BIOACTIVE FOOD COMPOUNDS OF MEDITERRANEAN AND NORDIC DIETS AND THEIR EFFECTS ON NUTRITIONAL AND CARDIOVASCULAR DISEASE.

Anna Pedret Figuerola

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Bioactive food compounds of Mediterranean and Nordic diets and their effects on nutritional and cardiovascular disease biomarkers.

INTERNATIONAL THESIS

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PhD in Biomedicine

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FAIG CONSTAR:

Que aquest treball, titulat "*Bioactive food compounds of Mediterranean and Nordic diets and their effects on nutritional and cardiovascular disease biomarkers*", que presenta **Anna Pedret Figuerola**, ha estat realitzat sota la meva direcció al Departament de Medicina i Cirurgia i que acompleix els requeriments per a poder optar al títol de Doctor.

Reus, 30 d'abril de 2014

El director de la Tesi doctoral

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- Hanhineva K, Lankinen M, Pedret A, Schwab U, Kolehmainen M, Paananen Ju, De Mello V, Solà R, Lehtonen M, Poutanen K, Uusitupa M, Mykkänen H. Non-targeted metabolite profiling discriminates diet-specific biomarkers for consumption of whole grains, fatty fish and bilberries – a randomized trial (Sysdimet-HealthGrain intervention). (editor submitted)

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"Look deep into nature, and then you will understand everything better"

Albert Einstein

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SUMMARY

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death globally, and atherosclerosis is the major biological process involved in these diseases. CVD biomarkers are tools used in clinical practice to identify individuals with a high risk of CVDs, to quickly diagnose the diseases and to accurately and effectively predict and treat patients. Currently, many CVD biomarkers already exist that help to identify vulnerable patients; however, the discovery of new biomarkers can provide us with valuable new information to understand the pathophysiology of CVDs, and to prevent and treat CVDs.

Currently, numerous epidemiological studies have shown several important links among Mediterranean and Nordic dietary bioactive compounds (such as polyphenols), and the occurrence of CVDs. The poor accuracy of measuring the intake of foods and bioactive compounds is one of the biggest challenges in nutrition research. This challenge could be overcome by using quantifiable biomarkers of dietary intake.

Omic technologies support detailed studies of molecular changes during CVD onset and progression, as well as during dietary interventions. In the last decade, proteomics and metabolomics have contributed substantially to our understanding of CVD biology and to the discovery of nutritional and molecular CVD biomarkers. In nutritional interventions, metabolome analyses focus on identifying new biomarkers that allow the intake of certain dietary bioactive compounds to be monitored and related to their expected biological effects. Moreover, recent advances in proteomic technologies permit the evaluation of systematic changes in protein expression in response to intrinsic or extrinsic perturbations to the biological system, for example, those changes that occur in CVDs or that occur after a dietary intervention. Proteomics is a key tool for exploring the molecular mechanisms involved in the cardioprotective activities of dietary bioactive compounds.

HYPOTHESIS

Our hypothesis is that the intake of certain dietary bioactive compounds of the Mediterranean and Nordic diets can be assessed and monitored by nutritional biomarkers, improving the accuracy of the dietary intake measure. Moreover, these dietary bioactive compounds could be associated with an important diversity of mechanisms of action and with healthy biological effects on molecular biomarkers involved in the development of CVDs, in both healthy and CVD risk subjects.

OBJECTIVES

The primary objective is to assess the effect of Mediterranean and Nordic dietary bioactive compounds, particularly phenolic compounds, on nutritional and molecular biomarkers, which allow mechanisms of action and biological effects involved in the development of CVDs to be determined.

The following specific objectives have been set:

Objective 1: To assess the relations between urinary total polyphenol excretion (TPE), a biomarker of total polyphenol intake (TPI), polyphenol-rich foods, and oxidative stress biomarkers in healthy adults of different ages following a Mediterranean dietary pattern **(Clinical Study 1)**.

Objective 2: To assess the modifications of plasma metabolite profiles after a healthy Nordic dietary intervention with whole grains, bilberries and fatty fish by applying a non-targeted metabolite profiling approach in subjects with metabolic syndrome. Furthermore, we aimed to identify novel nutritional biomarkers more specific than TPE, and to determine molecular biomarkers of endogenous metabolism modified by dietary bioactive compounds **(Clinical Study 2)**.

Objective 3: To assess the impact of a dietary intervention supplemented with a virgin olive oil (VOO) or two different functional VOOs enriched with their own phenolic compounds (PC; hydroxytyrosol derivatives) or

complemented with thyme PC (flavonoids, monoterpens, and phenolic acids) on the HDL protein cargo and on its cardiovascular profile, by applying a quantitative proteomics approach in hypercholesterolemic subjects from the Mediterranean area **(Clinical Study 3)**.

MATERIALS AND METHODS

Clinical Study 1:

A cross-sectional study in which 81 participants were classified into three age groups: 18 to 39, 40 to 54, and 55 to 72 years of age. The TPE was determined in urine samples using the Folin-Ciocalteau method. The TPI was quantified from 3-day dietary records using the Phenol-Explorer database. The extent of oxidative injury and the antioxidant capacities of plasma and enzymatic antioxidants were analyzed by immunoenzymatic, fluorometric and spectrophotometric techniques.

Clinical Study 2:

A randomized, controlled, parallel, dietary trial in which 106 participants were randomized into the following three 12- week diet interventions: (1) whole grain products, fatty fish and bilberries (healthy diet; HD); (2) whole grain enriched diet (WGED), with the same grain products as in the HD intervention, but with no changes in fish or berry consumption; and (3) refined wheat breads and restrictions on fish and berries (Control). A non-targeted LC-MS metabolite profiling analysis was performed on fasting plasma samples. Additionally, correlation analyses were performed between the nutritional biomarker candidates and the food intake data for identifying the causative food items.

Clinical Study 3:

A randomized, double blind, cross-over, controlled trial in which 33 hypercholesterolemic subjects were recruited. Participants were randomized to one of 3 sequences of administration of 25 mL/day of raw: (1) VOO, 80

ppm of phenolic content; (2) functional VOO enriched with its own PC, 500 ppm of phenolic content; and (3) functional VOO enriched with its own PC plus complementary phenols from thyme, 500 ppm of phenolic content (50% olive oil phenolic compounds and 50% thyme phenolic compounds). HDL fractions were obtained from plasma by sequential density ultracentrifugation in two steps using sodium bromide. A quantitative proteomic approach, based on iTRAQ-OFFGEL-LC-MS/MS analysis was performed to identify changes in the HDL proteome after the dietary interventions. Ingenuity Pathway Analysis (IPA) was used to analyze canonical pathways and protein networks that involve the differentially expressed proteins to approach the biological interpretation.

RESULTS

Clinical Study 1:

Urinary TPE increased with age ($p < 0.001$) and was inversely associated with urinary 8-hydroxydeoxyguanosine (8-OHdG; $p < 0.001$) and erythrocyte-oxidized glutathione concentrations ($p < 0.05$). A negative association between urinary 8-OHdG and the daily intake of polyphenols from vegetables and from fermented beverages, such as red wine, was observed ($p < 0.01$; $p < 0.05$, respectively).

Clinical Study 2:

Marked differences in fasting plasma metabolite profiles were observed after the intervention diets compared with the Control diet. In both intervention groups a significant increase was observed in signals identified as glucuronidated alk(en)ylresorcinols which correlated strongly with the intake of whole grain products. Additionally, HD intervention increased the signals for the furan fatty acid 3-carboxyl-4-methyl-5-propyl-2-furanopropionic acid (CMPF), hippuric acid and various lipid species incorporating polyunsaturated fatty acids (PUFAs). Plasma hippuric acid and CMPF correlated with intakes of bilberries and fish, respectively. WGED

intervention tended to increase endogenous betaine metabolites and certain amino acids.

Clinical Study 3:

In total, 127 HDL-associated proteins were identified. Among these proteins, 15 were commonly differentially expressed after the consumption of the three VOOs, although specific changes were observed for each intervention. The overlapping proteins among interventions were related with cardioprotective biological functions, and IPA signaling pathways revealed that these proteins were involved in a variety of canonical pathways, including LXR/RXR activation, acute phase response signaling, atherosclerosis signaling, interleukin 12 (IL-12) signaling and production in macrophages, production of Nitric oxide and reactive oxygen species in macrophages, clathrin-mediated endocytosis signaling and coagulation system.

CONCLUSIONS

1. Urinary TPE increased with age due to an increase in the consumption of vegetables, fruits, and coffee, and to a moderate intake of fermented beverages. Moreover, a higher urinary TPE is associated with a decrease in oxidative stress biomarkers, which may reflect an attenuation of oxidative damage in healthy individuals.

2. The beneficial effects of a dietary intake rich in vegetables and moderate red wine on healthy individuals, could potentially be explained by the attenuation of oxidative damage associated with these typical food items of the Mediterranean diet, which are rich in polyphenols.

3. A non-targeted metabolite approach based on LC-MS is an efficient technique in the discovery of specific novel nutritional biomarkers and

in reporting metabolic alterations in fasting plasma after dietary interventions.

4. Fasting plasma CMPF, glucuronidated alk(en)ylresorcinols and hippuric acid are suggested as novel candidate biomarkers of fatty fish, whole grain and bilberry consumption, respectively, because these candidates present a strong positive correlation with the proposed consumed foods.

5. Alterations in molecular biomarkers of endogenous metabolism, such as PUFA-containing lipids, amino acids and betaine metabolites, could explain the effect of whole grains, fatty fish and bilberry consumption on metabolic pathways, thus indicating the potential beneficial effects of a healthy Nordic diet.

6. A quantitative proteomics approach based on iTRAQ-LC-MS/MS potentially can better elucidate the effects of dietary interventions with olive oils rich in PC on the complex structure of the HDL protein cargo.

7. The consumption of VOO or phenol-enriched VOOs has an impact on the HDL proteome in a cardioprotective mode that could enhance HDL functionality by up-regulating proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation while down-regulating proteins involved in acute-phase response, lipid transport, and immune responses.

8. The common protein expression modifications reported after the consumption of the three VOOs indicate a major effect of the fatty acid and PC composition present in the common matrix of these VOOs on HDL remodeling.

OVERALL CONCLUSION

The use of nutritional biomarkers is a useful tool in assessing the mechanisms of action and the biological effects of dietary bioactive

compounds on CVD development. Thus, the use of omic sciences, such as metabolomics and proteomics, add relevant information to the discovery of novel nutritional and molecular biomarkers, which assist us in evaluating food bioactivity and food effects on human health and on disease prevention.

ABBREVIATIONS

8-OHdG - 8-hydroxydeoxyguanosine

A2M - Alpha-2-macroglobulin

ACN - Acetonitrile

AHSG - Alpha-2-HS-glycoprotein

ANOVA - Analysis of Covariance

ANPEP - Aminopeptidase N

APO - Apolipoprotein

APOA1 - Apolipoprotein A-I

APOA2 - Apolipoprotein A-II

APOA4 - Apolipoprotein A-IV

APOC1 - Apolipoprotein C-I

APOC2 - Apolipoprotein C-II

APOC3 - Apolipoprotein C-III

APOD - Apolipoprotein D

APOF - Apolipoprotein F

APOL1 - Apolipoprotein L1

APOL2 - Apolipoprotein L2

APOM - Apolipoprotein M

AR - Alk(en)ylresorcinol

AZGP1 - Zinc-alpha-2-glycoprotein

CAT - Catalase

CE - Capillary electrophoresis

CETP - Cholesteryl ester transfer protein

CHCA - α-cyano-4-hydroxycinnamic acid

CLU - Clusterin

CMPF - 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid

CNDP1 - Beta-Ala-His dipeptidase

CVD - Cardiovascular disease

DASH - Dietary Approaches to Stop Hypertension

DHA - Docosahexaenoic acid

DNA - Deoxyribonucleic acid

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EFSA - European Food Safety Authority

EPA - Eicosapentanoic acid

ESI - Electrospray ionization

FA - Formic acid

FDR - False discovery rate

FGA - Fibrinogen alpha chain

FGG - Fibrinogen gamma chain

FRAP - Ferric-reducing ability of plasma

FTMS - Fourier transform mass spectrometry

FVOO - Functional virgin olive oil

GAE - Gallic acid equivalent

GC - Gas chromatography

GC - Vitamin D-binding protein

GPx - Glutathione peroxidase

GSH - Reduced glutathione

GSSG - Oxidized glutathione

HD - Healthy diet

HDL - High-density lipoprotein

HILIC - Hydrophilic interaction

HP - Haptoglobin

HRG - Histidine-rich glycoprotein

hsCRP - High-sensitivity C-reactive protein

ICAM -1 - Intercellular adhesion molecule-1

IDL - Intermediate-density lipoprotein

IL-12 - Interleukin 12

IL-1β - Interleukine-1β

IL-6 - Interleukin 6

IPA - Ingenuity pathway analysis

IPG - Immobilized pH gradient

ITIH1 - Inter-alpha-trypsin inhibitor heavy chain H1

ITIH4 - Inter-alpha-trypsin inhibitor heavy chain H4

iTRAQ - Isobaric tagging for relative and absolute quantification

KNG1 - Kininogen-1

LC – Liquid chromatography

LCAT - Phosphatidylcholine-sterol acyltransferase

LDL - Low-density lipoprotein

LPA - Apolipoprotein(a)

LPC - Lysophosphatidylcholines

LPE - Lysophosphatidyl-ethanolamines

LXR/RXR - Liver X receptor/retinoid X receptor

MALDI - Matrix-assisted laser desorption/ionization

MD - Mediterranean diet

MetS - Metabolic syndrome

MS - Mass spectrometry

MUFA - Monounsaturated fatty acids

MW - Molecular weight

NMR - Nuclear magnetic resonance

NO - Nitric oxide

NR5A2 - Nuclear receptor subfamily 5, group A, member 2

Nrf2 - Nuclear factor erythroid 2-related 2

OGE - OFFGEL electrophoresis

OO - Olive oil

ORAC - Oxygen radical absorbance capacity

ORM1 - Alpha-1-acid glycoprotein1

ORM2 - Alpha-1-acid glycoprotein 2

oxLDL - Oxidized LDL

PAI-1 - Plasminogen activator inhibitor-1

PC - Phenolic compounds

PCh - Phosphatidylcholines

PF4 - Platelet factor 4

PIC - Protease inhibitor cocktail

PLS-DA - Partial least squares discriminant analysis

PON3 - Serum paraxonase/lactonase 3

PPBP - Platelet basic protein

ppm - Parts per million

PSM - Peptide-spectrum match

PUFA - Polyunsaturated fatty acids

QC - Quality control

RBP4 - Retinol-binding protein 4

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RP - Reversed phase

SAA1 - Serum amyloid A protein

SAA4 - Serum amyloid A protein 4

SCORE - Systemic Coronary Risk Evaluation

SCX - Strong-cation exchange

SDS - Sodium dodecyl sulfate

SERPINA5 - Plasma serine protease inhibitor

SERPINC1 - Antithrombin-III

SERPIND1 - Heparin cofactor 2

SERPINF2 - Alpha-2-antiplasmin

SMC - Smooth muscle cell

SOD - Superoxide dismutase

SPSS - Statistical package for the social sciences

TBARS - Thiobarbituric acid reactive substances

TCA - Tricloroacetic acid

TCEP - Tris-(2-carboxyethyl)phosphine

TFA - Trifluoroacetic acid

TMT - Tandem mass tags

TNF-α - Tumor necrosis factor-α

TOF - Time of flight

TPE - Total polyphenol excretion

TPI - Total polyphenol intake

UHPLC - Ultra high performance liquid cromatography

UNESCO - United Nations Educational, Scientific and Cultural Organization

VCAM-1 - Vascullar cell adhesion molecule-1

VIP - Variable influence on projection

VLDL - Very-low-density lipoprotein

VOO - Virgin olive oil

WGED - Whole grain enriched diet

WHO - World Health Organization

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INTRODUCTION

CHAPTER 1: CARDIOVASCULAR DISEASE

1.1 DEFINITION AND EPIDEMIOLOGY

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, including coronary heart disease, cerebrovascular disease and peripheral arterial disease. The World Health Organization (WHO, 2013) identifies CVDs as the leading cause of death worldwide and estimates that 17.3 million people died from CVDs in 2008, representing 30% of all global deaths. Of these deaths, an estimated 7.3 million were due to coronary heart disease, and 6.2 million were due to stroke. The number of people who die from CVDs, primarily from heart disease and from stroke, is predicted to increase and to reach 23.3 million by 2030. CVDs were once considered diseases that occur more frequently in industrialized countries. However, in recent years, there has been an increase in CVD risk factors globally, including obesity, hypertension and diabetes, reflecting significant global changes in behavior and lifestyle. The WHO states that over 80% of CVD deaths currently occur in low and middle income countries and almost equally in men and women. Atherosclerosis is the major biological process that causes both ischemic stroke and myocardial infarction and, thus, is the principal cause of cardiovascular death. Cardiovascular primary prevention, including identifying and treating at-risk individuals, remains a major public health priority.

1.2 PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Understanding the pathophysiology of atherosclerosis progression has been a major objective of CVD research in recent decades. This understanding is essential for improving strategies for CVD prevention and for developing new and effective treatments. Oxidative stress, inflammation, endothelial dysfunction and thrombosis are key mechanisms involved in the onset and progression of atherosclerosis.

The initiation of atherosclerosis is due to a qualitative change in the monolayer of endothelial cells that line the inner arterial surface. Endothelial

cells express adhesion molecules that capture leukocytes (monocytes and T lymphocytes) on their surfaces when subjected to pro-inflammatory mediators (**Figure 1B**; Libby et al., 2011). Vascular cell adhesion molecule-1 (VCAM-1) is one of the most important adhesion molecules in this interaction between the endothelium and leukocytes; however, other adhesion molecules, such as E-selectin, P-selectin and intercellular adhesion molecule-1 (ICAM-1) are also expressed in endothelial cells (Gui et al., 2012; Libby, 2012). In parallel, changes in endothelial permeability and the composition of the extracellular matrix stimulate the entrance and retention of low-density lipoprotein (LDL) particles in the artery wall. Then, LDL particles are oxidized into pro-inflammatory particles (oxidized LDL; oxLDL), which incite an inflammatory reaction within the intima, and consequently, several cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), are released, causing the expression of adhesion molecules in endothelial cells and, consequently, the binding of leukocytes to the arterial wall (Libby, 2006).

Once monocytes adhere to the arterial endothelium, these cells penetrate the endothelial lining and enter the intima of the vessel wall; this process requires chemoattractant mediators. Within the intima, monocytes differentiate into tissue macrophages. In the nascent atheroma, macrophages express scavenger receptors that permit the uptake of oxLDL and become foam cells that **develop fatty streaks** (Gui et al., 2012; Insull, 2009). Simultaneously, macrophages multiply and release several growth factors and cytokines, thus amplifying and sustaining pro-inflammatory signals and promoting thrombotic complications (Gui et al., 2012). T lymphocytes and mast cells also accumulate in atheroma plaques (**Figure 1B**). These early changes are microscopic and may progress to major visibility; additionally, these lipid changes can be reversed (Insull, 2009; Libby et al., 2011).

During the **progression of a complex atheromatous plaque**, smooth muscle cells (SMCs) are recruited from the tunica media into the tunica

intima and proliferate in response to mediators such as platelet-derived growth factor. In the intima, the SMCs produce extracellular matrix molecules, including collagen and elastin, and form a fibrous cap that covers the plaque, which is a collection of foam cells and released lipids that accumulate in extracellular regions (Libby et al., 2011). During this process, calcium deposition is produced in atheromatous plaques, initially as small aggregates and later as large nodules (**Figure 1C**; Insull, 2009).

Plaques generally cause clinical manifestations by producing flow-limiting stenosis that leads to tissue ischemia or by inciting thrombosis that can interrupt blood flow locally or in distal arteries. Thrombotic complications often appear after physical disruption of the plaque and, most commonly, after a fracture of the fibrous cap. Plaques that rupture typically have thin, collagen-poor fibrous covers with few SMCs but abundant macrophages (Libby et al., 2011). The inflammatory cells (T lymphocytes) may accelerate plaque disruption by producing collagenolytic enzymes that can degrade collagen and by generating mediators that provoke the death of SMCs, which are the source of arterial collagen (Libby, 2013). Macrophages that are in plaques also produce tissue factor, which is a procoagulant that promotes the thrombogenicity of the lipid core (Gui et al., 2012). When a **plaque ruptures**, a thrombus forms that is responsible for most of the acute complications of atherosclerosis (**Figure 1D**; Libby, 2006)

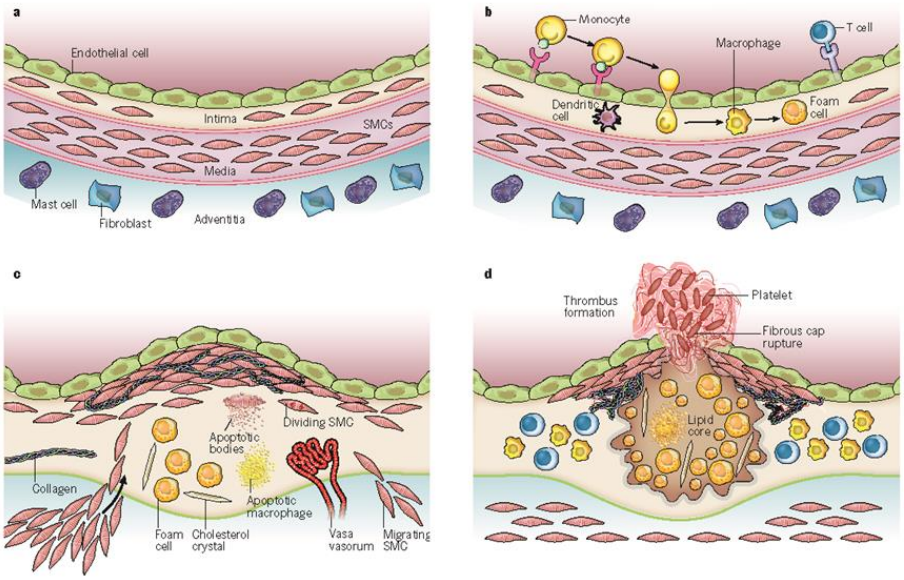

Figure 1. Development of atherosclerotic lesions. A) A normal artery contains three layers. The inner layer, or tunica intima, is lined by a monolayer of endothelial cells that is in contact with blood overlying a basement membrane. The middle layer, or tunica media, contains SMCs embedded in a complex extracellular matrix. The adventitia, which is the outer layer of arteries, contains mast cells, nerve endings and microvessels. **B)** The initial steps of atherosclerosis include adhesion of blood leukocytes to the activated endothelial monolayer, directed migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and lipid uptake by these machrophages, yielding foam cells. **C)** Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of resident intimal SMCs and media-derived SMCs, and the heightened synthesis of extracellular matrix macromolecules, such as collagen, elastin and proteoglycans. Plaque macrophages and SMCs can die, some by apoptosis, in advancing lesions. Extracellular lipids derived from dead and dying cells can accumulate in the central region of a plaque, often called the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels. **D)** Thrombosis, which is the ultimate complication of atherosclerosis. A fracture of the plaque's fibrous cap is shown, which has enabled blood coagulation components to access tissue factors in the plaque's interior, triggering thrombus that extends into the vessel lumen, where blood flow can be impede. Source: (Libby et al., 2011).

Oxidative stress plays an important role in the development of this thesis. Therefore, the specific function of oxidative stress in the atherosclerotic process is explained in greater detail below.

Oxidative stress is a potent inductor of endothelial dysfunction and is involved at all stages of atherosclerotic plaque evolution (Schnabel et al., 2007). Oxidative stress culminates due to an imbalance between pro-oxidants and antioxidants and a consequent excessive production of reactive oxygen species (ROS), contributing to the oxidative damage of lipids in cellular membranes, as well as in proteins and in deoxyribonucleic acid (DNA). Endogenous antioxidant enzymes and exogenous dietary antioxidants can prevent intracellular ROS concentrations from reaching levels at which damage occurs (Li et al., 2013). Risk factors for cardiovascular disease, such as hypertension, hypercholesterolemia, diabetes mellitus, and tobacco use, as well as CVD itself, are all associated with significant increases in ROS in the vascular wall, a situation that eventually culminates with oxidative stress. During atherosclerosis progression, ROS are produced by endothelial cells, SMCs and macrophages (**Figure 2**). Under oxidative stress, the enzymatic production of ROS exceeds the available antioxidant defense systems (Li et al., 2013). ROS overproduction under pathophysiological conditions is integral to the development of CVDs. ROS mediate various signaling pathways that cause vascular inflammation in atherogenesis: from the initiation of fatty streak development, through lesion progress, to ultimate thrombotic activity and plaque rupture. ROS reduce the production of nitric oxide (NO), which is an endothelium relaxant factor that modulates endothelial tone and reactivity. The reduced bioavailability of vascular NO is the most important mechanism of endothelial dysfunction observed in CVDs (Li et al., 2013). In addition, vascular oxidative stress promotes atherogenesis through numerous different mechanisms, including the activation of redox-sensitive transcription factors (which stimulate the expression of proinflammatory genes) and the induction of lipid peroxidation, protein oxidation, and mitochondrial and nuclear DNA damage (Li et al., 2013).

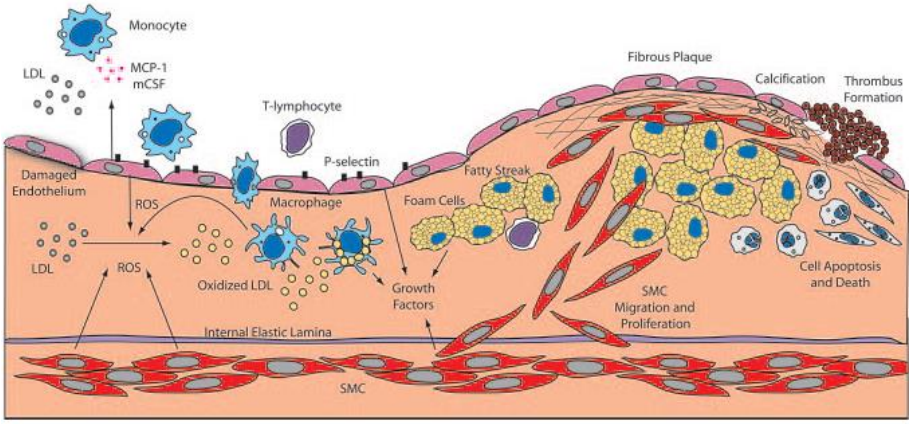

Figure 2. Pathophysiology of atherosclerosis. ROS produced by endothelial cells, SMCs, and macrophages oxidize LDLs in the subendothelial space, at the sites of endothelial damage, initiating events that culminate in the formation of a fibrous plaque. Fibrous plaque rupture leads to thrombus formation and vascular occlusion. Source: (Madamanchi et al., 2005).

1.3 CARDIOVASCULAR DISEASE RISK FACTORS

CVD prevention can improve the well-being of a population and possibly decrease healthcare spending. Therefore, CVD prevention should be the most important aspect of any sustainable health economy model. In recent decades, prevention strategies have contributed to a 50% decrease in CVD mortality observed in many high income countries (Perk et al., 2012).

CVD risk denotes the likelihood of a person developing a cardiovascular event over a defined period. CVD risk factors have emerged from a combination of epidemiological studies performed worldwide and are the elements that affect the probability and the risk of developing CVDs. The concepts of CVD risk factors and risk estimation have become integral parts of modern medical programs and have led to the development of effective treatments and preventive strategies in clinical practice (Framingham Heart Study, 2014).

CVD risk factors can be classified into two categories: non-modifiable risk factors and modifiable risk factors. The non-modifiable risk factors include

genetic factors, age and gender. There is evidence of strong heritability of many cardiovascular risk factors and of atherosclerotic disease (Perk et al., 2012). Increasing age and the male gender increase CVD risk and are stable characteristics used to stratify risk assessments. In contrast, CVDs are strongly connected to modifiable lifestyle risk factors, particularly the use of tobacco, unhealthy dietary habits, physical inactivity and psychosocial stress. The WHO has stated that 80% of CVD cases may be avoided with lifestyle changes. These unhealthy behaviours may cause an increase in the major intermediate biological risk factors that lead to CVDs, including blood pressure, blood glucose, blood lipids, overweight and obesity. These intermediate biological risk factors are considered the traditional or classical risk factors (Perk et al., 2012).

First, we focus our interest on classical risk factors for CVDs; however, emerging risk factors are also explained.

1.3.1 Classical risk factors

Traditional or classical risk factors are normally measured in primary care and are important risk assessment tools that have led to the development of predictive models of cardiovascular risk estimation, such as the Framingham 10-year risk score or the European Systematic Coronary Risk Evaluation (SCORE) (Artigao-Rodenas et al., 2013; Jørstad et al., 2013). The involvement of these classic risk factors in CVDs will be briefly explained:

- Elevated blood pressure: In several epidemiological studies, elevated blood pressure (BP) has been identified as a major risk factor for CVDs (Perk et al., 2012). Even small increases in BP are associated with an increased risk of cardiovascular events. BP is a heritable trait influenced by multiple biological pathways and responds to environmental stimuli, such as diet, use of tobacco and stress (Ehret et al., 2011; Lewington et al., 2002).
- Elevated glucose blood levels: Existing data indicate a relation between increased levels of glycaemia and cardiovascular events.

The risk of cardiovascular events or death increases progressively among individuals who are normoglycaemic, with impaired fasting glucose or impaired glucose tolerance, and newly diagnosed diabetics (Anand et al., 2012). A 1 mmol/L increase in the fasting plasma glucose level is associated with a 17% increase in the risk of future cardiovascular events or death. The intensive management of hyperglycaemia in diabetes reduces the risk of micro-vascular complications and, to a lesser extent, the risk of CVDs (Ton et al., 2013).

- Elevated lipid blood levels: Increased plasma cholesterol and LDL cholesterol levels are among the major risk factors for CVDs (Perk et al., 2012). Hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol concentrations are independent CVD risk factors. Because low concentrations of HDL cholesterol are independently associated with higher CVD risk, this parameter is also included in new SCORE charts (Chapman et al., 2011).

  A specific topic for this thesis is the cardioprotective effect of the HDL particle. Thus, this lipoprotein is explained in greater detail below.

HDL particle

In epidemiological studies, low plasma HDL cholesterol levels have shown an inverse relation to the CVD risk. HDL particles play a central role in the efflux of cholesterol from peripheral tissues, which may be vital to the atheroprotective effects of HDL (Annema et al., 2013). Therefore, the HDL particle is being intensely investigated as a potential therapeutic target in patients at high cardiovascular risk. However, several recent pharmacological and genetic studies have failed to demonstrate that increased HDL cholesterol plasma levels result in decreased CVD risk, creating controversy regarding whether HDL cholesterol plasma levels reflect HDL function. It is important to distinguish between HDL function and HDL cholesterol

plasma levels (Lüscher et al., 2014). In fact, it has been argued that low HDL cholesterol may only represent a marker for proatherogenic risk factors, rather than HDL being a mediator that protects against atherogenesis (Lüscher et al., 2014). Currently, a greater understanding of the complexity of HDL composition and biology has driven researchers to redefine the HDL particle. Thus, the focus of HDL has now begun to change from a cholesterol-centric view toward HDL particle number, subclasses and HDL composition. Many of the recently discovered functions of HDL are, actually not strictly conferred by its ability to promote cholesterol efflux but by the other molecules HDL transports, including a diverse set of proteins, small ribonucleic acids (RNAs), hormones, carotenoids, vitamins, and bioactive lipids (Vickers et al., 2014). Novel biological and atheroprotective activities are specifically related to HDL protein cargo. Currently, studying the HDL proteome has become important in the entire study of HDL functionality, and approximately one hundred different proteins, except for apolipoproteins (APOs), have been associated with the HDL particle (Shah et al., 2013). These new HDL-associated proteins have been linked with the following cardioprotective activities of the HDL particle: antioxidative, anti-inflammatory, antithrombotic, complement system regulation and protective effects on vascular endothelium and SMCs (Vickers et al., 2014; **Figure 3**).

For the above mentioned, it is important to demonstrate that novel therapeutic interventions not only increase HDL cholesterol plasma levels but also improve HDL functionality in patients at high cardiovascular risk, principally remodeling the HDL proteome (Lüscher et al., 2014).

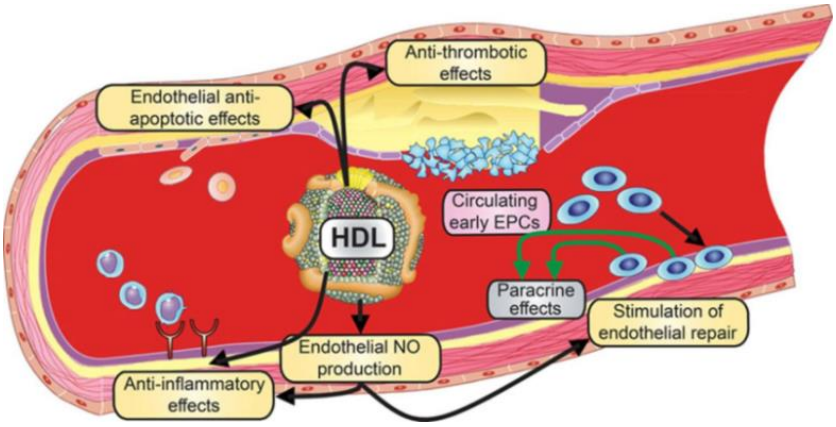

Figure 3. Proposed direct vascular protective and potentially antiatherogenic effects of HDL. HDL from healthy subjects stimulates NO release from human aorta endothelial cells and increases the expression of endothelial nitric oxide synthase. Furthermore, HDL suppresses the expression of adhesion molecules, such as VCAM-1, and inhibits the adhesion of white blood cells. HDL also exerts antithrombotic effects because this lipoprotein reduces tissue factor expression in endothelial cells exposed to cytokines and reduces platelet activation. Source: (Lüscher et al., 2014).

- Overweight and obesity: Obesity has become a major global contributor to CVD incidence and mortality. The visceral adipose tissue is a metabolically active endocrine organ capable of synthesizing and releasing an important variety compounds that may play a role in cardiovascular homeostasis into the bloodstream. This process affects CVD risk factors and hence on CVD risk (Perk et al., 2012). Overweight and obese individuals more commonly have other CVD risk factors, such as diabetes, insulin resistance, dyslipidemia and hypertension. This metabolic network is known as metabolic syndrome (MetS), which is a complex disorder that directly increases the CVD risk. MetS causes a high socioeconomic cost and is considered a worldwide epidemic (Kassi et al., 2011; Samson et al., 2014).

1.3.2 Emerging risk factors

Classical risk factors do not fully explain the inter-individual variation in cardiovascular risk. Intensive studies during the past decade have led to a deeper understanding of the biology and pathophysiology of atherosclerosis, which has provided new emerging risk factors (Ge et al., 2012). Oxidative stress, inflammation, endothelial dysfunction and thrombosis are processes that are involved in the pathogenesis of atherosclerosis and that have emerged as new risk factors for cardiovascular events before symptoms appear. Several studies have evaluated a series of candidate biomarkers of inflammation, oxidative stress, endothelial dysfunction and thrombosis as potential clinical tools for improving atherosclerosis prediction (Gui et al., 2012). Emerging validated biomarkers may add value in the context of specialized practice, for assessing CVD risk more precisely in specific subgroups of patients at moderate, unusual, or undefined levels of risk (Perk et al., 2012). Thus far, no biomarker has emerged as the best screening marker of CVD risk, and no single biomarker is sufficiently sensitive or specific for use on its own; instead, larger biomarker panels are used (Gilstrap et al., 2012). In Chapter 3, emerging CVD biomarkers will be explained in greater detail.

CHAPTER 2: DIET AND CARDIOVASCULAR DISEASE

Dietary habits are known to influence CVD risk, either through an effect on intermediate biological risk factors such as serum cholesterol, blood pressure, body weight, and diabetes, or through an effect independent of these risk factors. Currently, numerous epidemiological studies have shown several important links between diet and CVD occurrence (Engelfriet et al., 2010). A healthy dietary pattern can delay the development of atherosclerosis, thus reducing the incidence of coronary events, and is an important modifiable risk factor in CVD prevention (Perk et al., 2012). According to the European Guidelines on CVD prevention in clinical practice, the recommended characteristics of a healthy dietary pattern are described in **Table 1**.

Table 1. Characteristics of a healthy dietary pattern.

FOODS OR NUTRIENTS	HEALTHY DIETARY PATTERN RECOMMENDED
Saturated fatty acids	to account for < 10% of total energy intake, through replacement by polyunsaturated fatty acids
Trans-unsaturated fatty acids	to account for < 1% of total energy intake from natural origin, with preferably no intake from processed food
Salt	< 5 g per day
Fiber	30 - 45 g per day, from whole grain products, legumes, fruits and vegetables
Fruits	200 g per day (2 -3 servings per day)
Fish	at least twice a week, one of which should be fatty fish
Alcoholic beverages	should be limited to two glasses per day (20 g/ day of alcohol) for men and to one glass per day (10 g/ day of alcohol) for women

Source: (adapted from Perk et al., 2012).

The effect of diet on CVD risk can be studied at different levels, with the study of specific bioactive compounds as the most detailed method. Examining food groups is another method of assessing diet, and is more easily translated into dietary recommendations. Finally, there is growing interest in studying the effect of dietary patterns on CVD risk, and currently,

the Mediterranean diet (MD) is the most studied dietary pattern (Perk et al., 2012).

2.1 BIOACTIVE COMPOUNDS

In this section, we define and summarize the effects of some of the most important bioactive compounds that influence CVD risk. Moreover, we introduce a special section to discuss phenolic compounds (PC) because these compounds are bioactive molecules that have played a central role in the development of this thesis.

Bioactive compounds are defined as essential and non-essential compounds that occur in nature. These compounds are part of the food chain, and can provide human health benefits beyond the basic nutritional value of the food (Biesalski et al., 2009). A wide variety of foods offer a range of different bioactive compounds, including vitamins, minerals, fiber and phytochemicals (Liu, 2013). Increasing evidence suggests that CVD health benefits of plant foods are attributed to the additive and synergistic interactions of the bioactive compounds present in these foods by targeting multiple signal transduction pathways (Liu, 2013).

The effects of sodium and potassium intake on blood pressure are well established. The Dietary Approaches to Stop Hypertension (DASH) trial (Kwan et al., 2013) showed an inverse dose-response relation between sodium intake and blood pressure. In contrast, a higher potassium intake has been shown to reduce this classical risk factor. Moreover, a diet rich in magnesium and calcium has been related to favorable effects on blood pressure (Kwan et al., 2013).

Many case-control and prospective observational studies have observed inverse associations between levels of different vitamins, such as vitamin A, vitamin E, vitamin B6, vitamin B12, folic acid and vitamin D, and CVD risk (Perk et al., 2012). Consistent with these studies, in vitro studies performed

by our group showed that alpha-tocopherol, which is a form of vitamin E, prevents endothelial dysfunction (Catalán et al., 2012).

It is well established that the consumption of dietary fiber reduces CVD risk (Perk et al., 2012). One of the mechanisms involved in its effect is its capabilities of reducing the post-prandial glucose response after carbohydrate-rich meals and of lowering total and LDL cholesterol levels in the blood. Important sources of fiber include whole grain products, legumes, fruits and vegetables (Threapleton et al., 2013).

Phytochemicals are defined as bioactive non-nutrient plant chemicals present in fruits, vegetables, grains, legumes, nuts and other plant foods that may provide desirable health benefits beyond basic nutrition to reduce the risk of major chronic diseases, such as CVDs (Liu, 2004). Dietary phytochemicals can be classified into broad categories, such as PC, alkaloids, nitrogen-containing compounds, organosulfur compounds, phytosterols, and carotenoids (Liu, 2004). Of these phytochemical groups, PC are the most important and most studied in relation to CVDs.

2.1.1 Phenolic compounds

Several thousand molecules with polyphenol structures have been identified in higher plants, and several hundred have been found in edible plants. PC, which are also called polyphenols, are secondary metabolites of plants and are generally involved in the defense against ultraviolet radiation or aggression by pathogens. PC represent the most abundant antioxidants in human diets (Manach et al., 2004). An aromatic ring carrying one or more hydroxyl moieties is present in their structure, and different classes of PC can be considered according to the number of phenol rings and to the structural elements that bind these rings (Andrés-Lacueva et al., 2009).

In this context, PC have been classified into two primary groups:

- **Flavonoids:** these compounds are composed of all PC with a C6-C3-C6 structure (**Figure 4**). In this group we find the following subgroups:

flavanones, dihydroflavonols, flavan-3-ols, isoflavones, flavones, flavonols, anthocyanidins and proanthocyanidins (Andrés-Lacueva et al., 2009; Manach et al., 2004).

