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# DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS

Rosa M. Valls Zamora

European Thesis
Doctorat en Nutrició i Metabolisme
Departament de Medicina i Cirurgia
Unitat de Recerca en Lípids i Arteriosclerosi


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“Let your food be your medicine” said Hippocrates

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**INFORME DEL DIRECTOR DE TESI**

El/s (co)director/s sotasignats emeten l'informe de la tesi doctoral presentada a tràmit de dipòsit, en base a la revisió dels següents elements de qualitat:

| | SI | |
|---|---|---|
| La tesi consisteix en un treball original de recerca | X | |
| El títol reflecteix acuradament el contingut de la tesi | X | |
| Les hipòtesis i/o els objectius de la tesi estan clarament formulats | X | |
| La metodologia està descrita | X | |
| Hi consta el procediment | X | |
| Hi consten els resultats i la discussió dels mateixos | X | |
| Les conclusions de la tesi corresponen a les hipòtesis i/o objectius formulats | X | |
| La bibliografia està ben reflectida | X | |

D'aquesta tesi es deriven les següents aportacions científiques:

**- Effects of treatment with a soluble fiber (*Plantago ovata* husk) on biomarkers of cardiovascular disease risk in patients with hypercholesterolemia. A randomized trial.** Rosa Solà MD, PhD; Eric Bruckert MD; Rosa M Valls; et al. (sotmès a l'editor).

**- Beneficial effects of soluble fiber (*Plantago ovata* husk) on plasma triglycerides and apolipoprotein B to apolipoprotein A-I ratio in men in cardiovascular disease secondary prevention**
Rosa SOLÀ[1], Adriana ALVARO[1], Rosa M VALLS[1], Joan-Carles VALLVÉ[1], Anna ANGUERA[2]
(acceptat en 2009, com a capítol del llibre: Dietary Fiber: Sources, Functions and Benefits; www.novapublishers.com).

- **Effect of the long-term regular intake of virgin olive oil on the phenolic metabolites in human fasting plasma**
Valls RM[1*], Soler A[2*], Girona J[1], Heras M[1], Romero MP[2], Covas MI[3], Motilva MJ[2], Masana L[1], Solà R[1](sotmès a l'editor).

- **Antioxidant effect of virgin olive oil phenolic compounds and their identification in human plasma**
Josefa Girona[1*], Arantza Soler[2*], Cecilia González[1], Rosa-M Valls[1], Mercedes Heras[1], M-Paz Romero[2], María-Isabel Covas [3], M-José Motilva[2], Lluis Masana[1], Rosa Solà[1]
(sotmès a l'editor).

**- Improved method of microelution solid-phase extraction plate and liquid chromatography– tandem mass spectrometry to identification and quantification of olive oil phenols in human plasma**
Manuel Suárez1, Maria-Paz Romero1, Alba Macià1, Rosa M Valls2, Sara Fernández2, Rosa Solà2, Maria-José Motilva1 (sotmès a l'editor)

**- Cacao y Chocolate: ¿Un placer cardiosaludable?**
Vicente Pascual[1], Rosa M Valls[2], Rosa Solà[2] Clin Invest Arterioscl. 2009; 21:198-209.

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| |
|---|
| - **Effects of dietary supplements of cocoa cream on cardiovascular risk factors. A Randomized Trial**<br>Solà R, Valls RM, Godàs G, Pérez G, Ribalta J, Girona J, Heras M, Pons L, Cabré A, Castro A, Masana L, Anglès N, Reguant J, Ramírez B, Barriach JM. (sotmès a l'editor).<br><br>- **Informatics of nutritional intervention studies: Implementing CONSORT Statement**<br>Rosa-M VALLS, Rosa SOLÀ, Marta ROMEU, Montse GIRALT, Jordi ARAGONES, Josep-Mª GASTÓ, Xavier GÓMEZ (sotmès a l'editor) |
| Altres comentaris sobre la qualitat de la tesi: Menció Europea |

I en conclusió, s'emet l'informe FAVORABLE pel tràmit de dipòsit de la tesi doctoral i posterior defensa pública.

Reus, 21 de Juliol de 2009

Nom i cognoms. Rosa SOLÀ ALBERICH.
Director/a de la tesi

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# RESUMEN

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**Introducción:** Se han desarrollado diversas estrategias nutricionales para reducir los factores de riesgo o los biomarcadores de enfermedad cardiovascular (ECV). Los productos del cacao o la fibra soluble pueden ofrecer nuevas alternativas para modificar el riesgo de ECV, pero sus efectos sobre los biomarcadores de ECV aun no se han establecido. Además, algunos componentes bioactivos presentes en alimentos como los compuestos fenólicos o polifenoles del aceite de oliva virgen (AOV) deben ser identificados en plasma para relacionar los efectos biológicos con su presencia.

En la actualidad, los estudios de intervención deben ajustarse a los criterios de calidad definidos por el grupo CONSORT (Consolidated Standards of Reporting Trials) y su ejecución puede facilitarse mediante una aplicación informática.

**Hipótesis y objetivos:** Se proponen dos hipótesis.

Hipótesi 1:

Algunos componentes de la dieta como los productos del cacao, presentados en forma de cremas y que incluyen varios ingredientes (avellanas, fitosteroles y fibra soluble), o el tratamiento con fibra soluble, en particular, la cáscara de *Plantago ovata* (Po-husk), incorporados en una dieta baja en grasa saturada y en colesterol dietético, ayudan a controlar los niveles del colesterol de las lipoproteínas de baja densidad (c-LDL) y proporcionar nuevas dianas para reducir los biomarcadores de ECV.

Hipótesi 2:

La mejora de la metodología nutricional y técnica facilita la identificación de los compuestos fenólicos del aceite de oliva virgen (AOV) en plasma humano.

Por ello, los objetivos son los siguientes:

1) Evaluar los efectos de determinados productos del cacao, en forma de crema, sobre las concentraciones plasmáticas de los lípidos, la presión arterial y otros biomarcadores de ECV
2) Determinar los efectos de la fibra soluble (Po-husk) sobre el c-LDL y otros biomarcadores de ECV
3) Detectar y cuantificar los compuestos fenólicos del AOV en plasma humano

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4) Optimizar el proceso metodológico en los estudios de intervención nutricional para facilitar la aplicación de los criterios CONSORT

**Material y métodos:** La sección se describe en función de los objetivos a los que corresponden (1, 2, 3 y 4).

1) Estudio de intervención multicéntrico, randomizado, investigador ciego y de tipo paralelo con 4 productos diferentes de crema de cacao incorporados en una dieta isocalórica baja en grasa saturada y en colesterol, durante 4 semanas, con un período previo de 2 semanas de estabilización en que todos los participantes consumían el producto de la crema de cacao.

Los productos de cacao elaborados para el estudio han sido:

Producto A: crema de cacao (producto control)

Producto B: crema de cacao con avellanas

Producto C: crema de cacao con avellanas y fitosteroles

Producto D: crema de cacao con avellanas, fitosteroles y fibra soluble, el cual recibe el nombre de Portfolio.

2) Ensayo clínico multicéntrico, randomizado, comparativo, doble ciego y de tipo paralelo. El estudio fue diseñado para evaluar, en primer lugar, los efectos de Po-husk durante 8 semanas en comparación al placebo, y en segundo lugar, valorar el efecto de la combinación de la acción de la estatina en los pacientes no respondedores, mediante el análisis intragrupo durante 8 semanas más.

1 y 2) En el estudio de los productos del cacao y en el de Po-husk, los participantes son hombres y mujeres de más de 20 años de edad, con un c-LDL ≥ 3.35 mmol/L y ≤ 4.88 mmol/L y como mínimo con un factor de riesgo cardiovascular. Se considera factor de riesgo:

- edad superior a 45 años en el hombre y superior a 55 años en la mujer
- ser fumador
- hipertensión arterial
- colesterol de les lipoproteínas de alta densidad (c-HDL) ≤ 1.03 mmol/L en el hombre y ≤ 1.18 mmol/L en la mujer

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- antecedentes familiares de cardiopatía precoz (en hombres y mujeres, en familiares de primer grado, <55 años y <65 años de edad, respectivamente)

3) Consta de 3 partes: 1) se compararon los perfiles fenólicos entre consumidores habituales de AVO con no consumidores de AVO; 2), se estudió el perfil de los compuestos fenólicos en plasma postprandial después de ingerir 40 mL de AVO junto con 60 g de pan, en condiciones basales (después del ayuno de una noche) y a los 30, 60, 120 y 240 minutos de ingerir el AVO y 3) se validó el método de detección y cuantificación de los compuestos fenólicos del AOV en plasma humano.

1, 2 y 3) Los protocolos de los estudios realizados han sido aprobados por el Comité Ético de la Investigación Clínica (CEIC) del *Hospital Universitari Sant Joan* de Reus.

4) Se ha diseñado Reus – Informatics of Nutritional Intervention Studies (REUS-INIS), una herramienta metodológica para estandarizar y facilitar la presentación de los resultados de los proyectos, de forma transparente y con una valoración y interpretación crítica de la información, según los criterios de calidad del grupo CONSORT.

Los análisis estadísticos se adecuaron a cada estudio.

**Resultados:** Los resultados están descritos en función de los objetivos a los que corresponden (1, 2, 3 y 4).
1) 28 participantes han consumido el producto A, 28 el producto B, 30 el producto C y 27 el producto, en total 113 participantes.
La crema de cacao reduce la Presión Arterial Sistólica (PAS) media [95%, intervalo de confianza] un -7.89 mm Hg [-11.45 a -4.33] ($P$=0.0005) y la Presión Arterial Diastólica (PAD) -5.54 mm Hg [-7.79 a -3.29] ($P$=0.0001). En comparación

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con la crema control, el producto B reduce la PAD -3.38 mm Hg [-6.57 a -0.19] (*P*=0.03) y la Portfolio la disminuye -3.89 mm Hg [-7.15 a -0.63] (*P*=0.01).
En comparación con la crema control, el producto C reduce el c-LDL -11.2% (% diferencia del control) (*P*=0.0002), el colesterol total (CT) -7.4% (*P*=0.0001), la apolipoproteina B (Apo B-100) -8.1% (*P*=0.0016) y el cociente Apo B/Apo A -7.8% (*P*=0.0085). La Portfolio disminuye el c-LDL un -9.2% (*P*=0.0018), el CT -6.7% (*P*=0.0003), la Apo B-100 -8.5% (*P*=0.0012) y el cociente Apo B/Apo A -10.5% (*P*=0.0005).
En comparación con la crema control, la Portfolio reduce la Proteína C Reactiva ultrasensible (PCRus) -0.9 mg/L [-0.02 a 2.03] (*P*=0.0083) y la LDL oxidada (LDLox) -4.0 U/L [0.50 a 7.5] (*P*=0.02).
El peso corporal se mantuvo estable durante todo el estudio.

2) 101 participantes han consumido Po-husk y 108 participantes la fibra placebo (fibra insoluble). Después de 8 semanas, Po-husk reduce el c-LDL un 6.14%, los triglicéridos (TG) un 16% y Apo B-100 un 6%. Además, LDLox disminuye -5.3 U/l (95% intervalo de confianza -7.9 a -2.7), índice HOMA -0.31 µU/ml*mmol/L (0.42 a -0.20) y la PAS -3.8 mm Hg (-5.8 a -1.8) ($p<0.05$).

3) Los consumidores habituales de AOV, en condiciones basales, mostraron una mayor concentración de un tipo de flavonoide (2.90 ± 0.04 mmol/L vs 1.5 ± 0.04 mmol/L) y del derivado del catecol (0.70 ± 0.03 mmol/L vs 0.56 ± 0.03 mmol/L) que en el plasma de los no consumidores ($p<0.05$). Sin embargo, el análisis comparativo de los cromatogramas de los perfiles fenólicos en plasma entre el grupo de consumidores habituales y de los no consumidores de AOV no muestra diferencias cualitativas.
En plasma postprandial, después de ingerir 40 ml de AOV, se detectaron desde el minuto 60 al 240, la forma libre del hidroxitirosol, 3,4-DHPEA-EDA, luteolina y oleuropeina aglicona. Estos 4 compuestos fenólicos pertenecen a los 5 polifenoles con mayor actividad antioxidante observada en los ensayos i*n vitro*.
Finalmente, se ha validado la metodología para detectar y cuantificar los compuestos fenólicos del AOV en plasma humano.

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4) El acceso a la aplicación informática es a través de www.sortink.com. El programa incluye todas las secciones de los criterios CONSORT. Permite ver un resumen de cada nuevo proyecto que se introduce, junto con la duración y el equipo que forma parte de él. Además, el programa posee las siguientes características:

- Los datos se pueden clasificar fácilmente en las diferentes secciones
- Diferentes tipos de permisos y restricciones se pueden establecer para los usuarios
- Herramientas específicas que facilitan el traspaso de información entre el equipo investigador: correo, calendario/agenda y otros
- Herramienta para manejar y organizar la bibliografía
- Fácil almacenamiento de los datos: todo tipo de información, gráficos, datos e imágenes, que puede ser cargada y descargada

**Conclusión:** Los productos del cacao, presentados en forma de crema, y en particular la crema Portfolio, que es la combinación de algunos ingredientes cardioprotectores, y la fibra soluble (Po-husk) en una dieta baja en grasa saturada y en colesterol dietético, no solo inducen una reducción moderada de los niveles de c-LDL, sino que también tienen efectos beneficiosos sobre otros biomarcardores y factores de riesgo de ECV. Estos productos pueden mejorar la eficacia global de las dietas destinadas a reducir el riesgo de ECV.

Además de otros polifenoles, en el estudio del AOV, se ha detectado 3,4-DHPEA-EDA (un nuevo compuesto fenólico), en plasma humano.

Finalmente, incorporando los productos del cacao o la fibra soluble (Po-husk) o el AOV o la combinación de todos ellos, en una dieta baja en grasa saturada y en colesterol podría tenerse en cuenta como medida terapéutica de las ECV de forma individualizada en función del perfil de factores de riesgo de ECV de cada paciente.

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**Introduction:** Several nutritional strategies have been developed to reduce the risk factors, or biomarkers, of cardiovascular disease (CVD). Cocoa cream products and/or soluble fibre can offer new opportunities to modify CVD risk, but their effects on biomarkers of CVD remain to be established.

Further, some bioactive components present in a food items such as phenolic compounds or polyphenols of virgin olive oil (VOO), often present in very low concentrations, need to be identified in plasma in an approach to relate their presence to their biological effects.

Currently, intervention studies need to conform to the quality criteria defined by the CONSORT (Consolidated Standards of Reporting Trials) statement and this can be facilitated by the application of computer technology.

**Hypothesis and objectives:** we propose two hypotheses.

Hypothesis 1:

> Some dietary components such as cocoa cream products which include various ingredients (hazelnuts, phytosterols and soluble fibre), or soluble fibre treatment such as *Plantago ovata* husk (Po-husk), incorporated in a low saturated fat and low cholesterol diet can contribute to the control of plasma low density lipoprotein cholesterol (LDL-c) and to provide new targets to reduce CVD biomarkers.

Hypothesis 2:

> The nutritional and technical methodology improves VOO polyphenol identification in human plasma.

The objectives are:

1) To assess the effects of certain cocoa cream products on plasma lipids, blood pressure and other CVD biomarkers
2) To determine the effects of soluble fibre (Po-husk) on LDL-c and other CVD risk biomarkers
3) To detect and quantify VOO phenolic compounds in human plasma
4) To optimise the methodological processes to facilitate implementing the CONSORT criteria in interventional studies

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**Material and methods:** The section is described to correspond with the objectives 1, 2, 3 and 4 (above).

1) Multi-centred, randomised, investigador-blinded, controlled, parallel study in which 4 different cocoa cream products were introduced into a calorie-balanced diet for 4 weeks, with a prior stabilisation period of 2 weeks in which all participants received the cocoa cream product.

The cocoa cream products manufactured for the study were:

Product A: cocoa cream (control cream)

Product B: cocoa plus hazelnut cream

Product C: cocoa plus hazelnut and phytosterol cream

Product D: cocoa plus hazelnut, phytosterol and soluble fibre cream. The product was named Portfolio

2) Multi-centred, randomised, double-blind, placebo-controlled, parallel trial. The study was designed to evaluate, firstly, the effects of Po-husk for 8 weeks compared to placebo and, secondly, to assess the intra-group effect of the combined action of additional statin in non-responder patients for further 8 weeks.

1) and 2) In cocoa cream products and Po-husk studies, the participants were men and women between the ages of 20 and 65 years, with LDL-c ≥ 3.35 mmol/L and ≤ 4.88 mmol/L and at least one other major CVD risk factor, such as:

- Age, >45 years in men and >55 in women
- Current smoker
- High BP
- High density lipoprotein cholesterol (HDL-c) ≤ 1.03 mmol/L in men and ≤ 1.18 mmol/L in women
- Family history of premature heart disease (in males and females in first-degree relative <55 and <65 years of age, respectively)

3) The study was made up for 3 parts: 1) the phenolic profile in habitual consumers of VOO was compared with non-consumers of VOO; 2) the phenolic compound profiles in postprandial plasma were determined following the intake of 40 mL of

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VOO with 60 g of bread. The measurements were at baseline (following an overnight fast) and at 30, 60, 120 and 240 minutes post-intake; 3) the methodology was established and validated for the detection and quantification of the VOO phenolic compounds in plasma.

The protocols of the studies a) b) and c) were approved by the Clinical Research Ethical Committee of the *Hospital Universitari Sant Joan de Reus*.

4) Reus – Computer Technology in Nutritional Intervention Studies (REUS-INIS). A standard methodology to facilitate reporting of trial findings, complete and transparent reporting, and to add critical appraisal and interpretation according to the CONSORT statement.

**Results:** The results are described according to the objectives 1, 2, 3, and 4 (above).

1) Cocoa cream products study:

There were 28 participants in the product A group, 28 in the product B group, 30 in the product C group and 27 in the product D group in all, 113 participants.

Cocoa cream reduced systolic blood pressure (SBP) a mean of -7.89 mm Hg [95%CI: -11.45 to -4.33] (*P*=0.0005) and diastolic blood pressure (DBP) by -5.54 mm Hg [95%CI: -7.79 to -3.29] (*P*=0.0001). Compared with control, product B reduced DBP by -3.38 mm Hg [95%CI: -6.57 to -0.19] (*P*=0.03) and Portfolio decreased DBP by -3.89 mm Hg [95%CI: -7.15 to -0.63] (*P*=0.01).

Compared with control, product C reduced LDL-c by -11.2% (*P*=0.0002), total cholesterol (TC) by -7.4% (*P*=0.0001), apolipoprotein B (Apo B-100) by -8.1% (*P*=0.0016) and Apo B/Apo A ratio by -7.8% (*P*=0.0085); and Portfolio decreased LDL-c by -9.2% (*P*=0.0018), TC by -6.7% (*P*=0.0003), Apo B-100 by -8.5% (*P*=0.0012) and Apo B/Apo A ratio by -10.5% (*P*=0.0005).

Compared with control, Portfolio reduced high sensitive C-reactive protein (hsCRP) -0.9 mg/L [95%CI: -0.02 to 2.03] (*P*=0.0083) and oxidised LDL (oxLDL) by -4.0 U/L [95%CI: 0.50 to 7.5] (*P*=0.02).

Bodyweight was maintained stable during the study.

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2) Soluble fibre (Po-husk) study:

There were 101 participants in the Po-husk group and 108 in the placebo group. After 8 weeks, Po-husk reduced plasma LDL-c by -6.14% [adjusted mean: –0.29 mmol/L; 95%CI: –0.41 to –0.90] (*P* <0.0002), triglycerides (TG) by -0.22 mmol/L [95%CI: -0.34 to -0.11], Apo B-100 by -0.07 g/L [95%CI: 0.10 to -0.05], oxLDL by -5.3 U/L [95%CI: -7.9 to-2.7], HOMA-index by -0.31 μU/mlL*mmol/L [95%CI: 0.42 to -0.20] and SBP by -3.8 mm Hg [95%CI: -5.8 to -1.8] (*P*<0.05). At 16 weeks, 61 of 101 participants (60%) in the Po-husk group, 70 of 108 participants (65%) in placebo (*P*=0.5678), and the combined Po-husk + simvastatin group had similar lowering of LDL-c.

The bodyweight was kept stable during the study.

3) VOO polyphenols study:

For the 1st part: There were 20 participants in each group. Fasting plasma from VOO consumers showed higher concentration of a flavonoid type (2.90 ± 0.04 μmol/L vs 1.5 ± 0.04 μmol/L) and catechol derivative (0.70 ± 0.03 μmol/L vs 0.56 ± 0.03 μmol/L) than the plasma from non-consumers (*P*<0.05). However, the comparative analysis of the chromatographic profiles of the phenolic extracts of plasma samples between the habitual consumers and non-consumers of VOO showed no significant qualitative differences.

For the 2nd part: In postprandial plasma following 40 mL VOO intake, the free form of hydroxytyrosol, 3,4-DHPEA-EDA, luteolin and oleuropein aglycone were detected at 60 and up to 240 minutes post-intake. These 4 phenolic compounds belong to the 5 polyphenols with major antioxidant activity in the *in vitro* assays conducted.

For the 3rd part: The methodology to detect and quantify the phenolic compounds in human plasma was validated.

4) Access to the computer application is through www.sortink.com. The program included all sections of the CONSORT statement and includes a summary of a new trial that specifies the study duration and the teamwork involved. In addition, the program features the following aspects:

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- Data can be easily classified in sections
- Different types of permissions and restrictions for users can be set
- Specific tools for a collaborative framework are available: email and calendar, among other features
- Powerful bibliography manager tool
- Easy computerised data storage: various information charts, data, images that can be up- as well as down-loaded

**Conclusions:** We conclude that cocoa cream products (particularly the Portfolio type cream, which contain a combination of some cardioprotective ingredients), and Po-husk, as part of a low saturated fat and low cholesterol content diet, not only induced a moderate reduction in plasma LDL-c, but also had beneficial effects on other CVD biomarkers and risk factors. These products can improve the overall efficacy of diets designed to lower CVD risk.

Among other polyphenols, the VOO study detected 3,4-DHPEA-EDA (a new phenolic compound) in human plasma.

Finally, applying cocoa products or soluble fibre or VOO or in combination in a low saturated fat and low cholesterol diet warrants consideration as individualised therapeutic measures based on the individual's CVD risk factor profile.

UNIVERSITAT ROVIRA I VIRGILI
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# INDEX

UNIVERSITAT ROVIRA I VIRGILI
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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Abbreviations 11
1. Introduction 19
1.1. Cardiovascular disease 21
1.2. Atherosclerosis, oxidative stress, and inflammation 23
1.2.a. Defence against oxidative stress 29
1.2.b. Food items promoting optimal defence against oxidative stress 30
1.3. Gene-environment interactions 31
1.4. The concept of atherosclerosis risk factors and biomarkers 33
1.4.a. Definition and types 35
1.4.b. Characteristics of an ideal biomarker 37
1.4.c. Emerging blood biomarkers of CVD 43
1.4.d. Biomarkers in CVD risk assessment 44
1.5. Diet and CVD 45
1.6. Diet and biomarkers of CVD 46
1.6.a. High blood pressure 47
1.6.b. Integrity of artery lining 47
1.6.c. Elevated blood lipids 47
1.6.d. High homocysteine levels 48
1.6.e. Increased blood clot formation 48
1.6.f. Insulin resistance syndrome 48
1.7. Lifestyle modification in CVD 50
1.7.a. Dietary recommendations in CVD risk management 51
1.8. Mediterranean diet and olive oil 53
1.9. Cocoa 57
1.9.a. Stearic acid in chocolate 59
1.9.b. Cocoa polyphenols 60
1.9.c. Possible mechanisms underlying the protective effects of cocoa 63
1.9.c.i. Endothelial function and nitric oxide 65
1.9.c.ii. Antioxidant properties 67
1.9.c.iii. Platelet function 68

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



1.9.d. Antihypertensive effects of cocoa 68
1.9.e. Cocoa effects on blood lipids 70
1.9.f. Cocoa effects on insulin resistance 71
1.9.g. Considerations 71
1.10. Dietary fibre and definitions 72
1.10.a. Recommendations for fibre 73
1.10.b. Fibre and CVD 74
1.10.c. Fibre and risk factor prevalence 74
1.10.d. Fibre and effects on risk factors status 75
1.10.d.i. Lipoproteins 75
1.10.d.ii. Blood pressure 78
1.10.d.iii. Other risk factors 78
1.10.e. Proposed mechanisms underlying effects of fibre on CVD risk factors 79
1.11. Dietary components 79
1.11.a. Phenolic compounds 79
1.11.b. Types and classification of phenolic compounds 80
1.11.c. Classification and structure of major dietary polyphenols 82
1.11.d. Factors affecting choice of potential biomarkers of polyphenols intake 84
1.11.e. Bioavailability and bioefficacy of phenolic compounds 86
1.11.f. Phenolic molecules in VOO 87
1.11.f.i. Secoiridoids 89
1.11.f.ii. Phenolic acids 89
1.11.f.iii. Lignan 90
1.11.f.iv. Hydroxyl-isochromans 90
1.11.f.v. Flavonoids 91
1.11.f.vi. Luteolin 91
1.11.g. Health aspects linked to polyphenols in VOO 91
1.12. Other food components for a healthy hea 93
1.12.a. Antioxidants-rich diets 93

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



1.12.b. Folate and homocysteine 93
1.12.c. Minerals 94
1.12.d. Peptides from milk protein 94
1.12.e. Plant sterol and stanol esters 94
1.12.f. Soybean protein 94
1.12.g. Vitamin supplements 94
1.12.h. Dietary patterns 95
1.13. Methodological aspects 97
1.13.a. Randomised, controlled trials execution and description 97
1.13.b. Interventional control 109
1.13.b.i. Details of the interventions 109
1.13.b.ii. Details of the different components of the interventions 109
1.13.b.iii. Standardisation of the interventions 110
1.13.b.iv. Adherence of care providers to the protocol: assessment and enhancement 110
2. Hypothesis and objectives 111
3. Material and methods 117
3.1. Diet design 119
3.1.a. Cocoa cream products and soluble fibre studies 119
3.1.b. Olive oil polyphenols detection study 119
3.1.c. Monitoring of diets 120
3.2. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 120
3.2.a. Study participants 121
3.2.b. Flow of participants through the study 122
3.2.c. Cocoa cream products 123
3.2.d. Clinical and laboratory measurements 125
3.2.e. Diagram of visits 126
3.2.f. Sample size calculation 126
3.3. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD 128

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



3.3.a. Study participants 128
3.3.b. Flow of participants through the study 129
3.3.c. Soluble fibre and statin 130
3.3.d. Clinical and laboratory measurements 130
3.3.e. Diagram of visits 131
3.3.f. Sample size calculation 131
3.4. Cocoa and soluble fibre adverse effects 131
3.5. Interventional control 133
3.5.a. Cocoa cream products study 133
3.5.a.i. Details of the interventions 133
3.5.a.ii. Details of the different components of the interventions 133
3.5.a.ii.1. Diets 133
3.5.a.ii.2. Cocoa cream products 134
3.5.a.iii. Standardisation of the interventions 134
3.5.a.iv. Adherence of care providers to the protocol: assessment and enhancement 134
3.5.a.iv.1. Diets 134
3.5.a.iv.2. Cocoa cream products 135
3.5.b. Po-husk study 135
3.5.b.i. Details of the interventions 135
3.5.b.ii. Details of the different components of the interventions 135
3.5.b.ii.1. Diets 135
3.5.b.ii.2. Treatments 136
3.5.b.iii. Standardisation of the interventions 136
3.5.b.iv. Adherence of care providers to the protocol: assessment or enhancement 136
3.5.b.iv.1. Diets 136
3.5.b.iv.2. Treatments 136
3.6. Olive oil polyphenols detection in human plasma study 137

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



3.6.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers 137
3.6.a.i. Study participants 137
3.6.a.ii. Clinical and laboratory measurements 138
3.6.a.ii.1. Olive oil polyphenols detection and quantification in human plasma 139
3.6.a.iii. Diagram of visits 139
3.6.b. Olive oil polyphenols detection in postprandial human plasma 140
3.6.b.i. Study participants and VOO load 141
3.6.b.ii. Clinical and laboratory measurements 142
3.6.b.iii. Diagram of visits 142
3.6.c. Validation method to detect and quantify olive oil polyphenols in human plasma 143
3.7. Biomarkers 143
3.7.a. Lipid profile 144
3.7.b. Oxidative stress 144
3.7.c. Endothelial dysfunction 144
3.7.d. Inflammatory activity 144
3.7.e. Antithrombotic activity 144
3.7.f. Insulin resistance 145
3.7.g. Metabolic syndrome 145
3.7.h. Gene polymorphisms 145
3.7.i. Biomarkers evaluated in each study 146
3.8. Statistical analysis 148
3.8.a. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 149
3.8.b. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD 150
3.8.c. Olive oil polyphenols detection in human plasma study 152

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



3.8.c.i. Olive oil polyphenols detection in human plasma from habitual VOO consumers 152
3.8.c.ii. Olive oil polyphenols detection in human prostprandial plasma 152
3.9. Computerised systems application in interventional studies 152
3.10. Financial support 154
3.10.a. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 154
3.10.b. Effects of treatment with soluble fibre (Po-husk) on biomarkers of CVD 154
3.10.c. Olive oil polyphenols detection in human plasma study 154
3.10.d. Computerised applications for interventional studies 154
4. Results 155
4.1. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 157
4.1.a. Participants 157
4.1.b. Dietary adherence and consumption of cocoa cream products 158
4.1.c. Lipid profile 159
4.1.d. Anthropometric and clinical measures 159
4.1.e. CVD biomarkers 159
4.1.e.i. Endo-PAT subgroup 159
4.1.f. Adverse events 159
4.1.g. Patent 172
4.1.h. Clinical trials registration 172
4.2. Effects of treatment with soluble fibre (Po-husk) on biomarkers of CVD 172
4.2.a. Participants 172
4.2.b. Dietary and treatment adherence 172
4.2.c. Lipid profile 174
4.2.d. Anthropometric and clinical measures 175
4.2.e. CVD biomarkers 175
4.2.f. Insulin resistance biomarkers 175

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



4.2.g. Gene polymorphisms 175
4.2.h. Statin treatment 176
4.2.i. Adverse events 176
4.2.j. Patent 186
4.2.k. Clinical trials registration 186
4.2.l. CONSORT Statement checklist 186
4.3. Olive oil polyphenols detection in human plasma study 189
4.3.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers 189
4.3.a.i. Participants 189
4.3.a.ii. Dietary composition 190
4.3.a.iii. Phenolic compounds profile 190
4.3.b. Olive oil polyphenols detection in human postprandial plasma 191
4.3.b.i. Phenolic compounds concentration in VOO consumed 191
4.3.b.ii. VOO polyphenols detectection in postprandial plasma 192
4.3.c. Validation of methods to detect and quantify olive oil polyphenols in human plasma 193
4.4. Computerised applications for interventional studies 195
5. Discussion 199
5.1. General comments 201
5.2. Diet compliance and monitoring 201
5.3. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 202
5.4. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD 205
5.5. Olive oil polyphenols detection in human plasma 208
5.6. Computer technology applied in interventional studies 209
5.7. General 209
5.8. Overall limitations 211

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



6. Conclusions 213

6.1. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD 215

6.2. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD 215

6.3. Olive oil polyphenols detection in human plasma study 215

6.3.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers 215

6.3.b. Olive oil polyphenols detection in human postprandial plasma 216

6.4. Computer technology applied in interventional studies 216

6.5. Summary of conclusions 216

6.6. Overall conclusion 217

7. References 219

8. Appendix A: Contributions at Congresses and Conferences 235

9. Appendix B: Scientific papers 241

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

# ABBREVIATIONS

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Rosa Maria Valls Zamora
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**3,4-DHPEA:** hydroxytyrosol or (3,4-dihydroxyphenyl) ethanol

**3,4-DHPEA-EA:** oleuropein aglycon

**3,4-DHPEA-EDA:** decarboxymethyloleuropein aglycon

**A-I:** angiotensin I

**A-II:** angiotensin II

**ACE:** angiotensin-converting enzyme

**ACS:** American Cancer Society

**ADA:** American Diabetes Association

**ADMA:** asymmetric dimethylarginine

**ADO:** available data only

**ADP:** adenosine diphosphate

**AEMPS:** Agencia Española de Medicamentos y Productos Sanitarios

**AHA:** American Heart Association

**AI:** adequate intake

**AMDRs:** acceptable macronutrient distribution ranges

**ANCOVA:** analysis of covariance

**Apo A-1:** apolipoprotein A

**Apo A-2:** apolipoprotein A-2

**Apo A-5:** apolipoprotein A-5

**Apo B-100:** apolipoprotein B

**Apo B / Apo A:** ratio between apolipoprotein B-100 and apolipoprotein A-1

**Apo E:** apolipoprotein E

**$AT_1$:** angiotensin receptor

**ATP III:** Adult Treatment Panel III

**AU:** arbitrary units

**bET-1:** big endothelin-1

**BMI:** body mass index

**BP:** blood pressure

**CHD:** coronary heart disease

**CHO:** carbohydrate

**CI:** confidence interval

**cGMP:** cyclic guanosine monophosphate

**Cmax:** maximal plasma concentration

**CONSORT:** Consolidated Standards of Reporting Trials

**CREC:** Clinical Research Ethical Committee

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**CRP:** C reactive protein
**CT scan:** computer axial tomography
**CV:** coefficient of variation
**CVD:** cardiovascular disease
**d:** day
**DASH:** The Dietary Approaches to Stop Hypertension
**DBP:** diastolic blood pressure
**DDAH:** dimethylarginine dimethylaminohydrolase
**DNA:** deoxyribonucleic acid
**EA:** elenolic acid
**EAS:** European Atherosclerosis Society
**EC:** endothelial cells
**ECE:** endothelial-converting enzyme
**ECG:** electrocardiogram
**EDHF:** endothelium-derived hyperpolarising factor
**EDTA:** ethylenediaminetetraacetic acid
**eNOS:** endothelial NO synthase
**ESC:** European Society of Cardiology
**ESI:** electro spray ionisation
**ET-1:** endothelin 1
**$ET_{a/b}$:** endothelin receptor a and b
**FABP-4:** fatty acid binding protein-4
**Fbg:** fibrinogen
**FDA:** food and drug administration
**FFA:** free fatty acid
**F-HD:** family history of premature heart disease
**FPG:** fasting plasma glucose
**FSH:** Framingham Heart Study
**g:** gram
**GLM:** general linear models
**GSH-Px:** glutathione peroxidase
**HDL:** high density lipoprotein
**HDL-c:** high density lipoprotein cholesterol
**HPLC-MS/MS:** high performance liquid chromatography tandem mass spectometrer
**HPMC:** hydroxypropyl methylcellulose

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**HOMA-index:** homeostatic model assessment index
**hsCRP:** high sensitivity C reactive protein
**HSP:** heat shock protein
**ICAM-1:** intercellular adhesion molecule-1
**ICCO:** International Cocoa Organization
**IDF:** International Diabetes Federation
**IL:** interleukin
**IL-1:** interleukin-1
**IL-6:** interleukin-6
**IL-10:** interleukin-10
**IL-18:** interleukin-18
**IMA:** ischemia modified albumina
**IOM:** Institute of Medicine
**ITT:** intention to treat
**LDL:** low density lipoprotein
**LDL-c:** low density lipoprotein cholesterol
**LSMEANS:** least square means
**LOCF:** last observation carried forward
**Lp(a):** lipoprotein (a)
**LTB4:** leukotriene-B4
**MCP-1:** monocyte chemoattractant protein-1
**MedDiet:** mediterranean diet
**MedDra:** Medical Dictionary for Regulatory Activities
**MeSH:** medical subject heading
**MET:** metabolic equivalent unit
**MetS:** metabolic syndrome
**MI:** myocardial infarction
**MMP:** matrix metalloproteinase
**MMP-9:** matrix metalloproteinase-9
**MPO:** myeloperoxidase
**MRFIT:** Multiple Risk Factor Intervention Trial
**mRNA:** messenger RNA
**M-SCF:** macrophage colony-stimulating factor
**MUFA:** monounsaturated fatty acids
**MVC:** model-vista-control

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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**Myg:** myoglobin
**MySQL:** multithread structured query language
**n-3:** omega 3, polyunsaturated fatty acid
**n-6:** omega 6, polyunsaturated fatty acid
**N:** number of subjects
**NACB:** The National Academy of Clinical Biochemistry
**NCEP:** National Cholesterol Education Program
**NEFA:** nonesterified fatty acids
**NF-kB:** nuclear factor kappa-light-chain-enhance of activated B cells
**NIH:** National Institutes of Health
**NO:** nitric oxid
**NT-proBNP:** N-terminal proBNP
**OGGT:** oral glucose tolerance test
**OH:** hydroxide
**Omniheart:** The Optimal Macronutrient Intake Trial to Prevent Heart Disease
**oxHDL:** oxidised HDL
**oxLDL:** oxidised LDL
**P25:** percentile 25
**P75:** percentile 75
**PAI-1:** plasminogen activator inhibitor-1
**PAT:** peripheral arterial tonometry
**PCR:** polymerase chain reaction
**PDA:** photodiode array detector
**$PGI_2$:** prostacyclin
***p*-HPEA:** tyrosol or (*p*-hydroxyphenyl) ethanol
**p-HPEA-EA:** ligstroside aglycon
**p-HPEA-EDA:** decarboymethyl ligstroside aglycon
**PKC:** protein kinase C
**PPAP-A:** pregnancy-associated plasma protein-A
**PIGF:** placental growth factor
**Po-husk:** *Plantago ovata* husk
**PP:** per protocol
**PROCRAM:** The Prospective Cardiovascular Münster score
**PUFA:** polyunsaturated fatty acids
**RCTs:** randomized controlled trials

UNIVERSITAT ROVIRA I VIRGILI
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**REMARK:** Reporting recommendations for tumour MARKer prognostic studies

**REUS-INIS:** Reus- Informatics of Nutritional Intervention Studies

**RNA:** ribonucleic acid

**ROR:** ruby on rail

**ROS:** reactive oxygen species

**SAS:** statistical analysis system

**SBP:** systolic blood pressure

**sCD40L:** soluble CD40 ligand

**SCORE:** Systematic Coronary Risk Evaluation (from the European Society of Cardiology)

**SD:** standard deviation

**SEM:** standard error of the mean

**SFA:** saturated fatty acids

**SNP:** single nucleotide polymorphism

**SNPs :**

- **ABCG5:** ATP-binding cassette transporter G5 (rs6720173) → 1810C/G
- **ABCG8:** ATP-binding cassette transporter G8 (rs4148211) → 161A/G
- **ACE:** angiotensin-converting enzyme (rs4343) → 2350G/A
- **ApoA1:** apolipoprotein A-1(rs670) → -75G/A
- **APOA-V:** apolipoprotein A-V (rs662799) → -1131T>C
- **APOE-112:** apolipoprotein E (rs7412) → R158C (T/C Cys-Arg 176)
- **APOE-158:** apolipoprotein E (rs429358) → C112R (C/T Arg-Cys 130)
- **CETP:** cholesteryl ester transfer protein (rs2303790) → D442G (459 Gly-Asp)
- **CRABP2:** cellular retinoic acid binding protein-2 (rs2236795) → -394T/C
- **FABP-2:** fatty acid binding protein-2 (rs1799883) → Ala54Thr
- **FABP-4:** fatty acid binding protein-4 (rs8192688) → C2600T
- **FIBA:** fibrinogen de cadena alfa (rs2070024) → -500C/T
- **PAI-1:** plasminogen activator inhibitor-1 (rs1799768) → 4G/5G
- **PPARa:** peroxisome proliferator-activated receptor- alfa (rs1800206) → Leu162Val

**SOD:** superoxide dismutase

**SPE:** solid-phase-extraction

**SPSS:** Statistical Package for the Social Sciences

**SR:** scavenger receptor

**SRM:** selected reaction monitoring mode

**SSL:** secure sockets layer

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**STARD:** Standards for Reporting of Diagnostic accuracy

**STROBE:** Strengthening the Reporting of Observational Studies in Epidemiology

**TC:** total cholesterol

**TF:** tissue factor

**TFA:** trans-unsaturated fatty acid

**TG:** triglycerides

**TIMP:** tissue inhibitors of MMPs

**TNFα:** tumor necrosis factor-alpha

**TNI:** troponin I

**TNT:** troponin T

**t-PA:** tissue plasminogen activator

**TXB2:** thromboxane-B2

**UdL:** *Universitat de Lleida*

**UPLC-ESI-MS/MS:** ultra performance liquid chromatography employing electro spray ionization mass spectometry

**URLA:** *Unitat de Recerca en Lípids i Arteriosclerosi*

**URV:** *Universitat Rovira i Virgili*

**USA:** United States of America

**USDA:** United States Department of Agriculture

**USFA:** unsaturated fatty acids

**UV:** ultraviolet

**VCAM-1:** vascular cell adhesion molecule-1

**VLDL:** very low density lipoprotein

**VLDL-c:** very low density lipoprotein cholesterol

**VOO:** virgin olive oil

**vs:** *versus*

**VSCM:** vascular smooth muscle cell

**vWf:** von Willebrand factor

**WC:** waist circumference

**WHO:** World Health Organization

UNIVERSITAT ROVIRA I VIRGILI
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# INTRODUCTION

UNIVERSITAT ROVIRA I VIRGILI
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### 1.1. Cardiovascular disease

The World Health Organization (WHO) stresses the importance of a healthy diet in preventing non-communicable diseases. However, a diet can only be as healthy as the combination of individual foods contained in it.

A healthy diet is not merely limiting certain components-of-concern such as saturated or trans fatty acids, or simply delivering nutrient intake. It also concerns those elements that can provide an extra health benefit.

The best food items for a particular need should be based on scientific evidence.

It is certainly not sufficient to evaluate certain parameters simply because we are able to measure them. Rather, we need to ensure that the parameters we use to measure health benefit are indeed valid and are relevant biomarkers for the target functions being assessed.

Extensive clinical and statistical studies have identified several factors that increase the risk of coronary heart disease (CHD) and myocardial infarction (MI). Major risks factors are those that research has shown to significantly increase the risk of heart and blood vessel (cardiovascular) disease. Other factors, described as “contributing risk factors” by the American Heart Association (AHA) Scientific Position in 2009, are associated with increased risk of cardiovascular disease (CVD), but their significance and prevalence haven't as yet been clearly defined.

The propective, community-based Framingham Heart study (FHS) provides rigorous support for the concept that hypercholesterolaemia, hypertension and other factors correlate significantly with CVD risk (ATP III, 2001).

In primary prevention, traditional risk factors are a useful first step in the identification of those individuals who could be at risk for CVD events. In the era of “global risk assessment”, scores such as the Framingham score, the Prospective Cardiovascular Münster (PROCAM) score, or the European Society of Cardiology Systematic Coronary Risk Evaluation (SCORE), all of which are derived from multi-variable statistical models, should be used.

