes, *HOMA-IR* homeostatic model assessment of insulin resistance, *BMI* body mass index

different gene expression in lymphocytes. Interestingly, those subjects increasing their miR-15 and miR-21 values showed a significant inverse association with IL-6 expression in lymphocytes. Subjects lowering miR-29b values showed an inverse association with lymphocytes' SLC2A3 expression ($P < 0.05$). Moreover, those subjects in the group of lower miR-126 showed a significant positive association with IL-6 from lymphocytes.

Discussion

In the present study, we report for the first time that regular pistachio consumption is an effective strategy for modulating various plasma miRNAs related to insulin metabolism and type 2 diabetes.

Accumulating evidence suggests that circulating miRNAs are useful biomarkers of such diseases as T2D and cardiovascular disease [10]. However, the mechanisms regulating the circulating miRNA profile are still under debate, and little is known about the potential modulatory role of diet.

A previous study has described a specific miRNA plasma signature in patients with T2D consisting of low levels of miR-15a, miR-20b, miR-21, miR-24, miR-29b, miR-126, miR-191, miR-197, miR-223, miR-320 and miR-486 and the capacity of some miRNAs to predict the onset of T2D over a 10-year follow-up period [22]. Moreover, a recent meta-analysis of controlled profiling studies found that a total of 40 miRNAs were significantly deregulated in T2D, ten of which (miR-29a, miR-34a, miR-375, miR-103, miR-107, miR-132, miR-142-3p, miR-144, miR-199a-3p and miR-223) were identified as potential circulating or tissue T2D biomarkers [23]. The up-regulation of some of these diabetes-related miRNAs after pistachio consumption observed in our study reinforces current knowledge on the beneficial role of this type of nut on glucose and insulin metabolism and their potential role in lowering the risk of T2D [13]. Our results are in line with those showing that subjects with T2D and hypertension who consumed grape extract (containing resveratrol) for 12 months significantly up-regulated a set of miRNAs including miR-21 [11]. Moreover, the intake of polyphenols at nutritional doses modulated the expression of various murine miRNAs (e.g., miR-291b, miR-467b and miR-374) in the liver, revealing a new mechanism that explains the beneficial effects of polyphenols on glucose metabolism [24]. Finally, a recent clinical trial conducted in ten healthy women on the effect of an 8-week normocaloric diet enriched with almonds and walnuts demonstrated that the dietary polyunsaturated fatty acid content determined changes in circulating miRNAs [12].

Table 3 Correlations between the miRNAs (divided by up- or down-regulation) and expression of genes related to glucose metabolism and inflammation

↓ or ↑	*n*	TLR2	TLR4	SLC2A3	SLC2A4	IL-6	RETN
miR-15a							
↓	54	−0.077	0.210	−0.077	−0.164	0.064	−0.295
↑	44	0.208	0.034	0.148	−0.106	**−0.395**	−0.110
miR-21							
↓	56	−0.152	0.052	−0.062	0.060	0.021	0.119
↑	42	0.048	0.081	0.036	−0.157	**−0.321**	0.119
miR-29b							
↓	50	−0.180	−0.210	**−0.283**	0.029	−0.038	0.131
↑	48	−0.114	−0.089	−0.190	−0.065	−0.203	0.037
miR-126							
↓	45	−0.274	0.094	−0.088	−0.174	**0.309**	−0.242
↑	53	−0.157	0.004	0.066	−0.073	−0.137	−0.258
miR-192							
↓	54	−0.020	−0.246	−0.236	−0.031	0.027	0.120
↑	44	0.022	0.227	−0.240	0.038	0.119	−0.163
miR-223							
↓	59	−0.181	−0.027	−0.185	−0.085	0.082	−0.078
↑	39	−0.102	0.077	−0.146	0.006	−0.161	0.068
miR-375							
↓	52	−0.086	−0.094	−0.143	0.015	0.068	0.246
↑	46	0.098	−0.021	0.096	0.016	0.071	−0.013

Single associations were tested between the end/baseline ratio by Pearson's correlation coefficient for the whole cohort (49 subjects per intervention group, $n = 98$). Results represent the coefficient of correlation

Significant associations ($P < 0.05$) are shown in bold

miRNA microRNA, ↓ down-regulation, ↑ up-regulation

The exact mechanisms linking miRNAs with the glucose and insulin metabolism are still unknown. MiR-21, miR-29 and miR-223, which are up-regulated after pistachio intake, were related to glucose transporters and insulin signaling, while miR-15a seems to regulate insulin biosynthesis by targeting UCP-2 in mice [25]. MiR-29 could act on various target tissues involved in insulin signaling and its regulation, whereas in muscle, together with miR-223, it favors insulin signaling and glucose uptake [8]. Whereas miR-223 induces glucose transporter 4 (GLUT4) protein expression to restore normal glucose uptake [26], miR-21 reversed the induced insulin resistance in 3T3-L1 cells and significantly increased insulin-induced glucose uptake by translocating GLUT4 to the cell membrane [27]. Moreover, the inhibition of miR-29a in primary mouse islets contributes to the β-cell silencing of the MCT1 transporter which is involved in the insulin secretion [28] and the "mimic" restoration of miR-29b in induced diabetic rats may be related to some protection from diabetic neuropathy and insulin resistance [29]. Following this miRNA pattern, our study participants significantly increased their plasma levels of miR-15, miR-21, miR-29b and miR-223 after pistachio consumption, which was mirrored in an improvement in their IR status, as we have previously demonstrated [13]. Moreover, except for miR-15a, all of these miRNAs had decreased plasma levels in response to the control diet when we evaluated PD–CD sequence independently. Accordingly, those subjects with increased miR-15 and miR-21 also showed decreased lymphocyte IL-6 expression, which may lead to amelioration in the inflammatory status. Similarly, those subjects who down-regulated miR-29 had a significant increase of GLUT3 expression at lymphocyte level that could contribute to an excessive glucose uptake, increasing immune hyperactivity and compromising health [30].

Of the insulin-related miRNAs, miR-375 is one of the first pancreas-related miRNAs that has been shown to contribute to glucose and insulin metabolism, and which can also be detected in serum and plasma [31]. In fact, it has been suggested that high levels of plasma miR-375 are an indicator of higher islet miR-375 expression in subjects with T2D or with glucose or insulin impairments such as prediabetic subjects [32]. Because serum miR-375 levels are significantly higher in T2D patients [33] and newly diagnosed type 2 diabetic subjects [34] than in normoglycemic or prediabetic subjects, this miRNA could be used as a new biomarker to track treatment response for prediabetes. Recently, Erener et al. [35] reported a significant increase in plasma miR-375 concentrations 2 weeks before the onset of diabetes in a non-obese mouse model of autoimmune

diabetes. Our results showed a significant decrease in circulating miR-375 after pistachio consumption that could be interpreted as a delay or reversion in the progression from pre-D to T2D.

One of the miRNAs most consistently associated with T2D is miR-126. It has been shown to be a master regulator of endothelial function [36], and it is highly enriched in endothelial cells [37]. In fact, significant miR-126 down-regulation has been described in plasma and circulating cells of T2D patients [22, 38]. Moreover, values of miR-126 have been reported to be significantly lower in both plasma [39] and serum in subjects with prediabetes or newly diagnosed T2D than in normoglycemic subjects [40]. After a 6-month intervention (consisting of a control diet and exercise in IGT/IFG subjects, and insulin plus control diet and exercise in T2D patients), serum miR-126 levels increased significantly, which encourages the use of serum miR-126 as a biomarker for prediabetes, diabetes mellitus and therapeutic response [40].

Recently, miR-192 levels have been found to be elevated in subjects with prediabetes but invariant in T2D subjects, thus showing that the organism has adapted to the metabolic profile in prediabetes which will be lost or modified after the onset of T2D [41]. Moreover, miR-192 is also increased in the plasma of glucose-intolerant mice, thus reproducing human outcomes in animal models. Interestingly, normalizing metabolic parameters by exercise significantly improved miR-192 and miR-193b levels [41]. Accordingly, our pistachio nutritional intervention was also able to improve glucose-related parameters and miR-192 compared with the nut-free diet.

When we analyzed those genes potentially modulated by miR-192 and miR-375, we found that *BCL2* gene was significantly implicated in silico in three different pathways mainly related to apoptosis, but also to protein processing and tuberculosis. Surprisingly, *BCL2* is linked to different pathways related to T2D, as some BCL2 family proteins are important controllers of the mitochondrial β-cell apoptosis induced by pro-inflammatory cytokines or lipotoxicity [42]. This is of important relevance in the pathogenesis of prediabetes and T2D due to the β-cell dysfunction which is associated with these diseases.

Several strengths and limitations of our study deserve comment. Among its strengths are its crossover randomized design, its medium-term duration and its use of well-known and efficient TaqMan probes for RT and qPCR analysis. However, by design, an important limitation of this trial is that it focuses on prediabetic subjects, so the results can only be extrapolated with caution to healthy subjects or subjects with T2D until further studies are carried out. Moreover, as we used literature-based targeted miRNAs and not the whole array of all known miRNAs, we cannot exclude that other miRNAs—related or not to the insulin and glucose metabolism—are modulated.

Conclusion

This is the first study to demonstrate that the regular consumption of pistachios can modulate diabetes-related circulating miRNA toward a healthier profile. The results support the well-established beneficial effects of nut consumption, and pistachios in particular, on metabolic conditions such as prediabetes and help to elucidate one of the possible mechanisms involved. Future studies are needed if the specific molecular mechanisms and networks that explain our results are to be understood and if these results are to be extended to other chronic conditions.

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Authors' contribution MB and JS-S had full access to all the data in the study and take full responsibility for the integrity and accuracy of the data analysis. Study concept and design were performed by MB and JS-S. Acquisition of data was carried out by MB and PH-A. Analysis and interpretation of data were done by MB, PH-A, SG, JS-S and PA. Drafting of the manuscript was performed by PH-A, MB and JS-S. Critical revision of the manuscript for important intellectual content was done by MB and JS-S. Statistical analysis was conducted by MB and PH-A. MB and JS-S obtained funding. All the authors received administrative, technical or material support. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest Author disclosures are as follows: PH-A, SG, PA and MB have nothing to declare. JS-S is a non-paid member of the Scientific Advisory Council of the International Nut Council.

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G. DISCUSSION

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G. DISCUSSION

This thesis was designed to investigate the possible role of pistachio consumption on ameliorating different metabolic outcomes related to the pre-D state. First, we evaluated the effect of pistachio consumption on glucose metabolism and IR as the most powerful indicators of pre-D progression. Second, we measured the level of different inflammatory and other related risk markers, together with the expression of specific T2D-associated genes in lymphocytes to account for putative modulations. Third, we analyzed changes on lipoprotein subclasses' size and composition due to their novel association with CV risk. Fourth, to expand our knowledge, we also measure the expression of a set of specific miRNAs related to glucose and insulin metabolism, to investigate the effects of pistachios over systemic miRNA modulation. Our findings, presented in the current thesis, reinforce the well-known beneficial properties of nuts and extend the healthy properties of pistachio nuts to a particular metabolic condition: pre-diabetes.

Glucose metabolism, inflammation and lipid profile

Accumulating evidence from epidemiological studies and clinical trials suggests that nut intake decreases the risk of CVD and T2D[289,415]. These findings have generated proposed mechanisms for these associations including improved insulin sensitivity, increased antioxidant activity, and reduced concentrations of TC and LDL-C. Several studies have evaluated the effect of nut intake on glycemia and insulinemia[416–418], but few have focused on pistachio intake[291,292]. Only one previous study evaluated the beneficial effects of almond consumption on insulin sensitivity and other CV risk factors in pre-D[417]. However, inclusion criteria involved casual blood glucose ≥140-199 mg/dL, thus differing from our study. Our study is the first to evaluate the chronic effect of pistachio consumption on glucose metabolism, IR, inflammation, and related-metabolic risk markers, at systemic, cellular and molecular levels in IFG-pre-diabetic subjects.

Different acute clinical studies have investigated the effect of pistachio consumption on post-prandial glucose levels. In fact, a beneficial dose-response effect of pistachios has been observed on post-prandial glycemia and insulinemia when pistachios are consumed with CHO foods[306,307]. Moreover, some RCT have

observed a significant decrease in FPG and/or insulin levels[291–293] after pistachio intake in subjects with T2D or MetS. However, our study is the first to demonstrate a significant reduction in both FPG and fasting insulin levels, together with HOMA-IR, in pre-diabetic subjects.

Pistachios tend to have a beneficial effect on the inflammatory and oxidative status[291–293,295,311] attributed to their specific profile including healthy fat, high fiber content, phytosterols and other active components such as lutein, β-carotene, and γ-tocopherol[271]. Some studies have demonstrated an increased antioxidant capacity in healthy subjects after pistachio consumption[295] along with an improvement in the circulating levels of inflammation and oxidation markers (IL-6, CRP, TNF-α, adiponectin, ox-LDL) in healthy, T2D, MetS or mild-hypercholesterolemic subjects[291–293,311]. Similarly, we found a significant reduction in ox-LDL, fibrinogen and PF-4 during the pistachio diet although no change was observed in circulating IL-6 levels. Contrasting with circulating measures, lymphocytes' expression of IL-6 and resistin were significantly lowered in PD vs CD, suggesting a pistachio-mediated impact on classical inflammatory markers of glucose and insulin metabolism. These differences between systemic and cellular levels regarding IL-6 and resistin could be due to the fact that their protein levels are measured as circulating overall plasma levels, whereas *IL-6* and *RETN* mRNAs are evaluated at lymphocytes level. Therefore, it may suggest that lymphocytes could be an initial modulated target after pistachio consumption.