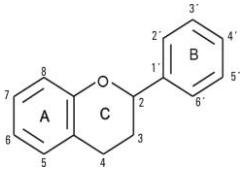

Figure 4. C6-C3-C6 structure from which all flavonoid compounds are derived.

- Non-flavonoids: these compounds are classified according to their number of carbons and include the following subgroups: simple phenols, benzoic acids, hydrolysable tannins, acetophenones and phenylacetic acids, cinnamic acids, coumarins, benzophenones, xanthones, stilbenes, chalcones, lignans and secoiridoids (Andrés-Lacueva et al., 2009; Manach et al., 2004).

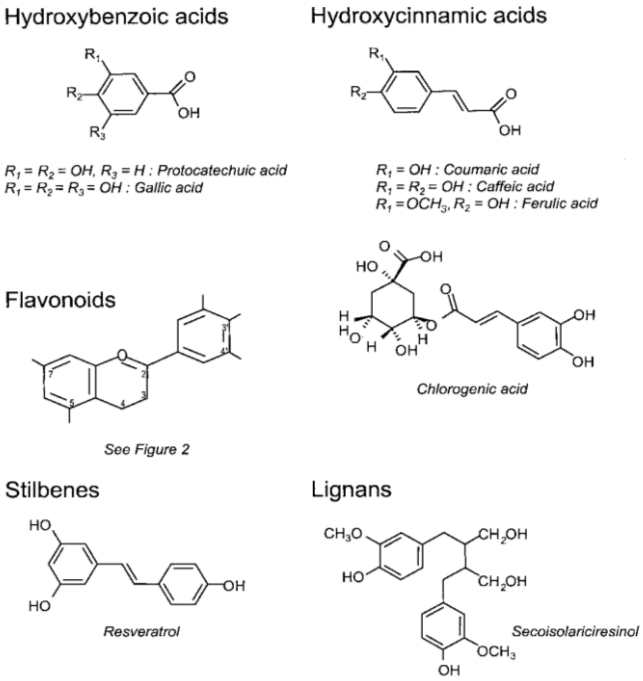

Figure 5. Chemical structures of some of non-flavonoids compounds. Source: (Manach et al., 2004)**.**

Occurrence of phenolic compounds in foods

Flavonoid and non-flavonoid compounds are present in significant amounts in many commonly consumed fruits, vegetables, grains, beverages and herbs. **Table 2** summarizes the PC classification (primary groups and subgroups), and provides examples of the primary dietary sources for each subgroup.

Table 2. Flavonoid and non-flavonoid compounds in foods.

	SUBGROUPS	PHENOLIC COMPOUNDS EXAMPLES	PRIMARY DIETARY SOURCES
FLAVONOIDS	Flavonols	quercitin and kaempferol	vegetables and some species, such as capers, onions, asparagus, berries and lettuce
	Flavones	apigenin and luteolin glycosides	species such as parsley and thyme
	Flavanones	hesperetin and naringenin	citric fruits
	Isoflavones	genistein and daidzein	almost exclusively in leguminous plants
	Anthocyanidins	cyanidin, delphinidin, malvidin, peonidin, and petunidin	berries and other red-blue fruits and vegetables
	Flavan-3-ols	catechin and epicatechin	Tea, chocolate, many types of fruit, red wine, beer and nuts
	Proanthocyanidins	procyanidins	cinnamon, sorghum, beans, red wine and chocolate
NON-FLAVONOIDS	Simple phenols	hydroxytyrosol and tyrosol	virgin olive oil
	Benzoic acids	ellagic and gallic acids	berries and grapes
	Hydrolysable tannins	ellagitannins and gallotannins	berries
	Acetophenones and phenylacetic acids	-	fermented soya bean
	Cinnamic acids	p-coumaric, caffeic, ferulic and sinapic acids	fruits and vegetables, with ferulic acid the most abundant in cereal grains
	Coumarins	scopoletin	fruits, green tea and other foods, such as chicory
	Benzophenones	-	distributed scarcely in foods
	Xanthones	-	mangosteen
	Stilbenes	resveratrol	red wine, grape and grape products
	Chalcones	chalconaringenin	apple juice, cider and pomace
	Lignans	pinoresinol	wine, beer, tea and coffee
	Secoiridoids	oleuropein	olive fruits

Source: (adapted from Andrés-Lacueva et al., 2009; Manach et al., 2004).

Bioavailability and cardiovascular health effects

Clinical and epidemiological studies have provided evidence that phenol-rich foods and phenol-rich diets have protective effects against chronic diseases, such as CVDs. Knowledge regarding the bioavailability of phenolic compounds is essential for researchers to understand their biological health effects. The most common PC in the human diet are not necessarily the most active *in vivo*, because these compounds either have a lower intrinsic activity than others or are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated (Manach et al., 2004). In foods, almost all PC are present in glycosylated forms; thus, these compounds cannot be absorbed directly in their native form. These substances must be hydrolyzed by intestinal enzymes or by the gut microbiota before absorption. During absorption, PC are conjugated in the small intestine and later in the liver, facilitating their biliary and urinary elimination by increasing their hydrophilicity. Circulating PC are conjugated derivatives that are extensively bound to albumin. PC that are secreted via the biliary route into the duodenum can be subjected to enterohepatic recycling, which may lead to a longer presence of PC within the body (Manach et al., 2004).

PC that are not absorbed in the small intestine reach the colon, where gut microbiota hydrolyze glycosides into aglycones and extensively metabolize aglycones into various aromatic acids. The majority of these acids are further metabolized to derivatives of benzoic acid and, finally, into hippuric acid (Moco et al., 2012). These microbial metabolites are absorbed and conjugated with glycine, glucuronic acid or sulfate and may have an important physiological effect. Plasma polyphenol microbial metabolites are generally much more abundant than the native intact PC from the diet and are proposed to exert systemic and beneficial effects on cardiovascular health (van Dorsten et al., 2010). However, great inter-individual variability exists regarding the capacity to produce active metabolites by the gut microbiota and depends on the colonic microbial population composition,

which, in turn, can be modulated by phenol-rich foods (Cardona et al., 2013; Moco et al., 2012).

Extensive literature based on *in vitro* and *in vivo* studies assess the health effects of certain PC subgroups, phenol-rich foods, and total PC intake on cardiovascular risk biomarkers. In the PREDIMED trial, a significant reduction of cardiovascular events and cardiovascular mortality has been shown with a higher total PC intake. Specifically, lignans, flavanols and hydroxybenzoic acids were the PC subgroups with greater involvement in this inverse association (Tresserra-Rimbau et al., 2014). PC have a wide range of biological activities beyond their direct antioxidant activity, including anti-allergenic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio protective and vasodilatory effects. Various mechanisms have been proposed to explain their biological activity, including signal transduction regulation and redox-sensitive transcription factors modulation (Hollman et al., 2011; Stevenson et al., 2007).

2.2 FOODS AND FOOD GROUPS

Diets are composed of foods rather than single bioactive compounds or macronutrients, and thus, dietary recommendations using foods are more likely to be understood and adopted by the population. Therefore, specific foods and food groups have been studied to assess their effect on CVD risk. We briefly explain some of the food groups in which beneficial effects on CVD risk have been studied. Those foods studied during the development of this thesis are specifically mentioned.

2.2.1 Fruits and vegetables

Observational studies have shown a protective effect of fruit and vegetable consumption on CVD prevention. The DASH trial has shown that increasing fruit and vegetable intake contributes to a decrease in blood pressure (Kwan et al., 2013; Perk et al., 2012). The protective effects of fruits and vegetables are because these foods are a major source of potassium, fiber, vitamins and antioxidants, such as PC.

Berries are specific types of fruits particularly abundant in PC, of which anthocyanidins are the most occurrence subgroup. The PC in berries are responsible for the many reported health benefits of these fruits. Berries have been reported to lower blood glucose, to have anti-inflammatory and lipid-lowering effects, to promote antioxidant defense and to lower oxidative stress (Chu et al., 2011).

2.2.2 Alcoholic beverages

Results from epidemiological studies have shown a protective effect of moderate alcohol consumption on CVD occurrence (Perk et al., 2012). The alcoholic beverage most studied because of its healthy properties is red wine; however, the interest in the study of beer is increasing. The protective cardiovascular effects of these fermented beverages are primarily related to their non-alcoholic fraction and, above all, to their phenolic composition; nevertheless, their alcoholic fraction is also related to protection against CVD (Arranz et al., 2012; Chiva-Blanch et al., 2014). A recent study demonstrated that resveratrol, which is the most important PC of wine, may promote health by maintaining and enhancing vasculature plasticity (Thompson et al., 2014).

2.2.3 Whole grain

Epidemiological evidence suggests that whole grain consumption lowers CVD risk. Whole grains present numerous bioactive components with healthy enhancing properties, such as fiber, magnesium, inulin, β-glucan, resistant starch, carotenoids, tocopherols, tocotrienols, betaines, PC and alkylresorcinols (Fardet, 2010; Ye et al., 2012). Many protective physiological mechanisms may be associated with whole grain cereal consumption because of the high number of protective bioactive compounds present (Fardet, 2010). Alkylresorcinols are plant-derived phenolic lipids, present in high concentrations in the outer layers of wheat, rye and barley grains, which were reported to show healthy biological activities (Ross et al.,

2010). Whole grains are the major dietary sources of betaine, which is a methyl donor that is involved in CVD protection (Fardet, 2010).

2.2.4 Fish

The evidence for the beneficial effects of fatty fish consumption on CVD risk is strong, particularly because of its n-3 polyunsaturated fatty acids content (PUFA; Raatz et al., 2013). Eicosapentaenoic fatty acids and docosahexaenoic fatty acids are representatives of the n-3 PUFA group. Current data provide evidence that n-3 PUFAs are bioactive fatty acids that reduce the risk of cardiac death by affecting molecular pathways and by regulating genes that are critical for controlling lipid homeostasis (Eftekhari et al., 2014; Mozaffarian et al., 2011). A meta-analysis showed that eating fish 2-4 times a week reduced the risk of stroke by 18% compared with eating fish less that once a month. A modest increase in fish consumption of 1-2 servings a week has been shown to reduce CVD mortality by 36% and all-cause mortality by 17% (Perk et al., 2012).

2.2.5 Olive oil

Olive oil (OO) is one of the most characteristic features of the MD and its consumption has been associated with beneficial effects on human health. Many of the health benefits reported for olives and for OO are thought to be associated with their phenolic contents and particularly with their hydroxytyrosol levels (Charoenprasert et al., 2012). The phenolic content of virgin OO is influenced by the variety, location, degree of ripeness and the type of oil extraction procedure. Hydroxytyrosol is one of the major PC present in virgin OO and shows a variety of pharmacological activities, such as antioxidant properties; anticancer, anti-inflammatory, neuroprotective activities; and beneficial effects on the cardiovascular system, demonstrating its potential for the development of dietary supplements (Hu et al., 2014). Recently, the European Food Safety Authority (EFSA) released a health claim concerning the protective effects of the ingestion of PC from OO on plasma lipid levels, oxidative damage, blood pressure and inflammatory

state. To support this claim, the EFSA panel recommends the daily consumption of 5 mg of hydroxytyrosol and its derivatives in OOs (European Food Safety Authority, 2011). However, the phenolic concentrations in most commercially available virgin olive oils (VOOs) are too low to allow the regular consumption of these amounts of hydroxytyrosol. Therefore, the enrichment of VOO with its own PC could be a possible strategy to increase and to standardize the daily intake of hydroxytyrosol without increasing the caloric intake (Rubió et al., 2012; Suárez et al., 2011). Moreover, the enrichment of VOOs with PC of aromatic herbs, such as thyme, is also used for improving the nutritional profile and sensorial characteristics of VOO (Rubió et al., 2012).

In addition, other health claims not related to OO PC are currently possible for OO, such as monounsaturated fatty acids (MUFAs) and vitamin E. MUFAs replace saturated fats in the diet and contribute to the maintenance of normal blood cholesterol levels. Elsewhere, vitamin E contributes to cellular protection against oxidative stress (Martín-Peláez et al., 2013).

2.2.6 Legumes and Nuts

Legumes and nuts are foods present in the MD that are rich in several bioactive compounds. Legumes are rich in lecithin, phytoestrogens, oligosaccharides and PC, which play cardiometabolic roles in humans (Bouchenak et al., 2013). There is convincing evidence that increasing the consumption of legumes confers cardioprotective benefits (Ros & et al., 2013).

Additionally, nuts are rich in unsaturated fatty acids, fiber, vitamins, minerals, phytoesterols and PC, all of which are related to CVD protective activity (Kris-Etherton et al., 2008). Observational studies and clinical trials have suggested that nut intake is beneficial for preventing CVD and has beneficial effects on intermediate CVD risk factors such as blood pressure, or total cholesterol (Sabaté et al., 2010). Consistent with these studies, in vitro studies performed by our group showed that a polyphenol-rich peanut

extract reduces extracellular TNF-α, suggesting anti-inflammatory effects (Catalán et al., 2012). Recently, the frequency of nut consumption was shown to be inversely associated with total and cause-specific mortality, independent of other predictors of death (Bao et al., 2013; Ruiz-Canela et al., 2014).

2.2.7 Coffee, tea and cocoa

Coffee and tea represent the most consumed non-alcoholic beverages worldwide and are important dietary sources of PC. Tea consumption may have beneficial effects on endothelial function and on total and LDL cholesterol. Although the regular consumption of moderate quantities of coffee appears to be associated with a weak protection against CVD, biological mechanisms by which this consumption might provide cardiovascular health benefits remain unclear (Larsson, 2014).

Cocoa beans and derived products, such as chocolate, contain different types of physiologically active compounds including PC such as catechins, epicatechins and procyanidins (Di Castelnuovo et al., 2012). In recent years, cocoa products have been shown to reduce inflammation, to decrease blood pressure and to increase endothelial function and insulin sensitivity (Larsson, 2014).

2.3 DIETARY PATTERNS

Corresponding to the change from evaluating and treating single risk factors to evaluating a person's total risk profile, many studies focus on dietary patterns instead of on single bioactive compounds. Studying the effect of a total dietary pattern theoretically shows the full preventive potential of a diet because this result yields a combined estimate of the effect of several favorable dietary habits. The benefits of the following two dietary patterns on CVD risk have been studied in this thesis: Mediterranean diet and healthy Nordic diet.

2.3.1 Mediterranean Diet

The MD is based on dietary patterns typical of Crete and other areas of Greece and southern Italy in the early 1960s, when the adult life expectancy of this region was among the highest in the world and when rates of coronary heart disease, certain cancers, and other diet-related chronic diseases were among the lowest (Keys et al., 1986). Thus, in 1986, Ancel Keys established the MD as the diet traditionally eaten by populations living in southern Europe and as a health protecting diet. Subsequently, in 1993, Willet presented the MD as a food pyramid reflecting the beneficial Mediterranean dietary traditions (Willett et al., 1995). Recently, the MD pyramid has been adapted to current lifestyles (Bach-Faig et al., 2011). Since these early observations concerning the MD, there have been many studies indicating a link between adherence to the MD and a reduced risk of overall mortality and of CVD (Tresserra-Rimbau et al., 2014). The MD was recommended by the United Nations Education, Scientific and Cultural Organization (UNESCO 2010) as a nutritional prototype of worldwide value. The MD exerts significant anticancer, cardioprotective, and anti-inflammatory activities, which are responsible for lower mortality from coronary heart disease and from cancer (Hu et al., 2014).

The MD is characterized by the following: 1) a high consumption of vegetables, legumes, fruit, and cereals; 2) a regular, but moderate, wine intake; 3) a moderate consumption of fish and white meat; 4) a moderate intake of dairy products; 5) a low consumption of red meat; and 6) a relatively high-fat consumption (up to 40% of total energy intake), primarily from MUFAs (up to 20% of energy) primarily provided by OO, which is the principal source of culinary fat and one of the primary properties of the MD (Martín-Peláez et al., 2013).

The MD is extremely rich in bioactive compounds, particularly in PC, which provide the major beneficial health effects related to this dietary pattern (Tresserra-Rimbau et al., 2014).

2.3.2 Healthy Nordic Diet

Because the Mediterranean dietary pattern has been associated with a decreased incidence of chronic diseases, the MD has been recommended as a better dietary alternative for populations in many other Western countries. Despite the wide promotion of the Mediterranean dietary pattern, the adherence to this diet remains low outside its traditional geographic regions. Regional and cultural differences limit adherence to food-based diets transferred between populations. Dietary habits vary greatly in different parts of Europe, and to be successful, dietary guidelines must be sensitive to the local food culture. Correspondingly, populations of northern countries have difficulties in integrating the MD into their families' eating habits. Traditionally, the Nordic diet has been and, to some extent, remains characterized by food items with positive nutritional profiles, and it can be easily adopted by the Nordic population. These traditional Nordic food items can, to some extent, be characterized by their origin from Nordic nature (Olsen et al., 2011). A healthy Nordic diet, is based on regional foods in season and contains rapeseed oil, whole grain, high-fiber cereal products, berries, root vegetables, fatty fish and low-fat dairy products (Roswall et al., 2014). The adherence to a healthy Nordic dietary pattern has been associated with lower mortality and has shown beneficial effects on lipid profile, low-grade inflammation, insulin sensitivity, blood pressure and, therefore, benefits against CVD (Olsen et al., 2011).

CHAPTER 3: BIOMARKERS

3.1 DEFINITION AND CLASSIFICATION OF BIOMARKERS

In 2001, the National Institutes of Health committee standardized the definition of a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group 2001). This definition includes not only circulating molecular biomarkers (in serum, plasma, urine, etc.) but also genetic, tissue, or cellular markers, imaging results, and physical or electronic measurements. However, circulating molecular biomarkers are the most attractive because these biomarkers are easy to obtain and generally reproducible (Ge et al., 2012).

Biomarkers are potentially useful along the entire spectrum of the disease process. As shown in **Figure 6**, biomarkers can be classified into five categories based on their application in different disease stages (Chen et al., 2011):

1. Antecedent biomarkers: to identify the risk of developing an illness.
2. Screening biomarkers: to screen for subclinical disease.
3. Diagnostic biomarkers: to recognize overt disease.
4. Staging biomarkers: to categorize disease severity.
5. Prognostic biomarkers: to predict future disease course, including recurrence, response to therapy, and monitoring efficacy of therapy.

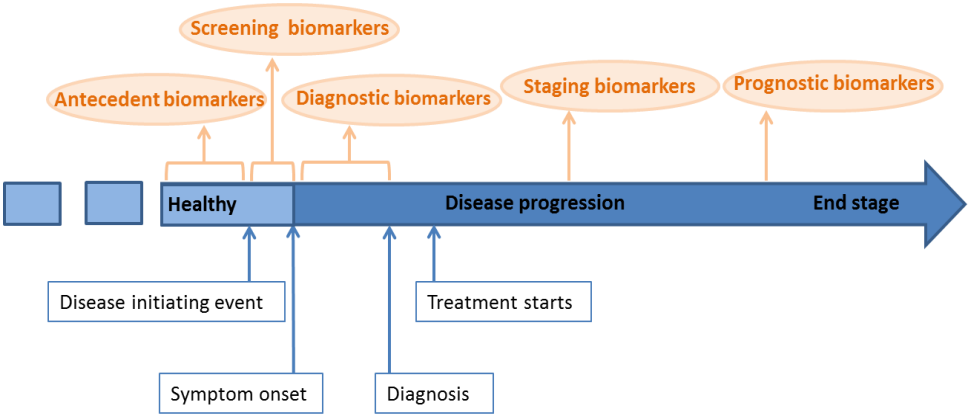

Figure 6. Biomarkers during disease evolution. Biomarkers can act at different levels of disease stages and, accordingly, can be classified as follows: antecedent biomarkers, screening biomarkers, diagnostic biomarkers, staging biomarkers and prognostic biomarkers.

Biomarkers can indicate a variety of health or disease characteristics, including the level and type of exposure to an environmental factor (such as chemicals or nutrients), genetic susceptibility, genetic responses to environmental exposures, markers of subclinical or clinical disease, or indicators of response to therapy (Chen et al., 2011).

Biomarkers have gained importance and interest in not only basic and clinical research but also in clinical practice (Strimbu et al., 2010). Technological advances in genomics, transcriptomics, proteomics and metabolomics has led to an increasing number of candidate biomarkers with potential clinical value. The critical evaluation of the multitude of proposed biomarkers is important for identifying those candidates with the strongest, most accurate, and reliable information. Accordingly, standards for the assessment of novel biomarkers have been proposed to prove clinical usefulness. These standards include the quality of the assay (precision, availability, and costs), the added value of the novel information in comparison with established methods, and the quantifiable value to support clinical decision-making (**Table 3**).

Table 3. Phases of evaluating a novel biomarker.

1 Proof of concept	Do novel marker levels differ between subjects with and without disease?
2 Prospective validation	Does the novel marker predict disease development in a prospective cohort or case-cohort study?
3 Incremental value	Does the novel marker add predictive information to the established risk marker?
4 Clinical utility	Does the novel marker change predicted risk sufficiently to change the recommended therapy?
5 Clinical outcome	Does using the novel marker improve clinical outcomes, particularly when tested in a randomized clinical trial?
6 Cost-effectiveness	Does the novel maker improve clinical outcomes sufficiently to justify the additional costs of testing and treatment?

Source: (Doehner, 2012).

In the future, the integration of biomarkers, identified using emerging high-throughput technologies, into medical practice will be required to achieve personalized treatment and disease prevention (Doehner, 2012).

3.2 BIOMARKERS FOR CARDIOVASCULAR DISEASE

As we previously mentioned in Chapter 2, the well-established risk factors for CVDs are not sufficient to identify all patients at risk. Certainly, not all patients with CVDs will have traditional risk factors and not all patients with risk factors will develop CVDs. Thus, the development of new circulating molecular biomarkers to diagnose and to prevent CVDs is an important public health goal worldwide. Cardiovascular biomarkers have the potential to augment clinical risk stratification by assisting in screening, diagnosing and assessing the prognosis of patients with and without known CVD risk (May et al., 2008).

Approaches to cardiovascular disease biomarker discovery

Two different complementary approaches can be used to discover new molecular biomarkers for CVDs. The first is a targeted approach based on

knowledge regarding underlying pathophysiology (deductive method), and the second is an unbiased approach (inductive strategy; May et al., 2008).

The knowledge-based strategy relies on a direct understanding of the biological processes that cause the development of atherosclerosis and its evolution. Targeted searches for CVD biomarkers are characterized by an important focus on molecules from pathways implicated in atherosclerosis, including mediators of inflammation, oxidative stress, endothelial function and components of the coagulation cascade (May et al., 2008; Vasan, 2006). A long list of molecular biomarkers related to these key mechanisms involved in the onset and progression of atherosclerosis has been discovered. Among these biomarkers, 8-hydroxydeoxyguanosine (8-OHdG) and oxLDL are commonly used as biomarkers of oxidative stress (Valavanidis et al., 2009) and high-sensitivity C-reactive protein (hsCRP) is used as an inflammatory biomarker that has been evaluated as potential tool for predicting cardiovascular events (Perk et al., 2012). Moreover, VCAM-1 and E-selectin are common biomarkers for endothelial dysfunction (Paulus et al., 2011) and homocysteine and plasminogen activator inhibitor-1 (PAI-1) play important roles as thrombotic biomarkers (Aispuru et al., 2012; Perk et al., 2012).

The unbiased approach involves searching through thousands of molecules using current technological advances to characterize the biomolecular profile of a disease stage. The use of platforms such as genomics, transcriptomics, proteomics and metabolomics has been indicated as an ideal method to identify new biomarkers in an “unbiased” manner because these platforms do not depend on preexisting knowledge of candidate pathways (May et al., 2008). These omic sciences are used for identifying putative CVD biomarkers that may be informative concerning various stages of atherogenesis, including overt CVD and its outcome. Technological advances will likely facilitate the use of multimarker profiling to individualize CVD treatments in the future (Vasan, 2006).

3.3 NUTRITIONAL BIOMARKERS

Nutritional biomarkers are another type of biomarkers not implicated in any disease stage. Research interest in these biomarkers has increased in recent years to reflect exposure to food intake.

Reliable dietary assessment methods are crucial when attempting to understand the links between specific foods or bioactive compound consumption and the risk of chronic diseases, such as CVDs. In nutritional studies, collecting valid dietary intake data remains one of the primary weaknesses of this type of research. Traditional methods of self-reported dietary intake assessment, such as food diaries, food frequency questionnaires or 24-hours recalls, present numerous challenges to obtaining accurate dietary intake and nutritional status data. This limitation can be overcome by the use of nutritional biomarkers, also called biomarkers of dietary intake, which are able to objectively assess dietary consumption without the bias of self-reported dietary intake errors (O'Sullivan et al., 2011).

Biomarkers of dietary intake are desirable because of their ability to more accurately assess nutritional intake or status versus self-reported methods. Dietary biomarkers can be used to validate self-reported intake measures, to assess the consumption of dietary items when food composition databases are inadequate, and to accurately associate dietary intake with disease risk and nutritional status. Although dietary biomarkers generally provide a more objective measure of dietary intake, factors that may not be present in traditional dietary assessment methods could skew biomarker measures of dietary intake. Such factors could include genetic variability, lifestyle or physiologic factors (e.g., smoking), dietary factors (e.g., nutrient-nutrient interaction), biological sample and analytical methodology (Hedrick et al., 2012). Therefore, the following are criteria that a nutritional biomarker should satisfy to validate its usefulness as a dietary biomarker in nutrition studies (Puiggròs et al., 2011):

1. Robustness of quantification and identification across diverse populations. Therefore, sensitive and specific techniques should be available to accurately quantify the biomarker of interest, which has been appropriately collected and stored to ensure minimum biomarker degradation.
2. Concentrations of the biomarker of interest should be sensitive to changes in the intake of the dietary component of interest.
3. High specificity. Variation in biomarker concentration should be due to changes in the intake of the dietary component of interest only.
4. Comprehension of the effect of physiological factors and whole diet composition on the absorption, metabolism and excretion kinetics of the putative biomarker.

Depending on its use, a nutritional biomarker should reflect recent intake (reflects compliance in shorter-term studies) or should reflect intake over a longer period (useful for dietary assessment in epidemiological studies or as a compliance marker in longer-term interventions). Hence, it is important to select an appropriate biomarker considering the aim of the study (Puiggròs et al., 2011).

In recent years, **metabolomics** has shown its utility for dietary assessment and for the identification of biomarkers of specific dietary intake and, consequently, its relation with health status (O'Sullivan et al., 2011). The emerging field of metabolomics may help to advance the development of nutritional biomarkers. The availability of biomarkers that estimate the intake of specific foods and dietary components could greatly enhance nutritional research targeting compliance to national recommendations for the population, as well as direct associations with disease outcomes. More research is required to refine existing biomarkers, to establish new indicators of specific food intake, and to develop techniques that are cost-effective, noninvasive, rapid and accurate measures of nutritional status.

During this thesis, we have worked with both types of biomarkers, circulating molecular biomarkers related to CVDs and nutritional biomarkers.

CHAPTER 4: OMIC TECHNOLOGIES

4.1 OMIC TECHNOLOGIES OVERVIEW

The growth of molecular biology studies, based on omic technologies has widely and rapidly expanded during recent decades. The concept beyond omic approaches is that a given biological system can be best determined and understood by considering this system in its globality rather than by studying its components individually (Rüegg et al., 2008). New high-throughput omic technologies (such as genomics, transcriptomics, proteomics and metabolomics) are allowing detailed studies of molecular changes during CVD onset and progression and also during dietary interventions (**Figure 7**). The use of these advanced methods will lead to the detection of new disease and nutritional biomarkers, which will provide a dynamic and powerful approach for researchers to understand CVD biology and the effects of the diet on CVD onset and progression. Moreover, these methods will have significant applications in analytic epidemiology, clinical trials and prevention, diagnosis, and management of disease (Barallobre-Barreiro et al., 2013).

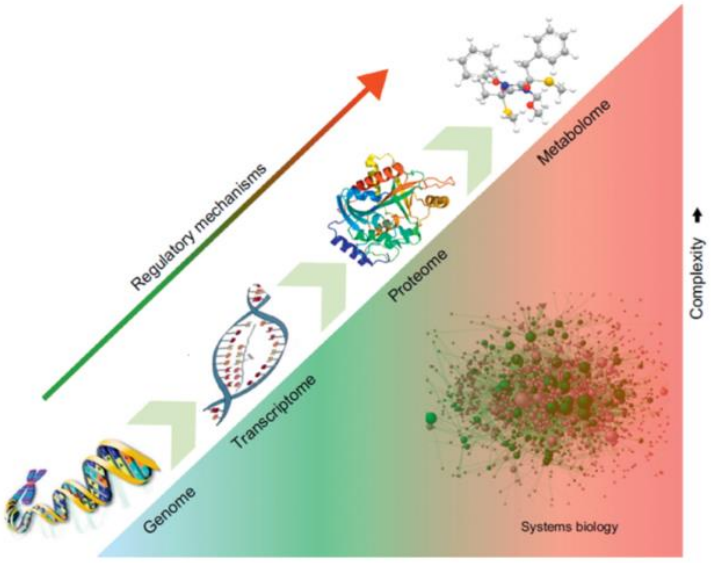

Figure 7. Systems biology. Regulatory processes at the DNA level affect the expression of

downstream molecules, including RNAs, proteins, and metabolites. The effects of the different regulatory elements are additive. Systems biology attempts to analyze the interactions among the different molecular entities to offer a holistic view of biological processes and pathological changes occurring in disease. Source: (Barallobre-Barreiro et al., 2013).

All omic studies have shown a typical scheme that has been broadly categorized into the following five primary steps: study design, sample collection and preparation, data acquisition, data analysis and data mining, and biological interpretation. An omic workflow is shown in **Figure 8**. Omic studies generate high-dimensional data sets, complicating the analysis and requiring the massive use of mathematical, statistical and computational efforts (bioinformatics) and the development of novel analytical tools (Rüegg et al., 2008). Data processing is an important step in the data mining process from any omic study because this step prevents the presence of irrelevant and redundant information, noise and unreliable data that could mislead biological interpretation (Ibáñez et al., 2013).

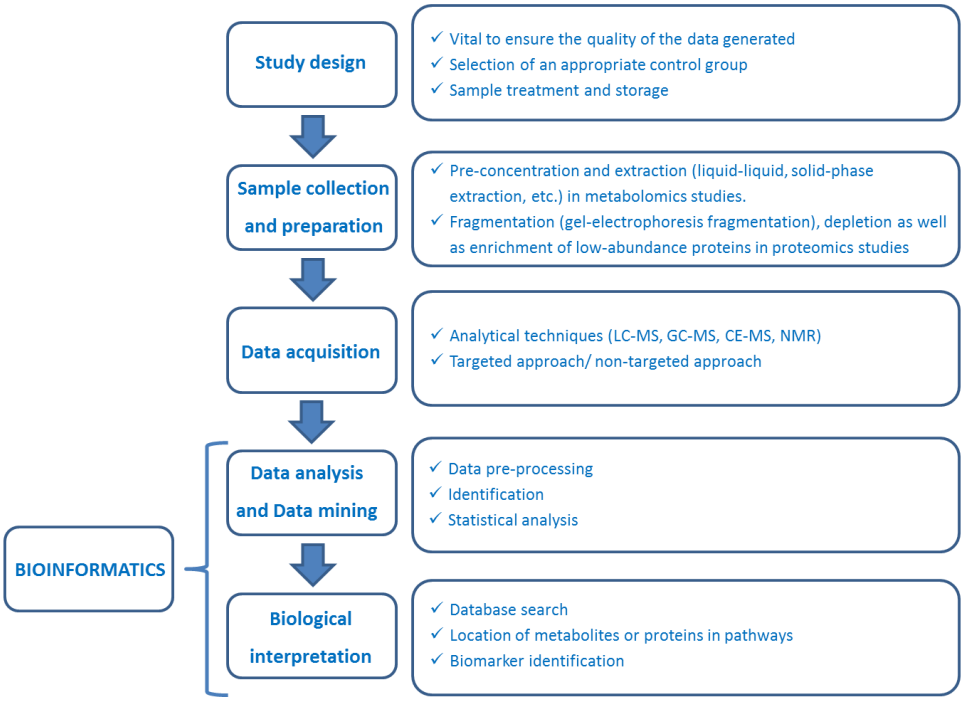

Figure 8. Omic workflow scheme. Source: (adapted from Llorach et al., 2012).

4.2 FOODOMICS CONCEPT

Foodomics has been defined as a new discipline that studies food and nutrition domains through applying advanced omic technologies to improve consumers' well-being, health, and confidence (Cifuentes, 2009). Foodomics is proposed as a global discipline that includes all of the emerging working areas in which food (including nutrition), advanced analytical techniques (primarily omics tools), and bioinformatics are combined (Herrero et al., 2010). Foodomics covers the development of new investigations of biomarkers discovery and of food bioactivity and its effect on human health (Herrero et al., 2012). The use of omic technologies is a requirement in this new discipline. By using this global strategy identifying all the small changes induced by bioactive food ingredients on a given system (cell, tissue, organ or organism) at different expression levels should be possible (Ibáñez et al., 2013). An ideal foodomics platform scheme is shown in **Figure 9**.

The importance of food for human health is not a new concept; however, currently, we are not simply thinking that a good diet is important for health, but we can demonstrate this fact, indicating the mechanisms underlying these health effects (Capozzi et al., 2013).

In the last decade, proteomics and metabolomics have contributed substantially to our understanding of CVDs and to nutritional biomarker discovery (Griffin et al., 2011). Combining proteomics and metabolomics to quantify changes in metabolites and proteins will advance our understanding of pathophysiological mechanisms for CVDs and the effects of diet on CVD onset and progression (Barallobre-Barreiro et al., 2013).

Metabolomics and proteomics are the omic sciences used for the realization of this thesis. Thus, these omic approaches and their applications in nutrition and CVD research are explained below in greater detail.

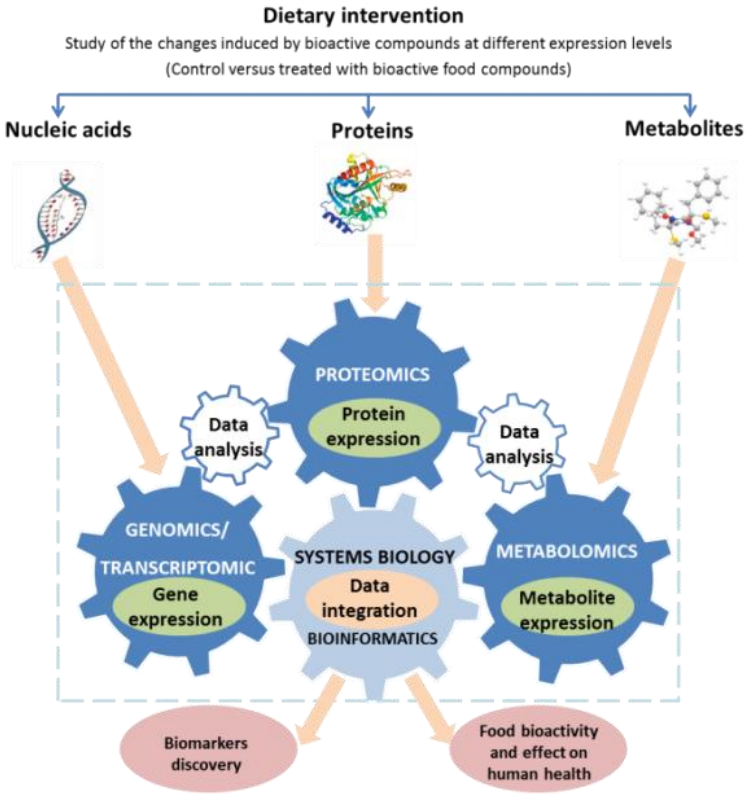

Figure 9: **Scheme of an ideal foodomics platform to investigate the health benefits of dietary constituents on a given biological system**. Source: (adapted from Ibáñez et al., 2012).

4.3 METABOLOMICS OVERVIEW

The Metabolomics Society defines metabolomics as a newly emerging field of omics research concerned with the comprehensive characterization of the small molecule metabolites in biological systems that can provide an overview of the metabolic status and global biochemical events associated with a cellular or biological system (Mishur et al., 2012; Theodoridis et al., 2012).

The term metabolome has been defined as the complete set of metabolites or chemicals that can be found in a cell, organ, or organism. In general, metabolites are the final downstream products of the genome most closely

reflecting the operation of the biological system and its phenotype (Griffin, 2004). A metabolite is defined as a molecule smaller than 1 kDa. However, these small molecules vary significantly in terms of their molecule weights, concentrations, polarity, solubility and volatility (Samuelsson et al., 2008). Two factors that influence the metabolome have been described: the endogenous metabolome, which refers to intrinsic metabolites involved in or resulting from metabolism formed under directly derived cell genome/proteome control, and the exogenous metabolome, which involves all metabolites directly derived from extrinsic factors such as diet, drugs, toxics and metabolites produced by the colonic flora (Llorach et al., 2012). Bioactive compounds consumption will induce changes in the endogenous metabolome, and these effects may be caused by the presence of many exogenous metabolites resulting from the digestion of food. These exogenous metabolites can be useful as biomarkers of dietary intake (Llorach et al., 2012).

Two analytical platforms are currently used for metabolomics analyses: mass spectrometry (MS) and nuclear magnetic resonance (NMR) -based systems. These techniques stand alone or are combined with separation techniques, such as liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE). Both MS- and NMR-based technologies are complementary and, therefore, often used in parallel in metabolomics research. Compared with NMR, MS is a more-sensitive technique. Additionally, MS coupled to GC, LC, or CE allows higher resolution and sensitivity for low-abundance metabolites. Thus, the number of MS-based metabolomics studies has grown quickly and now exceeds that of NMR-based studies (Herrero et al., 2012). The platform for MS analysis consists of different units, including the separation unit, which allows the separation of highly complex mixtures of analytes into single components before MS analysis; the ionization unit, which creates charged particles; the mass analyzer unit, which resolves ions formed in the ionization unit according to their mass to charge ratio (m/z); the detector unit, which measures the

electrical signal coming from the mass analyzer; and the analysis unit, which records and coverts signals into MS spectra (Napoli et al., 2013). The use of high and ultra-high resolution analyzers, such as, time of flight (TOF), Fourier transform mass spectrometry (FTMS), or orbitrap, is essential for obtaining accurate mass measurements for determining the elemental composition of metabolites (Herrero et al., 2012).

Metabolomics is an interesting tool for assessing the biological consequences of following a nutritional intervention and for assessing food consumption. In nutritional interventions, metabolome analysis focuses on identifying new biomarkers that allow the intake of certain dietary compounds to be monitored and related to their expected biological effect, as described in **Figure 10** (Llorach et al., 2012).

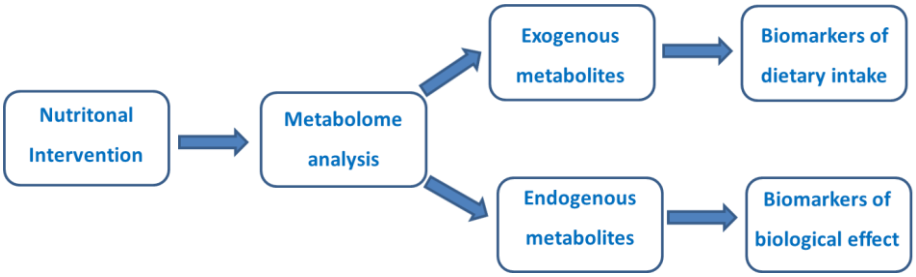

Figure 10. Metabolomic approach in nutritional interventions. Assessment of nutritional and dietary interventions. Source: (adapted from Llorach et al., 2012).

Two different basic approaches can be used in metabolomic studies: a targeted approach and a non-targeted profiling approach. Targeted metabolomics refers to a method in which a specified list of metabolites is measured, typically focusing on one or more related pathways of interest. Non-targeted metabolomics aim to simultaneously measure as many metabolites as possible from biological samples and to compare between samples without bias (Llorach et al., 2012).

Targeted analyses of pre-determined sets of clinical biomarkers after the consumption of specific health promoting nutrients, foods or diets can

provide important mechanistic information; however, these analyses fail to provide a larger picture of the altered metabolic status resulting from a variety of interactions between food constituents and endogenous metabolites (Brennan, 2013). Non-targeted metabolite profiling utilizing sensitive and high-throughput analytical technologies offers a wide window for monitoring changes in diet-derived and endogenous metabolite levels, thus aiding in identifying of novel biomarkers for both dietary intake and biological effects (O'Sullivan et al., 2011; Wishart, 2008). Metabolic profiling technologies provide a global overview of complex dietary processes, and this overview has generated novel insights into the molecular mechanisms that shape the relation between diet and CVD risk (Kinross et al., 2014).

Diet is an important modulator of the human metabolic phenotype, and the analysis of the nutritional metabolome will drive the future development of personalized nutritional interventions (Kinross et al., 2014).

