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EFFECTS OF HESPERIDIN ON CARDIOVASCULAR DISEASE RISK BIOMARKERS: AN OMICS SCIENCE APPROACH

INTERNATIONAL DOCTORAL THESIS

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FAIG CONSTAR que aquest treball, titulat “Effects of hesperidin on cardiovascular disease risk biomarkers: an omics science approach”, que presenta Laura Pla Pagà per a l’obtenció del títol de Doctor amb menció internacional, ha estat realitzat sota la meva direcció al Departament de Medicina i Cirurgia d’aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado “Effects of hesperidin on cardiovascular disease risk biomarkers: an omics science approach”, que presenta Laura Pla Pagà para la obtención del título de Doctor con mención internacional, ha sido realizado bajo mi dirección en el Departamento de Medicina y Cirugía de esta universidad.

I STATE that the present study, entitled “Effects of hesperidin on cardiovascular disease risk biomarkers: an omics science approach”, presented by Laura Pla Pagà for the award of the degree of Doctor with

an international distinction, has been carried out under my supervision at the Department of Medicine and Surgery of this university.

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“What we know is a drop,

what we don’t know is an ocean”

Isaac Newton

ABBREVIATIONS

AHA/ACA	American Heart Association/American College of Cardiology
AMPK	AMP-activated protein kinase
BCAA	branched-chain amino acids
BMI	body mass index
BP	blood pressure
CD	control drink
COVID-19	Coronavirus Disease 2019
CVDRFs	cardiovascular diseases risk factors
CVD	cardiovascular diseases
DBP	diastolic blood pressure
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EOJ	hesperidin-enriched orange juice
ESC	European Cardiology Society
FMD	flow-mediated dilation
GCPICH	Good Clinical Practice Guidelines of the International Conference of Harmonization
HDL-c	high-density lipoprotein-cholesterol
HS	hesperidin supplementation
HOMA	homeostasis model assessment
IL-6	interleukin-6
IPA	ingenuity pathway analysis
LDL-c	low-density lipoprotein-cholesterol
LDL-p	low-density lipoprotein particles
lncRNA	long non-coding ribonucleic acid
mRNA	messenger ribonucleic acid
M-S	mass spectrometry

NF-κB	nuclear factor-κB
NO	nitric oxide
OJ	orange juice
OPLS-DA	orthogonal projection to latent structures discriminant analysis
PBMCs	peripheral blood mononuclear cells
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PLS-DA	partial least squares discriminant analysis
PP	pulse pressure
PPAR-γ	peroxisome proliferator-activated receptor gamma
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-Analysis
RCT	randomized clinical trial
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
SBP	systolic blood pressure
sICAM-1	intercellular adhesion molecule-1
sVCAM-1	soluble vascular cell adhesion molecule-1
TMT	tandem mass tag
TC	total cholesterol
TG	triglycerides
HPLC	high-performance liquid chromatography
VIP	variable importance on projection
WHO	World Health Organization

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ABSTRACT

INTRODUCTION

Currently, cardiovascular diseases (CVDs) are still the main cause of mortality worldwide. Thus, there is a necessity to search for new strategies for the prevention and treatment of CVDs, and the flavonoid hesperidin could have cardioprotective effects. However, the literature is scarce, and some results are controversial. Moreover, the mechanisms of action by which hesperidin exerts its protective effects have not been fully defined, and omics science can study the impact of hesperidin consumption on metabolic pathways to determine how hesperidin exerts its health beneficial effects.

OBJECTIVES

The main objective is to evaluate the effects of the consumption of hesperidin in orange juice (OJ) and hesperidin-enriched orange juice (EOJ) on cardiovascular disease risk biomarkers, particularly on blood pressure (BP), and to elucidate the possible mechanisms of action through the application of different omics approaches in human samples: transcriptomics and metabolomics, and proteomics approach in experimental rat models to know its effects on key cardiovascular organs such as heart and kidney.

METHODS AND RESULTS

To achieve it, five projects were carried out: 1) a systematic review to known the scientific evidence available on the subject in humans and experimental rat models with cardiovascular disease risk factors (CVDRFs) following the PRISMA 2015 guidelines and PICOS criteria; 2) a randomized, parallel, double-blind, placebo-controlled clinical trial with 159 subjects with pre- and stage 1 hypertension who consumed

500 mL/day for 12 weeks of OJ (392 mg/day of hesperidin), EOJ (670 mg/day of hesperidin) or control drink, performing also two single dose studies of 6 hours, to evaluated its effects on BP (CITRUS study); 3) a transcriptomic analysis realised on peripheral blood mononuclear cells (PBMCs) by the Agilent' Microarray Platform in a subsample of 37 subjects from the CITRUS study after 12 weeks and also after the single dose studies; 4) metabolomics analysis that included targeted approach performed by high-performance liquid chromatography in plasma and urine samples in a subsample of 129 subjects of the CITRUS study, and nontargeted metabolomic approach performed by nuclear magnetic resonance spectroscopy in serum samples in a subsample of 52 subjects and in urine samples in a subsample of 129 subjects of the CITRUS study; 5) an experimental study with metabolic syndrome rats that consume 100 mg/kg body weight/day of hesperidin supplementation or not hesperidin supplementation was performed to realize a proteomics approach of heart and kidney tissues.

The results from the systematic review showed that hesperidin improve lipid profile and blood glucose levels in animal models with CVDRFs. However, no definitive conclusion can be drawn in humans. From CITRUS study, the results indicated that the consumption of hesperidin in OJ and EOJ has beneficial effects by reducing the levels of systolic BP and pulse pressures (PP) in a dose-dependent way in humans. Moreover, transcriptomic approach showed the ability of hesperidin to downregulate pro-inflammatory human genes; metabolomics approach showed the ability of hesperidin to decreases human metabolites related with BP, oxidative stress and inflammation; and finally, proteomics approach showed the ability of hesperidin to changes protein expression

related with an improvement of cardiovascular system in rat heart and kidney tissues.

CONCLUSIONS

Hesperidin reduces human BP in a dose-dependent way. Thus, the hesperidin enrichment achieved with EOJ, can be a useful co-adjuvant tool for BP and PP management in pre- and stage 1 hypertensive subjects. The mechanisms of action by which hesperidin exerts its beneficial effects can be explained through transcriptomics and metabolomics approaches in humans which demonstrated cardioprotective actions through decreases in pro-inflammatory genes and decreases in serum endogenous metabolites related to BP, oxidative stress and inflammation. Moreover, the proteomic approach realized in kidney and heart tissues of metabolic syndrome rats, showed that hesperidin changes proteomic profiles exerting positive effects on two main organs involved on BP regulation and cardiovascular system, reducing free radical scavenging and lipid and glucose metabolism.

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1. JUSTIFICATION

Currently, cardiovascular diseases (CVD) are the main cause of mortality worldwide.(1) Triggering these diseases is tobacco smoking, physical inactivity, and unhealthy diet. Thus, the main risk factors implicated in CVD development are modifiable, meaning that we can act on them and reduce the risk of developing it.(2) However, every year more than 17 million people die worldwide from CVD.(3)

There is an ongoing development of new drugs to treat CVD and their intermediate biological factors such as hypertension, hypercholesterolemia, diabetes and obesity, but the partial efficacy, partial intolerance and several adverse effects create the necessity to search for new strategies or coadjutant strategies for the prevention and treatment of CVD.(4) Therefore, the scientific world focuses their research on finding new bioactive compounds from food and beverages with beneficial effects on cardiovascular health. Moreover, the study of their impact on metabolic pathways through the omics science approach to determine their mechanisms of action and to identify new biomarkers of diseases or treatment response is also considered. Omics science refers to a field of study in biological sciences that includes transcriptomics, proteomics, metabolomics or genomics, with the objective of collecting a large number of biological molecules involved in the function of an organism at a defined moment and under certain conditions.(5)

It is well recognized that healthy dietary patterns have significant benefits for CVD treatment and CVD prevention.(6) In this sense, there is the Mediterranean diet, traditionally characterized by a high intake of seasonable vegetables, legumes, fruits, grains, fish and seafood, poultry protein, olive oil, and nuts and low intake of dairy products, red meat, processed meat and sugary drinks. The Mediterranean diet was linked

with the improvement in lipid profile, insulin sensitivity and endothelial function in subjects with cardiovascular disease risk factors (CVDRFs), acting on inflammation and antithrombotic and oxidative stress markers.(7) Another example is the DASH diet (Dietary Approaches to Stop Hypertension) characterized by a low consumption of salt and high intake of fruits, vegetables, whole grains, low-fat dairy and lean protein, which has demonstrated significant blood pressure (BP) reductions in subjects with hypertension,(8) which is one of the most important CVDRFs.

In this sense, most of these observed effects on cardiovascular health are attributable to bioactive compounds, and one of the most important are phenolic compounds, founded in plants products and mainly present in species, fruits, vegetables, olive oil, nuts and beverages.(9) Phenolic compounds are classified into two large families: flavonoids and nonflavonoids.

Phenolic compounds have gained much interest in this field because of their demonstrated capacity to exert beneficial effects on various diseases, including CVDs,(10) and they have been reported in several randomized controlled trials (RCT), the gold standard studies for efficacy evaluation in clinical research.(11) One of the most studied phenolic compounds related to the Mediterranean diet is phenolic compounds from olive oil. In this sense, it is reported that the consumption of a functional virgin olive oil enriched with olive oil and thyme phenolic compounds enhanced the expression of cholesterol efflux regulators, promoting major high-density lipoprotein function and having a beneficial effect on hypercholesterolaemic subjects.(12) Thus, another randomized controlled clinical trial also demonstrated the cardioprotective effect of virgin olive oil and virgin olive oil enriched

with phenolic compounds on oxidation, blood coagulation, lipid transport and immune response.(13) On the other hand, there are some promising phenolic compounds not largely studied until today, and one of them is the flavonoid hesperidin.

Hesperidin, naturally present in citrus fruits and the main flavonoid of orange and orange juice (OJ), has been shown in preclinical and clinical studies to have a therapeutic effect in several diseases, including CVDs, due to its anti-inflammatory and antioxidant effects, and its capacity to decrease lipids and improve insulin sensitivity.(14) Moreover, hesperidin could exert promising hypotensive activities.(15,16) However, the literature about hesperidin effects is scarce, and some results are unclear and controversial.

Besides, OJ is one of the most consumed beverages throughout the world(17) and is totally integrated into our dietary pattern. However, currently the health impact of fruit juices consumption is controversial.(18) Thus, assessing the beneficial effects of OJ on health can be interesting and relevant.

In addition, to the best of our knowledge, the mechanism of action by which hesperidin exerts its possible beneficial effects has not been clearly and fully defined. Finally, no omics approach has been performed to study the effects of hesperidin on the transcriptome, metabolome, or proteome profile, since omics science can shed light on the mechanisms of action.

Therefore, looking at the possible potential of the flavonoid hesperidin on cardiovascular health, the possibility of using hesperidin or hesperidin-rich foods and beverages as a coadjutant treatment or prevention strategy for CVDs, and particularly on BP, and due to the scarcity of the available literature, further research is needed.

Accordingly, a randomized controlled trial will be developed to know hesperidin effects on BP and the most efficient dose of hesperidin in humans. Moreover, to know the mechanisms of action and the biological effects of hesperidin, an omics science approach will be realized in human samples and in organs of experimental rat models.

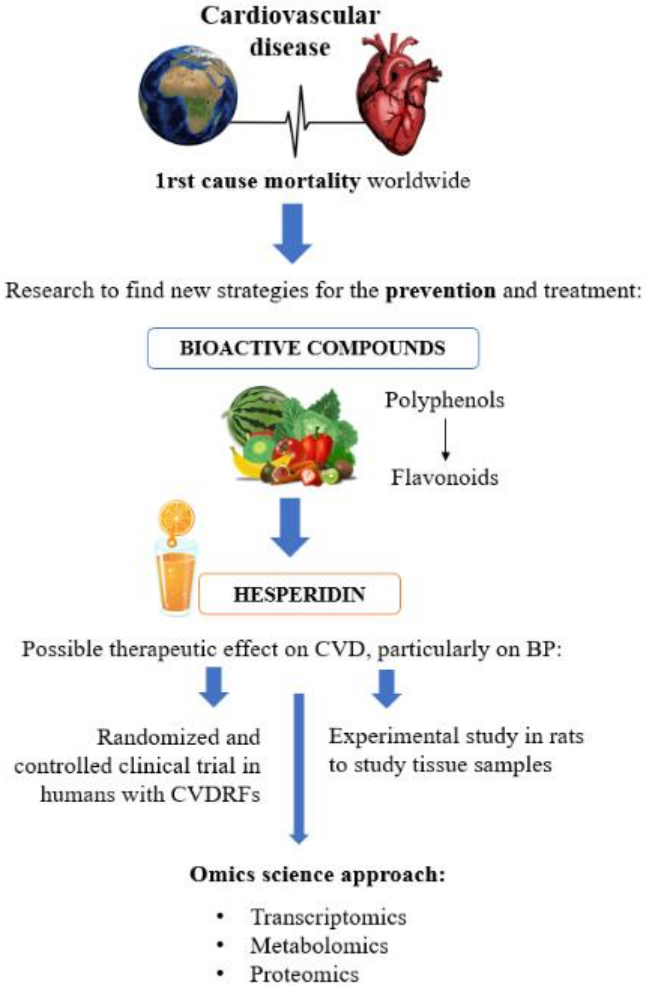


Figure 1. Graphical representation of the justification section of the present doctoral thesis. Abbreviations: BP, blood pressure; CVDRFs, cardiovascular disease risk factors (own source).

2. INTRODUCTION

CHAPTER 1. A worldwide health problem: cardiovascular disease.

The life expectancy of the world population has been increasing over the decades and currently, the average number of years of life in the world is 72(19), and in Spain it is 83.(20) The increase is due to the eradication of several diseases, the mortality reduction of usual pathologies and changes in daily life such as better food control and hygiene measures.

In this sense, CVDs are one of the most advanced fields in research and contribute to an increase in life expectancy. A few decades ago, heart attack and stroke involved fatal outcomes, but today, the prognosis has changed, and patients can recover and live normally.(21) However, CVDs continue to be the main cause of mortality in the world according to the World Health Organization (WHO) (1) and the main cause of mortality in Spain according to the *Instituto Nacional de Estadística*.(22) Thus, more people die every year from CVDs than from any other cause. The statistics show that in 2030 more than 23 million people will die from CVDs.(23)

Several factors influence the development of CVDs, but the experts mainly highlight the progressive ageing of the population and the loss of healthy lifestyle habits. Age is a risk factor itself, and the possibility of developing CVDs increases with advancing age.(21) However, a change towards a healthier and more active life opens a window of possibilities to transform the course of CVDs.

1. 1 Definition of cardiovascular disease.

CVD is a multifactorial disease consisting of a set of disorders of heart and blood vessels that include:(23)

- Hypertension.
- Coronary heart disease or heart attack.
- Cerebrovascular disease or stroke.
- Peripheral vascular disease.
- Heart failure.
- Rheumatic heart disease.
- Congenital heart disease.
- Cardiomyopathies.

1. 2 Pathophysiology of atherosclerosis.

Atherosclerosis, an inflammatory disease characterized by oxidative stress and systemic inflammation, is the main cause of the major incidence and mortality of CVD mentioned above.(24) It is known that the formation of atherosclerotic lesions occurs due to local inflammation in the vascular wall that is induced by dyslipidaemia, normally because of high low-density lipoprotein-cholesterol (LDL-c) levels,(25) and is produced because of lipid and leucocyte accumulation in blood vessels producing plaque formation.(26) Gradually, the plaque hardens and causes narrowing of the arteries, restricting blood flow. Later, the plaque can rupture and form a thrombus (blood clot) causing a further blockage of blood flow and therefore, the availability of oxygen to the body's organs.(26)

Thus, atherosclerosis is characterized by endothelial dysfunction and the accumulation of low-density lipoprotein particles (LDLp), immune cells and necrotic debris in the endothelial space, and endothelial activation triggers the expression of leukocyte adhesion molecules such as E-selectin and P-selectin, glycoproteins such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), chemokines such as monocyte chemoattractant protein-1 (MCP-1), etc.(27) Then, the accumulation of LDLp promotes the release of macrophage colony stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), facilitating monocytes maturation.

Additionally, LDLp turn into oxidized LDL (oxLDL) particles, and they can be recognized by macrophage receptors (CD36 or LOX-1). Consequently, the receptors activate nuclear factor kB (NF-kB) signaling, producing pro-inflammatory cytokines such as interleukin 1 beta and tumour necrosis factor (TNFα), generating foam cells. Foam cells capture oxLDL and lysosomal acid lipase converts esters of cholesterol into free cholesterol and fatty acids. Therefore, M1 macrophages, Th1 cells and some B cell subtypes produce pro-inflammatory cytokines and chemokines promoting atherosclerosis.
On the other hand, Bregs, Tregs and M2 macrophages suppress inflammation reducing the size of the plaque and stabilizing the atherosclerotic lesion.(27) The graphical representation of atherosclerosis pathogenesis is shown in Figure 2.

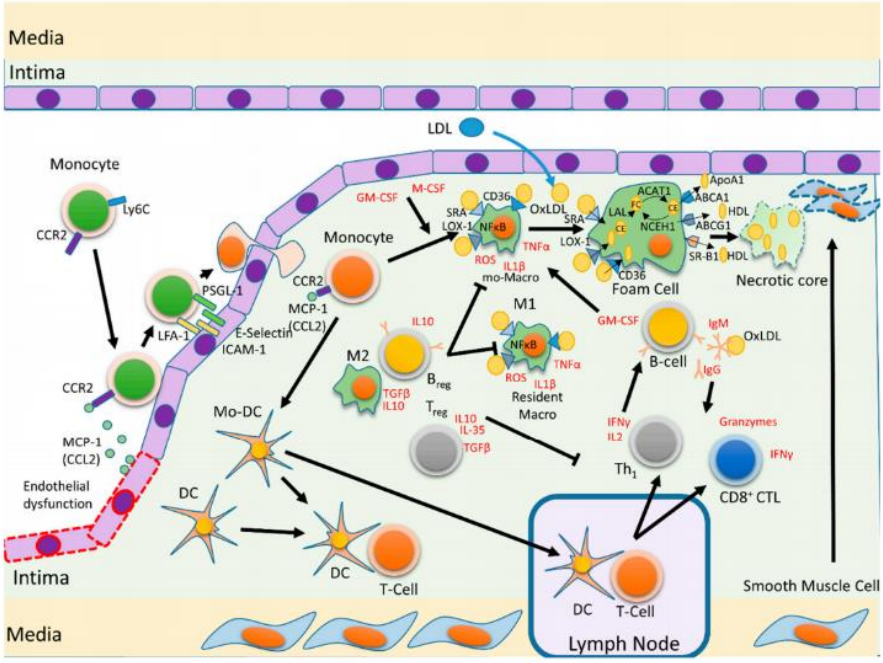


Figure 2. Pathogenesis of atherosclerosis. Source: (Herrero-Fernandez B et al. 2019).(27)

1. 3 Cardiovascular disease risk factors.

A risk factor is defined as the measurable characteristic that is causally associated with an increased rate of a disease; and that is an independent and significant predictor of the risk of presenting a disease.(28) Thus, CVDRFs are those that increase the possibility of developing CVDs.

In this sense, it is known that CVDRFs can be divided into two categories: non-modifiable risk factors and modifiable risk factors.

Non-modifiable risk factors are those that we cannot influence. Factors included in this category are the following:(2)

- Family history of CVDs.
- Age.
- Sex: the male sex has a higher risk of developing CVDs.
- Ethnicity: CVD mortality rates are especially high among black men.
- Socioeconomic status.

On the other hand, modifiable risk factors are those related to habits or behavior performed by humans, which are susceptible to being modified. In this category, the following factors are included:(2)

- Tobacco use.
- Physical inactivity.
- Physiological stress.
- Diet.

Smoking is one of the most preventable risk factors that contributes the most to the development of CVDs.(29) There are group therapies and medication approaches that can be used to stop smoking cigarettes, thus reducing the CVD risk. On the other hand, physical inactivity increases CVD risk by 1.5 times.(30) Regular physical activity protects against CVDs since it reduces body weight, lipid levels, blood glucose and BP levels. Moreover, reducing the progression of atherosclerosis decreases oxidative stress, increases sensitivity to insulin and decreases the incidence of coronary disease. Therefore, it reduces the total and CVD morbidity and mortality.(27)

Finally, the role of diet is crucial in the development and prevention of CVDs since the association between eating habits and CVDs is well known.(30) Unhealthy behavior increase the main risk factors that lead to CVDs such as BP, blood glucose, blood lipids, overweight, obesity and diabetes, resulting in hypertension, hypercholesterolaemia, hypertriglyceridaemia, or metabolic syndrome. Therefore, changes in diet are one of the keys to follow to impact CVDRFs.(31)

In this sense, in Table 1 there is a general description of risk goals and target levels for the most important CVDRFs according to the European Cardiology Society (ESC). Thus, achieving these target levels can prevent the development of CVDs.

Table 1. Risk goals and target levels for the most important cardiovascular diseases risk factors according to the ESC.

Smoking	No exposure to tobacco.
Diet	Healthy diet low in saturated fat with a focus on wholegrain products, fruits, vegetables, and fish.
Physical activity	3.5-7 hours moderately vigorous physical activity every week or 30 – 60 min most days.
Body weight	BMI 20-25 kg/m^2. Waist circumference <94 cm in men and <80 cm in women.
BP	<140/90 mm Hg.

Lipids LDL-c	-Very high-risk: <70 mg/dL, or a reduction of at least 50% if the baseline is between 70 and 135 mg/dL. -High-risk: <70 mg/dL, or a reduction of at least 50% if the baseline is between 100 and 200 mg/dL. -Moderate risk: <100 mg/dL. -Low risk: <116 mg/dL.
Non-HDL-c	-For very high risk: <85 mg/dL. -For high risk: 100 mg/dL. -Moderate risk: 130 mg/dL.
TG	<150 mg/dL.
Diabetes	HbA1c <7%. (<53 mmol/mol).

Abbreviations: BMI, body mass index; BP, blood pressure; LDL-c, low-density lipoprotein-cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; HbA1c, glycated hemoglobin. Adapted from: (Mach F, et al. 2020).(32)

1. 4 Prevention of cardiovascular diseases.

To prevent CVDs, a coordinated pool of activities at the individual or population level to eliminate or reduce CVD incidence to the lowest level is needed.(33) From 1994 to the present, the reference guidelines on CVD prevention are the guidelines of the ESC. The guidelines have been revised several times and the latest version was published in 2020.

These guidelines showed the role of lifestyle including diet in the prevention and treatment of CVDs since the probability of developing

CVDs is associated with unhealthy dietary patterns such as excessive intake of sodium, processed food, added sugars or unhealthy fats, and low intake of vegetables, fruits, and whole grains, among others.(34)

CHAPTER 2. Diet, nutrition and cardiovascular disease.

In this section we will explain the general perspective of the influence of dietary patterns such as the Western diet and Mediterranean diet, nutrients such as sodium and fibre, bioactive compounds such as flavonoids, and functional food such as citrus fruits, on CVDs and their main risk factors.

Dietary habits influence the risk of developing CVDs by influencing CVDRFs, such as blood cholesterol levels, body weight and blood glucose levels.(35) Vast scientific evidence has shown that nutrition might be the most significant preventive factor of CVD death and can reverse heart disease. Additionally, diet can be used to manage excess weight, hypertension, diabetes and dyslipidaemia.(36)

Thus, Table 2 summarizes the characteristics of a healthy diet according to the last update of the European Cardiology Society (ESC) for CVD prevention in clinical practice.

For that reason, as dietary-associated risk is the most important behavior factor influencing global health, there is increased experimentation with the use of "food is medicine" interventions to prevent, manage and treat chronic diseases such as CVDs. However, CVDs remain the leading cause of death and disability in developed countries, and the challenge through dietary interventions in CVDs is to create more effective strategies to motivate populations to change their diet and maintain it over time.

Table 2. Characteristics of healthy dietary habits.

Saturated fatty acids to account for <10% of total energy intake, through replacement by polyunsaturated fatty acids.
Trans unsaturated fatty acids: as little as possible preferably no intake from processed food, and <1% of total energy intake from natural origin.
<5 g of salt per day.
30–45 g of fiber per day, preferably from wholegrain products.
≥200 g of fruit per day (2–3 servings).
≥200 g of vegetables per day (2–3 servings).
Fish 1–2 times per week, one of which to be oily fish.
30 g unsalted nuts per day.
Consumption of alcoholic beverages should be limited to 2 glasses per day (20 g/day of alcohol) for men and 1 glass per day (10 g/day of alcohol) for women.
Sugar-sweetened soft drinks and alcoholic beverages consumption must be discouraged.

Adapted from: (Piepoli MF, et al. 2016). (34)

From the 1990s there has been a transition from a nutrient-based to a dietary-based approach for addressing nutritional interventions in CVDs because of the new evidence that emerged from randomized clinical trials and meta-analyses. There is evidence (with few exceptions such as sodium or trans-saturated fatty acids) that single nutrients have effects of limited magnitude on chronic diseases such as

CVDs compared with whole foods or with complex integrated dietary interventions expressed as dietary patterns.(31)

It is known that a westernized diet, characterized by a high intake of proteins derived from processed and read meats, saturated fats, refined grains, sugar, salt and alcohol and a low intake of vegetables and fruits, is associated with an increased risk of metabolic and chronic diseases such as CVDs.(37) On the other hand, the most well-studied dietary patterns that have beneficial effects on health, especially on cardiovascular health and hypertension, are the Mediterranean and Dietary Approach to stop Hypertension (DASH) diets. The composition of the Mediterranean diet and DASH diet are detailed in Table 3.

Both dietary patterns are associated with a lower risk of clinical cardiovascular events.(31,38) However, the first diet that demonstrated a reduced mortality risk for CVDs was the Mediterranean diet. It was realized through a clinical trial with nutritional intervention called the PREDIMED study.(39)

Table 3. The composition of Mediterranean and DASH diets.

Mediterranean diet	DASH diet
-High content in fresh fruits, vegetables, whole grains, and fatty fish (rich in ω-3 PUFA). -Low content in red meat. -Substituted lower-fat or fat-free dairy products for higher-fat dairy foods. -Olive oil, nuts, or margarines.	-High in vegetables, fruits, low-fat fermented dairy products, whole grains, poultry, fish, and nuts. -Low in sweets, sugar-sweetened beverages, and red meats. -Low in saturated fat, total fat, and cholesterol. -Rich in potassium, magnesium, and calcium. -Rich in protein and fiber.

Abbreviations: DASH, Dietary for Approach to Stop Hypertension; PUFA, poly-unsaturated fatty acids. Adapted from: (Ravera A et al. 2016).(40)

The Mediterranean diet, characterized as rich in whole grains, fruit, and vegetables and low in meat, with a considerable amount of fat from olive oil and nuts,(40) was first described in 1979 by the American biologist Ancel Keys when he observed that the population of the shores of the Mediterranean Sea (in Greece, south of Italy and Yugoslavia) had a lower incidence of CVDs. Later, it would be reported that a Mediterranean diet protects against coronary heart diseases and reduces the risk of the development of diabetes mellitus type 2 and metabolic syndrome.(41,42) This type of diet can reduce CVD risk by mechanisms that reduce BP, lipids, glucose, endothelial function, waist circumference and body mass index (BMI).(43) Additionally, it has

been reported that a Mediterranean diet can increase nitric oxide (NO) bioavailability and can have antioxidant and anti-inflammatory properties. Moreover, the Mediterranean diet seems to have beneficial effects on the synergy among various cardioprotective nutrients and foods.(43)

On the other hand, in the 1990s, a research group led by Lawrence Appel evaluated the effects of a diet rich in fruit, vegetables and low-fat dairy foods on BP levels in a randomized study called the Dietary Approach to Stop Hypertension (DASH) trial. The DASH diet has demonstrated efficacy in treating hypertension without anti-hypertensive medication. Moreover, the DASH diet decreases the risk of developing diabetes in randomized controlled clinical trials and reduces cardiovascular mortality in prospective cohort studies.(44)

Both the Mediterranean and DASH diets demonstrated an improvement in large CVDRFs, including long-term weight gain, BP levels, glucose-insulin homeostasis, lipid profile, inflammation and endothelial function.(43,44)

Last, vegetarian diets also showed beneficial effects on cardiovascular health. It is known that vegetarian patterns reduce CVD mortality and the risk of coronary heart disease.(45) In addition, in 2014 a non-controlled study showed that heart disease could be reversed by a low-fat vegan diet.(46) Moreover, recently published review concluded that a vegetarian diet exerts beneficial effects on BP levels, lipid profiles, platelet aggregation, obesity, metabolic syndrome and type 2 diabetes mellitus.(45) All its benefits are the result of the lower exposure to

harmful substances contained in animal products such as saturated fats and cholesterol, and greater consumption of whole plants rich in fibre and bioactive compounds.(45)

2. 1 Nutrition and cardiovascular health: nutrients.

The evidence shows that some beneficial effects on several CVDRFs are the higher intake of specific nutrients present in foods and the lower intake of another nutrients.

For example, the intake of long-chain ω-3 polyunsaturated fatty acids and their consequent higher circulant levels are protective against CVDs.(47) This is due to the anti-inflammatory and anti-atherosclerotic effects achieved by altering the fatty acid composition of inflammatory cells.(48) On the other hand, the reduced consumption of saturated fats and trans-fats also shows positive effects on cardiovascular health decreasing LDL-c levels.(49) For that reason, the American Heart Association/American College of Cardiology (AHA/ACA) published in guidelines for the management of reducing CVD risk that included the recommendation of ensuring the intake of polyunsaturated fatty acids and reducing the intake of saturated and trans-fat fatty acids.(50)

On the other hand, the AHA/ACA also reported that the consumption of salt and sodium present naturally in foods and added during cooking can affect the CVDs risk. In this sense, reduced sodium intake is related to lower BP levels in adults, which is useful in preventing or treating hypertension and decreasing the risk of developing CVDs.(50) It is known that a higher dietary sodium intake increases extracellular

volume and cardiac output, increasing BP levels. Moreover, combined with sodium intake, abnormal serum levels of potassium and magnesium may affect CVDs because of their influence on hypertension, arrhythmia, and myocardial infarction. This relation is one of the most studied, and in Figure 3, the metabolism of sodium, potassium and magnesium in the body and their relationship with BP levels are presented.

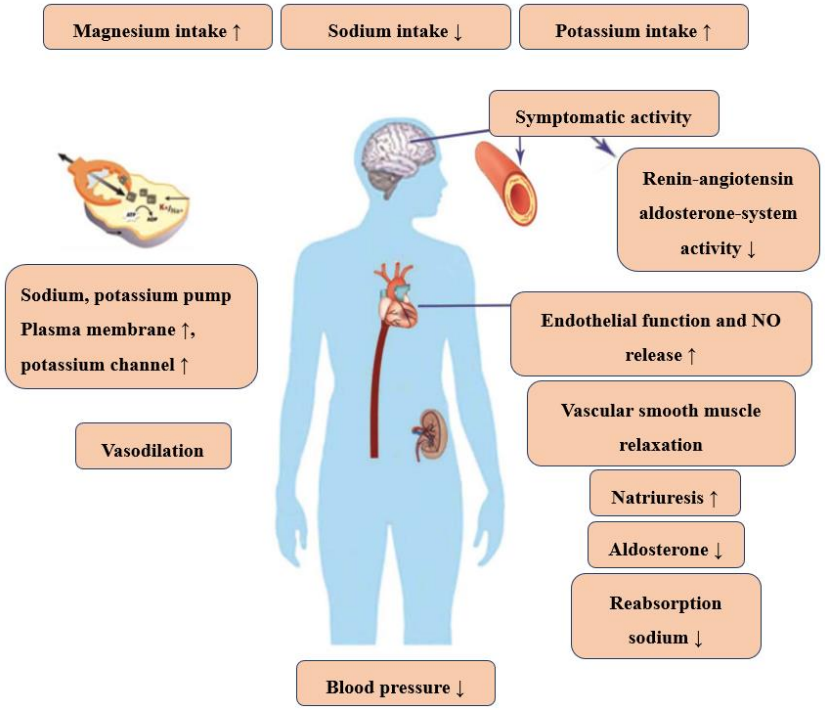


Figure 3. The metabolism of sodium, potassium and magnesium in the body and their relationship with BP levels. Adapted from: (Mohammadifard N et al. 2018).(51)

Moreover, several case-controls and prospective observational studies showed inverse associations between micronutrients such as the levels of vitamin A and vitamin E and the risk of CVDs.(34) Additionally, a correct intake of dietary calcium has beneficial effect on the cardiovascular system through the improvement in lipid profile, BP, obesity, insulin secretion, inflammation and anti-thrombotic agents.(51) It is known that micronutrients can exert their protective effect in three forms: decreasing endothelial cell damage, increasing NO production and/or inhibiting the oxidation of LDL-p. (24)

Additionally, prospective cohorts and meta-analyses observe that a higher intake of total fibre decrease the risk of coronary artery disease, stroke and diabetes mellitus type 2, decreasing blood cholesterol levels and BP, while a deficiency of fiber intake was associated with the development of CVDs.(24)

There is much scientific evidence that relates the beneficial effects of certain nutrients to CVDs and their risk factors. Therefore, the recognition of appropriate nutrients and their adequate intake have an important role in preventing CVDs. It is important to focus on the possible beneficial effects of specific nutrient intake to avoid possible deficiencies in nutrients that can lead to the development of atherosclerotic disease.

2. 2 Nutrition and cardiovascular health: bioactive compounds and functional foods.

The term “functional food” was coined in Japan and the USA in 1970.(52) Functional foods are foods fortified with different probiotics and microorganisms, or natural or processed foods that have biologically active compounds and specific health-promoting benefits that have been scientifically substantiated.(52)

Table 4 summarizes some of the most studied functional foods, their respective bioactive compounds, and their potential mechanism of action.

Table 4. Bioactive compounds, functional foods, and potential mechanisms.

Bioactive compound:	Functional food:	Potential mechanism:
Tocopherols, ω-3 fatty acids	Nuts	Lowering blood cholesterol
Fiber	Legumes	Inhibition of LDL-p oxidation
Genistein and daidzein	Soy proteins	Antioxidant action and platelet aggregation
Flavonoids	Dark chocolate	Lowering blood TG, decreasing BP and antioxidant action
Lycopene	Tomato	Antioxidant action
Ascorbic acid	Citrus	Antioxidant action

Anthocyanins and catechins	Grapes	Endothelial function, antioxidant action and platelet aggregation
Phytochemicals	Whole grains	Lowering blood TG and decreasing BP

Abbreviations: LDL-p, low-density lipoprotein particles; TG, triglycerides; BP, blood pressure. Adapted from: (Asgary S et al. 2018).(52)

The presence of some bioactive compounds present naturally in food and beverages is also responsible for the prevention and treatment of CVDs because of their beneficial effects on atherosclerosis development, reducing LDL-c levels, inflammation, and oxidative stress.(9)

In recent decades, the number of studies evaluating the physiological activities of bioactive compounds from food has markedly increased, and phenolic compounds are one of the most studied. However, due to the large types of phenolic compounds and controversial results reported, more research is needed in this area since the identification of beneficial effects on cardiovascular health through phenolic compound intake could be part of the new nutritional treatment tools.

CHAPTER 3. Phenolic compounds.

In this section, the definition and summary of the characteristics and effects of phenolic compounds will be reported, focusing on flavonoids since they are studied in the present doctoral thesis.

Phenolic compounds are secondary metabolites of plants present in high concentrations in several species, food, and beverages. In contrast to minerals, vitamins and other nutrients, the deficiency of phenolic compounds intake does not produce specific deficiency diseases, although adequate phenolic compounds intake has beneficial effects on health status, especially on chronic diseases.(53)

Currently, phenolic compounds are one of the most studied bioactive compounds because they are the most consumed antioxidant.(54) Total phenolic compounds ingestion, according to our previous results, ranged from 1196.7 to 1967.9 mg/day in an adult healthy Mediterranean population.(55) It is known that cocoa, tea, fruits, and vegetables are the main foods rich in phenolic compounds (56), and a high intake of specific phenolic compounds has been linked to reduced mortality from specific vascular diseases and cancers.(57)

3. 1 Classification of phenolic compounds.

There are more than 8000 different types of phenolic compounds that differ between them depending on the number of phenolic rings they have, and the substituent attached to the rings.(9) Phenolic compounds are classified into two large categories: flavonoids and nonflavonoids.

Flavonoids:

Flavonoids are the most common type in plants and consist of 15 carbons with 2 aromatic rings connected by a 3-carbon bridge (Figure 4).

The main subclasses of flavonoids are flavonols, flavones, flavan-3-ols, isoflavones, flavanones and anthocyanidins. Yellow and red onions are especially rich sources of flavonols containing high concentrations of quercetin; rooibos tea and caffeine-free beverages have high concentrations of flavones such as apigenin and luteolin; leguminous plants are especially rich sources of isoflavones containing high concentrations of daidzein and genistein; citrus fruits have high concentrations of flavanones such as naringenin and hesperetin; and colourful fruits have high concentrations of the anthocyanidins cyanidin and pelargonidin.(9)

Non-flavonoids:

The main non-flavonoids present in food are phenolic acids, and gallic acid is the most common. Non-flavonoids also include stilbenes and lignans (Figure 5).

The main stilbenes are resveratrol, present in red wine and blueberries, while secoisolariciresinol is the most common lignan, present in linseed, cereals, and grain.(9)

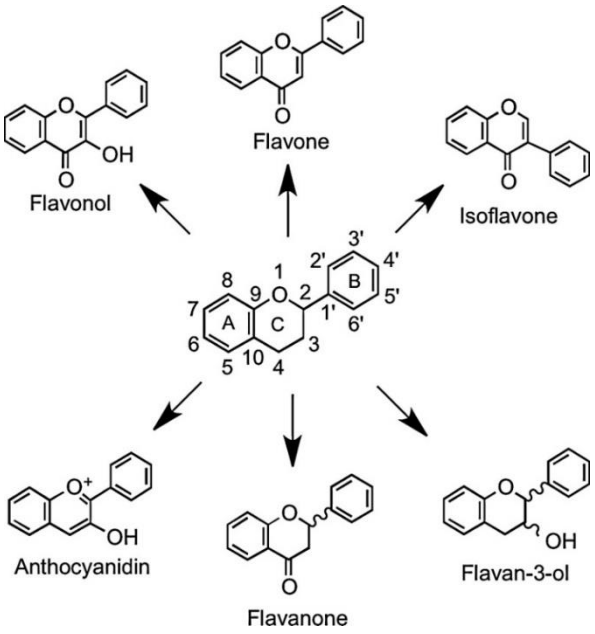


Figure 4. Structure of flavonoid and their subclasses. Source: (Del Rio D et al. 2013).(9)

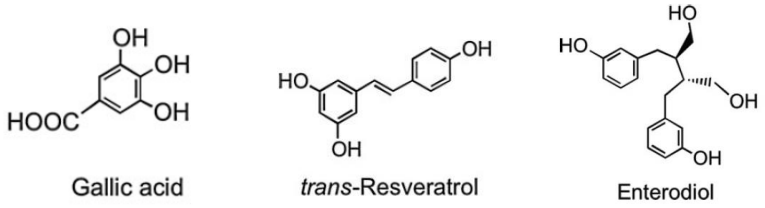


Figure 5. Structure of the main non-flavonoids: gallic acid (phenolic acid), resveratrol (stilbene) and enterodiol (lignan). Adapted from: (Del Rio D et al. 2013).(9)

3. 2 Absorption and metabolism of flavonoids.

Flavonoids present in the diet are mainly present in their glycoside form, and deglycosylation takes place in the small and large intestines, depending on the type of sugar moiety. Two enzymes have been reported to act as β-glucosidases in the small intestine against flavonoid monoglucosides: lactase-phlorizin hydrolase (LPH) and cytosolic β-glucosidase.(9)

An example of the absorption and metabolism of flavonoids, specifically apple flavonoids, is represented in Figure 6.

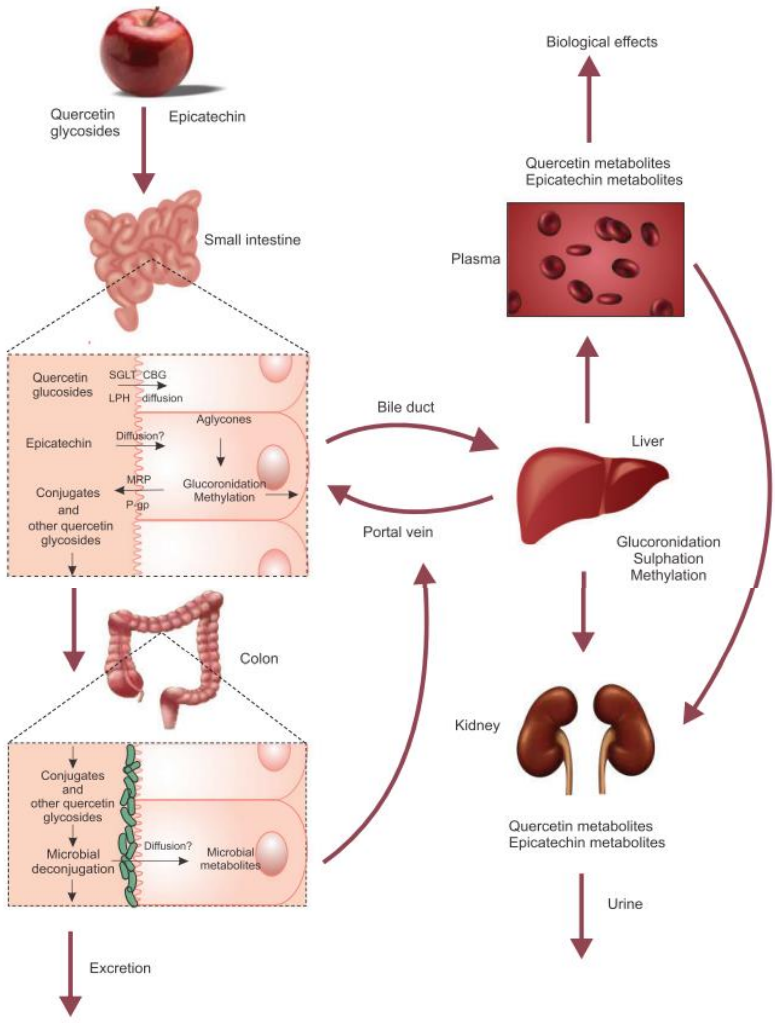


Figure 6. Absorption and metabolism of flavonoids. Quercetin glycosides represent the absorption and metabolism of flavonoid glycosides. Epicatechin represents the absorption and metabolism of the flavonoid subclass, the flavan-3-ols. Abbreviations: SGLT, sodium-dependent glucose transporter; LPH, lactase-phlohizin-hydrolase; CBG, cytosolic β-glucosidase; MRP, multidrug resistance protein; P-gp, P-glycoprotein. Source: (Bondonno CP et al. 2015).(58)

3. 3 Health effects of flavonoids.

At the end of the 19th century the chemical structure of flavonoids was identified and in the early years of the 20th century, flavonoids and their related substances were synthesized in the laboratory. Finally, in the 1930s Albert Szent-Györgyi focused his attention on the effects of specific flavonoids on human health.(59)

Since the effects of flavonoid consumption have been studied for decades, *in vivo* and *in vitro* studies were performed to determine their mechanisms of action and their implications on biological pathways. Additionally, epidemiological studies and clinical trials in humans were performed to evaluate the effects of flavonoid consumption.

Epidemiological evidence has demonstrated a reduction in the risk of fatal CVDs in subjects with a high flavonoid intake. Moreover, prospective studies observed an association between high flavonoid intake and a lower risk of CVD mortality and future cardiovascular events.(60)

On the other hand, human randomized clinical trials showed protective effects of flavonoid consumption against CVDs. For example, flavonoids of phenol-enriched olive oils modulate oxidative balance producing cardioprotective effects,(61) and flavonoids of flavonoid-rich apple improve endothelial function decreasing the risk for CVDs.(62)

In this sense, several studies have shown benefits of flavonoid consumption on the prevention and treatment of several lifestyle-related diseases,(56) including atherosclerosis,(63) coronary heart diseases(64) and metabolic syndrome.(65)

Meta-analyses reported that the consumption of flavonoid-rich foods is associated with a reduced risk of cause-specific mortalities such as those attributable to cancer, diabetes, and CVDs.(57) Additionally, observational studies showed a lower risk of cardiometabolic events with a high dietary intake of flavonoids.(66,67) In human clinical trials, flavonoid consumption also shows beneficial effects on several parameters related to chronic diseases. For example, RCT performed on subjects with CVDRFs such as hypertension, overweight or obesity, diabetes and dyslipidaemia, reported that the daily consumption of chocolate containing 70% cocoa, rich in gallic acid, for 6 weeks improved biochemical parameters (such as total cholesterol (TC), LDL-c and triglycerides (TG)) and waist circumference.(68) In another randomized clinical trial, both the single dose and chronic consumption of flavonoid-rich apple improved the endothelial function, an independent risk factor for CVDs.(62) Moreover, after daily consumption for 8 weeks of blueberry, rich in anthocyanins, the systolic BP levels were reduced in postmenopausal women with pre and stage 1 hypertension, due to the increase in NO production.(69) A recently published review shows that flavonoid-enriched foods decrease BP levels and improve endothelial function by promoting vascular dilation in geriatric patients, who are characterized by multiple chronic diseases.(70) Furthermore, consumption of OJ, which is naturally rich in hesperidin, for 4 weeks, increases endothelium-dependent microvascular reactivity and decreases diastolic BP.(71)

Thus, several clinical trials were performed in humans with one or more CVDRFs to evaluate the effects of different flavonoids. However, there are differences among different flavonoid and therefore, their effect on health cannot be generalized, and each type of flavonoid needs to be

studied. Thus, the effects of some of them are currently controversial, and some flavonoid classes have not been sufficiently studied to date. Thus, investigating how the consumption of specific flavonoids influences different conditions is useful to find new ways to prevent and treat diseases such as CVDs.

Figure 7 summarizes some of the known potential health benefits of dietary flavonoids.

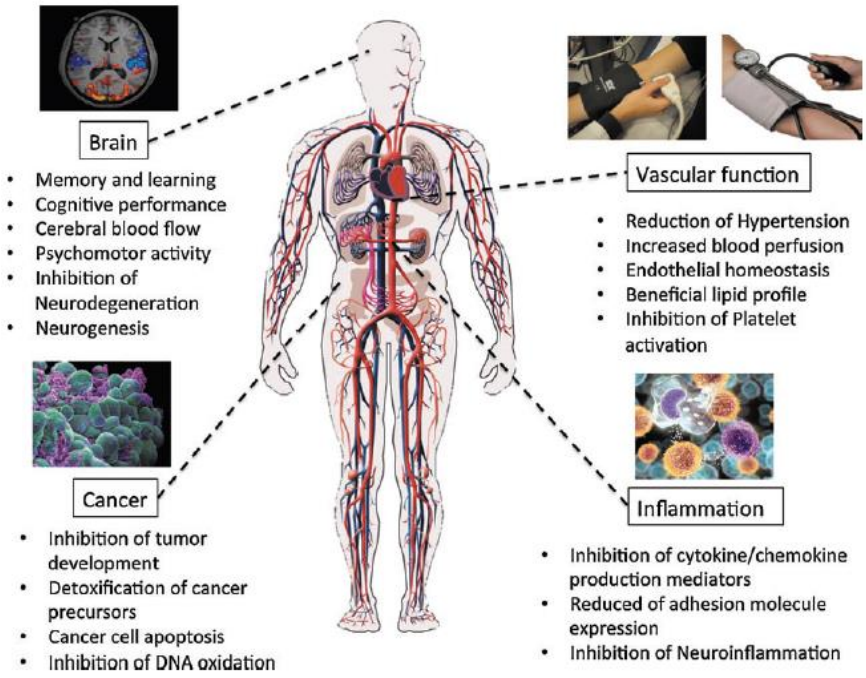


Figure 7. Potential health benefits of dietary flavonoids. Source: (Del Rio D et al. 2013).(9)

3. 4 Mechanisms of action.

The first mechanism of action described for flavonoids was focused on their direct antioxidant effects.(56) However, the concentrations of these compounds in most tissues do not reach the minimum needed to have a significant effect in terms of scavenging free radicals.(56) Additionally, other molecular mechanisms of action have been identified such as the implication on cellular signalling pathways, regulating nuclear transcription factors and lipid metabolism, and modulating inflammatory mediators synthesis such as interleukins and TNFα.(72) In this sense, *in vitro* and *in vivo* as well as epidemiological and experimental studies highlighted the anti-inflammatory activity of flavonoids.(73)

While the molecular mechanisms continue to be clarified, the identified signalling pathways include AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor gamma (PPAR-γ) and nuclear factor-κB (NF-κB). Nevertheless, the mechanism of action by which several flavonoids exert beneficial effects remains unclear. On the other hand, there are many identified flavonoids to date, and the observed effects on molecular pathways for some of them are unlikely to be generalizable to others because of their differential structure and their different ways of acting on organism.(74)

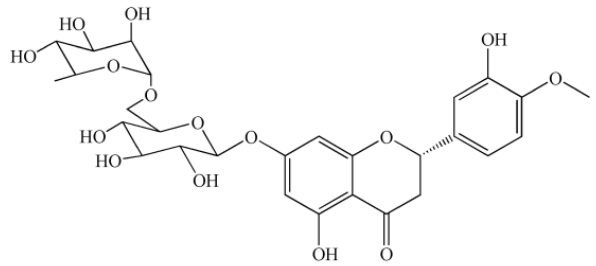
CHAPTER 4. The flavonoid hesperidin.

In this section, the definition, characteristics, metabolism, bioavailability, and health effects of the flavonoid hesperidin will be presented. Moreover, we introduce the actual evidence of the effects of hesperidin on cardiovascular health since it is the focus of the present thesis.

The flavonoid hesperidin (hesperetin 7-O-rutinoside) is founded in glycosylated form in food, and when it is absorbed in the organism is transformed into aglycated form called hesperetin. In bloodstream, hesperetin can be conjugated into sulfate and glucuronides forms.(14) The chemical structures of hesperidin and hesperetin are represented in Figure 8.

Hesperidin is found in the peel of citrus fruits representing 90% of citrus flavonoids but a considerable amounts are found in their juices.(75) Moreover, hesperidin is the main citrus flavonoid of orange fruit and its juice, and orange and OJ are the most common citrus fruit products consumed in Europe.(17) Furthermore, the consumption of hesperidin through supplements or capsules is considered safe and harmless.(76)

A



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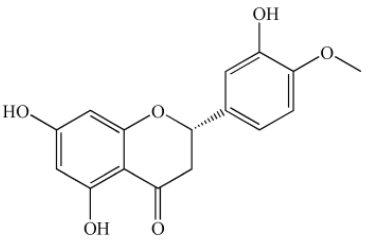

Figure 8. Chemical structures of hesperidin form (A) founded in food, and hesperetin form (B) founded when hesperidin is absorbed. Source: (Xiong H et al. 2019).(77)

4. 1 Health effects of the flavonoid hesperidin.

In recent years, the flavonoid hesperidin has gained much attention in the phenolic compounds research due to its reported beneficial effects on health. Observational studies have reported that citrus fruit consumption is associated with a lower risk of acute coronary events.(78) Moreover, preclinical and clinical studies have demonstrated the possible therapeutic action of the flavonoid hesperidin on several diseases, such as psychiatric disorders, neurological disorders, carcinoma and CVDs, because of its anti-inflammatory, antioxidant, and other interesting properties.(79)

There is evidence of the beneficial effect of hesperidin on the cardiovascular system.(80) Figure 9 shows the possible beneficial effects of hesperidin consumption on several CVDRFs.

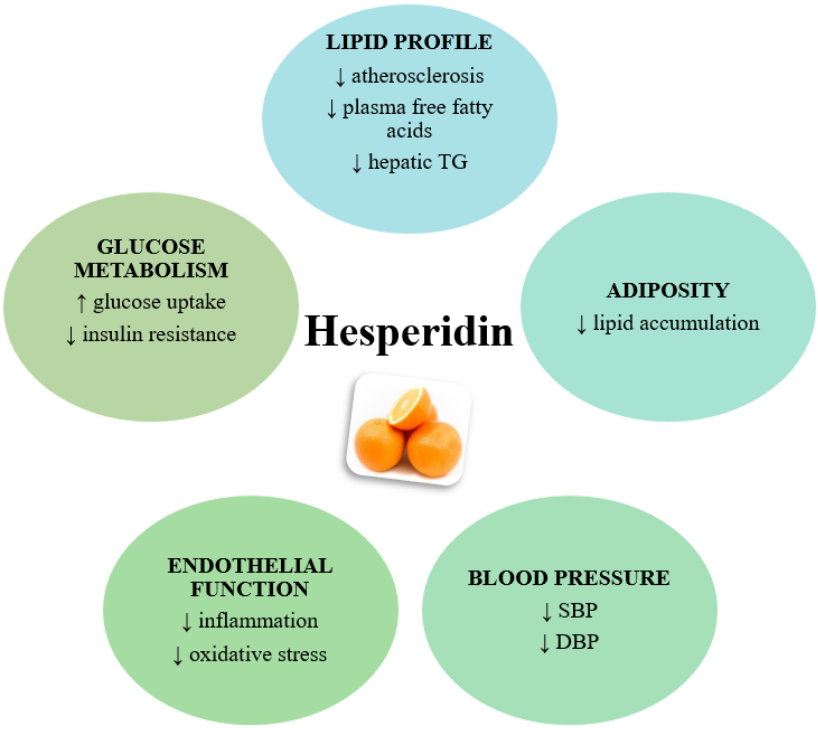

Figure 9. Effects of hesperidin consumption on cardiovascular disease risk factors in humans. Abbreviations: TG, triglycerides; SBP, systolic blood pressure; DBP, diastolic blood pressure (own source).

Lipid profile and adiposity:

First, in animal models, hesperidin administration improves the lipid profile in rats with type 2 diabetes and hypercholesterolaemia. In this sense, it is known that dyslipidaemia is an important and significant risk factor for the development of atherosclerosis. Additionally, hesperidin administration increases high-density lipoprotein-cholesterol (HDL-c) levels and decreases TG, TC and LDL-c levels in metabolic syndrome rats.(81) Moreover, in type 2 diabetic rats, hesperidin decreased the plasma free fatty acids and plasma and hepatic TG levels after 5 weeks of daily consumption.(82) The improvement is produced because of the downregulation of the synthesis of very low density lipoprotein (VLDL) in hepatocytes, the inhibition of lipogenesis and the promotion of beta oxidation of fatty acids.(83) Moreover, hesperidin can suppress hepatic fatty acid synthase and glucose-6-phosphate dehydrogenase and decrease fatty acid oxidation and carnitine palmitoyl transferase activity.(84)

Second, similar results were obtained in human studies. In hypercholesterolaemic subjects, hesperidin consumption through OJ improved lipid profiles increasing HDL-c concentrations and decreasing the LDL-c/HDL-c ratio.(85,86) Thus, hesperidin might benefit atherosclerosis by reducing lipid levels because of its anti-lipid peroxidation and antioxidant properties.(87)

However, unlike the studies in animals, in humans, there is controversy; some studies see similar results as those in animal studies, while other studies did not observe its effects.(88,89)

On the other hand, the alteration of adipose tissue and its dysfunction promotes the development of obesity, an independent risk factor for

CVDs, and hesperidin seems to be a possible therapeutic agent for obesity. It is known that the chemical structure of the flavonoid hesperidin is one of the most effective inhibitors of adipocyte formation since hesperidin can induce a decrease in TG concentration in preadipocytes.(90)

In animal models of obesity or metabolic syndrome, hesperidin reduces body weight and adipose tissue weight.(91,92) In contrast, in human studies the actual evidence is controversial. In some studies, daily hesperidin consumption reduces body weight in subjects with obesity or overweight and in hypercholesterolaemic subjects(93,94) and can increase adiponectin levels in patients with myocardial infarction.(86) However, other studies in humans did not show positive results for weight loss or obesity-related biomarkers.(95,96)

Blood pressure and endothelial function:

As we previously detailed, high BP levels are one of the most important CVDRFs, and endothelial dysfunction is also an important contributor to the pathobiology of atherosclerotic CVD.(97)

It has been demonstrated that hesperidin has an anti-hypertensive effect in renovascular hypertensive rats that involves the suppression of the renin-angiotensin system(98). Additionally, the anti-hypertensive effect of hesperidin was suggested to be mediated by the vascular NO synthase pathway and the reduction of oxidative stress by overexpression of NADPH oxidase, improving endothelial function in rats.(98)

In vitro studies have demonstrated an increased production of NO in endothelial cells after hesperidin administration. Additionally, *in vivo*

studies in rats showed that hesperidin can prevent NO deficiency and hypertension, and the possible mechanism involved its anti-inflammatory and antioxidant effects.(99) Animal studies also showed the capacity of hesperidin to exert antioxidant activity. In this sense, NO is an important vasodilator produced by the vascular endothelium with the objective of regulating the vascular tone.(99) Thus, decreased NO production results in higher vascular resistance and increased BP levels. Therefore, hesperidin seems to improve this aspect.

In animal models and *in vitro* studies, the effects of hesperidin consumption on BP and endothelial function are clear; however, in human studies, the effects are controversial. In subjects with at least one CVDRF, the sustained hesperidin consumption decreases systolic and diastolic BP levels and improves the endothelial function.(71,93,100,101) Nevertheless, there are studies that did not show beneficial effects on BP and endothelial function.(102,103)

Glucose metabolism:

The main complication of diabetes is CVDs, and there are several studies both *in vitro* and *in vivo* that have shown positive effects of hesperidin consumption on glucose homeostasis.

Recently, in an *in vitro* study, both hesperidin and hesperetin inhibited the non-enzymatic glycation of proteins, the main reaction involved in the formation of advanced glycation end-products which has an important role in the pathogenesis of diabetes.(104) On the other hand, hesperidin can affect the gene expression of glucose-regulating enzymes, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, influencing glucose metabolism and glucose

regulation.(105) Additionally, hesperidin can decrease the blood glucose concentration by upregulating hepatic glucokinase and PPAR-γ and adipocyte GLUT4.(106)

In human clinical trials, similar to the evidence for BP and endothelial function, there is no clear effect of hesperidin on glucose metabolism. To the best of our knowledge, to date no study has found a positive effect of hesperidin consumption on plasma glucose levels or insulin in humans.(101,103,107)

4. 3 Metabolism and bioavailability of the flavonoid hesperidin.

The absorption of flavonoid glycosides normally occurs in epithelial cells of the small intestine by the enzymes lactase phlorizin hydrolase and *B*-glycosidase, and the metabolites obtained are transported into the bloodstream.(9) However, bioavailability studies have shown that the flavonoid hesperidin is resistant to enzymes of the stomach and small intestine; therefore, hesperidin can arrive intact at the colon.(9) It is known that only 30% of hesperetin metabolites are absorbed in the small intestine and the other 70% are absorbed in the colon.(108)

At the colon, hesperidin is converted to glucuronides form by alpha-ramnosidase and microbiota.(109) Then, the hesperidin molecule realizes the aglycone form called hesperetin.

Hesperetin is released into the bloodstream in glucuronide form and sulfate conjugates.(110) Three of the most relevant metabolites of hesperidin founded in the organism are: hesperetin 7-O-B-D glucuronide, hesperetin 3-O-B-D glucuronide and hesperetin 7-O-sulfate.(111)

A schematic representation of hesperidin metabolization in the colon is represented in Figure 10.

The bioavailability of the flavanone hesperidin depends on the type of food matrix form in which it is ingested (juice, extract, capsules, etc.), the processing techniques, and the characteristics of the host, such as health status, age, sex, genetics and/or gut microbiota composition.(112) In this sense, it is known that the bioavailability of hesperidin is not different between orange fruit and OJ with higher doses of hesperidin. The similar bioavailability is likely due to the metabolism of flavanones being saturated when the intake exceeds a certain limit.(113) On the other hand, when hesperidin is consumed by capsules or supplements, the bioavailability is similar to hesperidin consumed by food or beverages.

Finally, it is known that subjects can be stratified into three categories depending on their capacity to absorb and excrete hesperidin metabolites: high, medium and low.(110) Therefore, as we previously mentioned, the bioavailability of the flavanone hesperidin depends on different factors that can be considered when the effects are studied.

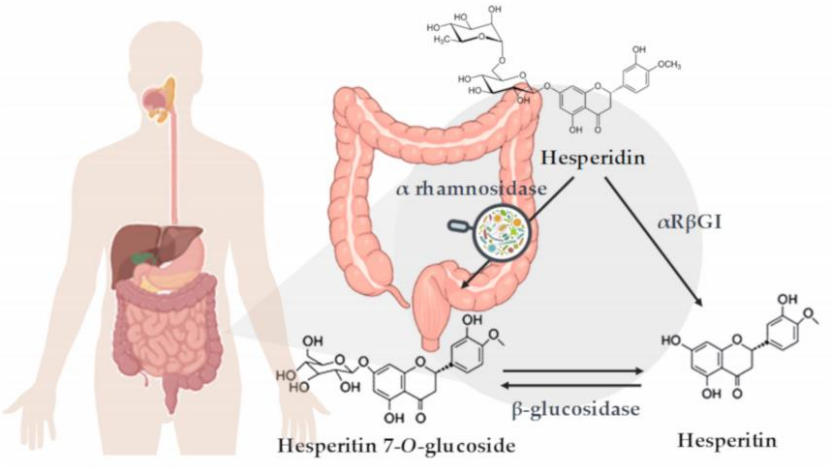

Figure 10. Graphical representation of hesperidin metabolization in the colon. Source: (Mas-Capdevila A, et al. 2020).(112)

CHAPTER 5. Omics science: the future of research.

The word omics refers to an area of study in biological sciences that includes various disciplines including genomics, transcriptomics, proteomics, and metabolomics (Figure 11), as well as other emergent omics such as metagenomics, lipidomics, glycomics or foodomics. The ending -ome is used to describe the object of study of each field, such as the genome, transcriptome, proteome, or metabolome.(5)

The omics sciences involve the analysis of a large volume of data using bioinformatics tools to bring great advances in the basic knowledge of biological issues and the study of organisms and their functions, all through genetic tracing.(114) The omics approach provides a good option for hypothesis-generating experiments, as holistic approaches analyse all available data to describe a hypothesis in situations when no hypothesis is known or prescribed due to a lack of data.(5) In addition, as the omics sciences allow for study at the molecular level of the different elements that include biological systems (cells, tissues, etc.) in all their complexity, omics also allows for the study of the interactions and relationships that occur between the internal components of the individual and the external elements.