However, it has been noted that a considerable number of at-risk individuals cannot be identified on the basis of traditional risk factors alone. This has prompted the search for novel markers of CVD risk to help improve risk prediction.

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Such markers could either represent various biomarkers in the circulation which are relevant to the pathophysiology of atherothrombosis (e.g. biomarkers of inflammatory response, coagulation, platelet aggregation, lipoproteins, or lipid-related variables, genetic mutations), or biomarkers of subclinical disease; all of which assist in improved risk prediction.

As greater insight into the basis of variation between the individual's physiological responses is gained from the application of "omics" (proteomics, genomics, etc), it may be possible quantify an individual's risk of, say, heart disease, and to choose food items and diets that would help defer, or delay, the onset of such adverse cardiovascular events. An interaction between developments in food science and "omics" may, in the future, result in individuals being able to make fully-informed choices regarind food items that provide the best opportunities for improved health, well-being and reduced risk of disease.

Measurement of global risk based on traditional risk factors allows categorisation of the individual into high (10-year risk, >20%), low (10-year risk, <10%), or intermediate risk (10-year risk, 10% to 20%). Subjects at high risk should have lifestyle changes recommended, or prescribed appropriate pharmacotherapy (currently, one of the statins). Subjects at low risk would be re-evaluated 3 to 5 years later. Those at intermediate risk, however, who comprise up to 40% of the population at risk, would be candidates for additional testing to decrease their risk status. A large panel of blood biomarkers are available for this purpose, but most of them are not yet applicable in standard clinical practice for reasons including economics, borderline relevance and current quality of measurement.

Often, established risk factors do not directly reflect the prognosis in CVD with respect to myocardium damage, left ventricular dysfunction, renal failure and inflammation. Hence, new biomarkers such as troponin I, N-terminal pro–brain natriuretic peptide, cystatin C, and C-reactive protein (CRP) have been explored in relation to clinical conditions that have been shown to be associated with an increased risk of CVD and death (Zethelius B, 2008).

Recently, in a study of elderly men with or without prevalent CVD, the simultaneous measurement of several biomarkers of cardiovascular and renal abnormalities substantially improved the stratification risk of death from

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



cardiovascular causes beyond that of a model based on the more established risk factors (Zethelius B, 2008).

Substantial data indicate that CVD is a life-course disease, the onset of which lies in a background of risk factors which then progresses subclinical atherosclerosis and, if left unchecked, to overt CVD (Vasan R, 2006).

The onset of CVD itself portends an adverse prognosis with increased risk of recurrent events, morbidity, and mortality. However, it is also increasingly clear that although frequent clinical assessment is the keystone of patient management, such evaluation has its limitations (Vasan R, 2006).

As suggested by a recent National Institutes of Health (NIH) panel report (Naghavi M, 2003) many additional tools have been used to aid clinical assessment and to enhance the ability to identify the “vulnerable” patient at risk of CVD. Biomarkers are one such tool which can be used to better identify high-risk individuals, to diagnose incipient disease conditions promptly and accurately in order to implement preventative measures, to derive a prognosis, and to treat patients who have the established disease.

In this context, the effect of diets on CVD biomarkers is a valuable approach to the manipulation of those dietary factors that could result in a reduction in the risk of the disease.

### 1.2. Atherosclerosis, oxidative stress, and inflammation

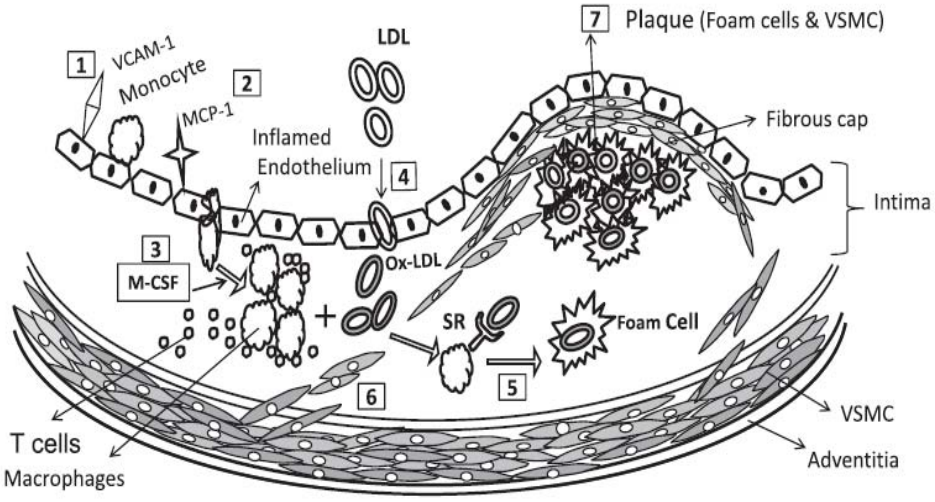
CVD is a major public health concern. Despite major progress in diagnosis and treatment over the past 25 years, CVD represents, according to the WHO, the most frequent cause of morbidity and mortality (28% of all deaths annually) in the developed world. In Europe, CVD is responsible for nearly half (49%) of all mortality – over 4 million each year – with CHD and stroke as the predominant causes of death. Atherothrombosis, which involves direct interaction between atherosclerotic plaque and arterial thrombosis, underlies the majority of cardiovascular events, independently of the specific vascular bed in which they occur (Chapman J, 2007).

Atherosclerosis, a major degenerative disease of arteries involves a series of inflammatory and oxidative modifications within the arterial wall (Fan J, 2003).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 1.** Oxidative stress and inflammation in initiation and progression of atherosclerosis




(1) VCAM-1 promoting monocyte adhesion. (2) MCP-1 mediating monocyte migration into intima. (3) M-CSF promoting monocyte maturation into macrophage. (4) LDL deposition in intima and oxidative modification to oxLDL. (5) Macrophages engulfing oxLDL through SR and converting as foam cells. (6) T cells promoting VSMC proliferation and migration into intima. (7) Plaque formation: foam cells surrounded by VSMC. Macrophages, T cells, foam cells, and VSMC secrete pro-inflammatory cytokines and collagenases that aggravate the process of atherosclerosis and lead to thrombotic event.

Abbreviations: VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; oxLDL, oxidised-LDL; SR, scavenger receptor; VSMC, vascular smooth muscle cell.

Source: Basu A, 2009.

Figure 1 depicts the putative roles of oxidative stress and inflammation in the initiation and progression of atherosclerosis, as postulated by existing research (Fan J, 2003). Emerging research shows that obesity, hypertension, diabetes mellitus, dyslipidaemia, smoking, aging, diets rich in saturated fats, and low physical activity are the established risk factors for atherosclerosis (ATP III, 2001), highlighted as inflammation status and oxidant burden (Singh U, 2006).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Oxidative stress, an imbalance between free radical formation and antioxidant status, is the major contributor to CVD, while inflammation itself is a manifestation of oxidative stress. Oxidative stress induces inflammation via pathways that generate inflammatory mediators such as adhesion molecules and pro-inflammatory cytokines (Basu A, 2009). Recent studies in humans have demonstrated significant positive associations between oxidative stress and inflammation and indicators of vascular damage including impaired endothelial function and increasing arterial rigidity (Lavi S, 2008).

Oxidative stress and inflammation also induce vascular smooth muscle cell (VSMC) activation and proliferation, as well as angiogenesis, lipid peroxidation, and platelet activation (Yung L, 2006).

CRP, vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-a (TNFa), interleukin-1 (IL-1), interleukin-18 (IL-18), soluble CD40 ligand (sCD40L), and monocyte matrix metalloproteinase 9 (MMP-9) are biomarkers of inflammation (Packard R, 2008).

Lipid peroxidation, oxidised-LDL (oxLDL), and urinary 8-isoprostane levels are the oxidative biomarkers of CVD risk (Steinberg D, 2002).

Oxidative stress and inflammation lead to endothelial dysfunction by reducing nitric oxide (NO) bioavailability and the formation of peroxynitrite, which is cytotoxic (Qamirani E, 2005).

Endothelial cells (EC) when exposed to inflammatory stimuli express VCAM-1 and promote monocyte adhesion, an initial step in atherosclerosis. Selectins, integrins, and monocyte chemoattractant protein-1 (MCP-1) expressed on EC surface mediate attachment and migration of monocytes into the intima layer of the artery, where they reside and continue to increase in numbers. The intima, under inflammatory conditions, expresses macrophage colony stimulating factor (M-CSF), which promotes monocyte maturation into macrophages (Basu A, 2009).

Atherogenic lipoproteins, such as low-density lipoproteins (LDL) and very-low-density lipoprotein (VLDL), enter the sub-intima space and undergo oxidative modifications resulting in the formation of oxLDL.

Macrophages express scavenger receptors (SR) which engulf oxLDL to form lipid laden foam cells. Macrophages further the process of atherosclerosis by secreting

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



pro-inflammatory cytokines such as TNF-α, and IL-1β. T cells join macrophages and promote plaque formation by inducing VSMC migration and proliferation. During the final stage, VSMC express enzymes that degrade collagen, weaken the fibrous plaque cap and make it prone to rupture (Basu A, 2009).

Inflammatory stimuli also increase the expression of pro-coagulant tissue factor (TF), which triggers thrombus formation when the plaque ruptures. Thrombus formation leads to acute coronary syndrome (Basu A, 2009).

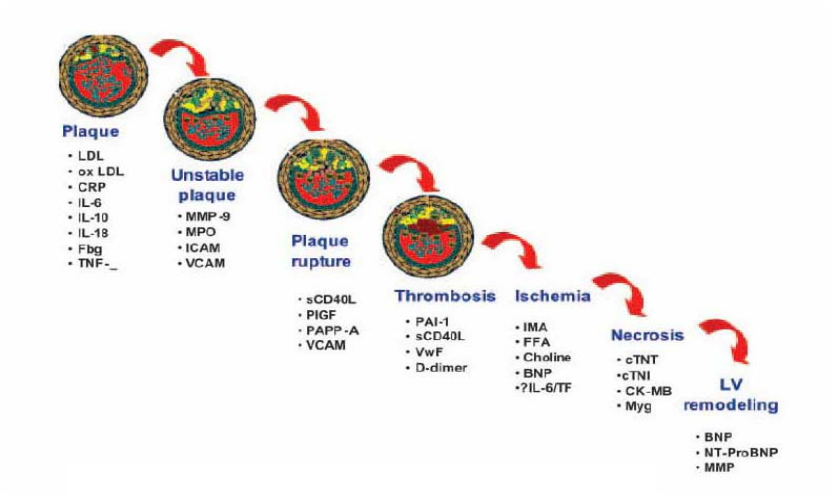
Thus, oxidative stress, inflammation initiation and endothelial dysfunction participate in, and enhance, the process of atherosclerosis. As such, they constitute the principal targets of therapeutic interventions (including diets) with the intention of preserving the endothelium and/or in reversing atherosclerosis (Basu A, 2009).

Each biomarker can be seen as an indicator of the stage of the arteriosclerosis process, as summarised in Figure 2.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 2**. Biomarkers of acute coronary syndromes




The arrows indicate the sequence of events during an acute coronary syndrome. Biomarkers that may be elevated at each phase of the disease are displayed.

Abbreviations: sCD40L, soluble CD40 ligand; Fbg, fibrinogen; FFA, free fatty acid; ICAM, intercellular adhesion molecule; IL-interleukin; IMA, ischemia modified albumina; MMP, matrix metalloproteinases; MPO, myeloperoxidase; Myg, myoglobin; NT-proBNP, N-terminal proBNP; oxLDL, oxidised low-density lipoprotein; PAI-1, plasminogen activator inhibitor; PPAP-A, pregnancy-associated plasma protein-A; PIGF, placental growth factor; TF, tissue factor; TNF, tumor necrosis factor; TNI, troponin I; TNT, troponin T; VCAM, vascular cell adhesion molecule; vWF, von Willebran factor.

Source: Vasan R, 2006.

Two types of serological biomarkers are proposed as indicators of vunerability:

a) those reflecting metabolic and immune disorders (Table 1)

b) those reflecting hypercoagulability (Table 2)

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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**Table 1.** Serological biomarkers of vulnerability (reflecting metabolic and immune disorders)

- Abnormal lipoprotein profile (eg. high LDL, low HDL, abnormal LDL and HDL size density, lipoprotein (a), etc)
- Nonspecific biomarkers of inflammation (eg. hsCRP, CD40L, ICAM-1, VCAM-1, P-selectin, leukocytosis, and other serological biomarkers related to the immune system; these biomarkers may not be specific for atherosclerosis or plaque inflammation)
- Serum biomarkers of metabolic syndrome (eg. diabetes or hypertriglyceridemia)
- Specific biomarkers of immune activation (eg. anti-LDL antibody, anti-HSP antibody)
- Biomarkers of lipid peroxidation (eg. ox-LDL and ox-HDL)
- Homocysteine
- PAPP-A
- Circulating apoptosis biomarker(s) (eg. Fas/Fas ligand, not specific to plaque)
- ADMA/DDAH
- Circulating nonesterified fatty acids (eg. NEFA)

Abbreviations: hsCRP, high-sensitivity C Reactive Protein; CD40L, CD40 ligand; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; MMP, matrix meatlloproteinases; TIMP, tissue inhibitors of MMPs; LDL, low-density lipoprotein; HDL, high-density lipoprotein; HSP, heat shock protein; ADMA, asymmetric dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; NEFA, nonesterified fatty acids.
Source: Naghavi M, 2003.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 2.** Blood biomarkers of vulnerability (reflecting hypercoagulability)

- Biomarkers of blood hypercoagulability (eg. fibrinogen, D-dimer, and factor V Leiden)
- Increased platelet activation and aggregation (eg. gene polymorphisms of platelet glycoproteins Iib/IIIa, Ia/Iia, and Ib/IX)
- Decreased anticoagulation factors (eg. proteins S and C, thrombomodulin, and antithrombin III)
- Decreased endogenous fibrinolysis activity (eg. reduced t-PA, increased PAI-1, certain PAI-1 polymorphisms)
- Prothrombin mutation (eg. G20210A)
- Other thrombogenic factors (eg. anticardiolipin antibodies, thrombocytosis, sickle cell disease, polycythemia, diabetes mellitus, hypercholesterolemia, hyperhomocysteinemia)
- Increased viscosity
- Transient hypercoagulability (eg. smoking, dehydration, infectioin, adrenergic surge, cocaine, estrogens, postprandial, etc)

Abbreviations: t-PA, tissue plasminogen activator; PAI, type 1 plasminogen activator inhibitor.
Source: Naghavi M, 2003.

### 1.2.a. Defence against oxidative stress

The paradox of oxygen is that although it is essential for metabolic processes upon which all life is predicated, it is also involved in toxic reactions and, as such, is a constant threat to the wellbeing of the organism. Most of the potentially harmful effects of oxygen are believed to be the result of the formation and the activity of reactive oxygen species (ROS). These act as oxidants which are considered the major contributors to ageing and to many of the diseases associated with ageing including CVD, cancer, cataracts, age-related decline in the immune system, and degenerative diseases of the nervous system such as Parkinson's and Alzheimer's disease (Howlett J, 2008).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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The human body has several mechanisms for defence against ROS. The various defences are complementary to one another since they act on different oxidants or in different cellular compartments.

One important line of defence is a system of antioxidant enzymes. Nutrition plays a key role in maintaining these enzymatic defences. Several essential minerals and trace elements, including selenium, copper, manganese and zinc, are involved in the structure of these enzymes and their catalytic activity. Enzymatic defences become impaired if the supply of these essential minerals is restricted or inadequate.

Another line of defence is the group of small-molecular weight compounds that act as antioxidants. These include glutathione and some vitamins (e.g. vitamins C and E) which regenerate the buffering capacity of the body's antioxidant systems.

If exposure to external sources of oxidants is high, for example from tobacco smoke or atmospheric pollution, the body's antioxidant defences come under pressure i.e. oxidative stress, which is an imbalance between pro- and anti-oxidant status.

In homeostasis, pro-oxidant factors are counterbalanced by antioxidant defences. An increase in the production of oxidants or a deficiency in the defence system could disturb this balance, causing oxidative stress (Naghavi M, 2003).

### 1.2.b. Food items promoting optimal defence against oxidative stress

The body's innate defences need to be supported by a wide variety of small-molecular-weight antioxidants found in the diet. The best known are vitamin E, vitamin C, carotenoids and polyphenols including flavonoids. Many of the antioxidant compounds in the diet are of plant origin. Since plant leaves are very exposed to visible and ultraviolet light and other radiation, they are especially susceptible to damage by activated forms of oxygen. Hence, they contain numerous natural antioxidant molecules that can counter ROS directly or boost regenerative systems to restore antioxidant capacity.

To-date, randomised controlled trials (RCT) with single antioxidants have not provided evidence of health-benefit effects. So, if benefit is to be gained, it might

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



be better to consume a wide range of antioxidants, such as occurs naturally in certain foods (Howlett J, 2008).

Examples of opportunities for modulation of target functions related to defence against oxidative stress, and the food components that might be used to achieve such benefits are summarised in Table 3.

**Table 3.** Examples of opportunities for modulation of target functions related to defence against oxidative stress by candidate food components

| Target functions | Possible biomarkers / measurement techniques | Candidate food components |
|---|---|---|
| Preservation of structural and functional activity of DNA | Measurement of damaged DNA components | Combinations of vitamins E and C, carotenoids and polyphenols including flavonoids |
| Preservation of structural and functional activity of polyunsaturated fatty acids | Measurements of lipid hydroperoxides or derivatives | As above |
| Preservation of structural and functional activity of lipoproteins | Measurements of lipid hydroperoxides and oxidised apoproteins | As above |
| Preservation of structural and functional activity or proteins | Measurement of damaged proteins or components | As above |

Source: Howlett J, 2008.

The chemistry, bioavailability, efficacy, and safety of novel phytochemicals are being actively researched. Nutritionists need to highlight the roles of these food ingredients in the prevention and treatment of different stages of CVD. The dissemination of this information to the general public can be a major contribution to primary care prevention of CVD.

### 1.3. Gene-environment interactions

Remarkable progress has been made over the recent past in identifying new genetic variants associated with metabolic diseases. The identification of these

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



variants offers us new insights into the pathogenesis of disease and suggests novel directions of investigation that will help in better understanding the causes of chronic diseases. Extensive deep phenotyping of individuals to identify functional variants at candidate gene loci helps to elucidate the pathways and metabolic variations in the genotype of individuals, and may provide insight into the physiologic bases of disease. However, there remain significant gene-environment interactions and epigenetic mechanisms which warrant extensive investigation. The rapid advances in the epidemiology of chronic diseases clearly indicate an environmental basis for these diseases, particularly in developing and in "occidentalised" countries (Ordovas J, 2008).

From an epidemiological perspective, studies of genetic and environmental sets of factors continue to underestimate the population-attributable risk associated with either set considered separately. Consideration of the combined effects of genetic and environmental factors can strengthen the observed association with the disease, and may enable the identification of risk factors that have small, marginal effects (Ordovas J, 2008).

Studies focusing on smoking, physical activity and the consumption of alcohol and coffee are observational and include relatively large sample sizes. They tend to examine single genes, however, and fail to address interactions with other genes and other related environmental factors. Studies examining gene–diet interactions include both observational and interventional designs. These studies are smaller, especially those involving dietary interventions. Of note among the reported gene–diet interactions, is the strengthened position of apolipoprotein A-5 (*Apo A5)* as a major gene involved in triglyceride metabolism, and modulated by dietary factors. Also of note is the identification of apolipoprotein A-2 (*Apo A2)* as a modulator of food intake and obesity risk (Ordovas J, 2008).

The primary goal of this research into gene–environment interactions is to gain a better understanding of the global impact of nature and over metabolic processes. However, the ultimate objective is to translate this knowledge into practical applications that benefit the individual and, as such, the overall population. The concept that nutrition can be personalised to optimise an individual's health requirements based on genotype has been gaining momentum among the general

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



population, in part driven by its frequent exposure in the popular press. This has resulted in some products being offered commercially to fit one's 'personal dietary advice'. The studies by Kornman K & Arkadianos I (2007) provided some support for the beneficial influence on some parameters of health of such 'genetic advice'. However, the studies were limited in size, and the products offered need to be further evaluated and considerably improved if they are to have a significant impact on the health of the population (Ordovas J, 2008).

The study of gene–environment interactions is an active and much needed area of research. Although technical barriers of genetic studies are rapidly being overcome, comprehensive and reliable environmental information still represent significant shortcomings of genetics studies (Ordovas J, 2008).

Progress in this area requires inclusion of larger population samples but also more comprehensive, standardised, and precise approaches to capturing environmental information. Despite current uncertainties and limitations, the concept of gene–environment interactions modulating common disease risk factors is well founded. Knowledge of such interactions could, in the future, provide the scientific understanding needed to address the major health problems in the population using molecular, and more individually targeted, approaches to disease prevention and therapy (Ordovas J, 2008).

Further, the world of proteomics, metabolomics and epigenetics offer new opportunities to explore underlying mechanisms and to develop new therapeutic targets to reduce CVD risk.

### 1.4. The concept of atherosclerosis risk factors and biomarkers

The systematic study of risk factors for atherosclerosis emerged from a coalescence of experimental results as well as cross-sectional and longitudinal studies in humans. The prospective, community-based FHS provided rigorous support for the concept that hypercholesterolaemia, hypertension, and other factors correlated well with CVD risk. Similar observational studies performed worldwide bolstered the concept of "risk factors" for CVD.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The CVD risk factors that have emerged from such studies fall into two categories: those modifiable by lifestyle and/or pharmacotherapy and those such as age and gender which are immutable. The weight of evidence supporting the various risk factors differs considerably. For example, hypercholesterolaemia and hypertension certainly predict coronary risk, but other so-called non-traditional risk factors, such as levels of homocysteine, lipoprotein (a) (Lp(a)), or infection, remain controversial. Moreover, the causality of some biomarkers that predict cardiovascular disease risk, such as CRP, remains uncertain (Harrison's online, 2009).

**Table 4.** Major risk factors (exclusive of LDL-c) that modify LDL goals from ATP III 2001

| |
|---|
| Cigarrette smoking |
| Hypertension (BP ≥ 140/90 mmHg or on antihypertensive medication) |
| Low HDL-c[a] [<1.0 mmol/L (<40 mg/dL)] |
| Diabetes Mellitus |
| Family history of premature CHD: |
| CHD in male first-degree relative <55 years |
| CHD in female first-degree relative <65 years |
| Age (men ≥ 45 years; women ≥ 55 years) |
| Lifestyle risk factors: |
| Obesity (BMI ≥ 30 Kg/$m^2$) |
| Physical inactivity |
| Atherogenic diet |
| Emerging risk factors: |
| Lipoprotein(a) |
| Homocysteine |
| Prothrombotic factors |
| Proinflammatory factors |
| Impaired fasting glucose |
| Subclinical atherogenesis |

[a] HDL-c ≥ 1.6 mmol/L (≥ 60 mg/dL) counts as a "negative" risk factor; its presence removes one risk factor from the total count. Abbreviations: LDL, low-density lipoprotein; BP, blood pressure; HDL, high-density lipoprotein; CHD, coronary heart disease; BMI, body mass index.

Source: Harrison's online 2009.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.4.a. Definition and types

The term biomarker (biological marker) was introduced in 1989 as a Medical Subject Heading (MeSH) term: "measurable and quantifiable biological parameters (e.g., specific enzyme concentration, specific hormone concentration, specific gene phenotype distribution in a population, presence of biological substances) which serve as indices for health- and physiology-related assessments, such as disease risk, psychiatric disorders, environmental exposure and its effects, disease diagnosis, metabolic processes, substance abuse, pregnancy, cell line development, and epidemiologic studies." (Vasan R, 2006; Biomarkers Definitions Working Group, 2001).

In 2001, an NIH working group standardised 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" and defined types of biomarkers (Biomarkers Definitions Working Group, 2001).

A biomarker may be:

- a measure of a variable on a biosample (blood, urine, or tissue)
- a recording obtained from a person (blood pressure, ECG, or Holter)
- an imaging test (echocardiogram or CT scan)

Biomarkers can indicate a variety of health or disease characteristics, including:

- the level or type of exposure to an environmental factor
- genetic susceptibility
- genetic responses to exposures
- markers of subclinical or clinical disease
- indicators of response to therapy

Thus, biomarkers are, essentially, indicators of:

- disease trait (risk factor or risk marker)
- disease state (preclinical or clinical)
- disease rate (progression-regression)

Accordingly, biomarkers can be classified as:

- antecedent biomarkers (identifying the risk of developing an illness)
- screening biomarkers (screening for subclinical disease)

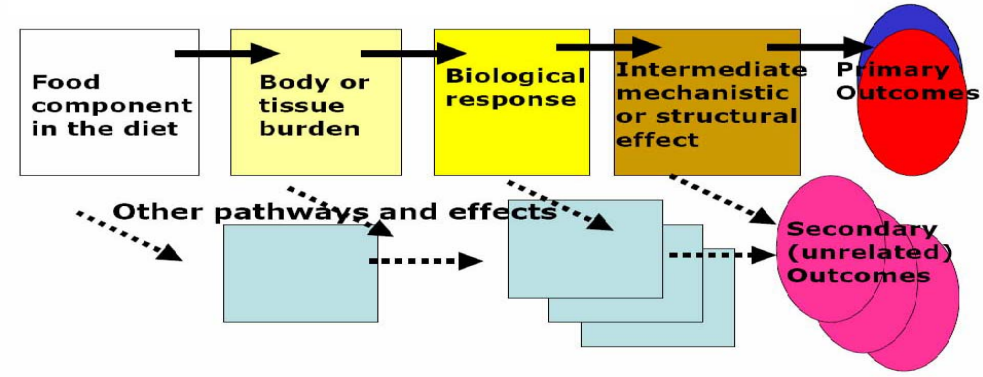
UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



- diagnostic biomarkers (recognising overt disease)
- staging biomarkers (categorising disease severity)
- prognostic biomarkers (predicting future disease course, including recurrence and response to therapy, and monitoring efficacy of therapy)

Thus, biomarkers can be related to different stages of a pathophysiological pathway, for example the CVD process, as can be seen in Figure 3.

**Figure 3.** Principal stages in the pathophysiological pathway (such as CVD)




Source: Agget P, ILSI 2007.

Biomarkers may also serve as surrogate endpoints. Although there is limited consensus on this issue, a surrogate endpoint is one that can be used as an outcome in clinical trials to evaluate safety and effectiveness of therapies instead of the measurement of the true outcome-of-interest (Vasan R, 2006; Biomarkers Definitions Working Group, 2001).

The underlying principle is that alterations in the surrogate endpoint track closely with changes in the outcome-of-interest. Surrogate endpoints have the advantage that they may be collated in a shorter time-frame and with less expense than endpoints such as morbidity and mortality, which require large clinical trials for evaluation.

Additional values of surrogate endpoints include being:

- closer to the exposure/intervention of interest

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



- easier to relate causally than more distant clinical events

A considerable disadvantage of surrogate endpoints is that if the clinical outcome-of-interest is influenced by numerous factors (in addition to the surrogate endpoint), the residual confounding factors may reduce the validity of the surrogate endpoint. The validity of a surrogate endpoint is greater if it can explain at least 50% of the effect of an exposure or intervention on the outcome-of-interest.

A risk factor is something that increases the chances of developing a disease.

An outcome measure is a variable-of-interest in the trial (also called an endpoint). Differences between groups with respect to outcome variables (following adjustment for confounding variables) are the result of the different interventions.

When the true endpoint of a health benefit cannot be measured directly, studies should use biomarkers to address:

- delayed impact or appearance of key benefit
- feasibility or ethical issues limiting access to tissues
- resource constraints: e.g. expensive assays

Moreover, biomarkers should be:

- biologically valid in that they have a known relationship to the final outcome and their variability within the target population is known
- methodologically valid with respect to their analytical characteristics

Recently, a new concept of nutrient exposure biomarker has been defined. Biological markers of nutrient exposure, as an alternative to the more traditional dietary assessment tools, have been gaining support. In this approach one or more biochemical moieties are measured in an accessible fluid or tissue to provide a semi-quantitative index of the exposure to individual food constituents (Spencer J, 2008).

### 1.4.b. Characteristics of an ideal biomarker

The overall expectation of a CVD biomarker is to enhance diagnosis and clinical management of the patient. For instance, in an individual with chronic or atypical chest pain, the measurement of a specific biomarker may be expected to relate the chest pain to ischaemic aetiology (Vasan R, 2006; Biomarkers Definitions Working Group, 2001).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Regardless of the purpose for which it is used, a new biomarker will be of clinical value only if:

- it is accurate
- it is reproducibly obtained in a standardised manner
- it is acceptable to the patient
- it is easy to interpret
- it has high sensitivity and high specificity for the outcome
- it is expected to associate clearly or unambiguously with the outcome
- it explains a reasonable proportion of the outcome, independent of established predictors, consistently in multiple studies

Data suggest that knowledge of biomarker levels changes patient management.

The desirable overall properties of biomarkers and of biomarkers of screening, diagnostic and prognostic purposes vary with their intended use.

- For screening biomarkers, features such as high sensitivity, specificity, and predictive values, large likelihood ratios (discussed below), and low costs are of primary importance.

- For diagnostic biomarkers of CVD (such as acute MI), in addition to the above-mentioned characteristics, those such as rapid sustained elevation, high tissue specificity (indicating myocardial origin), a release proportional to disease extent, and assay features conducive to point-of-care testing are critical.

- For biomarkers monitoring disease progression or response to therapy, features such as sensitivity or specificity are less important because the patient serves as his or her own control (baseline values are compared with follow-up values). Narrow intra-individual variations and tracking with disease outcome or therapy are critical. Costs may be less important for prognostic markers because only people with the disease are tested (Vasan R, 2006).

Some biomarkers (e.g., exercise stress test) may be used for both diagnostic and prognostic purposes. Establishing the prognostic utility of a biomarker is more challenging because it requires a larger sample and a prospective study design, whereas demonstrating its value as a diagnostic test requires a smaller sample and a cross-sectional study design (Vasan R, 2006).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Regardless of the intended use, it is important to remember that biomarkers that do not change disease management cannot affect patient outcome and, therefore, are unlikely to be cost-effective (judged in terms of quality-adjusted life-years gained).

Typically, for a biomarker to change patient management, it is important to have evidence that risk reduction strategies vary with biomarker levels, and a biomarker-guided approach translates into better patient outcomes, as compared to a management scheme (usually the current standard of care) without biomarker levels. It also means that biomarker levels should be directly, or indirectly, modifiable by therapy and based on evidence from prospective clinical trials. In other situations, biomarkers may serve as research tools by providing insights into disease mechanisms (Vasan R, 2006).

Biomarkers, defined as alterations in the constituents of tissues or body fluids, provide a powerful approach to understanding the spectrum of CVD, with applications in at least 5 areas:

- screening
- diagnosis
- prognostication
- prediction of disease recurrence
- therapeutic monitoring

Advances in functional genomics, proteomics, metabolomics, and bioinformatics have revolutionised unbiased inquiries into numerous putative markers that may be informative with regard to the various stages of atherogenesis, including overt CVD and its sequelae.

Prerequisite for the clinical use of biomarkers are:

- elucidation of the specific indications
- standardisation of analytical methods
- characterisation of analytical features
- assessment of performance characteristics
- incremental yield of different biomarkers for given clinical indications
- demonstration of cost-effectiveness

Technological advances will likely facilitate the use of multi-marker profiling to individualise treatment of CVD in the future (Vasan R, 2006).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 5.** Desirable features of biomarkers of CVD

| All biomarkers | "Screening" biomarkers to identify "vulnerable" patients | "Diagnostic" biomarkers to identify ischaemia or injury | "Prognostic / Treatment" biomarkers |
|---|---|---|---|
| General features<br><br>- Measure a specific pathology<br><br>- Add to clinical assessment<br>- Acceptable to patient<br>- Linear relation between change in marker and change in pathology<br><br>- Stable product<br><br>- Single measure representative<br><br><br><br><br><br>- Applicable to men and women, different ages, different ethnicities | Known "reference limits"<br><br>Add to known CHD index such as the FHS risk score<br>Change in *c* statistic or AUC (discrimination)<br>Acceptable calibration<br><br>"Rule in" strategy with high specificity more important to avoid mislabelling asymptomatic individuals<br>Account for a moderate or greater proportion of CHD in the community<br>Change management<br><br>Reclassify risk in patients at intermediate risk | High myocardial specificity<br><br>Not present in normal serum/noncardiac tissue<br><br>Zero baseline, immediate release (early detection)<br>Long t1/2<br><br>Permit long time window for diagnosis<br><br>But <24h to permit diagnosis of recurrent ischemia<br>Release proportional to injury size or ischaemic burden<br>Convenience for point-of-care testing<br><br>Rapid test (results available in <1h) | Known reference limits<br><br>Add to known prognostic index<br><br>Change in marker alters management<br>Affect choice of drug<br><br>Change dose of drug<br><br><br><br>Indicate tolerance<br><br>Indicate safety margin<br><br>Used for monitoring progression of disease<br><br>Trajectory of marker correlates with disease progression |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 5.** Desirable features of biomarkers of CVD (continued)

| All biomarkers | "Screening" biomarkers to identify "vulnerable" patients | "Diagnostic" biomarkers to identify ischaemia or injury | "Prognostic / Treatment" biomarkers |
|---|---|---|---|
| - Reproducible in multiple studies | Target individuals with increased levels of biomarker superior to conventional Rx for reducing Cost-benefit ratio favourable | No special sample preparation needed | "Rule out" strategy with high specificity more important to avoid mislabelling asymptomatic individuals |
| Assay/measurement features<br>- Internationally standardised<br>- Accuracy<br>- Precision | | Inexpensive<br>Readily available<br>Diagnostic cutoff well defined and accepted<br>Known discrimination limits or action thresholds<br>Change management, triage, or specific treatment | Cost-effective<br>REMARK guidelines |
| Assay application features<br>- Tested in a spectrum of people with varying degrees of pathology<br>- High sensitivity and specificity | | "Rule out" strategy<br>With high sensitivity; more important to avoid missing disease<br>Cost-effective | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 5.** Desirable features of biomarkers of CVD (continued)

| All biomarkers | "Screening" biomarkers to identify "vulnerable" patients | "Diagnostic" biomarkers to identify ischaemia or injury | "Prognostic / Treatment" biomarkers |
|---|---|---|---|
| Laboratory features<br>- Automation<br>- High throughput / short turnaround time<br>- Connectivity to laboratory information management systems<br>- Compatibility with existing laboratory processes | | STARD guidelines | |
| Desirable features for *in vitro* diagnostic industry<br>- Address unmet patient needs<br>- Return on investments<br>- Requirements for research and development phases<br>- Manufacturability | | | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 5.** Desirable features of biomarkers of CVD (continued)

| All biomarkers | "Screening" biomarkers to identify "vulnerable" patients | "Diagnostic" biomarkers to identify ischaemia or injury | "Prognostic / Treatment" biomarkers |
|---|---|---|---|
| - Marketability including barriers to entry<br>- Postsales customer support<br>- Other market features (acceptance competition, regulatory issues, reimbursement, etc) | | | |

Abbreviations: FHS, Framingham Heart Study; t1/2, half-life; REMARK, Reporting recommendations for tumour MARKer prognostic studies; and STARD, Standards for Reporting of Diagnostic accuracy.
Source: Vasan R, 2006

### 1.4.c. Emerging blood biomarkers of CVD

CVD and stroke continue to be the leading causes of death in the United States of America (USA). As a result, investigators continue to look for new and emerging biomarkers of disease risk. Because many of these emerging biomarkers are not as well documented as those of conventional lipid and lipoprotein risk factors, their value in clinical practice needs to be critically appraised, and appropriate guidelines developed for their proposed use (Vasan R, 2006; Biomarkers Definitions Working Group, 2001).

The National Academy of Clinical Biochemistry (NACB) convened a multidisciplinary expert panel to develop laboratory medicine practice guidelines for a selected subset of these emerging risk factors as applied in a primary prevention setting of heart disease and stroke (Myers G, 2008).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



A number of prospective studies in initially healthy subjects have convincingly demonstrated independent associations between even slightly elevated concentrations of various systemic markers of inflammation and important cardiovascular endpoints.

This may also apply to combinations of other biomarkers, though evidence so far is limited. Future expectations are for a biomarker profile that covers various aspects of the complex pathophysiology of the atherothrombotic process and, potentially, such a profile would help focus on biological patterns or systems, rather than on single biomarkers. To date, however, there is no sound evidence to suggest such a procedure for clinical practice, and there is continuing discussion as to whether any of the emerging blood biomarkers alone contribute significant incremental information over and above the information gained from available "global risk" scores (Vasan R, 2006; Biomarkers Definitions Working Group, 2001).

A new concept of "health biomarkers" through quantification of the robustness of homeostatic mechanisms involved in maintaining optimal health is progressively gaining credence since it is based on the assumption that the ability to maintain homeostasis in a continuously challenged environment and changing physiology is the key for healthy ageing.

**1.4.d. Biomarkers in CVD risk assessment**

Firstly, global risk assessment with traditional risk factors still represents the rational basis for CVD risk stratification.

Secondly, although theoretically attractive, currently available biomarkers, even the combination of a robust systemic biomarker of "disease activity" with a biomarker that provides information on structural changes of the arterial vasculature, which must be seen as a surrogate/precursor of clinical disease, does not appreciably improve risk prediction.

Thirdly, in the future, despite such somewhat disappointing information regarding single markers, the clinical application of multi-marker panels (for which the possibilities of model improvement are greater) may still prove to be a promising

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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approach, provided that such variables show low correlations with conventional risk factors and with each other, but provide strong associations with clinical events. Such emerging biomarkers will have to be rigorously evaluated in large cohorts for their clinical efficacy and effectiveness with innovative statistical analytical tools.
The world of proteomics, metabolomics and epigenetics, together with advanced imaging modalities such as functional molecular imaging, may provide just such promising candidates.

### 1.5. Diet and CVD

Varied and energy-balanced regimens together with regular exercise are critical to the preservation of a good cardiovascular status. Moreover, the effects of different dietary components on CVD risk are being established. Fatty acids regulate cholesterol homeostasis and concentrations of blood lipoproteins and, via a range of mechanisms, affect the levels of other CVD risk factors such as blood pressure (BP), haemostasis, and bodyweight. The levels of blood lipids can be influenced by dietary fatty acids; an influence usually related to the molecular size and shape and the degree of saturation of the hydrocarbon chains (ATP III, 2001 and EAS, 2007).
Saturated fatty acids (SFA) are those fatty acids with hydrocarbon chains that contain no double bonds. SFA with chain lengths of 12–16 carbon atoms increase plasma low-density lipoproteins (LDL-c) concentrations more effectively than plasma high-density lipoproteins (HDL-c) concentrations. In their favour, they are very much less easily oxidised.
Unsaturated fatty acids (USFA) are those in which the hydrocarbon chain contains at least one double bond. Monounsaturated fatty acids (MUFA) contain one double bond; polyunsaturated fatty acids (PUFA) contain two or more. Most naturally occurring USFA are cis-fatty acids, in which the two hydrogen atoms around the double bonds (one at each end) are positioned on the same side of the fatty acid chain. This causes a bend in the hydrocarbon chain at that point. In contrast, *trans*-fatty acids (TFA) have the hydrogen atoms at each of the double bonds on opposite sides of the fatty acid chain and, as a result, are straight and more like SFA. They are formed during some manufacturing processes and are, therefore, consumed in products such as hard margarines and baked food items. They are

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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also formed in the rumen of animals such as cows and, consequently, a portion of the TFA in the diet (around 20%) comes from the consumption of dairy products and meat.

Dietary TFA can increase plasma LDL-c and reduce HDL-c concentrations. Diets low in SFA and TFA could, therefore, reduce the risk of CVD.

There are strong, consistent, and graded relationships between saturated fat intake, blood cholesterol levels, and the occurrence of CVD. The consensus is that these relationships are causal.

Polyunsaturated n-3 fatty acids, in contrast, show protective effects against adverse (even fatal) events in patients who had suffered a previous MI.

Sodium intake, especially in the form of sodium chloride, influences arterial BP and, as a consequence, increases the risk of hypertension, stroke, cardiac heart disease and heart failure.

The *cis*-USFA with 18 carbon atoms – oleic (monounsaturated), linoleic and alpha-linolenic acids (polyunsaturated) – reduce plasma concentrations of LDL-c, and some may also raise plasma concentrations of HDL-c.

Like alpha-linolenic acid, the long-chain, highly unsaturated PUFA found in fish oils belong to the n-3 family. They can promote improvements in endothelial and arterial integrity, as well as counteract blood clotting and reduce BP. They also reduce plasma triglycerides (TG) levels (ATP III, 2001 and EAS, 2007).

### 1.6. Diet and biomarkers of CVD

To fully appreciate the potential role that foods can play in the prevention of CVD, it is necessary to understand the diversity of risk factors associated with onset and development of CVD. These factors include high BP, inflammation, inappropriate blood lipoprotein levels, insulin resistance and control of blood clot formation. The interdependence of these factors has not been fully demonstrated. Because only 50% of the incidence of CVD can be explained by these known risk factors, other contributory and interactive factors need to be invoked and identified. For example, genetic predispositions, smoking, and levels of physical activity play a role but the contribution to CVD risk has yet to be quantified (ATP III, 2001 and EAS, 2007).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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### 1.6.a. High blood pressure

CVD is directly related to high BP and any measures taken to reduce high BP should result in a lowering of the risk of coronary disease. High BP increases the risk of arterial injury. Genetic predisposition and obesity are involved in the aetiology of high BP, but diet and lifestyle also have a substantial impact; overweight, physical inactivity, high alcohol intake, high sodium intake and low potassium intake being among the main contributors.

### 1.6.b Integrity of artery lining

Damage to the EC that line the arteries, as well as more general structural damage at susceptible points in the arteries, increases the risk of CVD.

Oxidation is considered a major contributor to atherosclerosis because it converts LDL into an oxidised form (oxLDL) which has been identified in damaged arterial walls and, as well, has been shown to have several actions that could contribute to the initiation and progression of arterial damage. The extent of LDL oxidation is related to the extent of atherosclerosis.

### 1.6.c. Elevated blood lipids

Lipids, which are generally insoluble in aqueous medium, are transported in the blood in the form of lipoprotein particles composed of specific proteins and lipids (TG, cholesterol and phospholipids). LDL and VLDL contain high concentrations of TG and cholesterol, and are termed “low density” on the basis of their hydrated densities. Elevated levels of LDL and VLDL are recognised risk factors for CHD and other aspects of CVD. HDL contains lower concentrations of cholesterol and are believed to be protective against CVD.

A raised plasma concentration of LDL is a strong risk factor for CVD. High levels of other lipoproteins, high concentrations of TG concentrations and low levels of HDL are also risk factors. Raised postprandial levels of lipids, especially TG, appear to be better indicators of potential CVD than fasting levels.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.6.d. High homocysteine levels

Epidemiological data suggest that high plasma levels of the amino acid homocysteine are associated with increased risk of CVD. Several mechanisms to explain the effects of homocysteine on atherosclerosis and thrombosis have been suggested, but none has found general favour.

