TESIS DOCTORAL

Ácidos grasos omega 3, oleico y vitaminas B_6, B_9 y E.

Influencia sobre los marcadores de riesgo cardiovascular en sujetos sanos, con dislipemia moderada y con enfermedad vascular periférica.

Juan Jesús Carrero Roig
Granada, 2005

Editor: Editorial de la Universidad de Granada
Autor: Juan Jesús Carrero Roig
D.L.: Gr. 618 - 2005
ISBN: 84-338-3323-5

Ácidos grasos omega 3, oleico y vitaminas B_6, B_9 y E. Influencia sobre los marcadores de riesgo cardiovascular en sujetos sanos, con dislipemia moderada y con enfermedad vascular periférica.

Memoria que presenta el Ldo. Juan Jesús Carrero Roig para aspirar al grado de Doctor en Farmacia.

Los trabajos de investigación que se exponen en esta Memoria de Tesis Doctoral han sidos realizados en el Departamento de Nutrición y Salud de Puleva Biotech S.A. bajo la dirección de los doctores D. Eduardo López-Huertas León y D. Luis Baró Rodríguez, bajo la tutela de la Dra. C.U. María Dolores Suárez Ortega, del Departamento de Bioquímica y Biología Molecular de la Universidad de Granada y en colaboración con la Universidad de Southampton, Reino Unido.

Dr. Eduardo López-Huertas León

Dr. Luis Baró Rodríguez

Dra. María Dolores Suárez Ortega

Universidad de Granada.
Departamento de Bioquímica y Biología Molecular
Departamento de Nutrición y Salud, Puleva Biotech S.A.
Institute of Human Nutrition, University of Southampton, UK

1

Introducción, justificación y objetivos.

Aterosclerosis, enfermedad cardiovascular y factores de riesgo.

La aterosclerosis es una enfermedad progresiva caracterizada por la acumulación de lípidos en las capas íntima y media arterial que terminan por invadir la luz de las arterias dificultando la llegada de la sangre a los tejidos irrigados (*isquemia*). Esta isquemia se acompaña de un dolor muy intenso (*angina*), provocado por el exceso de lactato producido durante la anaerobiosis debida a la falta de oxígeno.

Cuando una de las placas fibrosas se lesiona, aparece el riesgo de formación de trombos y coágulos. La trombosis en la zona de las placas de ateroma puede provocar la oclusión del vaso. Cuando la oclusión es completa, cesa la irrigación del tejido, que se necrosa (*infarto*). El ateroma puede producir también un debilitamiento progresivo de la pared arterial, que se dilata (*aneurisma*), facilitando su rotura y consiguiente hemorragia.

Las consecuencias clínicas de la aterosclerosis pueden ser muy variadas, tanto más dependiendo de las arterias en que se forme la placa de ateroma; Cuando afecta a las arterias coronarias, constituye el proceso patológico subyacente en la cardiopatía isquémica, que incluye la angina de pecho y el infarto de miocardio; Cuando afecta a las arterias cerebrales, se produce ictus o apoplejía, caracterizado por la pérdida de la funcionalidad relacionada con la región afectada (parálisis motora, pérdida de memoria...etc.); Cuando afecta a la aorta, es frecuente la formación de aneurismas voluminosos, donde se facilita la formación de trombos, que pueden desprenderse y afectar a otras zonas adyacentes (*embolia*); Cuando la oclusión afecta a las extremidades inferiores (*enfermedad vascular periférica*), se produce una pérdida de funcionalidad durante el ejercicio que cede al descansar, cuando disminuye la demanda de oxígeno (*claudicación intermitente*) (1).

La aterosclerosis es la patología que habitualmente subyace a la enfermedad cardiovascular (ECV), que se desarrolla silenciosamente a lo largo de los años y que suele estar muy avanzada cuando aparecen los síntomas. Actualmente, la ECV constituye la primera causa de muerte en todos los países del mundo, a excepción de aquellos más pobres y de las regiones de África afectadas de SIDA. En el año 2002 fue responsable de aproximadamente 16.7 millones de defunciones, erigiéndose como uno de los grandes retos médicos del s. XXI, dada su gran y creciente prevalencia, su grave pronóstico, y la enorme carga que supone en consumo de recursos sanitarios (2).

En España observamos una tendencia parecida: la ECV permanece como primera causa de muerte en nuestro país representando, según las estimaciones del 2002, el 34,1% del total de defunciones. Dentro de este grupo, las enfermedades isquémicas del corazón (infarto agudo de miocardio, angina de pecho, etc.) se mantienen como la primera causa de muerte, con 39.400 fallecidos (**tabla 1**) (3-5). Se estima que su impacto sobre la salud, medido por el número de enfermos y el uso de servicios sanitarios, aumentará en los próximos años debido

al envejecimiento de la población. Por ello, es de máxima prioridad desarrollar estrategias encaminadas a incidir sobre las causas que desencadenan este tipo de enfermedades, tanto a nivel poblacional por las administraciones sanitarias públicas, como sobre los pacientes (6).

Tabla 1. Número de defunciones según las principales causas de muerte y sexo. INE, España 2002.

	Total	Varones	Mujeres
Todas las causas	368.618	193.269	175.349
Enf. isquémica del corazón	39.400	22.281	17.119
Enf. cerebrovascular	35.947	14.929	21.018
Insuficiencia cardiaca	18.986	6.336	12.650
Cáncer de bronquios y pulmón	18.095	15.979	2.116
Enf. crónicas de vías respiratorias	16.841	12.479	4.362

Aún no se ha podido establecer una causa precisa de la ECV. De lo único que podemos tener certeza, es que la ECV aparece cuando confluyen un número suficiente de factores desencadenantes o factores de riesgo. Los estudios epidemiológicos de los últimos 50 años, que se iniciaron con el estudio *Framingham* (7), identificaron a la diabetes, la hipertensión, el tabaquismo y las dislipemias como factores de riesgo independientes de la ECV. Posteriormente, la investigación experimental dio a conocer otros factores, como las alteraciones en el metabolismo del fibrinógeno, de la homocisteína, de las endotelinas...etc. La investigación de las últimas décadas se centra en la fuerte asociación de la ECV con problemas de tipo inflamatorio (8).

Aún hoy en día se siguen identificando nuevos factores de riesgo que afectan al desarrollo de la ECV, lo que hace difícil su agrupación y su clasificación. Tradicionalmente, estas evidencias epidemiológicas se vienen clasificando en función de si son corregibles o no y de la forma en que contribuyen a la aparición de la ECV.

Entre los factores no corregibles se encuentran la edad, los antecedentes familiares y el sexo. Este tipo de enfermedades son predominantemente masculinas. El estrógeno disminuye la concentración de colesterol unido a lipoproteínas LDL en grados variables según su relación con la progesterona, siendo esta una posible razón por la que las mujeres en edad de procrear sean menos propensas a las ECV (9,10). Entre los factores que pueden corregirse se encuentran el tabaquismo, la hipertensión, la obesidad, la diabetes, el tipo de alimentación, el estilo de vida y las dislipemias. Es en estos apartados donde las autoridades sanitarias inciden y aplican estrategias encaminadas a la prevención.

El avance en el conocimiento acerca de nuestra predisposición génica a la aparición de ciertas alteraciones y patologías nos permite hacer una clasificación más completa que incluye las evidencias experimentales, agrupándolas en factores genéticos o con un fuerte componente genético, y factores ambientales (**Tabla 2**).

Tabla 2: Factores genéticos y ambientales asociados a la aterosclerosis y a la ECV (11)

Factores con un componente genético	Factores ambientales
Niveles elevados LDL/ VLDL	Dieta rica en grasa saturada
Niveles reducidos de HDL	Tabaco
Niveles elevados de lipoproteína (a)	Bajos niveles de antioxidantes
Hipertensión	Sedentarismo
	Agentes infecciosos
Niveles elevados de homocisteína	
Antecedentes familiares	
Diabetes y Obesidad	
Factores hemostáticos	
Depresión y otros trastornos del comportamiento	
Sexo	
Inflamación sistémica	
Alteraciones Metabólicas	

Los factores genéticos determinan la susceptibilidad de un individuo a enfermar, mientras que los factores ambientales determinan que un sujeto de los genéticamente predispuestos, lo haga (12). En este sentido, el estilo de vida y los hábitos dietéticos son factores determinantes muy importantes. Los estudios en los aspectos evolutivos de la dieta indican que los cambios más importantes que han tenido lugar en nuestra dieta han ocurrido especialmente en el tipo y en la cantidad de grasa y de antioxidantes en los alimentos. Los cambios ocurridos en los últimos 100 años han contribuido de manera significativa a la elevada prevalencia de enfermedades crónicas como la aterosclerosis ya citada, la diabetes, la hipertensión arterial, la obesidad y determinados tipos de cáncer (13).

Aproximadamente la mitad de la población española presenta en la actualidad valores de colesterol en sangre elevados (más de 200 mg/dL). Sin embargo, la mayoría de las personas desconoce este hecho, e incluso la mayoría de los que tienen alto riesgo cardiovascular no recibe tratamiento hipolipemiante (14). Mediante una adecuada intervención sobre estos factores de riesgo modificables, sería posible contribuir a la disminución de la mortalidad cardiovascular.

Enfermedad cardiovascular y vascular periférica: una relación causal.

La iniciación de la lesión aterosclerótica en las extremidades inferiores define a la enfermedad vascular periférica (EVP). Los pacientes con EVP pueden cursar de manera asintomática o presentar claudicación intermitente, dolor isquémico en reposo y/o gangrena. Se ha estimado que aproximadamente el 12% de la población adulta padece esta enfermedad, y su incidencia aumenta con la edad, de manera que se calcula que un 20% de la población con más de 70 años la padece (15-17).

La sintomatología más frecuente en este tipo de pacientes es la claudicación intermitente, que está presente en un 15-40% de los enfermos sintomáticos (18). Se define como un dolor intenso producido por la isquemia que la placa de ateroma induce al andar. Este dolor

claudicante puede presentarse en una o en las dos piernas y desaparece con el reposo, al disminuir la demanda de oxígeno.

Una elevada proporción de enfermos de EVP acaban desarrollando un episodio de infarto u otro evento cardiovascular (19,20). Se calcula que los enfermos de EVP poseen un riesgo cardiovascular 5 veces mayor que sus controles (21-24). Este riesgo elevado, que parece ser independiente de los factores de riesgo clásicos (hiperlipemia, tabaquismo, obesidad…) y que solo se puede justificar de manera parcial mediante la asociación plausible de la EVP con la enfermedad cerebrovascular o coronaria, parece ser directamente proporcional a la severidad de la sintomatología que cursen (25).

La aterosclerosis que subyace en ambas enfermedades es la responsable de esta relación causal. El proceso aterosclerótico en sí debe entenderse como un proceso inflamatorio, y no únicamente como una acumulación pasiva de lípidos en la pared del vaso sanguíneo. Como tal, un gran número de estímulos (una molécula de LDL oxidada, radicales libres, agentes oxidantes) son responsables potenciales de esta cascada inflamatoria que desemboca en la formación de la placa de ateroma. La interacción de estos estímulos lesivos con el endotelio vascular desencadena una serie de cambios conocidos como "*activación endotelial*", que comprende la expresión de moléculas de adhesión y la síntesis de citocinas inflamatorias en la pared arterial (26).

Esta activación endotelial va a ser crucial en el desarrollo de la aterosclerosis, pues es responsable de la proliferación de las células del músculo liso, de la atracción de monocitos a la pared del vaso por acción de las quimiocinas y del proceso de diapédesis que los introducirá en la matriz subendotelial para que se diferencien en macrófagos. Allí, los macrófagos interaccionan con las LDL oxidadas, se transforman en células espumosas, e inician el desarrollo de la estría grasa que marca el comienzo de la oclusión del vaso (27). Cuando esta oclusión aparece en las extremidades inferiores, desarrolla lo que se denomina EVP. Cuando aparece en las arterias coronarias o en la aorta, desarrolla lo que se conoce como ECV.

El **Capítulo 6** del presente proyecto de tesis describe la relación causal entre ECV y EVP con más detalle, y muestra cómo, al compartir idéntico proceso patológico, comparten los mismos factores de riesgo. Ello lleva a sugerir que las estrategias de prevención válidas para la ECV pueden ser también efectivas en la EVP. Sin embargo, numerosos estudios en los últimos años han puesto de manifiesto la falta de acciones específicas encaminadas tanto a la paliación de los síntomas propios de la EVP, como a la prevención de los eventos coronarios que generalmente acaban desarrollando estos enfermos (24, 28-31).

Enfermedad y dieta

La relación entre componentes específicos de la dieta y ECV está bien establecida. Una dieta sana y equilibrada puede reducir el riesgo cardiovascular al incidir sobre factores de riesgo tales como el índice de masa corporal, el descenso de la presión arterial, la mejora del perfil lipídico, el control de la glicemia y la reducción de la predisposición de la trombosis. Los estudios epidemiológicos han puesto de manifiesto que los factores dietéticos son responsables de las grandes diferencias en la mortalidad cardiovascular de los distintos países (32). Sin embargo, la relación entre los componentes específicos de la dieta y la EVP no es tan robusta o al menos, por el momento, científicamente no tan evidente, a pesar de que la malnutrición sea bastante común en este tipo de pacientes (33).

El Estudio de los Siete Países (*Seven Countries Study*) fue el primero, en la década de los 50, en evidenciar la asociación dieta-ECV e introducir el concepto de Dieta Mediterránea. Este trabajo mostró por primera vez, que a pesar de una alta ingesta en grasas la población de la Isla de Creta (Grecia) tenía mayor longevidad y una muy baja incidencia de enfermedad coronaria y de ciertos tipos de cáncer cuando se comparó con países del Norte de Europa y los Estados Unidos. Desde entonces se consideró que el patrón dietético tradicional de la Isla de Creta, así como el del resto de Grecia y el Sur de Italia, era el posible responsable de la buena salud de que gozaban estas zonas geográficas (34).

A lo largo de los años siguientes, el patrón de Dieta Mediterránea fue considerado como un modelo de alimentación cardiosaludable. Sin embargo, existen más de 15 países bordeando el Mediterráneo, con una gran variabilidad en hábitos de vida, cultura y platos típicos (13). Faltaba encontrar la evidencia que mostrase las bondades de esta dieta y definir conceptos comunes que aglutinasen en una sola las dietas de estos 15 países mediterráneos.

Esta evidencia vino con los resultados del *Lyon Diet Heart Study* (35). 605 pacientes que habían sufrido un episodio de infarto se asignaron, de manera aleatoria, a una dieta tipo mediterránea o a una dieta control (basada en la dieta americana *tipo I*, caracterizada por: 8-10% de las calorías totales procedente de grasa saturada, 30% de las calorías totales o menos procedente de la grasa, menos de 300 mg de colesterol por día y las calorías mínimas para mantener un peso adecuado). Los pacientes adscritos a la dieta tipo mediterránea consumían una mayor cantidad de frutas, vegetales y pescado, y una menor cantidad de carne roja. Asimismo, se les recomendaba reemplazar la mantequilla y la nata por margarina rica en ácido a-linolénico, (para alcanzar el contenido en ácidos grasos poliinsaturados omega 3 que posee la dieta tradicional cretense). Tras 27 meses de seguimiento, la tasa de eventos coronarios se redujo en un 73% en este grupo, y la mortalidad total en un 70%.

Estos resultados multiplicaron en los años siguientes los estudios que trataban de encontrar denominadores comunes en las dietas de los países mediterráneos. Los numerosos

trabajos en el campo evidenciaron la presencia de una serie de nutrientes clave que se erigieron como principales contribuyentes al menor riesgo cardiovascular mediante una gran variedad de mecanismos de acción. Diversos organismos internacionales han enfatizado las ventajas de adoptar un patrón dietético mediterráneo, promoviendo la ingesta de los alimentos que la caracterizan y que han demostrado ser efectivos en la reducción del riesgo cardiovascular.

Uno de estos alimentos es el **aceite de oliva**, rico en *ácido oleico y antioxidantes* y máximo exponente de la Dieta Mediterránea. La evidencia científica sugiere que su consumo continuado es positivo para la salud en tanto en cuanto reduce los factores de riesgo de ECV y es capaz de modificar la respuesta inmune e inflamatoria. Las propiedades terapéuticas del aceite de oliva se atribuyen a menudo a sus elevados niveles en ácidos grasos monoinsaturados (ácido oleico), pero no podemos obviar la existencia de otros componentes minoritarios (antioxidantes, fenoles y otros compuestos naturales) que también influyen saludablemente. El consumo de aceite de oliva se ha asociado a una reducción de las concentraciones de colesterol y colesterol-LDL plasmático, y a un aumento de las concentraciones de colesterol-HDL, combinado con una protección antioxidante. El consumo de aceite de oliva también se ha asociado a efectos beneficiosos sobre la presión sanguínea, sobre el proceso de fibrinolisis, la coagulación sanguínea y la respuesta inmune (36-45).

También se recomienda el consumo de **pescado,** por su alto contenido en *ácidos grasos poliinsaturados omega-3* de larga cadena. Los estudios epidemiológicos y de casos-controles indican que el consumo de pescado, grasa de pescado o ácidos grasos de esta serie, en concreto ácido eicosapentaenoico (EPA) y docosahexaenoico (DHA), reduce el riesgo de mortalidad cardiovascular. Se ha descrito que los ácidos grasos poliinsaturados omega-3 disminuyen los niveles de triglicéridos en plasma; disminuyen la infiltración de macrófagos en la pared del vaso, la producción de moléculas quimiotácticas, factores de crecimiento, moléculas de adhesión, eicosanoides y citocinas inflamatorias; disminuyen la presión sanguínea; aumentan la producción de óxido nítrico; contribuyen a la distensibilidad del vaso y a la ralentización del desarrollo de la placa de ateroma; disminuyen la trombosis y aumentan la tasa cardiaca. Estos son los mecanismos de acción que en la actualidad parecen explicar la protección que la ingesta de ácidos grasos poliinsaturados omega-3 ejerce en la prevención primaria y secundaria de la ECV. El **Capítulo 2** recoge en más detalle la amplitud de estos efectos.

Las frutas y verduras, por ser ricas en *fibra, vitaminas y antioxidantes* también forman parte de esta lista de recomendaciones. De entre estos componentes, destacan por su papel en la prevención cardiovascular, el ácido fólico y las vitaminas B_6 y B_{12}, cuya ingesta combinada o por separado se asocia a un menor riesgo coronario. En concreto, estos nutrientes participan del metabolismo de la Homocisteína, un metabolito del aminoácido

cisteína, cuyos niveles en plasma están directamente relacionados con una mayor agregación plaquetaria, estrés oxidativo, proliferación de células del músculo liso en la pared del vaso, menor producción de óxido nítrico y alteración en la función endotelial. El **Capítulo 3** ofrece una mayor perspectiva de cómo el consumo continuado de ácido fólico y vitaminas B_6 y B_{12} ayuda a reducir los niveles plasmáticos de homocisteína.

Trichopoulou *et al.* (46) demostraron que la adopción del patrón dietético mediterráneo se asocia a una menor tasa de mortalidad en general y a una menor mortalidad coronaria en particular, especialmente en aquellas personas de más de 55 años de edad. Pero un aspecto notable de este trabajo es, que a pesar de esta robusta asociación dieta mediterránea-mortalidad, no se encontraron asociaciones significativas para ninguno de los nutrientes típicos de esta dieta por separado. Una explicación plausible a este hecho pudiera ser que el efecto que ejerce cada nutriente o cada alimento por separado puede ser demasiado pequeño como para detectarse, mientras que la ingesta conjunta de todos los alimentos y nutrientes esenciales que la caracteriza puede, de manera acumulativa, ejercer un efecto sustancial en la salud. Además, las múltiples interacciones entre los distintos alimentos y nutrientes, pueden ser las responsables de una sinergia en el efecto saludable producido. Esta observación refuerza la idea de que es la interacción conjunta de los nutrientes que constituyen la Dieta Mediterránea, y no la ingesta separada, la que posiblemente explique los beneficios saludables de ésta.

El *Edinburgh Artery Study* (47,48) es el único estudio epidemiológico que intenta relacionar componentes específicos de la dieta con la incidencia de la EVP. Este estudio, que incluye a 1581 hombres y mujeres con esta patología, describió una relación positiva entre el consumo de alimentos ricos en fibra, cereales, alcohol y vitaminas E y C, y una menor severidad en los síntomas. Por otro lado, el consumo de carne y grasas saturadas se asoció de manera negativa a esta patología. En las últimas décadas, diversos estudios de intervención nutricional en pacientes con EVP han descrito interesantes aportaciones que sugieren el papel potencial que los nutrientes pudieran tener en esta enfermedad. Sin embargo, estos estudios no han generado la creación de ninguna recomendación nutricional consensuada. El **Capítulo 6** del presente proyecto de tesis revisa la evidencia existente para cada uno de los nutrientes asociados a un patrón dietético mediterráneo y sugiere, con base en la literatura científica precedente, cómo también podrían ser efectivos en la paliación de los síntomas de la EVP.

Alejamiento del patrón Mediterráneo.

La Guía Europea de Prevención Cardiovascular (*The Third Joint Task Force*), sugiere la implantación de una dieta saludable como estrategia fundamental para la prevención de ECV (32,49), y empleando como base el patrón dietético mediterráneo establece que:

1. La dieta debe ser variada y proporcionar una ingesta calórica adecuada para el mantenimiento del peso ideal.

2. Los alimentos cuyo consumo debe fomentarse son los siguientes: frutas y verduras, cereales y pan integral, productos lácteos y bajos en grasa, pescado, carne magra y aceite de oliva.

3. La ingesta de productos vegetales puede tener un efecto positivo en la prevención cardiovascular, a través de un incremento en la ingesta de fibra, vitaminas y de diversas sustancias antioxidantes.

4. Por lo que a la prevención cardiovascular se refiere, parece más importante el tipo de grasa consumida que la cantidad total, cuyo límite superior podría situarse entre el 30 y el 35% de la ingesta calórica total, siempre que exista un claro predominio de los ácidos grasos monoinsaturados. Puesto que parece poco probable eliminar la grasa saturada de una dieta nutricionalmente equilibrada, lo más aconsejable sería mantener un consumo lo más bajo posible de la misma (<7% de la ingesta calórica total), tratar de eliminar o reducir al mínimo la ingesta de grasas hidrogenadas, estimular la ingesta de grasa monoinsaturada, procedente del aceite de oliva, y ácidos grasos omega-3, que proceden del aceite de pescado y poseen propiedades protectoras específicas.

Otros organismos internacionales, establecen recomendaciones muy similares:

1. La Organización Mundial de la Salud (*OMS*) recomienda consumir pescado de manera regular para conseguir una ingesta de EPA+DHA próxima a los 200-500 mg por semana. De igual manera, recomienda sustituir el consumo de grasa saturada por grasa monoinsaturada (enfatizando en el aceite de oliva) e incrementar la ingesta de frutas y verduras que contribuirá a mejorar nuestros niveles de folatos y antioxidantes naturales (50).

2. La Sociedad Americana del Corazón (*American Heart Association*) recomienda consumir 5 porciones de frutas y verduras y 6 porciones de cereales por día. Igualmente sugieren consumir pescado dos veces por semana como mínimo, acompañado de un descenso en la ingesta de grasas saturadas, colesterol y ácidos grasos *trans* (los aceites hidrogenados presentes en determinados alimentos preparados y en margarinas) (51).

Existe un gran interés en la Sanidad Pública y otros organismos internacionales por la adopción de un patrón dietético más saludable que constituiría una herramienta de prevención efectiva y aplicable a toda la población desde edades muy tempranas. Sin embargo, aunque los patrones dietéticos y hábitos de vida pueden corregirse y modificarse, es cierto que son

característicos e inherentes a nuestra cultura y costumbres. Quizás sea esta la razón por la que las diferentes estrategias que han intentado promover la Dieta Mediterránea en países no mediterráneos no han tenido el éxito esperado (52,53).

Además, no podemos olvidar que incluso en países mediterráneos como el nuestro, los patrones dietéticos han ido progresivamente alejándose del patrón inicial. En España existen importantes diferencias geográficas en lo que a mortalidad cardiovascular se refiere. Por ejemplo, los valores más altos se presentan en Canarias, Andalucía y levante. (14). Esta mayor mortalidad cardiovascular en las zonas más "mediterráneas" de nuestro país ha sido denominada la "paradoja española" de la mortalidad cardiovascular. Se manifiesta desde hace al menos 30 años y no se debe a factores metodológicos relacionados con la calidad de las estadísticas de mortalidad. Este mismo patrón geográfico es compartido con otras muchas enfermedades crónicas, por lo que los factores determinantes pueden ser comunes (54). Por otro lado, datos del estudio *IBÉRICA* ilustran que este patrón de mortalidad coincide a grandes rasgos con el de la incidencia de enfermedad coronaria (55). Es posible que entre los factores determinantes se encuentren el nivel socioeconómico, la actividad física, y factores dietéticos como el consumo de frutas, pescado y vino que progresivamente se alejan del prototipo tradicional mediterráneo (56).

En efecto, los diversos estudios sobre la dieta existente en España reflejan que aunque todavía se ajusta al patrón Mediterráneo, ha experimentado algunos cambios asociados al desarrollo económico que hacen que tienda a separarse en algunos aspectos de este patrón dietético Mediterráneo (57). España tenía en 1964-65 un patrón dietético muy próximo al recomendado. En 1990-91 se registró un aumento del porcentaje de energía aportado por las grasas (del 32% en 1964-65 al 42% en 1990-91), seguido de un mayor consumo de carne, huevos, leche y derivados lácteos, fundamentalmente en detrimento de los hidratos de carbono (del 53% de las calorías totales en 1964-65 a solo el 42% en 1990-91). También se registró un menor consumo de cereales y legumbres. La alimentación de los españoles pareció evolucionar hacia un modelo anglosajón. Durante la década de los 90, la alimentación Española prosiguió hacia una disminución progresiva en la ingesta energética y una inversión paulatina el aporte de energía a partir de las grasas (14,58), hasta situarse en torno al 37% en la actualidad. Las principales fuentes de lípidos en la dieta media de la población adulta española son las grasas visibles (aceites, margarinas y mantequillas) que contribuyen con un 49% al aporte graso total, las carnes (25%) y a mayor distancia la leche y los derivados lácteos (14%). Las grasas saturadas proceden sobre todo de las carnes, que aportan el 30% y de los lácteos, con un 27%. Sin embargo, la calidad de la grasa consumida aún es razonablemente mediterránea, porque aunque hay un exceso de grasas saturadas (el 12% de todas las calorías ingeridas), hay un predominio de las grasas monoinsaturadas, que casi alcanzan el 20% de las calorías totales de la dieta (59-61).

El perfil alimentario actual sugiere elevados consumos para el grupo de las carnes, pescados y lácteos. El consumo de cereales (pan fundamentalmente), vegetales y hortalizas ha disminuido de manera significativa, y el consumo de frutas y verduras expresa una tendencia hacia un mayor consumo de productos elaborados y procesados en detrimento de las frutas y verduras frescas. Por otro lado, existen deficiencias descritas para numerosos minerales y para las vitaminas A, D, E, B2, B6 y ácido fólico (62-65).

Justificación.

Algunos de los cambios que la dieta de los españoles ha sufrido en los últimos años son positivos (como puede ser la mayor accesibilidad a los alimentos), pero la tendencia general parece ser un progresivo alejamiento del patrón dietético saludable. Conocer la extensión de este alejamiento puede suponer el primer paso hacia la instauración de medidas correctoras o campañas de información acerca de los valores mediterráneos que hemos perdido en nuestra dieta. Y dado que esta tarea pudiera ser difícil de cumplir en un contexto de globalización y unión de fronteras y culturas, es necesario estudiar otras maneras de asegurar la ingesta de estos nutrientes "mediterráneos" y saludables en nuestras dietas. El objetivo que debe marcarse la ciencia de la nutrición para una adecuada prevención de la ECV y de otras enfermedades crónicas como la EVP, comienza por restaurar el desequilibrio en nuestra dieta.

Como ciencia joven en constante desarrollo, la Nutrición ha de adaptarse a estos cambios y necesidades. Conceptos que parecían inamovibles, como la dieta para el "mantenimiento" de una salud adecuada por simple cobertura de las necesidades establecidas, o la prescripción de auténticos listados de alimentos prohibidos o tolerados en función del tratamiento de patologías capaces de afectar al metabolismo o la estructura orgánica, dejan paso a conceptos como los de "nutrición óptima", "promoción de la salud" o "prevención de enfermedades" mediante la nutrición, dotando a los alimentos del matiz de potenciales promotores de la salud (66).

Para la consecución de este fin surge el concepto de alimento funcional. Un **alimento funcional** sería aquel que, además de la función de nutrir (dada su composición en nutrientes) y de proporcionar placer (con su sabor, aroma…) posee una tercera función, la de influir positivamente sobre la salud o sobre una determinada función fisiológica. De un modo general, se pueden describir tres condiciones que definen el carácter "funcional" de un alimento: 1) Ha de responder a las características de un alimento, 2) siempre debe ser consumido formando parte de la elaboración de los platos que integran los menús de las dietas alimenticias y 3) debe ejercer, una vez ingerido, un efecto positivo sobre una determinada función fisiológica (67,68).

El presente proyecto de tesis, justifica la necesidad de recuperar y/o incrementar la ingesta de estos nutrientes saludables mediante el uso de un alimento funcional de consumo diario

(Puleva Omega 3©, producto lácteo enriquecido en ácidos grasos poliinsaturados omega-3 de cadena larga, ácido oleico y vitaminas B_6, B_{12}, E y ácido fólico), como una herramienta más en la consecución de un patrón dietético saludable para la prevención del riesgo a padecer ECV en la población general, y como una acción coadyuvante al tratamiento farmacológico dentro del programa de alimentación y hábitos de vida que acompaña a las estrategias de prevención secundaria de aquellos pacientes no hospitalizados con EVP.

Objetivo general

Evaluar la eficacia del consumo continuado de una mezcla de nutrientes (ácidos grasos omega 3, ácido oleico, vitaminas B_6, B_{12}, E y ácido fólico) administrados de manera conjunta en una matriz láctea, en la reducción de diversos factores de riesgo de la enfermedad cardiovascular y de la enfermedad vascular periférica.

Objetivos específicos

1. Evaluar el efecto que el consumo de estos nutrientes ejerce sobre los factores de riesgo cardiovascular de una población sana o con hiperlipemia moderada.
2. Evaluar el posible papel del consumo de estos nutrientes como estrategia coadyuvante en el tratamiento la enfermedad vascular periférica.
3. Evaluar el posible papel del consumo continuado de ácidos grasos omega-3 en la sintomatología clínica de la enfermedad vascular periférica.

Para responder a estos objetivos específicos se planteó el siguiente plan de trabajo:

1. Realización de un estudio longitudinal de intervención a corto plazo (2 meses) que evalúa los efectos producidos por el consumo de un alimento funcional enriquecido con la mezcla de nutrientes objeto de estudio, sobre una cohorte de voluntarios sanos. Los resultados de este estudio se reflejan en el **Capítulo 4**.
2. Realización de un estudio longitudinal de intervención a corto plazo (2 meses) que evalúa los efectos producidos por el consumo de un alimento funcional enriquecido con la mezcla de nutrientes objeto de estudio, sobre una cohorte de voluntarios con hiperlipemia moderada. Los resultados de este estudio se reflejan en el **Capítulo 5**.
3. Realización de un estudio longitudinal, aleatorio y controlado que evalúa los efectos producidos por el consumo a largo plazo (1 año) de un alimento funcional enriquecido con la mezcla de nutrientes objeto de estudio, en una cohorte de pacientes afectados de enfermedad vascular periférica y que presentan sintomatología de claudicación intermitente. Los resultados de este estudio se presentan en el **Capítulo 7**.

4. Realización de un estudio *ex vivo* sobre monocitos estimulados con LPS procedentes de pacientes afectados de enfermedad vascular periférica, tras consumo de ácidos grasos omega 3 en forma de cápsulas, a fin de elucidar los posibles mecanismos de acción de los nutrientes por separado, responsables de la mejoría clínica observada en el capítulo 7. Los resultados de este estudio se presentan en el **Capítulo 8**.

Bibliografía

1. Gil A, Ramírez-Tortosa MC, Aguilera C, Mataix J. " Aceite de Oliva y sistema cardiovascular I: aterosclerosis". En: "Aceite de Oliva Virgen: nuestro patrimonio alimentario" Mataix, J (Ed) Universidad de Granada-Puleva Food, Granada, España. 2001: 79-108.
2. The World Health report 2004. Changing history. Geneva, World Health Organization, 2004.
3. Instituto Nacional de Estadistica. INE 2004. www.ine.es
4. Villar Alvarez F, Banegas Banegas JR, Rodriguez Artalejo F, del Rey Calero J. Cardiovascular mortality in Spain and its autonomous communities (1975-1992). Med Clin (Barc) 1998; 110(9):321-327.
5. Sans S, Kesteloot H, Kromhout D. The burden of cardiovascular diseases mortality in Europe. Task Force of the European Society of Cardiology on Cardiovascular Mortality and Morbidity Statistics in Europe. Eur Heart J. 1997;18(12):1231-1248.
6. Brotons C, Royo-Bordonada MA, Alvarez-Sala L, *et al.* Spanish adaptation of the European Guidelines on Cardiovascular Disease Prevention. Rev Esp Salud Publica. 2004; 78(4):435-438.
7. Dawber TR, Kannel WB. The Framingham study. An epidemiological approach to coronary heart disease. Circulation. 1966;34(4):553-555.
8. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999 14;340(2):115-126.
9. Koh KK, Son JW, Ahn JY, *et al.* Comparative effects of diet and statin on NO bioactivity and matrix metalloproteinases in hypercholesterolemic patients with coronary artery disease. Arterioscler Thromb Vasc Biol. 2002; 22(9):19-23.
10. Rosano GM, Fini M. Postmenopausal women and cardiovascular risk: impact of hormone replacement therapy. Cardiol Rev. 2002;10(1):51-60.
11. Lusis AJ. Atherosclerosis. Nature. 2000;407(6801):233-241.
12. Ghazalpour A, Doss S, Yang X, Aten J, Toomey EM, Van Nas A, Wang S, Drake TA, Lusis AJ. Thematic review series: The pathogenesis of atherosclerosis. Toward a biological network for atherosclerosis. J Lipid Res. 2004;45(10):1793-805.
13. Chahoud G, Aude YW, Mehta JL. Dietary recommendations in the prevention and treatment of coronary heart disease: do we have the ideal diet yet? Am J Cardiol. 2004;94(10):1260-1267.
14. Villar F, Banegas JR, Donado J, Rodríguez-Artalejo F. Las enfermedades cardiovasculares y sus factores de riesgo en España. Informe de la SEA 2003. www.searteriosclerosis.org.
15. Halperin JL. Evaluation of patients with peripheral vascular disease. Thrombosis Research. 2002;106: V303-V311.

16. Hiatt WR, Hoag S, Hamman RF. Effect of diagnostic criteria on the prevalence of peripheral arterial disease: the San Luis Valley Diabetes Study. Circulation 1995; 91:1472-1479.
17. Trans Atlantic Inter-Society Consensus (TASC) on the management of peripheral arterial disease. Eur J Vasc Endovasc Surg 2000; 19 (suppl A): 1-244.
18. Hirsch AT, Hiatt WR; PARTNERS Steering Committee. PAD awareness, risk, and treatment: new resources for survival--the USA PARTNERS program. Vasc Med 2001: 6, 9-12.
19. Leng GC, Fowkes FGR. The epidemiology of peripheral arterial disease. Vasc Med Rev 1993:4, 5-18.
20. Muluk SC, Muluk VS, Kelley ME, *et al.* Outcome events in patients with claudication: a 15-years study in 2777 patients. J Vasc Surg 2001; 33, 251-257.
21. Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR, McCann TJ, Browner D. Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med 1992: 326, 381-386.
22. Vogt MT, Wolfson SK & Kuller LH. Segmental arterial disease in the lower extremities: correlates of disease and relationship to mortality. J Clin Epidemiol 1993: 46, 1267-1276.
23. Leng GC, Fowkes FG, Lee AJ, Dunbar J, Housley E, Ruckley CV. Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. BMJ 1996: 313, 1440-1444.
24. Henke PK, Blackburn S, Proctor MC, Stevens J, Mukherjee D, Rajagopalin S, Upchurch GR Jr, Stanley JC, Eagle KA. Patients undergoing infrainguinal bypass to treat atherosclerotic vascular disease are underprescribed cardioprotective medications: effect on graft patency, limb salvage, and mortality. J Vasc Surg 2004: 39, 357-365.
25. Brevetti G, Chiariello M, Ferulano G, *et al.* Increases in walking distance in patients with peripheral vascular disease treated with L-carnitine: a double-blind, cross-over study. Circulation 1998: 77, 767–773.
26. Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr. 2001;73(4):673-686
27. Ross R. Atherosclerosis-an inflammatory disease. N Engl J Med. 1999; 340(2):115-126
28. McDermott MM, Mehta S, Ahn H, Greenland P. Atherosclerotic risk factors are less intensively treated in patients with peripheral arterial disease than in patients with coronary artery disease. J Gen Intern Med 1997; 12, 209-215.
29. Tornwall ME, Virtamo J, Haukka JK, Aro A, Albanes D, Huttunen JK. Prospective study of diet, lifestyle, and intermittent claudication in male smokers. Am J Epidemiol 2000; 151, 892-901.
30. Cassar K, Coull R, Bachoo P, Macaulay E, Brittenden J. Management of secondary risk factors in patients with intermittent claudication. Eur J Vasc Endovasc Surg 2003; 26, 262-266.
31. Mukherjee D, Lingam P, Chetcuti S, *et al.* Missed opportunities to treat atherosclerosis in patients undergoing peripheral vascular interventions: insights from the University of Michigan Peripheral Vascular Disease Quality Improvement Initiative (PVD-QI2). Circulation 2002: 106, 1909-1912.
32. De Backer G, Ambrosioni E, Borch-Johnsen K, *et al.* Third Joint Task Force of European and Other Societies on Cardiovascular Disease Prevention in Clinical Practice. European guidelines on cardiovascular disease prevention in clinical practice. Third Joint Task Force of European

and Other Societies on Cardiovascular Disease Prevention in Clinical Practice. Eur Heart J. 2003;24(17):1601-10

33. Spark JI, Robinson JM, Gallavin L, Gough MJ, Homer-Vanniasinkam S, Kester RC, Scott DJ. Patients with chronic critical limb ischemia have reduced total antioxidant capacity and impaired nutritional status. Eur J Vasc Endovasc Surg 2002; 24, 535-539.
34. Willett WC, Sacks F, Trichopoulou A, Drescher G, Ferro-Luzzi A, Helsing E, Trichopoulos D. Mediterranean diet pyramid: a cultural model for healthy eating. Am J Clin Nutr. 1995; 61(6 Suppl):1402S-1406S.
35. de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, Guidollet J, Touboul P, Delaye J. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. Lancet. 1994; 343(8911):1454-1459.
36. Panagiotakos DB, Pitsavos C, Polychronopoulos E, Chrysohoou C, Zampelas A, Trichopoulou A. Can a Mediterranean diet moderate the development and clinical progression of coronary heart disease? A systematic review. Med Sci Monit. 2004;10(8):RA193-198.
37. Visioli F, Grande S, Bogani P, Galli C. The role of antioxidants in the mediterranean diets: focus on cancer. Eur J Cancer Prev. 2004;13(4):337-343.
38. Moreno JJ, Mitjavila MT. The degree of unsaturation of dietary fatty acids and the development of atherosclerosis (review). J Nutr Biochem. 2003;14(4):182-195.
39. Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer.Am J Med. 2002;113 Suppl 9B:71S-88S.
40. Yaqoob P. Monounsaturated fatty acids and immune function. Eur J Clin Nutr. 2002;56 Suppl 3:S9-S13.
41. Massaro M, De Caterina R. Vasculoprotective effects of oleic acid: epidemiological background and direct vascular antiatherogenic properties. Nutr Metab Cardiovasc Dis. 2002;12(1):42-51.
42. Stark AH, Madar Z. Olive oil as a functional food: epidemiology and nutritional approaches. Nutr Rev. 2002;60(6):170-6.
43. Visioli F, Galli C. Biological properties of olive oil phytochemicals. Crit Rev Food Sci Nutr. 2002;42(3):209-221.
44. Owen RW, Giacosa A, Hull WE, Haubner R, Wurtele G, Spiegelhalder B, Bartsch H. Olive-oil consumption and health: the possible role of antioxidants. Lancet Oncol. 2000; 1:107-112.
45. Sanders TA. Olive oil and the Mediterranean diet. Int J Vitam Nutr Res. 2001; 71(3):179-184.
46. Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med. 2003; 348(26):2599-2608.
47. Donnan PT, Thomson M, Fowkes GR, *et al.* (1993) Diet as a risk factor for peripheral arterial disease in the general population: the Edinburgh Artery Study. Am J Clin Nutr 57, 917–921.
48. Jepson RG, Fowkes FG, Donnan PT, Housley E. Alcohol intake as a risk factor for peripheral arterial disease in the general population in the Edinburgh Artery Study. Eur J Epidemiol. 1995; 11(1):9-14.
49. De Backer G, Ambrosioni E, Borch-Johnsen K, *et al.* European guidelines on cardiovascular disease prevention in clinical practice. Third Joint Task Force of European and other Societies

on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of eight societies and by invited experts). Atherosclerosis. 2004;173(2):381-391.