4.4 PROTEOMICS OVERVIEW

Proteomics is the global analysis of the gene expression, function, and interaction of the complement of proteins in an organism (Arab et al., 2006). Recent advances in proteomic technologies permit the evaluation of systematic changes in protein expression in response to intrinsic or extrinsic perturbations to the biological system, for example, those perturbations that occur in CVDs or that occur after a dietary intervention. The proteome encompasses the entire set of proteins expressed by a cell, tissue or organism, including their posttranslational modifications (Barallobre-Barreiro et al., 2013).

The increasing popularity of proteomics has created a requirement for quantitative analysis methods. Thus, many different techniques are now available for performing gel-based or gel-free quantitative proteomics (Bruce et al., 2013). These techniques provide insight into global protein expression, from identification to quantification. MS is used in these strategies as the last analytical step for peptide detection and for protein

identification. Currently, MS has become fundamental in proteomic studies and represents the most-powerful tool in proteomics because MS requires no prior knowledge of the proteins to be identified (Bruce et al., 2013). MS also allow the analysis of proteins and peptides in large-scale and high-throughput modes. Improved mass spectrometers with better sensitivity and with superior mass accuracy and resolution aim to identify and to quantify complex protein (peptide) mixtures in a single experiment. As we previously mentioned in the metabolomics overview section, the versatility of MS technologies involves numerous different mass spectrometers, with matrix-assisted laser desorption/ionization (MALDI)-TOF-TOF, quadrupole-TOF, and orbitrap mass analyzers among the common technologies currently in use for discovery proteomics (Herrero et al., 2012; Langley et al., 2013). MS is not inherently quantitative because of differences in ionization efficiency. The most abundant ions will attract the most charges during electrospray ionizations, making it less likely for low-level peptides to be ionized. To avoid false-positive protein changes due to co-eluting high-abundant peptides, labeling techniques should be used for reliable quantification. Popular labeling methods in humans include: isobaric tagging for relative and absolute quantification (iTRAQ) and tandem mass tags (TMT; Langley et al., 2013).

Two primary workflows exist in proteomic studies using MS: top-down and bottom-up procedures (Napoli et al., 2013). In top-down procedures, intact proteins are pushed into MS-coupled systems, and important data for protein characterization are provided, such as molecular mass, post-translational modifications, and isoforms. In bottom-up procedures, proteins are purified, cut into fragments by chemicals or enzymes and analyzed using MS technologies (Napoli et al., 2013).

Proteomic studies can be performed by either global proteomics approaches (non-targeted approach), involving the analysis of many proteins, or the selection and analysis of specific types of proteins (targeted approach).

Each approach involves choosing the most appropriate experimental strategies and technologies (Napoli et al., 2013).

Proteomic analysis provides an opportunity to understand novel pathophysiological mechanisms of CVDs in a non-biased manner. New proteomic tools, with systems biology approaches, have the potential to unravel greater complexity in previously unexplored signaling networks, leading to improved biological understanding of CVD onset and progression (Bruce et al., 2013). Proteomic analysis permits the development of a suit of putative biomarkers for the increased precision in diagnosis, identification of susceptibility to complications, and prognosis personalized to individuals and subclassification of disease by pathophysiological manifestations (Bruce et al., 2013). Proteomics tools also allow the identification of novel therapeutic targets (Arab et al., 2006; Barallobre-Barreiro et al., 2013).

From a foodomics point of view, comparative proteomics strategies are primarily used to obtain an insight on the effect of specific bioactive compounds, or diet on the proteome of organisms, tissues, or cells. It is interesting to mention that, currently, limited studies remain concerning the effects of specific bioactive compounds, or diets on the human proteome (Herrero et al., 2012). Proteomics is a key tool for exploring the molecular mechanisms involved in the cardioprotective activities of bioactive compounds.

In combinations with other omic technologies, such as transcriptomics and metabolomics, proteomics searches different aspects of disease, and the different pillars of observations facilitate data integration in disease-specific networks. Finally, a systems biology approach may advance our understanding of CVD processes at the biological pathway level instead of the single molecule level and accelerate progress toward modifying interventions for disease prevention (Langley et al., 2013).

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

Our hypothesis is that the intake of certain dietary bioactive compounds of the Mediterranean and Nordic diets can be assessed and monitored by nutritional biomarkers, improving the accuracy of the dietary intake measure. Moreover, these dietary bioactive compounds could be associated with an important diversity of mechanisms of action and with healthy biological effects on molecular biomarkers involved in the development of CVDs, in both healthy and CVD risk subjects.

OBJECTIVES

The primary objective is to assess the effect of Mediterranean and Nordic dietary bioactive compounds, particularly phenolic compounds, on nutritional and molecular biomarkers, which allow mechanisms of action and biological effects involved in the development of CVDs to be determined.

The following specific objectives have been set:

Objective 1: To assess the relations between urinary total polyphenol excretion (TPE), which is a biomarker of total polyphenol intake (TPI), polyphenol-rich foods, and oxidative stress biomarkers in healthy adults of different ages following a MD pattern (**Clinical Study 1**).

Objective 2: To assess the modifications of plasma metabolite profiles after a healthy Nordic dietary intervention with whole grains, bilberries and fatty fish by applying a non-targeted metabolite profiling approach in subjects with metabolic syndrome. Furthermore, we aimed to identify novel nutritional biomarkers more specific than TPE, and to determine molecular biomarkers of endogenous metabolism modified by dietary bioactive compounds **(Clinical Study 2)**.

Objective 3: To assess the impact of a dietary intervention supplemented with a virgin olive oil (VOO) or two different functional VOOs enriched with their own PC (hydroxytyrosol derivatives) or complemented with thyme PC

(flavonoids, monoterpens, and phenolic acids) on the HDL protein cargo and on its cardiovascular profile, by applying a quantitative proteomics approach in hypercholesterolemic subjects from the Mediterranean area **(Clinical Study 3)**.

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METHODS AND RESULTS

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STUDY 1

Polyphenol-rich foods exhibit DNA antioxidative properties and protect the glutathione system in healthy subjects

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RESEARCH ARTICLE

Polyphenol-rich foods exhibit DNA antioxidative properties and protect the glutathione system in healthy subjects

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Scope: Polyphenols (ingested via food items) can decrease DNA, and oxidative damage of proteins and lipids. However, polyphenol effects in healthy populations have not been well defined. The aim of this study was to assess the relationship between urinary total polyphenol excretion (TPE), a biomarker of total polyphenol intake (TPI), polyphenol-rich foods, and oxidative stress biomarkers in healthy adults of different ages participating in the cross-sectional PAScual MEDicina study.

Methods and results: Urinary TPE was determined by Folin–Ciocalteau method in spot urine samples of 81 participants (46 women), classified into three age groups: 18 to 39, 40 to 54, and 55 to 72 years of age. TPI was quantified from 3-day dietary records using the Phenol-Explorer database. Urinary TPE increased with age ($p < 0.001$). Urinary TPE was inversely associated with urinary 8-hydroxydeoxyguanosine (8-OHdG; $p<0.001$) and erythrocyte-oxidized glutathione concentrations ($p < 0.05$). A negative association between urinary 8-OHdG and daily intake of polyphenols from vegetables and fermented beverages such as red wine was observed.

Conclusion: Urinary TPE increased with age and may reflect attenuation of oxidative damage. These results could explain the beneficial effects in healthy individuals of a diet rich in vegetables and moderate red wine; food items typical of the Mediterranean diet.

Keywords:
DNA oxidation / Fruits / Urinary phenol excretion / Vegetables / Wine

1 Introduction

Numerous epidemiological studies have shown several important links between diet and the occurrence of chronic diseases. Evidence is available to link several nutrients, minerals, food groups, and dietary patterns with an increased, or decreased, risk of diseases [1]. Oxidative stress is involved in the pathogenesis of several chronic diseases [2]. Oxidative stress

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Abbreviations: **CAT**, catalase; **CVD**, cardiovascular disease; **FRAP**, ferric-reducing ability of plasma; **GAE**, gallic acid equivalent; **GPx**, glutathione peroxidase; **GSH**, reduced glutathione; **GSSG**, oxidized glutathione; **Nrf2**, nuclear factor erythroid 2-related 2; **8-OHdG**, 8-hydroxydeoxyguanosine excretion; **ORAC**, oxygen radical absorbance capacity; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **TBARS**, thiobarbituric acid reactive substances; **TPE**, total polyphenol excretion; **TPI**, total polyphenol intake

culminates due to an imbalance between prooxidants and antioxidants and a consequent excessive production of reactive oxygen species (ROS), which contribute to oxidative damage of lipids in cellular membranes as well as in proteins and DNA [3]. Endogenous antioxidant enzymes can prevent intracellular ROS concentrations from reaching levels at which damage occurs. Further, important exogenous dietary antioxidants such as some vitamins, selenium, or phytochemicals are also effective against oxidation and offer protection against oxidative damage and physiological benefit accrues [4]. However, the evidence from animal and in vitro studies to date is not sufficient to predict such an in vivo protective effect of food or food constituents in humans [5]. As such, the measurement of biomarkers of oxidative damage can be used to evaluate the effects of various dietary compounds, such as polyphenols.

Polyphenols are a heterogeneous group of naturally occurring molecules distributed in fruits, vegetables, nuts, seeds, wine, tea, cocoa, and coffee. Clinical and epidemiological studies provide evidence that polyphenol-rich foods and polyphenol-rich diets have protective effects against chronic diseases [6]. Polyphenols have a wide range of biological activities beyond their direct antioxidant activity. Various mechanisms have been proposed to explain their biological activity, including regulation of signal transduction and modulation of redox-sensitive transcription factors [7]. To establish the health-benefit effects of dietary polyphenol consumption, it is essential to have quantitative information regarding their dietary intake [8]. A realistic option is to assay total urinary phenols as a first approach to the study of the polyphenol intake-excretion balance. The data may, then, be related to measured physiological antioxidant phenomena and, hence, to the potential health benefits of dietary-derived polyphenols [8–10]. The 24 h urinary total polyphenol excretion (TPE) and spot urinary TPE have been validated in intervention studies as biomarkers of dietary polyphenol-rich food ingestion [8–10]. The level of protection provided by dietary polyphenols would differ, depending on the population at cardiovascular disease (CVD) risk. However, this has not been well defined in a healthy population nor has the urinary TPE been explored in relation to age.

We hypothesized that polyphenol-rich food consumption results in high levels of urinary TPE which, in turn, can be used as a marker of protection against oxidative damage conferred by polyphenol intake, in healthy participants of different ages. The objective of current study was to assess, in healthy human subjects segregated with respect to age, the relationships between urinary TPE, polyphenol-rich food intake, and biomarkers of oxidative stress.

2 Materials and methods

2.1 Subjects and design

The PAScual MEDicina (PASMED) study is a cross-sectional study aimed at defining the concentrations of circulating intermediate metabolites of various CVD risk biomarkers, and their distributions in healthy volunteers. The study was conducted in the Hospital Universitari Sant Joan de Reus (Spain) from March to July 2008 and from February to July 2009. Eligible subjects included men and women aged 18 to 75 years who were nonsmokers, with no other major CVD risk factors, not using any medications and/or vitamin supplements, and ostensibly healthy according to clinical history as well as physical and biochemical examination.

Participants provided written informed consent prior to enrolment into the trial and eligibility or exclusion was assessed by the attending physician, followed by a screening visit. The study protocol was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (08-01-31/proj1). The study protocol was in accordance with the Declaration of Helsinki of 1975 (revised 1983) and good clinical practice guidelines of the International Conference of Harmonization (ICH GCP).

2.2 Sample size

To calculate the sample size, we took as a reference the SD of LDL-cholesterol measurement. In our laboratory, this value is 21.47 mg/dL. The sample of 82 subjects provides an accuracy of ±4.67 mg/dL LDL-cholesterol assuming an alpha risk of 0.95. We included at least ten participants (five women and five men) within each arbitrary 10-year age group, i.e. 18 to 29, 30 to 39, 40 to 49, 50 to 59, 60 to 69, and >70 years of age.

2.3 Measurements

2.3.1 Clinical history

All participants completed a detailed clinical history to provide sociodemographic data; family and personal history of illness; use of medications; level of ultraviolet exposure; and psychological, socioeconomic, and occupational status.

2.3.2 Physical examination

All data were collected by trained study personnel. Height and weight were measured while participants were wearing lightweight clothing and no shoes, using a calibrated balance and a well-mounted stadiometer, respectively. Waist circumference was measured as the point between the last rib and the iliac crest, at the end of normal exhalation. With the subject seated, blood pressure was measured three times at 1-min intervals using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain) and the mean value used in the statistical analyses.

2.3.3 Dietary intake and physical activity

Participants were provided with a questionnaire incorporating a 3-day 24-h food record (two work days and a holiday or weekend) and the Minnesota Leisure Time Physical Activity

Questionnaire validated in Spanish [11]. Trained dieticians explained how to complete these questionnaires.

2.3.4 Collection of blood and urine sample

Fasting blood samples were collected by venipuncture and the plasma (or serum) used to determine standard biochemistry analytes, biomarkers of oxidative stress, lipid profile, inflammation, endothelial dysfunction, antithrombotic activity, and insulin resistance. To minimize variability of analysis, the blood samples and supernatants of centrifuged spot urine samples were stored at –80°C in the central laboratory's Biobanc-REUS-IISPV (http://bancmb@grupsagessa.com) and thawed just prior to batched analyses.

2.3.5 Oxidative stress biomarkers

The extent of oxidative injury and the antioxidant capacity of plasma and enzymatic antioxidants were analyzed. Plasma was used to measure oxidized LDL by a monoclonal antibody-based immunoassay kit (Mercodia AB, Uppsala, Sweden); 8-isoprostan was analyzed using a commercial ELISA kit (Cayman Chemical Company, Ann Arbor, MI); 8-hydroxydeoxyguanosine (8-OHdG) was measured in urine using a competitive enzyme-linked immunosorbent assay (New 8-OHdG check, Japan Institute for the Control of Aging, Shizuoka, Japan) [12]. Carbonyl groups were measured by binding to dinitrophenylhydrazine and subsequent spectrophotometry [13]. Ferric-reducing ability of plasma (FRAP) was measured as the ability of plasma to reduce Fe^{3+} to Fe^{2+}. The Fe^{2+} forms a colored complex with 2,4,6-tripyridyl-s-triazine and which is measured by spectrophotometry [14]. Oxygen radical absorbance capacity (ORAC) and trichloroacetic acid ORAC ($ORAC_{TCA}$; plasma without protein) were measured as the antioxidant's capacity to scavenge peroxyl radicals of 2,2′-azobis(2-amidinopropane induced by dihydrochloride. The reaction was measured by fluorimetry [15]. Reduced glutathione (GSH) and oxidized glutathione (GSSG) in erythrocytes and plasma were also measured using fluorimetry [15] as were the levels of malondialdehyde and malondialdehyde-like substances (measured as thiobarbituric acid reactive substances; TBARS) [17]. Serum vitamin C was measured using a fluorimetric technique with a commercial ascorbic acid kit (Biovision Inc. San Francisco Bay Area, CA). The method used for the measurement of superoxide dismutase (SOD) activity in erythrocytes was based on the auto-oxidation of epinephrine, the product of which was measured by spectrophotometry [18]. Glutathione peroxidase (GP_x) in erythrocytes and plasma was determined via a spectrophotometric method that monitors the rate at which $NADP^+$ or NADPH is converted by the butyl hydroperoxide oxidation of GSH to GSSG and its subsequent reduction to GSH by glutathione reductase [19]. Catalase (CAT) in erythrocytes was measured via a spectrophotometric method based on the rate of hydrogen peroxide breakdown [20].

2.3.6 Determination of total polyphenol concentration in urine sample

To avoid degradation in the measurement of TPE, the urine samples were thawed in ice-water over 3 h. The analyses were performed immediately with the Folin–Ciocalteau method using an Oasis® MAX 96-well plate cartridge for solid phase extraction, as described by Medina-Remón et al. [9]. TPE was expressed as milligram of gallic acid equivalent (GAE) per gram creatinine. Creatinine measurement was used to adjust for variations in analyte concentration in urine. For analysis of creatinine in urine samples, the Jaffé alkaline picrate method was adapted to 96-well thermomicrotitre plates [9, 21].

2.3.7 Determination of total polyphenol intake

Total polyphenol intake (TPI) was quantified according to Phenol-Explorer database (http://www.phenol-explorer.eu) using the 3-day dietary record maintained by each participant.

2.4 Statistical analysis

Analyses were performed by available data only. Data were expressed as the mean and SD for variables with normal distribution. The geometric mean and antilog SD were used to describe log-transformed variables with normal distribution. The median and interquartile ranges were used to describe log-transformed variables that had nonnormal distribution. The Kolmogorov–Smirnov test was used to verify the distributions of the variables. The Mann–Whitney and Kruskal–Wallis tests were used for comparison of nonpaired and nonnormally distributed samples. ANOVA and Student *t*-tests were used for comparison of nonpaired and normally distributed samples. Pearson correlation coefficients were calculated for relationships between TPI and urinary TPE, between TPI and polyphenol from plant foods, between oxidative stress biomarkers and urinary TPE, and between oxidative stress biomarkers and TPI. Stepwise multivariate linear regression analyses were used to assess relationships between TPI and polyphenols from plant foods, between urinary TPE and oxidative stress biomarkers, and between polyphenols from plant food and oxidative stress biomarkers. The level of statistical significance was set at $p < 0.05$. All data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (17.0 version; IBM Corp., Armonk, NY, USA).

3 Results

3.1 Characteristics of subjects

From 117 eligible volunteers, 82 were enrolled in the PASMED study and, finally, data from 81 were analyzed. The

35 participants excluded during the screening process were because they did not fulfill all the inclusion criteria. The one volunteer excluded after enrolment was because the blood sample was not available. Thus, 81 participants entered the study; 46 women with a mean (SD) age of 44.6 (15.8) years and 35 men aged 42.3 (16.4) years. Participants were ostensibly healthy with no known CVD risk factors.

We classified our subjects into three age groups (18 to 39 years, 40 to 54 years, and 55 to 72 years) to evaluate the influence of age on urinary TPE and on oxidative stress biomarkers. Table 1 summarizes the mean values of oxidative stress biomarkers segregated according to age. In our study sample, the values of plasma-oxidized LDL, plasma 8-isoprostan, erythrocyte GSH, plasma GSSG, and plasma GSSG/GSH ratio significantly increased with age; mainly between the 18- to 39-year and the 40- to 54-year age groups. However, values of 8-OHdG, plasma ORAC, erythrocyte GSSG, erythrocyte GSSG/GSH ratio, plasma GSH, plasma FRAP, and erythrocyte GPx decreased significantly with age; especially between 18- to 39-year and 40- to 54-year age groups.

3.2 Urinary TPE and TPI

Overall, the mean (SD) total urinary TPE was 67.82 (1.83) mg GAE/g creatinine (range: 22.07 to 336.43). Segregated by gender, these values were 78.30 (1.68) and 55.84 (1.94) mg GAE/g creatinine in females and males, respectively ($p = 0.007$). Table 1 also summarizes the mean urinary TPE of study participants segregated according to age. A significant increase of TPE with age was observed ($p < 0.001$).

The mean daily TPI in the study population was estimated as 1564.56 (676.38) mg GAE/person/day (range: 422.02 to 3311.61 mg GAE/person/day). Segregated by gender, these values were 1600.24 (635.35) and 1496.61 (733.64) mg GAE/person/day in females and males, respectively. The differences were not statistically significant. TPI increased with age ($p < 0.001$). This variability in daily TPI among participants was also reflected in urinary TPE excretion. A significant Pearson correlation was observed between daily TPI and urinary TPE ($r = 0.281$, $p = 0.012$). Table 2 summarizes the plant food consumption and the polyphenol intake

Table 1. Oxidative stress biomarkers and urinary TPE segregated according to age groups

	18–39 years, $N = 34$	40–54 years, $N = 22$	55–72 years, $N = 25$	p^a	p^b	p^c	p^d
Plasma-oxidized LDL; U/L[a)]	55.45 (11.51)	75.87 (23.41)	86.03 (16.72)	<0.001	<0.001	<0.001	0.024
Plasma 8-isoprostan; pg/mL[b)]	1.61 (1.32)	3.59 (3.03)	4.54 (2.36)	<0.001	<0.001	<0.001	0.865
8-OHdG; ng/mg creatinine[b)]	85.69 (1.53)	22.57 (2.66)	25.63 (2.89)	<0.001	<0.001	<0.001	0.006
Carbonyl groups; nmol/mg protein[b)]	0.67 (1.76)	0.66 (1.69)	0.71 (1.83)	0.253	0.290	0.444	0.104
Plasma ORAC; μmol TE/mL[a)]	29.75 (5.89)	23 (7.75)	22.44 (14.12)	<0.001	0.001	<0.001	0.322
Plasma $ORAC_{TCA}$; μmol TE/mL[a)]	1.07 (0.44)	1.05 (0.29)	1.08 (0.27)	0.946	0.980	0.987	0.946
Erythrocyte GSH; μmol/g Hb[a)]	3.51 (0.88)	4.45 (1.2)	4.6 (0.75)	<0.001	0.003	<0.001	0.254
Erythrocyte GSSG; μmol/g Hb[a)]	0.74 (0.26)	0.57 (0.22)	0.51 (0.20)	0.001	0.027	0.001	0.653
Erythrocyte GSSG/GSH ratio[b)]	0.24 (1.76)	0.11 (1.61)	0.13 (1.67)	<0.001	0.001	<0.001	0.593
Plasma GSH; nmol/mL[c)]	9.06 (2.39)	7.35 (4.68)	4.39 (5.9)	<0.001	0.002	<0.001	0.862
Plasma GSSG; nmol/mL[a)]	22.58 (3.71)	33.37 (7.5)	37.34 (4.19)	<0.001	<0.001	<0.001	0.482
Plasma GSSG/GSH ratio[b)]	2.43 (1.50)	5.56 (2.11)	5.12 (2.76)	<0.001	<0.001	<0.001	0.006
Plasma TBARS; nmol/mL[a)]	1.01 (0.26)	0.97 (0.22)	1.07 (0.21)	0.365	0.856	0.624	0.380
Serum vitamin C; nmol/mL[a)]	291.65 (158.44)	245.24 (212.01)	352.98 (118.97)	0.075	0.287	0.039	0.091
Plasma FRAP; μmol TE/mL[a)]	0.56 (0.11)	0.50 (0.09)	0.45 (0.08)	<0.001	0.097	<0.001	0.249
Erythrocyte SOD; U/g Hb[a)]	1831.44 (722.72)	2004.95 (498.74)	1620.08 (271.33)	0.025	0.104	0.480	0.002
Erythrocyte GPx; U/g Hb[b)]	122.33 (1.30)	61.88 (1.32)	72.17 (1.41)	<0.001	<0.001	<0.001	0.241
Plasma GPx; U/L[b)]	159.42 (1.35)	148.42 (1.44)	149.10 (1.29)	0.038	0.034	0.034	0.633
Erythrocyte CAT; mmol/min/g Hb[a)]	234.63 (55.75)	237.59 (35.03)	252.31 (44.31)	0.347	0.974	0.372	0.572
Urinary TPE; mg GAE/g creatinine[b)]	46.14 (1.55)	69.7 (1.67)	110.12 (1.68)	<0.001	0.007	<0.001	0.016

Data are presented as a) mean (SD); b) geometric mean (antilog SD); c) median (IQR = interquartile range).
p^a: two-tailed test of significance between three age groups; p^b: two-tailed test of significance between 18- to 39-year-old group and 40- to 55-year-old group; p^c: two-tailed test of significance between 18-to 39-year-old group and 55- to 72-year-old group; p^d: test significance between 40- to 55-year-old group and 55- to 72-year-old group.

Table 2. Daily plant food intake and polyphenol intake from plant food sources segregated according to age groups

	Food intake; g portion/person/day						
Plant foods	18–39 years, $N = 34$	40–54 years, $N = 22$	55–72 years, $N = 25$	p^a	p^b	p^c	p^d
Cereals and tubers[a)]	190.85 (1.50)	243.85 (1.62)	219.16 (1.73)	0.597	0.310	0.868	0.482
Olive oil[b)]	41.00 (2.80)	45.64 (3.7)	41.76 (3.27)	0.583	0.590	0.960	0.779
Vegetables[b)]	208.05 (19.79)	302.75 (33.08)	302.52 (28.16)	0.012	0.040	0.044	0.992
Legumes[a)]	21.68 (2.32)	21.44 (3.29)	26.41 (2.34)	0.052	0.581	0.016	0.129
Fruits[b)]	230.77 (34.18)	449.93 (46.50)	421.66 (48.34)	<0.001	0.002	0.006	0.871
Nuts[a)]	6.43 (3.97)	10.71 (2.31)	9.11 (2.19)	0.302	0.135	0.592	0.302
Chocolate[a)]	10.26 (2.50)	7.51 (2.33)	7.10 (1.98)	0.006	0.076	0.002	0.153
Fruit juice[c)]	0.5 (66.67)	0.00 (66.67)	2.72 (79.17)	0.508	0.556	0.470	0.261
Coffee[b)]	76.03 (13.44)	149.17 (15.17)	88.39 (13.22)	0.002	0.003	0.608	0.057
Fermented alcoholic beverages[a)]	68.95 (2.57)	60.11 (10.62)	116.02 (2.20)	0.049	0.291	0.010	0.379
	Polyphenol intake; mg GAE/person/day						
Plant foods	18–39 years ($n = 34$)	40–54 years ($n = 22$)	55–72 years ($n = 25$)	p^a	p^b	p^c	p^d
Cereals and tubers[a)]	19.60 (2.46)	47.40 (2.07)	46.31 (2.30)	<0.001	0.001	0.001	0.790
Olive oil[b)]	20.82 (1.44)	23.99 (2.1)	22.12 (1.61)	0.405	0.410	0.783	0.823
Vegetables[b)]	246.70 (41.42)	367.49 (58.6)	409.31 (58.27)	0.071	0.254	0.099	0.922
Legumes[a)]	6.61 (3.38)	10.12 (4.55)	11.17 (3.67)	0.059	0.533	0.011	0.260
Fruits[b)]	428.03 (61.87)	769.12 (95.49)	650.07 (88.53)	0.009	0.012	0.140	0.570
Nuts[a)]	29.93 (4.62)	47.03 (2.63)	39.48 (2.40)	0.353	0.157	0.621	0.357
Chocolate[a)]	118.14 (2.45)	93.12 (2.76)	93.06 (2.26)	0.013	0.118	0.004	0.159
Fruit juice[c)]	0.9 (26.05)	0.00 (29.83)	4.89 (47.48)	0.508	0.587	0.412	0.287
Coffee[b)]	202.77 (35.85)	397.83 (40.47)	235.73 (35.25)	0.002	0.003	0.608	0.057
Fermented alcoholic beverages[a)]	51.32 (3.25)	71.23 (9.78)	135.48 (2.34)	0.021	0.299	0.003	0.324
Total polyphenols[b)]	1196.70 (90.45)	1967.90 (132.50)	1687.59 (140.19)	<0.001	<0.001	0.012	0.268

Data are presented as a) mean (SD); b) geometric mean (antilog SD); c) median (IQR = interquartile range).
p^a: two-tailed test of significance between three age groups; p^b: two-tailed test of significance between 18- to 39-year-old group and 40- to 55-year-old group; p^c: two-tailed test of significance between 18- to 39-year-old group and 55- to 72-year-old group; p^d: test significance between 40- to 55-year-old group and 55- to 72-year-old group. In fermented beverages category, red wine represents 73%, beer 8.8%, and cava/champagne 14.4% of total. GAE = gallic acid equivalent.

from plant food sources, segregated according to age. Vegetable and fruit consumption increased significantly between the 18- to 39- and 40-year to 54-year age groups ($p = 0.04$, $p = 0.002$) but no differences were observed between the 40- to 54-year and 55- to 72-year age groups. Coffee consumption increased significantly between the 18- to 39-year and 40- to 54-year age groups ($p = 0.003$) but it decreased again between the 40- to 54-year and 55- to 72-year age groups. Fermented beverage consumption was not significantly different between the 18- to 39-year and 40-to 54-year age groups, but a significant increase was observed starting with the 55- to 72-year age group ($p = 0.01$). We observed a significant decrease in chocolate consumption with age ($p < 0.006$).

Males consumed significantly more cereals and tubers ($p = 0.001$), olive oil (including virgin olive oil; $p = 0.048$), and fermented beverages than females ($p < 0.001$). Women consumed significantly more coffee than men ($p = 0.02$).

TPI was correlated with polyphenols from fruits ($p < 0.001$), vegetables ($p < 0.001$), coffee ($p < 0.001$), fermented beverages ($p = 0.013$), and olive oil ($p = 0.031$). Based on these Pearson correlations, we applied a stepwise multivariate linear regression analysis in which TPI was the dependent variable and polyphenol intake from fruits, vegetables, coffee, fermented beverages, and olive oil were the independent variables. Table 3 summarizes the standardized coefficients from stepwise multivariate model that showed that polyphenol intake from fruits is the largest contributor to TPI, followed by vegetables, coffee, and fermented beverages.

3.3 Urinary TPE and oxidative stress biomarkers

Stepwise multivariate linear regression analyses were performed with oxidative stress biomarkers as dependent variables and urinary TPE as principal independent variable. The models included other variables that can influence oxidation such as age, gender, physical activity, stress, ultraviolet exposure, and vitamin C intake as independent variables. As shown in Table 4, urinary 8-OHdG and erythrocyte GSSG exhibited an inverse association with urinary TPE. Other oxidative stress biomarkers tested showed no significant association. However, Pearson correlations showed significant negative relationships between urinary TPE and plasma FRAP

Table 3. Multivariate linear regression analysis with TPI (mg GAE/day) as the dependent variable and polyphenol intake from food sources as independent variables

Polyphenol source; mg GAE/day	β	SE	Beta	*p*	95% CI
From fruits	1.008	0.069	0.653	<0.001	0.869–1.146
From vegetables	819.457	80.396	0.460	<0.001	658.131–980.783
From coffee	0.751	0.160	0.209	<0.001	0.429–1.073
From fermented beverages	155.606	47.480	0.145	0.002	60.331–250.881

TPI = total polyphenol intake; β = nonstandardized coefficient (regression line coefficient); SE = standard error; Beta = standardized coefficient; *p* = two-side test of significance; CI = confidence interval; GAE = gallic acid equivalent.

($r = -0.432$, $p<0.001$), erythrocyte SOD ($r = -0.253$, $p < 0.05$), erythrocyte GPx ($r = -0.446$, $p < 0.001$), and plasma GPx ($r = -0.239$, $p<0.05$). However, the association was positive for GSH ($r = 0.257$, $p < 0.05$).

TPI, as assessed via the 3-day dietary record, showed a negative Pearson correlation with 8-OHdG and erythrocyte GSSG. However, there were no statistically significant associations with these oxidative stress biomarkers when assessed using multivariate linear regression analysis.

Stepwise multivariate linear regression analyses with oxidative stress biomarkers (urinary 8-OHdG and erythrocyte GSSG) as dependent variables and polyphenol intake from fruits, vegetables, coffee, and fermented beverages as independent variables were carried out. We chose these independent variables because they were those that showed a positive association with TPI. Urinary 8-OHdG exhibited an inverse association with polyphenol intake from vegetables ($\beta = -0.403$, $p = 0.006$, 95% CI = –0.687 to –0.120) and fermented beverages ($\beta = -0.209$, $p = 0.017$, 95% CI = –0.380 to –0.039). However, there were no statistically significant associations between polyphenol intake from plant food items and erythrocyte GSSG.

4 Discussion

The present study confirmed our hypothesis that, in healthy subjects of different ages, a high intake of dietary polyphenols would be reflected in a high urinary excretion, which would be related to changes in oxidative stress biomarkers as indicated by a significant inverse association with urinary 8-OHdG excretion and with erythrocyte GSSG concentrations.

Polyphenols can exert their effects, *in vitro*, by inactivating harmful free radicals, and by chelating divalent metal ions. They are also thought to prevent oxidation of food-associated lipids prior to consumption, which could reduce the prooxidant burden. However, it is suggested that polyphenols *in vivo* do not appear to be present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity, i.e. they are present in the circulation and tissues only in nano- to low-micromolar ranges and are predominantly present as conjugated metabolites. However, recent studies indicate that polyphenols may induce upregulation of endogenous antioxidant enzymes *in vivo* and exert an indirect antioxidant effect. Inductive or signaling effects may occur at concentrations much lower than required for effective radical scavenging [6, 22]. Polyphenols are capable of affecting two major redox-sensitive nuclear transcription factors, erythroid 2-related 2 (Nrf2) and Kappa B, which mediate antioxidant and inflammatory signaling [6, 22, 23]. Arola-Arnal et al. [24], proposed the influence of polyphenols on microRNA expression as a new mechanism of action. Hollman et al. [6], suggested that polyphenols should be considered as "versatile bioactive molecules" rather than mere antioxidants, and their role in gene transcriptional regulation need to be explored in more detail.

We observed that urinary 8-OHdG excretion was significantly, and inversely, associated with intake of polyphenols

Table 4. Multivariate linear regression analysis with oxidative stress biomarkers (urinary 8-OHdG and erythrocyte GSSG) as dependent variables and urinary TPE, age, gender, physical activity, stress, ultraviolet exposure, and vitamin C as independent variables

Variable	β	SE	Beta	*p*	95% CI
8-OHdG					
Age	−0.015	0.002	−0.511	<0.001	−0.020 to −0.011
Urinary TPE; mg GAE/g creatinine	−0.797	0.139	−0.444	<0.001	−1.074 to −0.521
Erythrocyte GSSG					
Age	−0.004	0.002	−0.232	0.071	−0.008 to 0.000
Gender; 1 = women; 2 = men	−0.196	0.059	−0.379	0.001	−0.313 to −0.079
Work and home stress	−0.062	0.028	−0.232	0.032	−0.118 to −0.005
Urinary TPE; mg GAE/g creatinine	−0.273	0.133	−0.276	0.044	−0.539 to −0.007

TPE = total polyphenol excretion; β = nonstandardized coefficient (regression line coefficient); SE = standard error; beta = standardized coefficient; CI = confidence interval; *p* = two-sided test of significance; GAE = gallic acid equivalent.

from vegetables and fermented beverages (mainly red wine). Thus, in healthy individuals, ingestion of some protective foods is associated with oxidative biomarker reduction even before these markers become relevant in assessing risk-factor status or in subclinical manifestations of CVD. However, the protective effects of polyphenols from foods would depend on their mechanisms of absorption, bioavailability, and metabolism [6, 25].

We need to take into account that assays for measuring oxidative stress have several shortcomings. Measurement of 8-OHdG is the commonest method of assessing DNA damage. Urinary 8-OHdG has been used to assess whole-body DNA damage. Measurements of urinary excretion rates of 8-OHdG alone should be interpreted with caution. Despite these limitations, 8-OHdG continues to be the most frequently studied biomarker in the measurement of endogenous oxidative DNA damage [26, 27]. Recently, an interventional study in young adult men showed that a moderate red wine intake significantly decreased 8-OHdG in DNA isolated from peripheral blood leukocytes [28, 29]. Di Wang et al. [29] also demonstrated that dietary supplementation with polyphenol extract from black tea was, possibly, a useful agent against functional disturbance caused by environmental xenobiotics. The mechanism proposed was through maintaining DNA double-helix architecture and mitigating oxidative stress. They suggested that the most plausible molecular mechanism was that tea polyphenols can up-regulate the activation of Nrf2.

Our present study demonstrated that age is related to changes of certain oxidative stress biomarkers. Urinary 8-OHdG was highest in the youngest subjects, and decreased with increasing age. Tamura et al. [31] also observed a significant inverse correlation between urinary 8-OHdG and age. However, their findings were based only on children and adolescents. Our results represent physiological changes associated with normal aging.

The glutathione system is an antioxidant system that helps to protect cells from ROS. We observed that individuals with higher TPE have lower levels of GSSG, which would indicate a greater capacity for detoxification of the glutathione system. In our study, concentrations of erythrocyte GSH also correlated positively and significantly with urinary TPE; albeit not statistically significantly on multivariate regression analysis. Urquiaga et al. [29] observed that moderate red wine consumption produced an increase in erythrocyte GSH. Also, Ya-Chen Y et al. [32] observed an induction of glutathione synthesis by flavonoids mediated via the Nrf2 pathway and protection against oxidative stress. Using Pearson correlation, we observed an inverse, and significant, association between urinary TPE and plasma FRAP, erythrocyte SOD, erythrocyte, and plasma GPx. In general, the antioxidant defense system seeks to maintain, or restore, redox homeostasis. To this end, endogenous enzymes and exogenous antioxidants function interactively and often synergistically [33]. The reduction of these enzymes could be a consequence of this attempt to regulate the body's redox balance. The studies of Covas et al. [34] and Estruch et al. [35] also reported that a steady consumption of a polyphenol-rich diet decreased scavenger enzymes such as SOD and GPx. The implication is that a regular diet rich in exogenous antioxidants could reduce the dependence on endogenous antioxidant defenses, probably by effects at the transcriptome level. Our data provide information on redox homeostatic regulation in a healthy population consuming its habitual diet. This is of considerable interest because, currently, there are data only on populations exposed to a high intake of polyphenols, or with a high degree of associated oxidative stress.

Our study also demonstrated that age, gender, and stress are related to erythrocyte GSSG. Further studies are needed to establish the mechanisms underlying these associations.

In the present study, the mean consumption of total polyphenol from all dietary constituents was estimated as 1564.56 mg GAE/person/day. TPI is documented as being higher than any other known dietary antioxidant, including dietary vitamin C, vitamin E, and carotenoid intake [36]. TPI, according to the observations of Medina-Remón et al. [36], ranged from 122.96 to 3298.17 mg/day in an elderly Mediterranean population considered at high risk of CVD. Also, Saura-Calixto et al. [37] estimated the TPI as 1171 mg/person/day in those consuming the Spanish Mediterranean diet. In our study participants, the mean consumption was 350.05 g/day fruits, 263.18 g/day vegetables, 103.00 g/day coffee, and 80.56 g/day fermented beverages and, in this ranking order, constituted the most important contributors to the TPI. Our results are similar to those of Medina-Remón et al. [36].

Of considerable note is our novel finding that age is a factor associated with the increase in TPE. This association was also observed by Medina-Remón et al. [36] but which had been established in an elderly population. Instead, the population we studied had a wide age range from 18 to 72 years. This enabled us to observe a significant increase in TPE with age. This association could be related to an increase in the consumption of vegetables, fruits, coffee, and moderate intake of fermented beverages with age, which are the greatest contributors to TPI. However, we observed that 40- to 54-year-old age group had a higher consumption of total polyphenol than the 55- to 72-year-old group while the urinary TPE excretion was higher in 55- to 72-year-old group. We observed that the 40- to 54-year-old group consumed more coffee but the 55- to 72-year-old group consumed more fermented beverages. Age alters the metabolism of common dietary polyphenols each of which has differing intrinsic activities, absorption, metabolism, and urinary elimination [38].

Our participants are healthy individuals with a low risk of CVD, and represent an optimal sample to identify relationships between polyphenols, diet, and oxidative stress. Such information represents an important phase in the understanding of the role of polyphenols in oxidative stress prevention and in optimizing dietary advice for the general population. Intervention studies would be necessary to confirm these intriguing data.

One limitation of our study is that urinary TPE is an indirect biomarker of overall circulating polyphenol status. Also, the study did not focus on clinical outcomes. Our objective was to assess, in general, the relationships between polyphenol-rich foods and oxidative stress biomarkers. To obtain this insight, we needed to determine the urinary TPE rather than individual polyphenols. However, as we have found associations between food groups and oxidative stress biomarkers, future studies are warranted to determine specific polyphenols and to identify specific biomarkers of each polyphenol-rich food. Further, their roles in signaling pathways need to be identified. Such information would be a valuable in future recommendations for dietary improvements and/or fortified food selection.

In conclusion, urinary TPE increased with age and may reflect an attenuation of oxidative damage. These results could potentially explain the beneficial effects in healthy individuals of a dietary intake rich in vegetables, and moderate red wine; typical food items of the Mediterranean diet.

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UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVE FOOD COMPOUNDS OF MEDITERRANEAN AND NORDIC DIETS AND THEIR EFFECTS ON NUTRITIONAL AND CARDIOVASCULAR DISEASE.
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Dipòsit Legal: T 1916-2014

STUDY 2

Non-targeted metabolite profiling discriminates diet-specific biomarkers for consumption of whole grains, fatty fish and bilberries -a randomized trial (Sysdimet-HealthGrain intervention)

Non-targeted metabolite profiling discriminates diet-specific biomarkers for consumption of whole grains, fatty fish and bilberries – a randomized trial (Sysdimet-HealthGrain intervention)[1-3]

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[5] ***Running head:*** *Sysdimet-HeathGrain trial: metabolite profiling*

[6] ***Study were registered at ClinicalTrials.gov NCT00573781***

[7] ***List of abbreviations:*** *AR, alk(en)ylresorcinol; CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ESI, electrospray ionization; FDR; false discovery rate; HD, Healthy Diet; HILIC, hydrophilic interaction; LC, liquid chromatography; LPC, lysophosphatidylcholines; LPE; lysophosphatidyl-ethanolamines; MS, mass spectrometry; PC, phosphatidylcholines; PLS-DA, partial least squares discriminant analysis; RP, reversed phase; VIP, variable influence on projection; WGED, whole grain enriched diet*

ABSTRACT

Background: Non-targeted metabolite profiling allows for concomitant examination of wide range of metabolite species elucidating the metabolic alterations caused by dietary interventions.