In fact, lymphocytes play an important role in inflammatory responses, thus they need to be tightly regulated to maintain health. Whilst hypoglycemia decreases the viability of peripheral blood cells, hyperglycemia leads to an excessive glucose uptake thus promoting immune hyperactivity and compromising health[419]. Further, significant increased GLUT-4 levels on the surface of lymphocytes has been described in both T2D subjects[420] and pre-D subjects[421]. We have demonstrated that pistachio consumption significantly decrease *SLC2A4* mRNA (codifying GLUT-4) which is mirrored in a lower increase in cellular glucose uptake during PD. This decrease can be explained by circulating insulin levels after dietary treatment. Therefore, these findings suggest a potential mechanism by which pistachios could lead to a healthier systemic inflammatory profile.

The effect of pistachios on insulin metabolism could be partly explained by an increase in GLP-1 levels, a gastric hormone that stimulate pancreatic insulin secretion and suppress glucagon secretion in a glucose-

dependent manner[54,422]. GLP-1 levels increased after almonds intake[423] and were also significantly increased in a feeding acute trial conducted on MetS participants consuming pistachios[306]. In the present study, we extend these results, and show that pistachios have a long-term stimulating effect on GLP-1 and insulin-sparing effects in patients with pre-D and support previous observations[424] suggesting that the high MUFA/PUFA content in nuts may improve β-cell efficiency through enhanced intestinal secretion of GLP-1[425].

The lipid-lowering effect of nut consumption has been observed in several large observational studies (reviewed by Ros[426]). Moreover, a systematic review and meta-analysis of 61 RCTs (N=2,582) has recently shown a significant reduction of TC, LDL-C, and triglycerides after nut consumption[427]. Consistently, in hyper-cholesterolemic, normolipidemic and healthy subjects the regular intake of nuts has shown to reduce the serum LDL-C, without significantly affecting TG or HDL-C[272]. However, the effect of pistachio consumption on LDL-C concentrations is less consistent, tending to decrease[291,295] but not always significantly[295]. In particular, consumption of pistachio has been reported to induce a significant reduction in TC, TC/HDL-C ratio and/or LDL-C/HDL-C ratio[291,292,294–298,300] and a significant increase in plasma HDL-C[295,297,300] particularly in mild hyper-cholesterolemic subjects. However, these lipid-lowering properties attributed to nuts are more controversial in obese subjects or IR subjects[414,428]. In our study we found no significant changes in TC, LDL-C or HDL-C after pistachio consumption supporting the hypothesis that the lipid profile of these subjects is less likely to undergo changes - compared with hyper-cholesterolemic subjects - because alterations in the cholesterol homeostasis inherent to their state makes them resistant to down-regulating cholesterol[382].

Novel risk markers of IR, T2D and CVD

Beyond the classical lipid profile, interest in the size and composition of lipoprotein subclass particles (small, medium and large) has been growing due to their potential effect on atherosclerosis and CV risk[366]. As we have previously mentioned, we did not report any significant modulation in the classic lipid profile. Therefore, we decided to investigate the variation of lipoproteins (i.e. particle concentration and size).

Discussion

Unlike large-LDL-P, small dense-LDL-P confers greater atherogenic risk because of its interaction with the arterial wall comprising: increased residence time in the circulation, easy penetration into the sub-endothelial space and greater susceptibility to oxidative modifications[366]. Moreover, recent studies have suggested that HDL particles (HDL-P) may be a more suitable and independent risk factor than HDL-C[429]or a combination of both parameters, HDL-C/HDL-P, can determine the anti-atherogenic function of HDLs, rather than either parameter alone[430]. Small-HDL-P possess multiple anti-atherogenic properties, including potent cholesterol efflux capacity and anti-oxidative, anti-inflammatory and anti-apoptotic activities[431]. However, the anti-atherogenic activities of small-HDL-P are defective in atherogenic dyslipidemia conditions (i.e. high small LDL-P, low HDL-C and high TG)[431,432]. Therefore, small-HDL-P has a dual role in CVD as their presence is anti-atherogenic except in the presence of atherogenic dyslipidemia. In fact, recent investigations have shown that in T2D subjects with atherogenic dyslipidemia[433], the reduction in the size of HDL-P, force cholesterol esters and triglycerides to emerge from the HDL core to the surface. It makes the outer surface of HDL more hydrophobic which may alter molecular interactions[434]. However, even though some of our subjects have treated dyslipidemia, none of them have atherogenic dyslipidemia, thus the increase in small-HDL-P following PD may reflect an atheroprotective activity which is in fact also exhibited by a reduction in small-LDL-P. Beyond this, lipoprotein subclass abnormalities have also been related to IR and T2D as concentrations of small-LDL-P are commonly higher in IR and T2D subjects[435,436]. The results found in the IRAS[437], were similar and suggested that dyslipidemia associated with IR or T2D is not detected when the traditional lipid profile is evaluated. The IRAS study also demonstrated for the first time that, independently of TG and HDL-C concentrations, lipoprotein subclasses were positively associated with the incidence of T2D at 5-year follow-up[334].

Although a considerable amount of research has been carried out on the conventional lipid profile, the modulatory effect of dietary fatty acids on the size and concentration of lipoprotein subclasses has been poorly analyzed. In fact, the effect of MedDiet on LDL-P remains controversial as some studies have found reduction in small-LDL-P following a MedDiet[438–440] whereas others failed to show any changes[441]. However, these studies varied in duration and sample size. Beyond MedDiet, few studies have evaluated the effect of specific nut consumption on the distribution and particle size of lipoprotein subclasses. First

in 2001, Almario et al. conducted a study in adults with combined hyperlipidemia who consumed 48g of walnuts for 6 weeks. Cholesterol decreased, particularly in small-LDL-P and large-HDL particles, which suggested that the beneficial properties of nut intake on CV risk may be affected by an additional mechanism[383]. Recent research on pistachio consumption has also found a significant decrease of small-LDL-P in subjects with elevated LDL levels (≥2.86 mmol/L)[309]. In the context of the PREDIMED trial, results showed that the MedDiet with nuts reduced the concentration of atherogenic small-LDL-P and increased LDL-P size[442]. In agreement with these results, our study demonstrates that chronic consumption of pistachios induces a significant decrease of small-LDL-P concentrations and HDL-P size, despite the absence of changes in TC, LDL-C or HDL-C. Moreover, we found a significant decrease in the concentration of non-HDL-particles, which are also strongly associated with CV risk[443].

Accumulating evidence suggests that circulating miRNAs are useful biomarkers of diseases such as T2D and CVD[400]. In this regard, a specific plasma miRNA signature has been described in patients with T2D consisting of low levels of miR-15a, miR-21, miR-29b, miR-126, miR-223, among others, together with the capacity of some miRNAs to predict the onset of T2D over a 10-year follow-up period[399]. Similarly, a recent meta-analysis of controlled profiling studies found that a total of 40 miRNAs were significantly deregulated in T2D, ten of which (including miR-375 and miR-223) were identified as potential circulating or tissue T2D biomarkers[444]. Additionally, peripheral blood and circulating miR-15a and miR-126 were found to be significantly decreased in patients with T2D and IFG/IGT individuals, compared with healthy control subjects[399,445–448], thus they may serve as potential biomarkers for T2D and pre-D. However, the mechanisms regulating the circulating miRNA profile are still under debate, and little is known about the potential modulatory role of diet. We selected seven miRNAs as strongly related to glucose homeostasis (e.g. GLUT-4), pancreatic β-cell viability and function and insulin metabolism (receptor and internal cascades). Five of these miRNAs (i.e. miR-15a, miR-21, miR-29b, miR-126, miR-223) have been found to be down-regulated, and two of our set of miRNAs (i.e. miR-192 and miR-375) has been found up-regulated in circulating levels comparing T2D and/or pre-D subjects with NGT subjects[449]. Up-regulation of some of these T2D-related miRNAs after PD observed in our study together with the improved IR status reinforces the beneficial role of this type of nut on glucose and insulin metabolism and their putative role in lowering the risk of T2D. These modulatory effects could be explained by the pistachios' content in polyphenols

(mainly resveratrol) and PUFAs as it has been previously suggested by other authors in similar food[401,402,450].

Nevertheless, the exact mechanisms linking miRNAs with the glucose and insulin metabolism are still unknown. MiR-21, miR-29 and miR-223, which are up-regulated after PD, were related to glucose transporters and insulin signaling, whilst miR-15a seems to regulates insulin biosynthesis by targeting UCP-2 in mice[451] and miR-126 has been shown to be a regulator of endothelial function[452,453]. MiR-29 could act on various target tissue involved in insulin signaling and its regulation, whereas in muscle, together with miR-223, it favors insulin signaling and glucose uptake[387]. Whereas miR-223 induces GLUT-4 protein expression to restore normal glucose uptake[454], miR-21 reversed the induced IR in 3T3-L1 cells and significantly increased insulin-induced glucose uptake by translocating GLUT-4 to the cell membrane[455]. Following this miRNA pattern, our study subjects significantly increased their plasma levels of miR-15, miR-21, miR-29b and miR-223 after pistachio consumption, which was mirrored in an improvement in their IR status. Accordingly, those subjects who increased miR-15 and miR-21, decreased lymphocytes' *IL-6* expression, which may lead to amelioration in the inflammatory state. Similarly, those subjects who down-regulated miR-29 had a significant increase of *SLC2A3* expression (codifying GLUT-3) at lymphocytes' level that, as we have mentioned, could contribute to an excessive glucose uptake, increasing immune hyperactivity and compromising health[419].

Of the insulin-related miRNAs, miR-375 is one of the first pancreas-related miRNAs that has been shown to contribute to glucose and insulin metabolism[397]. Both *in vivo* studies in mice and human studies suggest that miR-375 is specifically expressed in β-cells and that increased plasma levels of miR-375 are associated with β-cell death, predicting also hyperglycemia in pre-D and T2D subjects[332]. Because serum miR-375 levels are significantly higher in T2D patients[456,457] and newly diagnosed T2D subjects[330] compared to normoglycemic or pre-D subjects, this miRNA could be used as a new biomarker to track treatment response for pre-D. Recently, Erener et al. reported a significant increase in plasma miR-375 concentrations two weeks before the onset of diabetes in a non-obese mouse model of autoimmune diabetes[458]. Another miRNA recently found to be altered in T2D/pre-D is miR-192. In fact, miR-192 levels have been found to be elevated in subjects with pre-D but invariant in T2D subjects, thus showing that the organism has adapted to the metabolic profile in pre-D which will be lost or modified after the onset of

T2D[459]. Moreover, miR-192 is also increased in the plasma of glucose-intolerant mice, thus reproducing human outcomes in animal models. Interestingly, normalizing metabolic parameters by exercise significantly improved miR-192 levels[459]. Our results showed a significant decrease in circulating miR-375 after pistachio consumption and a significant increase of miR-192 following CD, together with an improvement in glucose-related parameters, which could be interpreted as a delay or reversion in the progression from pre-D to T2D during the pistachio intervention.

Taken together, our results support the beneficial role of pistachio consumption on insulin resistance and chronic inflammation through the modulation of several mechanisms including lipoprotein size and composition and modulation of miRNAs.

Strengths and Limitations

Several strengths and limitations of our study deserve comment in this thesis. Among its strengths are the cross-over randomized design, its medium-term duration, the presence of dietary compliance markers (i.e. lutein-zeaxanthin and γ-tocopherol) and different levels of approach: systemic, cellular and molecular. We have also used NMR to analyze lipoprotein subfractions, which it has been suggested as a better CHD estimator than non-denaturing polyacrylamide gradient gel electrophoresis[460], and we used efficient TaqMan probes for RT and qPCR analysis in both genes' and miRNAs' experiments. Importantly, our subjects were pre-diabetic that were not taking any drugs that may interfere with examined parameters. However, by design, an important limitation of this trial is precisely that it was focused on pre-D patients. Therefore, results may only be extrapolated to healthy subjects or subjects with T2D with caution pending further studies. Despite the cross-over design, other limitations could be related to the relatively heterogeneity of the subjects studied as the presence of dyslipidemia or the use of lipid-lowering treatments. Because data on HDL function was not measured, the implications of changes in HDL subtype distribution need to be further investigated. Moreover, as we used literature-based targeted miRNAs and not the whole array of all known miRNAs, we cannot exclude that other miRNAs – related or not to the insulin and glucose metabolism – were also modulated.