In this sense, omics applications can be used to prove the connections and interrelationships among the many scenarios in a complex physiologic state and to discover missing pieces in the current knowledge.(5)

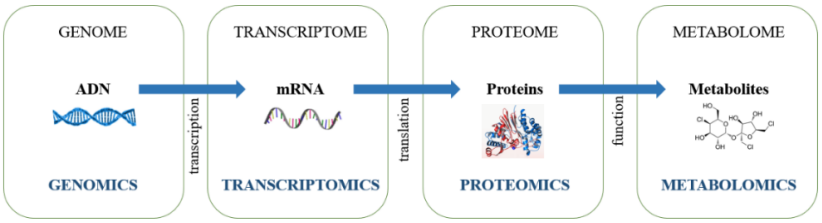

Figure 11. Graphical representation of the main omics sciences (own source).

In addition, there are two types of analyses: nontargeted and targeted. Nontargeted approaches have the objective of detecting, identifying, and quantifying as many genes, proteins or metabolites in a biological sample as possible. In contrast, a targeted approach identifies a group of genes, proteins or metabolites previously selected.(115) Thus, the wide coverage of nontargeted approaches has the potential to identify new metabolic pathways and new disease biomarkers.

In this section of the present doctoral thesis, we focus on transcriptomics, metabolomics, and proteomic approaches.

5. 1 Transcriptomics approach.

The starting point of molecular biology represents the flow of genetic information from genes to the respective functions in cells and organisms. This process begins with the transcription of deoxyribonucleic acid (DNA), the genetic information repository. DNA is transcribed by the ribonucleic acid (RNA) polymerase enzymes into RNA and after that, a subset of RNA is translated into protein.

However, not all RNAs are translated into proteins. Some of them will have a structural function (such as rRNAs), develop a regulatory function (such as siRNAs) or be long non-coding RNAs (lncRNAs), that they do not be translated into proteins but demonstrate their capacity to play important roles in human diseases such as cardiovascular and cancer disorders. Therefore, the transcriptome is a set of RNA molecules present in a cell, a set of cells or in an organism. However, transcriptomics also provides important data regarding the content of cell non-coding RNAs, such as lncRNAs.(116)

Transcriptomics analysis is the study of the transcriptome, the complete set of RNA transcripts, that is produced by the genome under specific conditions in a specific cell, using high-throughput methods, such as microarray analysis. A microarray is a tool used to detect the expression of thousands of genes at the same time. DNA microarrays are microscope slides that are printed with thousands of tiny spots in defined positions, with each spot containing a known sequence or gene. Then, the unknown DNA molecules are cut into fragments by restriction endonucleases and fluorescent markers are attached to these DNA fragments, allowing the reaction with probes of the DNA microarray.(5)

Transcriptomic analysis is used in research and biomedicine for disease diagnosis, biomarker discovery, risk assessment of new drugs, etc. Moreover, transcriptomics analysis also allows the identification of pathways and metabolic changes produced because of environmental stresses such as diet. In this sense, transcriptome analysis is commonly used to compare samples that received different external conditions to

identify genes that are differentially expressed in distinct cell populations, or in response to different treatments. Additionally, it is commonly used to compare healthy and disease states to determine, for example, the mechanisms of pathogenesis.(116)

5. 2 Proteomics approach.

Proteomics, first used in 1996 by Marc Wilkins, is the study and characterization of the proteome. The proteome is defined as all the protein content of a cell, tissue or biological fluid in specific conditions, characterized by their localization, interactions, expression, structure and functions.(117) The proteome changes from time to time, from cell to cell and in response to external factors.

Proteomics analysis is one of the most important methods to determine gene function, although it is more complex than genomics. Proteins are effectors of biological function, and their levels not only depend on mRNA levels but also depend on the control and regulation of translation.(117) Therefore, proteomics is considered the most important set of data to characterize a biological system. In this sense, proteomics offers complementary information to genomics and transcriptomics needed to understand complex biochemical processes.(118) Furthermore, the understanding of protein expression, and thereby what and how biological processes are regulated at the protein level allows the understanding of the molecular basis for diseases and the option to shed light on disease prevention, diagnosis, and treatment.

There is diverse proteomics approaches and one of the most relevant tools to identify, characterize and quantify proteins on a large scale, is mass spectrometry (M-S), which allows the analysis of complex protein mixtures with high sensitivity.(119) M-S is a technique that ionizes all chemical compounds in a sample, resulting in charged molecules that are analysed depending on their mass-to-charge ratios. For the simple pre-separation of complex protein mixtures before M-S analysis, one- or two-dimensional polyacrylamide gel electrophoresis was often used in the past.(5) Currently, off gel-nanoLC-(Orbitap) MS/MS is used because it is more efficient in identifying novel proteins in various biological systems.

The identification of differentially expressed proteins in healthy and diseased subjects allow the discovery of new biomarkers in biomedical research. For example, recent work demonstrated the implication of several proteins in heart failure in subjects through proteomic analysis, allowing new plasma biomarker discovery for heart failure.(120)
Moreover, as the application that will be realized in the present doctoral thesis, proteomics can provide insight into the effects of specific compounds, such as the flavonoid hesperidin, on the proteome to explore the molecular mechanisms involved in the cardioprotective effects of hesperidin.(121)

5. 3 Metabolomics approach.

Metabolomics emerged in the third millennium with the intention of measuring all the small molecule metabolites in a biological system, or at least most of them. For that reason, the improvement in instrument

technologies has improved the metabolomics analysis, most notably in the sensitivity and mass range of mass spectrometers.(122)

The metabolome is the global profile of metabolites in a biological sample. Metabolites are the natural intermediate products of metabolic reactions catalysed by enzymes that occur in cells, including an immense variety of endogenous small molecules such as amino acids, lipids, nucleic acids, sugars, fatty acids, etc., as well as exogenous chemicals such as pharmacological agents, toxins or xenobiotics. Thus, metabolomics is the study of metabolites in response to environmental stimuli and genetic alterations, giving a full-scale analysis of cellular and tissue metabolism and providing a more comprehensive understanding of biology.(122)
Typically, metabolomics uses high-resolution analysis together with statistical analysis such as principal component analysis, to obtain an integrated picture of the metabolome. The most commonly used methods by which the metabolome is assessed are nuclear magnetic resonance spectroscopy and M-S.

The metabolic profiles obtained permit the development of diagnostic and/or prognostic tools that have the potential to significantly alter the management of CVDs.(123) For example, several works identified both urinary and plasma metabolites correlated with BP levels and hypertension, such as alanine or hippuric acid.(124) On the other hand, branched-chain amino acids (BCAA) levels have been linked with insulin resistance and type 2 diabetes mellitus, which increase CVD risk.(125)

For that reason, knowing the metabolome profile of individuals can offer translation from research to clinical practice to substantially affect the diagnosis, prognosis, and treatment of cardiovascular medicine. In nutritional interventions, a metabolomics approach can offer the possibility of identifying new biomarkers to determine the intake of specific compounds such as hesperidin and to relate it to the biological effects observed. Biomarkers are constituents present in biofluids such as blood and urine that can be used to indicate dietary exposure to compare it to the recommended or estimated intake. Moreover, in human intervention studies, biomarkers can indicate dietary changes and can be exposure biomarkers. Furthermore, metabolomics offers away to evaluate the contribution of dietary factors by associating circulating metabolites with global CVDs but also with specific CVDRFs such as BP.(126)

5. 4 Omics approach for the prevention and treatment of diseases.

The molecular measurements from large-scale omics data could be integrated into models of disease risk prediction adding valuable information to traditional clinical tests to better stratify patients into high- or low-risk groups for the potential of developing a disease such as CVD. However, currently, the cost of performing an omics analysis is high.

Nevertheless, the integration of different omics techniques is expected to become increasingly powerful for disease treatment and prognosis and has even been suggested to be useful in disease regression.(127)

The simultaneous integration of science omics was called systems biology in the past, and foodomics when it was applied to the food,(128) but nowadays it is called multi-omics (Figure 12).

Multi-omics approach could be powerful in better understanding the mechanisms and pathways implicated to identify key drivers that have the largest contribution to diseases such as CVDs.

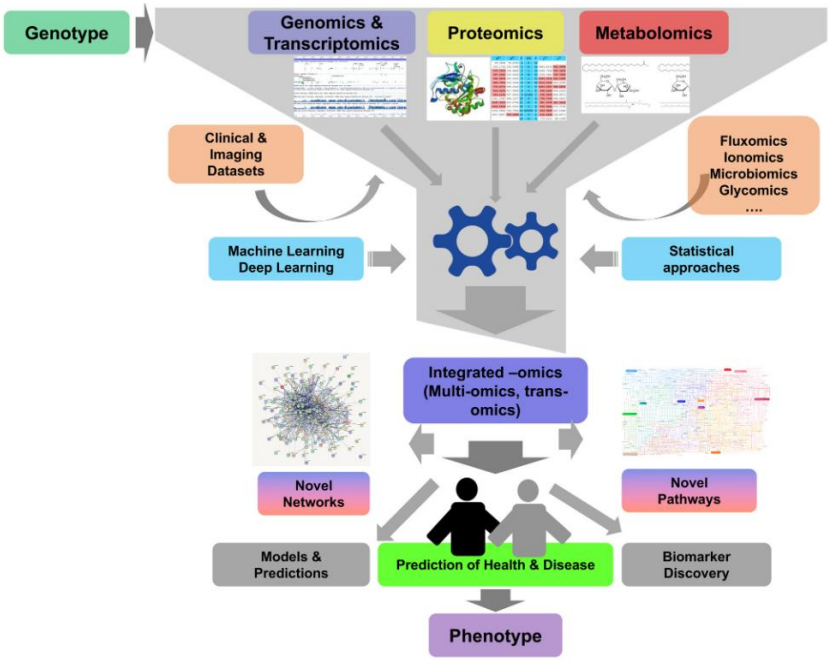

Figure 12. Workflow with integrated omics showing input datasets and results. Source: (Misra BB et al. 2018).(129)

3. HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Our hypothesis is that the intake of hesperidin, the main flavonoid of citrus fruits presents mainly in orange and OJ, might exert beneficial effects on CVDRFs, particularly reducing BP, in humans. Moreover, the mechanisms of action of hesperidin by which it could improve CVD can be elucidated through different omics approaches, such as transcriptomics, metabolomics, and proteomics, both in humans and in experimental animal models.

OBJECTIVES

The main objective is to evaluate the effects of the consumption of hesperidin in OJ on different CVDRFs on humans, particularly on BP, and to elucidate the possible mechanisms of action and the biological effects involved though the application of different omics approaches such as transcriptomics, metabolomics and proteomics, both in humans and animal models.

The following specific objectives have been set:

Objective 1:

To summarize and evaluate the current scientific evidence from animal studies and human RCTs to determine the effects of hesperidin on cardiovascular risk biomarkers.

Objective 2:

To assess the sustained and acute effects, and the sustained consumption influence on acute effects, of real-life doses of OJ and a

hesperidin-enriched dose on BP, PP, and cardiovascular risk biomarkers in pre- and stage-1 hypertensive individuals.

Objective 3:

To determine whether the sustained and single dose consumption of hesperidin in OJ and EOJ can change the transcriptomic profile of PBMCs of subjects with pre- and stage 1 hypertension to elucidate possible mechanisms of action of the hesperidin and their role in CVD.

Objective 4:

To determine new biomarkers of the biological effects of hesperidin in OJ applying nontargeted metabolomics approach in plasma/serum and urine samples after both single dose (6 hours) and sustained (12 weeks) consumption, validated through targeted metabolomics analyses of compliance biomarkers in subjects with pre- and stage 1 hypertension.

Objective 5:

To determine the changes in proteomic profiles of kidney and heart tissues, as key organs involved in BP regulation and cardiovascular system, in healthy and metabolic syndrome rats after hesperidin supplementation to shed light on the hesperidin mechanism of action.

4. METHODS AND RESULTS

Project 1: Effects of hesperidin consumption on cardiovascular risk biomarkers: a systematic review of animal studies and human randomized clinical trials.

Lead Article

Effects of hesperidin consumption on cardiovascular risk biomarkers: a systematic review of animal studies and human randomized clinical trials

L. Pla-Pagà, J. Companys, L. Calderón-Pérez, E. Llauradó, R. Solà, R. M. Valls, and A. Pedret

Context: *The cardioprotective effects of the flavonoid hesperidin, which is present in citrus products, are controversial and unclear. This systematic review was conducted in accordance with the PRISMA 2015 guidelines.* ***Objective:*** *To evaluate the current evidence from animal and human clinical studies and thus determine whether the consumption of hesperidin exerts beneficial effects on cardiovascular risk factors.* ***Data sources:*** *PICOS (Population, Intervention, Comparison, Outcome, and Study Design) criteria defined the research question. Searches of the PubMed and Cochrane Plus databases were conducted and studies that met the inclusion criteria and were published in English in the last 15 years were included.* ***Data extraction:*** *The first author, year of publication, study design, characteristics of animals and humans, intervention groups, dose of hesperidin, route of administration, duration of the intervention, cardiovascular risk biomarkers assessed, and results observed were extracted from the included articles.* ***Results:*** *A total of 12 animal studies and 11 randomized clinical trials met the inclusion criteria. In the animal studies, the glucose, total and LDL cholesterol, and triglyceride levels decreased with chronic flavonoid consumption. In the human studies, endothelial function improved with flavonoid consumption, whereas no conclusive results were observed for the other biomarkers.* ***Conclusions:*** *Animal studies have revealed that hesperidin and hesperetin consumption reduces glucose levels and various lipid profile parameters. However, a definitive conclusion cannot be drawn from the existing human clinical trials. Further research is needed to confirm whether the findings observed in animal models can also be observed in humans.* ***Systematic Review Registration:*** *Prospero registration number CRD42018088942.*

INTRODUCTION

Cardiovascular diseases (CVDs) constitute the main cause of mortality throughout the world.[1] The latest statistical data from the World Health Organization showed that ischemic heart disease and stroke caused 15 million deaths in 2015 worldwide.[1]

Currently, there is a growing interest in identifying new bioactive compounds with healthy effects on CVDs, which can then be used to develop functional

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Key words: cardiovascular risk biomarkers, citrus flavonoids, glucose, hesperidin, hesperetin, lipid profile.

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foods, and phenolic compounds have gained much interest in this field of research. Polyphenols are secondary metabolites of plants, and more than 8 000 different types exist, which can be classified into different groups depending on the number of phenolic rings they contain and the type of substituent attached to the rings.[2] Polyphenols are divided into two large families: flavonoids and non-flavonoids. Flavonoids are the most abundant type in plants, and the main subclasses include flavonols, flavones, isoflavones, flavanones, anthocyanidins, flavan-3-ols, and dihydrochalcones.[2] Flavonoids can be found in many commonly consumed fruits and vegetables, and numerous studies have shown their benefits for the prevention and treatment of different pathologies.[3–5] In recent years, citrus flavonoids, which are present in different citrus fruits, particularly in orange juice, have gained the attention of the food industry because they may exert beneficial effects on different cardiovascular risk factors (CVRFs)[6] and because orange juice is one of the most consumed beverages throughout the world.[7] In European adults, the mean flavonoid intake is 428 mg/day.[8]

The main citrus flavonoid of orange fruit and orange juice is hesperidin, which is found in greater quantities in the peel and represents 90% of citrus flavonoids.[9] Hesperidin (hesperetin-7-O-rutinoside) is a flavanone glycoside and the dietary form of the aglycone hesperitin.[6] Normally, the absorption of flavonoid glycosides such as hesperidin occurs in epithelial cells in the small intestine and is facilitated by the enzymes lactase phlorizin hydrolase or cytosolic β-glucosidase, resulting in the separation of the aglycone and its transportation into the bloodstream.[2] Then, the metabolites are transported to the liver for phase II metabolism, and they can be recycled by the enterohepatic recirculation in the small intestine. However, bioavailability studies show that only 30% of hesperetin metabolites are absorbed in the small intestine and the other 70% are absorbed in the colon,[2] via microbiota and alpha-rhamnosidase activity,[10] where the hesperidin is converted to glucuronides. In-vitro studies have revealed that hesperidin stimulates the production of nitric oxide (NO) in endothelial cells,[11,12] inhibits the secretion of endothelin-1[12] and inhibits platelet activity by inhibiting the activities of specific phospholipases and cyclooxygenase-1.[13] Animal studies have shown that hesperidin exhibits antioxidant capacity and endothelial protection against reactive oxygen species in spontaneously hypertensive rats, and improves hyperlipidemia and hyperglycemia in diabetic rats.[14] Conversely, other animal studies have not found that hesperidin exerts beneficial effects on glucose or insulin levels, lipid profile, or blood pressure.[15,16] In contrast, several observational studies have shown that citrus fruit consumption is associated with a lower risk of acute coronary events.[17,18] However, the findings from human randomized clinical trials (RCTs) are not consistent: some studies have found that daily consumption of orange juice decreases systolic blood pressure (SBP) and diastolic blood pressure (DBP),[19] and increases the total plasma antioxidant capacity or decreases lipid peroxidation,[20] but others have not reported any beneficial effects on blood pressure or the lipid profile after hesperidin consumption.[21,22] To the best of our knowledge, the current scientific evidence on the effects of hesperidin on cardiovascular risk biomarkers obtained from animal studies and human RCTs has not been systematically reviewed, and thus, no conclusive remarks can be drawn.

Therefore, the present systematic review aimed to determine whether hesperidin consumption might exert beneficial effects on cardiovascular risk biomarkers. The objective was to summarize and evaluate the current scientific evidence from animal studies and human RCTs to determine the effects of hesperidin on cardiovascular risk biomarkers.

METHODS

This systematic review was conducted according to the PRISMA 2015[23] (Preferred Reporting Items for Systematic reviews and Meta-Analysis) guidelines and was registered with PROSPERO on February 20, 2018, under the ID number CRD42018088942. The protocol can be accessed at http://www.crd.york.ac.uk/PROSPERO/display_record.php? ID=CRD42018088942.

Eligibility criteria

Animal studies and RCTs were eligible for the systematic review in accordance with the review's PICOS criteria. The complete PICOS criteria for inclusion and exclusion of studies are described in Table 1.

Information sources, search strategy, and study selection

A literature search of the PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) and Cochrane Plus (www.bibliotecaco-gov/pubmed) databases was performed using medical subject headings (MeSH). The complete search strategy is shown in Table 2. The literature search was restricted to English-language articles published between January 2003 and January 2018.

To ensure the accurate identification of eligible studies, a two-step selection process was used. To confirm the eligibility of the included articles, the titles and

Table 1 **PICOS criteria for inclusion and exclusion of studies**

Parameter	Inclusion criteria	Exclusion criteria
For the animal studies		
Participants	Rats or mice with at least one CVRF (obesity, dyslipidemia, hypertension, diabetes, or metabolic syndrome)	Studies performed on animal models that were not rats or mice
Intervention	Some type of intervention based on hesperidin	Combination of different classes of phenolic compounds (other than citrus flavonoids) and combination with other nutrients, components, or drugs (vitamin C, caffeine, or hypertension drugs)
Comparisons	Different doses of hesperidin and/or hesperidin consumption and non-consumption	
Outcomes	Studies that assessed the effects of hesperidin on biomarkers or risk factors related to CVDs: anthropometric parameters, vascular parameters, glucose and insulin levels, lipid profile and coagulation, inflammation and oxidation biomarkers	
Study design	Randomized and non-randomized, acute and chronic follow-up, published in English	Studies published before January 2003 and in any language other than English
For the RCTs		
Participants	Humans of all races, ages, and genders with at least one CVRF (obesity, dyslipidemia, hypertension, diabetes, or metabolic syndrome)	Humans with no CVRF
Intervention	Some type of nutritional intervention based on the consumption of hesperidin from food, drink, or supplement	Combination of different classes of phenolic compounds (other than citrus flavonoids) and combination with other nutrients, components, or drugs (vitamin C, caffeine, or hypertension drugs)
Comparisons	Different doses of hesperidin and/or hesperidin consumption and non-consumption	
Outcomes	Studies that assessed the effects of hesperidin consumption on biomarkers or risk factors related to CVD: anthropometric parameters, vascular parameters, glucose and insulin levels, lipid profile and coagulation, inflammation and oxidation biomarkers	
Study design	Randomized controlled clinical trials, parallel and crossover design, acute and chronic follow-up, published in English	Reviews, expert opinion, comments, letter to editor, case reports, conference reports, observational studies, animal studies, and studies published before January 2003 and in any language other than English

Abbreviations: CVDs, cardiovascular diseases; CVRF, cardiovascular risk factor; RCTs, randomized clinical trials.

abstracts of the studies identified using the search strategy were screened independently by two authors (LP-P and JC). The full text of the potentially eligible studies was then retrieved and independently assessed for eligibility by the same two authors. Any disagreement between the authors over the eligibility of a study was resolved through discussion with a third author (LC-P).

Data collection and extraction

From the total number of articles identified by assigning appropriate MeSH terms, any duplicate articles within and between the databases were removed. The remaining articles were assessed primarily according to their title and abstract, and then according to their full text, and those studies that did not meet the eligibility criteria were removed.

The following data were extracted from the included animal studies: first author, year of publication, study design, characteristics of the animals, intervention groups, dose of hesperidin, route of administration, duration of the intervention, cardiovascular risk (CVR) biomarkers assessed, and results observed.

The following data were extracted from the RCTs: first author, year of publication, study population, population age and health status, characteristics of the nutritional intervention, dose of hesperidin, consumption matrix, duration of the intervention, method used to confirm compliance with the intervention, CVR biomarkers assessed, and results observed.

Study quality and risk of bias in the individual studies

Assessments of the quality and possible risks of bias in each RCT included in the present systematic review were performed using Review Manager software (RevMan; version 5.3), a tool provided by the Cochrane Collaboration. The following items were included in the assessments: random sequence generation, allocation concealment, blinding of participants and personnel,

Table 2 **Search strategy and MeSH terms used**

For the animal studies	For the RCTs
Search strategy: -Electronic databases: PubMed and Cochrane Plus -Publication dates: January 2003 – January 2018 -Species: Other animals	Search strategy: -Electronic databases: PubMed and Cochrane Plus -Publication dates: January 2003 – January 2018 -Species: Humans
MeSH terms: hesperidin hesperetin and blood pressure endothelial function blood cholesterol high density lipoprotein low density lipoprotein apolipoprotein A1 apolipoprotein B100 triglycerides plasma no esterified reactive protein glucose insulin resistance diabetes C-reactive protein IL-6 IL-18 nitrates and nitrites platelet aggregation endothelin soluble intercellular adhesion molecule-1 soluble vascular cell adhesion molecule-1 E-selection serum amyloid A oxidized low density lipoprotein urinary creatinine oxidative stress nitric oxide homocysteine nitrotyrosine plasminogen activator inhibitor-1 von Willebrand factor fibrinogen body mass index body weight obesity overweight	MeSH terms: orange juice orange polyphenols citrus flavonoids citrus flavanones hesperidin hesperetin and blood pressure hypertension endothelial function blood cholesterol high density lipoprotein low density lipoprotein apolipoprotein A1 apolipoprotein B100 triglycerides plasma no esterified reactive protein glucose insulin resistance diabetes IL-6 IL-18 nitrates and nitrites platelet aggregation endothelin soluble intercellular adhesion molecule-1 soluble vascular cell adhesion molecule-1 E-selection serum amyloid A oxidized low density lipoprotein urinary creatinine oxidative stress nitric oxide homocysteine nitrotyrosine plasminogen activator inhibitor-1 von Willebrand factor fibrinogen body mass index body weight obesity overweight atherosclerosis cardiovascular risk factors

Abbreviation: RCTs, randomized clinical trials.

blinding of outcome assessments, incomplete outcome data, selective reporting, and other biases. The risk of bias in each study was classified as "low," "unclear," or "high." Two authors (LP-P and JC) evaluated the risk of bias in the RCTs, and any disagreement between them over the risk of bias of a study was resolved through discussion with a third author (LC-P).

RESULTS

Animal studies

Study selection. A total of 698 articles were identified from the two databases (643 in PubMed and 55 in Cochrane Plus). Of these, 367 duplicate articles were

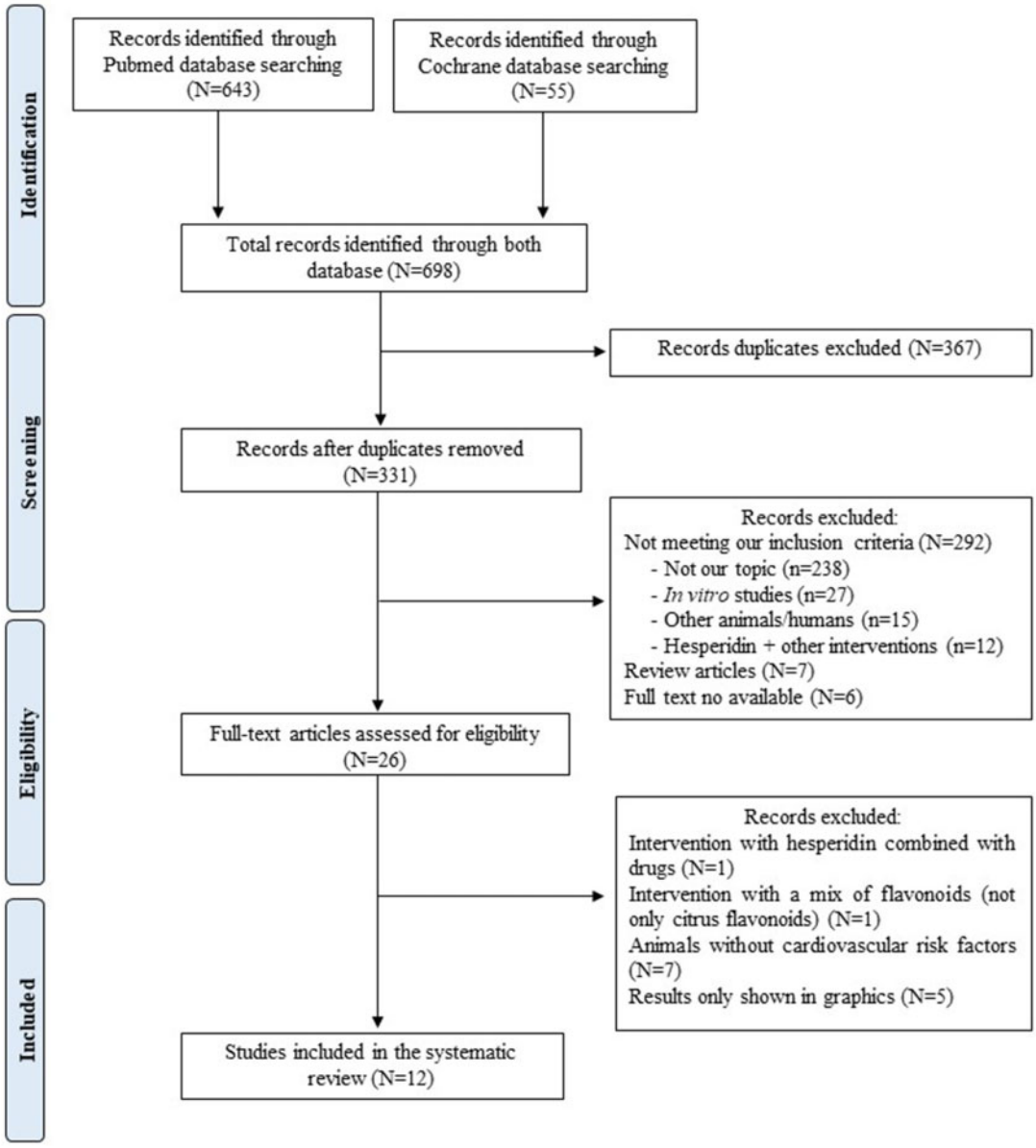

Figure 1 **Flow diagram of the literature search process for animal studies.**

removed and 292 of the remaining 331 articles were excluded because they did not meet the inclusion criteria, 7 were excluded because they were review articles, and 6 were excluded because no full text was available. As a result, 12 articles were included in the systematic review. Figure 1 shows the study selection process for the animal studies included in the review.

Study characteristics. Table 3 shows the general characteristics of the 12 animal studies included in the systematic review. Further details of each study are presented in Table S1 in the Supporting Information online. The 12 studies included in the systematic review were controlled animal studies involving an intervention group that was administered flavanone and a control group that was not administered flavanone. In 9 of the studies, hesperidin was orally administered,[15,21,22,24–29] while in 2 of the remaining 3 studies hesperidin was administered by gavage,[30,31] and in the other study hesperidin was administered intravenously.[32] The doses of hesperidin ranged from 5 mg/kg of body weight/day to 200 mg/kg of body weight/day in 10 studies and from 0.08% to 4.60% of the total calorie intake in the other 2 studies. The duration of the intervention ranged from 7 days to 24 weeks. All the animals had at least one CVRF, such as hypertension, myocardial ischemia, systemic inflammation, hypercholesterolemia, and type 2 diabetes. The sample size ranged from 4 to 16 animals in each group, and of the 12 studies, 8 were performed on rats and 4 on mice.

Table 3 **Characteristics and results of the animal studies included in the systematic review (n = 12)**

Author, year, reference	Experimental animal	Groups (n)	Dose of flavanone	Route	Duration	Results													
						Anthropometric parameters			Vascular parameters		Glucose and insulin levels		Lipid profile				INFL. BIOM	OXID. BIOM
						BW	WG	VF	SBP	DBP	GL	INS	TC	LDL-c	HDL-c	TG	IL-6	NO
Iskender et al (2017)[24]	Type 2 diabetic rats	Control group (n=10) vs Hesperidin group (n=10)	0 mg vs 100 mg/kg BW/d	Orally	15 d	NS	–	–	–	–	↓	–	–	–	–	–	–	–
Dobiaš et al (2016)[25]	Spontaneously hypertensive rats	Control group (n=13) vs Hesperidin group (n=13)	0 mg vs 50 mg/kg BW/d	Orally	4 wk	NS	–	–	NS	–	–	–	–	–	–	–	–	–
Ferreira et al (2016)[15]	Mice with systemic inflammation	Control group (n=10) vs Hesperidin group (n=10)	0 mg vs 100 mg/kg BW/d	Orally	4 wk	NS	NS	NS	–	–	NS	–	NS	↓	NS	NS	↓	–
Jia et al (2015)[31]	Type 2 diabetic mice	Control group (n=10) vs Neohesperidin group (n=10)	0 mg vs 50 mg/kg BW/d	By gavage	6 wk	NS	–	–	–	–	↓	–	↓	–	–	↓	–	–
Yamamoto et al (2013)[32]	Hypertensive rats	Control group (n=4) vs Hesperetin group (n=4) vs Hesperetin-7-O-β-D-glucuronide group (n=4) vs Hesperetin-3′-O-β-D-glucuronide group (n=4)	0 mg vs 5 mg/kg BW/d vs 5 mg/kg BW/d vs 5 mg/kg BW/d	Intravenous	3 min (acute s tudy)	–	–	–	↓ with hesperetin; ↓ with HESPT7G	NS	–	–	–	–	–	–	–	–
Kumar et al (2012)[30]	Type 2 diabetic rats	Control group (n=16) vs Hesperetin group (n=16)	0 mg vs 200 mg/kg BW/d	By gavage	24 wk	–	–	–	–	–	↓	–	–	–	–	–	–	–

(continued)

Table 3 **Continued**

Author, year, reference	Experimental animal	Groups (n)	Dose of flavanone	Route	Duration	Results													
						Anthropometric parameters			Vascular parameters		Glucose and insulin levels		Lipid profile				INFL. BIOM	OXID. BIOM
						BW	WG	VF	SBP	DBP	GL	INS	TC	LDL-c	HDL-c	TG	IL-6	NO
Mahmoud et al (2012)[26]	Type 2 diabetic rats	Control group (n=6) vs Hesperidin group (n=6)	0 mg 50 mg/kg BW/d	Orally	30 d	–	–	–	–	–	↓	↑	–	–	–	–	–	↓
Selvaraj and Pugalendi (2012)[22]	Rats with myocardial ischemia	Control group (n=6) vs Hesperidin group (n=6)	0 mg 200 mg/kg BW/d	Orally	7 d	–	–	–	–	–	–	–	↓	↓	↑	↓	–	–
Wang et al (2011)[16]	Hypercholesterolemic rats	Control group (n=15) vs Hesperidin (n=15)	0% 0.08% TCD/d	Orally	12 wk	NS	–	–	NS	NS	–	–	–	–	–	–	–	–
Akiyama et al (2009)[27]	Type 2 diabetic rats	Control group (n=6) vs Hesperidin group 1 (n=6) vs Hesperidin group 2 (n=6)	0% TCD/d 1% TCD/d 4.6% TCD/d	Orally	4 wk	↓ with 4.6%	–	–	–	–	↓ with 4.6%	↓ with 4.6%	↓	–	–	↓	–	–
Jung et al (2006)[28]	Type 2 diabetic mice	Control group (n=10) vs Hesperidin group (n=10)	0 mg 200 mg/kg BW/d	Orally	5 wk	–	–	–	–	–	↓	–	↓	–	NS	↓	–	–
Jung et al (2004)[29]	Type 2 diabetic mice	Control group (n=10) vs Hesperidin group (n=10)	0 mg 200 mg/kg BW/d	Orally	5 wk	–	–	–	–	–	–	↑	–	–	–	–	–	–

-, parameter not evaluated; ↓, significant decrease in intervention group vs control group; ↑, significant increase in intervention group vs control group.

Abbreviations: BW, body weight; DBP, diastolic blood pressure; GL, glucose; HDL-c, high density lipoprotein; IL-6, interleukin-6; INFL. BIOM, inflammation biomarkers; INS, insulin; LDL-c, low-density lipoprotein; NO, nitric oxide; NS, no significant differences between intervention group and control group; OXID. BIOM, oxidation biomarkers; SBP, systolic blood pressure; TC, total cholesterol; TCD, total calorie diet; TG, triglycerides; VF, visceral fat; WG, weight gain.

Results for anthropometric parameters. The effect of hesperidin consumption on body weight was evaluated in 6 studies.[15,21,24,25,27,30] Of these, 4 studies reported no significant changes,[15,16,24,25] 1 study reported a significant decrease,[27] and 1 study did not specify the outcome.[30] Akiyama et al[27] reported that the administration of a daily oral dose of hesperetin of 4.60% of total calorie intake to type 2 diabetic rats for 4 weeks prevented the weight gain, of 13.56 g, observed in the control group. In another study that examined the effect of hesperidin consumption on visceral fat,[15] no significant changes were observed.

Results for vascular parameters. The effect of hesperidin on SBP was evaluated in 3 studies[21,25,32]: 2 of these studies reported no significant changes[21,25] and 1 study reported a significant decrease in SBP.[32] Yamamoto et al[32] reported that intravenous administration of an acute dose of 5 mg/kg of body weight of hesperetin to hypertensive rats significantly decreased SBP by 9.90 ± 1.70 mmHg, compared with the control group. The same study[32] also reported that an acute dose of 5 mg/kg of body weight of hesperetin-7-O-β-D-glucuronide significantly decreased SBP by 8.70 ± 0.80 mmHg, compared with the control group. The effect of hesperidin on DBP was evaluated in 2 studies,[21,32] but no significant changes were observed.

Results for glucose and insulin levels. The effect of hesperidin on blood glucose was evaluated in 7 studies.[15,24,26–28,30,31] Six of these studies reported decreases in blood glucose[24,26–28,30,31] and 1 study found no significant changes.[15] Iskender et al[24] reported that the oral consumption of 100 mg/kg of body weight/day of hesperidin for 15 days significantly lowered blood glucose levels in type 2 diabetic rats by 9.25 mmol/L, compared with the control group. Jia et al[31] observed that the consumption of 50 mg/kg of body weight/day of neohesperidin (derived from hesperidin) by gavage for 6 weeks significantly lowered blood glucose levels in type 2 diabetic mice by 7.73 mmol/L, compared with the control group. Kumar et al[30] found that the consumption of 200 mg/kg of body weight/day of hesperetin by gavage for 24 weeks significantly lowered blood glucose levels in type 2 diabetic rats by 5.99 mmol/L, compared with the control group. Mahmoud et al[26] detected significant reductions – of 9.49 mmol/L – in the blood glucose levels of type 2 diabetic rats after oral consumption of 50 mg/kg of body weight/day of hesperidin for 30 days, compared with the control group. Akiyama et al[27] found that daily consumption of hesperetin at a dose of 4.60% of total calorie intake for 4 weeks significantly lowered blood glucose levels in type 2 diabetic rats by 1.61 mmol/L, compared with the control group. In addition, Jung et al[28] reported that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 5 weeks significantly lowered blood glucose levels in type 2 diabetic mice by 7.84 mmol/L, compared with the control group.

The effect of hesperidin on serum insulin levels was evaluated in 3 studies,[26,27,29] of which 2 reported significant increases in insulin levels[26,29] and 1 reported a significant decrease.[27] Mahmoud et al[26] reported that the oral consumption of 50 mg/kg of body weight/day of hesperidin for 30 days significantly raised insulin levels in type 2 diabetic rats by 6.05 μU/mL, compared with the control group. Jung et al[29] found a significant increase of 18.13 μU/mL in the insulin levels of type 2 diabetic mice after 5 weeks of oral consumption of 200 mg/kg of body weight/day of hesperidin, compared with the control group. Moreover, Akiyama et al[27] reported that oral daily consumption of hesperidin at a dose of 4.60% of total calorie intake for 5 weeks of intervention significantly lowered insulin levels in type 2 diabetic rats by 90.64 μU/mL, compared with the control group.

Results for lipid profile. The effect of hesperidin consumption on total cholesterol (TC) levels was evaluated in 4 studies.[15,22,27,28] Of these, 3 reported significant decreases in TC levels[22,27,28] and 1 study found no significant changes.[15] Selvaraj and Pugalendi[22] observed that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 7 days significantly lowered TC levels in rats with myocardial ischemia by 0.40 mmol/L, compared with the control group. Akiyama et al[27] noted that daily consumption of hesperetin at a dose of 1% and 4.60% of total calorie intake for 4 weeks significantly lowered TC levels in type 2 diabetic rats by 1.71 mmol/L and 2.51 mmol/L, respectively, compared with the control group. Moreover, Jung et al[28] reported that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 5 weeks significantly lowered TC levels in type 2 diabetic mice by 0.81 mmol/L, compared with the control group.

The effect of hesperidin consumption on high-density lipoprotein cholesterol (HDL-c) levels was evaluated in 3 studies.[15,22,28] Two of these studies reported no significant changes in HDL-c[15,28] and the other study reported a significant increase.[22] Selvaraj and Pugalendi[22] reported that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 7 days significantly increased HDL-c levels in rats with myocardial ischemia by 0.34 mmol/L, compared with the control group.

The effect of hesperidin consumption on low-density lipoprotein cholesterol (LDL-c) levels was assessed

in 2 studies,[15,22] and significant decreases were observed in both studies. Ferreira et al[15] observed that the oral consumption of 100 mg/kg of body weight/day of hesperidin for 15 days significantly lowered LDL-c levels in mice with systemic inflammation by 0.29 mmol/L, compared with the control group. In addition, Selvaraj and Pugalendi[22] observed that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 7 days significantly decreased LDL-c by 0.67 mmol/L, compared with the control group.

The effect of hesperidin consumption on triglyceride (TG) levels was evaluated in 5 studies.[15,22,27,28,31] Four of these studies reported significant decreases[22,27,28,31] and the other study reported no significant changes.[15] Jia et al[31] noted that the consumption of 50 mg/kg of body weight/day of neohesperidin by gavage for 6 weeks significantly lowered TG levels in type 2 diabetic mice by 2.05 mmol/L, compared with the control group. In rats with myocardial ischemia, Selvaraj and Pugalendi[22] observed that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 7 days significantly lowered TG levels by 0.18 mmol/L, compared with the control group. Akiyama et al[27] noted that daily consumption of hesperetin at a dose of 1% and 4.60% of total calorie intake for 4 weeks lowered TG levels in type 2 diabetic rats by 0.66 mol/L and 0.91 mmol/L, respectively, compared with the control group. Lastly, Jung et al[28] stated that the oral consumption of 200 mg/kg of body weight/day of hesperidin for 5 weeks of intervention lowered TG levels in type 2 diabetic mice by 1.74 mmol/L, compared with the control group.

Results for inflammation biomarkers. The effect of hesperidin on interleukin-6 (IL-6) levels was evaluated in a study by Ferreira et al.[15] Using a mouse model of systemic inflammation, this study reported a significant decrease of 58.64 pg/mL after the oral consumption of 100 mg/kg of body weight/day of hesperidin for 4 weeks, compared with the control group.

Results for oxidation biomarkers. The effect of hesperidin on nitric oxide levels was evaluated in a study by Mahmoud et al.[26] The study reported a significant decrease of 5.08 mg/dL after the oral consumption of 50 mg/of body weight/day of hesperidin for 30 days in type 2 diabetic rats, compared with the control group.

Human randomized controlled trials

Study selection. A total of 1917 articles were identified from the searches of the two databases (1 495 in PubMed and 422 in Cochrane Plus). Of these, 1 486 duplicate articles were removed and 393 were excluded because they did not meet the inclusion criteria. Thus, 11 articles were included in the systematic review. Figure 2 shows the study selection process for the RCTs included in the review.

Study characteristics. Tables 4 to 6 show the characteristics of the RCT studies included in this systematic review. Further details of each study are presented in Table S2 in the Supporting Information online. The 11 studies included in this review were RCTs involving some type of nutritional intervention. In fact, the interventions in 3 of the 11 included RCTs consisted of supplementation with a placebo capsule or a hesperidin capsule,[11,33,34] whereas those in the 3 other studies consisted of the administration of a control drink (CD) or orange juice (OJ).[35–37] In addition, the interventions in 2 other studies involved the consumption of different drinks with different hesperidin concentrations,[38,39] whereas those in 2 and 1 of the remaining RCTs consisted of no product intervention vs OJ administration[40,41] and supplementation with a placebo or hesperidin capsule or consumption of OJ, respectively.[42]

Four of the studies comprised a parallel design,[33,34,40,41] and the other seven comprised crossover designs.[11,35–39,42] Ten of the included RCTs involved a long-term follow-up, and one of these also involved a short-term follow-up. The other RCT involved only a short-term follow-up. The duration of the intervention in the long-term studies ranged from 1.5 to 13 weeks, and the duration in the short-term studies ranged from 4 to 5 hours. Nine of the studies were conducted with European populations, and the other 2 investigated South American populations. The sample sizes ranged from 22 to 194 subjects, and the ages of the subjects ranged from 18 to 69 years. All the subjects had at least one CVRF, such as dyslipidemia, overweight, obesity, and/or metabolic syndrome. The methods used to confirm intervention compliance involved keeping 3- or 5-day food records, maintaining 24-hour dietary records, returning all used and unused capsule boxes, and self-reporting.

Assessment of the quality and risk of bias. The risk of bias in each individual RCT is detailed in Figure 3. Six of the 11 RCTs used an adequate random sequence generator; 3 studies incorporated adequate allocation concealment; 5 studies performed adequate blinding of the participants, personnel, and outcome assessment; 9 studies presented completed data; and 6 studies presented their study protocol with all the reported outcomes. Regarding other types of bias, potential conflicts of interest were considered, and 8 studies reported a lack of conflicts of interest.

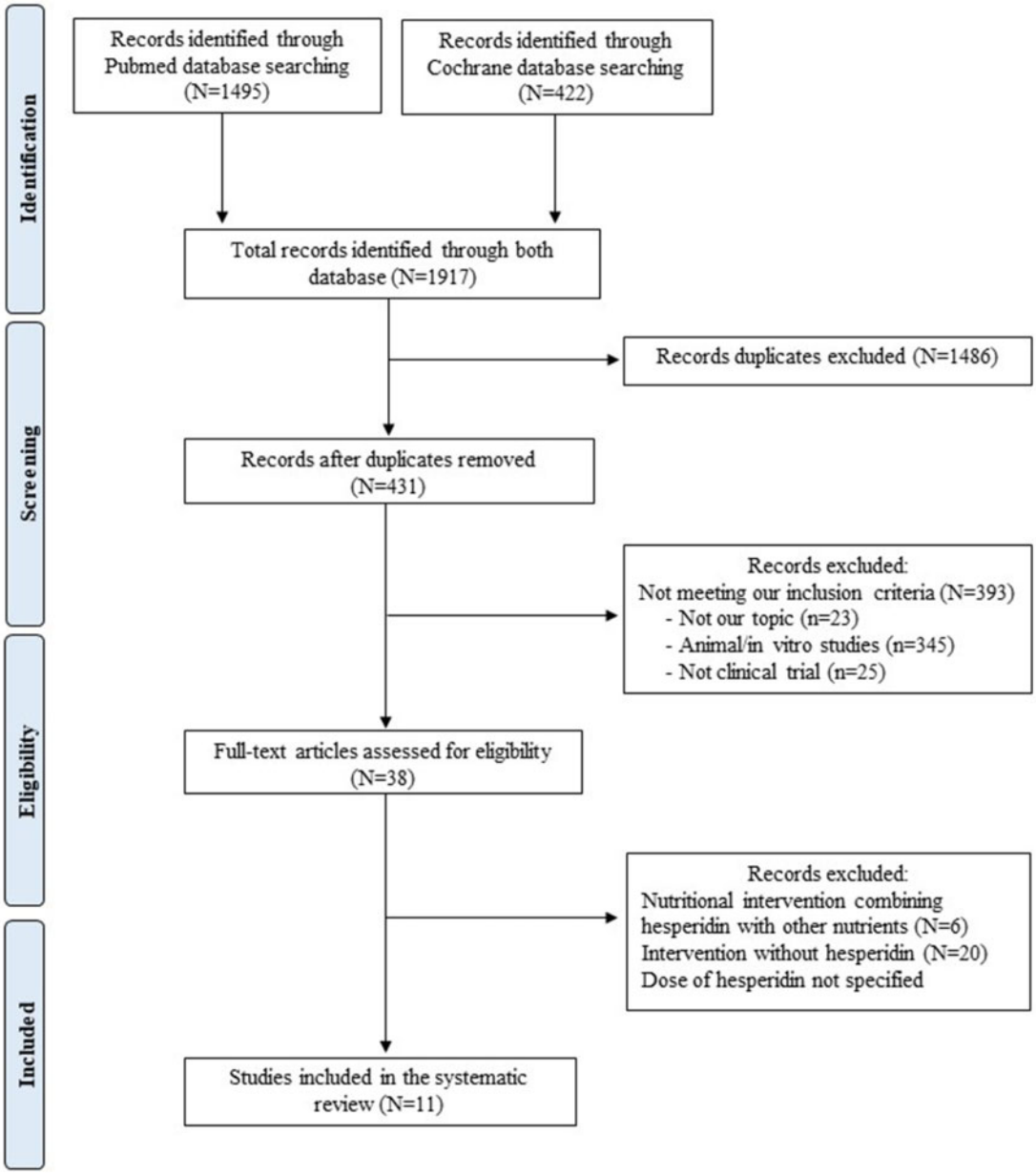

Figure 2 **Flow diagram of the literature search process for randomized clinical trials.**

Effects of chronic hesperidin consumption on cardiovascular risk biomarkers.

Results for anthropometric parameters. The characteristics of the long-term RCTs included in this review in relation to anthropometric parameters are detailed in Table 4.

The effect of hesperidin consumption on body weight was evaluated in 3 studies.[34,39,40] Of these, 2 studies reported significant decreases,[39,40] and 1 study found no significant changes.[34] Rangel-Huerta et al[39] observed that the consumption of 237 mg/day or 582.50 mg/day of hesperidin in 500 mL/day of OJ for 12 weeks reduced the body weight of overweight or obese subjects by 1.30 kg and 1.80 kg, respectively, compared with basal levels. No differences between the different hesperidin concentrations were observed. Aptekmann and Cesar[40] noted that the consumption of 54.60 mg/day of hesperetin in 500 mL/day of OJ for 13 weeks of intervention significantly reduced the body weight of hypercholesterolemic subjects by 1 kg, compared with basal levels. No significant differences were observed between the intervention and control groups.

The effect of hesperidin consumption on the body mass index (BMI) was evaluated in 5 studies[11,34,39–41]: 3 of these studies reported no significant changes[11,34,41] and 2 studies found significant decreases.[39,40] Rangel-Huerta et al[39] observed that the consumption of 237 mg/day and 582.50 mg/day of hesperidin in 500 mL/day

Table 4 **Characteristics of the long-term randomized clinical trials included in the systematic review with results for anthropometric and vascular parameters and glucose and insulin levels (n = 8)**

Author, year, reference	Sample size	Cardiovascular risk factors	Nutritional intervention				Results									
			Groups	Flavanone dose	Productdose	Duration	Anthropometric parameters			Vascular parameters				Glucose and insulin levels		
							BW, kg	BMI, kg/m2	BF, %	SBP, mm Hg	DBP, mm Hg	FMD, %	GTN, %	GLUC, mmol/L	INS, μU/mL	QUICKI
Salden et al (2016)[33]	n=65	Overweight or obesity	Placebo capsule vs Hesperidin capsule	0 mg/d 450 mg/d	500 mg/d 500 mg/d	6 wk	–	–	–	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	–	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)
Kean et al (2015)[38]	n=37	Overweight	Low hesperidin drink vs High hesperidin drink	64 mg/d 549 mg/d	500 mL/d 500 mL/d	8 wk	–	–	–	NS/NS (NS)	NS/NS (NS)	–	–	–	–	–
Constans et al (2015)[35]	n=25	Hypercholesterolemia	Control drink vs Orange juice (hesperidin)	0 mg/d 213 mg/d	600 mL/d 600 mL/d	4 wk	–	–	–	–	–	–	–	NS/NS(NS)	–	–
Rangel-Huerta et al (2015)[39]	n=100	Overweight or obesity	Orange juice (hesperidin) vs Enriched orange juice (hesperidin)	237 mg/d 582.5 mg/d	500 mL/d 500 mL/d	12 wk	↓1.3/↓1.8 (NS)	↓0.5/↓0.7 (NS)	–	↓4.00/NS (NS)	↓3.00/NS (NS)	–	–	↑0.3/↑0.2 (P <0.05)*	↓1.2/NS (P <0.05)*	–
Buscemi et al (2012)[37]	n=31	Metabolic syndrome	Control drink vs Orange juice (hesperidin)	0 mg/d 159.5 mg/d	500 mL/d 500 mL/d	1.5 wk	–	–	–	–	–	NS/↑2.2 (P <0.05)*	NS/NS(NS)	–	–	–
Rizza et al (2011)[11]	n=24	Metabolic syndrome	Placebo capsule vs Hesperidin capsule	0 mg/d 500 mg/d	1 capsule/d 1 capsule/d	3 wk	–	NS/NS (NS)	–	NS/NS (NS)	NS/NS (NS)	NS/↑2.5 (p=0.02)*	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)
Morand et al (2011)[42]	n=23	Overweight	Placebo capsule with control drink vs Hesperidin capsule with control drink vs Orange juice (hesperidin)	0 mg/d 292 mg/d 292 mg/d	1 capsule and 500 mL/d 1 capsule and 500 mL/d 500 mL/d	4 wk	–	–	–	NS/NS/NS (NS)	NS/↓5.3/↓4.5 (P <0.023)*	–	–	NS/NS/NS (NS)	NS/NS/NS (NS)	–
Demonty et al (2010)[34]	n=194	Overweight Hypercholesterolemia	Placebo capsule vs Hesperidin capsule	0 mg/d 800 mg/d	4 capsules/d 4 capsules/d	4 wk	NS/NS (NS)	NS/NS (NS)	–	–	–	–	–	–	–	–
Aptekmann and Cesar (2010)[40]	n=26	Overweight	No intervention vs Orange juice (hesperetin)	0 mg/d 54.6 mg/d	0 mL/d 500 mL/d	13 wk	↓1.8/↓1.0 (NS)	↓0.7/↓0.3 (NS)	↓5.5/↓4.3 (NS)	–	–	–	–	–	–	–
Cesar et al (2010)[41]	n=22	Hyperchole sterolemia	No intervention vs Orange juice (hesperetin)	0 mg/d 42 mg/d	0 mL/d 750 mL/d	8.5 wk	–	NS/NS (NS)	–	–	–	–	–	–	–	–

-, parameter not evaluated; ↓, significant decrease between basal and final value within each group; ↑, significant increase between basal and final value within each group; *, significant differences between groups.

Abbreviations: BF, body fat; BMI, body mass index; BW, body weight; DBP, diastolic blood pressure; FMD, flow-mediated dilatation; GLUC, glucose; GTN, glyceryl-nitrate dilation of the brachial artery; INS, insulin; NS, no significant differences between basal and final within each group; (NS); no significant differences between intervention group and control group; QUICKI, quantitative insulin-sensitivity check index; SBP, systolic blood pressure.

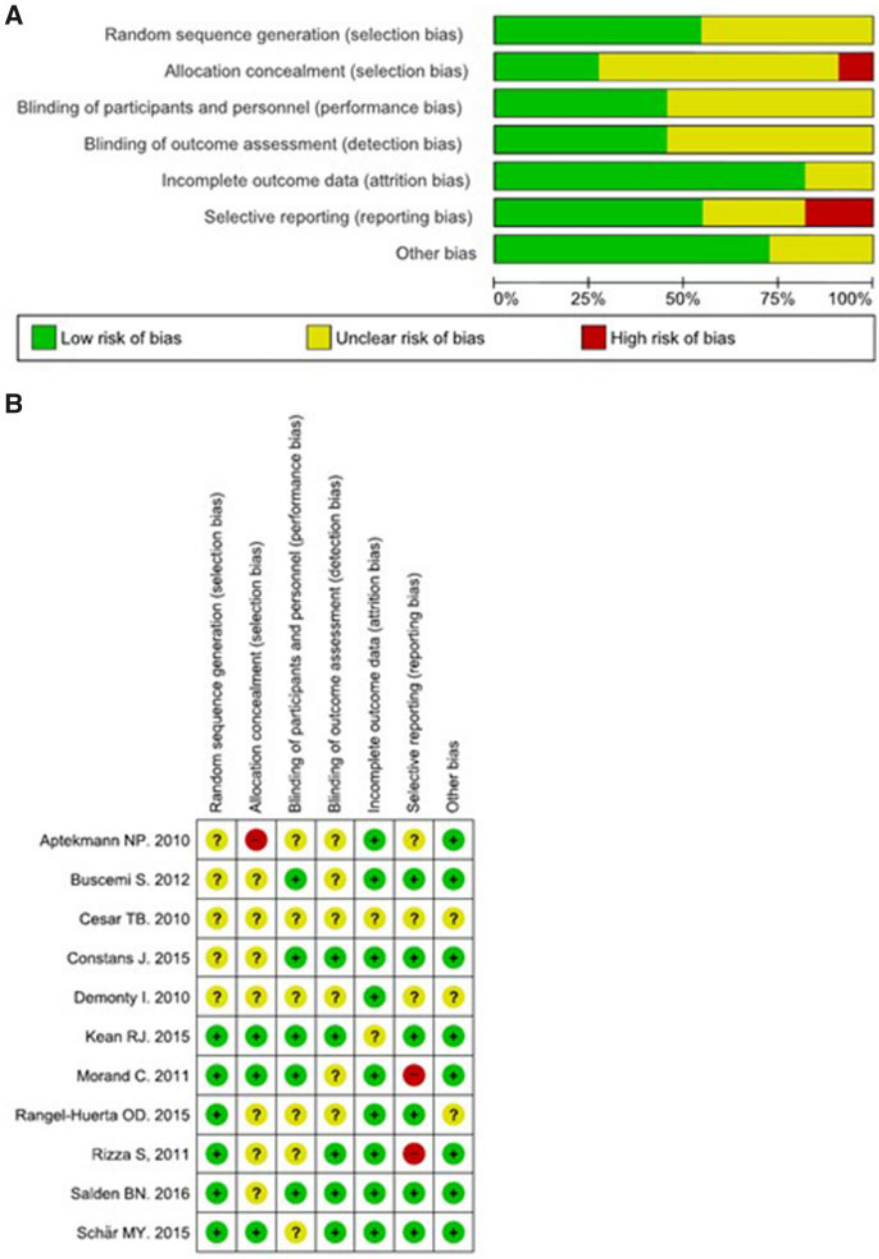

Figure 3 **Risk of bias graph (A) and summary (B) of the randomized clinical trials included.** + indicates a low risk of bias, - indicates a high risk of bias, and ? indicates an unclear risk.

of OJ for 12 weeks reduced the BMI of overweight or obese subjects by 0.50 kg/m^2 and 0.70 kg/m^2, respectively, compared with basal levels. No differences between the different hesperidin concentrations were observed. Aptekmann and Cesar[40] reported that the consumption of 54.60 mg/day of hesperetin in 500 mL/day of OJ significantly reduced the BMI of hypercholesterolemic subjects by 0.30 kg/m^2 after 13 weeks of intervention, compared with basal levels. No significant differences were observed between the intervention and control groups.

The effect of hesperidin consumption on body fat was evaluated in 1 study and a significant decrease was observed.[40] Specifically, Aptekmann and Cesar[40] reported that 54.60 mg/day of hesperetin in 500 mL/day of OJ significantly reduced the body fat of hypercholesterolemic subjects by 4.30% after 13 weeks of intervention, compared with basal levels. No significant differences were observed between the intervention and control groups.

Results for vascular parameters. The characteristics of the long-term RCTs included in this review in relation to vascular parameters are detailed in Table 4.

The effect of hesperidin consumption on SBP and DBP was evaluated in 5 studies.[11,33,38,39,42] Of these,

3 reported no significant changes[11,33,38] and significant decreases were detected in the other 2 studies.[39,42] Rangel Huerta et al[39] observed that the consumption of 237 mg/day of hesperidin for 12 weeks reduced the SBP and DBP of overweight or obese subjects by 4 mmHg and 3 mmHg, respectively, compared with basal levels. No significant differences were observed in a comparison with the group administered a lower concentration of hesperidin. Morand et al[42] reported that the consumption of 292 mg/day of hesperidin – in the form of pure hesperidin capsules or provided naturally with 500 mL/day of OJ for 4 weeks – reduced the DBP of overweight subjects by 5.30 mmHg and 4.50 mmHg, respectively, compared with basal levels. Significant differences were observed in a comparison with the control group.

The effect of hesperidin consumption on endothelial function was evaluated in 3 studies[11,33,36]: 2 of these studies reported significant increases[11,36] and the other study found no significant changes.[33] In subjects with metabolic syndrome, Buscemi et al[36] observed a significant increase in flow-mediated dilation (FMD) of 2.20% after 1.5 weeks of the consumption of 159.50 mg/day of hesperidin in 500 mL/day of OJ. Significant differences between the intervention group and the control group were observed. Similarly, in subjects with metabolic syndrome, Rizza et al[11] reported a significant increase in FMD of 2.48% after 3 weeks of the consumption of 500 mg/day of hesperidin in capsule form, and the differences between the intervention and control groups were significant.

Results for glucose and insulin levels. The characteristics of the long-term RCTs included in this review in relation to glucose and insulin levels are detailed in Table 4.

The effect of hesperidin consumption on plasma glucose levels was evaluated in 5 studies.[11,33,35,39,42] Of these, 4 reported no significant changes,[11,33,35,42] and a significant increase was observed in the other study.[39] Specifically, Rangel-Huerta et al[39] observed significant increases of 0.30 mmol/L and 0.20 mmol/L in the glucose levels of overweight and obese subjects after the consumption of 237 mg/day and 582.50 mg/day of hesperidin in OJ, respectively, for 12 weeks, compared with basal levels. Significant differences were observed between both intervention groups.

Four studies evaluated the effect of hesperidin consumption on plasma insulin levels[11,33,39,42]: 3 of these studies reported no significant changes,[11,33,42] whereas a significant decrease was detected in the other study.[39] Rangel-Huerta et al[39] noted a significant decrease of 1.20 μU/mL in the insulin levels of overweight or obese subjects after the consumption of 237 mg/day of hesperidin in OJ for 12 weeks, compared with basal levels. Significant differences were found between both intervention groups.

The effect of hesperidin consumption on the QUICKI index was evaluated in 2 studies; neither of these studies reported any significant changes.[11,33]

Results for lipid profile parameters. The characteristics of the long-term RCTs included in this review in relation to lipid profiles are detailed in Table 5.

The effect of hesperidin consumption on TC levels was evaluated in 8 studies.[11,33–35,39–42] Of these, 6 reported no significant changes[11,33,34,39,42] and 2 studies reported significant decreases.[35,36] Aptekmann and Cesar[40] found that the TC levels of overweight subjects were significantly decreased by 0.22 mmol/L, compared with basal levels after 13 weeks of consumption of 54.60 mg/day of hesperetin in OJ. No significant differences were observed between the intervention group and the control group. Cesar et al[41] reported a significant decrease of 0.46 mmol/L in the TC levels of hypercholesterolemic subjects who consumed 42 mg/day of hesperetin in 750 mL/day of OJ for 8 weeks, compared with the control subjects. No significant differences were observed between the intervention and control groups.

Eight studies evaluated the effect of hesperidin consumption on LDL-c levels.[11,33–35,39–42] Of these, 6 reported no significant changes,[11,33–35,39,42] while significant decreases were found in the other 2 studies.[40,41] Specifically, compared with the basal level, Aptekmann and Cesar[40] observed a significant decrease of 0.44 mmol/L in the LDL-c levels of overweight subjects after 13 weeks of the consumption of 54.60 mg/day of hesperetin in OJ. No significant differences were observed between the intervention and control groups. Cesar et al[41] observed a significant decrease of 0.49 mmol/L in the LDL-c levels of hypercholesterolemic subjects who consumed 42 mg/day of hesperetin in OJ 8.5 weeks. No significant differences were observed between the intervention and control groups.

The effect of hesperidin consumption on HDL-c levels was evaluated in 8 studies.[11,33–35,39–42] No significant changes were detected in 7 of these studies,[11,33–35,39,41,42] and the other study reported a significant increase.[40] In overweight subjects, Aptekmann and Cesar[40] found that the consumption of 54.60 mg/day of hesperetin in OJ for 13 weeks increased HDL-c levels by 0.23 mmol/L, compared with basal levels. No significant differences were observed between the intervention and control groups.