### 1.6.e. Increased blood clot formation

The control of blood clotting (thrombus formation) is likely to be an important element in the reduction of CVD risk. Risk factors include those that increase the aggregation of platelets and those that increase the activity of the clotting factors. These are counterbalanced by factors that promote the breakdown or disruption of the thrombus.

### 1.6.f. Insulin resistance syndrome

Apart from being associated with higher than normal levels of insulin and glucose, insulin resistance is also associated with characteristic changes in lipid metabolism.

Insulin resistance syndrome is characterised by increased concentrations of TG, decreased concentrations of HDL-c, and high BP. Seen in association with central obesity, as measured as waist circumference (WC), this risk profile is also referred to as “metabolic syndrome” (MetS). There are different definitions of MetS but the most used are the National Cholesterol Education Program (NCEP) ATP III criteria (2001) (Table 6) and the International Diabetes Federation (IDF) criteria (Alberti K, 2006) (Table 7).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 6.** ATP III clinical identification of the MetS

| Risk factor | Defining level |
|---|---|
| Abdominal obesity (given as WC): | |
| Men | >102 cm |
| Women | >88 cm |
| TG | ≥ 150 mg/dL |
| HDL-c: | |
| Men | <40 mg/dL |
| Women | <50 mg/dL |
| BP | ≥ 130 / ≥ 85 mm Hg |
| Fasting glucose | ≥ 110 mg/dL |

Abbreviations: WC, waist circumference; TG, triglycerides; HDL-c, high density lipoprotein cholesterol; BP, blood pressure.

Source: NCEP ATP III criteria (2001).

**Table 7.** The new IDF definition of MetS

| **Central obesity** (defined as WC* with ethnicity specific values) **plus** any two of the following four factors: | |
|---|---|
| Raised TG | ≥ 150 mg/dL (1.7 mmol/L)<br>or specific treatment for this lipid abnormality |
| Reduced HDL-c | <40 mg/dL (1.03 mmol/L) in males<br><50 mg/dL (1.29 mmol/L) in females<br>or specific treatment for this lipid abnormality |
| Raised BP | SBP ≥ 130 or DBP ≥ 85 mm Hg<br>or treatment of previously diagnosed hypertension |
| Raised FPG | FPG ≥ 100 mg/dL (5.6 mmol/L)<br>or previously diagnosed type 2 diabetes<br>If above 5.6 mmol/L or 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome. |

* If BMI is >30 Kg/m$^2$, central obesity can be assumed and WC does not need to be measured.

Abbreviations: WC, waist circumference; TG, triglycerides; HDL-c, high density lipoprotein cholesterol; BP, blood pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; OGTT, oral glucose tolerance test.

Source: Alberti K, 2006.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The Table 8 shows the ethnicity-specific values for WC as applied in diagnosis of MetS.

**Table 8.** Ethnicity-specific values for WC (from the IDF)

| Country/Ethnic group | | WC |
|---|---|---|
| Europids* | Male | ≥ 94 cm |
| | Female | ≥ 80 cm |
| South Asian** | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| Chinese | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| Japanese | Male | ≥ 90 cm |
| | Female | ≥ 80 cm |
| South and Central Americans | Use south Asian recommendations until more specific data are available | |
| Sub-Saharan Africans | Use European data until more specific data are available | |
| Eastern Mediterranean and Middle East (Arab) populations | Use European data until more specific data are available | |

* European and North American populations.

** Chinese, Malay and Asian-Indian population.

Abbreviations: WC, waist circumference

Source: Alberti K, 2006.

### 1.7. Lifestyle modification in CVD

The prevention of atherosclerosis presents a long-term challenge to all health-care professionals and for public health policy, as well. Individual practitioners as well as organisations providing health-care should strive to help patients optimise their risk-factor profile long before atherosclerotic disease becomes manifest. The current accumulation of CVD risk in youth and in certain minority populations presents a particularly vexing concern from a public health perspective (ATP III, 2001 and AHA position, 2009).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The care plan for all patients seen by internists should include measures to assess and minimise CVD risk. Physicians must counsel patients regarding the health risks of tobacco use and provide guidance and resources regarding smoking cessation. Likewise, physicians should advise all patients about prudent dietary and physical activity habits for maintaining ideal bodyweight. The NIH and the AHA statements recommend at least 30 min of moderate-intensity physical activity per day. Physicians should encourage their patients to take personal responsibility for behaviour-related, modifiable, risk factors in the development of premature atherosclerosis. Conscientious counselling and patient education may forestall the need for pharmacologic measures intended to reduce coronary disease risk (Harrison's online, 2009 and AHA position, 2009).

### 1.7.a. Dietary recommendations in CVD risk management

Dietetics is an integral part of CVD risk management. All patients presenting with evidence of CVD and those individuals at high risk should be given professional advice on food items and dietary options which reduce the CVD risk.

Dietary recommendations should be defined individually, taking into account the subject's risk factors such as dyslipidaemia, hypertension, diabetes, and obesity.

According to European guidelines on CVD prevention and managing CVD risk (EAS, 2007), all individuals should be advised about food choices that are associated with a lower CVD risk, and high-risk individuals should receive specialist dietary advice when feasible.

The following are the general recommendations, which should be modified to suit the local culture:

- a wide variety of foods should be eaten
- energy intake should be adjusted to avoid overweight
- encourage: fruits, vegetables, wholegrain cereals, bread, fish (especially oily fish), lean meat, low-fat dairy products
- replace saturated fats with the above foods and with monounsaturated and polyunsaturated fats from vegetable and marine sources to reduce total fat to <30% of energy, of which less than 1/3 is saturated

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



- reduce salt intake if BP is raised by avoiding table salt and salt in cooking, and by choosing fresh or frozen unsalted foods. Many processed and prepared foods, including bread, are high in salt

The AHA fixed diet and lifestyle goals for CVD risk reduction are summarised in Table 9.

**Table 9.** Diet and lifestyle goals for CVD risk reduction

| |
|---|
| Consume an overall healthy diet |
| Aim for a healthy bodyweight |
| Aim for recommended levels of LDL-c, HDL-c, and TG |
| Aim for a normal BP |
| Aim for a normal blood glucose level |
| Be physically active |
| Avoid use of, and exposure to, tobacco products |

Abbreviations: LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; BP, blood pressure.
Source: AHA, 2006.

Table 10 shows the diet and lifestyle recommendations for CVD risk reduction (according to AHA, 2006).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 10.** Diet and lifestyle recommendation for CVD risk reduction

| |
|---|
| Balance calorie intake and physical activity to achieve, or maintain, a healthy bodyweight |
| Consume a diet rich in vegetables and fruits |
| Choose whole-grain, high-fibre foods |
| Consume fish, especially oily fish, at least twice a week |
| Limit your intake of saturated fat to <7% of energy, *trans* fat to <1% of energy, and cholesterol to <300 mg/d by |
| Choose lean meats and vegetable alternatives |
| Selecting fat-free (skim), 1% fat, and low-fat dairy products |
| Minimizing intake of partially hydrogenated fats |
| Minimise intake of beverages and foods with added sugars |
| Choose and prepare foods with little or no salt |
| If you consume alcohol, intake should be moderate |
| When food is prepared outside the home, follow the AHA diet and lifestyle recommendations |

Source: AHA, 2006.

### 1.8. Mediterranean diet and olive oil

Olive oil is the most representative food in the traditional Mediterranean diet (MedDiet) and its most important source of MUFA. The healthy benefits of MUFA-rich diets on plasma cholesterol levels were the first to generate interest in this dietary model. In addition to the benefits conferred by its lipoprotein effects, olive oil has other biological effects, some of them also related to MUFA. Several epidemiologic and clinical studies suggests that the MedDiet, rich in virgin olive oil (VOO), improves the major CVD risk factors such as the lipoprotein profile, postprandial lipaemia, BP, bodyweight, insulin resistance, glucose metabolism and antithrombotic profile. Endothelial function, inflammation and oxidative stress are also positively modulated. Some of these effects are attributed to minor components of VOO. In countries where the populations follow a typical MedDiet, where VOO is the principal source of fat, cancer incidence rates are lower than in Northern Europeans. The experimental and human cellular studies have provided much new evidence on the potential protective effect of VOO in cancer. Several observational studies have shown that the intake of a MedDiet may be protective

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



against age-related cognitive decline and Alzheimer's disease. In view of these effects, it would appear that when olive oil is the basic source of dietary fat it has a major anti-atherogenic capacity, which is not shared to the same extent by other oils that are rich in oleic acid but lack the characteristic micronutrients of VOO.

A recent Consensus Report of II International Conference on Olive Oil and Health (Jaén and Córdoba, Spain) 2008 reported the following checklist of recent studies supporting the healthy effects of olive-oil-rich MedDiet rich (Table 11).

**Table 11**. Health benefit effects of olive-oil-rich MedDiet (based on the current literature)

| Level of evidence | Type of effect |
|---|---|
| Demonstrated by dietary intervention trials in different populations | - A favourable lipid profile, with a decrease in LDL-c plasma levels, and higher HDL/TC (total cholesterol) ratio versus saturated fats<br>- The reduction *in vitro* oxidation of LDL<br>- The improvement of glucose metabolism in normal subjects and type 2 diabetic patients. MUFA result in a lower insulin requirement and plasma glucose concentration that SFA, and at least as effective as carbohydrate (CHO)<br>- Control of BP<br>- Improvement in endothelial function<br>- The promotion of a less prothrombotic environment compared to SFA-rich diets influencing different thrombogenic factors: reduction of platelet aggregation, Thromboxane B2 production, decrease in VWf, TF, TF-pathway inhibitor, PAI-1, Factor VII, and Factor XII |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 11**. Health benefit effects of olive-oil-rich MedDiet (based on the current literature) (continued)

| Level of evidence | Type of effect |
|---|---|
| Suggested by few dietary intervention trials, observational studies or with *in vitro* experiments | - Lower NF-kB activation when compared to other types of diet, both in fasting and postprandial states<br>- Favourable effects on obesity<br>- Reduction of age-related cognitive decline and Alzheimer's Disease compared with SFA-rich diets<br>- Decreased Relative Risk for Parkinson's disease |

Source: Consensus Report of II International Conference on Olive Oil and Health, 2008.

The conclusions of the Consensus Report of II International Conference on Olive Oil and Health (2008) were:

1. Ageing represents a great concern in developed countries because the number of people involved and the pathologies related with it, like atherosclerosis, Parkinson´s disease, Alzheimer's disease, vascular dementia, cognitive decline, diabetes and cancer. In addition, the growing incidence and prevalence of obesity could negatively influence in the development of these pathological process.
2. Epidemiological studies suggest that the ingestion of a MedDiet (which is rich in VOO) is associated with a decrease in the risk of CVD, obesity, MetS, type 2 diabetes, hypertension and a lower morbi-mortality of CHD.
3. Several epidemiologic and clinical studies suggests that the MedDiet, rich in VOO, improves the major cardiovascular risk factors, such as the lipoprotein profile, postprandial lipemia, BP, bodyweight, insulin resistance, glucose metabolism and antithrombotic profile. Endothelial function, inflammation and oxidative stress are also positively modulated. Some of

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



these effects are attributed to minor components of VOO. Therefore, the definition of the MedDiet should include VOO.

4. The unsaponifiable components from VOO oil can modulate the characteristics of TG-rich lipoproteins. Phenols compound of VOO have shown antioxidant and antiinflammatory properties, prevent the lipoperoxidation, induce a protective lipid profile and improve the endothelial function. Furthermore they are also able to modify the haemostasis, showing antithrombotic properties.
5. In countries where the populations fulfilled a typical MedDiet, such as Spain, Greece and Italy, where VOO is the principal source of fat, cancer incidence rates are lower than in northern European countries. The experimental and human cellular studies have provided several new evidences on the potential protective effect of VOO on cancer.
6. Several observational studies have shown that the intake of a MedDiet may be protective against age-related cognitive decline and Alzheimer's disease.
7. The more recent studies consistently support that the MedDiet, based in VOO, is compatible with a healthier ageing and increased longevity.
8. Like to other healthy foods, the protective effect of VOO in the context of a MedDiet can be most important in the first decades of life, which suggests that the benefit of VOO intake should be initiated in the infancy, and maintained through life.
9. Furthermore, current studies should take advantage of the additional strengths provided by genetic epidemiology and functional genomics. Firstly, recent studies support that genetic variation is a relevant factor in CVD risk. Moreover, gene-diet interactions have shown to modulate this genetic predisposition. Secondly, other studies are providing mechanistic evidence to support the beneficial effects of the MedDiet and its main components such as VOO. The combination of new technologies and clinical expertise will provide more solid evidence about the action of a Mediterranean dietary pattern and their individual components on health. This information will represent a substantial boost to the knowledge

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



needed to successfully prevent and treat CVDs and will provide evidence based support for public health education to increase the adherence to a traditional MedDiet in which olive oil is an essential component.

### 1.9. Cocoa

The cocoa tree, native to Central and South America, produces cocoa pods containing cocoa beans which are used mainly in the manufacture of chocolate and chocolate drinks (ICCO, 2005).

For centuries, cocoa-rich chocolate has been known not only for its palatability but also for its health-benefit effects. The Incas considered it the drink of gods, an association that gave rise to the scientific name of the cocoa tree, *Theobroma cacao*, from the Greek *theo* (god) and *broma* (drink). The first hints of cocoa consumption date back to 1600 BC. In Honduras, archaeologists uncovered elaborately designed bowls of this period that are believed to have been used by the Aztecs to drink liquid cocoa. In the 16th Century AD, Aztec Emperor Montezuma was a keen admirer of cocoa, calling it a “divine drink, which builds up resistance and fights fatigue. A cup of this precious drink permits a man to walk for a whole day without food” (Hernán Cortés, 1519). In the language of the Aztecs, this drink was called *chocolatl*. With the discovery of the New World, cocoa came to Europe in the 16th Century AD. Since then, the modern chocolate industry has developed, and cocoa seeds are now processed in a multitude of different ways (Corti R, 2009).

Cocoa has a unique natural taste and colour and possesses a delicious aroma. It is used in many food products for extra flavour and colour. The natural properties of cocoa make chocolate pleasurable with a high “feel good factor”. Since ancient times, chocolate has been associated with pleasure, passion and energy (ICCO, 2005).

Several health-benefit effects of cocoa have been proposed, including improved heart function and relief of angina pectoris, stimulation of the nervous system, improved digestion as well as improved kidney and bowel function. In addition, cocoa has been used to treat anaemia, mental fatigue, tuberculosis, fever, gout, kidney stones, and even poor sexual appetite. In the 19th Century, chocolate

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



became a luxury item and, from a puritanical point of view, its consumption was considered a sin, rather than a remedy. More recently, however, chocolate consumption is associated with caries, obesity, high BP, and diabetes. As such, many health-care providers tend to warn patients about the potential health hazards of consuming excessive amounts of chocolate-based food items. However, the recent identification of biologically-active phenolic compounds in cocoa has changed this perception and has stimulated research into its effects on ageing, BP regulation, and atherosclerosis (Corti R, 2009).

A recent review based on clinically-relevant cardiovascular effects of cocoa, focusing on potential mechanisms involved in the response to cocoa and the potential clinical consequences associated with its consumption. It is important to differentiate between the natural product *cacao* and the processed product *chocolate*, the latter refers to the combination of cocoa, sugar, milk and other ingredients into a solid consumable product. Many of the health effects of cocoa may not be applicable to chocolate.

Epidemiologic data demonstrate that regular dietary intake of plant-derived foods and beverages reduce the risk of CHD and stroke, and are inversely associated with CVD risk (Corti R, 2009).

First evidence of a beneficial effect of cocoa was obtained in a study involving Kuna Indians, a native tribe living on islands off the coast of Panama. The Kuna belong to one of the few cultures protected against the age-dependent increase in BP and the development of hypertension. Interestingly, their daily consumption of cocoa in very high, and is sometimes even enriched with salt. Clinical studies revealed that the Kuna Indians have lower BP values and no age-dependent decline in kidney function. Also, CVD mortality is markedly lower compared to other Pan-American citizens ($9.2 \pm 3.1$ vs $83.4 \pm 0.7$ age-adjusted deaths per 100.000). The factors involved are clearly environmental rather than genetic because this protection is lost on migration to the urban environment of Panama City, where the original home-prepared cocoa is replaced by other manufactured foods with lower flavanol content (Hollenberg N, 1997).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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A prospective study in 34,489 postmenopausal women in the Iowa Women's Health Study with a 16-year follow-up showed that foods rich in flavonoids were associated with a decreased risk of death from CHD. Further, a borderline significant inverse association between chocolate intake and cardiovascular mortality was observed, following adjustment in multivariate analysis (Mink P, 2007). The Dutch Zutphen Study, a cross-sectional analysis, showed cocoa intake to be inversely related to BP and, in a prospective analysis, intake was associated with a reduction of cardiovascular and all-cause mortality. The CVD-related mortality risk for men in the highest tertile of cocoa intake was reduced by 50% compared with the lowest tertile ($P$=0.004) and the adjusted relative risk for all-cause mortality was 0.53 (95%CI: 0.39 to 0.72; $P$<0.001) (Buijsse B, 2006).

**1.9.a. Stearic acid in chocolate**

Saturated fat has long been thought to contribute to atherosclerosis and, thus, to CVD risk. However, stearic acid has been suggested to be a non-atherogenic type of dietary saturated fat. Stearic acid is a long-chain 18:0 SFA found commonly in meats and dairy products. Cocoa butter, a fat derived from cocoa plants and predominantly found in dark chocolate, contains an average of 33% oleic acid (cis-18:1 monounsaturated), 25% palmitic acid (16:0 saturated), and 33% of stearic acid. Although it is considered that saturated fats in general increase total cholesterol (TC) and LDL levels, early studies have also suggested stearic acid may be cholesterol-neutral (Ding E, 2006).

This has been confirmed in a series of studies. A meta-analysis of 60 controlled feeding trials concluded that stearic acid neither lowers HDL, nor increases LDL or TC. The meta-analysis estimated that for every 1% energy isocaloric replacement of stearic acid for CHO, stearic acid intake is predicted to beneficially lower serum TG by -17.0 nmol/L ($P$< 0.001) (Mensink R, 2003). The study by Thijseen M (2005) showed that the effects of stearic acid on lipids is even similar to oleic and linoleic acids.

Emerging studies have begun to explain how stearic acid in chocolate may be cholesterol-neutral. One suggested mechanism, observed in several animal and human studies, is stearic acid's lower absorption. These could be related to the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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position of stearate on the triglyceride molecule which may affect its relative absorption rate. This might also be explained by stearic acid from plants sources, such as cocoa, being different from animal-derived sources. Some feeding trials, though not others, found lower absorption of cocoa butter compared to corn oil (Ding E, 2006). However, this heterogeneity of effects may be due to the dual presence of calcium in chocolate since other trials found cocoa butter absorption was further decreased 13% when supplemented with calcium (1% by weight) (Shahkhalili Y, 2001), as is common practice in chocolate manufacture. Finally, another strongly supported protective mechanism may be related to the relatively high percentage desaturation of stearic acid to monosaturated oleic acid; a fat considered hypocholesterolaemic and protective against CHD (Ding E, 2006).

Two other pathways suggested as being potentially beneficial are stearic acid's potential anti-platelet and BP reducing actions. Feeding trials have shown that stearic acid reduces mean platelet volume, an index of platelet activation. However, mixed findings have been observed in relation to stearic acids and factor VIIc coagulation factor, a predictor of fatal CHD. Several trials have shown that stearic acid lowered the levels of factor VIIc coagulation factor compared to palmitic and other SFA (Ding E, 2006). As for the relationship between stearic acid and BP, stearic acid appears not to adversely affect systolic BP (SBP) (DIng E, 2006). Further, cross-sectional analysis within the Multiple Risk Factor Intervention Trial (MRFIT) even found stearic acid levels may be inversely associated with diastolic BP (DBP) (Simon J, 1996).

In summary, given the vast majority of studies showing stearic acid having beneficial or neutral effects on BP and clotting parameters, it appears unlikely that stearic acid intake would adversely affect CVD risk via these risk factors. Data indicate that stearic acid does not adversely affect traditional lipid risk factors, but even a favourable lowering of serum TG if used as isocaloric replacement of CHO (Ding E, 2006).

### 1.9.b. Cocoa polyphenols

Observations from epidemiologic studies have led to the hypothesis that health benefits from cocoa products might be linked, at least in part, to plant-derived

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



flavonoids; a large subgroup of the heterogeneous group of polyphenols. All flavonoids share a common chemical structure: C6-C3-C6. Flavanols (also termed flavan-3-ols) have attracted particular interest because they are found in high concentrations in certain fruits and vegetables such as certain teas, grape juice, wine, various berries and, especially, cocoa products, as shown in Table 12.

**Table 12.** Catechin/Epicatechin concentrations found in various food items

| Source | Flavanol content (mg/Kg or mg/L) |
|---|---|
| Chocolate | 460-610 |
| Beans | 350-550 |
| Apricots | 100-250 |
| Cherries | 50-220 |
| Peaches | 50-140 |
| Blackberries | 130 |
| Apples | 20-120 |
| Green tea | 100-800 |
| Black tea | 60-500 |
| Red wine | 80-300 |
| Cider | 40 |

Source: Manach C, 2004.

Flavanols occur as epicatechin and catechin monomers which are the main flavanols in fruits. These monomers can form links between C4 and C8 allowing them to assemble as dimers, oligomers, and polymers of catechins; the so-called procyanidins. Procyanidins are also known as condensed tannins which, through the formation of complexes with salivary proteins, are responsible for the bitter taste of cacao (Manach C, 2004).

A 100 g bar of milk chocolate contains 170 mg of flavonoid antioxidants, procyanidins and flavanols. It is estimated that chocolate is a leading source of procyanidin intake in Western nations (18–20%).

Various studies have quantitatively compared the content of the flavanoids in cocoa with other food items. Cocoa has been shown to have the highest content of polyphenols (611 mg/serving) and flavanoids (564 mg of epicatechin/serving)

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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which are quantitatively greater than even tea and wine. Per serving, dark chocolate contains substantially higher amounts of flavonoids than milk chocolate (951 mg of catechins per 40 g vs 394 mg, respectively) and the levels of epicatechin in dark chocolate is comparable to red wine and tea. Also of note, dark chocolate per serving contains significantly greater amounts of total phenols as well as catechins than milk chocolate (126 ± 7.4 μmol/g vs 52.2 ± 20.2 μmol/g) (Ding E, 2006).

After oral intake of cocoa, the flavanol content as well as the total antioxidant capacity in plasma increase. These effects appear to be markedly reduced when cocoa is consumed with milk, or if cocoa is ingested as milk chocolate. The highest plasma peak concentrations of flavanols are obtained 2 to 3 hours post-ingestion in a dose-dependent manner, and are still measurable after 8 hours. In addition, molecular size matters i.e. the smaller the polyphenol, the higher the concentration in blood.

There is a large inter-individual variation in absorption. Hence, a single measurement of plasma levels at 2 hours postprandial cannot be considered a measurement of bioavailability but, rather, a check on protocol compliance and, as such, its measurement is of limited value (Corti R, 2009).

Beside molecular size, there are other important factors modulating the *in vivo* efficacy of polyphenols that need to be taken into account. For example: the metabolic conversion in intestinal cells, liver, and other tissues; the binding to proteins; the accumulation in cells; and the urinary elimination rate (Schewe T, 2008).

Therefore, careful distinction between *in vitro* and *in vivo* effects of flavanols is mandatory. For example, although procyanidins are biologically active *in vitro*, they are barely absorbed in the intestine and, thus, are largely inactive *in vivo*.

An important point is that, during the conventional chocolate manufacturing process from fresh cocoa seeds to the final product, the concentration of flavanols markedly decreases (Andres-Lacueva C, 2008). The amount and type of flavanols in finished food products largely depends on the origin, post-harvest handling and processing of the flavanol-containing ingredient. In particular, cultivation type, growing region and agricultural practices undoubtedly play a role in determining the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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content of flavanols in raw cocoa. In contrast, the role of post-harvest handling in modifying flavanol content is much better understood. Following collection, seeds from cacao pods are allowed to ferment for a time period that may cover several days prior to drying and bulk packaging for shipping. Fermentation is a critical step for flavour generation in cocoa, and is widely practiced. The longer the fermentation period, the greater the reduction in flavanol content of the cocoa. After bulk cocoa is received by food manufacturers, it is roasted to further develop flavour but, since flavanols are heat labile, significant losses can occur during this step. In addition, an alkalising process modifies both flavour and appearance which further reduce flavanol concentrations in cocoa (Hollenberg N, 2004).

Finally, interactions with other chemical constituents such as milk proteins within the finished product may influence the bioactivity and bioavailability of flavanols in foods. In particular, milk chocolate has the lowest flavanol content compared to cocoa powder and dark chocolate (Miller K, 2006).

Other flavanol-containing ingredients widely used in the food industry are also routinely, and specifically, removed from wines, fruit juices and teas to modify taste and appearance to meet current consumer expectations. Great caution must be used when interpreting flavanol levels in specific finished food products based on information derived from raw ingredients or generic food composition tables.

As a result of these practices, cocoa and chocolate products currently on the market vary widely, and unpredictably, in flavanol content. Beverages make little, if any, contribution to flavanol intake due to their ingredient processing techniques (Hollenberg N, 2004).

Finally, the average daily consumption of flavanols has been estimated at approximately 60 mg/d (Gu L, 2004).

### 1.9.c. Possible mechanisms underlying the protective effects of cocoa

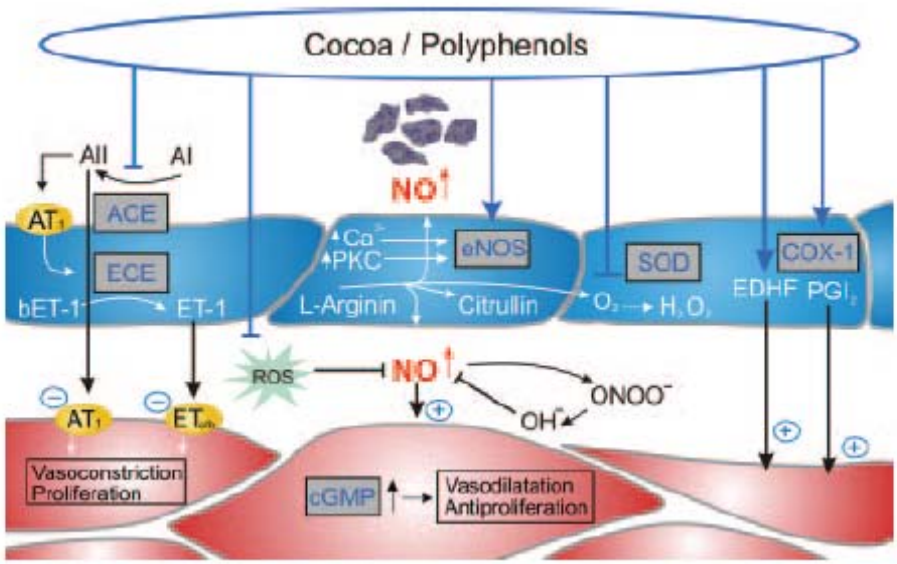
Although still being debated, a range of potential mechanisms through which flavanols and cocoa might exert their benefits on cardiovascular health have been proposed: activation of NO and antioxidant, antiinflammatory, and anti-platelet effects which, in turn, might improve endothelial function, lipid levels, BP, insulin resistance, and eventual clinical outcome (Ding E, 2006). Figure 4 summarises the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



relationships between cocoa polyphenols and the endothelium, with consequent cardiovascular benefits.

**Figure 4.** Endothelium-dependent effect of cocoa polyphenols




Abbreviations: AII, Angiotensin II; AI, Angiotensin I; PKC, protein Kinase C; SOD, superoxide dismutase; $PGI_2$, prostacyclin; ACE, angiotensin-converting enzyme; ECE, endothelin-converting enzyme; $AT_1$, angiotensin receptor; ET-1, endothelin 1; bET-1, big endothelin 1; $ET_{a/b}$, endothelin receptor a and b; cGMP, cyclic guanosine monophosphate; and ROS, reactive oxigen species.
Source: Corti R, 2009.

Figure 4 shows NO being released from EC mainly in response to shear stress elicited by the circulating blood or receptor-operated substances such as acetylcholine, bradykinin, or serotonin. NO is synthesised by endothelial NO synthase (eNOS) from L-argenine in the presence of the cofactor tetrahydrobiopterin. The activation may be due to an increase in $Ca^{2+}$ or a phosphorylation of eNOS by the PI3-kinase/Akt pathway. Cocoa also lowers vascular arginase activity in human edothelial cell *in vitro*, thus augmenting the local levels of L-arginine. Once released, NO increases intracellular cyclic

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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guanosine monophosphate (cGMP) concentrations and, in turn, induces a relaxation of VSMC. NO not only leads to vasodilatation but also prevents leukocyte adhesion and migration, smooth muscle cell proliferation, and platelet adhesion and aggregation. Other NO-mediated mechanisms are antioxidant effects that may reduce the production of ROS, thus contributing to an enhanced endothelial function. Cocoa polyphenols may activate endothelium-derived hyperpolarising factor (EDHF), increase endothelial prostacyclin release, or inhibit the synthesis of endothelin-1 (ET). Further, polyphenols may directly inhibit angiotensin-converting enzyme (ACE).

### 1.9.c.i. Endothelial function and nitric oxide

The endothelium is a continuous, smooth, non-thrombogenic surface of all blood vessels that exhibits a highly selective permeability in its healthy state. It synthesises and releases a broad range of vasoactive substances. Functional impairment of the vascular endothelium in response to injury occurs long before the development of structural atherosclerotic changes.

NO has an *in vivo* half-life of only a few seconds and has the capacity to cross biological membranes rapidly (Corti R, 2009).

Reduced eNOS expression and/or NO bioavailability is associated with endothelial dysfunction and, eventually, atherosclerosis.

In patients with coronary artery disease, eating food rich in flavanols, particularly short- and long-term consumption of black tea (Duffy S, 2001) and red wine (Karatzi K and Whelan A, 2004) improves endothelial function. In healthy smokers, green tea exerts similar effects (Nagaya N, 2004). In line with these findings, cocoa induces NO-dependent vasodilatation in the rat aorta and in the finger or forearm circulation of healthy humans, or patients with CVD risk factors, including diabetes (Corti R, 2009).

The underlying mechanisms have yet to be elucidated. In cultured EC and rat aorta, plant extracts rich in flavonoids increase eNOS activity. Incubation of EC with flavonoid-rich red wine up-regulates eNOS mRNA and protein expression, most likely via the stabilisation of eNOS mRNA. Further, EC produce up to 3 times more bioactive NO than control cells under similar conditions. Cocoa also lowers

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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vascular arginase activity in human EC *in vitro*, thus augmenting the local levels of L-arginine. Importantly, cocoa-derived flavanols induce NOS *in vitro* (Corti R, 2009). *In vivo*, in patients with CVD risk factors including smoking, a cocoa drink high in flavonol content (176 to 185 mg) rapidly enhances the circulating pool of bioactive NO by more than a third and, in turn, augments flow-mediated vasodilatation (Heiss C, 2003 and 2005). Moreover, infusion of *N*G-monomethyl-L-arginine, an inhibitor of NO synthesis, reverses the increase in NO and the increase in endothelial function associated with cocoa intake; the infusion of ascorbic acid has no such effect. Similarly, in isolated aortic rings, concentrations of flavanols comparable to those occurring in plasma after cocoa intake induce endothelium-dependent relaxation. Chronic consumption of a high-flavanol diet is associated with a high urinary excretion of NO metabolites, consistent with an increased NO production or diminished degradation. Finally, in humans, epicatechins closely mimic the vascular effects of flavanol-rich cocoa, suggesting that they represent the primary mediator of the beneficial effect of cocoa flavanols on vascular function (Schroeter H, 2006).

At the molecular level in EC, the short-term effects of epicatechin are due mainly to diminished inactivation of NO by free radicals through inhibition of NADPH oxidase by epicatechin metabolites while an increased generation of NO, as a consequence of higher protein eNOS expression, is involved in the long-term effects. Further, pure epicatechin ingestion not only augments NO bioavailability but also acutely reduces the plasma levels of endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor in healthy men. Of note is that drinking a flavonoid-enriched cocoa beverage results in regional changes in cerebral blood flow, and an overall increased blood flow to gray matter for up to 3 hours (as assessed by functional magnetic resonance imaging). In addition, in elderly humans, cerebral blood flow velocity in the middle cerebral artery increases (as measured by transcranial Doppler ultrasound) and which suggests that cocoa flavanols may protect against dementia and stroke (Corti R, 2009).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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### 1.9.c.ii. Antioxidant properties

Apart from their direct effects on eNOS expression and activity, cocoa flavanols and procyanidins exert strong antioxidant effects *in vitro*. First evidence came from an experiment in which extracted polyphenols from commercial cocoa delayed LDL oxidation (Waterhouse A, 1996). Other studies showed a reduction in the production of ROS in activated leukocytes, and an inhibition of ultraviolet-induced DNA oxidation (Ottaviani J, 2002). In humans, flavanol rich cocoa counteracts lipid peroxidation and, therefore, lowers the plasma level of F2-isoprostanes, *in vivo* markers of lipid peroxidation (Wiswedel I, 2004), and plasma levels of oxLDL in hypercholesterolaemic patients (Baba S, 2007). The overall antioxidant capacity is increased (Rein D, 2000). In young healthy smokers, commercially available dark chocolate (74% cocoa), but not white chocolate, markedly improves flow-mediated vasodilatation and improves plasma antioxidant status. This suggests that, under these conditions, an induction of eNOS and, in turn, elevated NO levels and a reduction in the production of ROS contribute to the enhanced endothelial function. Indeed, antioxidants may prevent NO transformation into peroxynitrite and protect against vasoconstriction and vascular damage. Oxidative stress and reduced antioxidant defense play a crucial role in the pathogenesis of atherosclerosis, particularly in transplant vasculopathy. A recent double-blind, randomised study assessed the effect of flavonoid-rich dark chocolate compared with cocoa-free control chocolate on coronary vasomotion in cardiac transplant recipients. The consumption of 40 g dark chocolate induced coronary vasodilatation, improved coronary vascular function, and decreased platelet adhesion. Again, these beneficial effects were paralleled by a reduction in serum oxidative stress (as assessed by plasma isoprostane measurement) and were positively related to serum epicatechin concentrations (Flammer A, 2007).

Nevertheless, the anti-oxidative effects of cocoa have been disputed recently. Sies H (2007) cautioned that, in addition to flavanols, fruits and vegetables contain many macronutrients and micronutrients that may directly, or through their metabolites, affect the total anti-oxidative capacity of plasma.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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### 1.9.c.iii. Platelet function

Platelet dysfunction is another hallmark of atherosclerotic vascular disease. In addition to providing antioxidant vitamins, certain fruits and vegetables may also protect against thrombosis because of their high flavanol content. Several studies have demonstrated platelet inhibitory properties of cocoa. Within hours of ingestion, cocoa reduces ADP/collagen-activated, platelet-related primary haemostasis. These effects were explained, at least in part, by a reduction in the ADP-induced expression of the activated conformation of glycoprotein IIb/IIIa surface proteins. Further, as with low-dose aspirin, *ex vivo* catechin and epicatechin reduce glycoprotein IIb/IIIa expression, thereby exerting anti-platelet effects (Pearson D, 2002). In healthy volunteers, consuming 100 g dark chocolate reduced platelet aggregation; an effect not observed following the ingestion of white chocolate or milk chocolate (Innes A, 2003). Cocoa decreases platelet aggregation as well as adhesion. In young healthy smokers, dark chocolate reduces platelet adhesion, as assessed by shear stress–dependent platelet test (Hermann F, 2006). Similarly, stearic acid, reduces mean platelet volume; an index of platelet activation, in humans (Corti R, 2009).

### 1.9.d. Antihypertensive effects of cocoa

Following from the initial observations in Kuna Indians, epidemiological support for the BP-lowering capacity of chocolate comes from the Zutphen Elderly Study. In this cohort of 470 men, cocoa intake was inversely related to BP. Even following multivariate adjustment, mean SBP was 3.8 mm Hg lower in the highest tertile of cocoa intake compared with the lowest tertile (Buijsse B, 2006). Another study evaluated the association between chocolate consumption and *de novo* onset of hypertension in a cohort of university graduates. The results indicated no protection due to cocoa (Alonso A, 2005).

More evidence on potential antihypertensive properties of cocoa comes from Taubert's trial (2007) which compared the long-term effect of dark compared with white chocolate consumption in patients with prehypertension, or stage I hypertension. A small amount of dark chocolate daily (6 g) in the evening

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



significantly reduced mean SBP by 2.9 ± 1.6 mm Hg and DBP by 1.9 ± 1.0 mm Hg with no changes in bodyweight, plasma lipid levels, glucose, and 8-isoprostane. However, serum levels of *S*-nitrosoglutathione, which is produced by unstable NO reacting with thiol groups to form a stable product, were increased in the dark chocolate group. Although preliminary in nature, these changes indicate an increase in NO production as a potential mechanism in the small reduction in BP seen with dark chocolate consumption.

Apart from increased eNOS activity, other mechanisms may contribute to the antihypertensive effect of cocoa-rich food. Indeed, flavanols on their own or in food items inhibit ACE activity *in vitro*. Whether such ACE inhibition also occurs *in vivo* needs further evaluation. Finally, stearic acid or theobromine may contribute to these effects. A cross-sectional linear regression analysis within the MRFIT study found that stearic acid levels are inversely associated with DBP (Corti R, 2009).

No matter the mechanism responsible, several independent, albeit small, studies indicate that ingestion of cocoa-rich chocolate has BP-lowering effects. One study reported reductions in systolic and diastolic BPs in hypertensive elderly subjects (Taubert D, 2003), and another study noted a decrease in daytime and night-time blood pressures, as assessed by ambulatory 24-hour measurements, following an intake of 100 g flavonoid-rich dark chocolate daily for 2 weeks (Grassi D, 2005). In the latter study, SBP decreased by 12 mm Hg following the consumption of dark chocolate, whereas white chocolate had no such effect. However, other studies showed no effect on BP (Fisher N, 2003 and Engler M, 2004). Since these studies were performed in a relatively small number of normotensive individuals, and with a lower chocolate intake over a shorter duration, any antihypertensive effect may have been missed as a result of the study design.

Taubert, D (2007) in a meta-analysis of RCT of cocoa administration (173 subjects; mean duration, 2 weeks), confirmed a significant reduction in pressure: mean systolic and diastolic BPs were reduced by 4.7 mm Hg (95%CI: 7.6 to 1.8; *P*=0.002) and 2.8 mm Hg (95%CI: 4.8 to 0.8; *P*=0.006), respectively. This finding is of note because the BP-lowering effects of currently-used antihypertensive drugs are within this range.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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Considering the small number of subjects studied so far, and the variable dose of flavanols and/or chocolate used, a large, well-controlled, interventional study appears warranted. Since the demonstration that treatment with candesartan of prehypertensive subjects reduced the risk of incident hypertension, the debate continues on the therapeutic need in this large population. Therefore, the response to flavanol-rich cocoa in subjects with prehypertension (120 to 139 mm Hg; according to the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High BP VII) or high-normal (130 to 139 mm Hg; according to the ESC) BP would be particularly interesting because it could have the potential to delay the onset of hypertension in this population.

### 1.9.e. Cocoa effects on blood lipids

Cocoa butter, a fat derived from cocoa plants and found predominantly in dark chocolate, contains an average of 33% monounsaturated oleic acid and 33% stearic acid. In general, plant stearic acid neither lowers HDL-c nor increases LDL-c or TC (Mensink R, 2003). Of note is that, in a study involving young healthy subjects, consumption of a milk chocolate bar (46 g) instead of a high-CHO snack, increased HDL-c and decreased plasma TG, but did not affect LDL despite an increase in total fat in the diet (Kris-Etherton P, 1994). In hypertensive patients, daily consumption of 100 g flavonoid-rich chocolate over a period of 2 weeks led to a significant 12% reduction in serum total and LDL cholesterol levels (Grassi, D 2005). Further, cocoa appears to inhibit LDL oxidation. In healthy subjects, daily consumption of 75 g polyphenol-rich dark chocolate over 3 weeks increases HDL-c by up to 14% and inhibits lipid peroxidation (Mursu J, 2004). A recent Japanese study demonstrated that, in hypercholesterolaemic patients, flavanol-rich cocoa lowers plasma levels of LDL-c and oxLDL, and increases HDL-c concentrations (Baba S, 2007).

Overall, the effects of chocolate and its various components on lipid levels are not conclusive. A large well-controlled study appears necessary. Importantly however, despite its high fat content, cocoa *per se* does not seem to exert untoward effects on lipid metabolism. It must be stressed that many chocolate products contain milk

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



or processed fats e.g. palm oils and the effects of these processed chocolates on blood lipids is not known, and may indeed be less favourable.

### 1.9.f. Cocoa effects on insulin resistance

Insulin sensitivity is partly dependent on insulin-mediated NO release. Thus, flavanols and dietary antioxidants may decrease insulin resistance by ameliorating NO bioavailability.

In line with this concept, Grassi, D (2005) reported reduced insulin resistance in patients with essential hypertension after a 15-day diet with daily 100 g flavonoid-rich chocolate. In hypertensive patients with impaired glucose tolerance, flavonoid-rich dark chocolate not only reduced BP and improved endothelial function but also ameliorated insulin sensitivity and ß-cell function. Since intravenous infusion of ascorbic acid improves not only flow-mediated dilation but also insulin sensitivity in subjects with impaired glucose tolerance and in smokers (Hirai N, 2000), the antioxidant properties of flavanols might contribute to these beneficial effects of coca on insulin sensitivity.

However, studies with cocoa in diabetics are scarce and diabetic subjects tend to be obese and, as such, recommending cocoa or flavonoid-rich chocolate consumption to such patients needs to be undertaken cautiously. Nevertheless, experimental evidence in obese diabetic mice suggests that cocoa prevents hyperglycaemia in a dose dependent manner (Tomaru M, 2007).

### 1.9.g. Considerations

Although many positive effects of chocolate and its ingredients have been documented with respect to the cardiovascular system, caution needs to be exercised. Indeed, the high caloric load of commercially available chocolate (about 500 Kcal/100 g) could induce weight gain which is a risk factor for HYPERTENSION, dyslipidaemia, and diabetes. Surprisingly, a study in 49 healthy women showed no weight gain following a daily consumption of 41 g chocolate, 60 g almonds, or almonds and chocolate together for 6 weeks (Kurlandsky S, 2006). Thus, weight gain may occur only with higher amounts of daily chocolate consumption and/or its intake over a protracted period. Further, the high sugar and

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



fat content of commercially-available chocolate has to be taken into account. High sugar intake is associated with obesity, dental caries, and diabetes and, as such, cocoa-based products with little or no sugar is to be preferred. Cocoa *per se,* unlike chocolate, can be recommended without hesitation because it is low in sugar and fat.