Objective: The effects of dietary modifications based on increasing consumption of whole grains, fatty fish and bilberries on plasma metabolite profiles were investigated, in order to identify applicable biomarkers for dietary intake and endogenous metabolism.

Design: Metabolite profiling analysis was performed on fasting plasma samples collected in a 12-week parallel group intervention with 106 participants randomized into three diet interventions: (1) whole grain products, fatty fish, and bilberries (Healthy Diet; HD), (2) Whole grain enriched diet (WGED), with the same grain products as in HD intervention, but with no change in fish or berry consumption, and (3) refined wheat breads and restrictions on fish and berries (Control). Additionally, correlation analyses were done between the putative biomarker candidates and the food intake data for identification of the causative food items.

Results: Non-targeted metabolite profiling revealed marked differences in fasting plasma after the intervention diets as compared to Control. In both intervention groups a significant increase was observed in two signals identified as glucuronidated alk(en)ylresorcinols and correlating strongly with intake of whole grain products. Additionally, HD intervention increased the signals for furan fatty acids (CMPF), hippuric acid, and various lipid species incorporating PUFAs. In particular, plasma CMPF correlated strongly with intake of fish, but not with any other foods.

Conclusions: Novel biomarkers of intake of health beneficial food items included in the Nordic diet were identified by metabolite profiling of fasting plasma and confirmed by correlation analyses with dietary records. The

most potential of these was CMPF, which was shown to be a highly specific biomarker for fatty fish intake.

1. INTRODUCTION

The Nordic diet is characterized by many food items with positive nutritional profiles, such as whole grain products, berries, root vegetables, and fatty fish. Adherence to a healthy Nordic dietary pattern has been associated with lower mortality (1), and controlled interventions using diets modified by single food items or combinations of Nordic foods have revealed beneficial cardiovascular health effects, *i.e*. improved lipid profile, glucose metabolism and anti-inflammatory properties (2–8).

The targeted analyses on pre-determined set of clinical biomarkers after consumption of specific health promoting nutrients, foods or diets can provide important mechanistic information, but they fail to give a broader picture of the altered metabolic status resulting from a variety of interactions between food constituents and endogenous metabolites. The non-targeted metabolite profiling utilizing sensitive and high-throughput analytical technologies offers a wide window to monitor changes in diet-derived and endogenous metabolite levels, thus aiding the identification of novel biomarkers for both dietary intake and biological effects (9–12).

The Sysdimet-HealthGrain intervention has provided new information of potential health effects of whole grain, in particular rye, and of a combination of whole grains with bilberries and fatty fish (6,7). Here we report metabolic alterations in fasting plasma, and especially novel biomarkers for consumption of whole grain, bilberries and fish, by applying liquid chromatography-mass spectrometry based non-targeted metabolite profiling approach. Moreover, the link between the observed biomarkers was refined to specific food groups by correlation analyses with the dietary intake based on repeated food record data.

2. MATERIALS AND METHODS

2.1 Subjects, study design, diets

The study population was from the Sysdimet-HealthGrain intervention, a Finnish randomized controlled dietary intervention (6,7) aiming to investigate the effects of the diet rich in whole grains, bilberries and fatty fish on glucose metabolism in high risk individuals with impaired glucose metabolism and features of the metabolic syndrome. A total of 131 volunteers were recruited for a 12-week intervention and randomized to three parallel interventions: Healthy Diet (HD), whole-grain-enriched diet (WGED), or control diet (Control) (Flow chart available as Online Supplemental Material, **Supplemental Figure 1**). The intervention groups were matched for sex and medians of BMI, age and fasting plasma glucose concentration. Altogether 106 participants completed the study. The baseline characteristics of the subjects are available as Online Supplemental Material (**Supplemental Table 1**).

The intervention diets have been described earlier in detail (6,7). In brief, in HD group, the participants were advised to replace their usual cereal products by whole grain breads and a bread with low postprandial insulin response, and by 3.5 deciliter (measured uncooked) of whole meal pasta per week. The breads recommended contributed 20-25% of the daily energy intake. In addition, they were instructed to eat fatty fish (100–150 g fish per meal) three times per week, and bilberries (*Vaccinium myrtillus*; frozen, pureed or dried powder) three portions per day (equivalent of 300 g fresh bilberries per day). In WGED group, the participants were instructed to consume the same cereal products as in HD group and not to change their current fish and berry consumption. In Control group, the participants were asked to avoid whole grain cereals and consume low-fiber products, intake of bilberries was not allowed, and consumption of fatty fish was restricted to once a week. Other dietary and lifestyle habits were kept unchanged in all groups.

The participants kept a 4-day dietary record (consecutive days including one weekend day) during the run-in period (baseline) and at weeks 3, 7 and 11 of the intervention period. The dietary data were analyzed using the MicroNutrica® software based on Finnish food composition analyses and international food composition tables(13).

2.2 Non-targeted LC-MS metabolite profiling analysis

Fasting EDTA plasma samples were collected for the LC-MS metabolite profiling analysis (106 at baseline and 106 at the end of the study). An aliquot of the sample, 100µL, was mixed with 400 µL of acetonitrile (ACN; VWR International, Leuven, Belgium) and mixed in vortex to precipitate the proteins, and centrifuged 10 min to collect the supernatant. The supernatant was filtered with a 96-well plate (PTFE 0.2 µm) to the collection plate. Aliquots of 2 µL were taken from at least half of the plasma samples, mixed together in one tube, and used as a quality control sample (QC) for the analysis. Additionally solvent blank was prepared in a same manner.

The samples were analyzed by the UHPLC-qTOF-MS system (Agilent Technologies, Waldbronn, Karlsruhe, Germany) that consisted of a 1290 LC system, a Jetstream electrospray ionization (ESI) source, and a 6540 UHD accurate-mass qTOF spectrometry. The samples were analyzed using two different chromatographic techniques, i.e. reversed phase (RP) and hydrophilic interaction (HILIC) chromatography. Data were acquired in both positive (+) and negative (-) polarity. Sample tray was kept at 4 °C during the analysis. Data acquisition software was MassHunter Acquisition B.04.00 (Agilent Technologies). The QC and the blank were injected after every 12 samples and also in the beginning of the analysis. The sample order for analysis was randomized.

In RP technique, four microliters of the sample solution was injected onto a column (Zorbax Eclipse XDB-C18, 2.1 × 100mm, 1.8 µm, Agilent Technologies, Palo Alto, CA, USA) kept at 50 °C. Mobile phases, delivered at 0.4 ml/min, consisted of water (eluent A, Milli-Q purified, Millipore, Milford,

MA) and methanol (eluent B; Sigma-Aldrich, St. Louis, MO), both containing 0.1 % (v/v) of formic acid (Sigma-Aldrich, St. Louis, MO). Following gradient profile was employed: 0–10 min: 2 → 100% B, 10–15 min: 100% B, 15–15.1 min: 100 → 2% B; 15.1–18 min: 2% B.

In HILIC technique, three microliters of the sample solution was injected onto a column (Acquity UPLC BEH Amide column, 2.1 × 100mm, 1.7 µm) (Waters Corporation, Milford, MA) kept at 45 °C. Mobile phases, delivered at 0.6 mL/min, consisted of 50% (v/v) (eluent A) and 90% (v/v) (eluent B) acetonitrile, respectively, both containing 20 mM ammonium formate, pH 3 (Sigma-Aldrich, St. Louis, MO). Following gradient profile was as employed: 0–2.5 min: 100% B, 2.5–10 min: 100% B → 0% B, 10–10.1 min: 0% B → 100% B; 10.1–14 min: 100% B.

The MS conditions were as follows after both chromatographies: Jetstream ESI source, operated in positive and negative ionization mode, conditions were drying gas temperature 325 °C and flow 10 L/min, sheath gas temperature 350 °C and flow 11 L/min, nebulizer pressure 45 psi, capillary voltage 3500 V, nozzle voltage 1000 V, fragmentor voltage 100 V, and skimmer 45 V. For data acquisition, 2 GHz extended dynamic range mode was used and the instrument was set to acquire over the *m/z* 50–1600. Data were collected in centroid mode at acquisition rate of 2.5 spectra/s (i.e. 400 ms/spectrum) with an abundance threshold 150. For automatic data dependent MS/MS analyses performed on the QC samples 4 most abundant ions were selected for fragmentation from every precursor scan cycle. These ions were excluded after 2 product ion spectra and released again for fragmentation after a 0.25-min hold. Precursor scan time was based on ion intensity, ending at 20000 counts or after 300 ms. Product ion scan time was 300 ms. Collision energies were 10, 20 and 40 V in subsequent runs. Continuous mass axis calibration was performed by monitoring two reference ions from an infusion solution throughout the runs. The reference ions were *m/z* 121.050873 and *m/z* 922.009798 in positive mode and *m/z* 112.985587 and *m/z* 966.000725 in negative mode.

2.3 Data analysis

2.3.1. Collection and statistical analysis of the LC-MS data

Data was collected using vendor's software (MassHunter Qualitative Analysis B.05.00, Agilent Technologies, USA) and output as compound exchange format (cef.files) into the Mass Profiler Professional software (MPP 2.2, Agilent Technologies, USA) for compound alignment and data preprocessing. Further data processing and selection for the most discriminating compounds between diets was performed in Excel (Microsoft 2007). In order to remove insignificant features, only features found in at least 80% of the samples in either at the beginning or at the end of the intervention in HD or WGED groups were included in the analysis. This resulted in dataset comprising altogether 3130 features found in RP ESI(-) (1415), RP ESI(+) (362), Hilic ESI(+) (632), and Hilic ESI(-) (721).

The difference in the peak area value between the beginning and end of the intervention for each participant was determined for each feature in all the three dietary groups. A Student's t-test was used to compare HD and WGED groups with Control group in order to find the discriminative features affected by either or both of the test diets when compared to the control. Benjamini–Hochberg false discovery rate (FDR) was used to adjust results for multiple comparisons (14) in each of the four analytical approaches separately, taking into account all the features included in each of the pre-processed four datasets, resulting in altogether 90 features with p_{corr}<0.05 (List of the differential features is available as Online Supplemental Material, Supplemental Table 2).

Additionally, the pre-processed datasets from each of the four analytical approaches were subjected to supervised clustering algorithm Partial Least-Squares Discriminant Analysis (PLS-DA; Simca-13, Umetrics, Sweden) including only the samples collected at the end of the intervention. Data were Log10-transformed, pareto-scaled and the model was validated by Simca-13 internal cross validation. Further visualization of the data was

performed on the RP ESI(-) data matrix containing the features exhibiting p_{corr} <0.05 (74 metabolic features) by the K-means cluster analysis with Pearson correlation as distance metric, followed by hierarchical clustering within each of the K-means clusters (http://www.tm4.org/mev.html).

2.3.2. Identification of differential features in the LC-MS data

The identification of the metabolites was based on MS/MS spectral comparison of pure standard compounds and the search of candidate compounds in databases including the Human Metabolome database, METLIN, ChemSpider and SciFinder, and verified with MS/MS spectral features included in the databases or reported in earlier publications. Lipids were identified based on MS/MS fragmentation when the metabolite was intensive enough to be caught in the automatic data dependent MS/MS analysis. The fragmentation pattern for phosphocholines (PCh) and phosphoetanolamines (PE) follows what has been published (15–17). Key elements for identification are the protonated head group (*m/z* 184.07 for PChs and *m/z* 196.03 for PEs) as well as the deprotonated PUFA fragments visible in the negative ionization mode (MS/MS fragmentation data for all of the identified metabolites is available as Online Supplemental Material, Supplemental Table 3.). The plasmalogen was identified based on the head group indicating PCh as well as the *m/z* 303 corresponding to arachidonic acid (C20:4) with links in Metlin solely for plasmalogens for the mass 765.579. Glucuronidated alk(en)ylresorcinols were identified based on MSMS fragmentation and compared to published data as described in the Online Supplemental Material (Supplemental Figure 2).

2.3.3. Clinical characteristics and dietary intake

Linear mixed-effect models were used to analyse group differences in clinical characteristics and dietary intake in the baseline and during the intervention as reported earlier (6). Analyses were performed using R Project for Statistical Computing version 2.7.2 (http://www.R-project.org) and nlme R-package version 3.1-96 (18). Interaction term between group and

intervention time-point (before or after intervention) was used to examine group related changes during the intervention. Control group was used as a reference group when comparing group differences. Benjamini–Hochberg false discovery rate (FDR) was used to adjust results for multiple comparisons (14).

2.3.4. Correlations between biomarker candidates and recorded dietary intake

Spearman rank correlation coefficients were calculated for relationships between the change in daily intake of whole grain bread, berries and fish, and change in peak abundance of selected biomarker candidates resulting from the statistical analysis. All subjects were included in the correlation analyses (n=106). Furthermore, analysis of variance was used (ANCOVA) with standardized values in order to study associations between selected biomarkers and food intake taking the effect of whole intervention diet into account (corrected by the intervention group). Stepwise linear regression model was applied in order to test the individual and combined associations of CMPF, EPA and DHA (independent variables) with the consumption of fish (dependent variable). The level of statistical significance was set at $p < 0.05$. Correlations were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (19.0 version; IBM Corp., Armonk, NY, USA).

3. RESULTS

3.1. Clinical characteristics and dietary intake

The clinical characteristics and dietary intakes have been reported in detail earlier, and there were no significant changes in measured characteristics during the study (**Supplemental Table 1**) (6,7). During the interventions, HD and WGED groups consumed the whole grain and low insulin response rye breads 7.7, and 7.9 portions per day, respectively, and Control group white wheat breads 6.8 portions per day, resulting in significantly higher fiber intake in HD and WGED groups as compared to Control group (**Supplemental Table 1**). In HD group, the mean fish consumption was 3.3

fish meals per week, resulting in an increased intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (**Supplemental Table 1**). Intake of bilberries in HD group was 3.2 portions per day (**Supplemental Table 1**). Thus the self-reported compliance with the diets was good.

3.2. Identification of statistically significant biomarkers

The non-targeted metabolite profiling revealed metabolic changes in the fasting plasma of the participants in HD and the WGED groups when compared to Control group. Out of the 3130 molecular features collected in the four different LC-MS modes, altogether 400 were found to have significant changes during the intervention with either one or both of the intervention diets when compared to Control group (Student's t-test, $p < 0.05$). After correcting for the multiple measurements within each of the four analytical approaches, the number of metabolic features with significant change was 90 (p_{corr} <0.05, **Supplemental Table 2**). The metabolites amenable for identification based on the automatic data dependent MS/MS analysis are listed in Table 1 with results from the statistical analysis, and the MS/MS spectral fragmentation data can be found in Supplemental Table 3. The metabolites with p_{corr} <0.05 are herein discussed as statistically significant features, whereas those having p<0.05 but not statistically significant after FDR correction are termed as metabolites having a trend.

A particularly clear change in the fasting plasma lipid profile after the HD period was the accumulation of 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) (2.56-fold; p_{corr}=1.98x10^{-6}). It was identified based on fragmentation match in Metlin and verified with the pure standard compound. Interestingly, in the near vicinity of the CMPF metabolite, there was another compound with *m/z* 267.1234 exhibiting the very same fragmentation pattern and also regarded as significant marker in RP ESI(-) analysis (1.22-fold; p_{corr} =0.033). The fragmentation and the elemental composition suggested a compound with an additional C_2H_4 in the molecular structure, which would refer to a two carbon units longer side chain in the

position 1 in the furan backbone of the metabolite. SciFinder browsing suggested such a compound, namely 3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid, reported earlier in sources including uremic serum (19) as well plant material (20).

Another prominent change in the fasting plasma in HD group was an increase in hippuric acid (21) (3.3-fold; p_{corr} =0.0006). Other phenolic metabolites, like pyrocatechol sulfate, were also increased in HD group (2.0-fold; p_{corr} =1.98x10^{-6}). The other metabolites affected by HD diet were phosphatidylcholines (PCh), lysophosphatidylcholines (LPC) and lysophosphatidyl-ethanolamines (LPE) with long chain PUFAs. HD intervention clearly increased *e.g.* LPC (20:5) with two isomers (both 1.7-fold; p_{corr} =0.0001), LPE(20:5) (1.6-fold; p_{corr} <0.0016), and LPE(20:6) (1.1-fold; p_{corr} =0.024). As expected, EPA (C20:5) and DHA (C22:6) fatty acids were also increased in HD group (1.5-fold; p_{corr} =0.0025 and 1.3-fold; p_{corr} =0.0039, respectively).

Whereas multiple metabolic markers were found in HD group, in WGED group there were only two metabolites with significant *p*-values after the strict FDR correction. These were statistically significant also after HD diet. The signals were two closely eluting peaks with *m/z* values of 551.3581 (1.2-fold; p_{corr}=0.0004) and 577.3737 (1.2-fold; p_{corr} =1.98x10^{-6}). Under MS/MS fragmentation both metabolites showed neutral loss of glucuronide, and the aglycon masses matched with alkylresorcinol (AR) 5-Nonadecyl-1,3-benzenediol for the compound with *m/z* 551.3581 and alkenylresorcinol 5-(16-Heneicosenyl)-1,3-benzenediol for the aglycon of the compound with m/z 577.3737. Further examination with the MS/MS spectra showed identical fragmentation with the published ones with these candidate metabolites (22,23), and although the exact position of the double bond in the molecule was not determined, the compounds were identified as Nonadecyl-benzenediol glucuronide (AR C19:0-Gln) and Heneicosenyl-benzenediol-glucuronide (AR C21:1-Gln) (**Table 1**, **Supplemental Figure 2**).

In addition to the glucuronidated alk(en)ylresorcinols, there was a trend towards altered metabolism of various betaine compounds in WGED group (**Table 1**). The retention between 2-3 minutes in the HILIC ESI(+) had numerous signals exhibiting betaine-like fragments in the MS/MS analysis, out of which a few could be identified based on earlier knowledge on occurrence of betaine in human biofluids (21,24,25). Among the identified betaine metabolites was pipecolic acid betaine which showed increased trend in both HD and WGED groups (1.3-fold; p=0.035 and 1.9-fold; p<0.0077, respectively). Interestingly, the peaks of two main betaine compounds, glycine-betaine and proline-betaine, with very large inter-individual variation did not differ between HD and WGED groups. On the contrary, analogous metabolites eluting shortly after both peaks with exactly same mass and similar fragmentation pattern, had differential trend in WGED group when compared to Control, namely *m/z* 144.1025, rt 3.95 and *m/z* 118.0619, rt 5.53 (**Table 1**). Propionylcarnitine and γ-butyrobetaine metabolites were increased in WGED group when compared with Control group (1.2-fold; p=0.043 and 1.1-fold; p=0.012, respectively). Furthermore, there were also increases in the levels of certain amino acids such as L-lysine, ornithine, and L-arginine in WGED group when compared with Control group (1.6-fold; p=0.0094, 1.2-fold; p=0.0043, and 1.2-fold; p=0.0097, respectively).

3.3. Classification of the LC-MS data by chemometrical analyses

The end-point samples from all of the intervention participants were subjected to the chemometric algorithm Partial Least Squares Discriminant Analysis (PLS-DA) as exemplified with the data from the RP ESI(-) mode (**Figure 1**). The clustering of the samples in the PLS-DA analysis was in line with the statistical analyses, since the samples collected at the end of HD intervention differ from the other diets (**Figure 1A**). Furthermore, as visualized by the loadings plot, the most discriminating metabolites for the study groups in the PLS-DA model are the same compounds that were identified as putative biomarkers between the study diets also by the

statistical analysis (**Figure 1B**). Variable influence on projection (VIP) values for *e.g.* hippuric acid, pyrocatechol sulphate and CMPF were 2.46, 2.56, 1.94, respectively, indicating that these compounds are among the most powerful discriminators between HD group and the two other groups. Additionally, from the loadings plot several unknown lipid metabolites were noticed based on mass and retention time, *e.g*. MW 869.539, rt 11.8, VIP 1.76, which contributed to the clustering of Control group, as well as a smaller metabolite of unknown identity MW 188.094, rt 3.3, VIP 1.81. These metabolites are most likely endogenous compounds that are at higher level in Control group than in the test groups.

74 features in RP ESI(-) had p_{corr} <0.05, and were subjected to K-means cluster algorithm followed by hierarchical cluster analysis with a heat map output (**Figure 2**). When the fold change values for the statistically significant features were included in the analysis, seven clusters were formed based on the similar response profile throughout the study participants (three markers did not cluster with any of those and were removed from the analysis). Cluster 1 contained two metabolites, hippuric acid and pyrocatechol sulfate, and cluster 2 held the two alk(en)ylresorcinol glucuronides. In cluster 3, there was a group of unknown lipids with retention time over 12 minutes increased in HD group, but tended to be increased also in the participants of WGED group. The fourth group clustered mainly the EPA and DHA fatty acids with several fragment ions, as well as the CMPF metabolite, likewise with several fragments. However, the other furan fatty acid (3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid) grouped together with the AR-metabolites in cluster 2. Cluster 5 contained various lipid metabolites that were increased in particular after the HD intervention, including the identified lipids LPE(20:5), LPE(22:6), and two isomers of LPC(20:5), as well as several unknown metabolites that had lower signal intensity and were not amenable for the identification based on the automatic data dependent MS/MS analysis. Cluster 6 had unknown lipid metabolites that were increased in HD group, and were completely

undetected in majority of the participants of WGED and Control groups. Cluster 7 was formed from unknown metabolites, mostly lipids based on the rt, that were seemingly decreased after the HD intervention.

3.4 Association between changes in plasma biomarkers and consumption of selected food items

After identifying the strongest putative biomarker metabolites for the intervention groups, we wanted to determine which foods specifically are associated with the increased levels of those metabolites by conducting correlation analyses with the food intake data collected during the intervention. Among the most significantly changed metabolites in the HD intervention was CMPF. We found very strong positive associations between plasma CMPF and recorded fish intake (**Table 2**). The association was stronger with total fish intake than with fatty fish intake only. However, there was no significant association between plasma CMPF and the intake of lean fish, possibly because the consumption of lean fish was limited due to the design of the original study.

Cross-sectionally, plasma CMPF associated also with the intake of berries at baseline (beta= 0.291, p=0.003) and at the end of the intervention (beta=0.363, p=0.001), (**Table 3**). However, there was no association between the changes in the intake of berries and changes in plasma CMPF when adjusted with the effect of intervention group (**Table 2)**. Plasma CMPF levels did not associate positively with the intakes of grain products, fruits, vegetables, milk products, meat, potatoes, nuts, eggs or oils and spreads (**Table 2**). Intakes of berries and fish correlated especially at the end of the study (r=0.524, p=8.2 x 10^{-9} vs. at baseline r=0.096, p=0.328), since the participants in the Healthy Diet group were advised to increase their intake of both fish and berries during the intervention. To account for this, the effect of intervention group was included into the model when analyzing associations between dietary intake and plasma CMPF cross-sectionally at the end of the study, and when comparing the respective changes during the

intervention period. Furthermore, we applied a linear regression model in which both the intake of berries and fish were included. In this analysis, changes in fish intake had an independent effect on changes in plasma CMPF when intake of berries was included in the model (beta=0.473 and $p=7 \times 10^{-8}$), but also berries seemed to have an independent effect on plasma CMPF (beta=0.290, p=0.001). However, in this model, the effect of fish intake on plasma CMPF level was 5-fold compared to the effect of berries.

We also tested the associations of plasma CMPF level with calculated dietary intakes of EPA and DHA and with plasma levels of EPA and DHA, the established biomarkers of fish intake. We found a strong positive correlation between plasma CMPF and EPA and DHA at baseline, at the end of the study, and between the respective changes during the intervention (**Table 3**). Using stepwise linear regression model we observed that plasma CMPF is even stronger independent predictor for fish consumption than plasma EPA (for CMPF beta=0.402, $p=7x10^{-5}$; for EPA beta=0.212, p=0.031). DHA did not predict fish consumption significantly. The adjusted R-square for the model that included only plasma CMPF was 0.261, and for the model including both CMPF and EPA 0.294, indicating that EPA improves the prediction of fish consumption for by only 3 %.

Associations between the consumption of specific food items were evaluated also for several other biomarker candidates including hippuric acid and alk(en)ylresorcinol glucuronides (**Table 4**). The associations between the respective biomarkers at the end of the intervention period are illustrated by plotting the peak area as a function of reported consumption of specific food (**Figure 3**). A significant correlation was observed between the change in peak abundance of plasma hippuric acid and change in daily intake of bilberries, but not with the change in whole grain intake (**Table 4**). The alkyl- and alkenylresorcinol-glucuronides, which were increased both in HD and WGED groups, associated strongly positively with the intake of whole grain bread, but also with the intake of berries (**Table 4**). The correlation with berry

consumption was, however, a consequence of the concurrent consumption of whole grain and berries in HD group, since after inclusion of the intervention group in the model, there was no longer association between the alk(en)ylresorcinol glucuronides and the intake of berries (beta=0.073 and p=0.510). Additionally changes in betaines correlated positively with the changes in the intake of whole grain bread (**Table 4**). Of all betaines, pipecolic acid betaine had the strongest correlation with the intake of whole grain bread ($r=0.343$, $p=4x10^{-4}$).

4. DISCUSSION

Using the non-targeted metabolite profiling with LC-MS analytics, we were able to show the concomitant changes in metabolite levels after two partially overlapping dietary modifications, *i.e*. the whole grain enriched diet (WGED) and the diet rich in bilberries and fatty fish in addition to whole grains (HD). After strict statistical filtering, 90 molecular features (out of 3130 detected) were found to have significant changes during either one or both of the intervention groups when compared to Control diet. In the HD intervention, the most important findings included the impact of fatty fish in particular on the plasma levels of furan fatty acids (CMPF) and on various PUFA containing lipids, as well as the remarkable increase in fasting plasma hippuric acid level (reflecting increased consumption of bilberries). Additionally, two novel biomarkers of whole grain consumption, the glucuronidated alk(en)ylresorcinols, were found in the interventions with whole grain products, particularly rye. Furthermore, we identified CMPF as a potential biomarker for fatty fish intake. The K-means cluster analysis on the fold change values classified the metabolic features having similar occurrence in all the study participants, further elaborating the general metabolic responses to the test diets. Betaine and amino acid metabolism also tended to be altered in both intervention groups.

CMPF correlated strongly with the intake of fish but not with other foods

The plasma level of CMPF was strongly affected by the HD diet, and correlation analyses with food intake data showed an association with the intake of fish but not with other foods. The association with intake of berries was most likely due to the concomitant increase in the consumption of fish and berries, since no correlations between the respective changes during the intervention were found. CMPF belongs to furan fatty acids, and fish is their richest source in human diet (26). However, small concentrations of furan fatty acids have also been measured in green plants, champignons, algae, vegetable oils and butter (20,26), but these foods were not associated with fasting plasma CMPF level in our study. The most likely origin for plasma CMPF are the fish-borne furan fatty acids, although the metabolic conversions are not precisely described (27).

In our study, plasma CMPF was a stronger marker for fish intake than the established biomarkers EPA and DHA. Furthermore, a model including both plasma CMPF and EPA was not able to predict fish intake considerably better than plasma CMPF alone. Analytically, plasma CMPF is a good candidate for a biomarker molecule to be quantified by a targeted measurement. It elutes in the C18 reversed phase LC column before the fatty acids and phospholipids, exhibiting excellent retention as evidenced by the sharp peak shape. Moreover, its ionization efficiency is free from the ion suppression risk associated with LC-MS analysis of metabolites co-eluting with the phospholipids. These facts support the potential application of plasma CMPF as an alternative for EPA and DHA. Indeed, several published targeted LC-MS protocols are available for measurement of plasma CMPF in renal failure (28,29), and they could be easily adjusted for utilization as straightforward biomarker analysis for nutritional assessment purposes.

Overall, the impact of the HD diet on the endogenous lipid profile was very extensive, as demonstrated here with the statistical and chemometric

analyses showing lipid species that were decreased in HD group. Several findings on the lipids also confirmed our earlier results of lipidomics analysis on the same sample set (6), *i.e*. increase in EPA, DHA, LPC(22:6), and LPC (20:5), but we discovered some additional lipid species with increase in response to the HD diet, such as LPE(22:6) and LPE(20:5).

Glucuronidated alk(en)ylresorcinols were the most prominent whole grain specific biomarkers

Glucuronidated alk(en)ylresorcinols were identified as significant biomarkers after both intervention diets, and AR C21:1-Gln was among the most important variables contributing to the PLS-DA model clustering of the end-point samples of the WGED intervention. Additionally, we found a strong positive correlation between the change in plasma levels of glucuronidated alk(en)ylresorcinol and change in consumption of whole grain bread.

The chromatographic conditions of our non-targeted analysis were most likely not suitable for analysing the intact alkylresorcinols, but the glucuronidated forms are more water–soluble and thus detectable in our analysis, and reported here for the first time in human plasma. Alkylresorcinols are phenolic lipids with 3,5-dihydroxy-5-alkylbenzene configuration found predominantly in the bran part of rye and wheat, and are well established biomarkers of intake of whole grain rye and wheat products (30). They can be measured by GC-MS method from urine and plasma following whole grain intake (31). The detection of glucuronidated alk(en)ylresorcinols in fasting plasma readily after protein precipitation in regular RP-MS conditions could offer a straightforward alternative to the presently applied analytical methods.

Interestingly, one of the glucuronidated AR markers has the C21:1 alkenyl side chain, thus exhibiting one double bond in the carbon tail. Both alkenylresorcinols and alkylresorcinols have been reported in rye and wheat (22,23), but thus far only alkylresorcinols have been discussed as dietary biomarkers. Our semi-quantitative analysis does not allow estimation of the

proportional amount of the glucuronidated ARs when compared to the regularly measured free forms (31).

We were unable to detect two main end products of endogenous metabolism of alkylresorcinols, 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-(3,5-dihydroxyphenyl)propanoic acid (3,5-DHPPA), which have been found in urine, plasma, and bile in either free or glucuronide or sulfate conjugated forms following intake of whole grain rye and/or wheat (32,33). Furthermore, glucuronidated and sulfated alkylresorcinol metabolites with novel structures, such as 5-(3,5-dihydroxyphenyl)pentanoic acid (3,5-DHPPTA) and 2-(3,5-dihydroxybenzamido)acetic acid (3,5-DHBA glycine) have been detected in human and mouse urine postprandially (34), and 2,6-DHBA has been reported as fasting plasma biomarker after a fiber-enriched dietary intervention (35). The estimated half-lives for alkylresorcinols in plasma range between 5 to 8 hours (36). Therefore it is possible that the glucuronidated forms of the alk(en)ylresorcinols reported here display different bioavailability, metabolism and kinetics in humans than the metabolites reported earlier.

Healthy diet induced increase in fasting plasma hippuric acid levels

Hippuric acid and pyrocatechol sulfate were among the most prominent metabolites of dietary related polyphenols that were increased in the fasting plasma in HD group. Since hippuric acid level was not altered in WGED group, bilberries and bilberry products were the likely sources for increased hippuric acid levels in HD group, and this was actually confirmed with the correlation analyses based on the food record data.

Several studies have reported elevated hippuric acid levels in urine after ingestion of polyphenol-rich foods and drinks, such as red wine, grape juice, black and green tea, cranberries, and blackcurrant juice (37–40), and the hippuric acid levels in 24-hour urine have been proposed as a potential biomarker for fruit and vegetables consumption (41). However, data on changes in plasma levels of hippuric acid and other phenolic acids in

response to diet are limited. An increasing trend has been reported following daily consumption of green tea over a 3-6 week period (42), and similarly after a single dose of blackcurrant juice (40). Earlier reports indicate that excretion of phenolic acids and hippuric acid is increased after intake of bilberry anthocyanins, indicating that the anthocyanins are most likely the source for the elevated levels of phenolics in fasting plasma also in our study (43,44).

We did not observe elevated levels of hippuric acid after WGED intervention, indicating different metabolism of the phenolics from this source than phenolics from berries. These results are in concordance with our earlier report, showing no differences in urinary excretion of hippuric acid after ingestion of rye bread and white wheat bread (45). Even though hippuric acid is regarded as biomarker for dietary intake of phenolics, polyphenol metabolism by intestinal microbiota and liver can produce numerous other metabolites into the circulation (46).

Whole grain consumption caused alterations in amino acid and betaine metabolism

WGED intervention tended to increase fasting plasma levels of several amino acids such as L-lysine, L-arginine, and ornithine in accordance with an earlier report on plasma amino acids as potentially useful biomarkers for intake of grain protein (47). Additionally, changes in essential amino acids such as phenylalanine and tyrosine have been observed postprandially after the intake of a sourdough fermented endosperm rye bread (48).

The diets enriched with whole grains tended to increase also betaine compounds in fasting plasma, and betaines associated positively with the intake of whole grain bread in the intervention groups. Whole grain products are known to be a major source of dietary betaine (49), and several metabolomics studies have reported increased plasma betaine levels and altered metabolism following whole grain intervention(50–53). In our study, the number of altered betaine-specific MS/MS signals was high, but many

were not identified and need to be investigated using modified analytical conditions. The high diversity of betaines and the large inter-individual variation is most likely due to altered endogenous metabolism of betaines after the increased consumption of whole grain products. Among the identified betaines, pipecolic acid betaine associated strongest with the consumption of whole grain bread, although it has not been reported as constituent of whole grains, but only in citrus genus plants (54). One potential metabolic route for the increase of pipecolic acid betaine level in fasting plasma in WGED group is the endogenous catabolism of lysine by intestinal bacteria, which was also increased in this study group (21,55). To our knowledge, pipecolic acid betaine has not been identified in human plasma earlier. Additionally, γ-butyrobetaine and propionylcarnitine were increased in the WGED group, as was reported also in our earlier work on betaine supplementation in mice (56).

Conclusion

Novel biomarkers for intakes of selected food items included in the Nordic diet were identified in fasting plasma using the non-targeted LC-MS metabolite profiling. Correlation analyses further verified the associations of 1) CMPF with increased consumption of fatty fish, 2) the glucuronidated alk(en)ylresorcinols with consumption of whole grains, and 3) hippuric acid with intake of bilberries, thus suggesting them as biomarkers of intakes of these healthy foods. Furthermore, the observations of altered endogenous metabolism of betaine, amino acids and lipids can indicate potential beneficial effects of a healthy Nordic diet, rich in whole grains, fatty fish and berries.

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7. LEGENDS FOR FIGURES

Figure 1. Partial least-squares discriminant analysis of the RP negative mode data. **A:** Score plot showing the individual end-point samples. Healthy Diet (black circle); Whole Grain Enriched Diet (gray square); Control (light grey triangle). **B:** Loading scatter plot showing the individual compounds. The depicted metabolites are: 1. Pyrocatechol sulfate, VIP 2.56; 2. CMPF, VIP 1.93; 3. Ar C21:1-Gln, VIP 1.79; 4. Hippuric acid, VIP 2.46; 5. Unknown, MW 188.094, rt 3.3, VIP 1.81; 6. Unknown lipid, MW 869.539, rt 11.8, VIP 1.76. VIP: Variable influence on projection, MW: molecular weight.

Figure 2. K-means cluster analysis with hierarchical clustering of the fold change values (beginning versus end of the intervention) for all study participants. Included are metabolic features having p_{corr} <0.05 in the RP ESI(-) data. The clusters are numbered one to seven as depicted in the figure. The color scale indicates fold change (fc) values between 0.12 and 2.1. Gray color in the heat map indicates not detected value.

Figure 3. Plots of peak areas of putative biomarkers as a function of reported consumption of selected foods in the Healthy Diet (black circle), Whole Grain Enriched Diet (dark grey square) and Control (light grey triangle) groups. A) Alkylresorcinol C21:1-Gly (AR C21:1-Gly) and the consumption of whole grain bread (at least 50 % of whole grain), B) Hippuric acid and the consumption of berries (includes whole berries, berry purées and berry juices). Three outliers in the Whole Grain Enrichment Diet group were excluded since their berry consumption included mainly juices with low polyphenol content and was more than two standard deviations away from the mean consumption of berries. C) 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and the consumption of fish.

Table 1. Annotated metabolites having p<0.05 in HD or WGED groups. Parameters for the LC-MS analysis include the chromatography (LC column), ionization mode in the mass spectrometry (MS mode), metabolite mass, identified ion (*m/z*) and retention time (Rt). The fold change values indicate the average fold change when compared against the control group, with p-values and FDR corrected p-values (n=106).