50. WHO Study Group. “Diet, nutrition and prevention of chronic diseases” Technical Report Series, 2003; No. 916.
51. Kris-Etherton PM, Harris WS, Appel LJ; AHA Nutrition Committee. American Heart Association. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. Arterioscler Thromb Vasc Biol. 2003;23(2):151-152.
52. Paffenbarger RS Jr, Hyde RT, Wing AL, Lee IM, Jung DL, Kampert JB. The association of changes in physical-activity level and other lifestyle characteristics with mortality among men. N Engl J Med. 1993; 328:538-545
53. Wannamethee SG, Shaper AG, Walker M. Changes in physical activity, mortality, and incidence of coronary heart disease in older men. Lancet. 1998; 351:1603-1608
54. Rodriguez-Artalejo F, Guallar-Castillon P, Banegas Banegas JR, del Rey Calero J. [Geographic variation in hospitalizations and mortality related to congestive heart failure in Spain, 1980-1993] Rev Esp Cardiol. 2000;53(6):776-782.
55. Marrugat J, Sala J, Masía R, Pavesi M, Sanz G, Valle V, Molina L, Seres L, Elosua R. Mortality differences between men and women following first myocardial infarction. RESCATE Investigators. JAMA 1998; 280: 1405-1409.
56. Rodríguez Artalejo F, Banegas JR, Graciani A, Hernández R, Rey Calero J. El consumo de alimentos y nutrientes en España en el periodo 1940-1988. Análisis de su consistencia con la dieta mediterránea. MedClin (Barc) 1996; 106:161-168.
57. Ros Rahola E, Fisac C, Pérez-Heras A. ¿Qué es realmente la dieta mediterránea? FMC 1998; 5: 557-571.
58. Aranceta J, Serra-Majem Ll (ed.). Guías Alimentarias para la población española. Madrid: Sociedad Española de Nutrición Comunitaria, 2001.
59. Varela G, Moreiras O, Carbajal A, Campo M. Estudio Nacional de Nutrición y Alimentación 1991. Tomo I. Encuesta de Presupuestos Familiares 1990-91. Madrid: Instituto Nacional de Estadística, 1995.
60. Serra-Majem L, Ribas L, Lloveras G, Salleras L. Changing patterns of fat consumption in Spain. Eur J Clin Nutr 1993; 47 (Supl 1): 13-20.
61. Rodríguez Artalejo F, Graciani MA, Banegas JR, Martín-Moreno JM, Sabaté J, Rey Calero J. El consumo de alimentos y nutrientes en España en el período 1940-1988 (y II). Un estudio comparativo de las principales fuentes de información sobre consumo alimentario. Med Clin (Barc) 1996; 107: 446-452.
62. Aranceta J, Perez Rodrigo C, Foz Sala M, Mantilla T, Serra Majem L, Moreno B, Monereo S, Millan J; Grupo Colaborativo para el estudio DORICA fase 2. Tables of coronary risk evaluation adapted to the Spanish population: the DORICA study. Med Clin (Barc). 2004;123(18):686-691
63. Ortega RM, Lopez Sobaler AM, Aranceta J, Serra Majem L. Are there any nutritional deficiencies in the Mediterranean Diet?Arch Latinoam Nutr. 2004 Jun;54(2 Suppl 1):87-91.
64. Ortega RM, Aranceta J, Serra-Majem L, Entrala A, Gil A, Mena MC. Nutritional risks in the Spanish population: results of the eVe study. Eur J Clin Nutr. 2003;57 Suppl 1:S73-75.
65. Aranceta J. Spanish food patterns. Public Health Nutr. 2001; 4(6A):1399-402

66. Milner JA. Functional foods: the US perspective. Am J Clin Nutr. 2000;71(6 Suppl):1654S-1659S.

67. FUFOSE. Scientific concepts of functional foods in Europe: consensus document. Br J Nutr 1999; 81 (Suppl. 1): 1-27

68. Diplock AT, Aggett PJ, Ashwell M, Bornet F, Fern EB, Roberfroid MB. Scientific concepts of functional foods in Europe: consensus document. Br J Nutr 1999; 81(s1): 1-27.

2

Efectos cardiovasculares de los ácidos grasos omega-3 y alternativas para incrementar su ingesta.

Nutrición Hospitalaria 2005; ***20(1):****56-62*

JJ Carrero*, E Martín-Bautista†, L Baró†, J Fonollá†, J Jiménez†, JJ Boza† y E López-Huertas†

*Departamento de Bioquímica y Biología Molecular. Universidad de Granada Spain
†PULEVA BIOTECH S.A. Granada, Spain

Resumen

Las enfermedades cardiovasculares son la principal causa de mortalidad en Europa, Estados Unidos y gran parte de Asia. Existen varios factores de riesgo asociados a las enfermedades cardiovasculares, como son el colesterol total, la homocisteína, los triglicéridos elevados, la hipertensión, la diabetes y unos niveles reducidos de colesterol HDL. Muchos de estos factores de riesgo son potencialmente modificables mediante dieta. Este artículo ofrece una visión sobre cómo la ingesta de ácidos grasos poliinsaturados n-3, presentes en el pescado y en el aceite de pescado, pueden contribuir a la reducción del riesgo cardiovascular.

Existe una gran cantidad de alimentos enriquecidos con ácidos grasos n-3 en el mercado. El consumo de este tipo de alimentos pudiera ser una opción eficaz en la reducción de factores de riesgo de enfermedades, complementando la ingesta de pescado en la dieta sin originar grandes cambios en los hábitos alimentarios y disminuyendo el uso de los suplementos dietéticos. Sin embargo, el conocimiento de los efectos originados por el consumo regular de estos alimentos supone aún un reto en la mayoría de los casos. También se presenta un estudio de intervención usando un alimento funcional de base láctea que contiene ácidos grasos n-3, ácido oleico y vitaminas.

Summary

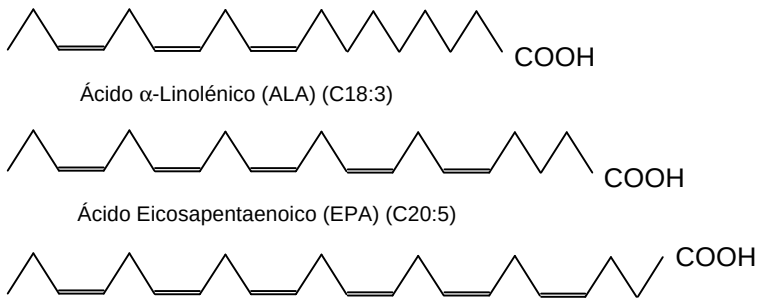
Cardiovascular disease is the leading cause of death in Europe, USA and many countries in Asia. There is a variety of risk factors associated to the development of cardiovascular diseases, such as total cholesterol, levels of Homocysteine, elevated triacylglycerols, hypertension, diabetes and reduced levels of HDL cholesterol. Many of these risk factors are modifiable and/or preventable by a healthy diet. This article reviews how the intake of n-3 fatty acids, present in fish and fish oil, can help to reduce the cardiovascular risk.

There are many foods products enriched with n-3 fatty acids in the market. The consumption of there enriched products could be a valid strategy for the reduction of cardiovascular risk, reducing the use of supplements without performing major changes in the dietary habits. However, it is still unknown whether the regular long-term consumption of these enriched products would produce any effect on cardiovascular health. We also present in this review the results from an intervention study using a functional dairy product enriched in n-3 fatty acid, oleic acid, folic acid and vitamins.

Introducción

Las enfermedades cardiovasculares (ECV) son el principal problema de salud pública de los países europeos debido a su elevada incidencia. (1). Los estudios epidemiológicos y de intervención indican que el consumo de ácidos grasos poliinsaturados (AGPI) n-3 de larga cadena producen cambios en variables homeostáticas que se asocian a efectos beneficiosos para la salud. Desde que Dyerberg estableció una relación entre la ingesta de AGPI n-3 y el riesgo de padecer ECV al estudiar el patrón dietético de la población esquimal en Groenlandia (2), se han publicado más de 6000 trabajos científicos sobre los efectos éstos en la ECV.

Fig. 1. Estructura química del ácido alfa-linolénico (ALA), ácido eicosapentaenoico (EPA) y ácido docosahexaenoico (DHA).



Ácido α-Linolénico (ALA) (C18:3)

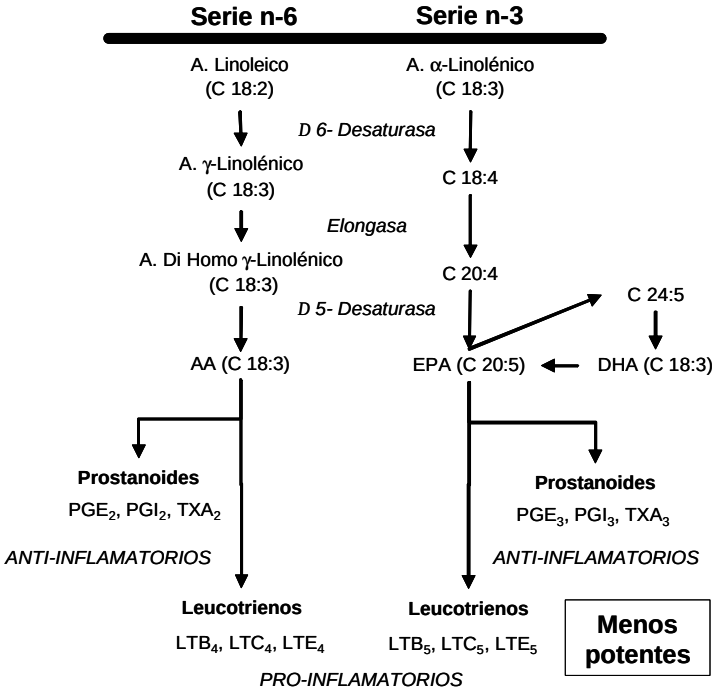
Ácido Eicosapentaenoico (EPA) (C20:5)

Ácido Docosahexaenoico (DHA) (C 18:3)

Existen dos familias de AGPI. La familia de AGPI n-6 deriva del ácido linoleico y se caracteriza por tener dobles enlaces alternos comenzando por el sexto carbono a partir del grupo metilo. La familia de AGPI n-3 deriva del ácido a-linolénico (ALA), y se caracteriza por tener dobles enlaces alternos comenzando por el sexto carbono (**Fig. 1**). Tanto el linoleico como el a-linolénico son ácidos grasos esenciales que deben ser aportados en la dieta, ya que no pueden ser sintetizados por el organismo (3). La diferente cantidad y posición de los dobles enlaces confiere a los ácidos grasos diferentes propiedades fisiológicas, lo que hace que la relación entre los ácidos grasos n-3 y n-6 de la dieta sea muy importante. El ácido linoleico se metaboliza a ácido araquidónico y el a-linolénico da lugar al ácido eicosapentaenoico (EPA) y al ácido docosahexaenoico (DHA). Todos ellos emplean las mismas rutas metabólicas y compiten por las mismas enzimas elongasas y desaturasas. Además de ser una fuente de energía, las familias de AGPI n-6 y n-3 se incorporan a las membranas celulares, donde son precursores de eicosanoides (prostaglandinas, prostaciclinas, tromboxanos y leucotrienos) que intervienen en numerosos procesos fisiológicos como la coagulación de la sangre o la respuesta inflamatoria (**Fig. 2**). En general, los eicosanoides sintetizados a partir de la familia de AGPI n-3 son menos activos (por ejemplo tienen menor actividad antiinflamatoria) que los eicosanoides derivados de la familia n-6. Al aumentar el consumo de AGPI n-3 en la dieta, también puede incrementarse la

producción de eicosanoides menos activos. El consumo de ácidos grasos n-6 y n-3 determina el tipo y cantidad de eicosanoides en el organismo, lo cual influye potencialmente en todos los procesos en los que intervienen.

Figura 2. Series de ácidos grasos poliinsaturados y rutas metabólicas de eicosanoides.



Esta revisión ofrece una perspectiva general sobre los efectos que el consumo de ácidos grasos n-3 produce en la salud, haciendo especial énfasis en la salud cardiovascular, y las opciones que la industria ofrece para incrementar la ingesta de éstos ácidos grasos.

Fuentes de ácidos grasos n-3

Entre los aceites vegetales, el aceite de linaza es considerado como la fuente más rica de ALA (57% de los ácidos grasos totales). La semilla de colza, la soja, el germen de trigo y las nueces contienen entre un 7% y un 13% de ALA. Las verduras también pueden ser una buena fuente de ALA (por ejemplo espinaca, lechuga), aunque su contenido graso es bastante bajo. La carne de origen animal, particularmente la de rumiantes, y los productos lácteos también proporcionan ALA. Sin embargo, la ganadería moderna, debido al uso de concentrados de cereales ricos en ácidos grasos n-6 para la elaboración de pienso, ha originado un descenso en el contenido de ácidos grasos n-3 de estas carnes (especialmente cordero y ternera) (4).

En cuanto al EPA y al DHA, las fuentes más ricas son los aceites de pescado y el pescado azul (**Tabla I**). El alto contenido de DHA y EPA en el pescado se debe al consumo de fitoplancton (rico en AGPI n-3). El contenido de AGPI n-3 varía en función de la especie de pescado, su localización, la estación del año y la disponibilidad del fitoplancton.

Tabla I. Contenido medio de AGPI n-3 de pescados y mariscos (6)

Marisco/pescado	g de AGPI n-3/100 g
Caballa	1.8-5.3
Arenque	1.2-3.1
Salmón	1.0-2.0
Trucha	0.5-1.6
Atun	0.5-1.6
Gamba	0.2-0.4
Bacalao, Halibut	Aprox. 0.2

Ingesta de ácidos grasos n-3 y recomendaciones dietéticas

Las estimaciones realizadas sobre la ingesta de ácidos grasos n-3 se basan principalmente en los datos sobre consumo de alimentos y los análisis químicos de las dietas. El consumo aproximado de ácido a-linolénico en los países europeos oscila entre 0,6 y 2,5 gramos / día (3, 5, 6). Sin embargo, hay pocos datos disponibles de la ingesta de DHA y EPA en Europa debido a la escasez de datos de consumo de alimentos fiables. Se ha estimado que el consumo de ácidos grasos n-3 en Europa oscila de 0,1 a 0,5 g/día (3). Estas cifras son elevadas en comparación con la ingesta de DHA y EPA estimada en Estados Unidos (0,1-0,2 g/día), pero reducidas con respecto a los datos de ingesta estimados en Japón (hasta 2 g/día) (7), donde el pescado es uno de los alimentos más consumidos.

En relación a las recomendaciones nutricionales de ingesta de ácidos grasos n-3, la Sociedad Internacional para el Estudio de Ácidos Grasos y Lípidos (“ISSFAL”) sugiere la cantidad de 0,65 g/día de DHA más 1g/día de ácido a-linolénico (8). Por otra parte, las nuevas recomendaciones de la Sociedad Americana del Corazón (“AHA”) son:

1. las personas adultas han de consumir pescado al menos 2 veces por semana,
2. para pacientes con enfermedad cardiaca crónica las recomendaciones de consumo son de 1 gramo diario de EPA + DHA procedente de aceites de pescado o suplementos y,
3. para pacientes con hipertrigliceridemia se recomienda el suplemento de 2 a 4 gramos diarios de EPA + DHA para disminuir en un 20-40 % los niveles de triglicéridos del plasma (9).

La Organización para Agricultura y Alimentación y la Organización Mundial de la Salud en su informe del año 2003 sobre dieta, nutrición y prevención de enfermedades crónicas,

recomiendan una ingesta de grasas saturadas menor al 10% y un consumo de grasa monoinsaturada que aporte entre un 15 y un 30 % de la energía total. El consumo de grasa poliinsaturada debe representar un 6-10 % de la energía y de manera particular, los ácidos grasos n-3 han de contribuir con un 1-2 % (10).

El consumo actual de AGPI n-3 está muy por debajo de las distintas recomendaciones antes citadas. En el Reino Unido, por ejemplo, sólo un tercio de la población adulta consume pescado azul y apenas una vez por semana (11). En estos países especialmente, donde el consumo de pescado es muy bajo, es necesario buscar estrategias válidas para conseguir aumentar el consumo de pescado que consiga proporcionar al individuo las cantidades de EPA y DHA recomendadas.

Ácidos grasos n-3 y la enfermedad cardiovascular.

Las ECV son la principal causa de mortalidad en los países occidentales y una parte importante de Asia. Numerosos estudios científicos avalan el papel que una dieta equilibrada tiene, en la reducción de alguno de los factores de riesgo asociados a estas enfermedades (12). En las últimas décadas, los efectos que el consumo de AGPI n-3 produce en la salud cardiovascular han sido y son, motivo de estudio por parte de la comunidad científica.

Algunos estudios epidemiológicos destacables en este sentido son: el estudio "*The Seven Countries*", de 20 años de duración y seguimiento, demostró que aquellos hombres que consumían 30 g/día de pescado reducían el riesgo de mortalidad por enfermedad coronaria en un 50% frente a los que no consumían pescado (13). El estudio "*The Western Electric*" determinó que los hombres que consumían más de 35 g/día de pescado presentaban un riesgo relativo de mortalidad por enfermedad coronaria de 0,62 frente a los que casi nunca consumían pescado (14). El estudio "*US Physicians' Health*" demostró que el consumo semanal de pescado estaba asociado a un riesgo relativo de 0,48 a padecer muerte súbita cardiaca (15). El estudio sobre "Prevención de Aterosclerosis Coronaria Mediante Intervención con Ácidos Grasos Omega-3 de Origen Marino" (también conocido por "*SCIMO*"), demostró una reducción en el desarrollo del proceso aterosclerótico al administrar dosis bajas de AGPI n-3 (1,65 g/día) (16).

Tres estudios de intervención han demostrado que el consumo de pescado o de aceite de pescado tiene efectos protectores importantes frente a las ECV. El "*Diet and Reinfarction Trial*" (DART) demostró que dosis relativamente bajas de AGPI n-3 (2,3 g/semana), equivalentes a 2-3 porciones de pescado azul a la semana, reducía el riesgo a sufrir un segundo evento coronario descendiendo un 30%, la mortalidad debida a ECV (17). En el estudio "*GISSI-Prevenzione*", el consumo de un suplemento nutricional de AGPI n-3 (1 g/día) disminuyó en un 17% el riesgo de mortalidad por ECV (18). Además, el estudio "*Lyon Heart*" demostró que una dieta de tipo mediterránea, que aportaba ácido oleico, antioxidantes

naturales, cantidades reducidas de ácidos grasos saturados y aproximadamente 2 g/día de ALA, redujo la aparición de episodios coronarios en un 70% y la mortalidad en un 80% (19).

Otros estudios no epidemiológicos han demostrado que dosis bajas de aceites de pescado (1 g/día de AGPI n-3) pueden disminuir la concentración de triglicéridos del plasma en ayunas y también en el estado postpandrial (20, 21), a partir de cuyos valores se ha sugerido se puede predecir el riesgo de sufrir infarto de miocardio (4, 11, 22).

Posibles mecanismos de acción de los ácidos grasos n-3

Aún no está claro el mecanismo exacto mediante el cual los ácidos grasos n-3 ejercen su efecto protector. Entre las distintas hipótesis propuestas, se ha descrito que tienen los ácidos grasos n-3 afectan a la coagulación sanguínea y a la trombosis, al perfil de los lípidos plasmáticos, a la presión sanguínea, la arritmia y la inflamación. Los efectos ateroprotectores de los AGPI n-3 se deben principalmente a su incorporación a los fosfolípidos de las membranas celulares, compitiendo con el ácido araquidónico como sustrato inicial para la producción de eicosanoides (11). Cuando las células vasculares sufren algún tipo de daño, se desencadena el proceso de agregación plaquetaria. Los intermediarios derivados del metabolismo de los AGPI n-3 son menos pro-trombóticos y menos vasoconstrictores que los derivados del araquidónico (n-6). El contenido en ácidos grasos de las plaquetas origina la producción de tromboxano A2 a partir de la familia n-6, o de tromboxano A3 a partir de la familia n-3. Este último posee un efecto pro-agregante menor que el tromboxano A2, reduciendo por tanto la agregación plaquetaria y la trombosis (23).

Por otra parte, un músculo cardiaco enfermo es susceptible de sufrir irregularidades en la actividad eléctrica (arritmias), que en muchas ocasiones son causa de muerte súbita cardiaca. La proporción de ácidos grasos n-3 /n-6 en el músculo cardiaco parece estar relacionada con el riesgo de muerte súbita cardiaca. Se ha sugerido que la ingesta moderada de AGPI n-3 puede reducir el riesgo de parada cardiaca debido a un efecto regulador sobre las propiedades eléctricas del miocardio, disminuyendo por tanto la susceptibilidad a las arritmias ventriculares y, por consiguiente, el riesgo de muerte súbita (24, 25).

El consumo de ácidos grasos n-3 afecta favorablemente al perfil lipídico (26), en concreto contribuye a reducir los triglicéridos plasmáticos. Los niveles de triglicéridos elevados constituyen un factor de riesgo independiente de las ECV, especialmente en individuos con valores reducidos de colesterol HDL. Tras consumir una comida rica en grasa, se produce un aumento característico de los triglicéridos sanguíneos que se conoce con el nombre de hiperlipemia postpandrial o respuesta postpandrial. La intensidad de esta respuesta también se considera un factor de riesgo de ECV y está relacionada con el tipo de grasa ingerida. Algunos estudios indican que la ingesta de DHA y EPA reduce el aumento postpandrial de los triglicéridos y, por tanto, produce un efecto beneficioso (27-29). Otros estudios también han

demostrado que el consumo de cantidades considerables de pescado o de aceite de pescado, puede disminuir los niveles de triglicéridos en sujetos sanos e hiperlipémicos (30-32). No existe suficiente evidencia científica para poder afirmar que el consumo de los AGPI n-3 contribuya a reducir el colesterol sanguíneo (33,34). En cambio, los aceites de pescado sí contribuyen a aumentar los niveles de colesterol HDL en hasta un 10%, dependiendo del alimento y de las cantidades de n-3 ingeridas (11).

La hipertensión es uno de los factores de riesgo de ECV más importantes. La hipertensión es responsable, entre otros efectos, de la activación del endotelio (35), que se traduce en un aumento de la producción de las moléculas de adhesión (como ICAM-1, VCAM-1) responsables de la infiltración de células sanguíneas a la pared vascular, contribuyendo al engrosamiento de la arteria y al desarrollo de la aterosclerosis (36). Existe evidencia científica de que los ácidos grasos n-3 pueden estimular la producción endotelial de óxido nítrico (37). Esta molécula provoca la relajación de las células del músculo liso, permitiendo la dilatación de los vasos sanguíneos, y reduciendo la presión sanguínea y la activación endotelial. Se ha demostrado que sólo cantidades elevadas de aceites de pescado (un mínimo de 3 g/día) producen un descenso significativo, aunque moderado, de la presión sanguínea (38, 39). Las cantidades de pescado que habría que consumir para obtener estas dosis efectivas son tan elevadas que en la práctica sólo se alcanzan mediante el consumo de suplementos o alimentos enriquecidos con AGPI n-3.

Ácidos grasos n-3 y salud

Se han descrito otros efectos beneficiosos del consumo de los ácidos grasos n-3 en procesos inflamatorios tales como la artritis reumatoide, la enfermedad de Crohn, el asma, la psoriasis y algunas nefropatías. Aunque se necesitan más estudios para demostrar los beneficios clínicos, en general el consumo de AGPI n-3 alivia alguno de los síntomas de estas enfermedades, debido a la producción de la cascada de eicosanoides menos potentes.

Durante el desarrollo fetal e infantil, los AGPI n-3 juegan un papel fundamental en el desarrollo del cerebro, el sistema nervioso, la retina y el crecimiento (40-44) y, por tanto, una ingesta adecuada es esencial (11). En este sentido, es destacable el hecho de que el contenido de DHA en la leche humana oscile alrededor de 30 mg por cada 100 g, mientras que en la leche de otros mamíferos, particularmente en la de vaca, oveja o cabra, el DHA casi inapreciable.

Los efectos que el consumo de AGPI n-3 produce en la función cerebral adulta aun no están claros. Algunos trabajos científicos han descrito que la cantidad de AGPI n-3 en las membranas celulares de individuos que padecen Alzheimer, depresión o esquizofrenia es muy baja. Algunos estudios epidemiológicos indican que existe una relación inversa entre el consumo de pescado y la aparición de enfermedades depresivas. Además, algunos estudios

realizados en individuos sanos muestran que una concentración plasmática baja de DHA afecta a los niveles del neurotransmisor serotonina, y los niveles bajos de serotonina están relacionados con el comportamiento suicida (45).

Alternativas para incrementar la ingesta de ácidos grasos n-3

Las autoridades sanitarias recomiendan un aumento del consumo de AGPI n-3, en especial los de cadena larga (EPA y DHA) (46). Sin embargo, las sociedades occidentales modernas tienden a incluir muy poco pescado en la dieta. Además, la escasez de pescado y su elevado precio hace que en muchas ocasiones el consumidor prefiera otros alimentos de mayor comodidad y menor precio (47). Una alternativa válida y eficaz para contribuir a un aumento de la ingesta de AGPI n-3 es la fortificación o la adición de AGPI n-3 a alimentos de uso cotidiano. La tecnología alimentaria hace posible hoy en día, que una gran cantidad de alimentos puedan enriquecerse en ácidos grasos n-3 y, de hecho, existe en todo el mundo una gran variedad de productos alimenticios enriquecidos. Algunos ejemplos de estos alimentos que se comercializan en la casi totalidad de los países europeos son el pan y los productos de panadería, margarinas, grasas untables, huevos y derivados, pastas, salsas, zumos y bebidas no alcohólicas, carnes, productos lácteos y leche (4). Una limitación importante al adicionar AGPI n-3 a los alimentos estriba en la química de los mismos. Estos ácidos grasos son muy susceptibles a la oxidación y reaccionan muy rápidamente cuando se exponen a condiciones o agentes oxidantes como el oxígeno del aire. Por esta razón, los aceites de pescado se adicionan a los alimentos con vitamina E y otros antioxidantes para prevenir la oxidación que, de lo contrario, produciría enranciamientos, malos olores e inestabilidad. Además, la producción de alimentos enriquecidos con ácidos grasos n-3 es técnicamente difícil y requiere de métodos especiales para producir un aceite de pescado adecuado, apropiado para la adición a alimentos, sin olor ni sabor a pescado.

A pesar del gran número de productos alimenticios enriquecidos con ácidos grasos n-3 disponibles en el mercado, los efectos sobre la salud derivados del consumo regular de estos productos supone aún un reto en muchos casos y son muy pocos los estudios llevados a cabo en este sentido (28, 47-49). Estos tienen especial importancia cuando se trata de alimentos como los huevos o la carne, en los que la presencia de antioxidantes para prevenir la oxidación no puede controlarse por completo. Resultados de una intervención nutricional con huevos enriquecidos con AGPI n-3 mostraron que el consumo regular de éstos no producía el aumento característico en los niveles plasmáticos de colesterol originado por el consumo de huevos no enriquecidos (50-52). En otro estudio realizado con mujeres en periodo de lactancia se demostró que el consumo de huevos enriquecidos con ácidos grasos n-3 producía un aumento en la concentración de AGPI n-3 en la leche (53).

Se ha demostrado que la leche es un vehículo eficaz para absorber las grasas, ya que la grasa de la leche se encuentra en forma de micelas y la superficie de absorción, en comparación con otros alimentos, es elevada. En nuestro laboratorio hemos estudiado los efectos derivados del consumo de un producto lácteo enriquecido con ácidos grasos n-3, ácido oleico, vitaminas E, B6 y ácido fólico (Puleva Omega 3©) en factores de riesgo de enfermedad cardiovascular de individuos sanos (54). El estudio fue controlado, aleatorio y se realizó con una población sana de edades comprendidas entre los 25 y 45 años. El estudio se diseñó como complemento a su dieta habitual con la única salvedad de no ingerir pescado o derivados durante el tiempo de intervención. Los sujetos bebieron 500 mL/día de leche semidesnatada enriquecida con vitaminas A y D durante cuatro semanas, conteniendo la misma cantidad de grasa total que el producto lácteo enriquecido, pero con diferente perfil de ácidos grasos. Transcurrido este periodo de tiempo, los sujetos pasaron a consumir 500 mL/día del producto enriquecido durante las ocho semanas siguientes. Se extrajeron muestras de sangre cada cuatro semanas desde el inicio de la intervención.

Las cantidades de ácido oleico, DHA y EPA contenidas en los 500 mL del producto lácteo enriquecido fueron de 5,12 g, 0,2 g y 0,13 g, respectivamente. En la leche semidesnatada, en cambio, existían 1,82 g de oleico y cantidades inapreciables de DHA y EPA. La leche enriquecida con n-3 contenía ocho veces más cantidad de AGPI que la leche semidesnatada y más del doble de la cantidad de ácidos grasos monoinsaturados, mientras que las cantidades de ácidos grasos saturados detectados en la leche enriquecida fueron de aproximadamente un tercio de la existente en la leche semidesnatada empleada en el estudio.

Los resultados obtenidos en el estudio mostraron que el consumo durante 8 semanas del producto lácteo enriquecido contribuyó a un incremento promedio del 30% en los niveles plasmáticos de DHA y EPA. El consumo de la leche enriquecida produjo además una disminución de la concentración de colesterol total en plasma (6%) y colesterol LDL (16%), y también un descenso de los niveles plasmáticos de homocisteína (13%), todos ellos factores de riesgo de ECV conocidos. El consumo de leche enriquecida no produjo cambios en la concentración de vitamina E del plasma ni de las lipopoproteínas LDL. Asimismo, tampoco se modificó la capacidad antioxidante del plasma ni la susceptibilidad de las lipoproteínas LDL a la oxidación, que se midió indirectamente como tiempo de retardo a la oxidación o “lag time”, y de forma directa con ayuda de un anticuerpo monoclonal que reconocía LDL oxidada.

El consumo de este producto lácteo originó un descenso en las formas solubles de las moléculas de adhesión VCAM-1 e ICAM-1, marcadores de activación endotelial implicadas en procesos de captación e infiltración de monocitos a la pared vascular. También de detectó un incremento notable de las concentraciones de folatos sérico y eritrocitario resultante de la absorción del ácido fólico existente en la leche enriquecida (150 µg/500 mL).

En este estudio, en comparación con otros, hemos utilizado cantidades pequeñas de ácidos grasos n-3 y oleico. Está descrito que la grasa de la leche se absorbe en el intestino con gran facilidad y eficiencia, por lo que quizá el uso de este vehículo para la administración de los AGPI n-3 y oleico sea responsable de los efectos beneficiosos cardiovasculares encontrados a bajas dosis.

La ingesta de alimentos enriquecidos en ácidos grasos poliinsaturados n-3 (EPA y DHA) es una opción disponible que puede ser eficaz en la reducción de factores de riesgo de enfermedades, sustituyendo a los suplementos y sin originar cambios en los hábitos alimentarios del consumidor. Aunque el mercado de alimentos funcionales está en constante expansión, se necesitan muchos más estudios que evalúen en qué medida el consumo de alimentos funcionales produce efectos beneficiosos para la salud, un área de investigación que con toda seguridad continuará desarrollándose en los próximos años.

Bibliografía

1. The European Heart Network (EHN) : Food, nutrition and cardiovascular disease prevention in the European region : challenges for the new millennium, 2002.
2. Bang HO, Dyerberg J, Sinclair HM : The composition of the Eskimo food in north western Greenland. Am J Clin Nutr, 1980, 33:2657-61.
3. Sanders TA: Polyunsaturated fatty acids in the food chain in Europe. Am J Clin Nutr, 2000, 71: 176S-180S.
4. Trautwein EA : n-3 Fatty acids-physiologycal and technical aspects for their use in food. Eur J Lipid Sci Technol , 2001, 103:45-55.
5. Hulshof KF, van Erp-Baart MA, Anttolainen M et al.: Intake of fatty acids in western Europe with emphasis on trans fatty acids: the TRANSFAIR Study. Eur J Clin Nutr, 1999, 53: 143-157.
6. Hepburn FN, Exler J, Weihrauch JL : Provisional tables on the content of omega-3 fatty acids and other fat components of selected foods. J Am Diet Assoc, 1986, 86: 788-793.
7. Kris-Etherton PM, Taylor DS, Yu-Poth S et al.: Polyunsaturated fatty acids in the food chain in the United States. Am J Clin Nutr, 2000, 71:179S-188S.
8. Simopoulos AP, Leaf A, Salem N Jr.: Essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. Ann Nutr Metab, 1999, 43: 127-130.
9. Kris-Etherton PM, Harris WS, Appel LJ; AHA Nutrition Committee. American Heart Association : Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. Arterioscler Thromb Vasc Biol, 2003, 23:151-152.
10. Report of a Joint Expert Consultation: Diet, Nutrition and the prevention of chronic diseases. WHO Technical Report Series 916. FAO/WHO , Ginebra, 2003.
11. Report of the British Nutrition Foundation's Task Force : n-3 fatty acids and health". The British Nutrition Foundation. Chapman & Hall. New York & London, 1999.
12. Lusis AJ: Atherosclerosis. Nature, 2000, 407: 233-241.

13. Kromhout D, Bosschieter EB, de Lezenne Coulander C: The inverse relation between fish consumption and 20-year mortality from coronary heart disease. N Engl J Med, 1985, 312: 1205-1209.
14. Daviglus ML, Stamler J, Orencia AJ et al.: Fish consumption and the 30-year risk of fatal myocardial infarction. N Engl J Med, 1997, 336: 1046-1053.
15. Albert CM, Hennekens CH, O'Donnell CJ et al.: Fish consumption and risk of sudden cardiac death. JAMA, 1998, 279: 23-28.
16. von Schacky C, Baumann K, Angerer P: The effect of n-3 fatty acids on coronary atherosclerosis: results from SCIMO, an angiographic study, background and implications. Lipids, 2001, 36: 99S-102S.
17. Burr ML, Fehily AM, Gilbert JF et al.: Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). Lancet, 1989, 2: 757-761.
18. Hopper L, Ness A, Higgins JP, Moore T, Ebrahim S : GISSI-Prevenzione trial. Lancet , 1999, 354: 447-455.
19. Kris-Etherton P, Eckel RH, Howard BV et al.: AHA Science Advisory: Lyon Diet Heart Study. Benefits of a Mediterranean-style, National Cholesterol Education Program/American Heart Association Step I Dietary Pattern on Cardiovascular Disease. Circulation, 2001, 103: 1823-1825.
20. Harris WS: n-3 fatty acids and serum lipoproteins : human studies. Am J Clin Nutr, 1997, 65 : 1645 S-1654 S.
21. Zampelas A, Roche H, Knapper JME et al. : Polyunsaturated fatty acids of the n-6 and n-3 series : effects on postprandial lipid and apoprotein levels in healthy men. Eur J Clin Nutr, 1994, 48: 842-848.
22. von Schacky C : n-3 fatty acids and the prevention of coronary atherosclerosis. Am J Clin Nutr, 2000, 71: 224S-227S.
23. Connor WE : Importance of n-3 fatty acids in health and disease. Am J Clin Nutr, 2000, 71: 171S-175S.
24. Siscovick DS, Raghunathan TE, King I, Weinman S : Dietary intake and cell membrane levels of chain n-3 polyinsaturated fatty acids and the risk of primary cardiac arrest. J Am Med Assoc, 1996, 274: 1363-1367.
25. de Deckere EA, Korver O, Verschuren PM, Katan MB : Health aspects of fish and n-3 polyunsaturated fatty acids from plant and marine origin. Eur J Clin Nutr, 1998, 52: 749-753.
26. The British Nutrition Foundation : Diet and heart disease, a round table of factors. Segunda Edición, Chapman & Hall, Londres, 1997.
27. Zampelas A, Roche H, Knapper JM et al.: Differences in postprandial lipemic response between Northern and Southern Europeans. Atherosclerosis, 1998, 139: 83-93.
28. Layne KS, Goh YK, Jumpsen JA, Ryan EA, Chow P, Clandinin MT. Normal subjects consuming physiological levels of 18:3(n-3) and 20:5(n-3) from flaxseed or fish oils have characteristic differences in plasma lipid and lipoprotein fatty acid levels. J Nutr, 1996, 266: 2130-2140.

29. Adler AJ, Holub BJ: Effect of garlic and fish-oil supplementation on serum lipid and lipoprotein concentrations in hypercholesterolemic men. Am J Clin Nutr, 1997, 65: 445-450.
30. Williams CM, Moore F, Morgan L, Wright J: Effects of n-3 fatty acids on postprandial triacylglycerol and hormone concentrations in normal subjects. Br J Nutr, 1992, 68: 655-666.
31. Visioli F, Rise P, Plasmati E, Pazzucconi F, Sirtori CR, Galli C : Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerol and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol Res, 2000, 41: 571-576.
32. Cobiac L, Clifton PM, Abbey M, Belling GB, Nestel PJ: Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n-3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr, 1991, 53: 1210-1216.
33. Schectman G, Boerboom LE, Hannah J, Howard BV, Mueller RA, Kissebah AH : Dietary fish oil decreases low-density-lipoprotein clearance in nonhuman primates. Am J Clin Nutr 1996, 64: 215-221.
34. Harris WS : n-3 fatty acids and serum lipoproteins: human studies. Am J Clin Nutr, 1997, 65: 1645S-1654S.
35. Brown AA, Hu FB : Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr , 2001, 73: 673-686.
36. Report of the British Nutrition Foundation‘s Task Force. Unsaturated fatty acids : Nutritional and physiological significance. Chapman & Hall. Londres, 1992.
37. Harris WS: n-3 fatty acids and lipoproteins: comparison of results from human and animal studies. Lipids, 1996, 31: 243-252.
38. Appel LJ, Miller ER 3rd, Seidler AJ, Whelton PK: Does supplementation of diet with 'fish oil' reduce blood pressure? A meta-analysis of controlled clinical trials. Arch Int Med, 1993, 153: 429-438.
39. Morris MC, Sacks F, Rosner B: Does fish oil lower blood pressure? A meta-analysis of controlled trials. Circulation, 1993, 88: 523-533.
40. Al MDM, van Houwelingen AC, Kester ADM et al. : Maternal essential fatty acid patterns during normal pregnancy and its relationship with the neonatal essential fatty acid status. Brit J Nutr, 1995, 7: 55-68.
41. Broadhurst CL, Cunnane SC & Crawford MA : Rift Valley lake fish and selfish provided specific nutrition for early Homo. Brit J Nutr, 1998, 79: 3-21.
42. Eaton SB & Konner N: Paleolithic nutrition: a consideration of its nature and current implications. New Eng J Med, 1985, 312: 283-289.
43. Innis SM: Essential fatty acids in growth and development. Prog Lipid Res, 1991, 30: 39-103.
44. Uauy R, Peirano P, Hoffman D, Mena P, Birch D, Birch E : Role of essential fatty acids in the function of developing nervous system. Lipids, 1996, 31: 167S-176S.
45. Hibbeln JR : Fish consumption and major depression. Lancet, 1998, 351: 1213.
46. Schmidt EB, Christensen JH, Aardestrup I, Madsen T, Riahi S, Hansen VE, Skou HA: Marine n-3 fatty acids: basic features and background. Lipids, 2001, 36: 65S-68S.
47. Mantzioris E, Cleland LG, Gibson RA, Neumann MA, Demasi M, James MJ: Biochemical effects of a diet containing foods enriched with n-3 fatty acids. Am J Clin Nutr, 2000, 72: 42-48.

48. Gustafsson IB, Ohrvall M, Ekstrand B et al.: Moderate amounts of n-3 fatty acids enriched seafood products are effective in lowering serum tryglycerides and blood pressure in healthy subjects. J Hum Nutr Diet, 1996, 9: 135-145.
49. Lovegrove JA, Brooks CN, Murphy MC, Gould BJ, Williams CM: Use of manufactured foods enriched with fish oils as a means of increasing long-chain n-3 polyunsaturated fatty acid intake. Br J Nutr, 1997, 78: 193-195.
50. Oh SK, Ryue J, Hsien CH, Bell DE: Eggs enriched in n-3 fatty acids and alterations in lipid concentrations in plasma and lipoproteins and in blood pressure. Am. J Clin Nutr, 1991, 54: 689-695.
51. Surai PF, MacPherson A, Speake BK and Sparks NH: Designer egg evaluation in a controlled trial. Eur J Clin Nutr, 2000, 54: 298-305.
52. Farrell D: Enrichment of hen eggs with n-3 long-chain fatty acids and evaluation of enriched eggs in humans. Am J Clin Nutr, 1998, 68: 538-544.
53. Cherian G, Sim J: Changes in the breast milk fatty acids and plasma lipids of nursing mothers following consumption of n-3 polyunsaturated fatty acid enriched eggs. Nutrition, 1996, 12: 8-12.
54. Baró L, Fonollá J, Peña JL, et al.: n-3 Fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans. Clin Nutr, 2003, 22: 175-182.

3

Health effects due to folic acid supplementation. Food fortification strategies to achieve adequate folate status..

Nutrafoods 2004; ***3(1):****5-13*

JJ Carrero*, J Fonollá†, M González-Santiago†, L Baró†, J Jiménez†, JJ Boza† and E López-Huertas†

*Departamento de Bioquímica y Biología Molecular. Universidad de Granada Spain
†PULEVA BIOTECH S.A. Granada, Spain

Resumen

El ácido fólico ha cobrado un mayor protagonismo en las últimas décadas por su presunto papel en la patogénesis de los defectos del tubo neural, en las enfermedades cardiovasculares, en el cáncer y en las alteraciones neuropsiquiátricas.