### 1.10. Dietary fibre and definitions

Dietary fibre provides many health benefits. A generous intake of dietary fibre reduces risk of developing: CHD, stroke, hypertension, diabetes, obesity, and certain gastrointestinal disorders (Anderson J, 2009; Petchetti L, 2007).

Further, increased consumption of dietary fibre improves serum lipid concentrations, lowers BP, improves blood glucose control in diabetes, promotes gastrointestinal regularity, aids in weight loss, and appears to improve immune function (Anderson J, 2009; Petchetti L, 2007).

Unfortunately, most individuals in developed countries consume less than half of the recommended daily levels of dietary fibre. This is consequence of suboptimal intake of whole-grain foods, vegetables, fruits, legumes, and nuts. Fibre supplements have the potential to play an adjunctive role in the health benefits provided by high-fibre foods (ATP III, 2001; Anderson J, 2009).

Until recently, there has been a wide variety of terms to define dietary fibre, and this has contributed to the difficulty in data interpretation the data.

Traditionally, dietary fibre was defined as the portions of plant foods, including polysaccharides and lignin, which were resistant to digestion by human digestive-tract enzymes. The definition has been expanded to include oligosaccharides, such as inulin, and resistant starches. Fibres have been classified as soluble (such as viscous or fermentable fibres e.g. pectin) that are fermented in the colon, and insoluble fibres (such as wheat bran) that have bulking action but may only be fermented to a limited extent in the colon (Anderson J, 2009).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.10.a. Recommendations for fibre

Current recommendations for dietary fibre intake are related to age, gender, and energy requirement. The general recommendation for an adequate intake (AI) is 14 g/1000 Kcal (Anderson J, 2009). This AI includes non-starch polysaccharides (analogous to CHO e.g. resistant starches), lignin, and associated substances. Using the energy guideline of 2000 Kcal/d for women and 2600 Kcal/d for men, the recommended daily dietary fibre intake is 28 g/d and 36 g/d, respectively.

A recent review summarised the research data related to the effects of dietary fibre on health. Most of the available data on disease prevalence and adverse events are from epidemiological studies.

While limited data are available on the effects of consumption of high-fibre foods or specific food sources of fibre, extensive data are available relating the effects of fibre supplements on serum lipid values, weight management, postprandial glycaemia, and gastrointestinal function.

Comments in this section are restricted to the use of soluble fibre supplements because the data generated have been from studies using the soluble fibre *Plantago ovata* husk (Po-husk).

The use of fibre supplements to the diet is not widely recommended by authoritative health organisations in the USA or in Europe. Standard dietary sources of fibre contain, as well, vitamins, minerals, water, and a variety of phyto-nutrients. However, fibre supplements may play an important role in helping some individuals achieve fibre intakes approaching the recommended levels, but which were not in their habitual diet.

The available clinical trial data suggest that the use of fibre supplements is more efficacious than the use of high-fibre foods for improving serum lipoprotein values, enhancing weight loss, and improving gastrointestinal function.

These improved health benefits for fibre supplements compared to high-fibre foods are probably related to better adherence to supplement use than undertaking substantial improvements in dietary practices.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.10.b. Fibre and CVD

High levels of dietary fibre intake are associated with significantly lower prevalence rates for CHD, stroke, and peripheral vascular disease (Anderson J, 2009). Major risk factors such as hypertension, diabetes, obesity, and dyslipidaemia are also less common in individuals with the highest levels of fibre consumption (Anderson J, 2009). The impact of dietary fibre or whole grain consumption on the prevalence of these conditions is summarised in Table 13.

**Table 13.** Dietary fiber intake related to relative risk for disease based on estimates from prospective cohort studies

| Disease | N of subjects (n of studies) | Relative risk* | 95% CI |
|---|---|---|---|
| CHD | 158.327 (7) | 0,71 | 0,47-0,95 |
| Stroke[+] | 134.787 (4) | 0,74 | 0,63-0,86 |
| Diabetes | 239.485 (5) | 0,81 | 0,70-0,93 |
| Obesity | 115.789 (4) | 0,70 | 0,62-0,78 |

*Relative risks adjusted for demographic, dietary, and non-dietary factors.

[+]Estamates related to whole-grain consumption, total dietary fiber, and cereal fiber.

Source: Anderson J, 2009.

### 1.10.c. Fibre and risk factor prevalence

The prevalence of hypertension or dyslipidaemia, as they relate to fibre intake, has not been well characterised.

In a small group of Chinese residents higher consumption of oats or buckwheat was associated with significantly lower body mass index (BMI), SBP and DBP, and serum LDL-c and TG values. Serum HDL-c values were also lower (He J, 1995).

Total dietary fibre intake was associated with significantly lower serum LDL-c values while soluble fibre was associated with lower SBP and TC values (He J, 1995; Anderson J, 2009). Among French adults, higher intakes of dietary fibre were associated with lower prevalence of hypertension and with lower TC serum and TG values (Lairon D, 2005).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.10.d. Fibre and effects on risk factor status

#### 1.10.d.i. Lipoproteins

Soluble or viscous fibres have significant hypocholesterolaemic effects (Brown L, 1999). Extensive studies with guar gum focused on diabetic control, bodyweight, and serum lipoproteins (Anderson J, 2009).

The meta-analysis of Brown (1999) provided estimates of effects of various soluble fibres on serum lipoproteins. For more data on efficacy, RCTs were reviewed and the net LDL-c effects (change with fibre treatment minus change with placebo treatment) were weighted by number of subjects per trial. The findings of the meta-analysis are summarised in Table 14.

**Table 14.** Effects of soluble fiber intake on serum LDL-c values in RCTs with weighted mean changes based on number of subjects

| Fiber | N of trials | N of subjects | Grams/d (median) | Baseline LDL-c | Weighted net change[+] |
|---|---|---|---|---|---|
| Barley ß-glucan | 9 | 129 | 5 | 4,1 | -11,1 |
| Guar gum | 4 | 79 | 15 | 4,4 | -10,6 |
| HPMC | 2 | 59 | 5 | 4,2 | -8,5 |
| Oat ß-glucan | 13[^] | 457 | 6 | 4 | -5,3 |
| Pectin | 5 | 71 | 15 | 3,9 | -13,0 |
| Psyllium | 9[^] | 494 | 6 | 3,9 | -5,5 |

[+] Net changes are treatment change minus placebo change.

[^] Analysis of high-quality clinical trials.

Abbreviation: HPMC, hydroxypropyl methylcellulose.

Sourece: Anderson J, 2009.

For guar gum, over 40 clinical trial publications were reviewed and RCTs in non-diabetic subjects were selected for analysis. Intakes ranged from 9 to 30 g/d, divided into at least three servings/d, were associated with a weighted mean reduction in LDL-c of 10.6%.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



For pectin, the acceptable-quality RCTs reviewed indicated that consumption of 12–24 g/d in divided amounts was associated with a 13% reduction in LDL-c values.

Barley b-glucan intake of 5 g/d in divided doses was associated with an 11.1% reduction in LDL-c values.

Limited information on hydroxypropyl methylcellulose (HPMC) indicated that 5 g/d in divided doses decreases LDL-c values by 8.5%. These LDL-c changes with soluble fibres occur without significant changes in HDL-c or TG concentrations.

Psyllium and oat b-glucan are the most widely used sources of soluble fibre and have been approved by the Food and Drug Adminsitration (FDA) from USA for health benefits related to protection from CHD. A recent review by Anderson J (2009) of RCTs published since the FDA recommendations yielded eight high-quality RCTs for psyllium and 11 high-quality studies for oat b-glucan. The meta-analysis compared the outcomes with respect to the LDL-c changes against the meta-analysis of Brown L (1999). The respective values were -5.5% and -5.2% for psyllium and -5.3% and -5.6% for oat b-glucan. These analyses indicate that psyllium or oat b-glucan do not significantly affect serum HDL-c or TG values.

The limited data available for Konjac mannan (glucomannan) indicates that it has a significant hypocholesterolaemic effect.

Gum arabic (acacia gum), partially hydrolysed guar gum, and methylcellulose appear to have only modest hypocholesterolaemic effects.

These short-term studies, with mean durations of 4–8 weeks, indicate that the widely-used psyllium or oat b-glucan decrease serum LDL-c values by about 5.5%. These reductions would be expected to reduce the risk for CVD by 7–11%.

The best long-term data available for soluble fibre are that obtained from the use of psyllium for 6 months and the use of guar for 12–24 months. Use of psyllium for 6 months maintains the LDL-c reduction of 6.7% (Anderson J, 2000) and long-term use of guar sustains the reductions of LDL-c values of 16.1% at 1 year, and 25.6% at 24 months (Anderson J, 2009).

While the levels of guar used in these studies may not be practical for general population use, these data do indicate that regular use of a soluble fibre can sustain significant hypocholesterolaemic effects over the long term. These changes

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



were maintained without changes in bodyweight, HDL-c, or serum TG values (Anderson J, 2009).

Our unit research has been researching Po-husk over a considerable period of time. The purpose of the present study was to assess, in a crossover design protocol, the therapeutic effects of 10.5 g/d soluble Po-husk fibre compared to 10.5 g/d of an equivalent insoluble fibre added to a diet low in saturated fat and cholesterol. The target variables measured were plasma lipids, lipoproteins and apolipoproteins in patients with established CHD but with only moderately elevated plasma LDL-c ≤ 3.35 mmol/L (130 mg/dL). The potential interactions with genes involved in the response to dietary fibre therapy were explored, as well.

The insoluble fibre used as control additive was hemicellulose and lignin Klason obtained from Po seeds (Madaus S.A., Barcelona, Spain). The two products are obtained from the same Po plant; Po husk consisting only of the epidermis and collapsed adjacent layers removed from the dried ripe Po seeds (Solà R, 2007).

In a controlled, single-blind, crossover study consisting of a 4 week dietary adaptation period, 28 patients were randomly assigned to two different fibre-supplement periods of 8 weeks each. We incorporated a washout period of 8 weeks between the 1$^{st}$ and 2$^{nd}$ periods of the study in order to correct for any possible interactions between treatment and sequence order (carry-over effect) (Solà R, 2007).

In men with CVD on a low saturated-fat and low-cholesterol diet, the incorporation of Po husk significantly reduced plasma TG concentrations by 6.7%, Apo B/Apo A ratio by 4.7% and increased the Apo A-I concentration by 4.3% (Solà R, 2007).

The plasma TG reduction observed with the Po husk treatment was approximately half (between 10% and 16%) that of the reduction observed with statin therapies (the most widely used LDL-c-lowering drugs) and was similar to that of ezetimib (7% TG reduction). With this modest hypotriglyceridaemic effect, Po husk can be considered as an adjuvant treatment in patients with moderate hypertriglyceridaemia (Brunzell JD, 2007).

The Apo B/Apo A ratio has been shown to be the best marker of atherogenic and anti-atherogenic particles in plasma. The lower the mean Apo B/Apo A ratio the lower the CVD risk. The Interheart study showed this ratio to be a marker of risk of

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



MI, irrespective of the geographic regions of the populations studied (Yusuf S, 2004). Recently, the Apo B/Apo A ratio was linked to the risk of fatal stroke in a similar manner to that of MI, and other ischemic events (Walldius G, 2006).

**1.10.d.ii. Blood pressure**

Increasing consumption of dietary fibre is often accompanied by a reduction in SBP and DBP. Early studies suggested that high fibre diets were associated with a significant reduction in BP, but these studies were not well-controlled clinical trials (Anderson J, 2009).

The effects of increasing oat fibre intake on BP have been reported in several studies. The net results suggest a modest-to-moderate reduction in SBP and DBP (Anderson J, 2009).

Two meta-analyses recently assessed the effects of fibre intake on BP. A meta-analysis of the effects of fibre supplements on BP in RCTs reported that the effects of fibre supplements averaging 11.5 g/d were modest, with decreases in SBP of 1.1 mm Hg and DBP of 1.3 mm Hg; reductions were greater in hypertensive subjects and in older individuals (Anderson J, 2009). Similarly, a meta-analysis of 25 RCTs indicated that dietary fibre intake was associated with non-significant changes in SBP (-1.2 mm Hg) and significant reductions in DBP (-1.7 mm Hg). Significant reductions in SBP (-6.0 mm Hg) and DBP (-4.2 mm Hg) occurred in hypertensive subjects, or those receiving treatment for more than 8 weeks (Whelton S, 2005). Thus, increasing dietary fibre intake or fibre supplements may have a modest independent effect in reducing BP, especially in hypertensive individuals.

**1.10.d.iii. Other risk factors**

In addition to having favourable effects on serum lipoproteins and BP, dietary fibre consumption has favourable effects on bodyweight, visceral adiposity, and insulin sensitivity, as well as on inflammatory biomarkers (Anderson J, 2009).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.10.e. Proposed mechanisms underlying effects of fibre on CVD risk factors

The hypocholesterolaemic effects of dietary fibre have been studied extensively, and are the best-characterised effects of fibre consumption on CVD risk. Soluble or viscous fibres appear to exert primary effects on serum cholesterol and LDL-c values by binding bile acids in the small intestine and increasing their excretion in the faeces (Anderson J, 2009).

Fermentation of fibres in the colon, with production of the short-chain fatty acid Propionate, may contribute to hypocholesterolaemia by attenuating cholesterol synthesis (Wright, R 1990; Alvaro A, 2008).

## 1.11. Dietary components

### 1.11.a. Phenolic compounds

Dietary flavonoids represent a diverse range of phenolic compounds that occur naturally in plant-derived foods. The range and structural complexity of flavonoids have led to their sub-classification as flavonols, flavones, flavanones, flavan-3-ols (and their oligomers, proanthocyanidins), isoflavones, and anthocyanins.

They are present in significant amounts in many commonly-consumed fruits, vegetables, grains, herbs, and beverages. These structurally diverse compounds exhibit a range of biological activities *in vitro* that may explain their potential cardioprotective properties, including antioxidant and antiinflammatory effects, and induction of apoptosis (Erdman J, 2007).

Epidemiologic evidence of the cardiovascular effects of diets rich in flavonoids is mixed, with some studies supporting positive effects, and others not (Arts I, 2001; Knekt P, 2002; Geleijnse J, 2002; Sesso H, 2003 and Lin J, 2007). A large prospective study of postmenopausal women with 16 years of follow-up recently showed that dietary intakes of foods rich in anthrocyanins and flavanones were associated with a lower risk of all-cause mortality as well as death due to CHD and death due to CVD (Mink P, 2007).

To date, a substantial number of studies have reported on the efficacy of individual plant foods or extracts in reducing biomarkers of CVD risk in acute and short-term interventions with healthy volunteers, as well as with at-risk population groups.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



However, these data have not been combined in a systematic examination of the relative effects of the different subclasses of flavonoids.

To examine the relative importance of the different flavonoid subclasses and flavonoid-rich foods, Hopper L (2008) proposed a systematic review of all published RCTs to determine the optimal doses, or food sources, to reduce CVD risk and to identify priorities for future research. There were 133 trials included. No RCTs studied effects on CVD morbidity or mortality. Significant heterogeneity confirmed differential effects between flavonoid subclasses and foods. Chocolate increased flow mediated dilation after acute (3.99%, 95%CI: 2.86 to 5.12; 6 studies) and chronic (1.45%, 95%CI: 0.62 to 2.28; 2 studies) intake and reduced SBP (-5.88 mm Hg, 95%CI: -9.55 to -2.21; 5 studies) and DBP (-3.30 mm Hg, 95%CI: -5.77 to -0.83; 4 studies).

Soy protein isolate (but not other soy products or components) significantly reduced DBP (-1.99 mm Hg, 95%CI: -2.86 to -1.12; 9 studies) and LDL-c (-0.19 mmol/L, 95%CI: -0.24 to -0.14; 39 studies).

Acute black tea consumption increased SBP (5.69mmHg, 95%CI: 1.52 to 9.86; 4 studies) and DBP (2.56 mm Hg, 95%CI: 1.03 to 4.10; 4 studies).

Green tea reduced LDL-c (-0.23 mmol/L, 95%CI: -0.34 to -0.12; 4 studies).

For many of the other flavonoids, there was insufficient evidence to draw conclusions regarding efficacy. The conclusions from this meta-analysis were that although the effects of flavonoids from soy and cocoa have been the main focus of attention. future studies should focus on other commonly-consumed subclasses (e.g., anthrocyanins and flavanones) to assess dose-response effects and need to be of long enough duration to allow assessment of clinically relevant endpoints (Hooper L, 2008).

### 1.11.b. Types and classification of phenolic compounds

To establish firm evidence for the health benefit effects of dietary polyphenol consumption, it is essential to have quantitative information regarding their dietary intake. The usefulness of the current methods, which rely mainly on the assessment of polyphenol intake using food records and food composition tables, is limited because they fail to assess total intake accurately (Spencer J, 2008).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Recently, a review by Spencer J (2008) highlighted the problems associated with such methods with regard to polyphenol-intake predictions. It is suggest that the development of biological biomarkers, measured in blood and urine, are essential for making accurate estimates of polyphenol intake. However, the relationship between dietary intakes and nutritional biomarkers are often highly complex. The review identifies the criteria that need to be considered in the development of such biomarkers. In addition, it provides an assessment of the limited number of potential biomarkers of polyphenol intake currently available.

In the last decade, there has been intense interest in the potential health benefits of dietary-derived plant polyphenols. An ever increasing number of studies have described their antioxidant properties, and linked this to their proposed role in the prevention of human disease. Plant polyphenols are abundant in the human diet, particularly in fruit, vegetables and pulses which have been consistently associated with a decreased risk of cancer, CVD, and a range of other chronic disorders (Terry P and Gardner C, 2001; Le Marchand L and Hu F, 2002; Jenkins D, 2003).

The likely active components of fruits, vegetables and pulses are a group of phytochemicals, known as polyphenols. However, for a variety of reasons, it has proved extremely difficult to quantitatively establish the benefit afforded by polyphenols:

- there is a great diversity of polyphenol content between foods
- there is limited data regarding the polyphenol content of specific foods within the commonly-used food composition databases
- there are challenges in characterising and quantifying habitual food intake
- there is a limited understanding regarding the extent of absorption and metabolic fate of individual polyphenols from particular foods

Biological markers of nutrient exposure, as an alternative to the more traditional dietary assessment tools, have been used for many years. In this approach one or more biochemical moieties are measured in an accessible fluid or tissue to provide a semi-quantitative index of the exposure to individual food constituents.

This could be an attractive approach for polyphenols. However, the relationship between dietary intake and resulting concentrations of biomarkers in body fluids is highly complex. Before a particular dietary component, or its metabolite, can be

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



used as a sensitive and accurate biomarker of exposure to a specific polyphenol, a number of factors need to be taken into account (Spencer J, 2008):

a) a full understanding of the metabolism of polyphenols in human subjects is required in order to select credible biomarkers

b) it is important to understand the time-response relationship between polyphenol intake and the appearance of the biomarker in biological fluids

c) the precise dose-response relationship between the intake of a specific polyphenol and the appearance of its biomarker is essential

d) there needs to be an understanding of the extent to which certain physiological and environmental factors affect the rate of polyphenol metabolism in human subjects.

### 1.11.c. Classification and structure of major dietary polyphenols

Currently, polyphenols are also termed phenolic compounds, and frequently both names apply to the same molecule or compound.

Polyphenols are naturally-occurring, non-nutritive, plant compounds which can be classified into several groups based on their structures. The main classes include flavonoids, phenolic acids, stilbenes and lignans. Figure 5 illustrates the different groups of polyphenols, with a representative compound from each group to highlight the characteristic chemical structure.

One of the major classes of polyphenols is the flavonoids, identified by having a common structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



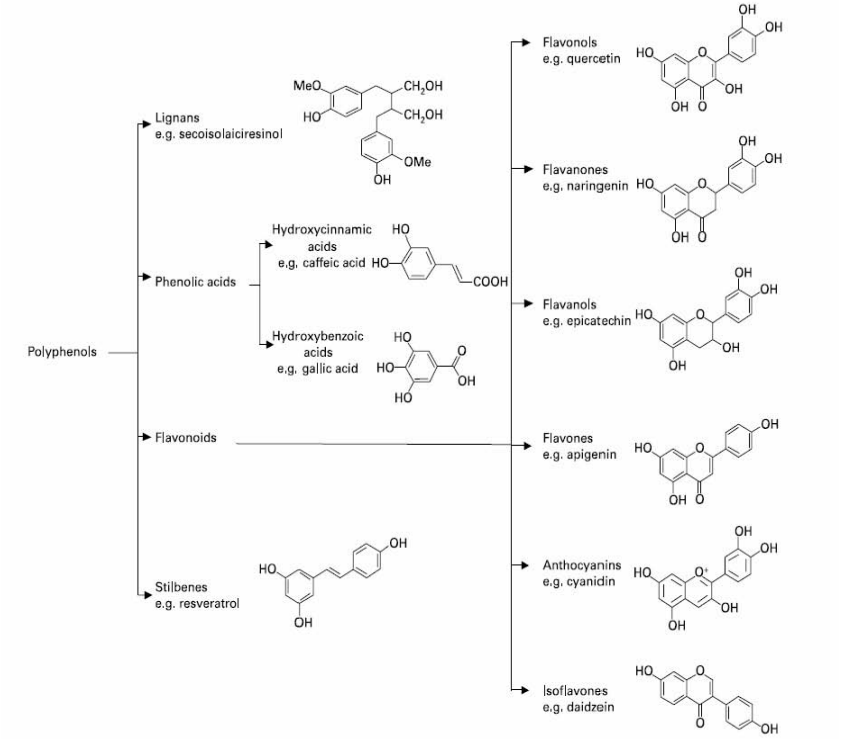



**Figure 5.** Classification and chemical structure of major classes of dietary polyphenols
Source: Spencer J, 2008

Based on the variation in the type of heterocycle involved, flavonoids may be divided into six subclasses (see Figure 5):

1. flavonols
2. flavones
3. flavanones
4. flavanols
5. anthocyanins
6. isoflavones

Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups, and their extent of alkylation and/or glycosylation. Phenolic acids are abundant in food and can be classified into

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



derivatives of benzoic acid and of cinnamic acid. Polyphenolic compounds also include stilbenes, which are compounds that possess 1,2-diarylethene structures and lignans characterised by a 1,4-diarylbutane structure (Spencer J, 2008).

**1.11.d. Factors affecting choice of potential biomarkers of polyphenols intake**

To serve as a useful biomarker of intake in nutrition studies, the following criteria need to be satisfied:

1. Measurement of the biomarker of interest should be qualitatively and quantitatively robust. Therefore, sensitive and specific techniques should be available to quantify accurately the biomarker-of-interest in tissue/biofluid, which has been appropriately collected and stored to ensure minimum biomarker degradation
2. Concentrations of the biomarker in the tissue/biofluid should be sensitive to changes in intake of the dietary component
3. The biomarker should be specific to the dietary component-of-interest i.e. variation in its concentration should be due to changes in the intake of the dietary component-of-interest only
4. The interpretation of dietary intake and biomarker relationships requires a clear understanding of the impact of physiological factors and whole-diet composition on kinetics of absorption, metabolism and excretion of the putative biomarker (Spencer J, 2008)

It is clear that the choice of biomarker for the estimation of polyphenol intake is complex, with different classes of flavonoids and possibly even individual polyphenols requiring different approaches.

For the majority of flavonoids, which possess short plasma half-lives, measurement of plasma concentrations will provide little information regarding even acute intakes, and quantification of their output in urine may be a better approach. In addition to scientific considerations, practical issues may determine the choice of method.

The quantification of a number of metabolites in 24h urine samples may be a suitable approach in small-scale human intervention trials, but may not be a

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



realistic possibility in large-scale epidemiological studies. This is due to problems in organising the collection of 24h urine samples from a large study population.

An intermediate approach may be the quantification of metabolites in spot urine samples, but there is little evidence on which to assess the suitability of this approach. This could be an important area for future research.

The Spencer J (2008) review focused on the identification and validation of individual polyphenols, or their metabolites, which may represent useful biomarkers of the intake of polyphenols in human subjects. The majority of potential biomarkers are those deriving from phase I and II metabolism of flavonoids and other polyphenols in the small intestine and liver. Metabolites such as glucuronides and sulphate conjugates (and related O-methylated forms) are commonly used to determine the extent of polyphenol absorption in human subjects, and represent excellent biomarkers for researchers to explore using precise analytical techniques.

In addition to these well characterised metabolic forms, there is great potential for cellular metabolites of polyphenols, such as glutathionyl and cysteinyl conjugates, to act as specific biomarkers of polyphenol intake since they are likely to be present in significant amounts in blood as well as urine. There are also a number of other candidates, such as valerolactones for flavanol intake, isoferulic acid for chlorogenic acid ingestion and homovanillic alcohol for the intake of hydroxytyrosol. In summary, quantification of specific flavonoid and polyphenol biomarkers in accessible body fluid/tissues holds great potential as an alternative approach to traditional dietary assessment techniques, and will undoubtedly provide invaluable information regarding the intake of polyphenols in human subjects.

Although most polyphenols are absorbed to some extent, this is very dependent on the type of polyphenol. The range of concentrations required for a demonstrable effect *in vitro* varies from <0.1 μmol/L to >100 μmol/L. Physiologic concentrations do not exceed 10 μmol/L and, hence, the effects of polyphenols *in vitro* at concentrations of <10 μmol/L (Williamson G, 2005).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.11.e. Bioavailability and bioefficacy of phenolic compounds

The specific definition of bioavailability is the fractional amount of a nutrient or other bioactive substance that, subsequent to ingestion, becomes available for use in target tissues (Howlett J, 2008).

One of the main objectives of bioavailability studies is to determine which, among the hundreds of dietary polyphenols, are better absorbed and which lead to the formation of active metabolites. Bioavailability of polyphenols varies widely from one compound to another. Most polyphenolic compounds are poorly absorbed from the intestine and are highly metabolised, or rapidly eliminated.

There are accurate methods of analysis to calculate several bioavailability measures. These include the maximal plasma concentration (Cmax), time to reach Cmax, area under the plasma concentration-time curve, elimination half-life, and relative urinary excretion.

The bioavailability and pharmacokinetics of polyphenols are governed by a plethora of factors such as their native form (glycosylated/aglycone), the type of sugar moiety present, and their physiochemical properties. Moreover, some of the metabolites still possess inherent biological activities.

Better knowledge of some variables of polyphenol bioavailability, such as the kinetics of absorption, accumulation, and elimination, will facilitate the design of such studies. Besides, more precise data on the nature of the circulating metabolites and on metabolism by the microflora can now to be used for better interpretations.

Bioavailability differs greatly from one polyphenol to another and, as such, the most abundant polyphenols in the diet are not necessarily those leading to the highest concentrations of active metabolites in target tissues.

Mean values for the Cmax, the time to reach Cmax, the area under the plasma concentration-time curve, the elimination half-life, and the relative urinary excretion have been calculated for 18 major polyphenols.

The observed variability in the effects and efficacy of polyphenols may well be due to a different bioavailability or tissue specificity of the polyphenols in the different *in vitro* and animals models, and different affinities for the primary target molecule/s (potency).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The data on the bioavailability of polyphenols summarised above takes into account only the presence of intact polyphenols in the blood i.e. the ingested compound or its conjugates. The microfloras in the colon also play a critical role in the metabolism of polyphenols. There are 2 possible routes available following microbial enzyme-catalysed deconjugation of any polyphenol conjugates that reach the colon:

a) absorption of the intact polyphenol through the colon epithelium

b) passage into the bloodstream (as free or conjugated forms) or breakdown of the original polyphenol structure into metabolites

The absorption data presented above include the contribution of absorption of intact polyphenols in the colon, but do not include the breakdown contribution. Microbial metabolism deserves special consideration because many of the diverse polyphenols are broken down into simpler phenolic compounds that are common to many different polyphenols. In addition, some of the microbial metabolites could have unique biological effects. For example, the isoflavone daidzein is converted to equol by gut microflora in ~30–40% of the population, and the equol is absorbed into the bloodstream in these individuals (Williamson G, 2005).

### 1.11.f. Phenolic molecules in virgin olive oil (VOO)

Table 15 summarises the major structures of phenolic compounds presents in VOO.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 15.** Phenolic compounds in VOO

| Compounds name | Substituent (MW) |
|---|---|
| Benzoic acid and derivatives: | |
| 3-Hydroxybenzoic acid | 3-OH (138) |
| *p*-Hydroxybenzoic acid | 4-OH (138) |
| 3,4-Dihydroxybenzoic acid | 3,4-OH (154) |
| Gentisic acid | 2,5-OH (154) |
| Vanillic acid | 3-$OCH_3$, 4-OH (168) |
| Gallic acid | 3,4,5-OH (170) |
| Syringic acid | 3,5-$OCH_3$, 4-OH (198) |
| Cinnamic acids and derivatives: | |
| *o*-Coumaric acid | 2-OH (164) |
| *p*-Coumaric acid | 4-OH (164) |
| Caffeic acid | 3,4-OH (180) |
| Ferulic acid | 3-$OCH_3$, 4-OH (194) |
| Sinapinic acid | 3,5-$OCH_3$, 4-OH (224) |
| Phenyl ethyl alcohols: | |
| Tyrosol [(*p*-hydroxyphenyl) ethanol] or *p*-HPEA | 4-OH (138) |
| Hydroxytyrosol [(*3,4*-dihydroxyphenyl) ethanol] or 3,4-DHPEA | 3,4-OH (154) |
| Other phenolic acids and derivatives: | |
| *p*-Hydroxyphenylacetic acid | 4-OH (152) |
| 3,4-Dihydroxyphenylacetic acid | 3,4-OH (168) |
| 3-(3,4-Dihydroxypheny) propanoic acid | 3-$OCH_3$, 4-OH (182) |
| 3-(3,4-Dihydroxypheny) propanoic acid | (182) |
| Dialdehydic forms of secoiridoids: | |
| Decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA) | $R_1$-OH (304) |
| Decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA) | $R_1$-H (320) |
| Secoiridoid aglycons | |
| Oleuropein aglycon or 3,4-DHPEA-EA | $R_1$-OH (378) |
| Ligstroside aglycon or *p*-HPEA-EA | $R_1$-H (362) |
| Aldehydic form of oleuropein aglycon | $R_1$-OH (378) |
| Aldehydic form ligstroside aglycon | $R_1$-H (362) |
| Flavonols: | |
| (+)-Taxifolin | (304) |
| Apigenin | $R_1$-OH, $R_2$-H (270) |
| Luteolin | $R_1$-OH, $R_2$-OH (286) |
| Lignans: | |
| (+)-Pinoresinol | R-H (358) |
| (+)-1-Acetoxypinoresinol | R-$OCOCH_3$ (416) |
| (+)-1-Hydroxypinoresinol | R-OH (374) |
| Hydroxyisochromans: | |
| 1-Phenyl-6,7-dihydroxyisochroman | $R_1$,$R_2$-H (242) |
| 1-(3'-Methoxy-4'-hydroxy) phenyl-6,7-dihydroxyisochroman | $R_1$-OH, $R_2$-$OCH_3$ (288) |

Abbreviations: MW, molecular weight.
Source: Bendini A, 2007.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.11.f.i. Secoiridoids

Oleuropein belongs to a specific group of coumarin-like compounds, the secoiridoids, which are abundant in *Oleaceae*. Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. The secoiridoids, found only in plants belonging to the family of *Olearaceae* that includes *Olea europaea* L., are characterised by the presence of elenolic acid (EA) in its glucosidic or aglyconic form in the molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), EA and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the EA glucoside (oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*). Secoiridoids of VOO in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the aqueous vegetative medium, and are more concentrated in the latter fraction because of their polar functional groups. During VOO storaged, hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols such as secoiridoids. The most abundant secoiridoids of VOO, identified for the first time by Montedoro G (1992), are the dialdehydic forms of EA linked to hydroxytyrosol or tyrosol (p-HPEA) respectively and termed 3,4-DHPEAEDA and *p*-HPEA-EDA, and an isomer of the oleuropein aglycon (3,4-DHPEA-EA). In 1999 another hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-DHPEA-AC) was identified in VOO (Bendini A, 2007).

### 1.11.f.ii. Phenolic acids

Phenolic acids are secondary aromatic plant metabolites that are widely distributed throughout the plant kingdom. These naturally occurring phenolic acids contains two distinguishing constitutive carbon frameworks, namely the hydroxycinnamic and hydroxybenzoic structures. Elucidation of their roles in plant life is only one of the many ongoing investigations into phenolic acids: a vast area of interest applied to food quality. Phenolic acids have been associated with colour and sensory

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



qualities, as well as with the health-related antioxidant properties of foods. One impetus for analytical investigations has been the role of phenolic compounds in the organoleptic properties of food (flavour, astringency, and texture). The content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives have been evaluated (Bendini A, 2007). Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables, against diseases that may be related to oxidative damage (CHD, stroke, and cancers). In particular, several phenolic acids such as gallic, protocatechuic, *p*- hydroxybenzoic, vanillic, caffeic, syringic, *p*- and *o*-coumaric, ferulic and cinnamic acid have been identified and quantified in VOO in concentrations <1 mg of analyte $kg^{-1}$ of olive oil. Carrasco P (2004) found that trans-cinnamic acid, sinapinic acid, caffeic acid and 3,4-dihydroxyphenylacetic acid were present in several monovarietal VOO of the six Spanish olive varieties analysed. Therefore, these compounds might be potential markers of geographical origin of the olive fruit (Bendini A, 2007).

**1.11.f.iii. Lignan**

(+)-Pinoresinol is a common component of the lignan fraction of several plants such as *Forsythia* species and *Sesamum indicum* seeds, whereas (+)-1-acetoxypinoresinol and (+)-1-hydroxypinoresinol and their respective glucosides have been detected in the bark of the olive tree (*Olea europaea L.*). According to Owen R (2000), the quantity of lignans in VOO may be up to 100 mg $kg^{-1}$ as simple phenols, and with considerable inter-oil variation. As suggested by Brenes M (2002), the amount of lignans may be used as a varietal marker. They reported a method to authenticate VOO produced by Picual olives based on the very low content of the lignan (+)-1-acetoxypinoresinol in these oils.

**1.11.f.iv. Hydroxyl-isochromans**

During the malaxation step of VOO extraction, hydrolytic processes through the activity of glycosidases and esterases augment the quantity of hydroxytyrosol and carbonylic compounds, thus favouring the presence of all compounds necessary for the formation of isochroman derivatives. Two hydroxy-isochromans, formed by

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



the reaction between hydroxytyrosol and benzaldehyde or vanillin, have been identified by HPLC-MS/MS technique and quantified in commercial VOO (Bendini A, 2007).

### 1.11.f.v. Flavonoids

As highlighted in section 1.11.c, flavonoids are widespread secondary plant metabolites. Over the past decade, an increasing number of publications have appeared on the health-benefit effects of flavonoids, such those related to cancer and CHD (Bendini A, 2007). Flavonoids are largely planar molecules and their structural variation derives, in part, from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 with an OH group at C-3, respectively. Several authors such as Carrasco-Pancorbo A (2006), Murkovic M (2004) and Morelló J (2005), have reported that flavonoids such as luteolin and apigenin are also phenolic components of VOO.

### 1.11.f.vi. Luteolin

Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. There are also several studies of note in which several flavonoids have been found in olive leaves as well as the fruits (Bendini A, 2007).

### 1.11.g. Health aspects linked to polyphenols in VOO

The nutritional value of VOO arises from high levels of oleic acid, and from minor components such as phytosterols, carotenoids, tocopherols and hydrophilic phenols (Pérez-Jimenez F, 2005). VOO contains at least 30 phenolic compounds of which the major ones are oleuropein derivatives based on hydroxytyrosol. These are strong antioxidants and RS. Recently there has been a surge in the number of publications that have investigated their biological properties.

Bisignano G (1999) found that hydroxytyrosol and oleuropein, which have antimicrobial activity against hydroxytyrosol, are likely to be bioavailable. Of note is

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



that acid hydrolysis of oleocanthal would produce elenolic acid, a dialdehyde compound even more similar to ibuprofen than oleocanthal itself (Beauchamp G, 2005).

In the context of the MedDiet and CHD, VOO rich in phenols increases the resistance of LDL to oxidation, *in vitro* and *ex vivo* (Bendini A, 2007). The study carried out by Bogani P (2007) confirmed the antithrombotic and antiinflammatory effects of VOO phenolic components, in a postprandial setting. The results showed significant reductions in serum concentration of inflammatory markers (Tromboxane B2 (TXB2) and Leukotriene B4 (LTB4)) at 2 hours and 6 hours post-consumption of VOO, but not after consumption of olive oil or corn oil. The effects of these different oils on *in vivo* indexes of oxidative stress (plasma antioxidant capacity and urinary hydrogen peroxide levels) were also evaluated. The results showed that the antioxidant activity of VOO phenolic compound post-ingestion increased plasma antioxidant capacity 2 hours after VOO consumption.

Foods containing high amounts of lignans, such as flaxseed, have been found to be protective against breast cancer and, specifically, to exert an anti-oestrogenic effect. This latter observation might be explained on the basis of the structural similarities between the lignans and the synthetic anti-oestrogen tamoxifen (Togna G, 2003).

Two hydroxyisochromans, 1-(3'-methoxy-4'-hydroxyphenyl)-6,7 dihydroxy-isochroman and 1-phenyl-6,7-dihydroxyisochroman, are formed by reaction (under mild conditions) between hydroxytyrosol and vanillin and benzaldehyde, respectively. They have only recently been identified in VOO and are active in inhibiting platelet aggregation and thromboxane release evoked by agonists (sodium arachidonate and collagen) that induce ROS-mediated platelet activation (Togna G, 2003).

As commented in a review by Covas M (2006), the precepts of evidence-based medicine require high-level scientific evidence to be provided before nutritional recommendations for the general public can be formulated. Scientific evidence required is provided by randomised, controlled, double-blind clinical trials (level I evidence) and, to some extent, by large cohort studies (level II evidence). Basic research, despite its usefulness in exploring a mechanistic approach, does not

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



provide evidence for nutritional recommendations. Finally, evidence is built on concordance of results from several similar studies.

In experimental studies, olive oil phenols have been shown to:

- have antioxidant effects, greater than those of vitamin E, on lipid and DNA oxidation
- prevent endothelial dysfunction by decreasing the expression of cell adhesion molecules, increasing NO production and inducible NO synthesis, and quenching vascular endothelium intracellular free radicals
- inhibit platelet-induced aggregation
- enhance mRNA transcription of the antioxidant enzyme glutathione peroxidase (GSH-Px) (Covas M, 2006).

Other potential activities of VOO phenolic compounds include chemopreventive activity. The anti-carcinogenic activity of phenols may be due not only to their antioxidant properties but also to their ability to reduce the bioavailability of food carcinogens, and to inhibit their metabolic activation (Bendini A, 2007).

### 1.12. Other food components for a healthy heart

#### 1.12.a. Antioxidant-rich diets

Diets rich in antioxidants, including plant flavonoids, can inhibit LDL oxidation and inhibit the formation of cell-cell adhesion factors which are implicated in damage to the arterial endothelium and in the formation of blood clots. However, their importance with respect to CVD remains to be established (ESC Guidelines, 2007).

#### 1.12.b. Folate and homocysteine

Evidence provides support for protecting vascular integrity through beneficial modulation of risk indicators such as plasma homocysteine concentrations and BP. Folate has the potential to reduce CVD risk by lowering the plasma level of homocysteine. Evidence supports its ability to reduce homocysteine levels but, to-date, its effectiveness in reducing CHD has not been confirmed in clinical trials (ESC Guidelines, 2007).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.12.c. Minerals

An increase in the intake of potassium and calcium and a reduction in sodium can help reduce BP (ESC Guidelines, 2007).

### 1.12.d. Peptides from milk protein

Consumption of certain fatty acids and peptides derived from milk proteins has also been reported to be beneficial (ESC Guidelines, 2007).

### 1.12.e. Plant sterol and stanol esters

Plant sterols are natural constituents of plants, including a number of common crops such as soya and maize. They play a similar role to that of cholesterol in animals i.e. as a metabolic precursor of several biologically active molecules such as hormones and, as well, as a cell structure molecule.

Early evidence, more than 50 years ago, indicated that plant sterols can interact with cholesterol in the intestinal tract to induce a reduction of cholesterol absorption and a subsequent reduction in blood cholesterol. More recently, a number of studies have confirmed the ability of plant sterols and stanols (their hydrogenated derivatives) to reduce LDL-c under a variety of conditions (Plana N, 2008).

They are present naturally in the diet at levels below. However, recent technological advances in the extraction and esterification of plant sterols, either as the sterols themselves or as stanols, enable them to be solublised in the matrix of food fat and, as such, to be easily incorporated into food products, at biologically effective levels (ESC Guidelines, 2007).

### 1.12.f. Soybean protein

Soybean protein reduces levels of LDL-c (ESC Guidelines, 2007).

### 1.12.g. Vitamin supplements

Intervention trials with vitamin supplements have failed to demonstrate any protection against CVD. Treatment with β-carotene, vitamin A, and vitamin E may even increase mortality risk. The potential roles of vitamin C and selenium on mortality warrant further investigation (Bjelakovic G, 2007).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.12.h. Dietary patterns

People do not eat isolated nutrients. Diets involve a complex combination of food items and nutrients that are likely to be interactive. The single nutrient approach may be inadequate to assess interactions (e.g. enhanced iron absorption in the presence of Vitamin C). High levels of correlations between some nutrients make it difficult to examine their separate effects. The effect of a single nutrient may be too small to be detectable, while the cumulative effects of multiple nutrients may be sufficiently large for effective measurement (Hu F, 2002).

Analysis of multiple isolated food items or nutrients may produce statistically sufficient association simply by chance. Single nutrient analysis may be confounded by the effect of other nutrients that are part of a dietary pattern. Adjustments may not remove all the confounding effects. In dietary pattern analysis, co-linearity of food items and nutrients could be used to advantage (Hu F, 2002).

Different patterns have been defined. In 2002, the Institute of Medicine (IOM) of the National Academies developed Acceptable Macronutrient Distribution Ranges (AMDRs) for total fat, n-6, and n-3, CHO, dietary fibre, and protein (IOM, 2002-2005). These guidelines were designed to move beyond ensuring mere adequacy i.e. determining an AI of these nutrients and to address the relationships of macronutrients to chronic disease in the context of adequate physical activity and energy expenditure required to maintain a healthy weight. In 2006, the AHA (Lichtenstein A, 2006), the American Diabetes Association (ADA) (Bantle J, 2006), and the American Cancer Society (ACS) (Kushi L, 2006) updated their diet and lifestyle recommendations with guidelines consistent with the IOM document. All of these organisations recommended a dietary pattern rich in vegetables and fruit and promoted the consumption of whole grains instead of refined grains. The AHA and ADA included specific targets for saturated fat and cholesterol. Against this background of health guidelines are popular diets with a limited evidence base of RCTs, several of which have shown a reduction in CVD risk factors, or short-term weight loss. The macronutrient content of some of these diets diverges markedly from guidelines, whereas others appear to be generally consistent with guidelines. These popular diets include very-low-fat vegetarian diets such as the Dean Ornish

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



diet; Mediterranean dietary patterns characterised by a higher intake of MUFAs; higher-protein, lower CHO diets that are low in fat such as the Zone diet; and higher protein, lower CHO diets that are more liberal with fat such as the South Beach and Atkins diets. The nutritional adequacy, long-term safety, sustainability, and impact of these popular diets on disease risk have not all been evaluated rigorously.