						HD			WGED		
LC column	MS mode	Mass	*m/z*	Rt (min)	Putative annotation	*p*-value	FDR-corr *p*-value	fold change	*p*-value	FDR-corr *p*-value	fold change
HILIC	ESI-	2.460.195	2.450.116	0.46	sulfated phenol derivative	0.01504	n.s.	1.36	0.11910	n.s.	1.24
HILIC	ESI-	220.004	2.189.961	0.47	sulfated phenol derivative	0.00317	n.s.	1.80	0.07858	n.s.	1.38
HILIC	ESI-	1.040.473	1.030.394	1.04	Hydroxyisobutiric acid	0.00860	n.s.	1.12	0.42560	n.s.	2.16
RPLC	ESI+	1.810.742	1.820.821	1.11	L-Tyrosine	0.03220	n.s.	1.20	0.00910	n.s.	1.20
HILIC	ESI+	1.130.593	1.140.672	1.25	Creatinine	0.59570	n.s.	0.94	0.00680	n.s.	1.23
HILIC	ESI+	1.360.386	1.370.465	1.41	Hypoxanthine	0.00170	n.s.	1.16	0.01110	n.s.	1.28
HILIC	ESI+	3.121.478	3.131.557	1.76	Phenylalanine- Phenylalanine	0.78590	n.s.	1.63	0.04440	n.s.	1.71
RPLC*	ESI-	1.899.934	1.889.855	1.91	Pyrocatechol sulfate	5.6E-09	1.98E-06	1.96	0.12520	n.s.	1.12
HILIC	ESI+	2.171.317	2.181.396	2.05	Propionylcarnitine	0.63470	n.s.	0.95	0.04270	n.s.	1.21
RPLC*	ESI-	2.130.096	212.002	2.39	Indoxylsulfuric acid	0.04410	n.s.	1.01	0.28630	n.s.	1.13
HILIC	ESI+	1.571.104	1.581.183	2.95	Pipecolic acid betaine	0.03504	n.s.	1.32	0.00765	n.s.	1.90
HILIC	ESI+	1.451.106	146.117	3.10	γ-Butyrobetaine	0.43000	n.s.	0.98	0.01219	n.s.	1.12
RPLC*	ESI+	179.058	180.067	3.14	Hippuric acid	9.89E-06	0.00060	3.31	0.64030	n.s.	1.19
HILIC	ESI+	1.290.792	130.087	3.66	Pipecolic acid	0.03780	n.s.	2.08	0.07200	n.s.	2.09
HILIC	ESI+	1.430.946	1.441.025	3.95	Proline betaine analogue	0.45340	n.s.	0.99	0.01232	n.s.	1.32
HILIC	ESI-	2.160.402	2.150.323	4.94	Hexose (Cl adduct)	0.85580	n.s.	1.11	0.02220	n.s.	1.27
HILIC	ESI+	117.054	1.180.619	5.53	Glycine-betaine analogue	0.09270	n.s.	1.04	0.01240	n.s.	1.08
HILIC	ESI+	1.871.685	1.881.764	6.03	N-acetyl Spermidine	0.13940	n.s.	0.93	0.02220	n.s.	1.20
HILIC	ESI+	1.690.853	1.700.932	6.18	3-Methyl-L-histidine	0.42970	n.s.	0.98	0.04000	n.s.	1.13
RPLC	ESI+	362.21	3.632.179	6.61	Cortisol	0.35770	n.s.	1.04	0.02940	n.s.	1.14
HILIC	ESI+	1.741.121	175.118	6.79	L-Arginine	0.45060	n.s.	0.97	0.00970	n.s.	1.19
HILIC	ESI+	1.461.055	1.471.128	6.94	L-lysine	0.24060	n.s.	0.97	0.00940	n.s.	1.63
HILIC	ESI+	1.320.899	1.330.973	6.99	L-Ornithine	0.13330	n.s.	0.96	0.00430	n.s.	1.24
RPLC*	ESI-	2.401.002	2.390.923	7.45	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF)	5.4E-09	1.98E-06	2.56	0.00174	n.s.	1.13
RPLC*	ESI-	268.131	2.671.234	8.52	3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid	0.00157	0.03322	1.22	0.11546	n.s.	1.33
RPLC	ESI+	5.413.173	5.423.252	9.57	LPC (20:5) minor isomer	1.52E-06	0.00011	1.73	0.23530	n.s.	1.13
RPLC	ESI+	5.413.178	5.423.257	9.69	LPC (20:5)	1.42E-06	0.00011	1.74	0.21420	n.s.	1.12
RPLC	ESI-	4.992.701	498.26	9.69	LPE (20:5)	0.00003	0.00164	1.57	0.29677	n.s.	1.20
RPLC	ESI+	4.933.181	494.326	9.77	LPC (16:1)	0.14292	n.s.	2.90	0.76132	n.s.	2.04
RPLC	ESI-	5.252.856	524.28	9.84	LPE (22:6)	0.00099	0.02374	1.13	0.42743	n.s.	1.03

						HD			WGED		
LC column	MS mode	Mass	*m/z*	Rt (min)	Putative annotation	*p*-value	FDR-corr *p*-value	fold change	*p*-value	FDR-corr *p*-value	fold change
RPLC	ESI+	5.673.332	5.683.411	9.84	LPC (22:6)	0.02360	n.s.	2.10	0.36440	n.s.	1.44
RPLC	ESI+	5.433.331	544.341	9.85	LPC (20:4)	0.17398	n.s.	5.84	0.34192	n.s.	2.69
RPLC	ESI-	3.022.249	301.217	10.30	EPA	5.34E-05	0.00252	1.46	0.37560	n.s.	1.08
RPLC	ESI-	3.282.408	327.24	10.48	DHA	0.00010	0.00386	1.30	0.46854	n.s.	1.05
RPLC	ESI-	552.366	5.513.581	10.84	Nonadecyl-1,3-benzenediol glucuronide (AR C19:0-Gln)	4.61E-06	0.00041	1.20	8.6E-07	0.00061	1.46
RPLC	ESI-	578.382	5.773.737	10.89	Heneicosenyl-1,3-benzenediol (AR C21:1-Gln)	3.68E-09	1.98E-06	1.18	4.19E-10	5.93E-07	1.38
RPLC	ESI+	7.795.608	7.805.687	11.84	PCh(16:0/20:5)	0.00092	n.s.	1.45	0.22833	n.s.	1.11
RPLC	ESI+	8.055.761	806.584	12.03	PCh 16:0/22:6)	0.02405	n.s.	2.35	0.71453	n.s.	0.94
RPLC	ESI+	7.315.588	7.325.667	12.04	PCh (16:0/16:1)	0.45513	n.s.	2.04	0.02411	n.s.	1.43
RPLC	ESI+	7.655.792	7.665.871	12.14	PCh (P-16:0/20:4)	0.05244	n.s.	1.60	0.11386	n.s.	1.34
RPLC	ESI+	8.075.924	808.6	12.34	PCh (20:5/18:0)	0.00180	n.s.	1.41	0.07600	n.s.	1.05

*Metabolite was detected in both chromatographic analyses and had $p<0.05$

Table 2. Associations between plasma CMPF and dietary intake (n=104-105)

Dietary intake	CMPF					
	Baseline [1]		End[2]		Change[2]	
	r	p-value	Beta	p-value	Beta	p-value
All fish	**0.254**	**0.009**	**0.472**	**$6x10^{-7}$**	**0.337**	**$4x10^{-5}$**
Fatty fish	0.228	0.190	**0.448**	**$2x10^{-5}$**	**0.280**	**0.001**
Lean fish [3]	0.098	0.317	**0.159**	**0.074**	0.112	0.116
Whole grain products	0.154	0.118	0.102	0.506	**-0.197**	**0.047**
Other grain products	-0.061	0.538	-0.179	0.200	0.166	0.094
Vegetables and root vegetables	0.135	0.169	0.069	0.437	-0.042	0.602
Fruits [4]	0.059	0.548	0.081	0.348	**-0.210**	**0.019**
Berries [4]	**0.291**	**0.003**	**0.363**	**0.001**	0.063	0.505
Milk products	-0.020	0.843	-0.078	0.360	0.078	0.343
Red meat	-0.048	0.629	0.089	0.307	-0.112	0.199
Poultry	0.099	0.314	-0.122	0.158	0.039	0.473
Potatoes	-0.052	0.597	-0.081	0.345	0.016	0.849
Nuts	-0.070	0.477	-0.064	0.454	0.060	0.457
Eggs	-0.038	0.699	-0.006	0.994	0.045	0.582
Vegetable oils and spreads	0.019	0.848	-0.056	0.519	-0.007	0.927
Dairy fat based spread	-0.056	0.572	-0.055	0.519	0.056	0.489

[1]Spearman Rank Correlation, [2] analysis of covariance (ANCOVA), intervention group included into the model, [3]consumption was low, [4]includes whole fruits/berries, purée and juice.

Table 3. Associations between plasma CMPF and markers of fish intake (n=104-106)

	CMPF					
	Baseline[1]		End[2]		Change [2]	
	r	p-value	Beta	p-value	Beta	p-value
Calculated dietary EPA intake[3]	**0.273**	**0.005**	**0.423**	**$3x10^{-5}$**	0.188	0.058
Calculated dietary DHA intake[3]	**0.254**	**0.009**	**0.389**	**$1x10^{-4}$**	**0.314**	**$2x10^{-4}$**
Plasma EPA[4]	**0.684**	**$6x10^{-16}$**	**0.376**	**$1x10^{-5}$**	**0.328**	**$1x10^{-4}$**
Plasma DHA[4]	**0.665**	**$8x10^{-15}$**	**0.363**	**$2x10^{-5}$**	**0.232**	**0.006**

[1]Spearman Rank Correlation, [2] analysis of covariance (ANCOVA), intervention group included into the model, [3]calculated from food records, [4]peak area in LC-MS analysis.

Table 4. Correlation[1] between changes in metabolites and changes in food intake (n=103-105).

CHANGE			**Intake of whole grain bread [2,3]**		**Intake of berries[3]**		**Intake of fish[3]**	
Metabolites	*mass*	*retention time*	r	p	r	p	r	p
Hippuric acid	*179.0580*	*3.14 RP(+)*	0.026	0.797	0.380	7×10^{-5}	0.119	0.228
AR C21:1-Gln	*578.3816*	*10.90 RP(-)*	0.627	1×10^{-12}	0.223	0.023	0.119	0.226
AR C19:0-Gln	*552.3660*	*10.85 RP(-)*	0.474	4×10^{-7}	0.174	0.077	0.093	0.347
Pipecolic acid betaine	*157.1104*	*2.95 HILIC(+)*	0.343	$4x10^{-4}$	0.138	0.164	-0.007	0.942
γ-butyrobetaine	*145.1106*	*3.10 HILIC(+)*	0.208	0.035	0.033	0.743	0.086	0.382
Proline betaine analogue	*143.0945*	*3.95 HILIC(+)*	0.275	0.005	-0.059	0.554	-0.012	0.901

[1]Spearman rank correlation, [2] at least 50 % whole grain, [3]Changes in dietary intake are based on 4-day food records, which were kept at study weeks 0, 3, 7 and 11.

Figure 1. Partial least-squares discriminant analysis of the RP negative mode data.

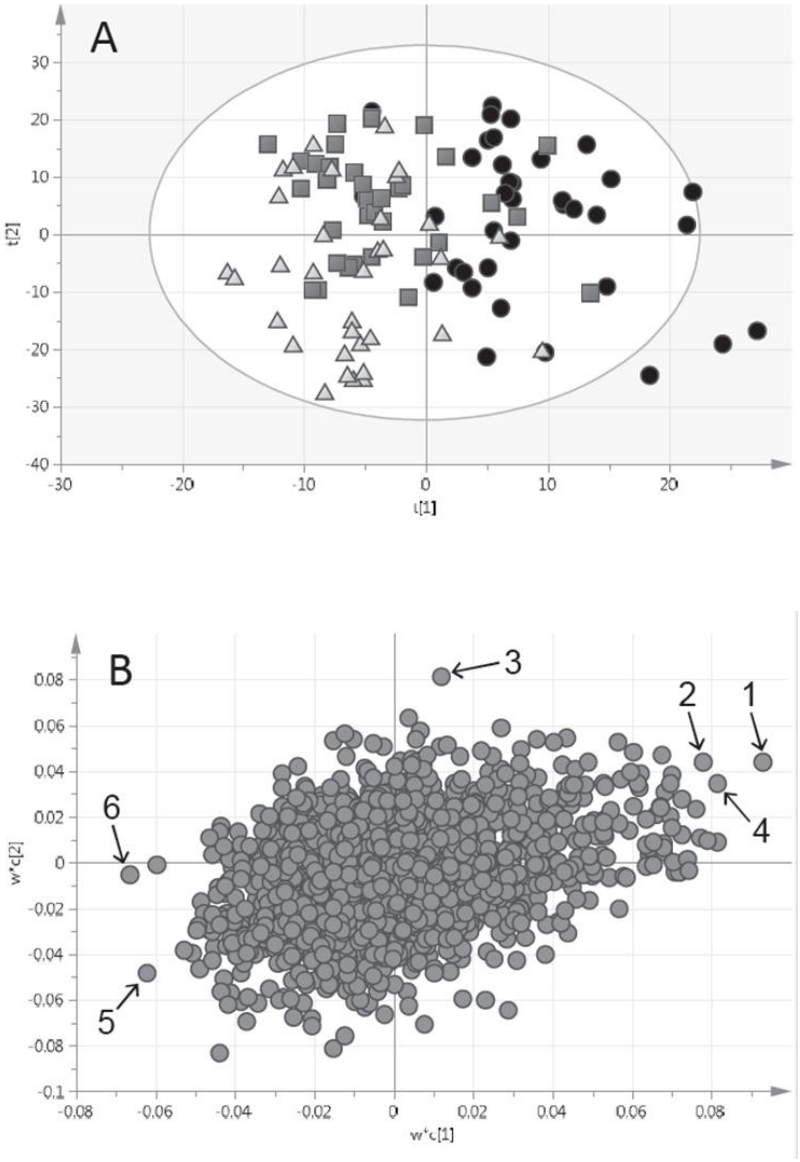


Figure 2. K-means cluster analysis with hierarchical clustering of the fold change values (beginning versus end of the intervention) for all study participants.

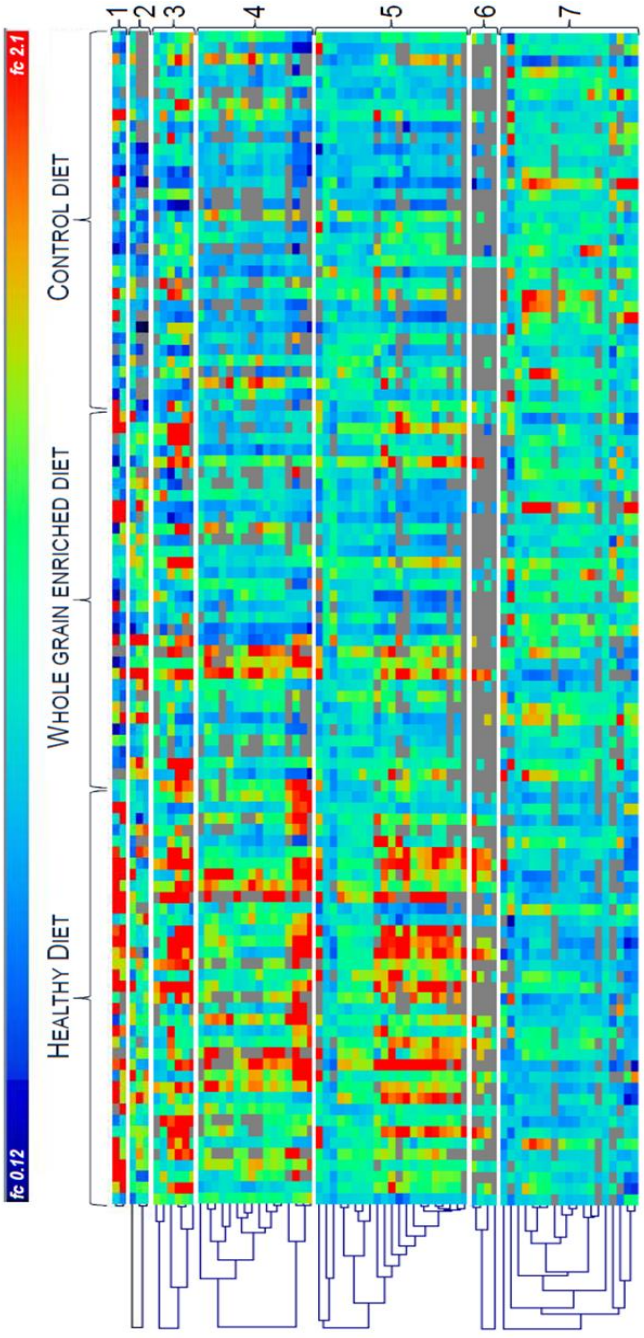
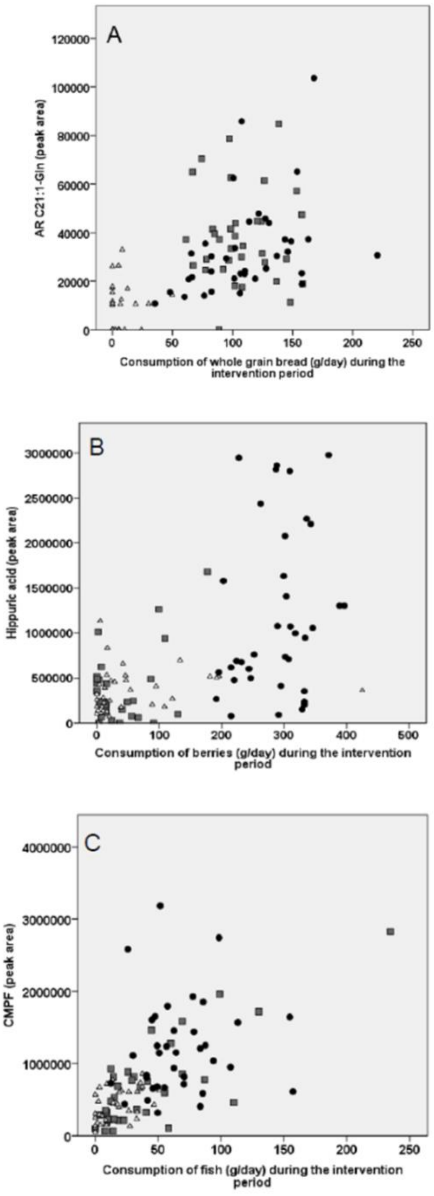


Figure 3. Plots of peak areas of putative biomarkers as a functional of reported consumption of selected foods in the HD (black circle), WGED (dark greq squares) and Control (light grey triangle) groups.



Supplemental Table 1. Clinical characteristics and the intake of energy and the most relevant nutrients (mean ± SD).

	Healthy Diet n=37				WGED n=34				Control n=35	
	0 wk[1]	12 wk[2]	p [3]	FDR p [3]	0 wk[1]	12 wk[2]	p [3]	FDR p [3]	0 wk[1]	12 wk[2]
Clinical characteristics										
Body mass index (kg/m^2)	31.1 ± 3.6	30.9 ± 3.4	0.860	0.966	31.4 ± 3.4	31.4 ± 3.4	0.171	0.675	31.0 ± 3.6	31.2 ± 3.7
Waist circumference (cm)	105.6 ± 9.5	105.0 ± 9.1	0.008	0.091	106.3 ± 11.1	105.9 ± 11.4	0.034	0.406	105.8 ± 10.0	106.3 ± 9.8
Systolic blood pressure (mmHg)	137 ± 13	132± 13	0.453	0.833	135 ±16	133 ± 15	0.453	0.805	139 ± 12	135 ± 13
Diastolic blood pressure (mmHg)	89 ± 7	86 ± 7	0.596	0.833	86 ± 8	84 ± 7	0.592	0.833	88 ± 7	86 ± 6
Serum total cholesterol (mmol/l)	5.1 ± 0.9	5.2 ± 0.8	0.396	0.793	5.1 ±1.0	5.3 ± 1.1	0.160	0.675	5.4 ± 1.0	5.4 ± 1.0
LDL cholesterol (mmol/l)	3.1 ± 0.7	3.1 ± 0.7	0.804	0.953	3.2 ±0.8	3.2 ± 0.9	0.334	0.712	3.4 ± 0.8	3.4 ± 0.8
HDL cholesterol (mmol/l)	1.3 ± 0.3	1.3 ± 0.3	0.966	0.966	1.2 ±0.4	1.2 ± 0.4	0.919	0.966	1.3 ± 0.3	1.3 ± 0.3
Serum triglycerides (mmol/l)	1.6 ± 0.6	1.7 ± 0.8	0.250	0.712	1.5 ± 0.8	1.7 ± 1.0	0.951	0.966	1.5 ± 0.8	1.6 ± 0.7
Fasting plasma glucose (mmol/l)	6.1 ± 0.5	6.1 ± 0.5	0.728	0.932	6.1 ±0.4	6.1 ± 0.5	0.506	0.833	6.2 ± 0.5	6.2 ± 0.5
2-h plasma glucose (mmol/l)	6.7 ± 1.7	6.0 ± 1.8	0.099	0.530	6.6 ± 1.6	6.1 ± 1.9	0.211	0.675	6.8 ± 1.9	6.7 ± 2.2
Dietary intake[4]										
Energy (kJ)	8386 ± 2196	9146 ± 2206	0.053	0.121	6995 ± 2373	7654 ± 2395	0.119	0.204	7282 ± 2011	8533 ± 1693
Total fat (E%)	32.5 ± 6.2	30.5 ± 4.6	0.064	0.129	33.6 ± 5.2	31.1 ± 6.3	0.012	0.043	31.3 ± 5.3	31.9 ± 5.9
SAFA (E%)	12.0 ± 3.1	10.8 ± 2.5	0.034	0.090	12.3 ± 2.4	11.1 ± 2.8	0.024	0.070	12.0 ± 2.6	12.1 ± 2.7
MUFA (E%)	10.6 ± 2.6	9.5 ± 1.9	0.171	0.261	11.3 ± 2.1	9.8 ± 2.7	0.020	0.065	10.2 ± 2.4	9.9 ± 2.6
PUFA (E%)	5.6 ± 1.5	5.4 ± 1.2	0.011	0.043	6.0 ± 1.6	5.0 ± 1.5	0.902	0.962	5.6 ± 1.5	4.6 ± 1.2
EPA	0.15 ± 0.13	0.22 ± 0.09	0.002	0.010	0.14 ± 0.24	0.12 ± 0.13	0.180	0.262	0.10 ± 0.10	0.06 ± 0.05
DHA	0.35 ± 0.28	0.59 ± 0.27	0.002	0.010	0.30 ± 0.35	0.31 ± 0.29	0.157	0.251	0.22 ± 0.24	0.17 ± 0.13
Protein (E%)	18.0 ± 3.1	18.5 ± 2.3	0.210	0.280	19.1 ± 3.2	18.8 ± 2.5	0.950	0.980	18.8 ± 3.7	18.3 ± 2.5
Carbohydrate (E%)	46.6 ± 7.1	48.1 ± 5.8	0.194	0.270	45.6 ± 6.3	47.2 ± 7.5	0.268	0.343	47.8 ± 5.6	47.3 ± 5.1
Fiber (g)	29.2 ± 8.2	36.4 ± 6.3	$4.3x10^{-13}$	$1.4x10^{-11}$	24.6 ± 7.0	26.5 ± 5.4	$2.7x10^{-7}$	$4.3x10^{-6}$	22.5 ± 7.0	18.0 ± 4.2

[1]Dietary intake is based on a 4-day food record at baseline, [2]Dietary intake is based on three 4-day food records (weeks 3, 7 and 11). [3] Based on a mixed model comparison, where the interaction of time and group compared to Controls (time*group) is analyzed, [4] In the HealthyDiet group n=37.

Supplemental Table 2. Metabolic features with FDR corrected p<0.05 in at least one of the test groups (HD, Healthy Diet; WGED, Whole grain enriched Diet).

mass	rt	HD p-value	HD FDR corr. p-value	WGED p-value	WGED FDR corr. p-value	LC-MS mode
189,9938	0,493355	4,84031E-08	1,77322E-05	0,133172913	0,962318162	HILIC ESI (+)
110,0368	0,49362028	5,10207E-08	1,77322E-05	0,119809792	0,962318162	HILIC ESI (-)
240,0994	0,5201768	7,37817E-08	1,77322E-05	0,002451289	0,441844842	HILIC ESI (+)
851,5463	0,588121	1,15116E-05	0,007275331	0,987412205	0,99687622	HILIC ESI (+)
851,5675	0,6144882	0,00018567	0,022311345	0,168055235	0,962318162	HILIC ESI (+)
587,3214	0,8136639	7,38487E-05	0,010648983	0,428098633	0,973444572	HILIC ESI (-)
179,058	1,0425574	4,80936E-05	0,008668871	0,653769303	0,977093398	HILIC ESI (-)
380,098	1,0518724	0,000345102	0,035545506	0,80108224	0,991020773	HILIC ESI (-)
189,9934	1,9126782	5,60359E-09	1,98227E-06	0,125196431	0,993631105	RP ESI (-)
179,0586	3,1450853	9,89231E-06	0,000596836	0,640296535	0,999338103	RP ESI (+)
104,0267	3,1460624	5,08147E-05	0,002299365	0,288974497	0,999338103	RP ESI (+)
179,0583	3,1497583	6,1464E-06	0,000511597	0,626483877	0,993631105	RP ESI (-)
222,0899	7,4488095	2,55087E-08	4,61707E-06	0,001888884	0,430376008	RP ESI (+)
180,0788	7,448404	5,02731E-09	1,81989E-06	0,005802385	0,999338103	RP ESI (+)
254,1158	7,448584	3,57443E-07	4,31315E-05	0,067309769	0,999338103	RP ESI (+)
502,1789	7,4503036	5,27113E-08	1,24311E-05	0,005558921	0,989136335	RP ESI (-)
196,1098	7,450658	9,54542E-10	1,35068E-06	0,001043689	0,369205087	RP ESI (-)
240,1002	7,450778	5,39598E-09	1,98227E-06	0,001742705	0,493185628	RP ESI (-)
308,083	7,4518	1,84434E-08	5,21948E-06	0,007819543	0,993631105	RP ESI (-)
188,1409	8,124126	0,00079082	0,020345642	0,544054638	0,993631105	RP ESI (-)
268,1313	8,521267	0,001573191	0,033224855	0,115462023	0,993631105	RP ESI (-)
587,3225	9,562276	8,64729E-07	0,000145833	0,3480684	0,993631105	RP ESI (-)
541,3173	9,569969	1,51497E-06	0,000109684	0,235320701	0,999338103	RP ESI (+)
1128,6385	9,674337	1,32014E-06	0,000173235	0,169718077	0,993631105	RP ESI (-)
527,3019	9,674516	0,000110726	0,004352147	0,727600613	0,993631105	RP ESI (-)
1282,248	9,6751	0,001488638	0,032406504	0,80238463	0,993631105	RP ESI (-)
577,2934	9,6753645	0,000514844	0,015837049	0,628794188	0,993631105	RP ESI (-)
587,3231	9,675394	9,27562E-07	0,000145833	0,319192256	0,993631105	RP ESI (-)
701,3023	9,675462	6,13068E-07	0,000123927	0,255575018	0,993631105	RP ESI (-)
723,2932	9,675517	0,000274669	0,009253729	0,420186493	0,993631105	RP ESI (-)
659,2523	9,675558	2,84749E-06	0,000309938	0,648771909	0,993631105	RP ESI (-)
655,3096	9,675588	1,3467E-06	0,000173235	0,413119971	0,993631105	RP ESI (-)
673,2683	9,675987	4,44787E-06	0,000407696	0,560310932	0,993631105	RP ESI (-)
541,3178	9,685477	1,4154E-06	0,000109684	0,214182196	0,999338103	RP ESI (+)
499,2703	9,6896	1,69456E-05	0,00087633	0,217939166	0,999338103	RP ESI (+)
499,2701	9,6897705	3,1281E-05	0,001639356	0,296767812	0,993631105	RP ESI (-)
687,247	9,692872	0,000052915	0,015930793	0,686036507	0,993631105	RP ESI (-)
613,3378	9,821916	0,000181559	0,006587333	0,770118392	0,993631105	RP ESI (-)
477,2855	9,834772	0,001979693	0,038906467	0,199138396	0,993631105	RP ESI (-)
525,2856	9,836399	0,000989846	0,023739527	0,427429093	0,993631105	RP ESI (-)
501,2857	9,839699	0,001182454	0,027886207	0,853397654	0,993631105	RP ESI (-)
613,3384	9,909094	5,14157E-05	0,002508732	0,816099144	0,993631105	RP ESI (-)

mass	rt	HD p-value	HD FDR corr. p-value	WGED p-value	WGED FDR corr. p-value	LC-MS mode
1108,67	9,925616	0,001512341	0,032423674	0,628947252	0,993631105	RP ESI (-)
525,2863	9,928424	6,05993E-05	0,002766065	0,18757731	0,993631105	RP ESI (-)
1044,6176	9,930789	0,000262477	0,009058657	0,239821214	0,993631105	RP ESI (-)
501,286	9,939335	0,000162072	0,006035049	0,520005407	0,993631105	RP ESI (-)
1539,9493	9,940709	3,51387E-05	0,001775759	0,648792973	0,993631105	RP ESI (-)
1020,6175	9,941704	0,000366633	0,012064784	0,478914697	0,993631105	RP ESI (-)
477,2862	9,947106	0,001854566	0,037488727	0,25434158	0,993631105	RP ESI (-)
996,6178	9,9475565	0,001650665	0,034348397	0,340184758	0,993631105	RP ESI (-)
1582,0175	10,1095	0,000391676	0,012595944	0,079348342	0,993631105	RP ESI (-)
1086,6855	10,113635	9,50381E-05	0,003856211	0,309680005	0,993631105	RP ESI (-)
503,3011	10,114712	0,001436264	0,032258945	0,686850757	0,993631105	RP ESI (-)
591,3538	10,119609	0,000134882	0,005158325	0,20952931	0,993631105	RP ESI (-)
613,3557	10,297085	1,60909E-05	0,00108422	0,394416763	0,993631105	RP ESI (-)
370,2121	10,301224	7,52131E-05	0,003324199	0,582259556	0,993631105	RP ESI (-)
302,2249	10,301281	5,34268E-05	0,002519964	0,37562021	0,993631105	RP ESI (-)
431,1538	10,30366	1,11083E-05	0,000786485	0,779037517	0,993631105	RP ESI (-)
1374,967	10,483133	3,41925E-06	0,000345588	0,215563689	0,993631105	RP ESI (-)
851,6043	10,484104	2,43721E-05	0,001436938	0,249229288	0,993631105	RP ESI (-)
739,403	10,484601	0,000984835	0,023739527	0,583284305	0,993631105	RP ESI (-)
328,2408	10,484611	9,53833E-05	0,003856211	0,46853979	0,993631105	RP ESI (-)
678,4621	10,485334	0,002279205	0,043582096	0,799436194	0,993631105	RP ESI (-)
396,2276	10,485919	0,000232904	0,008238979	0,429879936	0,993631105	RP ESI (-)
919,5907	10,487602	0,000671639	0,01719757	0,496847925	0,993631105	RP ESI (-)
444,2328	10,602536	0,000641514	0,017702983	0,185621849	0,993631105	RP ESI (-)
552,366	10,84702	4,60999E-06	0,000407696	8,5993E-07	0,0006084	RP ESI (-)
578,3816	10,891702	3,67808E-09	1,98227E-06	4,19E-10	5,92885E-07	RP ESI (-)
866,4715	11,476284	0,00057023	0,016466846	0,081262535	0,993631105	RP ESI (-)
823,5358	11,515031	0,000640765	0,017702983	0,893498329	0,993631105	RP ESI (-)
873,5517	11,556156	0,000564884	0,016466846	0,282209913	0,993631105	RP ESI (-)
811,5359	11,624635	0,000474362	0,01491605	0,133786606	0,993631105	RP ESI (-)
869,5388	11,779243	0,000650569	0,017702983	0,633884935	0,993631105	RP ESI (-)
801,5524	11,785342	1,77862E-05	0,001143976	0,32147294	0,993631105	RP ESI (-)
825,553	11,814418	2,1338E-05	0,001312751	0,351713984	0,993631105	RP ESI (-)
971,4816	11,820216	0,001726569	0,035407176	0,572238865	0,993631105	RP ESI (-)
1451,1385	11,866824	0,001485843	0,032406504	0,702546429	0,993631105	RP ESI (-)
1530,132	11,907536	0,001209767	0,028062628	0,207758209	0,993631105	RP ESI (-)
774,5887	11,974042	0,000916005	0,022739422	0,422685368	0,993631105	RP ESI (-)
721,5044	12,005768	1,05664E-05	0,000786485	0,131275965	0,993631105	RP ESI (-)
839,5671	12,048171	2,90008E-05	0,001578313	0,337879341	0,993631105	RP ESI (-)
747,5201	12,099164	2,56969E-05	0,001454445	0,01393611	0,993631105	RP ESI (-)
811,5725	12,124447	7,75255E-05	0,003324199	0,120321543	0,993631105	RP ESI (-)
1585,1285	12,12596	0,000676231	0,01719757	0,906639341	0,993631105	RP ESI (-)

mass	rt	HD p-value	HD FDR corr. p-value	WGED p-value	WGED FDR corr. p-value	LC-MS mode
835,5722	12,237691	1,11164E-05	0,000786485	0,166321217	0,993631105	RP ESI (-)
853,5845	12,332424	1,89759E-06	0,000223757	0,384146505	0,993631105	RP ESI (-)
919,4654	12,34315	0,000824694	0,02083825	0,0727673	0,993631105	RP ESI (-)
841,5237	12,343224	0,001920098	0,038266742	0,435234393	0,993631105	RP ESI (-)
843,5983	12,54822	0,002238856	0,043397003	0,288686262	0,993631105	RP ESI (-)
749,5357	12,585786	0,001341073	0,030606747	0,12956552	0,993631105	RP ESI (-)

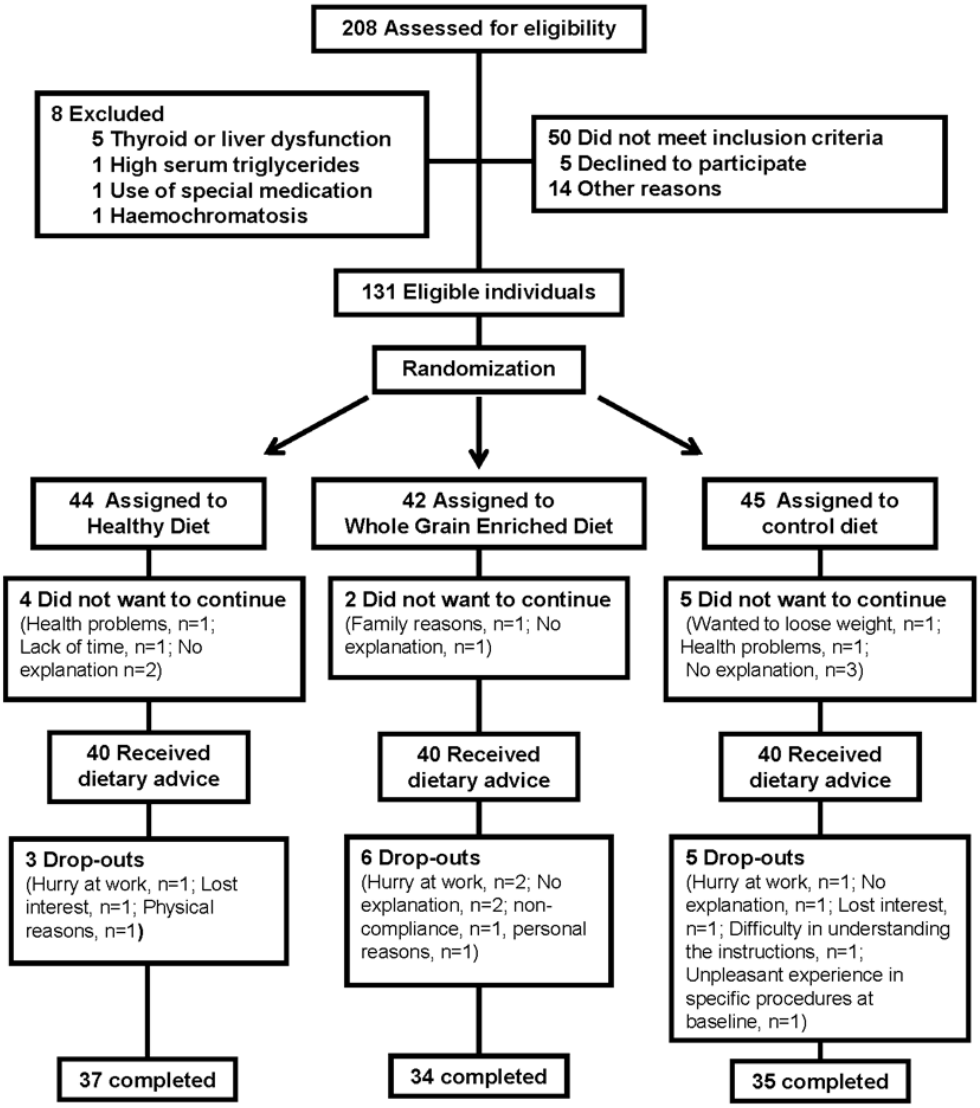
Supplemental Table 3. MS/MS fragmentation data for all of the identified metabolites.

Column	Ionization Mode	Mass	m/z	Retention Time (min) (RT)	Putative annotaion	CID	MS/MS fragmentation	Identification reference*
HILIC	ESI-	246,020	245,012	0,46	sulfated phenol derivative	10eV	165.092, 79.956	
HILIC	ESI-	220,004	218,996	0,47	sulfated phenol derivative	20eV	124.015, 139.039, 138.850, 79.954, 156.442, 59.930, 80.9627	Methoxycatechol standard
HILIC	ESI-	104,047	103,039	1,04	Hydroxyisobutyric acid			
RPLC	ESI+	181,074	182,082	1,11	L-Tyrosine	10eV	136.075, 147.044, 165.054, 123.045, 119.050	Standard
HILIC	ESI+	113,059	114,067	1,25	Creatinine	10eV	44.049, 86.071, 72.041	MID 8
HILIC	ESI+	136,039	137,047	1,41	Hypoxanthine	20eV	110.035, 119.036, 55.029, 94.041, 67.029, 82.039	Standard
HILIC	ESI+	312,148	313,156	1,76	Phenylalanine-Phenylalanine	10eV	120.081, 166.084, 186.893, 155.105, 267.144, 296.132	MID 23981
RPLC*	ESI-	189,993	188,986	1,91	Pyrocatechol sulfate	40eV	109.030, 108.021, 79.957, 81.034, 91.020, 53.041	Pyrocatechol standard
HILIC	ESI+	217,132	218,140	2,05	Propionylcarnitine	10eV	85.028, 60.080, 159.062, 144.100	Standard
RPLC*	ESI-	213,010	212,002	2,39	Indoxylsulfuric acid	10eV	80.965, 80.965, 132.0444	Metlin
HILIC	ESI+	157,110	158,118	2,95	Pipecolic acid betaine		71.050, 58.066, 97.085, 72.046, 112.462	Servillo *et al.*, 2012
HILIC	ESI+	145,111	146,117	3,10	γ-Butyrobetaine	20eV	87.044, 60.081, 45.034, 91.053	
RPLC*	ESI+	179,058	180,067	3,14	Hippuric acid	10eV	105.033, 77.039, 51.024	Standard
HILIC	ESI+	129,079	130,087	3,74	Pipecolic acid			Standard
HILIC	ESI+	143,095	144,103	3,95	proline betaine analogue	20eV	98.096, 70.062, 58.065	
HILIC	ESI+	117,054	118,062	5,53	glycine-betaine analogue	10eV	89.9052, 59.0703, 76.0376	
HILIC	ESI+	187,169	188,176	6,03	N-acetyl Spermidine	10eV	100.075, 72.080, 171.145, 122.212	MID 3323
HILIC	ESI+	169,085	170,093	6,18	3-Methyl-L-histidine	10eV	96.068, 109.075, 126.100, 83.059, 153.064, 170.130	MID 3293
RPLC	ESI+	362,210	363,218	6,61	Cortisol	20eV	121.065, 363.219, 241.157, 97.065, 297.185, 267.174, 309.183, 201.130, 135.080	MID272
HILIC	ESI+	174,112	175,118	6,79	L-Arginine	10eV	60.056, 116.071, 158.088, 130.097, 72.080	
HILIC	ESI+	146,106	147,113	6,94	L-lysine	10eV	84.081, 105.953, 147.113, 130.086	Standard
HILIC	ESI+	132,090	133,097	6,99	L-Ornithine	10eV	116.069, 70.065, 115.084	Standard
RPLC*	ESI-	240,100	239,092	7,45	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF)			Standard
RPLC*	ESI-	268,131	267,123	8,52	3-carboxy-4-methyl-5-pentyl-2-furanpropionic acid			MS/MS
RPLC	ESI+	541,317	542,325	9,57	LPC (20:5) minor isomer			MS/MS

Column	Ionization Mode	Mass	m/z	Retention Time (min) (RT)	Putative annotaion	CID	MS/MS fragmentation	Identification reference*
RPLC	ESI+	541,318	542,326	9,69	LPC (20:5)	20eV	184.073, 104.107, 524.314, 86.097, 60.080; ESI(-) 20eV: 585.988, 526.295, 301.215	MS/MS
RPLC	ESI-	499,270	498,260	9,69	LPE (20:5)	10eV	301.215, 453.179, 325.837, 236.166, 196.034	MS/MS
RPLC	ESI+	493,318	494,326	9,77	LPC (16:1)	20eV	184.073, 104.107, 476.313, 86.097; In ESI-: 538.316, 478.294, 253.217	MS/MS
RPLC	ESI-	525,286	524,280	9,84	LPE (22:6)	20eV	327.233, 283.243, 196.037, 78.960, 140.010	MS/MS
RPLC	ESI+	567,333	568,341	9,84	LPC (22:6)	20eV	184.073, 104.107, 550.331, 86.098, 60.081; ESI(-) 20eV: 528.309, 303.232, 588.331	MS/MS
RPLC	ESI+	543,333	544,341	9,85	LPC (20:4)	20eV	104.107, 184.073, 86.097; ESI(-) 20eV: 528.309, 303.232, 588.331	MS/MS
RPLC	ESI-	302,225	301,217	10,30	EPA	10eV	257.228, 100.933, 189.857, 86.040	
RPLC	ESI-	328,241	327,240	10,48	DHA	10eV	283.243, 229.195, 59.013	
RPLC	ESI-	552,366	551,35806	10,84	5-Nonadecyl-1,3-benzenediol glucuronide (AR C19:0-Gln)	20eV	375.326, 285.878, 227.90, 175.024, 113.023, 59.013	MS/MS
RPLC	ESI-	578,382	577,374	10,89	5-(16-Heneicosenyl)-1,3-benzenediol (AR C21:1-Gln)	20eV	401.341, 287.052, 175.026, 113.024, 85.028	MS/MS
RPLC	ESI+	779,561	780,569	11,84	PCh(16:0/20:5)	40eV	184.074, 86.097, 124.999, 60.081; ESI(-) 40eV: 303.217, 255.232, 824.548, 764.525	MS/MS
RPLC	ESI+	805,576	806,584	12,03	PCh 16:0/22:6)	40eV	184.074, 86.097, 125.000, 60.081; ESI(-) 40eV: 327.231, 255.233, 790.540, 850.566	MS/MS
RPLC	ESI+	731,559	732,567	12,04	PCh (16:0/16:1)	40eV	184.073, 86.096, 60.081, 125.000; ESI(-) 40eV: 253.216, 255.232, 716.523, 776.548	MS/MS
RPLC	ESI+	765,579	766,587	12,14	PCh (P16:0/20:4)	20eV	184.073, 86.096, 60.081; ESI(-) 20eV: 750.543, 303.232, 810.563	MS/MS
RPLC	ESI+	807,592	808,600	12,34	PCh (20:5/18:0)	40eV	184.074, 86.096, 124.999, 60.082; ESI(-) 40eV: 301. 217, 283.264, 792.552, 852.580	MS/MS

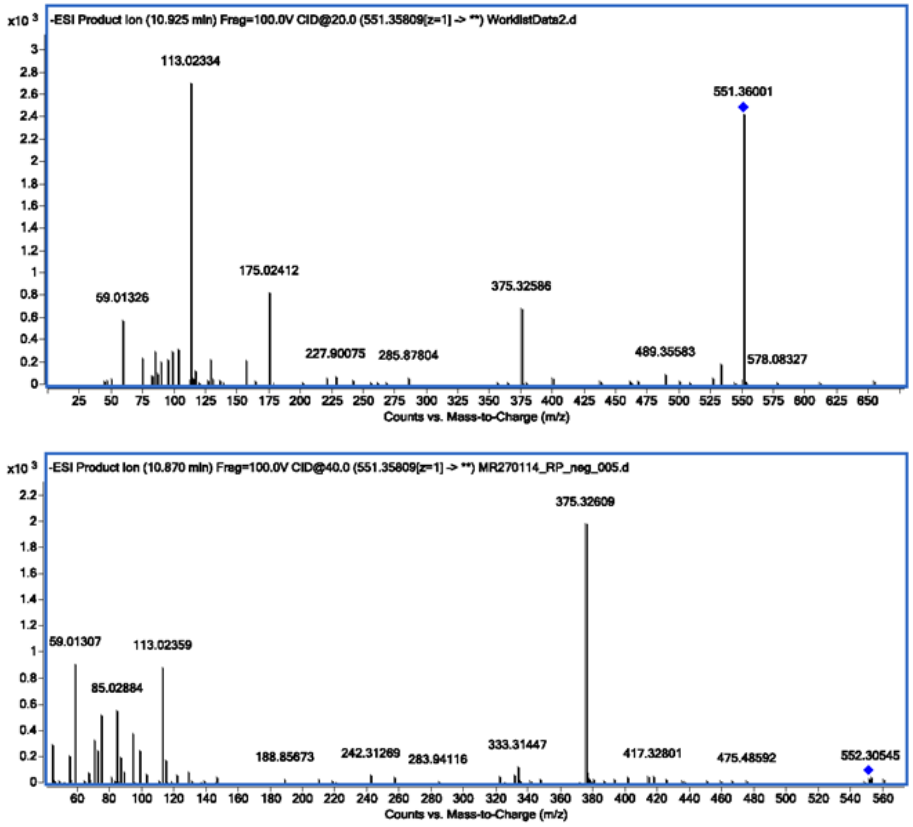
*Identification is based on manual MS/MS spectral interpretation, Metlin ID when MSMS spectrum available, or commercial standard compound. Servillo et al., 2012, Occurrence of pipecolic acid and pipecolic acid betaine (homostachydrine) in Citrus genus plants. J. Agric. Food Chem. 60:315-321.