Table 5 **Characteristics of long-term randomized clinical trials included in the systematic review with results for lipid profiles (n = 8)**

Author, year, reference	Sample size	Cardiovascular risk factors	Nutritional intervention				Results					
			Groups	Flavanone dose	Product dose	Duration	Lipid profile					
							TC, mmol/L	LDL-c, mmol/L	HDL-c, mmol/L	TG, mmol/L	APO A, mg/dL	APO B, mg/dL
Salden et al (2016)[33]	n=6	Overweight or obesity	Placebo capsule vs Hesperidin capsule	0 mg/d 450 mg/d	500 mg/d 500 mg/d	6 wk	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	–	–
Constans et al (2015)[35]	n=25	Hypercholesterolemia	Control drink vs Orange juice (hesperidin)	0 mg/d 213 mg/d	600 mL/d 600 mL/d	4 wk	↓0.35/NS (NS)	↓0.32/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/↑5.00 (NS)	NS/↑8.00 (NS)
Rangel-Huerta et al (2015)[39]	n=100	Overweight or obesity	Orange juice (hesperidin) vs Enriched orange juice (hesperidin)	237 mg/d 582.5 mg/d	500 mL/d 500 mL/d	12 wk	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	↓0.09/NS (NS)	NS/↓4.00 (NS)	↓4.00/NS (NS)
Rizza et al (2011)[11]	n=24	Metabolic syndrome	Placebo capsule vs Hesperidin capsule	0 mg/d 500 mg/d	1 capsule/d 1 capsule/d	3 wk	NS/NS ($P < 0.05$)*	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)
Morand et al (2011)[42]	n=24	Overweight	Placebo capsule with control drink vs Hesperidin capsule with control drink vs Orange juice (hesperidin)	0 mg/d 292 mg/d 292 mg/d	1 capsule and 500 mL/d 1 capsule and 500 mL/d 500 mL/d	4 wk	NS/NS/NS (NS)	NS/NS/NS (NS)	NS/NS/NS (NS)	NS/NS/NS (NS)	–	–
Demonty et al (2010)[34]	n=194	Overweight Hypercholesterolemia	Placebo capsule vs Hesperidin capsule	0 mg/d 800 mg/d	4 capsules/d 4 capsules/d	4 wk	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	NS/NS (NS)	–	–
Aptekmann and Cesar (2010)[41]	n=26	Overweight	No intervention vs Orange juice (hesperetin)	0 mg/d 54.6 mg/d	0 mL/d 500 mL/d	13 wk	NS/↓0.22 (NS)	NS/↓0.44 (NS)	NS/↓0.23 (NS)	NS/NS (NS)	–	–
Cesar et al (2010)[41]	n=22	Hypercholesterolemia	No intervention Vs Orange juice (hesperetin)	0 mg/d 42 mg/d	0 mL/d 750 mL/d	8.5 wk	NS/↓0.24 (NS)	NS/↓0.49 (NS)	NS/NS (NS)	↑0.38/NS (NS)	–	–

-, parameter not evaluated; ↓, significant decrease between basal and final value within each group; ↑, significant increase between basal and final value within each group; *, significant differences between groups.

Abbreviations: APO A, apolipoprotein A; APO B, apolipoprotein B; HDL-c, high density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; NS, no significant differences between basal and final within each group; (NS), no significant differences between groups; TC, total cholesterol; TG, triglycerides.

Table 6 **Characteristics of long-term randomized clinical trials included in the systematic review with results for coagulation, inflammation, and oxidation biomarkers (n = 6)**

Author, year, reference	Sample size	Cardiovascular risk factors	Nutritional intervention				Results									
			Groups	Flavanone dose	Product dose	Duration	Coagulation biomarkers		Inflammation biomarkers					Oxidation biomarkers		
							FIB	HOM	IL-6, pg/mL	sVCAM-1	sICAM-1	SAA PROTEIN	sE-SEL	sP-SEL	oxLDL, pg/mL	NO
Salden et al (2016)[33]	n=6	Overweight or obesity	Placebo capsule	0 mg/d	500 mg/d	6 wk	–	–	–	NS/NS (NS)	NS/NS (NS)	–	NS/NS (NS)	NS/NS (NS)	–	–
			vs													
			Hesperidin capsule	450 mg/d	500 mg/d											
Constans et al (2015)[35]	n=25	Hypercholesterolemia	Control drink	0 mg/d	600 mL/d	4 wk	NS/NS (NS)	NS/NS (NS)	–	NS/NS(NS)	NS/NS(NS)	–	NS/NS(NS)	–	–	–
			vs													
			Orange juice (hesperidin)	213 mg/d	600 mL/d											
Rangel-Huerta et al (2015)[39]	n=100	Overweight or obesity	Orange juice (hesperidin)	237 mg/d	500 mL/d	12 wk	–	–	–	–	–	–	–	–	NS/NS (NS)	–
			vs													
			Enriched orange juice (hesperidin)	582.5 mg/d	500 mL/d											
Buscemi et al (2012)[36]	n=31	Metabolic syndrome	Control drink	0 mg/d	500 mL/d	1.5 wk	–	–	NS/↓3.30 ($P < 0.05$)*	–	–	–	–	–	–	NS/NS (NS)
			vs													
			Orange juice (hesperidin)	159.5 mg/d	500 mL/d											
Rizza et al (2011)[11]	n=24	Metabolic syndrome	Placebo capsule	0 mg/d	1 capsule/d	3 wk	NS/NS (NS)	NS/NS (NS)	–	NS/NS (NS)	NS/NS (NS)	NS/NS ($P < 0.05$)*	NS/NS (NS)	–	–	–
			vs													
			Hesperidin capsule	500 mg/d	1 capsule/d											
Morand et al (2011)[42]	n=24	Overweight	Placebo capsule with control drink	0 mg/d	1 capsule and 500 mL/d	4 wk	–	–	NS/NS/NS (NS)	NS/NS/NS (NS)	NS/NS/NS (NS)	–	–	–	–	NS/NS/NS (NS)
			vs													
			Hesperidin capsule with control drink	292 mg/d	1 capsule and 500 mL/d											
			vs													
			Orange juice (hesperidin)	292 mg/d	500 mL/d											

-, parameter not evaluated; ↓, significant decrease between basal and final value within each group; *, significant differences between groups.

Abbreviations: FIB, fibrinogen; HOM, homocysteine; IL-6, interleukin-6; NO, nitric oxide; NS, no significant differences between basal and final within each group; (NS), no significant differences between groups; oxLDL, oxidized low-density lipoprotein; SAA protein, serum amyloid A protein; sE-SEL, soluble E-selectin; sICAM-1, soluble intercellular adhesion molecule-1; sP-SEL, soluble P-selectin; sVCAM-1, soluble vascular cell adhesion molecule-1.

Eight studies evaluated the effect of hesperidin consumption on TG levels.[11,33–35,39–42] Of these, 7 reported no significant changes[11,33–35,39,40,42] and the other study reported a significant decrease.[39] Compared with basal levels, Rangel-Huerta et al[39] observed a significant decrease of 0.09 mg/dL in the TG levels of overweight and obese subjects who consumed 237 mg/day of hesperidin in OJ for 12 weeks. No significant differences were observed between the intervention and control groups.

The effects of hesperidin consumption on apolipoprotein A-1 (Apo A-1) and apolipoprotein B (Apo B) were evaluated in 3 studies,[11,35,39] and different results were obtained. Specifically, compared with basal levels, Constans et al[35] reported a significant increase in Apo A-1 and Apo B levels of 5 mg/dL and 8 mg/dL, respectively, in hypercholesterolemic subjects after the consumption of 213 mg/day of hesperidin in OJ for 4 weeks. No significant differences between the intervention and control groups were observed. Rangel-Huerta et al[39] noted a significant decrease of 4 mg/dL in the Apo A-1 levels and also in the Apo B levels of overweight or obese subjects who consumed 237 mg/day and 582.50 mg/day of hesperidin in OJ for 12 weeks, compared with basal levels. No significant differences were observed between the intervention and control groups. In addition, Rizza et al[3311] found no significant changes between these two groups.

Results for coagulation, inflammation, and oxidative biomarkers. The characteristics of the long-term RCTs included this review in relation to the biomarkers of coagulation, inflammation, and oxidation are detailed in Table 6.

The effect of hesperidin consumption on coagulation biomarkers, assessed based on the plasma levels of fibrinogen and homocysteine, was explored in 2 studies,[11,35] but neither of these RCTs reported any significant changes.

In one study, the effect of hesperidin consumption on inflammation biomarkers was assessed according to plasma protein serum amyloid A (SAA) levels,[11] but no significant changes were observed. Inflammation was also assessed according to plasma IL-6 levels in 2 studies.[36,42] Of these, 1 study observed a significant decrease,[36] but no significant changes were detected in the other study.[42] Buscemi et al[36] found a significant decrease of 3.30 pg/mL in the IL-6 levels of subjects with metabolic syndrome after the consumption of 159.50 mg/day of hesperidin in OJ for 1.5 weeks, compared with basal levels. Significant differences were observed between the intervention and control groups. Four studies evaluated the effects of hesperidin consumption on the plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1) and soluble intercellular adhesion molecule-1 (s-ICAM-1),[11,33,35,42] and the plasma levels of sE-selectin (soluble E-selectin) and sP-selectin (soluble P-slectin) were evaluated in 3 studies[11,33,35] and 1 study,[33] respectively. None of these studies detected any significant changes.

The effect of hesperidin consumption on oxidative biomarkers was assessed according to plasma NOx levels in 2 studies,[36,42] but no significant changes were observed. Additionally, plasma oxidized low-density lipoprotein levels were assessed in 1 study, but no significant changes were detected.[39]

Effects of acute hesperidin consumption on cardiovascular risk biomarkers. The effects of acute consumption of hesperidin were evaluated according to vascular parameters (SBP, DBP, and endothelial function) and inflammation biomarkers (sVCAM-1, s-ICAM-1, sE-selectin, and sP-selectin) in 2 studies, but no significant changes in any of the investigated parameters were detected.

DISCUSSION

The current systematic review presents a summary of the available scientific evidence regarding the effects of hesperidin consumption on cardiovascular risk biomarkers obtained from animal studies and human RCTs.

The results from the animal studies included in the present systematic review showed that daily consumption of 50–200 mg/kg of body weight of hesperidin or hesperetin for a period ranging from 15 days to 24 weeks significantly lowered blood glucose levels in type 2 diabetic rats and mice. As possible mechanisms of action, other experimental studies with rats have suggested that hesperidin consumption may increase hepatic glycolysis and hepatic glucokinase activity and decrease hepatic gluconeogenesis and hepatic glucose-6-phospatase activity,[43] which would inhibit the gluconeogenic pathway in liver cells[44] and thus prevent the progression of hyperglycemia.[43,45] These beneficial effects on glucose and insulin levels were not observed in the human RCTs included in this systematic review. However, it is interesting to note that only 5 of the 11 RCTs included in the review assessed the effects of hesperidin consumption on blood glucose levels, and the population investigated in these RCTs were overweight, obese, or hypercholesterolemic, whereas the animal studies were performed on type 2 diabetic rats. Because the types of population investigated in the RCTs that evaluated glucose levels yielded no significant results and because only a few RCTs evaluated the possible effect of hesperidin on glucose, more RCTs should be

conducted with type 2 diabetic subjects to assess the effects of hesperidin consumption on glucose and insulin levels in order to confirm the results observed in animals. With respect to insulin levels, no relevant changes were observed in either the animal studies or the human RCTs.

The animal studies included in the present systematic review demonstrated that daily consumption of hesperidin or hesperetin at a dose of 50–200 mg/kg of body weight and 1% or 4.60% of total calorie intake improves the lipid profile by significantly reducing blood levels of TC, LDL-c, and TG in rats and mice with type 2 diabetes and myocardial ischemia. An in-vitro study showed that the possible mechanism through which hesperidin improves the lipid profile may involve the modulation of hepatic lipid metabolism and the inhibition of Apo B in HepG2.[46] In contrast, the results of the RCTs included in this review did not show the same conclusive results. In fact, only 2[40,41] of the 8 articles that assessed lipid profiles observed a decrease in TC and LDL-c levels. Interestingly, only one study[41] assessed the effect of hesperidin on lipid profile in hypercholesterolemic subjects. This RCT observed marked decreases of 0.47 mmol/L and 0.49 mmol/L in TC and LDL-c levels, respectively,[47] after the consumption of 42 mg/day of hesperidin in OJ for 8.5 weeks, and this finding was clinically relevant.[47] Thus, hypercholesterolemic subjects constitute an appropriate population for further evaluation of the specific effects of hesperidin on lipid profile. The differences between the doses of hesperidin administered in the animal and human studies (higher doses were used in the animal studies than in the human RCTs) may also have contributed to the difference in the results obtained from these two types of studies. Thus, more human RCTs are needed to better understand the effects of hesperidin consumption on lipid profile in humans.

The present systematic review showed that, in animal models, the consumption of hesperidin does not improve anthropometric parameters, such as body weight and visceral fat. However, it is important to note that the animal studies included in this review were conducted with rats or mice with normal body weight and anthropometric parameters for their age; future studies should investigate overweight or obese rats or mice to allow more relevant conclusions to be drawn. Similarly, in the human RCTs, there were no effects of hesperidin on body weight, BMI, and body fat, and only a limited number of studies have assessed these parameters. Two[39,40] of the 3 RCTs that evaluated the effect of hesperidin consumption on body weight and BMI observed reductions of 1.30–1.80 kg/m^2 and 0.30–0.70 kg/m^2, respectively, in overweight subjects after daily consumption of 54.60–582.50 mg/day of hesperidin in OJ for 12–13 weeks, compared with the basal values. However, both of these studies had some limitations: one was not a placebo-controlled clinical trial,[39] and the other study observed decreases in both the intervention and control groups,[40] probably owing to the fact that volunteers tend to pay more attention to their health when participating in a study.[48]

Hesperidin has aroused interest on account of its possible effect on blood pressure because it has been suggested that this compound exerts effects similar to those found with other flavonoids, such as quercetin.[49] In-vitro studies have shown that the improvements in blood pressure and endothelial dysfunction observed after hesperidin consumption may be mediated by a decrease in NADPH oxidase 2, increase in plasma NO metabolites, and an inhibitory effect on angiotensin-converting enzyme.[50,51] These data suggest that hesperidin may increase the secretion of NO by human endothelial cells, inhibit cyclic nucleotide phosphodiesterase, and increase cyclic AMP (adenosine monophosphate) and GMP (guanosine monophosphate), thereby exerting a vasorelaxant effect.[14,52,53] Nevertheless, according to the findings of the present review of animal studies and RCTs, the consumption of hesperidin has no clear effect on DBP and SBP levels. However, it is interesting to note that the subjects assessed in the included RCTs were overweight or obese, with no hypertension or elevated blood pressure levels. Therefore, studies that evaluate the effect of hesperidin on blood pressure in subjects with high blood pressure levels are needed for us to draw a definitive conclusion about this CVRF. Interestingly, 3 RCTs [11,33,36] included in the present review assessed the effects of hesperidin on endothelial function, and 2 of these[11,36] observed improvements in these parameters in subjects with metabolic syndrome and increased CVRFs after 1.5–3 weeks of intervention with 300–500 mg/day of hesperidin in OJ or capsule form. Although the available evidence is scarce, it appears that hesperidin consumption seems likely to increase endothelial function. Thus, more human RCTs are needed to determine whether hesperidin decreases blood pressure and improves endothelial function in hypertensive or type 2 diabetic populations.

The results obtained in the present review of RCTs showed that hesperidin has no significant effects on biomarkers of coagulation, inflammation, and oxidation. However, few studies have assessed the effect of hesperidin on these biomarkers in relation to CVDs because almost all studies have focused on cancer and other chronic diseases.[26,54,55]

One factor to consider is the interindividual variability in hesperidin bioavailability, which may, for example, depend on the microbiota composition of each subject.[56,57] Thus, it is possible that different individuals

administered the same dose of hesperidin can absorb this compound to different degrees, and therefore, these individuals would show different effects for the various cardiovascular biomarkers. This could also explain the differing results between the studies included in this review because none of the studies considered the bioavailability of hesperidin.

The RCTs included in the present review that observed more significant changes[39–41] presented many potential risks of bias, which were classified as unclear risk owing to insufficient information about allocation concealment and blinding of participants, personnel, and outcomes, or in terms of including a conflict of interest based on the Cochrane risk of bias criteria. These unclear risks of bias indicate potential problems related to the methodological quality of the studies and hence lead us to question the reliability of the results of the RCTs. Therefore, further RCTs are needed with a lower risk of bias and consequent improvement in quality.

One strength of this review concerns the standardized methodology that was used. In addition, the included studies were published recently and thus presented strong scientific evidence, such as RCTs, along with analyses of their individual risks of bias. Moreover, the novelty of this review lies in the fact that it was the first to evaluate the effects of hesperidin consumption on different CVRFs based on both animal models and human studies. However, the present review has several limitations that warrant discussion. The first is the scarce scientific evidence available from human and animal studies that assessed the effects of hesperidin on CVRFs. In most studies, the populations used to evaluate the effects of hesperidin on different CVRFs have not been the most appropriate for reaching definitive conclusions. Thus, if the objective of a study is to improve a specific cardiovascular risk factor – for example, to reduce high serum cholesterol concentrations in humans – the recommendation is to include subjects that present with symptoms associated with this specific CVRF, such as hypercholesterolemic patients.[58] In addition, the studies included in this review utilized different intervention durations, monitoring approaches, and methods of supplementation. However, the sample size in some of the animal studies was perhaps insufficient for a robust evaluation of the objectives, and in 2 studies, the doses of hesperidin or hesperetin were not estimated in milligrams, and therefore their dose-dependent effects could not be compared with those of other studies. In addition, dose- and time-dependent effects, as well as the physiological relevance of the dose used, were not evaluated in the animal studies. Also, the possibility of residual confounding related to hesperidin bioavailability cannot be excluded. Moreover, even though compliance with the nutritional intervention is necessary, dietary factors may not have been considered to a sufficient degree because only 3 RCTs controlled the participants' diet through validated dietary records, and no biomarkers for consumption were used in any of the included studies. Therefore, other polyphenol compounds present in the diet may have been responsible, either partially or entirely, for the observed health effects. In addition, with inadequate monitoring of the participants' diet, it is possible that some subjects had greater hesperidin intake than others because they consumed food or beverages with significant amounts of hesperidin, potentially affecting the study results of the study. Thus, in nutritional RCTs, monitoring of the participants' diet is necessary to avoid confounding between other dietary compounds and the dietary intervention. Limiting hesperidin intake as a dietary recommendation for all participants, monitoring their dietary intake, and the use of biomarkers for consumption are necessary to obtain robust results in this type of study. Lastly, most of the articles included in this review lacked statistical data, such as mean differences and their standard deviation and the standard error or confidence intervals for each intervention, as well as their p-values. Consequently, a meta-analysis, which would have provided more conclusive results, as well as a forest plot, which would have provided a clearer presentation of the results, could not be performed.

CONCLUSION

In conclusion, hesperidin consumption was found to improve glucose levels and various lipid profile parameters, such as TC, LDL-c, and TG, in animal models, but no definitive conclusion regarding the effects of hesperidin on different CVRFs in humans can be currently drawn. Further RCTs of greater quality are needed to confirm that the results observed in animal models can be translated to the human population and thus to evaluate whether the administration of hesperidin through the consumption of citrus food or as a supplement would serve as a new tool for the prevention and treatment of CVDs.

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Declaration of interest. None of the authors have relevant interests to declare.

Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

Table S1 **Characteristics and results of animal studies included in the systematic review (n = 12)**

Table S2 **Characteristics and results of randomized clinical trials included in the systematic review (n = 11)**

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Supporting Information 1. Table S1. Characteristics and results of animal studies included in the systematic review (N=12).

AUTHOR, YEAR, REFERENCE	STUDY DESIGN	EXPERIMENTAL ANIMAL	INTERVENTION GROUPS (N)	DOSE OF THE FLAVANONE AND ROUTE OF ADMINISTRATION	DURATION OF INTERVENTION	BIOMARKERS	RESULTS
Iskender H et al. (2017) [S1]	Controlled	4-6-week old male Wistar albino rats treated with STZ (type 2 diabetes)	Control group (n=10) vs intervention group (n=10)	0 mg vs 100 mg/kg body weight/day of hesperidin in aqueous suspension orally	15 days	Body weight, glucose	-Body weight (g): Control group = B: 235.80 ± 14.35; F: 190.51 ± 10.16. NDA Intervention group = B: 242.50 ± 8.36; F: 205.54 ± 11.18. NDA No significant differences between intervention group and control group at the end of the study. -Glucose (mmol/L): Control group = B: NDA; F: 33.45 ± 1.12. NDA Intervention group = B: NDA; F: 24.20 ± 0.79. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$
Dobias L et al. (2016) [S2]	Randomized, controlled, not blinded	15-week old spontaneously hypertensive male rats	Control group (n=13) vs intervention group (n=13)	0 mg (corresponding volume of distilled water) vs 50 mg/kg body weight/day of hesperidin orally suspended in distilled water	4 weeks	Body weight, SBP	-Body weight (g): Control group = B: 282.00 ± 2.50; F: 301.00 ± 2.40. NDA Intervention group = B: 290.00 ± 2.70; F: 313.00 ± 2.60. NDA No significant differences between intervention group and control group at the end of the study. -SBP (mm Hg): Control group = B: 169.00 ± 2.20; F: 168.00 ± 2.62. NDA Intervention group = B: 161.00 ± 1.70; F: 166.00 ± 2.12. NDA No significant differences between intervention group and control group at the end of the study.
Ferreira PS et al. (2016) [S3]	Randomized, controlled	9-week old male C57BL/6J mice with systemic inflammation caused by high fat diet	Control group (n=10) vs intervention group (n=10)	0 mg vs 100 mg/kg body weight/day of hesperidin orally added to the regular diet	4 weeks	Weight gain, visceral fat, glucose, TC, HDL-c, LDL-c, TG, IL-6	-Weight gain (g): Control group = B: NDA; F: 7.20 ± 3.60. NDA Intervention group = B: NDA; F: 5.60 ± 0.90. NDA No significant differences between intervention group and control group at the end of the study. -Visceral fat (%): Control group = B: NDA; F: 4.50 ± 1.90. NDA Intervention group = B: NDA; F: 3.80 ± 1.40. NDA No significant differences between intervention group and control group at the end of the study. -Glucose (mmol/L): Control group = B: NDA; F: 20.81 ± 3.27. NDA Intervention group = B: NDA; F: 18.70 ± 4.72. NDA No significant differences between intervention group and control group at the end of the study. -TC (mmol/L): Control group = B: NDA; F: 3.59 ± 0.05. NDA Intervention group = B: NDA; F: 3.17 ± 0.52. NDA No significant differences between intervention group and control group at the end of the study. -HDL-c (mmol/L): Control group = B: NDA; F: 2.16 ± 0.03. NDA Intervention group = B: NDA; F: 2.00 ± 0.39. NDA No significant differences between intervention group and control group at the end of the study. -LDL-c (mmol/L): Control group = B: NDA; F: 1.04 ± 0.31. NDA Intervention group = B: NDA; F: 0.75 ± 0.23. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -TG (mmol/L): Control group = B: NDA; F: 1.85 ± 0.16. NDA Intervention group = B: NDA; F: 2.18 ± 0.23. NDA No significant differences between intervention group and control group at the end of the study.

							-IL-6 (pg/mL): Control group = B: NDA; F: 64.40 ± 44.30. NDA Intervention group = B: NDA; F: 5.76 ± 4.32. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$
Jia S et al. (2015) [S4]	Randomized, controlled	8-weeks old male KK-A^y mice (type 2 diabetic)	Control group (n=10) vs Intervention group (n=10)	0 mg (water) vs 50 mg/kg body weight/day of neohesperidin (derived from hesperidin) by gavage	6 weeks	Body weight, glucose, TC, TG	-Body weight (g): Control group = B: 38.39 ± 1.12; F: 43.18 ± 0.80. NDA Intervention group = B: 38.52 ± 1.17; F: 42.16 ± 1.73. NDA No significant differences between intervention group and control group at the end of the study. -Glucose (mmol/L): Control group = B: 10.16 ± 0.44; F: 20.91 ± 1.86. NDA Interventional group = B: 10.28 ± 0.35; F: 13.18 ± 2.72. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -TC (mmol/L): Control group = B: NDA; F: 6.10 ± 0.26. NDA Intervention group = B: NDA; F: 4.60 ± 0.36. NDA Significant differences between intervention group and control group at the end of the study. $p<0.01$ -TG (mmol/L): Control group = B: NDA; F: 3.73 ± 0.21. NDA Intervention group = B: NDA; F: 1.68 ± 0.22. NDA Significant differences between intervention group and control group at the end of the study. $p<0.001$
Yamamoto M. (2013) [S5]	Controlled	14-18-weeks old male hypertensive rats	Control group (n=4) vs Hesperetin supplement (n=4) vs Hesperetin-7-O-β-D-glucuronide supplement (H7 supplement) (n=4) vs Hesperetin-3'-O-β-D-glucuronide supplement (H3 supplement) (n=4)	0 mg vs 5 mg/kg body weight/day of each flavanone by intravenous administration	3 minutes (acute study)	SBP, DBP	-SBP (mm Hg): Control group = B: 193.40 ± 4.20; F: -3.50 ± 0.40. NDA Hesperetin supplement = B: 194.10 ± 4.70; F: -9.90 ± 1.70. NDA H7 supplement = B: 197.00 ± 3.40; F: -8.70 ± 0.80. NDA H3 supplement = B: 201.30 ± 4.20; F: -4.10 ± 0.80. NDA Significant differences between hesperetin supplement and control group at the end of the study. $p<0.01$ Significant differences between H7 supplement and control group at the end of the study. $p<0.05$ -DBP (mm Hg): Control group = B: 174.90 ± 3.80; F: data not shown. NDA Hesperetin supplement = B: 173.80 ± 3.90; F: data not shown. NDA H7 supplement = B: 179.80 ± 2.80; F: data not shown. NDA H3 supplement = B: 186.90 ± 6.00; F: data not shown. NDA No significant differences between the 4 groups at the end of the study.
Kumar B et al. (2012) [S6]	Controlled	STZ treated Wistar albino rats (type 2 diabetes)	Control group (n=16) vs intervention group (n=16)	0 mg vs 200 mg/kg body weight/day of hesperetin by gavage	24 weeks	Weight gain, glucose	-Weight gain (%): Control group = F: 22.34%. NDA Intervention group = F: 45.35%. NDA NDA about differences between interventional group and control group. -Glucose (mmol/L): Control group = B: NDA; F: 27.67 ± 1.03. NDA Intervention group = B: NDA; F: 21.68 ± 2.06. NDA Significant differences between intervention group and control group at the end of the study. $p<0.001$
Mahmoud AM et al. (2012) [S7]	Controlled	STZ treated while male albino rats (type 2 diabetes)	Control group (n=6) vs intervention group (n=6)	0 mg vs 50 mg/kg body weight/day of hesperidin in aqueous suspension orally	30 days	Glucose, insulin, NO	-Glucose (mmol/L): Control group = B: NDA; F: 16.37 ± 0.23. NDA Intervention group = B: NDA; F: 6088 ± 0.22. NDA Significant differences between intervention group and control group at the end of the study. $p<0.01$ -Insulin (μU/mL): Control group = B: NDA; F: 15.50 ± 0.76. NDA Intervention group = B: NDA; F: 21.55 ± 1.13. NDA Significant differences between intervention group and control group at the end of the study. $p<0.01$

							-NO (µmol/L): Control group = B: NDA; 13.60 ± 0.53. NDA Intervention group = B: NDA; F: 8.52 ± 0.48. NDA Significant differences between intervention group and control group at the end of the study. $p<0.01$
Selvaraj P et al. (2012) [S8]	Randomized, controlled	Isoproterenol hydrochloride treated male albino Wistar rats (myocardial ischemia)	Control group (n=6) vs intervention group (n=6)	0 mg vs 200 mg/kg body weight/day of hesperidin dissolved in carboxyl methyl-cellulose post-orally	7 days	TC, HDL-c, LDL-c, TG	-TC (mmol/L): Control group = B: NDA; F: 2.72 ± 0.26. NDA Intervention group = B: NDA; F: 2.32 ± 0.18. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -HDL-c (mmol/L): Control group = B: NDA; F: 0.79 ± 0.06. NDA Intervention group = B: NDA; F: 1.13 ± 0.11. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -LDL-c (mmol/L): Control group = B: NDA; F: 1.54 ± 0.13. NDA Intervention group = B: NDA; F: 0.87 ± 0.05. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -TG (mmol/L): Control group = B: NDA; F: 0.87 ± 0.07. NDA Intervention group = B: NDA; F: 0.69 ± 0.04. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$
Wang X et al. (2011) [S9]	Controlled	8-weeks old male Wistar rats treated with high-cholesterol diet (2% TCD/day)	Control group (n=15) vs intervention group (n=15)	0 mg vs 0.08 % TCD/day of hesperidin	12 weeks	Body weight, SBP, DBP	-Body weight (g): Control group = B: NDA; F: 544.50 ± 4.90. NDA Intervention group = B: NDA; F: 542.00 ± 9.00. NDA No significant differences between interventional group and control group at the end of the study. -SBP (mm Hg): Control group = NDA. Intervention group = NDA. No significant differences between interventional group and control group at the end of the study. -DBP (mm Hg): Control group = NDA. Intervention group = NDA. No significant differences between interventional group and control group at the end of the study.
Akiyama S et al. (2009) [S10]	Randomized	3-week old male GK rats (type 2 diabetes)	Control group (n=6) vs intervention group 1 (n=6) vs intervention group 2 (n=6)	0% TCD/day vs 1% TCD/day of hesperidin vs 4.6% TCD/day of hesperidin	4 weeks	Body weight, glucose, insulin, TC, TG	-Body weight (g): Control group = B: 53.67 ± 1.48; F: 179.97 ± 1.82. NDA Intervention group 1 = B: 52.98 ± 1.45; F: 172.30 ± 5.83. NDA Intervention group 2 = B: 52.81 ± 1.57; F: 166.41 ± 2.36. NDA Significant differences between intervention group 2 and control group + intervention group 1 at the end of the study. $p<0.05$ -Glucose (mmol/L): Control group = B: 6.09 ± 0.19; F: 7.25 ± 0.35. NDA Intervention group 1 = B: 6.19 ± 0.18; F: 5.78 ± 0.22. NDA Intervention group 2 = B: 6.01 ± 0.14; F: 5.64 ± 0.17. NDA Significant differences between intervention group 2 and control group at the end of the study. $p<0.05$ -Insulin (µU/mL): Control group = B: NDA; F: 116.17 ± 55.53. NDA Intervention group 1 = B: NDA; F: 14.04 ± 9.36. NDA Intervention group 2 = B: NDA; F: 25.53 ± 21.91. NDA Significant differences between intervention group 2 and control group at the end of the study. $p<0.05$

							-TC (mmol/L): Control group = B: NDA; F: 4.76 ± 0.14. NDA Intervention group 1 = B: NDA; F: 3.05 ± 0.11. NDA Intervention group 2 = B: NDA; F: 2.25 ± 0.10. NDA Significant differences between intervention groups and control group at the end of the study. $p<0.05$ -TG (mmol/L): Control group = B: NDA; F: 1.30 ± 0.16. NDA Intervention group 1 = B: NDA; F: 0.64 ± 0.06. NDA Intervention group 2 = B: NDA; F: 0.39 ± 0.03. NDA Significant differences between intervention groups and control group at the end of the study. $p<0.05$
Jung UJ et al. (2006) [S11]	Controlled	5-weeks old male C57BL/KsJ-db/db mice (type 2 diabetes)	Control group (n=10) vs intervention group (n=10)	0 mg vs 0.20 g/kg body weight/day of hesperidin added to the regular diet	5 weeks	Glucose, TC, HDL-c	-Glucose (mmol/L): Control group = B: 21.06 ± 1.05; F: 39.66 ± 1.83. NDA Intervention group = B: 20.66 ± 1.07; F: 31.82 ± 1.11. NDA Significant differences between intervention group and control group at the end of the study. $p<0,05$ -TC (mmol/L): Control group = B: NDA; F: 5.62 ± 0.12. NDA Intervention group = B: NDA; F: 4.81 ± 0.19. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$ -HDL-c (mmol/L): Control group = B: NDA; F: 1.06 ± 0.07. NDA Intervention group = B: NDA; F: 1.17 ± 0.07. NDA No significant differences between intervention group and control group at the end of the study. -TG (mmol/L): Control group = B: NDA; F: 3.32 ± 0.19. NDA Intervention group = B: NDA; F: 1.58 ± 0.22. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$
Jung UJ et al. (2004) [S12]	Controlled	5-weeks old male C57BL/KsJ-db/db mice (type 2 diabetes)	Control group (n=10) vs intervention group (n=10)	0 mg vs 0.20 g/kg body weight/day of hesperidin added to the regular diet	5 weeks	Insulin	-Insulin (µU/mL): Control group = B: NDA, F: 29.10 ± 1.81. NDA Intervention group = B: NDA; F: 47.18 ± 0.59. NDA Significant differences between intervention group and control group at the end of the study. $p<0.05$

STZ, streptozotocin; NDA, no data available about *p*-valor between basal and final values within each group; SBP, systolic blood pressure; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; TG, triglycerides; IL-6, interleukin-6; DBP, diastolic blood pressure; NO, nitric oxide; TCD, total calorie diet.

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Supporting Information 2. Table 2S. Characteristics and results of randomized clinical trials included in the systematic review (N=11).

AUTHOR, YEAR, REFERENCE	STUDY DESIGN	POPULATION	SAMPLE SIZE, POPULATION AGE AND HEALTH STATUS	NUTRITIONAL INTERVENTION	DOSE OF THE FLAVANONE	DURATION OF INTERVENTION	METHOD TO CHECK INTERVENTION COMPLIANCE	BIOMARKERS	RESULTS
Salden BN et al. (2016)[S1]	Randomized, placebo-controlled, double-blind, parallel, chronic and acute	European (Holland)	65 subjects 34-69 years CVRF: overweight or obesity	500 mg/day of placebo capsule (n=32) vs 500 mg/day of hesperidin capsule (n=33)	0 mg/day in placebo capsule vs 450 mg/day in hesperidin capsule	6 weeks	-	SBP, DBP, FMD, glucose, insulin, QUICKI, TC, LDL-c, HDL-c, TG, sVCAM-1, sICAM-1, sE-selectin, sP-selectin	<u>CHRONIC FOLLOW-UP</u> <u>VASCULAR PARAMETERS:</u> -SBP (mm Hg): Placebo capsule = B: 131.00 ± 3.00; F: 129.00 ± 2.00. NS Hesperidin capsule = B: 135.00 ± 2.00; F: 130.00 ± 2.00. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -DBP (mm Hg): Placebo capsule = B: 80.00 ± 2.00; F: 81.00 ± 2.00. NS Hesperidin capsule = B: 83.00 ± 1.00; F: 81.00 ± 2.00. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -FMD (%): Placebo capsule = B: 5.57 ± 0.51; F: 5.43 ± 0.47. NS Hesperidin capsule = B: 4.50 ± 0.51; F: 4.29 ± 0.47. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. <u>GLUCOSE METABOLISM:</u> -Glucose (mmol/L): Placebo capsule = B: 5.00 ± 0.10; F: 5.00 ± 0.10. NS Hesperidin capsule = B: 4.90 ± 0.10; F: 5.00 ± 0.10. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -Insulin (µU/mL): Placebo capsule = B: 8.35 ± 3.02; F: 7.63 ± 2.88. NS Hesperidin capsule = B: 10.66 ± 3.02; F: 11.66 ± 3.02. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -QUICKI: Placebo capsule = B: 0.42 ± 0.01; F: 0.23 ± 0.01. NS Hesperidin capsule = B: 0.34 ± 0.01; F: 0.42 ± 0.01. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. <u>LIPID PROFILE:</u> -TC (mmol/L): Placebo capsule = B: 5.70 ± 0.20; F: 5.60 ± 0.20. NS Hesperidin capsule = B: 5.50 ± 0.20; F: 5.40 ± 0.20. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-LDL-c (mmol/L):
Placebo capsule = B: 3.60 ± 0.20; F: 3.60 ± 2.00. NS
Hesperidin supplement = B: 3.50 ± 0.20; F: 3.40 ± 0.20. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-HDL-c (mmol/L):
Placebo capsule = B: 1.50 ± 0.50; F: 1.50 ± 0.10. NS
Hesperidin capsule = B: 1.50 ± 0.10; F: 1.50 ± 0.10. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-TG (mmol/L):
Placebo capsule = B: 1.30 ± 0.10; F: 1.30 ± 0.10. NS
Hesperidin capsule = B: 1.30 ± 0.10; F: 1.30 ± 0.10. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

INFLAMMATION BIOMARKERS:
-sVCAM-1 (ng/mL):
Placebo capsule = B: 214.00 ± 10.00; F: 215.00 ± 10.00. NS
Hesperidin capsule = B: 210.00 ± 10.00; F: 190.00 ± 10.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sICAM-1 (ng/mL):
Placebo capsule = B: 107.00 ± 5.00; F: 107.00 ± 5.00. NS
Hesperidin capsule = B: 110.00 ± 5.00; F: 100.00 ± 5.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sE-selectin (ng/mL):
Placebo capsule = B: 12.00 ± 1.00; F: 11.00 ± 2.00. NS
Hesperidin capsule = B: 11.00 ± 1.00; F: 12.00 ± 2.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sP-selectin (ng/mL):
Placebo capsule = B: 78.00 ± 5.00; F: 83.00 ± 5.00. NS
Hesperidin capsule = B: 94.00 ± 5.00; F: 83.00 ± 5.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

ACUTE FOLLOW-UP
VASCULAR PARAMETERS:
-FMD (%) at baseline:
Placebo capsule = B: 5.57 ± 0.51; T2h: 5.08 ± 0.53. NS
Hesperidin capsule = B: 4.50 ± 0.51; T2h: 4.23 ± 0.51. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-FMD (%) after 6 weeks of supplementation:
Placebo capsule = B: 5.57 ± 0.51; T2h: 5.08 ± 0.53. NS

Hesperidin capsule = B: 4.21 ± 0.48; T2h: 4.38 ± 0.51. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

INFLAMMATION BIOMARKERS:
-Acute sVCAM-1 (ng/mL) at baseline:
Placebo capsule = B: 214.10 ± 10.00; T2h: 213.00 ± 9.00; T4h: 206.00 ± 10.00. NS
Hesperidin capsule = B: 210.00 ± 10.00; T2h: 207.00 ± 9.00; T4h: 209.00 ± 10.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sVCAM-1 (ng/mL) after 6 weeks of supplementation:
Placebo capsule = B: 215.00 ± 10.00; T2h: 208.00 ± 9.00; T4h: 200.00 ± 10.00. NS
Hesperidin capsule = B: 190.00 ± 10.00; T2h: 185.00 ± 9.00; T4h: 180.00 ± 10.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sICAM-1 (ng/mL) at baseline:
Placebo capsule = B: 107.00 ± 5.00; T2h: 105.00 ± 4.00; T4h: 109.00 ± 5.00. NS
Hesperidin capsule = B: 110.00 ± 5.00; T2h: 100.00 ± 4.00; T4h: 104.00 ± 5.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sICAM-1 (ng/mL) after 6 weeks of supplementation:
Placebo capsule = B: 107.00 ± 5.00; T2h: 105.00 ± 5.00; T4h: 102.00 ± 5.00. NS
Hesperidin capsule = B: 100.00 ± 5.00; T2h: 98.00 ± 5.00; T4h: 96.00 ± 5.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sE-selectin (ng/mL) at baseline:
Placebo capsule = B: 12.00 ± 1.00; T2h: 12.00 ± 2.00; T4h: 12.00 ± 2.00. NS
Hesperidin capsule = B: 11.00 ± 1.00; T2h: 10.00 ± 2.00; T4h: 11.00 ± 2.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sE-selectin (ng/mL) after 6 weeks of supplementation:
Placebo capsule = B: 11.00 ± 2.00; T2h: 9.00 ± 2.00; T4h: 10.00 ± 2.00. NS
Hesperidin capsule = B: 12.00 ± 2.00; T2h: 11.00 ± 2.00; T4h: 11.00 ± 2.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sP-selectin (ng/mL) at baseline:
Placebo capsule = B: 78.00 ± 5.00; T2h: NDA; T4h: 83.00 ± 4.00. NS
Hesperidin capsule = B: 94.00 ± 5.00; T2h: NDA; T4h: 90.00 ± 4.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-sP-selectin (ng/mL) after 6 weeks of supplementation:
Placebo capsule = B: 83.00 ± 5.00; T2h: NDA; T4h: 94.00 ± 5.00. NS
Hesperidin capsule = B: 83.00 ± 5.00; T2h: NDA; T4h: 89.00 ± 5.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

Kean RJ et al. (2015)[S2]	Randomized, double-blind, crossover, chronic	European (United Kingdom)	37 subjects 61-70 years CVRF: overweight	500 mL/day of low flavanone drink vs 500 mL/day of high flavanone drink	64 mg/day of hesperidin vs 549 mg/day of hesperidin	8 weeks		SBP, DBP	<u>VASCULAR PARAMETERS:</u> -SBP (mm Hg): Low flavanone drink on women = B: 126.00 ± 2.70; F: 125.00 ± 4.00. NS High flavanone drink on women = B: 123.00 ± 3.20; F: ± 124.00 ± 2.40. NS Low flavanone drink on men = B: 132.00 ± 4.30; F: 136.00 ± 4.00. NS High flavanone drink on men = B: 135.00 ± 5.60; F: 133.00 ± 3.40. NS Significant differences between sexes at the end of the study. $p<0.05$ -DBP (mm Hg): Low flavanone drink on women = B: 75.00 ± 1.60; F: 76.00 ± 1.60. NS High flavanone drink on women = B: 73.00 ± 1.70; F: 73.00 ± 1.60. NS Low flavanone drink on men = B: 81.00 ± 2.60; F: 82.00 ± 2.60. NS High flavanone drink on men = B: 79.00 ± 2.40; F: 79.00 ± 2.40. NS Significant differences between sexes at the end of the study. $p<0.05$
Constans J et al. (2015)[S3]	Randomized, controlled, single blind, crossover, chronic	European (France)	25 subjects 51-56 years CVRF: hypercholesterolemic	600 mL/day of control drink (CD) vs 600 mL/day of orange juice (OJ)	0 mg/day in 600 mL/day of CD vs 213 mg/day of hesperidin in 600 mL/day of OJ	4 weeks for one drink test + 5 weeks for wash-out + 4 weeks for the other drink test + 5 weeks for wash-out	5 days-food record	Glucose, TC, LDL-c, HDL-c, TG, Apo A-1, Apo B, fibrinogen, homocysteine, sVCAM-1, sICAM-1, sE-selectin	Differences compared to baseline: <u>GLUCOSE METABOLISM:</u> -Glucose (mmol/L): CD = F: -0.27. NS OJ = F: +0.06. NS No significant differences between CD and OJ at the end of the study. <u>LIPID PROFILE:</u> -TC (mmol/L): CD = F: -0.35. $p<0.05$ OJ = F: +0.20. NS No significant differences between CD and OJ at the end of the study. -LDL-c (mmol/L): CD = F: -0.32. $p<0.05$ OJ = F: +0.11. NS No significant differences between CD and OJ at the end of the study. -HDL-c (mmol/L): CD = F: -0.07. NS OJ = F: +0.00. NS No significant differences between CD and OJ at the end of the study. -TG (mmol/L): CD = F: +0.10. NS OJ = F: +0.24. NS No significant differences between CD and OJ at the end of the study. -Apo A-1 (mg/dL): CD = F: -3.00. NS OJ = F: +5.00. $p<0.05$ No significant differences between CD and OJ at the end of the study. -Apo B (mg/dL): CD = F: -1.00. NS OJ = F: +8.00. $p<0.05$ No significant differences between CD and OJ at the end of the study.

								COAGULATION BIOMARKERS: -Fibrinogen (mg/dL): CD = F: +11.00. NS OJ = F: +0.1. NS No significant differences between CD and OJ at the end of the study. -Homocysteine (μmol/L): CD = F: +0.64. $p<0.05$ OJ = F: +0.33. NS No significant differences between CD and OJ at the end of the study. INFLAMMATION BIOMARKERS: -sVCAM-1 (ng/mL): CD = F: 579.79 ± 50.23. NS OJ = F: 558.62 ± 47.36. NS No significant differences between CD and OJ at the end of the study. -sICAM-1 (ng/mL): CD = F: 168.34 ± 11.03. NS OJ = F: 172.10 ± 11.70. NS No significant differences between CD and OJ at the end of the study. -sE-selectin (ng/mL): CD= F: 121.90 ± 12.30. NS OJ = F: 110.12 ± 9.98. NS No significant differences between CD and OJ at the end of the study.
Rangel-Huerta OD et al. (2015)[S4]	Randomized, double-blind, crossover, chronic	European (Spain)	100 subjects 18-65 years CVRF: overweight or obese	500 mL/day of OJ vs 500 mL/day of OJ with a high hesperidin concentration	237 mg/day in 500 mL/day of OJ vs 582.50 mg/day of hesperidin in 500 mL/day of OJ	12 weeks for one intervention + 7 weeks for wash-out + 12 weeks for the other intervention	BW, BMI, SBP, DBP, glucose, insulin, TC, LDL-c, HDL-c, TG, Apo A-1, Apo B, oxLDL	ANTHROPOMETRIC PARAMETERS: -BW (kg): OJ = B: 90.40 ± 1.50; F: 89.10 ± 1.50. $p<0.05$ OJ with high hesperidin concentration = B: 90.60 ± 1.50; F: 88.80 ± 1.50. $p<0.05$ No significant differences between OJ and OJ with high hesperidin concentration at the end of the study. -BMI (kg/m^2): OJ = B: 32.50 ± 0.40; F: 32.00 ± 0.40. $p<0.05$ OJ with high hesperidin concentration = B: 32.60 ± 0.40; F: 31.90 ± 0.40. $p<0.05$ No significant differences between OJ and OJ with high hesperidin concentration at the end of the study. VASCULAR PARAMETERS: -SBP (mm Hg): OJ = B: 128.00 ± 1.00; F: 124.00 ± 2.00. $p<0.05$ OJ with high hesperidin concentration = B: 127.00 ± 1.00; F: 124.00 ± 1.00. NS No significant differences between OJ and OJ with high hesperidin concentration at the end of the study. -DBP (mm Hg): OJ = B: 79.00 ± 1.00; F: 76.00 ± 1.00. $p<0.05$ OJ with high hesperidin concentration = B: 78. 00 ± 1.00; F: 77.00 ± 1.00. NS No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

GLUCOSE METABOLISM:

-Glucose (mmol/L):

OJ = B: 4.90 ± 0.10; F: 5.20 ± 0.10. $p<0.05$

OJ with high hesperidin concentration = 5.00 ± 1.00; F: 5.20 ± 0.01. $p<0.05$

Significant differences between OJ and OJ with high hesperidin concentration at the end of the study. $p<0.05$

-Insulin (µU/mL):

OJ = B: 12.70 ±0.70; F: 11.50 ± 0.60. $p<0.05$

OJ with high hesperidin concentration = B: 13.80 ± 0.90; F: 12.70 ± 0.70. NS

Significant differences between OJ and OJ with high hesperidin concentration at the end of the study. $p<0.05$

LIPID PROFILE:

-TC (mmol/L):

OJ = B: 5.60 ± 0.10; F: 5.60 ± 0.10. NS

OJ with high hesperidin concentration = B: 5.60 ± 0.10; F: 5.60 ± 0.10. NS

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

-LDL-c (mmol/L):

OJ = B: 3.39 ± 0.08; F: 3.47 ± 0.08. NS

OJ with high hesperidin concentration = B: 3.41 ± 0.08. NS

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

-HDL-c (mmol/L):

OJ = B: 1.29 ± 0.03; F: 1.32 ± 0.03. NS

OJ with high hesperidin concentration = B: 1.32 ± 0.03; F: 1.29 ± 0.03. NSD

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

-TG (mmol/L):

OJ = B: 1.49 ± 0.07; F: 1.40 ± 0.07. $p<0.05$

OJ with high hesperidin concentration = B: 1.54 ± 0.07; F: 1.47 ± 0.06. NS

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

-Apo A-1 (mg/dL):

OJ = B: 147.00 ± 2.00; F: 147.00 ± 2.00. NS

OJ with high hesperidin concentration = B: 149.00 ± 2.00; F: 145.00 ± 2.00. $p<0.05$

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

-Apo B (mg/dL):

OJ = B: 95.00 ± 2.00; F: 91.00 ± 2.00. $p<0.05$

OJ with high hesperidin concentration = B: 96.00 ± 2.00; F: 93.00 ± 2.00. NS

No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.

									OXIDATION BIOMARKERS: -OxLDL (pg/mL): OJ = B: 335.00 ± 40.00; F: 343.00 ± 38.00. NS OJ with high hesperidin concentration = B: 322.00 ± 39.00; F: 326.00 ± 40.00. NS No significant differences between OJ and OJ with high hesperidin concentration at the end of the study.
Schär MY et al. (2015)[S5]	Randomized, placebo-controlled, crossover, acute	European (United Kingdom)	28 men 51-69 years CVRF: 10-20% of cardiovascular risk the next 10 years according to British Hypertension Society	767 mL/day of CD (n=15) vs 767 mL of OJ (n=13)	0 mg/day in CD vs 320 mg/day of hesperidin in OJ	5 hours	24 hours-dietary recall	SBP, DBP, RH-PAT index	VASCULAR PARAMETERS: -SBP (mm Hg): CD = B: 128.20 ± 2.20; T5h: 123.60 ± 1.80. NS OJ = B: 126.30 ± 1.80; T5h: 123.60 ± 2.90. NS No significant differences between control drink and OJ at the end of the study. -DBP (mm Hg): CD = B: 80.20 ± 1.60; T5h: 75.10 ± 1.80. NS OJ = B: 77.90 ± 1.80; T5h: 73.60 ± 1.90. NS No significant differences between CD and OJ at the end of the study. -RH-PAT index: CD = B: 2.78 ± 0.18; T5h: 2.66 ± 0.17. NS OJ= B: 2.77 ± 0.13; T5h: 2.68 ± 0.19. NS No significant differences between CD and OJ at the end of the study.
Buscemi S et al. (2012)[S6]	Randomized, placebo-controlled, single blind, crossover, chronic	European (Italy)	31 subjects 19-67 years CVRF: metabolic syndrome	500 mL/day of CD (n=12) vs 500 mL/day of OJ (n=19)	0 mg/day in placebo drink vs 159.50 mg/day of hesperidin in 500 mL/day of OJ	1.5 weeks	Food diary 24 hours the day before the visit	FMD, GTN, IL-6, NOx	VASCULAR PARAMETERS: -FMD (%): CD = B: 5.70 ± 2.40; F: 5.00 ± 1.80. NS OJ = B: 5.70 ± 2.40; F: 7.90 ± 2.70. $p<0.05$ Significant differences between CD and OJ at the end of the study. $p<0.05$ -GTN (%): CD = B: 17.90 ± 4.90; F: 17.90 ± 4.80. NS OJ = B: 17.90 ± 4.90; F: 18.80 ± 4.60. NS No significant differences between CD and OJ at the end of the study. INFLAMMATION BIOMARKERS: -IL-6 (pg/mL): CD = B: 33.90 ± 2.60; F: 32.90 ± 3.20. NS OJ = B: 33.90 ± 2.60; F: 30.60 ± 2.60. $p<0.05$ Significant differences between CD and OJ at the end of the study. $p<0.05$ OXIDATION BIOMARKERS: -NOx (µmol/L): CD = B: 19.80 ± 4.60; F: 20.80 ± 6.20. NS OJ = B: 19.80 ± 4.60; F: 20.70 ± 7.20. NS No significant differences between CD and OJ at the end of the study.
Rizza S et al. (2011)[S7]	Randomized, placebo-controlled, double-blind, crossover, chronic	European (Italy)	24 subjects 21-65 years CVRF: metabolic syndrome	1 placebo capsule/day vs 1 hesperidin capsule/day	0 mg/day in placebo capsule vs 500 mg/day in 1 hesperidin capsule	3 weeks		BMI, SBP, DBP, FMD, GTN, TC, LDL-c, HDL-c, TG, Apo-A1, Apo-B, glucose, insulin, QUICKI, fibrinogen, homocysteine, SAA protein,	ANTHROPOMETRIC PARAMETERS: -BMI (kg/m^2): Placebo capsule = B: 34.70 ± 1.50; F: 34.70 ± 1.50. NS Hesperidin capsule = B: 34.70 ± 70.00; F: 34.70 ± 1.50. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. VASCULAR PARAMETERS: -SBP (mm Hg):

	VCAM, ICAM, sE-selectin	Placebo capsule = B: 138.00 ± 3.00; F: 132.00 ± 2.00. NS Hesperidin capsule = B: 138.30 ± 3.00; F: 134.00 ± 3.00. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -DBP (mm Hg): Placebo capsule = B: 89.00 ± 2.00; F: 90.00 ± 2.00. NS Hesperidin capsule = B: 89.00 ± 2.00; F: 90.00 ± 2.00. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -FMD (%): Placebo capsule = B: 8.24 ± 0.88; F: 7.78 ± 0.76. NS Hesperidin capsule = B: 8.24 ± 0.88; F: 10.26 ± 1.19. p=0.05 Significant differences between placebo capsule and hesperidin capsule at the end of the study. p=0.02 -GTN (%) Placebo capsule = B: 13.98 ± 1.32; F: 14.40 ± 1.02. NS Hesperidin capsule = B: 13.98 ± 1.32; F: 14.04 ± 1.08. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. <u>GLUCOSE METABOLISM:</u> -Glucose (mmol/L): Placebo capsule = B: 7.33 ± 0.67; F: 7.16 ± 0.39. NS Hesperidin capsule = B: 7.33 ± 0.67; F: 6.99 ± 0.33. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -Insulin (μU/mL): Placebo capsule = B: 21.30 ± 2.10; F: 21.10 ± 1.90. NS Hesperidin capsule = B: 21.30 ± 2.10; F: 20.20 ± 2.10. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -QUICKI: Placebo capsule = B: 0.298 ± 0.004; F: 0.297 ± 0.003. NS Hesperidin capsule = B: 0.298 ± 0.004; F: 0.300 ± 0.004. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. <u>LIPID PROFILE:</u> -TC (mmol/L): Placebo capsule = B: 4.65 ± 0.21; F: 4.81 ± 0.21. NS Hesperidin capsule = B: 4.65 ± 0.21; F: 4.52 ± 0.21. NS Significant differences between placebo capsule and hesperidin capsule at the end of the study. p<0.05 -LDL-c (mmol/L): Placebo capsule = B: 3.09 ± 0.21; F: 3.17 ± 0.16. NS Hesperidin capsule = B: 3.09 ± 0.21; F: 2.99 ± 0.16. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-HDL-c (mmol/L):
Placebo capsule = B: 0.96 ± 0.05; F: 0.88 ± 0.05. NS
Hesperidin capsule = B: 0.96 ± 0.05; F: 0.91 ± 0.05. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-TG (mmol/L):
Placebo capsule = B: 1.80 ± 0.13; F: 2.04 ± 0.19. NS
Hesperidin capsule = B: 1.80 ± 0.13; F: 1.87 ± 0.11. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-Apo A1 (mg/dL):
Placebo capsule = B: 134.00 ± 5.00; F: 136.00 ± 7.00. NS
Hesperidin capsule = B: 134.00 ± 5.00; F: 137.00 ± 6.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-Apo B (mg/dL):
Placebo capsule = B: 90.00 ± 4.00; F: 93.00 ± 4.00. NS
Hesperidin capsule = B: 90.00 ± 4.00; F: 88.00 ± 4.00. NS
Significant differences between placebo capsule and hesperidin capsule at the end of the study. $p<0.05$

COAGULATION BIOMARKERS:
-Fibrinogen (mg/dL):
Placebo capsule = B: 320.00 ± 14.00; F: 330.00 ± 16.00. NS
Hesperidin capsule = B: 320.00 ± 14.00; F: 331.00 ± 15.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

-Homocysteine (μM/L):
Placebo capsule = B: 11.90 (10.30 – 14.90); F: 13.60 (10.60 – 16.70). NS
Hesperidin capsule = B: 11.90 (10.30 – 14.90); F: 13.00 (10.20 – 15.50). NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

INFLAMMATION BIOMARKERS:
-SAA protein (mg/L):
Placebo capsule = B: 7.30 (5.60 – 6.10); F: 8.00 (5.60 – 11.20). NS
Hesperidin capsule = B: 7.30 (5.60 – 6.10); F: 5.60 (3.20 – 7.80). NS
Significant differences between placebo capsule and hesperidin capsule at the end of the study. $p<0.05$

-VCAM (ng/mL):
Placebo capsule = B: 956.00 ± 29.00; F: 976.00 ± 30.00. NS
Hesperidin capsule = B: 956.00 ± 29.00; F: 950.00 ± 27.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.
-ICAM (ng/mL):
Placebo capsule = B: 291.00 ± 6.00; F: 299.00 ± 7.00. NS
Hesperidin capsule = B: 291.60 ± 6.00; F: 294.00 ± 7.00. NS
No significant differences between placebo capsule and hesperidin capsule at the end of the study.

									-sE-selectin (ng/mL): Placebo capsule = B: 31.00 ± 2.00; F: 31.00 ± 2.00. NS Hesperidin capsule = B: 31.00 ± 2.00; F: 27.00 ± 2.00. NS Significant differences between placebo capsule and hesperidin capsule at the end of the study. $p<0.05$
Morand C et al. (2011)[S8]	Randomized, placebo-controlled, crossover, chronic	European (France)	23 men 51-63 years CVRF: overweight	500 mL/day of CD and placebo supplement vs 500 mL/day of CD and hesperidin supplement vs 500 mL/day of OJ	0 mg/day in 500 mL/day of CD and 1 placebo supplement vs 0 mg/day in 500 mL/day of CD and 292 mg/day of pure hesperidin capsule vs 292 mg/day of hesperidin in 500 mL/day of OJ	4 weeks	3 days-food record	SBP, DBP, glucose, insulin, TC, LDL-c, HDL-c, TG, IL-6, sVCAM, sICAM-1, NOx	<u>VASCULAR PARAMETERS:</u> -SBP (mm Hg): CD and placebo supplement = F: 133.70 ± 2.10. NS CD and placebo supplement vs CD and hesperidin supplement = F: +2.00 ± 2.90. NS CD and placebo supplement vs OJ = F: -2.00 ± 2.80. NS No significant differences between the three interventions at the end of the study. -DBP (mm Hg): CD and placebo supplement = F: 84.90 ± 2.10. NS CD and placebo supplement vs CD and hesperidin supplement = F: -5.30 ± 2.00. $p<0.05$ CD and placebo supplement vs OJ = F: -4.50 ± 2.00. $p<0.05$ Significant differences between the three interventions at the end of the study. $p<0.023$ <u>GLUCOSE METABOLISM:</u> -Glucose (mmol/L): CD and placebo supplement = F: 6.10 ± 0.20. NS CD and placebo supplement vs CD and hesperidin supplement = F: +0.20 ± 0.20. NS CD and placebo supplement vs OJ = F: +0.00 ± 0.20. NS No significant differences between the three interventions at the end of the study. -Insulin (µU/mL): CD and placebo supplement = F: 16.90 ± 1.20. NS CD and placebo supplement vs CD and hesperidin supplement = F: -1.30 ± 1.50. NS CD and placebo supplement vs OJ = F: -1.20 ± 1.50. NS No significant differences between the three interventions at the end of the study. <u>LIPID PROFILE:</u> -TC (mmol/L): CD and placebo supplement = F: 5.40 ± 0.20. NS CD and placebo supplement vs CD and hesperidin supplement = F: +0.40 ± 0.20. NS CD and placebo supplement vs OJ = F: +0.30 ± 0.20. NS No significant differences between the three interventions at the end of the study. -LDL-c (mmol/L): CD and placebo supplement = F: 3.50 ± 0.20. NS CD and placebo supplement vs CD and hesperidin supplement = F: +0.40 ± 0.20. NS CD and placebo supplement vs OJ = F: +0.30 ± 0.20. NS No significant differences between the three interventions at the end of the study. -HDL-c (mmol/L): CD and placebo supplement = F: 1.40 ± 0.10. NS CD and placebo supplement vs CD and hesperidin supplement = F: 0.00 ± 0.10. NS CD and placebo supplement vs OJ = F: 0.00 ± 0.10. NS No significant differences between the three interventions at the end of the study.

									-TG (mmol/L): CD and placebo supplement = F: 1.30 ± 0.10. NS CD and placebo supplement vs CD and hesperidin supplement = F: +0.10 ± 0.10. NS CD and placebo supplement vs OJ = F: +0.10 ± 0.10. NS No significant differences between the three interventions at the end of the study. INFLAMMATION BIOMARKERS: -IL-6 (pg/mL): CD and placebo supplement = F: 1.98 ± 0.25. NSD CD and placebo supplement vs CD and hesperidin supplement = F: -0.19 ± 0.29. NS CD and placebo supplement vs OJ = F: +0.11 ± 0.29. NS No significant differences between the three interventions at the end of the study. -sVCAM-1 (ng/mL): CD and placebo supplement = F: 119.00 ± 119.00. NS CD and placebo supplement vs CD and hesperidin supplement = F: -283.00 ± 157.00. NSD CD and placebo supplement vs OJ = F: -302.00 ± 154.00. NS No significant differences between the three interventions at the end of the study. -sICAM-1 (ng/mL): CD and placebo supplement = F: 360.00 ± 19.00. NS CD and placebo supplement vs CD and hesperidin supplement = F: +16.00 ± 27.00. NSD CD and placebo supplement vs OJ = F: +28.00 ± 26.00. NS No significant differences between the three interventions at the end of the study. OXIDATION BIOMARKERS: -NOx (µmol/L): CD and placebo supplement = F: 37.90 ± 5.60. NS CD and placebo supplement vs CD and hesperidin supplement = F: -0.80 ± 7.00. NS CD and placebo supplement vs OJ = F: +13.50 ± 6.90. NS No significant differences between the three interventions at the end of the study.
Demonty I et al. (2010)[S9]	Randomized, placebo-controlled, double blind, parallel, chronic	European (Holland)	194 subjects 51-69 years CVRF: overweight, hypercholesterolemic	4 placebo capsules/day vs 4 hesperidin capsules/day	0 mg/day in 4 placebo capsules vs 800 mg/day in 4 hesperidin capsules	4 weeks	Participants returned all used an unused capsule boxes	BW, BMI, TC, LDL-c, HDL-c, TG	ANTHROPOMETRIC PARAMETERS: -BW (kg): Placebo capsule = B: 74.00 ± 12.30; F: +0.09 ± 0.09. NS Hesperidin capsule = B: 74.00 ± 9.50; F: +0.19 ± 0.10. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -BMI (kg/m^2): Placebo capsule = B: 25.10 ± 2.30; F: +0.02 ± 0.03. NS Hesperidin capsule = B: 25.10 ± 2.10; F: +0.07 ± 0.03. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. LIPID PROFILE: -TC (mmol/L): Placebo capsule = B: 6.18 ± 0.85; F: 6.22 ± 0.05. NS Hesperidin capsule = B: 6.18 ± 0.83; F: 6.19 ± 0.05. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study.