El ácido fólico se encuentra, de manera natural, en alimentos como el zumo de naranja, vegetales de hoja verde, habas y guisantes, espárragos, fresas y cacahuetes. Sin embargo, la deficiencia en folato es aún la deficiencia vitamínica más común de los países desarrollados.

En esta separata se repasan someramente los efectos beneficiosos sobre la salud atribuidos al consumo de ácido fólico, con especial énfasis en las recomendaciones nutricionales que existen hoy en día y en las estrategias de fortificación de alimentos que se han puesto en marcha para aproximar la ingesta de folatos a la Cantidad Diaria Recomentada (CDR). Estas estrategias de fortificación son alternativas válidas y efectivas para conseguir un estatus nutricional de folato adecuado. El enriquecimiento de ciertos alimentos con folato puede contribuir a este fin, constituyendo una estrategia sana y efectiva para ayudar a prevenir estas patologías. También se presenta diversos resultados de un estudio de intervención usando un alimento funcional de base láctea que contiene ácidos grasos n-3, ácido oleico, ácido fólico y vitaminas del complejo B, en lo referente a la reducción de los niveles de homocisteína.

Summary

Folic acid has gained a considerable attention in the last decades because of its presumed role in the pathogenesis of birth defects, cardiovascular diseases, cancer and neuropsychiatric disorders.

Food folates are concentrated in selected foods such as orange juice, dark green leafy vegetables, dried beans and peas, asparagus, strawberries and peanuts. However, deficiency in folate is still the most common vitamin deficiency in developed countries.

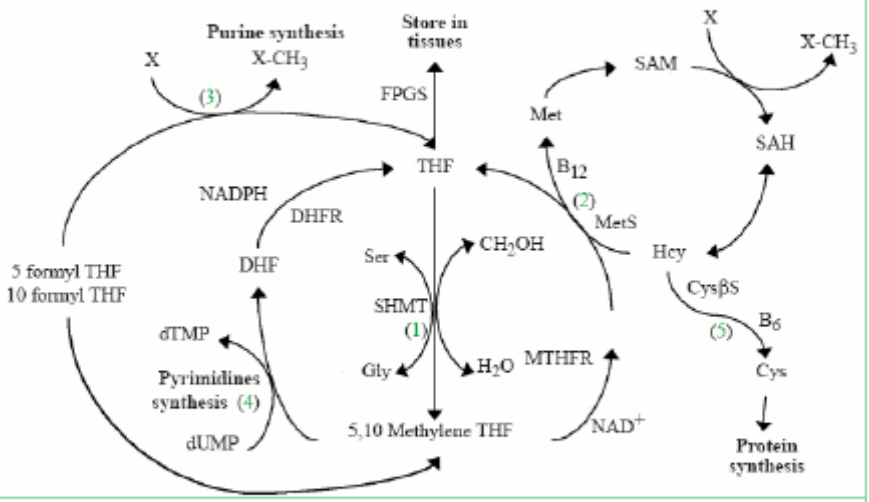
In this article we will review the health effects derived from the consumption of folic acid, focusing on nutritional recommendations and fortification strategies implemented in order to enhance the daily intake of the population so as to meet Dietary Reference Intake (DRI) levels. These fortification policies constitute an effective way to achieve an adequate folate status. In addition, fortifying foods with folic acid may be an easy and safe strategy in preventing these severe pathologies. We also present in this review the results from an intervention study using a functional dairy product enriched in n-3 fatty acids, oleic acid, folic acid and B vitamins regarding the reduction of tHcy levels.

Introduction

Folic acid is a water soluble vitamin which, in the last decades, has gained a considerable attention because of its presumed role in the pathogenesis of birth defects (1), cardiovascular diseases (2), cancer (3) and neuropsychiatric disorders (4).

The various enzymes of the folate cycle, facilitate methylation reactions as well as the transfer of “one-carbon units” from donor molecules needed for the remethylation of homocysteine to generate methionine, the formation of purines and pyrimidines in the biosynthesis of DNA, and many other biological methylation reactions. They also mediate the interconversion of serine and glycine, and play a role in histidine catabolism (**Fig. 1 Folate Metabolic Cycle**).

Figure 1 *Folate cycle metabolism, representing the major pathways in which folate facilitates methylation reactions as well as the transfer of 'one-carbon units' from several donors. Folate participates in the methylation process needed to regenerate serine from glycine (1) methionine from homocysteine (2), the formation of purines (3) and pyrimidines (4) in the biosynthesis of DNA, and many other biological methylation reactions (5)*



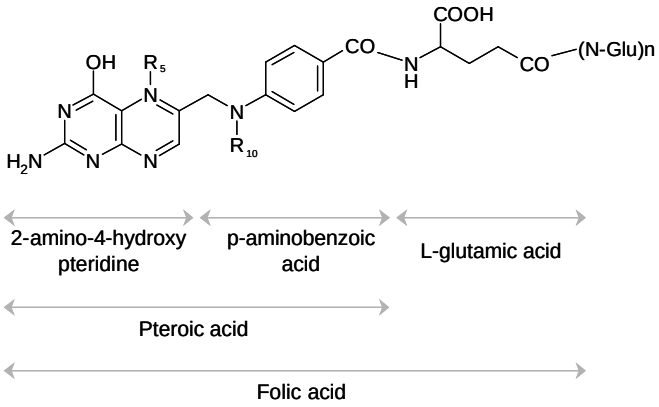
Abbreviations: THF: Tetrahydrofolic acid; DHF: Dihydrofolate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; FPGS: Folylpolyglutamate synthase; MetS: Methionine synthase; CysβS: Cystathionine synthase; THFR: 5,10-Methylenetetrahydrofolate reductase; SHMT: Serinehydroxymethyl transferase; DHF: hydrofolate reductase

Although folate is present in a variety of foods, deficiency in folate is the most common vitamin deficiency in developed countries (5). In this article we will review the health effects derived from the consumption of folic acid, focusing on fortification strategies in order to enhance the daily intake of the population so as to meet Dietary Reference Intake (DRI) levels. In the case of folate and its demonstrated benefit in prevention and against progression of several diseases, health authorities recommend providing greater levels than the current DRIs.

Food sources and bioavailability.

Folic acid or pteroylglutamate (PteGlu) is a stable and synthetic analog of the vitamin, considered as the parent structure of a large family of vitamin coenzymes. Folate is the naturally occurring form of the vitamin. Mammals cannot synthesize the pteriding ring of PteGlu, so they have to obtain it from their diets or from microorganisms in their intestinal tract (**Fig. 2 Folate structure**).

Figure 2. Chemical structure of folate family.



Folic acid species	R_5	R_{10}
Tetrahydrofolic acid (THF)	-H	-H
5-methyl THF	$-CH_3$	-H
5,10-methyleneTHF	$-CH_2-$	$-CH_2-$
5,10-methenyl THF	$-CH=N^+-$	$-CH=N^+-$
5-formyl THF	-CHO	-H
10-formyl THF	-H	-CHO
10-formimino THF	-H	-HCNH

Food folates are concentrated in selected foods such as orange juice, dark green leafy vegetables, dried beans and peas, asparagus, strawberries and peanuts (**Table 1**.). The chemical structure of folate consists of a pteroylglutamate molecule linked to a chain of glutamates. However, the estimation of the dietary intake of folate by food composition tables may not reflect the actual intake because naturally occurring folate in the diet may be rendered inactive by cooking and food processing.

The synthetic form of the vitamin, folic acid, exists in the monoglutamate form. The bioavailability of this form is significantly greater than the natural folate polyglutamate presumably because the natural form requires the cleavage of the glutamate chain prior to absorption. The enzyme responsible for this cleavage activity is the folate conjugase, which

may be specifically inhibited by food factors in yeast and beans and also by low pH (6). Finally, the monoglutamate forms of folate are transported across the proximal small intestine via saturable pH-dependent process.

It is widely recognised that for typical mixed diets, the bioavailability of naturally occurring folate is incomplete. Several studies (7) indicate that the bioavailability of natural food folate in a typical mixed diet is not more than 50% when compared with a formula diet containing the same amount of synthetic folic acid (8). A similar study with free-living subjects observed that folate from foods fortified with folic acid and folic acid supplements was more bioavailable than natural food folate. Other authors have stated an 85% bioavailability for synthetic folic acid (9). Taking into consideration the differences in bioavailability between natural an synthetic folate, the current DRIs express folate requirements in terms of Dietary Folate Equivalents (DFEs) (10).

It should be recognized that not all food sources of folate exhibit poor bioavailability. For example, natural folate from fruits and vegetables exhibited 60-90% of the bioavailability shown for synthetic folic acid given as such (11). Regarding the bioavailability of folic acid from supplements, very little published data is available. Incomplete in vitro dissolution of certain commercial supplements may be indicative of reduced bioavailability, but no in vivo data has been published regarding this issue. However, in the context of long-term nutrition, minor differences in the rate of absorption of folate from supplements are of little or no consequence (12).

Table 1. Folate content of selected non-fortified foods.

Food item	Amount (g)	Folate content (µg DFEs)
Beef liver	85	185
Chickpeas, boiled	150	81
Beans, cooked	180	60
Peas, boiled	180	90
Spinach, raw	180	160
Spinach, boiled	180	150
Tomatoes	85	15
Potatoes, boiled	180	50
Broccoli, cooked	180	100
Asparagus, boiled	50	78
Orange	160	60
Avocado	150	100
Banana	100	20
Melon	180	50
Peanuts	180	198
Whole grain bread	100	90

Abbreviations: DFEs = dietary folate equivalent
Adapted from (47)

Folic acid and prevention of Neural Tube Defects (NTDs).

NTDs are birth defects of the spine that occur when the neural tube, the precursor of the spine, fails to close properly in the developing fetus. NTDs constitute one of the more important contributors to infant mortality and childhood morbidity, affecting approximately 4000 infants every year in the United States and one in 100 births in Spain. In these cases the evidence is irrefutable: numerous trials confirm so strongly that supplements containing folic acid that are taken periconceptionally dramatically reduce the risk of NTDs (13-15), that various public health policies have been implemented worldwide (16-18). These studies suggest that intakes ranging from 360 to 4000 µg per day of periconceptional folate can prevent approximately 70% of NTDs first occurrence and recurrence (1).

The US Public Health Service recommended that all women of childbearing age consume 400 µg/day of folic acid (16), and the UK Department of Health also suggested that an extra 400 µg/day of folic acid, from either dietary or supplemental sources, should be consumed before conception and during the early months of pregnancy (19).

Finally, in 1998, the Food and Drug Administration of the US Department of Health implemented a new legislation mandating the fortification of enriched cereal-grain products with folic acid at a concentration of 140 µg/100g of product, aimed to increase the population´s daily consumption of folic acid by 100 µg per day. In contrast, other research studies did not find a reduction in the incidence of NTDs in England and Wales upon education of the population about folic acid supplementation (20). In addition, a 1999 survey in the USA showed that less than third of women were taking a vitamin supplement with folate; only 13% knew that folic acid helped to prevent birth defects; and only 7% knew that it should be taken before pregnancy (21). This data strengthens the argument for Health Authorities to increase folic acid fortification to meet the recommended intake levels to 400 µg/day.

Folic acid and cardiovascular diseases.

Numerous epidemiological studies support an inverse association between dietary folate intake and vascular disease risk. The metabolic basis for the observed inverse association appears to be related to the fact that folate is a coenzyme involved in the regulation of normal plasma homocysteine (Hcy) concentrations through a key remethylation reaction in which vitamins B12 and B6 are also involved cofactors (Fig. 1). Hcy is a thiol-containing amino acid from methionine metabolism, and hyperhomocysteinemia (>15 µmol/L) has been identified as an independent risk factor for the development of atherothrombotic, cardiovascular and cerebrovascular diseases, as promotes damage to the vascular endothelium, proliferation of smooth muscle cells, and enhances platelet and leukocyte aggregation (22). Because increased folate intake levels are associated with decreases in Hcy levels, some authors have

hypothesized that increased folate intake would reduce mortality and morbidity from vascular disease and have proposed increases in the fortification level to achieve “maximal” reduction in serum Hcy levels (23).

The homocysteine-lowering effects of folic acid have been well documented (24, 25). Bronstrup *et al.* (26), also describes that although folic acid is mainly responsible for Hcy decrease, the addition of vitamins B12 and B6 to folic acid supplements or enriched foods may maximize the reduction of homocysteine. To give an idea of the importance of reducing this novel cardiovascular risk factor, a very recent meta-analysis suggests that lowering homocysteine concentrations by 3 µmol/L from current levels would reduce the risk of ischaemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24% (27).

In these cases, folate was only seen as an Hcy reducing agent, but it has also been proposed a beneficial effect on vessels independent from Hcy and attributed to folate itself, as beneficial effects from folate were also observed in subjects without elevated Hcy concentration (28-30).

Stanger *et al.* (31) attempted to separate the effects of folic acid from those derived from Hcy decrease, by administering an identical dose of folic acid for 12 weeks to coronary heart disease (CHD) patients measuring vessel resistance activity. Results showed that improvement of vessel reactivity was exclusively observed in individuals which reduced the plasma Hcy concentration by more than 2 µmol/L. In subjects which did not show significant changes in Hcy levels, folic acid administration was associated with increased antioxidant capacity, suggesting an antioxidative potential for folate.

Only a few months ago, FAO/OMS associated folate intake to a “probable” evidence of diminishing cardiovascular risk (32).

Folic acid and cancer prevention.

Several population-based studies have proposed an inverse relation between folate status and diverse types of cancers, but is more clearly defined for colorectal cancer and colorectal adenomas (33).

Currently, it is believed that folate deficiency affects DNA stability mainly via two potential pathways (Fig. 1). The first is through altered DNA methylation, as folate participates in the remethylation of Hcy to S-adenosylmethionine (SAM). SAM methylates specific cytosines in DNA, and its depletion induced DNA hypomethylation and potentially pro-oncogene expression leading to cancer. The second pathway implies folate as a donor of methyl group to uracil converting it to thymine, which is used for DNA synthesis and repair. If folate is limited, uracil misincorporation into DNA may occur. Then uracil in DNA is excised to repair it and may lead to a double-strand breaks, chromosomal damage and cancer (34).

Giovannucci *et al.* (35) evaluated the risk of colorectal cancer adenomas in the Nurses' Health Study (15,984 women) and Health Professionals Follow-Up Study (9,490 men) cohorts. A 30-40% decreased risk was found with median energy-adjusted total folate intakes > 700 µg/d compared with the lowest folate intake group (166 and 241 µg/d for women and men, respectively). Fuchs *et al.* (36) recently evaluated the influence of folate and multivitamin use on the familial risk of colon cancer in women (n=88,758). They observed that colon cancer risk was reduced by 52% in women with a family history who consumed >400 µg/d compared with women with a similar family history who consumed = 200 µg/d. This risk was substantially attenuated in women consuming multivitamins for >5 years together with a low alcohol consumption.

A lower risk of disease is better observed in individuals with higher folate intake or status, mostly derived from the consumption of multivitamins or supplements. This suggests that folic acid from supplement sources may be an important contributor to overall folate intake in the diet, and could play a role in contributing to cancer risk reduction.

However, the present evidence does not yet support public health recommendations regarding folic acid and cancer prevention. The optimal dose, the duration of treatment and the stage of carcinogenesis for folate are not still defined (37).

Recommended intake and needs of dietary fortification.

As a result of the demonstrated beneficial effects derived from folic acid consumption, several Proffesional Health Associations currently recommend that women of childbearing age consume at least 400 µg per day of synthetic folic acid to prevent NTDs (38,16). For those women who have had a previous NTD-affected pregnancy, it is recommended that they consume 4 mg of folic acid per day (16).

In terms of cardiovascular disease and cancer prevention, no official recommendations have been released. At the moment, we can only refer to the different RDIs of several countries (**Table 2**). However, van Oort *et al.* (39) concluded in a dose response-study, that a daily dose of approximately 400 µg is the minimum dose required for adequate homocysteine reduction.

As commented by Law (40), nutritionists may prefer naturally folate-rich food rather than folic acid enriched products. However, the amount of folate-rich food needed to provide 400 µg per day is unrealistic: 4 servings of Brussels sprouts, 10 servings of broccoli, or 8 glasses of orange juice per day, for example. Futhermore, we should remember the 50% bioavailability of naturally occurring folates versus 85% of synthetic folic acid. Nowadays, two effective ways of supplementing folic acid are available: folic acid supplements and folic acid fortified foods. Though folic acid supplements (e.g. multivitamin pills) constitute an effective strategy,

implementing the consumption by Public Health Education Programmes have only resulted in a limited proportion of people taking supplements daily.

Table 2: Recommended dietary intake (RDI) for folate for the adult population.

Country	Minimun Requirements	Recommended	
		Male	Female
FAO/WHO	60	400	400
USA		320	320
UK	100	300	300
The Netherlands	50	200-300	200-300
France	50	300	300
Germany	150	300	300
Spain		300-400	300-400

Adapted from (48,49)

Folic acid Fortified foods.

Folic acid fortified foods constitute an easy and inexpensive way of supplementing folic acid to the general population, and several approaches have proven to be effective. For instance, breakfast cereals fortified with 400 µg per serving increased folate status and reduced Hcy levels (41).

The mandatory flour fortification programme in the US has resulted in an increase of serum folate and a decrease of NTDs (42,43). The prevalence of spina bifida decreased 31% from the pre- to the mandatory fortification period and the prevalence of anencephaly decreased 16% (44). Indeed, flour is an excellent food product to fortify, as is widely consumed and provides folic acid to other commercially available manufactured foods which include flour.

Other ways of supplementing folic acid in the diet should be explored. In countries like Spain, where mandatory flour fortification has not been established, other daily-used foods could also be studied as a means of folate fortification.

Milk could also be another effective strategy for food fortification. Milk fortification may offer certain advantages such as 1) being an every day used drink, it may help to overcome the problem of compliance with supplements, 2) producing a minor impact in the life-style of the population, specially in regular milk drinkers, and 3) giving as well the possibility of fortification with other nutrients that may potentially be beneficial (n-3 fatty acids, vitamins B6, B12…etc).

Based on this hypothesis, we designed a multivitamin/mineral fortified dairy product enriched in folic acid, vitamins B6 and B12 (Puleva Mamá®), in order to provide the nutritional requirements for women of childbearing age and those in the first three months of pregnancy. In a recent study, we assessed whether the administration of folic acid and other vitamins and

minerals through this dairy vehicle would be effective in the improvement of folate status in women of reproductive age (45). Thirty-one healthy non-pregnant women (age: 28.2±1.0; range: 21-37 years) consumed 500 ml/day of the fortified milk for two months, providing 400 µg/d of folic acid to meet nutritional requirements (16,17). The daily consumption lead to a statistically significant 80% increase in plasma folate and another significant 80% increase in red blood cell folate concentration. At the same time, tHcy concentrations significantly decreased by 30% after the 8 week period.

Another study carried out in our laboratory (46) tested possible beneficial effects derived from the consumption of a similar enriched dairy product thought to help in the prevention of cardiovascular risk factors (Puleva Omega3®). In this case, 30 volunteers consumed 500 mL/day of the enriched dairy product for a further two months which provided with 150 µg/d of folic acid (75% RDI). Plasma folate levels rose up to 91% at the end of the study (3.37 ±0.35 vs 6.10±0.45 µg/L). Consistent with this increase, total plasma homocysteine showed a significant 13% reduction (12.9±07 vs 10.9±0.6 µmol/L).

We believe that milk fortification is an easy and effective way to increase the intake of dietary folate. These two studies show that consumption of only 500 mL/d of the dairy products, can be enough to significantly increase folate status and easily reach nutritional recommendations.

Scientific evidence suggests that food fortification policies constitutes an effective way to achieve an adequate folate status. In addition, fortifying foods with folic acid may be an easy and safe strategy in preventing severe pathologies including NTDs, cardiovascular events and cancer.

References

1. Van Dyke DC, Stumbo PJ, Mary JB, Niebyl JR. (2002) Folic acid and prevention of birth defects.Dev Med Child Neurol. 44(6):426-9.
2. Lucock M. (2000) Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab. 71(1-2):121-38.
3. Bailey LB, Rampersaud GC, Kauwell GP. (2003) Folic acid supplements and fortification affect the risk for neural tube defects, vascular disease and cancer: evolving science. J Nutr.133(6):1961S-1968S.
4. Reynolds EH. (2002) Folic acid, ageing, depression, and dementia. BMJ. 324(7352):1512-5.
5. Stanger O. (2002) Physiology of folic acid in health and disease. Curr Drug Metab. 3(2):211-23.
6. Fitzpatrick A. (2003) Folate (Folic acid): implications for health and disease. Agrofood ind hi-tech. May/June, 4545-48.
7. Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC.(1987) Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr. 46(6):1016-28.

8. Cuskelly GJ, McNulty H, Scott JM. (1996) Effect of increasing dietary folate on red-cell folate: implications for prevention of neural tube defects. Lancet. 347(9002):657-9.
9. Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF 3rd. (1997) Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. Am J Clin Nutr. 66(6):1388-97.
10. Suitor CW, Bailey LB. (2000) Dietary folate equivalents: interpretation and application. J Am Diet Assoc. 100(1):88-94.
11. Brouwer IA, van Dusseldorp M, West CE, Meyboom S, Thomas CM, Duran M, van het Hof KH, Eskes TK, Hautvast JG, Steegers-Theunissen RP. (1999) Dietary folate from vegetables and citrus fruit decreases plasma homocysteine concentrations in humans in a dietary controlled trial. J Nutr.129(6):1135-9.
12. Gregory 3rd JF (2001) Case Study: folate bioavailability. Case study: folate bioavailability. J Nutr. 131(4 Suppl):1376S-82S.
13. Czeizel AE, Dudas I. (1992) Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation.N Engl J Med. 327(26):1832-5.
14. Khoury MJ, Shaw GM, Moore CA, Lammer EJ, Mulinare J. (1996) Does periconceptional multivitamin use reduce the risk of neural tube defects associated with other birth defects? data from two population-based case-control studies. Am J Med Genet. 61(1):30-6.
15. Manning SM, Jennings R, Madsen JR. (2000) Pathophysiology, prevention, and potential treatment of neural tube defects. Ment Retard Dev Disabil Res Rev. 6(1):6-14.
16. Centers for Disease Control and prevention. (1992) Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. MMWR 41: 1-47.
17. Institute of Medicine (1998) Folate. In: Dietary reference Intakes for thiamine, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline. Pp. 196-305. National Academy Press, Washington DC.
18. Lumley J, Watson L, Watson M, Bower C. (2001) Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects. Cochrane Database Syst Rev. (3):CD001056.
19. Report from Expert Advisory Group on folic acid and the prevention of neural tube defects (1992). London: Department of Health.
20. Kadir RA, Sabin C, Whitlow B, Brockbank E, Economides D. (1999) Neural tube defects and periconceptional folic acid in England and Wales: retrospective study. BMJ. 319(7202):92-3.
21. Centers for Disease Control and Prevention. (1999) Knowledge and use of folic acid by women of childbearing age-United States, 1995 and 1998. MMWR. 48:325-327.
22. Graham IM, O'Callaghan P. (2002) Vitamins, homocysteine and cardiovascular risk. Cardiovasc Drugs Ther. 16(5):383-9.
23. Wald DS, Bishop L, Wald NJ, Law M, Hennessy E, Weir D, McPartlin J, Scott J. (2001) Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med. 161(5):695-700.
24. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analisys of randomised trials. BMJ 1998; 316: 894-98.
25. van den Berg M, Franken DG, Boers GH, *et al.* Combined vitamon B6 plus folic acid in young patients with atherosclerosis and hyperhomocysteinaemia. J Vasc Surg 1994; 20: 933-40.

26. Bronstrup A, Hages M, Prinz-Langenohl R, Pietrzik K. Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women. Am J Clin Nutr 1998; 68(5):1104-10
27. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002; 325(7374):1202
28. Verhaar MC, Wever RM, Kastelein JJ, van Loon D, Milstien S, Koomans HA, Rabelink TJ. (1999) Effects of oral folic acid supplementation on endothelial function in familial hypercholesterolemia. A randomized placebo-controlled trial. Circulation. 100(4):335-8.
29. Wilmink HW, Stroes ES, Erkelens WD, Gerritsen WB, Wever R, Banga JD, Rabelink TJ. (2000) Influence of folic acid on postprandial endothelial dysfunction. Arterioscler Thromb Vasc Biol. 20(1):185-8.
30. Doshi SN, McDowell IF, Moat SJ, Payne N, Durrant HJ, Lewis MJ, Goodfellow J. (2002) Folic acid improves endothelial function in coronary artery disease via mechanisms largely independent of homocysteine lowering. Circulation. 105(1):22-6.
31. Stanger O, Semmelrock HJ, Wonisch W, Bos U, Pabst E, Wascher TC. (2002) Effects of folate treatment and homocysteine lowering on resistance vessel reactivity in atherosclerotic subjects. J Pharmacol Exp Ther. 303(1):158-62.
32. FAO/OMS. March 2003. Diet, Nutrition, and the Prevention of Chronic Disease. Rome-Geneva, March 2003. p, 64.
33. Choi SW, Mason JB. (2002) Folate status: effects on pathways of colorectal carcinogenesis. J Nutr. 132(8 Suppl):2413S-2418S.
34. Duthie SJ, Narayanan S, Brand GM, Pirie L, Grant G. (2002) Impact of folate deficiency on DNA stability. J Nutr. 132(8 Suppl):2444S-2449S.
35. Giovannucci E, Stampfer MJ, Colditz GA, Rimm EB, Trichopoulos D, Rosner BA, Speizer FE, Willett WC. (1993) Folate, methionine, and alcohol intake and risk of colorectal adenoma. J Natl Cancer Inst. 85(11):875-84.
36. Fuchs CS, Willett WC, Colditz GA, Hunter DJ, Stampfer MJ, Speizer FE, Giovannucci EL. (2002) The influence of folate and multivitamin use on the familial risk of colon cancer in women. Cancer Epidemiol Biomarkers Prev. 11(3):227-34.
37. Eichholzer M, Luthy J, Moser U, Fowler B. (2001) Folate and the risk of colorectal, breast and cervix cancer: the epidemiological evidence. Swiss Med Wkly. 131(37-38):539-49.
38. American Academy of Pediatrics. (1999) Folic acid for the prevention of neural tube defects. American Academy of Pediatrics. Committee on Genetics. Pediatrics 104: 325-327.
39. van Oort FV, Melse-Boonstra A, Brouwer IA, Clarke R, West CE, Katan MB, Verhoef P. (2003) Folic acid and reduction of plasma homocysteine concentrations in older adults: a dose-response study. Am J Clin Nutr. 77(5):1318-23.
40. Law M. (2000) Fortifying food with folic acid. Sem Thromb Hemost. 26(3): 349-352.
41. Malinow MR, Duell PB, Irvin-Jones A, Upson BM, Graf EE. (2000) Increased plasma homocyst(e)ine after withdrawal of ready-to-eat breakfast cereal from the diet: prevention by breakfast cereal providing 200 microg folic acid. J Am Coll Nutr. 19(4):452-7.
42. Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong LY. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. JAMA. 285(23):2981-6.

43. Lawrence JM, Petitti DB, Watkins M, Umekubo MA. (1999) Trends in serum folate after food fortification. Lancet. 354(9182):915-6.
44. Williams LJ, Mai CT, Edmonds LD, Shaw GM, Kirby RS, Hobbs CA, Sever LE, Miller LA, Meaney FJ, Levitt M. (2002) Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. Teratology. 66(1):33-9.
45. Baró L, Martínez-Férez A, Rodríguez C, Valero A, Fonollá J, Lucena A, Jiménez J, Boza J and López-Huertas E. (In Press) The administration of a multivitamin/mineral fortified dairy product improves folate status and reduces plasma homocysteine concentration in women of reproductive age. Int J Vit Nutr Res.
46. Baro L, Fonollá J, Peña JL, Martinez-Férez A, Lucena A, Jimenez J, Boza JJ, Lopez-Huertas E. (2003) n -3 Fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans. Clin Nutr. 22(2):175-82.
47. Rampersaud GC, Kauwell GP, Bailey LB (2003) Folate: a key to optimizing health and reducing disease risk in the elderly J Am Coll Nutr 22(1) 1-8
48. Krishnaswamy K, Madhavan Nair K (2001) Importance of folate in human nutrition Br J Nutr 85(Suppl 2) S115-S124
49. Serra-Majem L, Ortega R, Aranceta J, Entrala A, Gil A, GRAN Group (2001) Fortified foods. Criteria for vitamin supplementation in Spain Public Health Nutr 4(6A) 1331-1334

4

n3-fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans.

Clinical Nutrition (2003). **22(2):** 175-182.

L Baró*, J Fonollá*, JL Peña*, A Martínez-Férez†, A Lucena‡, J Jiménez*, JJ Boza* and E López-Huertas*.

* PULEVA BIOTECH S.A. Granada, Spain
†Departamento de Ingeniería Química. Universidad de Granada Spain
‡Hospital Universitario “San Cecilio” 16, Av Dr Oloriz, Granada, Spain

Resumen

Un gran número de estudios científicos sugiere que la ingesta de ácidos grasos polinsaturados n-3 y de ácido oleico ejerce una serie de efectos beneficiosos para la salud cardiovascular. El propósito de este estudio es evaluar el efecto que una leche semidesnatada comercial enriquecida con ácidos grasos n-3, ácido oleico y vitaminas E, B_6, B_{12} y ácido fólico (Puleva Omega3 ®) ejerce sobre diversos factores de riesgo cardiovascular.

30 voluntarios sanos tomaron 55 mL/día de leche semidesnatada durante 4 semanas, seguido de 500 mL/día de la leche enriquecida durante las 8 semanas siguientes. Se extrajeron muestras de plasma y de lipoproteínas LDL al principio del estudio y tras 4, 8 y 12 semanas.

El consumo de la leche enriquecida produjo una disminución significativa en la concentración plasmática de colesterol total y LDL, seguida de una reducción en los niveles de homocisteína. Tanto la oxidabilidad plasmática y de las LDL como la concentración de vitamina E permaneció inalterada durante el tiempo de estudio. Se apreció un aumento en los niveles plasmáticos de folatos y una reducción significativa de la molécula de adhesión VCAM-1. La ingesta de esta leche enriquecida en ácidos grasos n-3, ácido oleico, ácido fólico y vitaminas ejerció un efecto favorable en ciertos factores de riesgo cardiovascular de la población objeto.

Summary

Numerous studies suggest n-3 polyunsaturated fatty acids (n-3 PUFA) and oleic acid intake have beneficial effects on health including risk reduction of coronary heart disease. The purpose of this study was to evaluate the effect of a commercially available skimmed milk supplemented with n-3 PUFA, oleic acid, and vitamins E, B_6, B_{12} and folic acid (Puleva Omega3 ®) on risk factors for cardiovascular disease.

Thirty volunteers were given 500 ml per day of semi-skimmed milk for 4 weeks and then 500 ml/day of the n-3 enriched milk for 8 further weeks. Plasma and LDL lipoproteins were obtained from volunteers at the beginning of the study (Tpre), and at 4, 8 and 12 weeks.

The consumption of n-3 enriched milk produced a significant decrease in plasma concentration of total and LDL cholesterol accompanied by a reduction in plasma levels of homocysteine. Plasma and LDL oxidability and vitamin E concentration remained unchanged throughout the study. A significant reduction in plasma levels of vascular cell adhesion molecule 1, and an increase in plasma concentration of folic acid were also observed. Daily intake of n-3 PUFA and oleic acid supplemented skimmed milk plus folic acid and B type vitamins has favourable effects on risk factors for cardiovascular disease.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in Europe, the US and a major part of Asia. A variety of risk factors are associated with CVD, including high cholesterol levels, high plasma levels of homocysteine, hypertension, diabetes, low HDL-cholesterol levels and low levels of antioxidants, most of them influenced by diet (1). Beneficial effects of the Mediterranean diet on CVD are related to reduced saturated fat and high olive oil consumption (rich in oleic acid) and also to a high intake of fruit and vegetables, all of them rich in antioxidants. The consumption of monounsaturated fatty acids (MUFAs), especially oleic acid, has been shown to decrease plasma triacylglycerol and cholesterol concentrations, without affecting plasma HDL-cholesterol levels in healthy normolipidemic subjects (2,3).

A considerable number of research studies focus on cardiovascular disease prevention by n-3 polyunsaturated fatty acids (n-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). n-3 PUFAs favourably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioural disorders (4). Dietary n-3 PUFA have been reported to prevent cardiovascular disease through a variety of actions. As they are incorporated into the cellular phopholipids, they produce less active forms of eicosanoids precursors with important physiological implications. Although the exact mechanism by which n-3 fatty acids exert an atheroprotective effect is still unclear, they present anti-inflammatory properties, prevent arrythmia, inhibit the synthesis of cytokines and mitogens, stimulate endothelial-derived nitric oxide, lower blood lipids and also inhibit atherosclerosis and thrombosis (5-8).

Atherosclerosis and inflammation share similar mechanisms in their early phases, involving increased interactions between vascular endothelia and circulating leukocytes, where vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) play major roles (9).

Several studies suggest plasma levels of these adhesion molecules constitute a good marker for long-term prediction of cardiovascular events (10,11). In this sense, there is growing evidence regarding the effect of fish oils showing they act modulating endothelial function (11) by reducing expression of endothelial adhesion molecules (VCAM-1 and ICAM-1) (12, 48).

Epidemiological studies have shown high blood concentration of homocysteine appears to be associated with higher risks of coronary, cerebral, and peripheral vascular disease and are inversely related to blood levels of folate and of vitamins B_{12} and B_6 (13). This indicates coronary heart disease risk being reduced by dietary vitamin supplementation (14). Furthermore, a study carried out in healthy young women showed doses of folic acid as low as 250 µg per day, in addition to usual dietary intakes of folic acid, significantly decreased plasma levels of homocysteine (15).

As a result, health authorities have recommended increases in the consumption of PUFAs (16-18), in which fish oil is specially rich. However, modern western societies tend to include very little fish in their diets and increasing fish consumption would involve major dietary changes, which makes this approach not very effective. Therefore, other ways to increase consumption of PUFAs have to be explored and assessed at a community or clinical level (19).

Milk has been shown to be the most efficient vehicle for fat absorption because milk fat appears highly dispersed in micelles. We have used milk as a vehicle for n-3 PUFA, oleic acid, and vitamins E, B_6, and folic acid supplementation. The objective of the present work was to study the effects of this product on risk factors for cardiovascular disease in young healthy normolipidemic subjects.

Materials and Methods

Subjects and study design

Thirty volunteers (15 men and 15 women, age 33.1± 7.2, range: 20-45 y) who were resident in Spain participated in the study. Volunteers were defined as healthy normolipidaemic subjects after consulting medical history and carrying out a physical examination. No subject was taking any medication known to influence lipid metabolism one month before the study and they were not suffering from any chronic or metabolic disease. Women volunteers were not using oral contraceptives. Volunteers were instructed not to change their physical activity and their usual diet, but only to avoid eating fish from the beginning until the end of the study. The protocol was approved by the Ethical Committee of Puleva Biotech S.A., and informed written consent was obtained from the subjects. All of the subjects completed the study and not one missed a sampling date. Volunteers were also requested to fill in a food diary according to instructions from the principal investigator where they registered all food consumption during the study. The amounts of oleic acid and n-3 fatty acids coming from sources other than the milks were calculated using the food composition tables published in (49) as reference values.

The subjects drank 500 mL/day of semi-skimmed milk enriched in vitamins A and D from the beginning of the study (Tpre) for four weeks (T_0). At time T_0, subjects replaced the semi-skimmed milk with 500 ml/day of the n-3 enriched milk, a dairy product containing oleic acid and polyunsaturated fatty acids of the n-3 series, vitamins A,D, E, B_6 and folic acid (Tables 1-4). After an overnight fast lasting 10 hours, a blood sample (30 mL) was taken at times Tpre (beginning of the study), T_0 (after consumption of the semi skimmed milk) and then, at 4 and 8 weeks during consumption of the n-3 enriched milk (T_4 and T_8, respectively).

Table 1. Composition of semi skimmed milk and n-3 PUFA enriched milk.

	Semi-skimmed milk	Enriched milk
Energy (kcal-kJ/100 ml)	46.5-195.5	52-218
Protein (g/100ml)	3.1	3.5
Carbohydrates (g/100ml)	4.7	5.2
Fat (g/100ml)	1.9	1.9
Calcium (mg/100ml)	120	132
Vitamin A (µg/100ml)	120	120
Vitamin D (µg/100ml)	0.75	0.75
Vitamin E (mg/100ml)	ND	1.50
Vitamin B-6 (mg/100ml)	ND	0.30
Vitamin B-12 (µg/100ml)	0.38	0.38
Folic acid (µg/100ml)	ND	30

ND, not detected

Plasma and LDL isolation

Blood was withdrawn in EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany) and plasma was obtained by centrifugation at 1 000 x g for 10 min at 4°C. For LDL isolation, 10 ml of fresh plasma were transferred to ultracentrifuge tubes and density was adjusted to 1.30 g/ml by addition of solid KBr. Tubes were then filled up dropwise with a 0.15 M NaCl solution and centrifuged at 242 000 x g for 2.5 h at 4°C in a VTi50 rotor as described in (20). LDL particles typically sedimented at density range 1.006-1.063 g/ml as described elsewhere. LDL fractions were pooled and dialysed in the dark for 24 h against three changes of 2 l each of 0.01M phosphate buffered saline (PBS) 0.15 M NaCl, pH 7.4, and then frozen at –80°C under nitrogen atmosphere until needed.

Plasma and lipoprotein lipid peroxidation

Fresh plasma (50 µl) was incubated with 100 mM of the free radical generator AAPH (2, 2´-azobis-2-amidinopropane hydrochloride, Wako Chemical Industries Ltd, Japan), at 37°C for 2 h. AAPH is a widely used azo compound that produces peroxyl radicals at a constant rate. Plasma lipid peroxidation was determined by measuring TBARS as described in (21).

For lag time measurements, 50 µg of dialysed LDL in 1 ml PBS were incubated with 10 µM $CuSO_4$ for several hours at 37°C. The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm every 10 min. Lag time was determined according to (22). LDL lipid peroxidation was determined before and after AAPH induction as described in (23).

Total antioxidant capacity

Total antioxidant capacity was measured in plasma using Trolox as standard (24). Briefly, 20 µl of fresh plasma were 1:1 diluted in PBS and incubated with 1 ml of ABTS+ for 20 min. Absorbance was read at 734 nm. ABTS cation was prepared by addition of 88 µl of 140 mM potassium persulfate to 5 ml of a 7 mM solution of ABTS (Sigma A-1808) in water and incubation for 12-14 h. Working solution was obtained by dilution of the former with PBS until the aborbance at 734 nm was 0.7 ± 0.02 as described in (25).

Lipids, Homocysteine, vitamin B_{12}, vitamin E, folate, VCAM-1 and ICAM-1 determinations

Plasma triacylglycerols, total cholesterol, and HDL cholesterol were measured using commercial kits purchased from Biosystems (Barcelona, Spain) according to manufacturer´s instructions. Plasma fatty acid profile was determined by gas-liquid chromatography as described in (26). Total plasma homocysteine concentration was measured by HPLC with fluorescence detection (27). Plasma vitamin E concentration was determined by HPLC with ultraviolet detection following the method described by Thurnham *et al.* (1988) (28). Plasma folic acid and vitamin B_{12} concentration were measured by immunoassay using a commercial kit (SimulTRAC-SNB Radioassay Kit, ICN Pharmaceuticals, USA). VCAM-1 and ICAM-1 were measured using a commercial kit from Biosource International (USA) according to manufacturer´s instructions.

Statistical analysis

All data are expressed as means ± SEMs. Comparisons between groups at the different time points were assessed by one-way analysis of variance (ANOVA). When this analysis indicated a significant difference ($p<0.05$) paired Student's t-test analyses followed by Bonferroni corrections for multiple analyses were perfomed. The data was analysed using SPSS statistical software package (SPSS for Windows 10.1; SPSS Chicago, IL, USA).

Results

Both types of milk were well accepted and compliance was good. There was no significant body weight change in the volunteers throughout the study.

Dietary intake of fatty acids.

Composition and fatty acid profile of the semi-skimmed and n-3 enriched milks used in the study are given in **Tables 1 and 2**. The amounts of oleic acid, DHA and EPA supplemented in 500 ml of the n-3 enriched milk were 5.12 g, 0.13 g, and 0.2 g, respectively, whereas the semi-skimmed milk contained only 1.82 g per 500 ml, and no detectable levels of DHA and EPA. The n-3 enriched milk contained more than 8 times the amounts of PUFAs contained in the

semi-skimmed milk and more than twice the amount of MUFAs, whereas levels of saturated fatty acids detected in the n-3 enriched milk are reduced by 3 times compared to the semi-skimmed milk used in the study. Excluding the test milk, the diet consumed by volunteers throughout the study contained negligible levels of DHA and EPA as they were instructed to avoid fish consumption or fish derived products. Dietary contribution calculated for oleic acid and linolenic acid coming from foods other than the milks were 22 g and 300 mg per day in average, respectively.

Table 2. Relative fatty acid composition (as total percentage in milk fat), and amounts of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs) (also as total percentage) occurring in semi-skimmed and n-3 enriched milks.