Future insights

Research focused on the effects of nuts on different health outcomes have increased since the first epidemiological studies demonstrating their beneficial role, thereafter corroborated in several specific RCT. Investigation has widely focused on the beneficial effects of nuts on already-established diseases such as T2D and MetS. However, little information is currently available on the potential role that nuts play in preventing the development of chronic diseases such as T2D. In this thesis and for the first time, we have demonstrated a beneficial effect of pistachio intake on insulin resistance through modulation of insulin and glucose levels, along with other biological mediators. However, long term preventive studies should be addressed to contrast our results. Moreover, due to their content in specific vitamins and minerals, nuts could also have a protective role regarding neurodegenerative diseases such as Alzheimer disease. Beyond classical metabolic research, recent findings have shown that nuts have a potential prebiotic effect. Therefore, there is a huge need for research aiming to evaluate the distribution of microbiota composition, as it is clear that nut consumption may be changing different metabolites present in plasma and excreted in urine and feces.

Subjects with T2D or at high cardiovascular risk - in whom nut consumption could be even more beneficial compared to the general population - are commonly overweight or obese, as these metabolic disorders are highly correlated. Even though no study has demonstrated an increase in body weight after nut consumption, some studies have reported a reduction following nut consumption. However, general population still considers nuts as a fatty food with deleterious effects on body weight, thus they remove them from their usual diets. However, nuts have satiating properties mainly attributed to its high content of fiber; their demonstrated GLP-1 modulatory effects; and due to the fact that energy in form of fatty acids could not be totally metabolized and differs from energy calculated by the Atwater general factors. Therefore, well-conducted *ab libitum* trials are needed to investigate the real effect of nuts on weight management.

H. CONCLUSIONS

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H. CONCLUSIONS

The main finding of this thesis is that a chronic pistachio diet is able to ameliorate pre-diabetes state by modulating the insulin resistance state.

The specific conclusions of the EPIRDEM study are:

1. The inclusion of pistachios into a balanced diet modulates plasma glucose and insulin levels which is reflected in a significant reduction in the insulin resistance of the subjects.

2. Pistachio intake is able to improve inflammatory markers and other metabolic risk markers (fibrinogen, platelet factor-4 and ox-LDL) increasing also the levels of satiety biomarkers (GLP-1).

3. The inclusion of pistachios significantly reduces lymphocyte's expression of inflammatory genes (*IL-6* and *RETN*), the expression of *SLC2A4* (codifying GLUT-4) and overall glucose uptake.

4. Chronic consumption of pistachios has a beneficial effect on emergent cardiovascular risk factors such as lipoprotein composition (size and concentration), even though it has little or no effect on classic lipid risk markers in pre-diabetic subjects.

5. Chronic pistachio consumption supports a healthier miRNA profile by modulating the expression of those miRNAs associated to glucose and insulin metabolism.

This study's findings build on the literature by describing the health-enhancing properties of pistachios, along with nuts in general, and in particular its benefits for glucose metabolism and cardiovascular health.

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I. REFERENCES

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J. ANNEX

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J. ANNEX

Annex I. Review Article 1

Title: Nutrition attributes and health effects of pistachio nuts.

Authors: Mònica Bulló; Martí Juanola-Falgarona; Pablo Hernández-Alonso and Jordi Salas-Salvadó.

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Impact factor: 3.311; 18/77; Q1; Nutrition & Dietetics.

Abstract:

Epidemiological and/or clinical trials have suggested that nut consumption has a beneficial impact on health outcomes such as hypertension, diabetes, CVD, cancer, other inflammatory conditions and total mortality. Nuts are nutrient-dense foods with a healthy fatty acid profile, as well as provide other bioactive compounds with recognised health benefits. Among nuts, pistachios have a lower fat and energy content and the highest levels of K, γ-tocopherol, vitamin K, phytosterols, xanthophyll carotenoids, certain minerals (Cu, Fe and Mg), vitamin B_6 and thiamin. Pistachios have a high antioxidant and anti-inflammatory potential. The aforementioned characteristics and nutrient mix probably contribute to the growing body of evidence that consumption of pistachios improves health. The present review examines the potential health effects of nutrients and phytochemicals in pistachios, as well as epidemiological and clinical evidence supporting these health benefits.

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Nutrition attributes and health effects of pistachio nuts☆

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Abstract

Epidemiological and/or clinical trials have suggested that nut consumption has a beneficial impact on health outcomes such as hypertension, diabetes, CVD, cancer, other inflammatory conditions and total mortality. Nuts are nutrient-dense foods with a healthy fatty acid profile, as well as provide other bioactive compounds with recognised health benefits. Among nuts, pistachios have a lower fat and energy content and the highest levels of K, γ-tocopherol, vitamin K, phytosterols, xanthophyll carotenoids, certain minerals (Cu, Fe and Mg), vitamin B_6 and thiamin. Pistachios have a high antioxidant and anti-inflammatory potential. The aforementioned characteristics and nutrient mix probably contribute to the growing body of evidence that consumption of pistachios improves health. The present review examines the potential health effects of nutrients and phytochemicals in pistachios, as well as epidemiological and clinical evidence supporting these health benefits.

Key words: Pistachios: CVD: Blood glucose: Insulin resistance: Polyphenols: Antioxidants: Body weight

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The health benefits of nuts, mainly in relation to CVD as well as to other chronic conditions, have been widely demonstrated in both epidemiological[1] and clinical[2,3] trials. For this reason, the American Heart Association[4,5], the Canadian Cardiovascular Society[6] and the US Food and Drug Administration[7] recommend the regular consumption of nuts to the general population, in the context of a healthy diet, to prevent the risk of CVD. Recently, nut consumption has also been inversely associated with total mortality[8,9]. Nuts are the rich sources of unsaturated fatty acids, fibre and protein, along with many vitamins (vitamins E and B_6, niacin or folic acid), minerals (Mg, K and Cu) and other phytochemical constituents (stigmasterol, campesterol, resveratrol and catechins)[10]. Compared with other nuts, pistachios have a lower fat (mostly from PUFA and MUFA) and energy content, and higher levels of fibre (both soluble and insoluble), K, phytosterols, γ-tocopherol, vitamin K, and xanthophyll carotenoids[10] (Table 1). Pistachios are among the top fifty foods with a high antioxidant potential[11]. In addition, pistachios are the only nut that contains significant amounts of lutein and zeaxanthin[10]. Polyphenols, xanthophylls and tocopherols from pistachios have been demonstrated to be rapidly accessible in the stomach, thus maximising the possibility of absorption in the upper small intestine, thereby contributing to the beneficial relationship between pistachio consumption and health-related outcomes[12].

The present review examines the potential health effects of compounds in pistachios as well as epidemiological and

Abbreviations: BP, blood pressure; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; T2DM, type 2 diabetes.

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Table 1. Macronutrient contents of the selected nuts per 100 g (raw and dry roasted)*

	Almonds	Hazelnuts	Macadamia nuts	Peanuts	Pecans	Pistachio nuts	Walnuts
Energy (kJ)							
Raw nuts	579	628	718	567	691	562	654
Dry roasted nuts	598	646	718	585	710	567	NA
Total lipids (g)							
Raw nuts	49·93	60·75	75·77	49·24	71·97	45·39	65·21
Roasted nuts	52·54	62·40	76·08	49·66	74·27	44·82	NA
SFA (g)							
Raw nuts	3·80	4·46	12·06	6·83	6·18	5·56	6·13
Roasted nuts	4·09	4·51	11·94	6·89	6·28	5·45	NA
PUFA (g)							
Raw nuts	12·39	7·92	1·5	15·56	21·61	13·74	47·17
Roasted nuts	12·96	8·46	1·5	15·69	20·57	13·44	NA
MUFA (g)							
Raw nuts	31·55	45·65	58·88	24·43	40·8	23·82	8·93
Roasted nuts	33·08	46·60	59·27	24·64	43·95	23·67	NA
Proteins (g)							
Raw nuts	21·15	14·95	7·91	25·8	9·17	20·27	15·23
Roasted nuts	20·96	15·03	7·79	23·6	9·50	20·95	NA
Carbohydrates (g)							
Raw nuts	21·55	16·70	13·82	16·13	13·86	27·51	13·71
Roasted nuts	21·01	17·60	13·38	21·51	13·55	29·38	NA
Fibre (g)							
Raw nuts	12·5	9·7	8·6	8·5	9·6	10·3	6·7
Roasted nuts	10·9	9·4	8·0	8·0	9·4	9·9	NA

NA, not available.
* US Department of Agriculture, Nutrient Database for Standard Reference, Release 26, 2013[10].

clinical evidence supporting the health benefits of pistachio consumption.

Bioactive components of pistachios

Nuts and diet quality

Recent epidemiological studies conducted in children and adults have demonstrated a significant association between nut consumption and a higher diet quality score or improved nutrient intakes[13,14]. O'Neil *et al.*[13], in a study of 13 292 adults participating in the 1999–2004 National Health and Nutrition Examination Survey, observed that tree nut consumers, defined as those consuming more than 7·09 g/d of nuts or tree nut butters, had a significantly higher intake of several nutrients as fibre, vitamins and minerals, and also a higher Total Healthy Eating Index-2005 score. Similarly, in an analysis including concatenated data from adults aged 2+ years participating in the National Health and Nutrition Examination Survey 1999–2000, 2001–02 and 2003–04, consumption of more than 7·08 g/d was associated with a healthier nutrient profile and higher Total Healthy Eating Index-2005 score in consumers of all age groups. Moreover, adult consumers showed a better metabolic risk profile[14]. Furthermore, the results of a clinical trial conducted on 124 obese subjects demonstrated that nutritional dietary quality among nut consumers (those eating 42 g hazelnuts/d for 12 weeks) was appreciably improved compared with other groups consuming chocolate, potato crisps or no additional foods[15]. Finally, the inclusion of nuts in energy-restricted diets reduced attrition and increased weight loss, supporting that nuts enhance palatability and compliance with diets without compromising beneficial health effects[16].

Fat content

Pistachios, compared with other nuts, are relatively low in fat, containing 45·4 g total fat per 100 g pistachio kernel and consisting of 5·6 g SFA, 13·7 g PUFA and 23·8 g MUFA (Table 1)[10]. Within fatty acids, oleic and linoleic fatty acids, both recognised for their cardiovascular-preventive properties[17], represent more than 60 % of the total fat content in pistachios.

The USA (California, Arizona and New Mexico), Iran and Turkey are the largest producers of pistachios, growing varieties that differ slightly in nutritional composition. Whereas US pistachios have less energy and contain higher amounts of lutein and zeaxanthin, Iranian pistachios are richer in linoleic acid[18] and Turkish pistachios in Ca[19] (Table 2). Fatty acid composition and nutritional profile characteristics also depend on the climate in which the pistachios are grown. For example, cultivars of pistachio nuts grown in hot temperatures (over 25°C) tend to produce a lower amount of a saturated fat such as palmitic acid[20].

Protein

Pistachios are a good source of vegetable protein, which comprises about 20 % of total weight, with approximately 2 % L-arginine[21]. This amino acid, also present in other nuts, is a precursor to the endogenous vasodilator NO, an important molecule involved in the cardiovascular system as a key regulator of vascular tone and in numerous pathological conditions such as hypertension, CVD and neurodegenerative disorders due to its pro-oxidant capacity[22,23]. NO synthase inhibitors based on arginine have been of special interest for experimental as well as clinical applications[24]. Therefore, pistachios could

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Table 2. Comparison of nutrient contents of pistachio seeds by country of origin

	US pistachios* (roasted/salted, 28 g)		Iranian pistachios† (roasted/salted, 28 g)		Turkish pistachios‡ (roasted/salted, 28 g)	
Nutrients	Absolute value	% DV	Absolute value	% DV	Absolute value	% DV
Total energy (kJ)	669·44		761·48		790·77	
Total fat (g)	12·7	20 %	15·1	23 %	16·4	25 %
Energy from fat (kJ)	120		136		147	
Saturated fat (g)	1·5	8 %	1·5	8 %	1·8	9 %
Monounsaturated fat (g)	6·7		9·1		11·1	
Polyunsaturated fat (g)	3·8		3·9		2·8	
Linoleic acid (18 : 2) (g)	3·7		4·0		2·9	
Linolenic acid (18 : 3) (g)	0·07		0·06		0·05	
Trans-fat (g)	0		<0·02		<0·01	
Cholesterol (mg)	0	0 %	<0·28	0 %	<0·28	0 %
Na (mg)	121	5 %	163	7 %	162	7 %
Total carbohydrate (g)	8·1	3 %	5·3	2 %	4·4	1 %
Sugars (g)	2·2		1·4		0·8	
Fibre (g)	2·8	11 %	3·1	12 %	2·8	11 %
Protein (g)	5·9	12 %	6·1	12 %	5·9	12 %
Vitamin A (μg)	43·8	1 %	<5·95	0 %	34·38	1 %
β-Carotene (μg)	44		<5·7		34·3	
α-Carotene (μg)	0		<5·7		<5·7	
β-Cryptoxanthin (μg)	0		<5·7		<5·7	
Lycopene (μg)	0		<5·7		<5·7	
Lutein + zeaxanthin (μg)	329		<127·7		204	
Vitamin C (mg)	0·9	2 %	<0·28	0 %	<0·28	0 %
Ca (mg)	30	3 %	35·7	4 %	45·9	5 %
Fe (mg)	1·1	6 %	0·64	4 %	0·78	4 %

% DV, % daily value.
* US Department of Agriculture National Nutrient Database for Standard Reference, Release 26, 2013[10].
† Covance Certificate of Analysis[18].
‡ Covance Certificate of Analysis[19].

play an important protective role in NO synthase-related diseases. On a per serving basis (28·35 g), pistachios provide 10·6 % US RDA of adult men and 12·9 % of adult women[25]. Compared with the FAO- and WHO-recommended essential amino acid pattern for an adult, pistachios contain adequate amounts of all of the essential amino acids[26]. Pistachios have an essential amino acid ratio (essential amino acid:total amino acid) of 39·1, higher than most of all the commonly consumed nuts (almonds, walnuts, pecans and hazelnuts). Pistachios also provide a high percentage of branched-chain amino acids (1·599 g leucine, 0·932 g isoleucine and 1·262 g valine per 100 g), higher than other tree nuts.