									-LDL-c (mmol/L): Placebo capsule = B: 3.97 ± 0.71; F: 4.00 ± 0.04. NSD Hesperidin capsule = B: 3.99 ± 0.77; F: 3.99 ± 0.04. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -HDL-c (mmol/L): Placebo capsule = B: 1.51 ± 0.45; F: 1.54 ± 0.02. NS Hesperidin capsule = B: 1.52 ± 0.40; F: 1.53 ± 0.02. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study. -TG (mmol/L): Placebo capsule = B: 1.44 ± 0.60; F: 1.26 ± 0.03. NS Hesperidin capsule = B: 1.31 ± 0.54; F: 1.24 ± 0.04. NS No significant differences between placebo capsule and hesperidin capsule at the end of the study.
Aptekmann NP et al. (2010)[S10]	Randomized, controlled, parallel, chronic	South American (Brazil)	26 premenopausal women 30-48 years CVRF: overweight	0 mL/day (n=13) vs 500 mL/day of OJ (n=13)	0 mg/day vs 54.60 mg/day of hesperetin in 500 mL/day of OJ	13 weeks	Self-report	BW, BMI, body fat, CT, LDL-c, HDL-c, TG	<u>ANTHROPOMETRIC PARAMETERS:</u> -BW (kg): Control group = B: 76.30 ± 15.30; F: 74.50 ± 15.90. p<0.05 OJ group = B: 74.60 ± 13.00; F: ± 73.60 ± 12.40. p<0.05 No significant differences between control group and OJ group at the end of the study. -BMI (kg/m^2): Control group = B: 29.00 ± 5.33; F: 28.30 ± 5.81. p<0.05 OJ group = B: 28.40 ± 4.46; F: 28.10 ± 4.47. p<0.05 No significant differences between control group and OJ group at the end of the study. -Body fat (%): Control group = B: 39.30 ± 7.33; F: 33.80 ± 7.98. p<0.05 OJ group = B: 37.70 ± 7.56; F: 33.40 ± 7.42. p<0.05 No significant differences between control group and OJ group at the end of the study. <u>LIPID PROFILE:</u> -TC (mmol/L): Control group = B: 5.03 ± 0.70; F: 4.95 ± 0.76. NS OJ group = B: 4.82 ± 0.74; F: 4.60 ± 0.74. p<0.05 No significant differences between control group and OJ group at the end of the study. -LDL-c (mmol/L): Control group = B: 3.50 ± 0.87; F: 3.33 ± 0.85. NS OJ group = B: 3.03 ± 0.64; F: 2.59 ± 0.79. p<0.05 No significant differences between control group and OJ group at the end of the study. -HDL-c (mmol/L): Control group = B: 1.53 ± 0.29; F: 1.44 ± 0.32. NS OJ group = B: 1.27 ± 0.28; F: 1.50 ± 0.31. p<0.05 No significant differences between control group and OJ group at the end of the study. -TG (mmol/L): Control group = B: 1.02 ± 0.30; F: 0.95 ± 0.27. NS OJ group = B: 1.14 ± 0.49; F: 1.12 ± 0.32. NS No significant differences between control group and OJ group at the end of the study.

Cesar TB et al. (2010)[S11]	Randomized, controlled, parallel, chronic	South American (Brazil)	22 subjects 28-56 years CVRF: hypercholestero-lemic	0 mL/day (n=8) vs 750 mL/day of OJ (n=14)	0 mg/day vs 42 mg/day of hesperetin in 750 mL/day of OJ	8.5 weeks	BMI, TC, LDL-c, HDL-c, TG	ANTHROPOMETRIC PARAMETERS: -BMI (kg/m^2): Control group = B: 30.00 ± 6.00; F: 30.00 ± 5.00. NS OJ = B: 28.00 ± 5.00; F: 28.00 ± 5.00. NS No significant differences between control group and OJ at the end of the study. LIPID PROFILE: -TC (mmol/L): Control group = B: 5.51 ± 0.73; F: 5.75 ± 1.04. NS OJ = B: 5.95 ± 0.55; F: 5.49 ± 0.78. $p<0.05$ No significant differences between control group and OJ at the end of the study. -LDL-c (mmol/L): Control group = B: 3.69 ± 0.68; F: 3.82 ± 0.94. NS OJ = B: 4.16 ± 0.44; F: 3.67 ± 0.68. $p<0.05$ No significant differences between control group and OJ at the end of the study. -HDL-c (mmol/L): Control group = B: 1.14 ± 0.34; F: 1.14 ± 0.31. NS OJ = B: 1.12 ± 0.18; F: 1.17 ± 0.18. NS No significant differences between control group and OJ at the end of the study -TG (mmol/L): Control group = B: 1.46 ± 0.60; F: 1.84 ± 0.73. $p<0.05$ OJ = B: 1.48 ± 0.49; F: 1.66 ± 0.59. NS No significant differences between control group and OJ at the end of the study.

CVRF, cardiovascular risk factors; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow-mediated dilatation; QUICKI, quantitative insulin-sensitivity check index; TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; sVCAM-1, soluble vascular cell adhesion molecule-1; sICAM-1, soluble intercellular adhesion molecule-1; sE-selectin, soluble E-selectin; sP-selectin, soluble P-selectin; NS, no significant differences between basal and final values; CD, control drink; OJ, orange juice; Apo A-1, apolipoprotein A-1; Apo B, apolipoprotein B; BW, body weight; BMI, body mass index; oxLDL, oxidized low density lipoprotein; RH-PAT index, reactive hyperemia-peripheral arterial tonometry index; NDA, no data available; GTN, glyceryl-nitrate dilation of the brachial artery; IL-6, interleukin 6; NOx, nitric oxide; SAA protein, serum amyloid A protein.

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Projects 2, 3 and 4: Summarizing the CITRUS randomized controlled trial.

The CITRUS study

This section explains the RCT called CITRUS, a competitive project, from which 3 projects were realized and reported in the present thesis.

First, we will start with the evaluation of hesperidin consumption in OJ and hesperidin-enriched OJ (EOJ) in pre- and stage 1 hypertensive subjects on BP and PP (Project 2). Second, we evaluated the effects of hesperidin consumption in OJ and EOJ on the transcriptomic profile of PBMCs (Project 3). Finally, we evaluated the effects of hesperidin consumption in OJ and EOJ on plasma, serum, and urine metabolomic profiles (Project 4).

Study design:
A randomized, parallel, double-blinded, and placebo-controlled clinical trial was performed. All the participants were randomly assigned to one of the three intervention groups, namely control drink (CD), OJ and EOJ, and they consume 500 mL/day of the corresponding drink for 12 weeks. Moreover, two single dose studies, one at the beginning of the study and the other one at the end of the study after 12 weeks, were performed. For single dose studies participants consumed 500 mL of the corresponding intervention drink in the postprandial state.

After 1 week with control dietary habits following nutritionist recommendations to limit the total intake of flavonoid-rich foods and citrus-containing foods and maintain their normal dietary habits, the participants started the clinical trial. Moreover, 48 hours before the two

single dose studies, the participants realized a phenolic compounds-free diet.

During the sustained study the subjects attended 7 visits (V), and the two single dose studies were performed at V1 and V7. The first single dose study (V1) was realized to determine the postprandial effects of hesperidin in OJ, and the second single dose study (V7) was realized to assess the postprandial effects of hesperidin in OJ after the sustained consumption.

At V1, V3, V5 and V7 a 3-day food record was obtained to determine their dietary habits, and blood and urine samples in fasting conditions were collected. Moreover, at each visit, a physical examination, physical activity questionnaire class and anthropometric measurements were performed.

Study population:

From the subjects who attended to the preselection, 159 (53 women and 106 men) were included. Participants has pre- or stage 1 hypertension.

Inclusion criteria were:

- Age from 18 to 65 years old.
- SBP ≥120 mm Hg.
- No family history of CVDs or chronic diseases.
- Willingness to provide informed consent before starting the study.

Exclusion criteria were:

- BMI ≥ 35 kg/m^2.
- Fasting glucose > 125 mg/dL.
- SBP ≥ 160 mm Hg
- DBP ≥ 100 mm Hg

- Taking antihypertensive medications.
- Hyperlipemia or antilipemic medication.
- Smoking.
- Pregnancy or intending to become.
- Use of medications, antioxidants, vitamin supplements or adherence to a vegetarian diet.
- Chronic alcoholism.
- Physical activity > 5 hours/week
- Intestinal disorders
- Anemia (hemoglobin ≤13 mg/dL in men and ≤12 mg/dL in women).
- Consumption of a research product in the 30 day prior to inclusion in the present study.
- Failure to follow the study guidelines.

The clinical trial was approved by the Clinical Research Ethical Committee of Hospital Sant Joan (14-12-18/12aclaassN1), Reus, Spain; was conducted in accordance to Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization and were reported as CONSORT criteria. Finally, the clinical trial was registered at Clinical-Trials.gov: NCT02479568.

Intervention drinks:

The three intervention drinks were supplied by the Florida Department of Citrus of the United States of America. The intervention drinks were CD with no hesperidin content, OJ containing 392 mg/500 mL hesperidin, and EOJ containing 670 mg/500 mL hesperidin.

Ferrer HealthTech of Murcia (Spain) provided the micronized 2S hesperidin used to enrich the EOJ intervention because it is the form

that is naturally present in OJ and the most bioavailable.(103) The detailed composition of the three drinks is reported in Supporting Information Table S1 of the published version of Project 2.

Parameters measured:

The following parameters were obtained and measured during the CITRUS study and reported in 3 different projects depending on the objective:

-In the Project 2, the following were evaluated at V1, V3, V5 and V7:

- SBP and DBP levels, and pulse pressure (PP).
- Serum levels of homocysteine, F2α-isoprostanes, ICAM-1, VCAM-1 and uric acid.
- For the single-dose study, SBP, DBP, PP and homocysteine were evaluated at baseline and after 2, 4 and 6 hours.

-In the Project 3, the following were obtained to realize transcriptomic analysis at V1 and V7:

- Blood samples to obtain PBMCs.

-In the Project 4, the following were obtained to realize the metabolomics analysis:

- Plasma samples at weeks 4, 8 and 12, and after 2, 4 and 6 hours of the single dose.
- Serum and urine samples at V1 and V7.
- Serum samples were obtained at baseline and after 2, 4 and 6 hours of a single dose of hesperidin.

Finally, Figure 13 presents a schema of the CITRUS study that includes the 3 projects realized in subjects with pre- or stage 1 hypertension to evaluate the effects of hesperidin consumption in OJ and EOJ.

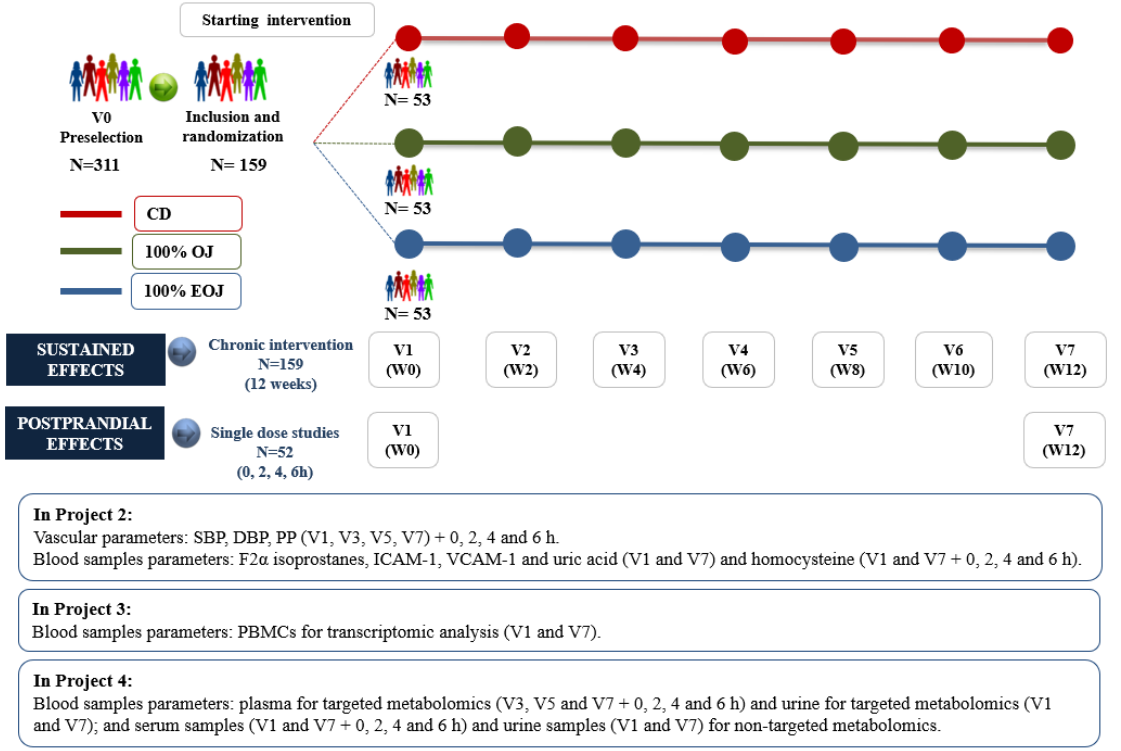

Figure 13. Schema of the CITRUS randomized controlled trial. Abbreviations: CD, control drink; OJ, orange juice; EOJ, hesperidin-enriched orange juice.

Project 2: Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized clinical trial (CITRUS study).

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ORIGINAL CONTRIBUTION

Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial (Citrus study)

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Abstract

Purpose To assess the sustained and acute effects, as well as the influence of sustained consumption on the acute effects, of orange juice (OJ) with a natural hesperidin content and hesperidin-enriched OJ (EOJ) on blood (BP) and pulse (PP) pressures in pre- and stage-1 hypertensive individuals.

Methods In a randomized, parallel, double-blind, placebo-controlled trial, participants ($n = 159$) received 500 mL/day of control drink, OJ, or EOJ for 12 weeks. Two dose–response studies were performed at baseline and after 12 weeks.

Results A single EOJ dose (500 mL) reduced systolic BP (SBP) and PP, with greater changes after sustained treatment where a decrease in diastolic BP (DBP) also occurred ($P < 0.05$). SBP and PP decreased in a dose-dependent manner relative to the hesperidin content of the beverages throughout the 12 weeks ($P < 0.05$). OJ and EOJ decreased homocysteine levels at 12 weeks versus the control drink ($P < 0.05$). After 12 weeks of EOJ consumption, four genes related to hypertension (PTX3, NLRP3, NPSR1 and NAMPT) were differentially expressed in peripheral blood mononuclear cells ($P < 0.05$).

Conclusion Hesperidin in OJ reduces SBP and PP after sustained consumption, and after a single dose, the chronic consumption of EOJ enhances its postprandial effect. Decreases in systemic and transcriptomic biomarkers were concomitant with BP and PP changes. EOJ could be a useful co-adjuvant tool for BP and PP management in pre- and stage-1 hypertensive individuals.

Keywords Orange juice · Hesperidin · Blood pressure · Pulse pressure · Pre-hypertension

Introduction

Flavonoid compounds are the most abundant phenolic compounds in plants, and citrus flavonoids, particularly present in orange juice (OJ), are attracting attention due to their beneficial effects on cardiovascular risk factors [1].

OJ is a main dietary source of flavanones, a subclass of flavonoids, and hesperetin-7-*O*-rutinoside (hesperidin) and naringenin-7-*O*-rutinoside (narirutin) are the main citrus flavanone components [2].

Data from cohort studies reported an inverse association between citrus fruit/flavanone consumption and cerebrovascular disease [3–5] and cardiovascular mortality [6–8]. Antihypertensive, antithrombotic, anti-inflammatory, antilipemic, vasodilator, and antioxidant effects of hesperidin have been reported in animal models [9–11]. Similar outcomes have been observed for narirutin [12].

Recently, hesperidin has been shown to reduce the atherosclerotic plaque area and macrophage foam cell formation in low density lipoprotein (LDL) receptor-deficient mice [11]. The aforementioned properties of hesperidin have been considered to be the mechanisms responsible for the beneficial effects of citrus flavanone consumption on cardiovascular disease in humans [13].

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Concerning the antihypertensive effects of citrus flavanones, data from animal studies showed that hesperetin, a biological metabolite of hesperidin [10], exerts an antihypertensive effect in hypertensive rats but not in normotensive rats [9, 14]. The antihypertensive effect, as well as vasodilatory and anti-inflammatory activities, has been reported to be mediated by the hesperetin-7-*O*-β-D-glucuronide conjugate [10]. In humans, a natural OJ, but not a hesperidin-enriched beverage, decreased blood pressure (BP) in overweight and obese individuals [15]. Similarly, chronic consumption of hesperidin reduced BP in type 2 diabetes patients [16], although no hypotensive effect was observed in healthy or overweight individuals [17, 18]. In individuals at moderate risk of cardiovascular disease (CVD), no changes in BP or other cardiovascular risk biomarkers were observed after a single dose of OJ or a hesperidin supplement at 5 h post intake [19]. Among flavonoids subclasses, flavone and flavan-3-ol compounds, but not flavanones, were related to the prevention of hypertension in a cohort of 87,242 women from the Nurses' Health Study [20].

Thus, data of the antihypertensive effect of hesperidin consumption in humans remain controversial. Therefore, we assessed both the sustained and acute effects, as well as the influence of sustained consumption on acute effects, of real-life doses of OJ and a hesperidin-enriched dose on BP, pulse pressure (PP), and cardiovascular risk biomarkers in pre- and stage-1 hypertensive individuals. Our hypothesis was that hesperidin in OJ would provide benefits on BP and PP not only after sustained consumption but also at postprandial level after a single dose.

Materials and methods

Study population

Participants from the general population were recruited by means of news in the newspapers, social networks, and tableaux advertisements in the Hospital Universitari Sant Joan (HUSJ)-Eurecat, Reus, Spain, between January 2016 and June 2017. From 311 subjects assessed for eligibility, 159 (53 women and 106 men) pre- or stage-1 hypertensive individuals, according to current guidelines [21], were recruited. Inclusion criteria were as follows: age from 18 to 65, systolic blood pressure (SBP) ≥ 120 mmHg, no family history of cardiovascular disease or chronic disease, and willingness to provide informed consent before the initial screening visit. Exclusion criteria were: body mass index (BMI) ≥ 35 kg/m^2, fasting glucose > 125 mg/dL, SBP ≥ 160 mmHg and diastolic blood pressure (DBP) > 100 mmHg or taking antihypertensive medications, hyperlipemia or antilipemic medication; smoking, pregnancy or intending to become pregnant, use of medications, antioxidants, vitamin supplements or adherence to a vegetarian diet, chronic alcoholism, physical activity > 5 h/week, intestinal disorders, anemia (hemoglobin ≤ 13 mg/dL in men and ≤ 12 mg/dL in women), consumption of a research product in the 30 days prior to inclusion in the study, or failure to follow the study guidelines. Participants signed informed consent prior to their participation in the study, which was approved by the Clinical Research Ethical Committee of HUSJ (14-12-18/12aclaassN1), Reus, Spain. The protocol and trial were conducted in accordance with the Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization (GCP ICH) and were reported as CONSORT criteria. The trial was registered at ClinicalTrials.gov: NCT02479568.

Intervention products

Intervention beverages (supplied by the Florida Department of Citrus, USA) were control drink (CD), an OJ containing 690 mg/L of hesperidin (the natural hesperidin content), and an enriched orange juice (EOJ) containing 1200 mg/L of hesperidin. Ferrer HealthTech (Murcia, Spain) provided the Micronized 2S Hesperidin used in EOJ enrichment. The 2S form, the one present naturally in the OJ, is the most bioavailable [18]. Beverages were analyzed for hesperidin and narirutin content using chromatography–mass spectrometry (LC–MS/MS) (Supporting Information Table S1). Daily doses of 500 mL of CD, OJ and EOJ, provided 0 mg/day, 345 mg/day, and 600 mg/day of hesperidin, and 0 mg/day, 64 mg/day, and 77.5 mg/day of narirutin, respectively. Intervention drinks were similar in appearance and smell, and were differentiated only by a code assigned by an independent researcher not related to the study to guarantee blinding. Flavanone contents of the OJ and the EOJ were stable throughout the study.

Study design

A randomized, parallel, double-blind, placebo-controlled clinical trial was performed (Supporting Information Fig. S1). Participants were randomly assigned to one of the three intervention groups—CD, OJ, or EOJ—to consume 500 mL/day of the corresponding beverage for 12 weeks. Nested within the sustained consumption study were two dose–response studies, one at baseline and the other after 12 weeks of sustained consumption, where the 500 mL/dose was administered all at once and changes in the outcomes were recorded in the postprandial state. Participants were randomly allocated to the three intervention groups by a computerized random-number generator made by an independent statistician. PROC PLAN (SAS 9.2, Cary, NC:

83 SAS Institute Inc.) with a 1:1:1 allocation using random block sizes of 2, 4, and 6 was used. Participants, researchers and the statistician remained blinded to the type of product administered throughout the study.

After enrolment and following a 1-week run-in period with a control diet consisting of a maintained lifestyle and normal dietary habits based on nutritionist recommendations, the participants started the intervention trial. However, during the intervention period, the participants were instructed to also maintain their dietary habits, to completely refrain from consuming citrus-containing foods and to limit their total intake of flavonoid-rich foods (tea, coffee, cocoa, wine and other fruit juices) to reduce the possible masking effects that can exert these foods on BP [22, 23]. During the sustained study, participants attended seven visits (V) at the HUSJ-Eurecat. Dose–response postprandial studies, performed at V1 and V7, lasted from 08:00 a.m. to 02:00 p.m., and participants received a light meal before leaving. In addition to the baseline (0 h), blood samples were collected at 2 h, 4 h, and 6 h after the single dose of 500 mL. The adherence of the volunteers to their dietary habits throughout the study was assessed by a 3-day food record at V1, V3, V5, and V7. At each visit, subjects underwent a physical examination by a general practitioner and completed a Physical Activity Questionnaire Class AF [24], and anthropometric measurements were recorded. Samples were stored at -80ºC in the central laboratory's Biobanc of HUSJ-Eurecat (biobanc.reus@iispv.cat) until required for batch analyses.

Compliance measures

The plasma levels of the following biomarkers of nutrient exposures were measured by LC–MS/MS in the plasma samples: hesperetin-7-*O*-β-D-glucuronide, hesperetin-3-*O*-β-D-glucuronide, hesperetin-7-*O*-sulfate, naringin-4-*O*-β-D-glucuronide, naringin-glucuronide and naringin sulfate. The extraction was carried out with a semi-automated process using Agilent Bravo Automated Liquid Handling Platform. Briefly, 20 µL of internal standard (Hesperetin d4) was mixed with 125 µL of plasma and 750 µL of methanol. The mixture was vortexed and centrifuged at 4700 rpm at 4 °C, and then 900 µL was evaporated in a Speed-Vac at room temperature. Residues were reconstituted in 25 µL of MeOH and 75 µL of H_2O (1% of HFor) and injected in the LC–MS/MS, an Agilent 1200 series ultra-high-performance liquid chromatography (UHPLC) system coupled to a 6490 Triple Quad mass spectrometer, with electrospray source ionization (ESI) operating in negative mode.

Main outcome measures

SBP and DBP were measured twice after 2–5 min of respite, with the patient in a seated position, with 1-min interval between, using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). The mean values were used for statistical analyses. Office PP, which represents the force that the heart generates each time it contracts, was determined by the difference between SBP and DBP [25]. The main outcomes were measured in both dose–response and sustained consumption studies.

Secondary outcomes

Homocysteine in serum samples was determined by LC–MS/MS. F2α isoprostanes were determined by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) (Caymanchem, MI, USA) in 24-h urine. Soluble Intercellular Adhesion Molecule-1 (ICAM-1) and Soluble Vascular Cell Adhesion Molecule-1 (VCAM-1) were determined in serum by the Luminex™xMAP technology with the EPX010-40,232-901 kit eBioscience (Thermo Fisher Scientific, Waltham, Massachusetts, USA), in the the Bio-Plex™ 200 instrument (Bio-Rad, Hercules, California, USA). Uric acid was measured by standardized methods on an autoanalyzer 182 (Beckman Coulter-Synchron, Galway, Ireland) in serum samples. All biological biomarkers were measured in the sustained consumption study. Homocysteine was additionally measured after the single 500-mL dose of the corresponding intervention product in both dose–response studies.

Transcriptomic analyses

Gene expression was assessed in peripheral blood mononuclear cells (PBMCs) with an Agilent Microarray Platform (Agilent Technologies, Santa Clara, California, USA) in a subsample ($n = 37$) of participants (11, 15, and 11, in CD, OJ, and EOJ groups, respectively) at baseline and after 12 weeks. PBMC RNA was isolated using Ficoll gradient separation GE Healthcare Bio Sciences, Barcelona, Spain), RNA yield was quantified with a Nanodrop UV–VIS Spectrophotometer and integrity was measured with an Agilent 2100 Bioanalyzer using the Total RNA Nano kit and the Eukaryote Total RNA Nano (Agilent Technologies, Santa Clara, California, USA). Total RNA from the PBMCs was labeled with one color (Cy3) (ref: 5190-2305, Agilent Technologies, Santa Clara, California, USA) and hybridized using a Gene Expression Hybridization Kit (ref: 5188-5242, Agilent Technologies, Santa Clara, California, USA). Image scanning was performed with an Agilent Microarray Scanner System with SureScan

High Resolution Technology (Agilent Technologies, Santa Clara, California, USA). Differentially expressed genes were subjected to functional and biochemical pathway analysis using Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg) and PANTHER (protein annotation through evolutionary relationship classification system (https://www.pantherdb.org/) [26] biochemical pathway databases. The analysis was performed using GeneCodis (https://www.genecodis.dacya.ucm.es [27] software.

Selected genes related to hypertension were validated by PCR. Briefly, to analyze the expression of the genes and validate the DNA array results, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4 Barcelona, Spain) and MyGene Series Peltier Thermal Cycler (LongGene Scientific, Zhejiang, China) and used for reverse transcription. The cDNA was subjected to quantitative reverse transcription-polymerase chain reaction amplification using LightCycler 480 SYBR Green I Master (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain) and a LightCycler 480 II system (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain).

Sample size and power analyses

A sample size of 159 individuals was calculated assuming an expected dropout rate of 20% and a type I error of 0.005 (two sided), which allows at least 80% power for the detection of statistically significant differences in the SBP of 4 mmHg among the groups. The population standard deviation of the SBP was estimated to equal 6 mmHg [28].

Statistical analyses

Descriptive data were expressed as the mean 95% confidence interval (CI). The normality of variables was assessed by the Kolmogorov–Smirnov test. Non-parametric variables were log transformed. ANOVA was used to determine differences in baseline characteristics. Analyses were made by intention-to-treat. Multiple imputation was made by linear regression analysis. Intra-treatment comparisons were performed by means of a general linear model with Bonferroni correction and age and sex as covariables. Inter-treatment comparisons were carried out by analysis of covariance (ANCOVA) model adjusted for age and sex. For transcriptomic analyses, quality control was performed through principal component analyses. Statistical comparisons were performed by Student's *t* test or Welch's *t* test if proceeded. Multiple testing correction was performed using the Benjamini–Hochberg False Discovery Rate (FDR) control procedure. Probes were assumed to be differentially expressed if they presented a P value < 0.05 and a fold change ≤ -0.58 or ≥ 0.58 in log2 scale (corresponding to 1.5-fold difference in natural scale). Calculations were performed using the R statistical language. Comparisons among treatments were carried out by an ANCOVA model adjusted by age and sex and baseline values. Statistical significance was defined as a P value ≤ 0.05 for a two-sided test. Analyses were performed using SPSS for Windows, version 21 (IBM corp., Armonk, NY, USA). All data were analyzed according to the prespecified protocol.

Results

Study participants

Of the 311 subjects who were assessed for eligibility, 152 did not meet the inclusion criteria and were excluded. The remaining 159 participants were randomly allocated to the CD, OJ, and EOJ groups, ($n = 53$ in each group). Ultimately, 129 participants completed the study (43 in the CD, 46 in the OJ, and 40 in the EOJ groups) (Fig. 1). For the dose–response study, of the 52 allocated participants, three discontinued the intervention from the beginning, and six were lost for the second dose–response study. Thus, 52 participants (17 in the CD, 21 in the OJ, and 14 in the EOJ groups) were available for the first dose–response study, and 43 (13 in the CD, 18 in the OJ, and 12 in the EOJ groups) were available for the second dose–response study (Fig. 1). No differences in baseline characteristics were observed among the groups (Supporting Information Table S2). The baseline characteristics of participants in the dose–response study were similar to those of the whole sample. No changes in the level of physical activity were observed from the beginning to the end of the study in any group (data not shown). No differences in dietary intake were observed among groups with exception of protein (% energy) intake, which was greater in the OJ group than in the EOJ one ($P = 0.031$) (Supporting Information Table S3).

Compliance biomarkers

The volunteer compliance intervention was considered optimal because all the compliance biomarkers (hesperetin-7-*O*-β-D-glucuronide, hesperetin-3-*O*-β-D-glucuronide, hesperetin-7-*O*-sulfate, naringerine-4-*O*-β-D-glucuronide, naringenin-glucuronide and naringenin-sulfate) increased significantly during the OJ and EOJ intervention compared with the baseline values and the CD group intervention. At 12 weeks, the metabolite hesperetin-7-β-D-glucuronide was the main differentially expressed metabolite between the OJ and EOJ groups and the CD group ($P < 0.001$).

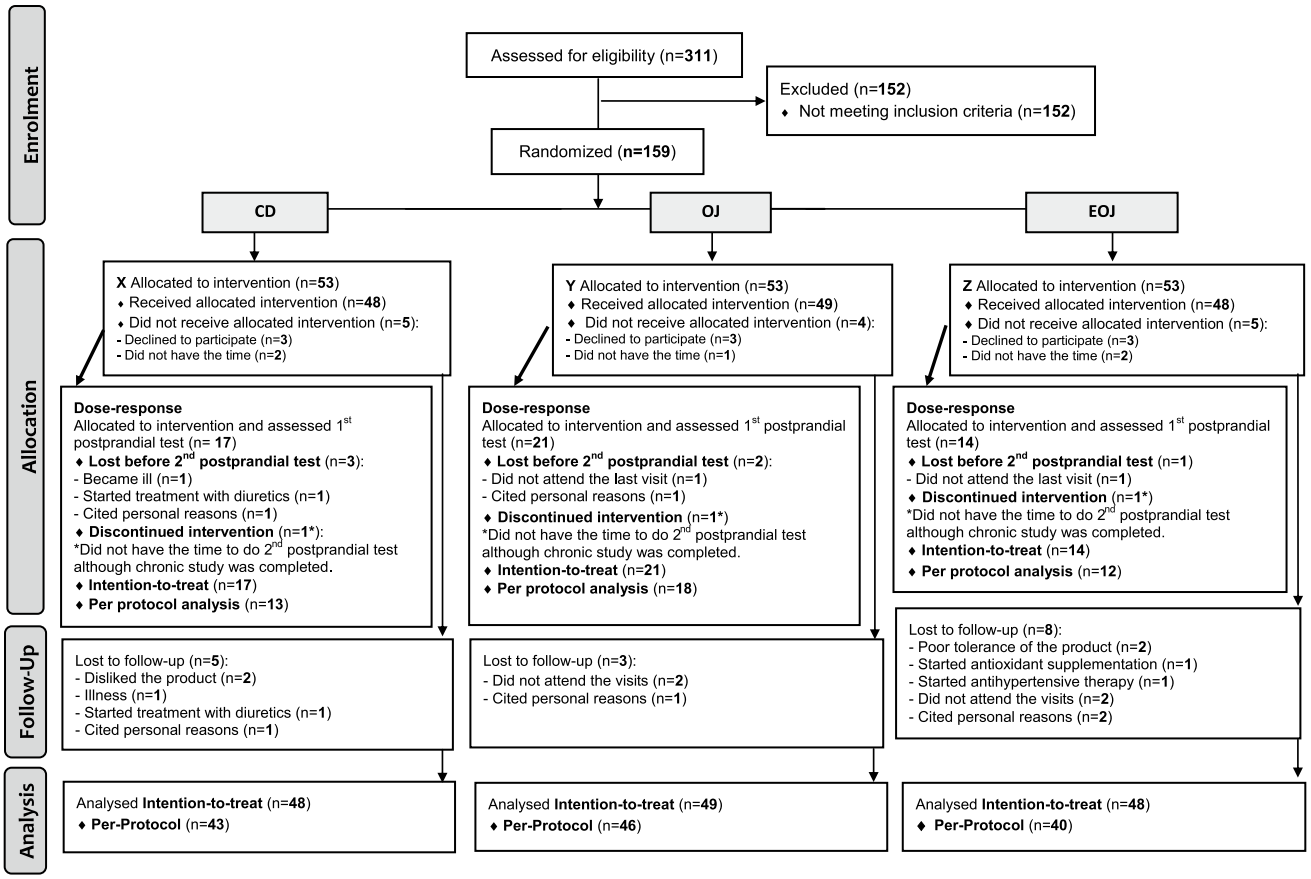

Fig. 1 Flow chart of the study

After 12 weeks of treatments (Fig. 2a), plasma hesperetin-7-β-D-glucuronide increased in a dose-dependent manner with the hesperidin content of the beverage administered ($P < 0.001$ for linear trend), and the increase in the EOJ group was significantly higher than that of the OJ group ($P < 0.05$). In the dose–response studies, plasma hesperetin-7-β-D-glucuronide increased at 4 and 6 h after OJ and EOJ ($P < 0.005$ versus changes in CD), both at the beginning (Fig. 2b) and at the end of the study. The individual changes in plasma hesperetin-7-β-D-glucuronide are depicted in Supplementary Fig. 4 .

Main outcomes

Changes in SBP at 2, 6, 10 and 12 weeks are shown in Fig. 3. SBP decreased in a dose-dependent manner with the hesperidin content of the beverage administered ($P < 0.05$ for linear trend). SBP decreased at weeks 4, 8, and 12 after OJ consumption, the decreases reaching significance versus changes.

in the CD at week 4 and 12 ($P < 0.05$) by mean 95% IC −5.58 (−9.8; −1.3) mmHg and −5.06 (−8.8; −1.3) mmHg, respectively. A borderline significance at week 8 was also observed ($P = 0.056$). After consumption of EOJ, SBP decreased in all evaluated weeks compared to CD, the decreases reaching significance ($P < 0.05$) at all weeks, with the only exception of week 8 in which a borderline significance ($P = 0.078$) was observed. The average of all decreases through the study was −6.35 and −7.36 mmHg for OJ and EOJ interventions, respectively. DBP decreased similarly after all interventions and in all weeks ($P < 0.05$) (data not shown). Changes in PP through the study are shown in Fig. 4. PP decreased in a dose-dependent manner with the hesperidin content of the beverage administered ($P < 0.05$ for linear trend) in all weeks, but in the 12 week, the trend did not reach significance ($P = 0.125$). Concerning dose–response studies, at the beginning of the study (Fig. 5a), significant decreases were observed in SBP at 2 h and in PP at all evaluated times ($P < 0.05$) after a single dose of 500 mL only in the case of EOJ. No changes were observed in DBP values. After 12 weeks of treatment (Fig. 5b), a single dose of 500 mL resulted in changes in BP and PP also only in the EOJ group (Panel B). DBP decreased versus baseline at all evaluated times ($P < 0.05$), and the decrease at 2 h and 6 h reached significance versus changes in CD ($P < 0.05$). Additionally, the observed

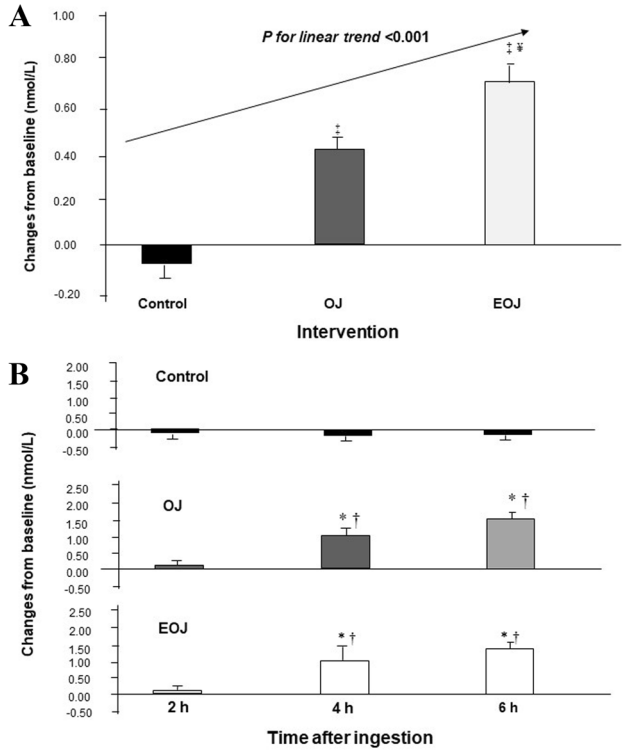

Fig. 2 Changes in plasma hesperitin-7-β-D-glucuronide after ingestion of control, orange juice (OJ), and enriched OJ. **a** After sustained consumption for 12 weeks (500 mL/day). **b** At the beginning of the study after a single dose of 500 mL. $^{*}P<0.05$ versus baseline; $^{\dagger}P<0.05$ versus control group; $^{\ddagger}P<0.001$ versus control; $^{¥}P<0.05$ versus OJ

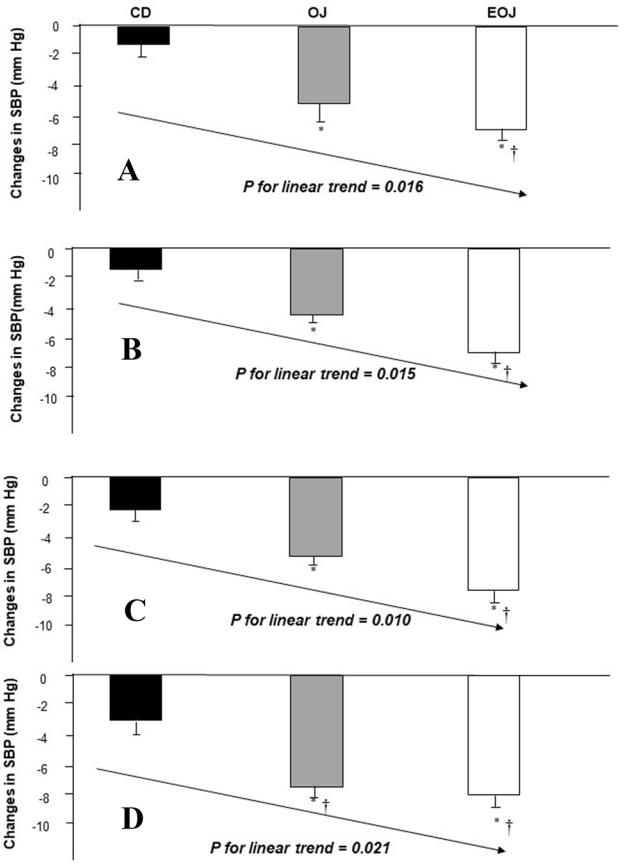

Fig. 3 Changes in systolic blood pressure (SBP) at 2 (**a**), 6 (**b**), 10 (**c**), and 12 (**d**) weeks after sustained consumption of control drink (CD), orange juice (OJ), and hesperidin-enriched OJ (EOJ). $^{*}P<0.05$ versus baseline; $^{\dagger}P<0.05$ versus CD

decreases in SBP at 6 h and those of PP at all evaluated times reached significance versus changes in CD ($P<0.05$). At 4 h and 6 h postprandial after our, an inverse relationship was observed between hesperidin-7-β-D-glucuronide values and those of PP ($R=-0.354$, $P=0.023$, and $R=-0.377$, $P=0.015$, respectively). At 6 h, an inverse relationship was also observed between the increase in hesperidin-7-β-D-glucuronide and SBP values ($R=-0.353$, $P=0.024$).

Secondary outcomes

At week 12 after sustained consumption, homocysteine values decreased after OJ and EOJ treatments, and the decreases reached significance versus changes after the CD treatment. At this time point, homocysteine plasma values decreased in the postprandial state at 2 h and 4 h after OJ and after 2 h of EOJ ingestion ($P<0.05$) (Fig. S2 in the online-only Data Supplement). Uric acid decreased at 12 weeks after EOJ treatment, reaching significance versus changes in the CD treatment ($P=0.044$). ICAM-1 decreased at week 12 after EOJ treatment ($P=0.032$), but no changes were observed between treatments. At week 12 after sustained consumption, uric acid concentrations were directly related to SBP, DBP, and PP ($P<0.05$). No changes were observed in other secondary outcomes. After 12 weeks sustained consumption, the values of SBP directly correlated with those of ICAM-1 ($R=0.251$, $P=0.004$) and VCAM ($R=0.185$, $P=0.036$) (Fig. S3 in the online-only Data Supplement), and the decrease in F2-isoprostanes, although without significance, were directly correlated with the decreases in SBP ($R=0.178$, $P=0.042$).

No adverse events were reported. All products were well tolerated.

In transcriptomic analyses, after the sustained consumption study, four genes related to hypertension were identified: Pentraxin-3 (*PTX3*); NLR family, pyrin domain containing 3 (*NLRP3*); neuropeptide S receptor 1(NPSR1); and nicotinamide phosphoribosyl transferase (*NAMPT*), which were differentially expressed after 12 weeks of treatment. The expressions of the *PTX3* and *NAMPT* genes decreased significantly in PBMC after the EOJ intervention versus the control treatment ($P<0.05$). Figure 6 shows the comparisons among interventions considering the dot axis at

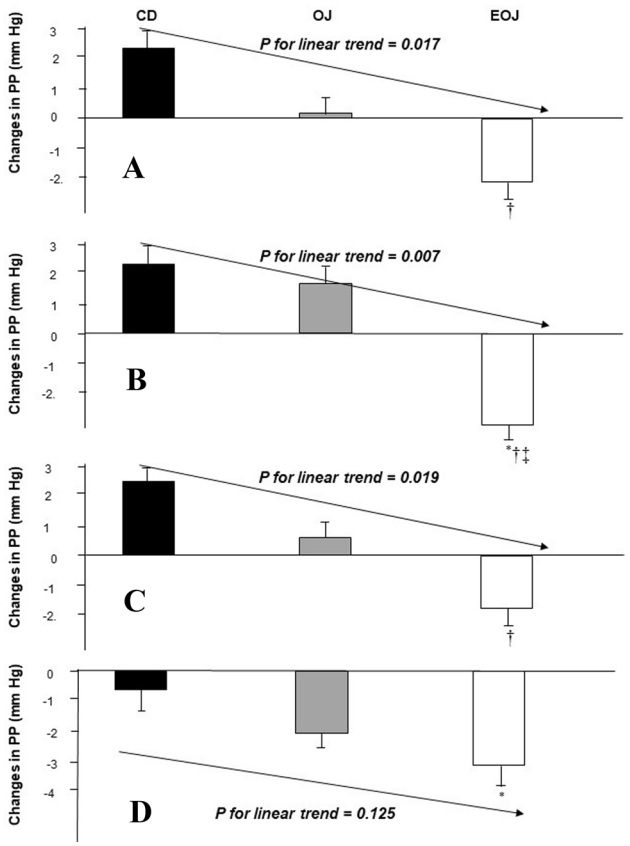

Fig. 4 Changes in pulse pressure (PP) at 2 (**a**), 6 (**b**), 10 (**c**), and 12 (**d**) weeks after sustained consumption of control drink (CD), orange juice (OJ), and hesperidin-enriched OJ (EOJ). $^{*}P<0.05$ versus baseline; $^{\dagger}P<0.05$ versus CD; $^{\ddagger}P<0.05$ versus OJ

$P<0.05$ to be of significance. The decreases in SBP and PP at week 12 were directly related to the decrease in *PTX3* expression ($R=0.393$, $P=0.016$ and $R=0.487$, $P=0.002$, respectively). The decrease in PP at week 12 was directly related to that of *NAMPT* expression ($R=0.344$, $P=0.037$). Although no significance was observed, changes in *NRLP3* expression were inversely related to those of PP at week 12 ($R=-0.420$, $P=0.010$). In turn, expression of *PTX3* at week 12 was directly related to that of *NAMPT* ($R=0.759$, $P<0.001$).

Discussion

In the present study, on the one hand, SBP and PP decreased in a dose-dependent manner with the hesperidin content of the beverage administered throughout the 12 weeks of the study. On the other hand, a single dose of 500-mL EOJ, but no other treatment, reduced SBP, and PP, greater changes when the dose was administered at the end of the study after 12 weeks of sustained treatment where DBP changes were also observed. Thus, these suggested that sustained consumption of hesperidin optimizes acute BP-lowering effects.

After 12 weeks, sustained EOJ consumption-related decreases in uric acid and ICAM were observed. Homocysteine decreased at 12 weeks after OJ and EOJ, and postprandial decreases in homocysteine were also present after single doses of OJ and EOJ at the end of the study. In agreement with the decrease in SBP and PP, *PTX3* and *NAMPT* gene expression decreased in PBMCs at 12 weeks after sustained EOJ treatment.

Currently, the worldwide prevalence of hypertension exceeds 1.3 billion [29] and is the main risk factor for death and disability-adjusted life-years lost during 2010 [30]. A 10-mmHg SBP decrease is associated with reductions of 22 and 41% in coronary heart disease and stroke, respectively [31]. Decreases in SBP with medical therapies range from 5 to 15 mmHg [32]. The average reductions in SBP throughout our study were −6.35 and −7.36 mmHg for the OJ and EOJ interventions, respectively. Our data are in agreement with those obtained after 8–12 weeks of a treadmill exercise program in hypertensive individuals (6.2 mmHg) [33] and after consumption of the Dietary Approaches to Stop Hypertension (DASH) diet (6.74 mmHg) [34] as well as with the results of a meta-analysis reporting a mean reduction of 8 mmHg SBP by regular endurance exercise in hypertensive patients [35]. PP, a surrogate marker of aortic stiffness, is recognized as a powerful and independent risk factor for CVD with prognostic utility beyond BP measurements [36, 37]. Throughout our study, the average PP reductions was −2.41 mmHg after EOJ. A 10-mmHg increase in PP is associated with a 13% increase in all-cause mortality and >20% increase in recurrent myocardial infarction [36].

Throughout the 12 weeks of sustained intervention with 345 mg/day of hesperidin in OJ and 600 mg/day of hesperidin EOJ, we observed decreases in SBP and PP, but not in DBP. Our results are opposite of those reported in overweight men, with a DBP-lowering effect but not an SBP one, after 4 weeks of OJ or a hesperidin-rich capsule providing 292 mg and 146 mg of hesperidin/day, respectively [23]. No benefits on BP were reported after sustained high hesperidin consumption of 549 mg/L/day over 8 weeks in healthy elderly individuals [17]; 6 weeks at 420 mg/day in healthy volunteers [18]; or 3 weeks at 500 mg/day in individuals with metabolic syndrome [38]. In type 2 diabetes patients, however, consumption (500 mg/day) of hesperidin over 6 weeks led to decreases in SBP and DBP [16]. Differences in populations and lengths of treatment could account for discrepancies among studies. If the objective of a study is to improve a specific cardiovascular risk factor, subjects that present symptoms associated with the specific cardiovascular risk factor should be included in the study [39]. Thus, the present study constitutes the first RCT that assesses the effects of hesperidin on BP and PP in pre- and

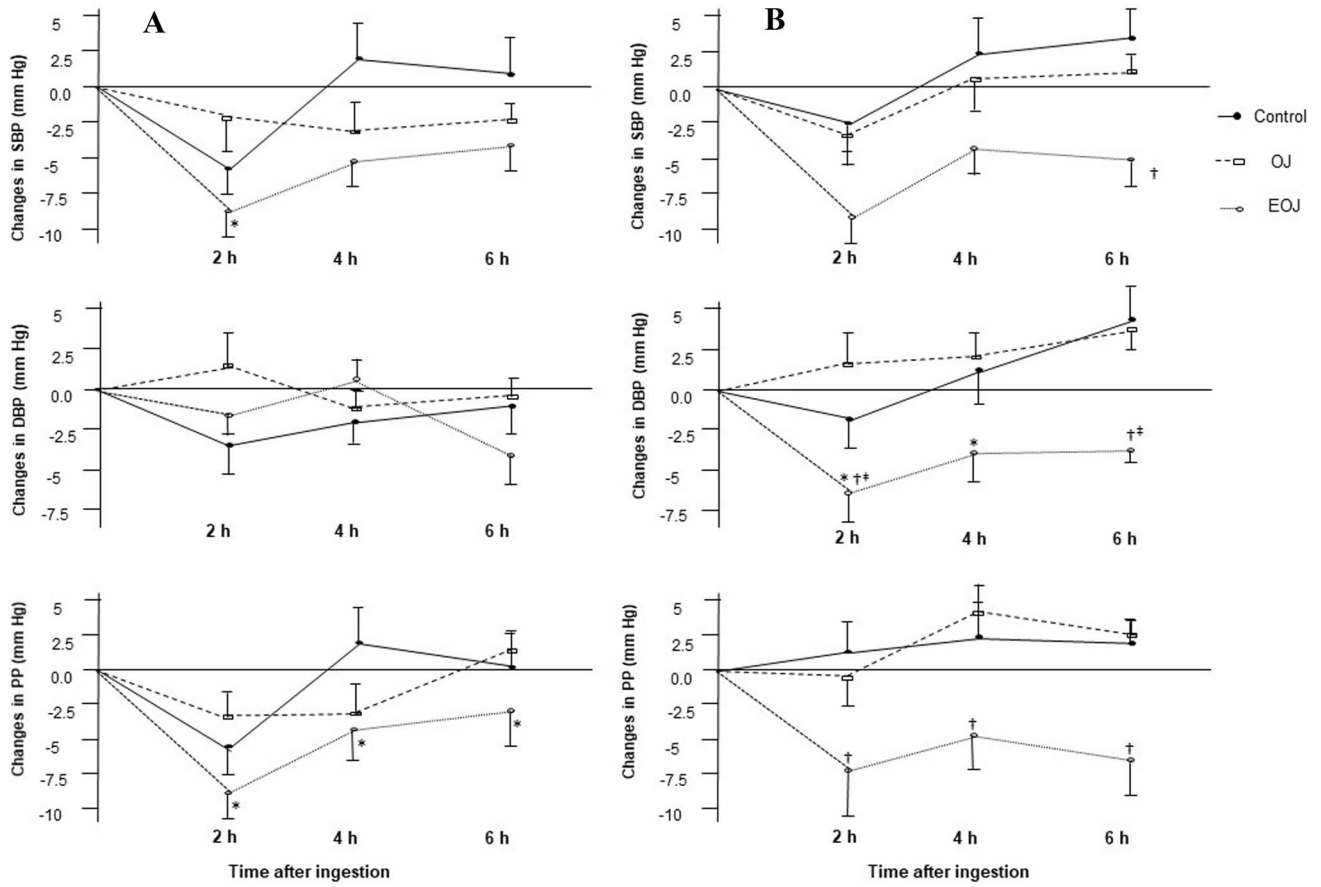

Fig. 5 Changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and pulse pressure (PP) after a single dose of 500 mL of control drink, orange juice (OJ), and enriched OJ at the beginning (**a**) and at the end of the study (**b**)[a]

stage-1 hypertensive subjects; whereas, the populations studied in other RCTs were overweight, obese or diabetic with no hypertension or elevated BP levels [40]. At present, few reports exist concerning the effect of hesperidin on PP. Hesperidin reversed aortic stiffness in mice [41]. Although the beneficial effect of flavonoids on arterial stiffness is emerging [42], our data are the first available to supporting the effect of dietary flavanones on human arterial stiffness.

Mechanisms by which hesperidin could contribute to the control of BP and PP are associated with improvements on endothelial function, oxidative stress, and inflammation [8]. Homocysteine is associated with these risk factors and with a renin–angiotensin system activation to induce a BP increase [43]. In agreement with this, we observe a decrease in homocysteine concomitant with decreases in BP and PP after hesperidin treatments. After 12-week EOJ consumption, ICAM-1 values decreased, and this decrease and those of other inflammatory and oxidative markers were directly related to the SBP decrease. In our work, plasma uric acid decreased after 12-week EOJ consumption. Hyperuricemia is strongly associated with hypertension and arterial stiffness through activation of the NLPR3 inflammasome [44]. Accordingly, in our study changes in *NLPR3* gene expression after 12 weeks were inversely associated with those of PP. After 12 weeks of EOJ consumption, we observed a decrease in PBMC expression of two key hypertension-related genes: *PTX3* and *NAMPT*. Serum levels of PTX3, a marker of inflammation activation, are elevated in hypertensive patients [45], and experimental studies reported a direct role of PTX3 in vascular function and BP homeostasis [46]. NAMPT, also called visfatin, is secreted by visceral fat and is a stimulator of proinflammatory cytokines [47]. NAMPT is elevated not only in hypertensive patients but also in pre-hypertensive patients [48, 49], leading to the proposal that NAMPT is a marker for damage in the pre-hypertensive state [48]. Thus, in our study, the decrease in biochemical and transcriptomic markers could account for the decreases in BP and PP after intake of hesperidin-rich beverages.

One factor that could minimize differences among sustained hesperidin interventions could be the similar contribution of the narirutin present in these treatments. In experimental and human studies, naringin and narirutin showed a hypotensive effect [12, 50, 51]. When comparing the dose–response results on the main outcomes, however, only

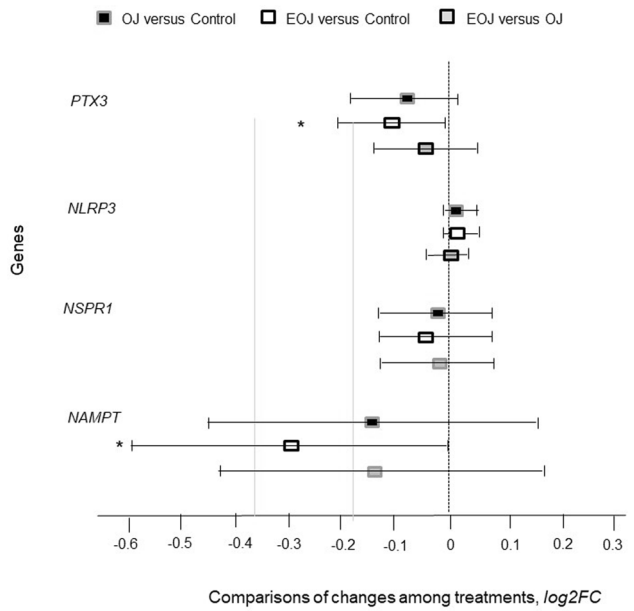

Fig. 6 Comparisons of changes in gene expression (*log2FC*, mean (95%CI) among treatments after interventions. *PTX3,* Pentraxin-3; *NLRP3,* NLR family, pyrin domain containing 3; NPSR1, neuropeptide S receptor 1; and *NAMPT,* nicotinamide phosphoribosyl transferase. The dot axis displays the significance between orange juices at the $P < 0.05$ level. $*P < 0.05$

the EOJ single-dose intervention was capable of decreasing SBP, DBP, and PP at the postprandial level. At present, few reports exist concerning the dose–response effect of hesperidin consumption in humans. No changes in SBP or DBP have been reported at 5 h after OJ or hesperidin supplementation (containing 320 mg of hesperidin) in healthy elderly individuals, despite an increase in hesperidin at this time point [19]. The fact that a unique measurement was obtained after a single dose could explain differences between studies. To the best of our knowledge, our data are the first to report the postprandial benefits of a hesperidin-enriched beverage to BP and PP, as well as the fact that its sustained consumption enhances these benefits.

The study has strengths and limitations. As a strength, the participants' diets were monitored throughout the entire study, and avoiding hesperidin intake and limiting the consumption of flavonoid-rich foods were given as dietary recommendations to all the participants, which is of special interest in nutritional RCTs because these guidelines would limit confounding between other dietary compounds and the dietary intervention [40]. The dietary recommendations were established equally for all the intervention groups (CD, OJ and EOJ), and thus, the possible changes in the metabolome profile and consequently the downstream effects on BP due to these dietary modifications would be equally observed in all the groups, which would result in the control of these changes. Another important strength is that this study constitutes the first human RCT that assessed a compliance marker, hesperitin-7-β-D-glucuronide metabolite, which is associated with PP and SBP values, and thus, these results add robustness to our study.

Multiple measurements throughout the study permitted the assessment of the homogeneity of the results. One limitation is the inability to assess potential interactions between the interventions and other dietary components. Additionally, a larger sample size could have permitted detection of significant differences between both hesperidin treatments. Although BP measurements were performed with maximal care, a 24-h ambulatory BP monitoring could have been more accurate. The fact that participants were pre- and stage-1 hypertensive individuals limits the extrapolation of the results to the general population. Whether additional or different effects would have been observed over longer time periods is unknown, but longer intervention periods could have affected the compliance of the individuals.

In summary, our results show that the intake of hesperidin in OJ decreases SBP and PP after sustained consumption in a dose-dependent manner with the hesperidin content of the beverage administered. Chronic consumption of hesperidin-rich OJ enhances the postprandial response of decreasing SBP, DBP and PP. Decreases in homocysteine, uric acid and inflammatory markers at the systemic level and in *PTX3* and *NAMPT* at the transcriptomic level could account for the observed changes in BP and PP.

Perspectives

In a randomized, controlled clinical trial with pre- and stage-1 hypertensive individuals, we showed that sustained consumption of hesperidin promoted a dose-dependent decrease in SBP and PP with the hesperidin content of the beverage administered. Our data are the first to support an effect of dietary flavanones on human arterial stiffness. Additionally, we report for the first time the postprandial benefits of a hesperidin-enriched beverage to BP and PP, as well as the fact that its sustained consumption enhances these benefits. Regular consumption of OJ, particularly hesperidin-rich OJ, could be a useful co-adjuvant tool for BP management in pre- and stage-1 hypertensive individuals. This fact has public health implications in preventive medicine for reducing the secondary effects of long-term medical treatment of mild hypertension.

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Author contributions RMV, FP, AC, JPdB, LLA, RS: designed the research (project conception, development of overall research plan, and study oversight); RMV, AP, LC, ELL, LPP, JC, AM, FML, YO, MG, MR, LR, JMP, NC, AC, RS: conducted the research (hands-on performance of the experiments and data collection); RMV, AP, LC, ELL, LPP, JMP, NC: provided essential reagents or provided essential materials (applies to authors who contributed by providing animals, constructs, databases, etc., necessary for the research); RMV, AP, MR, JMP, NC, FP, AC, JMdB, RS: analysed data or performed statistical analysis; RMV, AP, LC, ELL, LPP, JC, JMP, NC, FP, AC, JMdB, LLA, RS: wrote the paper (only authors who made a major contribution); RMV, AP, LLA, RS: had primary responsibility for final content.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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Online Supplemental Tables for the manuscript

Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial.

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Table S1. Composition of the intervention products calculated by 500 mL/day*

	Intervention		
	CD	OJ	EOJ
Acidity, %	2.49	2.49	2.49
Sugar, g	43.1	37.7	37.7
Vitamin C, mg	235.3	235.3	235.3
Citric acid, g	3.40	3.40	3.40
Narirutin, mg	ND	64	77
Hesperidin, mg	ND	392	670

*In frozen concentrated canned drink, once diluted 3.4:1 (water to syrup). Abbreviations: CD, control drink; OJ, orange juice; EOJ, enriched orange juice; kcal, kilocalories; ND, non-detectable.

Table S2. Baseline characteristics of participants by intervention group

Variable	CD (n = 53)	OJ (n= 53)	EOJ (n= 53)	P
Age, *y*	45.4 ±13.0	43.3 ± 12.0	43.6 ± 11.8	0.629
Females, *%*	34.0	32.1	34.0	0.981
SBP, *mm Hg*	132 ± 9.94	132 ± 9.11	134 ± 9.82	0.687
DPB, *mm Hg*	79 ± 8.14	80 ± 8.42	79 ± 10.2	0.868
Pulse pressure, *mm Hg*	53 ± 9.09	52 ± 8.05	54 ± 6.74	0.261
Weight, *kg*	77.3 ± 15.4	78.8 ± 12.2	75.9 ± 11.6	0.523
BMI, kg/m^2	26.1 ± 3.8	26.4 ± 3.6	26.1 ± 3.3	0.858
Waist circumference, *cm*	93.0 ± 11.0	91.7 ± 10.9	91.4 ± 10.7	0.766
Waist/height, *cm*	0.54 ± 0.06	0.53 ± 0.07	0.54 ± 0.07	0.790
Conicity index	1.50 ± 0.76	1.30 ± 0.35	1.39 ± 0.62	0.269
Glucose, *mg/dL*	91.6 ± 9.2	93.6 ± 11.6	93.6 ± 9.6	0.517
Cholesterol, *mg/dL*				
Total	196 ± 30.1	198 ± 32.7	196 ± 31.6	0.937
LDL	124 ± 26.4	125 ± 31.5	127 ± 25.1	0.900
HDL	50.9 ±13.4	51.0 ± 14.7	49.8 ± 13.0	0.889
Triglycerides*, *mg/dL*	82 (67-118)	85 (65-121)	81 (63-116)	0.624
Physical activity, *AU*	3.08 ± 0.06	3.12 ± 1.38	3.12 ± 1.26	0.986

Data are expressed as the mean ± standard deviation, or percentages. CD, control drink; OJ, orange juice; EOJ, enriched orange juice; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pulse pressure = SBP-DBP; BMI, body mass index; LDL, low-density lipoproteins; HDL, high-density lipoproteins * median (25^{th} -75^{th} percentiles). AU, arbitrary units: 0, inactive; 1, very low activity; 2, low activity; 3, moderately active; 4, very active. *P* for ANOVA with logarithmic transformation for triglycerides.

Table S3. Energy, nutrients, fibre and alcohol after 12 weeks of intervention.

Variable	Treatment						P*
	Control	P	OJ	P	Enriched OJ	P	
Energy, *kcal/day*							
Baseline	2443 ± 638		2422 ± 618		2488 ± 627		
12-week	2270 ± 691	0.033	2154 ± 610	0.001	2293 ± 565	0.019	NS
HC, *% energy*							
Baseline	37.5 ± 6.3		37.9 ± 5.8		37.5 ± 6.1		
12-week	34.0 ± 8.4	0.008	35.8 ± 7.5	0.042	36.8 ± 7.9	0.533	NS
HC, *grams*							
Baseline	224 ± 55		227± 67		227 ± 66		
12-week	187± 66	<0.001	190 ± 62	<0.001	205 ± 61	0.001	NS
Protein, *% energy*							
Baseline	17.6 ± 2.9		16.6 ± 2.4		17.6 ± 3.8		
12-week	17.9 ± 3.6	0.578	17.7 ± 3.5	0.028	16.8 ± 2.9	0.142	0.031†
Protein, *grams*							
Baseline	104 ± 29.5		99.0 ± 26.0		106 ± 28.9		
12-week	100 ± 32.6	0.287	93.8 ± 28.6	0.204	94.3 ± 2.,3	0.003	NS
Total fat, % *energy*							
Baseline	43.0 ± 5.2		43.1 ± 5.7		41.9 ± 4.8		
12-week	45.5 ± 7.7	0.036	43.8 ± 6.1	0.496	43.1 ± 5.2	0.230	NS
Total fat, *grams*							
Baseline	120 ± 42.4		118 ± 33.8		118 ± 34.7		
12-week	118 ± 44.8	0.759	107 ± 36.7	0.032	112 ± 32.6	0,310	NS

SFA, *% energy*							
Baseline	12.1± 2.4		12.4 ± 2.8		12.0 ± 2.7		
12-week	12.6 ± 3.1	0.233	13.3 ± 3.3	0.092	11.9 ± 2.3	0.886	NS
SFA, *grams*							
Baseline	34.7± 14.8		34.2 ± 130		34.2 ± 12.0		
12-week	33.8 ± 15.4	0.662	33.1 ± 14.2	0.572	30.8 ± 9.2	0.111	NS

Table S3 (cont.)