The Dietary Approaches to Stop Hypertension (DASH) eating pattern —a high-CHO diet rich in fruit, vegetables, and low-fat dairy foods— reduced BP and serum lipid risk factors in average- and high-risk subgroups (Sacks FM, 2001). The Optimal Macronutrient Intake Trial to Prevent Heart Disease (OmniHeart) extended the approach of the DASH diet to determine the effect of macronutrients on CVD risk factors. Substitution of 10% of energy from CHO with either protein or unsaturated fat enhanced the dietary effects on BP, and lipid risk factors, and increased the accuracy of estimation of CHD risk (Appel L, 2005 and de Souza R, 2008).

The NCEP ATP III guidelines advocate effective combinations of cholesterol-lowering dietary components. This approach termed “dietary portfolio” produces large reductions in serum cholesterol. Prompted by current dietary recommendations for the control of serum cholesterol and new targets to reduce the risk of CHD, together with the CHD risk reduction claims made for certain foods or food components, studies are now being undertaken using combinations of cholesterol-lowering foods in a single diet as a “dietary portfolio” rather than single foods to achieve more effective dietary control of serum cholesterol. Moreover, such a dietary portfolio reduced plasma inflammatory biomarkers as CRP (Jenkins D, 2007).

A dietary portfolio includes plant sterols at 2 g/d, high soy protein content (22.5 g/1000 Kcal), viscous fibres (10 g/1000 Kcal) and almonds (23 g/1000 Kcal) was evaluated by Jenkins D (2000, 2003, 2005). The ideal contribution of individual components remains to be established. However, in combination with other cholesterol-lowering foods, and against the background of a low-saturated fat diet, plant sterols contributed to over one third of the LDL-c reduction seen with the dietary portfolio after 1 year of this diet (Jenkins D, 2008). This approach has

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



increased the potential relevance of dietary therapy, and may yield nutrition strategies that bridge the gap between a good diet and drug therapy.

In conclusion, apart from micro- and macro-nutrients, dietary patterns that include fruit and vegetables, MUFA-rich oil (such as olive oil), and low fat dairy products have been associated with decreased incidence of adverse cardiovascular events (ESC Guidelines, 2007).

### 1.13. Methodological aspects

#### 1.13.a. Randomised, controlled trials (RCTs): execution and description

Well-designed and properly executed RCTs provide the best evidence on the efficacy of health-care interventions, while trials with inadequate methodology are associated with exaggerated treatment effects. Biased results from poorly-designed and inadequately-reported trials can mislead decision making in health-care provision at all levels, from treatment decisions for the individual patient to formulation of national public health policies. Critical appraisal of the quality of clinical trials is possible only if the design, conduct, and analysis of RCTs are thoroughly and accurately described in published articles. Far from being transparent, the reporting of RCTs is often incomplete, compounding problems arising from poor methodology.

Appropriate level of reporting of RCTs is necessary to enable accurate and critical appraisal of the validity and applicability of the results (Moher D, 2001).

The Consolidated Standards of Reporting Trials (CONSORT) Statement, published in 1996 and revised in 2001, is a set of guidelines designed to improve the reporting of RCTs. Use of these evidence-based guidelines is associated with improved quality of reporting of RCTs. The CONSORT statement has been extended to cover other designs such as non-inferiority and equivalence trials; types of interventions such as herbal therapies; and data such as the reporting of harms. However, despite the wide dissemination of the CONSORT statement, inadequate reporting remains common (Boutron I, 2008).

The CONSORT statement, a 22-item checklist and flow diagram, is intended to address this problem by improving the reporting of RCTs. However, some specific issues that apply to trials of non-pharmacologic treatments (e.g. surgery, technical

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



interventions, devices, rehabilitation, psychotherapy, and behavioural intervention) are not specifically addressed in the CONSORT statement. Also, considerable evidence suggests that the reporting of non-pharmacologic trials needs further improvement. Therefore, the CONSORT group developed an extension of the CONSORT statement for trials assessing non-pharmacologic treatments (Boutron I, 2008).

A consensus meeting of 33 experts was organised in Paris in February of 2006 to develop an extension of the CONSORT statement for trials of non-pharmacologic treatments. The participants in the meeting extended 11 items of the CONSORT statement, added 1 further item and developed a modified flow diagram.

To enable understanding and implementation of the CONSORT extension, the CONSORT group developed this elaboration (together with an explanatory document) from a review of the literature to provide examples of appropriate reporting. This extension, in conjunction with the main CONSORT statement and other CONSORT extensions, includes non-pharmacologic treatments such as surgery, technical procedures, devices, rehabilitation, psychotherapy, behavioural interventions, and complementary and alternative medicine which should help to improve the reporting of RCTs, as originally described by Boutron I (2008), and to facilitate the execution and description of CONSORT statement.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 16.** Checklist of items to include when reporting a randomised trial

| Paper Section and Topic | Item | Descriptor | Reported on Page |
|---|---|---|---|
| Title and abstract | 1 | How participants were allocated to interventions (e.g., “random allocation”, “randomised” or “randomly assigned”. | |
| Introduction Background | 2 | Scientific background and explanation of rationale. | |
| Methods - Participants | 3 | Eligibility criteria for participants and the settings and locations where the data were collected. | |
| - Interventions | 4 | Precise details of the interventions intented for each group and how and when they were actually administered. | |
| - Objectives | 5 | Specific objectives and hypothesis. | |
| Outcomes | 6 | Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors). | |
| - Sample size | 7 | How sample size was determined and, when aapplicable, explanation of any interim analyses and stopping rules. | |
| - Randomisation | | | |
| * Sequence generation | 8 | Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification). | |
| * Allocation concealment | 9 | Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned. | |
| *Implementation | 10 | Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups. | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 16.** Checklist of items to include when reporting a randomised trial (continued)

| Paper Section and Topic | Item | Descriptor | Reported on Page |
|---|---|---|---|
| - Blinding (masking) | 11 | Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. If done, how the success of blindig was evaluated. | |
| - Statistical methods | 12 | Statistical methods used to compare groups for primary outcome(s); methods for additional analyses, such as subgroup analyses and adjusted analyses | |
| Results | | | |
| - Participant flow | 13 | Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analysed for the primary outcome. Describe protocol deviations from study as planned, together with reasons. | |
| - Recruitment | 14 | Dates defining the periods of recruitment and follow-up. | |
| - Baseline data | 15 | Baseline demographic and clinical characteristics of each group. | |
| - Numbers analysed | 16 | Number of participants (denominator) in each group included in each analysis and whether the analysis was by “intention to treat”. State the results in absolute numbers when feasible (e.g., 10 of 20, not 50%). | |
| - Outcomes and estimation | 17 | For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval). | |
| - Ancilliary analyses | 18 | Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory. | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 16.** Checklist of items to include when reporting a randomised trial (continued)

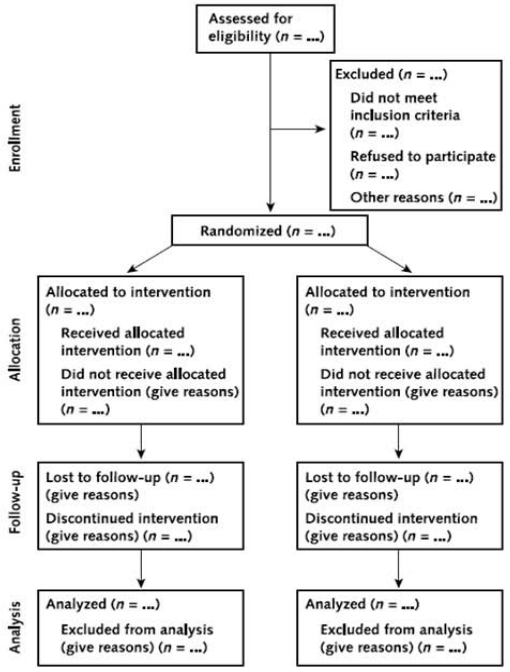
| Paper Section and Topic | Item | Descriptor | Reported on Page |
|---|---|---|---|
| - Adverse events | 19 | All important adverse events or side effects in each intervention group | |
| Discussion<br>- Interpretation | 20 | Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes. | |
| -Generalisability | 21 | Generalisability (external validity) of the trial findings. | |
| - Overall evidence | 22 | General interpretation of the results in the context of current evidence. | |

Source: CONSORT statement, Checklist-2001.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 6.** CONSORT diagram showing the flow of participants through each stage of a randomised trial.




Source: Altman D, 2001.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 17.** Checklist of items for reporting trials of non-pharmacologic treatments*

| Section | Item | Standard CONSORT description | Extension for non-pharmacologic trials |
|---|---|---|---|
| **Title and abstract** | 1 | How participants were allocated to interventions (e.g., "random allocation", "randomised" or "randomly assigned". | In the abstract, description of the experimental treatment, comparator, care providers, centres, and blinding status |
| **Introduction** | | Scientific background and | |
| Background | 2 | explanation of rationale. | |
| **Methods** | | | |
| Participants | 3 | Eligibility criteria for participants and the settings and locations where the data were collected. | When applicable, eligibility criteria for centres and those performing the interventions |
| Interventions | 4 | Precise details of the interventions intented for each group and how and | Precise details of both the experimental treatment and comparator |
| | 4A | when they were actually administered. | Description of the different components of the interventions and, when applicable, descriptions of the procedure for tailoring the interventions to individual participants |
| | 4B | | Details of how the interventions were standardised |
| | 4C | | Details of how adherence of care providers with the protocol was assessed or enhanced |
| Objectives | 5 | Specific objectives and hypothesis | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 17.** Checklist of items for reporting trials of non-pharmacologic treatments* (continued)

| Section | Item | Standard CONSORT description | Extension for non-pharmacologic trials |
|---|---|---|---|
| **Methods** | | | |
| Outcomes | 6 | Clearly defined primary and secondary outcomes measures and, when applicable, any methods to enhance the quality of measurements (e.g., multiple observations, training of assessors) | |
| Sample size | 7 | How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules | When applicable, details of whether and how the clustering by care providers or centers was addressed |
| Randomisation | | | |
| Sequence generation | 8 | Method used to generate the random allocation sequence, including details of any restriction (e.g., blocking, stratification) | When applicable, how care providers were allocated to each trial group |
| Allocation concealment | 9 | Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned | |
| Implementation | 10 | Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 17.** Checklist of items for reporting trials of non-pharmacologic treatments* (continued)

| Section | Item | Standard CONSORT description | Extension for non-pharmacologic trials |
|---|---|---|---|
| Blinding (masking) | 11A | Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment | Whether or not those administering co-interventions were blinded to group assignment |
| | 11B | | If blinded, method of blinding and description of the similarity of interventionist |
| Statistical methods | 12 | Statistical methods used to compare groups for primary outcomes(s); methods for additional analyses, such as subgroup analyses and adjusted analyses | When applicable, details of whether and how the clustering by care providers or centres was addressed |
| **Results** | | | |
| Participant flow | 13 | Flow of participants through each stage (a diagram is strongly recommended)- specifically, for each group, report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analysed for the primary outcome; describe protocol deviations from study as planned, together with reasons | The number of care providers or centers performing the intervention in each group and the number of patients treated by each care provider or in each centre |
| Implementing of intervention | New | | Details of the experimental treatment and comparator as they were implemented |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 17.** Checklist of items for reporting trials of non-pharmacologic treatments* (continued)

| Section | Item | Standard CONSORT description | Extension for non-pharmacologic trials |
|---|---|---|---|
| Recruitment | 14 | Dates defining the periods of recruitment and follo-up | When applicable, a description of care providers (case volume, qualification, expertise, etc.) and centres (volume) in each group |
| Baseline data | 15 | Baseline demographic and clinical characteristics of each group | |
| Numbers analyzed | 16 | Number of participants (denominator) in each group included in each analysis and whether analysis was by "intention-to-treat"; state the results in absolute numbers when feasible (e.g., 10/20, not 50%) | |
| Outcomes and estimations | 17 | For each primary and secondary outcome, a summary of results for each group and the estimated effect size and its precision (e.g., 95% confidence interval) | |
| Ancillary analyses | 18 | Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those prespecified and those exploratory | |
| Adverse Events | 19 | All important adverse events or side effects in each intervention group | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 17.** Checklist of items for reporting trials of non-pharmacologic treatments* (continued)

| Section | Item | Standard CONSORT description | Extension for non-pharmacologic trials |
|---|---|---|---|
| **Discussion** | | | |
| Interpretation | 20 | Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision, and the dangers associated with multiplicity of analyses and outcomes | In addition, take into account the choice of the comparator, lack of partial blinding, and unequal expertise of care providers or centres in each group |
| Generalisability | 21 | Generalisability (external validity) of the trial findings | Generalisability (external validity) of the trial findings according to the intervention, comparators, patients, and care providers and centres involved in the trial |
| Overall evidence | 22 | General interpretation of the results in the context of current evidence | |

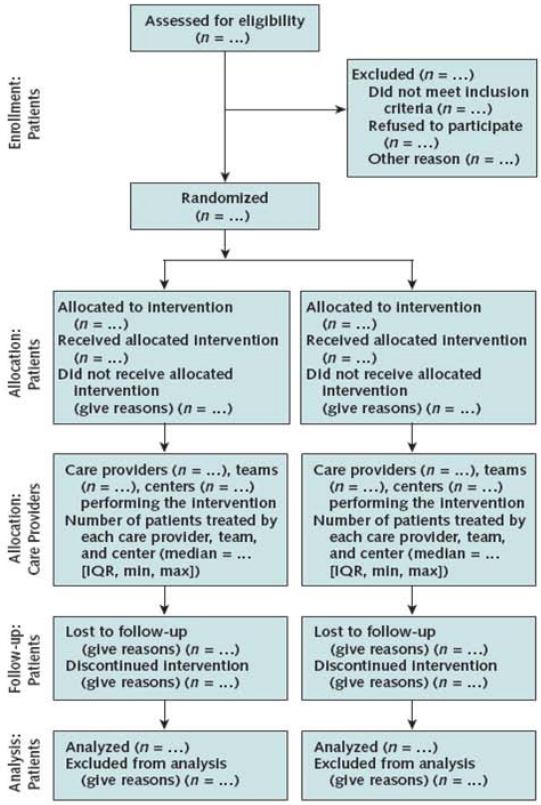
* Additions or modifications to the CONSORT statement Checklist-2001.

Source: Boutron I, 2008.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 7.** Modified CONSORT flow diagram for individual RCTs of non-pharmacologic treatment




An extra box per intention group relating to care providers has been added. For cluster RCTs, authors should refer the appropriate extension.

Abbreviations: IQR, interquartile range; max, maximum; min, minimum.

Source: Boutron I, 2008.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.13.b. Interventional control

According to criteria of the CONSORT statement, is necessary to demonstrate that the intervention treatment had a control arm.

#### 1.13.b.i. Details of the interventions

Precise details are needed of the interventions intended for each group together with how and when they were actually administered. In addition, non-pharmacologic trials require precise details of the experimental treatment and the comparator.

#### 1.13.b.ii. Details of the different components of the interventions

Descriptions of the different components of the interventions are needed and, when applicable, description of the procedure for tailoring the interventions to individual participants.

It is important to provide a detailed description of non-pharmacologic treatments since these are usually complex interventions involving several components, each of which may influence the estimation of the treatment effect. All the different components of the treatment procedure must be reported. These descriptions will help introduce the safest and most effective treatments into clinical practice. They are also necessary to facilitate study comparison, reproducibility, and inclusion in systematic reviews (Herbert R, 2005).

In non-pharmacologic trials, the control treatment can be placebo, usual-care, an active treatment, or a waiting list. If the control treatment is usual-care, all the components received by the control group must be reported. This information will allow readers to compare the intensity of usual-care with the experimental intervention and with what is usually provided to participants in their own setting.

Interventions in non-pharmacologic trials are often poorly described. The information that is required for a complete description of non-pharmacologic treatments depends on the type of intervention being tested.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 1.13.b.iii. Standardisation of the interventions

Assessments of non-pharmacologic treatments in RCTs present special difficulties because of the complexity of the treatment and the variability found across care providers and centres (Feldon S, 2003). The variety of settings that characterise multi-centre trials only exacerbates these problems (Herbert R, 2005).

Any method used to standardise the intervention across centres or practitioners need to be described. In pragmatic trials (i.e. trials attempting to show whether an intervention works under the usual conditions in which it will be applied), standardisation might consist of simply informing care providers to perform the treatment as they usually do.

In efficacy trials (i.e. trials aimed at determining whether an intervention works when administered under ideal circumstances), standardisation is likely to be more stringent with, for example, a requirement for a certification process (Feldon S, 2003).

The description of any standardisation methods is essential to enable replication of the non-pharmacologic treatment. Interested readers must be allowed access to the materials used to standardise the interventions, either by including a Web appendix with the article or a link to a stable Web site. Such materials include written manuals, specific guidelines, and materials used to train care-providers to uniformly deliver the intervention.

### 1.13.b.iv. Adherence of care providers to the protocol: assessment and enhancement

Assessing treatment adherence is essential in appraising the feasibility and reproducibility of the intervention in clinical practice. The use of any adherence-improving strategies must be reported and such strategies should enhance treatment adherence and influence the treatment effect. These methods and strategies need to be clear in order to accurately transpose the results of the trial into clinical practice and to appraise the applicability of the trial's results (Davidson K, 2003).

UNIVERSITAT ROVIRA I VIRGILI
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# HYPOTHESIS AND OBJECTIVES

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Nutritional strategies have been developed to reduce risk factors of CVD. The best evidence on the efficacy of CVD dietary interventions have been provided by RTCs. Currently, nutritional intervention studies need to conform to the quality criteria defined by the CONSORT statement, and this can be facilitated by the application of computer technology.

The identification of some bioactive food components such as polyphenols in bio-fluids (including plasma and urine), cells or tissues can highlight their nutritional effects on risk factors or biomarkers of CVD. The detection of polyphenols in human plasma is methodologically complex, and needed to be refined.

Thus, we propose two hypotheses:

**Hypothesis 1:**

Some dietary components such as cocoa cream products which include various ingredients (hazelnuts, phytosterols and soluble fibre), or soluble fibre treatment such as Po-husk, incorporated in a low saturated fat and low cholesterol diet can contribute to the control of plasma LDL-c and to provide new targets to reduce CVD biomarkers.

**Hypothesis 2:**

The nutritional and technical methodology improves VOO polyphenol identification in human plasma.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Objectives:**

1. To asses the effects of cocoa cream products on plasma lipids, BP and others CVD biomarkers
    a. To evaluate the effect on biomarkers of oxidative stress
    b. To evaluate the effect on biomarkers of inflammation activity
    c. To evaluate the effect on biomarkers of antithrombotic activity
    d. To evaluate the effect on biomarkers of metabolic syndrome
    e. To evaluate the possible variations in effects of the different cocoa cream products in relation to the study population sample characteristics including LDL-c, gender and BMI

2. To determine the effects of soluble fibre (Po-husk) on LDL-c and other biomarkers of CVD risk
    a. To assess the capacity of combined treatment with Po-husk and statins to achieve the cholesterol-lowering objective
    b. To analyse the effect of Po-husk on BP
    c. To study the effect of the combination of statins with Po-husk on BP
    d. To assess whether the plasma lipid response is modulated by the polymorphisms of candidate genes
    e. To evaluate the effect on biomarkers of oxidative stress
    f. To evaluate the effect on biomarkers of inflammation activity
    g. To evaluate the effect on biomarkers of antithrombotic activity
    h. To evaluate the effect on biomarkers of insulin resistance
    i. To evaluate possible variations in effects of the soluble fibre and the statin in relation to the study population sample characteristics including LDL-c, gender and BMI.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



3. To detect and quantify the olive oil polyphenols in human plasma
    a. To determine the phenolic profile in fasting human plasma from habitual VOO consumers, using the fasting plasma of non-consumers as control
    b. To assess whether the phenolic compounds with the highest LDL antioxidant activities were identifiable in plasma following an oral load of VOO
    c. To assess the phenolic profile in fasting human plasma to develop and compare two metodologies to extract phenolic compounds from plasma samples.

4. To optimise the methodological process to facilitate the implementing of CONSORT criteria in interventional studies

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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UNIVERSITAT ROVIRA I VIRGILI
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# MATERIAL AND METHODS

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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### 3.1. Diet design

Each part of the interventional study had a specific diet designed to assess the main objective in the trial.

There were two types of diets, one was the stabilisation diet and the other was the intervention diet.

The energy requirement of each participant was calculated with Harris-Benedict formula (Harris J, 1918) and the result was adjusted for a correction factor since this formula overestimates the energy requirement. We took into account the physical activity which was estimated via an interview, or the metabolic equivalent unit (METs) test.

#### 3.1.a. Cocoa cream products and soluble fibre studies

Both studies were designed to have stabilisation and interventional diets.

In the stabilisation period of 2 weeks duration, the nutrient intake target was the average Mediterranean diet, as consumed in the Catalan population (*Generalitat de Catalunya*, 2003); 39% of total energy as fat and, of which, 13% was a SFA.

In the intervention period, the diet prescribed was 35% of total energy as fat of which <7% was SFA, 50% of total energy as CHO, 15% of total energy as protein and <200 mg/d of cholesterol. This diet conformed to the consensus recommendations of the several expert committees for the prevention of CVD. In the cocoa study the duration was 4 weeks and in fibre study the duration was 8 weeks.

#### 3.1.b. Olive oil polyphenols detection study

The day before the postprandial test, a diet free of polyphenols was prescribed to assess whether the olive oil polyphenols could be detected in fasting plasma samples.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.1.c. Monitoring of diets

All the diets were designed to include the nutrient-of-interest. For example, the cocoa study, when designing the diet, the nutritional composition of cocoa was included as any other basic food of diet such as bread, vegetables or milk; not as a supplement. The diets were isocaloric and the energy intake was adjusted to maintain stable bodyweight.

Three-day food records at the end of each period were used to monitor dietary compliance, and a 24h dietary recall every 2 weeks in the intervention period to assess adherence to the recommended diet. The participants were weighed in indoor clothing without shoes using calibrated scales and wll-mounted stadiometer (Añó sayol S.A., Huelva, Spain) and, if the weight varied by >1 Kg, the caloric intake was modified to maintain basal bodyweight.

The nutrient composition of the diet was calculated using the *Répertoire Géneral Des Aliments* (1995).

## 3.2. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers CVD

The study was a controlled, investigator-blind, parallel, multi-centred study in which the 4 different cocoa cream products were introduced into a calorie-balanced diet for 4 weeks with a prior stabilisation period of 2 weeks in which all participants received the cocoa cream product.

The trial was conducted in one clinical centre (*Hospital Universitari Sant Joan de Reus*) and 3 peripheral coordinated Primary-Care Centres.

The study was approved by the Clinical Research Ethical Committee (CREC) of the *Hospital Universitari Sant Joan de Reus*. The study protocol was in accordance with the Declaration of Helsinki and good clinical practice guidelines. The protocol was fully explained to the participants and all provided written consent to participation at the time of enrolment.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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### 3.2.a. Study participants

Between April and December 2005, eligible participants were recruited in Reus (coordinating centre), Alcover, Vic and Centelles (3 surrounding villages). The participants were men and women >20 years of age with LDL-c ≥ 3.35 mmol/L (130 mg/dL) and ≤ 4.88 mmol/L (189 mg/dL), and at least one of the following major CVD risk factors:

- Age, > 45 years in men and > 55 years in women
- Current smoker
- Hypertension or on antihypertensive treatment
- HDL-c ≤ 1.03 mmol/L (40 mg/dL) in men and ≤ 1.18 mmol/L (46 mg/dL) in women
- Family history of premature CVD (in male and female first-degree relative < 55 and 65 years of age, respectively)

The exclusion criteria were:

- LDL ≥ 4.88 mmol/L (189 mg/dL)
- TG > 3.97 mmol/L (350 mg/dL) (threshold that permits LDL-c to be calculated from the Friedewald formula)
- Chronic alcoholism
- BMI > 35 Kg/$m^2$
- History of CVD
- Treatment with statins prior to the start of the trial and having dropped-out of treatment at least 2 months before the beginning of the study
- Diabetes mellitus (at least 2 fasting hyperglycaemic values > 126 mg/dL)
- Renal disease (plasma creatinine levels > 133 µmol/L (1.5 mg/dL) for men and > 124 µmol/L (1.4 mg/dL) for women
- Acute infectious diseases, neoplasms, severe liver failure, chronic respiratory failure, or associated endocrine diseases
- Other conditions, such as special nutritional requirements or medications that may affect lipid metabolism or BP

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



- Participation in a clinical trial in the previous 3 months
- Incapacity to understand and to follow the study objectives and requirements
- Allergy to chocolate or nuts
- History of gastrointestinal disease that may alter the absorption of nutrients
- Depression syndrome or self-injuring proclivities

There was no monetary inducement to participation in the trial.

**3.2.b. Flow of participants through the study**

Eligibility for participation was ascertained in the initial screening visit. Those who met the inclusion criteria at the baseline visit were randomised into two dietary sequences. All participants began a stabilisation diet which included 6 doses of cocoa cream (total of 465 Kcal/d) over 2 weeks. This was to assess the subject's cocoa tolerance. The participants were then randomly assigned to one of 4 different cocoa cream products (465 Kcal/d).

Randomised assignment was with a computer-generated random number sequence, in gender-stratified blocks of 4 persons each. This was the responsibility of the Barcelona Randomisation Unit, which played no further part in the study.

Allocation was concealed in sealed folders which were held in a central, secure location until informed consent had been obtained. The dietician who scheduled the study visits did not have access to the randomisation list. The laboratory personnel responsible for the analyses were blinded with respect to patient-group assignment.

The participants attended the outpatient clinic where a full clinical history was recorded and anthropometrical measurements made. To increase adherence to the dietary requirements of the study, each individual received expert dietary counselling from the dietician at the start of the stabilisation period and twice during the intervention period. Participants were encouraged to maintain their habitual physical activity.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.2.c. Cocoa cream products

Based on the published literature, 4 different cocoa cream products were designed. Each cream was used as a control for the others. Table 18 summarises the nutritional composition and the diet components. Table 19 summarises the fatty acids ratios of each cocoa cream product used in the trial.

A low saturated fat calorie-balanced diet included 6 units (13g/unit, 465 Kcal/d) of product A cocoa (approximately 1g per unit); product B, cocoa plus hazelnut (30g/d hazelnuts; 5 g in each unit); product C, cocoa, hazelnuts plus phytosterols (2 g/d); and product D, cocoa, hazelnuts, phytosterols and soluble fibre (20g/d). This last was named “Portfolio”

All the different cocoa cream products were manufactured by *La Morella Nuts* (Reus, Spain) specifically for the trial.

The compositions of the creams were provided by the manufacturers and the nutrient values were calculated from the USDA food composition tables (Table 18).

To blind the participants with respect to the type of cream being consumed, the four creams were especially manufactured to present the same texture and visual characteristics.

The individual doses were consumed during the day as snacks or as additions at mealtimes. The only stipulation was that the creams were not to be consumed in conjunction with milk or other dairy products; the purpose being not inhibit the polyphenol cocoa effects and to preclude these food items being confounding variables in the subsequent analyses.

Compliance was monitored by empty wrapper counting and any non-consumed doses were collected at follow-up visits. We defined non-compliance as a consumption of <80% of the creams.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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**Table 18.** Nutritional composition of each cream dose (13 g)[a]

| Content per dose | Product A | Product B | Product C | Product D |
|---|---|---|---|---|
| Energy, Kcal | 77 | 77 | 73 | 73 |
| Carbohydrate, g | 6.6 | 5.6 | 5.4 | 4.5 |
| Protein, g | 0.2 | 0.9 | 0.8 | 0.9 |
| Total fat, g | 6.0 | 6.1 | 5.8 | 6.1 |
| Saturated fat, g | 1.2 | 1.3 | 1.2 | 1.4 |
| Stearic fatty acid, g | 0.3 | 0.6 | 0.6 | 0.5 |
| Monounsaturated, g | 3.0 | 3.6 | 3.0 | 3.4 |
| Polyunsaturated, g | 1.4 | 0.9 | 1.3 | 1.0 |
| n-6, g | 3.0 | 1.3 | 1.3 | 1.0 |
| Fiber, g | 0.3 | 0.7 | 0.7 | 2.7 |
| Vitamin E, mg | 2.0 | 1.3 | 1.3 | 1.3 |

Abbreviation: Kcal, kilocalories; Product A, cocoa cream; Product B, cocoa plus hazelnut s cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fiber cream; code name “Portfolio”.

[a]Nutrient composition were calculed from data provided by the manufacturers and with USDA food composition tables.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 19.** Fatty acids ratios of each cocoa cream product.

| | Ratio | Product A | Product B | Product C | Product D |
|---|---|---|---|---|---|
| Portfolio recommendations (Jenkins D, 2003) | | | | | |
| SFA /USFA | 1:3.5 | 1:3.5 | 1:3.32 | 1:3.44 | 1:3.22 |
| MUFA / PUFA | 1:1 | 1:2.06 | 1:0.42 | 1:0.46 | 1:0.40 |
| SFA / MUFA | 1:2 | 1:1.14 | 1:2.33 | 1:2.35 | 1:2.48 |
| SFA / PUFA | 1:1.5 | 1:2.35 | 1:0.98 | 1:1.09 | 1:0.73 |
| SEA recommendations, 2005 | | | | | |
| SFA / USFA | 1:4 | 1:3.5 | 1:3.32 | 1:3.44 | 1:3.22 |
| MUFA/ PUFA | 3:1 | 3:6.18 | 3:1.26 | 3:1.39 | 3:0.88 |

Abbreviations: SFA, saturated fatty acids; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; Product A, cocoa cream; Product B, cocoa plus hazelnut s cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fiber cream; code name “Portfolio”.

### 3.2.d. Clinical and laboratory measurements

Trained personnel measured weight and height using calibrated scales and well-mounted stadiometer, respectively. WC was measured midway between the lowest rib and the iliac crest using an anthropometric tape. Bodyweight was measured every 2 weeks during the study.

BP was measured at each screening visit, during the stabilisation period as well as before and after each intervention period. At least 2 measurements were made at least 1-2 minutes apart and additional measurements were taken if the first two were substantially different. The subjects were seated and the BP measurements were made after at least 5 minutes of repose. BP was determined using the OMRON HEM-907 (Peroxfarma, Barcelona, Spain).

Blood for biochemical analyses was drawn from each patient following an overnight fast. To reduce intra-individual day-to-day variability, the blood sampling was performed on two separate days at the end of the stabilisation period and at the

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



end of the intervention periods. Samples of serum and plasma were stored at -80ºC for batched analyses. Technical aspects of biomarker measurements are described in section 3.7.

In a subgroup of participants, endothelial function was measured as microvascular reactivity using fingertip tonometry (Peripheral Arterial Tonometry (PAT); Hamar Medical, Caesarea, Israel). Endo-Pat was a non-invasive and reproducible tool to identify individuals with microvascular endothelial dysfunction. It was based on a measure of volume changes in the fingertip during reactive hyperaemia. A BP cuff was placed on the upper arm while the contra-lateral arm was used as control. PAT probes were placed on one finger of each hand for continuous recording of the PAT signal. After 5 min, the cuff was inflated to supra-systolic pressure and maintained for 5 minutes. The cuff was deflated while PAT recording was continued for 5 min. The Endo-PAT index was calculated as the ratio of the digital pulse volume during reactive hyperaemia divided by that at baseline. An Endo-PAT index of <1.6 Arbitrary Units (AU) was indicative of endothelial dysfunction.

### 3.2.e. Diagram of visits

See Table 20.

### 3.2.f. Sample size calculation

Sample size calculation was based on being able to detect differences in the change (Δ) LDL between the treatment group with the biggest weighted reduction (product C) and the group with product A (the control). Results from previous studies (Volpe R, 2001) were used to obtain the desired value of ΔLDL in phytosterols product.

The outcome effect was to detect (ΔLDL= 12.75 mg/dL) i.e. the equivalent of a reduction of 8.5% LDL-c in patients with basal values of 150 mg/dL LDL-c.

The working assumption is the following:

H0: ΔLDL sterols - ΔLDL control = 0

H1: ΔLDL sterols - ΔLDL= 12.75 mg/dL

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



To achieve a statistical power of 80% and α value of 0.05 with a standard deviation of ΔLDL of 21.5 mg/dL, the sample size needed to detect significant differences of 12.75 mg/dL between the group control and the group with product C, was for 25 participants per group. Adjusting for up to 10% loss to the study, it was necessary to recruit 27 participants for each treatment group i.e. 108 participants in all.

**Table 20.** Study evaluations diagram

| Variable / Day or Week | W-2 | D-3 | D0 | W2 | D25 | W4 |
|---|---|---|---|---|---|---|
| Informed consent | X | | | | | |
| Medical history | X | | X | | | X |
| Examination | X | | X | | | X |
| Weight | X | | X | | | X |
| Height | X | | | | | |
| BMI | X | | X | | | X |
| BP | X | | X | | | X |
| Physical activity | X | | X | | | X |
| Lipid profile | | X | X | | X | X |
| Biochemistry | | | X | | | X |
| Haemogram | | | X | | | X |
| CVD risk biomarkers | | | X | | | X |
| SNPs | | | X | | | |
| Delivery of dietary record | X | | X | X | | |
| Collection of dietary record | | | X | | | X |
| 24 h dietary recall | | | | X | | |
| Delivery of creams | X | | X | X | | |
| Collection of creams | | | X | | | X |
| Collection of AE data | | | X | | | X |

Abbreviation: D, day; W, week; BMI, body mass index; BP, blood pressure; SNPs, single nucleotide polymorphisms; AE, adverse effect

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.3. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD

The study was a phase IV-II, multi-centred, randomised, double-blind, placebo-controlled, parallel trial. The protocol was designed to evaluate, firstly, the effects of Po-husk (14 g/d) for 8 weeks compared to placebo and, secondly, to assess the intra-group effect of the combined action of 20 mg/d co-adjuvant treatment with a statin (simvastatin) in individuals who were non-responders.

The trial was conducted in clinical centres in Spain, France and Holland.

The study was approved by the CRECs of all participating centres as well as the AEMPS (Spanish Medicines Agency). The study protocol was in accordance with the Declaration of Helsinki and good clinical practice guidelines. The study protocol was fully explained to the participants and all provided written consent to participation at the time of enrolment.

#### 3.3.a. Study participants

Between September 2005 and June 2007, eligible participants were recruited in Reus (coordinating centre), Málaga, San Vicente de Raspeig, Petrer, Alcover, Vic, Centelles, Benidorm (from Spain), París (France) and Rotterdam (Holland). The participants were men and women > 20 and <65 years of age, with LDL-c ≥ 3.35 mmol/L and ≤ 4.88 mmol/L, and at least one other of the following major CVD risk factors:

- Age > 45 years in men and > 55 years in women
- Smoker
- High BP
- HDL-c ≤ 1.03 mmol/L (40 mg/dL) in men and ≤ 1.18 mmol/L (46 mg/dL) in women
- Family background of premature heart disease

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The exclusion criteria were:

- LDL-c ≥ 4.88 mmol/L (189 mg/dL)
- TG 3.97 mmol/L (350 mg/dL) (threshold that permits LDL-c to be calculated using the Friedewald formula)
- Chronic alcoholism
- BMI > 35 Kg/$m^2$
- History of CVD
- Treatment with statins prior to the beginning of the trial and who have not dropped-out at least 2 months before the beginning of the present study
- Diabetes mellitus (at least 2 fasting hyperglycaemia levels, > 126 mg/dL)
- Renal disease (plasma creatinine levels > 133 µmol/L (1.5 mg/dL) for men and > 124 µmol/L (1.4 mg/dL) for women
- Acute infectious diseases, neoplasms, severe liver failure, chronic respiratory failure or associated endocrine diseases
- Other conditions, such as special nutritional requirements or medications that may affect lipid metabolism or BP
- Participating or having participated in a clinical trial in the previous 3 months
- Incapacity to understand the objectives and to adhere to the study requirements
- Allergy to *Plantago ovata*
- History of gastrointestinal disease that may alter the absorption of nutrients
- Depression syndrome or self-injuring proclivities

There was no monetary inducement to participate in the trial.

### 3.3.b. Flow of participants through the study

The list of potential candidates was obtained from computer-based records of patients who attended each participating centre. Eligibility or exclusion was assessed by the attending physician based on review of clinical records followed by a screening visit.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



After 2 weeks of stabilisation, screened patients were randomised to receive Po-husk (Plantaben®, Madaus S.A., Barcelona, Spain) treatment (14g/d) or placebo (14g/d) for 8 weeks. Blinding was maintained using matching placebo sachets which did not differ from the active fibre with respect to colour, appearance, or any other physical characteristic.

The randomisation code was computer generated and participant assignment to treatment or placebo arm was at a ratio of 1:1. The number sequence for the subject, centre, and treatment assignment were allocated via an interactive electronic response system. This was the responsibility of the Barcelona Randomisation Unit, which played no further part in the study.

Participants were encouraged to maintain their habitual physical activity.

### 3.3.c. Soluble fibre and statin

Po-husk was manufactured as a palatable, orange-flavoured, sugar-free product, distributed in 4 sachets of 5 g each (70% soluble fibre). The dosage was one sachet dissolved in 200 mL of water, taken 15 minutes before breakfast and lunch and 2 sachets before the evening meal. A 20 mg/d simvastatin tablet (Cinfa Laboratorios, Spain) was taken 2 hours after the last meal at night.

Treatment compliance was monitored by counting the unopened sachets and/or simvastatin tablets remaining in the blister pack returned at follow-up visits. Consumption > 80% was considered acceptable.

### 3.3.d. Clinical and laboratory measurements

Patients attended the outpatient clinic where a full medical history was taken and physical examination made. Each subject received expert dietary counselling from the dietician to reinforce the dietary requirements of the study. Patients attended the clinic every 4 weeks post-randomisation.

Participants' height and weight were measured in indoor clothing without shoes by trained personnel using calibrated scales and well-mounted stadiometer, respectively. Bodyweight was measured every 2 weeks during the study.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



BP was measured 3 times at 1-minute intervals using an Omron automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain) and mean taken. The measurements were performed with the participant seated and relaxed.

Fasting blood sample was taken every 8 weeks. The standard clinical chemistry analyses and haemogram were performed in each of the participating centres. All lipids and other biomarkers were measured centrally at *Hospital Universitari Sant Joan de Reus*. Samples were stored at -80ºC in the central laboratory and batch-analysed to minimise variability in measurements. Technical aspects of the biomarker measurements are described in section 3.7.

### 3.3.e. Diagram of visits

See Table 21.

### 3.3.f. Sample size calculation

The sample size calculation was based on the assumption of a reduction of 0.19 mmol/L LDL-c with treatment. This was equivalent to a reduction of 5% in the LDL-c levels in patients with LDL-c baseline values of 3.88 mmol/L. With 112 valid patients per group, the study had 80% statistical power to detect 0.19 mmol/L differences in ΔLDL between the group treated with Po-husk and the group receiving placebo, with a SD of 20.0 mg/dL and a two-sided type I error protection level of 5%. Although 270 patients were sufficient to cope with a 15% drop-out rate, the study planned to include 290 patients to guarantee the follow-up of into the extension (simvastatin) period of those patients not achieving the therapeutic objective with Po-husk.

## 3.4. Cocoa and soluble fibre adverse effects

Adverse events were coded according to the MedDra dictionary (version 8.0) and categorised by body system and preferred term.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 21.** Participant evaluations during the study

| Variable / Day or week | W-2 | D-3 | D0 | W2 | W4 | W6 | W7 | W8 | W10 | W12 | W14 | W15 | W16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Informed consent | X | | | | | | | | | | | | |
| Clinical history | X | | X | | X | | | X | | X | | | X |
| Physical examination | X | | X | | X | | | X | | X | | | X |
| Weight, BMI | X | | X | X | X | X | | X | X | X | X | | X |
| Height | X | | | | | | | | | | | | |
| BP | X | | X | | X | | | X | | X | | | X |
| Physical activity | X | | X | | | | | X | | | | | X |
| Lipid profile | | X | X | | | | X | X | | | | X | X |
| Biochemistry, Haemogram | | | X | | | | | X | | | | | X |
| CVD risk biomarkers | | | X | | | | | X | | | | | X |
| SNPs | | | X | | | | | | | | | | |
| Delivery of dietary record | X | | | X | | X | | | X | | X | | |
| Collection of dietary record | | | X | | X | | | X | | X | | | X |
| 24 h dietary recall | | | | X | | X | | | X | | X | | |
| Delivery of medication | | | X | | X | | | X | | X | | | |
| Collection of medication | | | | | X | | | X | | X | | | X |
| Collection of AEs | | | | | X | | | X | | X | | | X |

Abbreviation: D, day; W, week; BMI, body mass index; BP, blood pressure; SNPs, single nucleotide polymorphisms; AEs, adverse effects

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.5. Interventional control

To assess the intervention monitoring, the criteria of the CONSORT statement were applied to the two nutrition intervention studies done (cocoa cream products and Po-husk studies).

#### 3.5.a. Cocoa cream products study

#### 3.5.a.i. Details of the interventions

This study was a non-pharmacologic trial and the details of the experimental treatment and comparator were specified.

From the start of the run-in period, the participants consumed the recommended diet and, for the intervention period, a low-saturated-fat and low-cholesterol diet under strictly-controlled conditions in which compliance was assessed by a research nutritionist.

All participants began a run-in dietary period of 2 weeks and which included 6 doses of cocoa cream (465 Kcal/d) in order to assess the subject's tolerance to the product.

The participants were then randomly assigned to cocoa cream (product A) or cocoa plus hazelnuts cream (product B) or cocoa plus hazelnuts and phytosterols cream (product C) or cocoa plus hazelnuts, plus phytosterols and soluble fibre cream (product D).

The effects of products B, C and D were compared against product A.

#### 3.5.a.ii. Details of the different components of the interventions

The detailed characteristics of the study are described so as to facilitate the tailoring of the interventions to the individual participants.

#### 3.5.a.ii.1. Diets

For each participant, an isocaloric diet was calculated using the Harris-Benedict formula with a correction factor and activity physical questionnaire.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



During the run-in period, the percentage of SFA in the diet was between 10 to 15% and, in the intervention period, the SFA set at ≤ 7% within an isocaloric diet.