Carbohydrates and fibre

The amount of carbohydrate in pistachios, as in other nuts, is low to moderate (about 27·5 % by weight), but pistachios are rich in fibre, containing 10 % by weight of insoluble forms and 0·3 % of soluble forms. Pistachios provide 3 g or 12 % of RDA per serving basis (Table 1)[10]. According to the US Department of Agriculture food composition tables, of all nuts, only almonds have similar amounts of fibre, with 13 % of weight. Fibre content is important because epidemiological and clinical studies have consistently demonstrated that fibre intake is inversely associated with weight gain[27], diabetes[28], CVD[29] and some types of cancer[28]. Moreover, pistachios have a low glycaemic index, which contributes to maintaining satiety longer and lowering postprandial blood glucose concentrations[30,31].

Vitamins and minerals

Pistachios are rich in Cu, Mg, Mn, vitamin A, vitamin C and B vitamins, with the exception of vitamin B_{12} (cyanocobalamin)[32], compared with other nuts (Table 3). In particular, pistachios contain relatively high amounts of thiamin (vitamin B_1), which is involved in intermediary carbohydrate metabolism, with 0·87 mg/100 g of pistachios (providing up to 50 % of the RDA). The amount of pyridoxine (vitamin B_6) that is involved in the metabolism of amino acids and in the production of niacin is about 1·7 mg/100 g of pistachios, exceeding the RDA. Finally, the amount of folic acid in pistachios provides approximately 25 % of the RDA. Folic acid is necessary for the formation of structural proteins and Hb, and deficiency leads to an increase in the risk of CVD[33]. Among nuts, pistachios also stand out for high vitamin K content, with approximately 13·2 μg/100 g (16 % of the RDA; Table 3). Beyond its role in bone metabolism[34–36], a higher dietary intake of vitamin K has been associated with a lower risk of several chronic diseases such as type 2 diabetes (T2DM)[34], cancer[37,38] and CVD[38], thus expanding the potential health benefits of pistachio consumption. The beneficial role of pistachios in inflammatory-related diseases may also be explained by the relatively high amount of γ-tocopherol they contain[39].

Pistachios are rich in several minerals such as K, Mg, Ca, Cu and Mn. Because of their mineral profile, pistachios could play a beneficial role in blood pressure (BP) regulation or in bone-related diseases. Pistachios also contain significant

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Table 3. Micronutrient contents of the selected nuts per 100 g (raw and dry roasted)*

	Almonds	Hazelnuts	Macadamia nuts	Peanuts	Pecans	Pistachio nuts	Walnuts
Vitamin A (μg)							
Raw nuts	0·6	12	0	0	33·6	249	12
Roasted nuts	0·6	36·6	0	0	84	155·4	NA
Vitamin C (mg)							
Raw nuts	0	6·3	1·2	0	1·1	5·6	1·3
Roasted nuts	0	3·8	0·7	0	0·7	3·0	NA
Vitamin K (μg)							
Raw nuts	0	14·2	NA	0	3·5	13·2	2·7
Roasted nuts	0	NA	0	0	NA	13·2	NA
Vitamin B_6 (mg)							
Raw nuts	0·14	0·56	0·27	0·34	0·21	1·70	0·53
Roasted nuts	0·13	0·62	0·35	0·25	0·19	1·12	NA
Vitamin B_{12} (mg)							
Raw nuts	0·14	0	0	0	0	0	0
Roasted nuts	0·00	0	0	0	0	0	NA
Folate (μg)							
Raw nuts	44	113	11	240	22	51	98
Roasted nuts	55	88	10	145	16	51	NA
Thiamin (mg)							
Raw nuts	0·20	0·73	1·19	0·64	0·66	0·87	0·34
Roasted nuts	0·07	0·33	0·71	0·43	0·45	0·69	NA
Riboflavin (mg)							
Raw nuts	1·13	0·11	0·16	0·13	0·13	0·16	0·15
Roasted nuts	1·20	0·12	0·08	0·09	0·10	0·23	NA
Niacin (mg)							
Raw nuts	3·61	1·80	2·47	12·06	1·16	1·30	1·12
Roasted nuts	3·64	2·05	2·27	13·52	1·16	1·37	NA
Ca (mg)							
Raw nuts	264	114	85	92	70	105	98
Roasted nuts	268	123	70	54	72	107	NA
Fe (mg)							
Raw nuts	3·72	4·7	3·7	4·6	2·5	3·9	291
Roasted nuts	3·73	4·3	2·7	2·3	2·8	4·0	NA
Mg (mg)							
Raw nuts	270	163	130	168	121	121	201
Roasted nuts	279	173	118	178	132	109	NA
K (mg)							
Raw nuts	705	680	368	376	410	1025	441
Roasted nuts	713	755	363	658	424	1007	NA
Na (mg)							
Raw nuts	1	0	5	18	0	1	2
Roasted nuts	3	0	4	6	1	6	NA
Total phenol (mg)							
Raw nuts	287	687	126	406	1284	867	1576
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Flavonoids (mg)							
Raw nuts	15	12	NA	0·7	34	14	3
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Procyanidins (mg)							
Raw nuts	184	500	NA	16	494	237	67
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Tocopherols (mg)							
Raw nuts	25	33	4	8	4	7	6
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Carotenoids (μg)							
Raw nuts	2	106	NA	NA	55	332	NA
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Lutein + zeaxanthin (μg)							
Raw nuts	1	92	0	0	17	1405	9
Roasted nuts	NA	NA	NA	NA	NA	NA	NA
Total phytosterols (mg)							
Raw nuts	120	0	116	220	102	214	108
Roasted nuts	NA	NA	NA	NA	NA	NA	NA

NA, not available.
* US Department of Agriculture, Nutrient Database for Standard Reference, Release 26, 2013[10]. Polyphenol data were obtained from the Phenol-Explorer database (http://www.phenol-explorer.eu)[32].

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amounts of Zn and Se, both minerals with recognised antioxidant effects that are involved in the prevention of CVD and some types of cancer[40,41].

Phenol content

Pistachios, pecans and walnuts are rich sources of phenolic compounds, including anthocyanins, flavonoids, proanthocyanidins, flavonols, isoflavones, flavanones, stilbenes, phenolic acids and hydrolysable tannins, which are important as antioxidants and also for their chemopreventive, cardioprotective and vasoprotective properties[42,43]. Phenolic compounds may have protective effects against diseases related to free radical overproduction, such as CVD and cancer. A randomised, double-blinded, cross-over study with placebo *v.* a supplement of 640 mg anthocyanins daily during 4 weeks in pre-hypertensive men showed a significant increase in HDL-cholesterol (HDL-C) levels and also blood glucose levels after anthocyanin *v.* placebo treatment[44]. Furthermore, the hydrophilic extract from pistachios, which has high antioxidant activity, increases the resistance of human LDL-cholesterol (LDL-C) from healthy subjects to Cu-induced oxidation after 2 h of incubation[45].

According to Tomaino *et al.*[46], all phenolic groups found in pistachios, and in other nuts, are present in higher amounts in the skins than in the seeds. *Pistacia vera* L. (variety Bronte) skins contain cyanidin-3-*O*-galactoside (5865 mg/g), gallic acid (1453 mg/g), catechin (377 mg/g) and eriodictyol-7-*O*-rutinoside (366 mg/g). Pistachio kernels contain quercetin-3-*O*-rutinoside (98·1 mg/g), genistein (69·1 mg/g), genistein-7-*O*-glucoside (47·0 mg/g) and daidzein (42·4 mg/g). Therefore, the final content of total flavonoids in the skins is 70·27 (SD 5·42) mg of catechin equivalents/g of fresh weight, whereas in the seeds, it is only 0·46 (SD 0·03) mg of catechin equivalents/g of fresh weight[46]. Pistachios are the only nut containing anthocyanins, phenolic compounds, in the skin. These phenolic compounds are known to bind metals through binding with *o*-diphenol groups, which is important in the inhibition of metal-induced lipid oxidation[47]. Nonetheless, in a simulated human digestion model, more than 90 % of the pistachio polyphenols were released to the gastric compartment without differences between raw or roasted pistachios[12,48].

Carotenoids

Lutein and zeaxanthin are two xanthophyll carotenoids responsible for giving colour to pistachio nuts. Raw pistachios contain 1405 μg lutein + zeaxanthin/100 g, about thirteen times more than the next highest nut type, hazelnuts, which contain only 92 μg (Table 3). The bioavailability of carotenoids depends on the source and interaction with other dietary components. Van Het Hof *et al.*[49] demonstrated that the interaction of β-carotene and lycopene with the lipid matrix increases the bioavailability of carotenoids. Notably, almost 100 % of the bioaccessibility of lutein was found after *in vitro* duodenal digestion[12]. Carotenoids have antioxidant properties and have been associated with a reduced risk of CVD and some types of cancer[49]. Moreover, lutein and zeaxanthin are concentrated in the retina where they thought to function as antioxidants and/or as a blue light filter, to protect the underlying tissues from phototoxic damage[50]. This has been proposed as an important factor in the pathophysiology of age-related macular degeneration[51].

Total phytosterols

Among nuts, pistachios have the highest phytosterol content, with 214 mg/100 g, including stigmasterol, campesterol and β-sitosterol. Phytosterols, structurally similar to cholesterol, have the same basic cyclopentanoperhydrophenanthrene ring structure but differ in the side chain at C24 and/or the position and configuration of unsaturated double bonds and the optical rotation at chiral carbons. Several studies have demonstrated a dose–response reduction of cholesterol mediated by phytosterols, even at lower levels similar to those found in plant-based diets with pistachios[52]. Although 500 mg of phytosterols per serving are needed to support the Food and Drug Administration (FDA) health claim, the levels of phytosterols in pistachio nuts may be sufficient to play a synergistic role with unsaturated fatty acids and the low SFA levels in helping to maintain normal cholesterol levels.

Effects of processing and storage on the final level of bioactive compounds

Roasting and steam roasting

Roasting and steam roasting are a common method of processing pistachios to increase the overall safety and palatability and enhance the flavour, colour, texture and appearance of the nuts[48]. However, this process may alter the bioactive compounds in pistachios[46]. In this sense, it was demonstrated that the antioxidant capacity and total phenol content were reduced by 60 % in the same lot of Bronte's pistachio nuts, before and after exposure at 160°C for 40 min. Proanthocyanidin content was reduced by about 90 % and loss of vitamin C was observed, whereas isoflavones were not modified[45]. Other antioxidants could be modified during the roasting processes as has been demonstrated in vegetables, in which, during a thermal process, the *trans* double bonds predominately present in carotenoids become susceptible to isomerisation, creating a *cis* configuration[53] and lowering the total antioxidant content[54]. Lutein, however, seems to be more stable with respect to degradation compared with other types of carotenoids.

Pistachios, as well as other types of nuts, contain several protein allergens that may trigger type I hypersensitivity reactions[55]. Noorbakhsh *et al.*[56] showed that the IgE-binding activity of pistachio nuts could be reduced by a steam-roasting process without any significant changes in the sensory quality of pistachios, due to the heat-induced denaturation of some proteins and/or reaction of these proteins to the food matrix.

Storage

Oxidation is one of the most serious problems in the storage of nuts. Oxidation causes the formation of hydroperoxides,

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which are colourless, tasteless and odourless. In addition, hydroperoxides increase water and soluble antioxidants by a degradation reaction of polymerised polyphenols to monomers. Fatty acid oxidation can be controlled by the application of antioxidants, using processing techniques that minimise the losses of tocopherols and other natural antioxidants; inactivate pro-oxidant metals and enzymes; reduce the exposure of nuts to oxygen, heat and light; promote hydrogenation of PUFA; and use an inert gas or vacuum packaging to expel atmospheric oxygen before long-term storage[57,58].