MUFA ,*% energy*							
Baseline	19.1 ± 4.1		19.9 ± 2.9		19.4 ± 4.3		
12-week	20.1 ± 4.2	0.217	19.6 ± 3,8	0.566	19.5 ± 3.8	0.869	NS
MUFA, *grams*							
Baseline	53.3 ± 21.0		53.8 ± 15.5		53.0 ± 16.6		
12-week	51.9 ± 21.2	0.641	46.5 ± 13.3	0.003	50.0 ± 14.3	0.270	NS
PUFA, *% energy*							
Baseline	8.3 ± 3.6		7.4 ± 2.5		7.6 ± 2.3		
12-week	8.9 ± 2.8	0.140	7.6 ± 2.6	0.549	8.1 ± 2.9	0.320	NS
PUFA, *grams*							
Baseline	22.8 ± 9.9		19.8 ± 7.6		21.4 ± 9.4		
12-week	22.1 ± 9.2	0.612	18.9 ± 10.4	0.431	23.3 ± 16.9	0.495	NS

Fibre, *g/day*[a]							
Baseline	23.1 (16.1-30.2)		21.0 (15.9-27.4)		24.0 (19.4-28.6)		
12-week	18.5 (13.3-23-.7) <0.001		17.0 (13.3-21.2)	< 0.001	19.0 (15.6-27.2)	0.028	NS
Alcohol, *g/day*[a]							
Baseline	1.69 (0.00-8.8)		4.51 (0,56-10.7)		5.97 (0.36-22.8)		
12-week	3.60 (0.02-13.6)	0.068	6.43 (0.66-13.1)	0.279	6.30 (0.00-14.9)	0.589	NS

Data are expressed as the mean ± standard deviation: a median (25-75th percentile).
H C, carbohydrates; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Intra-treatment comparisons by Student's t test and Wilcoxon test for related samples.
* P value for differences among treatments. ANOVA and Mann-Whitney tests. †P for Enriched OJ versus OJ.

Online Supplemental Figures for the manuscript

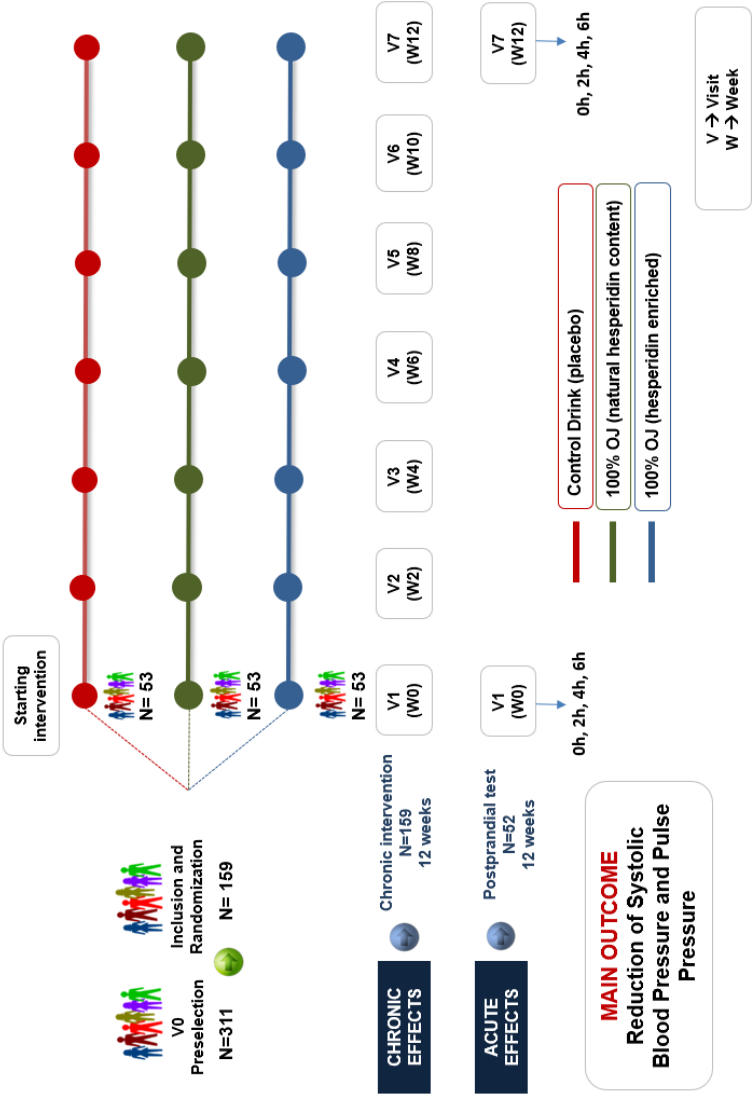
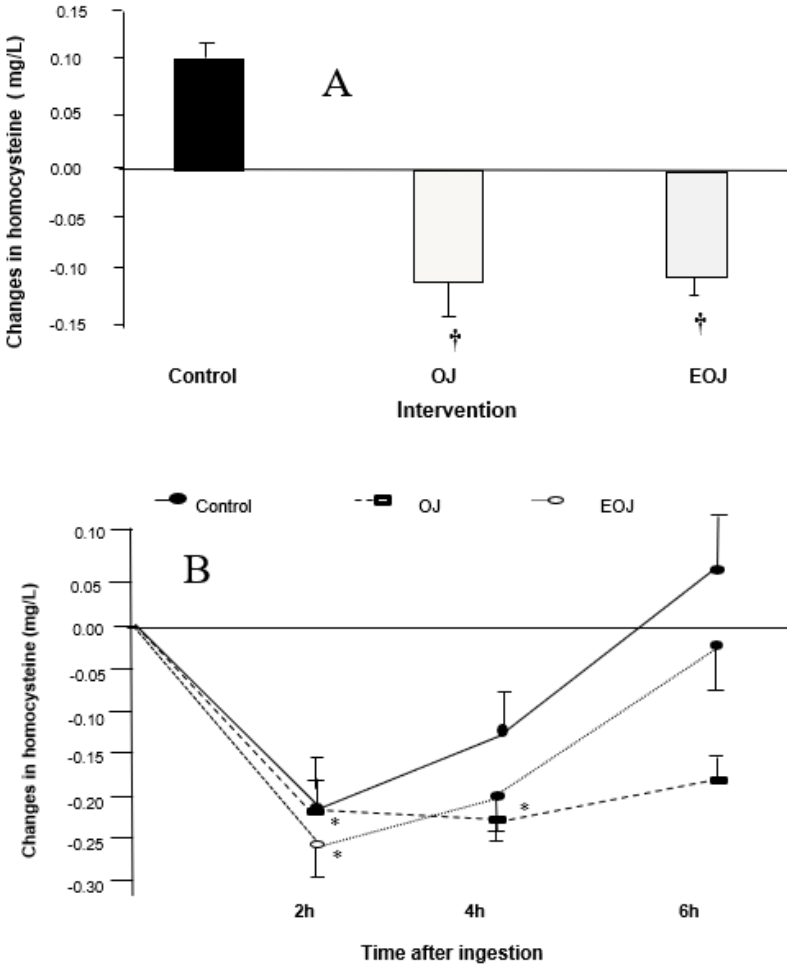


Figure S1. Schema of the study interventions



* $P< 0.05$ versus its baseline; † $P<0.05$ versus control group; ‡ $P<0.05$ versus OJ.

Figure S2. Changes in homocysteine at 12 weeks:(A) after sustained consumption; (B) after a single 500 mL dose

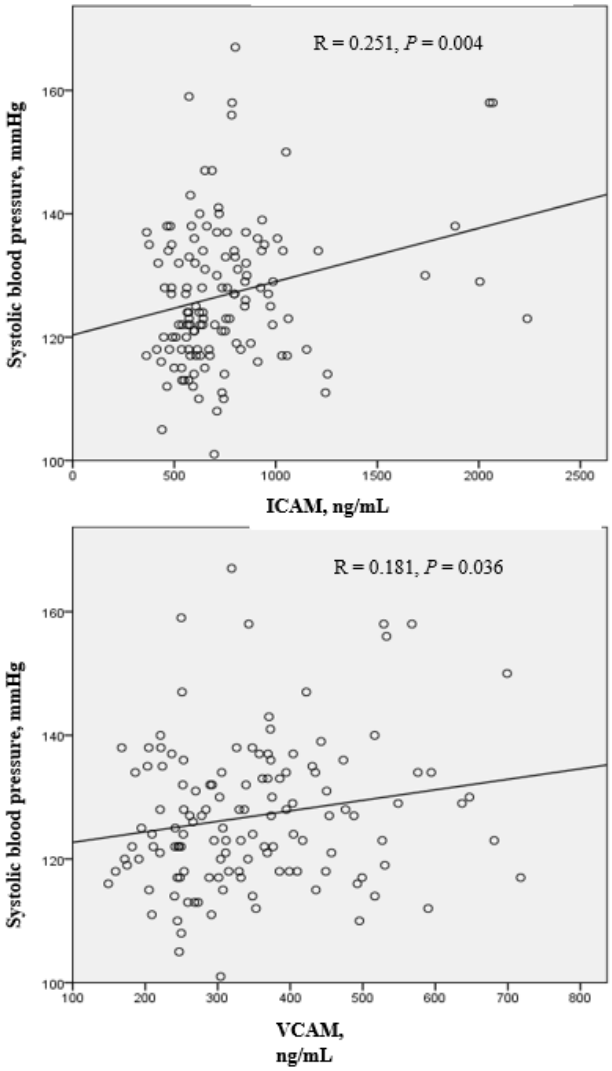
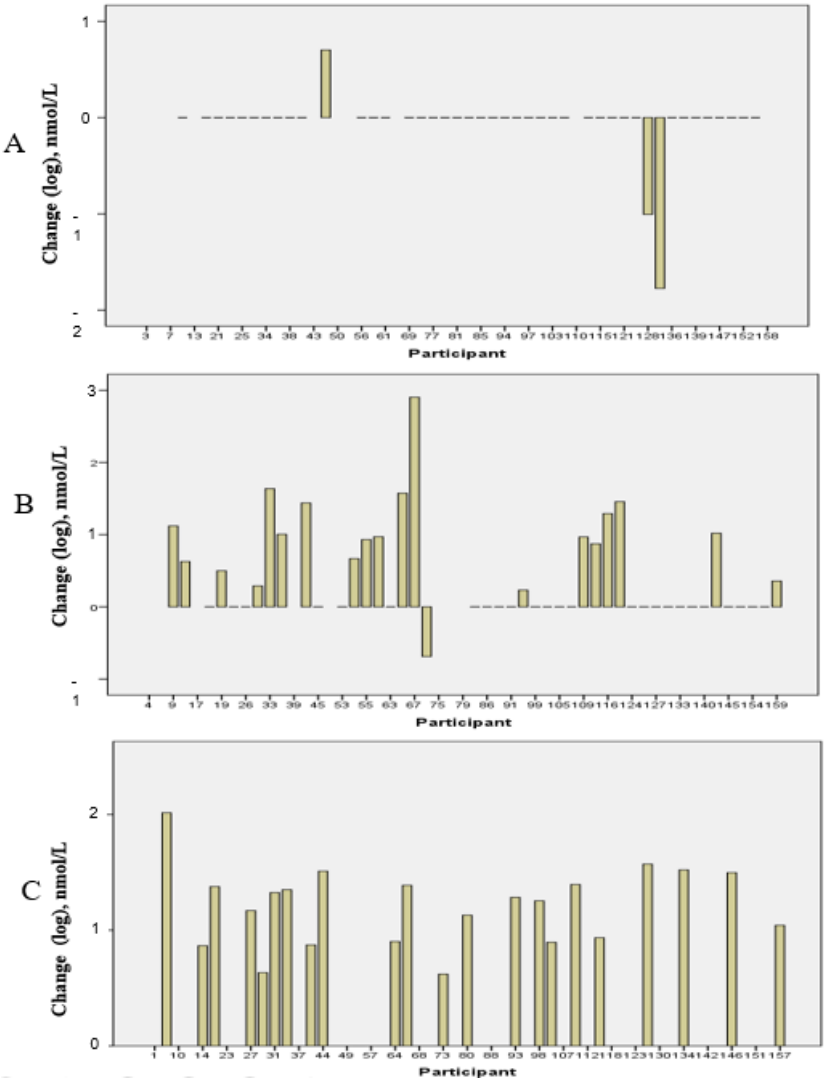


Figure S3. Relationship of systolic blood pressure and ICAM and VCAM after 12 weeks of sustained consumption of treatments

Sup Fig 4. Individual changes in plasma hesperitin-7-b-D-glucuronide after: A, control; B, oranje juice (OJ); and C, enriched OJ



Project 3: Effect of the consumption of hesperidin in orange juice on the transcriptomic profile of subjects with pre- and stage 1 hypertension: a randomized controlled trial (CITRUS study) (Editor submitted)

Effect of the consumption of hesperidin in orange juice on the transcriptomic profile of subjects with pre- and stage 1 hypertension: a randomized controlled trial (CITRUS study)

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Abbreviations

CD:	control drink
CVD:	cardiovascular diseases
CVDR:	cardiovascular diseases risk
CVDRFs:	cardiovascular diseases risk factors
DBP:	diastolic blood pressure
EOJ:	hesperidin-enriched orange juice
IPA:	ingenuity pathway analysis
LncRNA:	long non-coding RNA
OJ:	orange juice
PBMCs:	peripheral blood mononuclear cells
SBP:	systolic blood pressure

Keywords: transcriptomic analysis, hypertensive subjects, hesperidin, orange juice, PBMCs, plasma.

ABSTRACT

Scope

Hesperidin exerts cardiovascular beneficial effects, but its mechanisms of action remain undefined. The aim of this work was to determine whether the sustained and single dose consumption of hesperidin in orange juice (OJ) and hesperidin-enriched orange juice (EOJ) can change the transcriptomic profile of subjects with pre- and stage 1 hypertension to elucidate possible mechanisms of action of the hesperidin.

Methods and results

For transcriptomic analysis, peripheral blood mononuclear cells were obtained from 37 subjects with pre- and stage 1 hypertension from CITRUS study who were randomized to receive for 12 weeks: control drink (CD; n=11), OJ (containing 392 mg of hesperidin; n=15) or EOJ (containing 670 mg of hesperidin; n=11). At baseline, a single dose 6-hour study in each group was also performed. After the single dose consumption, EOJ versus OJ, downregulated DHRS9 gene which is related with insulin resistance. Compared to CD, sustained consumption of EOJ downregulated 6 proinflammatory while after OJ consumption only 1 proinflammatory gene was downregulated. Moreover, sustained consumption of EOJ versus OJ, downregulated acute coronary syndrome gene related (SELENBP1).

Conclusion

A single dose consumption of EOJ could protect from insulin resistance. Moreover, EOJ decrease the expression of proinflammatory genes after sustained consumption providing a possible mechanism of action on inflammation pathway.

1. INTRODUCTION

Hesperidin is a naturally occurring flavonoid present in citrus fruits and is found at particularly high concentrations in orange fruits and orange juice (OJ).(9) The beneficial effects of hesperidin on cardiovascular risk factors have been elucidated and integrated in a recently published systematic review of animal studies and randomized human clinical trials.(130) The beneficial effects of hesperidin consumption on the lipid profile and glucose levels have been observed in rats, but the results from human studies remain unclear.(130) Some studies have related hesperidin to hypotensive,(98,131) hypolipemiant,(132) anti-inflammatory(131) and antioxidant(99) effects. Consistently, we recently reported that the sustained and acute consumption of hesperidin in OJ decreases the systolic blood pressure (SBP) and pulse pressures in subjects with pre- and stage 1 hypertension, and the sustained consumption of hesperidin-enriched OJ (EOJ) enhances the postprandial effect of hesperidin compared with that obtained with a single dose.(133) Thus, the consumption of hesperidin via OJ could be an interesting strategy for the SBP levels and treatment of cardiovascular disease risk factors (CVDRFs).(134)

In contrast, the impacts of different bioactive compounds on gene expression have been clearly established and have gained much interest in research because better comprehension of these effects can be used to prevent, detect and treat chronic diseases.(135) Therefore, knowledge about nutrient-gene interactions is key for obtaining more information about new mechanisms of action, and transcriptomics can provide such knowledge because compares the transcriptomes, which are sets of RNA transcripts of cells, tissues or organisms under specific conditions. For complex diseases, such as cardiovascular disease

(CVD) and cancer, a transcriptomic approach offers the possibility to identify novel mechanisms and thus further characterize disease pathophysiology.

Different families of phenolic compounds have the ability to modify gene expression profiles.(53) Specifically, phenolic compounds from virgin olive oil improved cholesterol efflux gene expression in humans(12) and can modify the whole transcriptome to exert beneficial effects on CVD and cancer.(136) Similarly, other phenolic compounds such as flavonoids, such as resveratrol and hesperidin, have been linked to effects on the transcriptome. Thus, previous studies have shown that resveratrol consumption improves vascular function in older adults.(137) Moreover, the chronic consumption of hesperidin can change the expression of leucocyte genes to exert an anti-atherogenic and anti-inflammatory effects in overweight subjects.(138) However, the molecular mechanism through which hesperidin influences metabolic pathways via transcriptome changes remains unclear. To the best of our knowledge, a transcriptomic analysis that simultaneously assesses the impacts of sustained and acute hesperidin consumption in human peripheral blood mononuclear cells (PBMCs) of subjects with hypertension has not been performed. PBMCs are highly useful for demonstrating the capacity of cell systems to interact with nutrients and bioactive food compounds.(139,140)

The aim of this work was to determine whether the sustained and single dose consumption of hesperidin in OJ and EOJ can change the transcriptomic profile of PBMCs of subjects with pre- and stage 1 hypertension to elucidate possible mechanisms of action of the hesperidin and their role in CVD.

2. EXPERIMENTAL SECTION

2.1 Subjects

The study population was included in our previous randomized clinical trial.(133) The subjects were 159 men and women with pre and stage 1 hypertension who did not smoke, had no family history of CVD, SBP ≥ 120 mm Hg and < 159 mm Hg, diastolic blood pressure (DBP) < 100 mm Hg,(34) and were not taking any anti-hypertensive medications.

2.2 Test drinks

The three interventional drinks were provided by the Florida Department of Citrus from the USA: a control drink (CD), OJ with the natural content of hesperidin, and EOJ, which contains the 2S form of hesperidin, the naturally form in OJ and a bioavailable form (micronized 2S hesperidin; Ferrer Health Tech, Murcia, Spain).(103) The composition of the three test drinks is detailed in Supporting Information Table S1.

2.3 Dosage information

The subjects orally consumed 500 mL/day of the corresponding test drink: CD (0 mg/day of hesperidin), OJ (345 mg/day of hesperidin) and EOJ (600 mg/day of hesperidin).

2.4 Study design

A randomized, parallel, double-blind, placebo-controlled clinical trial was performed and registered at Clinical-Trials.gov under NCT02479568. All volunteers were randomly assigned to one of the three intervention groups: CD, OJ and EOJ groups. The sustained intervention was conducted for 12 weeks and nested, two 6-hour single dose studies, one at baseline and the other after 12 weeks, were performed.(133) For the transcriptomic analysis in the sustained study,

PBMCs were obtained from blood samples collected under fasting conditions at baseline and after 12 weeks. For the transcriptomic analysis in the single dose study, PBMCs were obtained at baseline and after 6 hours only in the single dose study conducted at the beginning of the study.

2.5 Transcriptomic analysis

Whole gene expression in PBMCs from whole blood samples collected under fasting conditions in both the dose-response and sustained studies was assessed using the Agilent Microarray Platform (Agilent Technologies, Santa Clara, California, USA). Total PBMC RNA was isolated by Ficoll gradient separation (GE Healthcare Bio Sciences, Barcelona, Spain). The RNA yield was quantified with a Nanodrop UV-VIS spectrophotometer, and the RNA integrity was measured with an Agilent 2100 Bioanalyzer using the Total RNA Nano kit and the protocol Eukaryote Total RNA Nano (Agilent Technologies, Santa Clara, California, USA). Total RNA (100 ng) from the PBMCs was labelled with Cy3 (ref: 5190-2305, Agilent Technologies, Santa Clara, California, USA) and hybridized using a Gene Expression Hybridization Kit (ref: 5188-5242, Agilent Technologies, Santa Clara, California, USA), on an Agilent SurePrint G3 Human Gene Expression 8x60K v3 Microarray (Design ID 072363) with SurePrint Technology (Agilent Technologies, Ref. G4851C). Image scanning was performed with a 3 μm resolution using an Agilent Microarray Scanner System with SureScan High-Resolution Technology (ref: G2565CA, Agilent Technologies, Santa Clara, California, USA). The Feature Extraction version 12.0.1.1 software (Agilent Technologies) was used for data extraction.

Gene expression and real-time PCR analysis

To analyse the gene expression in the samples and validate the DNA array results, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4 Barcelona, Spain). A MyGene Series Peltier Thermal Cycler (LongGene Scientific, Zhejiang, China) was used for reverse transcription. The reaction was performed according to the manufacturer's instructions. The cDNA was amplified by quantitative reverse transcriptase polymerase chain reaction using LightCycler 480 SYBR Green I Master (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain) and a LightCycler 480 II system (Roche Diagnostic, Sant Cugat del Vallès, Barcelona, Spain). The primers used for the different genes are described in Supporting Information Table S2 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as percentages compared with the control group (CD) using the $-2^{\Delta\Delta Ct}$ method (141) and the RPLP0 gene was used as an endogenous control. Real-time PCR analysis was performed in a subset of six genes: DHRS9, DSP, TNF, NAMPT, PTX3 and IER3; and in a subsample of the samples collected: 22 samples for DHRS9, 18 for DSP, 24 for TNF, 19 for NAMPT, 17 for PTX3 and 20 for IER3.

2.7 Data analysis

Quality control was performed through principal component analyses. Statistical comparisons were performed by Student's t-test or Welch's t-test. Differentially expressed genes were genes with that met the following criteria: P-value < 0.05 and fold change <= -0.58 or >= 0.58 in the log2 scale (corresponding to a 1.5-fold difference in natural scale). Treatment comparisons were performed with an ANCOVA model adjusted for age, sex and baseline values. Statistical significance

was defined by a P value ≤ 0.05 from a two-sided test. SPSS for Windows (version 21; IBM Corp., Armonk, New York, USA) was used for the analyses. Differentially expressed genes were subjected to functional and biochemical pathway analyses using Gene Ontology and the KEGG (Kyoto Encyclopedia of Genes and Genomes (www.genome.jp/kegg) and PANTHER (protein annotation through evolutionary relationship classification system (http://www.pantherdb.org/) biochemical pathway databases, respectively. The analysis was performed using GeneCodis (http://genecodis.dacya.ucm.es) software. For biological interpretation, the functions, pathways and networks of the results that showed significant differences between the groups were analysed by Ingenuity pathway analysis (IPA; www.ingenuity.com), which explores the possible metabolic cell signalling pathways that were up- and downregulated after the intervention.

3. RESULTS

3.1 Baseline characteristics of the participants

PBMC samples were collected from 37 of the 159 subjects included in the original randomized clinical trial after both the single dose and sustained interventions, and these subjects were thus candidates for transcriptomic profile evaluation.(133) These 37 participants were assigned to the three groups: CD, N = 11; OJ, N = 15; and EOJ, N = 11. The baseline characteristics of the participants of each group are detailed in Table 1. The volunteers ranged in age from 41 to 65 years, had a normal weight or were overweight, and had pre- and stage 1 hypertension. No differences in the baseline characteristics were found among the three groups.

3.2 Clinical results

In accordance with our previously published results,(133) the consumption of hesperidin in OJ and EOJ for 12 weeks decreased the SBP of subjects with pre- and stage 1 hypertension by an average of -6.35 mm Hg and -7.36 mm Hg, respectively. Additionally, the pulse pressure, which is a recognized independent risk factor for CVD, decreased by -2.41 mm Hg after the consumption of EOJ. No significant changes in the DBP were observed. Additionally, the homocysteine and uric acid levels decreased after the consumption of OJ and EOJ. In contrast, the acute consumption at baseline of a single dose of hesperidin in EOJ reduced the SBP, pulse pressure and homocysteine levels, and after 12 weeks of treatment resulted in greater changes in these variables as well as a decrease in the DBP.

Moreover, as a new result, the plasminogen activator inhibitor type 1 (PAI-1) levels decreased after EOJ consumption versus CD (Mean change: -0.191; 95% CI: -0.32; -0.06; P=0.004) and also versus OJ (Mean change: -0.199; 95% CI: -0.33; -0.07; P=0.002).

3.3 Gene expression profile

After sustained and acute consumption, the number of statistically or borderline differentially expressed genes ($P < 0.05$ or $P < 0.10$, respectively) after OJ and EOJ compared with the levels obtained with the CD are represented in Figure 1. Compared with their expression in the CD group, 3 genes were commonly differentially or potentially differentially expressed after consumption of a single dose of hesperidin in OJ and EOJ. In addition, compared with their expression in the CD group, after the consumption of hesperidin in OJ and EOJ for 12 weeks, 16 genes were commonly differentially or potentially differentially expressed compared with their levels in the CD group.

Gene expression after dose-response intervention

Comparisons of the single dose gene expression changes in PBMCs of individuals with pre-a and stage 1 hypertension among the three treatment groups are shown in Table 2. Three differentially expressed genes ($P < 0.05$) were observed after the single dose consumption of OJ compared with the CD. Two of these three genes, DHRS9 and PKDL1, were significantly upregulated, while the other gene, TNFAIP3, was downregulated. Moreover, 5 genes were borderline differentially expressed ($P < 0.10$) after OJ consumption. The comparison of the EOJ and CD groups revealed no differentially expressed genes, but 6 genes were borderline differentially expressed ($P < 0.10$). Finally, the comparison of the EOJ with OJ group revealed that DHRS9 gene was significantly downregulated ($P < 0.05$). All these genes are mainly related to the inflammation pathways and insulin resistance.

Gene expression after sustained intervention

Comparisons of the gene expression changes in PBMCs from individuals with pre- and stage 1 hypertension among the three treatment groups after 12 weeks of sustained consumption of hesperidin are shown in Table 3. After 12 weeks, we identified 12 genes that were differentially expressed in the OJ group compared with the CD group, and these included seven upregulated genes (CCL20, FAM53B, LINC 01220, lncRNA SNRPD3-2, lncRNA NFKBID-1, lncRNA PDE3B-1, and LOC101929524) and five downregulated genes (TNF, TMPO-AS1, BPIFB3, lncRNA ACOT-13 and CCT8-1). Moreover, 13 genes were borderline differentially expressed ($P < 0.10$) after OJ consumption. After 12 weeks, 18 genes showed differential expression in the EOJ group compared with the CD group, and these included four upregulated genes (DSP, FAM53B, lncRNA SNRPD3-2 and lncRNA

SLC39A8-1) and 14 downregulated genes (TNF, IL1B, CCL3L3, CXCL2, CXCL8, PTGS2, IER3, PTX3, KMT22-AS1, ATP2B1-AS1, lncRNA CCT8-1, lncRNA GRK3-1, lncRNA CCDC117-1 and LOC644090). Moreover, 17 genes were borderline differentially expressed ($P < 0.10$) after EOJ consumption. Finally, after 12 weeks, 4 genes showed differential expression between the EOJ and OJ groups: one gene (lncRNA IYD) was upregulated, and 3 genes (SELENBP1, ALAS2 and BTBD19) were downregulated.

The 12 differentially expressed genes after OJ were related to inflammation (2 genes), cancer (1 gene) and unknown functions (9 genes). After EOJ, the 18 differentially expressed genes were related to inflammation (6 genes), endothelial function (2 genes), hypertension (1 gene), and unknown functions (9 genes).

A summarized table of results are detailed in Supporting Information Table S4.

Pathway analysis

IPAs were performed only with the genes that showed significantly and borderline significantly differential expression after sustained consumption of hesperidin because the number of differentially expressed genes after acute consumption was insufficient for the pathway analysis. None of the long noncoding RNAs (lncRNAs) that were showed significantly or borderline significantly differential expression after 12 weeks of hesperidin consumption could be used for the pathway-based IPA due to the scarcity of the related literature. The biological networks based on the differentially expressed genes after sustained consumption of OJ and EOJ are represented in Figure 2. The overlapping graphical representation of the two most important

networks identified by the IPAs of the differentially expressed genes after OJ and EOJ consumption compared to CD consumption, are shown in panels A and B, respectively, and the up- and downregulated genes are shown in red and green colours, respectively. The top canonical pathways, diseases and functions regulated by sustained consumption of OJ and EOJ and the implicated genes are shown in Table S3 in the Supplementary Information. The top diseases and functions related to the differentially expressed genes identified after the consumption of hesperidin in OJ for 12 weeks were the inflammatory response, cell-to-cell signalling and interaction, organismal injury and abnormalities, and the haematological and cardiovascular systems. After the consumption of hesperidin in EOJ for 12 weeks, the top diseases and functions were the inflammatory response, organismal injury and abnormalities, cellular growth and proliferation, lipid metabolism and the haematological system.

3.4 Results of the real-time PCR analysis

Figure 3 shows the real-time PCR validation of the arrays results for a subset of 6 genes, which demonstrated that the directions of the differences induced by the interventions were consistent with both techniques.

4. DISCUSSION

In the present work, we demonstrated that the consumption of 500 mL/day for 12 weeks of OJ and EOJ can change the transcriptomic profile of PBMCs of subjects with pre- and stage 1 hypertension. Our results showed the single dose consumption of EOJ compared to OJ, downregulated the gene DHRS9 reducing the insulin resistance.

Moreover, the sustained consumption of EOJ, which provides a higher dose of hesperidin, can induce greater changes in the expression profile of PBMCs, compared with those obtained with the lower dose of hesperidin in OJ, since EOJ downregulated more pro-inflammatory genes.

Interestingly, the differential expression of a gene related to insulin resistance was only observed after the single dose consumption of hesperidin in OJ and EOJ but not after sustained consumption for 12 weeks. The consumption of a single dose of hesperidin in OJ significantly upregulated the expression of DHRS9, whereas the consumption of a single dose of hesperidin in EOJ downregulated DHRS9 gene expression compared to CD. DHRS9, an oxidoreductase/decarboxylase, promotes insulin resistance by activating lipid metabolism.(142) Accordingly, we hypothesized that the presence of fructose in OJ could increase DHRS9 expression and thereby increases fructose metabolism, the plasma triglyceride levels and insulin resistance;(142) whereas the higher doses of hesperidin in EOJ could counteract the effects of the fructose content to induce downregulation of the DHRS9 gene. The evidence suggests that the consumption of 50-100 mg/kg body weight/day hesperidin improves insulin resistance and decreases the glucose plasma levels in rats.(143,144) Because this dose range of hesperidin for animals is equivalent to the hesperidin dose present in EOJ (670 mg/day), the results might explain the different gene expression profiles detected after a single dose between the two test drinks, whereas positive results were observed after EOJ consumption.

Moreover, the consumption of a single dose of hesperidin in EOJ induced the borderline significant upregulation of different pro-inflammatory genes, including the gene encoding the chemokine CXCL2. CXCL2, which is also called macrophage inflammatory protein 2-alpha, produces endogenous signals during the first steps of inflammasome activation to generate pro-inflammatory cytokines(145) A recently published review reported that CXCL2 overexpression contributes to atherosclerotic plaque formation, inflammation in obesity and the induction of diabetes, which demonstrates that CXCL2 might be a therapeutic target in CVD.(146) Additionally, the consumption of a single dose of EOJ borderline significantly downregulated the expression of the CCL3-encoding gene. CCL3 is a chemokine with pro-inflammatory activity and serves as an independent risk factor in subjects with acute coronary syndromes because high levels of CCL3 are associated with short-term mortality.(147) Additionally, high levels of this chemokine are associated with future ischaemia events in subjects with acute myocardial infarction (148) Thus, downregulated CCL3 gene expression can suggest a role for this chemokine in the prevention of inflammation related to cardiovascular disease risk (CVDR).

The sustained consumption of hesperidin in OJ and EOJ induces significantly differential expression of different pro-inflammatory genes. In this sense, the consumption of OJ and EOJ for 12 weeks significantly downregulated TNF gene expression. Experimental studies have shown that the cytokine TNF is associated with hypertension and renal injuries in hypertensive rats and mice.(149) In addition, the intracerebroventricular administration of TNF to

normotensive and hypertensive rats increases their blood pressure and thereby influences angiotensin type 1 receptors.(149) Therefore, the downregulation of TNF might be beneficial for the improvement of high blood pressure levels and thereby influences the angiotensin II levels and oxidative stress in hypertension.(150) If these effects can be translated to humans, they can at least partly explain the decrease in SBP observed in subjects with pre- and stage 1 hypertension.(133) IL1B was also significantly downregulated after 12 weeks of EOJ compared with CD consumption. IL1B is a cytokine with cogent links to atherosclerosis and other inflammatory diseases.(151) Previous studies have shown that high levels of ILB1 promote atherothrombosis and thereby increase CVDR.(151) Additionally, increased levels of ILB1 suggest an important role of this cytokine in hypertension pathogenesis.(152) In addition, PTGS2 was borderline significantly downregulated after 12 weeks of EOJ consumption. PTGS2 (also named COX-2) induces pro-inflammatory cytokines and thereby stimulates cell proliferation and increases metastatic potential through inflammatory pathways.(153) PTGS2 is also an important enzyme in prostaglandin synthesis, and its overexpression increases the risk of ischaemic stroke.(154) In this sense, CITRUS study showed decreased levels of PAI-1 after EOJ consumption and reduced homocysteine levels after OJ and EOJ consumption for 12 weeks(133), and both results are related with an improvement of inflammation, that which observed also at transcriptome level in the present analysis.

In contrast, unlike our previously positive results regarding decreases in the expression of pro-inflammatory genes, some anti-inflammatory-related genes were significantly, or borderline significantly

downregulated after the single dose and sustained consumption of hesperidin in OJ and EOJ. Specifically, TNFAIP3, which is related to vascular disease, was significantly downregulated after a single dose of OJ compared with CD. The overexpression of the TNFAIP3 gene is related to suppression of adhesion protein expression at sites of atherosclerotic plaque formation; therefore, lower levels of TNFAIP3 are associated with an increased risk of atherosclerosis in subjects with type 2 diabetes.(155) An aspect that should be considered is that the decrease in the expression of anti-inflammatory genes could be explained by the fructose content of fruit juices, although natural and enriched juices were used in the present study. A high consumption of fructose can increase CVDR due to increases in inflammation pathways,(142) but some controversial studies have only showed an association between cardiometabolic risk factors and fructose content though the consumption of artificially sweetened beverages rather than fruit juices.(156) The unclear conclusions might be due to the presence of fibres and phenolic compounds, such as hesperidin, or other beneficial bioactive compounds, such as vitamins and minerals, in fruit juices, including OJ. Hence, more studies in this field are needed to obtain conclusive results regarding the effects of fruit juices and the fructose content on inflammation and CVD factors.

Moreover, our results showed that the gene selenium binding protein 1 (SELEBP1) was downregulated after EOJ consumption for 12 weeks. The elevated levels of SELENBP1 was related with a high risk of adverse cardiac events and death(157). Thus, the downregulation of these gene could be beneficial since it is considered a biomarker for cardiac events. Additionally, the genes PTX3 and NAMPT previously

published in CITRUS study(133), were changed after 12 weeks of OJ and EOJ consumption, related with the improvement of blood pressure levels. Also, the genes DSP and IER3, which are related with an improvement of endothelial function, were differentially expressed after EOJ sustained consumption.

Moreover, our findings suggest that both the single dose and sustained consumption of hesperidin in OJ and EOJ for 12 weeks can change the expression of several lncRNAs. lncRNAs, which are RNA transcripts that do not encode proteins, have been implicated in numerous biological processes and diseases.(158) LNCipedia, a comprehensive compendium of human lncRNAs with an integrated database that offers the annotation of thousands of lncRNA transcript sequences, was searched for the differentially expressed lncRNAs, including both up- and downregulated lncRNAs, after the consumption of OJ and EOJ compared with the CD. However, although some of these lncRNAs were identified in previous studies, most of the identified lncRNAs are unknown, and their functions and involvement in diseases have not yet been assessed. Accordingly, further research is needed in this topic because some studies have shown that lncRNAs can act as key regulators of the inflammatory response(159) and can play an important role in the cardiovascular system.(160)

One strength of the present work is that the study constitutes the first evaluation of the transcriptomic profile of human PBMCs after the consumption of hesperidin in OJ and EOJ. The assessment of gene expression in human PBMCs can demonstrate their capacity to reflect the distinct gene expression signatures of several diseases linked to

cardiovascular health(140,161) and the gene expression signatures after interaction with bioactive food compounds, such as flavonoids. In contrast, this work provides the first evaluation of the transcriptomic profile of human PBMCs after the acute consumption of hesperidin in OJ and EOJ. The evidence demonstrates the capacity of phenolic compounds to exert postprandial effects on several systems, and the accumulation of acute functional changes can induce chronic physiological alterations,(162) and thus has a positive impact on the target systems. The insights on postprandial actions can offer the possibility to study unknown mechanisms for the beneficial effects of hesperidin in human health. However, whether the changes in the transcriptomic profile of PBMCs are maintained over time and whether the observed changes might improve long-term CVDRFs remain unclear. Therefore, larger clinical trials and trials that include patients with hypertension and other CVDRFs are needed to better understand these clinical findings. Moreover, in the present work, the transcriptomic analysis of the single dose intervention was only performed at the beginning of the clinical trial. Thus, a second PBMC analysis in the second single dose study conducted at 12 weeks could provide evidence regarding the effects of chronic phenolic compounds intake on the possible optimization of the acute intake observed in previous studies.(133)

In conclusion, the single dose and sustained consumption of hesperidin in OJ and EOJ changed the transcriptome of PBMCs of subjects with pre- and stage 1 hypertension. The single dose consumption of higher doses of hesperidin could induce a better response than the consumption of the naturally occurring doses of hesperidin in OJ because of their

improvement of insulin resistance. Moreover, the sustained consumption of hesperidin in EOJ decrease the expression of proinflammatory genes providing a possible mechanism of action on inflammation pathway and thereby could induces beneficial effects on the cardiovascular system. However, some results are unclear and controversial; therefore, more studies are needed to confirm and clarify the biological effects of hesperidin consumption on the transcriptomic profile.

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Author contributions

The human randomized clinical trial was conducted by LP-P, LCP, EL, JC, RMV, AP and RS. LP-P wrote the manuscript and RMV, AP and RS provided feedback on the manuscript. CD and LP-P realized the qRT-PCR analysis. All the authors have read the manuscript. We thank the Centre for Omics Sciences (COS) Joint Unit of the Universitat Rovira i Virgili-Eurecat, for their contribution to the processing of the samples and transcriptomic analysis. We thank Nutritional Projects Assessment (Nuproas.es) for their contribution to the statistical analysis.

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Declaration of interest

The authors declare that they have not conflicts of interest with the content of this article.

Table 1. Baseline characteristics of the participants by intervention group (N=37).

Variable	CD (n=11)	OJ (n=15)	EOJ (n=11)	P
Age	51.9 ± 12.8	41.0 ± 14.2	49.3 ± 5.9	0.061
Females, %	18.2	33.3	7.3	0.691
SBP, mm Hg	130.4 ± 9.8	134.9 ± 11.9	139.9 ± 11.8	0.156
DBP, mm Hg	74.6 ± 9.8	79.9 ± 9.2	82.1 ± 9.8	0.182
Pulse pressure, mm Hg	58.4 ± 9.7	55.0 ± 10.7	57.8 ± 7.2	0.623
Weight, kg	75.6 ± 10.0	76.9 ± 11.9	77.8 ± 11.8	0.905
BMI, kg/m^2	25.5 ± 3.2	26.3 ± 3.5	26.3 ± 3.1	0.812
Waist circumference, cm	92.3 ± 9.1	91.8 ± 11.8	92.8 ± 7.7	0.966
Glucose, mg/dL	95 ± 5.6	94 ± 9.3	97 ± 2.8	0.559
Cholesterol, mg/dL				
Total	201 ± 42	187 ± 28	194 ± 19	0.507
LDL	134 ± 33	111 ± 27	129 ± 16	0.078
HDL	49.2 ± 11.9	54.6 ± 21.5	44.7 ± 13.9	0.347
Triglycerides, mg/dL	88 ± 20	104 ± 49	102 ± 39	0.594
Physical activity, UA	2.91 ± 1.7	3.33 ± 1.2	3.63 ± 0.5	0.398

Data expressed as mean ± standard deviation, or percentages. Abbreviations: CD, control drink; OJ, orange juice; EOJ, hesperidin-enriched orange juice; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; UA, arbitrary units: 0, inactive. 1, very low activity. 2, low activity. 3, moderately active. 4, very active.

Table 2. Comparisons of dose-response gene expression changes among treatments in PBMCs of mildly hypertensive individuals.

Gene Symbol	Gene Name	Post-int values mean (SE)			Changes among treatments					
		Control (n=11)	OJ (n=15)	EOJ (n=11)	OJ vs Control		EOJ vs Control		EOJ vs OJ	
					Mean (95%CI)	P	Mean (95%CI)	P	Mean (95%CI)	P
Inflammation related genes										
Anti-inflammatory										
NFKB1A	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	7.19 (0.37)	6.23 (0.32)	6.23 (0.35)	-0.968 (-2.0;0.07)	0.066	-0.962 (-2.0;0.06)	0.064	0.006 (-0.98;1.0)	0.991
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	4.00 (0.35)	2.82 (0.31)	3.11 (0.34)	-1.171 (-2.2; -0.18)	0.022	-0.889 (-1.9;0.09	0.073	0.282 (-0.67;1.2)	0.549

TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	-2.45 (0.27)	-2.80 (0.24)	-2.92 (0.26)	-0.356 (-1.1;0.42)	0.357	-0.471 (-1.2;0.29)	0.219	-0.115 (-0.86;0.63)	0.755
DUSP2	Dual specify phosphatase 2	1.29 (0.20)	0.86 (0.27)	0.92 (0.19)	-0.422 (-0.99;0.14)	0.138	-0.364 (-0.92;0.19)	0.192	0.058 (-0.48;0.60)	0.827
TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-gamma-GT	-3.70 (0.07)	-3.55 (0.06)	-3.56 (0.07)	0.144 (-0.05;0.34)	0.140	0.134 (-0.06;0.32)	0.161	-0.009 (-0.19;0.18)	0.917
TMIGD3	Transmembrane and immunoglobulin domain containing 3	-3.80 (0.07)	-3.70 (0.04)	-3.68 (0.05)	0.098 (-0.04; 0.24)	0.167	0.112 (-0.03;0.25)	0.110	0.014 (-0.12;0.15)	0.831
Pro-inflammatory										
CCL3	Chemokine (C-C motif) ligand 3	6.05 (0.53)	4.95 (0.44)	4.45 (0.49)	-1.094 (-2.5;0.35)	0.133	-1.278 (-2.7;0.13)	0.073	-0.204 (-1.6;1.2)	0.765

CCL3L3	Chemokine (C-C motif) ligand 3-like 3	2.72 (0.58)	1.39 (0.50)	1.22 (0.56)	-1.333 (-3.0;0.30)	0.106	-1.502 (-3.1;0.11)	0.066	-0.169 (-1.7;1.4)	0.827
CCL4L2	Chemokine (C-C motif) ligand 4-like 2	4.85 (0.89)	4.11 (0.25)	4.11 (0.28)	-0.739 (-1.5;0.07)	0.074	-0.741 (-1.5;0.06)	0.069	-0.002 (-0.78;0.78)	0.996
CXCL2	Chemokine (C-X-C motif) ligand 2	1.22 (0.56)	0.09 (0.49)	-0.34 (0.54)	-1.132 (-2.7;0.46)	0.158	-1.566 (-3.1;0.005)	0.051	-0.434 (-31.9;1.1)	0.566
EREG	Epiregulin	-1.81 (0.52)	-2.67 (0.45)	-2.66 (0.50)	-0.868 (-2.3; 0.60)	0.239	-0.853 (-2.3; 0.60)	0.240	0.015 (-1.4;1.4)	0.691
CLEC5A	C-type lectin domain family 5, member A	-4.40 (0.17)	-3.94 (0.15)	-4.21 (0.16)	0.454 (-0.01; 0.93	0.056	-0.191 (-0.27;0.66)	0.409	-0.268 (-0.72;0.18)	0.236
FKBP5	FK506 binding protein 5	1.51 (0.06)	1.46 (0.05)	1.46 (0.05)	-0.059 (-0.22;0.10)	0.465	-0.050 (-0.21;0.11)	0.529	0.009 (-0.15;0.16)	0.908
DDIT4	DNA-damage-inducible transcript 4	3.20 (0.08)	3.13 (0.07)	3.22 (0.08)	-0.069 (-0.30;0.16)	0.547	0.022 (-0.21;0.25)	0.847	0.091 (-0.19;0.84)	0.409

Cardiovascular related										
FMN1	Formin 1	-4.37 (0.27)	-4.90 (0.23)	-4.84 (0.26)	-0.532 (-1.3;0.23)	0.163	-0.470 (-1.2;0.28)	0.209	0.062 (-0.66;0.79)	0.864
Obesity related										
AREG	Amphiregulin	-3.21 (0.28)	-3.52 (0.25)	-3.28 (0.27)	-0.318 (-1.1;0.48)	0.425	-0.079 (-0.87;0.71)	0.841	0.239 (-0.53;1.0)	0.529
Insulin resistance										
DHRS9	Dehydrogenase/reductase (SDR family) member 9, transcript variant 3	0.031 (0.08)	0.323 (0.07)	0.038 (0.08)	0.292 (0.05;0.53)	0.019	0.057 (-0.23;0.24)	0.992	-0.285 (-0.51;-0.05)	0.017
Others (Unknown function)										
SLED1	Proteogly can 3 pseudogene non-coding RNA	0.190 (0.45)	-0.956 (0.40)	-0.769 (0.44)	-1.146 (-2.4;0.14)	0.078	-0.958 (-2.2;0.31)	0.132	0.188 (-1.0;1.4)	0.757

GOS2	G0 /G1 switch gene 2	1.95 (0.65)	0.638 (0.56)	0.699 (0.62)	-1.317 (-3.1;0.51)	0.152	-1.256 (-3.0;0.54)	0.165	0.061 (-1.7;1.8)	0.944
PKDL1	Polycystic kidney disease2 -like 1	-2.51 (0.19)	-1.96 (0.17)	-2.24 (0.18)	0.549 (-0.007; 1.1)	0.047	0.263 (-0.27;0.80)	0.324	-0.286 (-0.80;0.23)	0.269
LncRNA CEP44-1	Long non coding RNA Centrosomal Protein 44-1	-3.89 (0.17)	-3.67 (0.15)	-3.88 (0.17)	0.219 (-0.27;0.71)	0.368	0.012 (-0.47;0.49)	0.961	-0.207 (-0.67;0.26	0.372
LncRNA ATOH8-2	Long non coding RNA Atonal homolog 8-2	-2.77 (0.09)	-3.01 (0.07)	-2.96 (0.08)	0.231 (-0.47;0.01)	0.062	-0.188 (-0.43;0.05)	0.121	0.043 (-0.19;0.28)	0.709
LncRNA ERP44-3	Long non coding RNA endoplasmic reticulum protein 44-3	-4.05 (0.10)	-4.04 (0.09)	-4.06 (0.10)	0.017 (-0.26;0.30)	0.905	-0.005 (-0.28;0.27)	0.972	-0.021 (-0.29;0.25)	0.872

XLOC-l2_0132293	Long non coding XLOC-l2-0132293	-4.69 (0.21)	-4.87 (0.18)	-4.77 (0.20)	-0.184 (-0.78;0.41)	0.535	-0.084 (-0.67;0.50)	0.772	0.100 (-0.47;0.67)	0.725

Data expressed as mean ± standard error (ES) or mean (95% confidence interval, CI). Inter treatment comparisons by ANCOVA Model adjusted by sex and age. Analyses performed with the IBM SPSS Statistics 21 Package. Abbreviations: OJ, orange juice; EOJ, hesperidin-enriched orange juice.

Table 3. Comparisons of gene expression changes among treatments after 12 weeks of sustained consumption in PBMCs of mildly hypertensive individuals.

Gene Symbol	Gene Name	Post-intervention values mean (SE)			Changes among treatments					
		Control (n=11)	OJ (n=15)	EOJ (n=11)	OJ vs Control		EOJ vs Control		EOJ vs OJ	
					Mean (95%CI)	P	Mean (95%CI)	P	Mean (95%CI)	P
Inflammation related										
Anti-inflammatory										
NFKBIA	Nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	8.42 (0.09)	8.20 (0.08)	8.20 (0.09)	-0.220 (-0.47;0.03)	0.085	-0.236 (-0.48;0.01)	0.062	-0.016 (-0.26;0.22)	0.894
NFKBIZ	Nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.41 (0.06)	3.29 (0.05)	3.27 (0.06)	-0.125 (-0.30;0.05)	0.165	-0.141 (-0.32;0.04)	0.115	-0.016 (-0.19;0.15)	0.854

TNFAIP3	Homo sapiens TNF, alpha-induced protein3	5.04 (0.03)	5.03 (0.03)	5.04 (0.03)	-0.007 (-0.02;0.01)	0.095	-0.006 (-0.01;0.03)	0.186	0.002 (-0.007;0.01)	0.686
FFAR3 GPR41	Free fatty acid receptor 3 (FFAR3)	-2.18 (0.07)	-2.16 (0.06)	-2.18 (0.07)	-0.051 (-0.26;0.16)	0.621	-0.071 (-0.27;0.13)	0.483	-0.020 (-0.22;0.18)	0.834
KRT1	Keratin 1, type II	-0.715 (0.64)	0.047 (0.54)	-0.186 (0.62)	0.761 (-1.0;2.5)	0.394	0.528 (-1.2;2.3)	0.547	-0.233 (-1.9;1.5)	0.783
Pro-inflammatory										
IL6	Interleukin 6 (interferon, β 2)	-0.78 (0.08)	-0.61 (0.07)	-0.63 (0.08)	0.175 (0.06;0.41)	0.134	0.153 (-0.08;0.38)	0.183	-0.022 (-0.24;0.20)	0.838
TNF	Tumor necrosis factor	4.59 (0.18)	4.08 (0.15)	3.98 (0.17)	-0.510 (-1.0;-0.005)	0.048	-0.610 (-1.1;-0.11)	0.018	-0.100 (-0.58;0.38)	0.677
IL1B	Interleukin 1, beta	5.93 (0.15)	5.58 (0.12)	5.52 (0.14)	-0.350 (-0.76;0.06)	0.092	-0.414 (-0.82;-0.01)	0.045	-0.065 (-0.46;0.33)	0.739
ICAM (log)	Intercellular adhesion molecule 1	0.37 (0.08)	0.35 (0.07)	0.35 (0.08)	-0.016 (-0.04;0.007)	0.160	-0.021 (-0.04;0.002)	0.075	-0.004 (-0.03;0.02)	0.681

CCL3	Chemokine (C-C motif) ligand 3	5.98 (0.49)	4.89 (0.42)	4.73 (0.48)	-1.086 (-2.5;0.30)	0.121	-1.252 (-2.6;0.12)	0.071	-0.167 (-1.5;1.1)	0.799
CCL20	Chemokine (C-C motif) ligand 20	-1.42 (0.05)	-1.22 (0.05)	-1.29 (0.05)	0.193 (0.04;0.35)	0.016	0.128 (-2.5;1.2)	0.098	-0.065 (-0.21;0.08)	0.377
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	2.29 (0.10)	2.13 (0.09)	2.00 (0.10)	-0.160 (-0.44;0.12)	0.261	-0.288 (-057;-0.008)	0.044	-0.129 (-0.40;0.14)	0.340
CCL4L2	Chemokine (C-C motif) ligand 4-like 2	5.81 (0.06)	5.94 (0.05)	5.94 (0.05)	0.129 (-0.03;0.29)	0.104	0.130 (-0.02;0.28)	0.097	0.001 (-0.15;0.15)	0.990
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activityα)	-0.03 (0.14)	-0.29 (0.12)	-0.37 (0.13)	-0.286 (-0.68;0.11)	0.150	-0.364 (-0.75;0.02)	0.066	-0.078 (-0.45;0.30)	0.674
CXCL2	Chemokine (C-X-C motif) ligand 2	3.08 (0.26)	2.60 (0.22)	2.32 (0.25)	-0.485 (-1.2;0.23)	0.180	-0.755 (-1.5;-0.04)	0.038	-0.270 (-0.95;0.41)	0.428

CXCL3	Chemokine (C-X-C motif) ligand 3 [	-0.39 (0.14)	-0.66 (0.12)	-0.71 (0.13)	-0.278 (-0.66;0.19)	0.149	-0.324 (-0.70;0.05)	0.091	-0.046 (-0.41;0.32)	0.800
CXCL8	Chemokine (C-X-C motif) ligand 8	1.43 (0.30)	0.859 (0.32	0.220 (0.36)	-0.543 (-1.6;0.52)	0.305	-1.182 (-2.2;-0.14)	0.028	-0.639 (-1.6;0.37)	0.207
EREG	Epiregulin	-0.10 (0.11)	-0.28 (0.10)	-0.30 (0.11)	-0.176 (-0.50;0.15)	0.277	-0.192 (-0.51;0.13)	0.229	-0.016 (-0.32;0.29)	0.914
PTGS2 (COX2)	Prostaglandin-endoperoxide synthase 2 (cyclooxygenase 2)	1.05 (0.25)	0.57 (0.21)	0.13 (0.24)	-0.484 (-1.2;0.21)	0.167	-0.922 (-1.6;-0.23)	0.010	-0.438 (-1.10;0.23)	0.189
RasGEF1B	Ras GEF domain family member 1 B	0.238 (0.03)	0.303 (0.02)	0.274 (0.03)	0.065 (-0.01;0.14)	0.108	0.036 (-0.04;0.011)	0.356	-0.029 (-0.10;0.05)	0.450
Cardiovascular disease related										
FMN1	Formin 1	-3.23 (0.03)	-3.35 (0.03)	-3.42 (0.03)	-0.069 (-0.17;0.03)	0.164	-0.063 (-0.16;0.03)	0.199	0.006 (-0.09;0.10)	0.893

Acute coronary syndrome										
SELENBP1	Selenium binding protein 1	-2.19 (0.26)	-1.77 (0.22)	-2.51 (0.25)	0.352 (-0.38;1.1)	0.338	-0.392 (-1.1;0.33)	0.280	-0.744 (-1.4;-0.04)	0.038
Obesity related										
PPP1R15A GADD34	Homo sapiens protein phosphatase 1, regulatory subunit 15A	2.93 (0.08)	2.76 (0.07)	2.72 (0.08)	-0.167 (-0.40;0.07)	0.163	-0.210 (-044;0.02)	0.077	-0.044 (-0.27;0.18)	0.696
BCL2A1	BCL2-related protein A1	3.80 (0.08)	3.71 (0.06)	3.63 (0.07)	-0.087 (-0.30;0.13)	0.421	-0.165 (-0.38;0.05)	0.127	-0.078 (-0.28;0.13)	0.449
Hemoglobin related										
HBB	Hemoglobin, beta	9.30 (0.14)	9.35 (0.12)	9.09 (0.14)	0.044 (-0.36;0.45)	0.826	-0.213 (-0.61;0.19)	0.289	-0.257 (-0.64;0.13)	0.187
ALAS2	5'-aminolevulinate synthase 2	0.67 (0.27)	1.04 (0.22)	0.31 (0.26)	0.370 (-0.37;1.1)	0.319	-0.357 (-1.1;0.38)	0.330	-0.727 (-1.4;-0.02)	0.045
HBA2	Hemoglobin, alpha 2	7.32 (0.18)	7.39 (0.16)	6.98 (0.18)	0.063 (-0.46;0.58)	0.808	-0.341 (-0.85;0.17)	0.185	-0.404 (-0.90;0.09)	0.107

HBD	Hemoglobin, delta	6.40 (0.21)	6.59 (0.18)	6.13 (0.20)	0.100 (-0.50;0.60)	0.737	-0.360 (-0.95;0.23)	0.223	-0.460 (-1.0;0.11)	0.110
SLC4A1	Solute carrier family 4 (anion exchanger), member 1	-3.69 (0.26)	-3.67 (0.22)	-4.07 (0.25)	0.019 (-0.71;0.74)	0.958	-0.379 (-1.1;0.34)	0.289	-0.398 (-1.1;0.29)	0.250
Neuronal differentiation										
FOXD4	Forkhead box D4	-3.33 (0.02)	-3.81 (0.02)	-3.84 (0.02)	0.028 (-0.03;0.09)	0.361	-0.004 (-0.06;0.06)	0.892	-0.032 (-0.09;0.03)	0.273
CD83	CD83 molecule	4.65 (0.06)	4.50 (0.05)	4.50 (0.06)	-0.150 (-0.32;0.02)	0.077	-0.145 (-0.31;0.02)	0.083	0.005 (-0.15;0.16	0.945
Cancer										
Prostate										
TMPO-AS1	TMPO antisense RNA 1 long non-coding RNA	-2.27 (0.02)	-2.33 (0.01)	-2.33 (0.02)	-0.058 (-0.11;-0.03)	0.038	-0.053 (-0.11;0.01)	0.053	0.005 (-0.05;0.06)	0.854

Esophageal squamous cell										
CFLAR-AS1	CFLAR antisense RNA 1 long non-coding RNA	-2.49 (0.01)	-2.51 (0.01)	-2.49 (0.01)	-0.019 (-0.05;0.02)	0.277	-0.005 (-0.04;0.03)	0.780	0.015 (-0.02;0.05)	0.393
Gastric										
LncRNA GPR65-1	Long non-coding RNA G protein-coupled receptor 65-1	-3.38 (0.04)	-3.45 (0.03)	-3.43 (0.04)	-0.066 (-0.18;0.04)	0.227	-0.046 (-0.15;0.06)	0.392	0.020 (-0.08;0.12)	0.696
Unknown										
PLA1A	Phospholipase A1 member A	-3.03 (0.04)	-3.14 (0.04)	-3.14 (0.04)	-0.115 (-0.24;0.007)	0.065	-0.111 (-0.23;0.01)	0.071	0.004 (-0.11;0.12)	0.942
FAM53B	Family with sequence similarity 53, member B	-2.55 (0.02)	-2.16 (0.02)	-2.17 (0.02)	0.087 (0.02;0.15)	0.011	0.074 (0.01;0.14)	0.025	-0.013 (-0.07;0.05)	0.682
SLED1	Proteoglycan 3 pseudogene non-coding RNA	1.40 (0.05)	1.27 (0.04)	1.28 (0.05)	-0.129 (-0.28;0.02)	0.090	-0.115 (-0.26;0.03)	0.125	0.014 (-0.13;0.16)	0.841
GOS2	G0 /G1 switch gene 2	4.28	3.66	3.64	-0.620	0.171	-0.635	0.155	-0.015	0.972

		(0.32)	(0.27)	(0.31)	(-1.5;0.28)		(-1.5;0.25)		(-0.87;0.84)	
BPIFB3	BPI fold containing family B, member 3	-2.46 (0.04)	-2.60 (0.04)	-2.58 (0.04)	- 0.132 (-0.25; -0.01)	0.032	-0.113 (-0.023;0.06)	0.062	0.020 (-0.09;0.13)	0.728
BTBD19	BTB (POZ) domain containing 19	-3.93 (0.07)	-3.83 (0.06)	-4.09 (0.07)	0.100 (-0.11;0.31)	0.331	-0.157 (-0.36;0.05)	0.129	-0.257 (-0.45;-0.06)	0.012
LINC 01220	Long intergenic non-protein coding RNA 1220	-3.47 (0.11)	-3.14 (0.09)	-3.37 (0.11)	0.330 (0.02;0.64)	0.040	0.100 (-0.21;0.41)	0.516	-0.230 (-0.53;0.07)	0.128
KMT2E-AS1	KMT2E antisense RNA 1(ncRNA)	0.90 (0.16)	0.74 (0.14)	0.43 (0.16)	-0.151 (-0.61;0.31)	0.508	-0.465 (-0.92;-0.01)	0.044	-0.314 (-0.75;0.12)	0.155
ATP2B1-AS1	ATP2B1 antisense RNA 1 (ncRNA)	0.14 (0.10)	-0.04 (0.09)	-0.17 (0.10)	-0.177 (-0.46;0.11)	0.218	-0.309 (-0.59;-0.02)	0.034	-0.131 (-0.41;0.14)	0.338
LncRNA BTBD19-1	Long non-coding BTB domain containing 19_1	-1.90 (0.07)	-2.04 (0.06)	-2.08 (0.07)	-0.142 (-0.33;0.05)	0.145	-0.177 (-0.37;0.01)	0.067	-0.036 (-0.11;0.31)	0.696
LncRNA ACOT-13	Long non-coding Acyl-CoA thioesterase 13	-1.49 (0.11)	-1.86 (0.10)	-1.76 (0.11)	- 0.373 (-0.68;-0.06)	0.020	-0.272 (-0.58;0.03)	0.080	0.101 (-0.22;0.15)	0.493

LncRNA ARRCD3-1	Long non-coding arresting containing domain 3-1	-0.83 (0.08)	-0.87 (0.07)	-0.88 (0.07)	-0.039 (-0.26;0.18)	0.718	-0.054 (-0.27;0.16)	0.610	-0.015 (-0.22;0.19)	0.882
LncRNA IYD	Long non-coding iodotyrosine deiodinase	-2.30 (0.06)	-2.46 (0.05)	-2.19 (0.06)	-0.161 (-0.34;0.01)	0.070	0.106 (-0.07;0.28)	0.220	0.268 (0.10;0.43)	0.003
LncRNA KDM5A-3	Long non-coding lysine demethylase 5A-3	-1.85 (0.12)	-2.07 (0.11)	-2.16 (0.12)	-0.216 (-0.56;0.13)	0.212	-0.307 (-0.65;0.03)	0.076	-0.091 (-0.42;0.24)	0.579
LncRNA SNRPD3-2	Long non-coding small nuclear ribonucleoprotein D3 polypeptide-2	-0.52 (0.05)	-0.30 (0.04)	-0.37 (0.05)	0.220 (0.07;0.37)	0.004	0.146 (0.002;0.29)	0.047	0.074 (-0.10;0.06)	0.285
LncRNA SLC39A8-1	Long non-coding solute carrier family 39 member 8	0.06 (0.12)	-0.27 (0.11)	-0.28 (0.12)	0.330 (-0.67;0.01)	0.060	0.345 (-0.68;-0.007)	0.046	-0.016 (-0.34;0.31)	0.923
LncRNA IL1B1	Long non-coding Interleukin 1, beta	-1.28 (0.11)	-1.01 (0.10)	-1.09 (0.11)	0.284 (-0.04;0.89)	0.066	0.189 (-0.12;0.50)	0.224	-0.085 (-0.39;0.21)	0.567
LncRNA NFKBID-1	Long non-coding NFKB inhibitor delta 1	-3.56 (0.02)	-3.49 (0.02)	-3.52 (0.02)	0.074 (0.02;0.12)	0.006	0.041 (-0.009;0.09)	0.107	-0.033 (-0.08;0.02)	0.178

LncRNA PDE3B-1	Long non-coding phosphodiesterase 3B-1	-3.10 (0.06)	-2.93 (0.05)	-2.97 (0.06)	0.175 (0.009;0.34)	0.039	0.129 (-0.03;0.29)	0.118	-0.046 (-0.20;0.11)	0.557
LncRNA MKI67-2	Long non-coding marker of proliferation Ki-67-2	-2.52 (0.01)	-2.53 (0.01)	-2.54 (0.01)	-0.010 (-0.04;0.02)	0.567	-0.021 (-0.05;0.01)	0.232	-0.011 (-0.04;0.02)	0.519
LncRNA CCT8-1	Long non-coding chaperoning containing TCP1 subunit 8	-2.76 (0.09)	-3.07 (0.08)	-3.07 (0.09)	-0.314 (-0.057;-0.05)	0.020	-0.310 (-0.057;-0.05)	0.020	0.003 (-0.25;0.25)	0.979
LncRNA LRRC61-2	Long non-coding leucine rich repeat containing 61-2	-1.91 (0.03)	-2.00 (0.03)	-1.98 (0.03)	-0.091 (-0.19;0.005)	0.062	-0.073 (-0.17;0.02)	0.125	0.018 (-0.07;0.11)	0.694
LncRNA GRK3-1	Long non-coding G protein-coupled receptor kinase 3-1	-3.00 (0.17)	-3.31 (0.14)	-3.47 (0.16)	-0.313 (-0.78;0.16)	0.186	-0.470 (-0.093;-0.005)	0.048	-0.157 (-0.61;0.29)	0.483
LncRNA TOMM20-2	Long non-coding RNA translocase of outer mitochondrial membrane 20-2	-2.37 (0.01)	-2.39 (0.01)	-2.38 (0.01)	-0.014 (-0.03;0.003)	0.095	-0.011 (-0.03;0.005)	0.179	0.003 (-0.01;0.02)	0.706

LncRNA RP11-701P16.2.1-2	Long non-coding RNA RP11-701P16.2.1-2	-2.35 (0.17)	-2.78 (0.14)	-2.82 (0.16)	-0.426 (-0.90;0.04)	0.075	-0.466 (-0.93;-0.001)	0.050	-0.039 (-0.49;0.41)	0.860
LncRNA CCDC117-1	Long non-coding coiled-coil domain containing 117-1	-2.00 (0.04)	-2.09 (0.03)	-2.12 (0.03)	-0.088 (-0.19;0.01)	0.090	-0.118 (-0.22;-0.02)	0.024	-0.029 (-0.13;0.07)	0.547
LOC101929524	Uncharacterized LOC101929524	-3.31 (0.03)	-3.20 (0.03)	-3.25 (0.03)	0.106 (0.01;0.20)	0.030	0.057 (-0.04;0.15)	0.223	-0.049 (-0.14;0.04)	0.280
LOC644090	Uncharacterized LOC644090	-2.47 (0.07)	-2.64 (0.06)	-2.66 (0.06)	-0.163 (-0.35;0.02)	0.087	-0.187 (-0.04;0.002)	0.048	-0.024 (-0.20;0.16)	0.789

Data expressed as mean ± standard error (SE) or mean (95% confidence interval, CI). *logarithmic transformation of the variable. Inter treatment comparisons by ANCOVA Model adjusted by sex and age. Significant differences values were set for $p<0.05$. Abbreviations: OJ, orange juice; EOJ, hesperidin-enriched orange juice.