Fatty acid	Semi-skimmed milk	enriched milk
4:0	3.5	0.2
6:0	2.3	0.1
8:0	1.4	0.1
10:0	3.0	0.2
12:0	3.25	0.3
14:0	10.9	1.3
16:0	31.0	11.1
16:1	1.4	0.7
18:0	11.9	8.7
18:1	21.5	54.4
18:2n-6	2.3	14.9
18:3n-3	ND	0.6
20:5n-3	ND	1.4
22:6n-3	ND	2.1
SFA (%)	70.5	23.7
MUFAs (%)	27.2	56.8
PUFAs (%)	2.3	19.5

ND, not detected

Table 3. Values of major plasma fatty acids (as total percentage) found in the subjects of the study at the time points tested.

Fatty acid	Tpre	T_0	T_4	T_8
16:0	22.9±0.4	23.3±0.4	19.6±0.4	22.9±0.4
16:1n-7	1.4±0.2	1.3±0.1	1.3±0.1	1.3±0.1
18:0	6.9±0.1	7.1±0.2	7.4±0.3	7.0±0.1
20:0	0.22±0.01	0.21±0.01	0.20±0.01	0.20±0.01
22:0	0.56±0.02	0.52±0.02	0.52±0.02	0.54±0.02
18:1n-9	21.0±0.5	20.6±0.6	21.1±0.6	21.1±0.1
18:2n-6	29.8±0.9	30.3±0.9	29.4±1.1	30.5±0.7
18:3n-6	0.34±0.02	0.35±0.02	0.4±0.1	0.39±0.03
20:4n-6	6.2±0.2	6.4±0.3	5.9±0.2	6.3±0.2
18:3n-3	0.31±0.03	0.28±0.03	0.28±0.02	0.30±0.02
20:3n-3	1.3±0.1	1.3±0.1	1.25±0.05	1.3±0.05
20:5n-3	0.57±0.05 a	0.50±0.04 a	0.62±0.05 a	0.74±0.05 b
22:5n-3	0.25±0.02	0.25±0.01	0.27±0.01	0.27±0.01
22:6n-3	1.8±0.1 a	1.6±0.1 a	1.7±0.1 a	2.4±0.08 b

Average ± SEM (n=30). Values with different letters are significantly different, $p < 0.05$. Tpre, initial values; T_0, after one month consumption of semi skimmed milk; T_4 and T_8, after four and eight weeks consumption of n-3 enriched milk.

Fatty acid composition of plasma.

Average levels of major plasma fatty acids detected in the volunteers of the study are given in **Table 3**. Abstinence from fish consumption during 1 month during the first period of the study did not significantly change plasma fatty acid profile although a moderate decrease in plasma levels of EPA and DHA was found. The first 4-week period of semi-skimmed milk supplementation, together with dietary exclusion of fish throughout the study are likely to be responsible for the reported decrease, as the subjects of the study were recruited from southern Spain where fish consumption is usually moderate to high. However, 8-week supplementation with the n-3 enriched milk used in the study not only restored initial levels of plasma EPA and DHA but increases of 33% and 30% were obtained, respectively. Plasma levels of the rest of fatty acids measured did not significantly change at the time points tested, compared to levels detected at the beginning of the study (Table 3). Fatty acid composition in LDL particles was also measured at the times of the study but no significant change in the fatty acid profile was found compared to fatty acid composition of plasma (not shown).

Plasma Lipids

Plasma lipid values measured in volunteers at the different times of the study are given in **Table 4**. Four weeks of semi-skimmed milk consumption produced mild increase, although no significant, in values of total and LDL cholesterol and there was a decrease in HDL cholesterol levels, most likely due to the effect of the saturated fat contained in this type of milk (ca. 70%). The two months consumption of the n-3 enriched milk was associated with a total cholesterol significant reduction of about 6% ($p<0.05$). The effect on LDL-cholesterol was more pronounced as values were significantly reduced at T_8 by more than 16% ($p<0.05$) compared to initial values (Tpre), and more than 19% when compared to T_0. The LDL cholesterol decrease observed at times T_4 and T_8 was linear from T_0 which suggests the decrease might have been more pronounced if the study had been carried out for a longer period of time. n-3 enriched milk consumption also produced a mild but not statistically significant linear increase on HDL-cholesterol values at times T_4 and T_8, which becomes clearer when compared to T_0. With regard to triglyceride levels found in plasma, periods of either semi-skimmed or n-3 enriched milk consumption produced no significant changes at the times of the study.

Vitamin E, plasma and LDL oxidation parameters

Total antioxidant capacity, TBARS induced with the free radical generator AAPH, and vitamin E were measured in plasma (**Table 4**) and no significant differences were found in any of these parameters at the times of the study. The effect of the n-3 enriched milk consumption on oxidability of LDL particles isolated from volunteers of the study differ from the ones obtained in plasma (**Table 5**). Two months consumption of n-3 enriched milk resulted in a significant 12% ($p<0.05$) reduction of LDL hydroperoxides. Lag time, or the time required for an

oxidant to induce the propagation phase of oxidation in LDL, indicates susceptibility of LDL to oxidation. Lag time values were significantly increased by 27% ($P<0.05$) at T_8, suggesting LDL particles do not become more prone to oxidation upon consumption of n-3 enriched milk. However, vitamin E concentration measured in LDL showed mild but no statistically significant increase at T_8.

Table 4. Biochemical data obtained from volunteers at the different times of the study.

Parameter	Tpre	T0	T4	T8
TG (mmol/L)	1.22±0.05 a	1.24±0.05 a	1.25±0.04 a	1.27±0.05 a
TC (mmol/L)	4.56±0.10 a	4.61±0.12 a	4.39±0.10 ab	4.30±0.11 b
LDL- C (mmol/L)	2.35±0.12 ab	2.45±0.11 a	2.13±0.11 bc	1.97±0.11 c
HDL-C (mmol/L)	1.65±0.07 a	1.60±0.06 a	1.69±0.08 a	1.75±0.08 a
Vitamin E (mg/L)	37.4±2.4 a	34.5±1.7 a	33.7±1.5 a	35.8±1.9 a
CAP (µM Trolox)	20.7±0.2 a	20.4±0.2 a	20.7±0.2 a	20.4±0.2 a
TBARS (µmol/L MDA)[1]	16.7±0.8 ab	15.2±0.8 a	18.3±1.5 b	16.8±0.9 ab
ICAM-1 (µg/L)	170.2±15.6 a	168.6±17.6 a	154.4±13.6 a	152.2±13 a
VCAM-1 (µg/L)	510±30 a	505±30 a	475.0±30 ab	430±29.5 b

Average±SEM (n=30). Values with different letters are significantly different, $p < 0.05$. Tpre, initial values; T0, values obtained after one-month consumption of semi skimmed milk; T4 and T8, values obtained after four and eight weeks consumption of n-3 enriched milk, respectively. TG, triacylglycerol; TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL-cholesterol; CAP, plasma total antioxidant capacity; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1. [1] TBARS as µmol/L malondialdehide.

Plasma levels of ICAM-1, VCAM-1

Plasma concentrations of soluble forms of ICAM-1 and VCAM-1 at the different times of the study are shown in **Table 4**. We observed a significant 16% ($p<0.05$) gradual reduction in VCAM-1 values after two months consumption of n-3 enriched milk (T_8), compared to initial levels and T0. This type of milk also tended to decrease the expression of ICAM-1 by more than 10% but this decrease was not statistically significant when compared to values at Tpre or T_0.

Table 5. Vitamin E, hydroperoxide concentrations and lag time measured in LDL particles isolated from the subject of the study.

Parameter	Tpre	T0	T4	T8
Vitamin E (mg/g protein)	21.2±1.0	19.5±1.0	20.4±0.9	23.1±1.4
µM hydroperoxides / mg LDL protein	19.8±1.1 a	19.9±1.0 a	17.9±0.9 ab	16.7±0.6 b
Lag time (min)	155.3±8.5 a	184.4±11.5 b	183.2±10.9 b	198.2±15.6 b

Average±SEM (n=30). Values with different letters are significantly different, $p < 0.05$. Tpre, initial values; T0, after one month of consumption of semi skimmed milk; T4 and T8, after four and eight weeks of consumption of n-3 enriched milk.

Homocysteine and folic acid.

Results show two-months intake of the n-3 enriched milk produced a significant 13% reduction ($p<0.05$) in plasma levels of homocysteine (Table 6). Results also show folic acid from the supplemented milk was bioavailable and produced a significant 75% and 91% increase of folic acid concentration in plasma at times T_4 and T_8, respectively. Plasma vitamin B_{12} concentrations were increased at time T_8 compared to those observed at times Tpre, T_0 and T_4.

Table 6. Concentrations of plasma total homocysteine, plasma folic acid and vitamin B_{12}.

Parameter	Tpre	T0	T4	T8
Homocysteine (µg/L)	12.5±0.6 a	12.9±0.7 a	11.8±0.6 ab	10.9±0.6 b
Plasma folic acid (µg/L)	3.18±0.26 a	3.37±0.35 a	5.58±0.45 b	6.10±0.45 b
Vitamin B_{12} (ng/L)	380±21 a	363±20 a	412±22 a	474±20 b

Average±SEM (n=30). Values with different letters are significantly different, $p < 0.05$. Tpre, initial values; T0, after one month of consumption of semi skimmed milk; T4 and T8, after four and eight weeks of consumption of the n-3 enriched milk.

Discussion

Milk has been shown to be a very efficient vehicle for fat absorption because milk fat appears highly dispersed in very small micelles. In this sense we used milk, an every day used drink, as a carrier for PUFAs, MUFAs and vitamin supplementation to try to increase the intake of those, and further studied their effects on risk factors for cardiovascular disease in healthy volunteers.

Total amounts of EPA and DHA contained in 500 ml of the n-3 enriched milk were 133 mg and 200 mg, respectively (daily intake for 2 months). These levels roughly exceed the amount of EPA + DHA contained in the UK average serving portion of cod (29). Two months administration of the n-3 enriched milk resulted in a significant (about 30%) increase in DHA and EPA plasma levels. Similar results were obtained by others when semi-skimmed milk was supplemented with similar amounts of PUFAs (30). Fatty acid composition of plasma fatty acids is consistent with LDL fatty acid profile.

PUFAs on their own are very susceptible to being oxidised and quickly react when oxidants or oxidising conditions are present. The n-3 supplemented milk used in the study contained 7.5 mg vitamin E per 500 ml which represents 75% of the recommended vitamin E daily intake. We addressed the question of whether regular intake the PUFAs enriched milk used in the study would make plasma and LDL particles more prone to oxidation, or else, if they would reduce levels of endogenous antioxidants to consider possible deleterious effects derived from consumption. n-3 supplemented milk consumption did not produce an increase in any of the plasma oxidation parameters analyzed. Possible explanations for these results

could either be due to the amount of PUFAs being too low to induce changes in plasma oxidability and/or a compensation effect derived from vitamin E supplementation, which in turn may have counteracted oxidation effects. Although vitamin E was supplemented in the n-3 enriched milk to provide ca. 75% of the recommended daily intake, we did not detect any significant increase in plasma vitamin E levels. This is not surprising as several other research studies show that much higher levels of vitamin E either supplemented in milk or in capsules do not produce significant increase in plasma levels (31).

Plasma concentration of triglycerides did not change upon consumption of the n-3 enriched milk whereas a modest non-significant increase on HDL cholesterol was found. Average plasma triglyceryde values obtained for volunteers at the beginning of the study (1.22±0.05 mmol/L) were below average levels reported for normolipidemic subjects (1.58 mmol/L, range 0.34-2.82 mmol/L). This suggests daily supplementation with 500 ml of n-3 enriched milk, only containing 333 mg of EPA+DHA, is not enough to reduce triglyceride levels in the volunteers of the study, and that there was probably a threshold level we should have gone beyond to have been able to exert an effect. Visioli et al (30) carried out a similar research study describing reductions of up to 19% in plasma triglyceride values and 19% increase in HDL-cholesterol levels after 6-week consumption of milk enriched only with n-3 PUFA that supplemented 400 mg of n-3 fatty acids per day. Interestingly, a similar effect in plasma triglyceride reduction and HDL increase was only reached by Cobiac et al (32) over a 5 week period of daily administration of 4.5 g DHA+EPA in mildly hyperlipidemic males. Fat milk is highly dispersed in micelles and is known to be very efficiently absorbed in the gut, so the administration of n-3 PUFA supplemented in the test milk may be responsible for the relatively high levels found in plasma and the effects shown.

Moreover, the n-3 enriched milk used in our the study contained almost 3 times the amount of oleic acid occurring in standard milk which, in combination with PUFAs, produced desirable LDL and total cholesterol lowering effects. Particularly, the LDL cholesterol 16% reduction found in our study has, to our knowledge, never been reported before for any supplemented milk. Total and LDL cholesterol lowering effects of oleic acid and PUFAs have been extensively described (3, and references therein) and are associated with a wide range of physiological effects. Indeed, free radical generation and lipid peroxidation are positively correlated with plasma total cholesterol concentration (33) and LDL cholesterol is also associated with increased susceptibility of LDL particles to oxidation (34). This may also explain the increase found in LDL lag time values (27%) found at T_8 of the study, when vitamin E concentration measured in LDL remained mostly unchanged throughout the study. In this sense, alpha-tocopherol is in quantitative terms the major antioxidant among those present in LDL and is therefore considered the first line of defence against oxidation (35). It has been shown that LDL resistance to oxidation not only depends on alpha tocopherol content but also

in other variables yet to be identified (36). In this sense, retinol has also been shown to have important effects on increasing LDL resistance to oxidation (37). Both types of milk used in the study were enriched in equal amounts of vitamin A (see Table 1), and intake of this vitamin by the volunteers of the study supposed more than 75% of the recommended daily intake, over and above that already present in their diets. This fact may explain the 18% increase in lag time values also detected during the first period of semi-skimmed milk consumption, and the constant levels of LDL vitamin E throughout the study. However, the fact that LDL lag time is increased by 27%, though statistically significant, is perhaps too low to accept a meaningful clinical correlation (38), so we conclude the n-3 enriched milk tested does not increase significantly plasma oxidability of LDL.

The folic acid supplemented in the n-3 enriched milk produced an increase of more than 90% in plasma levels of this vitamin at T_8 of the study. Vitamin B_6 was also supplemented in n-3 enriched milk but not in semi-skimmed milk. Homocysteine-lowering effects of folic acid and vitamin B_6 have been well documented (39, 40), and also folic acid has been shown to reverse endothelial disfunction in animal models (41), suggesting vitamins supplemented in n-3 milk may have significantly contributed to reducing homocysteine levels in plasma.

Many clinical and epidemiological studies have shown high plasma levels of homocysteine is an independent risk factor for cardiovascular disease (1). Possible mechanisms of homocysteine–induced atherosclerosis include endothelial dysfunction, promotion of lipoprotein oxidation and increased cholesterol synthesis in hepatocytes (42-44). Recently, homocysteine has been described to stimulate the transcription factor nuclear factor kB (NF-kB), causing a pleiotropic response which involves upregulation of endothelial activation factors such as VCAM-1. Reactive oxygen species (ROS) and oxidative stress are well known activators of NF-kB (9). Homocysteine has been demonstrated to promote ROS production by a number of mechanisms including inhibition of glutathion peroxidase, one of the most important antioxidant defences (45). Consistent with this, the significant reduction in plasma concentration of homocysteine found in our study may influence: 1) VCAM-1 levels, as the latter seems to be induced by the former via ROS and NF-kB, as described above; 2) the absence of changes in plasma and LDL oxidability found in the study, and 3) at least in part, the cholesterol reduction.

In agreement with our results, Yaqoob *et al.* (46) observed a decrease in plasma levels of adhesion molecules after 2 months consumption of a MUFA rich diet. However, we found differences in the expression pattern of VCAM-1 and ICAM-1. A major role for VCAM-1 but not for ICAM-1 has been found in early atherosclerosis (47 and references therein). Both VCAM-1 and ICAM-1 are expressed by aortic endothelium in regions predisposed to atherosclerosis and are both up-regulated in hypercholesterolemic animals. Their expression patterns are different however which suggests different functions for both molecules in lesion initiation. This

may also explain the differences in the plasma concentration of VCAM-1 and ICAM-1 found in our study, which were increased by 16% ($p<0.05$) and 10%, respectively. A very recent study also reported that fish oil supplementation (1.2 g DHA+EPA) in human volunteers for 12 weeks significantly decreased (20% in average) plasma VCAM-1 concentrations whereas ICAM-1 concentrations were not affected by the fish oil treatment (48).

Various research studies show the intake of a range of foods and ingredients, including milk, enriched with alpha linoleic acid, EPA and DHA can be used to achieve desired biochemical effects without the ingestion of supplements or changing dietary habits (19, 30).

In conclusion, dietary consumption of the n-3 supplemented milk tested in our study is an effective way to increase consumption of PUFAs also having favourable effects on risk factors for CVD. Further studies with this type of milk investigating their effects in older populations and in patients with high risk factors for cardiovascular disease are under way in our laboratory.

Acknowledgements

Supported by Puleva Food, S.A. Authors wish to thank Mr Carlos Rodrigez and Mr Antonio D. Valero for technical assistance, and Mr Jose Andres Fernández for help with blood extractions.

References

1. Lusis, A.J. (2000) “Atherosclerosis” Nature 407, 233-241.
2. Kris-Etherton PM, Pearson TA, Wan Y, Hargrove RL, Moriarty K, Fishell V, Etherton TD. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. Am J Clin Nutr 1999; 70: 1009-15.
3. Feldman EB. Assorted monounsaturated fatty acids promote healthy hearts. Am J Clin Nutr 1999; 70: 953-4.
4. Connor WE. Importance of n-3 fatty acids in health and disease. Am J Clin Nutr 2000; 71: 171S-5S.
5. Connor We. n-3 fatty acids and heart disease. In: Kritchevsky D, Carroll KK, eds. Nutrition and disease update: heart disease. Champaign, IL: American Oil Chemists’ Society, 1994: 7-42.
6. Harris WS. Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. J Lipid Res 1989; 30: 785-807.
7. Kang JX, Leaf A. Prevention of fatal cardiac arrhythmias by polyunsaturated fatty acids. Am J Clin Nutr 2000; 71: 202S-7S.
8. von Shacky C. n-3 fatty acids Fatty acids and the prevention of coronary atherosclerosis. . Am J Clin Nutr 2000; 71: 224S-7S.
9. De Caterina R. Endothelial dysfunctions: common denominators in vascular disease. Curr Opin Clin Nutr Metab Care 2000; 3: 453-67.
10. Morris MC, Manson JE, Rosner B, Buring JE, Willett WC, Henekens CH. Fish consumption and cardiovascular disease in the Physicians’ Health Study: a prospective study. Am J Epidemiol 1995; 142: 166-75.

11. Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr 2001; 73: 673-86.
12. De Caterina R, Liao JK, Libby P. Fatty acid modulation of endothelial activation. Am J Clin Nutr 2000; 71: 213S-23S.
13. Clarke R, Armitage J. Vitamin supplements and cardiovascular risk: review of the randomized trials of homocysteine-lowering vitamin supplements. Semin Thromb Hemost 2000; 26: 341-8.
14. Chambers JC, Obeid OA, Refsum H, Ueland P, Hackett D, Hooper J, Turner RM, Thompson SG, Kooner JS. Plasma homocysteine concentrations and risk of coronary heart disease in UK Indian Asian and European men. Lancet 2000 12; 355: 523-7.
15. Brouwer IA, van Dusseldorp M, Thomas CMG, Duran M, Hauvast JGAJ, Eskes TKAB, Steegers-Theunissen RPM. Low-dose folic acid supplementation decrease plasma homocysteine concentrations: a randomised trial. Am J Clin Nutr 1999; 69:99-104.
16. Scientific Review Committee. Nutrition Recommendations. Ottawa: Minister of National Health and Welfare 1990. (H49-42/1990E).
17. The British Nutrition Foundation. Unsaturated fatty acids-nutritional and physiological significnce: the report of the british nutrition foundation's Task Force. London: Chapman and Hall 1992.
18. National Health and Medical Research Council. Report of the NHMRC working party: the role of polyunsaturated fats in the Australian diet. Canberra, Australia: Australian Governent Publishing Service 1992.
19. Mantzioris E, Cleland LG, Gibson RA, Neumann MA, Demasi M, James MJ. Biochemical effects of a diet containing foods enriched with n-3 fatty acids. Am J Clin Nutr 2000; 72: 48-8.
20. Chung BH, Wilkinson T, Geer JC, Segrest JP. Preparative and quantitative isolation of plasma lipoprotein: rapid, single, discontinuous density gradient centrifugation in a vertical rotor. J Lipid Res 1980; 21: 284-291.
21. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978; 52: 302-310.
22. Esterbauer H, Striegl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Rad Res Comms 1989; 6: 67-75.
23. Jiang Z-Y, Woollard, ACS, Wolff, SP. Lipid hydroperoxide measurement by oxidation of Fe2+ in the presence of xylenol orange. Comparison with the TBA assay and an iodometric method. Lipids 1991; 26: 853-856.
24. Miller JN, Rice-Evans C, Davies, MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 1993; 84: 407-412.
25. Pellegrini N, Re R, Yang M, Rice-Evans C. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2´-azinobis 3-ethylenebenzothiazoline-6-sulfonic acid radical cation decolorization assay. Methods Enzymol 1999; 299: 379-389.
26. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986;27:114-20.
27. Araki A and Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography 1987; 422: 43-52

28. Thurnham DI, Smith E, Flora PS. Concurrent Liquid-Chromatographic assay of retinol, alfa toopherol, beta-carotene, alfa-carotene, lycopene and beta-cryptoxanthin in plasma with tocopherol acetate as Internal Standard. Clinical Chemistry 1988; 34 (2): 377-381
29. The British Nutrition Foundation. n-3 fatty acids and health 1999. Briefing Paper. London BNF.
30. Visioli, F, Rise P, Plasmati E, Pazzucconi F, Sirtori C, and Galli C. Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerols and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol. Res 2000; 41: 571-576.
31. Hayes KC, Pronckuk A and Perlman D. Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins. Am J Clin Nutr 2001; 74: 211-8.
32. Cobiac L, Clifton PM, Abbey M, Belling GB and Nestel PJ. Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n-3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr 1991; 53: 1210-16.
33. Ohara Y, Peterson TE and Harrison DG. Hipercolesterolemia increases endotelial superoxide anion production. J Clin Invest 1993; 91: 2546-51.
34. Kritchevsky SB, Hughes TA, Belcher J and Gross M. Carotenoid and lipid/lipoprotein correlates of the susceptibility of low-density lipoproteins to oxidation in humans. In: Basu TK, Temple NJ and Gard ML, Eds. Antioxidants in human health and disease. CAB International, USA, 1999: 71-82 .
35. Reaven PD, Grasse BJ and Tribble DL. Effects of linoleate enriched and oleate enriched diets in combination with alpha-tocopherol on the susceptibility of LDL and LDL subfractions to oxidative modifications in humans. Aterioscler Tromb 1994; 14: 557-66.
36. Estebauer H, Gebiski J, Puhl H and Jurgens G. The role of lipid peroxidation and antioxidant in oxidative modification of LDL. Free Rad Biol Med 1992; 13: 341-90.
37. Livrea MA, Tesoriere L, Bongiorno A, Pntaudi AM, Ciaccio M and Riccio A. Contribution of vitamin A to the oxidation resistance of human low density lipoprotein. Free Rad Biol Med 1995; 18: 401-9.
38. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997; 272: 20963-66.
39. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analisys of randomised trials. BMJ 1998; 316: 894-98.
40. van den Berg M, Franken DG, Boers GH, *et al.* Combined vitamin B6 plus folic acid in young patients with atherosclerosis and hyperhomocysteinaemia. J Vasc Surg 1994; 20: 933-40.
41. Miller A, Mujumdar V, Palmer L, Bower JD and Tyagi SC. Reversal of endocardial endothelial dysfunction by folic acid in homocysteinemic hypertensive rats. AJH 2002; 15: 157-63.
42. Brand K, Page S, Rogler G, Bartsch A, Brandl R, Knuechel R, Page M, Kaltshmidt C, Baeuerle PA and Neumeier D. Activated transcription factor nuclear factor-kB is present in the atherosclerotic lesion. J Clin Invest 1996; 97: 1715-22.
43. Brand K, Page S, Walli AK, Neumeier D, and Baeuerle PA. Role of nuclear factor-kB in atherogenesis. Exp Physiol 1997; 82: 297-304.
44. Thurberg BL and Collins T. The nuclear factor-kB/inhibitor of kB autoregulatory system and atherosclerosis. Curr Opin Lipidol 1998; 9: 387-96.
45. Upchurch GR, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF and Loscalzo J. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997; 272: 17012-17.

46. Yaqoob P, Knapper JA, Webb DH, Williams CM, Newsholme EA and Calder P. Effect of olive oil on immune function in middle-aged men. Am J Clin Nutr 1998; 67: 129-35.
47. Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos JC, Connelly PW and Milstone DS. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001; 107: 1255-62.
48. Miles EA, Thies F, Wallace FA, Powell JR, Hurst TL, Newsholme EA and Calder PC. Influence of age and dietary fish oil on plasma soluble adhesion molecule concentrations. Clin Sci 2001; 100: 91-100.
49. Hernández M and Sastre A. Tratado de Nutrición. Díaz de Santos, Spain, 1999.

5

Cardiovascular effects of milk enriched with n-3 polyunsaturated fatty acids, oleic acid folic acid and vitamins E, B6 and B12 in volunteers with mild hyperlipidaemia.

Nutrition 2004; ***20:****521-527*

JJ Carrero*, L Baró†, J Fonollá†, M González-Santiago†, A Martínez-Férez?, R Castillo‡, J Jiménez†, JJ Boza† and E López-Huertas†

*Departamento de Bioquímica y Biología Molecular. Universidad de Granada Spain
†PULEVA BIOTECH S.A. Granada, Spain
? Departamento de Ingeniería Química. Universidad de Granada. Granada 18071 Spain
‡, Hospital Universitario “San Cecilio”. 16, Av Dr Oloriz, Granada 18012, Spain

Resumen

Un gran número de estudios epidemiológicos sugiere que la ingesta de ácidos grasos polinsaturados n-3 y de ácido oleico ejerce una serie de efectos beneficiosos para la salud como es la reducción del riesgo cardiovascular. El propósito de este estudio es evaluar el efecto que una leche semidesnatada comercial enriquecida con ácidos grasos n-3, ácido oleico y vitaminas E, B_6, B_{12} y ácido fólico ejerce sobre diversos factores de riesgo cardiovascular.

30 voluntarios de edad comprendida entre los 45-65 años 51.3± 5.3 años) tomaron 500 mL/día de leche semidesnatada durante 4 semanas, seguido de 500 mL/día de la leche enriquecida durante las 8 semanas siguientes. Se extrajeron muestras de plasma y de lipoproteínas LDL al principio del estudio y tras 4, 8 y 12 semanas.

El consumo de la leche enriquecida durante 8 semanas produjo un aumento en la proporción de DHA y EPA junto con una reducción significativa ($p<0.05$) en la concentración plasmática de triglicéridos (24%), colesterol total (9%) y LDL (13%). Tanto la oxidabilidad plasmática y de las LDL como la concentración de vitamina E permaneció inalterada durante el tiempo de estudio. Se apreció un aumento en los niveles plasmáticos de la molécula de adhesión VCAM-1 (9%) y homocisteína (17%), acompañada de un aumento en los niveles séricos de folato del 98%.

Los resultados de este estudio muestran que estrategias de enriquecimiento dietético con ácidos grasos n-3, ácido oleico, ácido fólico y vitaminas a través de un vehículo lácteo puede ser de utilidad en la reducción del riesgo cardiovascular.

Summary

Results from epidemiological studies and clinical trials indicate that n-3 fatty acids, oleic acid and folic acid consumption have beneficial effects on health, including risk reduction of cardiovascular disease. The purpose of this study was to evaluate the combined effects of these nutrients through the consumption of milk enriched with n-3 polyunsaturated fatty acids (PUFAs), oleic acid, vitamins E, B6 and folic acid on risk factors for cardiovascular disease in volunteers with mild hyperlipidaemia.

Thirty subjects aged 45-65 (51.3± 5.3 y.) were given 500 mL per day of semi-skimmed milk for 4 weeks and then 500 mL/day of the enriched milk for 8 further weeks. Plasma and LDL lipoproteins were obtained at the beginning of the study and at 4, 8 and 12 weeks.

Consumption of enriched milk for 8 weeks produced an increase in the plasma concentrations of DHA and EPA together with a significant ($P<0.05$) 24% decrease in the plasma concentration of triacylglycerol, total (9%) and LDL cholesterol (13%). Plasma and LDL

oxidisability and vitamin E concentration remained unchanged throughout the study. Significant reductions in plasma concentrations of vascular cell adhesion molecule-1 (9%) and homocysteine (17%) were found, accompanied by a 98% increase in plasma concentration of folic acid.

Results from this study show that dairy supplementation strategies with n-3 PUFA, oleic acid and vitamins may be useful for reducing risk factors for cardiovascular disease.

Introduction

There is a wealth of evidence from epidemiological and clinical studies suggesting that modifications of dietary fat composition affect the risk of cardiovascular disease (CVD) (1). The consumption of n-3 fatty acids (n-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has several beneficial properties which prevent CVD, including anti-inflammatory, antiarrhythmic and antihypertensive effects, and are specially valued for their capacity to lower blood lipids, to inhibit the synthesis of cytokines and mitogens, to modulate endothelial function, to stimulate endothelial-derived nitric oxide and also to inhibit atherosclerosis and thrombosis (2-5). Olive oil is also considered a healthy source of fat and international nutritional guidelines recommend its consumption due to the cardiovascular beneficial effects reported.

The supplementation with certain nutrients like folic acid, vitamins B6 and B12 has also come to be regarded as potentially protective against CVD. For instance, plasma homocysteine concentration, a novel risk factor for CVD, is reduced when the intake of these vitamins is increased (6).

Health authorities have recommended an increase in the consumption of PUFAs (7), in which fish oil is especially rich. The latest WHO report (8) recommends a regular fish consumption to provide about 200-500 mg of EPA and DHA per week, a replacement of saturated fat by monounsaturated fat, and an increase in the consumption of fruits and vegetables in order to achieve proper antioxidant and vitamin status. However, modern western societies tend to include very little fish, vegetable and fruit in their diets and therefore ways to increase consumption of PUFAs and folic acid, have to be explored and assessed at a community or clinical level.

An oil blend containing n-3 PUFAs, olive oil and vitamins B6 , B12 and folic acid was produced and included in skimmed milk to create a dairy product with the palatability of the semi-skimmed milk but a healthier fatty acid and vitamin profile. Milk, an every day drink, is a very efficient vehicle for fat and lipid-soluble compounds absorption because of its dispersion in micelles. In this 8-week study, we tested the hypothesis that the substitution of regular milk

(ca 70% saturated fat) by this dairy product has the potential to lower cardiovascular risk factors in free-living mildly hyperlipidaemic subjects.

Experimental Methods

To ensure analytical consistency, samples T-4 to T8 from the same volunteer(s) were processed at the same time and analysed in one batch when techniques involving HPLC, gas-liquid chromatography or spectrophotometry were used. For ELISA determinations all the analysed samples were processed and run in one batch.

Table 1. Calculated dietary intake of nutrients at baseline and at week 11th in the experimental period excluding the milks tested.

	week 1		week 11	
	Men n=15	**Women n=15**	**Men n=15**	**Women n=15**
Energy (Kcal)	2398±46	1880±32	2412±37	1869±24
Proteins (g)	96.1±3.2	78.1±1.8	95.2±1.5	77.3±1.8
Carbohydrates (g)	257.4±6.2	205.4±5.0	265±5.8	202.8±3.4
Total Fat (g)	98.1±4.5	77.7±2.0	97.2±3.6	79.3±1.9
SFAs (g)	32.5±1.0	24.4±0.9	31.1±0.9	24.7±1.0
MUFAs (g)	42.6±0.8	31.5±0.8	41.6±0.9	31.8±0.6
PUFAs (g)	14.4±0.4	10.4±0.2	14.9±0.3	10.7±0.3
Fiber (g)	17.6±0.5	16.0±0.4	17.6±0.3	15.8±0.2
Calcium (mg)	874.5±10.2	800.6±9.5	880±9.8	807±11.3
Iron (mg)	13.2±0.2	10.4±0.1	13.5±0.4	10.6±0.6
Sodium (mg)	1942.5±16.6	1393.2±20.8	1950±34.2	1375±24.8
Vit A (µg)	865±97	857±74	882±103	825±74
Thiamin (mg)	1.21±0.06	1.10±0.09	1.14±0.08	1.16±0.10
Riboflavin (mg)	2.0±0.1	1.8±0.3	1.7±0.2	1.6±0.6
Vitamin B_6 (mg)	2.20±0.09	1.75±0.10	2.29±0.11	1.84±0.14
Vitamin B_{12} (µg)	11.1±0.72	8.4±0.65	10.8±0.86	9.1±0.72
Vitamin C (mg)	125.4±14.3	137.4±20.8	145.2±25.2	128.4±14.7
Vitamin D (µg)	4.8±0.68	3.4±0.34	5.4±0.84	3.1±0.47
Vitamin E (mg)	9.8±0.35	7.2±0.47	9.6±0.89	8.1±0.51
Niacin (mg)	29.8±0.54	22.7±1.5	31.1±0.86	23.7±64
Folate (µg)	199.4±6.4	196.7±7.9	189.2±8.4	200.0±5.9

Average ± SEM (n=30)

Subjects and study design

Thirty subjects (15 men and 15 women, age 51.3± 5.3, range: 45-65 y) were recruited in Granada (Spain) from volunteers who responded to an advertisement about dietary intervention studies. We advertised for subjects within the age range 45 to 65 years, preferably

with high blood triacylglycerols. The subjects were given a physical examination and their medical history was consulted before they were included in the study. The subjects were not suffering from any chronic or metabolic disease and did not take any medication known to influence lipid metabolism from at least 1 month before the beginning of the study until the end of it. The subjects were instructed not to change their physical activity or their usual diet, but only to avoid eating fish from the beginning until the end of the study. The study was conducted according to the Helsinki Declaration, the protocol was approved by the Ethical Committee of Puleva Biotech S.A., and informed written consent was obtained from the subjects. Dietary intake was assessed at baseline and again at week 11 of the study with a 7 day-self-administered food-frequency questionnaire. They were also requested to fill in a food diary according to instructions from the principal investigator where they registered all food consumption during the study. Compliance with the consumption of the product during the intervention period was assured and checked upon by regular telephone call and weekly collection of the containers consumed. The dietary analysis of the average intake of nutrients during the 12-week intervention period is given in **Table 1**, using the food composition tables published in (9) as reference values.

Table 2. Composition of semi-skimmed milk and enriched milk, showing relative fatty acid composition of specific fatty acids (as total weight percentage in milk fat), amounts of saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated fatty acids (PUFAs).

	Semi-skimmed milk	Enriched milk
Energy (kcal /100 mL)	46.5	52
Protein (g/100mL)	3.1	3.5
Carbohydrates (g/100mL)	4.7	5.2
Fat (g/100mL), where	1.9	1.9
18:1 (%)	21.5	54.4
18:3n-3 (%)	U	0.6
20:5n-3 (%)	U	1.4
22:6n-3 (%)	U	2.1
SFAs (%)	70.5	23.7
MUFAs (%)	27.2	56.8
PUFAs (%)	2.3	19.5
Calcium (mg/100mL)	120	132
Vitamin A (µg/100mL)	120	120
Vitamin D (µg/100mL)	0.75	0.75
Vitamin E (mg/100mL)	U	1.50
Vitamin B_6 (mg/100mL)	U	0.30
Vitamin B_{12} (µg/100mL)	0.38	0.38
Folic acid (µg/100mL)	U	30

U, undetected

The milks used in the study were a semi-skimmed milk enriched with vitamins A and D, and a commercial dairy product (Puleva Omega 3©) containing n-3 polyunsaturated fatty acids, oleic acid, folic acid and vitamins A, D, E, B6 (enriched milk). The dairy product was prepared

by adding a mixture of fish and vegetable oils to skimmed milk, resulting in a product containing a total fat comparable to that contained in standard semi-skimmed milk (1.9 g/100 mL), but with a different fatty acid profile. Vitamins A, D, E, B6 and folic acid were also added to the final product. The composition and the fatty acid profile of the semi-skimmed milk and the enriched milk are given in **Table 2**.

The subjects drank 500 mL/day of semi-skimmed milk from the beginning of the study (T-4) for four weeks (T0). At time T0, the subjects replaced the semi-skimmed milk with 500 mL/day of the enriched milk described above, that was consumed for a period of 8 weeks. After an overnight fast lasting 10 hours, a blood sample (30 mL) was taken at times T-4 (beginning of the study), T0 (after 4 weeks consumption of semi-skimmed milk) and then after 4 and 8 weeks consumption of the enriched milk (T4 and T8, respectively).

Plasma and LDL isolation

Blood was withdrawn in EDTA-containing vacutainers (S-Monovette, Sarstedt, Germany). Plasma was obtained by centrifugation at 3.500 x g for 5 min at 4°C, and immediately frozen at –80ºC until further analyses. For LDL isolation, 10 mL of fresh plasma were transferred to ultracentrifuge tubes and lipoprotein fractions were isolated as described in (10). LDL particles typically sedimented at density range 1.006-1.063 g/mL. LDL fractions were pooled and dialysed in the dark for 24 h in 10 mM phosphate buffered saline (PBS) 0.15 M NaCl, pH 7.4, and finally frozen at –80°C under nitrogen atmosphere until needed.

Oxidized LDL and lag time measurements.

Oxidized LDL in plasma was quantified using a commercial ELISA kit (Mercodia, Sweden), according to the manufacturer's instructions. For lag time measurements, 50 ? g protein of dialyzed LDL in 1 mL PBS were incubated with 10 µM CuSO4 for several hours at 30°C. The formation of conjugated dienes was monitored continuously by measuring the increase in absorbance at 234 nm every 10 min. Lag time was determined according to (11).

Total antioxidant capacity and malondialdehyde (MDA)

Total antioxidant capacity was measured in plasma using Trolox as standard. Briefly, 20 µl of fresh plasma were 1:1 diluted in PBS and incubated with 1 mL of ABTS+ for 20 min. Absorbance was read at 734 nm. ABTS cation was prepared by addition of 88 µl of 140 mM potassium persulfate to 5 mL of a 7 mM solution of ABTS in water and incubation for 12-14 h. Working solution was obtained by dilution of the former with PBS until the absorbance at 734 nm was 0.7 ± 0.02 as described in (12).

Plasma malondialdehyde concentrations were measured by using a HPLC separation described in (13), which is based on the thiobarbituric acid (TBA) reaction and reverse-phase separation with fluorescence detection.

Plasma lipids, total Homocysteine, vitamin E, plasma folate, Lipoprotein (a), VCAM-1 and ICAM-1 determinations

Plasma triacylglycerols, cholesterol and HDL-cholesterol were measured at the hospital central laboratory. All the plasma samples from the volunteers of the study were defrosted at the same time before they were analysed. Blood lipids were measured by colorimetry in triplicates in one batch for each determination, using commercial reagents obtained from Biosystems (Barcelona, Spain). Plasma fatty acid profile was determined by gas-liquid chromatography as described in (14). Total fasting plasma homocysteine (tHcy) concentration was measured by HPLC with fluorescence detection (15). Plasma vitamin E concentration was determined by HPLC with ultraviolet detection following the method described in (16). Lipoprotein (a) (Lp(a)) levels were quantified by using a commercial kit based on kinetic nephelometry (LPAX immunochemical systems IMMAGE, Beckman Coulter Inc., USA). Plasma folate concentration was measured by immunoassay using a commercial kit (SimulTRAC-SNB Radioassay Kit, ICN Pharmaceuticals, USA). VCAM-1 and ICAM-1 were measured using commercial ELISA kits from Biosource International (USA), according to the manufacturer´s instructions.

Statistical analysis

All the data is expressed as means ± SEMs. Comparisons across time were assessed by a one-way analysis of variance (ANOVA). When this analysis indicated a significant difference ($P<0.05$), paired Student's t-test analyses followed by Bonferroni corrections for multiple comparisons were perfomed. The data was analysed using SPSS statistical software package (SPSS for Windows 10.1; SPSS Chicago, IL, USA).

Results

The milks used in the study were well accepted and compliance was good. No gender differences were found in the parameters measured, therefore all the data is presented as pooled. There was no significant body weight change in the volunteers throughout the study (72.74 ± 2.32 Kg at T-4 vs. 72.52 ± 2.37 Kg at T8).

Dietary intake of fatty acids

Total amounts of oleic acid, DHA and EPA supplemented in 500 mL of the enriched milk were 5.12 g, 0.13 g, and 0.2 g respectively, whereas the semi-skimmed milk contained only 1.82 g oleic acid per 500 mL and no detectable levels of DHA and EPA. The enriched milk contained more than 8 times the amounts of PUFAs, and more than twice the amount of monounsaturated fatty acids (MUFAs) compared to the semi-skimmed milk used in the study. The amounts of saturated fatty acids detected in the enriched milk were approximately a third compared to the semi-skimmed milk. Excluding the test milks, the diet consumed by the

subjects during the study contained negligible levels of DHA and EPA. Dietary contribution calculated for oleic acid and a-linolenic acid coming from food other than the milks were 23 g and 500 mg per day in average, respectively (**Table 1**).