Storage of nuts requires particular temperature, humidity/moisture and ventilation conditions. Bellomo *et al.*[59] tested the stability of lutein and oil in pistachio (*P. vera* L., variety Bronte) kernels stored up to 14 months at three temperatures: 10, 25 and 37°C. The samples were hermetically packaged using two films (nylon and ethylene vinyl alcohol) with and without oxygen scavengers. For each temperature, reference samples were packaged in open bags. After 14 months, the oil showed only a slight increase in acidity and peroxide value irrespective of storage temperature. As for lutein stability, the lowest concentrations were observed at 37°C with a degradation of about 57·5 %. At 10 and 25°C, the samples showed slight differences in lutein concentrations with a 37 % of degradation. Therefore, controlled storage is important for preserving pistachio quality. Oil stability is influenced only by the length of storage; lutein stability is also influenced by storage temperature and kinetic degradation. During storage, lutein showed good stability both at 10 and 25°C. In particular, a low storage temperature, such as 10°C, was the most important parameter because it guarantees good pistachio quality both for pigment and oil (acidity) stability and the absence of mould and bugs.

In vitro and animal studies

Recent *in vitro* studies and studies conducted on animals have suggested that the healthy properties of pistachios can be attributed partially to the content of the nut's dietary antioxidants. Gentile *et al.*[60] evaluated the effects of a hydrophilic extract of *P. vera* L. on the production of reactive oxygen species in RAW 264.7 macrophage cells. A dose-dependent decrease in the production of Lipopolysaccharide (LPS)-induced reactive oxygen species was observed when the cells were incubated with different concentrations of hydrophilic extract, indicating proanthocyanidins as the bioactive components responsible for this effect. Similarly, the incubation of RAW 264.7 murine macrophages with a pistachio oil extract for 24 h decreased some LPS-induced inflammatory markers such as Ifit-2, TNF-α and IL-6[61]. This pistachio oil extract also reduced the expression of Ifirt-2, TNF-α, IL-6 and IL-1β by 78, 55, 58 and 35 %, respectively, in response to LPS stimulation of the same cells. In two studies on rats, increased antioxidant enzymatic activity was found in animals fed pistachios for 8 weeks[62,63]. In the first study, rats were divided into three groups of twelve animals and assigned to a control group fed a standard diet and two pistachio groups fed with a standard diet containing 20 or 40 % of the energy in the form of pistachios. A significant increase in the activities of Paraoxonase 1 (PON1) and arylesterase, both markers of antioxidant capacity, was shown in both groups supplemented with pistachios compared with the control group after 10 weeks of intervention[62]. In the second study, rats were assigned to a control diet (standard commercial chow); a control diet supplemented with 1·26 % of the total energy intake in the form of pistachios; a control diet with 1·63 % of cholesterol, 0·41 % of cholic acid and 16·3 % of sunflower oil (hyperlipidaemic diet); or a hyperlipidaemic diet supplemented with 1·26 % of the total energy intake in the form of pistachios. After 8 weeks, rats fed with the hyperlipidaemic diet supplemented with pistachios had higher total antioxidant activity, determined by thiobarbituric acid-reactive substances, than rats fed with the hyperlipidaemic diet alone[63]. In another study, feeding 19-month-old rats with a 6 or 9 % walnut diet, which was approximately equivalent to a human eating 28 or 42 g, significantly inhibited the activation or phosphorylation of P38-Mitogen-activated protein kinase (MAPK) and the transcription factor NF-κB in brain tissues. Because both molecules are involved in the inflammatory response, these results suggest the potential attenuation of several inflammatory genes mediated by walnuts[64].

Clinical trials in human subjects

Satiety and body-weight control

Despite the fact that nuts, including pistachios, contain a significant amount of fat and are energy-dense foods, several epidemiological studies have provided strong evidence that nut consumption is associated with neither weight gain nor an increased risk of obesity[65–67]. In addition, different clinical trials evaluating the effect of nuts on body weight have been conducted, but only a few have been designed to evaluate body weight as the main outcome. One of them, a 6-month cross-over study, assessed the impact of supplementing the habitual diet with 28–56 g of walnuts per d. In this study, walnut supplementation resulted in a much lower than expected weight gain[68]. Similar results were shown in a parallel, randomised, controlled trial conducted on 123 overweight and obese subjects assigned to an almond-enriched/low-energy diet (containing 56 g almonds to consume daily) or a free-nut/low-energy diet. After 6 months of follow-up, subjects in the almond-enriched diet lost slightly but significantly less weight than those in the free-nut diet, but no significant differences in body composition were observed after 18 months of follow-up[69]. Most of the clinical trials that have assessed the influence of nuts on classical or emergent cardiovascular risk factors have also gathered and evaluated body-weight changes[70,71]. However, review of the available data suggests that adding nuts to habitual diets of free-living individuals does not lead to any appreciable weight gain[72–78].

In three randomised, controlled clinical trials, the effect of pistachio consumption on body weight was evaluated[31,73,76]. In a 12-week weight-loss programme with hypoenergetic diets providing 2092 kJ less than energy recommendations, seventy overweight or obese individuals were randomly allocated to a pistachio-diet group (eating 53 g/d of pistachios) or to a pretzel-enriched diet group (eating 56 g/d of salted pretzels).

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The pistachios or pretzels were consumed as an afternoon snack. During the intervention, a significant reduction in BMI in the pistachio-supplemented group was observed (−4·3% of the BMI). This reduction was higher than that observed in the pretzel-supplemented group (−2% of the BMI)[73]. Similarly, Wang *et al.*[76] evaluated the impact of a 12-week normoenergetic diet intervention supplemented or not with two different doses of pistachio nuts (70 or 42 g/d) on total body-weight maintenance in ninety subjects with the metabolic syndrome. The results indicated that the consumption of any dosage of pistachios resulted in no changes in BMI or waist:hip ratio compared with the group of individuals following the American Heart Association Step I recommendations. More recently, a 24-week, randomised controlled trial, including sixty metabolic syndrome subjects randomised to either the pistachio (20% of total energy in the form of pistachio nuts daily) or control group for 6 months, failed to find significant differences in body weight. However, Gulati *et al.*[31] observed a significant decrease in waist circumference and a trend towards a reduction in subcutaneous adipose tissue in the pistachio group compared with the control group.

Furthermore, five randomised feeding trials evaluated the effect of pistachio consumption on body weight and/or BMI as a secondary outcome. In all five studies, participants consumed at least 15% of the total energy intake in the form of pistachio nuts. No significant effect on body weight and/or BMI was observed compared with participants assigned to the control diet group[79–83] (Table 4).

Several biological mechanisms may explain the unexpected null effect of nut consumption on adiposity. Nuts are rich in unsaturated fatty acids, and evidence suggests that MUFA and PUFA are more readily oxidised[84] and have a greater thermogenic effect[85] than SFA, which can lead to less fat accumulation. Several lines of evidence also demonstrate that nuts have high satiety properties. Nuts are energy dense and a good source of fibre, protein and unsaturated fats, dietary factors that increase satiety ratings. Nuts exert a strong suppression of hunger and therefore subsequent food intake is curtailed[86–88]. In fact, two recent published studies have evaluated the satiating properties of pistachio nuts. The impact of consuming in-shell pistachios or pistachio kernels on fullness and energy intake was evaluated in a randomised, cross-over, controlled feeding trial including 140 university students aged 18–24 years. Consumption of in-shell pistachios resulted in a lower energy intake than consumption of kernels[89]. The same authors, in a second cross-over feeding trial with 118 healthy individuals (mean age 47 (SD 10) years), demonstrated that the visual cue of the empty pistachio shells may have helped the participants to consume fewer pistachios and about 18% less energy[90] (Table 5).

The physical structure of nuts may also contribute to their satiety effect; they are crunchy and must be mechanically reduced to particles small enough for swallowing. Mastication activates mechanical, nutrient and sensory signalling systems that may modify appetitive sensations[91].

Furthermore, a small degree of fat malabsorption has been reported after nut intake, which is attributed to the fat being contained within walled cellular structures that are incompletely digested in the gut, an effect that can be compounded by incomplete mastication[92]. In fact, a cross-over trial conducted on sixteen healthy volunteers consuming pistachios (42 and 84 g/d) or a free-nut diet for 3 weeks, as part of a controlled diet, demonstrated that the metabolisable energy of pistachios, calculated from differences in faecal energy excretion during the different dietary treatments, is 5% less than the energy calculated by the Atwater general factors, suggesting that the energy from pistachios is not totally utilisable[93].

Classical markers of CVD

In a pooled analysis of twenty-five intervention trials, participants who consumed an average of 67 g/d of nuts saw a 5% decrease in total cholesterol, a nearly 7·5% decrease in LDL-C levels and an 8% decrease in the LDL-C:HDL-C ratio. The effects of nut consumption were dose-related, and different types of nuts had similar effects on blood lipid levels[94]. The effect of pistachio consumption on cardiovascular risk markers has been evaluated in five randomised clinical trials as a primary outcome[79–83] and in other studies as a secondary outcome[73,76,95], giving from 10 to 20% of energy or from 42 to 100 g/d as pistachios *v.* diets avoiding the consumption of nuts (Table 4). From them, in a total of five studies, the authors found significant reductions in plasma total cholesterol concentrations in the pistachio-supplemented group[79,80,82,83,95], and in six of them, they found a significant reduction in the total cholesterol:HDL-C ratio and LDL-C:HDL-C ratio[79–83,95]. Moreover, LDL-C concentrations were decreased in the pistachio-supplemented group in three studies[82,83,95], whereas two studies reported no significant reductions in this recognised major cardiovascular risk factor[79,80], although the levels decreased but not significantly in Kocyigit *et al.*[80]. Only Wang *et al.*[76], in a study of Chinese subjects with the metabolic syndrome, found an increase in plasma LDL-C levels after a 12-week period of dietary intervention with a normoenergetic diet including different amounts of pistachios compared with the normoenergetic diet alone. According to Wang *et al.*[76], the nutrient content of the diet was underpowered to show changes in the secondary analyses of risk factors such as blood lipids. Notably, dietary intake was not controlled or reported, so it is difficult to ascertain the reason why LDL-C levels increased in the high-pistachio group. With respect to plasma HDL-C, only Sheridan *et al.*[81] found a significant increase in this parameter in those subjects supplemented with pistachios.

A beneficial effect of pistachios on BP has also been demonstrated recently in a randomised, cross-over, clinical trial conducted on twenty-eight dyslipidaemic individuals. Participants were randomised to three 4-week interventions: a low-fat control diet; a diet containing 10% of the total energy content in the form of pistachios; a diet containing 20% of the total energy as pistachios. A dose-dependent reduction in systolic BP was observed in those subjects supplemented with pistachios, and a decrease in peripheral vascular dilation was observed in those supplemented with higher doses of pistachios[96]. The BP-lowering effects of pistachios have also been evaluated in three additional

Table 4. Summary of cross-over, parallel and sequential intervention studies and their characteristics

References	Subjects (*n*) (M/F)	Type of subjects (age)	Study design (length of the intervention)	Control group	Intervention group(s)	Primary outcome	Secondary outcomes
Edwards *et al.*[79]	10 (4/6)	Moderate hypercholesterolaemic (28–64 years)	Cross-over (3 weeks each period)	RD	20 % of energy in the form of pistachios (PD)	Significant decreases in TC, TC:HDL-C ratio and LDL-C:HDL-C ratio in the PD group compared with the RD group. Non-significant changes in LDL-C, TAG and HDL-C in the PD group compared with the RD group	Non-significant changes in body weight and blood pressure between the interventions
Sheridan *et al.*[81]	15	Moderate hypercholesterolaemic subjects (36–75 years)	Cross-over (4 weeks each period)	RD	15 % of energy in the form of pistachios (PD)	Significant decreases in TC:HDL-C ratio and LDL-C:HDL-C ratio, and increases in HDL-C in the PD group compared with the RD group. Non-significant changes in TC, TAG, LDL-C and VLDL-C	Non-significant changes in BMI and blood pressure between the interventions
Gebauer *et al.*[82]	28 (10/18)	Subjects with elevated LDL-C (≥2·8 mmol/l) (35–61 years)	Cross-over (4 weeks each period)	CD	PD1: 10 % of energy in the form of pistachios PD2: 20 % of energy in the form of pistachios	Both PD interventions significantly decreased TC, LDL-C and non-HDL-C compared with the CD intervention	Non-significant changes in body weight between the interventions
Kay *et al.*[97]	28 (10/18)	Subjects with elevated LDL-C (≥2·8 mmol/l) (35–61 years)	Cross-over (4 weeks each period)	CD	PD1: 10 % of energy in the form of pistachios PD2: 20 % of energy in the form of pistachios	Both PD interventions significantly decreased oxidised LDL and increased serum antioxidants (γ-tocopherol, lutein and β-carotene)	
Baer *et al.*[93]	16 (8/8)	Healthy subjects (29–64 years)	Cross-over (3 weeks each period)	CD Traditional American diet	PD1: CD + 42 g/d of pistachios PD2: CD + 84 g/d of pistachios	Pistachios contain significantly 5 % less energy than the value calculated from the Atwater factors	
West *et al.*[96]	28 (10/18)	Subjects with elevated LDL-C (≥2·8 mmol/l) (35–61 years)	Cross-over (4 weeks each period)	CD	PD1: CD + 10 % of energy in the form of pistachios PD2: CD + 20 % of energy in the form of pistachios	Significant reduction in SBP in the PD1 *v.* PD2 intervention. Significant decrease in peripheral vascular dilatation and heart rate in the PD2 *v.* CD intervention	
Kocyigit *et al.*[80]	44 (24/20)	Healthy subjects (24–40 years)	Parallel (3 weeks of follow-up)	RD	20 % of energy in the form of pistachios (PD)	Significant decreases in TC, TC:HDL-C ratio and LDL-C:HDL-C ratio in the PD intervention compared with the RD intervention. Non-significant changes in LDL-C, TAG and HDL-C in the PD intervention compared with the RD intervention	Non-significant changes in body weight between the interventions. Significant improvement in oxidative status (increases in AOP and AOP:MDA ratio, and decreases in MDA) in the PD intervention compared with the RD intervention
Li *et al.*[73]	52 (M/F)	Healthy obese subjects (20–65 years)	Parallel (12 weeks)	2092 kJ energy-restricted diet with 56 g of pretzels included (CD)	2092 kJ energy-restricted diet with 53 g/d of pistachios included (PD)	Significant reduction in body weight and BMI in the PD intervention compared with the CD intervention	Decrease in TAG in the pistachio group. Non-significant changes in glucose or insulin levels
Wang *et al.*[76]	90 (41/49)	Subjects with the metabolic syndrome (25–65 years)	Parallel (12 weeks)	CD	PD1: CD + 42 g/d of pistachios PD2: CD + 70 g/d of pistachios	Non-significant changes in body weight, BMI or waist:hip ratio	Non-significant changes in blood pressure, fasting glucose and blood lipid levels in the PD1 or PD2 intervention compared the with CD intervention