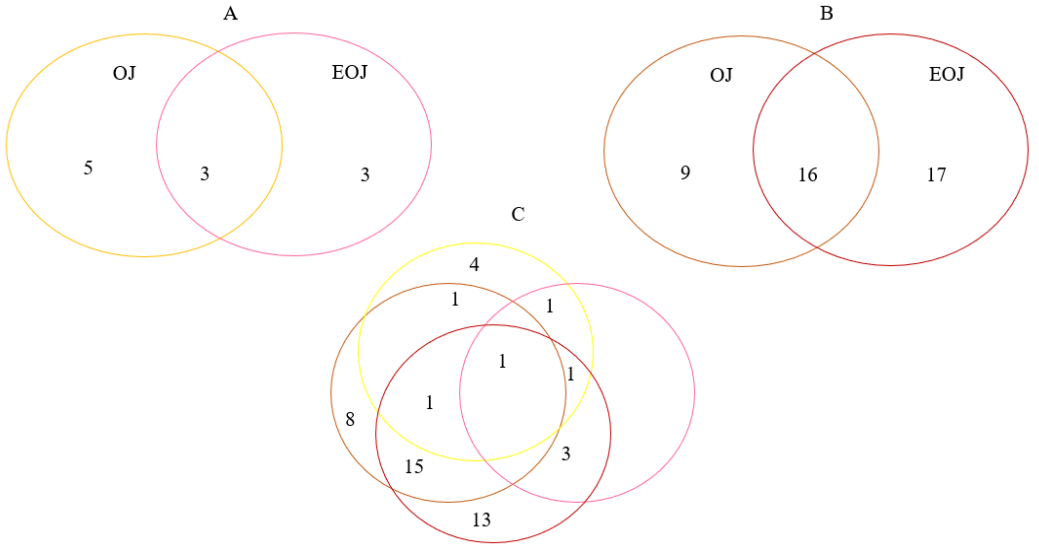
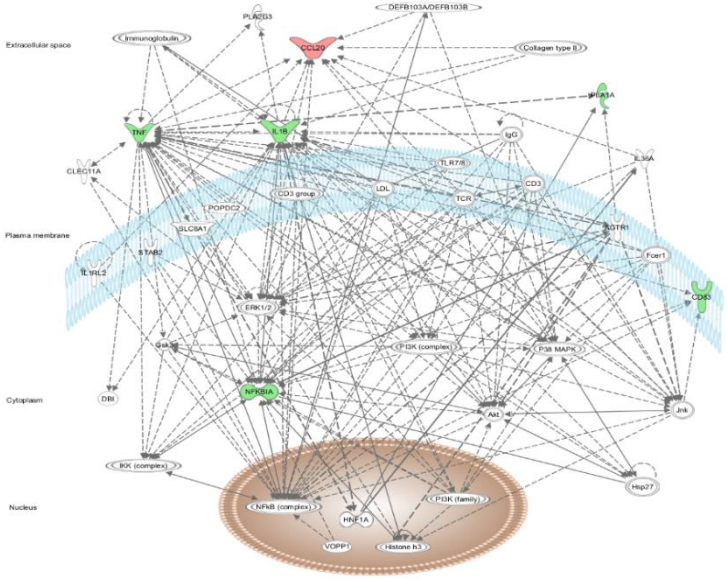


Figure 1. Venn diagram of the number of differentially (P < 0.05) and borderline (P > 0.10) expressed genes in PBMC of stage 1 and pre-hypertensive individuals: 6 h after the consumption of 500 mL (A) and after 12-week consumption of orange juice (OJ) and hesperidin-enriched orange juice (EOJ) (B) compared to control drink, and the differentially expressed genes after both consumptions (C). Yellow, postprandial OJ; pink, postprandial EOJ; brown, sustained OJ; red, sustained EOJ.

Panel A



Panel B

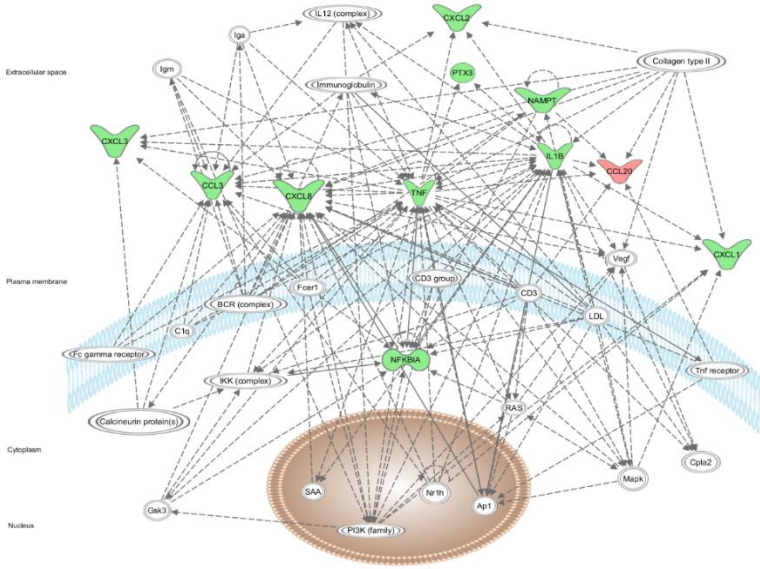


Figure 3. Biological networks based on the differentially expressed gens after sustained consumption of orange juice (panel A) and hesperidin-enriched orange juice (panel B). Interaction between the differently expressed genes and other important related gens. Down- and up-regulated genes are symbolized in red and green color, respectively.

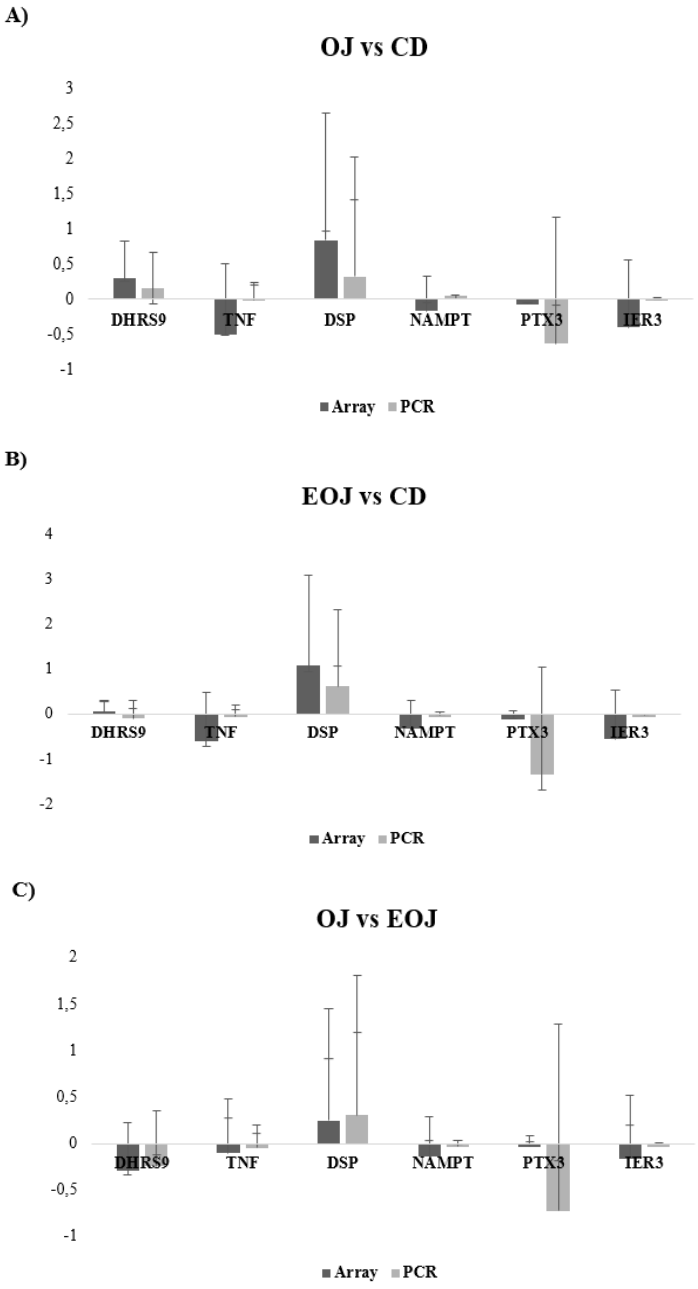


Figure 2. Real-time PCR validation of the arrays results for a subset of 6 genes.

Supporting Information Table S1. Composition of the three test drinks.

	Dose per day in mg/500 mL		
	Control drink	Orange juice	Hesperidin-enriched orange juice
Hesperidin	ND	392	670
Narirutin	ND	64	77
Vitamin C	235	235.3	235
Glucose	14407	938.2	9382
Fructose	7357	10123	10123
Sucrose	13197	18158	18158

Composition determined by State of Florida, Department of Citrus.

Abbreviations: ND, no detected.

Supporting Information Table S2. Nucleotide sequences of primers used for PCR amplification.

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')	Ref. or Acc. No.
DHRS9	TGGACCACGCTCTAACAAGT	CACACTGCCTTGGGATTAGC	NM_001142270.2
DSP	TACCCCTGCGACAAGAACAT	TCTGGGTTACGAGGCTTCAG	NM_004415.4
IER3	GGCTTCTCTTTCTGCTGCTC	GAGGGCTCCGAAGTCAGATT	NM_003897.4
NAMPT	TGGAGGAAGGAAAAGGAGACC	CACACAACACACACCCAGTC	NM_005746.3
PTX3	GGTAAATGGTGAACTGGCGG	TGACAAGACTCTGCTCCTCC	NM_002852.4
RPLP0	ATGGCAGCATCTACAACCCT	AGGACTCGTTTGTACCCGTT	NM_001002.4
TNF	CAGAGGACCAGCTAAGAGGG	TCAGTGCTCATGGTGTCCTT	NM_000594.4

Supporting Information Table S3. Top canonical pathways, diseases and functions regulated by sustained consumption (12 weeks) of orange juice and hesperidin-enriched orange juice in PBMCs of pre- and stage 1 hypertensive individuals compared to control drink.

Treat-ment	Top canonical pathways	P-value	Top diseases and functions	P-value range
EOJ	Granulocyte adhesion and diapedesis: CCL3, CCL20, CCL3L3, CCL4L2, CXCL1, CXCL2, CXCL3, CXCL8, IL1B, TNF.	8.85^{-17}	Inflammatory response: CXCL1, CXCL2, CXCL3, CXCL8, IL1B, PTGS2, PTX3, TNF, CCL20, CCL3, CCL3L3, CD83, NFKB1A, NAMPT, DSP.	6.75^{-04}–4.17^{-16}
	Agranulocyte adhesion and diapedesis: CCL3, CCL20, CCL3L3, CCL4L2, CXCL1, CXCL2, CXCL3, CXCL8, IL1B, TNF.	1.71^{-16}	Cell–to-cell signaling and interaction: CCL20, CCL3, CCL3L3, CXCL1, CXCL2, CXCL3, CXCL8, IL1B, PTGS2, TNF, CD83, PTX3, NFKB1A, DSP, NAMPT.	6.75^{-04}–3.57^{-13}
	Role of IL-17A in arthritis: CCL20, CXCL1, CXCL3, CXCL8, NFKB1A, PTGS2.	8.20^{-12}	Organismal injury and abnormalities: CCL20, CCL3, CD83, CXCL1, CXCL2, CXCL3, CXCL8, IL1B, NAMPT, NFKB1A, PTGS2, TNF.	9.41^{-04}–2.42^{-12}
	TREM1 signaling: CCL3, CD83, CXCL8, IL1B, TNF.	3.68^{-11}	Hematological system: CCL20, CCL3, CCL3L3, CXCL1, CXCL2, CXCL3, CXCL8, IL1B, TNF, CD83, NFKB1A, PTX3.	8.86^{-04}–3.12^{-12}
	Communication between innate and adaptive immune cells: CCL3, CCL3L3, CD83, CXCL8, IL1B, TNF.	1.40^{-10}	Cardiovascular system: CCL3, CXCL1, CXCL2, CXCL8, IL1B, PTGS2, PTX3, TNF, NFKB1A.	7.36^{-04}–2.53^{-09}
OJ	Dendritic cell maturation: CD83, IL1B, NFKB1A, TNF.	1.84^{-07}	Inflammatory response: CCL20, CD83, IL1B, NFKB1A, TNF.	1.39^{-03}–7.24^{-11}
	IL-23 signaling pathway: IL1B, NFKB1A, TNF.	3.61^{-07}	Organismal injury and abnormalities: CCL20,	1.39^{-03}–7.24^{-11}

		CD83, IL1B, NFKB1A, TNF.	
IL-10 signaling: IL1B, NFKB1A, TNF.	1.33^{-06}	Cellular growth and proliferation: CCL20, CD83, IL1B, NFKB1A, TNF.	1.22^{-03}– 3.21^{-08}
TREM1 signaling: CD83, IL1B, TNF.	1.39^{-06}	Lipid metabolism: CD83, IL1B, NFKB1A, TNF, PLA1A.	1.04^{-03}– 3.98^{-08}
Toll-like receptor signaling: IL1B, NFKB1A, TNF.	1.71^{-06}	Hematological system: CD83, IL1B, NFKB1A, TNF, CCL20.	1.39^{-03}– 3.00^{-08}

Analysis performed with the Ingenuity Pathway Analysis (IPA). Abbreviations: PBMCs, peripheral blood mononuclear cells; EOJ, hesperidin-enriched orange juice; OJ, orange juice.

Supporting information Table S4. Statistically significant and borderline differentially expressed genes after the single dose study and after 12 weeks of OJ, EOJ and CD consumption.

	After 6h (single dose study)			After 12 weeks (sustained consumption)		
Genes	OJ vs CD	EOJ vs CD	EOJ vs OJ	OJ vs CD	EOJ vs CD	EOJ vs OJ
Anti-inflammatory						
NFKB1A	↓[b]	↓[b]		↓[b]	↓[b]	
TNFAIP3	↓[a]	↓[b]				
Pro-inflammatory						
TNF				↓[a]	↓[a]	
IL1B				↓[b]	↓[a]	
ICAM					↓[b]	
CCL3		↓[b]			↓[b]	
CCL20				↑[a]	↑[b]	
CCL3L3		↓[b]			↓[a]	
CCL4L2	↓[b]	↓[b]			↑[b]	
CXCL1					↓[b]	
CXCL2		↓[b]			↓[a]	
CXCL3					↓[b]	
CXCL8					↓[a]	
CLEC5	↑[b]					
PTGS2					↓[a]	
Insulin resistance						
DHRS9	↑[a]		↓[a]			
Acute coronary syndrome						
SELENBP1						↓[a]

Endothelial function related				
DSP			↑[a]	
IER3			↓[a]	
Improvement of hypertension				
PTX3			↓[a]	
NAMPT			↓[b]	
Obesity related				
PPP1R15AGADD34			↓[b]	
Hemoglobin related				
ALAS2				↓[a]
Neuronal differentiation				
CD83		↓[b]	↓[b]	
Cancer: prostate				
TMPO-AS1		↓[a]	↓[b]	
Unknown function				
SLED1	↓[b]	↓[b]		
BPIFB3		↓[a]	↓[b]	
PKDL1	↑[a]			
ATP2B1-AS1			↓[a]	
PLA1A		↓[b]	↓[b]	
FAM53B		↑[a]	↑[a]	
BTBD19				↓[a]
KMT2E-AS1			↓[a]	
LINC01220		↑[a]		
LncRNA ATOH8-2	↑[b]			
LncRNA ACOT-13		↓[a]	↓[b]	
LncRNA KDM5A-3			↓[b]	
LncRNA SNRPD3-2		↑[a]	↑[a]	

	LncRNA SLC39A8-1	↑[b]	↑[a]	
	LncRNA CCT8-1	↓[a]	↓[a]	
	LncRNA GRK3-1		↓[a]	
	LncRNA RP11-701P16.2.1-2	↓[b]	↓[b]	
	LncRNA CCDCC117-1	↓[b]	↓[a]	
	LOC101929524	↑[a]		
	LOC644090	↓[b]	↓[a]	
	LncRNA IYD	↓[b]		↑[a]
	LncRNA BTBD19-1		↓[b]	
	LncRNA IL1B1	↑[b]		
	LncRNA NFKBID-1	↑[a]		
	LncRNA PDE3B-1	↑[a]		
	LncRNA LRRC61-2	↓[b]		
	LncRNA TOMM20-2	↓[b]		

[a], $p<0.05$; [b], $p<0.10$. Abbreviations: OJ, orange juice; EOJ, hesperidin-enriched orange juice; CD, control drink.

Project 4: The consumption of hesperidin in orange juice modulates the plasma and urine metabolic profiles in pre- and stage 1 hypertensive subjects to promote beneficial effects on the cardiovascular system: targeted and nontargeted metabolomic approaches (CITRUS study) (Editor submitted)

The consumption of hesperidin in orange juice modulates the plasma and urine metabolomic profiles in pre- and stage 1 hypertensive subjects to promote beneficial effects on the cardiovascular system: targeted and nontargeted metabolomic approaches (CITRUS study)

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Abbreviations

CD:	control drink
CVDs:	cardiovascular diseases
CVDRFs:	cardiovascular diseases risk factors
DMA:	dimethylamine
DMG:	dimethylglycine
EOJ:	hesperidin-enriched orange juice
GPC:	glycerophosphocholine
NAG:	N-acetylglycoproteins
OJ:	orange juice
PAG:	phenylacetylglutamine
2-HIB:	2-hydroxyisobutyrate

ABSTRACT

Scope

The aim of the present work was to determine new biomarkers of the biological effects of hesperidin in orange juice (OJ) applying a nontargeted metabolomics approach validated by targeted metabolomics analyses of compliance biomarkers.

Methods and results

Plasma/serum and urine targeted (HPLC-MS/MS) and untargeted (^{1}H-NMR) metabolomics signatures were explored in a subsample with pre- and stage-1 hypertension subjects of the CITRUS study (N=159). Volunteers received 500 mL/day of control drink, OJ, or hesperidin-enriched OJ (EOJ) for 12-weeks. A 6-h postprandial study was performed at baseline. Targeted analyses revealed plasma and urine hesperetin 7-O-□-D-glucuronide as the only metabolite differing between OJ and EOJ groups after both single dose and 12-weeks consumption, and in urine is correlated with a decreased systolic blood pressure level. The nontargeted approach showed that after single dose and12-weeks consumption of OJ and EOJ changed several metabolites related with an anti-inflammatory and antioxidant actions, lower blood pressure levels and uraemic toxins.

Conclusions

Hesperetin 7-O-□-D-glucuronide could be a candidate marker for distinguishing between the consumption of different hesperidin doses as well as a potential agent mediating blood pressure reduction. Moreover, changes in different endogenous metabolites could explain the mechanisms of action and the biological effects of hesperidin consumption.

1. INTRODUCTION

The metabolome comprises all the metabolites found in an organ, cell, biofluid or organism under certain conditions. Metabolites have several functions in cells and systems,(69) and their levels in organism are directly associated with human health.(163) Notably, changes at the metabolome level can appear in biofluids before the appearance of clinical symptoms.(164) Therefore, when assessing nutritional studies, metabolomics approaches provide information about changes in diet-derived and endogenous metabolites to allow the identification of novel biomarkers related to dietary intake and biological effects.(165)

The relationship between bioactive compounds in food and the development of several chronic conditions has been widely studied. In this sense, the effects of phenolic compounds, that are naturally present in plant-based foods, have emerged as potential nutritional strategy against on cardiovascular disease (CVD). In particular, hesperidin, which is the main flavanone in citrus fruits and is present mainly in oranges and orange juice (OJ), has demonstrated beneficial effects on different cardiovascular risk biomarkers in animal and human studies.(130) Nevertheless, the bioavailability of the flavanone hesperidin depends on the body's capacity to absorb this metabolite, and subjects can be stratified into three categories: low, moderate and high absorbers.(110)

Through omics analyses, our research group recently showed that hesperidin consumption can modify the heart and kidney tissue proteome(166) and the plasma metabolome profile of rats with metabolic syndrome.(167) Specifically, our metabolomics analysis showed that hesperidin consumption decrease the levels of several

plasma metabolites related to inflammation and oxidative stress and thereby, reduces the blood pressure levels, influences on the cardiovascular system, and improves the conditions of metabolic syndrome in rats.(167) In humans, hesperidin consumption decreases the body weight(94) and diastolic blood pressure levels,(107) and improves lipid profile(93) and postprandial microvascular endothelial reactivity.(107) In fact, our research group recently demonstrated a beneficial effect of hesperidin consumption in OJ on blood pressure levels in subjects with pre- and stage 1 hypertension.(133)

The study of the metabolic profiles can offer important insights into cardiovascular system and CVDs pathogenesis as well as the possible identification of new CVDs biomarkers. However, to the best of our knowledge, no study has combined a targeted and nontargeted metabolomics approach after the consumption of hesperidin in OJ in humans with cardiovascular disease risk factors (CVDRFs).

Therefore, the aim of the present work was to determine new biomarkers of the biological effects of hesperidin in OJ applying nontargeted metabolomics approach in plasma/serum and urine samples after both single dose (6 hours) and sustained (12 weeks) consumption, validated through targeted metabolomics analyses of compliance biomarkers in subjects with pre- and stage 1 hypertension.

2. METHODS

2.1 Study population and study design

The study population was obtained from the CITRUS study (N= 159), a randomized, parallel, double-blind and placebo controlled clinical trial (registration number in Clinical-Trials.gov: NCT02479568).(133)

Moreover, the targeted metabolomics analysis was performed in 129 subjects who completed sustained study, and nontargeted metabolomics was performed in 52 subjects who completed both the single dose and sustained studies. The subjects were women and men with pre- or stage 1 hypertension, systolic blood pressure ≥ 120 mm Hg and < 159 mm Hg and diastolic blood pressure < 100 mm Hg, who are not taking any anti-hypertensive or anti-hyperlipemia medication. The subjects, who were recruited between January 2016 and June 2017, were randomly assigned to one group of the three intervention groups: control drink (CD), OJ and hesperidin-enriched orange juice (EOJ) groups. Detailed information on the study population and the design of the study are described in Supplementary Information Methods S1.

2.2 Test drinks

The following three test drinks were provided by The Florida Department of Citrus from the USA: the CD (without hesperidin), OJ containing hesperidin at its natural level, and EOJ, which was OJ enriched with hesperidin. The intervention drinks were similar in smell and appearance and can be differentiated by a code assigned by an independent researcher not related directly to the clinical trial.

2.3. Dosage information

All subjects drank 500 mL of the corresponding test drink daily: the CD, provided 0 mg/day of hesperidin, OJ provided 345 mg/day of hesperidin, and EOJ provided 600 mg/day of hesperidin. The composition of the three test drinks is detailed in Supporting Information Table S1.

2.4 Targeted metabolomics analysis of compliance biomarkers

Sample collection

To assess whether the compliance biomarkers in plasma supply more information than those in urine, plasma samples were collected at baseline and after 2, 4 and 6 h in the single dose study. In the sustained study, plasma samples were collected at baseline and at 4, 8 and 12 weeks after sustained consumption. Moreover, 24-h urine samples were collected at the beginning of the study and after 12 weeks of treatment.

Determination of hesperidin and naringenin metabolites in urine and plasma samples by UHPLC-MS/MS

Urine and plasma samples were thawed at 4 °C. Subsequently, 50 μL of the urine samples was mixed with 100 μL of water containing 1% HF or the internal standard (rac-hesperetin-d3). This dilution was performed through a semi-automated process using the Agilent Bravo Automated Liquid Handling Platform. In contrast, 20 internal standards (hesperetin-d4) were mixed with 125 μL of the plasma samples and 750 μL of methanol, and then, mixture was then vortexed for 10 mins and centrifuged at 4700 rpm at 4°C for 10 min. A total of 900 μL was transferred and evaporated in a Speed-Vac at ambient temperature. The residues were reconstituted in 25 μL of MeOH and 75 μL of H2O (1% HFor) and injected into an LC-MS/MS instrument. The extraction was performed through a semi-automated process using the Agilent Bravo Automated Liquid Handling Platform. The analyses were performed with an Agilent 1200 series UHPLC coupled to a 6490 Triple Quad LC-MS mass spectrometer, and the source electrospray source ionization (ESI) was operated in the negative mode. A reversed-phase Eclipse Plus column (C18, 1.8 μm, 2.1 mm × 150 mm) from Agilent Technologies was used for chromatographic separation.

The assignment of the hesperetin and naringenin metabolites was performed by direct comparison with commercial standards (hesperidin, narirutin, hesperetin, naringenin, hesperetin 3-O-β-D-glucuronide, hesperetin 7-O- β -D-glucuronide, naringenin 4'-O-β-D-glucuronide and hesperetin 7-O-sulfate). The tentative identification of the other hesperetin and naringenin metabolites was based on the precursor ion mass, chromatographic behaviour on a similar system, and published data from plasma samples. The method was validated using a pool of samples collected during the study and was based on standard addition. The method was validated by determining the limit of detection (MDL), and the limit of quantification (MQL), the repeatability (expressed as relative standard deviation, RSD), the accuracy (%) and the coefficient of determination of the calibration curve (Supplementary Information Table S2). The total flavanone content was obtained by summing the plasma or urine levels of all identified hesperidin and naringenin metabolites.

2.5 Nontargeted metabolomics analysis of biological effects biomarkers

Sample collection

Serum samples were collected at baseline and 6 h after treatment in the single dose study (N=129). Nontargeted metabolomics analyses were performed at 6-h, corresponding with the reported maximal flavanone plasma concentration.(168) For the sustained study, serum samples were collected at the beginning and after 12 weeks of treatments only for those subjects that participated in single dose study (N=52). Twenty-four hours urine samples were also collected at the beginning of the study and after 12 weeks of treatments (N=129).

NMR sample preparation and acquisition

Urine and serum samples were allowed to reach room temperature. Subsequently, 400 μL of urine was mixed with 200 μL of phosphate buffer (pH 7.4; 100% D2O) containing the internal standard/L, 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP), at a concentration of 1 mM and 2 mM sodium azide. In addition, 200 μL of each serum sample was mixed with 400 μL of phosphate buffer containing 2 mM TSP, and the resulting mixture was vortexed and centrifuged at 10,000 x g for 10 min. A 550-μL aliquot was then transferred into a 5-mm NMR tube.

All NMR spectra were recorded at 300 K with a Bruker Avance III 600 spectrometer operating at 600.20 MHz for protons and equipped with a 5-mm PABBO BB-1H/D Z-GRD probe and an automatic sample changer with a cooling rack at 4°C. For the urine samples, a standard one-dimensional (1D) NOESY presaturation pulse sequence (RD-90°-t1-90°-tm-90°-acquire) was used with water suppression. The data from all the samples were acquired using a recycle delay (RD) of 5 s, a mixing time (tm) of 100 ms, an acquisition time of 3.41 s, and a 90° pulse of 10.99 μs. For each sample, 128 scans were collected after four dummy scans to obtain 64K data points with a spectral width of 16 ppm. For all serum samples, 1D-NOESY and Carr-Purcell-Meiboom-Gill (CPMG) spin echo experiments with water suppression were performed to obtain the corresponding metabolic profile. The settings for the NOESY experiments were the same as those used for the urine samples. Low-molecular-weight-filter CPMG spectra were then obtained using a CPMG sequence (RD [90°x-(t-180°y-t)n-collect FID) with a spin-echo delay of 400 μs (for a total T2 filter of 210 ms), which allowed efficient attenuation of the lipid NMR signals. The total acquisition time

was 2.73 s with an RD of 2 s, and the 90° pulse length was automatically calibrated for each sample at approximately 11.1 μs. For each sample, eight dummy scans were followed by 128 scans, and 64K points over a spectral width of 20 ppm were collected.

NMR spectra processing

Prior to the data analysis, all FIDs were multiplied by an exponential function equivalent to a 0.3-Hz line broadening factor before applying a Fourier transform. The transformed spectra were phased, corrected for baseline distortions, and calibrated using the reference standard TSP in Topspin 3.5 (Bruker). Each spectrum in the range between 0 and 10.0 ppm was digitized into consecutive integrated spectral regions (32.768) of equal width (0.00033 ppm) using MATLAB (MathWorks). The region containing the residual water in both the urine and serum samples was removed to minimize the baseline effects arising from improper water suppression. Chemical shift variation was minimized across the dataset by applying a recursive segment-wise peak alignment algorithm to each spectrum. Each spectrum was then normalized using a probabilistic quotient normalization.

2.6 Statistical analysis

To express the changes in plasma compliance biomarkers after 4, 8 and 12 weeks of the treatments, intra-treatment comparisons were evaluated by a general linear model with Bonferroni correction and age and sex as covariables, and inter-treatment comparisons were evaluated with the ANCOVA model adjusted by sex and age. To express the changes in urine compliance biomarkers after 12 weeks of the treatments, intra-treatment comparisons were evaluated by Wilcoxon test and inter-treatment comparison by Mann-Whitney test for independent samples.

The relationship of hesperetin 7-O-B-glucuronide and systolic blood pressure levels was assessed by Pearson's and Spearman's correlation coefficients since this metabolite seems to be the responsible of hypotensive, vasodilatory and anti-inflammatory activities in literature.(15)

Multivariate modelling of the results from the nontargeted metabolomics analysis was performed in MATLAB using in-house scripts. First, outlying samples were identified by PCA, and the significant metabolites that can be used to discriminate between groups were identified using orthogonal partial least squares discriminant analysis (O-PLS-DA). Hence, NMR metabolic profiles served as the descriptor matrix (X) and the experimental groups were used as the response variable (Y). The O-PLS model loadings were back-transformed by multiplying all the values by their standard deviation (covariance) and colour-plotted based on their model weights (R^2). The important variables for between-class discrimination are highlighted by the correlation colour scale, and the red colour indicates high significance. The predictive performance (Q^2Y) of the models was calculated using a 7-fold cross-validation approach, and the model significance was assessed using 1000 permutations. Large interindividual variability has been observed in the bioavailability and excretion of hesperidin among subjects.(169,170) Therefore, we also build O-PLS models using the maximum total flavanone (hesperidin + naringenin metabolites) content, independently from the intervention group, as a continuous predictor variable (Y).

3. RESULTS

3.1 Volunteer characteristics and results from the human randomized clinical trial

Of the 159 volunteers with pre- and stage 1 hypertension who were randomly allocated, 129 completed the sustained study (N = 43, 46 and 40 in CD, OJ and EOJ groups, respectively), and 52 of these volunteers participated in the baseline single-dose study (N = 17, 21 and 14 in the CD, OJ and EOJ groups, respectively). The flow diagram of the volunteers is detailed in Figure S1 in the Supplementary Information, and the basal characteristics of the volunteers are detailed in Table S3 in the Supplementary Information.

3.2 Targeted metabolomics for compliance biomarkers

Plasma targeted metabolomics analysis

The changes in the six studied plasma metabolites, namely, hesperetin 7-O-β-D-glucuronide, hesperetin 3-O-β-D-glucuronide, hesperetin 7-O-sulfate, naringenin 4-O-β-D-glucuronide, naringenin glucuronide and naringenin sulfate, after 4, 8 and 12 weeks of the treatments are detailed in Table 1 (N = 129). Compared with those observed in the CD group, significantly increased levels of the six exposure biomarkers were observed after 12 weeks of OJ and EOJ consumption. In particular, the metabolite hesperetin 7-O-B-D-glucuronide was the main metabolite that showed differential expression between the OJ and EOJ groups.

The changes in the six studied plasma metabolites, namely, hesperetin 7-O-B-D-glucuronide, hesperetin 3-O-B-D-glucuronide, hesperetin 7-O-sulfate, naringenin 4-O-B-D-glucuronide, naringenin glucuronide and naringenin sulfate, detected after 2, 4 and 6 h in the single dose study are presented in Figure 1 (N = 52). After 6 h, OJ and EOJ

consumption significantly increased the plasma levels of hesperetin 7-O-B-D-glucuronide, hesperetin 3-O-B-D-glucuronide, hesperetin 7-O-sulfate, naringenin glucuronide and naringenin sulfate compared with the levels obtained with the CD.

High interindividual variability has been observed in absorption and excretion in hesperidin-based acute and chronic interventions.(112) Consistently, we also observed a large interindividual variability in total plasma flavanone pharmacokinetics upon beverage intake in each treatment group (Supplementary Information Figure S2). Some participants had the maximal total flavanone concentration after 6-h of consumption, while other had the maximum peak at 4-h or even earlier (2-h). In addition, the total amount of flavanones absorbed in each hesperidin supplemented group had a high variability, with high-, medium-, and low-absorbers in each group.

Urine targeted metabolomics analysis:

The changes in the six studied urine metabolites, namely, hesperetin 7-O-β-D-glucuronide, hesperetin 3-O-β-D-glucuronide, hesperetin 7-O-sulfate, naringenin 4-O-β-D-glucuronide, naringenin glucuronide and naringenin sulfate, after sustained consumption of the intervention drinks are detailed in Table 2 (N = 129). After 12 weeks of CD intervention, no differences were observed in the six studied urine metabolites. Compared to CD, after OJ and EOJ interventions, significant increased levels of the six studied metabolites were observed. Moreover, the EOJ consumption compared to OJ, increased the levels of only one metabolite in urine: the hesperetin 7-O-β-D-glucuronide.

In Figure 2 is represented the relationship between the levels of urine hesperetin 7-O-β-D-glucuronide and changes on blood pressure levels

of the participants of CITRUS study. Our results showed that increased levels of hesperetin 7-O-β-D-glucuronide in urine are correlated with lower blood pressure levels after 12 weeks of treatment (R=-0.223; P=0.011).

3.3 Nontargeted metabolomics analysis of biomarkers of the biological effect in the serum and urine samples

Pairwise O-PLS-DA models were built to compare the metabolic profiles of the three study groups at 6-h in the single dose study. Significant models were obtained for the comparison of the metabolic profiles between CD and OJ group (Q^2Y=0.36, P=0.001) and between CD and EOJ groups (Q^2Y=0.35, P=0.001) after the 6 hours of the single dose consumption. The serum levels of proline betaine and dimethylglycine (DMG) were significantly increased and the level of leucine were significantly decreased after 6 h, in the OJ and EOJ groups compared with the CD group. Moreover, 6 h after EOJ consumption, the isoleucine urine levels were also significantly decreased compared with those in the CD group (Figure 3, panels A and B).

To take into account that hesperidin absorption varied largely in both OJ and EOJ groups (Figure 3, panel C), we also built an O-PLS model considering the the maximal total flavanone concentration observed during the single dose study for each participant independent of the treatment group. Interestingly, we obtained a more significant model (Q^2Y=0.20, P<0.001) that revealed additional differences between high and low absorbers (Figure 3, panel D). Consistent with the previous models, high hesperidin absorbers had higher plasma levels of DMG and proline betaine. However, this model also revealed that high absorbers had higher levels of ketone bodies (3-hydroxybutyrate, acetoacetate) but lower levels of BCAA, alanine, lysine, and glutamine.

Pairwise O-PLS-DA models were also constructed to compare the metabolic profiles between treatment groups at the end of the study (week 12) for those participants that completed the single dose study. Again, we obtained significant models differentiating the metabolic profiles of CD *vs* OJ (Q^2Y=0.35, P=0.02) and CD *vs* EOJ (Q^2Y=0.28, P=0.05) (Figure 4). After the sustained consumption study, a nontargeted metabolomics was performed with a subsample of 52 subjects (N = 17 in CD, 21 in OJ and 14 in EOJ) who completed both the single dose study and the sustained study, and differences in the metabolic profiles were observed after sustained consumption. As a result, increased serum levels of proline betaine and decreased serum levels of glycerophosphocholine (GPC), N-acetyl glycoproteins (NAGs), acetate, valine, isoleucine, and leucine were observed after 12 weeks of OJ consumption compared with the levels founded in the CD group (Figure 4, panel A). Similarly, increased serum levels of proline betaine and decreased plasma levels of GPC, aspartate, glutamate, valine, isoleucine, and leucine were observed after 12 weeks of EOJ consumption compared with the levels detected in the CD group (Figure 4, panel B).

The nontargeted analysis of urine samples from the sustained study (N=129) also revealed significant differences between the metabolic profiles of the OJ and CD (Q^2Y=0.79, P<0.001) and between the EOJ and CD (Q^2Y=0.80, P<0.001) groups after 12 weeks. Significantly increased levels of proline betaine in urine were observed after 12 weeks of OJ consumption compared with the levels found in the CD group, whereas the levels of 4-hydroxyhippurate, pseudouridine, phenylacetylglutamine (PAG), 4-cresyl sulfate, creatinine,

dimethylamine (DMA), NAGs, alanine and 3-methyl-2-oxovalerate were significantly decreased (Figure 5, panel A). Compared with the levels found in the CD group, significantly increased levels of proline betaine in urine were observed after 12 weeks of EOJ consumption, and the levels of 4-cresyl sulfate, pseudouridine, uracil, creatinine, creatine, NAGs, alanine, 2-hydroxyisobutyrate (2-HIB) and 3-MOV were significantly decreased (Figure 5, panel B).

No differences were found between the OJ and EOJ groups in any of the untargeted analyses.

4. DISCUSSION

The current study comprises the first evaluation of the effects on the metabolome of single dose and sustained consumption of hesperidin in OJ and EOJ on subjects with pre- and stage 1 hypertension through targeted and nontargeted approaches to finally determine new biomarkers of the biological effects of hesperidin. Both targeted and nontargeted analyses revealed that OJ and EOJ consumption induced significant changes on plasma/serum and urine metabolome compared to the CD group.

The targeted metabolomics approach for compliance biomarkers revealed increased plasma and urine of six flavanone catabolites after 12 weeks of OJ and EOJ consumption and 6 h after consumption of the single dose. As previously reported in plasma(133) and in the present work also in urine, the six compliance biomarkers were significantly increased after OJ and EOJ consumption, which demonstrate compliance with the interventions Moreover, hesperetin 7-O-B-D-glucuronide, both in plasma and urine, was the only metabolite that

showed differences between the OJ and EOJ groups, which indicated that hesperetin 7-O-B-D-glucuronide could be a candidate marker for distinguishing between different doses of hesperidin consumed for at least 12 weeks. Additionally, urine levels of hesperetin 7-O-B-D-glucuronide was correlated with lower systolic blood pressure levels, demonstrating a possible role on blood pressure control in humans. In this sense, its appreciation was only previously observed in rat models, where hesperetin 7-O-B-D-glucuronide exerts antihypertensive effects and exhibits vasodilatory and anti-inflammatory activities.(15) Thus, to the best of our knowledge, this is the first study to show a negative correlation between the urine metabolite hesperetin 7-O-B-D-glucuronide and the levels of systolic blood pressure.

Metabolic profiles provide a direct functional read-out of the physiological status of an individual. As a result, they are closely related to the phenotype. Therefore, we also applied an NMR-based untargeted metabolomics approach to identify metabolites and metabolic pathways associated with the phenotypes after hesperidin consumption to understand the mechanisms underlying these phenotypes. We found a significant increase in plasma and urine levels of proline betaine after both the single dose study and sustained consumption of OJ and EOJ compared to CD. Proline betaine is a direct marker of citrus fruit intake and it reflects a good treatment adherence.(171) Moreover, the levels of leucine, valine and isoleucine were significantly decreased in the serum and urine samples collected after 12 weeks of sustained consumption of OJ and EOJ and after consumption of a single dose of OJ and EOJ, which supported the effect of hesperidin consumption independent of the dose. Valine, leucine and isoleucine are branched-chain amino acids

(BCAA) associated with several cardiometabolic risk factors such as insulin resistance,(125) obesity, atherogenic dyslipidaemia, elevated blood pressure,(172,173) and increased incident of CVDs events.(124) The effects of BCAA in general, and leucine in particular, on mTOR activation could partly explain these associations.(172,173) Intracerebroventricular administration of leucine has shown to active the mTORC1 signalling pathway specifically in the mice hypothalamus and cause a significant increase in arterial pressure.(174) In this sense, previous studies have reported elevated circulating leucine levels in hypertensive subjects.(175) Notably, the circulating levels of BCAA, and leucine in particular, were significantly decreased after 12 weeks of OJ or EOJ consumption compared to the CD. In addition, the urinary levels of 3-methyl-2-oxovalerate (3-MOV), a microbial-derived metabolite from leucine degradation, were also decreased after chronic consumption of both OJ and EOJ treatments. These findings could partly explain the decrease in systolic blood pressure that we observed in the participants consuming OJ or EOJ. Therefore, decreased plasma levels of BCAA could point to a possible protective role of hesperidin in OJ on hypertension.

Another potential mechanism by which hesperidin could reduce systolic blood pressure involves an improvement of the inflammatory and oxidative stress status. We observed decreased serum and urine levels of N-acetyl glycoproteins (NAGs) after 12 weeks of OJ and EOJ consumption. NAGs is considered a novel biomarker of systemic inflammation and cardiovascular disease risk.(176) In addition, the levels of pseudouridine, a metabolite used as a biomarker of oxidative stress in DNA,(177) were decreased in the urine samples collected after 12 weeks of OJ and EOJ consumption. Higher pseudouridine excretion

increases oxidative stress and inflammatory processes.(178) As a result, hesperidin in OJ and EOJ could improve the inflammatory state of subjects with pre- or stage 1 hypertension. Moreover, decreased plasma levels of GPC, which are related to decreases in oxidative and inflammatory tissue damage, were also observed after 12 weeks of OJ and EOJ consumption.(179) However, few studies have broadly discussed its role as a CVD risk factor. Therefore, decreased levels of NAG, pseudouridine and GPC, following hesperidin consumption reflects an improvement in the oxidative and inflammatory status of pre- and stage 1 hypertensive subjects, which could contribute to the amelioration of systolic blood pressure.

Hesperidin consumption also resulted in significant alterations in choline metabolism. Choline is rapidly absorbed in the small intestine and catabolized to betaine. Betaine plays a pivotal role in the one-carbon metabolism as a methyl-donor by reacting with homocysteine to generate methionine and DMG. However, gut bacteria compete with the host for choline. Hence, choline can be alternatively metabolized by the gut microbiota to TMA and dimethylamine (DMA), thereby depleting metabolites involved in one-carbon metabolism. Alterations in one-carbon metabolism has been related with metabolic diseases and hypertension-related health outcomes.(180) Notably, OJ and EOJ participants had higher plasma levels of DMG and lower urinary excretions of DMA, which suggests a lower microbial metabolism of choline following hesperidin consumption, that could explain the improvement of systolic blood pressure in these patients.

DMA is also a microbial-derived uremic toxin that has shown to contribute to CVD(181) and is considered a potential uraemic

cardiovascular toxin according to the European Uraemic Toxin Working Group.(182) Other uraemic toxins, namely, 4-cresyl sulfate, creatinine and PAG, were significantly decreased in urine after OJ and EOJ consumption compared with their levels in the CD group. Lower serum levels of these toxins are beneficial to the cardiovascular and renal systems because the accumulation of their metabolites could produce vascular inflammation, endothelial dysfunction and vascular calcifications.(183) Moreover, PAG have also been negatively associated with systolic blood pressure (184) and with increased risk of incident of coronary artery disease.(185) In this sense, the lower urinary excretion of uraemic toxins observed after 12 weeks of OJ and EOJ consumption could be due to the minor serum levels derived from their lower production, which is likely related to an improvement in oxidative stress.(186) Thus, the lower oxidative stress obtained after OJ and EOJ consumption could result in the production of uraemic toxins.

Additionally, our results suggest that the individual capacity of flavanone absorption is an important aspect to consider. We observed a large interindividual variability in flavanone absorption, as reported in the literature,(111) and identify high and low total flavanone absorbers regardless of their consumption of OJ or EOJ. We considered the maximum absorption values of total flavanones in all the samples regardless of their intervention group at the end of the single dose study (6 h) and found that the subjects who absorbed more flavanones showed significant changes in their proline betaine, 3-hydroxybutyrate, leucine, isoleucine, valine, lysine, and alanine levels compared with the levels found in the CD group. This subanalysis was performed because a large interindividual variability was observed after the single-dose study,

which resulted in the identification of high and low absorbers in the OJ and EOJ groups. Moreover, this large interindividual variability in the absorption of flavanones, which includes the group of phenolic compounds to which hesperidin belongs, could at least partly explain why similar and non-significant changes in biomarkers of the biological effects of hesperidin were observed after OJ and EOJ consumption, although the doses of hesperidin consumed were different.

Consistently, the volunteers who participated in the single dose study (N = 52) and presented higher plasma levels of total flavanones, showed higher plasma levels of ketone bodies, such as 3-hydroxybutyrate and acetoacetate. The decrease in the activation of mTORC1 activity in the hypothalamus achieved with ketogenic diets and induced by ketone bodies plays a critical role in blood pressure control, which results in vasodilation and improvements in the blood pressure levels.(187) Therefore, this findings could indicate another possible mechanism through which hesperidin induced blood pressure improvements in subjects with pre- and stage 1 hypertension.

Moreover, participants consuming hesperidin also excreted lower urinary amounts of 2-HIB compared to those consuming the CD. This metabolite is also a microbial-derived metabolites resulting from the degradation of dietary proteins.(188) Higher levels of 2-HIB have been identified in the urine of obese people and have been associated with a reduced microbiota diversity. Recently, 2-HIB has also been associated with an increased risk of type 2 diabetes.(189)

In this sense, the present study has a limitation and it is that stool samples were not collected in the CITRUS randomized clinical trial, and the use of these type of samples would have provided more

information about metabolites related to microbiota, such as 2-HIB and PAG, and their relationship with the cardiovascular system. In contrast, the subanalysis performed considering the absorption of flavanones is a strength of the study because not all of the subjects have the same characteristics, such as their nutritional status, medication, and microbiota, which could influence the absorption of hesperidin by increasing or decreasing the bioavailability of hesperidin. Thus, whether high hesperidin levels in plasma exert a stronger effect than lower hesperidin levels should be investigated. Moreover, these results highlight the importance of volunteer stratification in clinical trials with hesperidin due to the large interindividual variability in flavanone absorption.

In conclusion, the plasma and urine metabolite hesperetin 7-O-B-D-glucuronide is the only metabolite that differentiated between responses to different hesperidin doses, while urine hesperetin 7-O-B-D-glucuronide metabolite is correlated with low systolic blood pressure levels. In addition, independent of the hesperidin dose, the consumption of hesperidin in OJ and EOJ for 12 weeks has an impact on the serum metabolomic profile by decreasing the levels of endogenous metabolites related to blood pressure (leucine and isoleucine) and inflammation (NAGs). In contrast, hesperidin in OJ and EOJ can also decrease the urinary excretion of uraemic toxins (DMA, NAG and 4-cresuyl sulfate) and endogenous metabolites suggesting an antioxidant effect (pseudouridine). Moreover, 6 h after the consumption of a single dose of hesperidin in OJ and EOJ, changes in the serum levels of metabolites related to reduced blood pressure levels and anti-inflammatory effects (proline betaine, DMG, leucine and isoleucine)

were detected. Thus, the nontargeted metabolomics approach offered the possibility of identifying changes in different endogenous metabolites induced by hesperidin consumption that could indicate a beneficial cardiovascular effect of hesperidin and expand our knowledge regarding its potential mechanism of action or biological effects.

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Author contributions

The human randomized clinical trial was conducted by LP-P, RMV, AP, LCP, EL, JC, RS, AM, and FM-L. JM-P realized the statistical analysis of metabolomics data. LP-P wrote the manuscript and JM-P, RMV, AP, and RS provided feedback on the manuscript. All the authors have read the manuscript. We thank Nutritional Projects Assessment (Nuproas.es) for their contribution to the statistical analysis.

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Declaration of interest

None of the authors report a conflict of interest related to the study.

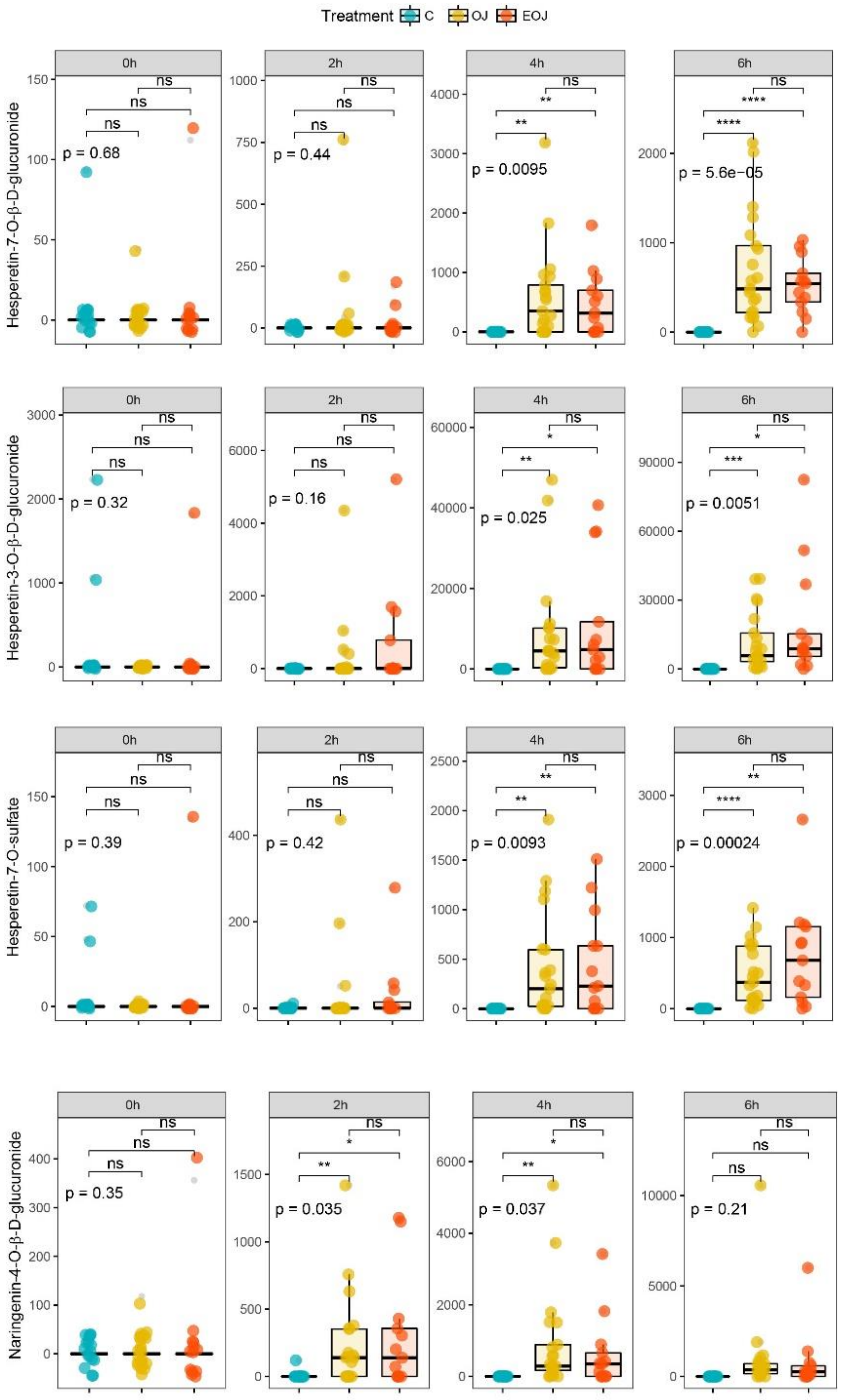
Table 1. Changes in plasma compliance biomarkers at 4, 8 and 12 weeks after treatments.

	Treatment						Changes among treatments					
	Control drink		OJ		EOJ		OJ vs Control drink		EOJ vs Control drink		EOJ vs OJ	
Variable	Post-int	Change	Post-int	Change	Post-int	Change	Mean (95% CI)	P	Mean (95% CI)	P	Mean (95% CI)	P
Hesperetin 7-O-B-D glucuronide, nM/L, (log)												
Week 4	1.26 ± 0.05	-0.032 (-0.15;0.08)	1.70 ± 0.09	0.444 (0.19;0.70)	1.78 ± 0.09	0.566 (0.31;0.82)	0.461 (0.24;0.86)	<0.001	0.618 (0.38;0.84)	<0.001	0.157 (-0.38;0.06)	0.164
Week 8	1.23 ± 0.03	-0.031 (-0.15;0.08)	1.69 ± 0.09	0.443 (0.17;0.72)	1.73 ± 0.10	0.515 (0.22;0.81)	0.451 (0.21;0.69)	<0.001	0.542 (0.30;0.69)	<0.001	0.090 (-0.15;0.33)	0.460
Week 12	1.21 ± 0.02	-0.048 (-0.19;0.09)	1.68 ± 0.10	0.426 (0.14;0.70)	1.88 ± 0.11	0.664 (0.38;0.95)	0.474 (0.23;0.71)	<0.001	0.731 (0.48;0.98)	<0.001	0.257 (0.01;0.50)	0.040
Hesperetin 3-O-B-D glucuronide, nM/L, (log)												
Week 4	0.50 ± 0.00	-0.049 (-0.18;0.09)	2.33 ± 0.23	0.971 (0.59;1.30)	2.59 ± 0.22	1.177 (0.7;1.60)	1.021 (0.68;1.30)	<0.001	1.203 (0.87;1.50)	<0.001	0.183 (-0.15;0.52)	0.281
Week 8	0.54 ± 0.04	-0.06 (-0.19; 0.18)	2.61 ± 0.20	1.249 (0.50;2.00)	2.55 ± 0.23	1.131 (0.51;1.70)	1.257 (0.69;1.0)	<0.001	1.040 (0.47;1.60)	<0.001	-0.217 (-0.78;0.35)	0.448
Week 12	0.50 ± 0.00	-0.048 (-0.18; 0.09)	2.39 ± 0.22	1.028 (0.41;1.60)	2.48 ± 0.25	1.059 (0.40;1.70)	1.089 (0.55;1.60)	<0.001	1.099 (0.56;1.60)	<0.001	0.010 (-0.52;0.54)	0.971

Hesperetin 7-O-sulfate, nM/L, (log)													
Week 4	-0.77 ± 0.07	-0.188 (-0.72;0.34)	0.88 ± 0.22	1.257 (0.56;1.90)	0.93 ± 0.21	1.215 (0.36;2.10)	1.537 (0.84; 2.20)	<0.001	1.685 (0.99;2.40)	<0.001	0.148 (-0.54;0.84)	0.671
Week 8	-0.88 ± 0.00	-0.302 (-0.77;0.17)	1.03 ± 0.21	1.404 (0.45;2.40)	1.02 ± 0.22	1.303 (0.44;2.20)	1.738 (0.95;2.50)	<0.001	1.689 (0.89;2.50)	<0.001	-0.049 (-0.84;0.74)	0.902
Week 12	-0.89 ± 0.00	-0.302 (-0.78;0.17)	0.75 ± 0.22	1.122 (0.35;1.90)	1.01 ± 0.25	1.293 (0.38;2.20)	1.485 (0.77;2.20)	<0.001	1.679 (0.94;2.40)	<0.001	0.194 (-0.54;0.92)	0.599
Naringerin 4-O-B-D glucuronide, nM/L, (log)													
Week 4	1.22 ± 0.00	-0.074 (-0.22;0.07)	1.82 ± 0.12	0.490 (0.14;0.83)	1.52 ± 0.09	0.259 (-0.005;0.52)	0.516 (0.24;0.79)	<0.001	0.420 (0.14;0.70)	0.004	-0.096 (-0.18;0.37)	0.493
Week 8	1.21 ± 0.03	-0.073 (-0.14;0.08)	1.66 ± 0.11	0.335 (-0.04;0.71)	1.59 ± 0.12	0.330 (-0.009;0.67)	0.386 (0.08;0.69)	0.012	0.414 (0.11;0.72)	0.008	0.028 (-0.27;0.33)	0.855
Week 12	1.22 ± 0.00	-0.074 (-0.22;0.07)	1.62 ± 0.11	0.296 (-0.03;0.62)	1.71 ± 0.13	0.457 (0.08;0.83)	0.412 (0.11;0.71)	0.007	0.555 (0.25;0.86)	0.001	0.142 (-0.16;0.44)	0.353
Naringerin glucuronide, nM/L, (log)													
Week 4	1.24 ± 0.00	-0.074 (-0.22;0.07)	1.77 ± 0.09	0.443 (0.18;0.71)	1.66 ± 0.09	0.392 (0.12;0.66)	0.511 (0.28;0.74)	<0.001	0.559 (0.32;0.79)	<0.001	0.048 (-0.18;0.28)	0.682
Week 8	1.21 ± 0.03	-0.073 (-0.14;0.08)	1.75 ± 0.10	0.428 (0.09;0.77)	1.59 ± 0.10	0.322 (0.02;0.62)	0.509 (0.24;0.78)	<0.001	0.431 (0.16;0.70)	0.002	-0.079 (-0.35;0.19)	0.561

Week 12	1.22 ± 0.00	-0.074 (-0.22;0.07)	1.70 ± 0.09	0.380 (0.11;0.65)	1.75 ± 0.09	0.485 (0.19;0.78)	0.488 (0.25;0.73)	<0.001	0.586 (0.34;0.83)	<0.001	0.098 (-0.15;0.34)	0.429
Naringerin sulfate, nM/L, (log)												
Week 4	-0.81 ± 0.04	-0.101 (-0.36;0.16)	-0.17 ± 0.15	0.549 (0.15;0.95)	-0.35 ± 0.16	0.418 (0.04;0.79)	0.640 (0.28;1.00)	0.001	0.660 (0.30;1.00)	<0.001	0.019 (-0.33;0.37)	0.914
Week 8	-0.79 ± 0.06	-0.081 (-0.32;0.16)	-0.24 ± 0.17	0.486 (-0.06;1.00)	-0.38 ± 0.16	0.387 (-0.01;0.79)	0.577 (0.16;0.99)	0.006	0.526 (0.11;0.94)	0.014	-0.051 (-0.46;0.36)	0.807
Week 12	-0.85 ± 0.00	-0.140 (-0.37;0.10)	-0.30 ± 0.15	0.417 (0.04;0.79)	-0.25 ± 0.18	0.521 (0.09;0.95)	0.635 (0.28;0.99)	0.001	0.696 (0.33;1.10)	<0.001	0.061 (-0.30;0.42)	0.739

Data expressed as mean ± standard error or mean (95% confidence interval, CI). Intra treatment comparisons evaluated by General Lineal Model with Bonferroni correction and age and sex as covariables. Inter treatment comparisons by ANCOVA Model adjusted by sex and age. P<0.05. Abbreviations: Post-in, post-treatment values; Change, change from baseline; OJ, orange juice; EOJ, hesperidin-enriched orange juice.