Supplemental Figure 1. Participants Flow chart.



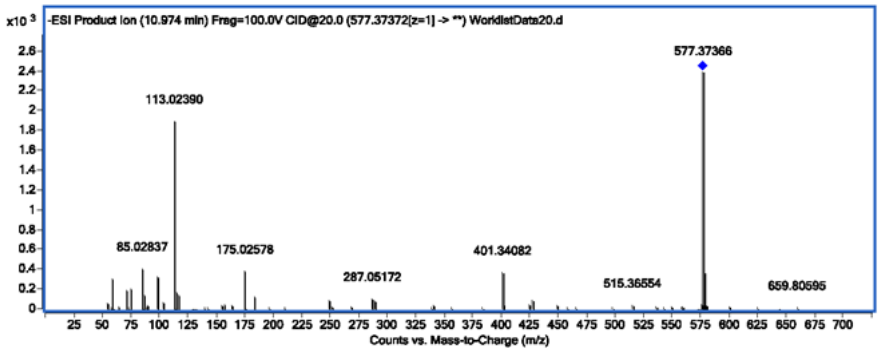
Supplemental Figure 2. LC-qTOF-MS/MS fragmentation of two alk(en)ylresorcinol glucuronides.

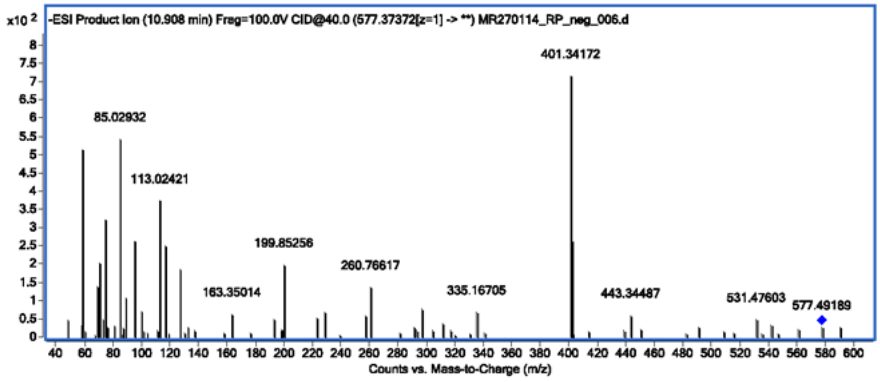
***m/z* 551.3581 at Rt 10.84**



The first neutral loss 176 refers to glucuronide unit. The peaks *m/z* 175.0241 and *m/z* 113.0233 result from the deprotonated glucuronide unit. The aglycon ion gives molecular formula of C25H44O2, which matches with Nonadecyl-1,3-benzenediol, *i.e*. alkylresorcinol with C19:0 tail unit. Analysis with higher collision energy (CID 40eV) shows further fragmentation with neutral loss of 42 amu, which is visible twice: *m/z* 375.3261 to *m/z* 333.3145, and *m/z* 283.941 to *m/z* 242.3127 referring to shortening of the tail by two carbon-units. The minor fragments include *m/z* 122.035 and *m/z* 81.0306, which have been detected in the LC-MS(-) analysis of intact alkylresorcinol C19:0 earlier (Dr. Alastair Ross, personal communication) (1, 2).

***m/z* 577.3737 at Rt 10.89**





The neutral loss of glucuronide was observed for the molecule *m/z* 577.3737. The aglycon ion gives Metlin hit to 5-(16-Heneicosenyl)-1,3-benzenedion – an alkylresorcinol with the C21:1 configuration in the carbon tail. The fragmentation pattern of the aglycon follows essentially the one seen for *m/z* 551.3600, with numerous smaller fragments in the region 60 to 130 amu (1, 2).

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UNIVERSITAT ROVIRA I VIRGILI
BIOACTIVE FOOD COMPOUNDS OF MEDITERRANEAN AND NORDIC DIETS AND THEIR EFFECTS ON NUTRITIONAL AND CARDIOVASCULAR DISEASE.
Anna Pedret Figuerola
Dipòsit Legal: T 1916-2014

STUDY 3

Impact of virgin olive oil and phenol-enriched virgin olive oils on the high-density lipoprotein proteome in hyper_ cholesterolemic subjects. A double blind, randomized, controlled, cross-over clinical trial (VOHF study)

Impact of virgin olive oil and phenol-enriched virgin olive oils on the high-density lipoprotein proteome in hypercholesterolemic subjects. A double blind, randomized, controlled, cross-over clinical trial (VOHF study).

First author's surname: Pedret

Short title: Impact of olive oil phenols on HDL proteome.

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ABSTRACT

Background: The effects of olive oil phenolic compounds (PC) on the complex structure of high-density lipoprotein (HDL)-proteome, with respect to new aspects of cardioprotective properties, are still unknown. The aim of the current study was to assess the impact on the HDL protein cargo of the intake of virgin olive oil (VOO) and two functional VOOs, enriched with their own PC (FVOO) or complemented with thyme PC (FVOOT), in hypercholesterolemic subjects.

Methods and Results: The VOHF study is a double blind, randomized, cross-over trial in which HDL fractions were obtained from 33 hypercholesterolemic subjects who received for 3 weeks 25 mL/day of: VOO, FVOO, and FVOOT. Using a quantitative proteomics approach, based on iTRAQ-OFFGEL-LC-MS/MS analysis, 127 HDL-associated proteins were identified. Among these, 15 were commonly differently expressed after the three VOO interventions compared to baseline, with specific changes observed for each intervention. Ingenuity pathway analysis revealed that the overlapping proteins, with cardioprotective functions, among the interventions were involved in the following pathways: LXR/RXR activation, acute phase response, atherosclerosis, IL-12 and production in macrophages, production of nitric oxide and reactive oxygen species in macrophages, clathrin-mediated endocytosis, and coagulation system.

Conclusions: Consumption of VOO, or phenol-enriched VOOs, has an impact on the HDL proteome in a cardioprotective mode by up-regulating proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation while down-regulating proteins implicated in acute-phase response, lipid transport, and immune response. The common observed protein expression modifications after the three VOOs indicate a major matrix effect.

Clinical Trial Registration: ISRCTN77500181.

Key words: cardiovascular disease, high-density lipoprotein, olive oil, phenolic compounds, proteomics.

[Word count = 250]

INTRODUCTION

Phenolic compounds (PC) are versatile, heterogeneous, bioactive compounds naturally distributed in a number of edible plant products including fruit, vegetables, nuts, olive oil (OO), wine, tea, cocoa, and coffee. Epidemiologic studies suggest that a high dietary intake of PC is associated with beneficial effects on human health and provides protection against a number of chronic conditions including neurodegenerative and cardiovascular diseases (CVD), and some cancers[1–3]. Most of these PC health effects have been attributed to their antioxidant and radical scavenging activities, signaling cascade modifications, transcriptional networks[2], and gene expression coding of key metabolic proteins[1,4]. OO is a food item typical of the Mediterranean diet, and several studies have revealed that it has a unique phenolic profile with specific biological properties. Results from the European EUROLIVE study[5] showed that an increase in plasma high-density lipoprotein (HDL) cholesterol, and a decrease of low-density lipoprotein (LDL) oxidation, took place in a direct relationship with the PC content of the OO administered. These findings provided evidence to recommend the use of phenol-rich OO, i.e. virgin olive oil (VOO), in order to achieve beneficial effects. To date, studies have been focused on hydroxytyrosol because it has been reported as being the most biologically active PC in OO[6]. In fact, the European Food Safety Authority (EFSA) has recently released a health claim concerning the protective effects of the ingestion of PC from OO on lipid oxidative damage. The EFSA panel considers that the claim may be used only for OO which contains at least 5mg of hydroxytyrosol and its derivatives per 20g of OO. Thus, 20g of OO should be consumed daily in order to confer protection to LDL particles from oxidative damage[6]. As the phenolic concentration in most of the VOOs available on the market is too low to provide this daily amount, the enrichment of VOO with its own PC could be a possible approach to assure and standardize the consumption without increasing caloric intake[7,8]. Furthermore, the enrichment of VOOs with hydroxytyrosol derivatives

combined with complementary phenols from aromatic herbs, such as thyme used for oil flavoring, might be a good strategy to provide the optimum balance amongst the different kinds of OO PC such as flavonoids, monoterpens, and phenolic acids[9].

As previously mentioned, several of the beneficial effects of the OO PC have been linked to a lipid profile improvement and, more specifically, to the HDL molecule. HDL has traditionally been recognized as playing a central role in cholesterol reverse transport to the liver. In concrete, the efflux of cholesterol from peripheral tissues promoted by HDL is considered to be its main anti-atherosclerotic function. In this context, Farràs M, et al. (2013)[10] have described the key role of OO PC in the up-regulation of genes involved in the cholesterol efflux from cells to HDL in humans. In addition to cholesterol efflux, more novel biological and atheroprotective activities are currently being ascribed to the HDL particle, in particular to its protein cargo. Approximately one hundred different proteins, not considered to be apolipoproteins (APO), have been identified as being associated with the HDL particle[11] and linked to its cardioprotective properties. These include the antioxidant, anti-inflammatory, antithrombotic, complement system regulation and protective effects on vascular endothelium and smooth muscle cells[12,13]. Such new data promise to better elucidate the relationship of HDL with atherosclerosis and contribute to the development of biomarkers of HDL functionality[14]. The considerable number of proteins and functions identified within the HDL proteome suggest that mere determination of HDL cholesterol, which is the current clinical marker related with HDL mass, probably do not provide accurate information about HDL functionality. Analysis of the HDL protein cargo is, however, still in its early stages, and data concerning the HDL proteome as a biomarker for disease or functionality are limited. Moreover, studies with respect to the impact of dietary interventions on the HDL proteome are scarce[15] , and the effects of OO PC on the complex structure of the HDL protein cargo have not yet been evaluated.

In this context, we hypothesized that sustained consumption of PC from VOO, or functional phenol-enriched VOO, could modify the HDL proteome, which in turn could be related to their cardioprotective benefits. The objective of the current study was to assess the impact on the HDL protein cargo of a dietary intervention supplemented with a VOO or two different functional VOOs enriched with their own PC (hydroxytyrosol derivatives) or complemented with thyme PC (flavonoids, monoterpens, and phenolic acids), in hypercholesterolemic subjects from the VOHF study.

METHODS

Subjects and Study design

The VOHF study aimed at assessing whether functional VOOs, enriched with their own PC or with them plus complementary phenols from thyme, could have a nutraceutical effect on HDL lipoprotein functionality. The present clinical trial was conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community. All participants provided written informed consent, and the local institutional ethics committees approved the protocol (CEIC-IMAS 2009/3347/I) registered with the International Standard Randomized Controlled Trial register (www.controlled-trials.com; ISRCTN77500181).

The VOHF study was a randomized, controlled, double-blind, crossover clinical trial with 33 hypercholesterolemic volunteers (total cholesterol>200 mg/dL; 19 men and 14 women), aged 35 to 80 year. Exclusion criteria included the following: BMI >35 Kg/m^2, smokers, athletes with high physical activity (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or any other disease or condition that could worsen compliance. The study was conducted at IMIM- Hospital del Mar Medical Research Institute (Spain) from April 2012 to September 2012. The participants' flow chart is described in **Supplemental Figure 1**.

Subjects were randomly allocated to one of 3 sequences of administration of 25 mL/day of raw: a) VOO; 80 mg of PC/kg oil, b) functional VOO enriched with its own PC (FVOO; 500 mg of PC/kg oil), and c) functional VOO enriched with its own PC plus complementary phenols from thyme (FVOOT; 500 mg of PC/kg; 50% of OO PC and 50% of thyme PC) (Sequence 1: FVOO, FVOOT and VOO, Sequence 2: FVOOT, VOO and FVOO, Sequence 3: VOO, FVOO and FVOOT) (**Figure 1**). The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the random sequence was done by a physician. Due to the fact that all participants received each one of the three VOOs, restrictions such as blocking were unnecessary. Intervention periods were of 3 weeks and VOOs were consumed daily distributed among meals. There was a 2-week washout period prior to VOO interventions during which a common OO was consumed. To avoid an excessive intake of PC, the participants were advised to limit their consumption of polyphenol-rich foods during the clinical trial. A set of portable containers with the corresponding 25 mL of OO for each day of consumption were delivered to the participants at the beginning of each OO administration period. The participants were instructed to return the containers to the center after the corresponding OO consumption period in order to register the amount consumed.

The procedure to obtain the phenolic extracts and the enriched oils has been previously described[9]. **Supplemental Table 1** shows the phenolic compounds, the fat soluble micronutrients and the fatty acids daily intake with 25mL of VOO, FVOO, and FVOOT.

Sample size and power analysis

A sample size of 30 individuals allows a power of at least 80% to detect a statistically significant difference among groups of 3 mg/dL of HDL cholesterol, assuming a dropout rate of 15% and a Type I error of 0.05 (2-sided).

Collection of blood samples

Fasting blood samples were taken from 33 participants before the first washout period (first visit-baseline) and after each VOO intervention period (visits 3, 5, and 7), through a catheter in an antecubital vein (**Figure 1**). Blood was collected in Vacutainer® tubes with K_2EDTA anticoagulant. Blood samples were centrifuged at 1 500 x*g* for 15 minutes and 2.8 mL of plasma were finally recovered. Protease Inhibitor Cocktail (PIC; Sigma-Aldrich, Tres Cantos, Spain) was added to plasma at a concentration 1/100 (1 µL of PIC for 100 µL of plasma). All samples were stored at -80ºC until processing. A total of 123 plasma samples were used for proteomic analyses (33 participants per 4 visits minus 9 missing). See proteomic study flow chart (**Figure 2**).

Human plasma HDL isolation

The HDL fraction (d=1.036-1.21 g/mL) from each sample was isolated from 2.5 mL of plasma by sequential density ultracentrifugation in two steps using sodium bromide (NaBr; Sigma-Aldrich, Tres Cantos, Spain), as previously described by Havel RJ, et al.[16]. Briefly, the VLDL, IDL, and LDL, were removed by ultracentrifugation at 140 000 x*g* for 21 h at 10 ºC after adjustment of the plasma density to 1.063mg/mL with NaBr. The HDL fraction was obtained by adjusting the infranatant density to 1.21 mg/mL with NaBr prior to ultracentrifugation at 140 000 x*g* for 40 h at 10 ºC. 2 mL of HDL fraction were recovered and later stored at -80ºC.

HDL biochemical characterization

Apo A-I, Apo A-II, and Apo B-100 were determined in HDL fractions using routine clinical chemistry assays (Horiba, Montpellier, France) in an autoanalyzer Cobas-Mira Plus (Roche Diagnostic System, Madrid, Spain). Total protein was quantified in HDL fractions by the Bradford method (BioRad, Hercules, CA, USA). HDL fraction purity was assessed through the

measurement of Apo B-100, almost all samples exhibited concentrations below the limit of detection (2 mg/dL).

Statistical analysis of biochemical data

The Kolmogorov-Smirnov test was used to verify the distributions of the variables. A paired Student's t-test was employed for the comparison of paired and normally distributed variables. Wilcoxon signed-rank test was used for the comparison of paired and non-normally distributed variables. The level of statistical significance was set at $p < 0.05$. All statistical analyses were performed with Statistical Package for the Social Sciences (SPSS) for Windows (20.0 version; IBM corp., Armonk, NY, USA).

Proteomic sample preparation and quantitative analysis

For the proteomics studies (detailed in Supplementary Data) the 123 isolated HDL samples were reduced to ten pools: 1 from first visit- baseline, 3 after third visit, 3 after fifth visit, and 3 after seventh visit (**Figure 2**). The pools were then dialyzed and delipidated by methanol/ diethyl ether extraction, followed by a trichloroacetic acid/acetone protein precipitation before an in-solution tryptic digestion. Samples were subjected to an iTRAQ labeling for quantitative analysis, the resulting labeled peptides were separated according to their isoelectric point on an OffGel fractionator. Finally, two aliquots of the sample were analyzed by nLC-MALDI MS/MS and nLC-ORBITRAP-ESI MS/MS (**Figure 2**). The files generated with the two mass spectrometers were combined in order to identify the HDL-associated proteins. The final identified proteins were required to present more than one peptide-spectrum match (PSM), or to have a minimum confidence score > 30% and coverage > 10%, in order to ensure accuracy in the assignment of protein identifications. In addition, intracellular and cell surface proteins were removed as being possible contaminants. In order to identify the differentially expressed proteins after interventions with respect to first visit- baseline, only samples analyzed by nLC-ORBITRAP-ESI MS/MS were used due to the precision of this method. Relative expression

levels were calculated for each protein as a ratio by Proteome Discoverer; biological replicates from the three iTRAQ sequences were then combined in an Excel spreadsheet and the mean of the ratios of all proteins calculated. Proteins with a differential expression of at least 0.8-fold change, or 1.3-fold change relative to baseline, were considered differentially expressed. In addition, a cut-off inferior to 0.5, or superior to 1.5, was applied to establish the most relevant protein expression changes observed after each intervention, defined as stronger effects. A detailed description of the methods and analysis is provided in the online-only Data Supplement.

Clustering and Pathway analysis

Various bioinformatic tools were employed for the biological interpretation of the results. Proteins are referred to by their gene encode symbol.

Hierarchical clustering of protein expression data to investigate overall similarities of the proteome samples and identify the main biological functions involved was performed in web-tool STRING 9.1 (http://string-db-org). Ingenuity Pathway analysis (IPA; Ingenuity System Inc., Redwook, CA, USA, www.ingenuity.com) was used to analyze canonical pathways and protein networks involving the differentially expressed proteins for biological interpretation. Significance levels were assessed with Fisher's exact tests (p<0.01). The differentially expressed proteins were overlaid with IPA-curated canonical pathways to explore possible metabolic and cell signaling pathways that were over- or under-represented by the experimentally determined genes. Specifically, we analyzed the proteins that overlapped the three VOO interventions in order to investigate potential common OO PC mechanisms. In addition, possible connections between mapped genes were evaluated and graphical networks were algorithmically generated. Nodes representing genes and gene products were linked by biological relationships. Networks were ranked by a score that defines the probability of a collection of nodes being equal to or greater than the number in a network achieved by chance alone.

RESULTS

Participants' characteristics

Table 1 summarizes the baseline characteristics of the study participants, significant differences were not observed among groups. The three VOOs were well tolerated by all participants and no adverse events were reported.

Biochemical characterization of HDL

Compared to baseline, significant increases in Apo A-I (p = 0.027; p = 0.021; p = 0.006, respectively), Apo A-II (p = 0.029; p = 0.019; p = 0.004, respectively) and total protein (p = 0.073; p = 0.045; p = 0.037, respectively) were observed after the VOO, FVOO, and FVOOT intervention periods.

HDL Proteomic results

Identification of HDL-associated proteins

A total of 155 HDL-associated proteins were initially identified by applying shotgun proteomics using Maldi and Orbitrap MS/MS to HDL fractions isolated by ultracentrifugation. Data were subsequently screened to ensure protein identification accuracy and 28 possible false-positives removed. The final list of HDL-associated proteins was refined to 127 proteins (**Supplemental Table 2**). The majority (80/127) were consistent with well-established HDL-associated proteins determined in at least 3 different MS studies[11] and 32 had been previously identified by at least 1 MS study[11,17]. A total of 15 novel proteins were identified: Beta-Ala-His dipeptidase, BPI fold-containing family B member 1, Carbonic anhydrase 6, CD209 antigen, CD44 antigen, CD5 antigen-like, Ig heavy chain V-I region HG3, Ig lambda-1 chain C regions, Ig lambda-2 chain C regions, Ig lambda-7 chain C region, Indian hedgehog protein, Integrin alpha-2, Integrin beta-1, Multimerin-2, and Thymidine phosphorylase. Six well-established HDL-associated proteins were not detected: Apo O, Ceruloplasmin, Complement factor B, Inter alpha trypsin inhibitor 2, Plasma Kallikerein and Plasminogen[11].

Quantitative analyses

Compared to baseline values (first visit), the HDL protein cargoes of the differentially expressed proteins differed after the interventions according to the VOO received. The complete list of the proteins differentially expressed after each VOO intervention, and their principal biological functions, are shown in **Supplemental Table 3**. The proteins were associated with a broad range of biological functions, principally cholesterol homeostasis, lipid transport, acute-phase response, blood coagulation, immune response, protection against oxidation, and proteolysis. The proteins differently modulated after VOO, FVOO, and FVOOT interventions and associated with these main functions are represented in **Figure 3**. The overlapping among interventions indicates that 15 proteins were commonly up- or down-regulated after the three VOO interventions. These proteins were: Serum paraxonase/lactonase 3 (PON3), Apo A-II, Apo A-I, Apo D, Retinol binding protein 4 (RBP4), Heparin cofactor 2 (SERPIND1), zinc-alpha-2-glycoprotein (AZGP1), alpha-2-antiplasmin (SERPINF2), alpha-2-HS-glycoprotein (AHSG), clusterin (CLU), alpha-2-macroglobulin (A2M), haptoglobin (HP), alpha-1-acid glycoprotein (ORM1), Beta-Ala-His dipeptidase (CNDP1), and Aminopeptidase N (ANPEP). A protein-protein interaction network was generated for the 15 common, differentially expressed proteins using database and web-tool STRING 9.1 (**Supplemental Figure 2**). The common up-regulated proteins were related to cholesterol homeostasis, blood coagulation and protection against oxidation; the common down-regulated proteins were implicated in acute-phase response, lipid transport, immune response, and proteolysis. All proteins except for ANPEP and CNDP1, which are involved in proteolysis, appeared in the center of the functional network intersection indicating their key role in protein interactions.

In addition to these 15 commonly modulated proteins, other specific protein expression changes were observed after each VOO intervention. The stronger effects for each VOO were related to the following biological functions linked to atherosclerosis protection: transport, homeostasis

cholesterol, antioxidant protection, blood coagulation, innate immune response, acute phase, and cellular adhesion. A stronger protein expression change was observed after VOO intervention in the following 6 proteins: Apo C-I, Apo A-I, Cholesteryl ester transfer protein (CETP), PON3, histidine-rich glycoprotein (HRG), and Ig heavy chain V-I region HG3, which were up-regulated while Ig lambda chain V-III region LOI was down-regulated. A stronger effect after FVOO was observed on 2 protein expression changes, PON3 and platelet basic protein (PBP) which were up-regulated. Finally, a stronger up-expression change was noticed after FVOOT in the following 7 proteins: Apo A-I, Apo A-IV, Apo C-II, Apo C-III, serum amyloid A protein 4 (SAA4), afamin (AFM) and integrin beta-3 (ITGB3).

Clustering and Pathway Analysis

IPA analysis was performed to reveal the canonical pathways significantly affected (p<0.01) by OO phenols. The top 7 signaling pathways modified by the differentially expressed common proteins after all VOO interventions included: LXR/RXR activation, acute phase response signaling, atherosclerosis signaling, IL-12 signaling and production in macrophages, production of nitric oxide (NO) and reactive oxygen species (ROS) in macrophages, Clathrin-mediated endocytosis signaling, and coagulation system (**Figure 4**). Our results showed that OO phenols tended to have a strong effect on LXR/RXR activation (p=2.39E-16; ratio=0.071), followed by acute phase response signaling (p=4.91E-15; ratio=0.052). The highest scoring associated network generated by IPA, and the differentially expressed proteins common for all VOO interventions, are shown in **Figure 5**. The top scoring significantly associated network (score=30) included 12 focused proteins and several associated genes, and was related to the following biological functions: lipid metabolism, small molecule biochemistry, and molecular transport. The nuclear receptor subfamily 5, group A, member 2 (NR5A2) and interleukin 6 (IL-6) were associated genes appearing in this network and representing the significantly affected top upstream regulators (p<0.01). NR5A2, which is implicated in the canonical pathway of FXR/RXR

activation and the biological process of cholesterol homeostasis, was predicted from our results to be activated. Likewise, IL-6, which is involved in the acute phase response canonical pathway, was expected to be inhibited.

DISCUSSION

The present study revealed that, in hypercholesterolemic subjects, a supplementary consumption of 25 mL/day of raw VOO, FVOO, or FVOOT has an impact on the HDL proteome by changing the expression of a number of proteins with biological functions related to the cardioprotective function of the HDL particle. The greatest expression modifications were observed in 15 of the 127 proteins identified in the HDL fractions which were commonly up- or down- regulated after the three VOO interventions. A finding that encouraged us to highlight the effect of the fatty acid composition and the role of the PC of the common matrix of the three VOO tested on HDL remodeling. After the three VOO interventions, the commonly up-regulated proteins were mainly related to the following biological functions: cholesterol homeostasis, blood coagulation, and protection against oxidation. Down-regulated ones were involved in acute-phase response, lipid transport, and immune response leading to a CVD protective HDL profile. Moreover, IPA analysis revealed that signaling pathway LXR/RXR activation, acute phase response signaling, atherosclerosis signaling, IL-12 signaling and production in macrophages, production of NO and ROS in macrophages, Clathrin-mediated endocytosis signaling, and coagulation system were affected by OO PC consumption. These data emphasize the key role of OO PC in modifying pathways and conferring cardioprotective properties. To our knowledge, this is the first HDL proteomic study in humans that assesses the effects of OO PC on the HDL protein cargo.

Common effects of VOO interventions

Two well-known proteins related to cholesterol homeostasis, Apo A-I and Apo A-II, were significantly augmented after the three VOO interventions.

Both proteins participate in reverse cholesterol transport and present anti-inflammatory and antioxidant properties[13,18]. Our results are reinforced by the fact that these increases were also observed in our biochemical characterization of HDL determined by autoanalyzer. The increase in Apo A-I concurred with the results observed by Solà et al.[19] in high CVD risk subjects after following a Mediterranean diet rich in VOO.

Two proteins related to blood coagulation, SERPIND1 and A2M, were up-regulated after the three VOO interventions thus increasing the cardioprotective activity of the HDL particle. SERPIND1 inactivates thrombin action in the subendothelial layer of the vascular wall whilst A2M inhibits proteinases regulating the extracellular proteolytic activity resulting from clotting and fibrinolysis[20,21].

PON3 was up-regulated after all VOO interventions. It is one of the three 3 known genotypic forms of the paraoxonase family and very similar in activity to PON1 although it has been less studied [22]. PON3 acts as an antioxidant inhibiting LDL oxidation and enhancing the capacity of the HDL particle to protect against oxidation. The effect of up-regulating PON3 could be indicative of an improvement in the oxidative status of the HDL particle promoted by the interventions.

AHSG, ORM1, SERPINF2, and HP are acute-phase response proteins which were down-regulated after all VOO interventions. Acute-phase proteins are increased during inflammation and could be used as inflammatory biomarkers related to CVD[23,24]. Our results are in concordance with those of Santos-González et al.[25] who observed a decrease in acute-phase response proteins after a VOO diet in rats.

Plasma Apo D is a component of HDL and is structurally similar to the lipocalins, a diverse family of lipid-binding proteins that are responsible for transporting lipids and other small hydrophobic molecules. RBP4, which also belongs to the lipocalin family, is an adipokine that delivers retinol from the

liver stores to the peripheral tissues. These two proteins, which have been reported to be up-regulated during CVD development[26,27], decreased after the three VOO interventions.

CLU, or Apo J, is a component of the HDL particle which participates in diverse physiological processes such as the complement system, innate immune response, lipid transport, cholesterol efflux, apoptosis, oxidation, and inflammation[28]. It has been suggested that CLU might play a compensatory protective role by acting as an inflammatory modulator in myocardial infarction[29] . In the same way, AZGP1, which is mainly related to immune response, lipid metabolic process, and anti-inflammatory effects acting as an adipokine[30], has been observed to be up-regulated to counteract metabolic stress situations in humans[30]. Both proteins were down-regulated after all VOO interventions. Our results concur with Santos-González et al.[25] who showed down-regulated levels of CLU after a VOO diet in rats.

Stronger effects of each VOO intervention

In addition to the commonly modulated proteins, specific strong effects on the HDL protein cargo were also observed after each intervention. For instance, after the intervention with VOO, Apo C-I, Apo A-I, CETP, and PON3 were markedly up-regulated. Among these changes the up-regulation of CETP is noteworthy. This protein facilitates the transport of cholesteryl ester from HDL to Apo B-100 containing lipoproteins, and its plasma activity has been suggested to be inhibited after a PC intervention [31]. To our knowledge, data concerning the effects of PC on the expression and activity of CETP directly determined in HDL samples instead of plasma samples are limited. Our results should be complemented with further studies assessing the activity and functionality of this enzyme in HDL in order to determine whether the observed expression changes are relevant for the biological processes involved. In addition, a major increase in histidine-rich glycoprotein (HRG), which plays a role in modulating coagulation, innate

immunity, and inflammation, was detected after VOO[32]. Changes in some immunoglobulins after VOO intervention were also reported. Immunoglobulins, which are secreted proteins related to innate immune response, could be plasmatic contaminants and, as a result, several HDL proteomic studies have discarded them as being HDL-associated[17]. The literature with respect to their functioning as HDL-associated proteins is, however, scarce. Nevertheless, the possibility that the HDL particle could act as a transporter of these proteins cannot be discarded and their role should be further studied.

From all the marked expression changes observed after the FVOO intervention, we would like to emphasize the up-regulation of the PON3 and platelet basic protein (PBP). Up-regulation of PON3 has also been described as a major effect after VOO and further studies are needed in order to determine the activity and functionality of this enzyme in HDL. PBP is a small platelet-derived cytokine that is released from activated platelets and is related to platelet activation and immune response, which could be of interest in atherosclerosis research.

We also highlight the strong up-regulation of Apo A-I, Apo A-IV, Apo C-II, and Apo C-III among the changes observed after the FVOOT intervention. Apo A-IV is mainly related to cholesterol homeostasis and oxidative protection, and has been associated with the cardioprotective properties of the HDL particle[33]. Although Apo C-III protein is present in HDL, it is also a major protein in VLDL. Chang et al.[34] showed positive associations between HDL- Apo C-III and the presence of CVD, suggesting that high levels of Apo C-III in the HDL particle may represent a class of dysfunctional HDL. Such results are, however, still controversial. A redistribution of APOs between proatherogenic lipoproteins such as LDL to cardioprotective HDL could have been possible during the FVOOT intervention. We also observed a relevant increase in Serum amyloid A protein 4 (SAA4) which is an acute-phase response protein and a biomarker of inflammatory response associated with atherosclerosis[35,36]. Controversially, it has been suggested that SAA proteins

might exert an atheroprotective function during acute-phase response by improving HDL cholesterol efflux activity from macrophages[37]. Further studies focused on the effects of phenol-enriched VOO intake on the HDL functionality are needed. Other observed changes were the clear increase in afamin (AFM) and integrin beta-3 (ITGB3) proteins after the FVOOT intervention. Afamin, which is related to vitamin E transport[38], has been identified in several HLD proteomic studies and suggested as being a well-established HDL-associated protein[11]. Integrin beta-3, which plays a critical role in platelet aggregation and normal hemostasis[39], has been identified in only a few HDL proteomic studies[13] and data concerning its presence and function in the HDL particle are limited.

Limitations and strengths

One of the strengths of this study is its randomized and crossover design, which permitted the participants to ingest all olive oils types. In this proteomic analysis a pooling sample approach was employed in order to focus primarily on the whole protein response and also to optimize the associated research expenses. HDL isolation is a key methodological point for proteomic studies. Whilst sequential ultracentrifugation may alter HDL functionality, composition, and lead to a loss of lipid-poor Apo A-I it continues to be the most commonly used procedure in these studies[40]. Moreover, the possibility exists that non-HDL plasmatic contaminants could have been present in the isolated HDL fraction. We identified HDL-associated proteins with two different MS techniques, Orbitrap and Maldi, which provide reliability and robustness to our identification results.

In conclusion, consumption of VOO, or phenol-enriched VOOs, has an impact on the HDL proteome in a cardioprotective mode that could enhance HDL functionality by up-regulating proteins related to cholesterol homeostasis, protection against oxidation, and blood coagulation while down-regulating proteins involved in acute-phase response, lipid transport, and immune response. The common protein expression modifications

reported after the three VOOs indicate an important effect of the fatty acid and PC composition present in the common matrix of these VOO on the HDL remodeling. Further studies are needed, however, in order to assess the specific effects of each VOO incorporating different phenolic contents.

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DISCLOSURES

None.

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FIGURES LEGEND

Figure 1. Experimental protocol for the intervention study. VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own PC; FVOOT: Functional Virgin Olive Oil enriched with its own PC plus complementary phenols from thyme. Blood collection for proteomic analysis: Visit 1, baseline; Visit 3, post-first intervention; Visit 5, post-second intervention; Visit 7, post-third intervention.

Figure 2. Proteomic study flow chart. S1: Sequence 1; S2: Sequence 2; S3: Sequence 3; HDL: High-density lipoprotein; P.: Pool; SCX: Strong Cation Exchange. Visits with n lower than 11 was due to loss of participants for the following reasons: dropout, did not participate in all interventions, did all the interventions but not all the tests, or sample loss during laboratory processing.

Figure 3. Venn diagram showing intersections of proteins differentially expressed after VOO, FVOO, and FVOOT interventions. Proteins are presented with their gene encode symbol. Red proteins: up-regulated; green proteins: down-regulated. VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own PC; FVOOT: Functional Virgin Olive Oil enriched with its own PC plus complementary phenols from thyme.

Figure 4. The top 7 signaling pathways that were significantly affected by the 15 common proteins differentially expressed after all VOO interventions. The pathways were ranked by *p* value. Blue bars indicate the negative log value (*p*-value). The ratio was calculated as the number of molecules in a given pathway that meet cutoff criteria divided by total number of molecules that make up that pathway.

Figure 5. Top scored associated network generated by IPA describing common differentially expressed proteins after all VOO interventions. Proteins were presented with their gene encode symbol. The proteins indicated in red and green are those whose expression levels were

significantly up- or down-regulated, respectively. Proteins indicated in white are those available in the IPA database, but not detected as differentially expressed in the present study. The shapes of the symbols denote the molecular class of proteins. Solid lines indicate direct molecular interactions, whereas dashed lines indicate indirect molecular interactions.

Table 1. Characteristics of the study participants at baseline.

Variable	Sequence 1 (n=11)	Sequence 2 (n=11)	Sequence 3 (n=11)
Gender (male/female)	5/6	7/4	7/4
Age, years	54.91 ± 12.57	55.27 ± 11.88	55.45 ± 7.84
Body weight, kg	74.75 ± 16.80	74.60 ± 18.49	84.45 ± 17.74
BMI, kg/m2	25.63 ± 3.68	26.31 ±5.25	27.85 ± 4.71
SBP, mm Hg	125.09 ± 18.70	128.27 ± 16.69	130.45 ± 17.93
DBP, mm Hg	68.09 ± 13.53	72.27 ± 9.31	71.91 ± 13.43
Glucose, mg/dL	88.55 ± 11.63	93.00 ± 13.33	90.91 ± 10.53
Total cholesterol, mg/dL	228.36 ± 42.70	231.91 ± 32.70	218.82 ± 31.21
LDL cholesterol, mg/dL	150.38 ± 32.33	152.08 ± 28.46	142.26 ± 25.72
HDL cholesterol, mg/dL	52.78 ±11.75	52.96 ±12.82	53.39 ± 9.55
Tryglicerides, mg/dL	94.00 (75.00; 149.00)	119.00 (95.00; 168.00)	117.00 (81.00; 126.00)

Values expressed as mean ± standard deviation (SD) or median (25^{th} to 75^{th} percentile). Sequence 1= FVOO, FVOOT and VOO; Sequence 2= FVOOT, VOO and FVOO; Sequence 3= VOO, FVOO and FVOOT. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high-density lipoprotein.