Table 3. Values of major plasma fatty acids (as total percentage) found in the subjects of the study at the time points tested.

Fatty acid	T-4		T0		T4		T8	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM
C 16:0	20,05 a	0,39	19,93 a	0,24	19,36 b	0,30	19,22 b	0,21
C 16:1n-7	1,00	0,08	1,24	0,08	1,19	0,08	1,20	0,08
C 18:0	7,32	0,17	7,39	0,20	7,27	0,21	7,24	0,23
C 18:1n-9	20,66	0,84	21,14	0,71	21,03	0,77	20,97	0,68
C 18:1n-7	1,34	0,03	1,42	0,03	1,37	0,03	1,35	0,02
C 18:2n-6	26,41	0,75	25,94	0,63	25,56	0,54	26,21	0,57
C 18:3n-3	0,32	0,02	0,34	0,03	0,32	0,02	0,36	0,02
C 20:3n3	1,55	0,07	1,89	0,11	1,75	0,08	1,61	0,09
C 20:4n-6	8,25	0,47	8,43	0,45	8,54	0,41	8,42	0,37
C 22:5-n 3	0,81	0,09	0,67	0,07	0,69	0,07	0,72	0,07
EPA	0,56 a	0,05	0,39 b	0,03	0,68 c	0,06	0,75 c	0,08
DHA	1,83 a	0,09	1,83 a	0,09	2,02 b	0,07	2,20 b	0,06

Average ± SEM (n=30). Values with different letters are significantly different, $P< 0.05$. T-4, initial values; T0, after four weeks consumption of semi-skimmed milk; T4 and T8, after four and eight weeks consumption of the enriched milk.

Lipid Profile.

The average values of major plasma fatty acids detected in the subjects are given in **Table 3**. Average plasma values of lipids before the intervention were around the borderline-high range, as defined by the NCEP-Adult Treatment Panel III, 2002 (17). An interim analysis of blood samples at T-4 showed none of the subjects enrolled in the study had lipid values in the range advised for pharmacological treatment. The consumption of the corresponding semi-skimmed milk during the first four weeks did not change the plasma fatty acid profile with the exception of a significant decrease in EPA concentration. However, the 8-week supplementation with the enriched milk used in the study not only restored initial levels of plasma EPA, but a significant ($P<0.05$) increase of 33% was found at T8, and also a sustained 20% ($P<0.05$) increase in DHA plasma concentration. Plasma concentration of the rest of the fatty acids measured did not change at the time points tested, except for a significant decrease in palmitic acid (C16:0). Fatty acid composition in LDL particles was also measured at the times of the study but no significant change in the fatty acid profile was found compared to fatty acid composition of plasma (not shown).

Table 4. Plasma lipid concentration data obtained from subjects at the different times of the study.

Parameter	T-4	T0	T4	T8
TG (mmol/L)	2.35±0.24a	2.42±0.29 a	1.96±0.14 b	1.79±0.13 b
TC (mmol/L)	6.16±0.25 a	6.03±0.23 a	5.38±0.14 b	5.60±0.15 b
LDL- C (mmol/L)	4.23±0.25 a	4.47±0.24 a	3.51±0.14 b	3.68±0.14 b
HDL-C (mmol/L)	1.06±0.04	1.07±0.05	1.09±0.05	1.18±0.06

Average ± SEM (n=30). Values with different letters are significantly different, $P < 0.05$. T-4, initial values; T0, values obtained after four weeks consumption of semi-skimmed milk; T4 and T8, values obtained after four and eight weeks consumption of enriched milk, respectively. TG, triacylglycerol; TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol.

Plasma lipid parameters measured at the different times of the study are given in **Table 4**. The four week consumption of semi-skimmed milk produced a small increase in concentration of triacylglycerols, probably as a result of the saturated fat contained in this type of milk (ca. 70%). The consumption of the enriched milk during the 8 weeks was associated with a significant total cholesterol reduction of about 9% ($P<0.05$). The effect on LDL-cholesterol was more pronounced as the concentration was reduced at T8 by more than 13% ($P<0.05$). The enriched milk consumption also produced a mild but not significant linear increase on HDL-cholesterol concentration at times T4 and T8. With regard to triacylglycerols concentration in plasma, consumption of the enriched milk for 8 weeks produced a 24% ($P<0.05$) decrease at the times of the study compared with the initial levels at T-4.

Table 5. Plasma vitamin E, total antioxidant capacity (TAC), malondialdehyde, oxidized LDL, and lag time measured in LDL particles isolated from the subjects of the study. Concentrations of plasma ICAM-1, VCAM-1 and Lp(a) measured in plasma.

Parameter	T-4	T0	T4	T8
Vitamin E (µmol/L)	99.82±6.40	95.27±6.09	105.50±6.98	105.41±6.00
TAC (mM Trolox)	173,63±2.75	174,53±2.70	173,88±2.69	172,99±2.62
Lag time (min)	86.32±3.01	85.08±2.72	89.76±2.49	82.36±2.49
Malondialdehyde (µmol/L)	0.767±0.126	0.966±0.093	0.929±0.084	1.028±0.144
Oxidized LDL (U/L)	28.47±2.82	24.60±2.45	ND	24.97±2.17
Vit E (µmol/L)/TG (mmol/L)	22.01±2.02a	18.43±2.01a	26.54±2.33b	28.08±2.11b
VCAM-1 (µg/L)	664 ± 36 a	848 ± 65 b	488 ± 47 c	585 ± 33 d
ICAM-1 (µg/L)	212 ± 13.5	203 ± 14	234 ± 14	229 ± 17
Lp(a) mg/100 mL	36.64±5.57	32.32±4.86	34.05±5.30	33.49±5.08

Average ± SEM (n=30). Values with different letters are significantly different, $P < 0.05$. T-4, initial values; T0, after four weeks consumption of semi-skimmed milk; T4 and T8, after four and eight weeks of consumption of enriched milk. ND = not determined.

Vitamin E, plasma and LDL oxidation parameters

Malondialdehyde, total antioxidant capacity and vitamin E were measured in plasma (**Table 5**). No significant differences were found in any of these parameters at the times of the study. To study the effect of n-3 PUFA supplementation on LDL oxidizability, LDL particles

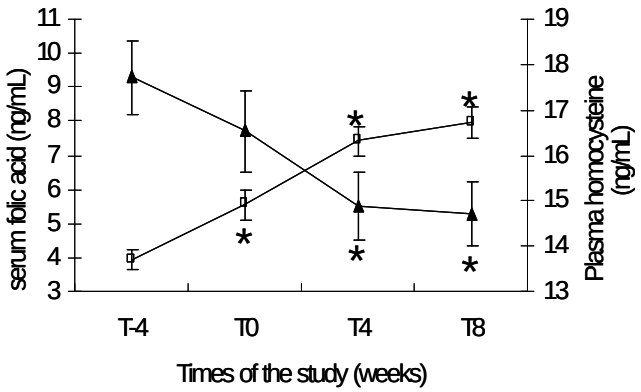
were isolated from the subjects of the study and lag time, or the time required for an oxidant (copper) to induce the propagation phase of oxidation, was measured. We also measured levels of oxidized LDL in plasma using a monoclonal antibody raised against the oxidized LDL. None of these parameters changed at the times tested.

Plasma concentration of ICAM-1, VCAM-1, Lp(a), folate and total fasting homocysteine.

The plasma concentration of Lp(a) and soluble forms of ICAM-1 and VCAM-1 at the different times of the study are shown in Table 5. We observed a significant 30% ($P<0.05$) reduction in VCAM-1 concentration after the 8 week consumption of enriched milk (T8), compared with values at time T0 of the study. ICAM-1 and Lp(a) concentration measured in plasma remained unchanged throughout the study.

The subjects of the study consumed 150 µg folic acid per day coming from the enriched milk. An increase in plasma folate concentration ($P<0.05$) of 88% and 98% was found at times T4 and T8, respectively. Simultaneously, plasma levels of tHcy significantly decreased by 16% and 18% ($P<0.05$) at times T4 and T8 , compared with levels detected at the beginning of the study. The decrease was more prominent during the first 4 weeks of consumption of the enriched milk and was sustained for the following 4 weeks. In this sense, the increase in plasma folate concentration was also more pronounced during the first four weeks (**Fig. 1**).

Figure 1. Plasma folate (?), and plasma total homocysteine concentrations (?) at the times of the study (n=30). T_{-4}, initial values; T_0, after four weeks consumption of semi-skimmed milk; T_4 and T_8, after four and eight weeks consumption of the enriched milk.
* Significantly different from T_{-4} ($P< 0.05$).



Discussion

The influence of enriched milk consumption on risk factors for cardiovascular disease in middle-aged hyperlipidaemic subjects was studied. The 8-week administration of the enriched milk resulted in a significant 20% and 33% increase in plasma levels of DHA and EPA respectively, which shows the compliance with the consumption of the product was good. The absorption of EPA and DHA from fish oil is improved when associated to other fats and spread out in small doses during the day (18). The fact that milk fat is highly dispersed in very small micelles increasing the surface of absorption of fats and lipid-soluble compounds (19) may explain the significant increases in plasma levels of DHA and EPA detected, when only small amounts were supplemented in the diet through the enriched milk.

The prevalence of hypercholesterolemia in Spain (defined as total Cholesterol >5.2 mmol/L) in the age range 35-64 years is about 60% (56.7% males and 58.6% females). In addition, around 20% of the Spanish population has cholesterol values above 6.5 mmol/L (20). The changes in lifestyle and dietary patterns, with the very extended use of convenience foods we believe are responsible for the prevalence of hypercholesterolemia in Spain.

Plasma concentrations of total cholesterol, LDL- and HDL-cholesterol and triacylglycerols did not differ significantly between men and women at the beginning of the study (Table 4). We advertised for subjects within the age range 45 to 65 years, preferably with high blood triacylglycerols, likely to have blood lipid values in the moderate to high range. In fact, the initial concentrations at baseline were beyond reference values reported for normolipidaemic subjects (total cholesterol > 6.21 mmol/L; LDL-cholesterol > 4.14 mmol/L; triacylglycerols >2.25 mmol/L) (17). The 8-week consumption of the enriched milk brought plasma triacylglycerol concentration down to normal values (<1.70 mmol/L). In addition, total and LDL-cholesterol concentration at the end of the intervention were close to normal (total cholesterol <5.17 mmol/L; LDL-cholesterol <3.36 mmol/L). The lipid-lowering effect was more prominent during the first 4 weeks of the enriched milk consumption and then was maintained for the following 4 weeks. We have previously reported the cardiovascular effects derived from consumption of this enriched milk in young normolipidaemic volunteers (21). In that study, consumption of the milk lowered plasma cholesterol concentration but did not affect triacylglycerol values. In contrast, a similar study with normolipidemic volunteers was carried out describing reductions of up to 19% in plasma triacylglycerol values and 19% increase in HDL-cholesterol levels upon 6-week consumption of milk enriched with similar amounts of n-3 PUFA (19). A similar effect found in plasma triacylglycerol reduction and HDL increase was also reached by other authors (22) over a 5 week period of daily administration of pharmacological amounts of PUFAs (4.5 g DHA+EPA) in capsules. These results show the vehicle of administration also plays a role in the effects produced.

Total and LDL-cholesterol lowering effects of oleic acid have been extensively described and are associated with a wide range of physiological effects. However, this study was carried out in a Mediterranean life-style context, where the average intake of oleic acid was 23 g/d (Table 1). In other populations and dietary patterns, the administration of this enriched milk may expect higher influence at this level.

We addressed the question of whether regular intake of enriched milk used in the study, because of its PUFAs content, would make plasma and LDL particles more prone to oxidation, or else, if they would reduce levels of endogenous antioxidants in order to consider possible negative effects. In our study, the consumption of the enriched milk did not produce an increase in any of the LDL or plasma oxidation parameters analysed (Table 5). Possible explanations for these results could either be due to the amount of PUFAs being too low to induce changes in plasma oxidisability and/or a compensation effect derived from vitamin E supplementation, which may have counteracted oxidation effects. Schnell et al. (23) suggested that vitamin E may reduce lipoprotein oxidation susceptibility in vivo when administered together with PUFAs. Although vitamin E was supplemented in the enriched milk to provide ca. 75% of the recommended daily allowance (RDA), we did not detect a significant increase in plasma vitamin E concentration. Other research studies show that much higher levels of vitamin E either supplemented in milk, or in capsules, are needed produce a significant increase in plasma levels (24, 25). One possible explanation might be the remarkable decrease in blood lipids found in our study that at the same time may have limited the concentration of vitamin E in plasma. In fact, the actual ratio of vitamin E to plasma lipids significantly increased (Table 5). The ratio vitamin E to plasma lipids (triacylglycerol or cholesterol) has been very recently proposed as a better oxidative stress marker than plasma vitamin E alone (26). Our results are in accordance with other authors showing n-3 PUFAs in moderate amounts do not increase LDL oxidizability when provided in the context of a diet rich in MUFAs (27).

Hyperhomocysteinaemia is an important risk factor for atherosclerosis. Possible mechanisms for homocysteine–induced atherosclerosis include endothelial dysfunction, promotion of lipoprotein oxidation and increased cholesterol synthesis in hepatocytes (28). Homocysteine-lowering effects of folic acid and vitamin B6 have been well documented. Although folic acid has been described as the main responsible for tHcy decrease, the addition of vitamin B12 and B6 to folic acid supplements or enriched foods may maximize the reduction of homocysteine (29). The intake of 500 mL/day of the enriched milk, contributes to more than 70% of the EUR RDAs of folic acid and vitamins B12 and B6 (30). Subjects in our study, varied from a suboptimal folate status (i.e., plasma folate <15 nmol/L) (31) at the beginning of the study, to an optimal folate status (18 nmol/L at T8) after the enriched milk supplementation period. Average tHcy plasma concentration of the subjects of the study (17,72 µmol/L) indicated the group was slightly hiperhomocysteinemic, (normal range 5-15 µmol/L) (32). The

8-week supplementation with the enriched milk produced a significant 18% reduction, which restored tHcy levels to those within the normal range (14,69 µmol/L)

A major role for VCAM-1 but not for ICAM-1 has been found in early atherosclerosis (33). Both VCAM-1 and ICAM-1 are expressed by aortic endothelium in regions predisposed to atherosclerosis and are both up-regulated in hypercholesterolemic animals. However, their expression patterns are different, suggesting different functions for both molecules in lesion initiation and different mechanisms that are not equally sensitive to n-3 PUFA (4) or Hcy-related interventions (35). This may explain the different behaviour of VCAM-1 and ICAM-1 found in our study. The VCAM-1 significant decrease is in agreement with other studies linking n-3 fatty acids to decreases in soluble markers of endothelial function (21,35). The plasma concentration of ICAM-1 did not change throughout our study. A recent study reported that fish oil supplementation (1.2 g EPA+DHA) in human volunteers for 12 weeks significantly decreased (20%) plasma VCAM-1 concentration whereas ICAM-1 concentration was not affected (4).

Finally, high blood concentration of Lp(a) has been proposed as an independent risk factor for CVD. Enriched milk consumption did not change Lp(a) concentration in our study, as occurred in other n-3 PUFAs intervention studies with similar doses (36).

In this study we show that the consumption of PUFAs, oleic acid and vitamins administered in a dairy product may be an effective way to reduce risk factors for cardiovascular disease. Supplemented food approaches could play an important role in CVD prevention without involving major dietary changes in the population.

Consumption of milk enriched with n-3 PUFAs, oleic acid, vitamins E, B6 and folic acid by 30 mildly hyperlipidaemic volunteers for a period of 8 weeks, produced a reduction in blood lipids, tHcy and VCAM-1, accompanied by an increase in plasma concentration of folate, therefore reducing risk factors for CVD.

Acknowledgements

Authors wish to thank Mr Jose A. Fernández for help with blood extractions, Ms Ruth Wilson for revising the English language and specially Mr. Carlos Rodriguez and Mr. Antonio D. Valero for technical assistance.

References

1. Kris-Etherton PM, Etherton TD, Carlson J, Gardner C. Recent discoveries in inclusive food-based approaches and dietary patterns for reduction in risk for cardiovascular disease. Curr Opin Lipidol 2002; 13(4):397-407.
2. Connor WE. Importance of n-3 fatty acids in health and disease. Am J Clin Nutr 2000; 71: 171S-5S.

3. von Shacky C. n-3 fatty acids and the prevention of coronary atherosclerosis. Am J Clin Nutr 2000; 71: 224S-227S.
4. Miles EA et al. Influence of age and dietary fish oil on plasma soluble adhesion molecule concentrations. Clin Sci 2001; 100: 91-100.
5. Yaqoob P, Knapper JA, Webb DH, Williams CM, Newsholme EA and Calder P. Effect of olive oil on immune function in middle-aged men. Am J Clin Nutr 1998; 67: 129-135.
6. Nakano E, Higgins JA, Powers HJ. Folate protects against oxidative modification of human LDL. Br J Nutr 2001; 86: 637-639.
7. Kris-Etherton PM, Harris WS, Appel LJ. AHA Nutrition Committee. American Heart Association. Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. Arterioscler Thromb Vasc Biol. 2003: 23(2):151-152.
8. WHO Study Group. "Diet, nutrition and prevention of chronic diseases" Technical Report Series, 2003; No. 916.
9. Hernández M and Sastre A. Tratado de Nutrición. Díaz de Santos Eds, 1999; Spain.
10. Chung BH, Wilkinson T, Geer JC, Segrest JP. Preparative and quantitative isolation of plasma lipoprotein: rapid, single, discontinuous density gradient centrifugation in a vertical rotor. J Lipid Res 1980; 21: 284-291
11. Esterbauer H, Striegl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. Free Rad Res Comms 1989; 6: 67-75.
12. Pellegrini N, Re R, Yang M, Rice-Evans C. Screening of dietary carotenoids and carotenoid-rich fruit extracts for antioxidant activities applying 2,2´-azinobis 3-ethylenebenzothiazoline-6-sulfonic acid radical cation decolorization assay. Methods Enzymol 1999; 299: 379-389.
13. Fukunaga K, Yoshida M, Nakazono N. A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high-performance liquid chromatography. Biomed Chromatogr 1998; 12(5):300-303
14. Lepage G and Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986; 27:114-120.
15. Araki A and Sako Y. Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. Journal of Chromatography 1987; 422: 43-52
16. Thurnham DI, Smith E, Flora PS. Concurrent Liquid-Chromatographic assay of retinol, alfa tocopherol, beta-carotene, alfa-carotene, lycopene and beta-cryptoxanthin in plasma with tocopherol acetate as Internal Standard. Clinical Chemistry 1988; 34 (2): 377-381
17. Third Report of the National Cholesterol Education Programme Expert Panel on "Detection, evaluation and treatment of high blood cholesterol in adults (ATP III)". 2002; National Institutes of Health (NIH), publication number 02-5215.
18. Lawson LD, Hughes BG. Absorption of eicosapentaenoic acid and docosahexaenoic acid from fish oil triacylglycerols or fish oil ethyl esters co-ingested with a high-fat meal. Biochem Biophys Res Commun 1988; 156: 960-963.
19. Visioli, F, Rise P, Plasmati E, Pazzucconi F, Sirtori C, and Galli C. Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerols and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol. Res 2000; 41: 571-576

20. Plaza, I., et al. Control de la colesterolemia en España, 2000. Un instrumento para la prevención cardiovascular. Rev Esp Cardiol, 2000; 53: 815-837.
21. Baro L et al. n -3 fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans. Clin Nutr 2003; 22(2):175-182.
22. Cobiac L, Clifton PM, Abbey M, Belling GB and Nestel PJ. Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n-3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr 1991; 53: 1210-1216.
23. Schnell JW, Anderson RA, Stegner JE, Schindler SP, Weinberg RB. Effects of a high polyunsaturated fat diet and vitamin E supplementation on high-density lipoprotein oxidation in humans. Atherosclerosis 2001; 159: 459-466.
24. Hayes KC, Pronckuk A and Perlman D. Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins. Am J Clin Nutr 2001; 74: 211-218.
25. Meydani SN et al. Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. Am J Clin Nutr 1998: 68(2):311-318
26. Kadiiska MB et al. Biomarkers of oxidative stress study: are plasma antioxidants markers of CCl(4) poisoning? Free Radic Biol Med 2000; 28: 838-845.
27. Kratz M et al. Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. Eur J Clin Nutr 2002; 56(1):72-81.
28. Brand K et al. Activated transcription factor nuclear factor-kB is present in the atherosclerotic lesion. J Clin Invest 1996; 97: 1715-1722.
29. Bronstrup A, Hages M, Prinz-Langenohl R, Pietrzik K. Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy young women. Am J Clin Nutr 1998; 68(5):1104-1110
30. Expert group on vitamins and minerals (EVN). Safe upper levels for vitamins and minerals: report of the expert group on vitamins and minerals. ISBN 1-904026-11-7. Food Standards Agency Publications. May 2003.
31. Rader JI. Folic acid fortification, folate status and plasma homocysteine. J Nutr 2002; 132(8):2466S-2470S
32. Nygard O, Nordrehaug JE, Refsum H, Ueland PM, Farstad M, Vollset SE. Plasma homocysteine levels and mortality in patients with coronary artery disease. N Engl J Med 1997; 337(4):230-236
33. Cybulsky MI et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001; 107: 1255-1262.
34. Koga, T., Claycombe, K. & Meydani, M. Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. Atherosclerosis 2002; 161(2):365-374.
35. Collie-Duguid ES, Wahle KW. Inhibitory effect of fish oil n-3 polyunsaturated fatty acids on the expression of endothelial cell adhesion molecules. Biochem Biophys Res Commun 1996; 220:969-74.
36. Haglund O, Mehta JL, Saldeen T. Effects of fish oil on some parameters of fibrinolysis and lipoprotein(a) in healthy subjects. Am J Cardiol 1994; 74:189-192.

6

Does nutrition have a role in peripheral vascular disease? Potential implications of nutrient interactions.

Submitted for publication

JJ Carrero* and RF Grimble†

*Departamento de Bioquímica y Biología Molecular. Universidad de Granada Spain
†School of Medicine, University of Southampton, Southampton, United Kingdom

Resumen

La Enfermedad Vascular Periférica (EVP) es una manifestación clínica de un proceso de aterosclerosis sistémica en las extremidades inferiores. EVP está íntimamente relacionada con un elevado riesgo a padecer un evento coronario agudo, y los pacientes con EVP poseen un riesgo de mortalidad cardiovascular de 3 a 5 veces mayor cuando se comparan con sus controles. Sin embargo, los pacientes con EVP no reciben el mismo tratamiento que los pacientes de enfermedad coronaria, en lo que a la reducción de los factores de riesgo cardiovascular o uso de medicación hipolipemiante o antiplaquetaria se refiere.

El uso de una dieta equilibrada puede influir sobre la progresión del proceso aterosclerótico no sólo mediante la modulación del perfil lipídico, sino mediante una acción directa sobre los procesos que se desarrollan en el endotelio vascular y que se conocen están íntimamente implicados en el desarrollo de esta enfermedad. Existe certeza epidemiológica que demuestra los efectos beneficiosos que ciertos nutrientes y hábitos dietéticos tienen en la prevención de las enfermedades cardiovasculares, pero el papel potencial de los nutrientes en la EVP no ha sido estudiado con la misma profundidad.

En las últimas décadas, diversos estudios de intervención realizados en pacientes con EVP han ofrecido interesantes resultados para ciertos nutrientes aislados, pero no nos dan una evidencia lo suficientemente consistente como para elaborar una guía nutricional. El propósito de la presente revisión es ofrecer una visión general del conocimiento que tenemos en la actualidad acerca de cómo los alimentos o sus nutrientes bioactivos pudiera ser capaces de afectar a la EVP. Además, se sugieren los mecanismos potenciales por los que esta interacción nutricional podría contribuir a la reducción de los síntomas de la EVP.

Summary

Peripheral vascular disease (PVD) is a manifestation of systemic atherosclerosis in the lower limbs. PVD is closely associated with high risk for myocardial infarction and stroke, and PVD patients have a 3 to 5 fold increase risk of cardiovascular mortality compared with age-matched controls. Nevertheless, PVD patients are undertreated with regard to risk factor reduction and the use of lipid-lowering or antiplatelet drugs, as compared with patients with coronary heart disease.

Diet can affect the development of atherosclerosis not only through modulation of serum lipids but also by influencing the immune and inflammatory processes present in the endothelium and known to be associated with the development of this disease. There is appreciable epidemiologic evidence that demonstrates the beneficial effects of certain nutrients and dietary habits in the prevention of cardiovascular diseases, but there is little attention on the role of nutrients in PVD.

In the last decades, several nutritional intervention studies in PVD patients have reported interesting findings for isolated nutrients, but the studies of which do not provide consistent evidence such as to suggest a specific dietetic guideline. The purpose of this review is to provide an overview of our present understanding of how foods and their bioactive nutrients could possibly benefit PVD. Furthermore, the potential mechanisms by which this nutrient-interaction could contribute to the reduction of PVD are briefly explored.

1. Introduction

Atherosclerosis is the common form of arteriosclerosis in which deposits of yellowish plaques (atheromas) containing cholesterol, lipoid material and lipophages are formed within the intima and inner media of large and medium sized arteries. Atherosclerosis is also the most common cause of chronic arterial narrowing that reduces blood flow to the lower limbs at rest or during exercise. Atherosclerosis of lower extremities defines what is known as peripheral vascular disease (PVD). Patients with PVD may be asymptomatic or present with intermittent claudication (IC), ischemia rest pain, and/or gangrene. It is estimated that PVD including asymptomatic stages, occurs in approximately 12% of the adult population, and the incidence of PVD increases with age, such that almost 20% of people over the age of 70 years have this disease (Hiatt *et al.* 1995; Halperin, 2002; TASC consensus, 2003).

IC is the most common symptom, present in 15 to 40 % of patients with PVD (Hirsch *et al.* 2001). It is defined as walking-induced pain in one or both legs that does not go away with continued walking and is relieved only by rest, as is associated with a diminished ability to perform daily activities. In approximately 25% of patients with IC, there is a progression to critical ischemia, eg, rest pain and gangrene, that may eventually necessitate amputation (Hertzer, 1991). A commonly used noninvasive test for PVD diagnosis is the measurement of systolic blood pressures in the ankles and arms with a Doppler ultrasonic instrument, from which the ankle-brachial index (ABI) is derived. A low ABI is highly predictive not only of the presence of arterial occlusive disease but also of subsequent cardiovascular mortality (Hiatt *et al.* 1995). An ABI greater than 0.90 is considered normal, 0.70 to 0.89 is considered mild disease, 0.5 to 0.69 moderate disease, and less than 0.5 severe disease (Tabet *et al.* 1996). On the other hand, the degree of functional impairment is established according to the distance that the patients can walk without pain or without onset of claudication, that is pain-free walking distance (PFWD).

PVD is closely associated with high risk for myocardial infarction and stroke (Leng & Fowkes, 1993; Muluk *et al.* 2001), and PVD patients have a 3 to 5 fold increase risk of cardiovascular mortality compared with age-matched controls (Criqui *et al.* 1992; Vogt *et al.* 1993; Leng *et al.* 1996; Henke *et al.* 2004). This increased risk, which appears to be independent of classic risk factors (Leng & Fowkes, 1993; Brevetti *et al.* 1998a), and is only

partially explained by the expected association of PVD with coronary and cerebrovascular disease (Leng & Fowkes, 1993; Muluk *et al.* 2001), is strongly related to the severity of PVD itself (Leng 1993; Brevetti 1998b). Because the systemic nature of atherosclerosis and the high risk if ischemic events, patients with PVD should be considered candidates for secondary prevention strategies that include antiplatelet-drug therapy and aggressive atherosclerotic risk factor modification (Hiatt, 2001; ATPIII, 2002). Nevertheless, patients with PVD are undertreated with regard to risk factor reduction and the use of lipid-lowering or antiplatelet drugs, as compared with patients with coronary heart disease (McDermott *et al.* 1997; Tornwall *et al.* 2000). Consensus guidelines for the specific management of PVD patients should be considered, as well as strategies to ensure their implementation (Cassar *et al.* 2003, Mukherjee *et al.* 2002; Henke *et al.* 2004).

Atherosclerosis is an inflammatory disease of the vascular system. An initial impairment in the functional properties of the endothelium of the affected limb vessels could explain the origin of the PVD, which would elicit a series of changes directly related to initiation, progression, and clinical complications of atheromatous plaque. Diet can affect the development of this plaque not only through modulation of serum lipids but also by influencing the immune and inflammatory processes present in the endothelium and known to be associated with the development of this disease (Ross, 1999). There is appreciable epidemiologic evidence that demonstrates the beneficial effects of certain nutrients and dietary habits in the prevention of cardiovascular diseases (CVD). Based on this evidence, International Health Societies have produced a number of nutritional recommendations that should be taken into consideration for primary as well as for secondary cardiovascular risk prevention. The latest WHO report (2003), for instance, recommends a regular fish consumption to provide about 200-500 mg/wk of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), a replacement of saturated fat by monounsaturated fat (oleic acid), and an increase in the consumption of fruit and vegetables.

However, there is little attention on the role of nutrients in PVD. It has been shown that malnutrition is common in patients with PVD and is associated with changes in other markers that predict an increase in complication (Spark *et al.* 2002). Although many factors including chronic vascular illness, diabetes, sedentary lifestyle, nutritional deficiencies and ageing itself may contribute to the progression of PVD and the ischemia-induced muscle weakness, only skeletal muscle disuse and undernutrition are potentially reversible with targeted interventions. The Edinburgh Artery Study (Donnan *et al.* 1993) described a positive association between a higher consumption of fiber-containing foods with greater mean ABI in PVD males. Higher consumption of meat was also associated with low mean ABI in PVD males and females, together with positive associations with cereal fiber, alcohol and vitamins E and C. In the last decades, several nutritional intervention studies in PVD patients have reported interesting

findings for isolated nutrients, but the studies of which do not provide consistent evidence such as to suggest a specific dietetic guideline (Hooper *et al.* 2004). The purpose of this review is to provide an overview of our present understanding of how foods and their bioactive nutrients could possibly benefit PVD. Furthermore, the potential mechanisms by which this nutrient-interaction contributes to the reduction of PVD are briefly explored.

2. Nutritional targets

Atherosclerosis, underlying PVD complications, is an inflammatory disease, not merely the passive accumulation of lipids within lower limbs artery walls. A variety of initiating agents and multiple pathogenic mechanisms (e.g. hyperlipidemia), initiate an inflammatory response that contributes to the development of atheromatous plaques (Ross, 1999). Endothelial dysfunction, by predisposing to thrombosis, impairment of the flow, leukocyte adhesion, and smooth muscle cell proliferation, plays a pivotal role in the development, progression, and clinical manifestations of atherosclerosis. Endothelial dysfunction originates an inflammatory cascade that includes the interaction of pro- and anti-inflammatory cytokines within the arterial wall.

Patients with PVD have an impaired endothelial function, which is related to the severity of the circulatory failure in the affected limb and to increased plasma markers of inflammation (Yataco *et al.* 1999; Brevetti *et al.* 2003a; Gokce *et al.* 2003). PVD patients have been reported to have elevated levels of cytokines, adhesion molecules (Signorelli *et al.* 2003), selectins (Blann *et al.* 1997a, Signorelli *et al.* 2003) von Willebrand factor (vWf) (Philipp *et al.* 1997; Blann *et al.* 2000a), tissue factor (Blann *et al.* 2000b), C-reactive protein (Rossi *et al.* 2002) and fibrinogen (Violi *et al.* 1996).

Moreover, PVD patients also have reduced RBC deformability, increased RBC aggregation and increased blood viscosity (Lowe *et al.* 1993), which may impede blood flow through various regions of the microcirculation in the lower limbs (Simchon *et al.* 1987). Pathological changes in RBC structure and hemodynamic functions may hinder blood flow in large vessels and occlude microvessels, facilitating endothelial and platelet activation by modulating shear stress and attenuating flow rate, and promoting white cell migration and adhesion to vessel wall endothelium.

Finally, systemic atherosclerosis narrows limb vessels and impairs blood flow to exercising leg muscles causing claudication, which is brought on by exercise and relieved by rest. As vessel narrowing increases, critical limb ischemia can develop when the blood flow does not meet the metabolic demands of tissue at rest. A reduction of the symptoms produced by this ischemic process would also ameliorate PVD progression.

Three major targets in PVD for which nutrients could play an important role have been summarised: 1) restoring the endothelial dysfunction; 2) improving RBC deformability, aggregation and blood flow; 3) improving oxygen perfusion in atherosclerosis-induced muscle ischemia. The existing literature suggests certain evidence of how selected nutrients could diminish PVD symptoms by affecting these three targets.

3. Dietary fats

3.1 Polyunsaturated fatty acids (fish oil)

n-3 Long-Chain Polyunsaturated fatty acids (n-3 PUFA), namely EPA and DHA, are found in fatty fish and in fish oils. Evidence from epidemiological and case-control studies indicate that consumption of fish, fatty fish and long-chain n-3 PUFA reduces the risk of cardiovascular mortality (Carrero *et al.* 2005). Studies using n-3 PUFA in patients post-myocardial infarction have shown a reduction in total and cardiovascular mortality. n-3 PUFA have been shown to decrease blood triacylglycerol concentrations, to decrease production of chemoattractants, growth factors, adhesion molecules, inflammatory eicosanoids and inflammatory cytokines, to lower blood pressure, to increase nitric oxide production, endothelial relaxation and vascular compliance, to decrease thrombosis and cardiac arrhythmias and to increase heart rate variability (Calder, 2004). These mechanisms most likely explain the primary and secondary cardiovascular protection afforded by long-chain n-3 PUFAs consumption and suggest that may be beneficial in PVD too.

Few randomised controlled trials have studied the effects of n-3 PUFAs on PVD. The very first of them described rheological changes (a decrease in whole blood viscosity) and a fall in triglycerides after supplementation for 7 wk with 1.8 g EPA/d in the form of fish oil capsules (Woodcock *et al.* 1984). No effect on clinical outcomes was reported in such short study though, and the changes in blood viscosity were not enough such as to give consistency to the hypothesis. A later double-blind study (Gans *et al.* 1990) reported that 4-mo daily administration of fish oil capsules containing 1.8 g EPA and 1.2 g DHA, significantly increased HDL-cholesterol and decreased triglycerides, blood viscosity and blood pressure in the intervention group. However, clinical outcomes were controversial: the mean PFWD increased by 18% in the intervention group and by 41% in the control group. Large standard deviations prevented results from being statistically significant, as it happened in another trial (Leng *et al.* 1998), that reported a decrease in blood pressure and a non-significant 81% increase of the mean PFWD in the intervention group compared to a 26% in the control group after to 2 y. treatment with a combination of ?-linolenic acid (GLA) and EPA (280 mg GLA + 45 mg EPA/d).

Randomized controlled trials suggest that n-3 PUFAs supplementation in PVD patients results in a lowering of diastolic blood pressure, blood viscosity and triglycerides, but an

increase in LDL cholesterol levels. Methodological shortcomings (small sample sizes and short length) though, may have resulted in a failure to detect significant clinical effects in these trials (Sommerfield & Hiatt, 2004). The effects described should theoretically reduce the elevated risk of coronary heart disease possessed by individuals with PVD, but further research is needed to evidence the clinical implications of this supplementation.

Modern research regarding n-3 PUFAs supplementation on other atherosclerotic disorders suggests other mechanisms by which these fatty acids could play a potential role in PVD. EPA and DHA exert an anti-inflammatory effect within the vessel wall (Calder, 2004). EPA and DHA compete with arachidonic acid for the insertion at the sn-2 position of membrane phospholipids producing less potent eicosanoids than those produced by arachidonic acid. In fact, fish oil decreased neutrophil leukotriene B4 in PVD patients, while leukotriene B5 levels increased significantly (Mori *et al.* 1992). Supporting this idea we have recently suggested how fish oil might influence inflammation in PVD patients by altering the balance between leukotriene B4 and prostaglandin E2 production. As the former stimulates and the latter inhibits pro-inflammatory cytokine production respectively, a fall in the ratio will lead to a decrease in cytokine production and vice-versa (Carrero et al, 2004a). In addition, EPA is able to promote vascular endothelial cell migration and simultaneously block the smooth muscle cell migration (Kanayasu-Toyoda *et al.* 1993, 1996) which are beneficial for repair of blood vessel injuries and inhibition of atherosclerotic plaque formation, respectively. Moreover, we also reported a rapid incorporation of dietary EPA and DHA in atherosclerotic plaques, resulting in increased plaque stability and reduced macrophage infiltration, slowing the progression of the vascular lesion (Thies *et al.* 2003) and perhaps the onset of clinical events.

On the other hand, EPA and DHA have been shown to increase RBC deformability (Ernst, 1989) and reduce their aggregation (Ho *et al.* 1999), maybe as result of modifying the cell membrane lipid content. RBC aggregation results in a net increase in ‘cell’ size (of the aggregate) and increased sludging in capillaries. Therefore reduced platelet and RBC aggregation can potentially increase blood flow (Vicaut, 1995).

Fish oil could theoretically and potentially play a significant role in PVD by a variety of actions. However, more research is required with regard to PVD management, and trials with bigger cohorts, lengths and dosages might be needed to assess potential plausible benefits on clinical outcomes. Two studies are being carried out in our laboratories that hopefully will contribute to clarify this topic.

3.2 Monounsaturated fatty acids (olive oil)

Olive oil in the Mediterranean diet, commonly replaces saturated fats consumed in the Western countries. Accumulating evidence suggests that it may have health benefits that include reduction of risk factors of coronary heart disease and modification of immune and

inflammatory responses. The therapeutic properties of olive oil are often attributed to its high levels of monounsaturated fatty acids, but other minor components (e.g. antioxidants and phytochemicals) are also responsible for its health effects. It has the advantage of being less susceptible to oxidation than oils rich in PUFA and in most studies olive oil has shown to lower plasma total and LDL cholesterol to a similar degree as PUFA, combined with a possible protective antioxidant effect (Stark & Madar, 2002). Olive oil has also been shown to have beneficial effects on blood pressure (Ferrara *et al.* 2000; Ruiz-Gutierrez *et al.* 1996) and modify immune response (Alarcon de la Lastra *et al.* 2001).

This evidence suggests that olive oil consumption could be indicated in PVD management. However, very few trials have tested its potential role in IC. Olive oil reduced total cholesterol levels after 4-wk supplementation in PVD patients, accountable to a significantly decrease in LDL-cholesterol levels, together with an increase in serum thromboxane B2 (Mori *et al.* 1992). Another small 3-mo trial in PVD patients using a combination of olive oil and fish oil resulted in a fall in triglycerides and a lower susceptibility of LDL to oxidation (Ramirez-Tortosa *et al.* 1999). In both trials, no effect was found in clinical outcomes, perhaps due to short duration of the studies or short sample size. Since well-sustained evidence recommends its consumption for cardiovascular risk prevention and no deleterious or side effects have been reported, it might be useful in reducing risk factors and worth trying in future trials.

4. Antioxidants

Free radical activity and oxidative damage have been implicated in the initiation of vascular disease, and antioxidants provide the first line of defence against free radicals. Several studies have shown that episodes of ischemia-reperfusion can reduce the total antioxidant capacity (Khaira *et al.* 1995, 1996). It is possible, therefore, that the longer or more severe the bouts of ischemia, the greater the reduction in the total antioxidant capacity and subsequent increase in the risk of developing infective complications. In fact, it has been shown that antioxidants in PVD patients are lower than age matched controls (Duthie *et al.* 1989; Spark *et al.* 2002). If these patients have an unimpaired nutritional status with low total antioxidant capacity, they may benefit from antioxidant supplementation. It their nutritional status is impaired, then nutritional supplementation could also be required.

4.1 Vitamin E

Vitamin E (tocopherol) is a fat-soluble vitamin which functions solely as a membrane-bound antioxidant that prevents cell membrane damage by inhibiting peroxidation of membrane phospholipids and disrupting free radical chain reactions induced by formation of lipid peroxides. As the only membrane-bound lipid-soluble antioxidant, Vitamin E plays a key role in preventing cellular injury from oxidative stress associated with premature ageing,

cataracts, uncontrolled diabetes, cardiovascular disease, inflammation, and infection. (Morrisey, 1999). Vitamin E is naturally present in fruits and vegetables.

It has been hypothesized that vitamin E consumption could benefit PVD. Vitamin E might improve tolerance to the ischemia that occurs in the lower limbs, if indeed it eliminates free radicals (Ferrari *et al.* 1983). It also might influence the process of atherosclerosis by stopping further deterioration. It has been shown that patients with ischemic heart disease and patients with PVD have higher plasma lipid peroxide concentrations than controls (Stringer *et al.* 1989). Inhibition of peroxidation by vitamin E might influence beneficially the balance between peroxidative damage and the body's repair mechanisms. Finally, it may influence platelet aggregation (Steiner & Mower 1982) and affect RBCs (Farrel *et al.* 1977), improving blood flow, which might account for some beneficial effect on the symptoms of IC (Kleijnen & Mackerras, 2004).