Treatment C OJ EOJ
0h
2h
4h
6h
Hesperetin-7-O-β-D-glucuronide
p = 0.68
p = 0.44
p = 0.0095
p = 5.6e−05
Hesperetin-3-O-β-D-glucuronide
p = 0.32
p = 0.16
p = 0.025
p = 0.0051
Hesperetin-7-O-sulfate
p = 0.39
p = 0.42
p = 0.0093
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Naringenin-4-O-β-D-glucuronide
p = 0.35
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p = 0.037
p = 0.21
ns

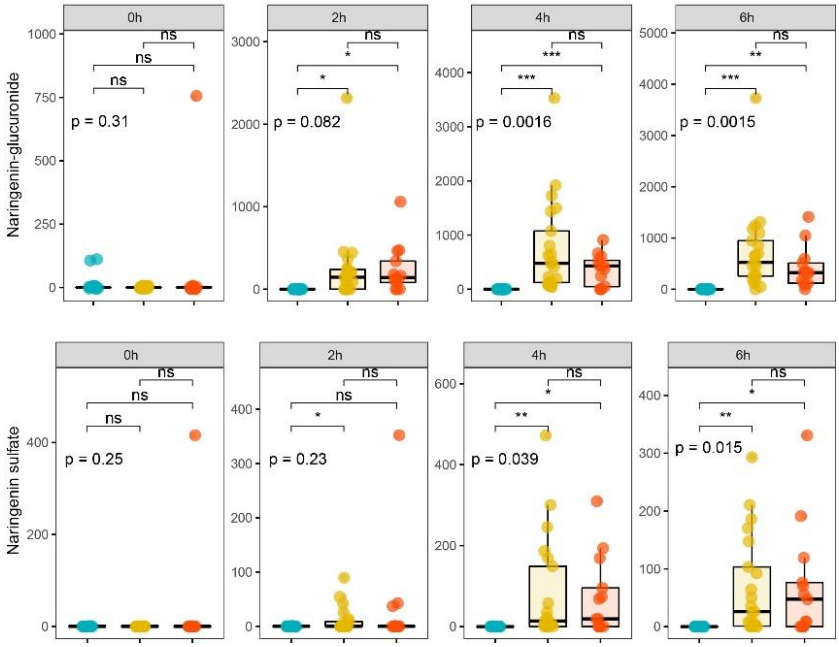


Figure 1. Targeted metabolomics results in plasma samples after the single dose consumption of control drink, orange juice and hesperidin-enriched orange juice (N=52). P<0.05; **, P<0.01; **, P<0.001; ns, nonsignificant.

Table 2. Changes in urine of compliance biomarkers after sustained consumption of treatments (N=129).

Treatment						Changes among treatments					
CD		OJ		EOJ		OJ vs CD		EOJ vs CD		EOJ vs OJ	
Change	Post-int	Change	Post-int	Change	Post-int	Median (25^{th}-75^{th} p)	P	Median (25^{th}-75^{th} p)	P	Median (25^{th}-75^{th} p)	P
Hesperetin 7-O-β-D glucuronide, nM											
0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	0.00‡ (0.00; 137)	0.00 (0.00; 137)	116‡ (0.00; 347)	116 (0.00; 347)	0.00 (0.00;137	<0.001	116 (0.00;137	<0.001	116 (0.85;210)	0.030
Hesperetin 3-O-β-D glucuronide, nM											
0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	495‡ (0.00; 2234)	1493 (0.00; 2870)	1281‡ (0.00; 6344)	1499 (0.00; 6344)	495 (0.00;2234)	<0.001	1281 (0.00; 6344)	<0.001	786 (0.00;4110)	0.265
Hesperetin 7-O-sulfate, nM											
0.00 (0.00; 0.00)	0.00 (0.00; 0.00)	2.16† (0.00; 90)	24 (0.00; 119)	43‡ (0.00; 209)	43 (0.00; 218)	2.16 (0.00; 90)	<0.001	43 (0.00; 209)	<0.001	41 (0.00;119)	0.136

Naringerin 4-O-β-D glucuronide, nM											
0.00	0.00	0.00	0.00	0.00†	0.00	0.00	0.004	0.00	<0.001	0.00	0.569
(0.00; 0.00)	(0.00; 0.00)	(0.00; 0.00)	(0.00; 140)	(0.00; 308)	(0.00; 308)	(0.00; 0.00)		(0.00; 308)		(0.00; 308)	
Naringerin glucuronide, nM											
0.00	0.00	0.00†	0.00	0.00†	0.00	0.00	<0.001	0.00	<0.001	0.00	0.413
(0.00; 0.00)	(0.00; 0.00)	(0.00; 205)	(0.00; 232)	(0.00; 195)	(0.00; 195)	(0.00; 205)		(0.00; 195)		(-5;0.00)	
Naringerin sulfate, nM											
0.00	0.00	0.00*	0.00	0.00†	0.00	0.00	0.001	.00	0.001	0.00	0.601
(0.00; 0.00)	(0.00; 0.00)	(0.00; 0.11)	(0.00; 1.08)	(0.00; 3.12)	(0.00; 3.12)	(0.00; 0.11)		(0.00; 3.12)		(0.00; 3.01)	

Data expressed as median (25^{th}-75^{th} percentiles) /median (minimum-maximum) Intra-treatment comparisons by Wilcoxon test, * P<0.05; †P<0.005, ‡P<0.001. Inter-treatment comparisons by Man-Whitney test for independent samples**.** Abbreviations: CD, control drink; OJ, orange juice; EOJ, hesperidin-enriched orange juice.

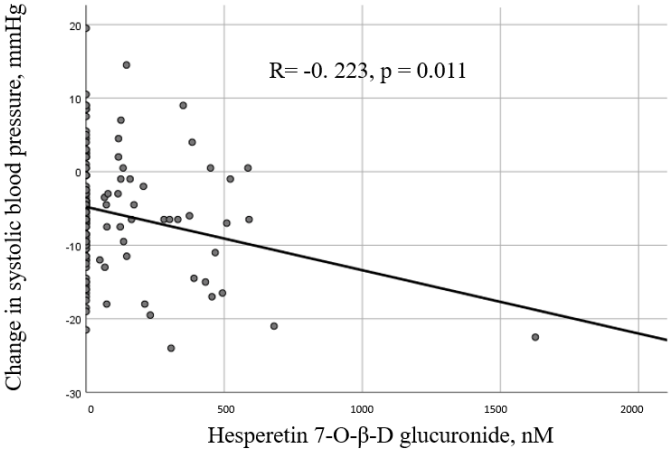


Figure 2. Relationship between levels of hesperetin-7-O-β-d glucuronide in urine and changes in systolic blood pressure after 12 weeks of treatments.

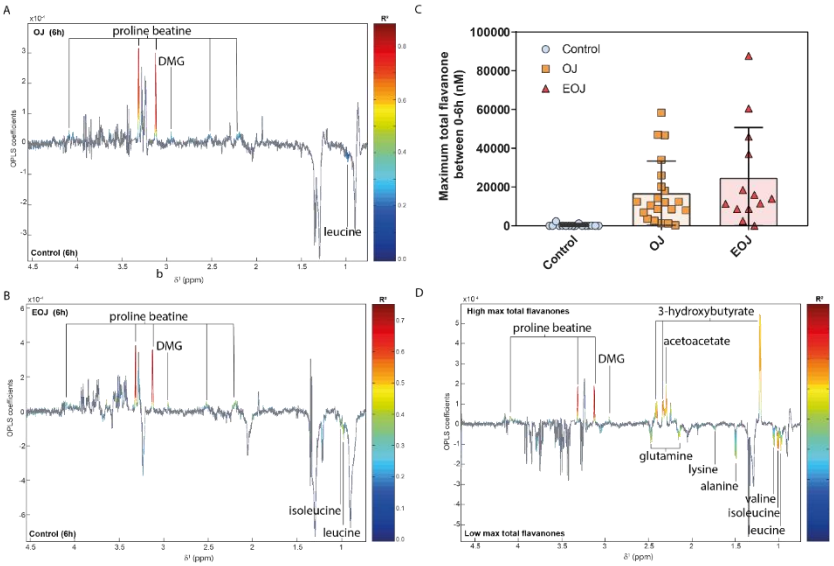


Figure 3. O-PLS coefficients plots for the nontargeted plasma metabolomics analyses after 6-h of beverage consumption in the single dose study (N=52). (A) Orange juice vs control drink. (B) Hesperidin-enriched orange juice vs CD. (C) Maximal total flavanone concentration during the single dose study for each participant in the three treatment groups. (D) O-PLS coefficients plot considering the maximal total flavanone concentration as the response variable. Abbreviations: OJ, orange juice; DMG, dimethylglycine; EOJ, hesperidin-enriched orange juice.

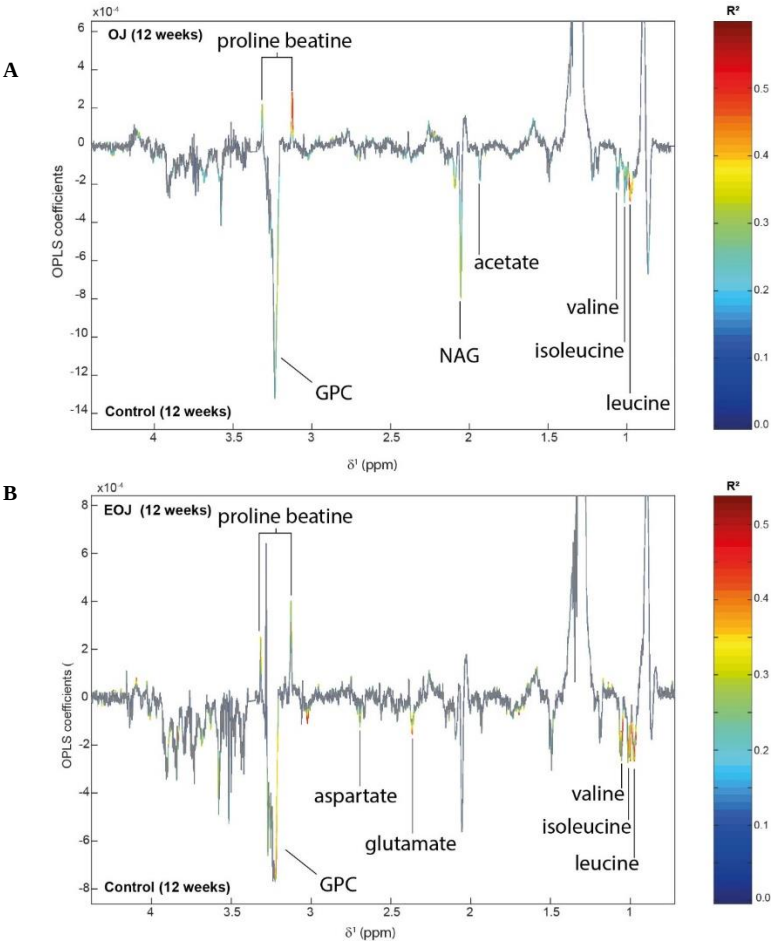


Figure 4. O-PLS-DA coefficient plots for the plasma nontargeted metabolomics analyses at the end of the chronic study (N=52). (A) Orange juice *vs* Control Drink. (B) Hesperidin-enriched orange juice *vs* Control Drink. Abbreviations: OJ, orange juice; GPC, glycerophosphocholine; NAG, N-acetylglycoproteins; EOJ, hesperidin-enriched orange juice.

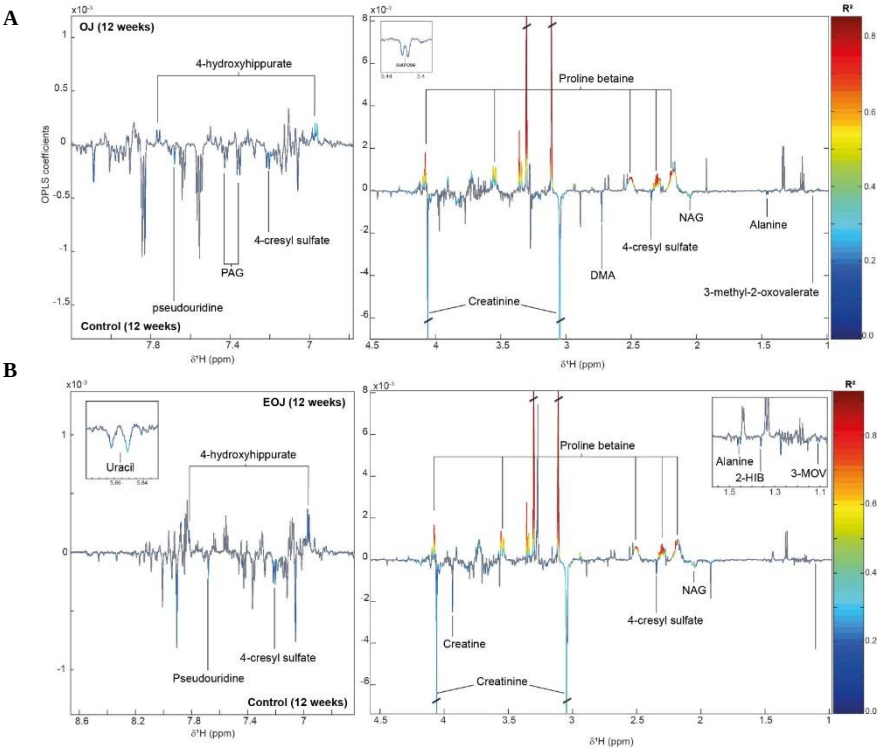


Figure 5. O-PLS-DA coefficient plots for the urine nontargeted metabolomics analyses at the end of the chronic study (N=129). (A) Orange juice *vs* Control Drink. (B) Hesperidin-enriched orange juice *vs* Control Drink. Abbreviations: OJ, orange juice; PAG, phenylacetylglutamine; DMA, dimethylamine; NAG, N-acetylglycoproteins; EOJ, hesperidin-enriched orange juice.

Supplementary Information Methods S1

Study population:

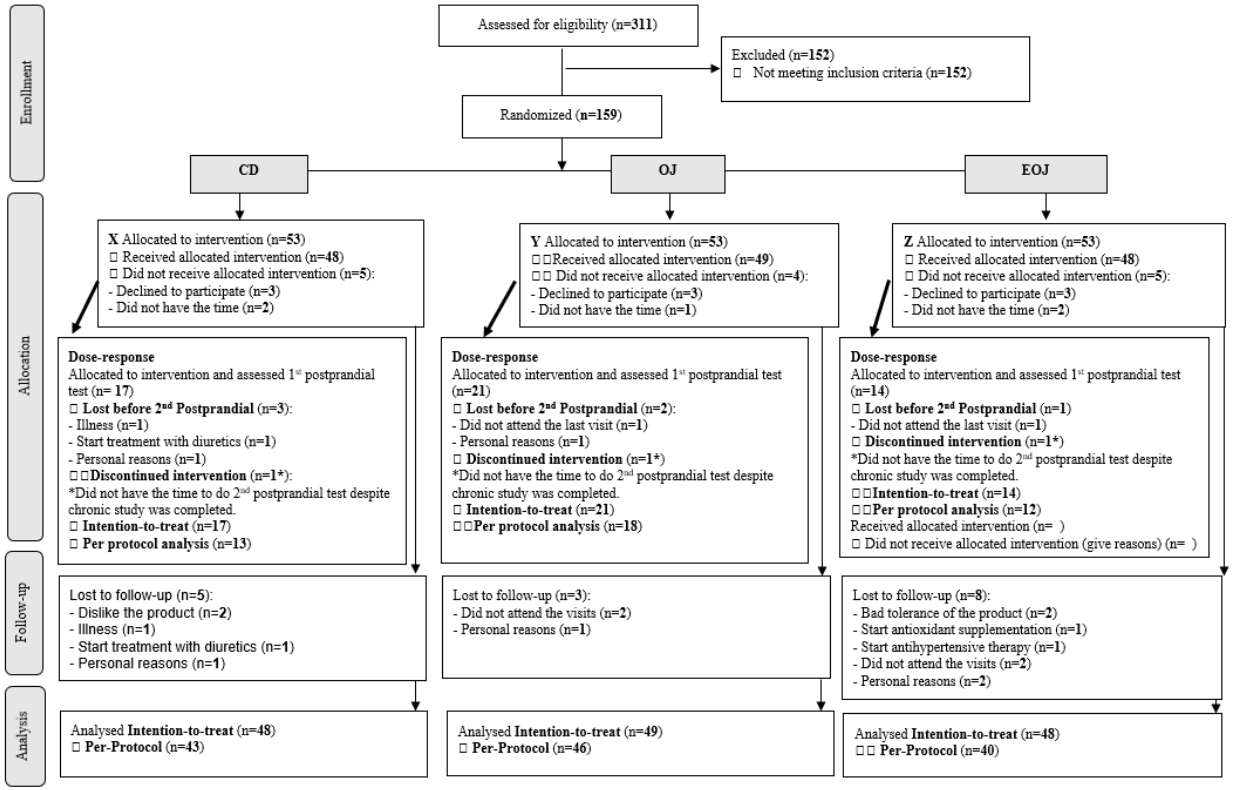
Participants were recruited between January 2016 and June 2017 thought social networks and newspapers in the Hospital Universitari Sant Joan of Reus (Spain). The inclusion criteria were adults from 18 years to 65 years, with systolic blood pressure level ≥ 120 mm Hg and no family history of CVDs or other chronic diseases. The exclusion criteria were subjects with: body mass index ≥35 kg/m^2, fasting glucose >125 mg/dL, hemoglobin ≤13 mg/dL in men and ≤12 mg/dL in women, systolic blood pressure ≥160 mm Hg and diastolic blood pressure >100 mm Hg or taking anti-hypertensive medications, smoking, alcoholism, pregnancy, use of antioxidants or vitamin supplements, and following a vegetarian diet.

All the volunteers signed the informed consent before their inclusion in the clinical trial. The randomized clinical trial received the approbation by the Clinical Research Ethical Committee of Hospital Universitari Sant Joan of Reus (14-12-18/12aclaassN1). The study was conducted in accordance with Helsinki Declaration and Good Clinical Practice Guidelines of the International Conference of Harmonization and was registered at Clinical-Trials.gov: NCT02479568.

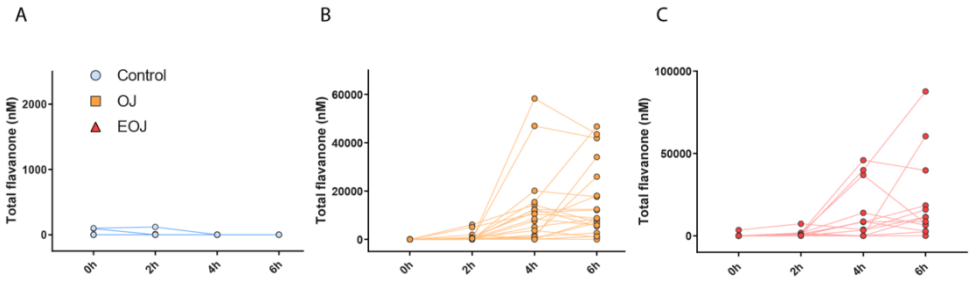
Study design:

The study was a randomized, parallel, double-blind, and placebo-controlled clinical trial. The subjects were randomly assigned by a computerized random-number generator to 1 group of the 3 intervention groups: control drink (CD), orange juice (OJ) and hesperidin-enriched

orange juice (EOJ). All the participants consume daily 500 mL of the corresponding test drink for 12 weeks, and 2 dose-responses studies were performed at baseline and after 12 weeks. Only in the baseline dose-response study were collected plasma samples for targeted and non-targeted metabolomics approach.



Supplementary Information Figure S1. Flow diagram of participants of the study.



Supplemental Information Figure S2. Total absorption of flavanones in baseline conditions and at 2, 4 and 6 hours after each treatment. Abbreviations: OJ, orange juice; EOJ, hesperidin-enriched orange juice.

Supporting Information Table S1. Composition of the 3 test drinks.

	Test drink (for 500 mL/day)		
	Control drink	Orange juice	Hesperidin-enriched orange juice
Acidity, %	2.49	2.49	2.49
Sugar, g	43.1	37.7	37.7
Vitamin C, mg	235.3	235.3	235.3
Citric acid, g	3.40	3.40	3.40
Narirutin, mg	ND	64	77
Hesperidin, mg	ND	392	670

The 3 test drinks were in frozen concentrated canned drink, once diluted 3.4:1 (water to syrup). Abbreviations: ND, non-detectable.

Supplementary Information Table S2. Results from the method validation for targeted metabolomics by UHPLC-MS/MS.

Metabolites	Abbreviation	MDL (µM)	MQL (µM)	Repeatability (RSD) (n=20) (%)
Hesperetin 7-O-beta-D-glucuronide	H7G	20.9	62.8	10.8
Hesperetin 3-O-beta-D-glucuronide	H3G	85.3	255.9	6.7
Hesperetin 7-O-sulfate	H7S	0.2	0.45	4.6
Naringenin 4'-O-beta-D-glucuronide	N4G	51.4	154.1	10.1
Naringenin-glucuronide	NG	-	-	7.5
Naringenin-sulfate	N-S	-	-	4.5

Supplementary Information Table S3. Baseline characteristics of participants by intervention group.

Variable	CD (n=53)	OJ (n=53)	EOJ (n=53)	P
Age, *y*	45.4 ±13.0	43.3 ± 12.0	43.6 ± 11.8	0.629
Females, *%*	34.0	32.1	34.0	0.981
SBP, *mm Hg*	132 ± 9.94	132 ± 9.11	134 ± 9.82	0.687
DPB, *mm Hg*	79 ± 8.14	80 ± 8.42	79 ± 10.2	0.868
Pulse pressure, *mm Hg*	53 ± 9.09	52 ± 8.05	54 ± 6.74	0.261
Weight, *kg*	77.3 ± 15.4	78.8 ± 12.2	75.9 ± 11.6	0.523
BMI, kg/m^2	26.1 ± 3.8	26.4 ± 3.6	26.1 ± 3.3	0.858
Waist circumference, *cm*	93.0 ± 11.0	91.7 ± 10.9	91.4 ± 10.7	0.766
Waist/height, *cm*	0.54 ± 0.06	0.53 ± 0.07	0.54 ± 0.07	0.790
Conicity index	1.50 ± 0.76	1.30 ± 0.35	1.39 ± 0.62	0.269
Glucose, *mg/dL*	91.6 ± 9.2	93.6 ± 11.6	93.6 ± 9.6	0.517
Cholesterol, *mg/dL*				
Total	196 ± 30.1	198 ± 32.7	196 ± 31.6	0.937
LDL	124 ± 26.4	125 ± 31.5	127 ± 25.1	0.900
HDL	50.9 ±13.4	51.0 ± 14.7	49.8 ± 13.0	0.889
Triglycerides*, mg/dL	82 (67-118)	85 (65-121)	81 (63-116)	0.624
Physical activity, A	3.08 ± 0.06	3.12 ± 1.38	3.12 ± 1.26	0.986

Data are expressed as the mean ± standard deviation, or percentages. CD, control drink; OJ, orange juice; EOJ, hesperidin-enriched orange juice; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pulse pressure = SBP-DBP; BMI, body mass index; LDL, low-density lipoproteins; HDL, high-density lipoproteins * median (25^{th}-75^{th} percentiles). AU, arbitrary units: 0, inactive; 1, very low activity; 2, low activity; 3, moderately active; 4, very active. *P* for ANOVA with logarithmic transformation for triglycerides.

Project 5: Proteomic analysis of heart and kidney tissues in healthy and metabolic syndrome rats after hesperidin supplementation.

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Proteomic Analysis of Heart and Kidney Tissues in Healthy and Metabolic Syndrome Rats after Hesperidin Supplementation

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Scope: Proteomics has provided new strategies to elucidate the mechanistic action of hesperidin, a flavonoid present in citrus fruits. Thus, the aim of the present study is to determine the effects of hesperidin supplementation (HS) on the proteomic profiles of heart and kidney tissue samples from healthy and metabolic syndrome (MS) rats.

Methods and results: 24 Sprague Dawley rats are randomized into four groups: healthy rats fed with a standard diet without HS, healthy rats administered with HS (100 mg kg^{-1} day^{-1}), MS rats without HS, and MS rats administered with HS (100 mg kg^{-1} day^{-1}) for eight weeks. Heart and kidney samples are obtained, and proteomic analysis is performed by mass spectrometry. Multivariate, univariate, and ingenuity pathways analyses are performed. Comparative and semiquantitative proteomic analyses of heart and kidney tissues reveal differential protein expression between MS rats with and without HS. The top diseases and functions implicated are related to the cardiovascular system, free radical scavenging, lipid metabolism, glucose metabolism, and renal and urological diseases.

Conclusion: This study is the first to demonstrate the protective capacity of hesperidin to change to the proteomic profiles in relation to different cardiovascular risk biomarkers in the heart and kidney tissues of MS rats.

1. Introduction

Diets rich in fruits and vegetables are known to protect against cardiovascular diseases (CVDs),[1,2] which include heart failure, stroke, and chronic kidney diseases and are the leading cause of mortality worldwide.[3] Several studies have aimed to elucidate the beneficial role of bioactive compounds present in food, such as phenolic compounds, which have shown beneficial effects on different cardiovascular risk factors (CVRFs)[4] and on the prevention of CVDs,[5] to determine their mechanisms of action and identify biomarkers of disease or treatment response. In this sense, omics sciences have gained attention since they can provide important biological information on many biomolecules. Proteomics, one of the most common omics sciences involves large-scale protein identification to study the proteome of a tissue or organ under certain conditions. In

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cardiovascular research, proteomics can help to elucidate the signaling mechanisms involved in CVDs[6] and facilitate prevention and drug development.[7]

In recent years, citrus, particularly orange and orange juice, has been investigated in cardiovascular research. Citrus is rich in polyphenols, mainly flavonoids, and lignans, with hesperidin (hesperetin-7-O-rutinoside) being the most abundant. Hesperidin has been assessed in studies on rats and humans and shown to have beneficial effects on several parameters related to the cardiovascular system and the improvement of CVRFs, such as decreasing blood pressure,[8–11] improving endothelium-dependent vasodilation during hypertension,[12] decreasing total cholesterol and triglyceride levels,[13] improving glucose and insulin levels and the homeostasis model assessment index,[14] decreasing inflammatory markers,[9] decreasing kidney damage markers,[15] and decreasing oxidative stress.[16]

However, proteomic analyses of the effects of citrus on health are scarce. Some studies analyzed the orange proteome but only for technological purposes.[17,18] To the best of our knowledge, only one study performed an interventional analysis of orange juice consumption in humans to evaluate the proteomic changes in the peripheral blood mononuclear cells of healthy humans after consumption of a high-fat and high-carbohydrate meal to evaluate oxidative stress and inflammatory markers.[19] In this study, orange juice suppressed diet-induced inflammation. Furthermore, only one study evaluated the effects of hesperidin on the proteomic profile of human HepG2 cells in relation to cell death.[20] Therefore, no studies have examined the effects of hesperidin on the human or rat tissue proteome in relation to CVD or CVRFs despite several publications reporting a beneficial effect of hesperidin. Furthermore, the biological processes by which hesperidin can induce cardioprotective effects have not been elucidated through proteomic analyses.

Therefore, the aim of this work was to determine the changes in the proteomic profiles of heart and kidney tissues in healthy and metabolic syndrome (MS) rats after hesperidin supplementation to shed light on the hesperidin mechanism of action.

2. Experimental Section

2.1. Animals and Experimental Design

The animal procedures were conducted in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments and approved by the Government of Catalonia and the Animal Ethics Committee of the University Rovira i Virgili (number 10 061). 24 eight-week-old male Sprague Dawley rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were randomly assigned to one of the following four groups ($n = 6$ per group): healthy rats fed a standard diet and supplemented with milk as a vehicle (STDV), healthy rats fed a standard diet and supplemented with milk containing hesperidin (STDH), MS-rats fed a cafeteria diet supplemented with milk as a vehicle (CAFV), and MS-rats fed a cafeteria diet supplemented with milk containing hesperidin (CAFH); all of the treatments occurred over eight weeks. The experimental design was the same as that described by Guirro M et al.[21]

2.2. Dosage Information

Hesperidin was administered daily and orally via low-fat condensed milk over eight weeks at a dose of 100 mg kg^{-1} of body weight per day. The rationale for choosing this dose was based on the beneficial effects of hesperidin reported in previous works focused on MS factors in rat models.[14,22] Accordingly, the human equivalent dose of 100 mg kg^{-1} hesperidin was 1350 mg per day for a 60 kg human,[21,23] a dose achievable with hesperidin-rich orange juice.

2.3. Kidney and Heart Proteomic Analysis

Heart and kidney tissue samples were obtained immediately after the animals were sacrificed, snap-frozen in liquid nitrogen, and stored at −80 °C until the analyses were performed.

2.3.1. Protein Extraction and Quantification

Sample tissue was weighed (25–30 mg) to perform cell lysis, realized according to the radioimmunoprecipitation assay buffer (RIPA) protocol (ThermoFisher Scientific, Barcelona, Spain). First, the samples were frozen in liquid nitrogen for complete lysis. The samples were then mixed with 1 mL of RIPA buffer, homogenized completely with a BlueBender via freeze thaw cycles, agitated for 1 h at 4 °C, and centrifuged. After centrifugation, the samples were sonicated with a 30 s pulse at 50% amplitude. The samples were then centrifuged at 21 130 relative centrifugal force for 15 min, and the supernatants were collected for protein precipitation with the addition of 10% trichloroacetic acid/acetone. The protein pellets were resuspended in 6 M urea per 50 mM ammonium bicarbonate and quantified by Bradford's method.

2.3.2. Protein Digestion and Peptide 10-Plex Tandem Mass Tag Labeling

Thirty micrograms of total protein was reduced with 4 mM 1.4-dithiothreitol for 1 h at 37 °C and alkylated with 8 mM iodoacetamide for 30 min at 25 °C in the dark. Afterward, the samples were digested overnight (pH 8, 37 °C) with sequencing-grade trypsin (Promega, Wisconsin, USA) at an enzyme:protein ratio of 1:50. Digestion was quenched by acidification with 1% v/v formic acid, and peptides were desalted on an Oasis HLB SPE column (Waters, California, USA) before TMT 10-plex labeling (Thermo Fisher, Scientific, Massachusetts, USA) according to the manufacturer's instructions.

For normalization of all samples in the study along with the different TMT-multiplexed batches, a pool containing all the samples was labeled with a TMT-126 tag and included in each TMT batch. The different TMT 10-plex batches were desalted on Oasis HLB SPE columns before nanoLC-MS analysis.

2.3.3. Off Gel-NanoLC-(Orbitrap) MS/MS Analysis

Labeled and multiplexed samples were fractionated by off-gel technology (Agilent, California, USA) according to the manufacturer's protocol. Samples were fractioned on 12 cm non-linear pH 3–10 strips in 12 fractions. Thus, fraction 1 (F1) was mixed with fraction 7 (F7), F2 was mixed with F8, and this protocol was repeated with all fractions. In total, 6 fractions were obtained, and they were separated on a C-18 reversed-phase (RP) nanocolumn (75 μm I.D; 15 cm length; 3 μm particle diameter, Nikkyo Technos Co., Ltd., Japan) on an EASY-II nanoLC made by Thermo Fisher Scientific (Massachusetts, USA). The chromatographic separation was performed with a 90-min gradient using Milli-Q water (0.10% formic acid) and acetonitrile (0.10% formic acid) as the mobile phase at a flow rate of 300 nL min^{-1}.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro made by Thermo Fisher via an enhanced FT-resolution MS spectrum (R = 30 000 FHMW) followed by a data-dependent FT-MS/MS acquisition (R = 15 000 FHMW, 40% HCD) of the 10 most intense parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min.

2.3.4. Protein Identification and Quantification

Protein identification and quantification were performed with Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific, Massachusetts, USA) using multidimensional protein identification technology, combining the six raw data files obtained after off-gel fractionation. For protein identification, all MS and MS/MS spectra were analyzed using the Mascot search engine (v.2.5). Mascot was set up to search SwissProt_2018_03.fasta database (557 012 entries), restricted to *Rattus norvegicus* taxonomy (8003 sequences) and assuming trypsin digestion. Two missed cleavages were allowed, and errors of 0.02 Da for an FT-MS/MS fragmentation mass and 10 ppm for an FT-MS parent ion mass were allowed. TMT-10plex was set as the quantification modification, oxidation of methionine and acetylation of N-termini were set as dynamic modifications, and carbamidomethylation of cysteine was set as the static modification. For protein quantification, the ratios between each TMT-label and the 126-TMT label were used, and quantification results were normalized based on the protein median.

2.4. Statistical Analysis

The proteins present in ≥67% of the samples in the four groups were considered for the statistical analysis. After the proteomic analysis, the data were log base 2 transformed, mean centered, and Pareto scaled. The multivariate statistical analysis was performed using Metaboanalyst 4.0 (https://www.metaboanalyst.ca/). The modeling included the use of unsupervised methods such as principal component analysis (PCA), supervised methods such as partial least squares discriminant analysis (PLS-DA), and an orthogonal projection to latent structures discriminant analysis (OPLS-DA). For the univariate statistical analysis, the distribution of normality was assessed by Kolmogorov-Smirnov tests, and a *t*-test or a Wilcoxon test was performed for pairwise comparisons. The proteins that were statistically significant in univariate analysis with a p-value < 0.01 and a PLS-DA variable importance on projection (VIP) score > 1.5 in multivariate analysis were considered differentially expressed between groups.

2.5. Pathway Analysis

The UniProt Database was used to obtain the gene symbols and protein description (https://www.uniprot.org/). Ingenuity pathway analysis (IPA; www.ingenuity.com) was used to analyze the protein networks via the statistically significant results from both multivariate and univariate analyses for biological interpretation. IPA was used to explore the possible metabolic cell signaling pathways that were over- or underrepresented by the experimentally-determined proteins.

3. Results

3.1. Biochemical Analyses

In previous studies,[21,24] the feeding of a cafeteria diet to Sprague Dawley rats induced an obesogenic pattern with significant increases in body weight and fat mass, elevated systolic blood pressure, hypertriglyceridemia, hyperglycemia, and high levels of low-density lipoprotein cholesterol (LDL-c), which are criteria required for MS diagnosis. Consequently, when applying the harmonized human definition of MS to Sprague Dawley rats consuming a cafeteria diet, the rats exhibited a reflex of fat mass, elevated triglyceride (TG) levels (drug treatment for elevated TG is an alternate indicator), reduced high-density lipoprotein cholesterol (HDL-c) levels (drug treatment for reduced HDL-c is an alternate indicator), elevated blood pressure (systolic or diastolic (antihypertensive drug treatment in a person with a history of hypertension is an alternate indicator), and/or elevated fasting plasma glucose levels (drug treatment of elevated glucose is an alternate indicator).[25]

We also report that the consumption of hesperidin (100 mg kg^{-1} body weight per day) for eight weeks improved lipid metabolism and the insulin response and decreased the systolic blood pressure in MS rats. In this sense, hesperidin supplementation can improve most of the MS criteria.[24]

3.2. Proteomic Analysis Results

From the proteomic analysis of heart and kidney tissue rat samples, 1127 and 1753 proteins were identified, respectively, and the total proteins identified from each tissue are detailed in Tables S1 and S2, Supporting Information. The tables contain information on the protein IDs from UniProt, descriptions of the proteins, coverage, unique peptides identified, total peptides identified, peptide-to-spectrum matches, molecular weights, and the value for each protein.

3.2.1. Proteomic Analysis of Heart Rat Tissue

For statistical analyses only those proteins that were present in ≥67% of the samples in the four groups were considered.

After filtering, a total of 872 proteins were considered for statistical analysis. The identified proteins with a p-value < 0.01 and a VIP score from PLS-DA > 1.5 were considered significantly different between the STDV and STDH groups, and between the CAFV and CAFH groups.

Hesperidin Effects in Healthy Rats Fed with a Standard Diet: In multivariate analysis, no clear differences were observed between the two STD treatment groups (STDV vs STDH) in the unsupervised analysis. The supervised analysis showed that none of the assessed models was significant due to the negative performance measurement Q^2, indicating that the models were not predictive at all or were overfitted and that a random model would perform better. Moreover, the univariate analysis showed no significant differences between the treatment groups (STDV vs STDH) in any of the 872 considered proteins.

Hesperidin Effects in MS Rats Fed with a Cafeteria Diet: In multivariate analysis, no clear differences between the two treatment groups (CAFV vs CAFH) were observed in CAF rats from the unsupervised analysis. The PLS-DA from the supervised analysis showed that a model including one component provided the best performance, as determined by the Q^2 measure. However, after permutation testing, the model was not found to be significant. Nevertheless, a borderline significant model with a strong predictive ability ($Q^2Y = 0.58$, $p = 0.053$) was obtained for the comparison between CAFH and CAFV rats. The univariate analysis showed 65 differentially expressed proteins between CAF groups with a p-value < 0.01.

The proteins with a p-value < 0.01 and a VIP score from PLS-DA > 1.5 were considered to be differentially expressed between the group without hesperidin supplementation (CAFV) and the group with hesperidin supplementation (CAFH). A total of 35 proteins considered to be significantly different between the two groups met the two criteria of univariate and multivariate analyses. The information about the 35 proteins is detailed in **Table 1**. In total, 19 proteins were downregulated and 16 were upregulated after hesperidin treatment for eight weeks compared to those in the CAFV group.

Pathway Analysis of the Heart Tissue Proteome: IPA analysis was performed and the top signaling pathways that were significantly affected after hesperidin supplementation were obtained. The top five significant signaling pathways were: production of nitric oxide and reactive oxygen species in macrophages (p = 4.08E−04; ratio = 0.021), which involved: APOA4, APOC2, CDC42, and RBP4; clathrin-mediated endocytosis signalling (p = 4.50E−04; ratio = 0021), which involved: APOA4, APOC2, CLTC, and RBP4; LXR/RXR activation (p = 1.07E−03; ratio = 0.028), atherosclerosis signaling (p = 1.19E−03; ratio = 0.027); and FXR/RXR activation (p = 1.25E−03; ratio = 0.026) which involved: APOA4, APOC2, and RBP4 in the last three canonical pathways.

Network of the Heart Tissue Proteome: The top networks found by IPA were "Molecular transport, carbohydrate metabolism, and small molecule biochemistry" (score = 16), "Cardiac arrhythmia, cardiovascular disease, and metabolic disease" network (score = 2), and "Cancer, cardiovascular disease, and cell cycle" (score = 2). The graphical representation of the top overlapping networks and the up- and downregulated proteins, symbolized in red or green, respectively, is shown in **Figure 1**. The proteins implicated in the top networks after hesperidin treatment for eight weeks were: APOA4, SOD1, ATP5F1D, ATPA1A, SLC27A1, SLC25A3, OGDH, FLNA, ALDH7A1, CLTC, and CDC42. The other proteins implicated in the top networks related to the proteins analyzed in the present study were: IL-1B, LAMC1, TNF, KCNJ11, ATP1B1, OTOF, SLC1A2, AP2B1, EPB41L2, GRM4, CACNA1B, F11R, TNFRSF1B, KCNJ2, CPT1A, PIN, MYO1C, PP2A, PRKCD, STAT3, NEFH, ITSN1, SRC, ABR, PRKCZ, CAMKII, FILIP1, AR, RCAN1, GSK3B, NFKB complex, and SIPI.

3.2.2. Proteomic Analysis of Kidney Rat Tissue

After filtering, a total of 1341 proteins were considered for the statistical analysis of kidney tissue samples. The identified proteins with a p-value < 0.01 and a VIP score from PLS-DA > 1.5 were considered significantly different between the STDV and STDH groups, and between the CAFV and CAFH groups.

Hesperidin Effects in Healthy Rats Fed a Standard Diet: From the multivariate analysis no clear separation between the two STD groups (STDV versus STDH) was evident from the unsupervised or supervised analysis. From the univariate analysis, no statistically significant proteins were observed between the two STD groups among any of the 872 considered proteins.

Hesperidin Effects in MS Rats Fed a Cafeteria Diet: From unsupervised analysis, a clear separation between the two CAF groups (CAFV versus CAFH) was evident from the PCA, although it was not very clear in the hierarchical clustering analysis. A PLS-DA from the supervised analysis showed that a model including one component provided the best performance, as determined by the Q^2 measurement. The OPLS-DA analysis showed a significant model with a strong predictive ability ($Q^2Y = 0.70$, $p = 0.046$) for the comparison between CAFH and CAFV rats. The univariate analysis showed 75 proteins that were differentially expressed between the two CAF groups with a p-value < 0.01.

The proteins with a p-value < 0.01 and a VIP score from PLS-DA > 1.5 were considered to be differentially expressed between the groups with and without hesperidin supplementation. A total of 53 proteins were considered to be significantly different between the two groups met the two criteria from univariate and multivariate analyses. The information on the 53 proteins is detailed in **Table 2**. In total, 33 proteins were downregulated and 20 were upregulated after hesperidin treatment for eight weeks compared to those in the CAFV group.

Pathway Analysis of the Kidney Tissue Proteome: IPA analysis was performed and the top signaling pathways that were significantly affected in kidney tissue after hesperidin supplementation were obtained. The top five significant signaling pathways were: mitochondrial dysfunction (p = 4.65E−06; ratio = 0.039), which involved ATP5PF, COX6B1, CPT1A, OGDH, TXN2, and VDAC3; the sirtuin signalling pathway (p = 9.37E−05; ratio = 0.023), which involved: ATP5PF, CPT1A, HIST2H3C, SOD1, TIMM8B, and VDAC3; xanthine and xanthosine salvage (p = 2.82E−03; ratio = 1), guanine and guanosine salvage I (p = 5.62E−03; ratio = 0.5); and adenine and adenosine salvage I (p = 5.62E−03; ratio = 0.5), which involved PNP.

Table 1. 35 proteins differentially expressed in heart tissue after hesperidin treatment in CAFH compared to CAFV rats.

UniProt code	Gene symbol	Protein description	MW [kDa]	Univariate analysis		Multivariate analysis
				FC	*p*-value[a)]	VIP[b)]
M0RDK9	ACAD8	Acyl-CoA dehydrogenase family, member 8	45.10	1.4409	0.0044	1.8469
F1LN92	AFG3L2	AFG3-like matrix AAA peptidase subunit 2	89.30	1.3967	0.0060	1.7448
Q64057	ALDH7A1	Alpha-aminoadipic semialdehyde dehydrogenase	58.70	1.2888	0.0004	1.6283
P02651	APO A4	Apolipoprotein A-IV	44.40	−1.5095	0.0093	1.9047
P19939	APO C1	Apolipoprotein C-I	9.90	1.7180	0.0033	2.2661
G3V8D4	APO C2	Apolipoprotein C-II	10.70	−1.8310	0.0006	2.4926
P06685	ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1	113.00	1.3297	0.0020	1.6668
G3V7Y3	ATP5F1D	ATP synthase subunit delta, mitochondrial	17.60	−1.3813	0.0047	1.7317
D4A305	CCDC58	Coiled-coil domain containing 58, isoform CRA_c	16.70	1.7327	0.0065	2.2324
Q8CFN2	CDC42	Cell division control protein 42 homolog	21.20	1.2746	0.0025	1.5289
Q5XIM5	CDV3	Protein CDV3 homolog	24.30	−1.6055	0.0040	2.1074
M0RC65	CFL2	Cofilin 2	18.70	−1.3380	0.0054	1.635
F1M779	CLTC	Clathrin heavy chain	191.40	1.2960	0.0024	1.5829
Q5BJQ0	COQ8A	Atypical kinase COQ8A, mitochondrial	72.20	−1.2667	0.0012	1.5373
P11240	COX5A	Cytochrome c oxidase subunit 5A, mitochondrial	16.10	−1.5030	0.0056	1.9324
P60841	ENSA	Alpha-endosulfine	NA	−1.2770	0.0034	1.5225
C0JPT7	FLNA	Filamin A	280.30	1.5440	0.0007	2.1071
D3ZT90	GCDH	Glutaryl-CoA dehydrogenase	49.70	1.2996	0.0050	1.5552
Q5I0P2	GCSH	Glycine cleavage system H protein, mitochondrial	18.50	−1.5136	0.0097	1.9064
D4ADD7	GLRX5	Glutaredoxin 5	16.40	−1.2978	0.0019	1.5977
D4A4L5	ISCA2	Iron-sulfur cluster assembly 2	16.70	−1.3870	0.0098	1.6936
G3V6P7	MYH9	Myosin, heavy polypeptide 9, nonmuscle	226.30	1.8328	0.0019	2.4356
A0A0G2KAQ5	MYOZ2	Myozenin 2	29.80	−1.4006	0.0084	1.7298
Q5XI78	OGDH	2-oxoglutarate dehydrogenase, mitochondrial	116.20	1.3004	0.0043	1.5656
P04916	RBP4	Retinol-binding protein 4	23.20	−1.3708	0.0037	1.7246
G3V8R0	RGD1311703	Similar to sid2057p	19.90	−1.5060	0.0053	1.9425
F1LSW7	RPL14	60S ribosomal protein L14	23.30	1.3440	0.0021	1.6981
Q6IRH6	SLC25A3	Phosphate carrier protein, mitochondrial	39.60	1.3677	0.0098	1.5331
P97849	SLC27A1	Long-chain fatty acid transport protein 1	71.20	1.2016	0.0037	1.5028
P07632	SOD1	Superoxide dismutase[Cu-Zn]	15.90	−1.39760	0.0040	1.7717
P62078	TIMM8B	Mitochondrial import inner membrane translocase subunit Tim8 B	9.30	−1.5168	0.0073	1.9335
P62074	TIMM10	Mitochondrial import inner membrane translocase subunit Tim10	10.30	−1.2840	0.0043	1.5285
Q5XIK2	TMX2	Thioredoxin-related transmembrane protein 2	33.80	1.4949	0.0031	1.9565
B0K010	TXNDC17	Thioredoxin domain-containing 17	14.10	−1.3020	0.0052	1.5612
Q5M9I5	UQCRH	Cytochrome b-c1 complex subunit 6 mitochondrial	10.40	−1.4631	0.0037	1.8937

CAFH, metabolic syndrome rats with hesperidin supplementation; CAFV, metabolic syndrome rats without hesperidin supplementation; MW, molecular weight; FC, fold change; NA, not available. [a)] Results from Wilcoxon tests and *t*-test. A p-value < 0.01 was considered statistically significant. [b)] VIP score was from PLS-DA.

Network of the Kidney Tissue Proteome: The top network found by IPA was "Cellular compromise, free radical scavenging, cell death, and survival" (score = 14), followed by "Cell death and survival, free radical scavenging, organismal injury, and abnormalities" (score = 6), and "Cardiovascular system development and function, immunological disease, inflammatory disease" (score = 1). The overlapping graphical representation of the two more important networks "Cellular compromise, free radical scavenging, cell death, and survival" and "Cardiovascular system development and function, immunological disease, inflammatory disease", as well as the up- and downregulated proteins (in red and green, respectively) are represented in **Figure 2**. The proteins implicated in the top networks after hesperidin treatment for eight weeks were EPB41L3, TXN2, SOD1, TPM3, NUCB2, VDAC3, MYO1D, SLC25A3, OGDH, CPT1A, MME, CDC42, ABCG2, and RACK1. The other proteins implicated in the top networks related to the proteins analyzed in the present study were TNF, AGT, INSULIN, OTOF, SLC27A1, FRIN2B, TPM1, CD36, ITGB1, KCNJ11, CHMP2B, PLEC, KRT8, TPM2, MAPK14, HSPA5, PRKCZ, PAR6, NCF1, ERN1, EIF2AK3, CTSV, and TP53.

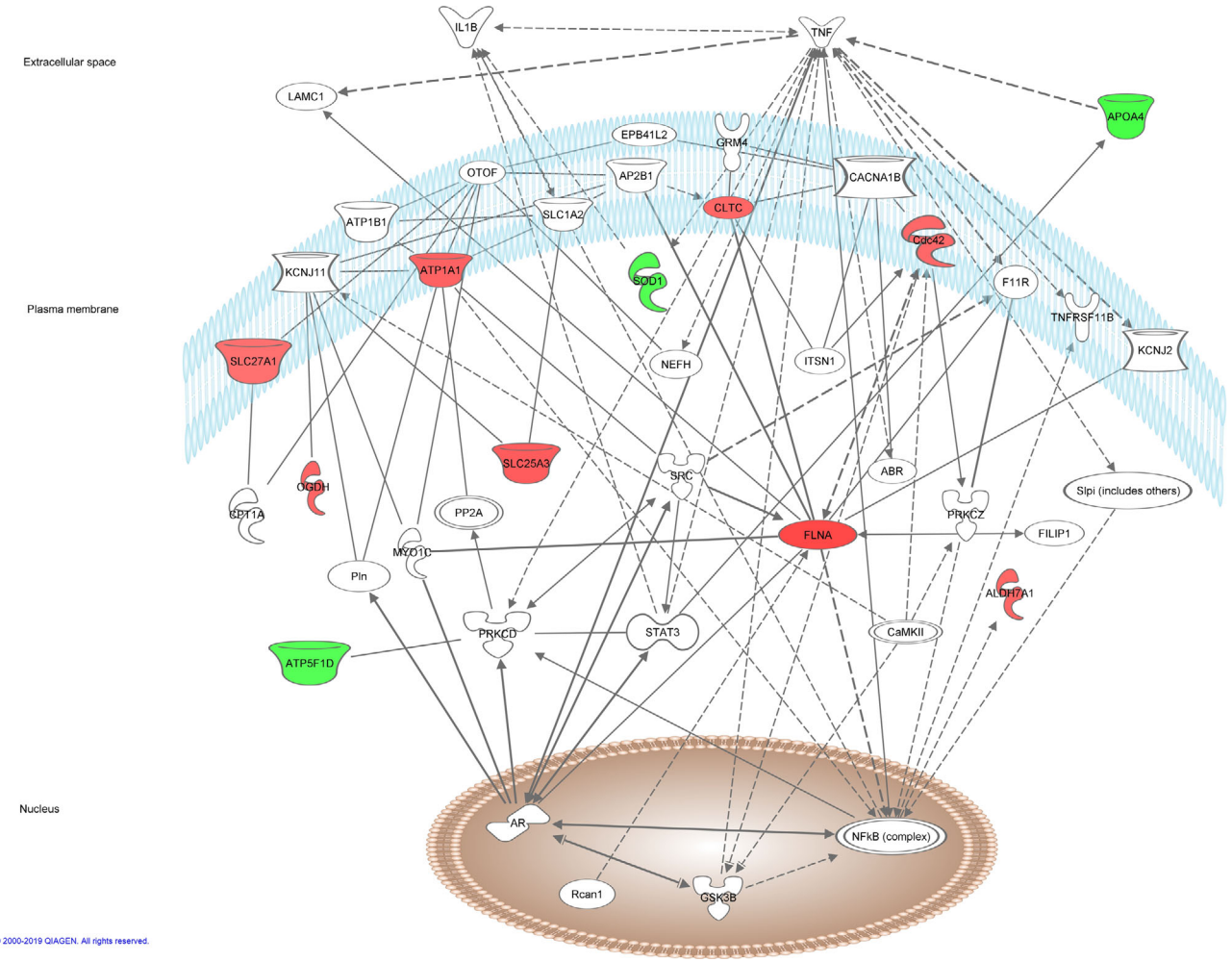

Figure 1. Graphical representation of the top networks of the heart tissue proteome after hesperidin supplementation. Interaction between the differentially expressed proteins and other important proteins. Down- and upregulated proteins are symbolized in red and green, respectively. ABR, active breakpoint cluster region-related protein; ALDH7A1, aldehyde dehydrogenase 7 family member 1; AP2B1, AP-2 complex subunit beta; APOA4, apolipoprotein A4; ATP1A1, ATPase Na+/K+ transporting subunit alpha 1; ATP1B1, sodium/potassium-transporting ATPase subunit beta-1; ATP5F1D, ATP synthase F1 subunit delta; CACNA1B, voltage-dependent N-type calcium channel subunit alpha-1B; CAMKII, calcium/calmodulin-dependent protein kinase type II alpha chain; CDC42, cell division cycle 42; CLTC, clathrin heavy chain; CPT1A, carnitine palmitoyltransferase 1A; EPB41L2, erythrocyte membrane protein band 4.1-like 2; F11R, junctional adhesion molecule A; FILIP1, filamin-A-interacting protein 1; GRM4, metabotropic glutamate receptor 4; IL1B, interleukin-1 beta; ITSN1, intersection-1; FLNA, filamin A; KCNJ11, ATP-sensitive inward rectifier potassium channel 11; KCNJ2, inward rectifier potassium channel 2; LAMC1, laminin subunit gamma 1; MYO1C, unconventional myosin-Ic; NEFH, neurofilament heavy polypeptide; NFKB, nuclear factor NF-kappa-B p105 subunit; OGDH, oxoglutarate dehydrogenase; OTOF, otoferlin; PIN, peptidyl-propyl cis-trans isomerase NIMA-interacting 1; PP2A, serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform; PRKCD, protein kinase C delta type; SRC, proto-oncogene tyrosine-protein kinase Src; PRKCZ, protein kinase C zeta type; RCAN1, calcipressin-1; GSK3B, glycogen synthase kinase-3 beta; SIPI, secretory leukocyte protease inhibitor; AR, androgen receptor; SLC1A2, excitatory amino acid transporter 2; SLC25A3, solute carrier family 25 member 3; SLC27A1, long-chain fatty acid transport protein 1; SOD1, superoxide dismutase 1; STAT3, signal transducer and activator of transcription 3; TNF, tumor necrosis factor; TNFRSF11B, tumor necrosis factor receptor superfamily member 11B. Figure reproduced with permission under the terms of the CC-BY 4.0 license. Copyright 2019, QIAGEN Silicon Valley.

3.3. Top Diseases and Functions Determined from the Heart and Kidney Tissue Proteomes

Table 3 details the top relevant diseases and functions involving the significant proteins in both heart and kidney tissues. The top categories are the cardiovascular system, free radical scavenging, lipid metabolism, glucose metabolism, and renal and urological diseases.

4. Discussion

The current study presents the effects of hesperidin supplementation of 100 mg kg^{-1} body weight per day for eight weeks on the proteomic profiles of heart and kidney tissues in rats with or without MS. Proteomic analysis revealed significant changes in the proteomic profiles of MS rats fed a cafeteria diet with and without hesperidin supplementation in both tissues after eight weeks.

Table 2. 53 proteins differentially expressed in kidney tissue after hesperidin treatment in CAFH compared to CAFV rats.

UniProt code	Gene symbol	Protein description	MW [kDa]	Univariate analysis		Multivariate analysis
				FC	*p*-value[a)]	VIP[b)]
Q80W57	ABCG2	ATP-binding cassette subfamily G member 2	72.90	1.2570	0.0031	1.6966
Q6P2A5	AK3	GTP:AMP phosphotransferase AK3, mitochondrial	25.50	−1.2561	0.0094	1.7174
Q9WUC4	ATOX1	Copper transport protein ATOX1	7.30	−1.2888	0.0021	1.8082
Q03344	ATP5IF1	ATPase inhibitor, mitochondrial	12.20	−1.3651	0.0005	2.0549
P21571	ATP5PF	ATP synthase−coupling factor 6, mitochondrial	12.50	−1.6119	0.0028	2.4703
B2GUV5	ATP6V1G1	V−type proton ATPase subunit G	13.70	−1.4949	0.0014	2.2956
Q5I0M1	APO H	Apolipoprotein H	38.40	−1.5042	0.0008	2.3359
F1LRS8	CD2AP	CD2−associated protein	70.40	−1.2746	0.0088	1.6887
D3ZD09	COX6B1	Cytochrome c oxidase subunit	10.10	−1.4006	0.009	1.9883
P32198	CPT1A	Carnitine O−palmitoyltransferase 1, liver isoform	88.10	1.4794	0.0043	2.1983
P97829	CD47	Leukocyte surface antigen CD47	33.00	1.2059	0.0038	1.5247
Q8CFN2	CDC42	Cell division control protein 42 homolog	21.20	1.2033	0.0033	1.5261
D3ZUX5	CHCHD3	MICOS complex subunit	26.40	−1.3168	0.0022	1.8771
A0A0H2UHL6	CTSH	Pro−cathepsin H	32.90	−1.6982	0.0038	2.5675
P07154	CTSL	Cathepsin L1	37.60	1.5305	0.0035	2.3057
Q68FR9	EEF1D	Elongation factor 1−delta	31.30	−1.2527	0.0035	1.6775
A3E0T0	EPB41L3	Erythrocyte membrane protein band 4.1−like 3	96.90	−1.5757	0.0065	2.3375
P80299	EPHX2	Bifunctional epoxide hydrolase 2	62.30	4.2663	0.0087	4.1318
C0JPT7	FLNA	Filamin A	280.30	1.2067	0.005	1.5157
P19468	GCLC	Glutamate-cysteine ligase catalytic subunit	72.60	1.3370	0.0008	1.9709
D3ZK97	H3F3C	Histone H3	15.30	1.2772	0.0097	1.691
F1M9B2	IGFBP7	Insulin-like growth factor binding protein 7, isoform CRA_b	28.90	−1.6947	0.0024	2.593
D4A4L5	ISCA2	Iron-sulfur cluster assembly 2	16.70	−1.3918	0.0055	2.0032
B2RZ79	ISCU	Iron-sulfur cluster assembly enzyme	18.00	−1.3077	0.0013	1.8767
D3ZCZ9	LOC100912599	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	13.00	−1.2978	0.0037	1.8041
Q562C6	LZTFL1	Leucine zipper transcription factor-like protein 1	34.60	−1.5810	0.0012	2.4546
D3Z900	MARC2	Mitochondrial amidoxime reducing component 2	38.20	1.2501	0.0009	1.7259
A0A0H2UHX5	MME	Neprilysin	78.60	1.2870	0.0062	1.745
Q63357	MYO1D	Unconventional myosin-Id	116.00	1.4044	0.0072	2.0134
G3V8R1	NUCB2	Nucleobindin 2. isoform CRA_b	50.10	−1.3538	0.0042	1.9359
Q5XI78	OGDH	2-oxoglutarate dehydrogenase, mitochondrial	116.20	1.2142	0.0003	1.6381
P51583	PAICS	Multifunctional protein ADE2	47.10	1.4550	0.0078	2.1091
D3ZD40	PAPLN	Papilin. proteoglycan-like sulfated glycoprotein	138.50	−1.4040	0.0046	2.0433
B0BN18	PFDN2	Prefoldin subunit 2	16.60	−1.2527	0.0065	1.6467
P85973	PNP	Purine nucleoside phosphorylase	32.30	1.3718	0.0005	2.0718
P10960	PSAP	Prosaposin	61.10	−1.4641	0.0057	2.151
P63245	RACK1	Receptor of activated protein C kinase 1	35.10	1.2130	0.0005	1.6195
Q6TXG7	SHMT1	Serine hydroxymethyltransferase	75.30	1.2142	0.0042	1.5493
F1LZW6	SLC25A13	Solute carrier family 25 member 13	54.10	1.2303	0.0023	1.6277
G3V741	SLC25A3	Phosphate carrier protein. mitochondrial	39.50	1.2050	0.0057	1.5022
Q9JJ19	SLC9A3R1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	38.80	−1.2588	0.0093	1.6398
G3V6D9	SLC9A3R2	Na(+)/H(+) exchange regulatory cofactor NHE-RF	37.30	−1.2467	0.0061	1.6295
P07632	SOD1	Superoxide dismutase[Cu-Zn]	15.90	−1.5801	0.0036	2.3891
A0A0G2K9 × 1	SPP2	Secreted phosphoprotein 24	14.90	−1.5094	0.004	2.2601
O70257	STX7	Syntaxin-7	28.80	−1.3122	0.0085	1.7901
P62078	TIMM8B	Mitochondrial import inner membrane translocase subunit Tim8 B	9.30	−1.5241	0.0058	2.2588
Q63610	TPM3	Tropomyosin alpha-3 chain	29.00	−1.4241	0.0036	2.1009

(*Continued*)

Table 2. Continued.

UniProt code	Gene symbol	Protein description	MW [kDa]	Univariate analysis		Multivariate analysis
				FC	*p*-value[a)]	VIP[b)]
P09495	TPM4	Tropomyosin alpha-4 chain	28.50	−1.3918	0.0056	2.0044
P63029	TPT1	Translationally controlled tumor protein	19.40	−1.4459	0.0027	2.163
P97615	TXN2	Thioredoxin. mitochondrial	18.20	−1.3698	0.0075	1.9353
Q5M9I5	UQCRH	Cytochrome b-c1 complex subunit 6, mitochondrial	10.40	−1.4015	0.0027	2.0703
Q9Z269	VAPB	Vesicle-associated membrane protein-associated protein B	26.90	−1.3168	0.0097	1.7924
A0A0G2JSR0	VDAC3	Voltage-dependent anion-selective channel protein 3	30.80	1.2226	0.0006	1.6488

CAFH, metabolic syndrome rats with hesperidin supplementation; CAFV, metabolic syndrome rats without hesperidin supplementation; MW, molecular weight; FC, fold change. [a)] Results from Wilcoxon tests and *t*-tests. A p-value < 0.01 was considered statistically significant. [b)] VIP score was from PLS-DA.

In the heart tissues of MS rats, 35 proteins were differentially expressed: 19 proteins were downregulated and 16 were upregulated. Moreover, in the kidney tissues of MS rats, 53 proteins were differentially expressed: 33 proteins were downregulated and 20 were upregulated.

Currently, there are no data on the effects of hesperidin polyphenol on the proteomes of heart and kidney tissues in healthy or MS rats. However, several studies have shown that polyphenols, such as resveratrol found in red wine and grapes and secoiridiods and hydroxytyrosol present in olive oil, can change the proteomes of cells, in rats and humans, improving different cardiovascular risk parameters such as inflammation, cholesterol homeostasis, oxidation, and blood coagulation.[26–28]

Our results suggest that, in MS rats, hesperidin supplementation could exert cardioprotective effects by upregulating the expression of proteins related to the cardiovascular system such as ATP1A1 (1.32-fold). ATP1A1, found in heart tissue, is associated with ischemic acute renal failure when it is downregulated and with a decrease in blood pressure levels when it is upregulated.[29] In addition, hesperidin can be cardioprotective by downregulating the expression levels of proteins known to affect blood pressure, such as ATP5PF (−1.61-fold), which is found in kidney tissue and related to vasoconstriction, hypertension, and cardiac hypertrophy in rats and humans.[30,31] Another related protein that appeared to be downregulated in the kidney tissues of MS rats was TPT1 (−1.44-fold). TPT is implicated in the pathogenesis of atherosclerosis and pulmonary artery hypertension to prevent macrophage apoptosis in the artery intima.[32] IGFBP7 was also downregulated by hesperidin in kidney tissue (−1.69-fold). At high concentrations, this molecule is related to poor diastolic function and ventricular systolic pressure. Thus, elevated IGFBP7 levels could be a biomarker of diastolic dysfunction and functional capacity in humans with heart failure.[33]

Moreover, our findings suggest that hesperidin could also exert cardioprotective effects in MS rats by upregulating the expression levels of proteins related to free radical scavenging, such as ALDH7A1 (1.28-fold). ALDH7A1, found in heart tissue, protects cells against oxidative stress by metabolizing lipid peroxidation-derived aldehydes produced during oxidative stress and xenobiotics metabolism.[34] During lipid peroxidation, large quantities of aldehydes are produced, and they can covalently bind to proteins and DNA, inactivating different proteins and damaging DNA. However, aldehydes are related to several diseases, such as atherosclerosis.[35] In the heart and kidney tissues of MS rats, another protein upregulated by hesperidin was FLNA, a large cytoplasmic protein (1.54-fold in heart tissue and 1.20-fold in kidney tissue). FLNA can promote or suppress cell processes important for heart development.[36] FLNA is downregulated in rats with coronary microembolization,[37] and the lack of FLNA demonstrates its importance during morphogenesis of several organs, such as the heart; the lack of FLNA can result in cardiovascular malformations.[38] Moreover, the lack of editing in FLNA increased RhoA/Rock and PLC/PKC signaling, increased aortic hypercontraction and induced cardiomyocyte hypertrophy, increasing the diastolic blood pressure.[39]

Our results suggested that hesperidin downregulated CD2AP inducing changes in the expression of proteins related to glucose metabolism. CD2AP, a cytosolic protein that interacts with signaling molecules,[40] was downregulated in the kidney tissues (−1.27-fold) of MS rats. Evidence suggests that high levels of CD2AP increase the risk of renal disease in patients with diabetes.[41] Another differentially expressed and upregulated protein in kidney tissue was EPHX2 (4.26-fold). Decreased EPHX2 expression is related to increased insulin sensitivity in humans with MS.[42] Accordingly, hesperidin supplementation could exert preventive effects on glucose metabolism in MS rats, but the effects of hesperidin intake on humans with CVRFs such as diabetes and MS need to be confirmed.