Figure 1. Experimental protocol for the intervention study

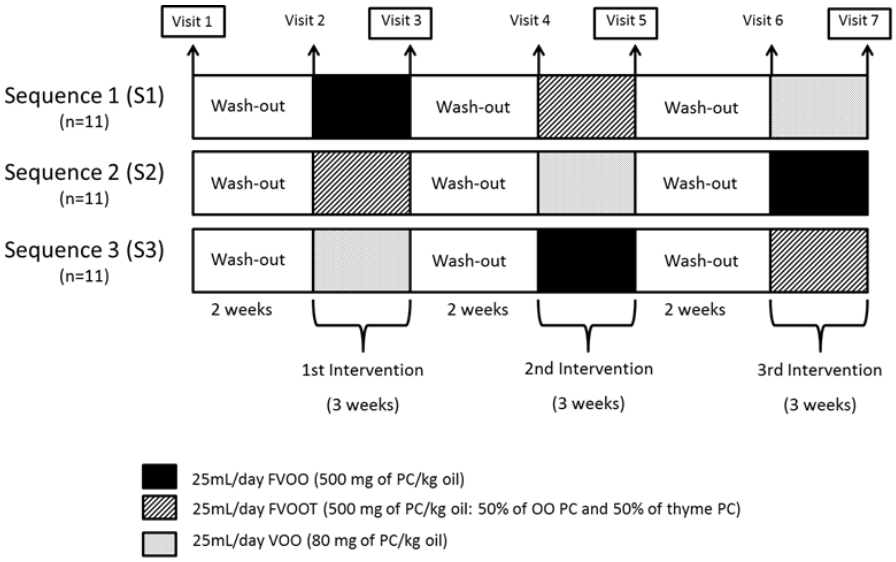


Figure 2. Proteomic study flow chart

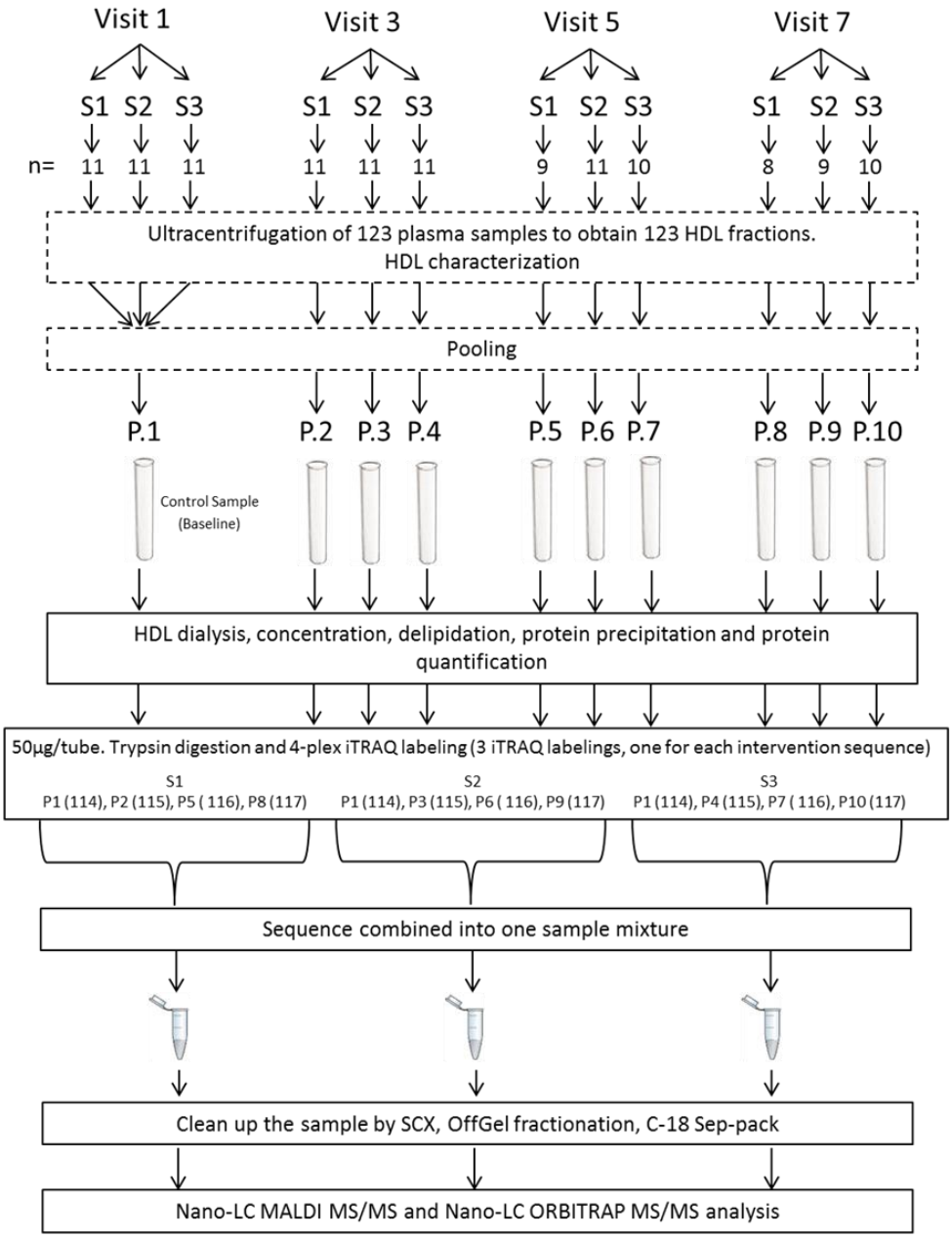


Figure 3. Venn diagram showing intersections of proteins differentially expressed after VOO, FVOO and FVOOT

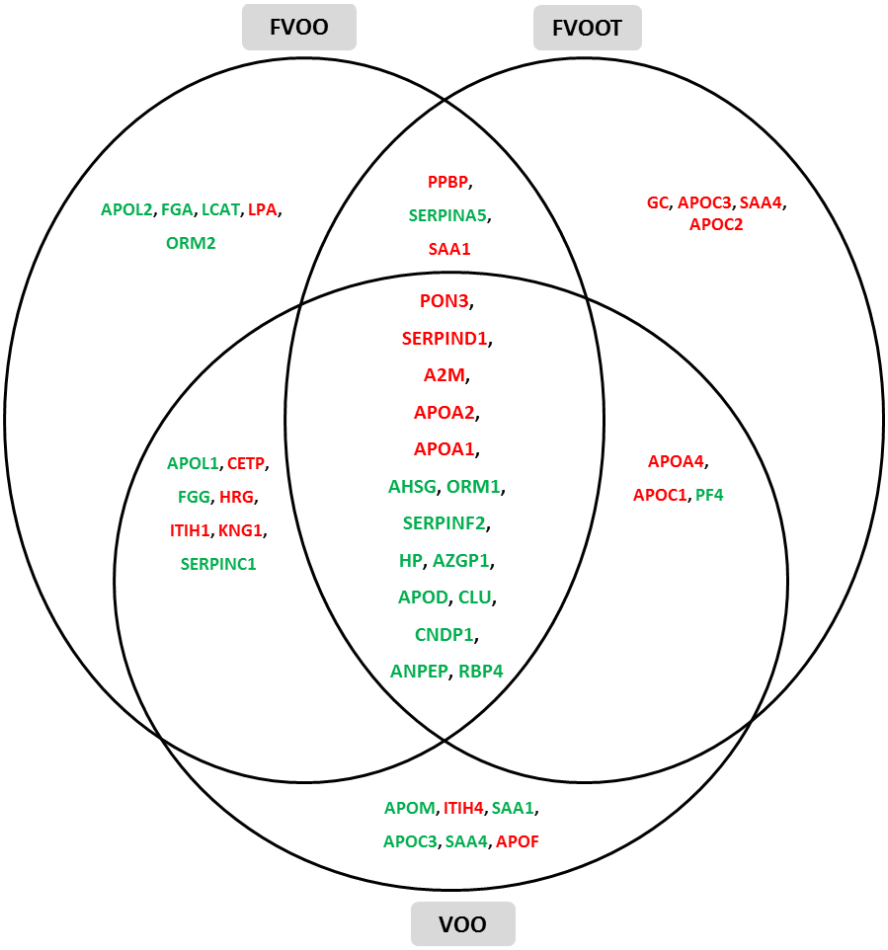


Figure 4. The top 7 signalling pathways that were significantly affected by the 15 common proteins differentially expressed after all VOO interventions

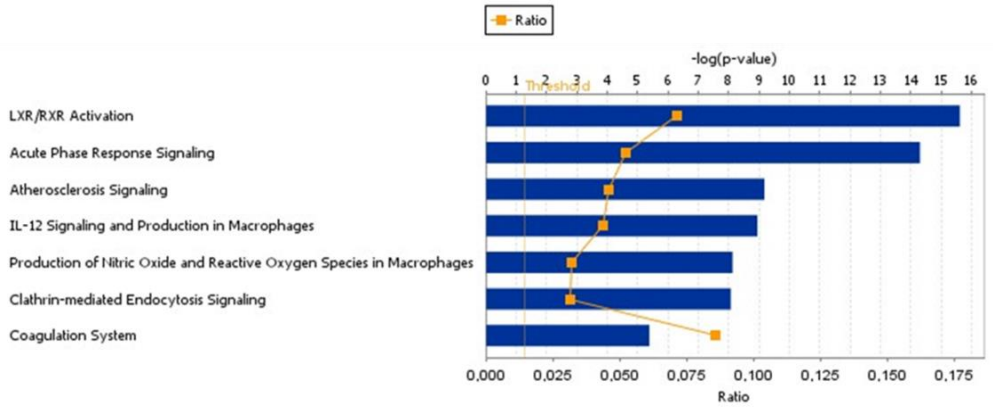
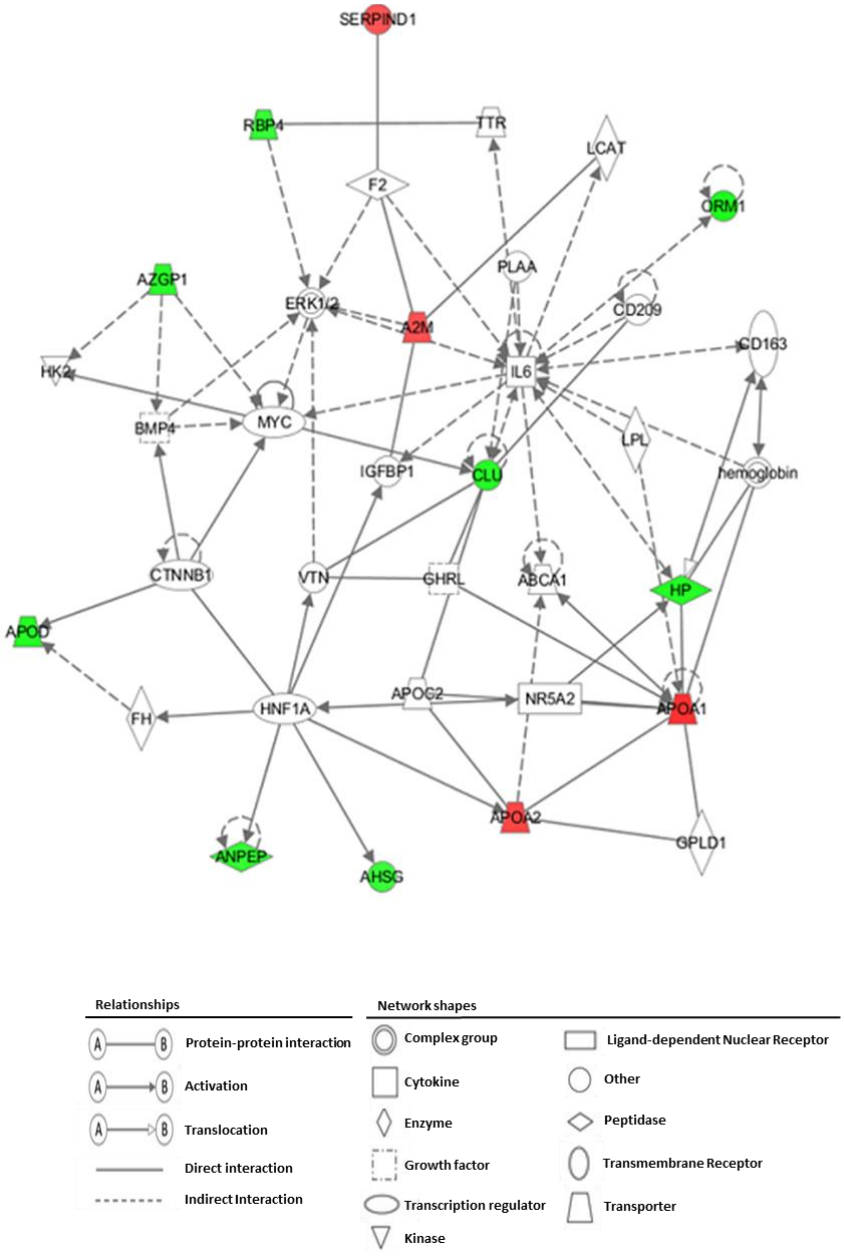


Figure 5. Top scored associated network generated by IPA describing common differentially expressed proteins after all VOO interventions.



SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Proteomic sample preparation and analysis

Pooling

The 123 isolated HDL samples were divided into ten pools taking into consideration the randomized sequence detailed in Figure 1 and the visit number. Pool 1 includes the HDL fraction from visit 1 of all participants (n=33) in order to normalize the data obtained between the three groups (Figure 2). Protein pools were dialyzed using Amicon Ultra-4 10K filters (Millipore, Co. Cork, IRL) at 4 000 x*g* for 20 minutes at 4 ºC.

HDL delipidation and protein precipitation

After concentration, pools were delipidated and precipitated. Briefly, concentrated HDL samples were dripped over 3 mL of methanol (Sigma-Aldrich, Tres Cantos, Spain) and then 7 mL of diethyl ether (-20°C; Sigma-Aldrich, Tres Cantos, Spain) were added. Samples were incubated for 10 minutes on ice, centrifuged at 1 000 rpm for 5 minutes at 4 °C, and supernatants were aspired. The step was repeated with 10 mL of diethyl ether (-20°C) and finally pelleted samples were suspended into 200 µL of sodium dodecyl sulfate- dithiothreitol (SDS-DTT) solution (62.5 mM Tris-HCl, 2%, SDS, 40 mM DTT) and stored at -80ºC. Samples were precipitated with trichloroacetic acid (TCA) (Sigma-Aldrich, Tres Cantos, Spain) and acetone (AppliChem GmbH , Darmstadt, Germany), to concentrate and purify them. The pellet was resuspended in dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5 and 0.1% SDS). Total protein concentration was calculated by Bradford assay (Sigma, St Louis, MO, USA).

In-solution protein digestion

50 µg of each sample was reduced for 1 h at 60 ºC with 5 mM tris-(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich, Tres Cantos, Spain) and alkylated for 30 min at room temperature in the dark with 4mM iodoacetamide (Sigma-Aldrich, Tres Cantos, Spain). Sequencing grade trypsin (Promega Corporation, Madison, USA) was added in a 1:100 (w/w) ratio and the incubation was carried out overnight at 48 °C.

iTRAQ labeling

Samples were labelled following the manufacturer's instructions of iTRAQ 4-plex (Applied Biosystems, Foster City, CA, USA), using 3 separate 4-plex iTRAQ kits, one for each randomized sequence (Figure 2). Control (pool 1) was labeled with 114 tag, and the rest of the samples were labelled by alternating 115, 116, and 117 tags to avoid bias. The excess of iTRAQ reagents was removed using a strong cation exchange (SCX)-cartridge (Strata, Phenomenex, Torrence, CA, USA). Peptides were eluted with a volatile buffer containing 5% ammonia and 30% methanol and subsequently dried in SpeedVac system prior to OffGel electrophoresis (OGE) fractionation.

OffGel fractionation

The resulting labeled peptides were separated according to their isoelectric point using 24 cm IPG strips (pH 3-10) on an Agilent 3100 OFFGEL fractionator, the separated components were then recovered in liquid fractions. The three sample sequences (three sets of iTRAQ labelling) we processed in parallel, diluting the dried mixtures in 1.8 ml of the focusing buffer containing only 5% (v/v) of glycerol contrary to the supplier's protocol. IPG strips (24cm, pH 3-10) were rehydrated by adding 40 µl of peptide IPG strip rehydration solution per well for 15 minutes. Then, the dried peptide mixtures samples were dissolved directly in the peptide OFFGEL stock solution, pH 3-10 and 150 µl of sample were loaded in each well. Peptide

focusing was performed until it reached 50 kVh with a maximum voltage of 8 000 V and maximum current of 50 µA. After focusing, the 24 liquid fractions were carefully recovered and well rinsed twice with 150 µl of 0.1% Formic Acid (FA) for 15 minutes, to recover the maximum sample solution for each well. 3x24 peptide fractions were finally obtained.

All fractions were checked by MALDI-TOF, those with higher mass intensities were kept alone whilst fractions with similar or lower intensities were combined, resulting 10 different fractions for each set of iTRAQ labelling. Samples were desalted and purified using Sep-Pack 100 mg C18 cartridge (Waters, Elstree, UK). The eluent was concentrated to dryness and reconstituted in 50 µL 0.1% FA.

Nano-LC separation and MALDI MS/MS

A 15 µl-aliquot of each fraction was analyzed by LC–MALDI-TOF/TOF. An Easy-nLC system (Proxeon, Thermo Scientific) coupled to a Proteinner-fc fraction collector (Bruker) was used for the nano-LC with a C18 pre-column (EASY-Column, 2 cm, ID100 µm, 5 µm, C18-A1) and a C18 analytical column (EASY-Column, 10cm, ID75µm, 3µm, C18-A2). Mobile phases were water (A) and acetonitrile (ACN) (B), both containing 0.1% (v/v) trifluoroacetic acid (TFA). Peptides were separated over 35 min at a flow rate of 0.3 µL/min as follows: 0−60% solvent B within 10 min; 10 min 100% solvent B; and held for 15 min 2% solvent B before ramping back down to the initial solvent conditions. During gradient, 16'' fractions were collected and directly deposited on a Maldi Target plate PACII 384, pre-spotted with α-cyano-4-hydroxycinnamic acid matrix. MALDI plates were rinsed with 10 mM ammonium phosphate monobasic buffer (Sigma-Aldrich, Tres Cantos, Spain) and immediately analyzed on a MALDI-TOF/TOF UltrafleXtrem (Bruker Daltonics, Bremen, Germany) instrument. Automated data acquisition was done using Flex Control v 3.4 and Warp-LC v 1.3 softwares (Bruker) , and was constituted to a full scan (m/z 700-5000) in positive reflector ion mode using 2 000 laser shots for every MS spectra. The twenty

most abundant peptide precursor ions, with a signal-to-noise ratio greater than or equal to 10, were selected for MS/MS analysis with LIFT detection mode using 4 000 laser shots.

Data analysis (MALDI)

The raw data files for protein identification were processed using FlexAnalysis v 3.4 via ProteinScape v 3 softwares (Bruker). Searches were performed with Mascot v 2.4.0 (Matrix Science, London, UK), against the SwisProt (released on 03/2013) human database. The following search parameters were selected: Homo sapiens for organism, trypsin as the digestion enzyme, with up to 2 missed cleavages allowed, cysteine carbamidomethylation as a fixed modification, whilst oxidation of methionine was set as variable. Tolerance settings were 50 ppm for full scan and 0.9 for MS/MS. Protein identifications were accepted with a minimum peptide Mowse score of 10 and a minimum Protein Mascot Score of 20.

Nano-LC separation and ORBITRAP MS/MS

Samples were analyzed on an Orbitrap Velos PRO instrument (Thermo Fisher Scientific, Bremen) connected to an Easy-nLC system (Proxeon, Thermo Scientific) with the same configuration columns as the nLC for MALDI, but coupled to a nanoelectrospray source with a stainless steel emitter. Solvent A consisted of 0.1 % FA in deionized water (Milli-Q, Millipore), and solvent B consisted of 0.1 % FA in 100% ACN. The flow rate was 0.3 µL/min with a 130 min long separation gradient running from 5% to 100% B. The mass spectrometry detection constituted a full scan (m/z 350–2 000) with Orbitrap detection at resolution R=30,000 (at m/z 400) followed by up to ten data-dependent acquisition MS/MS scans with Orbitrap detection of the most intense ions. The signal threshold for triggering an MS/MS event was set to 10 000 counts. The low mass cutoff was set to 100 m/z. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 were excluded. Dynamic exclusion of 30

s was used with an activation time of 0.1 s. For efficient fragmentation and detection of iTRAQ reporter ions, HCD normalized collision energy of 45 was used since reported optimization experiments showed that it gave the highest number of identified peptides with iTRAQ signal.

Data analyses (ORBITRAP)

Peak lists were searched against Swissprot (released on 03/2013), taxonomy filter was set to Homo sapiens using Mascot software version 2.4.04 (Matrix Science, UK). Proteome Discover v 1.4 was used for protein identification and iTRAQ reporter quantification. Trypsin was chosen as cleavage specificity with a maximum number of three missed cleavages allowed. Carbamidomethylation (C) was set as a fixed modification whereas those originating from iTRAQ protocol (iTRAQ4Plex on the N-terminal residue, iTRAQ4Plex on tyrosine (Y), iTRAQ4Plex on lysine (K)) and oxidation (M) were used as variable modifications. The searches were performed using a peptide tolerance of 7 ppm and a product ion tolerance of 0.02 Da (Orbitrap readout). For further filtering the decoy search options was enabled and only unique peptides were accepted. The resulting data files were exported and filtered for <1% false discovery rate at peptide. Hence there is no fixed Mascot score cutoff because peptide matches are accepted until a FDR rate of 1% is reached. However, only PSMs with Mascot scores >20 were accepted to ensure that only high quality data were employed for this study.

The files generated with Maldi and Orbitrap MS/MS were combined in order to identify the HDL-associated proteins. The final identified proteins were required to present more than one peptide-spectrum match (PSM) or have a confidence score at least >30% and a coverage >10%.

Quantification and statistics (ORBITRAP)

Samples analyzed by nLC-ORBIRAP-ESI MS/MS were used for the quantitative analyzes. In the quantitative calculations, only protein isoforms

with iTRAQ values in at least two of the analyses were included. For each protein, the relative expression levels were calculated as a ratio between the sample and the 114 labeling controls. Biological replicates from the three iTRAQ sequences were combined in an Excel document where the mean of the ratios of each protein were calculated. Proteins identified as differentially expressed were those with a differential expression of at least 0.8-fold change, or 1.3-fold change relative to the baseline. In addition, a cut-off inferior to 0.5 or superior to 1.5 was applied to establish the more relevant protein expression changes observed after each intervention, which were defined as stronger effects.

Supplemental Table 1. Virgin olive oils composition. Phenolic compounds, fat soluble micronutrients and fatty acids daily intake through 25 mL of VOO, FVOO and FVOOT.

	VOO	FVOO	FVOOT
PHENOLIC COMPOUNDS (mg/25 mL/day)			
hydroxytyrosol	0,01 ± 0,00	0,21 ± 0,02	0,12 ± 0,00
3,4-DHPEA-AC	n.d.	0,84 ± 0,06	0,39 ± 0,04
3,4-DHPEA-EDA	0,04 ± 0,00	6,73 ± 0,37	3,43 ± 0,29
3,4-DHPEA-EA	0,26 ± 0,04	0,71 ± 0,06	0,36 ± 0,03
Total hydroxytyrosol derivates	***0,30***	***8,49***	***4,30***
p-hydroxybenzoic acid	n.d.	0,02 ± 0,00	0,06 ± 0,00
vanillic acid	n.d.	0,07 ± 0,00	0,13 ± 0,01
caffeic acid	n.d.	0,00 ± 0,00	0,06 ± 0,00
rosmarinic acid	n.d.	n.d.	0,41 ± 0,03
Total phenolic acids	-	***0,09***	***0,65***
thymol	n.d.	n.d.	0,64 ± 0,05
carvacrol	n.d.	n.d.	0,23 ± 0,02
Total monoterpenes	-	-	***0,86***
luteolin	0,04 ± 0,00	0,18 ± 0,02	0,21 ± 0,02
apigenin	0,02 ± 0,00	0,06 ± 0,00	0,10 ± 0,00
naringenin	n.d.	n.d.	0,20 ± 0,02
eriodictyol	n.d.	n.d.	0,17 ± 0,01
thymusin	n.d.	n.d.	1,22 ± 0,09
xanthomicrol	n.d.	n.d.	0,53 ± 0,06
7-methylsudachitin	n.d.	n.d.	0,53 ± 0,09
Total flavonoids	***0,06***	***0,23***	***2,95***
pinoresinol	0,05 ± 0,00	0,12 ± 0,00	0,10 ± 0,05
acetoxipinoresinol	2,47 ± 0,19	3,66 ± 0,31	3,24 ± 0,28
Total lignans	***2,52***	***3,78***	***3,34***
FAT SOLUBLE MICRONUTRIENTS (mg/25 mL/day)			
α-tocopherol	3,27 ± 0,01	3,40 ± 0,02	3,44 ± 0,01
lutein	0,05 ± 0,00	0,06 ± 0,00	0,07 ± 0,00
β-cryptoxanthin	0,02 ± 0,00	0,03 ± 0,00	0,02 ± 0,00
β-carotene	0,01 ± 0,00	0,02 ± 0,00	0,02 ± 0,00
FATTY ACIDS (relative area %)			
Palmitic acid	11,21	11,20	11,21
Stearic acid	1,92	1,92	1,92
Araquidic acid	0,36	0,36	0,36
Behenic acid	0,11	0,11	0,11
Total saturated	***13,75***	***13,74***	***13,75***
Palmitoleic acid	0,70	0,70	0,69
Oleic acid	76,74	76,83	76,75
Gadoleic acid	0,27	0,27	0,27
Total monounsaturated	***77,71***	***77,80***	***77,72***
Linoleic acid	7,43	7,36	7,43
Timnodonic acid	0,36	0,36	0,35
Linolenic acid	0,43	0,43	0,43
Total polyunsaturated	***8,22***	***8,15***	***8,22***

Values expressed as mean ± standard deviation (SD). 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone

Supplemental Table 2. HDL-associated proteins identified by MALDI and ORBITRAP MS techniques.

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	SEQUENCE COVERAGE (%)	NUMBER OF UNIQUE PEPTIDES IDENTIFIED	NUMBER OF PSMs IDENTIFIED	MASCOT SCORE
P60709	ACTB	ACTB_HUMAN	Actin, cytoplasmic 1	17.87	4	5	56
Q9HDC9	APMAP	APMAP_HUMAN	Adipocyte plasma membrane-associated protein	6.97	3	6	54.4
P43652	AFM	AFAM_HUMAN	Afamin	3.01	2	8	98.2
P02763	ORM1	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	18.91	3	12	105.1
P19652	ORM2	A1AG2_HUMAN	Alpha-1-acid glycoprotein 2	12.94	2	6	64.4
P01011	SERPINA3	AACT_HUMAN	Alpha-1-antichymotrypsin	16.08	7	36	388.9
P01009	SERPINA1	A1AT_HUMAN	Alpha-1-antitrypsin	36.84	13	133	1499.3
P04217	A1BG	A1BG_HUMAN	Alpha-1B-glycoprotein	12.53	4	11	120
P08697	SERPINF2	A2AP_HUMAN	Alpha-2-antiplasmin	23.63	6	18	166
P02765	AHSG	FETUA_HUMAN	Alpha-2-HS-glycoprotein	21.8	9	76	704.4
P01023	A2M	A2MG_HUMAN	Alpha-2-macroglobulin	0.75	1	1	12.6
P15144	ANPEP	AMPN_HUMAN	Aminopeptidase N	2.17	2	4	47.4
P01019	AGT	ANGT_HUMAN	Angiotensinogen	21.24	8	38	422.3
P01008	SERPINC1	ANT3_HUMAN	Antithrombin-III	17.03	5	10	163.1
P02647	APOA1	APOA1_HUMAN	Apolipoprotein A-I	59.55	24	455	3876.5
P02652	APOA2	APOA2_HUMAN	Apolipoprotein A-II	67	7	50	410.7
P06727	APOA4	APOA4_HUMAN	Apolipoprotein A-IV	33.59	14	27	266.7
Q6Q788	APOA5	APOA5_HUMAN	Apolipoprotein A-V	16.9	2	-	64.3
P04114	APOB	APOB_HUMAN	Apolipoprotein B-100	36.03	157	794	7600.1
P02654	APOC1	APOC1_HUMAN	Apolipoprotein C-I	13.25	1	1	12.6
P02655	APOC2	APOC2_HUMAN	Apolipoprotein C-II	49.5	4	21	217.7
P02656	APOC3	APOC3_HUMAN	Apolipoprotein C-III	37.37	4	153	1652.9
P55056	APOC4	APOC4_HUMAN	Apolipoprotein C-IV	50.4	6	-	137.9
P05090	APOD	APOD_HUMAN	Apolipoprotein D	23.81	5	69	613.3
P02649	APOE	APOE_HUMAN	Apolipoprotein E	40.38	12	62	617
Q13790	APOF	APOF_HUMAN	Apolipoprotein F	7.36	2	2	20.7
O14791	APOL1	APOL1_HUMAN	Apolipoprotein L1	14.82	8	57	564
Q9BQE5	APOL2	APOL2_HUMAN	Apolipoprotein L2	2.08	1	2	20.1

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	SEQUENCE COVERAGE (%)	NUMBER OF UNIQUE PEPTIDES IDENTIFIED	NUMBER OF PSMs IDENTIFIED	MASCOT SCORE
O95445	APOM	APOM_HUMAN	Apolipoprotein M	54.79	6	75	536.8
P08519	LPA	APOA_HUMAN	Apolipoprotein(a)	44.13	32	188	1624.6
P02730	SLC4A1	B3AT_HUMAN	Band 3 anion transport protein	1.43	1	2	28
P02749	APOH	APOH_HUMAN	Beta-2-glycoprotein 1	17.39	4	10	79.2
Q96KN2	CNDP1	CNDP1_HUMAN	Beta-Ala-His dipeptidase	12.23	4	9	92.6
Q8TDL5	BPIFB1	BPIB1_HUMAN	BPI fold-containing family B member 1	15.5	5	20	196.6
P00915	CA1	CAH1_HUMAN	Carbonic anhydrase 1	7.28	1	1	14.9
P23280	CA6	CAH6_HUMAN	Carbonic anhydrase 6	7.14	1	1	15.3
Q9NNX6	CD209	CD209_HUMAN	CD209 antigen	13.37	1	1	11.2
P16070	CD44	CD44_HUMAN	CD44 antigen	2.7	2	2	23.2
O43866	CD5L	CD5L_HUMAN	CD5 antigen-like	4.03	1	2	33.3
P11597	CETP	CETP_HUMAN	Cholesteryl ester transfer protein	19.27	5	8	85.1
P10909	CLU	CLUS_HUMAN	Clusterin	22.27	10	43	372.6
P00736	C1R	C1R_HUMAN	Complement C1r subcomponent	3.85	2	-	66.1
P06681	C2	CO2_HUMAN	Complement C2	4.8	2	-	67.8
P01024	C3	CO3_HUMAN	Complement C3	26.64	32	81	796.6
P0C0L4	C4A	CO4A_HUMAN	Complement C4-A	15.14	15	41	394.1
P0C0L5	C4B	CO4B_HUMAN	Complement C4-B	46.6	73	-	2419.4
P02748	C9	CO9_HUMAN	Complement component C9	19.8	9	-	332.8
P00746	CFD	CFAD_HUMAN	Complement factor D	26.88	3	6	79.9
P08603	CFH	CFAH_HUMAN	Complement factor H	10.3	5	-	147.7
P01034	CST3	CYTC_HUMAN	Cystatin-C	19.18	2	15	167.3
P81605	DCD	DCD_HUMAN	Dermicidin	7.27	1	1	16.2
Q08554	DSC1	DSC1_HUMAN	Desmocollin-1	1.45	1	1	13.7
P27105	STOM	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	3.47	1	2	25.3
P02671	FGA	FIBA_HUMAN	Fibrinogen alpha chain	17.44	13	76	676.8
P02675	FGB	FIBB_HUMAN	Fibrinogen beta chain	36.7	12	-	389.6
P02679	FGG	FIBG_HUMAN	Fibrinogen gamma chain	3.31	1	3	45.4

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	SEQUENCE COVERAGE (%)	NUMBER OF UNIQUE PEPTIDES IDENTIFIED	NUMBER OF PSMs IDENTIFIED	MASCOT SCORE
P02751	FN1	FINC_HUMAN	Fibronectin	2.3	2	-	76.2
P21333	FLNA	FLNA_HUMAN	Filamin-A	0.34	1	1	14.9
P06396	GSN	GELS_HUMAN	Gelsolin	11.51	5	10	122.2
P00738	HP	HPT_HUMAN	Haptoglobin	23.89	2	52	498.5
P00739	HPR	HPTR_HUMAN	Haptoglobin-related protein	43.1	5	84	770.3
P69905	HBA1	HBA_HUMAN	Hemoglobin subunit alpha	10.56	1	1	12.4
P68871	HBB	HBB_HUMAN	Hemoglobin subunit beta	15.65	2	2	21
P02790	HPX	HEMO_HUMAN	Hemopexin	9.74	2	6	82.5
P05546	SERPIND1	HEP2_HUMAN	Heparin cofactor 2	5.41	2	3	40.1
P04196	HRG	HRG_HUMAN	Histidine-rich glycoprotein	5.71	2	2	20.3
P10412	HIST1H1E	H14_HUMAN	Histone H1.4	7.31	1	1	16.4
P04439	HLAA	1A03_HUMAN	HLA class I histocompatibility antigen. A-3 alpha chain	13.7	2	15	147.9
P30464	HLAB	1B15_HUMAN	HLA class I histocompatibility antigen. B-15 alpha chain	10.5	1	10	109.8
P01876	IGHA1	IGHA1_HUMAN	Ig alpha-1 chain C region	36.83	9	31	290.8
P01857	IGHG1	IGHG1_HUMAN	Ig gamma-1 chain C region	20.3	3	14	131.7
P01859	IGHG2	IGHG2_HUMAN	Ig gamma-2 chain C region	16.26	2	7	68.3
P01743		HV102_HUMAN	Ig heavy chain V-I region HG3	5.98	1	2	22.6
P01777		HV316_HUMAN	Ig heavy chain V-III region TEI	15.97	1	4	49.7
P01834	IGKC	IGKC_HUMAN	Ig kappa chain C region	66.98	4	22	293.6
P01619		KV301_HUMAN	Ig kappa chain V-III region B6	16.67	1	2	24.4
P01625		KV402_HUMAN	Ig kappa chain V-IV region Len	15.79	1	1	15.8
P80748		LV302_HUMAN	Ig lambda chain V-III region LOI	7.21	1	1	10.7
P0CG04	IGLC1	LAC1_HUMAN	Ig lambda-1 chain C regions	63.2	4	-	169.4
P0CG05	IGLC2	LAC2_HUMAN	Ig lambda-2 chain C regions	81.13	4	24	239
A0M8Q6	IGLC7	LAC7_HUMAN	Ig lambda-7 chain C region	38.68	1	12	148.9
P01871	IGHM	IGHM_HUMAN	Ig mu chain C region	14.16	5	21	223.2
Q14623	IHH	IHH_HUMAN	Indian hedgehog protein	13.38	3	7	76.6

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	SEQUENCE COVERAGE (%)	NUMBER OF UNIQUE PEPTIDES IDENTIFIED	NUMBER OF PSMs IDENTIFIED	MASCOT SCORE
P35858	IGFALS	ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	4.13	2	3	39.3
P17301	ITGA2	ITA2_HUMAN	Integrin alpha-2	1.86	1	3	50.5
P05556	ITGB1	ITB1_HUMAN	Integrin beta-1	4.01	4	5	41.9
P05106	ITGB3	ITB3_HUMAN	Integrin beta-3	1.4	1	1	10.3
P19827	ITIH1	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	1.43	1	1	10.7
Q14624	ITIH4	ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	7.2	4	8	82.4
P29622	SERPINA4	KAIN_HUMAN	Kallistatin	7.73	2	4	67.7
P01042	KNG1	KNG1_HUMAN	Kininogen-1	4.97	3	5	61.9
P02750	LRG1	A2GL_HUMAN	Leucine-rich alpha-2-glycoprotein	2.59	1	2	29.8
P18428	LBP	LBP_HUMAN	Lipopolysaccharide-binding protein	10.2	4	-	93.7
P51884	LUM	LUM_HUMAN	Lumican	14.2	4	-	70.6
P61626	LYZ	LYSC_HUMAN	Lysozyme C	14.19	2	9	93.6
Q9H8L6	MMRN2	MMRN2_HUMAN	Multimerin-2	0.95	1	3	37
Q96PD5	PGLYRP2	PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	16.32	4	11	115.3
P04180	LCAT	LCAT_HUMAN	Phosphatidylcholine-sterol acyltransferase	18.64	7	42	353.4
P80108	GPLD1	PHLD_HUMAN	Phosphatidylinositol-glycan-specific phospholipase D	5	4	11	137.5
P55058	PLTP	PLTP_HUMAN	Phospholipid transfer protein	25.35	9	36	295.3
P36955	SERPINF1	PEDF_HUMAN	Pigment epithelium-derived factor	37.32	14	54	536.6
P05155	SERPING1	IC1_HUMAN	Plasma protease C1 inhibitor	7.8	3	5	63.1
P05154	SERPINA5	IPSP_HUMAN	Plasma serine protease inhibitor	2.71	1	2	30.3
P02775	PPBP	CXCL7_HUMAN	Platelet basic protein	32.81	3	13	131.4
P02776	PF4	PLF4_HUMAN	Platelet factor 4	19.8	2	3	36.5
Q13093	PLA2G7	PAFA_HUMAN	Platelet-activating factor acetylhydrolase	12.47	3	13	157.4
Q9UHG3	PCYOX1	PCYOX_HUMAN	Prenylcysteine oxidase 1	20.2	8	25	232.3
P41222	PTGDS	PTGDS_HUMAN	Prostaglandin-H2 D-isomerase	12.11	2	4	40.5
P02760	AMBP	AMBP_HUMAN	Protein AMBP	19.6	6	19	222.9
Q9UK55	SERPINA10	ZPI_HUMAN	Protein Z-dependent protease inhibitor	5.41	2	5	69.7

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	SEQUENCE COVERAGE (%)	NUMBER OF UNIQUE PEPTIDES IDENTIFIED	NUMBER OF PSMs IDENTIFIED	MASCOT SCORE
P00734	F2	THRB_HUMAN	Prothrombin	9.9	3	-	82.9
P07988	SFTPB	PSPB_HUMAN	Pulmonary surfactant-associated protein B	8.14	1	2	33
P02753	RBP4	RET4_HUMAN	Retinol-binding protein 4	29.85	4	20	119.3
P02787	TF	TRFE_HUMAN	Serotransferrin	6.16	4	4	42.7
P02768	ALB	ALBU_HUMAN	Serum albumin	66.83	45	970	9096.3
P0DJI8	SAA1	SAA1_HUMAN	Serum amyloid A protein	46.72	5	44	536.9
P35542	SAA4	SAA4_HUMAN	Serum amyloid A-4 protein	39.23	6	40	367.9
P02743	APCS	SAMP_HUMAN	Serum amyloid P-component	13.9	2	-	60.6
P27169	PON1	PON1_HUMAN	Serum paraoxonase/arylesterase 1	79.44	18	191	1652.3
Q15166	PON3	PON3_HUMAN	Serum paraoxonase/lactonase 3	25.42	6	48	379.5
P05452	CLEC3B	TETN_HUMAN	Tetranectin	16.34	2	2	23.3
P19971	TYMP	TYPH_HUMAN	Thymidine phosphorylase	3.11	1	1	16
P02766	TTR	TTHY_HUMAN	Transthyretin	34.01	5	67	683.8
Q9BUN1	MENT	CA056_HUMAN	Uncharacterized protein C1orf56	8.5	2	5	50.6
P02774	GC	VTDB_HUMAN	Vitamin D-binding protein	35.02	11	46	434.3
P04004	VTN	VTNC_HUMAN	Vitronectin	9.62	4	46	440.8
P25311	AZGP1	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	16.44	3	10	105.7

A total of 127 proteins were identified. Of those, 80 appeared in at least 3 different MS studies (red), 32 appeared in at least 1 different MS study (green) and 15 were newly described (black). UniProt accession number, entry name and gene symbol information were from UniProt database (http://www.uniprot.org/).

Supplemental Table 3. Proteins differentially expressed after each VOO intervention.