Diet compliance was monitored using 3-day dietary records and interviews at the end of each period. Between the 2 clinical visits, diet compliance was monitored by an interview with the dietician using a 24h dietary recall to reinforce the dietary requirements of the study.

At each visit, participants were weighed without shoes and, if weight varied by > 1 Kg, the energy intake was modified accordingly.

The participants were encouraged to maintain their usual physical activity.

**3.5.a.ii.2. Cocoa cream products**

The participants were randomly assigned one or other of 4 cocoa cream products (A, B, C and D) and the effects of each cocoa cream product were compared with cocoa cream (the control) product A.

Adverse events were coded according to the MedDra dictionary (version 8.0) and categorised by body system and preferred term.

**3.5.a.iii. Standardisation of the interventions**

The nutritionists underwent training for standardisation for this particular study. All dietary records were reviewed by 2 nutritionists in order to reduce inter-observers variation.

The nutrient composition of the diet was calculated with *Répertoire Géneral Des Aliments* database, and the cocoa cream products from the USDA food composition tables.

**3.5.a.iv. Adherence of care providers to the procol: assessment and enhancement**

**3.5.a.iv.1. Diets**

If SFA content exceeded 10% of total energy, the diet was adjusted to a lower percentage.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



#### 3.5.a.iv.2. Cocoa cream products

Compliance was monitored by empty wrapper counting and any non-consumed doses were collected at follow-up visits. Compliance was defined as a product consumption >80%.

### 3.5.b. Po-husk study

#### 3.5.b.i. Details of the interventions

This study was a non-pharmacologic trial and the details of the experimental treatment and comparator were provided.

From the start of the run-in period, the participants consumed the recommended diet and, for the intervention period, a low-saturated-fat and low-cholesterol diet was consumed under strictly-controlled conditions in which compliance was assessed by a research nutritionist.

The experimental treatment was 14 g/d of Po-husk and the comparator was 14 g/d of placebo (microcrystalline cellulose) added to a low-saturated-fat and low-cholesterol diet for 8 weeks. Subsequently, if LDL-c remained > 3.35 mmol/L, participants continued with treatment plus 20 mg/d of statin added for a further 8 weeks.

#### 3.5.b.ii. Details of the different components of the interventions

The characteristics of the study are provided so as to describe the procedure for tailoring the interventions to the individual participants.

#### 3.5.b.ii.1. Diets

For each participant, an isocaloric diet was calculated using the Harris-Benedict formula and applying a correction, and with a METs physical activity test.

During the run-in period, the percentage SFA in the diet was between 10 to 15% and, in the intervention period, the SFA set at ≤ 7% within an isocaloric diet.

At each of 4 clinical visits, diet compliance was monitored using 3-day dietary records and interviews with dietician to reinforce the dietary requirements of the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



study. Further, in the additional weeks of the intervention period, the participants were interviewed and invited to fill-in a 24h dietary recall.

At each visit, participants were weighed without shoes and, if weight varied by > 1 Kg, the energy intake was modified accordingly.

The participants were encouraged to maintain their usual physical activity.

**3.5.b.ii.2. Treatments**

After a 2 week run-in period, screened patients were randomised to receive 14 g/d of Po-husk (Plantaben®, Madaus S.A., Barcelona, Spain) treatment or 14 g/d of placebo for 8 weeks.

Subsequently, at week 8, those individuals who did not achieve plasma LDL-c levels ≤ 3.35 mmol/L, had 20 mg/d simvastatin prescribed, and the combined action of co-adjuvant treatments was assessed up to week 16.

Adverse events were coded according to the MedDra dictionary (version 8.0) and categorised by body system and preferred term.

**3.5.b.iii. Standardisation of the interventions**

The nutritionists underwent standardisation training and all dietary records were revised by 2 nutritionists in order to reduce observer variation.

The nutrient composition of the diet was calculated with *Répertoire Géneral Des Aliments* database.

**3.5.b.iv. Adherence of care providers to the procol: assessment and enhancement**

**3.5.b.iv.1. Diets**

If SFA content exceeded 10% of total energy, the diet was modified to lower the percentage.

**3.5.b.iv.2. Treatments**

Treatment compliance was monitored by counting the unopened sachets and/or pills in the blister package returned at follow-up visits. Consumption >80% was considered acceptable.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.6. Olive oil polyphenols detection in human plasma study

This project was divided into different phases, and each phase was essential to the succeeding one. Based on the design the project can be described as 3 subprojects:

- Olive oil polyphenol detection in plasma from habitual VOO consumers
- Olive oil polyphenols detection in postprandial plasma
- Methodological validation to detect and quantify olive oil polyphenols in plasma

The future step will be to design a functional olive oil with the polyphenols detected *in vitro* with major antioxidant activity and to conduct an interventional study with hypertensive subjects to demonstrate the functional benefit of the olive oil on BP in hypertensive populations.

The project is currently underway in collaboration with the group of Motilva M at the *Universitat de Lleida* (UdL).

### 3.6.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers

The study was cross-sectional and the participants were invited to describe their usual diet and to provide blood sample in which differences in the phenolic profiles were evaluated between habitual VOO consumers and non-consumers.

The study design was approved by the CREC of *Hospital Universitari Sant Joan de Reus*. The study protocol was fully explained to the patients and they gave their written informed consent on enrolment.

#### 3.6.a.i. Study participants

Eligible participants were recruited in Reus and people came from Catalonia and Rumanian nationalities. The participants were males and females > 20 years of age, ostensibly healthy, and fulfilled one of the two inclusion criteria:

- a habitual long-term consumer of VOO
- a non-consumer of olive oil

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The exclusion criteria were:

- Consumer group: not consume more than 23 g/d regularly over the past 10 years
- Non-consumer group: use olive oil over the past 10 years
- LDL-c ≥ 4.88 mmol/L (189 mg/dL)
- TG > 3.97 mmol/L (350 mg/dL) (threshold that permits LDL-c to be calculated using the Friedewald formula)
- Alcohol abuse
- History of CVD
- High BP
- Diabetes mellitus (at least 2 fasting hyperglycaemic measurements > 126 mg/dL)
- Renal disease (plasma creatinine levels > 133 μmol/L (1.5 mg/dL) for men and > 124 μmol/L (1.4 mg/dL) for women
- Acute infectious diseases, neoplasms, severe liver failure, chronic respiratory failure or associated endocrine diseases
- Other conditions such as special nutritional requirements or medications that may affect lipid metabolism or BP
- Current participation or participation in a clinical trial in the past 3 months
- History of gastrointestinal disease that may alter the absorption of nutrients
- Depression syndrome or self-injuring proclivity
- Current use of prescription medications and/or vitamins

The group of non-consumers of olive oil were the Rumanian people who are strongly averse to olive oil consumption because of organoleptic as well as cultural issues.

**3.6.a.ii. Clinical and laboratory measurements**

Trained personnel measured weight and height using calibrated scales and well-mounted stadiometer, respectively.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Following an overnight (12 hours) fast, blood samples were collected in Vacutainer® tubes containing EDTA as anticoagulant. The samples were light-protected by being enveloped in aluminium foil, centrifuged for 15 minutes at 1500 xg and at 4° C (Kokusan, H-103RS, Japan). The plasma was immediately separated from cells and stored at –80 °C until analysis.

Technical aspects of biomarker measurements are described in section 3.7. The methodology for the detection of VOO polyphenols in human plasma was performed in collaboration with the UdL group and is described briefly (below).

**3.6.a.ii.1. Olive oil polyphenols detection and quantification in human plasma**

The first step was to obtain phenolic extracts of fasting human plasma using a solid-phase extraction procedure and the method of Ruiz-Gutierrez V (2000) with some modifications incorporated by the UdL group. The next phase was identify and quantify the phenolic fraction using high pressure liquid chromatography (HPLC) based on equipment for Waters Inc. (Milford, USA) which comprised a Waters 600 E pump, Waters 717 plus auto sampler (loop 20µL) and Waters 996 photodiode array detector (PAD). Finally, to tentatively identify the phenol metabolites in plasma, the mass spectrum analysis was applied to the chromatographic peaks obtained with a TQD™ mass spectrometer (Waters, Milford, MA, USA) using the electrospray ionisation (ESI) source (Z-spray™). All these procedures were performed by the UdL group.

**3.6.a.iii. Diagram of visits**

See Table 22.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 22.** Study evaluations

| Variable / Visit | V1 | V2 |
|---|---|---|
| Informed consent | X | |
| Clinical history | X | |
| Physical examination | X | |
| Weight | X | |
| Height | X | |
| BMI | X | |
| Lipid profile | X | |
| Biochemistry | X | |
| Haemogram | X | |
| VOO polyphenols | X | |
| Delivery of dietary record | X | |
| Collection of dietary record | | X |

Abbreviation: V, visit; BMI, body mass index; VOO, virgin olive oil

### 3.6.b. Olive oil polyphenols detection in postprandial human plasma

Figure 8 summarises the different phases in the detection of postprandial plasma VOO polyphenols; collaboration between the *Unitat de Recerca en Lípids i Arteriosclerosis* (URLA) of the *Universitat Rovira i Virgili* (URV) and the UdL groups.

The bioavailability of the polyphenols of VOO in human plasma was assessed in fasting plasma. Samples were obtained at several postprandial time-points. After blood sample processing, the URV group were to study the effects of polyphenols on clinical biomarkers, and the UdL group to analyse the levels and distributions of the polyphenols in plasma, using HPLC analyses. In this phase, URV group recruited the participants and provide the blood samples to UdL group.

The study was approved by the CREC of *Hospital Universitari Sant Joan de Reus*. All participants provided written informed consent prior to enrolment.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



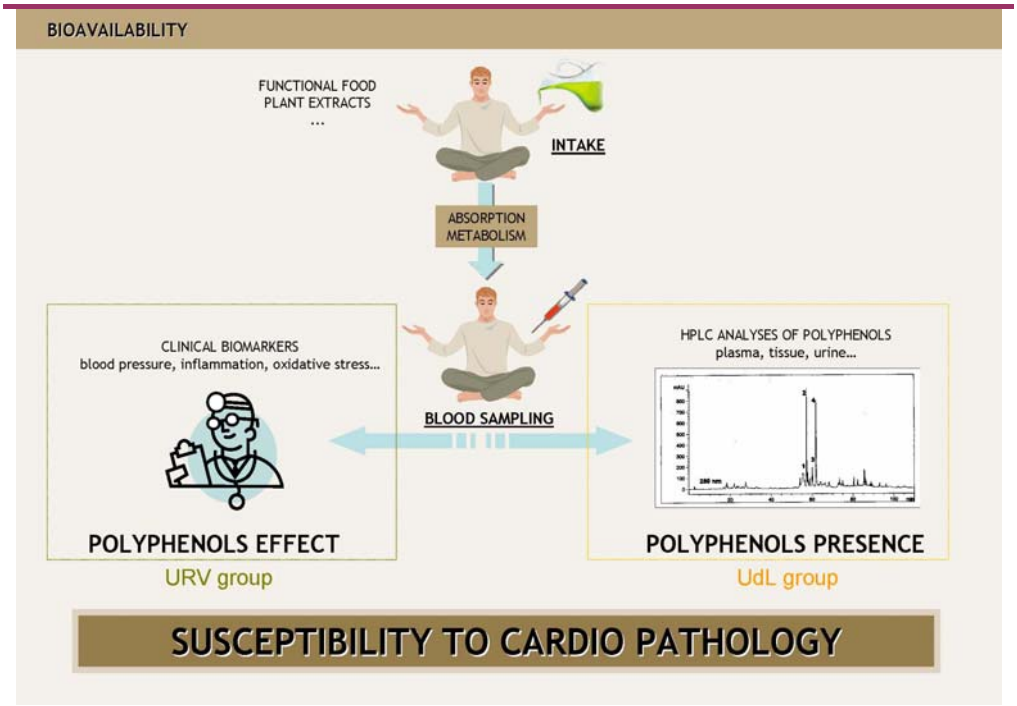



**Figure 8.** Descripcion of the study of bioavailability of phenolic compounds

Source: Romeu M and Valls RM.

### 3.6.b.i. Study participants and VOO load

Two healthy individuals ingested a single 40 mL dose of VOO spread on 60 g of bread. The phenolic compound profile in postprandial plasma taken from these individuals was determined at 0, 30, 60, 120 and 240 minutes following the VOO ingestion.

The VOO samples, from the variety of Arbequina olive, were obtained from an olive oil mill in Catalonia during the harvest season. The selected oil had a high level of phenolic compounds; a concentration of total phenols of around 300 mg/Kg. The total phenolic content of the oils was determined using the Folin-Ciocalteau method by spectrophotometry at 725 nm and were conducted in UdL group.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.6.b.ii. Clinical and laboratory measurements

Trained personnel measured weight and height using calibrated scales and well-mounted stadiometer, respectively.

Plasma samples were obtained by venipuncture after the ingestion of 40 mL of VOO. Blood samples were collected at basal conditions (following a 12 hours overnight fast, and a polyphenol-free diet the day before) and at 30, 60, 120 and 240 minutes post-consumption of the VOO. Blood was recollected in Vacutainer™ tubes containing EDTA as anticoagulant. They were protected from the light with aluminium foil, centrifuged for 15 minutes at 1500 *g* and at 4 ºC (Kokusan, H-103RS, Japan). The plasma was immediately separated from the cells and stored at –80 ◦C until analysis.

The process of detection and quantitation of VOO polyphenols in human plasma was as described in section 3.6.a.ii.1, and performed by the UdL group.

### 3.6.b.iii. Diagram of visits

**Table 23.** Study evaluations

| Visit / Variable | V1 | V2 | | | | |
|---|---|---|---|---|---|---|
| | | T BASAL | T 30 | T 60 | T 120 | T 240 |
| Informed consent | X | | | | | |
| Clinical history | X | | | | | |
| Physical examination | X | | | | | |
| Weight | X | | | | | |
| Height | X | | | | | |
| BMI | X | | | | | |
| VOO polyphenols | | X | X | X | X | X |
| Delivery of dietary record | X | | | | | |
| Collection of dietary record | | | | | X | |

Abbreviation: T, minutes; BMI, body mass index; VOO, virgin olive oil.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.6.c. Validation method to detect and quantify olive oil polyphenols in human plasma

The UdL group created an olive oil enriched with some polyphenols that had been demonstrated to have major antioxidant activity *in vitro*. The olive oil was given to healthy participants who followed the same protocol as that of the VOO administered to the two healthy volunteers (section 3.6.b.i.).

Plasma samples were obtained by venipuncture following the ingestion of 30 mL of polyphenol-enriched olive oil. Blood samples were collected at basal conditions (after 12 hours overnight fast, and a polyphenol-free diet the day before) and 60 and 120 minutes post-consumption of olive oil. Blood (50 mL) was collected in Vacutainer™ tubes containing EDTA as anticoagulant. They were protected from light with aluminium foil, centrifuged for 15 min at 1500 *g* and at 4 ºC (Kokusan, H-103RS, Japan) The plasma was immediately separated from the cells and stored at –80 ºC until analysis.

The human study was approved by the CREC at the *Hospital Universitari Sant Joan de Reus*.

The validation method, as conducted by the UdL group is described in more detail the article “Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectrometry” (Suarez M, 2009 (submitted for publication).

### 3.7. Biomarkers

All lipids and others biomarkers were measured centrally at *Hospital Universitari Sant Joan de Reus* (Catalonia, Spain) and in the URLA from URV. The blood samples had been stored at -80 ºC in the central laboratory’s Biobanc (bancmb@grupsagessa.com) and thawed just prior to analysis to minimise variability in measurements.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.7.a. Lipid profile

TC, TG, VLDL, HDLD-c, Apo A-1 and Apo B-100 were performed using standard methods on an autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

LDL-c was calculated by means of the Friedewald formula (Friedewald W, 1972)

LDL-c = CT – (HDL-c + TG/5) mg/dL

LDL-c = CT – (HDL-c + TG/2.21) mmol/L

### 3.7.b. Oxidative stress

OxLDL in EDTA plasma was measured by immunoassay (Mercodia AB, Uppsala, Sweden).

### 3.7.c. Endothelial dysfunction

VCAM-1 and ICAM-1 were determined by ELISA kits in plasma (R&D Systems, Minneapolis, USA).

### 3.7.d. Inflammatory activity

Leucocyte distributions were measured by routine methods on by autoanalyzer in EDTA plasma (Beckman Coulter-Synchron, Galway, Ireland).

hsCRP was determined by autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

IL-6 was analyzed by immunoassay systems by autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

### 3.7.e. Antithrombotic activity

PAI-1 was measured by antigen ELISA kit in citrate plasma (Technoclone GmbH, Vienna, Austria).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Folic acid concentrations were measured by routine methods on by autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

### 3.7.f. Insulin resistance

Glucose was determined by standard methods on an autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

Ultransensitive insuline was measured by immunoassay system on an autoanalyzer in serum (Beckman Coulter-Synchron, Galway, Ireland).

Homeostasis model assessment method (HOMA-Index) was calculated for each participant (Matthews D, 1985).

Homa-index = [Fasting insulin (μU/mL) x casting glucose (mmol/L)] / 22.5

Homa-index = [Fasting insulin (μU/mL) x casting glucose (mg/dL)] / 405

### 3.7.g. Metabolic syndrome

Fatty acid binding protein 4 (FABP-4), adipocyte was determined by ELISA kits in EDTA plasma (BioVendor Laboratory Medicine Inc., Brno, Czech Republic).

### 3.7.h. Gene polymorphisms

DNA for gene polymorphism studies was obtained from each subject using the cell package commercial kit (Servicios Hospitalarios, Spain). A region which contains 13 genes: apo E (APOE), angiotensin converting enzyme (ACE), PAI-1, fibrinogen, apo A-V (APO A-V), cholesterol ester transfer protein (CETP), fatty-acid-binding-protein (FABP)-2, CRABP-2, FABP-4, ABCG5, ABCG8, Apo A-1 (APO A-1) and PPARα genes) was amplified by PCR, and 14 single nucleotide polymorphisms (SNP), analysed using enzyme restriction according to Iplex Gold Sequenom technology (coordinacion.cegen@upf.edu).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.7.i. Biomarkers evaluated in each study

Each study was designed with specific objectives and the biomarkers evaluated differed according to these goals. Table 24 summarises the biomarkers evaluated in the separate studies.

Cocoa cream products study: TC, TG, HDLD-c, LDL-c, Apo A-1, Apo B-100, Apo B/Apo A, oxLDL, hsCRP, IL-6, VCAM-1, ICAM-1, PAI-1 and FABP-4

Soluble fibre study: TC, TG, HDLD-c, LDL-c, Apo A-1, Apo B-100, Apo B/Apo A, oxLDL, leucocytes, hsCRP, IL-6, VCAM-1, ICAM-1, PAI-1, folic acid, glucose, ultrasensitive insulin, HOMA-index and gene polymorphisms

VOO polyphenols bioavailability study: TC, TG, VLDL, HDLD-c, LDL-c–and VOO polyphenols detection in human plasma.

**Table 24.** Biomarkers evaluated in each study

| | Cocoa cream products | Soluble fibre (Po-husk) | VOO polyphenols |
|---|---|---|---|
| **Lipid profile:** | | | |
| TC | ●●● | ●●● | ●●● |
| LDL-c | ●●● | ●●● | ●●● |
| HDL-c | ●●● | ●●● | ●●● |
| VLDL-c | | | ●●● |
| TG | ●●● | ●●● | ●●● |
| Apo A-1 | ●●● | ●●● | |
| Apo B-100 | ●●● | ●●● | |
| Apo B/Apo A | ●●● | ●●● | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 24.** Biomarkers evaluated in each study (continued)

| | Cocoa cream products | Soluble fibre (Po-husk) | VOO polyphenols |
|---|---|---|---|
| **Oxidative stress:** | | | |
| oxLDL | ●●● | ●●● | |
| **Endothelial dysfunction:** | | | |
| VCAM-1 | ●●● | ●●● | |
| ICAM-1 | ●●● | ●●● | |
| **Inflammation activity:** | | | |
| IL-6 | ●●● | ●●● | |
| hsCRP | ●●● | ●●● | |
| Leucocytes | | ●●● | |
| **Antithrombotic activity:** | | | |
| PAI-1 | ●●● | ●●● | |
| Folic acid | | ●●● | |
| **Insulin resistance:** | | | |
| Glucose | | ●●● | |
| Insulin | | ●●● | |
| HOMA-index | | ●●● | |
| **Metabolic syndrome:** | | | |
| FABP-4 | ●●● | | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 24.** Biomarkers evaluated in each study (continued)

| | Cocoa cream products | Soluble fibre (Po-husk) | VOO polyphenols |
|---|---|---|---|
| **Gene polymorphisms:** | | | |
| SNPs | | ●●● | |
| **VOO polyphenols:** | | | |
| Detection in plasma | | | ●●● |

### 3.8. Statistical analysis

The statistical analyses were performed by the Laboratory of Biostatistics & Epidemiology from *Universitat Autònoma de Barcelona* and by the Statistics & Methodology Support (USEM; Barcelona) for the cocoa cream products and the soluble fibre (Po-husk) studies.

Initially, 3 subsets of subjects in each study were defined:

- The Intention to Treat (ITT) population was defined as all randomised subjects who satisfied the entry criteria, had a baseline efficacy measurement, and at least one corresponding post-baseline efficacy measurement.
- The Per Protocol (PP) population was defined as all randomised subjects who satisfied the entry criteria, had a baseline efficacy measurement, had at least one corresponding post-baseline efficacy measurement, and did not present any major violations of the protocol.
- The Safety population was defined as all randomised subjects who took at least one dose of the study medication.

The sample size for each study was calculated separately.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.8.a. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD

The subsets were analysed according to the time and treatment groups involved:

a) analysis performed on the data from 4 weeks of on-treatment period. The comparisons were between placebo (cocoa cream) and treatment creams (cocoa plus hazelnut, phytosterols and portfolio cream).

b) analysis performed on the complete follow-up data i.e. from baseline to week 4 and comparisons between the different cocoa creams.

Efficacy subsets were defined as:

- ITT-W4: all ITT subjects
- PP-W4: all PP subjects

The main efficacy analysis was performed using the ITT subjects for the primary and secondary end-points.

Results were expressed as mean ± Standard Deviation (SD), baseline adjusted least square means (95% confidence intervals; 95%CI), median (inter-quartile range; $25^{th}$ percentile, $75^{th}$ percentile) or frequencies and percentages (%).

The continuous efficacy variables were analysed by ANCOVA (ANalysis of COVAriance) including treatment and baseline in the model. Non-Gaussian continuous variables were analysed using rank transformation (non-parametric ANCOVA).

Categorical variables were analysed using the Fisher exact test.

Last Observation Carried Forward (LOCF) approach was used for missing values and the Available Data Only (ADO) approach was used for sensitivity analysis purposes.

The primary analysis was performed with the ITT subset and repeated with the PP to test the robustness of the results. The sensitivity analyses performed by the ADO approach and the PP population gave very similar results.

The efficacy outcomes analyses were carried out with the absolute values and with the absolute difference from baseline. The analysis of efficacy was performed

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



using the ADO approach. For LDL-c, the analysis was also performed with LOCF approach. Finally, only descriptive analyses were applied to the percentage of LDL-c reduction. Secondary efficacy laboratory variables presenting higher or lower values from set cut-off limits were analysed using a non-parametric approach. Suitable test of hypotheses were applied to the rest of the variable according to the nature of each variable: Fisher exact test for categorical variables, Student's *t*-Test for continuous variables, and Mann-Whitney U test for ordinal scale variables.

Two-sided $p< 0.05$ significance level was applied to all statistical analyses.

Descriptive statistics were performed for each variable per treatment group. No inferential statistics were performed.

All statistical analyses were with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

### 3.8.b. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD

The subset analyses were according to the time and treatment groups involved:

a) Analysis performed using the first 8 weeks of on-treatment period. The comparisons were between placebo and fibre groups.

b) Analysis performed using the complete follow-up data i.e. from baseline to week 16. This included the subgroup of individuals who were non-responders to placebo or fibre at week 8.

Efficacy subsets were defined as:

- ITT-W8: all ITT subjects.
- ITT-W16: ITT subjects not reaching the therapeutic objective (LDL-c <3.35 mmol/L at week 8).
- PP-W8: all PP subjects.
- PP-W16: PP subjects not reaching the therapeutic objective (LDL-c < 3.35 mmol/L at week 8).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Results were expressed as mean ± SD, baseline adjusted least square mean (95% CI), median (inter-quartile range; 25$^{th}$ percentile, 75$^{th}$ percentile) or frequencies and percentages (%).

The continuous efficacy variables were analysed by ANCOVA including treatment and baseline values in the model. Non-Gaussian continuous variables were analysed by applying a rank transformation (non-parametric ANCOVA).

Categorical variables were analysed using the Fisher exact test.

LOCF approach was used for missing values and the ADO approach was used for sensitivity purposes.

The primary analysis was performed with the ITT subset and repeated with the PP subset to test the robustness of the results. The sensitivity analyses performed by the ADO approach and the PP population gave very similar results. Data from the non-responder subgroup were analysed at week 16 using intra-group comparisons for exploratory purposes only, as planned in the protocol.

The analyses of efficacy outcomes were performed with the absolute values and with the absolute difference from baseline. The analysis of efficacy was performed using the ADO approach. For LDL-c, the analysis was also performed with LOCF approach. Finally, descriptive analysis alone was applied to the percentage of LDL-c reduction. Secondary efficacy laboratory variables presenting higher or lower values from set cut-off limits were analysed by means of a non-parametric approach. For the rest of variables, a suitable hypothesis test was applied according to the nature of each variable: Fisher exact test for categorical variables, Student's T-Test for continuous variables and Mann-Whitney U test for ordinal scale variables.

Two-sided $p< 0.05$ significance level was applied to all statistical analyses.

Descriptive statistics, but no inferential tests, for each variable per treatment group were performed.

All analyses were with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 3.8.c. Olive oil polyphenols detection in human plasma study

The study subset analysis was not applicable to the study design.

### 3.8.c.i. Olive oil polyphenols detection in human plasma from habitual VOO consumers

Data are expressed as the mean ± standard error of the mean (SEMs). Consumer and non-consumer groups were assumed to be random samples drawn from a normally-distributed population. An analysis of variance was performed using the General Linear Models (GLM) procedure ($\alpha = 0.05$) to compare the concentrations of phenols among consumers and non-consumers. The variances of the two groups were similar. The effect of factors such as age, gender and olive oil consumption, together with their interactions, were tested against the concentrations of the phenol compounds. If two factors interacted, a Least Square Means (LSMEANS) analysis was carried out. TUKEY's multiple range tests were performed to determine significant differences between treatments.

All statistical analyses were performed with the Statistical Package for Social Science software package (SPSS version 15.0).

### 3.8.c.ii. Olive oil polyphenols detection in human postprandial plasma

Results are expressed as mean ± SEM.

Statistical significance was set at $p<0.05$. All statistical analyses were performed with the Statistical Package for the Social Science (SPSS version 15.0).

### 3.9. Computerised systems application in interventional studies

The informatic's application to design intervention nutritional studies is an ambitious concept that hopes to provide advice and technological help to the scientific community and food companies when designing and developing new intervention studies, or in following-up of the tasks entrusted to a research group. Currently we are laying the foundations for this application.

For example, we have designed a structural and conceptual software application to facilitate and standardise the provision of information, and to create a useful tool that better organises and speeds-up the management of data. "Sortink" is an online

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



application that assists the handling of all kinds of information that may be required in managing projects. This "virtual project manager" can simultaneously administer 50 active projects with 10 gigabytes of memory available to store closed project data. There are an unlimited number of users for the project and access is secure via Secure Sockets Layer (SSL).

The setting language used is Ruby on Rail (RoR) following the Model-Vista-Control (MVC) standards and using, as well, multi-thread structured query language (MySQL).

The main characteristics of the software's application are summarised in Table 25.

**Table 25.** Characteristics of Reus- Informatics of Nutritional Intervention Studies (REUS-INIS)

| |
|---|
| Data can be easily classified in sections |
| Different types of permissions and restrictions for users can be set |
| Specific tools for a collaborative framework are available including e-mail and calendar |
| Powerful bibliography manager tool |
| Easy online data storage: all kind of information charts, data, images, can be up- as well as down-loaded |

Sortink's design allows creating predefined structures of folders where documents can be easily stored. Therefore, the structure of the CONSORT statement items can be easily adapted to Sortink. The result is REUS-INIS, a specific template especially designed to follow the CONSORT guidelines.

Following the CONSORT statement, the main structure of the program has been designed to help edit a new project with respect to requirements of clinical trials as well as to the recommendations for non-pharmacologic treatment interventions.

REUS-INIS has been designed to offer facilities in a standardised manner for authors to prepare reports of trial findings, to facilitate complete and transparent reporting, and to aid critical appraisal and interpretation.

Additionally, with the objective of better follow-up and control of information, the program incorporates a system of document searching. When the user uploads any information on the project, the program solicits keywords related to the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



document. With the help of the built-in keywords, the computer application creates a database and finds any document related to the project.

### 3.10. Financial support

#### 3.10.a. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD

This project was supported by PROFIT schedule from Ministry of commerce, industry and tourism and CDTI schedule from Ministry of education and science; 2002-2005, Spain.

#### 3.10.b. Effects of treatment with soluble fibre (*Plantago ovata* husk) on biomarkers of CVD

This work was supported by MADAUS S.A., the Center of innovation and business development (CIDEM) from *Generalitat de Catalunya* and Ministry of industry, Spain.

#### 3.10.c. Olive oil polyphenols detection in human plasma study

This grant was financed by the National plan for scientific research, development and technological innovation from Ministry of education and science (AGL2005-07881-CO2-02/ALI), Spain.

#### 3.10.d. Computerised applications for interventional studies

This project was supported by CENIT schedule from Minsitry of education and innovation (MET-DEV-FUN 1321 U07 E10), Spain.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

# RESULTS

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.1. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD

#### 4.1.a. Participants

There were 283 participants who were considered eligible for the study. The Figure 9 described the flow of participants through the study. The 113 remaining participants were randomised in ITT analysis. The 46 men and 67 women completed the two dietary periods of the study. Finally, 11 participants were excluded from the PP analysis. The 4 cocoa cream product groups did not differ with respect to baseline characteristics (Table 26). The results at 4 weeks of the intervention period, from anthropometric measures, lipid profile, CVD biomarkers and dietary adherence were showed at Tables 27, 28, 29 and 30.

**Table 26.** Baseline characteristics of study participants

| | Product A (n=28) | Product B (n=28) | Product C (n=30) | Product D (n=27) | Total (n=113) |
|---|---|---|---|---|---|
| Age, years | 49.79±9.53 | 56.79±10.46 | 56.03±10.06 | 53.33±8.42 | 54.03±9.93 |
| BMI, Kg/m$^2$ | 28.31±3.25 | 27.30±3.01 | 28.53±3.22 | 28.28±3.56 | 28.12±3.25 |
| Gender, male | 11 (39.3%) | 12 (42.9%) | 13 (43.3%) | 10 (37.0%) | 46 (40.7%) |
| CV risk factor: | | | | | |
| Age | 16 (57.1%) | 23 (82.1%) | 22 (73.3%) | 20 (74.1%) | 81 (71.7%) |
| Smoker | 8 (28.6%) | 8 (28.6%) | 7 (23.3%) | 5 (18.5%) | 28 (24.8%) |
| High BP | 12 (42.9%) | 8 (28.6%) | 13 (43.3%) | 11 (40.7%) | 44 (38.9%) |
| HDL-c* | 0 (0.0%) | 1 (3.6%) | 2 (6.7%) | 4 (14.8%) | 7 (6.2%) |
| F-HD$^+$ | 6 (21.4%) | 2 (7.1%) | 3 (10.0%) | 4 (14.8%) | 15 (13.3%) |

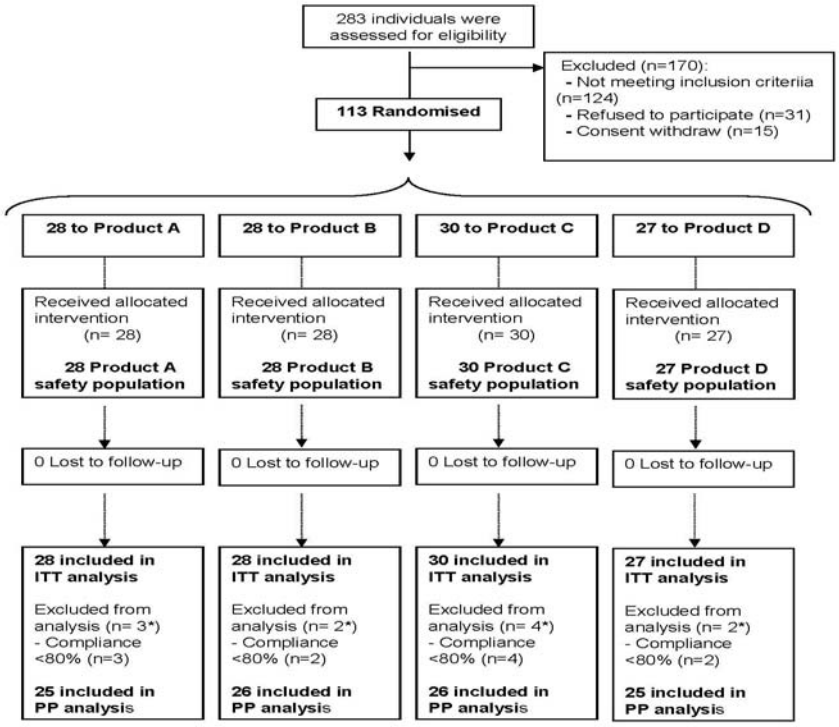
Results are expressed as means ± SD or frequencies (%)

Abbreviations: Product A, cocoa cream alone; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; BMI, body mass index, calculated as weight in kilograms divided by height in meters squared; CV, cardiovascular; HDL-c, high density lipoprotein cholesterol, * <1.0 mmol/L (40 mg/dL) and <1.18 mmol/L (46 mg/dL) for men and women, respectively; F-HD, family history of premature heart disease, $^+$ in male <55 years, in female <65 years in first-degree relative.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 9.** Flow diagram of participants




Abbreviations: Product A, cocoa alone cream; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phyitosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; ITT, intention to treat; PP, per protocol.

### 4.1.b. Dietary adherence and consumption of cocoa cream products

Participants consumed the Mediterranean-type diet (as consumed in Catalonia) up to the start of the intervention period (Table 30). During the intervention period, the 4 groups consumed approximately 10% of energy as SFA. Compared with the control, the Product B had higher intake of MUFA (11.7%) ($P$=0.0079). There were significant differences in intake of PUFA between the Product B (-29.8%), C (-36.2%) and Portfolio (-30.3%), compared with control cream ($P$=0.000, respectively). Portfolio group showed a higher intake of dietary fibre, compared with the control cream (33.4%) and the product B and C ($P$<0.0001) (Table 30).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The difference observed with respect to dietary fibre intake can be explained by the fibre supplement in the product D.

At 94%, compliance with the 4 different cocoa cream products was high. The compliance to the intervention diet was 98%.

#### 4.1.c. Lipid profile

After 4 weeks, product C compared with control reduced LDL-c by -11.8% and Apo B/Apo A ratio by -1.2%. Portfolio decreased LDL-c by -10.1% and Apo B/Apo A ratio by -10.5% (*P*=0.01, respectively) (Table 28).

#### 4.1.d. Anthropometric and clinical measures

After 4 weeks of the intervention period, no changes in bodyweight and WC measurements were observed (Table 27).

Compared with control, product B reduced SBP by -4.9 mm Hg (95%CI: -9.88 to 0.06) and DBP by -3.38 (95%CI: -6.57 to -0.19; *P*= 0.05) and Portfolio decreased DBP by -3.89 mm Hg (95%CI: -7.15 to -0.63; *P*=0.05) (Table 27).

#### 4.1.e. CVD biomarkers

Compared with control, Portfolio reduced hsCRP by 0.9 mg/L (95%CI: -0.02 to 2.03; *P* =0.0083) and oxLDL by -4.0 U/L (95%CI: 0.50 to 7.5; *P*=0.02) (Table 29).

#### 4.1.e.i. Endo-PAT subgroup

From the participants that consumed the control product, a subgroup of participants (n=14) were tested for endothelial function pre (1.800)- and 2-week post-intervention (2.173) AU using the Endo-PAT system. We observed that the consumption of cocoa improved the vasodilator response, as measured by fingertip tonometry by increasing 0.373 AU [-0.702 to -0.044] (*P*=0.0293).

#### 4.1.f. Adverse events

To assess cocoa tolerance, all participants consumed the cocoa cream during the stabilisation period and none of them reported any adverse effects. During the

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



intervention period, two participants reported a bloating feeling and one reported poor appetite. None of participants received any medications during the study.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 27.** Anthropometrics and clinical measures

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* |
|---|---|---|---|---|---|---|---|
| Weight, Kg | A | 77.32±10.83 | 0.2366 [-0.2755 to 0.7487] | 0.3% | | | |
| | B | 72.13±10.88 | 0.0083 [-0.5109 to 0.5275] | 0.0% | 0.2282 [-0.5059 to 0.9623] | -0.3% | 0.5386 |
| | C | 73.55±10.55 | -0.0048 [-0.4837 to 0.4741] | 0.0% | 0.2413 [-0.4612 to 0.9439] | -0.3% | 0.4969 |
| | D | 73.43±12.23 | -0.4014 [-0.9416 to 0.1389] | -0.5% | 0.638 [-0.108 to 1.3839] | -0.9% | 0.0928 |
| BMI, Kg/m$^2$ | A | 28.31±3.255 | 0.0823 [-0.0956 to 0.2602] | 0.3% | | | |
| | B | 27.30±3.016 | -0.0224 [-0.2056 to 0.1608] | -0.1% | 0.1047 [-0.151 to 0.3604] | -0.4% | 0.4183 |
| | C | 28.53±3.223 | 0.000 [-0.1684 to 0.1684] | 0.0% | 0.0823 [-0.1625 to 0.3271] | -0.3% | 0.5061 |
| | D | 28.28±3.568 | -0.1514 [-0.3412 to 0.0385] | -0.5% | 0.2336 [-0.0264 to 0.4937] | -0.8% | 0.0777 |
| WC, cm | A | 95.52±8.138 | -0.5794 [-1.7762 to 0.6174] | -0.6% | | | |
| | B | 93.66±7.871 | -1.8995 [-3.0963 to -0.7028] | -2.0% | 1.3202 [-0.3748 to 3.0151] | -1.4% | 0.1255 |
| | C | 95.68±8.867 | -1.7378 [-2.9154 to -0.5602] | -1.9% | 1.1584 [-0.5171 to 2.8339] | -1.2% | 0.1734 |
| | D | 93.18±9.720 | -1.6479 [-2.8688 to -0.4271] | -1.8% | 1.0686 [-0.6449 to 2.782] | -1.2% | 0.2191 |

ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 27.** Anthropometrics and clinical measures (continued).

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | Adjusted Mean [95%CI] (*% Placebo difference*)* | *P* |
|---|---|---|---|---|---|
| SBP, mm Hg | A | 124.7±12.65 | -7.8945 [-11.4508 to -4.3382] | | |
| | B | 127.4±13.07 | -2.9829 [-6.4998 to 0.5339] | -4.9116 [-9.8887 to 0.0655] | 0.0530 |
| | C | 131.9±16.32 | -4.4298 [-7.84 to -1.0196] | -3.4647 [-8.4332 to 1.5038] | 0.1698 |
| | D | 132.3±12.24 | -3.8718 [-7.4705 to -0.2731] | -4.0227 [-9.1287 to 1.0832] | 0.1213 |
| DBP, mm Hg | A | 84.86±7.847 | -5.5447 [-7.7949 to -3.2945] | | |
| | B | 83.38±8.619 | -2.1571 [-4.4207 to 0.1066] | -3.3876 [-6.5759 to -0.1994] | 0.0375 |
| | C | 83.87±7.570 | -3.1265 [-5.3076 to -0.9455] | -2.4181 [-5.5493 to 0.713] | 0.1287 |
| | D | 88.85±8.808 | -1.6502 [-3.9972 to 0.6969] | -3.8945 [-7.153 to -0.6361] | 0.0196 |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

* Mean mm Hg change= ([Mean baseline] – [Adjusted Mean at week 4]) / [Mean baseline]

Abbreviations: Product A, cocoa alone cream; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; BMI, body mass index, calculated as weight in kilograms divided by height in meters squared; WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 28.** Lipid profile variables

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant p |
|---|---|---|---|---|---|---|---|---|
| TC, mmol/L | A | 6.141±0.860 | -0.0878 [-0.2422 to 0.0667] | -1.4% | | | | |
| | B | 6.098±0.801 | 0.0075 [-0.1469 to 0.1619] | 0.1% | -0.0953 [-0.3137 to 0.1232] | 1.6% | 0.3892 | C, D |
| | C | 6.005±0.809 | -0.5412 [-0.6907 to -0.3916] | -8.9% | 0.4534 [0.2382 to 0.6685] | -7.4% | 0.0001 | |
| | D | 6.189±0.692 | -0.501 [-0.6585 to -0.3435] | -8.1% | 0.4132 [0.1928 to 0.6337] | -6.7% | 0.0003 | |
| LDL-c, mmol/L | A | 4.244±0.673 | -0.0028 [-0.1735 to 0.1679] | -0.1% | | | | |
| | B | 4.195±0.720 | 0.0687 [-0.102 to 0.2394] | 1.7% | -0.0715 [-0.3129 to 0.17] | 1.7% | 0.5586 | C, D |
| | C | 4.150±0.911 | -0.4716 [-0.6367 to -0.3066] | -11.2% | 0.4689 [0.2313 to 0.7064] | -11.2% | 0.0002 | |
| | D | 4.278±0.557 | -0.396 [-0.57 to -0.2221] | -9.3% | 0.3932 [0.1496 to 0.6368] | -9.2% | 0.0018 | |
| HDL-c, mmol/L | A | 1.219±0.305 | -0.0193 [-0.0776 to 0.039] | -1.6% | | | | |
| | B | 1.254±0.323 | -0.048 [-0.1063 to 0.0103] | -3.9% | 0.0287 [-0.0538 to 0.1111] | -2.3% | 0.4918 | |
| | C | 1.224±0.355 | -0.0514 [-0.1077 to 0.0049] | -4.1% | 0.0321 [-0.0489 to 0.1131] | -2.5% | 0.4337 | |
| | D | 1.264±0.419 | -0.0231 [-0.0824 to 0.0363] | -1.8% | 0.0038 [-0.0795 to 0.087] | -0.2% | 0.9288 | |

ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 28.** Lipid profile variables (continued)

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant P |
|---|---|---|---|---|---|---|---|---|
| TG, mmol/L | A | 1.419±0.894 | -0.1028 [-0.3203 to 0.1148] | -7.2% | | | | |
| | B | 1.374±0.958 | -0.036 [-0.2534 to 0.1815] | -2.8% | -0.0668 [-0.3743 to 0.2407] | 4.4% | 0.6676 | |
| | C | 1.288±0.892 | -0.0313 [-0.2414 to 0.1789] | -2.3% | -0.0715 [-0.3742 to 0.2312] | 5.0% | 0.6406 | |
| | D | 1.327±0.920 | -0.1394 [-0.3608 to 0.082] | -10.5% | 0.0366 [-0.2739 to 0.3471] | -3.3% | 0.8156 | |
| Apo B-100, g/L | A | 1.158±0.192 | 0.0346 [-0.0069 to 0.076] | 3.0% | | | | |
| | B | 1.161±0.160 | 0.0392 [-0.0014 to 0.0798] | 3.5% | -0.0047 [-0.0627 to 0.0533] | 0.5% | 0.8733 | C, D |
| | C | 1.116±0.202 | -0.059 [-0.0984 to -0.0197] | -5.1% | 0.0936 [0.0365 to 0.1508] | -8.1% | 0.0016 | |
| | D | 1.171±0.193 | -0.064 [-0.1055 to -0.0226] | -5.5% | 0.0986 [0.04 to 0.1572] | -8.5% | 0.0012 | |
| Apo A-1, g/L | A | 1.676±0.322 | 0.0089 [-0.0407 to 0.0585] | 0.5% | | | | |
| | B | 1.742±0.252 | 0.0221 [-0.0266 to 0.0709] | 1.3% | -0.0132 [-0.0829 to 0.0565] | 0.8% | 0.7074 | |
| | C | 1.661±0.295 | 0.0104 [-0.0366 to 0.0574] | 0.6% | -0.0015 [-0.0697 to 0.0668] | 0.1% | 0.9660 | |
| | D | 1.717±0.243 | 0.036 [-0.0136 to 0.0857] | 2.1% | -0.0271 [-0.0973 to 0.0431] | 1.6% | 0.4452 | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 28.** Lipid profile variables (continued)

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant P |
|---|---|---|---|---|---|---|---|---|
| Apo B / Apo A ratio | A | 0.710±0.153 | 0.0203 [-0.0086 to 0.0492] | 2.9% | | | | |
| | B | 0.683±0.155 | 0.0119 [-0.0164 to 0.0403] | 1.7% | 0.0084 [-0.0321 to 0.0488] | -1.2% | 0.6832 | C, D |
| | C | 0.698±0.205 | -0.0335 [-0.0608 to -0.0062] | -4.9% | 0.0538 [0.0141 to 0.0935] | -7.8% | 0.0085 | |
| | D | 0.704±0.187 | -0.0537 [-0.0826 to -0.0249] | -7.6% | 0.074 [0.0332 to 0.1148] | -10.5% | 0.0005 | |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

* Mean relative change= ([Mean baseline] – [Adjusted Mean at week 4]) / [Mean baseline]

Abbreviations: Product A, cocoa cream alone; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; Apo B-100, apolipoprotein B; Apo A-1, apolipoprotein A.