Although the present study showed that hesperidin supplementation can change the proteomic profile to exert positive effects on different parameters in MS rats, some differentially expressed proteins showed negative effects. For example, in MS rats, the following two antioxidant proteins were downregulated: SOD1 (−1.39-fold in heart tissue; 1.58-fold in kidney tissue) and TXN2 (−1.36-fold in kidney tissue), which likely decreased the protection against oxidative stress in MS rats. In addition, SLC9A3R1 was downregulated in kidney tissue (−1.25-fold). Decreased expression of SLC9A3R1 was found in hypertensive rats,[43] indicating that hesperidin supplementation does not exert a beneficial effect on it, at least not by this pathway. However, in MS rats, some proteins related to lipid metabolism were downregulated in the heart, such as APOA4 (−1.50-fold) and APOC2 (−1.83-fold). APOA4 increases triglyceride production and reduces hepatic lipids;[44] therefore, a decrease in its expression would be expected to interfere with the correct absorption and elimination of dietary fats. However, actual

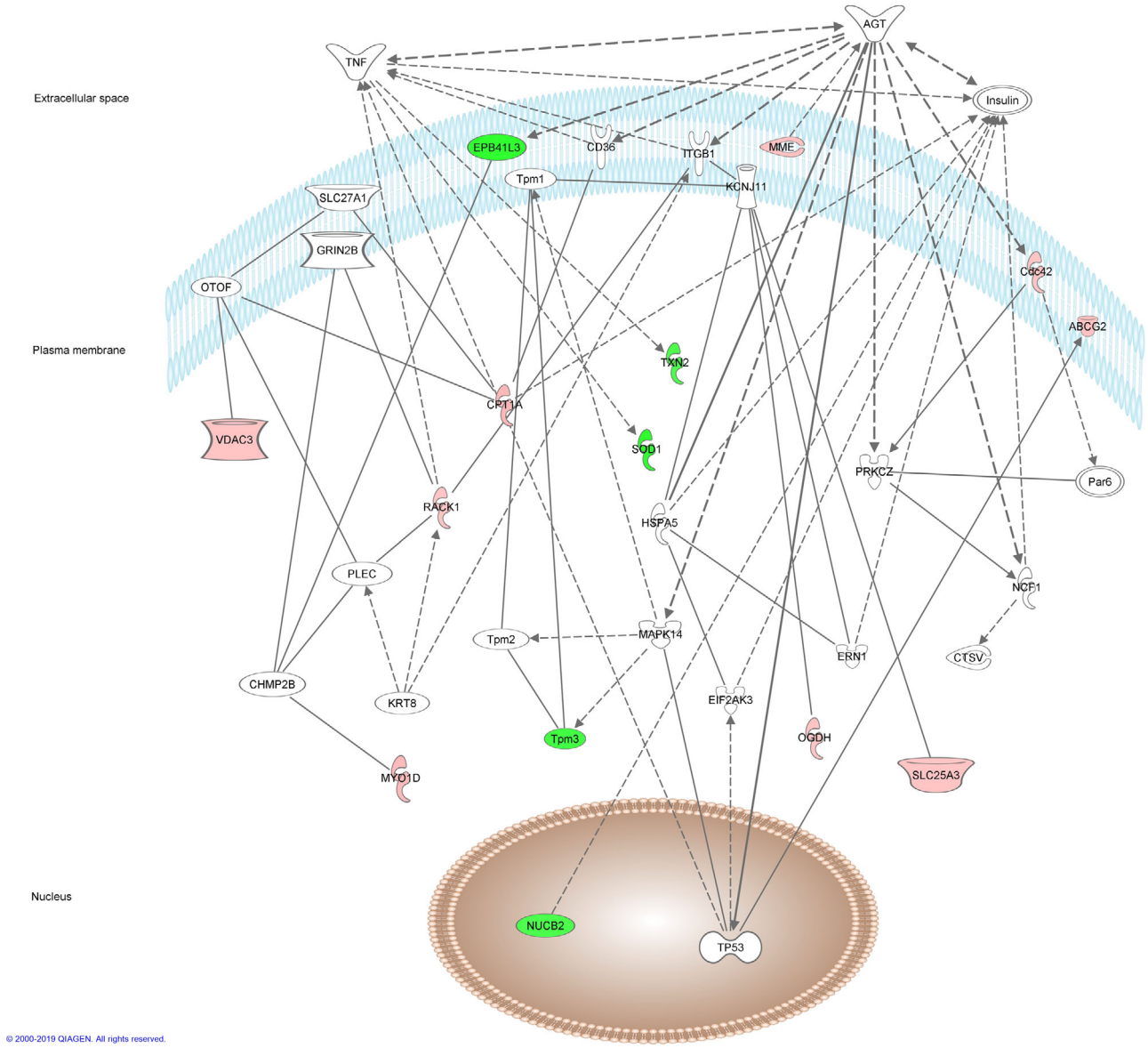

Figure 2. Graphical representation of the top networks of the kidney tissue proteome after hesperidin supplementation. Interaction between the differentially expressed proteins and other important proteins. Down- and upregulated proteins are symbolized in red and green, respectively. ABCG2, ATP binding cassette subfamily G member 2; AGT, angiotensinogen; CD36, CD36 molecule (thrombospondin receptor); CDC42, cell division cycle 42; CHMP2B, charged multivesicular body protein 2B; CPT1A, carnitine palmitoyltransferase 1A; CTSV, cathepsin V; EIF2AK3, eukaryotic translation initiation factor 2-alpha kinase 3; EPB41IL3, erythrocyte membrane protein band 4.1-like 3; ERN1, endoplasmic reticulum to nucleus-signaling 1; GRIN2B, glutamate receptor ionotropic NMDA 2B; HSPA5, endoplasmic reticulum chaperone BiP; ITGB1, integrin beta-1; KCNJ11, ATP-sensitive inward rectifier potassium channel 11; KRT8, keratin type II cytoskeletal 8; MAPK14, mitogen-activated protein kinase 14; MME, neprilysin; MYO1D, unconventional myosin-Id; NCF1, neutrophil cytosolic factor 1; NUCB2, nucleobindin 2; OGDH, oxoglutarate dehydrogenase; OTOF, otoferlin; PAR6, partitioning defective 6 homolog alpha; PLEC, plectin; PRKCZ, protein kinase C zeta type; RACK1, receptor of activated protein C kinase 1; SLC25A3, solute carrier family 25 member 3; SLC27A1, solute carrier family 27 member 1; SOD1, superoxide dismutase 1; TNF, tumor necrosis factor; TP53, cellular tumor antigen o53; TPM1, tropomyosin alpha-1 chain; TPM2, tropomyosin beta chain; TPM3, tropomyosin alpha-3 chain; TXN2, thioredoxin mitochondrial; VDAC3, voltage-dependent anion-selective channel protein 3. Figure reproduced with permission under the terms of the CC-BY 4.0 license. Copyright 2019, QIAGEN Silicon Valley.

evidence has demonstrated that decreased levels of APOA4 increase the chylomicron size, delaying its clearance from the blood and indicating it is not required for triglyceride absorption in the mouse intestine.[45] Thus, more studies of hesperidin supplementation are needed to clarify these effects on the proteomic profile.

Additionally, in MS rats after administered hesperidin supplementation, some of the proteins differentially expressed in the heart and kidney tissues were related to cancer. In the heart tissues of MS rats, CCDC58 and SLC25A3 were upregulated (1.73-fold and 1.36-fold, respectively). CCDC58 is a biomarker of breast, endometrial, and urethral cancer, and SLC25A3 is

Table 3. Top diseases and functions determined by heart and kidney tissue proteomic analyses in CAFH compared to CAFV rats.

Categories		Heart tissue proteins	Kidney tissue proteins
Cardiovascular system	Development of neointima	–	↓TPT1
	Blood pressure	–	↓ATP5PF, ↑EPHX2, ↓SOD1
	Density of microvessel	–	↑EPHX2
	Angiogenesis	–	↑Cdc42, ↑EPHX2, ↓TPT1
	Binding of endothelial cells	–	↑CD47
	Sprouting angiogenesis	↑Cdc42	↑CDC42
	Heart rate	↑ATP1A1, ↓SOD1	–
	Ischemic acute renal failure	↑ATP1A1	–
Free radical scavenging	Metabolism, removal, and quantity of superoxide	↓SOD1	↓SOD1
	Biosynthesis of hydrogen peroxide	↓SOD1	–
Lipid metabolism	Fatty acid metabolism		
		↑OGDH, ↓APOA4, ↑APOC1, ↑GCDH, ↓RBP4, ↑SLC27A1	↑OGDH, ↓APOH, ↓ATP5PF, ↑CPT1A, ↑EPHX2, ↓IGFBP7
	Transport of lipid	↓APOA4, ↑APOC1, ↓RBP4, ↑SLC27A1	–
	Metabolism of lipoprotein	↑APOC1	–
	Synthesis of epoprostenol	–	↓ATP5PF, ↓IGFBP7
	Synthesis of prostaglandin	–	↓ATP5PF, ↑EPHX2, ↓IGFBP7
	Binding of eicosapentenoic acid and malonyl-coenzyme A	–	↑CPT1A
	Beta-oxidation of oleic acid	–	↑CPT1A
	Transport of triacylglycerol	–	↓APOH
	Quantity of long-chain acyl-coenzyme A	–	↑CPT1A
	Metabolism of succinyl-coenzyme A	↑OGDH	↑OGDH
	Accumulation of triacylglycerol	–	↑CPT1A
	Oxidation of fatty acid	↑GCDH, ↑SLC27A1	↑CPT1A, ↓NUCB2
	Metabolism of long chain fatty acids	–	↑CPT1A
	Esterification, transport and oxidation of palmitic acid	↑SLC27A1	↑CPT1A
	Synthesis and metabolism of acyl-coenzyme A	↑GCDH, ↑OGDH	–
	Transport of retinol	↓RBP4	–
Glucose metabolism	Insulin sensitivity index	–	↓NUCB2
	Secretion of glucagon	–	↓NUCB2
Renal and urological disease	Nephrosis	↑CLTC	–
	Apoptosis of kidney cells	↑ATP1A1, ↑CDC42	–

CAFH, metabolic syndrome rats with hesperidin supplementation; CAFV, metabolic syndrome rats without hesperidin supplementation; —, no protein identified; ↑, protein upregulated; ↓, protein downregulated.

overexpressed in cervical carcinomas.[46] In the kidney tissues of MS rats, the proteins UQCRH and LZTFL1 were downregulated (−1.40-fold and −1.58-fold, respectively). In the literature, UQCRH overexpression is associated with a poor prognosis for lung cancer and hepatocellular carcinoma patients.[47,48] Finally, LZTFL1 is a tumor suppressor and an independent prognostic marker for the survival of gastric cancer patients when it is elevated.[49] In this sense, several studies in cancer models have shown that hesperidin can delay cell proliferation,[50] inhibit cell viability, and induce apoptosis in cancer cells.[51] Therefore, studies of hesperidin supplementation in rats with cancer and its effects on the proteome are needed to provide more robust evidence and to clarify whether hesperidin could be a tumor suppressor.

The present study has several limitations that warrant discussion. One limitation is the small sample size. Based on the results obtained in the present work, further studies with larger sample sizes and other experimental models, such as diabetes and cancer, will be performed to confirm the observed effects. Additionally, the dose of hesperidin should be increased or decreased to observe the different effects of various doses, which will be based on the natural doses of hesperidin present in citrus fruits or juice to extrapolate the results from rats to humans.

In conclusion, hesperidin supplementation for eight weeks can change the proteomic profiles of the heart and kidney tissues in MS rats and has a beneficial impact on the cardiovascular system, free radical scavenging, and lipid and glucose metabolism. Therefore, the identification of proteins involved in metabolic

pathways can help to understand the molecular basis of hesperidin in MS rats. However, further research is needed to confirm the results reported in the present study in humans.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.G., A.G.-G., A.G.R., and J.M.-P. performed the experimental study in rats. N.C. and E.F.-R. performed the proteomic analysis. L.P.-P. analyzed the proteomic results under the supervision of U.C. and L.P.-P. wrote the manuscript. U.C., N.C., J.M.-P., R.M.V., L.L.A., R.S., and A.P. provided feedback on the manuscript.

Keywords

hesperidin, kidneys, metabolic syndrome, proteomics

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5. SUMMARY OF RESULTS

5. 1 Project 1. Effects of hesperidin consumption on cardiovascular risk biomarkers: a systematic review of animal studies and human randomized clinical trials.

From 698 articles, after the study selection according to our eligibility criteria and search strategy, twelve animal studies conducted in rats and mice published between January 2003 and January 2018 were included in the systematic review. All the animals (N= from 4 to 16) had at least one CVDRF (hypertension, type 2 diabetes, systemic inflammation, myocardial ischaemia, hypercholesterolaemia) and they consumed a daily dose of hesperidin of 5-200 mg/kg/body weight from 7 days to 24 weeks. Further details of each study are presented in Table 3 of the published version.

The results for anthropometry parameters showed that 6 studies evaluated these parameters, and only 1 of them showed a significant decrease in body weight after 4.6% of the total calories in the diet was composed of hesperidin.

The results for inflammation and oxidation biomarkers showed that 2 studies evaluated these parameters, and they showed a significant decrease in interleukin-6 (IL-6) levels after treatment with 100 mg/kg body weight hesperidin for 4 weeks in rats with in systemic inflammation, and showed significant decreases in NO levels after treatment with 50 mg/kg body weight hesperidin for 30 days in type 2 diabetic rats.

The results for glucose and insulin levels showed that 7 studies evaluated these parameters and 6 of them showed significantly lowered

blood glucose levels in type 2 diabetic rats and mice after 50-200 mg/kg body weight hesperidin for 15-24 days. On the other hand, no significant changes were observed in insulin levels after hesperidin consumption.

The results for the lipid profile showed that 4 studies evaluated these parameters and 3 and 2 studies showed significant decreases in TC levels and LDL-c levels, respectively, after 50-200 mg/kg body weight of hesperidin. Moreover, no significant changes were observed in HDL-c levels after hesperidin consumption.

From 1917 articles, after the study selection according to our eligibility criteria and search strategy, eleven human randomized controlled clinical trials with nutritional intervention published between January 2003 and January 2018 were included in the systematic review. All the subjects (N= from 22 to 194) had at least one CVDRF (overweight, obesity, metabolic syndrome, hypercholesterolaemia) and they consumed a dose of hesperidin of 50-200 mg/kg body weight for 1.5-13 weeks. Further details of each study are presented in Table 4 and Table 5 of the published version (page 103 of the present doctoral thesis).

The results for anthropometric parameters showed that 3 studies evaluated the effects of hesperidin consumption on body weight, and 2 of them reported significant decreases after 54.60-582.50 mg/day of hesperidin in OJ for 12-13 weeks. Moreover, 5 studies evaluated the effects of hesperidin consumption on BMI and 2 of them reported significant decreases after 54.60-582.50 mg/day of hesperidin in OJ for

12-13 weeks in hypercholesterolaemic and overweight or obese subjects.

Finally, 1 study evaluated the effect of hesperidin consumption on body fat and significant decreases were observed after 13 weeks of 54.60 mg/day hesperidin in OJ in hypercholesteremic subjects.

The results for vascular parameters showed that 5 studies evaluated systolic and diastolic BP levels, and 2 of them reported significant decreases after 12 weeks of 237-292 mg/day of hesperidin consumption in OJ but also in the form of pure hesperidin capsules in overweight or obese subjects. On the other hand, 5 studies evaluated the effects of hesperidin consumption on endothelial function and 2 of them reported significant increases after 1.5-3 weeks of 159.60-500 mg/day hesperidin in OJ in subjects with metabolic syndrome.

The results for glucose and insulin levels showed that 5 and 4 studies evaluated plasma glucose levels and plasma insulin levels, respectively, and no significant changes were reported.

The results for lipid profile parameters showed that 8 studies evaluated the effect of hesperidin consumption on TC levels and LDL-c and 2 of them reported significant decreases in both parameters after 42-64.50 mg/day hesperidin or hesperetin in OJ for 8-13 weeks in overweight and hypercholesterolaemic subjects. Moreover, the effect of hesperidin consumption on HDL-c levels was evaluated in 8 studies and 1 of them reported a significant increase after 54.60 mg/day of hesperetin in OJ for 13 weeks in overweight subjects. Furthermore, 8 studies evaluated the effects of hesperidin consumption on TG levels and 1 of them reported a significant decrease after 237 mg/day of hesperidin in orange

juice for 12 weeks in overweight or obese subjects. Finally, the effect of hesperidin consumption on apolipoprotein A-1 and B was evaluated in 3 studies and 1 of them observed a significant increase after 213 mg/day of hesperidin in OJ for 4 weeks in hypercholesterolaemic subjects; while 1 of them reported a significant decrease after 237 mg/day of hesperidin in OJ for 12 weeks in overweight or obese subjects.

The results for coagulation biomarkers showed that 2 studies evaluated the plasma levels of fibrinogen and homocysteine after hesperidin consumption and no significant changes were observed.

The results for inflammation biomarkers showed that 1 study evaluated the effect of hesperidin consumption on plasma protein serum amyloid A, sVCAM-1, sICAM-1, sE-selectin and sP-selectin, and no significant changes were observed. On the other hand, 1 study evaluated the effect of hesperidin consumption on plasma IL-6 levels and after 159.50 mg/day of hesperidin in OJ for 1.5 weeks, the levels significantly decreased in subjects with metabolic syndrome.

Finally, the results for oxidative biomarkers showed that 2 studies evaluated NO levels, and no significant changes were observed.

5. 2 Project 2. Effects of hesperidin in orange juice in blood pressure and pulse pressure in mildly hypertensive individuals: a randomized clinical trial (CITRUS study).

Participants and intervention compliance:

A total of 159 adult participants with pre- and stage 1 hypertension completed the 12 weeks of the randomized clinical trial: N=43 in the control group, N=46 in the OJ group and N=40 in the EOJ group; 52 participants completed the single dose study: N= 17 in CD, N=21 in OJ and N=14 in EOJ. Further details with the flow diagram are presented in Figure 1 of the published version.

Regarding the baseline characteristics, no differences were observed among the groups or in the level of physical activity at the end of the study. Regarding dietary intake, only an increased intake of protein was observed in the OJ group compared with the EOJ group.

All the subjects complied with the nutritional intervention since 6 compliance biomarkers namely hesperetin-7-O-β-d-glucuronide, hesperetin-3-O-β-d-glucuronide, hesperetin-7-O-sulfate, naringerine-4-O-β-d-glucuronide, naringenin-glucuronide and naringenin-sulfate, were significantly increased with OJ and EOJ consumption compared with the control group.

Changes in blood pressure, the main outcome:

Hesperidin consumption in the OJ and EOJ groups significantly decreased the SBP levels compared to the control group. The average decreases in all SBP levels during the 12 weeks of hesperidin consumption in OJ and EOJ were -6.35- and 7.36-mm Hg, respectively.

Additionally, PP decreased in a dose-dependent manner with the hesperidin dose of the drinks consumed.

On the other hand, after the first single dose study significant decreases were observed in SBP levels at 2 hours after EOJ, and no effects were observed for DBP levels. After the second single dose study realized after 12 weeks of nutritional intervention, EOJ showed decreases in BP and PP levels.

Changes in secondary outcomes: homocysteine, uric acid, ICAM-1 and VCAM levels:

After 12 weeks of OJ and EOJ consumption, the levels of homocysteine decreased significantly compared to CD. After the second single dose study, the homocysteine levels also decreased after 2 hours and 4 hours of OJ consumption and after 2 hours of EOJ consumption. Additionally, after 12 weeks of EOJ consumption, uric acid levels significantly decreased compared to CD. Moreover, after 12 weeks of EOJ the levels of ICAM-1, VCAM and F2-isoprostanes significantly decreased.

All these changes were observed without changes in body weight and blood glucose levels.

Transcriptomic analysis:

After 12 weeks of EOJ the expression of the genes PTX3 and NAMPT in PBMCs was significantly decreased compared to CD. The decreases in SBP and PP were directly correlated with the decreased expression of both genes.

5. 3 Project 3. Effect of hesperidin consumption in orange juice on the transcriptomic profile of subjects with pre- and stage 1 hypertension: a randomized controlled trial (CITRUS study).

Baseline characteristics of the participants:

Of the 159 subjects included in the original clinical trial, 37 subjects had PBMCs collected and completed both single dose and sustained studies, making them candidates for transcriptomic profile evaluation. The 37 subjects were 41-65 years old and had pre- or stage 1 hypertension. They were from the 3 intervention groups: N=11 in the CD, N=15 in the OJ group and N=11 in the EOJ group.

Gene expression profile after single dose intervention:

After 6 hours of OJ consumption in the single dose study, 3 genes were significantly different ($P < 0.05$) compared to CD. Of these, 2 genes were upregulated (DHRS9, related with an increase of insulin resistance; and PKDL1, with unknown function) and 1 gene was downregulated (TNFAIP3, an anti-inflammatory gene). Moreover, several genes were borderline ($P < 0.10$) differentially expressed (such as NFKBIA, CCL3 and CCL4L2) after OJ and EOJ consumption related to the inflammation pathways.

Gene expression profile after sustained intervention:

After 12 weeks of hesperidin consumption in OJ, 12 genes were significantly different compared to CD. From these, 7 genes were upregulated: CCL20, FAM53B, LINC 01220, LncRNA SNRPD3-2, LncRNA NFKBID-1, LncRNA PDE3B-1, LOC101929524; and 5

genes downregulated: TNF, TMPO-AS1, BPIFB3, LncRNA ACOT-13 and CCT8-1. Moreover, 13 genes were borderline differentially expressed after OJ consumption (such as NFKBIA and IL1B).
After 12 weeks of hesperidin consumption in EOJ, 18 genes were significantly different compared to CD. From these, 4 genes were upregulated: DSP, FAM53B, LncRNA SNRPD3-2 and LncRNA SLC39A8-1; and 14 genes downregulated: TNF, IL1B, CCL3L3, CXCL2, CXCL8, PTGS2, IER3, PTX3, KMT22-AS1, ATP2B1-AS1, LncRNA CCT8-1, LncRNA GRK3-1, LncRNA CCDC117-1 and LOC644090. Moreover, 17 genes were borderline differentially expressed after OJ consumption (such as ICAM, CCL20 and CXCL3).

The significant changes observed in the genes after OJ and EOJ consumption were related to an improvement in the following top diseases and functions: inflammation, cardiovascular system, acute coronary syndrome, obesity, haemoglobin, neuronal differentiation and cancer.

5. 4 Project 4. Hesperidin consumption in orange juice modulates plasma and urine metabolic profiles in pre- and stage 1 hypertensive subjects promoting beneficial effects on cardiovascular system: targeted and non-targeted metabolomic approach (CITRUS study).

Volunteers:

A total of 159 subjects with pre- and stage 1 hypertension completed the 12 weeks of the CITRUS randomized clinical trial: N=43 in the CD, N=46 in the OJ group and N=40 in the EOJ group; 52 participants completed the single dose study: N= 17 in CD, N=21 in OJ and N=14 in EOJ. However, the targeted metabolomics analysis was performed in plasma and urine samples of 129 subjects who completed the sustained study. Nontargeted metabolomics was performed in serum samples of 52 subjects who completed both the single dose and sustained studies and in urine samples of 129 subjects.

Results of targeted metabolomics in plasma and urine samples:

After 12 weeks of OJ and EOJ consumption, the plasma and urine metabolites hesperetin 7-O-B-D-glucuronide, hesperetin 3-O-B-D-glucuronide, hesperetin 7-O-sulfate, naringenin 4-O-B-D-glucuronide, naringenin glucuronide and naringenin sulfate were statistical significantly increased. Moreover, plasma and urine hesperetin 7-O-B-D-glucuronide was the main differentially expressed metabolite between both OJ and EOJ interventions. Additionally, urine hesperetin 7-O-B-D-glucuronide metabolite was inversely correlated with SBP level.

Nontargeted metabolomics in serum:

Single dose study: After 6 hours of OJ and EOJ consumption, increased levels of proline betaine and dimethylglycine, and decreased levels of leucine were observed. Moreover, after single-dose EOJ consumption, decreased levels of urine isoleucine were observed. On the other hand, after an analysis to classify the subjects into low- and high-flavanones absorbers, increased serum levels of proline betaine, 3-hydroxybutyrate, DMG, acetoacetate and glutamine and decreased serum levels of leucine, isoleucine, valine, lysine, and alanine were observed in high flavanone absorbers.

Chronic study: After the analysis performed in a subsample of 52 subjects, who completed both the single dose study and the sustained study, increased serum levels of proline betaine and decreased serum levels of glycerophosphocholine (GPC), N-acetylglycoproteins (NAG), acetate, valine, isoleucine, and leucine were observed after OJ and EOJ consumption compared to CD.

Nontargeted metabolomics in urine:

After the analysis performed in a sample of 129 subjects, the consumption of OJ for 12 weeks increased urine levels of proline betaine and decreased levels of hydroxyhippurate, pseudouridine, PAG, 4-cresyl sulfate, creatinine, DMA, NAG, alanine and 3-methyl-2-oxovalerate were observed compared to CD. After 12 weeks of EOJ consumption increased urine levels of proline betaine and decreased levels of 4-cresyl sulfate, pseudouridine, uracil, creatinine, creatine, NAG, alanine, 2-HIB and 3-MOV were observed.

5. 5 Project 5. Proteomic analysis of heart and kidney tissues in healthy and metabolic syndrome rats after hesperidin supplementation.

The feeding of a cafeteria diet to Sprague Dawley rats produces an obesogenic dietary pattern and increases body weight, fat mass, SBP, TG, glucose, and LDL-c, producing metabolic syndrome.

After proteomic analysis of heart tissue samples, 1127 proteins were identified. After filtering, 872 proteins were candidates for the statistical analysis. After the analysis, in healthy rats fed a standard diet no significant differences were observed between the treatment groups (between the group that received hesperidin by the vehicle and the group that did not receive hesperidin). On the other hand, in metabolic syndrome rats (the rats fed with cafeteria diet) 35 proteins were differentially expressed between the treatment groups ($p<0.01$ and VIP score from PLS-DA >1.5). After hesperidin consumption in metabolic syndrome rats, 19 proteins were downregulated in heart tissue samples: APOC2, COQ8A, GLRX5, ENSA, RBP4, UQCRH, SOD1, CDV3, TIMM10, ATP5F1D, TXNDC17, RGD1311703, CFL2, COX5A, TIMM8B, MYOZ2, APOA4, GCSH, ISCA2; and 16 proteins were upregulated: ALDH7A1, FLNA, MYH9, ATP1A1, RP114, CLTC, CDC42, TMX2, APOC1, SLC27A1, OGDH, ACAD8, GCDH, AFG312, CDC58 and SLC25A3.

The most important and significantly affected pathways for hesperidin supplementation in heart tissue of metabolic syndrome rats, analysed by Ingenuity Pathway Analysis (IPA), were the following:

- Production of NO and reactive oxygen species in macrophages.

- Clathrin-mediated endocytosis signaling.
- LXR/RXR activation.
- Atherosclerosis signaling.
- FXR/RXR activation.

On the other hand, the top networks founded by IPA were the following:

- Molecular transport, carbohydrate metabolism and small molecule biochemistry.
- Cardiac arrhythmia, CVD and metabolic disease.
- Cancer, cardiovascular and cell cycle.

After proteomic analysis of kidney tissue samples, 1753 proteins were identified. After filtering, 1341 proteins were candidates for the statistical analysis. After the analysis, in healthy rats fed a standard diet no significant differences were observed between the treatment groups. On the other hand, in metabolic syndrome rats, 53 proteins were differentially expressed between the treatment groups ($p<0.01$ and VIP score from PLS-DA >1.5). After hesperidin consumption in metabolic syndrome rats, 33 proteins were downregulated in kidney tissue samples: ATP5IF1, APOH, LZTFL1, ISCU, ATP6V1G1, ATOX1, CHCHD3, IGFBP7, TPT1, UQCRH, ATP5PF, EF1D, SOD1, TPM3, LOC100912599, CTSH, SPP2, NUCB2, PAPLN, ISCA2, TPM4, PSAP, TIMM8B, SLC9A3R2, EPB4113, PFDN2, TXN2, STX7, CD2AP, COX6B1, SLC9A3R1, AK3, VAPB; and 20 proteins were upregulated: OGDH, RACK1, PNP, VDAC3, GCLC, MARC2, SLC25A13, ABCG2, CDC42, CTSL, CD47, SHTM1, CPT1A, FLNA, SLC25A3, MME, MYOLD, PAICS, EPHX2 and H3F3C. The detailed information of each protein is presented in Table 2 of the published version.

The most important and significantly affected pathways for hesperidin supplementation in kidney tissue of metabolic syndrome rats, analysed by IPA, were the following:

- Mitochondrial dysfunction.
- Xanthine and xanthosine salvage.
- Guanine and guanosine salvage I.
- Adenine and adenosine salvage I.

On the other hand, the top networks founded by IPA were the following:

- Cellular compromise, free radical scavenging, cell death and survival.
- Cell death and survival, free radical scavenging, organismal injury and abnormalities.
- Cardiovascular system development and function, immunological disease, inflammatory disease.

Finally, the significant changes observed in the expressed proteins in both heart and kidney tissues of metabolic syndrome rats after hesperidin consumption were related with to an improvement in these top diseases and functions:

- Cardiovascular system.
- Free radical scavenging.
- Lipid metabolism.
- Glucose metabolism.
- Renal and urological diseases.

6. DISCUSSION

The present work provides evidence that hesperidin consumption has beneficial effects reducing BP in humans, whereas genes expression and metabolites changes suggests effects also on others CVD risk biomarkers having protective capacity on cardiovascular health.

In this sense, the CITRUS randomized controlled trial showed the capacity of hesperidin to decrease SBP and PP, in a dose-dependent manner, in subjects with pre- and stage 1 hypertension after 12 weeks of OJ (392 mg/day of hesperidin) and EOJ (670 mg/day of hesperidin) consumption. Additionally, after single dose studies hesperidin decreased SBP and PP levels. On the other hand, markers of oxidation and inflammation (homocysteine, uric acid, ICAM-1 and VCAM) were reduced after 12 weeks of hesperidin consumption, and uric acid concentrations were directly related to SBP, DBP and PP at week 12. Moreover, daily consumption for 12 weeks and single dose consumption of hesperidin in OJ and EOJ significantly changed the transcriptome profile of PBMCs of subjects with pre- and stage 1 hypertension, with hesperidin being an anti-inflammatory agent because its capacity to downregulate pro-inflammatory genes and decrease the insulin resistance in higher doses. Furthermore, daily consumption for 12 weeks of hesperidin in OJ and EOJ changed the metabolome profile of urine and plasma/serum samples in subjects with pre- and stage 1 hypertension. Hesperidin changes endogenous metabolites related to BP, oxidative stress, inflammation and uraemic toxins, indicating anti-inflammatory and antioxidant actions, and lower SBP levels and ureamic toxins, providing beneficial effects on the cardiovascular system. Finally, daily hesperidin consumption for 8 weeks changes the proteome profile of kidney and heart tissue samples

of experimental rat models with metabolic syndrome, which has beneficial effects on the cardiovascular system, free radical scavenging and lipid and glucose metabolism.

In **Project 1**, we summarize by a systematic review the available scientific evidence of the effects of hesperidin consumption on cardiovascular risk biomarkers. Animal studies and human RCT published between January 2003 and January 2017 were included in the systematic review. Mice and rats and human subjects had at least one CVDRF, they participated in sustained and single dose studies and consumed a hesperidin dose through OJ or capsule; and orally, by gavage and intravenous administration in the case of animal models.

In animal studies, hesperidin consumption improves blood glucose, TC, LDL-c, and TG levels. No significant changes were observed in anthropometric parameters, BP, inflammation, or oxidative biomarkers. However, the sample size in some of the studies may be insufficient to obtain conclusive results. On the other hand, a conclusive inference cannot be drawn from the included RCT: most of the studies did not have the most appropriate population to evaluate the effects of different CVDRFs, and only 3 of the 11 included articles evaluated the diet of the subjects through a validated method and no one used consumption biomarkers. There may be factors that influence hesperidin bioavailability or other phenolic compounds of the diet that can influence the observed effects. Therefore, further RCT with higher quality are needed to clarify the effects of hesperidin on CVD biomarkers.

To the best of our knowledge, the present study is the first to systematically review the effects of hesperidin on CVD risk biomarkers in animal studies and human RCT. In this sense, no systematic review or meta-analysis about the effects of hesperidin on CVDs was published after our work. However, in 2020, a review has been published reporting the scientific evidence of hesperidin consumption on CVDRFs and its role in intestinal microbiota.(112) In this review the mechanism of action by which hesperidin can exert beneficial effects was discussed together with the results of *in vivo*, *in vitro* and human clinical trial studies. In this work, although they demonstrate the influence of the intestinal microbiota on the absorption and subsequent effects of hesperidin consumption, more animal and human studies are needed to clarify this relationship. In this sense, their conclusion about the actual evidence of the hesperidin effects is the same as what we reported in our systematic review.

As there is a need to realize more randomized and controlled clinical trials to confirm the effects of hesperidin on CVD risk biomarkers, the CITRUS study was carried out.

In **Project 2**, we assessed the sustained and single dose effects of hesperidin consumption in OJ and EOJ on BP levels and PP in pre- and stage 1 hypertensive subjects. After 12 weeks of daily hesperidin consumption in 500 mL of OJ (392 mg of hesperidin) and EOJ (670 mg of hesperidin), decreased SBP and PP levels were observed in a dose-dependent manner. Therefore, regular consumption of OJ, especially EOJ, could be a co-adjuvant tool for BP and PP management.

Similarly, an open-labelled randomized controlled trial that evaluated the clinical effects of hesperidin in metabolic syndrome subjects was recently published.(190) In this study, after 12 weeks of hesperidin powder consumption (1 g/day), significant reductions in SBP of -5.68 mm Hg and serum TG of 50.06 mg/dL were observed. Moreover, a randomized, double-blinded, placebo-controlled clinical trial also performed in patients with metabolic syndrome showed that the consumption of 500 mg/day hesperidin by 2 capsules for 12 weeks significantly decreased the levels of blood glucose, TG, SBP and TNFα.(191) Therefore, hesperidin can improve metabolic abnormalities and inflammatory status in subjects with metabolic syndrome. Moreover, hesperidin can exerts anti-hypertensive effects increasing NO production,(192) improving endothelium-dependent vasodilatation and improving potassium channel activity.(193)

Furthermore, in the CITRUS study the genes that were significantly and differently decreased after 12 weeks of hesperidin consumption in EOJ related to BP levels were PTX3 and NAMPT. PTX3 is a marker of inflammation activation and is increased in hypertensive subjects.(194) Additionally, NAMPT is implicated in inflammation since it is a stimulator of pro-inflammatory cytokines, and it is increased in subjects with prehypertension.(195) Therefore, NAMPT could be a marker of risk and damage in prehypertensive subjects. Accordingly, the decreased expression of both genes could be partially responsible for the decreased BP and PP levels after hesperidin consumption because their expression was correlated with BP and PP.

Therefore, the CITRUS study provides further evidence of the effects of hesperidin consumption on CVDRFs such as BP. However, more in-depth analyses are necessary to better understand how hesperidin can exert its beneficial effects and understand its mechanisms of action. In this sense, an omics science approach through transcriptomics and metabolomics analysis was performed in subjects with pre- and stage 1 hypertension from the CITRUS study.

In **Project 3**, additionally, we determined whether the sustained and single dose consumption of hesperidin in OJ and EOJ can change the transcriptome profile of PBMCs of pre- and stage 1 hypertensive subjects. According to our results, after 12 weeks of OJ and EOJ consumption, pro-inflammatory genes were downregulated. However, after EOJ consumption more pro-inflammatory genes were differentially and borderline downregulated than after the consumption of OJ (such as CXCL2, CCL3, TNF, IL1B and PTGS2). These differentially expressed genes after hesperidin consumption influence the inflammatory response and the communication between immune cells preventing atherosclerotic plaque formation, inflammation in obesity, short-term mortality and hypertension, thus decreasing the risk of developing CVDs.

Additionally, the differential expression of a gene related to insulin resistance was only observed after the single dose consumption of hesperidin in OJ and EOJ. The OJ upregulated the DHRS9 gene compared to CD, increasing insulin resistance, but EOJ downregulated it compared to OJ. The different behaviour between the two hesperidin interventions could be due to the presence of fructose: fructose can increase the expression of DHRS9, increasing fructose metabolism,

plasma TG levels and insulin resistance. In contrast, after EOJ consumption, the gene expression of DHRS9 decreased, which could be because hesperidin can counteract with the fructose content since the evidence suggests that higher doses of hesperidin in rats improve the insulin resistance and decrease blood glucose levels.(143,144)

On the other hand, and contrary to expectations, hesperidin in OJ and EOJ also showed the capacity to downregulate anti-inflammatory related genes such as NFKB1A and TNFAIP3. The downregulated effect observed could also be explained by the fructose content of the fruit juices, although the product of the present RCT was natural juice or enriched juice. Some evidence suggests that a high consumption of fructose increases CVD risk because of its influence on inflammatory response.(196) However, other evidence is controversial because its pro-inflammatory effects are only observed after artificially sweetened beverage consumption but not after fruit juice consumption.(156)

The present work is the first study to realize a transcriptomic approach of PBMCs in subjects with pre- and stage 1 hypertension after sustained and single dose consumption of hesperidin in OJ and EOJ. Transcriptomic analysis offers a unique opportunity to determine the effect of bioactive compounds such as hesperidin on metabolic and biological pathways. Additionally, transcriptomic analysis provides a way of knowing how the regulation of some pathways impacts the progression of chronic diseases.(197) Moreover, the transcriptomic approach allows for the identification of genes as biomarkers.

Thus, the new information provided through our transcriptomic approach would allow us to better understand how hesperidin

consumption can influence cardiovascular health, providing an explanation of the observed health effects. However, although our results are promising, more studies are needed to corroborate this hypothesis to better understand the mechanism of action of hesperidin and its action on gene expression.

In **Project 4**, we evaluated the effects of a single dose and sustained consumption of hesperidin for 12 weeks in OJ and EOJ in subjects with pre- or stage 1 hypertension on plasma, serum and urinary metabolomic profiles through targeted and nontargeted approach. After 12 weeks of hesperidin consumption in OJ and EOJ, compared to CD, the targeted metabolomics approach showed that the 6 compliance biomarkers were significantly increased in urine and plasma, but hesperetin 7-O-B-D-glucuronide was the only metabolite that showed differences between OJ and EOJ, indicating that hesperetin 7-O-B-D-glucuronide could be a candidate marker to distinguish between different hesperidin doses consumed in the long-term. Additionally, we observed a negative correlation between the urine levels of hesperetin 7-O-B-D-glucuronide and SBP levels, suggesting that this metabolite can have hypotensive actions. To the best of our knowledge, this is the first study to show a negative correlation between the urine metabolite hesperetin 7-O-B-D-glucuronide and the levels of SBP.

On the other hand, nontargeted metabolomics approach showed that after hesperidin consumption in OJ and EOJ for 12 weeks and after 6 hours of a single dose study, serum and urine metabolites that are inversely associated with BP levels,(173) such as proline betaine, were significantly increased. Moreover, decreased serum and urine levels of

the metabolites valine, leucine and isoleucine were reported after OJ and EOJ consumption, which were related to lower BP levels and improvement in insulin resistance.(125) Additionally, metabolites such as NAG and pseudouridine, related to inflammatory and oxidative stress status,(176) were decreased in serum and urine, after 12 weeks of OJ and EOJ consumption. Also, hesperidin consumption in OJ and EOJ, changes the choline metabolism changing related metabolites such as trimethylamine and dimethylglycine, which are increased, suggesting a lower microbial choline metabolism and thereby alterations in one-carbon metabolism that could improve the SBP levels in these patients. Furthermore, uraemic toxins such as dimethylamine, 4-cresyl sulfate and creatinine were decreased in urine after OJ and EOJ consumption, which is beneficial for the cardiovascular system because their accumulation produces vascular inflammation and endothelial dysfunction.(183)

On the other hand, and as reported in the literature, we observed a large interindividual variability in flavanone absorption and for that reason we divided the subjects into two groups in the single dose study: high and low total flavanone absorbers regardless of their consumption of OJ and EOJ. In this sense, we observed that the subjects who absorbed more flavanones showed higher plasma levels of ketone bodies such as acetoacetate and 3-hydroxybutyrate 6 h hours postprandially. It is known that increased ketone bodies produced by a ketogenic diet produce vasodilation and lower BP levels.(187) Therefore, this could be another mechanism through which hesperidin can improve BP levels in mildly hypertensive subjects.

In **Project 5**, we evaluated the effects of hesperidin supplementation at 100 mg/kg body weight/day for 8 weeks on the proteomic profiles of heart and kidney tissue samples from healthy and metabolic syndrome rats. The human equivalent dose of 100 mg/body weigh/day hesperidin was 1350 mg per day for a 60 kg human,(198) a dose achievable with an orange juice enriched with hesperidin.

After 8 weeks of hesperidin supplementation by diet, the metabolic syndrome rats had changes in their proteome in both heart and kidney tissues. In the heart and kidney tissue samples, 35 proteins and 53 proteins were differentially expressed, respectively. To the best of our knowledge, there are no data about the capacity of hesperidin to change the proteome profile of tissue samples of metabolic syndrome rats. Therefore, this is the first study to observe it.

According to our results, hesperidin supplementation showed positive effects on different parameters in metabolic syndrome rats, upregulating the expression of proteins related to the cardiovascular system (ATP1A1), BP and endothelial function (ATP5PF and IGFBP7, respectively), and atherosclerosis pathogenesis (TPT). Additionally, hesperidin supplementation exerts positive effects by downregulating the expression of proteins related to free radical scavenging (ALDH7A1), heart development (FLNA), glucose metabolism (CD2AP) and insulin sensitivity (EPHX2). However, hesperidin can also change the expression of some proteins with negative or controversial effects on cardiovascular health: hesperidin downregulated the expression of antioxidant proteins (SOD1 and TXN2), proteins related negatively to BP (SLC9A3R1) and proteins related negatively to lipid metabolism (APOA4).

In this sense, and contrary to expectations, we observed controversial results after the proteomics approach in rats as in the transcriptomics approach in humans. Therefore, more studies are needed in this area to explain and clarify the effects of hesperidin on these differentially expressed proteins.

On the other hand, some studies have performed proteomics analysis in cells and rats treated with flavonoids such as quercetin and observed interesting and positive results. After quercetin treatment in K562 cells (a cellular model of human chronic myeloid leukaemia), several proteins related to RNA metabolism, the antioxidant defense system and lipid metabolism changed, influencing the early stages of the apoptosis response.(199) On the other hand, in rats with cerebral ischaemia, quercetin treatment can change the expression of proteins related to cellular differentiation, metabolism and oxidative stress, reducing ischaemic injury.(200)

Thus, promising effects of hesperidin capacity to influence the proteome profile to exert beneficial effects on interestingly expressed proteins to promote cardiovascular health were reported and need to be verify in humans.

OVERALL DISCUSSION

We can observe that higher doses of hesperidin (670 mg/day in 500 mL/day in EOJ) have more beneficial effects than usual doses (392 mg/day in 500 mL/day in OJ). This is suggested because of the results from the CITRUS study regarding BP and PP, the transcriptomic approach performed in PBMCs which observed interestingly actions

decreasing proinflammatory genes, and the metabolomic approach realised in urine and plasma/serum samples which suggests changes on several metabolites related with lower levels of BP and beneficial effects on cardiovascular system.

Therefore, hesperidin consumption in OJ and EOJ can decrease BP levels and PP in a dose-dependent manner in subjects with pre- and stage 1 hypertension, and the mechanism of action of the flavonoid hesperidin, which exerts its beneficial effects, can be explained through transcriptomics and metabolomics analyses.

Hesperidin showed the ability to decrease the expression of pro-inflammatory genes and higher doses can improve glucose metabolism by interfering with the expression of genes related to insulin resistance. Furthermore, hesperidin in OJ and EOJ has an impact on the serum metabolomic profile, decreasing endogenous metabolite levels related to BP and inflammation, and decreasing urinary excretion of uraemic toxins and metabolites related to oxidative stress.

Moreover, an experimental study in rats with metabolic syndrome also showed the capacity of hesperidin to influence kidney and heart proteome profiles by proteomics analysis. In this sense, hesperidin can change the expression of proteins related to cardiovascular health, improving endothelial function and BP homeostasis. Additionally, hesperidin showed the capacity to change protein expression of heart and kidney tissues to exert antioxidant effects and improve of glucose metabolism in metabolic syndrome rats.

Thus, in the present work we reported promising effects of hesperidin consumption to promote cardiovascular health (Figure 14).

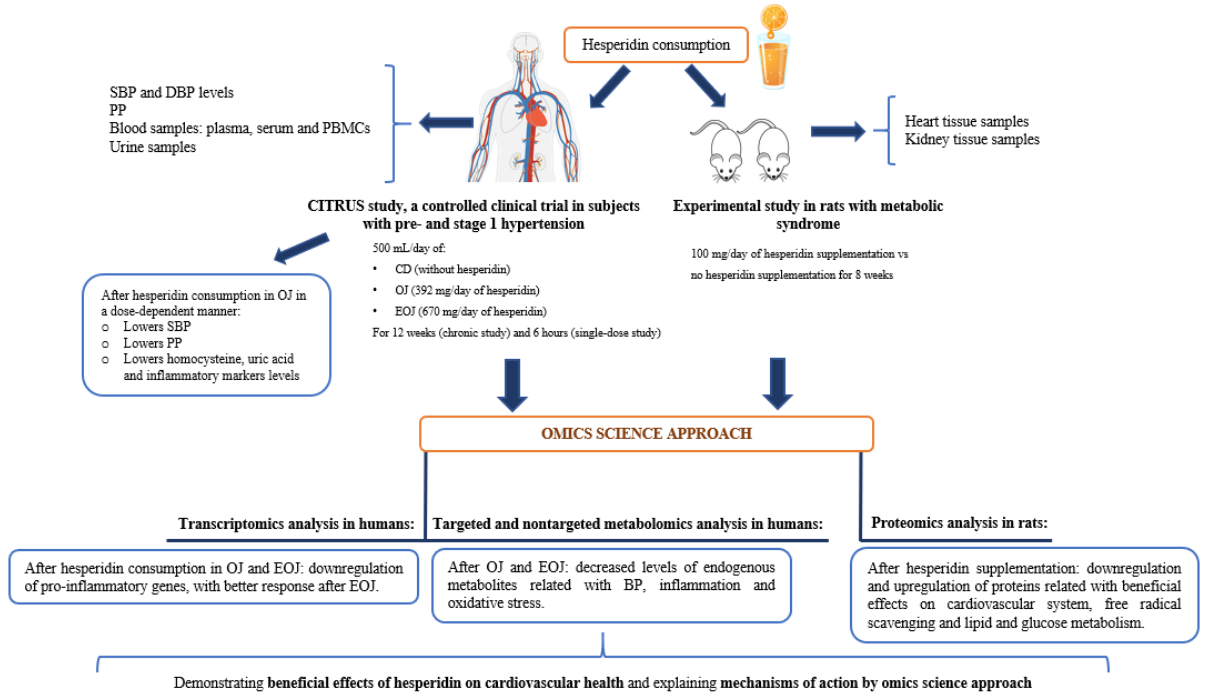

Figure 14. Omics science approach to evaluate the effects of the hesperidin consumption on CVD risk biomarkers. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressures; CD, control drink; OJ, orange juice; EOJ, hesperidin-enriched orange juice.

7. PERSPECTIVES

First, although our results are promising and indicate beneficial effects of hesperidin consumption reducing SBP linked to changes in gene expression, metabolites in humans and proteins in syndrome metabolic rats, more studies are needed to confirm our results.

Moreover, to assess the effects of hesperidin on other CVDRFs biomarkers such as lipid metabolism, endothelial function, glucose metabolism and anthropometric parameters, further research is needed.

Additionally, reviewing the types of studies performed with hesperidin consumption, the participants included in the RCT must be the proper population to ensure the observed positive effects on specific cardiovascular biomarkers. For example, if the hesperidin effects on TC plasma levels are to be evaluated, adult subjects with hypercholesterolaemia must be selected; or if the hesperidin effects on body weight want are to be evaluated, overweight or obese subjects must be selected.

On the other hand, there is a bidirectional relationship between phenolic compounds and the microbiota of the human gut since phenolic compounds can change the microbiota population (Figure 15).(56)

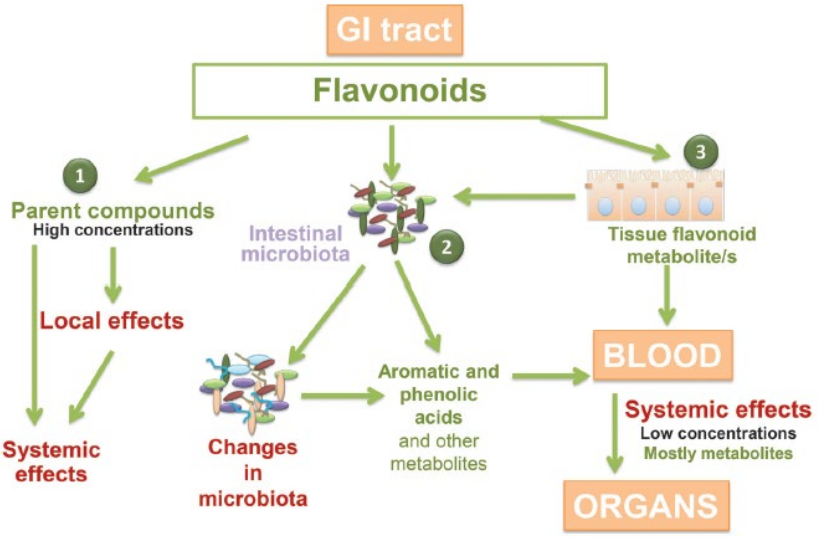

Figure 15. Flavonoids metabolism in the colon and metabolite absorption. 1. Dietary flavonoids can exert local effects in gastrointestinal tract, 2. Can interact with microbiota changing its profile and produce mainly aromatic and phenolic acids that will be absorbed producing systemic effects or exerting local effects; 3. and be metabolized by intestinal epithelial cells. Later metabolites will be absorbed or excreted. Abbreviations: GI, gastrointestinal. Source: (Fraga CG, et al. 2019).(56)

The microbiota is a complex ecosystem that depends on individual characteristics and their environmental conditions, and plays a very important role in health status by modulating the immune system and protecting against pathogenic microorganisms.(201) Therefore, the microbiota influences on the development of several diseases.

In this sense, in other human studies OJ consumption showed a prebiotic effect in the intestinal mucosa by decreasing pathogenic microorganisms and increasing positive effects on the intestine.(202) Moreover, hesperidin has demonstrated *in vivo* and *in vitro* the capacity to inhibit the growth of gram-positive bacteria, thereby inhibiting the progression of pathogenic bacteria, and the ability to promote the growth of beneficial bacteria, thus demonstrating immunomodulatory action on the gut.(203)
Furthermore, regarding BP, a recent study that was carried using the same rats as in Project 5 of the present thesis evaluated the effects of hesperidin consumption on the microbiota in metabolic syndrome rats, showing that hesperidin supplementation alters microbiota by acting as a hypertension modulator and modifying protein expression related to pathways metabolism such as amino acids and energy metabolism.(204)

For that reason, since gut microbiota have a crucial role in disease development, such as CVDs, and also phenolic compounds is metabolized by gut microbiota, stool samples should also be collected in future RCT to evaluate the hesperidin effects on microbiota and their implication for CVDs. Furthermore, a metagenomic approach could be useful to understand the complex relationship between CVDs, the gut microbiome and hesperidin intake since metagenomics can analyse the genomic content of all microorganisms present in an ecosystem to define their biodiversity in each experimental condition.

On the other hand, to the best of our knowledge, no proteomics approach has been realized in humans to evaluate the effects of

hesperidin consumption on proteomic profiles. Therefore, blood samples can also be collected in RCT to determine changes in serum and plasma proteins and to relate them to cardiovascular health.

Finally, further analysis from the CITRUS study results will be realized to integrate all the information generated and to better relate the changes at the molecular level to the clinical changes observed. In this sense, more correlations between clinical parameters such as BP, PP, or endothelial function with inflammation biomarkers, differentially expressed genes and endogenous metabolites can be realized.

Second, a multi-omics approach will be proposed thanks to the omics approach undertaken in the present work through transcriptomics, metabolomics and proteomics analysis, it has been possible to know the impact of the flavanone hesperidin on the transcriptome, metabolome and proteome to understand how hesperidin exerts beneficial effects on the cardiovascular system and CVD biomarkers. As results, each omics science provides specific insight into one study factor; however, an integrative new analysis proposal is currently emerging in the world of science and bioinformatics, and it is called the "multi-omics" approach.

Multi-omics is an integrative analysis of omics data from different omics levels with the objective of better understanding their interrelation and combined influence on molecular function, disease aetiology and disease development.(129) However, the integrative analysis of different omics data is not straightforward and has several logistic and computational changes.(205) Nevertheless, realizing a multi-omics approach is one more step that future studies can perform

to integrate multi-omics profiles into the investigation of the mechanisms associated with CVDs.

Third, considering that hesperidin has the ability to act on multiple factors, such as BP, lipid and glucose metabolism, as well as on inflammation and oxidative stress, studies could be carried out to determine the effects of hesperidin consumption in other chronic diseases such as cancer.

In this sense, there is literature that reported that hesperidin consumption has beneficial effects on oxidation and other biological pathways related to metastasis and better prognosis described in tumor cells.(206,207)

Figure 16 shows the role of hesperidin in apoptosis and the cell cycle: hesperidin generates reactive oxygen species in cancer cells, activates mitochondrial pathways inducing apoptosis, and arrests the cancer cell cycle.

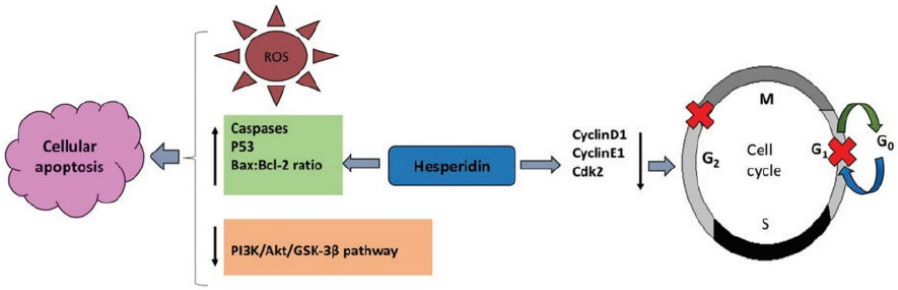

Figure 16. **Role of hesperidin on apoptosis and cell cycle.** Source: (Aggarwal V et al. 2020).(207)

Finally, with the currently active worldwide Coronavirus Disease 2019 (COVID-19) pandemic, studies have been carried out linking the consumption of hesperidin with positive effects on COVID-19. The ability of hesperidin to inhibit angiotensin-converting enzyme 2, transmembrane protease serine 2 and binding immunoglobulin protein receptors is one of the reasons for its possible beneficial effects since these receptors are the most noticeable receptors causing COVID-19.(208) Furthermore, flavonoids can have antiviral action because of their modulation of the immune system. Hesperidin showed the ability to bind to key proteins of severe acute respiratory syndrome coronavirus (SARS-CoV-2) with effective antiviral action that inhibited virus-induced cellular and systemic pathology.(209)

Therefore, there is evidence that supports the promising use of hesperidin in the prophylaxis and treatment of COVID-19: hesperidin can improve host cellular immunity against infection and anti-inflammatory action helping to control cytokine storms, and hesperidin mixed with specific drugs (diosmin co-administered with heparin) can protect against venous thromboembolism, which prevents the progression of the disease.

Figure 17 shows the possible effect of hesperidin on the prophylaxis and treatment of COVID-19.(210)

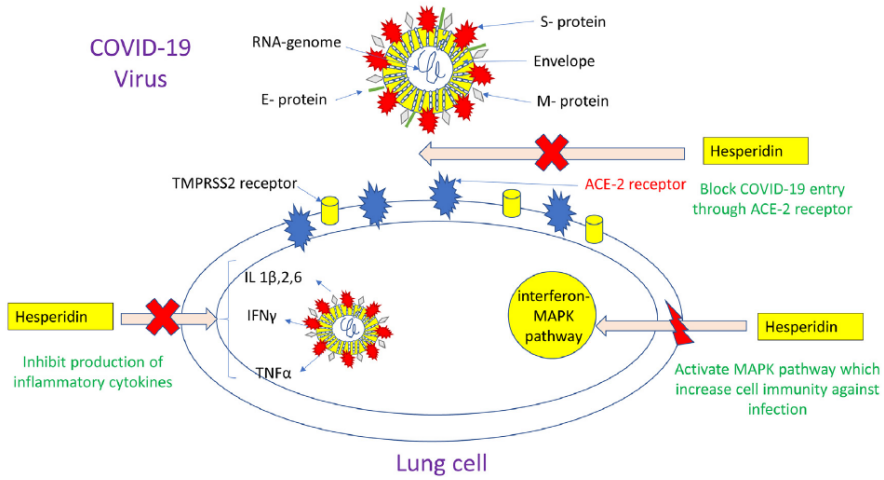

Figure 17. Possible effect of hesperidin on the prophylaxis and treatment of COVID-19. Abbreviations: ACE-2, angiotensin-converting enzyme 2. Source: (Haggag YA et al. 2020).(210)

In summary, it can be observed that there are future study routes in relation to hesperidin and its capacity to protect against chronic diseases that can be followed and continued. Further RCT with higher quality with the collection of stool samples for the metagenomics approach, the collection of plasma samples for the proteomics approach in humans, realizing a multi-omics analysis to integrate all the biological information and evaluate the effects of hesperidin consumption on other chronic diseases such as cancer, can be future goals in the world of nutrition and health for the prevention and treatment of diseases. Moreover, studies that relate hesperidin consumption and COVID-19 pathogenesis will be interesting given the possible applications it may have.

8. CONCLUSIONS

According to the results obtained, the conclusions about the effects of hesperidin consumption on CVD risk biomarkers are as follows:

-After the systematic review, hesperidin consumption was found to improve glucose levels and lipid profiles in animal models, but no definitive conclusion regarding the effects of hesperidin in humans can currently be drawn (Objective 1 and Project 1).

-After the CITRUS study, the intake of hesperidin in OJ decreased SBP and PP after sustained consumption in a dose-dependent manner with the hesperidin content of the beverage administered. Chronic consumption of EOJ enhances the postprandial response of decreasing SBP and PP. Decreases in homocysteine, uric acid and inflammatory markers at the systemic level and in PTX3 and NAMPT at the transcriptomic level could account for the observed changes in BP and PP (Objective 2 and Project 2).

-After the transcriptomic analysis from CITRUS study, the single dose consumption of higher doses of hesperidin could induce a better response than the consumption of the naturally occurring doses of hesperidin in OJ because of their improvement of insulin resistance. Moreover, the sustained consumption of hesperidin in EOJ decrease the expression of proinflammatory genes providing a possible mechanism of action on inflammation pathway and thereby could induces beneficial effects on the cardiovascular system. (Objective 3 and Project 3).

-The plasma and urine metabolite hesperetin 7-O-B-D-glucuronide is the only metabolite that reported hesperidin dose response differences,

and in urine it is inversely correlated with SBP levels. In addition, hesperidin consumption in OJ and EOJ for 12 weeks changed the serum metabolomic profile, decreasing the levels of endogenous metabolites related to BP, inflammation, and oxidative stress, and decreasing the urinary excretion of uraemic toxins. Additionally, after a single dose of hesperidin, changes in the serum levels of metabolites related to reduced BP levels and anti-inflammatory effects were observed (Objective 4 and Project 4).

-After proteomic analysis in metabolic syndrome rats, hesperidin supplementation changed the proteomic profiles of the heart and kidney tissues and had a beneficial impact on the cardiovascular system, free radical scavenging, and lipid and glucose metabolism (Objective 5 and Project 5).

OVERALL CONCLUSION

Finally, the overall conclusion is that hesperidin reduces human BP and PP in a dose-dependent manner. Thus, the hesperidin enrichment achieved with EOJ can be a useful co-adjuvant tool for BP and PP management in pre- and stage 1 hypertensive subjects. The mechanisms of action by which hesperidin exerts its beneficial effects can be explained through transcriptomics and metabolomics approaches in humans which demonstrated cardioprotective actions through decreases in pro-inflammatory genes (transcriptomic approach), decreases in serum endogenous metabolites related to BP and oxidative stress and decreases in the urinary excretion of uraemic toxins (metabolomic approach). Moreover, the proteomic approach realized in kidney and

heart tissues of metabolic syndrome rats, showed that hesperidin changes proteomic profiles exerting positive effects on two main organs involved on BP regulation and cardiovascular system.

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ANNEX

VISIT TO OTHER INTERNATIONAL CENTRE:

Visit to the International Agency for Research on Cancer / World Health Organization (IARC/WHO) of Lyon, France. Period: October 8th, 2018 – February 21st, 2019.

International Agency for Research on Cancer

World Health Organization

International PhD stay

Name:	Laura Pla Pagà, PhD student
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	Nutrition and Metabolism Section – Biomarkers Group
Start date:	8th October 2018
End date:	21st February 2019
Projects:	-CITRUS project in humans and the HESPERIDIN project in animals from our studies in Spain. Learning how analyse the proteomics, metabolomics, metagenomics and transcriptomics data obtained through the clinical trial in humans and the experimental study in rats. -EPIC project. Working and learning with the cancer data obtained through a prospective study in humans carried out by IARC. -Attendance to courses, seminars and meetings of interest about cancer, nutrition, epidemiology, bioinformatics and omics science in IARC. -Attendance to English course in IARC.
Signature and date:	

Supervisor

25 Feb 2019

PhD student