The treatment of IC with vitamin E was originally proposed in the 1940's by Shute *et al.* (1948), and led to several controlled trials in the following decades. These trials lasted for between 12 wk and 18 mo and they all showed to increase both PFWD and blood flow through arteries (in most of the cases measured as ABI) of the lower legs in people with IC (Hamilton *et al.* 1953; Livingstone & Jones, 1958; Boyd & Marks, 1963; Williams *et al.* 1971; Haeger, 1974; Westheim *et al.* 1975). The dosage used varied from 400 to 600 IU/d, although one study used 2,400 IU/d. Increasing dietary intake of vitamin E was also associated with better blood flow to the legs (Donnan *et al.* 1993). Possibly, more effect would have been noticed with longer duration of the studies (Livingstone & Jones, 1958) as one review article suggested that a minimum of 4-6 mo of vitamin E supplementation may be necessary before significant improvement is seen (Piesse, 1984). In the Rotterdam Study, vitamin E intake was inversely associated with PVD in men, and a 10 mg increase in intake was associated with a 0.015 ABI increase (Klipstein-Grobush *et al.* 2001).

Big methodological differences in these trials make comparison of studies and meta-analysis difficult to comply (Kleijnen & Mackerras, 2004): they all had different study lengths and dosages, they measured four different physical outcomes in small sample sizes, which means that baseline were not necessarily equal between the trials, and finally the active period for conducting vitamin E trials in IC was about 30 y. ago, with different techniques and understanding of the disease. There might not be enough consistent data still to recommend using vitamin E in patients with IC. However, the existing evidence always shows a positive beneficial effect and is in favor of vitamin E consumption. Since synthetic vitamin E is very cheap and since to date no serious side effects have been reported, it might be worth trying.

A combined strategy of n-3 PUFA and vitamin E supplementation has proved to be effective in CVD prevention, as an important function of vitamin E in the body is the protection

of PUFA from oxidation. Vitamin E could improve the role of n-3 PUFA through protection from lipid peroxidation, by acting independently on the same or closely related atherogenic and thrombotic mechanisms, or both (Meydani *et al.* 1991; Steinberg, 1991; Morrisey, 1999). n-3 PUFA are highly susceptible to oxidation by endogenous free radicals which are formed and needed in normal cell metabolism. In PVD, the cell damage that occurs in ischemic periods in the calf muscle and lower limbs, is probably caused by free radicals. Deformability of red blood cells, for instance, may be enhanced by vitamin E, since n-3 PUFA incorporated in the membranes are protected from oxidation. However, there are not studies available with this combination of nutrients in PVD patients.

4.2 Vitamin C

Vitamin C (ascorbic acid) is a water-soluble antioxidant capable of scavenging free radicals and is the first-line defence in the control of the redox state, sparing other antioxidants from consumption. Vitamin C preferentially concentrates in leucocytes and attenuates reperfusion-induced muscle injury (Kearns *et al.* 2004). It has been postulated that vitamin C may be effective in PVD management by restoring endothelial function. A small study showed that vitamin C prevented endothelial function induced by exercise in PVD patients with IC, together with a decrease in TBARS and sICAM-1 levels (Silvestro *et al.* 2002). Pre-treatment with vitamin C preserved muscle function and reduced the expression of sICAM-1, infiltration of the neutrophils and oedema in a study with skeletal muscle ischemic patients (Kearns *et al.* 2004), and acted as a potent acute vasodilator in the radial arteries of preoperative coronary patients (Drossos *et al.* 2003). In fact, a prospective study (Langlois *et al.* 2001) showed that vitamin C status is depleted in PVD patients, and is associated with the grade of inflammation (measured as C-reactive protein levels) and the severity of the disease (measured as shorter PFWD).

The question arises whether antioxidant vitamin supplements would be useful to address reduction of oxidative stress in PVD. The studies revised in this section give certain evidence to recommend its use or at least to suggest the necessity of more specific trials to clarify this topic. However, not all trials describe positive effects. It has been described a pro-oxidant effect of dietary vitamin C in humans, which may give rise to paradoxical effects in clinical intervention trials (Podmore *et al.* 1998). Recently, the long-term effect of combined vitamins E and C did not show any improvement on peripheral endothelial function (Kinlay *et al.* 2004). An ongoing multicenter European trial, the Critical Leg Ischemia Prevention Study (CLIPS), investigates the effectiveness of low-dose aspirin and antioxidant vitamins (vitamin E, vitamin C, ß-carotene) with a 2x2 factorial design.

5. Folic acid and B vitamins

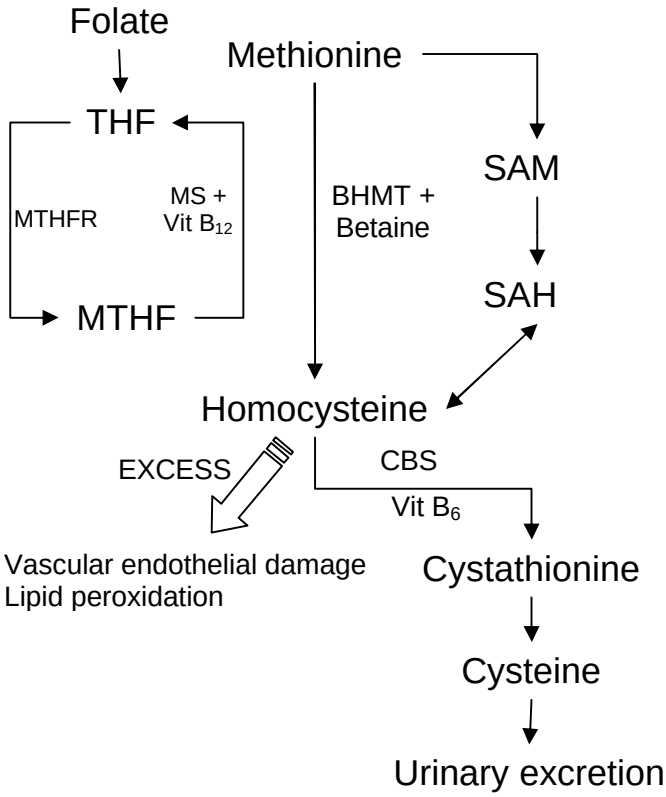
Elevated levels of plasma homocysteine (Hcy) increase platelet aggregation (Welch & Loscalzo, 1988), oxidative stress (Nappo *et al.* 1999) and vascular smooth muscle proliferation, decreases nitric oxide production (Tsai *et al.* 2000) and impairs endothelial function. Consistent with these adverse cardiovascular effects, elevated concentrations of Hcy have been positively associated with the risk of coronary heart disease (Cleophas *et al.* 2000) and PVD (Cheng *et al.* 1997).

Hyperhomocysteinemia (HHcy) is associated with an increased risk of developing PVD independent of established risk factors (smoking, hypercholesterolaemia, diabetes and hypertension) (Kuan *et al.* 2002). The relative risk of developing PVD has been estimated to vary from 2.0 to 11.0 for elevated fasting Hcy levels in several studies (Boers, 1997; de Jong *et al.* 1999; Taylor *et al.* 1999). In addition, there is evidence that post methionine load HHcy is an independent risk factor for PVD (Graham *et al.* 1997; Refsum *et al.* 1988). This risk increases when other factors like hypertension, smoking and hypercholesterolaemia are included. HHcy is present in 30% of PVD patients (Taylor *et al.* 1991), and the existing plasma levels of Hcy are associated with the severity of the PVD disease in type 2 diabetic patients, since PVD is the most prevalent expression of vascular atherosclerosis in this kind of patients (Ciccarone *et al.* 2003).

Figure 1 shows Hcy metabolism. A low intake of folate limits the remethylation of Hcy to methionine and increases the concentration of plasma Hcy (Verhoef *et al.* 1996). Vitamins B6 and B12 are cofactors that contribute to the conversion of Hcy to cysteine or methionine, respectively (Verhoef *et al.* 1996); low intakes of these vitamins can potentially increase Hcy. Therefore, it can potentially contribute to the regression of PVD by indirectly reducing Hcy levels and their effects.

Folate is a water soluble vitamin that mammals cannot synthesize and have to obtain from their diets. Folate is present in selected foods such as orange juice, dark green leafy vegetables, dried beans and peas, asparagus, strawberries and peanuts (Carrero *et al.* 2004b). Vitamin B6 is present in foods such as potatoes, breakfast cereals, bread, meat, fish, eggs, bananas, nuts and seeds. Vitamin B12 is naturally found in all animal foods – meat, meat products, milk, fish, eggs – and certain algae, such as seaweed. Recently, folate and B6 intake have been identified as independent predictors of PVD in men aged over 50 years (Wilmink *et al.* 2004).

Figure 1. Homocysteine metabolism.



THF, tetrahydrofolate; MTHF, methyltetrahydrofolate; MTHFR, methyltetrahydrofolate reductase; MS, methionine synthase; BHMT, betaine homocysteine methyltranferase; CBS, cysthionine beta synthase; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Very few intervention studies on PVD patients have been found on the literature. The existing data refers to nutritional epidemiology. A study recently held in the Health Professionals Follow-up Study population revealed that men in the top category of folate intake (median = 840 µg/d) were at 33% lower risk of PVD than men in the bottom category (median = 244 µg/d). Besides, there was a weak inverse association between intake if vitamin B6 and PVD risk (p=0.06) (Merchant *et al.* 2003a). These results suggest that higher consumption of folate and B vitamins may contribute to the prevention of PVD.

The Hcy-lowering effects of folate have been well documented (van den Berg *et al.* 1994), and the addition of vitamins B12 and B6 to folic acid supplements or enriched foods may maximize the reduction of Hcy in about a 7% more (Bronstrup *et al.* 1998; HLTC *et al.* 1998). To give an idea of the importance of Hcy reduction, a recent meta-analysis suggests that lowering homocysteine concentrations by 3 µmol/l from current levels would reduce the risk of

ischemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24% (Wald *et al.* 2002).

The mechanisms by which Hcy exerts its deleterious effects are not fully known, and in vitro studies demonstrate the multi-factorial nature of Hcy-induced vascular disease. Regarding PVD symptoms, a reduction of Hcy could be related to reduced endothelial cell injury (Wall *et al.* 1980), reduced adhesion molecule expression (Silverman *et al.* 2002), reduced monocyte and T-cell binding to endothelial cells (Koga *et al.* 2002) reduced endothelium-dependent relaxation (Weiss *et al.* 2002), or reduced Factor V activation (Rodgers *et al.* 1986). In addition, several studies have highlighted homocysteine-induced changes in coagulation response (Lentz & Sadler, 1991; Mujumdar *et al.* 2001), and a recent trial ex vivo suggests that the presence of IC alone does not influence platelet function but if complicated with mild HHcy, there appears an increased platelet activation (Riba *et al.* 2004).

In PVD patients, flow-mediated endothelium-dependent dilatation of the peripheral arteries is decreased (Poredos *et al.* 2002), and a reduction of Hcy is associated with an improved endothelial function by mediating in the three vasodilator pathways: the NO synthesis (Stamler *et al.* 1993), prostacyclin production (Wang *et al.* 1993) and the endothelium-derived hyperpolarizing factor, which is a major determinant of vascular tone in small resistance cells (de Vriese *et al.* 2004). In summary, a Hcy decrease is strongly related to vasodilatation, reduced endothelial dysfunction and reduced platelet reactivity, and can potentially be useful in ameliorating the flow in the lower limbs when in the context of PVD. However, more evidence is needed again such as to suggest dosages of these nutrients, fortification levels, suitability of supplements…etc.

6. Fiber

It has been hypothesized that the apparent protective effect of fiber intake against coronary heart disease is mediated by lowered cholesterol (Hunninghake *et al.* 1994) and lowered plasminogen activator inhibitor type 1 and factor VII activity (Marckmann *et al.* 1993). PVD results mainly from atherosclerotic narrowing of the blood vessel lumen. LDL are taken up by monocytes in the intima of the blood vessels, becoming foam cells, and leading to the formation of plaque. Increased cytosolic triglycerides are associated with oxidative stress and can cause endothelial dysfunction (Bakker *et al.* 2000). Thus, increased serum HDL and triglycerides increase the risk of PAD (Drexel *et al.* 1996). Few studies have studied the association of fiber and PVD. The Edinburgh Artery Study (Donnan *et al.* 1993) was pioneer in describing a positive association between ceral fiber-containing foods with greater mean ABI in PVD males. Later on, a greek case-control study in 100 PVD patients showed the same association with total fiber (Katsouyanni *et al.* 1991) which was confirmed in a cohort study conducted among finish smokers (Tornwall *et al.* 2000). Finally, based in the male population

of the Health professionals Follow-up Study, an inverse association with PVD risk was found for cereal fiber intake and not for total fiber intake, suggesting that it is important to evaluate the different types of fiber in relation to PVD risk, because associations vary considerably (Merchant *et al.* 2003b)

No interventional studies in PVD patients are available such as to provide an idea of possible improvements in clinical outcomes, but research literature suggests that fiber intake might reduce PVD risk by reducing serum cholesterol reduction, as described above, and by increasing insulin sensitivity, as fiber intake improves insulin sensitivity by slowing the absorption of nutrients from the gut (Jenkins & Jenkins, 1985), reducing serum glucose levels (Jenkins *et al.* 2000), producing short chain fatty acids by gut bacteria and consequently improving glucose metabolism (Thorburn *et al.* 1993), which is associated with lower LDL, blood pressure and triglycerides, and higher HDL (Hunninghake *et al.* 1994).

Finally, foods naturally containing fiber also contain other positive nutrients in PVD management such as vitamin E or folate, whose benefits are discussed in this review. It might be plausible to say that increasing cereal fiber intake in the diet could contribute, to some extent, to the prevention of PVD.

7. L-Carnitine

L-Carnitine (LC) or propionyl-L-Carnitine (PLC) is a nonessential dietary amino acid that humans endogenally synthesize from lysine and methionine. It is mainly found in meat, poultry, fish, avocados and dairy products. We will consider LC as a nutrient, though it is not easy to make a clear distinction or classification for a substance, which, on one hand, is synthesized endogenously and, on the other hand, has to be taken with food.

LC is a metabolic agent and an important co-factor for normal skeletal muscle bioenergetics in at least three reactions: First, LC is required for long-chain fatty acid oxidation; second, it assists in removing accumulated acyl groups from the mitochondria; and third, it plays an important role in detoxification. Muscles require optimum performance of these metabolic processes during impaired exercise. Theoretically, carnitine availability may be the limiting factor for fatty acid oxidation, or the removal of acyl-Coas during exercise (Karlic & Lohninger, 2004). Patients with PVD have been shown to accumulate acylcarnitines in their skeletal muscle, and this abnormal accumulation of acylcarnitines is directly correlated with impaired exercise performance (Smit, 1992; Hiatt, 1994). It has therefore been suggested that oral supplementation with LC in patients with IC could help to improve their symptoms

In double-blind trials, supplementation with either LC or PLC has increased PFWD in people with IC. PFWD was 75% greater after 3-wk of 2 g twice/d of LC supplementation (Brevetti *et al.* 1988b). In the study using PLC, improvement occurred only in those with

severely limited walking capacity (PFWD<250 m), where PFWD increased by 78% with PLC supplementation compared with a 44% increase in the placebo group (Brevetti *et al.* 1997). The amount of PLC used was 1 g/d, increasing to 2 g/d after 2-mo, and 3 g/d after an additional 2-mo, if needed. The results of this trial have been confirmed in a large European trial involving 485 IC patients, where PLC supplementation in the subgroup of severe PVD patients resulted in a 98% PFWD increase compared to a 54% PFWD increase in the placebo group (Brevetti *et al.* 1999). Oral LC has been associated to increased leg muscle strength (Barker *et al.* 2001) increased blood flow velocity, plasminogen activator inhibitor-1 activity and RBC deformity (Dal Lago *et al.* 1999).

Studies with athletes show, in the majority of the cases, an improvement in muscle function exercise performance and/or recovery after dietary LC (Karlic & Lohninger, 2004). Though there have not been later studies with oral LC, evidence suggests a potential use of this nutrient in diminishing IC symptoms by targeting the ischemia-induced in the calf muscle.

8. Other nutritional approaches

Several studies have used Ginkgo biloba extracts (GBE) for treatment of IC (Scheneider, 1992; Peters *et al.* 1998). Ginkgo biloba is rich in flavonoids and terpene trilactones, such as ginkgolide B, which inhibits platelet activation factor, releases nitric oxide, decreases aggregation and blood viscosity and shows anti-ischemic effects (Pittler & Ernst, 2000). Oral GBE tablets increased PFWD in three controlled but not randomized studies (Bauer, 1984: Blume *et al.* 1996; Blume *et al.* 1998) and these improvements are more pronounced when the dose is higher (160 mg/d) (Schweizer & Hautmann, 1999). Non-randomization, however, may have resulted in a substantial overestimation of the effect size (Kleijnen & Knipschild, 1992), and the size of the overall treatment effect is modest and of uncertain clinical relevance such as to recommend its general consumption for this disease, as suggested in a meta-analysis (Pitler & Ernst, 2000).

Garlic has also been tested as a treatment for IC, since its primary active component, allicin, has been reported to have some beneficial effects on serum cholesterol and platelet aggregation (Jepson *et al.* 1997). Only one study tested this hypothesis, supplementing 400 mg of a garlic powder extract twice per day for 12 wk. Although no significant improvement was found overall, the authors report that there was a significant increase in PFWD, but this only occurred in the last weeks of therapy. (Kiesewetter *et al.* 1993).

It has been hypothesized that moderate alcohol consumption exerts a protective effect on IC risk. The rationale for this hypothesis stands in the fact that alcohol raises HDL cholesterol (van Tol *et al.* 1998). HDL plays an important role in LDL transport from the bloodstream to the liver, where it is degraded (Reichl & Miller, 1989). Oxidized LDL is a key element in the pathophysiology of atherosclerosis, and an inverse association between HDL and IC has also

been reported (Fowkes *et al.* 1992). Alcohol intake favorably influences fibrinogen (Mennen *et al.* 1999), plasminogen activator inhibitor 1 (Ridker *et al.* 1994), factor VII (Gorinstein *et al.* 1997) and lowers platelet aggregation (Renaud & Ruf, 1996), all of them mechanisms that may prevent thrombogenesis or improve fibrinolysis in PVD patients. However, limited data is available on the effects of alcohol on IC. Most of the few observational studies that have evaluated this relation have yielded weak and inconsistent results: In the Edinburgh Artery Study, alcohol was positively associated with the ABI in males (Jepson *et al.* 1995), and another prospective study showed that moderate alcohol consumption was associated with decreased risk in male PVD patients (Camargo *et al.* 1997). Finally, this relation was studied in the Framingham cohort (Djousse *et al.* 2000), finding a protective effect of moderate alcohol consumption on IC risk, with lowest risk observed in men consuming 13 to 24 g/d (1 to 2 drinks/d) and in women consuming 7 to 12 g/d (0.5 to 1 drink/d). However, nonalcoholic components of certain drinks such as wine and beer may also contribute to IC risk reduction. Wine and beer contain polyphenols with antioxidant properties; phenolic compounds may delay the onset of atherosclerosis by preventing oxidation of LDL (Frankel *et al.* 1993). Phytoalexin, an antifungal compound found in grape skin (found in higher concentration in red wine), may raise HDL and reduce platelet aggregation (Jepson *et al.* 1995).

Finally, L-Arginine has been shown to induce NO formation and improve endothelial-dependent vasodilatation in patients with atherosclerosis. Intravenous injections of L-arginine have been shown to be effective at improving IC (Boger *et al.* 1998). However, to date no trials have examined the effects of oral arginine supplementation, except for a nutritional food bar enriched with L-arginine. In this 2-wk double-blind controlled trial, IC patients who consumed the food bar improved their PFWD in about 66% (Maxwell *et al.* 2000).

9. Conclusion

The potential role of nutrition and PVD is important. Data referred in this review suggest that quite modest levels of dietary modification could have significant effects. We have identified several nutrients that, together with appropriate lifestyle (such as daily exercise or smoking cessation) could be suitable in the prevention of this disease. It could be beneficial for PVD patients to increase in their diets the intake of fish or fish oil (n-3 PUFA) and replace saturated fat by monounsaturated fat (olive oil). A bigger consumption of fruits (antioxidants) and vegetables (fiber and folic acid) is also recommended.

These observations do not differ much from the nutritional consensus made for coronary heart disease. Though ATP III recommendations emphasise lifestyle and dietary changes in CVD prevention (ATPIII, 2002), it has been evidenced in the last years that more attention should be paid to dietary approaches in the management of PVD patients, as PVD risk factors are still and often mismanaged (Henke *et al.* 2004): after a hospital discharge, only 50% of the

PVD patients would modify their diet for lipid control (Mukherjee *et al.* 2002) and only 18% of the General Practitioners would consider cholesterol lowering therapy to be primary prevention (Cassar *et al.* 2003).

However, its been clear in all these approaches, that more longitudinal intervention studies are required to determine whether, indeed, this is the case and work towards a nutritional recommendation strategy for this impairment condition.

10. Acknowledgements

JJ Carrero has a PhD educational grant from University of Granada, Spain. We are grateful to Puleva Biotech S.A. for supporting the visit of JJ Carrero to Southampton University.

11. References

Alarcon de la Lastra C, Barranco MD, Motilva V & Herrerias JM. (2001) Mediterranean diet and health: biological importance of olive oil. *Curr Pharm Des* **7**, 933-950.

ATPIII (2002) Third Report of the National Cholesterol Education Programme Expert Panel on "Detection, evaluation and treatment of high blood cholesterol in adults (ATP III)". National Institutes of Health (NIH), publication number 02-5215.

Bakker SJ, IJzerman RG, Teerlink T, Westerhoff HV, Gans RO & Heine RJ. (2000) Cytosolic triglycerides and oxidative stress in central obesity: the missing link between excessive atherosclerosis, endothelial dysfunction, and beta-cell failure? *Atherosclerosis* **148**, 17-21.

Barker GA, Green S, Askew CD, Green AA & Walker PJ. (2001) Effect of propionyl-L-carnitine on exercise performance in peripheral arterial disease. *Med Sci Sports Exerc* **33,** 1415-1422.

Bauer U. (1984) Six-month double-blind randomised clinical trial of *Ginkgo biloba* extract versus placebo in two parallel groups in patients suffering from peripheral arterial insufficiency. *Arzneimittelforsschung* **34,** 716–720.

Blann AD, Amiral J, McCollum CN & Lip GY. (2000b) Differences in free and total tissue factor pathway inhibitor, and tissue factor in peripheral artery disease compared to healthy controls. *Atherosclerosis* **152,** 29–34.

Blann AD, Farrell A, Picton A & McCollum CN (2000a) Relationship between endothelial cell markers and arterial stenosis in peripheral and carotid artery disease. *Thromb Res* **97**, 209–216.

Blann AD, Seigneur M, Steiner M, Boisseau MR & McCollum CN. (1997) Circulating endothelial cell markers in peripheral vascular disease: relationship to the location and extent of atherosclerotic disease. *Eur J Clin Invest* **27,** 916–921.

Blume J, Kieser M & Hölscher U. (1996) Placebo-controlled, double-blind study on the efficacy of Ginkgo biloba special extract EGb 761 in maximum-level trained patients with intermittent claudication. *Vasa* **25,** 265–274.

Blume J, Kieser M & Hölscher U. (1998) Efficacy of *Ginkgo biloba* special extract EGb 761 in peripheral occlusive disease. *Fortschr Med* **116**, 137–143.

Boers GH. (1997) Hyperhomocysteinemia as a risk factor for arterial and venous disease. A review of evidence and relevance. *Thromb Haemost* **78,** 520-522.

Boger RH, Bode-Boger SM, Thiele W, *et al.* (1998) Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. J *Am Coll Cardiol* **32**, 1336–1344.

Boyd AM & Marks J. (1963) Treatment of intermittent claudication. A reappraisal of alpha-tocopherol. *Angiology* **14,** 198-208

Brevetti G, Chiariello M, Ferulano G, *et al.* (1998b) Increases in walking distance in patients with peripheral vascular disease treated with L-carnitine: a double-blind, cross-over study. *Circulation* **77,** 767–773.

Brevetti G, Diehm C & Lambert D. (1999) European multicenter study on propionyl-L-carnitine in intermittent claudication. *J Am Coll Cardiol* **34**, 1618–1624.

Brevetti G, Martone VD, Perna S, *et al.* (1998a) Intermittent claudication and risk of cardiovascular events. *Angiology* **49**, 843-848.

Brevetti G, Perna S, Sabba C, *et al.* (1997) Effect of propionyl-L-carnitine on quality of life in intermittent claudication. *Am J Cardiol* **79**, 777–780.

Brevetti G, Silvestro A, Di Giacomo S, *et al.* (2003) Endothelial dysfunction in peripheral arterial disease is related to increased plasma markers of inflammation and severity of peripheral circulatory impairment, but not to classic risk factors and atherosclerotic burden. *J Vasc Surg* **38,** 374–379

Brevetti G, Silvestro A, Schiano V & Chiariello M. (2003b) Endothelial dysfunction and cardiovascular risk prediction in peripheral arterial disease: additive value of flow-mediated dilation to ankle-brachial pressure index. *Circulation* **108**, 2093-2098

Bronstrup A, Hages M, Prinz-Langenohl R & Pietrzik K. (1998) Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women. *Am J Clin Nutr* **68,** 1104-1110

Calder PC. (2004) n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. *Clin Sci (Lond)* **107**, 1-11.

Camargo CA Jr, Stampfer MJ, Glynn RJ, Gaziano JM, Manson JE, Goldhaber SZ & Hennekens CH. (1997) Prospective study of moderate alcohol consumption and risk of peripheral arterial disease in US male physicians. *Circulation* **95**, 577-580.

Carrero JJ, Fonolla J, González-Santiago M, Baro L, Jimenez J, Boza J & Lopez-Huertas E. (2004b) Health effects due to folic acid supplementation. Food fortification strategies to achieve adequate folate status" *Nutrafoods* **3,** 5-13.

Carrero JJ, Madden J, Hadley J, *et al.*.(2004a) Fish oil alters the balance between LPS stimulated leukotriene B4 and prostaglandin E2 production by PBMCs in patients with peripheral vascular disease but not in healthy controls. *Clin Nutr* **23,** 789-790.

Carrero JJ, Martin-Bautista E, Baro L, Fonolla J, Jimenez J, Boza JJ & López-Huertas E. (2005) Cardiovascular effects of omega-3 fatty acids and alternatives to increase intake. *Nutr Hosp* **20,** 56-62.

Cassar K, Coull R, Bachoo P, Macaulay E & Brittenden J. (2003) Management of secondary risk factors in patients with intermittent claudication. *Eur J Vasc Endovasc Surg* **26,** 262-266.

Ciccarone E, Di Castelnuovo A, Assanelli D, *et al.* (2003) Homocysteine levels are associated with the severity of peripheral arterial disease in Type 2 diabetic patients. *J Thromb Haemost* **1,** 2540-2547.

Cleophas TJ, Hornstra N, van Hoogstraten B & van der Meulen J. (2000) Homocysteine, a risk factor for coronary artery disease or not? A meta-analysis. *Am J Cardiol* **86,** 1005-1009.

Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR, McCann TJ & Browner D. (1992) Mortality over a period of 10 years in patients with peripheral arterial disease. *N Engl J Med* **326,** 381-386.

Cheng SW, Ting AC & Wong J. (1997) Fasting total plasma homocysteine and atherosclerotic peripheral vascular disease. *Ann Vasc Surg* **11**, 217-223.

Dal Lago A, De Martini D, Flore R, *et al.* (1999) Effects of propionyl-L-carnitine on peripheral arterial obliterative disease of the lower limbs: a double-blind clinical trial. *Drugs Exp Clin Res* **25**, 29-36

de Jong SC, Stehouwer CD, van den Berg M, Geurts TW, Bouter LM & Rauwerda JA. (1999) Normohomocysteinaemia and vitamin-treated hyperhomocysteinaemia are associated with similar risks of cardiovascular events in patients with premature peripheral arterial occlusive disease. A prospective cohort study. *J Intern Med* **246**, 87-96.

de Vriese AS, Blom HJ, Heil SG, Mortier S, Kluijtmans LA, Van de Voorde J & Lameire NH. (2004) Endothelium-derived hyperpolarizing factor-mediated renal vasodilatory response is impaired during acute and chronic hyperhomocysteinemia. *Circulation* **109**, 2331-2336.

Djousse L, Levy D, Murabito JM, Cupples LA & Ellison RC. (2000) Alcohol consumption and risk of intermittent claudication in the Framingham Heart Study. *Circulation* **102**, 3092-3097.

Donnan PT, Thomson M, Fowkes GR, *et al.* (1993) Diet as a risk factor for peripheral arterial disease in the general population: the Edinburgh Artery Study. *Am J Clin Nutr* **57,** 917–921.

Drexel H, Steurer J, Muntwyler J, Meienberg S, Schmid HR, Schneider E, Grochenig E & Amann FW. (1996) Predictors of the presence and extent of peripheral arterial occlusive disease. *Circulation*. **94,** 199-205.

Drossos GE, Toumpoulis IK, Katritsis DG, Ioannidis JP, Kontogiorgi P, Svarna E & Anagnostopoulos CE. (2003) Is vitamin C superior to diltiazem for radial artery vasodilation in patients awaiting coronary artery bypass grafting? *J Thorac Cardiovasc Surg* **125,** 330-335.

Duthie GC, Wahle KWJ & James PWT. (1989) Oxidants, antioxidants and cardiovascular disease. *Nutr Res Rev* **2,** 51-62.

Ernst E. (1989) Effects of n-3 fatty acids on blood rheology. *J Intern Med Suppl* **225**, 129-132.

Farrell PM, Bieri JG, Fratantoni JF, Wood RE & di Sant'Agnese PA. (1997) The occurrence and effects of human vitamin E deficiency. A study in patients with cystic fibrosis. *J Clin Invest* **60,** 233-241.

Ferrara LA, Raimondi AS, d'Episcopo L, Guida L, Dello Russo A & Marotta T.(2000) Olive oil and reduced need for antihypertensive medications. *Arch Intern Med* 160, 837-842.

Ferrari R, Visioli O, Guarnieri C & Caldarera M. (1983) Vitamin E and the heart: possible role as antioxidant. *Acta Vitaminol Enzymol* **5,** 11-22.

Fowkes FG, Housley E, Riemersma RA, Macintyre CC, Cawood EH, Prescott RJ & Ruckley CV. (1992) Smoking, lipids, glucose intolerance, and blood pressure as risk factors for peripheral atherosclerosis compared with ischemic heart disease in the Edinburgh Artery Study. *Am J Epidemiol* **135,** 331-340.

Frankel EN, Kanner J, German JB, Parks E & Kinsella JE. (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. *Lancet* **341,** 454-457.

Gans RO, Bilo HJ, Weersink EG, Rauwerda JA, Fonk T, Popp-Snijders C & Donker AJ. (1990) Fish oil supplementation in patients with stable claudication. *Am J Surg* 160, 490-495.

Gokce N, Keaney JF, Hunter LM, *et al.* (2003) Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. *J Am Coll Cardiol* **41**, 1769–1775

Gorinstein S, Zemser M, Berliner M, Goldstein R, Libman I, Trakhtenberg S & Caspi A. (1997) Moderate beer consumption and positive biochemical changes in patients with coronary atherosclerosis. *J Intern Med* **242,** 219-224.

Graham IM, Daly LE, Refsum HM, *et al.* (1997) Plasma homocysteine as a risk factor for vascular disease. The European Concerted Action Project. *JAMA* **277,** 1775-1781.

Haeger K. (1974) Long-time treatment of intermittent claudication with vitamin E. *Am J Clin Nutr* **27,** 1179–1181.

Halperin JL. (2002) Evaluation of patients with peripheral vascular disease. *Thrombosis Research* **106,** 303-311.

Hamilton M, Wilson GM, Armitage P & Boyd JT. (1953) The treatment of intermittent claudication with vitamin E. *Lancet* **I,** 367-370.

Henke PK, Blackburn S, Proctor MC, Stevens J, Mukherjee D, Rajagopalin S, Upchurch GR Jr, Stanley JC & Eagle KA. (2004) Patients undergoing infrainguinal bypass to treat atherosclerotic vascular disease are underprescribed cardioprotective medications: effect on graft patency, limb salvage, and mortality. *J Vasc Surg* **39,** 357-365.

Hertzer NR. (1991) The natural history of peripheral vascular disease: implications for its management. *Circulation* **83,** 12-19.

Hiatt WR, Hoag S & Hamman RF. (1995) Effect of diagnostic criteria on the prevalence of peripheral arterial disease: the San Luis Valley Diabetes Study. *Circulation* **91,** 1472-1479.

Hiatt WR. (1994) Medical treatment of the patient with intermittent claudication. *J Vasc Tech* **18,** 311-315

Hiatt WR. (2001) Medical treatment of peripheral arterial disease and claudication. *N Engl J Med* **344,** 1608-1621.

Hirsch AT, Hiatt WR; PARTNERS Steering Committee. (2001) PAD awareness, risk, and treatment: new resources for survival--the USA PARTNERS program. *Vasc Med* **6,** 9-12.

HLTC. (1998) Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. Homocysteine Lowering Trialists' Collaboration. *BMJ* **316,** 894-898.

Ho M, Maple C, Bancroft A, McLaren M & Belch JJ. (1999) The beneficial effects of omega-3 and omega-6 essential fatty acid supplementation on red blood cell rheology. *Prostaglandins Leukot Essent Fatty Acids* **61,** 13-17.

Hooper L, Griffiths E, Abrahams B, *et al.* (2004) Dietetic guidelines: diet in secondary prevention of cardiovascular disease (first update, June 2003). *J Hum Nutr Diet* **17,** 337-349.

Hunninghake DB, Miller VT, LaRosa JC, Kinosian B, Brown V, Howard WJ, DiSerio FJ & O'Connor RR. (1994) Hypocholesterolemic effects of a dietary fiber supplement. *Am J Clin Nutr* **59,** 1050-1054.

Jenkins DJ & Jenkins AL. (1985) Dietary fiber and the glycemic response. *Proc Soc Exp Biol Med* **180,** 422-431.

Jenkins DJ, Axelsen M, Kendall CW, Augustin LS, Vuksan V & Smith U. (2000) Dietary fibre, lente carbohydrates and the insulin-resistant diseases. *Br J Nutr* **83,** 157-163.

Jepson RG, Fowkes FG, Donnan PT & Housley E. (1995) Alcohol intake as a risk factor for peripheral arterial disease in the general population in the Edinburgh Artery Study. *Eur J Epidemiol* **11**, 9-14.

Jepson RG, Kleijnen J & Leng GC. (2004) Garlic for peripheral arterial occlusive disease. The Cochrane library, **Issue 3,** Chichester, UK; John Wiley & Sons, Ltd.

Kanayasu-Toyoda T, Morita I & Murota S. (1996) Docosapentaenoic acid (22:5, n-3), an elongation metabolite of eicosapentaenoic acid (20:5, n-3), is a potent stimulator of endothelial cell migration on pretreatment in vitro. *Prostaglandins Leukot Essent Fatty Acids* **54,** 319-325.

Kanayasu-Toyoda T, Morita I, Hibino H, Nakao-Hayashi J, Ito H & Murota S. (1993) Eicosapentaenoic acid abolishes the proatherogenic effects of cholesterol: effects on migration of bovine smooth muscle and endothelial cells in vitro. *Prostaglandins Leukot Essent Fatty Acids* **48,** 463-468.

Karlic H & Lohninger A. (2004) Supplementation of L-carnitine in athletes: does it make sense? *Nutrition* **20,** 709-715.

Katsouyanni K, Skalkidis Y, Petridou E, Polychronopoulou-Trichopoulou A, Willett W & Trichopoulos D. (1991) Diet and peripheral arterial occlusive disease: the role of poly-, mono-, and saturated fatty acids. *Am J Epidemiol* **133,** 24-31.

Kearns SR, Daly AF, Sheehan K, Murray P, Kelly C & Bouchier-Hayes D. (2004) Oral vitamin C reduces the injury to skeletal muscle caused by compartment syndrome. *J Bone Joint Surg Br* **86,** 906-911.

Khaira HS, Maxwell SR & Shearman CP. (1995) Antioxidant consumption during exercise in intermittent claudication.*Br J Surg* **82,** 1660-1662.

Khaira HS, Maxwell SR, Thomason H, Thorpe GH, Green MA & Shearman CP. (1996) Antioxidant depletion during aortic aneurysm repair. *Br J Surg* **83,** 401-403.

Kiesewetter H, Jung F, Jung EM, *et al.* (1993) Effects of garlic coated tablets in peripheral arterial occlusive disease. *Clin Investig* **71,** 383–336.

Kinlay S, Behrendt D, Fang JC, *et al.* (2004) Long-term effect of combined vitamins E and C on coronary and peripheral endothelial function. *J Am Coll Cardiol* **43,** 629-634.

Kleijnen J & Knipschild P. (1992) Ginkgo biloba. *Lancet* **340,** 1136-1139.

Kleijnen J & Mackerras D. (2004) Vitamin E for intermittent claudication (Cochrane review). In: The Cochrane library, **Issue 3**, Chichester, UK; John Wiley & Sons, Ltd.

Klipstein-Grobusch K, den Breeijen JH, Grobbee DE, Boeing H, Hofman A & Witteman JC. (2001) Dietary antioxidants and peripheral arterial disease : the Rotterdam Study. *Am J Epidemiol* **154,** 145-149.

Koga T, Claycombe K & Meydani M. (2002) Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. *Atherosclerosis* **161,** 365-374.

Kuan YM, E Dear A & Grigg MJ. (2002) Homocysteine: an aetiological contributor to peripheral vascular arterial disease. *ANZ J Surg* **72,** 668-671.

Langlois M, Duprez D, Delanghe J, De Buyzere M & Clement DL. (2001) Serum vitamin C concentration is low in peripheral arterial disease and is associated with inflammation and severity of atherosclerosis. *Circulation* **103,** 1863-1868.

Leng GC & Fowkes FGR. (1993) The epidemiology of peripheral arterial disease. *Vasc Med Rev* **4,** 5-18.

Leng GC, Fowkes FG, Lee AJ, Dunbar J, Housley E & Ruckley CV. (1996) Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. *BMJ* **313,** 1440-1444.

Leng GC, Lee AJ, Fowkes FG, Jepson RG, Lowe GD, Skinner ER & Mowat BF. (1998) Randomized controlled trial of gamma-linolenic acid and eicosapentaenoic acid in peripheral arterial disease. *Clin Nutr* **17,** 265-271.

Lentz SR & Sadler JE. (1991) Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. *J Clin Invest* **88,** 1906-1914.

Livingstone PD & Jones C. (1958) Treatment of intermittent claudication with vitamin E. *Lancet* **ii,** 602–664

Lowe GD, Fowkes FG, Dawes J, Donnan PT, Lennie SE & Housley E. (1992) Blood viscosity, fibrinogen, and activation of coagulation and leukocytes in peripheral arterial disease and the normal population in the Edinburgh Artery Study. *Circulation* **87,** 1915–1920

Marckmann P, Sandstrom B & Jespersen J. (1993) Dietary effects on circadian fluctuation in human blood coagulation factor VII and fibrinolysis. *Atherosclerosis* **101,** 225-234.

Maxwell AJ, Anderson BE & Cooke JP. (2000) Nutritional therapy for peripheral arterial disease: a double-blind, placebo-controlled, randomized trial of HeartBar. *Vasc Med* **5,** 11-19.

McDermott MM, Mehta S, Ahn H & Greenland P. (1997) Atherosclerotic risk factors are less intensively treated in patients with peripheral arterial disease than in patients with coronary artery disease. *J Gen Intern Med* **12,** 209-215.

Mennen LI, Balkau B, Vol S, Caces E & Eschwege E. (1999) Fibrinogen: a possible link between alcohol consumption and cardiovascular disease? DESIR Study Group. *Arterioscler Thromb Vasc Biol* **19,** 887-892.

Merchant AT, Hu FB, Spiegelman D, Willett WC, Rimm EB & Ascherio A. (2003a) The use of B vitamin supplements and peripheral arterial disease risk in men are inversely related. *J Nutr*. 133, 2863-2867.

Merchant AT, Hu FB, Spiegelman D, Willett WC, Rimm EB & Ascherio A. (2003b) Dietary fiber reduces peripheral arterial disease risk in men. *J Nutr* **133,** 3658-3663.

Meydani M, Natiello F, Goldin B *et al.* (1991) Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *J Nutr* **121,** 484–491.

Mori TA, Vandongen R, Mahanian F & Douglas A. (1992) Plasma lipid levels and platelet and neutrophil function in patients with vascular disease following fish oil and olive oil supplementation. *Metabolism* **41,** 1059-1067

Morrissey PA & Sheehy PJ. 1999 Optimal nutrition: vitamin E. *Proc Nutr Soc* **58,** 459-468.

Mujumdar VS, Aru GM & Tyagi SC. (2001) Induction of oxidative stress by homocyst(e)ine impairs endothelial function. *J Cell Biochem* **82,** 491-500.

Mukherjee D, Lingam P, Chetcuti S, *et al.* (2002) Missed opportunities to treat atherosclerosis in patients undergoing peripheral vascular interventions: insights from the University of Michigan Peripheral Vascular Disease Quality Improvement Initiative (PVD-QI2). *Circulation* **106,** 1909-1912.

Muluk SC, Muluk VS, Kelley ME, *et al.* (2001) Outcome events in patients with claudication: a 15-years study in 2777 patients. *J Vasc Surg* **33,** 251-257.

Nappo F, De Rosa N, Marfella R, De Lucia D, Ingrosso D, Perna AF, Farzati B & Giugliano D. (1999) Impairment of endothelial functions by acute hyperhomocysteinemia and reversal by antioxidant vitamins. *JAMA* **281,** 2113-2118.