Conversions units to convert international units to conventional units (mg/dL): total, LDL and HDL cholesterol, multiply by 38,7; TG, multiply by 88,5; Apo A-1 and Apo B-100, multiply by 100.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 29.** Oxidative stress, endothelial dysfunction, inflammation, antithrombotic activity and metabolic syndrome biomarkers

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant P |
|---|---|---|---|---|---|---|---|---|
| oxLDL, U/L | A | 70.32±15.30 | -2.0927 [-4.5556 to 0.3702] | -3.0% | | | | |
| | B | 65.10±10.08 | -1.1222 [-3.585 to 1.3407] | -1.7% | -0.9705 [-4.4702 to 2.5291] | 1.3% | 0.5837 | D |
| | C | 66.75±12.51 | -4.2657 [-6.6354 to -1.8961] | -6.6% | 2.173 [-1.2509 to 5.597] | -3.6% | 0.2111 | |
| | D | 68.75±15.44 | -6.1012 [-8.5991 to -3.6032] | -8.9% | 4.0084 [0.5071 to 7.5098] | -5.9% | 0.0252 | |
| VCAM-1, ng/mL | A | 550.7±178.6 | -16.3532 [-53.5202 to 20.8138] | -3.0% | | | | |
| | B | 640.8±396.8 | -14.8957 [-51.9732 to 22.1818] | -2.4% | -1.4575 [-54.1488 to 51.2338] | 0.5% | 0.9564 | |
| | C | 614.5±191.4 | -21.1461 [-56.8802 to 14.5879] | -3.3% | 4.793 [-46.8254 to 56.4113] | -0.3% | 0.8543 | |
| | D | 605.2±224.9 | -6.074 [-43.7314 to 31.5834] | -1.0% | -10.2792 [-63.2002 to 42.6419] | 2.0% | 0.7010 | |
| ICAM-1, ng/mL | A | 316.0±107.4 | -0.3474 [-24.7371 to 24.0423] | -0.1% | | | | |
| | B | 318.8±115.3 | -2.6899 [-27.0693 to 21.6895] | -0.8% | 2.3425 [-32.1201 to 36.8052] | -0.7% | 0.8931 | |
| | C | 343.0±143.5 | -3.994 [-27.5981 to 19.61] | -1.3% | 3.6466 [-30.3466 to 37.6399] | -1.1% | 0.8320 | |
| | D | 325.5±115.9 | -5.079 [-29.8942 to 19.7361] | -1.6% | 4.7316 [-30.061 to 39.5242] | -1.5% | 0.7880 | |

ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 29.** Oxidative stress, endothelial dysfunction, inflammation, antithrombotic activity and metabolic syndrome biomarkers (continued)

| Variables | Product | Baseline Mean±SD or (95%CI) | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant P |
|---|---|---|---|---|---|---|---|---|
| IL-6, pm/mL | A | 0.301 (0.168 to 0.484) | -0.017 (-0.0102 to 0.037) | -5.6% | | | | |
| | B | 0.296 (0.214 to 0.398) | 0.035 (-0.068 to 0.148) | 11.8% | -0.067 (-0.262 to 0.090) | 17.5% | 0.1407 | |
| | C | 0.290 (0.180 to 0.480) | 0.023 (-0.050 to 0.239) | 7.9% | -0.080 (-0.290 to 0.060) | 13.6% | 0.0606 | |
| | D | 0.265 (0.140 to 0.416) | 0.024 (-0.130 to 0.152) | 9.1% | -0.052 (-0.229 to 0.139) | 14.7% | 0.3869 | |
| hsCRP, mg/L | A | 1.635 (0.765 to 3.240) | 0.115 (-0.435 to 0.900) | 7.0% | | | | |
| | B | 2.780 (1.280 to 5.300) | -0.190 (-1.080 to 0.115) | -6.8% | 0.59 (-0.475 to 1.640) | -13.9% | 0.0592 | |
| | C | 2.130 (1.380 to 3.020) | -0.025 (-0.460 to 0.280) | -1.2% | 0.375 (-0.415 to 1.220) | -8.2% | 0.2435 | |
| | D | 2.120 (1.250 to 5.190) | -0.560 (-1.80 to 0.030) | -26.4% | 0.955 (-0.02 to 2.0350) | -33.4% | 0.0083 | |

ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 29.** Oxidative stress, endothelial dysfunction, inflammation, antithrombotic activity and metabolic syndrome biomarkers (continued)

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95CI] (*% Baseline change*)* | | Adjusted Mean [95CI] (*% Placebo difference*)* | | p | Other significant P |
|---|---|---|---|---|---|---|---|---|
| PAI-1, ng/mL | A | 45.05±18.40 | 6.8934 [0.1935 to 13.5934] | 15.3% | | | | |
| | B | 42.99±20.46 | 3.2949 [-3.4078 to 9.9975] | 7.0% | 3.5986 [-5.881 to 13.0781] | 8.3% | 0.4534 | |
| | C | 47.27±26.37 | -0.1685 [-6.6566 to 6.3195] | -0.4% | 7.062 [-2.2593 to 16.3832] | -15.7% | 0.1361 | |
| | D | 41.87±19.80 | 4.9327 [-1.9007 to 11.766] | 11.8% | 1.9608 [-7.6136 to 11.5352] | -3.5% | 0.6856 | |
| FABP-4, ng/mL | A | 26.88±10.03 | 0.9964 [-1.4252 to 3.4179] | 3.7% | | | | |
| | B | 24.50±10.95 | 0.7373 [-1.6353 to 3.1099] | 2.9% | 0.2591 [-3.1365 to 3.6547] | -0.8% | 0.8800 | |
| | C | 25.19±10.23 | 1.6033 [-0.6805 to 3.8871] | 6.5% | -0.6069 [-3.9374 to 2.7235] | 2.8% | 0.7184 | |
| | D | 25.63±10.82 | 2.79 [0.3733 to 5.2066] | 10.9% | -1.7936 [-5.2143 to 1.627] | 7.2% | 0.3007 | |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI] for all parameters except IL-6 and hsCRP. For the latter variables the analyses were non-parametric and the estimators were median ($25^{th}$ percentile – $75^{th}$ percentile).

* Mean relative change= ([Mean baseline] – [Adjusted Mean at week 4]) / [Mean baseline]

Abbreviations: Product A, cocoa cream alone; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; oxLDL, oxidised LDL; VCAM-1, vascular adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6, hsCRP, high sensitivity C reactive protein; PAI-1, plasminogen activator inhibitor-1; FABP-4, fatty acid binding protein-4.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 30.** Composition diets for each group after 4 weeks of treatment

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant p |
|---|---|---|---|---|---|---|---|---|
| Energy, Kcal/d | A | 2347±514.3 | -238.4722 [-368.8103 to -108.1342] | -10.2% | | | | |
| | B | 2408±659.9 | -344.4643 [-474.807 to -214.1215] | -14.5% | 105.992 [-78.4079 to 290.392] | -4.3% | 0.2571 | |
| | C | 2381±549.1 | -230.0918 [-355.965 to -104.2187] | -9.6% | -8.3804 [-189.5851 to 172.8243] | 0.6% | 0.9271 | |
| | D | 2371±477.0 | -342.0286 [-474.7114 to -209.3458] | -14.4% | 103.5564 [-82.4222 to 289.535] | -4.3% | 0.2722 | |
| CHO, % of total energy | A | 37.60±8.738 | 3.15 [0.803 to 5.498] | 8.4% | | | | |
| | B | 38.45±6.559 | 1.235 [-1.093 to 3.563] | 3.2% | 1.915 [-1.365 to 5.196] | -5.2% | 0.2497 | |
| | C | 41.86±4.656 | 2.691 [0.419 to 4.963] | 6.4% | 0.459 [-2.852 to 3.77] | -1.9% | 0.784 | |
| | D | 40.73±5.702 | 2.941 [0.574 to 5.308] | 7.2% | 0.209 [-3.145 to 3.564] | -1.2% | 0.9019 | |
| Protein, % of total energy | A | 14.70±2.538 | 0.324 [-0.422 to 1.07] | 2.2% | | | | |
| | B | 13.99±1.881 | 1.107 [0.366 to 1.848] | 7.9% | -0.783 [-1.837 to 0.271] | 5.7% | 0.1437 | |
| | C | 13.67±1.985 | 1.493 [0.774 to 2.213] | 10.9% | -1.169 [-2.212 to -0.126] | 8.7% | 0.0284 | |
| | D | 14.20±2.855 | 1.324 [0.57 to 2.079] | 9.3% | -1 [-2.06 to 0.06] | 7.1% | 0.0642 | |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 30.** Composition diets for each group after 4 weeks of treatment (continued)

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant p |
|---|---|---|---|---|---|---|---|---|
| Total fat, % of total energy | A | 44.17±4.938 | -3.528 [-5.642 to -1.415] | -8.0% | | | | |
| | B | 45.55±4.801 | -3.469 [-5.587 to -1.351] | -7.6% | -0.059 [-3.056 to 2.937] | 0.4% | 0.9688 | |
| | C | 43.30±4.447 | -4.376 [-6.431 to -2.322] | -10.1% | 0.848 [-2.092 to 3.788] | -2.1% | 0.5688 | |
| | D | 45.72±8.581 | -4.369 [-6.528 to -2.21] | -9.6% | 0.841 [-2.186 to 3.868] | -1.6% | 0.583 | |
| SFA, % of total energy | A | 12.65±1.891 | -3.025 [-3.926 to -2.124] | -23.9% | | | | |
| | B | 13.22±2.343 | -3.042 [-3.943 to -2.14] | -23.0% | 0.017 [-1.26 to 1.294] | 0.9% | 0.9795 | |
| | C | 12.86±2.113 | -3.056 [-3.926 to -2.186] | -23.8% | 0.031 [-1.221 to 1.283] | 0.1% | 0.9609 | |
| | D | 12.98±3.431 | -2.708 [-3.625 to -1.792] | -20.9% | -0.317 [-1.603 to 0.969] | 3.1% | 0.6265 | |
| MUFA, % of total energy | A | 20.13±4.005 | -0.82 [-2.063 to 0.422] | -4.1% | | | | |
| | B | 20.86±2.294 | 1.583 [0.341 to 2.826] | 7.6% | -2.404 [-4.163 to -0.644] | 11.7% | 0.0079 | |
| | C | 19.47±2.538 | -0.086 [-1.299 to 1.127] | -0.4% | -0.734 [-2.463 to 0.995] | 3.6% | 0.4019 | |
| | D | 21.57±4.460 | 0.343 [-0.936 to 1.622] | 1.6% | -1.163 [-2.954 to 0.627] | 5.7% | 0.2005 | |

ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 30.** Composition diets for each group after 4 weeks of treatment (continued)

| Variables | Product | Baseline Mean±SD | Baseline change at 4 weeks Adjusted Mean [95%CI] (*% Baseline change*)* | | Adjusted Mean [95%CI] (*% Placebo difference*)* | | *P* | Other significant p |
|---|---|---|---|---|---|---|---|---|
| PUFA, % of total energy | A | 7.327±1.005 | 0.426 [0.032 to 0.819] | 5.8% | | | | |
| | B | 7.654±1.352 | -1.835 [-2.229 to -1.441] | -24.0% | 2.261 [1.703 to 2.818] | -29.8% | 0.0000 | |
| | C | 7.121±1.638 | -2.161 [-2.544 to -1.778] | -30.3% | 2.587 [2.04 to 3.134] | -36.2% | 0.0000 | |
| | D | 7.796±1.107 | -1.911 [-2.314 to -1.507] | -24.5% | 2.336 [1.771 to 2.901] | -30.3% | 0.0000 | |
| Dietary fibre, g/d | A | 21.93±5.934 | 0.1967 [-2.386 to 2.7794] | 0.9% | | | | |
| | B | 21.84±8.112 | 2.1777 [-0.4054 to 4.7607] | 9.7% | -1.981 [-5.6329 to 1.6709] | 8.8% | 0.2847 | D |
| | C | 22.47±8.829 | 2.8735 [0.3783 to 5.3687] | 13.2% | -2.6769 [-6.2687 to 0.9149] | 12.3% | 0.1425 | D |
| | D | 22.53±6.773 | 7.7223 [5.092 to 10.3527] | 34.3% | -7.5257 [-11.2127 to -3.8386] | 33.4% | <0.0001 | |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

* Mean relative change= ([Mean baseline] – [Adjusted Mean at week 4]) / [Mean baseline]

Abbreviations: Product A, cocoa cream alone; Product B, cocoa plus hazelnut cream; Product C, cocoa, hazelnut plus phytosterols cream; Product D, cocoa, hazelnut, phytosterols and soluble fibre cream; Kcal, kilocalories; CHO, carbohydrates; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



#### 4.1.g. Patent

Title: *Bombón con efectos cardiovasculares* [Snack with cardiovascular benefit]
Inventors (signature): Ramírez B, Anglès MN, Reguant J, Solà R, Godàs G
Title: *Alimento funcional con efectos positivos en la prevención de enfermedades cardiovasculares* [Functional food item with positive effects in the prevention of cardiovascular disease]
Application number: 20080487
First priority country: United States
Date of priority: 2008

#### 4.1.h. Clinical trials registration

This trial is registered in ClinicalTrials.gov with the identifier number: NCT00511420.

### 4.2. Effects of treatment with a soluble fibre (*Plantago ovata* husk) on biomarkers of CVD

#### 4.2.a. Participants

From the participants considered potentially eligible, 394 attended a screening appointment and 254 were randomised; 232 (91.3%) were included in the safety assessment population, 209 (82.3%) in the ITT analysis, 187 (73.6%) in the PP analysis. Figure 10 depicts the flow of participants through the study.
Demographic variables of participants at baseline are shown in Table 31. There were no differences between the placebo and Po-husk groups.
The results at 8 weeks of the intervention period, from anthropometric measures, lipid profile, CVD biomarkers, insulin resistance and dietary adherence were showed at Tables 32, 33, 34, 35 and 36.

#### 4.2.b. Dietary and treatment adherence

Although the low saturated fat and cholesterol diet prescribed was the same for both treatment groups, by week 8 the Po-husk group had consumed a lower percentage of total fat (-4.4%; 95%CI: 5.5 to -3.2; *P*=0.03) and comparison with placebo group showed a -1.8 % difference [95%CI: 0.1 to 3.4; *P*=0.03) (Table 36).

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The adherences to Po-husk and placebo treatment were high (96.7% and 94.2%, respectively). Adherence to the intervention diet was 96%.

**Figure 10.** Flow diagram of participants

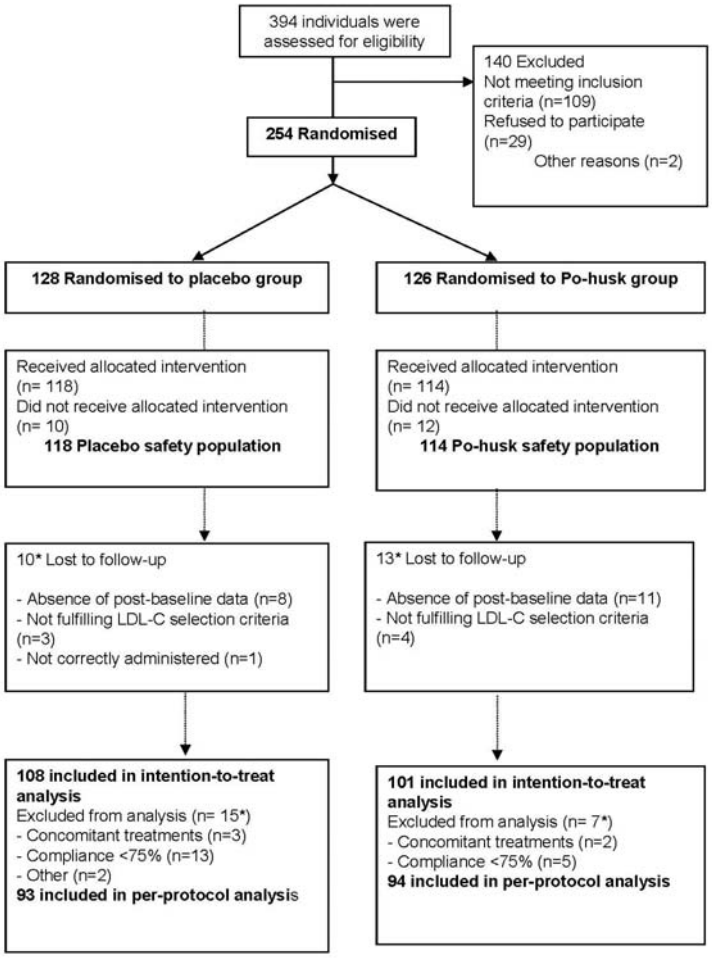

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**Table 31.** Baseline characteristics of study participants

| Variables | Placebo (n=108) | Po-husk (n= 101) |
|---|---|---|
| Age (years) | 55.59±11.5 | 54.24±9.96 |
| Weight (Kg) | 72.72±11.24 | 72.18±13.14 |
| Height (m) | 1.63±0.09 | 1.64±0.11 |
| BMI (Kg/m$^2$) | 27.45±3.54 | 26.82±3.72 |
| Gender (male) | 46(42.6%) | 45(44.6%) |
| Current smoker (n, %) | 22(20.4%) | 23(22.8%) |

Results are expressed as means ± SD and frequencies (%)
Abbreviations: BMI, body mass index, calculated as weight in kilograms divided by height in meters squared.

### 4.2.c. Lipid profile

In the ITT analysis at week 8, LDL-c decreased by 6% in the Po-husk group relative to the placebo group (*P*=0.0002). After 8 weeks, Po-husk reduced plasma LDL-c by 6.14% (adjusted mean: -0.29 mmol/L, 95%CI: -0.41 to -0.90; *P*<0.0002), TG by -0.22 mmol/L (95%CI: -0.34 to -0.11), Apo B-100 by -0.07 g/L (95%CI: 0.10 to -0.05) (Table 33).

LDL-c between baseline and week 8 had a reduction of 6.1% in the Po-husk group was statistically significant relative to the placebo group in which the reduction of 0.1% was statistically non-significant.

For those subjects not achieving the therapeutic objective, the mean adjusted reduction between week 8 and week 16 was -59.0 mg/dL (95%CI; -65.0 to 53.1) for placebo and - 63.9 mg/dL (95%CI: -70.3 to 57.6) for Po-husk.

The rate of subjects achieving a 5% reduction in LDL-c was 24.6% higher in the fibre group. In the placebo group, the rate of patients achieving the therapeutic objective was 26.9% while, in the Po-husk group, the average for subjects reaching the objective was 51.5% (*P*= 0.0004) in the ITT analysis at week 8.

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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The statistical differences for the rest of the lipid profile in relation to the absolute change from baseline were: Apo B-100 ($P$<0.0001), TC ($P$<0.0001), TG ($P$=0.0004) (Table 33). No other statistical differences between treatment groups were found.

**4.2.d. Anthropometric and clinical measures**

No significant differences were observed in the bodyweight between groups. Both maintained the bodyweight over the whole course of the study (Table 32).

SBP was statistically significantly lower -4.013 mm Hg (95%CI: 1.262 to 6.765; $P$=0.0045) in the Po-husk group compared to placebo at week 8. There were no significant differences in DPB (Table 32).

**4.2.e. CVD biomarkers**

There were significant differences in Po-husk group relative to baseline with respect to oxLDL ($P$=0.0003) (Table 34).

There were no statistically significant differences between groups at week 8 in inflammatory biomarkers (hsCRP, IL-6, leukocytes), endothelial dysfunction (ICAM-1, VCAM-1) and thrombotic activity (folic acid and PAI-1) (Table 33).

**4.2.f. Insulin resistance biomarkers**

Po-husk reduced insulin plasma concentrations by -7.10 pmol/L (95%CI: -9.97 to -4.23; $P$=0.021) and HOMA-index by -0.31 µU/mL*mmol/L (95%CI: -0.42 to-0.20; $P$=0.005), with no changes in plasma glucose concentrations (Table 35).

**4.2.g. Gene polymorphisms**

No association was observed between plasma LDL-c concentrations and any polymorphism studied.

In Po-husk consumers, plasma TG concentrations showed a greater reduction in homozygotes G and DEL (-38.7 ± 17.0 and -1.7 ± 5.2 mg/dL respectively, $P$=0.005) of PAI-1 gene variants and in homozygote A and G (-74.8 ± 8.7 and -18.5 ± 8.8 mg/dL respectively, $P$=0.031) of FABP-2 gene variants.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.2.h. Statin treatment

From the subgroup of ITT non-responder subjects at week 8 (ITT-W16 subset), 70/108 (65%) in the placebo group and 61/101 (60%) in the Po-husk (*P*=0.5678) were analysed for intra-group change at week 16 relative to week 8.

Results showed that LDL-c, Apo B/Apo A ratio, Apo B-100 and TC improved after 8 weeks of co-adjuvant simvastatin treatment, in both treatment arms, as well as TG in the placebo group (Figures 11 and 12).

### 4.2.i. Adverse events

No statistically significant differences were found between treatment groups for the number (%) of subjects reporting one or more adverse events, as assessed by body system according to the MedDRA classification (version 8.0). Only one patient in the placebo group had a serious event (diagnosis of type 2 diabetes) which was considered as not being related to the trial.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 32.** Anthropometric and clinical variables

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| Weight, Kg | Placebo | 72.72±11.236 | -0.543 [-0.863 to -0.223] | | |
| | Po-husk | 72.18±13.14 | -0.839 [-1.17 to -0.508] | 0.295 [-0.165 to 0.756] | 0.2075 |
| SBP, mm Hg | Placebo | 126.15±15.422 | 0.231 [-1.676 to 2.139] | | |
| | Po-husk | 129.51±17.474 | -3.782 [-5.755 to -1.809] | 4.013 [1.262 to 6.765] | 0.0045 |
| DBP, mm Hg | Placebo | 79.63±9.999 | -1.442 [-2.842 to -0.041] | | |
| | Po-husk | 81.257±11.084 | -2.775 [-4.224 to -1.327] | 1.334 [-0.685 to 3.352] | 0.1941 |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

*Means relative change= ([Mean baseline] – [Adjusted Mean at week 8]) / [Mean baseline].

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 33.** Lipid profile variables

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| TC, mmol/L | Placebo | 6.576±0.785 | 0.028 [-0.094 to 0.15] *(+0.43%)* | | |
| | Po-husk | 6.699±0.712 | -0.433 [-0.559 to -0.306] *(-6.46%)* | 0.46 [0.284 to 0.637] *(-6.89%)* | <0.0001 |
| LDL-c, mmol/L | Placebo | 4.748±0.641 | -0.008 [-0.113 to 0.098] *(-0.17%)* | | |
| | Po-husk | 4.873±0.624 | -0.299 [-0.408 to -0.19] *(-6.14%)* | 0.291 [0.139 to 0.443] *(-5.97%)* | 0.0002 |
| HDL-c, mmol/L | Placebo | 1.201±0.325 | 0.004 [-0.039 to 0.047] *(+0.33%)* | | |
| | Po-husk | 1.207±0.368 | -0.03 [-0.075 to 0.015] *(-2.49%)* | 0.034 [-0.029 to 0.096] *(-2.82%)* | 0.2893 |
| TG, mmol/L | Placebo | 1.38±0.742 | 0.069 [-0.044 to 0.181] *(+5.00%)* | | |
| | Po-husk | 1.361±0.916 | -0.227 [-0.343 to -0.11] *(-16.68%)* | 0.295 [0.133 to 0.458] *(-21.68%)* | 0.0004 |

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**Table 33.** Lipid profile variables (continued)

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| Apo A-1, g/L | Placebo | 1.776±0.284 | -0.011 [-0.056 to 0.035] *(-0.62%)* | | |
| | Po-husk | 1.779±0.303 | -0.067 [-0.113 to -0.02] *(-3.77%)* | 0.056 [-0.009 to 0.121] *(-3.15%)* | 0.0919 |
| Apo B-100, g/L | Placebo | 1.164±0.16 | 0.006 [-0.016 to 0.029] *(+0.52%)* | | |
| | Po-husk | 1.184±0.132 | -0.074 [-0.098 to -0.051] *(-6.25%)* | 0.081 [0.048 to 0.113] *(-6.77%)* | <0.0001 |
| Apo B / Apo A ratio | Placebo | 0.67±0.138 | 0.013 [-0.013 to 0.04] *(+1.94%)* | | |
| | Po-husk | 0.688±0.157 | -0.01 [-0.037 to 0.017] *(-1.45%)* | 0.024 [-0.014 to 0.061] *(-3.39%)* | 0.2134 |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

*Means relative change= ([Mean baseline] – [Adjusted Mean at week 8]) / [Mean baseline].

Abbreviations: TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c, high density lipoprotein cholesterol; TG, triglycerides; Apo A-1, apolipoprotein A; Apo B-100, apolipoprotein B.

To convert SI units to conventional units (mg/dL): total, LDL and HDL cholesterol, multiply by 38.7; TG, multiply by 88.50; Apo A-1 and Apo B-100, multiply by 100.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 34.** Oxidative stress, endothelial dysfunction, antithrombotic and inflammatory activity biomarkers

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| oxLDL, U/L | Placebo | 80.395±16.821 | 1.529 [-1.017 to 4.075] | | |
| | Po-husk | 80.288±18.869 | -5.292 [-7.925 to -2.659] | 6.821 [3.158 to 10.483] | 0.0003 |
| ICAM-1, ng/mL | Placebo | 226.35±55.255 | 1.771 [-3.413 to 6.956] | | |
| | Po-husk | 229.17±74.16 | 0.484 [-4.877 to 5.845] | 1.287 [-6.172 to 8.746] | 0.7341 |
| VCAM-1, ng/mL | Placebo | 734.43±181.49 | -0.709 [-19.3 to 17.887] | | |
| | Po-husk | 739.31±192.16 | 7.158 [-12.07 to 26.388] | -7.867 [-34.62 to 18.885] | 0.5627 |
| PAI-1, ng/mL | Placebo | 36.41±27.705 | -3.571 [-7.619 to 0.477] | | |
| | Po-husk | 38.026±29.179 | -6.759 [-10.94 to -2.573] | 3.188 [-2.636 to 9.011] | 0.2818 |

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 34.** Oxidative stress, endothelial dysfunction, antithrombotic and inflammatory activity biomarkers (continued)

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD or [95%CI] | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| Folic acid, nmol/L | Placebo | 11.43 [8.375 to 17.58] | -0.27 [-2.14 to 1.345] | | |
| | Po-husk | 11.16 [9.27 to 16.72] | -0.87 [-2.66 to 1.06] | | 0.1357 |
| hsCRP, mg/L | Placebo | 1.885 [0.785 to 3.54] | 0.06 [-0.465 to 0.69] | | |
| | Po-husk | 1.23 [0.51 to 2.53] | -0.02 [-0.33 to 0.325] | | 0.1412 |
| IL-6, pm/mL | Placebo | 1.66 [1.25 to 2.67] | 0.03 [-0.445 to 0.6] | | |
| | Po-husk | 1.74 [1.17 to 2.38] | 0.07 [-0.43 to 0.52] | | 0.7125 |
| Leukocytes, 10^9/L | Placebo | 6.302±1.565 | 0.160 [-0.09 to 0.409] | | |
| | Po-husk | 6.395±1.726 | 0.04 [-0.218 to 0.298] | 0.119 [-0.24 to 0.478] | 0.5129 |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI], To folic acid, hsCRP and IL-6 variables the analyses were non-parametric and the estimators were median ($25^{th}$ percentile – $75^{th}$ percentile).

Abbreviations: oxLDL, oxidised LDL; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PAI-1 plasminogen activator inhibitor-1; hsCRP, high sensitivity C reactive protein; IL-6, interleukin-6.

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 35.** Plasma glucose, insulin concentrations and HOMA-index.

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences Adjusted Mean | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| Glucose, nmol/L | Placebo | 5.182±0.607 | -0.015 [-0.093 to 0.063] | | |
| | Po-husk | 5.163±0.686 | -0.087 [-0.168 to -0.007] | 0.072 [-0.04 to 0.184] | 0.2046 |
| Insulin, pmol/L | Placebo | 50.891±21.737 | -2.424 [-5.198 to 0.351] | | |
| | Po-husk | 49.385±30.174 | -7.103 [-9.972 to -4.234] | 4.68 [0.688 to 8.672] | 0.0218 |
| HOMA-index, μU/mL*mmol/L | Placebo | 1.657±0.826 | -0.095 [-0.201 to 0.011] | | |
| | Po-husk | 1.656±1.386 | -0.311 [-0.42 to -0.202] | 0.215 [0.063 to 0.368] | 0.0058 |

Results are expressed as means ± SD and baseline adjusted least square means [95%CI].

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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

**Table 36.** Nutritional profile of low-SFA diets in the ITT population (n=209)

| | | Baseline | Change at 8 weeks relative to baseline | Treatment Differences | |
|---|---|---|---|---|---|
| Variables | Group | Mean ± SD | Adjusted Mean [95%CI] *(% Baseline change)** | Adjusted Mean [95%CI] *(% Placebo difference)** | *P* |
| Energy, Kcal/d | Placebo | 2021.6±533.57 | -209.2 [-286.1 to -132.4] | | |
| | Po-husk | 2007.4±589.9 | -230.8 [-310 to -151.7] | 21.581 [-88.76 to 131.92] | 0.7002 |
| CHO, % | Placebo | 43.547±6.933 | 2.616 [1.27 to 3.961] | | |
| | Po-husk | 41.979±6.807 | 4.236 [2.85 to 5.622] | -1.62 [-3.558 to 0.317] | 0.1007 |
| Proteins, % | Placebo | 17.604±3.51 | 0.166 [-0.322 to 0.654] | | |
| | Po-husk | 17.602±2.913 | 0.395 [-0.107 to 0.897] | -0.229 [-0.929 to 0.47] | 0.5187 |
| Total fats, % | Placebo | 35.524±5.786 | -2.581 [-3.733 to -1.429] | | |
| | Po-husk | 35.808±5.539 | -4.406 [-5.592 to -3.22] | 1.824 [0.171 to 3.478] | 0.0307 |
| SFA, % | Placebo | 10.385±2.899 | -1.846 [-2.201 to -1.49] | | |
| | Po-husk | 10.503±2.587 | -2.049 [-2.419 to -1.679] | 0.203 [-0.31 to 0.717] | 0.4361 |

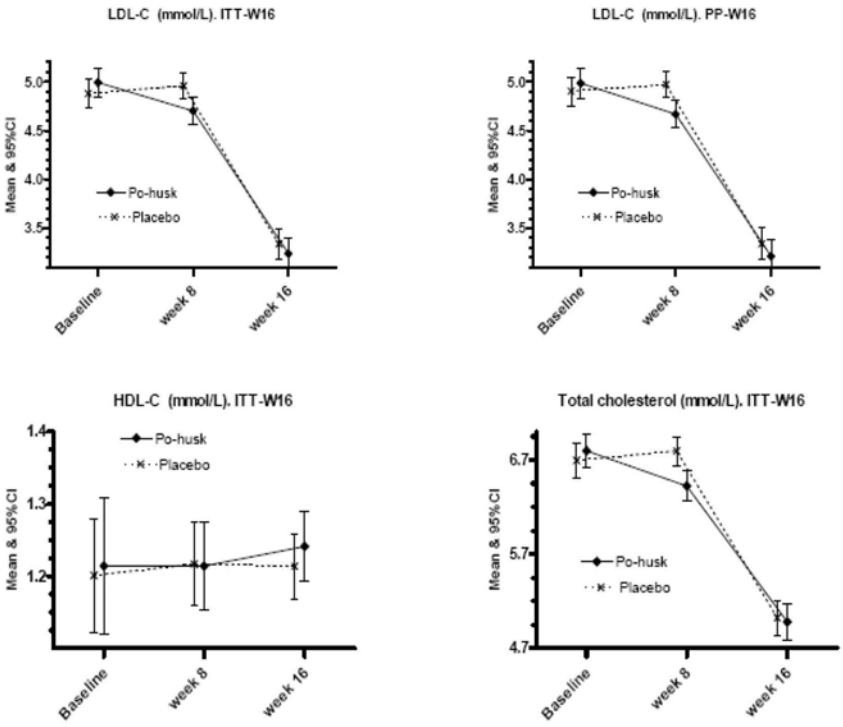
All results are expressed are means ± SD and baseline adjusted least square means [95%CI].

Abbreviations: Kcal, kilocalories; CHO, carbohydrates; SFA, saturated fatty acids.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 11.** Assessment of the co-adjuvant effect of simvastatin 20 mg/d on LDL-c, HDL-c and TC in non-responder patients

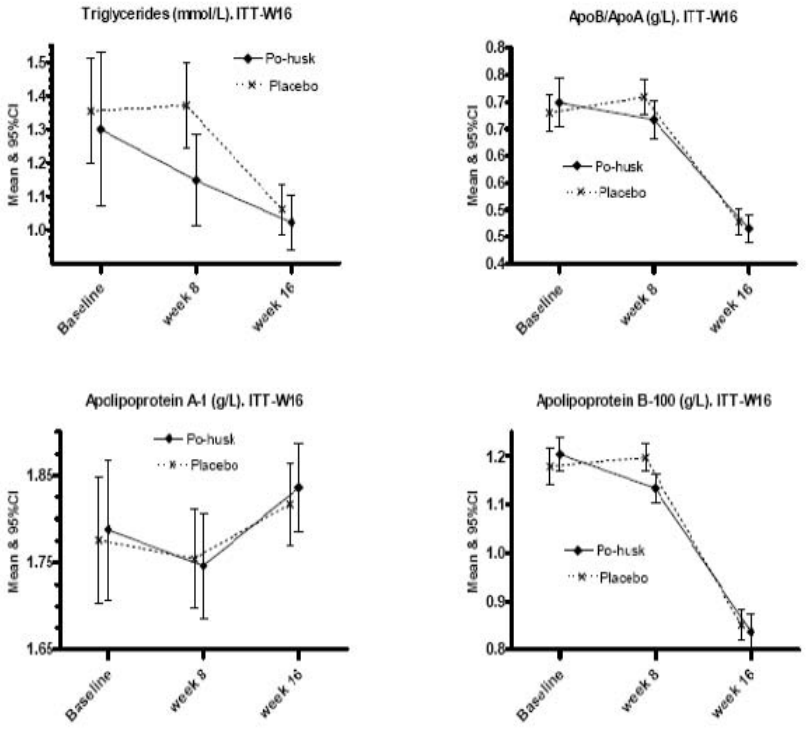



Analysis performed with ITT-W16 subset, 131 non-responder patients (i.e.: no plasma LDL-c levels below 130 mg/dL or 3.367 mmol/L at week 8), 70/108 (65%) in placebo and 61/101 (60%) in the Po-husk. LDL-c is also shown with the PP-W16 subset (64/93 (69%) in the placebo and 57/95 (61%) in the Po-husk).
LDL-c and TC showed statistically significant improvement in both arms at week 16.
Abbreviations: HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; TC, total cholesterol; ITT, intention to treat; PP, per protocol.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 12.** Assessment of the co-adjuvant effect of simvastatin 20 mg/d on TG, Apo B/Apo A, Apo A-1 and Apo B-100 in non-responder patients




Analysis performed with ITT-W16 subset, 131 non-responder patients (i.e.: no plasma LDL-c levels below 130 mg/dL or 3.367 mmol/L at week 8), 70/108 (65%) in placebo and 61/101 (60%) in the Po-husk. LDL-c is also shown with the PP-W16 subset (64/93 (69%) in the placebo and 57/95 (61%) in the Po-husk).
Apo B/Apo A ratio and Apo B-100 showed statistically significant improvement in both arms, and also TG in the placebo group at week 16.
Abbreviations: TG, triglycerides; Apo A-1, apolipoprotein A; Apo B-100, apolipoprotein B; ITT, intention to treat; PP, per protocol.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.2.j. Patent

Title: Fibra soluble con efectos metabólicos *[Soluble fibre with metabolic effects]*

Inventors (signature): César Molinero, Anna Anguera, Rosa Solà

Application number: P200802053

First priority country: Spain

Date of priority: 2008

### 4.2.k. Clinical trials registration

This trial is registered with ClinicalTrials.gov with the identifier number: NCT00502047 and EudraCT No. year-2004-002184-24.

### 4.2.l. CONSORT Statement checklist

The Table 37 shows the application of the CONSORT Statement checklist (2001) to reporte the trial.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 37.** CONSORT statement Checklist – 2001. Items to include when reporting a randomised trial

Effects of treatment with a soluble fibre (Po-husk) on biomarkers of cardiovascular disease risk in patients with hypercholesterolaemia. A multi-centre, randomised double-blind clinical trial

| *PAPER SECTION* And topic | *Item* | Descriptor | Reported on Page |
|---|---|---|---|
| TITLE & ABSTRACT | 1 | How participants were allocated to interventions (*e.g.*, "random allocation", "randomised", or "randomly assigned"). | 1-2-3 |
| *INTRODUCTION* Background | 2 | Scientific background and explanation of rationale. | 4 |
| *METHODS* Participants | 3 | Eligibility criteria for participants and the settings and locations where the data were collected. | 5-6 |
| Interventions | 4 | Precise details of the interventions intended for each group and how and when they were actually administered. | 5-6 |
| Objectives | 5 | Specific objectives and hypotheses. | 4-5 |
| Outcomes | 6 | Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (*e.g.*, multiple observations, training of assessors). | 6, 7,8 |
| Sample size | 7 | How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules. | 8 |
| Randomisation -- Sequence generation | 8 | Method used to generate the random allocation sequence, including details of any restrictions (*e.g.*, blocking, stratification) | 6 |
| Randomisation -- Allocation concealment | 9 | Method used to implement the random allocation sequence (*e.g.*, numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned. | 6 |
| Randomisation -- Implementation | 10 | Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups. | 6 |
| Blinding (masking) | 11 | Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. If done, how the success of blinding was evaluated. | 6 |
| Statistical methods | 12 | Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses. | 8 |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 37.** CONSORT Statement Checklist – 2001. Items to include when reporting a randomized trial (continued)

Effects of treatment with a soluble fibre Po-husk on biomarkers of cardiovascular disease risk in patients with hypercholesterolaemia. A multi-centre, randomised double-blind clinical trial

| *PAPER SECTION* And topic | *Item* | Descriptor | Reported on Page |
|---|---|---|---|
| *RESULTS*<br>Participant flow | 13 | Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analysed for the primary outcome. Describe protocol deviations from study as planned, together with reasons. | 10 and Figure 1 |
| Recruitment | 14 | Dates defining the periods of recruitment and follow-up. | 5 |
| Baseline data | 15 | Baseline demographic and clinical characteristics of each group. | 9 and Tables 1, 2 and 3 |
| Numbers analyzed | 16 | Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat". State the results in absolute numbers when feasible (*e.g.*, 10/20, not 50%). | 10, 11 and Figure 1 |
| Outcomes and estimation | 17 | For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (*e.g.*, 95% confidence interval). | 10,11, Tables 2,3,4 and Figure 2 |
| Ancillary analyses | 18 | Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory. | 10, 11 and Figure 2 |
| Adverse events | 19 | All important adverse events or side effects in each intervention group. | 11,12 and Table 5 |
| *DISCUSSION*<br>Interpretation | 20 | Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision and the dangers associated with multiplicity of analyses and outcomes. | 12 |
| Generalisability | 21 | Generalisability (external validity) of the trial findings. | 12-14 |
| Overall evidence | 22 | General interpretation of the results in the context of current evidence. | 12-14 |

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UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



## 4.3. Olive oil polyphenols detection in human plasma study

### 4.3.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers

#### 4.3.a.i. Participants

The anthropometric characteristics and lipid profile of the participants are shown in Table 38.