Table 4. *Continued*

References	Subjects (*n*) (M/F)	Type of subjects (age)	Study design (length of the intervention)	Control group	Intervention group(s)	Primary outcome	Secondary outcomes
Gulati *et al.*[31]	60 (30/30)	Subjects with the metabolic syndrome (42·5 (SD 8·2) years)	Parallel (24 weeks)	CD	PD: 20 % of energy in the form of pistachios	Non-significant differences in body weight (P=0·7). Significant decrease in waist circumference (P=0·02) and trend towards reduction in subcutaneous adipose tissue (P=0·07) in the PD *v.* CD intervention	Significant decrease in glucose levels and non-significant insulin (P=0·54) reduction in the PD *v.* CD intervention. Significant reduction in TC and LDL-C levels, non-significant reduction in TAG levels, and non-significant increase in HDL-C levels in the PD *v.* CD intervention
Sari *et al.*[83]	32 (M)	Healthy subjects (21–24 years)	Sequential feeding trial (4 weeks on the Mediterranean diet followed by 4 weeks on the PD)	No CD	Mediterranean-type diet with 20 % of energy in the form of pistachios (PD)	Significant decrease in TC, TAG, LDL-C, TC:HDL-C ratio, LDL-C:HDL-C ratio and non-significant increase in HDL-C in the PD intervention compared with the MD intervention	Non-significant changes in body weight and blood pressure between the groups. Decrease in IL-6 concentrations and improvement of antioxidant capacity in the PD *v.* MD intervention. Significant decrease in fasting glucose levels in the PD *v.* MD intervention
Aldemir *et al.*[95]	17 (M)	Individuals with erectile dysfunction (38–59 years)	Sequential feeding trial (3 weeks of intervention diet)	No CD	Pistachio diet: 100 g	Improvements of erectile function measured by the IIEF-15 score after the pistachio intervention	Significant decrease in TC, LDL-C, TC:HDL-C ratio and LDL-C:HDL-C ratio throughout the intervention, and significant increase in HDL-C

M, male; F, female; RD, regular diet; PD, pistachio diet; TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; VLDL-C, VLDL-cholesterol; CD, control diet; SBP, systolic blood pressure; AOP, antioxidant potential; MDA, malondialdehyde; IIEF, International Index of Erectile Function.

Table 5. Summary of acute intervention studies and their characteristics

References	Subjects (*n*) (M/F)	Type of subjects (age)	Study design (length of the intervention)	Control group	Intervention group(s)	Primary outcome	Secondary outcomes
Kendall *et al.*[105]	10 (3/7)	Overweight healthy subjects (48·3 (SD 6·4) years)	Acute postprandial trial	Study 1: WB1	Study 1: pistachio (28, 56 and 84 g) (PD1a) WB1 + pistachio (28, 56 and 84 g) (PD1b)	Pistachios had a significant dose-dependent glycaemic response: 56 and 86 g of pistachios + WB significantly resulted in the reduction of glycaemic responses compared with the WB1 intervention	
				Study 2: WB2 and SM2	Study 2: meal + 56 g pistachios (PD2)	PD2 resulted in significantly reduced glycaemic responses compared with the WB2 and SM2 interventions	
Honselman *et al.*[89]	140 (25/93 and 23 subjects with no specified sex)	Healthy subjects (18–24 years)	Acute feeding trial	No control diet	(1) In-shell pistachios (2) Unshelled pistachios	Select in-shell pistachios significantly reduced energy consumption	No differences in fullness or satisfaction
Kennedy-Hagan *et al.*[90]	118 (16/102)	Healthy subjects (47 ± 10·8 years)	Acute feeding trial	No control diet	(1) Pistachio shells piled up in bowls next to the participants (2) Pistachio shells removed	Energy consumption significantly decreased when the shells remained as the visual cue	
Kendall *et al.*[30]	20 (8/12)	Subjects with the metabolic syndrome (40–65 years)	Acute postprandial trial (cross-over)	Study 1: 50 g of available CHO − WB1, butter and cheese	Study 1: WB + 85·046 g pistachios (PD1)	Both PD1 and PD2 interventions significantly reduced postprandial glycaemia compared with the WB1 and WB2 interventions, respectively	
				Study 2: 12 g of available CHO − WB2	Study 2: pistachios (PD2)	PD1 and PD2 interventions increased GLP-1 compared with the WB1 and WB2 interventions, respectively	

WB, white bread; SM, specific meal; PD, pistachio diet; CHO, carbohydrate; GLP-1, glucagon-like peptide 1.

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controlled feeding trials as a secondary outcome showing non-significant differences in the changes in systolic or diastolic BP between those subjects supplemented with pistachios and those who did not receive supplementation[76,81,83].

In conclusion, some evidence suggests that pistachios may improve the blood lipid profile and reduce BP, which could contribute to decreased cardiovascular risk.

Emerging risk factors of CVD

Pistachios are a rich matrix of fat-soluble antioxidants that could have important effects on the control of oxidative stress and a reduced risk of chronic diseases. In a study conducted on forty-four healthy men and women, half of the subjects were randomised to a regular diet group and the other half to a pistachio group (accounting for 20 % of their daily energy intake in the form of pistachios) for 3 weeks. The study showed an increased blood antioxidant potential determined by the production of thiobarbituric acid-reactive substances and decreased malondialdehyde levels, which is an important indicator of lipid peroxidation, in those volunteers consuming pistachios compared with those following a free-nut diet[80]. A cross-over, randomised, controlled feeding trial conducted by Kay *et al.*[97] on twenty-eight hypercholesterolaemic adults showed that the consumption of diets containing 10 and 20 % of energy from pistachios (32–63 and 63–126 g/d, respectively) increased antioxidant concentrations in serum, such as γ-tocopherol, lutein and β-carotene, whereas it decreased oxidised LDL concentrations relative to the consumption of a control diet without pistachios. Finally, in a prospective study, Sari *et al.*[83] assessed the effect of a traditional Mediterranean diet supplemented with pistachios by replacing the monounsaturated fat content constituting approximately 20 % of daily energy intake on thirty-two healthy young men for 4 weeks. They found a significant improvement in endothelium-dependent vasodilation, whereas endothelium-independent vasodilation remained unchanged compared with the Mediterranean diet. An increase in total antioxidant status and superoxide dismutase and a decrease in inflammation and other oxidative markers were also observed. Taken together, these results provide evidence of the beneficial effects of pistachios on the risk of CVD beyond the lipid-lowering effect.

Insulin resistance and type 2 diabetes

Diabetes mellitus is one of the most common diseases worldwide, largely the result of an increase in the prevalence of obesity and physical inactivity. Moreover, T2DM is a recognised risk factor for CVD and other chronic conditions and diseases, and is thus becoming a serious public health burden[98,99]. Data from epidemiological and interventional studies suggest that the frequency of nut consumption is inversely related to an increased risk of T2DM, mainly attributed to the fibre, healthy fats, antioxidants and anti-inflammatory compounds[72,100–104] in nuts. In addition, among all nuts, pistachios have a low glycaemic index, suggesting a possible effect on reducing postprandial glycaemia and insulinaemia, thereby potentially decreasing the risk of diabetes. The effect of pistachios, consumed alone or combined with meals, on postprandial glycaemia has been evaluated[30,105] (Table 5). Thus, whereas pistachios consumed alone had a minimal effect on postprandial glycaemia, the addition of pistachios (56 g) to foods with a high glycaemic index (pasta, parboiled rice and instant mashed potatoes) reduced, in a dose-dependent manner, the total postprandial glycaemic response by 20–30 %[105]. In a recent randomised, cross-over study conducted on twenty subjects with the metabolic syndrome, 85·04 g of pistachios consumed with bread reduced postprandial glycaemia levels and increased glucagon-like peptide levels compared with bread alone[30].

In three clinical studies, the effect of pistachio supplementation on glucose concentrations as a secondary outcome was evaluated, with contradictory results. In a controlled, cross-over, clinical trial, participants were randomised to a Mediterranean diet or a Mediterranean diet supplemented with 20 % of energy intake as pistachios for 4 weeks in each arm. Subjects in the intervention period showed a significant decrease in fasting plasma glucose concentrations in comparison to the control period[83]. The second study evaluated the effect of the American Heart Association Step I diet supplemented with 42 or 70 g/d of pistachios compared with the effect of a control diet (American Heart Association Step I), in Chinese subjects with the metabolic syndrome using a randomised, parallel-group, controlled study design. After 12 weeks of intervention, no differences in fasting plasma glucose or insulin levels were observed between the groups, although compared with baseline values, blood glucose levels increased significantly in the control group at week 12 but not in the two pistachio groups[76]. Finally, in a third parallel study conducted on sixty subjects with the metabolic syndrome randomised to either an unsalted pistachios diet (20 % energy) or a control diet for 24 weeks, a significant decrease in glucose levels but not in blood insulin levels was observed[31].

In addition to the fibre, healthy fats and low available carbohydrate content, the effect of pistachios on glucose metabolism may be a result of the rich content of carotenoids. A 9-year longitudinal study conducted on 1389 healthy elderly volunteers demonstrated a 58 % lower risk for the development of impaired fasting glucose levels or T2DM mellitus in subjects in the highest quartile of total plasma carotenoids than in those in the lowest quartile, even after adjusting for possible confounding variables[106]. In a randomised controlled study, the intake of 75 g/d of mixed nuts (including pistachios) in 117 T2DM subjects during 3 months as a replacement for carbohydrate-containing foods in comparison to the intake of healthy whole-wheat muffins, or half portions of both, demonstrated for the first time a significant decrease in HbA_{1c} levels, even though the subjects were on oral antidiabetic medication. Additionally, and despite the subjects consuming statins, an improvement in total cholesterol was observed[3].

Despite the positive results observed for glucose metabolism in fasting conditions or postprandial status, more studies are necessary to evaluate the long-term effects of pistachio consumption on insulin resistance, secretion or diabetes control.

Summary and conclusions

Pistachios are nutrient-dense nuts with a healthy nutritional profile including fibre, healthy fats, phytosterols and antioxidant compounds, contributing to a reduced risk of heart disease. Growing evidence suggests that consumption of nuts, including pistachios, improves diet quality and provides several bioactive compounds with recognised properties for weight management, glycaemic control and vascular health.

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HEALTH BENEFITS OF PISTACHIO CONSUMPTION IN PRE-DIABETIC SUBJECTS
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Annex II. Review Article 2

Title: Pistachios for Health: What Do We Know About This Multifaceted Nut?

Authors: Pablo Hernández-Alonso; Mònica Bulló and Jordi Salas-Salvadó.

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Abstract:

Human beings have known about pistachio nuts since 6000 BC. Since then, pistachios have been systematically incorporated into the diet of various cultures. They are nutrient-dense nuts with a healthy nutritional profile that contains fiber, unsaturated fatty acids and antioxidant compounds.

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Pistachios for Health

What Do We Know About This Multifaceted Nut?