Peters H, Kieser M & Hölscher U. (1998) Demonstration of the efficacy of Ginkgo biloba special extract EGb 761® on intermittent claudication—a placebo-controlled, double-blind multicenter trial. *VASA* **27,** 106–110.

Philipp CS, Cisar LA, Kim HC, Wilson AC, Saidi P & Kostis JB. (1997) Association of hemostatic factors with peripheral vascular disease. *Am Heart J* **134,** 978–984

Piesse JW. (1984) Vitamin E and peripheral vascular disease. *Int Clin Nutr Rev* **4,** 178–182

Pittler MH & Ernst E. (2000) Ginkgo biloba extract for the treatment of intermittent claudication: a meta-analysis of randomized trials. *Am J Med* **108,** 276-281.

Podmore ID, Griffiths HR, Herbert KE, Mistry N, Mistry P & Lunec J. (1998) Vitamin C exhibits pro-oxidant properties. *Nature* **392,** 559.

Poredos P, Golob M & Jensterle M. (2003) Interrelationship between peripheral arterial occlusive disease, carotid atherosclerosis and flow mediated dilation of the brachial artery. *Int Angiol* **22,** 83-87.

Ramirez-Tortosa C, Lopez-Pedrosa JM, Suarez A, Ros E, Mataix J & Gil A. (1999) Olive oil- and fish oil-enriched diets modify plasma lipids and susceptibility of LDL to oxidative modification in free-living male patients with peripheral vascular disease: the Spanish Nutrition Study. *Br J Nutr* **82,** 31-39

Refsum H, Ueland PM, Nygard O & Vollset SE. (1998) Homocysteine and cardiovascular disease. *Annu Rev Med* **49,** 31-62.

Reichl D & Miller NE. (1989) Pathophysiology of reverse cholesterol transport. Insights from inherited disorders of lipoprotein metabolism. *Arteriosclerosis* **9,** 785-797.

Renaud SC & Ruf JC. (1996) Effects of alcohol on platelet functions. *Clin Chim Acta* **246,** 77-89.

Riba R, Nicolaou A, Troxler M, Homer-Vaniasinkam S & Naseem KM. (2004) Altered platelet reactivity in peripheral vascular disease complicated with elevated plasma homocysteine levels. *Atherosclerosis* **175,** 69-75.

Ridker PM, Vaughan DE, Stampfer MJ, Glynn RJ & Hennekens CH. (1994) Association of moderate alcohol consumption and plasma concentration of endogenous tissue-type plasminogen activator. *JAMA* **272,** 929-933.

Rodgers GM & Kane WH. (1986) Activation of endogenous factor V by homocysteine-induce vascular endothelial cell activator. *J Clin Invest* **11,** 1909–1916

Ross R. (1999) Related Atherosclerosis-an inflammatory disease. *N Engl J Med* **340,** 115-126.

Rossi E, Biasucci LM, Citterio F, *et al.* (2002) Risk of myocardial infarction and angina in patients with severe peripheral vascular disease: predictive role of C-reactive protein. *Circulation* **105,** 800-803.

Ruiz-Gutierrez V, Muriana FJ, Guerrero A, Cert AM & Villar J. (1996) Plasma lipids, erythrocyte membrane lipids and blood pressure of hypertensive women after ingestion of dietary oleic acid from two different sources. *J Hypertens* **14,** 1483-1490.

Schneider B. (1992) Ginkgo biloba extract in peripheral arterial disease. Meta-analysis of controlled clinical trials. *Arzneimittelforschung* **42,** 428–436.

Schweizer J & Hautmann C. (1999) Comparison of two dosages of Ginkgo biloba extract EGb 761 in patients with peripheral arterial occlusive disease Fontaine's stage IIb. *Arzneimittelforschung* **49,** 900–904.

Shute EV, Vogelsang Ab, Skelton FR & Shute WE. (1948) The influence of vitamin E on vascular disease. *Surg Gynaecol Obst* **86,** 1-8.

Signorelli SS, Mazzarino MC, *et al.* (2003) High circulating levels of cytokines (IL-6 and TNFalpha), adhesion molecules (VCAM-1 and ICAM-1) and selectins in patients with peripheral arterial disease at rest and after a treadmill test. *Vasc Med* **8,** 15-19

Silverman MD, Tumuluri RJ, Davis M, Lopez G, Rosenbaum JT & Lelkes PI. (2002) Homocysteine upregulates vascular cell adhesion molecule-1 expression in cultured human aortic endothelial cells and enhances monocyte adhesion. *Arterioscler Thromb Vasc Biol* **22,** 587-592.

Silvestro A, Scopacasa F, Oliva G, de Cristofaro T, Iuliano L & Brevetti G. (2002) Vitamin C prevents endothelial dysfunction induced by acute exercise in patients with intermittent claudication. *Atherosclerosis* **165,** 277-83.

Simchon S, Jan KM & Chien S. (1987) Influence of reduced red cell deformability on regional blood flow. *Am J Physiol* **253,** 898-903

Smit AJ. (1992) Medical treatment of peripheral vascular disease: are there new perspectives?. *Vasa Supplemental* **34,** 20-24

Sommerfield T & Hiatt WR. (2004) Omega-3 fatty acids for intermittent claudication (Cochrane review). In: The Cochrane libray, **Issue 3,** Chichester, UK: John Wiley & Sons, Ltd

Spark JI, Robinson JM, Gallavin L, Gough MJ, Homer-Vanniasinkam S, Kester RC & Scott DJ. (2002) Patients with chronic critical limb ischemia have reduced total antioxidant capacity and impaired nutritional status. *Eur J Vasc Endovasc Surg* **24,** 535-539.

Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D & Loscalzo J. (1993) Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. *J Clin Invest* **91,** 308-318.

Stark AH & Madar Z. (2002) Olive oil as a functional food: epidemiology and nutritional approaches. *Nutr Rev* **60,** 170-176.

Steinberg D. (1991) Antioxidants and atherosclerosis: a current assessment. *Circulation* **84,** 1420–1425.

Steiner M & Mower R. (1982) Mechanism of action of vitamin E on platelet function. *Ann N Y Acad Sci* **393,** 289-299.

Stringer MD, Gorog PG, Freeman A & Kakkar VV.(1989) Lipid peroxides and atherosclerosis. *BMJ* **298,** 281-284.

Tabet S, Berg AO, for the US Preventive Services task Force. (1996) Screening for peripheral arterial disease. In: Guide to clinical preventive services: report of the US preventive Services Task Force. Baltimore: Williams & Wilkins.

TASC (2000) Trans Atlantic Inter-Society Consensus on the management of peripheral arterial disease. *Eur J Vasc Endovasc Surg* **19,** 1-244.

Taylor LM Jr, DeFrang RD, Harris EJ Jr, & Porter JM. (1991) The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. *J Vasc Surg* **13,** 128-136.

Taylor LM Jr, Moneta GL, Sexton GJ, Schuff RA & Porter JM. (1999) Prospective blinded study of the relationship between plasma homocysteine and progression of symptomatic peripheral arterial disease. *J Vasc Surg* **29,** 8-21.

Thies F, Garry JM, Yaqoob P, *et al.* (2003) Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. *Lancet* **361,** 477-485.

Thorburn A, Muir J & Proietto J. (1993) Carbohydrate fermentation decreases hepatic glucose output in healthy subjects. *Metabolism* **42,** 780-785.

Tornwall ME, Virtamo J, Haukka JK, Aro A, Albanes D & Huttunen JK. (2000) Prospective study of diet, lifestyle, and intermittent claudication in male smokers. *Am J Epidemiol* **151,** 892-901.

Tsai MY, Arnett DK, Eckfeldt JH, Williams RR & Ellison RC. (2000) Plasma homocysteine and its association with carotid intimal-medial wall thickness and prevalent coronary heart disease: NHLBI Family Heart Study. *Atherosclerosis* **151,** 519-524.

van den Berg M, Franken DG, Boers GH, *et al.* (1994) Combined vitamin B6 plus folic acid in young patients with atherosclerosis and hyperhomocysteinaemia. *J Vasc Surg* **20,** 933-940.

van Tol A, van der Gaag MS, Scheek LM, van Gent T & Hendriks HF. (1998) Changes in postprandial lipoproteins of low and high density caused by moderate alcohol consumption with dinner. *Atherosclerosis* **141,** 101-103.

Verhoef P, Stampfer MJ, Buring JE, *et al.* (1996) Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B6, B12, and folate. *Am J Epidemiol* **143,** 845-859.

Vicaut E. (1995) Opposite effects of red blood cell aggregation on resistance to blood flow. *J Cardiovasc Surg* **36,** 361-368.

Violi F, Criqui M, Longoni A & Castiglioni C. (1996) Relation between risk factors and cardiovascular complications in patients with peripheral arterial disease: results from the A.D.E.P. study. *Atherosclerosis* **120,** 25–35

Vogt MT, Wolfson SK & Kuller LH. (1993) Segmental arterial disease in the lower extremities: correlates of disease and relationship to mortality. *J Clin Epidemiol* **46,** 1267-1276.

Wald DS, Law M & Morris JK. (2002) Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. *BMJ* **325,** 1202-1206

Wall RT, Harlan JM, Harker LA & Striker GE. (1980) Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. *Thromb Res,* **18,** 113-121.

Wang J, Dudman NP & Wilcken DE. (1993) Effect of homocysteine and related compounds on prostacyclin production by cultured human vascular endothelial cells. *Thromb. Haemost* **70,** 1047–1052.

Weiss N, Keller C, Hoffmann U & Loscalzo J. (2002) Endothelial dysfunction and atherothrombosis in mild hyperhomocysteinemia. *Vasc Med* **7,** 227-239.

Welch GN & Loscalzo J. (1998) Homocysteine and atherothrombosis. *N Engl J Med* **338,** 1042-1050.

Westheim AS, Brox D & Selvaag OAW. (1975) D-alpha-tokoferol ved claudication intermittens. *T Norske laegeforen* **95,** 13-15.

WHO Study Group. (2003) "Diet, nutrition and prevention of chronic diseases" Technical Report Series; No. 916

Wilmink AB, Welch AA, Quick CR, *et al.* (2004) Dietary folate and vitamin B6 are independent predictors of peripheral arterial occlusive disease. *J Vasc Surg* **39,** 513-516.

Williams HT, Fenna D & Macbeth RA. (1971) Alpha tocopherol in the treatment of intermittent claudication. *Surg Gynecol Obstet*, 662–666.

Woodcock BE, Smith E, Lambert WH, Jones WM, Galloway JH, Greaves M & Preston FE. (1984) Beneficial effect of fish oil on blood viscosity in peripheral vascular disease. *Br Med J (Clin Res Ed)* **288,** 592-594

Yataco AR, Corretti MC, Gardner AW, *et al.* (1999) Endothelial reactivity and cardiac risk factors in older patients with peripheral arterial disease. *Am J Cardiol* **83,** 754–758.

7

Daily supplementation with (n-3) PUFAs, oleic acid, folic acid and vitamins B-6 and E increases pain-free walking distance and improves risk factors in men with peripheral vascular disease.

Journal of Nutrition (In Press).

JJ Carrero[1], E López-Huertas[2], LM Salmerón[3], L Baró[4], and E Ros[3].

1. Department of Biochemistry and Molecular Biology, University of Granada, Spain
2. PULEVA BIOTECH S.A. Granada, Spain
3. Service of Angiology and Vascular Surgery, University "San Cecilio" Hospital. Granada, Spain.
4. Distrito Sanitario Costa del Sol, Servicio Andaluz de Salud, Málaga, Spain.

Resumen

Ciertos nutrientes han demostrado ser efectivos en la prevención de las enfermedades cardiovasculares. Investigamos si la ingesta diaria de pequeñas cantidades de estos nutrientes ejercen un efecto positivo sobre los factores de riesgo y los parámetros clínicos de pacientes con enfermedad vascular periférica y que presentan claudicación intermitente. (PVD-IC).

60 hombres PVD-IC fueron distribuidos de manera aleatoria en 2 grupos: El Grupo S ingirió 500 mL/día de una leche enriquecida en ácido eicosapentanoico (EPA), ácido docosahexanoico (DHA), ácido oleico, ácido fólico y vitaminas A, D, E, B6. El Grupo C consumió 500 mL/día de leche semidesnatada. Los pacientes recibieron recomendaciones acerca de sus hábitos dietéticos y estilo de vida, y debían ingerir el producto además de su dieta habitual. La extracción de sangre y exploraciones clínicas se realizaron tras 0, 6 y 12 meses.

Tras la intervención, hubo un aumento de las concentraciones plasmáticos de los nutrientes enriquecidos ($p<0.05$). El colesterol total y la concentración de ApoB disminuyo en el grupo S. La homocisteína total disminuyo en aquellos pacientes con valores elevados. El grupo S aumentó su distancia de claudicación ($p<0.001$) y su índice brazo-tobillo.

La inclusión en la dieta de ciertos nutrientes con un efecto beneficioso conocido en las enfermedades cardiovasculares produjo, en un grupo de pacientes PVD-IC, una mejora significativa en los parámetros clínicos mientras que redujo una serie de factores de riesgo. De esta manera, el presente trabajo sugiere nuevas evidencias acerca del papel de los nutrientes en la reducción de los síntomas de PVD-IC.

Summary

A number of nutrients have been shown to be effective in the prevention of cardiovascular (CV) disease. We investigated if a daily intake of low amounts of these nutrients would exert positive effects on risk factors and clinical parameters of peripheral vascular disease patients suffering from intermittent claudication (PVD-IC).

60 male PVD-IC patients were randomly allocated into 2 groups: the supplemented (S) group was provided with 500 mL/d of a fortified dairy product containing eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid, folic acid and vitamins A, D, E and B-6. The control (C) group consumed 500 mL/d of semi-skimmed milk added with vitamins A and D. The patients received lifestyle and dietary recommendations and they were instructed to consume the products in addition to their diet. Blood extractions and clinical explorations were performed after 0, 3, 6, 9 and 12 months.

The plasma concentration of EPA, DHA, oleic acid, folic acid, Vitamins B-6 and E increased after supplementation ($p<0.05$). Plasma total cholesterol and ApoB concentrations decreased in the S group, and total homocysteine was decreased in those patients with high initial concentration. The S group increased their walking distance before the onset of claudication ($p<0.001$) and their ankle-brachial pressure index value ($p<0.05$).

The inclusion of certain nutrients known to promote CV health in the everyday diet of a group of PVD-IC men, improved clinical outcomes while reducing a variety of risk factors, bringing new evidence of the potential role of nutrition in the reduction of PVD-IC symptoms.

Introduction

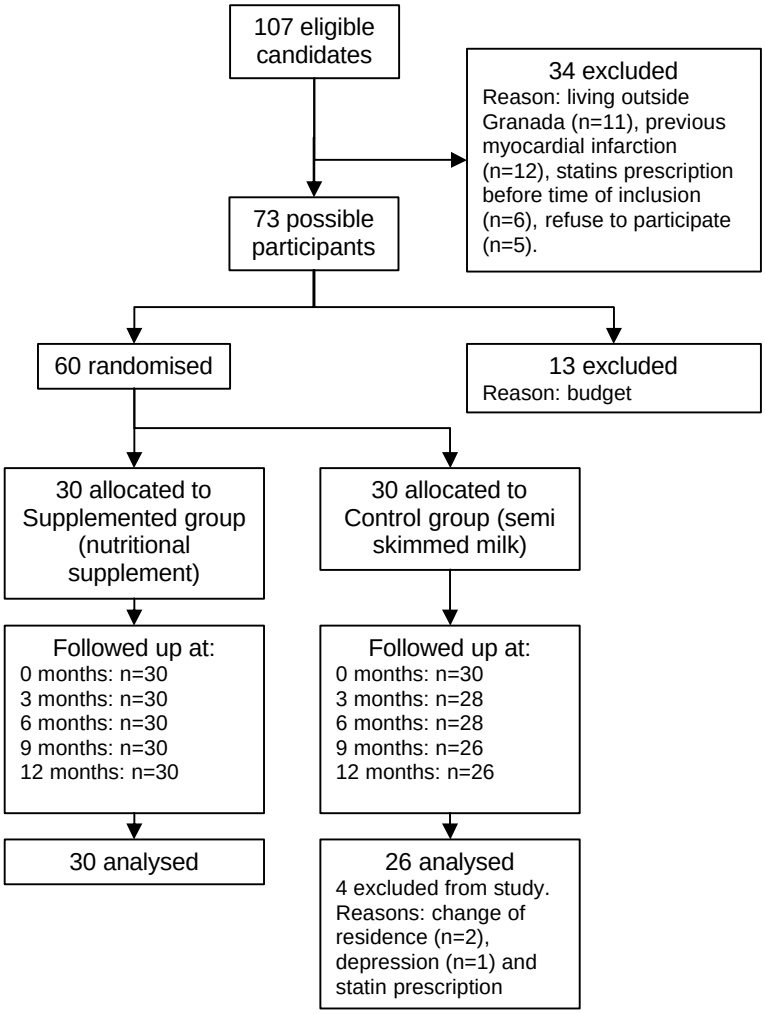
Peripheral vascular disease (PVD) is a manifestation of systemic atherosclerosis caused by the occlusion of the arteries to the legs. Patients with PVD may be asymptomatic or suffer from intermittent claudication (IC), rest pain, and/or gangrene (1), and have a 3 to 5 fold increased risk of cardiovascular mortality (2,3). IC is the most common symptom, present in approximately 40% of patients with PVD (4). Ischemia of the calf muscles cause exercise-induced lower leg discomfort which classically resolves with rest, and is associated with a diminished ability to perform daily activities. The degree of functional impairment of PVD patients is established according to the distance that the patients can walk without pain, or pain-free walking distance (PFWD). The treatment of this condition focuses on decreasing functional impairment caused by symptoms (5), and treating the underlying systemic atherosclerosis by targeting risk factors (6). The major risk factors for PVD are age (over 40 years), cigarette smoking, and diabetes. Hyperlipidemia, hypertension and hyperhomocysteinemia are also important PVD (7) and cardiovascular disease (CVD) risk factors. In fact, there is a clear association between IC and the risk of developing CVD, which can be detected in as many as 90% of IC patients (8). However, several studies have shown how risk factors for this disease are often overlooked (9,10), and suggest that guidelines for the specific management of these patients are needed together with strategies to ensure their implementation.

Diet has proved to play a major role in the prevention of CVD, and there is a wealth of evidence regarding the benefits produced by changing lifestyle habits, dietary patterns and nutrients in CVD prevention. International Health Societies have established nutritional guidelines in this sense. The latest WHO report (11), recommends regular fish consumption to provide about 200-500 mg of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) per week, a replacement of saturated fat by monounsaturated fat (oleic acid), and an increase in the consumption of fruit and vegetables in order to achieve proper antioxidant and folate status.

Earlier intervention studies have reported discrete benefits on clinical outcomes and risk factors after dietary supplementation with (n-3) polyunsaturated fatty acids (PUFAs; 12), olive oil (13), sunflower oil (14), vitamin E (15), folic acid or vitamin B-6 (16,17) in IC patients. However, results from these studies have not been developed enough to provide specific dietary guidelines for PVD patients (18).

In this study we carry out a longitudinal, controlled, randomized and double blind 12-month intervention in which we supplement the diet of PVD patients with a dairy product containing low amounts of EPA, DHA, oleic acid, folic acid and vitamins A, D, E and B-6. We have studied the effects of this supplement on cardiovascular risk factors and clinical outcomes.

Figure 1. Flow of participants in the study.



Materials and Methods

Subjects recruitment.

Patient's recruitment was conducted at the Service of Angiology and Vascular Surgery at the University "S. Cecilio" Hospital, Granada (Spain). All male patients diagnosed with PVD (Ankle-Brachial Index (ABI) at least <0.70, mild disease) and presenting with IC (Fontaine stage IIb, claudication distance <200 m) where candidates for inclusion in the study. They were not admitted to the study if any of the following criteria were present: (1) eligible for revascularization surgery, (2) presenting with endocrine or metabolic disturbances (such as hypothyroidism or obesity), (3) affected by cardiac episodes (such as angina pectoris) or previous acute myocardial infarction (both confirmed from personal interview and with hospital records) and (4) residence outside the city of Granada.

Study protocol and diets.

We carried out a longitudinal, randomized, controlled, double-blind intervention study, that aimed to investigate the effects of a nutritional supplement in PVD-IC patients..

From May 2003 to July 2003, a total of 107 possible candidates were recruited. 34 of them did not fulfill the inclusion criteria because of: living outside the metropolitan area of Granada (n=11), previous history of myocardial infarction (n=12), statin prescription already before time of inclusion (n=6), no willingness to participate (n=5). The remaining eligible candidates were randomly assigned to two intervention groups by using a table of random numbers till completion of 30 patients per group (**Figure 1**). Informed written consent was obtained from the patients of the study. The protocol was approved by the Ethical Committee of "S. Cecilio" University Hospital and conducted according to the Helsinki Declaration.

The study lasted for 12 months and was performed during August 2003-September 2004. All patients were prescribed with a baseline treatment consisting of an antiplatelet (Triflusal) and a hemorrheologic agent (Pentoxifylline).

The supplemented group (S, n = 30) was provided with 500 mL/d of a fortified dairy product (Puleva Omega3©, Puleva Food S.L. Granada, Spain) containing the following nutrients: EPA, DHA, oleic acid, folic acid and vitamins A, D, E and B-6. The dairy supplement was prepared by adding a mixture of fish and vegetable oils to skimmed milk, resulting in a product containing a total fat content comparable to that contained in standard semi-skimmed milk (1.9 g/100 mL), but with a different fatty acid profile. Vitamins A, D, E, B-6 and folic acid were also added to the final product. The control group (C, n = 30) was provided with 500 mL/d of regular semi-skimmed milk added with vitamins A and D (**Table 1**). The dairy products were produced and packaged in white 500 mL Tetra Bricks by Puleva Biotech S.A., so that neither the patients nor the researchers would know what was consumed. The patients were

instructed to consume the dairy products in 2x250 mL doses at the beginning and at the end of the day. The dairy products were home-delivered to the patients monthly and compliance with their consumption during the intervention period was assured and checked upon by regular telephone calls and collection of the containers consumed.

Table 1. Nutritional composition of the dairy products used in the study

	Semi-skimmed milk	Enriched milk
Energy, kJ/L	19,5	21.8
Protein, g/L	31	35
Carbohydrates, g/L	47	52
Total Fat, g/L	19	19
SFAs, g/100 g total fat	70.5	23.7
MUFAs, g/100 g total fat	27.2	56.8
PUFAs, g/100 g total fat	2.3	19.5
Specific fatty acids	*as g/100 g total fat*	
Oleic acid	21.5	54.4
ALA	U	0.6
EPA	U	1.4
DHA	U	2.1
Calcium, mmol/L	29.9	32.9
Vitamin A (Retinyl acetate), µmol/L	4.19	4.19
Vitamin D-3 (Cholecalciferol), nmol/L	19	19
Vitamin E (a-tocopherol acetate), µmol/L	U	34.8
Vitamin B-6, mol/L	U	0.12
Vitamin B-12, pmol/L	2800	2800
Folic acid, nmol/L	U	680

U, undetected.

At the beginning of the study, the patients and their partners attended a dietary counseling session about general aspects of food composition, food processes, adequate portions, the effects of alcohol consumption and the beneficial effects of the Mediterranean diet. Patients were advised to increase the consumption of fruit, legumes and vegetables to ensure adequate intake of fibre and vitamins. They were recommended not to eat fast food or precooked meals, and were encouraged to exercise daily (a 1-hour walk or walking until onset of pain) and to stop smoking. Dietary intake was assessed at baseline and again at the end of the study with a 7-day self-administered food-frequency questionnaire following instructions from the principal investigator. Spanish food composition tables (19) were used to estimate dietary intake.

Blood extraction and clinical examination.

The patients were interviewed in the hospital at the beginning of the study (T_0) and after 3, 6, 9 and 12 months (T_3, T_6, T_9 and T_{12}). In every visit, a blood sample (30 mL) was collected by venipuncture into EDTA-containing vacutainer tubes after an overnight fast lasting at least 10 hours. Samples were kept on ice before centrifugation at 1700 x g for 15 min at 4°C to obtain

plasma. To ensure analytical consistency, plasma samples from T_0 to T_{12} from the same patient(s) were processed at the same time and analyzed in one batch.

A complete clinical and vascular exploration was also given to the patients, including an anamnesis. PFWD was measured using a treadmill set at 3 km/h speed and 10% slope and is expressed as the mean of two consecutive tests performed before and after ABI calculation (approximately 40 minutes of interval between each test). In order to calculate the ABI, an air-filled plethysmography was placed on the lower limbs to record pulse volume and segmental pressure by continuous Doppler. The ABI, or the ratio of the ankle systolic pressure to the brachial artery systolic pressure, is useful in assessing disease severity. An ABI greater than 0.90 is considered normal, 0.70 to 0.89 is considered mild disease, 0.5 to 0.69 moderate disease, and less than 0.5 severe disease (20).

Biochemical measurements.

The plasma concentration of triacylglycerols (TG), total cholesterol (TC) and HDL cholesterol (HDL-C) were measured at the hospital central laboratory by colorimetry using commercial kits (Biosystems, Barcelona, Spain). Analyses were carried out in triplicates and in one batch, following the ptocolos provided by the manufacturer. LDL cholesterol (LDL-C) was calculated according to the Friedewald formula (21). Plasma fatty acid profile was determined by gas-liquid chromatography (22). Apolipoprotein B (ApoB) was measured using an immuno-turbidimetry test (Olympus Diagnostica, GmbH, Ireland). Plasma concentrations of total homocysteine (tHcy), vitamin E and malondialdehyde (MDA) were quantified by HPLC with fluorescence detection (23,24 and 25 respectively). Vitamin B-6 was also measured by HPLC following instructions from a commercial kit (Immundiagnostik AG, Germany). Plasma and red blood cell (RBC) folate and plasma vitamin B-12 concentrations were measured using immunoassay commercial kits from ICN Pharmaceuticals, USA. Plasminogen Activator Inhibitor 1 (PAI-1), E-selectin, soluble vascular adhesion molecule 1 (sVCAM-1) and soluble intercellular cell adhesion molecule 1 (sICAM-1) were measured by ELISA commercial kits from Biosource International, USA. High sensitivity C-reactive protein concentrations (CRP) were quantified by immuno-nephelometry (Dade Behring, Marburg, Germany). Oxidized LDL in plasma was quantified using an ELISA kit (Mercodia, Sweden). Apo B, CRP and all the vitamins were measured in one batch at Balagué Center Laboratories (Barcelona, Spain).

Statistical analysis

The data were analyzed using SPSS software (version 12.0, Chicago, USA). Data are expressed as means ± SEM. P values <0.05 were considered significant. Normality was assessed by Kolmogorov-Smirnov test. Between-group comparisons at the beginning of the study were assessed by independent *t* test or Mann-Whitney test for the non-gaussian variables. The longitudinal effect of each dairy product within each group at the different time

points of the study was analyzed by one-way repeated measures ANOVA followed by Tukey's HSD *post hoc* test (within-group comparison). Two-way repeated measured ANOVA was used to analyze statistical differences produced by the consumption of each dairy product. For the non-gaussian variables, Wilcoxon and Krustal-Wallis comparisons were performed to assess differences within- and between-groups, respectively. When between-group comparisons showed significant differences, independent *t* test or Mann-Whitney test were applied to asses the time points at which the groups differed. Two-tailed Pearson's bivariate correlations were used to asses the relation among nutrients plasma increase and PFWD improvements.

Results

Baseline characteristics of the patients included in the groups (**Table 2**) did not change at the end of the study. The dairy products used were well accepted and compliance was good. No changes were found in the dietary intake of nutrients, assessed at the beginning and the end of the study (not shown). Four patients in the C group did not complete the study (Figure 1) due to change of residence (n=2), depression (n=1) and statin prescription (n=1).

Table 2. Baseline characteristics of the control (C) and supplemented (S) men included in the study.

	Group S	Group C
Age, y	62.4±1.6	65.6±1.7
BMI, kg/m2	27.8±0.6	28.04±0.8
Smokers, n (%)	13 (43)	14 (47)
Type-II diabetes, n (%)	5 (16)	6 (20)
Hypertension, n (%)	12 (40)	14 (47)

Values are means +/- SEM unless noted otherwise. n=30.

The amounts of oleic acid, DHA and EPA daily supplemented in 500 mL of the enriched product were 5.12 g, 0.13 g, and 0.2 g respectively, whereas the semi-skimmed milk contained only 1.82 g oleic acid per 500 mL and no detectable levels of DHA and EPA While no changes were found in the plasma fatty acid profile of the patients from the C group, consumption of the fortified dairy product significantly increased the plasma percentages of EPA, total PUFAs and the ratio araquidonic acid (AA) to EPA (**Table 3**). Increases of oleic acid and DHA were also found within the S group but were not significant when compared between the groups. Other percentages of plasma fatty acids did not vary (**Supplemental Table 1**).

The patients from the S group increased their PFWD at the end of the study by up to 3.5 times the initial values (**Figure 2**). This increase was and significant from T_3 through to T_{12}. Increases in the PFWD of the subjects were directly associated with the increase in plasma % of EPA (Pearson correlation coefficient r=0.37; p=0.006), RBC folate concentration (r=0.28; p=0.040), but only a trend was observed for the plasma % of oleic acid (r=0.24; p=0.083). The ABI also increased in the S group at T_{12}, while no changes were found in the C group.

Table 3. Relevant plasma fatty acids in the control (C) and supplemented (S) men before (T_0) and after 6 (T_6) and 12 (T_{12}) months of intervention

	Group	T_0	T_6	T_{12}
		g/100 g total fatty acids		
Oleic acid	S	24.37±0.82	25.41±0.91	26.85±0.89***
	C	24.34±0.93	24.20±0.98	25.84±0.84
AA	S	6.03±0.25	5.99±0.27	5.94±0.28
	C	6.35±0.37	6.14±0.29	6.28±0.29
EPA	S	0.74±0.05	0.98±0.08***†	1.22±0.11***††
	C	0.81±0.09	0.70±0.12	0.76±0.09
DHA	S	1.96±0.10	2.18±0.17†	2.39±0.13***††
	C	1.80±0.11	1.86±0.11	1.83±0.12
SFAs	S	35.08±0.45	34.70±0.47	34.04±0.56
	C	35.92±0.50	35.73±0.44	35.34±0.42
MUFAs	S	29.55±0.94	30.26±0.93	30.14±0.88
	C	29.22±0.95	29.42±1.04	30.93±0.86
PUFAs	S	35.66±0.91	35.24±0.83	36.62±0.83*†
	C	34.83±1.26	35.28±1.16	34.20±0.97
Ratio AA/EPA	S	8.95±0.75	6.88±0.59***†	5.90±0.59***††
	C	9.69±1.05	12.64±2.29	10.02±1.24

Values are means +/- SEM. n=30 (S) or n=26 (C). Asterisks indicate different from T_0: *p<0.05, **p<0.01, ***p<0.001. Crosses indicate different from the control group: †p<0.05, ††p<0.01.

Table 4. Plasma lipid data and ApoB plasma concentration in the control (C) and supplemented (S) men before (T_0) and after 3 (T_3), 6 (T_6), 9 (T_9) and 12 (T_{12}) months of intervention.

Parameter	Group	T_0	T_3	T_6	T_9	T_{12}
TC, mmol/L	S	5.43±0.17	5.19±0.13	5.13±0.16	5.07±0.14*	5.15±0.13*
	C	5.42±0.21	5.35±0.20	5.42±0.21	5.40±0.22	5.48±0.23
TC >5.12 mmol/L	S	6.06±0.16	5.50±0.15***	5.45±0.22*	5.34±0.19***†	5.53±0.15***
(1)	C	5.97±0.17	5.87±0.16	5.90±0.25	5.79±0.27	5.80±0.3
LDL-C, mmol/L	S	3.30±0.15	3.12±0.13	3.16±0.14	3.10±0.13	3.21±0.15
	C	3.10±0.16	3.05±0.2	3.17±0.18	3.29±0.17	3.30±0.19
HDL-C, mmol/L	S	1.27±0.05	1.22±0.07	1.17±0.06**	1.14±0.05***	1.17±0.06***
	C	1.30±0.05	1.24±0.06	1.21±0.05***	1.17±0.04***	1.22±0.05*
TG, mmol/L	S	1.81±0.13	1.75±0.11	1.84±0.14	2.07±0.20	1.88±0.17
	C	1.87±0.18	1.90±0.19	1.80±0.19	1.78±0.19	1.93±0.18
ApoB, g/L	S	10.9±4.1	nd	10.3±3.1	nd	10.1±2.8***
	C	10.2±3.5	nd	10.0±3.5	nd	10.1±3.2

Values are means +/- SEM. n=30 (S) or n=26 (C). Asterisks indicate different from T0: *p<0.05, **p<0.01, ***p<0.001. Crosses indicate different from the control group: †p<0.05. nd, not determined.
(1) Selection of men with hypercholesterolemia (TC>5.12 mmol/L; 26) before the intervention. n=18 (S) or n=17 (C).

The plasma TC concentration decreased within the S group at T_9 and T_{12} but no variations on LDL-C or TG concentration were found during the study (Table 4). The HDL-C concentration showed similar decreases in both groups from T_6 to T_{12}. The plasma concentration of ApoB decreased at T_{12} within the S group only. When the patients with high initial TC concentration were investigated (> 5.12 mmol/L; 26), TC decreased from T_3 within the S group.

The amounts of folic acid and vitamin B-6 daily supplemented in 500 mL of enriched product were 150 µg and 1,5 mg respectively. Vitamin B-6, plasma and RBC folate concentration increased in the S group, while no changes were found in the C group (**Table 5**). No changes in the plasma concentration of total homocysteine (tHcy) were found when all the data were considered together. However, when subjects with elevated tHcy values were investigated (>15 µmol/L, 27), tHcy concentration decreased from T_6 to T_{12} in the patients from the S group. The vitamin E supplemented in the dairy product increased the plasma concentration of vitamin E and the ratio Vitamin E/TC.

The plasma concentrations of vitamin B-12, plasma MDA, oxidized LDL, CRP, PAI-1, sICAM-1, sVCAM-1 and E-selectin remained unchanged throughout the study (**Supplemental Tables 2,3**).

Discussion

In the present study we show that the inclusion of certain nutrients (EPA, DHA, oleic acid, folic acid, Vitamins B-6 and E) in the daily diet may improve clinical outcomes and reduce cardiovascular risk factors in PVD-IC patients.

The percentages of the plasma fatty acids varied in response to the dietary fats supplemented: In the S group the levels of oleic acid, DHA and EPA increased by 10%, 64% and 21%, respectively, together with the total PUFAs. The vitamins contained in the supplement also increased serum and RBC folate and vitamins B-6 and E in plasma. Similar increases in plasma have been reported for those fatty acids and vitamins when they were supplemented in a dairy product (28-30), and show good compliance with the intake of the supplement. The way of administration of the nutrients (in an every day used drink) may have contributed to the very good compliance obtained.

Although the prescription of the drugs and the recommendations of lifestyle and a Mediterranean dietary pattern resulted in improvements in PFWD in both groups, the increase found in the S group was outstanding: while the C group increased their mean PFWD by 44 m, the S group increased theirs by 280 m.. The increases in PFWD obtained in the S group were significant already after 3-months consumption of the fortified dairy product, indicating an early response. The correlation of increased PFWD with plasma % of EPA and RBC folate concentration suggest that the supplemented nutrients are likely to be responsible for the improvements obtained at the clinical level. These results agree with the increase in the ABI, suggesting an improvement of the blood flow to the lower limbs in the patients from the S group.

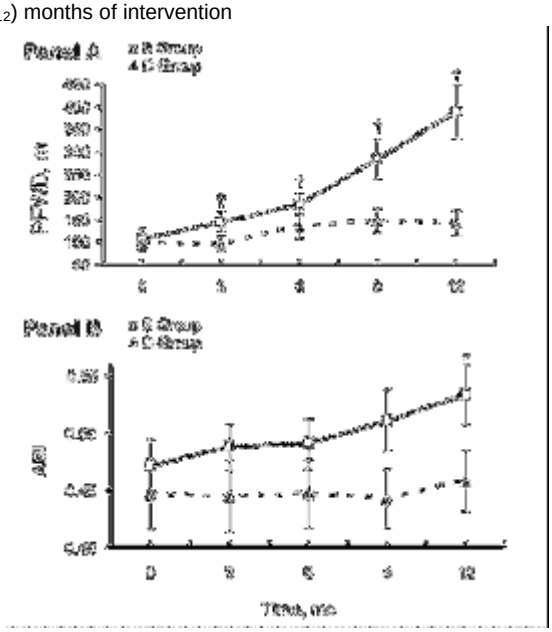
Table 5. Relevant plasma vitamins and total homocysteine concentrations in the control (C) and supplemented (S) men before (T_0) and after 3 (T_3), 6 (T_6), 9 (T_9) and 12 (T_{12}) months of intervention

Parameter	Group	T_0	T_3	T_6	T_9	T_{12}
Serum Folate,	S	10,94±1,58***	24.37±3.74***†	24.35±3.79***††	19.39±1.70***†††	20.98±1.7***†††
nmol/L	C	13.62±3.7	13.71±7.8	12.8±1.4	12.32±1.23	11.51±1.16
RBC Folate, nmol/L	S	1270±139	2033±241***†	2051±235***†	1985±242***††	2058±235***†††
	C	1148±144	1289±170	1359±142	1096±113	1009±89
tHcy, µmol/L	S	12.87±0.61	11.83±0.69*	12.32±0.36	12.76±0.5	12.28±0.38
	C	13.57±0.71	13.17±0.60	14.18±0.64	14.42±0.59	14.00±0.51
tHcy >15 µmol/L (1)	S	17.45±0.41	16.38±1.07	14.83±0.77**†	14.98±1.22*††	14.84±0.79*††
	C	17.27±0.67	16.24±0.72	16.92±0.98	16.76±0.73	16.01±0.69
Vitamin B-6, nmol/L	S	56.84±6.38	nd	79.07±8.26***†††	nd	70.86±7.20**†††
	C	36.49±3.00	nd	39.13±4.78	nd	39.60±3.51
Vitamin E, µmol/L	S	22.52±2.13	nd	25.04±2.07	nd	27.02±2.07 **†
	C	20.28±1.84	nd	22.43±2.25	nd	21.01±1.99
Ratio vitamin E / TC	S	4.12±0.32	nd	4.94±0.40*	nd	5.22±0.28 ***†
	C	3.81±0.84	nd	4.23±0.38	nd	3.94±0.36

Values are means +/- SEM. n=30 (S) or n=26 (C). Asterisks indicate different from T0: *p<0.05, **p<0.01, ***p<0.001. Crosses indicate different from the control group: †p<0.05. nd, not determined.
(1) Selection of men with hyperhomocysteinemia (tHcy>15 µmol/L; 27) before the intervention. n=8 (S) or n=9 (C).

The inflammatory process that occurs within the vessel wall, is now recognized to be a major contributory factor in atherosclerosis, and an anti-inflammatory effect has been described for DHA and EPA (31). In our study, the AA: EPA ratio was reduced and the % of EPA and DHA in plasma increased in the S group. EPA and DHA compete with AA for the insertion at the sn-2 position of membrane phospholipids producing less potent eicosanoids. Protaglandin I3 (PGI_3) formed from EPA in the endothelium is a more active vasodilator and inhibitor of platelet aggregation compared with PGI_2 formed from AA. Therefore, a decreased plasma AA: EPA ratio may be related to vasodilatation and inhibition of platelet aggregation (32). In addition, EPA and DHA have been shown to increase RBC deformability (33) and reduce their aggregation (34), maybe as result of modifying the cell membrane lipid content. Therefore reduced platelet and RBC aggregation can potentially increase blood flow (35). Recent reports have shown that atherosclerotic plaques may quickly incorporate dietary EPA and DHA resulting in increased plaque stability and reduced macrophage infiltration, slowing the progression of the vascular lesion (36) and perhaps the onset of clinical events. These effects are likely to influence the PFWD in the PVD-IC patients of the study. Oleic acid, DHA and EPA have been described to influence endothelial function and the production of endothelial adhesion molecules (31). No effects on these or other markers of inflammation were detected in the S group.

Figure 2. Pain-free walking distance (Panel A) and ankle-brachial index (Panel B) in the control (C) and supplemented (S) men before (T_0) and after 3 (T_3), 6 (T_6), 9 (T_9) and 12 (T_{12}) months of intervention



Values are means +/- SEM. n=30 (S) or n=26 (C). *Different from 0 mo, $p<0.05$; † Different from the control group, $p<0.05$

The average plasma lipid values of the subjects when they entered the study were in the area of borderline-high (25). Though no effect was observed on LDL-C, ApoB clearly decreased in this group. ApoB has been reported to be a better index of CVD risk than LDL, as ApoB is a marker for all the potential atherogenic particles (37). In fact, this reduction in ApoB indicates a reduction in the number of pro-atherogenic small and dense LDL particles that would not have been evidenced by observing only LDL-C. TC decreased in the S group, but major decreases were found in those patients with high initial TC (26). These results suggest that the nutrients supplemented might have contributed to stabilise blood lipids when in the context of a blood-lipid misbalance. Previous studies (29,30) describe a similar lipid-lowering effect in TC and LDL-C, but in contrast to those, the present work does not reveal any effect on the TG concentration. Almost half the patients included in the study were smokers (>15 cigarettes/day) and could explain the ca. 7% reduction in plasma HDL-C found in both study groups (38,39).