**Table 38.** Anthropometric measurements and lipid profiles of the participants

| Variables | VOO consumer group (n=20) | Non-consumer group (n=20) |
|---|---|---|
| Age, years | 45.5 ± 9.5 | 35.0 ± 6.5[a] |
| Weight, Kg | 76.2 ± 16.6 | 86.0 ± 16.1 |
| Height, cm | 164.6 ± 7.9 | 166.8 ± 5.7 |
| BMI, Kg/m$^2$ | 28.1 ± 5.6 | 30.8 ± 4.9 |
| TC, mmol/L | 5.1 ± 1.0 | 5.0 ± 0.8 |
| LDL-c, mmol/L | 3.5 ± 0.9 | 3.2 ± 0.7 |
| HDL-c, mmol/L | 1.3 ± 0.4 | 1.3 ± 0.5 |
| VLDL-c, mmol/L | 0.5 ± 0.2 | 0.6 ± 0.4 |
| TG, mmol/L | 0.9 ± 0.5 | 1.2 ± 1.0 |

Results are expressed as means ± SEM.

[a] $p<0.05$.

Abbreviations: BMI, body mass index calculated as weight in kilograms divided by height in meters squared; TC, total cholesterol; LDL-c, low density lipoprotein cholesterol; HDL-c; high density lipoprotein cholesterol; VLDL-c, very low density lipoprotein cholesterol; TG, triglycerides.

To convert SI units to conventional units (mg/dL): total, LDL, HDL and VLDL cholesterol, multiply by 38.7; TG, multiply by 88.50.

No significant differences were observed between the two groups, except for age, which was lower in the non-consumers (mean ± SEM; 35.0 ± 6.5 years) group than consumers (45.5 ± 9.5 years) ($p<0.05$).

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.3.a.ii. Dietary composition

The dietary compositions of the participants are shown in Table 39. The intakes were similar except for higher PUFA in the non-consumer group (P<0.05) and higher MUFA in the VOO consumer group (P<0.001).

**Table 39.** Nutritional composition of the participants' diets

| Variables | VOO consumer group (n=20) | Non-consumer group (n=20) |
|---|---|---|
| Energy, Kcal/d | 2099 ± 846 | 1547 ± 883 |
| CHO, % of energy | 38.8 ± 9.4 | 43.6 ± 5.3 |
| Protein, % of energy | 16.4 ± 2.8 | 17.9 ± 6.9 |
| Total fat, % of energy | 41.4 ± 7.5 | 35.1 ± 4.4 |
| SFA, % of energy | 12.4 ± 3.6 | 10.1 ± 2.1 |
| MUFA, % of energy | 20.6 ± 4.1[a] | 10.0 ± 2.1 |
| PUFA, % of energy | 5.1 ± 1.5[b] | 11.4 ± 4.8 |
| Dietary fibre, g/d | 20.8 ± 10.2 | 15.0 ± 8.8 |

Results are expressed as means ± SEM.

[a] p<0.05; [b]p<0.001.

Abbreviations: Kcal, kilocalories; CHO, carbohydrates; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

### 4.3.a.iii. Phenolic compounds profile

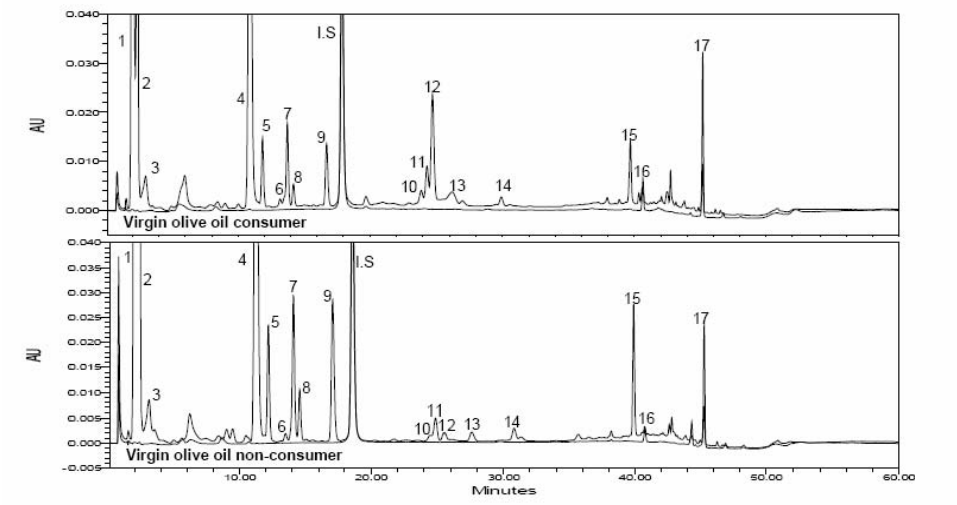
Fasting plasma from VOO consumers showed higher concentration of a flavonoid type (2.90 ± 0.04 µmol/L vs 1.5 ± 0.04 µmol/L) and catechol derivative (0.70 ± 0.03 µmol/L vs 0.56 ± 0.03 µmol/L) than the plasma from non-consumers (*P*<0.05).

Figure 13 presents the phenolic compounds profile of fasting plasma from chronic VOO consumers and non-consumers.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 13.** Chromatograms of phenols identified in fasting plasma samples from VOO consumers and non-consumers




Abbreviations: UV, ultraviolet spectrum.

A total of 17 chromatographic peaks with the characteristic UV spectrum of the phenolic structures were selected for tentative identification and quantification. The compounds selected were confirmed to be the same compound in all the plasma samples from each group by HPLC-MS/MS. The comparative analysis of the chromatographic profiles of the phenolic extracts of plasma samples from the two volunteer groups show no qualitative differences. Thus, no specific compound has been detected in plasma samples from chronic VOO consumers that could be used as a biomarker of olive oil consumption.

### 4.3.b. Olive oil polyphenols detection in human postprandial plasma

#### 4.3.b.i. Phenolic compounds concentration in VOO consumed

Table 40 shows the phenolic compound composition of VOO consumed by the volunteers. The concentrations are expressed in μM.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.3.b.ii. VOO polyphenols detection in postprandial plasma

Of the 5 phenolic compounds with antioxidant activity evaluated in the *in vitro* assay (Luteolin, 3,4-DHPEA-EDA, oleuropein aglycone, hydroxytyrosol and rutin), 4 are present in human plasma at 240 minutes post-ingestion of 40 mL V00 (Figure 13).

The free form of hydroxytyrosol, 3,4-DHPEA-EDA, together with luteolin and oleuropein aglycon were observed from 60 minutes to 240 minutes post-ingestion of VOO. Oleuropein is derived exclusively from plants but it is possible to detect the derivatives in plasma following VOO intake.

**Table 40.** Concentration of phenolic compounds in the VOO used in the study

| Compound | | Concentration (μM) |
|---|---|---|
| **Phenolic acids:** | | |
| | Cinnamic acid | 0.29 |
| | Acid cumaric | 1.14 |
| | Acid ferulic | 0.28 |
| | Vanillic acid | 0.78 |
| | Vanillin | 1.77 |
| **Phenyl ethyl alcohols:** | | |
| | Hydroxytyrosol | 13.01 |
| | Tyrosol | 20.85 |
| **Secoiridoid aglycons:** | | |
| | 3,4-DHPEA-AC | 0.26 |
| | 3,4-DHPEA-EDA | 76.11 |
| | 3,4-DHPEA-EA | 45.34 |
| | Methyl 3,4-DHPEA-EA | 7.05 |
| | Oleuropein derivative | 1.48 |
| | *p*-HPEA-EDA | 21.76 |
| | *p*-HPEA-EA | 28.39 |
| | Ligstroside derivative | 2.50 |

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 40.** Concentration of phenolic compounds in the VOO used in the study (continued)

| Compound | | Concentration (μM) |
|---|---|---|
| **Flavonoids:** | | |
| | Apigenin | 1.39 |
| | Luteolin | 6.96 |
| **Lignans:** | | |
| | Pinoresinol | 2.60 |
| | Acetoxipinoresinol | 29.71 |
| **TOTAL PHENOLS** | | 261.75 |

### 4.3.c. Validation of methods to detect and quantify olive oil polyphenols in human plasma

These results were presented in the article “Improved method for identifying and quantifying olive oil phenolic compounds and their metabolites in human plasma by microelution solid-phase extraction plate and liquid chromatography-tandem mass spectrometry” (Suarez M, 2009). The analyses were performed by the UdL.

Table 41 presents the results optimising and validating the technique. Two transitions are required for each phenolic compound; one to quantify and the other to confirm.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 41.** Optimised SRM conditions for the analyses of the studied phenolic compounds by UPLC-MS/MS

| Peak | Compound | MW | Quantification | | | Confirmation | | |
|---|---|---|---|---|---|---|---|---|
| | | | $SRM_1$ | Cone voltage (V) | Collision energy (eV) | $SRM_2$ | Cone voltage (V) | Collision energy (eV) |
| 1 | Hydroxytyrosol | 154 | 153 > 123 | 35 | 10 | 153 > 95 | 35 | 25 |
| 2 | Tyrosol | 138 | 137 > 106 | 40 | 15 | 137 > 119 | 40 | 15 |
| 3 | Homovanillic acid | 182 | 181 > 137 | 25 | 10 | 181 > 122 | 25 | 15 |
| 4 | *p*-coumaric acid | 164 | 163 > 119 | 35 | 10 | 163 > 93 | 35 | 25 |
| 5 | 3,4-DHPEA-EDA | 319 | 319 > 195 | 40 | 5 | 319 > 183 | 40 | 10 |
| 6 | Luteolin | 286 | 285 > 133 | 55 | 25 | 285 > 151 | 55 | 25 |
| 7 | Pinoresinol | 358 | 357 > 151 | 40 | 30 | 357 > 136 | 40 | 25 |
| 8 | *p*-HPEA-EDA | 303 | 303 > 285 | 30 | 5 | 303 > 179 | 30 | 5 |
| 9 | Acetoxypinoresinol | 415 | 415 > 151 | 45 | 15 | 415 > 235 | 45 | 15 |
| 10 | Apigenin | 270 | 269 > 117 | 60 | 25 | 269 > 151 | 60 | 25 |

Abbreviations: MW, molecular weight; SRM, selected reaction monitoring mode; V, voltage; eV, voltage of collision.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 4.4. Computerised applications for interventional studies

Access to the computer application was via www.sortink.com. From this website, and by means of an e-mail and password, the user accessed his own site where all the projects involved could be seen. The information was provided in a table format that facilitated the location of any project. In addition, it was possible to check on the general status of any project more easily.

This section presented the logo, title, key name, current phase, state (open or closed to modifications), leader, start-date and deadline of each project.

The home page facilitated editing, deleting, working on a project, or adding a new project to those already on the site. When a new project was created it was necessary to write a brief description, similar to an abstract, and to specify the study duration together with phases of the study, the teamwork involved, and applying the CONSORT guidelines.

At this stage, our software application (REUS-INIS) provided the opportunity to create a free project without structure, or to work within the predefined structure of the CONSORT statement.

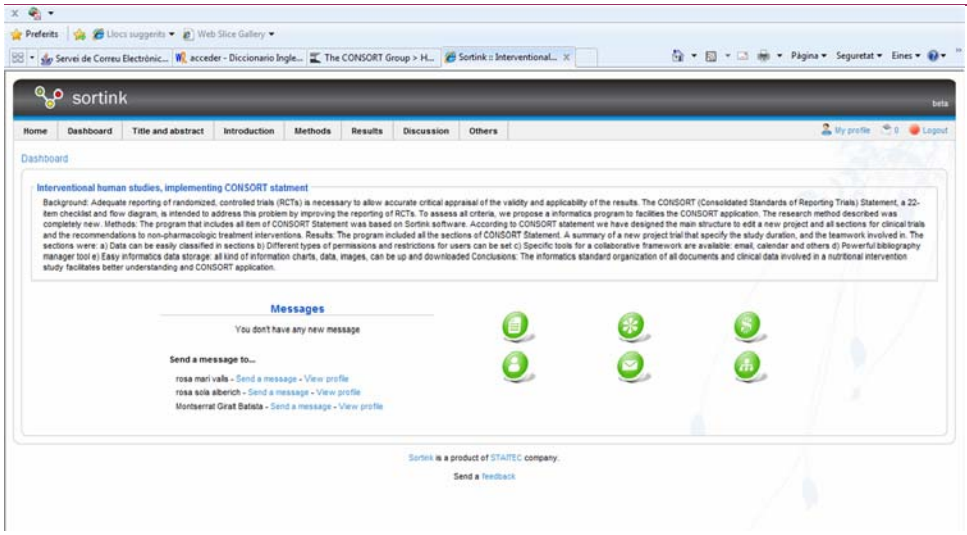
This predefined structure contained the 22-item checklist and flow diagram of the CONSORT statement, together with some specific issues that apply to trials of non-pharmacologic treatments (for example, surgery, technical interventions, devices, rehabilitation, psychotherapy, and behavioural intervention) that were not specifically addressed in the CONSORT statement (Boutron I, 2008).

Briefly, the sections were described in Figure 15.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 15.** Predefined structure according to the CONSORT statement

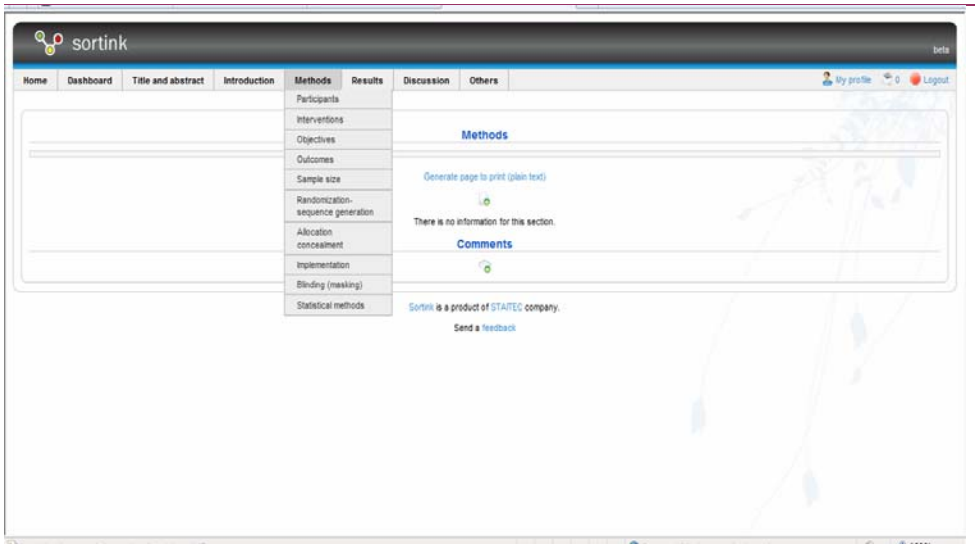



- Title and abstract. Specify the information to the section and the recommendation to use the CONSORT guide in writing the Abstract.
- Introduction
- Methods. This section was divided into 11 submenus: participants, interventions, objectives and outcomes, sample size, randomisation-sequence generation, allocation, concealment, implementation, blinding (masking) and, statistical methods (showed in Figure 16).
- Results. The section was divided into 8 submenus: participant flow (with flow diagram file available), implementation of intervention, recruitment, baseline data, numbers analysed, outcomes and estimation, ancillary analyses and, adverse events (showed in Figure 17).
- Discussion. This was structured in 3 parts: interpretation, generalisability and overall evidence.
- Others. This section allows admittance to the bibliography, calendar and events, finances, members, messages, and project files.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 16.** Methods section and submenus

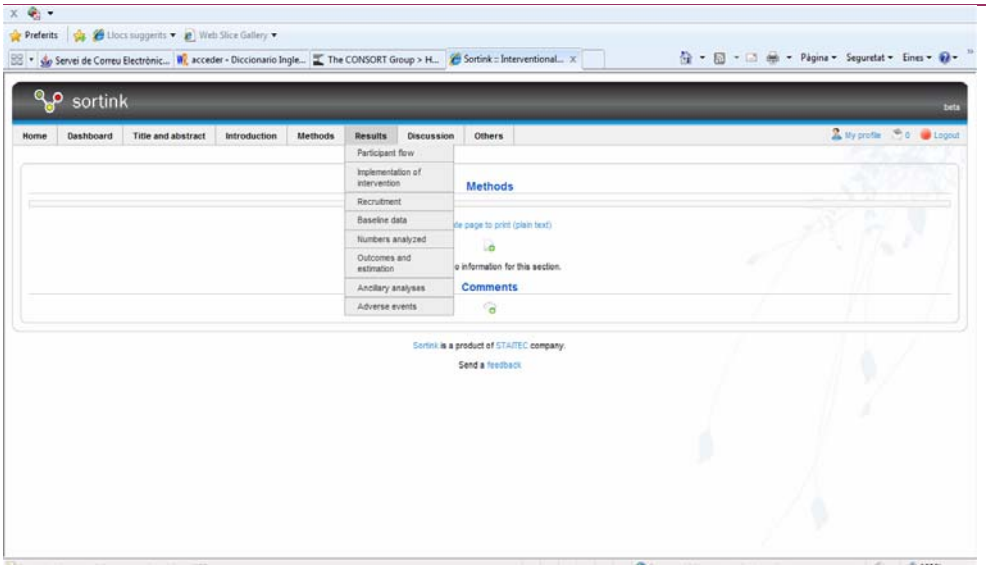



This software facilitated follow-up and monitoring of information with a system of document searches. When uploading information on any project, Sortink solicited keywords related to the document. With the built-in keywords, the software application creates a database. It was possible to find any key-word related document from the Sortink homepage.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Figure 17.** Results sections and submenus




REUS-INIS structure enabled reports to be created systematically and transparently. The current status of the study (data being analysed, documentation being prepared, article in preparation, report submitted, article completed). It is necessary to regularly verify the bibliography because the guidelines from the CONSORT statement are constantly being updated. REUS-INIS enables updating and modifying at any time in any of the sections within the initial structure of the project.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

# DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



### 5.1. General comments

In our dietary-intervention studies, we found that the cocoa products and a soluble fibre (Po-husk) when included in a low saturated fat and low cholesterol diet, produced beneficial effects on traditional risk factors and biomarkers of CVD (specific beneficial effects of these products on biomarkers are in the Detailed Comment section).

The results suggest that applying these dietary strategies could have value in clinical practice, and that these diets can be individualised according to personal preferences as well as to metabolic requirements.

Further, these products were safe with little or no adverse effects.

The cocoa cream products we evaluated and the Po-husk can modify a series of circulating, functional, structural, and genomic biological markers that reflect arterial vulnerability, and which have been proposed as potential novel risk factors in the development of CVD (Vasan R, 2006). Among the biomarkers, those reflecting oxidation (Fraley A, 2006), inflammation (Ferri N, 2006), and endothelial dysfunction (Deanfield J, 2007) have received increasing attention.

Thus, the present work provides further scientific evidence, based on data from the RCTs we conducted, to support the beneficial effects of certain dietary modifications on cardiovascular health. The products we evaluated have been patented.

### 5.2. Diet compliance and monitoring

The rates of adherence or compliance to dietary recommendations during interventional periods were high as were the adherence levels in cocoa cream product and Po-husk consumption.

Dietary changes remain the cornerstone for prevention and treatment of CVD. The landmark Seven Countries Study (Keys, 1980) showed associations between high SFA and cholesterol content of the diet and increases in serum cholesterol concentrations and the risk of CVD in cohorts from different countries.

In our studies, a low saturated fat and low cholesterol diet was the diet into which we incorporated the ingredients or products that we wished to investigate.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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ISBN:978-84-692-9052-1/DL: T-2070-2009



The diet was isocaloric to keep bodyweight constant since weight reduction induces similar metabolic changes such as those we observed with our diet modifications, or with natural product additions.

Control of the participant's diet is a key point with respect to consistency of results; an aspect that is frequently poorly attended-to. In our studies, diet compliance was monitored using 3-day dietary records, interviews with the nutritionist in all clinical visits to reinforce the dietary requirements and, in the additional weeks of the intervention period, the participants were interviewed by telephone without warning, and invited to fill-in a 24 hours dietary recall.

In the control of diets, methodological improvements are crucial and biomarkers of exposure or consumption of a food item or ingredient need to be identified and to be measured reproducibly.

### 5.3. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD

In the present study, cocoa products were manufactured to contain a combination of ingredients such as fatty acids, sugar, hazelnuts, phytosterols and soluble fibre and presented as a palatable cream. Product D, in particular, includes the ingredients of the "Portfolio" diet, although the amount of each constituent is different. For example, in the present studies, soluble fibre in each of 13g/unit is about 2.7g/unit and that supplied was 14 g/d; the impact in the diet is an increase of 8 g/d and provides about 30-35 g/d of total dietary fibre.

The cocoa alone product lowered SBP by -7.8 mm Hg and DBP by -5.4 mm Hg.

Compared with cocoa alone cream, cocoa plus hazelnuts and the Portfolio cream decreased DBP by -3.38 and -3.89 mm Hg, respectively.

The SBP reduction obtained in the present study was similar to that usually achieved with pharmacotherapy using β-blockers (-5 mmHg) or ACE inhibitors (-8 mm Hg) (Morgan T, 2001).

A small amount of dark chocolate daily (6 g) and 30 mg of polyphenols in the evening significantly reduced mean SBP by -2.9 ± 1.6 mm Hg and DBP by -1.9 ± 1.0 mm Hg with no changes in bodyweight, plasma lipid levels, glucose, and 8-isoprostane (Taubert D, 2007). In our study, while the 4 cream products contained

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



the same amount of cocoa and polyphenols, the overall compositions were different, and implied an impact of the food matrix composition. Lifestyle factors are widely recognised to affect BP and/or cardiovascular disease risk. Recommendations are to quit smoking, reduce weight (and to stabilise weight), reduce excessive alcohol intake, increase physical exercise, reduce salt intake increase fruit and vegetable intake, and to reduce saturated fat and total fat intake (Krauss R, 2000 and Mancia G, 2007). Over the past decade, increased potassium intake and dietary patterns based on the DASH diet (a diet rich in fruits, vegetables, and low-fat dairy products, with a reduced content of dietary cholesterol as well as saturated and total fat) (Sacks F, 2001) have been shown to have BP lowering effects, as well. Several small clinical trials and subsequent meta-analyses have documented that high-dose n-3 supplements (derived from fish oils) can lower BP in hypertensive individuals; albeit the effects can usually be achieved only at relatively high doses (3 g/d) (Dickinson H, 2006). Reduced intake of SFA is widely recommended for prevention of CVD. The type of macronutrient that should replace SFA in the diet has been evaluated in the OMNIHEART study. In the setting of a healthy diet, partial substitution of carbohydrate with either protein or MUFA can further lower BP, improve lipid levels, and reduce estimated CVD risk (Appel L, 2005). However, the percentage of the different types of fatty acids to be recommended remains unclear.

The LDL-c reductions observed (control cream vs cocoa plus hazelnuts and phytosterols of -11.8% vs Portfolio of -10.1%) are of similar magnitude to that of sterols and stanols (2 g/d) or of Po-husk (14 g/d). Further, these 2 cream products reduce Apo B/Apo A ratio to 0.645 and 0.630, respectively.

Apo B/Apo A ratio has been shown to be the best marker of atherogenic and anti-atherogenic particles in plasma. The INTERHEART study, a large international case-control study of myocardial infarction risk factors (McQueen M, 2008) found that the Apo B/Apo A ratio was a better indicator of CVD risk than the best conventional LDL-c measure, including other ratios (McQueen M, 2008 and Walldius G, 2004). Among the negative studies is a report from the FHS which, despite the rigorous statistical evaluation of new biomarkers including discrimination, calibration, and reclassification, failed to support the value of Apo

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



B/Apo A ratio as a marker of CVD risk. If clinical trials confirm that values for the Apo B/Apo A ratio are better than those of LDL-c to reflect the effect of therapy, then treatment with diet, exercise and drugs should seek to reduce the Apo B/Apo A ratio, preferably to < 0.7 (McQueen M, 2008 and Walldius G, 2006). This is the case for our 4 cream products evaluated, especially the Portfolio cream product.

In our study, the cocoa plus hazelnuts and phytosterols cream reduced this ratio by about -11.8% and Portfolio by -10.5%, which was mainly due to a moderate reduction in plasma Apo B-100. This could be clinically relevant in view of the results of the INTERHEART study in which the mean difference in Apo B/Apo A between CVD cases (0.85) and controls (0.80) was 6%.

Compared to the control cream, Portfolio reduced oxLDL by -6%. The added ingredient of soluble fibre reduced oxLDL plasma concentrations by about -8%. Also, cocoa appears to inhibit LDL oxidation (Kondo K, 1996) in healthy subjects following a daily consumption of 75 g polyphenol-rich dark chocolate over 3 weeks (Mursu J, 2004). A Japanese study demonstrated that, in hypercholesterolaemic patients, flavanol-rich cocoa lowers plasma levels of oxLDL (Baba S, 2007). The antioxidant lipid effect may be the result of interactions between these ingredients.

Compared with control, Portfolio reduced hsCRP by -33.4%, which suggests an antiinflammatory effect of this product.

The effect of the food matrix is a relevant aspect. When one or more bioactive ingredients are incorporated in a fat or protein or CHO food item, the final product can maximise beneficial effects, or minimise adverse effects or vice versa.

The cocoa products studied induced different beneficial effects on CVD biomarkers. For example, cocoa alone cream produces significant reduction in SBP and DBP while the cocoa plus hazelnuts and phytosterols cream reduced LDL-c and Apo B/Apo A ratio. The Portfolio cream lowered LDL-c, Apo B/Apo A ratio, DBP, oxLDL and hsCRP and, as such, induced an even more beneficial CVD biomarker profile. The different outcomes achieved can be attributed to the variation in cream product matrix. Long-term studies on these cocoa cream products, particularly the Portfolio, are currently underway.

It is important to differentiate between natural products that include *cacao* and the processed product *chocolate*; the latter is a combination of cocoa, sugar, milk and

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



other ingredients into a solid food product. Hence, the benefit of the cocoa intake needs to be traded-off against sugar, fat and calorie count if a high consumption of the product is to be legitimately encouraged (Taubert D, 2007).

A critical aspect is bodyweight control when cocoa products are consumed since overweight and obesity are associated with insulin resistance and the metabolic syndrome (ATP III, 2001).

In the present study, the quantity of cocoa present in each of the cream products is similar i.e. about 1 g/unit with 10 mg/unit of phenolic compounds within a food matrix composed of 10% of SFA. The control cream provided about 10% MUFA and 22% of PUFA. The inclusion of hazelnuts in the 3 other cream products changed the MUFA contribution to 24% and PUFA to about 10%. The sugar content was 50% or less of total energy.

Although cocoa has a composition that suggests healthy cardiovascular effects, the cocoa cream products that were created especially for the current evaluation need more detailed composition description.

Our study has several limitations. The intake of the cocoa cream products was over a short period and did not focus on clinical outcomes. Determining the appropriate daily intake of the products remains to be defined. Information on adherence to diet in our free-living study individuals was limited. To find an appropriate product to use as control remains undefined since, as our results showed, even cocoa cream alone, although effective as control for LDL-c plasma level outcome, had a BP lowering effect.

### 5.4. Effects of treatment with a soluble fibre (Po-husk) on biomarkers of CVD

Po-husk reduces LDL-c. In the present study, soluble fibre (Po-husk) lowered TG, Apo B-100, oxLDL and SBP. Po-husk has been described as lowering plasma TG concentrations, but new beneficial effects have been added. In the proprietary formulation used in this study and administered at 14 g/d for 8 weeks to individuals with mild-moderate hypercholesterolemia, Po-husk induced a mean reduction of 6.1% in LDL-c plasma concentration. Further, 24.6% more Po-husk consumers achieved the target 5% reduction of LDL-c compared to placebo. Similarly, Po-husk lowered plasma TC by 6.4% and Apo B-100 by 6.2%.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



A considerable lowering (16%) of plasma TG was observed and, when compared with placebo, this reduction with Po-husk was 21%. The magnitude of the TG-lowering effect is clinically significant since the reduction was similar to that which can be obtained by 1200 mg/d nicotinic acid (17-26%); an agent that has several side effects (Brunzell J, 2007).

Our data suggest that Po-husk has beneficial effects on plasma lipids when an appropriate daily dose is administrated (Jenkins D, 2003). In patients who are intolerant to hypocholesterolaemic agents (Dale K, 2007), Po-husk therapy can be considered as an alternative for the treatment of hypercholesterolaemia.

Additionally, Po-husk lowered the concentration of oxLDL in plasma and, as such, improves the patient's status with respect to the commonly-used marker of oxidative damage involved in CVD (Fitó M, 2007 and Meisinger C, 2005), and in MetS (Holvoet P, 2008).

In our study, Po-husk treatment, as part of a low salt intake, reduced SBP by -3.7 mmHg. This effect was greater than the non-significant -1.8 mmHg achieved in a study involving 12 weeks of water-soluble fibre (8 g/d) derived from oat bran (He J, 2004).

BP reduction could be related to the lowering of oxLDL observed, since oxidative stress decreases NO availability and increases BP (Ceriello A, 2008; Landmesser U, 2007 and Guxens M, 2009). Thus, the reduction in the degree of oxLDL may contribute, at least in part, to the decrease in BP observed in our study.

The magnitude of the hypotensive effect of Po-husk is clinically significant; the BP reduction of -3.7 mmHg being close to that usually achieved with pharmacotherapy using β-blockers (-5 mmHg). However, β-blockers are known to have more side effects, such as exacerbation of asthma and bronchospasm (Morgan T, 2001).

Po-husk reduced plasma TG concentration, SBP, insulin concentration (Tuan C, 2003) and HOMA-index. These beneficial effects suggest a possible role for Po-husk in improving MetS clinical status.

In our study, Po-husk consumption did not influence inflammation biomarkers of CVD, as has been described previously for other soluble fibres (North C, 2009). Neither did Po-husk modify biomarkers of endothelial dysfunction.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



The design of the present study was the sequential (step-by-step) management of individuals in a program of prevention of CVD i.e. "therapeutic lifestyle change" over 6 weeks and, if LDL-c remained ≥ 3.35 mmol/L, a cholesterol-lowering drug to be prescribed. Our strategy was that, following 8 weeks post-fibre treatment, participants who still had LDL-c > 3.35 mmol/L received 20 mg/d of simvastatin for a further 8 weeks. At 16 weeks, the intra-group combined action of Po-husk and simvastatin showed similar LDL-c reduction as did placebo plus simvastatin. However, in the study by Moreyra A (2005), when 15g/d of soluble fibre and 10 mg/d of simvastatin were administered simultaneously for 8 weeks, the LDL-c concentration reduction achieved was similar to that achieved by 20 mg/d simvastatin alone; the type of diet applied was not specified (Moreyra A, 2005). A possible explanation is that the low dose simvastatin (10 mg/d) can produce an additive effect with soluble fibre while 20 mg/d simvastatin, being much more effective in reducing LDL-c concentration, masks the cholesterol-lowering effect of the fibre.

In our study, despite the same dietary intake being prescribed to all participants, the Po-husk group had, at 8 weeks, consumed about 2% less lipids; albeit with the same amount of SFA consumption in both groups. This aspect implies a Po-husk palatability effect. The possible consequences on bodyweight need to be explored in future studies. This cannot be assessed in the present study because the intervention protocol ensured that the participants maintained constant bodyweight.

The level of significance of the data was confirmed by the PP population assessment. Po-husk intake was safe and the prevalence of adverse events was similar to placebo.

The gene polymorphisms studied showed no relationships with the reduction of LDL-c by Po-husk. However, in the Po-husk group, the TG-lowering effect was magnified, such that the response was about 30-50% in individuals homozygous for G and DEL variants of PAI-1 gene, and 20-50% in homozygote A and G variants of the FABP-2 gene. If the high frequency of these gene variants of about 0.5 is confirmed in a general population, Po-husk consumption could induce an optimised plasma TG lowering response.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



Our study has several limitations. The Po-husk intake was over a short period and did not focus on clinical outcomes. The appropriate daily dose of soluble fibre remains to be defined. Information on adherence to diet in our free-living study individuals was limited.

Clinically relevant findings associated with Po-husk treatment are a result of a combination of effects on more than one of the CVD pathways. Thus, the candidate patients to receive Po-husk can be those who present a cluster of various CVD risk factors, especially if they have drug intolerances as well. Po-husk reduces LDL-c, Apo B-100, oxLDL, insulin and triglyceride concentrations, as well as SBP in individuals with mild-moderate hypercholesterolaemia.

### 5.5. Olive oil polyphenols detection in human plasma

Fasting plasma from VOO consumers showed higher concentration of a flavonoid type (2.90 ± 0.04 μmol/L vs 1.5 ± 0.04 μmol/L) and catechol derivative (0.70 ± 0.03 μmol/L vs 0.56 ± 0.03 μmol/L) than the plasma from non-consumers ($P$<0.05). This suggests an indirect protective mechanism of long-term VOO consumption with respect to the endogenous antioxidant system.

However, the comparative analysis of the chromatographic profiles of the phenolic extracts of plasma samples between the habitual consumers and non-consumers of VOO showed no significant qualitative differences.

Conversely, the phenolic compounds of VOO having the highest LDL antioxidant effect *in vitro* were detected in human postprandial plasma. These preliminary data on their presence in plasma is the first step in explaining their effects on CVD biomarkers.

The measurement of phenolic compounds in postprandial plasma required the development of more precise detection methodology and, in this respect, constitutes an important contribution to the identification and quantification of VOO phenolic compounds in human plasma.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 5.6. Computer technology applied in interventional studies

Informatic's application (Reus-INIS) was introduced to improve the implementation the CONSORT statement in nutritional intervention studies and to increase appropriate reporting levels. It is clear that the CONSORT statement aims to increase the quality of reports of randomised trials. However, despite the wide dissemination of the CONSORT statement, inadequate reporting remains common. The difficulties in implementing the CONSORT statement criteria and their implications in publications are clear (Hopewell S, 2008; Berwanger O, 2009 and EQUATOR Network). In the case of a commercial enterprise that contracts a research group to conduct a trial, this software application facilitates the follow-up and clinical evolution of the trial.

Without wide endorsement, the CONSORT statement cannot fully yield the benefits for which it is intended.

Our purpose has been to adapt the CONSORT statement to a computer tool to facilitate the work of the researcher at the time of reporting a study. The aim is to help the researcher to focus on specific, and added value, tasks by appropriately organising the information. Similar adjustments could be applied to other existing guidelines of quality such as Standards for Reporting of Diagnostic Accuracy (STARD) and Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

### 5.7. General

The effects of the products studied provided new data on biomarkers involved in a complex pathology. Atherosclerosis has evolved beyond the original concept of accumulation of cholesterol in the arterial wall. A series of circulating, functional, structural, and genomic biological markers that reflect arterial vulnerability have been proposed as potential novel risk factors for the development of CVD (Vasan R, 2006). Among them, biomarkers of oxidation (Fraley A, 2006), inflammation (Ferri N, 2006), and endothelial dysfunction (Deanfield J, 2007) have received increasing attention.

The different effects on these biomarkers of products under study in the present body of work are summarised in Table 42.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



**Table 42**. Summary of the effects on novel biomarkers of CVD risk of the dietary compounds studied

| Effect | Soluble fiber (Po-husk) | Portfolio cream product |
|---|---|---|
| Loweried LDL-c | ●●● | ●●● |
| Reduced TG | ●●● | |
| Reduced Apo B/Apo A | | ●●● |
| Reduced oxidative stress | ●●● | ●●● |
| An antiinflammatory effect | | ●●● |
| Reduced Insulin resistance | ●●● | |
| Reduced BP | ●●● | ●●● |

Abbreviations: LDL-c, low density lipoprotein cholesterol; TG, triglycerides; BP, blood pressure.

Knowledge of inter-individual genetic variation is transforming the approach not only towards investigating nutritional mechanisms but also towards personalising nutritional advice tailored to individuals or population subgroups (Ordovas J, 2008). This is relevant for major dietary macronutrients and, as recently described, for micronutrients such as vitamin A, selenium and zinc (Lietz G, 2009).

In the future, because of the somewhat disappointing information to-date regarding single biomarkers, the clinical application of multi-marker panels may prove to be a promising approach (Zethelius B, 2008). The possibilities for improving the model are greater, provided that such variables show low correlations with conventional risk factors and with each other, but provide strong associations with clinical events. Such emerging biomarkers will need to be rigorously evaluated for their clinical efficacy and effectiveness in large cohorts or RCTs, with innovative statistical analytical tools. The world of proteomics, metabolomics and epigenetics, together with advanced imaging modalities such as functional molecular imaging, may offer such promising candidates.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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### 5.8. Overall limitations

One limitation is the short-period of intervention. Monitoring of the intervention diet was an indirect method; albeit the current method-of-choice. The products studied will need to be assessed over a longer term if a reduced risk of CVD events is to be demonstrated.

UNIVERSITAT ROVIRA I VIRGILI
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# CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
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Rosa Maria Valls Zamora
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UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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### 6.1. Effects of dietary supplements of 4 different cocoa cream products on plasma lipids, blood pressure and other biomarkers of CVD

The cocoa products studied induced different beneficial effects on the measured CVD biomarkers.

The cocoa alone product lowered SBP by -7.8 mm Hg and DBP by -5.4 mm Hg. Compared with cocoa alone cream, cocoa plus hazelnuts and the Portfolio cream decreased DBP by -3.38 and -3.89 mm Hg, respectively. The cocoa plus hazelnuts plus phytosterols (product C) reduced LDL-c and Apo B/Apo A ratio. The Portfolio (product D) reduced LDL-c, Apo B/Apo A ratio, DBP, oxLDL and hsCRP and, as such, induced the most beneficial CVD biomarker profile. The cocoa product chosen to be included in a low saturated fat would depend on the individual patient's CVD risk factor profile.

### 6.2. Effects of treatment with a soluble fibre (*Plantago ovata* husk) on biomarkers of CVD

Po-husk, a soluble fibre, was effective in lowering LDL-c, Apo B-100, oxLDL, insulin-resistance, gene-variant related TG levels, and SBP in subjects with moderate hypercholesterolaemia.

Thus, the target patients to receive Po-husk would be those who present a cluster of several CVD risk factors.

### 6.3. Olive oil polyphenols detection in human plasma study

#### 6.3.a. Olive oil polyphenols detection in human plasma from habitual VOO consumers

Fasting plasma from VOO consumers showed higher concentration of a flavonoid type and catechol derivative than the plasma from non-consumers. No specific phenolic structure related to VOO ingestion was detected in the fasting plasma from habitual VOO consumers. Of special note, however, is that the compounds quantified were at a higher concentration in the fasting plasma from habitual VOO consumers.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
ISBN:978-84-692-9052-1/DL: T-2070-2009



### 6.3.b. Olive oil polyphenols detection in human postprandial plasma

The novel identification of 3,4-DHPEA-EDA in postprandial human plasma, together with confirmation of luteolin, hydroxytyrosol and oleuropein (in the aglycone form), support the *in vivo* antioxidant potential of these compounds derived from VOO.

### 6.4. Computer technology applied in interventional studies

This research tool facilitates the implementation of the CONSORT statement and its related extensions, to improve the quality of reports of randomised trials and, most importantly, to enable authors to comply with the guidelines which are becoming a prerequisite for publication of data in reputable journals.

### 6.5. Summary of conclusions:

1. The cocoa cream products induced beneficial effects on several CVD biomarkers.
   The soluble fibre Po-husk lowered LDL-c; an effect that is beneficial among CVD risk factors.
2. The food matrix is a key aspect in optimising the effects of food items on CVD biomarkers.
3. The identification of VOO-derived phenolic compounds in human plasma is a first step in defining their dose-benefit effects on health.
4. The information technology application in standardising the organisation of all documents and clinical data involved in a nutritional intervention study facilitate better application of the CONSORT statement.

UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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### 6.6. Overall conclusion

We conclude that cocoa cream products which contain a combination of cardio-protective ingredients, particularly the Portfolio type cream, as well as Po-husk, when part of a low saturated fat and low cholesterol content diet, induced not only a moderate reduction in plasma LDL-c, but also had beneficial effects on other CVD biomarkers and risk factors. These products can improve the overall efficacy of diets designed to lower CVD risk.

The VOO study was able to detect 3,4-DHPEA-EDA, a new phenolic compound in human plasma.

Finally, the incorporation of cocoa products or soluble fibre or VOO or all in combination in a low saturated fat and low cholesterol diet could have considerable application in therapeutic dietary recommendations based on the individual's CVD risk factor profile.

UNIVERSITAT ROVIRA I VIRGILI
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Rosa Maria Valls Zamora
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UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
Rosa Maria Valls Zamora
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UNIVERSITAT ROVIRA I VIRGILI
DIETARY EFFECTS ON CARDIOVASCULAR DISEASE RISK BIOMARKERS
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# APPENDIX A: Contributions at Congresses and Conferences

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## POSTERS:

**Valls RM**, Pérez G, Godàs G, Solà R. **La incorporación de cacao en una dieta cardiosaludable no modifica el peso corporal.** III Congreso de la Asociación Española de Dietistas-Nutricionistas, I Congreso Hispano-Luso de Alimentación, Nutrición y Dietética. Madrid, 19-21 October 2006.

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UNIVERSITAT ROVIRA I VIRGILI
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Romeu M, **Valls RM**, Albaladejo R, Sánchez-Martos V, Catalán U, Fernández S, Aranda N, Espinel A, Delgado MA, Arija V, Giralt M, Solà R. **Marcadores de estrés oxidativo relacionados con las enfermedades cardiovasculares en humanos sanos.** XXII Congreso Nacional de la Sociedad Española de Arteriosclerosis. Pamplona, 26-29 May 2009.

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**Valls RM**, Macià A, Girona J, González C, Heras M, Romero MP, Covas MI, Motilva MJ, Masana L, Solà R. **Detection of phenolic compounds with verified in vitro LDL antioxidant activity in postprandial human plasma following a virgin olive oil oral load.** II Congreso Internacional sobre aceite de oliva y salud. Córdoba-Jaen, 20-22 November 2008.

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# APPENDIX B: Scientific papers

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Arija V, Giralt M, Solà R, **Valls RM,** Albaladejo R, Simón D, et al. **Programa EDAL de Educación en Alimentación: Enseñar aprendiendo. Promoción de estilos de vida saludables para prevenir la obesidad infantil.** Rev Esp Nutr Comunitaria. 2007; 13:46-47.

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**Valls RM**[*], Soler A[*], Girona J, Heras M, Romero MP, Covas MI, Motilva M, Masana L, Solà R. **Effect of the long-term regular intake of virgin olive oil on the phenolic metabolites in human fasting plasma** (editor submitted).

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Suárez M, Romero MP, Macià A, **Valls RM**, Fernández S, Solà R, Motilva MJ. **Improved method of microelution solid-phase extraction plate and liquid chromatography– tandem mass spectrometry to identification and quantification of olive oil phenols in human plasma** (editor submitted).

Solà R, **Valls RM**, Godàs G, Pérez G, Ribalta J, Girona J, Pons L, Heras M, Cabré A, Castro A, Masana L, Anglès N, Reguant J, Ramírez B, Barriach JM. **Effects of dietary supplements of cocoa cream on cardiovascular risk factors. A Randomized Trial** (editor submitted).

**Valls RM**, Solà R, Romeu M, Giralt M, Aragonès J, Gastó JM, Gómez X. **Informatics of nutritional intervention studies: Implementing CONSORT statement** (editor submitted)

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