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Human beings have known about pistachio nuts since 6000 BC. Since then, pistachios have been systematically incorporated into the diet of various cultures. They are nutrient-dense nuts with a healthy nutritional profile that contains fiber, unsaturated fatty acids and antioxidant compounds. Nutr Today. 2016;51(3):133–138

THE HISTORY OF PISTACHIOS

Pistachio (from the Greek word *pistákion* [πιστάκιον]), "the green nut," is widely cultivated in the Mediterranean region, even though it probably originated in central and southwest Asia. Evidence of its consumption has been found in archaeological excavations, which proves that it has long been associated with human activities.[1] Remains of pistachio nuts dating from the sixth millennium BC have been found in both Afghanistan and southeastern Iran, where pistachio (*Pistacia vera L*) was probably first cultivated in regions close to where it grew wild. It was widely cultivated in the ancient Persian Empire, from where it gradually expanded to the west. For example, legend has it that the Queen of Sheba (Assyria, ca 10th century BC) monopolized a limited crop of nuts for her exclusive use.[2] However, the Assyrians and the Greeks knew that pistachios could be used as medicines, aphrodisiacs, and antidotes. By the end of his reign, Emperor Tiberius, the Roman consul of the province, introduced pistachios into Italy. From Italy, they had spread into Mediterranean regions in southern Europe and North Africa. Around the 10th century, pistachios were also cultivated in China and more recently in Australia, New Mexico and California.[1]

NUTRITIONAL VALUE OF PISTACHIOS

Compared with other nuts (Table 1), dry roasted pistachios have a lower fat content (43.4 g/100 g), which is composed mainly of saturated fatty acid (5.6 g), polyunsaturated fatty acid (13.3 g), and monounsaturated fatty acid (24.5 g)[3] (Figure 1A). Of the fatty acids, oleic and linoleic acids represent more than half of the total fat content in pistachios. Pistachios are also a good source of vegetable protein (about 21% of total weight), with an essential amino acid ratio higher than most other commonly consumed nuts (ie, almonds, walnuts, pecans, and hazelnuts), and they have a high percentage of branched chain amino acids.[4] The amount of total carbohydrates is low to moderate (about 29% by weight), but they are richer in fiber than other nuts with a 10% by weight of insoluble forms and 0.3% of soluble forms (Table 1). Pistachios also contain significant amounts of minerals (ie, potassium, phosphorus, magnesium, calcium; Figure 1B) and vitamins such as vitamin A, vitamin E (especially γ-tocopherol), vitamin C, vitamin B (except B_{12}), vitamin K, and folate (Table 2), with relatively high amounts of these compounds compared with other nuts.[5] Moreover, pistachios are also a rich source of lutein and zeaxanthin (xanthophyll carotenoids) and phenolic compounds, including anthocyanins, flavonoids, and proanthocyanidins, and their antioxidant capacity is considerable. Pistachios are the nuts that have

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TABLE 1 Nutritional Composition of Nuts (Dry Roasted)

	Almonds	Hazelnuts	Macadamia Nuts	Peanuts	Pecans	Pistachio Nuts	Walnuts
Energy, kcal	598	646	718	587	710	572	643
SFA, g	4.1	4.5	11.9	7.7	6.3	5.6	5.4
PUFA, g	13.0	8.5	1.5	9.8	20.6	13.3	44.2
MUFA, g	33.1	46.6	59.3	26.2	44.0	24.5	8.4
Proteins, g	21.0	15.0	7.8	23.6	9.5	21.0	14.3
Carbohydrates, g	10.1	8.2	5.4	12.9	4.2	28.3	10.8
Fiber, g	10.9	9.4	8.0	8.4	9.4	10.3	7.1
Water, g	2.4	2.5	1.6	1.8	1.1	1.9	4.4
Ashes, g	3.1	2.5	1.1	2.9	1.6	3.0	2.8

Data obtained from US Department of Agriculture (USDA), Nutrient Database for Standard Reference, Release 28.[3]
Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

the highest content of phytosterols, including stigmasterol, campesterol, and β-sitosterol.[6] This complete and diverse set of micronutrients and macronutrients means that pistachio nuts are potentially one of the more health-promoting foods.

HEALTH BENEFITS OF PISTACHIO

As their nutritional profile suggests, pistachios can play an important role in improving such metabolic conditions as overweight, type 2 diabetes mellitus (T2DM), or metabolic syndrome. This review aims to analyze current knowledge on the relationship between pistachio intake and several metabolic risk markers (Figure 2).

Satiety Regulation and Weight Management

Because nuts are energy-dense foods with a high fat content, one of the main concerns regarding the regular consumption of nuts in a worldwide pandemic of overweight and obesity is that nuts are believed to be fattening. To date, however, epidemiological studies have failed to find any association between nut or pistachio consumption and either weight gain or an increased risk of obesity.[7–9] Likewise, controlled feeding trials confirm that adding nuts to usual diets does not induce weight gain.[10–17] Several studies that have evaluated pistachios' effect on body weight as a secondary outcome have reiterated their null effect on body weight and body mass index.[18–22] Only one recent study conducted in T2DM subjects has found a significant reduction of body mass index after pistachio consumption.[23]

These findings may be explained by the energy density of pistachios; their content in fiber, protein, and unsaturated fatty acids; and their crunchy physical structure, which may induce satiety and therefore reduce subsequent food intake.[24] It has been speculated that various signaling systems (ie, mechanical, nutrient, and sensory) are activated by mastication, which may modify appetitive sensations.[25] To date, only 2 studies have evaluated the satiating properties of pistachio nuts in humans. The conclusions are that the consumption of in-shell pistachios led to lower calorie intake than the consumption of kernels[26] and that the visual cue of empty pistachio shells helped the participants to consume fewer calories during the day.[27] In addition, the monounsaturated and polyunsaturated fatty acids that nuts contain have a greater thermic effect that will induce higher thermogenic effect[28] than saturated fatty acids, which can lead to less fat accumulation by an increase in sympathetic activity in brown adipose tissue.[29] Finally, after nut intake, fat is malabsorbed to a slight extent largely because the fat in the walls of nut cells is not completely digested in the gut,[30] suggesting that energy from nuts is poorly absorbed. Therefore, the metabolized energy contained in these types of nut is less than predicted by the Atwater general factors, which is the system used for the calculation of the available energy of foods developed from experimental studies in the early years of the 20th century.[31]

Lipid Profile

Pistachio consumption has been widely studied in terms of its possible protective cardiovascular disease role. Significant improvements in plasma total cholesterol concentrations,[18,19,21,22,32] Total cholesterol (C)/high-density lipoprotein C (HDL-C) ratio and low-density lipoprotein C (LDL-C)/HDL-C ratio[18–22,32] have been observed in several trials in the pistachio-supplemented group compared with the control group. Some studies have shown that LDL-C concentrations also decrease significantly in the

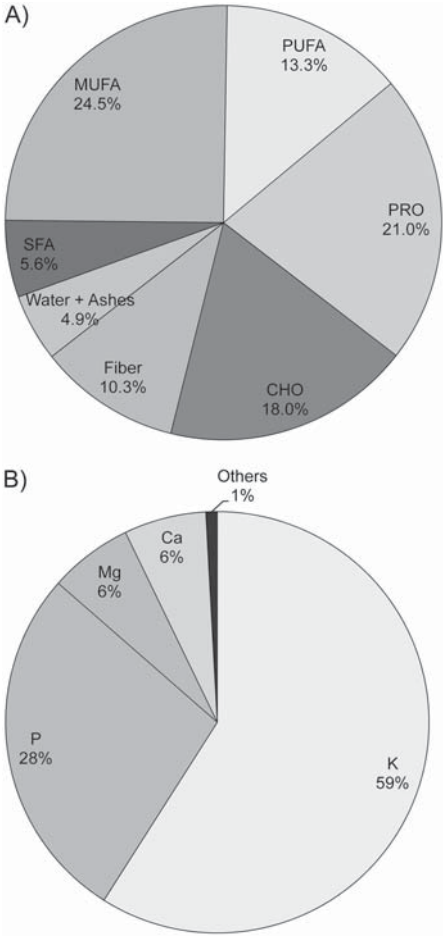

FIGURE 1. Macronutrient and mineral composition of pistachio nuts (dry roasted). (A) Macronutrient and (B) mineral composition of pistachios. Values are expressed as grams of macronutrient per 100 g of pistachios (A) and percentage of specific mineral from total mineral amount (B). ''Others'' includes copper, iron, manganese, selenium, sodium and zinc. Data obtained from United States Department of Agriculture, Nutrient Database for Standard Reference, Release 28.[3] MUFA indicates monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; CHO, carbohydrates regardless of fiber; PRO, protein.

pistachio-supplemented group,[21,22,32,33] whereas others have observed a nonsignificant reduction.[18,19,34] However, Sheridan and coworkers[20] found significant increases in circulating HDL-C concentrations after pistachio intake.

It is currently considered that the conventional lipid profile cannot completely explain the atherogenic damage of cardiovascular diseases. In fact, the non-HDL-C fraction (ie, LDL-C plus very low-density lipoprotein C) has been strongly associated with an increased risk of coronary heart disease,[35] which is even greater than that attributed to LDL-C.[36] In addition, small dense LDL particles have been associated with an increased risk of ischemic heart disease in men independently of the concomitant variation in lipoprotein-lipid concentrations.[37] Therefore, novel research has taken advantage of new methodologies (ie, nuclear magnetic resonance) to evaluate the concentration and size of lipoprotein subclasses (ie, small, medium, large) rather than only the classical lipid profile. In this regard, only 3 studies have analyzed the effect of nut consumption on modulating lipoprotein subclasses. The effect of walnut consumption on lipoprotein subclasses was evaluated in 2001,[38] and the effect of pistachio on lipoprotein metabolism has been evaluated only recently.[33,39] These clinical trials found a significant antiatherogenic modulation of lipoprotein subclasses following nut interventions. In conclusion, evidence suggests that pistachios may improve well-established and novel blood lipid markers of atherosclerosis and therefore help decrease cardiovascular

TABLE 2 Vitamin Content of Pistachios per 100 g (Dry Roasted)

	Pistachio Nuts
Vitamin A, IU	266
Vitamin B_6, mg	1.12
Vitamin B_{12}, mg	0
Vitamin C, mg	3.0
Vitamin D, IU	0
α-Tocopherol, mg	2.17
β-Tocopherol, mg	0.13
γ-Tocopherol, mg	23.42
δ-Tocopherol, mg	0.55
Vitamin K, μg	13.2
Folate, μg	51
Choline, mg	71.4
Betaine, mg	0.8
Thiamine, mg	0.70
Riboflavin, mg	0.23
Niacin, mg	1.37
Pantothenic acid, mg	0.51
Lutein + zeaxanthin, μg	1160
Alpha carotene, μg	0
Beta carotene, μg	159

Data obtained from the US Department of Agriculture, Nutrient Database for Standard Reference, Release 28.[3]
Abbreviation: IU, international units.

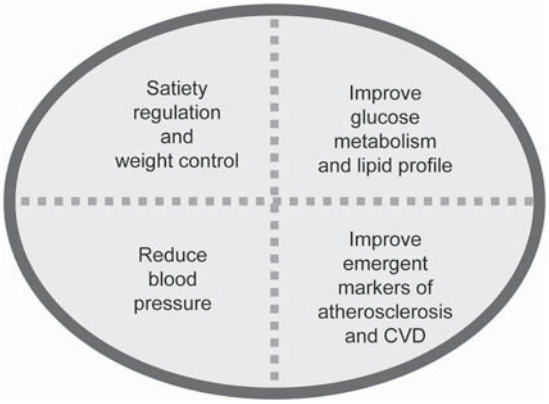

FIGURE 2. Possible health benefits of pistachio consumption. CVD indicates cardiovascular disease.

risk. Although they are difficult, very expensive, and time consuming, clinical trials need to be carried out in the future to fully establish the potential effects of pistachio consumption on the prevention of cardiovascular events.

Blood Pressure and Endothelial Function

Several prospective studies have shown an inverse association between nut consumption and blood pressure or hypertension. However, the results of clinical trials are more controversial. The intake of 10% of energy in the form of pistachios for 1 month significantly reduced systolic blood pressure (SBP) and made no difference in diastolic blood pressure (DBP) compared with the control nut-free group.[40] Similarly, a recent study conducted in T2DM subjects showed a reduction in SBP after 4 weeks consuming a diet with 20% energy from pistachios.[41] Moreover, a recent systematic review and meta-analysis of more than 20 randomized controlled trials found that although DBP was reduced by the intake of mixed nuts, pistachios alone seemed to have the strongest effect on reducing both SBP and DBP.[42]

Walnut, hazelnut, and pistachio consumption also improves the circulating concentrations of endothelial markers and endothelial function.[43–45]

In conclusion, chronic pistachio consumption has proved to have a beneficial effect on blood pressure and endothelial function, which may help to improve cardiovascular risk.

Glucose and Insulin Metabolism

Pistachios have more total carbohydrates (29% w/w) than do other nuts, but their consumption has no deleterious effect in subjects with abnormal glucose and insulin metabolism.

Data from several epidemiological studies and clinical trials suggest that the frequency of nut consumption is inversely related to an increased risk of T2DM. This may be because of the fact that nuts are relatively high in fiber, healthy fats, antioxidants, and anti-inflammatory content.[6,10,46,47] In addition, among all nuts, pistachios have a low glycemic index, suggesting that they may reduce postprandial glycemia and insulinemia and therefore contribute to reducing the T2DM risk.[48] Pistachios consumed alone had a minimal effect on postprandial glycemia, but the addition of pistachios to a meal containing foods rich in carbohydrates with a high glycemic index (eg, pasta, parboiled rice, or instant mashed potatoes)[49] or bread[48] reduces postprandial glycemia in a dose-dependent response.