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
VOO					
UPREGULATED					
Q15166	PON3	PON3_HUMAN	Serum paraoxonase/lactonase 3	Antioxidant protection	1.56
P04196	HRG	HRG_HUMAN	Histidine-rich glycoprotein	Blood coagulation	1.55
P05546	SERPIND	HEP2_HUMAN	Heparin cofactor 2	Blood coagulation	1.27
P01023	A2M	A2MG_HUMAN	Alpha-2-macroglobulin	Blood coagulation	1.25
P01042	KNG1	KNG1_HUMAN	Kininogen-1	Blood coagulation	1.30
P02647	APOA1	APOA1_HUMAN	Apolipoprotein A-I	Cholesterol homeostasis	1.69
P11597	CETP	CETP_HUMAN	Cholesteryl ester transfer protein	Cholesterol homeostasis	1.58
P02652	APOA2	APOA2_HUMAN	Apolipoprotein A-II	Cholesterol homeostasis	1.45
Q13790	APOF	APOF_HUMAN	Apolipoprotein F	Cholesterol homeostasis	1.34
P02654	APOC1	APOC1_HUMAN	Apolipoprotein C-I	Cholesterol homeostasis	2.96
P06727	APOA4	APOA4_HUMAN	Apolipoprotein A-IV	Cholesterol homeostasis and Antioxidant protection	1.36
P81605	DCD	DCD_HUMAN	Dermcidin	Defense response	1.31
P01743		HV102_HUMAN	Ig heavy chain V-I region HG3	Innate immune response	1.55
P0CG04	IGLC1	LAC1_HUMAN	Ig lambda-1 chain C regions	Innate immune response	1.37
P01871	IGHM	IGHM_HUMAN	Ig mu chain C region	Innate immune response	1.32
Q14624	ITIH4	ITIH4_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H4	Transport	1.37
P19827	ITIH1	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	Transport	1.36
P02730	SLC4A1	B3AT_HUMAN	Band 3 anion transport protein	Transport	1.25
P10412	HIST1H1E	H14_HUMAN	Histone H1.4	nucleosome assembly	1.27

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			VOO		
DOWNREGULATED					
P02765	AHSG	FETUA_HUMAN	Alpha-2-HS-glycoprotein	Acute-phase response	0.84
P35542	SAA4	SAA4_HUMAN	Serum amyloid A-4 protein	Acute-phase response	0.84
P02763	ORM1	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	Acute-phase response	0.75
P0DJI8	SAA1	SAA_HUMAN	Serum amyloid A protein	Acute-phase response	0.74
P08697	SERPINF2	A2AP_HUMAN	Alpha-2-antiplasmin	Acute-phase response	0.75
P00738	HP	HPT_HUMAN	Haptoglobin	Acute-phase response	0.56
P21333	FLNA	FLNA_HUMAN	Filamin-A	Binding	0.74
P02671	FGG	FIBG_HUMAN	Fibrinogen gamma chain	Blood coagulation	0.83
P02776	PF4	PLF4_HUMAN	Platelet factor 4	Blood coagulation	0.56
P01008	SERPINC1	ANT3_HUMAN	Antithrombin-III	Blood coagulation	0.75
P16070	CD44	CD44_HUMAN	CD44 antigen	Cell adhesion	0.64
O95445	APOM	APOM_HUMAN	Apolipoprotein M	Cholesterol homeostasis	0.83
P02656	APOC3	APOC3_HUMAN	Apolipoprotein C-III	Cholesterol homeostasis	0.84
P10909	CLU	CLUS_HUMAN	Clusterin	Complement pathway and innate immune response	0.61
P04439	HLAA	1A03_HUMAN	HLA class I histocompatibility antigen. A-3 alpha chain	Immune response	0.84
P25311	AZGP1	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	Immune response	0.69
P30464	HLAB	1B15_HUMAN	HLA class I histocompatibility antigen. B-15 alpha chain	Immune response	0.56
Q96PD5	PGLYRP2	PGRP2_HUMAN	N-acetylmuramoyl-L-alanine amidase	Innate immune response	0.79
P80748		LV302_HUMAN	Ig lambda chain V-III region LOI	Innate immune response	0.47
Q8TDL5	bpifb1	BPIB1_HUMAN	BPI fold-containing family B member 1	Lipid binding	0.84
P05090	APOD	APOD_HUMAN	Apolipoprotein D	Lipid transport	0.61
O14791	APOL1	APOL1_HUMAN	Apolipoprotein L1	Lipid transport	0.79
Q96KN2	CNDP1	CNDP1_HUMAN	Beta-Ala-His dipeptidase	Proteolysis	0.82
P15144	ANPEP	AMPN_HUMAN	Aminopeptidase N	Proteolysis	0.80

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			VOO		
DOWNREGULATED					
P02753	RBP4	RET4_HUMAN	Retinol-binding protein 4	Transport	0.78
P68871	HBB	HBB_HUMAN	Hemoglobin subunit beta	Transport	0.81
P69905	HBA1	HBA_HUMAN	Hemoglobin subunit alpha	Transport	0.65
P04217	A1BG	A1BG_HUMAN	Alpha-1B-glycoprotein	Extracellular region	0.83
Q9HDC9	APMAP	APMAP_HUMAN	Adipocyte plasma membrane-associated protein	Membrane protein	0.76
P27105	STOM	STOM_HUMAN	Erythrocyte band 7 integral membrane protein	Membrane protein	0.82

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			FVOO		
UPREGULATED					
P0DJI8	SAA1	SAA_HUMAN	Serum amyloid A protein	Acute-phase response	1.26
Q15166	PON3	PON3_HUMAN	Serum paraoxonase/lactonase 3	Antioxidant protection	1.55
P04196	HRG	HRG_HUMAN	Histidine-rich glycoprotein	Blood coagulation	1.39
P02775	PPBP	CXCL7_HUMAN	Platelet basic protein	Blood coagulation	1.77
P05546	SERPIND1	HEP2_HUMAN	Heparin cofactor 2	Blood coagulation	1.27
P01023	A2M	A2MG_HUMAN	Alpha-2-macroglobulin	Blood coagulation	1.33
P01042	KNG1	KNG1_HUMAN	Kininogen-1	Blood coagulation	1.29
P02652	APOA2	APOA2_HUMAN	Apolipoprotein A-II	Cholesterol homeostasis	1.40
P02647	APOA1	APOA1_HUMAN	Apolipoprotein A-I	Cholesterol homeostasis	1.37
P11597	CETP	CETP_HUMAN	Cholesteryl ester transfer protein	Cholesterol homeostasis	1.30
P01871	IGHM	IGHM_HUMAN	Ig mu chain C region	Innate immune response	1.54
P01743		HV102_HUMAN	Ig heavy chain V-I region HG3	Innate immune response	1.47
P0CG04	IGLC1	LAC1_HUMAN	Ig lambda-1 chain C regions	Innate immune response	1.32
P0CG05	IGLC2	LAC2_HUMAN	Ig lambda-2 chain C regions	Innate immune response	1.26
Q9NNX6	CD209	CD209_HUMAN	CD209 antigen	Innate immune response	1.32
P08519	LPA	APOA_HUMAN	Apolipoprotein(a)	Lipid transport	1.28
P19827	ITIH1	ITIH1_HUMAN	Inter-alpha-trypsin inhibitor heavy chain H1	Transport	1.32

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			FVOO		
DOWNREGULATED					
P02765	AHSG	FETUA_HUMAN	Alpha-2-HS-glycoprotein	Acute-phase response	0.80
P19652	ORM2	A1AG2_HUMAN	Alpha-1-acid glycoprotein 2	Acute-phase response	0.76
P02763	ORM1	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	Acute-phase response	0.53
P08697	SERPINF2	A2AP_HUMAN	Alpha-2-antiplasmin	Acute-phase response	0.72
P00738	HP	HPT_HUMAN	Haptoglobin	Acute-phase response	0.80
P02671	FGG	FIBG_HUMAN	Fibrinogen gamma chain	Blood coagulation	0.83
P02671	FGA	FIBA_HUMAN	Fibrinogen alpha chain	Blood coagulation	0.79
P01008	SERPINC1	ANT3_HUMAN	Antithrombin-III	Blood coagulation	0.80
P35858	IGFALS	ALS_HUMAN	Insulin-like growth factor-binding protein complex acid labile subunit	cell adhesion and signalling	0.82
P04180	LCAT	LCAT_HUMAN	Phosphatidylcholine-sterol acyltransferase	Cholesterol homeostasis	0.83
P10909	CLU	CLUS_HUMAN	Clusterin	Complement pathway and innate immune response	0.78
P81605	DCD	DCD_HUMAN	Dermcidin	Defense response	0.53
P25311	AZGP1	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	Immune response	0.75
Q9BQE5	APOL2	APOL2_HUMAN	Apolipoprotein L2	Lipid transport	0.81
O14791	APOL1	APOL1_HUMAN	Apolipoprotein L1	Lipid transport	0.72
P05090	APOD	APOD_HUMAN	Apolipoprotein D	Lipid transport	0.71
P05154	SERPINA5	IPSP_HUMAN	Plasma serine protease inhibitor	Lipid transport	0.71
Q96KN2	CNDP1	CNDP1_HUMAN	Beta-Ala-His dipeptidase	Proteolysis	0.69
P15144	ANPEP	AMPN_HUMAN	Aminopeptidase N	Proteolysis	0.78
P69905	HBA1	HBA_HUMAN	Hemoglobin subunit alpha	Transport	0.72
P02753	RBP4	RET4_HUMAN	Retinol-binding protein 4	Transport	0.78
Q9BUN1	MENT	CA056_HUMAN	Uncharacterized protein C1orf56	Extracellular region	0.71
Q9HDC9	APMAP	APMAP_HUMAN	Adipocyte plasma membrane-associated protein	Membrane protein	0.79

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			FVOOT		
UPREGULATED					
P35542	SAA4	SAA4_HUMAN	Serum amyloid A-4 protein	Acute-phase response	1.51
P0DJI8	SAA1	SAA_HUMAN	Serum amyloid A protein	Acute-phase response	1.47
Q15166	PON3	PON3_HUMAN	Serum paraoxonase/lactonase 3	Antioxidant protection	1.39
P02775	PPBP	CXCL7_HUMAN	Platelet basic protein	Blood coagulation	1.41
P05546	SERPIND1	HEP2_HUMAN	Heparin cofactor 2	Blood coagulation	1.32
P01023	A2M	A2MG_HUMAN	Alpha-2-macroglobulin	Blood coagulation	1.39
P05106	ITGB3	ITB3_HUMAN	Integrin beta-3	Cell adhesion	1.74
P02655	APOC2	APOC2_HUMAN	Apolipoprotein C-II	Cholesterol homeostasis	1.78
P02647	APOA1	APOA1_HUMAN	Apolipoprotein A-I	Cholesterol homeostasis	1.60
P02652	APOA2	APOA2_HUMAN	Apolipoprotein A-II	Cholesterol homeostasis	1.34
P02656	APOC3	APOC3_HUMAN	Apolipoprotein C-III	Cholesterol homeostasis	1.70
P02654	APOC1	APOC1_HUMAN	Apolipoprotein C-I	Cholesterol homeostasis	1.25
P06727	APOA4	APOA4_HUMAN	Apolipoprotein A-IV	Cholesterol homeostasis and Antioxidant protection	1.52
P01743		HV102_HUMAN	Ig heavy chain V-I region HG3	Innate immune response	1.39
P01859	IGHG2	IGHG2_HUMAN	Ig gamma-2 chain C region O	Innate immune response	1.34
P02774	GC	VTDB_HUMAN	Vitamin D-binding protein	Transport	1.49
P43652	AFM	AFAM_HUMAN	Afamin	Transport	2.38
P68871	HBB	HBB_HUMAN	Hemoglobin subunit beta	Transport	1.37

UNIPROT ACCESSION NUMBER	GENE SYMBOL	ENTRY NAME	PROTEIN NAME	PRINCIPAL BIOLOGICAL FUNCTION	FOLD CHANGE
			FVOOT		
DOWNREGULATED					
P02765	AHSG	FETUA_HUMAN	Alpha-2-HS-glycoprotein	Acute-phase response	0.76
P02763	ORM1	A1AG1_HUMAN	Alpha-1-acid glycoprotein 1	Acute-phase response	0.58
P08697	SERPINF2	A2AP_HUMAN	Alpha-2-antiplasmin	Acute-phase response	0.77
P00738	HP	HPT_HUMAN	Haptoglobin	Acute-phase response	0.65
P21333	FLNA	FLNA_HUMAN	Filamin-A	Binding	0.75
P02776	PF4	PLF4_HUMAN	Platelet factor 4	Blood coagulation	0.64
P16070	CD44	CD44_HUMAN	CD44 antigen	Cell adhesion	0.69
P10909	CLU	CLUS_HUMAN	Clusterin	Complement pathway and innate immune response	0.64
P04439	HLAA	1A03_HUMAN	HLA class I histocompatibility antigen. A-3 alpha chain	Immune response	0.84
P25311	AZGP1	ZA2G_HUMAN	Zinc-alpha-2-glycoprotein	Immune response	0.63
P30464	HLAB	1B15_HUMAN	HLA class I histocompatibility antigen. B-15 alpha chain	Immune response	0.53
P05154	SERPINA5	IPSP_HUMAN	Plasma serine protease inhibitor	Lipid transport	0.72
P05090	APOD	APOD_HUMAN	Apolipoprotein D	Lipid transport	0.68
Q96KN2	CNDP1	CNDP1_HUMAN	Beta-Ala-His dipeptidase	Proteolysis	0.72
P15144	ANPEP	AMPN_HUMAN	Aminopeptidase N	Proteolysis	0.68
P02753	RBP4	RET4_HUMAN	Retinol-binding protein 4	Transport	0.77
Q9HDC9	APMAP	APMAP_HUMAN	Adipocyte plasma membrane-associated protein	Membrane protein	0.80

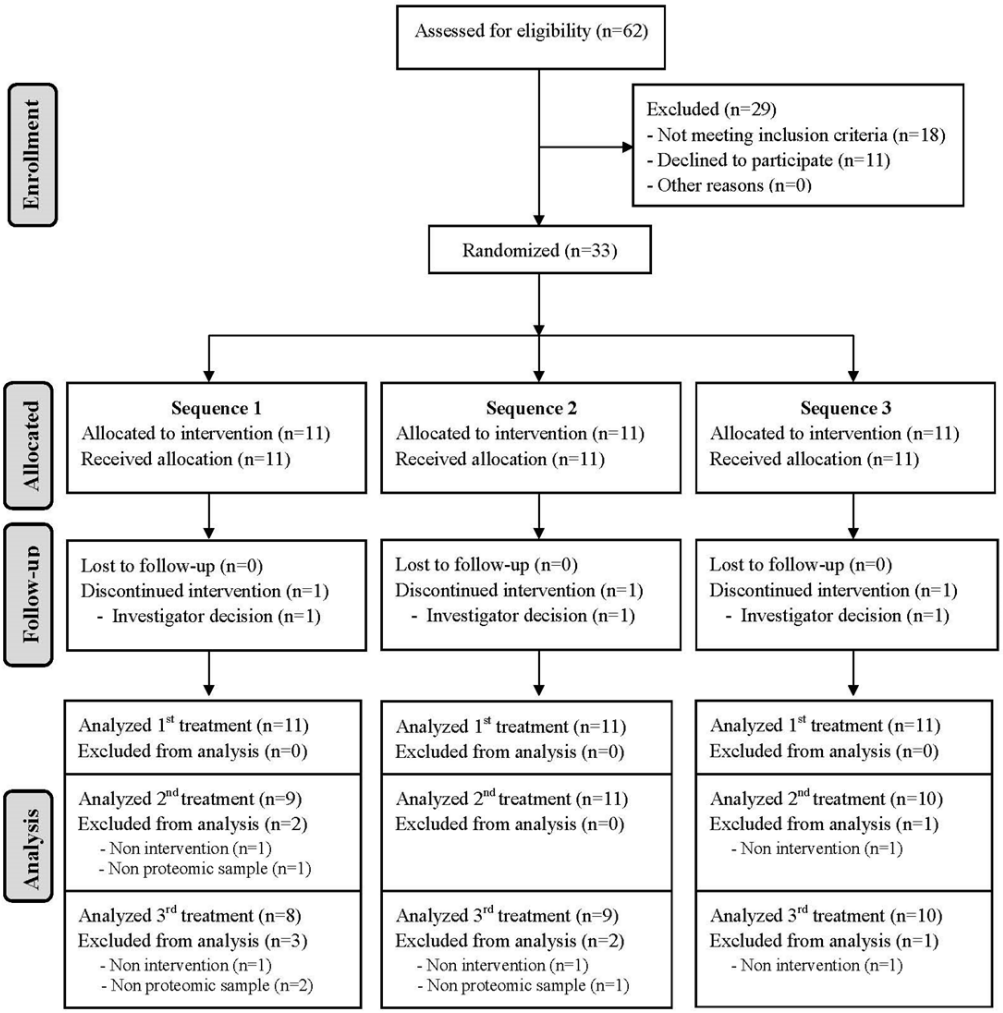
Fold change > 1.3 denotes proteins up-regulated while fold change < 0.8 denotes decrease protein expression after the VOO supplementations relative to the control-baseline. UniProt accession number, entry name, gene symbol and principal biological function information were from UniProt database (http://www.uniprot.org/).

SUPPLEMENTAL FIGURE LEGENDS

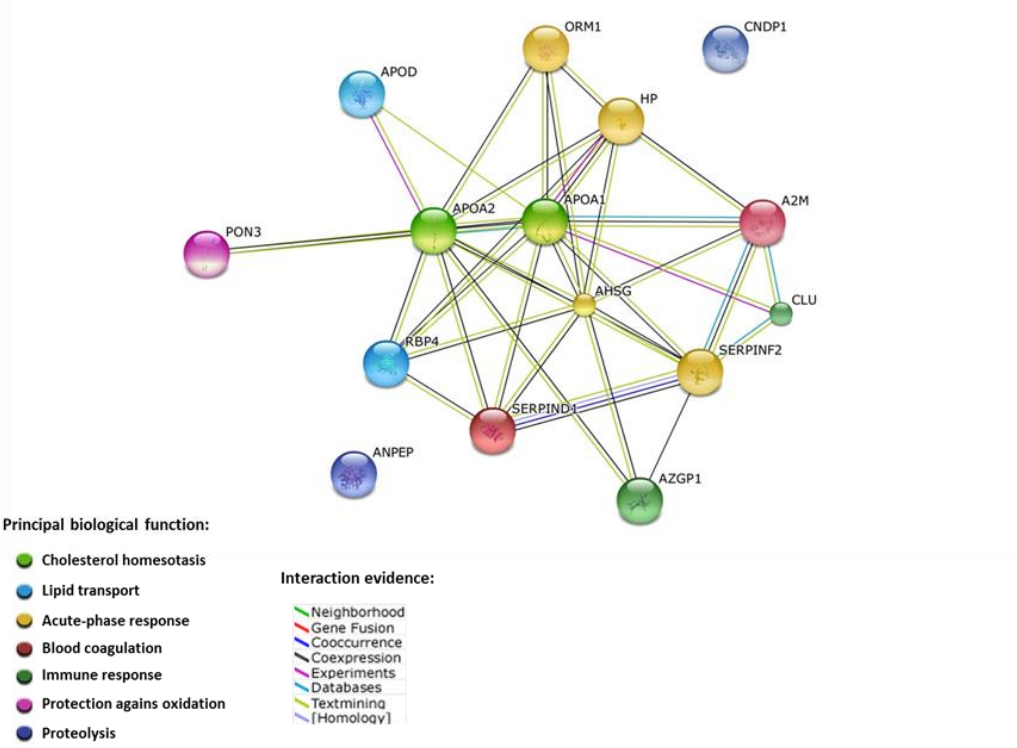
Supplemental Figure 1. Participants Flow-chart based on post-interventions of HDL proteomics variables. Sequence 1: FVOO, FVOOT and VOO; Sequence 2: FVOOT, VOO and FVOO; Sequence 3: VOO, FVOO and FVOOT.

Supplemental Figure 2. Protein-protein interaction network for the 15 commonly differentially expressed proteins after the three VOO interventions (http:www.string-db.org). Proteins were presented with their gene encode symbol. Nodes represent genes encoding interacting proteins and the lines between them represent known and predicted interactions.

Supplemental Figure 1. Participants Flow-chart.



Supplemental Figure 2. Protein-protein interaction network for the 15 commonly differentially expressed proteins after the three VOO interventions (http:www.string-db.org).



OVERALL DISCUSSION

The present work provides evidence that certain bioactive compounds of the Mediterranean and Nordic diets can be assessed and monitored by nutritional biomarkers, improving the accuracy of the dietary intake measure. Moreover, the Mediterranean and the Nordic dietary bioactive compounds have been associated with protective effects on the development of CVDs, in both healthy and CVD risk subjects. Thus, TPE was used as a biomarker of the TPI and for assessing the relation between polyphenol intake and oxidative stress biomarkers. By applying a LC-MS-based non-targeted metabolite profiling approach, novel and more specific biomarkers of the consumption of certain foods were identified. Moreover, using an iTRAQ-LC-MS/MS quantitative proteomic approach, molecular biomarkers were identified and related to the mechanisms of action and healthy biological effects of the studied bioactive compound-rich foods on CVDs.

In **Study 1**, we evaluated the effects of the TPI on oxidative stress, which is a known risk factor for developing CVDs (Li et al., 2013). This study was conducted in healthy subjects of different ages following a MD pattern. The TPI was assessed by the urinary TPE, which is a validated biomarker for the dietary intake of polyphenol-rich foods and which represents a first approach to study the TPI through nutritional biomarkers (Medina-Remón et al., 2009). Interestingly, the studied population had a wide age range from 18 to 72 years, which enabled us to observe a significant increase in the TPE with age. This association could be related to an increase in the consumption of vegetables, fruits, and coffee and to a moderate intake of fermented beverages with age, which are the greatest contributors to the TPI in our population. Moreover, our results confirm that the urinary TPE is related to changes in oxidative stress biomarkers. We observed that individuals with a higher urinary TPE had lower levels of 8-OHdG in urine and of GSSG in erythrocytes, indicating an attenuation of oxidative damage and consequent protection regarding the development of CVDs (Valavanidis et al., 2009; Yang et al., 2011). We also observed that polyphenols from vegetables and

fermented beverages (red wine being the most significant) were more related to the decrease in urinary 8-OHdG. Some studies indicate that polyphenols might exert inductive or signaling effects, which mediate inflammatory and antioxidant signals (Hollman et al., 2011; Stevenson et al., 2007). However, in this study, we did not study the specific role of polyphenols on particular signaling pathways to produce their protective effect against oxidative damage. The objective of this study was to assess the general relation between polyphenol-rich foods and oxidative stress biomarkers. To obtain this insight, we needed to determine the urinary TPE rather than individual polyphenols or nutritional biomarkers of particular polyphenol-rich foods. However, our results noted that there were associations between specific food groups and oxidative stress biomarkers; however, unfortunately, the urinary TPE biomarker did not allow the detailed effect of each polyphenol-rich food on oxidative protection to be differentiated. Therefore, we found an increasing need to study specific biomarkers for bioactive food compound intake and to explore the specific effects that these compounds exert on signaling pathways and the mechanisms of action involved. This new information would allow us to better understand how bioactive food compounds act and provide the observed health effects. To further achieve these new profiling objectives, Study 2 and Study 3 were conducted by applying omic technologies.

In **Study 2**, we assessed the effect of two partially overlapping dietary modification interventions, with whole grain or with whole grain plus bilberries and fatty fish, on plasma metabolite profiles. In this study, we assessed the effect of a dietary intervention with bioactive foods typical of the healthy Nordic diet on a Finnish population with MetS. The healthy Nordic diet, as well as the MD, has been associated with beneficial effects on CVDs (Olsen et al., 2011).

Through non-targeted metabolomic analyses, specific biomarkers for whole grain, bilberries and fatty fish intake were identified. In our study, the fasting plasma hippuric acid metabolite level correlated positively with the

consumption of bilberries, thus suggesting this metabolite as a novel biomarker for bilberry intake. In contrast, glucuronidated alk(en)ylresorcinols were identified for the first time in fasting human plasma, and we found a strong positive correlation between their changes in plasma levels and the consumption of whole grain, thus suggesting these molecules as new biomarkers for whole grain intake. Finally, we identified CMPF furan fatty acid as a potential biomarker for fatty fish intake, and in our study, this fatty acid was a stronger biomarker for fish intake than the established biomarkers EPA and DHA. In addition to these potential biomarkers of food intake, we identified molecular biomarkers of endogenous metabolism that could explain the effect of the consumed bioactive compounds on metabolic pathways and, thus, explain their beneficial effects on CVD onset and progression. The plasma levels of several amino acids, such as L-lysine, L-arginine, and ornithine, increased after whole grain consumption. Additionally, essential amino acids, such as phenylalanine and tyrosine, also increased after whole grain consumption. L-arginine is a cationic amino acid involved in several key metabolic processes and might improve postprandial glycemia, insulin sensitivity, hypertension, visceral obesity, endothelial dysfunction and inflammation (Lucotti et al., 2009; Morris, 2006). Thus, the beneficial effects of whole grain on CVD onset and progression might be partially explained by mechanisms involving several changes in plasma amino acid concentrations. Similarly, the diet enriched with whole grains increased betaine compounds in fasting plasma. Betaine enhances carnitine metabolism, and because carnitine is essential for transporting long-chain fatty acids into the mitochondria for energy production via β-oxidation, a link between betaine and lipid metabolism has been suggested (Pekkinen et al., 2013). Betaine homeostasis could be essential in terms of beneficial health implications related to whole grain consumption. In addition, after fatty fish consumption there was an observed effect on the endogenous lipid profile. We observed an increase in PUFA-containing lipids, which have been receiving attention because of their role as intracellular signaling molecules (Picq et al., 2010). All identified molecular biomarkers of endogenous

metabolism, were involved in the beneficial effect of the healthy Nordic diet on the development of CVDs.

Related to **Study 3**, we assessed the impact of a dietary intervention supplemented with a VOO or two different functional VOOs enriched with their own PC or complemented with thyme PC on HDL protein cargo. The study was conducted in hypercholesterolemic subjects of the Mediterranean area by applying a quantitative proteomic approach. Our results confirm the proposal that an intervention with different VOOs rich in PC remodels the HDL proteome changing the expression of different proteins with biological functions directly related to the cardioprotective activity of the HDL particle. Notably, although specific changes in the HDL protein cargo were observed after each intervention, the majority of the observed protein expression modifications were common after the three VOOs consumed. This result caused us to consider the effects of the fatty acid composition and the PC of the common matrix of the three VOOs tested on HDL remodeling. Proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation were up-regulated after the interventions, whereas proteins involved in acute-phase response, lipid transport, and immune response were down-regulated. The IPA analysis also demonstrated the effect of certain signaling pathways after OO phenolic compounds consumption thus conferring cardioprotective properties. In this study, we observed how PC from OO are involved in regulating the transcription of specific genes, and consequently, certain proteins present in the HDL particle were up- or down-regulated, thus inducing an improvement of HDL cardioprotective properties. The use of proteomics and new bioinformatics tools for data mining and biological interpretation such as IPA, allowed us to better understand the relation between the intake of VOO PC and the functionality of the HDL particle and to discover new therapeutic targets.

With the results derived from the three presented studies, we can confirm that the use of nutritional biomarkers is a necessary and useful tool for assessing the effects of dietary bioactive compounds on CVDs. In addition,

the use of omic sciences, such as metabolomics and proteomics, within the concept of foodomics, add relevant information to the discovery of new nutritional and molecular biomarkers, which assist us in evaluating food bioactivity and food effects on human health and on disease prevention. Metabolomics and proteomics allow us to better understand the biology of CVDs and the effects of the diet on CVD onset and progression, providing an integrated view of biological systems (**Figure 11**).

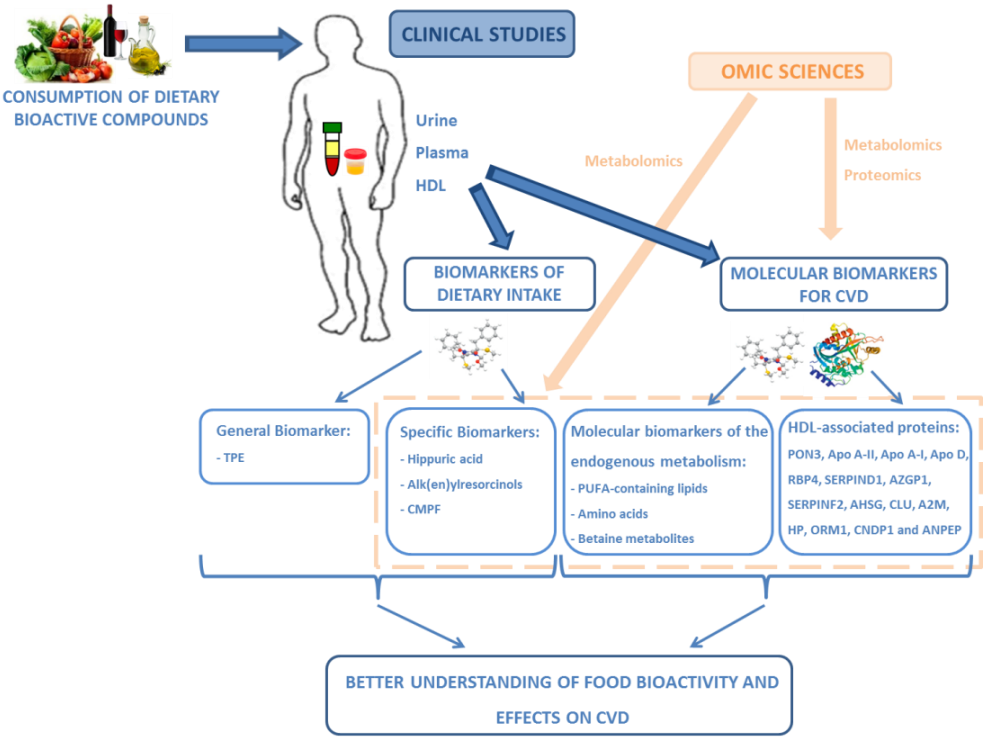


Figure 11. Approaches used for assessing the effects of dietary bioactive compounds on CVDs.

Future outlook:

Validating and verifying the usefulness and the applicability of fasting plasma hippuric acid, glucuronidated alk(en)ylresorcinols and CMPF metabolites as potential biomarkers for bilberries whole grain, and fatty fish dietary intake, respectively, in further nutritional studies and with other populations is of considerable interest. Likewise, assessing whether these nutritional biomarkers are involved in the reduction of molecular biomarkers related to CVDs is of important clinical relevance. Moreover, studying the specific mechanisms of action of the endogen metabolism biomarkers discovered using *in vitro* studies is necessary. Finally, one more step that should be performed is to determine the activity and functionality of some of the HDL-associated proteins to define whether the observed expression changes are relevant for the biological processes involved in CVDs.

CONCLUSIONS

1. Urinary TPE increased with age due to an increase in the consumption of vegetables, fruits, and coffee, and to a moderate intake of fermented beverages. Moreover, a higher urinary TPE is associated with a decrease in oxidative stress biomarkers, which may reflect an attenuation of oxidative damage in healthy individuals.

2. The beneficial effects of a dietary intake rich in vegetables and moderate red wine on healthy individuals, could potentially be explained by the attenuation of oxidative damage associated with these typical food items of the Mediterranean diet, which are rich in polyphenols.

3. A non-targeted metabolite approach based on LC-MS is an efficient technique in the discovery of specific novel nutritional biomarkers and in reporting metabolic alterations in fasting plasma after dietary interventions.

4. Fasting plasma CMPF, glucuronidated alk(en)ylresorcinols and hippuric acid are suggested as novel candidate biomarkers of fatty fish, whole grain and bilberry consumption, respectively, because these candidates present a strong positive correlation with the proposed consumed foods.

5. Alterations in molecular biomarkers of endogenous metabolism, such as PUFA-containing lipids, amino acids and betaine metabolites, could explain the effect of whole grains, fatty fish and bilberry consumption on metabolic pathways, thus indicating the potential beneficial effects of a healthy Nordic diet.

6. A quantitative proteomics approach based on iTRAQ-LC-MS/MS potentially can better elucidate the effects of dietary interventions with OOs rich in PC on the complex structure of the HDL protein cargo.

7. The consumption of VOO or phenol-enriched VOOs has an impact on the HDL proteome in a cardioprotective mode that could enhance HDL functionality by up-regulating proteins related to cholesterol homeostasis, protection against oxidation and blood coagulation while down-regulating proteins involved in acute-phase response, lipid transport, and immune responses.

8. The common protein expression modifications reported after the consumption of the three VOOs indicate a major effect of the fatty acid and PC composition present in the common matrix of these VOOs on HDL remodeling.

OVERALL CONCLUSION

The use of nutritional biomarkers is a useful tool in assessing the mechanisms of action and the biological effects of dietary bioactive compounds on CVD development. Thus, the use of omic sciences, such as metabolomics and proteomics, add relevant information to the discovery of novel nutritional and molecular biomarkers, which assist us in evaluating food bioactivity and food effects on human health and on disease prevention.

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ANNEX

1. SCIENTIFIC PAPERS

Catalán Ú, Rodríguez MÁ, Ras MR, Maciá A, Mallol R, Vinaixa M, Fernández-Castillejo S, Valls RM, **Pedret A**, Griffin JL, Salek R, Correig X, Motilva MJ, Solà R. **Biomarkers of food intake and metabolite differences between plasma and red blood cell matrices; a human metabolomic profile approach**. Mol Biosyst. 2013;9(6):1411-22.

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Pedret A, Valls RM, Fernández-Castillejo S, Catalán Ú, Romeu M, Giralt M, Lamuela-Raventós RM, Medina-Remón A, Arija V, Aranda N, Espinel A, Delgado MA, Solà R. **Polyphenol-rich foods exhibit DNA antioxidative properties and protect the glutathione system in healthy subjects.** Mol Nutr Food Res. 2012;56(7):1025-33.

Solà-Alberich R, Valls-Zamora RM, Fernández-Castillejo S, Catalán-Santos Ú, **Pedret-Figuerola A,** Giralt-Batista M and Konstantinidou V. **Do phenolic compounds exercise their effects by new pathways or mechanisms that would explain the healthy heart effects of virgin olive oil?.** Clin Invest Arterioscl. 2011;23(6)275-277.

Hanhineva K, Lankinen M, **Pedret A**, Schwab U, Kolehmainen M, Paananen Ju, De Mello V, Solà R, Lehtonen M, Poutanen K, Uusitupa M, Mykkänen H. **Non-targeted metabolite profiling discriminates diet-specific biomarkers for consumption of whole grains, fatty fish and bilberries –**

a randomized trial (Sysdimet-HealthGrain intervention). (editor submitted)

Pedret A, Catalán Ú, Fernández-Castillejo S, Farràs M, Valls RM, Rubió L, Canela N, Aragonés G, Romeu M, Castañer O, de la Torre R, Covas MI, Fitó M, Motilva MJ, Solà R. **Impact of virgin olive oil and phenol-enriched virgin olive oils on the high-density lipoprotein proteome in hypercholesterolemic subjects. A double blind, randomized, controlled, cross-over clinical trial (VOHF study).** (editor submitted)

Solà R, Valls RM, Puzo J, Calabuig JR, Brea A, **Pedret A**, Moriña D, Villar J, Millán J, Anguera A. **Effects of poly-bioactive compounds on lipid profile and body weight in a moderately hypercholesterolemic population with low cardiovascular disease risk: a multicenter randomized trial.** (editor submitted)

Solà R, Valls RM, Martorell I, Giralt M, **Pedret A**, Taltavull N, Romeu M, Rodríguez À, Moriña D, Lopez de Frutos V, Montero M, Casajuana MC, Pérez L, Faba J, Bernal G, Astilleros A, González R, Puiggrós F, Arola L, Chetrit C, Martinez-Puig D. **Effects of a low-fat yoghurt supplemented with a rooster comb extract, on muscle-joint function in adults with mild knee pain: a randomized trial.** (editor submitted)

Hernáez Á, Fernández-Castillejo S, Farràs M, Catalán Ú, Subirana I, López-Sabater MC, Solà R, Muñoz-Aguayo D, Gelabert-Gorgues A, Díaz-Gil Ó, Nyyssönen K, Zunft HF, de la Torre R, Martín-Peláez S, **Pedret A**, Covas MI, Fitó M. **Olive oil polyphenols enhance the cholesterol efflux capacity of HDL and induce in vivo a low-cardiovascular risk HDL profile (a randomized trial).** (editor submitted)

2. CONTRIBUTIONS AT CONGRESSES AND CONFERENCES

2.1 Posters

Pedret A, Lankinen M, Kolehmainen M, Paananen J, Schwab U, De Mello V, Sola R, Lehtonen M, Poutanen K, Uusitupa M, Mykkänen H, Hanhineva K. **Fatty fish, bilberries and whole grain products affect the metabolomic profile in subjects of the Sysdimet Study. A non-targeted metabolomic approach.** 82nd European Atherosclerosis Society Congress. Madrid, 31 May – 3 June 2014.

Solà R, Valls RM, Farràs M, Suárez M, Fernández-Castillejo S, Fitó M, Konstantinidou V, **Pedret A**, Fuentes F, López-Miranda J, Giralt M, Covas MI, Motilva MJ. **Functional olive oils functions.** 82nd European Atherosclerosis Society Congress. Madrid, 31 May – 3 June 2014.

Lankinen M, **Pedret A**, Kolehmainen M, Lappi J, Solà R, Mykkänen H, Poutanen K, Törrönen R, Gylling H, Schwab U, Uusitupa M, Hanhineva K. **Plasma 3-Carboxy-4-methyl-5-propyl-2-furanopropionic acid (CMPF)- a new biomarker for fish intake.** SYSDIET/NordFOOD workshop “Health effects of the Nordic diet”. Oslo, 13-15 November 2013.

Hanhineva K, Pekkinen J, Lappi J, Keski-Rahkonen P, **Pedret A**, Savolainen O, Auriola S, Mykkänen H, Poutanen K.**The accumulation of cereal bran derived metabolites in plasma and tissues and impact on endogenous metabolism in two human dietary studies and an animal feeding trial.** 9th Annual International Conference of the Metabolomics Society. Glasgow, 1-4 July 2013.

Pedret-Figuerola A, Valls-Zamora RM, Fernández-Castillejo S, Catalán-Santos Ú, Lamuela-Raventós RM, Medina-Remón A, Espinel A, Solà R. **Polyphenol-rich foods exhibit DNA anti-oxidative properties and protect the gluthatione system in Healthy Subjects.** XXV Congreso Nacional de la Sociedad Española de Arteriosclerosis. Reus, 6-8 June 2012.

Catalán Ú, Rodríguez MA, Ras MR, Macià A, Mallol R, Vinaixa M, Fernández-Castillejo S, Valls RM, **Pedret A**, Griffin JL, Salek R, Correig X, Motilva MJ, Solà R. **Biomarkers of food intake and metabolite differences between plasma and red blood cell matrices; a human metabolomics profile approach.** XXV Congreso Nacional de la Sociedad Española de Arteriosclerosis. Reus, 6-8 June 2012.

Pedret-Figuerola A, Valls RM, Fernández-Castillejo S, Catalán Ú, Romeu R, Lamuela-Raventós RM, Arija V, Solà R. **Polyphenol-rich foods exhibit DNA anti-oxidative properties and protect the gluthatione system in healthy subjects.** 5th International Conference on Polyphenols and Helath. Sitges, 17-20 October 2011.

Rubió L, Valls RM, Macià A, **Pedret-Figuerola A**, Romero MP, Solà R, Motilva MJ. **Selection of the best phenol-enriched olive oil: a dose-response pharmacokinetic study in human.** 5th International Conference on Polyphenols and Health. Sitges, 17-20 October 2011.

Catalán Ú, Macià A, Fernández-Castillejo S, Martorell I, Valls RM, **Pedret A**, Giralt M, Motilva MJ, Solà R. **Is the phenolic profile in erythrocytes a nutritional biomarker?** 5th International Conference on Polyphenols and Health. Sitges, 17-20 October 2011.

Pedret-Figuerola A, Valls RM, Fernández-Castillejo S, Catalán Ú, Romeu M, Lamuela-Raventós RM, Arija V, Solà R. **Fermented alcoholic beverages, urinary total polyphenol excretion and DNA oxidative damage biomarker in healthy subjects.** Congreso Nacional de la Sociedad Española de Arteriosclerosis. Sevilla, 25-27 May 2011.

2.2 Oral Communications

Hanhineva K*, Lankinen M*, **Pedret A**, Schwab U, Kolehmainen M, Paananen J, De Mello V, Sola R, Lehtonen M, Poutanen K, Uusitupa M, Mykkänen H. **Non-targeted metabolite profiling discriminates diet-specific biomarkers for consumption of whole grains, fatty fish and bilberries.** 10th Annual International Conference of the Metabolomics Society. Tsuruoka, 23-26 June 2014.

Valls RM, Farràs M, **Pedret A**, Tormos MC, de la Torre R, Motilva MJ, Covas MI, Solà R. **Biodisponibilidad y bioactividad de aceites de oliva virgen funcionales en humanos.** XXV Congreso Nacional de la Sociedad Española de Arteriosclerosis. Reus, 6-8 June 2012.

Fernández-Castillejo S, Farràs M, Catalán Ú, **Pedret A**, Konstatinidou V, Muñoz-Aguayo D, Solà R, Fitó M. **Efecto del Consumo del acetie de oliva y sus compuestos fenólicos sobre la fluidez de la HDL.** XXV Congreso Nacional de la Sociedad Española de Arteriosclerosis. Reus, 6-8 June 2012.

3. VISITS TO OTHER INTERNATIONAL CENTRES

Visit to the Institute of Public Health and Clinical Nutrition at the University of Eastern Finland. Kuopio, Finland. First period: May 14th – August 16th, 2012; Second period: April 15th – April 26th, 2013.

Faculty of Health Sciences
School of Medicine
Institute of Public Health and Clinical Nutrition

UNIVERSITY OF EASTERN FINLAND

Kuopio, August 16th 2012

re: Visit of Ms Anna Pedret Figuerola

To Whom It May Concern

I can confirm that Ms **Anna Pedret Figuerola** has visited the Institute of Public Health and Clinical Nutrition at the University of Eastern Finland, Kuopio, between 14th May and 16th August to learn methodologies in metabolomics analysis of human dietary intervention samples. During her visit Ms Pedret Figuerola participated in the data-analysis of a sample set consisting of 212 samples. She participated in the data collection, data cleaning and statistical analysis to find the discriminative markers between the different dietary groups at baseline and after intervention. She also started the identification of the observed markers by extracting the elemental composition and comparing it to web-based databases. Ms Pedret Figuerola composed the preliminary tables of biomarker identification, which will be used in subsequent MS/MS analysis for the metabolite identification. Ms Pedret Figuerola has performed all the steps of the data analysis with very high precision, and she has accustomed easily with all the demanding steps involved in large-scale metabolomics assay.

Yours faithfully,

Hannu Mykkänen
Professor

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Visit of Ms Anna Pedret Figuerola

To whom it may concern,

I can confirm that Ms **Anna Pedret Figuerola** has visited the Institute of Public Health and Clinical Nutrition at the University of Eastern Finland, Kuopio, between 15th and 26th of April 2013 to learn methodologies in metabolomics analysis of human dietary intervention samples. During her visit Ms Pedret Figuerola has continued the data-analysis of a sample set consisting of 212 samples that she initiated on her previous visit last summer. She has now familiarized with the interpretation of MSMS spectral fragmentation patterns of the potential biomarkers of the intervention study. She has searched information for the metabolite identification from various databases and published literature. Ms Pedret Figuerola has performed all the steps of the data analysis with very high precision, and she has accustomed easily with all the demanding steps involved in large-scale metabolomics assay.

Yours faithfully,

Kati Hanhineva
Coordinator, Metabolomics unit of Biocenter Kuopio

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