Several clinical studies have investigated the effect of pistachio consumption on glucose concentrations. They observed a significant decrease in fasting plasma glucose (FPG),[22] in glucose but not in insulin blood levels,[17,23] and in both FPG and insulin levels[34] after pistachio intake. Only 1 cross-over study conducted on metabolic syndrome subjects free of type 2 diabetes has shown no significant changes in FPG or in insulin concentrations during the pistachio-enriched diet period compared with the intervention period without pistachios.[15]

Pistachio consumption has also proved to have beneficial effects on diabetes control. In a randomized controlled study, the intake of mixed nuts (including pistachios) for 3 months in T2DM subjects, as a replacement for carbohydrate-containing foods, demonstrated for the first time a significant decrease in hemoglobin A_{1c} in a full-nut dose compared with a half-nut and control-muffin doses.[50] Results were similar in a recent crossover trial with 48 diabetic participants after 3 months of pistachio consumption.[23] Pistachio intake has also recently been found to significantly enhance the glucose and insulin metabolism of prediabetic patients[34] and improve insulin resistance status and other cardiovascular risk factors.

Despite the positive results observed for glucose metabolism, more studies need to be made to evaluate the long-term effects of pistachio consumption on insulin resistance and T2DM prevention and control.

NEW TRENDS IN PISTACHIO RESEARCH

Pistachio in the Prevention of T2DM and Other Metabolic Diseases

Research has focused widely on the beneficial effects of pistachios on such conditions as T2DM and metabolic syndrome.[17,23] However, very little information is currently available on the potential role that nuts play in preventing the development of chronic diseases such as T2DM.

Pistachios in Cancer or Neurodegenerative Diseases

Vitamin E and other antioxidants provide some protection against certain forms of cancer. Therefore, foods such as pistachios, with a high content of γ-tocopherol (a form of vitamin E) and other antioxidants may reduce the risk of

different types of cancer.[51] Moreover, the skin of nuts contains considerable amounts of resveratrol,[52] which has been widely studied for its role in cancer, but new research is now changing this focus to other diseases such as Alzheimer's or Parkinson's.[53]

Pistachio and Gut Microbiota

Recent findings have shown that both pistachios and almonds have a potential prebiotic effect in healthy populations, and that the effect of the former is greater.[54] Thereby, pistachios' microbiota modulation increased the number of butyrate-producing bacteria, identified as potentially beneficial, whereas bifidobacteria was not affected. However, new investigations should be performed to contrast and further explore these findings. Regulation of the phyla composition or the production of regulatory and protective molecules (eg, butyrate) by our gut microbiota could be mediators of the well-established beneficial properties of pistachios and other nuts.

PRACTICE IMPLICATIONS

A common pistachio serving is about 28 g (1 oz) or 49 kernels of pistachio nut, in which there are almost 160 kcal. Pistachios are globally distributed and consumed as a healthy snack. Pistachios can also be added to many savory dishes such as pastas, marinades and crusts for meat entrees, salsas, and stir-fries as well as a topping for salads, yogurts, and dips. Their beneficial properties, based on pistachios' specific macronutrient, micronutrient and bioactive molecules will remain unchanged even after cooked. Moreover, other properties such as their contribution to the glycemic index and glycemic load of a particular meal would be improved by their inclusion.

To prevent fatty acids in pistachios from oxidation, store pistachios in an airtight container in the refrigerator at 40°F (4°C) for up to 1 year. At room temperature 68°F (20°C), they should be kept in a dry environment and will last several months.

Therefore, the inclusion of a handful of pistachios is a taste snack that may confer health benefits in the context of a healthy diet.

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Annex III. Scientific contribution

Scientific Articles belonging to this Doctoral Thesis

Authors: Pablo Hernández-Alonso; Jordi Salas-Salvadó; Mònica Baldrich-Mora; Martí Juanola-Falgarona and Mònica Bulló.
Title: Beneficial effect of pistachio consumption on glucose metabolism, insulin resistance, inflammation, and related metabolic risk markers: a randomized clinical trial.
Journal information: *Diabetes care*. **2014**; 37(11): 3098-3105.
IF: 8.420; 7/128; Q1; Endocrinology & Metabolism.

Authors: Pablo Hernández-Alonso; Jordi Salas-Salvadó; Mònica Baldrich-Mora; Roger Mallol; Xavier Correig and Mònica Bulló.
Title: Effect of pistachio consumption on plasma lipoprotein subclasses in pre-diabetic subjects.
Journal information: *Nutrition, Metabolism and Cardiovascular Diseases*. **2015**; 25: 396-402.
IF: 3.390; 22/78; Q2; Nutrition & Dietetics.

Authors: Pablo Hernández-Alonso; Simona Giardina; Jordi Salas-Salvadó; Pierre Arcelin and Mònica Bulló.
Title: Chronic pistachio intake modulates circulating microRNAs related to glucose metabolism and insulin resistance in prediabetic subjects.
Journal information: *European Journal of Nutrition*. **2016**; [Epub ahead of print].
IF: 3.239; 27/78; Q2; Nutrition & Dietetics.

Other Scientific Articles

Authors: Martí Juanola-Falgarona; Jordi Salas-Salvadó; Núria Ibarrola-Jurado; Antoni Rabassa-Soler; Andrés Díaz-López; Marta Guasch-Ferré; Pablo Hernández-Alonso; Rafael Balanza and Mònica Bulló.
Title: Effect of the glycemic index of the diet on weight loss, modulation of satiety, inflammation, and other metabolic risk factors: a randomized controlled trial.
Journal information: *American Journal of Clinical Nutrition*. **2014**; 100(1): 27-35.
Impact Factor: 6.770; 3/77; Q1; Nutrition & Dietetics.

Authors: Raquel Garijo; Pablo Hernández-Alonso; Carmen Rivas; Jean-Simon Diallo and Rafael Sanjuán.
Title: Experimental evolution of an oncolytic vesicular stomatitis virus with increased selectivity for p53-deficient cells.
Journal information: *PloS one*. **2014**; 9(7): e102365.
Impact Factor: 3.234; 9/57; Q1; Multidisciplinary Sciences.

Annex

Authors: Mònica Bulló; Martí Juanola-Falgarona; Pablo Hernández-Alonso and Jordi Salas-Salvadó.
Title: Nutrition attributes and health effects of pistachio nuts.
Journal information: *British Journal of Nutrition*. **2015**; 113 (Suppl 2): S79-93.
Impact Factor: 3.311; 18/77; Q1; Nutrition & Dietetics.

Authors: Pablo Hernández-Alonso; Raquel Garijo; José M Cuevas and Rafael Sanjuán.
Title: Experimental evolution of an RNA virus in cells with innate immunity defects.
Journal information: *Virus Evolution*. **2015**; 1(1): vev008.
Impact Factor: it will be available on 2017.

Authors: Nerea Becerra-Tomás; Marta Guasch-Ferré; Joan Quilez; Jordi Merino; Raimon Ferré; Andrés Díaz-López; Mònica Bulló; Pablo Hernández-Alonso; Antoni Palau-Galindo and Jordi Salas-Salvadó.
Title: Effect of functional bread rich in potassium, γ-aminobutyric acid and angiotensin converting enzyme inhibitors on blood pressure, glucose metabolism and endothelial function: a double-blind randomized crossover clinical trial.
Journal information: *Medicine*. **2015**; 94(46): e1807.
Impact Factor: 5.723; 15/154; Q1; Medicine, General & Internal.

Authors: Pablo Hernández-Alonso; Jordi Salas-Salvadó; Miguel Ruiz-Canela; Dolores Corella; Ramón Estruch; Montserrat Fitó; Fernando Arós; Enrique Gómez-Gracia; Miquel Fiol; José Lapetra; Josep Basora; Lluis Serra-Majem; Miguel Ángel Muñoz; Pilar Buil-Cosiales; Carmen Saiz and Mònica Bulló.
Title: High dietary protein intake is associated with an increased body weight and total death risk.
Journal information: *Clinical Nutrition*. **2016**; 35(2): 496-506.
Impact Factor: 4.487; 10/78; Q1; Nutrition & Dietetics.

Not Indexed Scientific Articles

Authors: Jordi Salas-Salvadó and Pablo Hernández-Alonso.
Title: Pistachio nut consumption improves insulin resistance and reduces glucose levels in pre-diabetic subjects.
Journal information: *The Cracker*. **2014**; 63 - 3: 86 - 87.

Authors: Pablo Hernández-Alonso; Mònica Bulló and Jordi Salas-Salvadó.
Title: Nut consumption and microbiota modulation: nuts are not only good food for humans.
Journal information: *The Cracker*. **2015**; 64 - 1: 85 - 86.

Authors: Pablo Hernández-Alonso and Jordi Salas-Salvadó.
Title: Marine and vegetable ω-3 fatty acids act synergistically and are partners: updates from the PREDIMED study.
Journal information: *Nutfruit*. **2016** - July: 38-39.

Authors: Pablo Hernández-Alonso; Mònica Bulló and Jordi Salas-Salvadó.
Title: Pistachios for Health: What Do We Know About This Multifaceted Nut?
Journal information: *Nutrition Today*. **2016**; 51(3):133-138.

Participation in National and International Congresses

Congress: 21st European Congress on Obesity.
Date and Place: 28-31 May 2014; Sofia (Bulgaria).
Authors: Pablo Hernández-Alonso; Mònica Baldrich-Mora; Jordi Salas-Salvadó; Pierre Arcelin; Antoni Palau-Galindo; Marta Ciutat; Jesús San Miguel; Josep María Paris; Juan José Cabré; María Teresa Acera and Mònica Bulló.
Title: Effects of pistachio nut intake on glucose metabolism in individuals at high risk of type 2 diabetes mellitus: The EPIRDEM study.
Format: Oral communication (Pablo Hernández-Alonso).

Congress: VI Symposium CIBER de Fisiopatología de la Obesidad y Nutrición.
Date and Place: 20-21 November 2014; Madrid (Spain).
Authors: Pablo Hernández-Alonso, Jordi Salas-Salvadó, Mònica Baldrich-Mora, Martí Juanola-Falgarona, Mònica Bulló.
Title: Effects of pistachio nut intake on glucose metabolism in pre-diabetic subjects: the EPIRDEM study.
Format: Poster.

Congress: 22nt European Congress on Obesity.
Date and Place: 6-9 May 2015; Prague (Czech Republic).
Authors: Pablo Hernández-Alonso, Jordi Salas-Salvadó, Miguel Ruiz-Canela, Dolores Corella, Ramón Estruch, Montserrat Fitó, Fernando Arós, Enrique Gómez-Gracia, Miquel Fiol, José Lapetra, Josep Basora, Lluis Serra-Majem, Miguel Ángel Muñoz, Pilar Buil-Cosiales, Carmen Saiz, Mònica Bulló.
Title: Association of high dietary protein intake with the risk of weight gain and total death in subjects at high risk of cardiovascular disease.
Format: Oral communication (Mònica Bulló).

Annex

Congress: 22nt European Congress on Obesity.
Date and Place: 6-9 May 2015; Prague (Czech Republic).
Authors: Pablo Hernández-Alonso, Jordi Salas-Salvadó, Mònica Baldrich-Mora, Roger Mallol, Xavier Correig, Mònica Bulló.
Title: Regular consumption of pistachio modulates plasma lipoprotein subclasses in pre-diabetic subjects.
Format: Poster.

Congress: VII Symposium CIBER de Fisiopatología de la Obesidad y Nutrición "Obesity and Nutrition in the 21st Century".
Date and Place: 15-17 October 2015. Madrid (Spain).
Authors: Pablo Hernández-Alonso, Simona Giardina, Jordi Salas-Salvadó, Mònica Bulló.
Title: Pistachio consumption modulates microRNAs related with glucose metabolism and insulin resistance in pre-diabetic subjects.
Format: Poster.

Congress: European Obesity Summit (EOS).
Date and Place: 01-04 June 2016. Goteborg (Sweden).
Authors: Simona Giardina, Pablo Hernández-Alonso, Jordi Salas-Salvadó, Mònica Bulló.
Title: Effects of Glycemic index on the expression of selected miRNAs in adipose tissue: The GLYNDIET study.
Format: Poster.

Congress: 34th International Symposium on Diabetes & Nutrition.
Date and Place: 29 June - 01 July 2016. Prague (Czech Republic).
Authors: Pablo Hernández-Alonso, Simona Giardina, Jordi Salas-Salvadó, Mònica Bulló.
Title: Pistachio Consumption Modulates MicroRNAs Related with Glucose Metabolism and Insulin Resistance in Pre-diabetic Subjects.
Format: Oral communication (Mònica Bulló).

Mobility

Institution: Metagenopolis - Institut National de la Recherche Agronomique (INRA) (Paris, France).
Supervisors: Dr. Joël Doré and Florence Levenez.
Objective: To compare different protocols for extracting DNA from samples with low-biomass and learn the techniques related with metagenomics approaches.
Length: 3 months (January-April 2016).

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