We also addressed the question of whether the regular intake of small amounts of PUFAs, together with vitamin E would make plasma and LDL particles more prone to oxidation. In our study, plasma and LDL oxidizability, remained unchanged throughout the intervention period but the S group increased their plasma vitamin E concentration and the ratio vitamin E/TC to values > 5.2 µmol/L. This ratio is regarded as more useful when describing vitamin E status, and is considered optimal when >5.2 µmol/L (40). Increased RBC deformability produced by antioxidant protection of PUFAs at the cell membranes and amelioration of the ischaemia-induced oxidative stress at the lower limbs are possible beneficial effects of vitamin E in PVD-IC patients described in a recent meta-analysis (41).

The intake of the supplement contributed to more than 70% of the European Recommended Dietary Allowances (RDAs) for folic acid and vitamin B-6 (42). The S group changed their plasma folate concentration from suboptimal levels (<15 nmol/L; 43) at T0, to an optimal folate status at T12, as opposed to the C group. A similar response was observed for plasma vitamin B-6 and RBC folate concentration. Folate and B-6 intake are independent predictors of PVD in men aged over 50 years (17), and are the main responsible for lowering hyperhomocysteinemia, which is considered itself an independent risk factor for PVD-IC present in 30% of PVD patients (44). In our study, only the patients from the S group with hyperhomocysteinaemia (>15 µmol/L; 27) were seen to reduce their plasma tHcy (15% decrease). Folate and vitamin B-6 status, together with a reduction in plasma tHcy, are associated with changes in the coagulation response, reduced endothelium-dependent relaxation, reduced NO synthesis and prostacyclin production (45 and references therein) and may influence endothelium-derived hyperpolarizing factor, a major determinant of vascular tone in small resistance vessels (46).

The isolated effects of the nutrients used in the supplement have been previously described in the literature, but no dietary intervention using a combination of them has been reported before. Previous studies with olive oil in IC patients (13,14) only described a protection against LDL oxidation. The intake of (n-3) PUFAs appears to have some beneficial effects in PVD-IC patients, but no clear evidence for improved clinical outcomes is yet reported (12).

Though ATP III recommendations emphasize lifestyle and dietary changes in CVD prevention, recent reports suggest that more attention should be paid to dietary approaches in the management of PVD patients: after a hospital discharge, only 50% of the PVD patients would modify their diet for lipid control (9) and only 18% of the General Practitioners would consider cholesterol lowering therapy to be primary prevention (10).

In this study we report that the inclusion of specific nutrients (EPA, DHA, oleic acid, folic acid and vitamins E and B-6) in the everyday diet of a group of PVD-IC male patients, improved clinical outcomes while reducing a variety of risk factors, bringing new evidence of the potential role of nutrition in the reduction of PVD-IC symptoms.

Acknowledgments

Authors wish to thank M Gómez-Lopera and VE Ramos for their help in data recruitment, C Rodríguez and AD Valero for valuable technical assistance and Dr. JA Maldonado for helpful discussion and advice in statistics analyses..

Supplemental Table 1. Other plasma fatty acids in the control (C) and supplemented (S) men before (T_0) and after 6 (T_6) and 12 (T_{12}) months of intervention

	Group	T_0	T_6	T_{12}
		g/100 g total fatty acids		
C16:0	S	21.68 ± 0.43	21.72 ± 0.37	20.9 ± 0.84
	C	22.72 ± 0.64	22.32 ± 0.49	22.16 ± 0.42
C18:0	S	6.66 ± 0.11	6.49 ± 0.10	6.70 ± 0.13
	C	6.64 ± 0.13	6.74 ± 0.12	6.66 ± 0.10
C18:1 (n-7)	S	1.34 ± 0.03	1.40 ± 0.03	1.37 ± 0.03
	C	1.33 ± 0.03	1.35 ± 0.03	1.36 ± 0.04
C18:2 (n-6)	S	22.33 ± 0.86	22.11 ± 0.78	21.87 ± 0.69
	C	22.49 ± 1.09	23.20 ± 1.06	21.71 ± 0.91
C18:3 (n-3)	S	0.28 ± 0.01	0.29 ± 0.02	0.29 ± 0.02
	C	0.25 ± 0.01	0.27 ± 0.03	0.27 ± 0.01
C20:5 (n-3)	S	0.67 ± 0.07	0.70 ± 0.06	0.69 ± 0.07
	C	0.64 ± 0.06	0.63 ± 0.07	0.64 ± 0.07

Values are means +/- SEM. n=30 (S) or n=26 (C).

Supplemental Table 2. Plasma vitamin B-12 concentration and markers of plasma and LDL oxidizability in the control (C) and supplemented (S) men before (T_0) and after 6 (T_6) and 12 (T_{12}) months of intervention.

	Group	T_0	T_6	T_{12}
Vitamin B-12, pmol/L	S	315±20	279±18	324±38
	C	290±21	264±23	285±21
MDA, µmol/L	S	0.896±0.06	0.920±0.06	0.944±0.06
	C	0.872±0.05	1.081±0.08	0.900±0.08
Oxidized LDL, U/L	S	45883±3041	49841±3913	47878±2846
	C	43716±2217	47803±3975	44861±3547

Values are means +/- SEM. n=30 (S) or n=26 (C).

Supplemental Table 3. Markers of inflammation in the control (C) and supplemented (S) men before (T_0) and after 6 (T_6) and 12 (T_{12}) months of intervention.

	Group	T_0	T_6	T_{12}
CRP, mg/L	S	3,93± 0,62	3.66±0.46	4.21±0.47
	C	3.96±0.91	5.75±1.26*	5.73±0.95**
sVCAM-1, µg/L	S	593±78	620±67	596±59
	C	534±61	565±56	692±81*
sICAM-1, µg/L	S	576±35	585±48	610±44
	C	528±32	509±29	534±34
E-Selectin, µg/L	S	30.6±2.44	29.5±1.96	30.3±2.3
	C	29.0±1.83	30.0±2.99	30.0±2.88
PAI-1, mg/L	S	10.36±1.34	10.8±1.19	10.59±1.40
	C	11.54±1.66	12.87±2.55	12.02±1.99

Values are means +/- SEM. n=30 (S) or n=26 (C). Asterisks indicate different from T_0: *p<0.05, **p<0.01.

References

1. Halperin, J.L. (2002) Evaluation of patients with peripheral vascular disease. Thromb. Res.106:303-311.
2. Criqui, M.H., Langer, R.D., Fronek, A., Feigelson, H.S., Klauber, M.R., McCann, T.J. & Browner, D. (1992) Mortality over a period of 10 years in patients with peripheral arterial disease. N. Engl. J. Med. 326:381-386.
3. Leng, G.C., Fowkes, F.G., Lee, A.J., Dunbar, J., Housley, E. & Ruckley CV. (1996) Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. BMJ. 313:1440-1444.
4. Hirsch, A.T., Hiatt, W.R. & PARTNERS Steering Committee. (2001) PAD awareness, risk, and treatment: new resources for survival--the USA PARTNERS program. Vasc. Med. 6:9-12.
5. Ouriel, K. (2001) Detection of peripheral arterial disease in primary care. JAMA. 286:1380-1381.
6. Regensteiner, J.G. & Hiatt, W.R. (2002) Current medical therapies for patients with peripheral arterial disease: a critical review. Am. J. Med. 112:49-57.
7. Hiatt, W.R. (2001) Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA. 286:1317-1324.
8. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC) Working Group. (2000) J. Vasc. Surg. 31: 5-14, 93-101.

9. Mukherjee, D., Lingam, P., Chetcuti, S., Grossman, P.M., Moscucci, M., Luciano, A.E. & Eagle, K.A. (2002) Missed opportunities to treat atherosclerosis in patients undergoing peripheral vascular interventions: insights from the University of Michigan Peripheral Vascular Disease Quality Improvement Initiative (PVD-QI2). Circulation. 106:1909-1912.
10. Cassar, K., Coull, R., Bachoo, P., Macaulay, E. & Brittenden, J. (2001) Management of secondary risk factors in patients with intermittent claudication. Eur. J. Vasc. Endovasc. Surg. 26:262-266.
11. WHO Study Group. (2003) "Diet, nutrition and prevention of chronic diseases" Technical Report Series, No. 916.
12. Sommerfield, T. & Hiatt, W.R. (2004) Omega-3 fatty acids for intermittent claudication (Cochrane review). In: The Cochrane library, Issue 3, Chichester, UK: John Wiley & Sons, Ltd
13. Ramirez-Tortosa, M.C., Urbano, G., Lopez-Jurado, M., Nestares, T., Gomez, M.C., Mir, A., Ros, E., Mataix, J. & Gil, A. (1999) Extra-virgin olive oil increases the resistance of LDL to oxidation more than refined olive oil in free-living men with peripheral vascular disease. J. Nutr. 129:2177-2183.
14. Aguilera, C.M., Mesa, M.D., Ramirez-Tortosa, M.C., Nestares, M.T., Ros, E. & Gil, A. (2004) Sunflower oil does not protect against LDL oxidation as virgin olive oil does in patients with peripheral vascular disease. Clin. Nutr. 23:673-681.
15. Kleijnen, J. & Mackerras, D. (2004) Vitamin E for intermittent claudication (Cochrane review). In: The Cochrane library, Issue 3, Chichester, UK: John Wiley & Sons, Ltd
16. Robinson, K., Arheart, K., Refsum, H., Brattstrom, L., Boers, G., Ueland, P., Rubba, P., Palma-Reis, R., Meleady, R., Daly, L., Witteman, J. & Graham, I. (1998) Low circulating folate and vitamin B6 concentrations: risk factors for stroke, peripheral vascular disease, and coronary artery disease. European COMAC Group. Circulation. 97:437-443.
17. Wilmink, A.B., Welch, A.A., Quick, C.R., Burns, P.J., Hubbard, C.S., Bradbury, A.W. & Day, N.E. (2004) Dietary folate and vitamin B6 are independent predictors of peripheral arterial occlusive disease. J. Vasc. Surg. 39:513-516.
18. Hooper, L., Griffiths, E., Abrahams, B., et al. (2004) Dietetic guidelines: diet in secondary prevention of cardiovascular disease (first update, June 2003). J. Hum. Nut.r Diet. 17:337-349.
19. Mataix, J., Mañas, M., Llopis, J., Martinez-Victoria, E. (1998) Tabla de composición de alimentos españoles. Universidad de Granada ed., Granada. Spain.
20. Tabet, S. & Berg. A.O., for the US Preventive Services task Force. (1996) Screening for peripheral arterial disease. In: Guide to clinical preventive services: report of the US preventive Services Task Force. Baltimore: Williams & Wilkins.
21. Friedewald W.T., Levy R.I. & Fredrickson D.S. (1972) Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin. Chem. 18:499-502.
22. Lepage, G., Roy, C.C. (1986) Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid. Res. 27:114-120.
23. Araki, A. & Sako, Y. (1987) Determination of free and total homocysteine in human plasma by high-performance liquid chromatography with fluorescence detection. J. Chromatogr. 422: 43-52.
24. Thurnham, D.I., Smith, E. & Flora, P.S. (1998) Concurrent Liquid-Chromatographic assay of retinol, alfa tocopherol, beta-carotene, alfa-carotene, lycopene and beta-cryptoxanthin in plasma with tocopherol acetate as Internal Standard. Clin. Chem. 34: 377-381.

25. Fukunaga, K., Yoshida, M. & Nakazono, N. (1998) A simple, rapid, highly sensitive and reproducible quantification method for plasma malondialdehyde by high-performance liquid chromatography. Biomed. Chromatogr. 12:300-303
26. Third Report of the National Cholesterol Education Programme Expert Panel on "Detection, evaluation and treatment of high blood cholesterol in adults (ATP III)". (2002) National Institutes of Health (NIH), publication number 02-5215.
27. Nygard, O., Nordrehaug, J.E., Refsum, H., Ueland, P.M., Farstad, M. & Vollset, S.E. (1997) Plasma homocysteine levels and mortality in patients with coronary artery disease. N. Engl. J. Med. 337:230-236
28. Visioli, F., Rise, P., Plasmati, E., Pazzucconi, F., Sirtori, C. & Galli, C. (2000) Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerols and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol. Res. 41: 571-576.
29. Baro, L., Fonolla, J., Pena, J.L., Martinez-Ferez, A., Lucena, A., Jimenez, J., Boza, J.J. & Lopez-Huertas, E. (2003) n-3 Fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans. Clin. Nutr. 22:175-182.
30. Carrero, J.J., Baro, L., Fonolla, J., Gonzalez-Santiago, M., Martinez-Ferez, A., Castillo, R., Jimenez, J., Boza, J.J. & Lopez-Huertas, E. (2004) Cardiovascular effects of milk enriched with omega-3 polyunsaturated fatty acids, oleic acid, folic acid, and vitamins E and B6 in volunteers with mild hyperlipidemia. Nutrition. 20:521-527.
31. Calder, P.C. (2004) n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clin. Sci. (Lond). 107:1-11.
32. Singleton, C.B., Walker, B.D. & Cambell, T.J. (2000) N-3 polyunsaturated fatty acids and cardiac mortality. Aust. N.Z. J. Med. 30:246-251.
33. Ernst, E.(1989) Effects of n-3 fatty acids on blood rheology. J. Intern. Med. 225:129-32.
34. Ho, M., Maple, C., Bancroft, A., McLaren, M. & Belch, J.J. (1999) The beneficial effects of omega-3 and omega-6 essential fatty acid supplementation on red blood cell rheology. Prostaglandins Leukot. Essent. Fatty Acids. 61:13-17.
35. Vicaut, E. (1995) Opposite effects of red blood cell aggregation on resistance to blood flow. J. Cardiovasc. Surg. 36:361-368.
36. Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C. & Grimble, R.F. (2003) Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet. 361:477-485.
37. Grundy, S.M. (2002) Low-density lipoprotein, non-high-density lipoprotein, and apolipoprotein B as targets of lipid-lowering therapy. Circulation. 106:2526-2529.
38. Senti, M., Nogues, X., Pedro-Botet, J., Rubies-Prat, J. & Vidal-Barraquer, F. (1992) Lipoprotein profile in men with peripheral vascular disease. Role of intermediate density lipoproteins and apoprotein E phenotypes. Circulation. 85:30-36.
39. Garrison, R.J., Kannel, W.B., Feinleib, M., Castelli, W.P., McNamara, P.M. & Padgett, S.J. (1928) Cigarette smoking and HDL cholesterol: the Framingham offspring study. Atherosclerosis. 30:17-25.
40. Morrissey, P.A & Sheehy, P.J. (1999) Optimal nutrition: vitamin E. Proc. Nutr. Soc. 58:459-468.

41. Kleijnen, J. & Mackerras, D. (2004) Vitamin E for intermittent claudication (Cochrane review), In: The Cochrane Library, Issue 3. Chichester, UK: John Wiley & Sons, Ltd.
42. Bronstrup, A., Hages, M., Prinz-Langenohl, R. & Pietrzik, K. (1998) Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy young women. Am. J. Clin. Nutr. 68:1104-1110
43. Expert group on vitamins and minerals (EVN). (2003) Safe upper levels for vitamins and minerals: report of the expert group on vitamins and minerals. ISBN 1-904026-11-7. Food Standards Agency Publications.
44. Taylor, L.M. Jr., DeFrang, R.D., Harris, E.J. Jr. & Porter, J.M. (1991) The association of elevated plasma homocyst(e)ine with progression of symptomatic peripheral arterial disease. J. Vasc. Surg.13:128-136.
45. Riba, R., Nicolau, A., Troxler, M., Homer-Vaniasinkam, S. & Naseem, K.M. (2004) Altered platelet reactivity in peripheral vascular disease complicated with elevated plasma homocysteine levels. Atherosclerosis. 175:69-75.
46. De Vriese, A.S., Blom, H.J., Heil, S.G., Mortier, S., Kluijtmans, L.A., Van de Voorde, J. & Lameire, N.H. (2004) Endothelium-derived hyperpolarizing factor-mediated renal vasodilatory response is impaired during acute and chronic hyperhomocysteinemia. Circulation.109:2331-2336.

8

Fish oil alters the balance between leukotriene B4 and prostaglandin E2 production by LPS stimulated PBMCs in patients with peripheral vascular disease but not in healthy controls.

Submitted for publication

JJ Carrero[1], J Madden[2], J Hadley[3], N Dastur[3], NT Luu[4], E Rainger[4], G Nash[4], CP Shearman[3], WM Howell[2], PC Calder[2], E Lopez-Huertas[5] and RF Grimble[2].

[1]Department of Biochemistry and Molecular Biology, University of Granada, Spain
[2]School of Medicine, University of Southampton, UK
[3]Department of Surgery, Southampton University of Medical School, UK
[4]Department of Physiology, Medical School, University of Birmingham, UK
[5]PULEVA BIOTECH S.A. Granada, Spain

Resumen

PGE2 y LTB4 son moduladores de la respuesta inflamatoria. El aceite de pescado inhibe la aterosclerosis de manera parcial a través de sus efectos antinflamatorios, pudiendo alterar la producción de estos moduladores mediante la sustitución de n-6 PUFAs por n-3 PUFAs en las membranas celulares. Los pacientes con EVP padecen un proceso inflamatorio crónico. El efecto del aceite de pescado sobre la producción estimulada con LPS de PGE2 y LTB4 en EVP no ha sido objeto de estudio.

30 hombres con PVD y 73 controles sanos ingirieron 6 g/d de aceite de pescado durante 12 semanas. Se les extrajo sangre antes y después de la intervención. Se aisló la fracción de monolitos y cultivó en medio RPMI con 5% de plasma antólogo y 10 µg/ml de LPS durante 24h. Los niveles de IL-6, TNF-a, PGE2 y LTB4 fueron cuantificados por ELISA.

Antes de la intervención, Los monocitos de los pacientes con EVP producían mayores cantidades de IL-6 (11,60±6,56 vs 8,60±4,89 pg/L; pacientes vs controles p=0.027) y relativamente mayores cantidades de LTB4 que de PGE2 (pg/pg) que los monocitos de los controles (0.67±0.70 vs 0.50±1.29 respectivamente; p=0.035). El aceite de pescado redujo este ratio en los pacientes pero no en los controles (p=0.027 pacientes; p=0.325 controles). En los pacientes, el aceite de pescado aumentó la producción de PGE2 (39±4.57 vs 3.99±3.77 ng/ml; p=0.034). Variaciones en la producción de LTB4 pueden también modular la respuesta de las citocinas frente al aceite de pescado. Los sujetos fueron divididos en dos grupos, dependiendo de si el aceite de pescado reducía la producción de IL-6 o no. En aquellos casos en que el aceite de pescado redujo la producción de IL-6, hubo reducciones parejas en la producción de TNF-a y LTB4. En aquellos sujetos en que el aceite de pescado no fue capaz de disminuir la producción de IL-6 se observó un aumento en la producción de LTB4 y una tendencia a aumentar la producción de TNF-a. La “normalización” del ratio de producción de LTB4 frente a PGE” observado en los monocitos cultivados durante un esters inflamatorio, puede ser uno de los mecanismos por los que se disminuye la inflamación en aquellos pacientes con enfermedades con un elevado componente inflamatorio, puesto que LTB4 estimula la respuesta inflamatoria mientras que PGE2 ejerce un efecto contrario.

Summary

PGE2 and LTB4 are inflammation modulators. Fish oil (FO) inhibits atherosclerosis partly through its anti-inflammatory actions, and may alter modulator production by substitution of n-6 PUFAs for n-3 PUFAs in cell membranes. Patients with peripheral vascular disease (PVD) have chronic inflammation. The effect of FO on LPS stimulated PGE2 and LTB4 production in PVD is unknown.

Men (30) with PVD and healthy men (73) were given 6g/d FO for 12 weeks. Blood was taken before and after supplementation. PBMCs were cultured in RPMI containing 5% autologous plasma and 10 µg/ml LPS for 24h. IL-6, TNF-a, PGE2 and LTB4 were assayed by ELISA.

Before supplementation, PBMCs from men with PVD produced larger amounts of IL-6 (11,60±6,56 vs 8,60±4,89 pg/L; patients vs controls p=0.027) and relatively larger amounts of LTB4 than PGE2, (pg/pg) compared to control PBMCs (0.67±0.70 vs 0.50±1.29 respectively; p=0.035). FO reduced the ratio in patients but not in controls (p=0.027 patients; p=0.325 controls). In patients, FO increased PGE2 production, 3.39±4.57 vs 3.99±3.77 ng/ml (p=0.034). Changes in LTB4 production may also modulate the cytokine response to FO. Subjects were split into groups, according to whether FO reduced IL-6 production or not. Where FO decreased IL-6 production, significant reductions in TNF-a and LTB4 accompany each other. In subjects failing to show a fall in IL-6 production, LTB4 increases and non-significant TNF-a increases occur. The 'normalisation' of the ratio of LTB4 and PGE2 produced by PBMCs, during an inflammatory challenge, may reduce inflammation in patients with diseases in which inflammation plays a part, since LTB4 may enhance pro-inflammatory cytokine production while PGE2 has the opposite effect.

Introduction

Atherosclerosis, leading to cardiovascular disease, is a multifactorial, multistep disease that involves chronic inflammation at every stage, from initiation to progression and, eventually, plaque rupture (1). In atherosclerosis, the normal homeostatic functions of the endothelium are altered, promoting an inflammatory response. A variety of inflammatory mediators such as cytokines, lipid mediators and adhesion molecules are secreted, which elicit a series of changes that induce monocyte adhesion and are directly related to initiation, progression, and clinical complications of atheromatous plaque (2, 3).

There is appreciable epidemiologic evidence demonstrating that fish oil consumption, which is rich in n-3 polyunsaturated fatty acids (PUFAs), protects against atherosclerosis by a variety of mechanisms (4-8). n-3 PUFAs have been shown to have anti-inflammatory effects in animal models of inflammation (9, 10), in rheumatoid arthritis (11), Crohn's disease (12) and psoriasis (13). An underlying mechanism for the anti-inflammatory influence of n-3 PUFAs may lie in its ability to reduce pro-inflammatory cytokine production. In human volunteers fish oil supplementation reduced the ex vivo capacity of LPS stimulated peripheral blood mononuclear cells (PBMCs) to produce TNF-a and IL-6 (14). However, in a number of studies, the influence of fish oil on cytokine production is inconsistent. For example, only five out of 8 human studies, investigating the effect of fish oil on TNF-a and IL-6 production (14-21) report a suppressive effect (14,16,17,18,20).

The heterogeneous genetic background of the subjects in each study may have contributed to the variability in response. In an earlier studies, we demonstrated that individuals who exhibit an inherently high capacity for TNF-a production were more sensitive to the anti-inflammatory effects of fish oil than individuals who exhibit a low capacity (22). This sensitivity might by influenced by the body mass index (BMI) and the possession of certain alleles (23).

Prostaglandins (PG) or leukotrienes (LT) are the main initiators of the inflammatory cascade, and their production is directly influenced by dietary unsaturated fatty acids, which compete for insertion at the sn-2 position of membrane phospholipids. In place of the eicosanoids generated from dietary n-6 PUFAs (PGE2, LTB4), others with a lower potency arise from n-3 PUFAs (PGE3, LTB5) (24). Thus, fish oil supplementation has been reported to decrease the production of both LTB4 and PGE2 (25, 26).

Studies have demonstrated that PGE2 inhibits the production of pro-inflammatory cytokines such as TNF-a or IL-ß, while LTB4 enhances their production (27,28). If fish oil lowers PGE2 production, enhanced production of pro-inflammatory cytokines may occur. However this is not the case, and fish oil reduces ex-vivo PGE2, TNF-a and IL-ß production (29). The situation may be more complex than this, because fish oil decreases LTB4 production. The overall effect would be a decrease in both stimulatory and inhibitory factors. Therefore, the precise effect of dietary fish oil upon production of cytokines might be related to the changed balance in production of LTB4 and PGE2.

Atherosclerosis of lower extremities defines what is known as peripheral vascular disease (PVD). This disease is common and PVD patients have a 3 to 5 fold increased risk of cardiovascular mortality (30-32). Recent investigations identify a strong underlying inflammatory process in PVD which constitutes a good prognostic factor for cardiovascular mortality (33-36). Fish oil decreased LTB4 in PVD patients, while LTB5 levels increased (37) but, to our knowledge, the ex vivo effects or fish oil on eicosanoids and inflammation markers in LPS-stimulated PBMCs of PVD patients have not been studied before, and may contribute to explain inconsistencies in the literature regarding clinical improvements in this kind of patients after fish oil supplementation (38). Furthermore, we hypothesized that the protective effects of fish oil in atherosclerosis may be mediated by changes in the balance of LTB4 and PGE2 production, which would determine the intensity of the inflammatory cascade. Therefore, we measured eicosanoid production by LPS-stimulated PBMCs in PVD and healthy men before and after 3 mo of fish oil supplementation, and related it to the individual's capacity to reduce, or not, inflammation in terms of a decrease / increase in IL-6 production.

Materials and Methods

Subjects recruitment and study design.

A total of 30 PVD patients and 73 healthy volunteers were recruited in the study. Only male subjects were used, as constancy in individual cytokine production has been demonstrated in males but not pre-menopausal females (39). Subjects were not taking any lipid lowering drugs or fish oil supplements from at least 3 mo before commencement of the study.

The patients studied were those affected by PVD of the lower limbs, as a model of chronic inflammation. Patients were recruited from the vascular clinics in Southampton, Winchester and the Isle of Wight. Inclusion criteria were diagnosed PVD (confirmed from their clinical history and examination), presenting with intermittent claudication (IC; Stage II in Fontaine classification), ankle-brachial index (ABI) <0.70 and total cholesterol > 5.2 mmol/L. Patients on NSAIDs, with malignancy, or other inflammatory diseases such as arthritis, or diabetes mellitus were excluded. Patients who have recently had interventional treatment (within 3 months) for PVD were also excluded. The control subjects were healthy volunteers recruited from Southampton University Staff Club and other local institutions. Control subjects were matched for age and as closely as possible for smoking. The presence of PVD was excluded by undertaking a full cardiovascular history and examination, measuring ABI and recording ECGs. Individuals who were diabetic, hypertensive or had other significant medical problems were also be excluded from the control group. A full clinical history of cardiovascular events and risk factors was recorded.

Subjects maintained their usual lifestyles and diets but consumed 6 g of encapsulated fish oil/d, which provided 1.8 g n-3 PUFAs/d (MaxEPA; Seven Seas Ltd, Hull, United Kingdom), for 12 wk. Before providing blood samples, subjects fasted overnight for =12 h. Three separate blood samples were collected sequentially at the commencement and completion of fish oil supplementation. First, 20-mL blood samples were collected and drawn into citrate-phosphate-dextrose solution with adenine-formula 1 (CPDA-1); These samples were used to prepare PBMCs. Next, 10-mL blood samples were collected into coagulant-free evacuated tubes; these samples were used to prepare serum. Finally, 5-mL blood samples were collected into evacuated tubes containing EDTA; these samples were used to prepare plasma for the measurement of the fatty acid composition in the phosphatidylcoline fraction. This measurement shows incorporation of n-3 PUFAs into membrane phospholipids and compliance with the consumption of the fish oil supplements. Full compliance was encouraged

by frequent contact with the subjects of the study and payment of a small inconvenience honorarium. The habitual diet of all subjects in the study was measured by means of a seven day weighed record. The study was approved by the Southampton and South West Hampshire Joint Ethics Committee.

Isolation and culture of PBMCs

CPDA-1 blood was transferred in Starsted EDTA tubes, and PBMCs were isolated by sedimentation with 6% dextran-500 (w/v) in PBS to remove red cells, followed by density gradient centrifugation and flotation of PBMCs on Nycoprep 1068. Cells were counted by Coulter Counter and diluted in RPMI 1640 containing 5% autologous plasma, 1% Penicillin and Streptomycin (w/v), 2 mmol/L L-Glutamine and 10 µg/mL lipopolysaccharide (LPS), at a known cellular concentration (3×105 cells/mL). They were incubated for 24 hours at 37°C in a humidified 5% CO2 atmosphere. At the end of the incubation period, the cells were frozen at - 80°C in their incubation medium until needed.

Compliance with fish oil supplementation: phospholipid fatty acid composition analysis.

Subjects were contacted at at least three points during the study to check that there were no problems with capsule consumption. Fasting plasma phospholipids fatty acid composition was measured by gas liquid chromatography (40): total plasma (1 mL) lipids were isolated by extraction with chloroform/methanol (2:1 vol/vol) containing 50 mg/L butylated hydroxytoluene. Plasma phosphatidylcholine (PC) fraction was purified by solid phase extraction on aminopropylsilica cartridges (Varian, Surrey, UK). Plasma PC fatty acids were converted to methyl esters by incubation with methanol containing 2% (vol/vol) sulphuric acid at 50°C for 18 h. Fatty acid methyl esters were separated, re-dissolved in hexane and analysed by capillary gas chromatography using a Hewlett-Packard 5890 GC (Hewlett-Packard, Stockport, Ches., UK) equipped with an HP7686 GC autosampler using a BPX-70 fused silica capillary column (50 m×0.25 mm×0.32 m) with flame ionisation detection. Peaks were identified by retention times relative to standards. Fatty acids are reported as proportionate values (g/100g total fatty acids). Co-efficient of variation was less than 5% for determination of fatty acid composition.

Analysis of cytokine and eicosanoid production by PBMCs

LTB4 and PGE2 concentration were measured in culture medium by commercial immunoassay kits (R&D Systems Europe Ltd, Oxon, UK; Cross reactivity of PGE2 kit with PGE3 16.3%) and TNF-a and IL-6 concentration were measured in culture medium by standard commercial ELISA kits (Biosource International, Nivelles, Belgium) according to manufacturer's instructions.

Statistical analysis

The data was analysed using SPSS software (version 12.0, Chicago, IL), and is expressed as means ± SD unless indicated otherwise. Statistical comparisons within and between groups were performed using paired and two-sample t-tests for parametric variables or Wilcoxon signed-rank and Mann–Whitney U tests for non-parametric variables. Associations between IL-6 production and LTB4, PGE2 production or its ratio were examined for statistical significance by Pearson's correlation. P values pf <0.05 were judged to show a statistically significant effect.

Results.

Plasma phospholipid fatty acid composition.

The proportions of EPA and DHA in plasma phospholipids increased in all subjects after fish oil supplementation, with mean increase of 244% and 152% in patients and 229% and 154% in controls, respectively, at the end of the supplementation period (**Table 1**). An increase in fish oil-derived n-3 PUFAs was accompanied by a significant decrease in the proportion of arachidonic acid in plasma phospholipids.

Table 1. Influence of fish oil supplementation on proportions of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) in plasma phospholipids.

		Before supplementation	After supplementation
		% by wt of total fatty acids	
EPA	Patients	1.68 ± 0.95	4.11 ± 1.29*
	Controls	1.80 ± 1.09	4.13 ± 1.25*
DHA	Patients	3.13 ± 1.46	4.77 ± 2.12*
	Controls	4.14 ± 1.46	6.38 ± 1.87*
AA	Patients	9.15 ± 2.26	7.39 ± 1.14*
	Controls	8.90 ± 1.54	7.43 ± 1.35*

Average ± SD; n=30 in patients and n=73 in controls; *Significantly different from presupplementation value, p<0.001.

Influence of fish oil on eicosanoids and cytokine production.

LTB4, PGE2, IL-6 and TNF-a production by LPS stimulated PBMCs before and after fish oil supplementation is shown in **Table 2**. Prior to fish oil supplementation, IL-6 was greater in patients than in controls, and significant positive correlations (Pearson's rho) were present between IL-6 and PGE2 or LTB4 production. While mean values of TNF-a production were lower in both groups after fish oil supplementation, the effect did not reach statistical significance. In patients, PGE2 production increased significantly after fish oil supplementation.

Influence of fish oil supplementation on ratio of LTB4 to PGE2 (pg/pg) production.

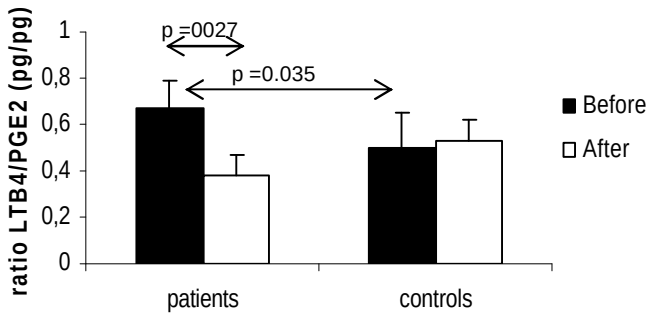
The changes on ratio of LTB4 to PGE2 (pg/pg) production before and after fish oil supplementation is shown in **Figure 1**. Before supplementation, PBMCs from men with PVD produced relatively larger amounts of LTB4 than PGE2, (pg/pg) compared to control PBMCs, and fish oil significantly reduced the ratio in patients but not in controls.

Table 2. Influence of fish oil supplementation on interleukin 6 (IL-6), tumor necrosis factor a (TNF-a), prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) production by LPS stimulated PBMCs.

		Before supplementation	p	After supplementation	p
			pg/L		
IL-6	Patients	11,60 ± 6,56	0.027†	11,49 ± 6,12	0.089
	Controls	8,60 ± 4,89		9,40 ± 4,63	
TNF-a	Patients	1,07 ± 1,09	0.130	0,87 ± 0,77	0.149
	Controls	0,83 ± 1,34		0,64 ± 0,44	
PGE2	Patients	2,90 ± 2,36‡	0.544	2,95 ± 3,56	0.034†
	Controls	3,39 ± 4,57‡		3,99 ± 3,76*	
LTB4	Patients	0,69 ± 0,05‡	0.173	0,66 ± 0,15	0.061
	Controls	0,57 ± 0,25		0,59 ± 0,22	

Average ± SD; n=30 in patients and n=73 in controls; *Significantly different from presupplementation value, p<0.001. †Significant difference between patients and controls. ‡Significant correlation with IL-6 production: Pearson's rho; 0.419, p=0.03 (PGE2 patients); 0.489, p<0.001 (PGE2 controls); 0.464, p=0.01 (LTB4 patients).

Figure 1. Effect of fish oil supplementation on ratio of leukotriene B4 (LTB4) : prostaglandin E2 (PGE2) (pg/pg) released from LPS-stimulated PBMCs of patients with PVD and healthy controls.



Average ± SEM; n=30 in patients and n=73 in controls.*Significant effect after fish oil supplementation; †Significant difference between patients and controls.

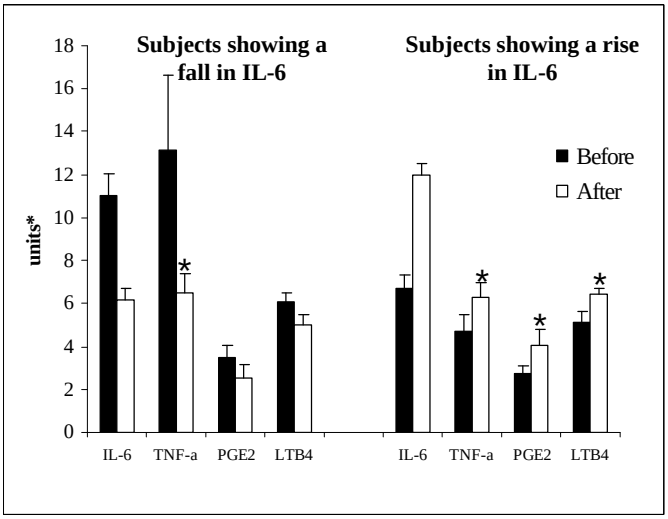
Relationship between changes in IL-6, TNF-a, LTB4 and PGE2 production by LPS-stimulated PBMCs after fish oil supplementation.

Figure 2 shows the changes in TNF-a, LTB4 and PGE2 production by LPS-stimulated PBMCs, when subjects were split into groups by whether fish oil reduced IL-6 production or not in patients (**Panel A**) or controls (**Panel B**). Where fish oil decreased IL-6 production, TNF-a decreased (p=0.006, p=0.010, controls and patients respectively), and mean LTB4 production showed a decreasing trend (NS; 611±228 vs 505±279 and 712±46 vs 665±168 pg/mL, controls and patients respectively). In subjects failing to show a fall in IL-6 production, mean LTB4 production was significantly increased (p=0.037, p=0.003, controls and patients

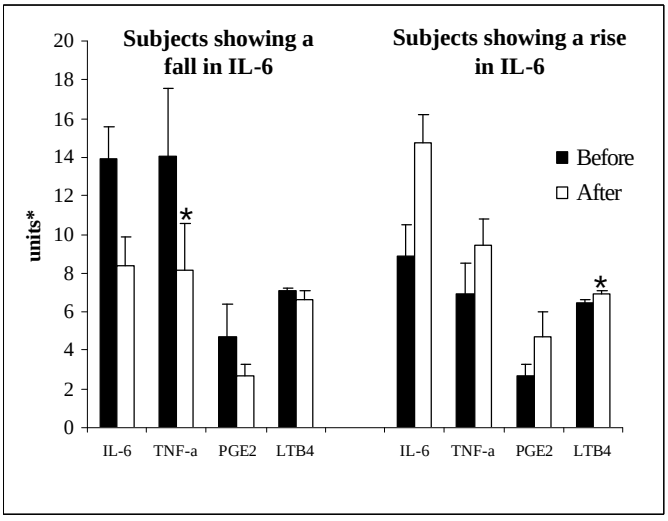
respectively) and TNF-a and PGE2 production increased only in the control group failing to show a fall in IL-6 production (p=0.027 and p=0.009 respectively). As shown in figure 1 patients had an inherantly higher capacity for LTB4 than PGE2 production. Fish oil resulted in changes in ratio of LTB4 to PGE2. The magnitude of the change in the production of IL-6 after fish oil supplementation, showed a positive correlations, with the change in ratio both in patients and controls, as illustrated in **Figure 3**.

Figure 2. Relationship between changes in IL-6, TNF-a, LTB4 and PGE2 production by LPS-stimulated PBMCs after fish oil supplementation.

Panel A: Controls



Panel B: Patients

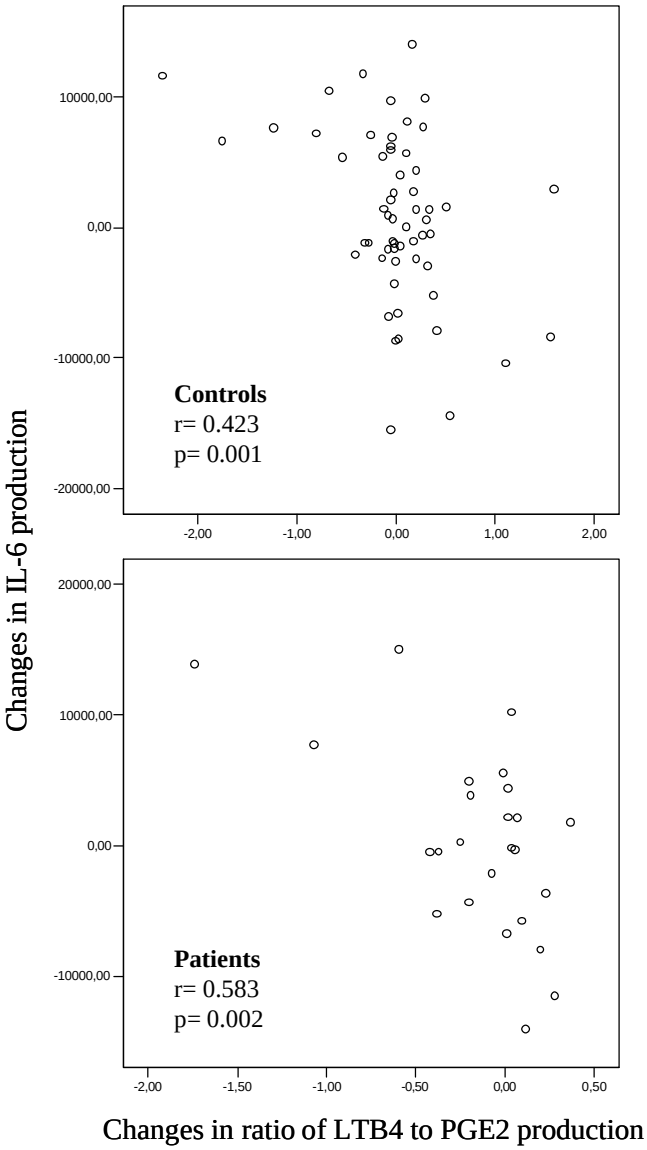


Average ± SEM; #Units are pg/L (IL-6 and PGE2), 10x pg/L (TNF-a and LTB4).
*Significant effect after fish oil supplementation.

Discussion

Our data suggests a relationship between fish oil supplementation and inhibition of inflammation through the "normalisation" of the ratio LTB4/PGE2 produced by PBMCs during an inflammatory challenge in PVD patients. Furthermore, in healthy subjects, the ability of fish oil to change ex-vivo IL-6 production is related to concommitant changes in the proportion of production of the two eicosanoids.

Figure 3. Association of the influence of fish oil supplements with the relationship between changes in ratio of LTB4 to PGE2 and the magnitude of the change in the production of IL-6 in LPS stimulated PBMCs.



Two-tailed Pearson's correlation. r stands for Pearson's rho.

Dietary supplementation with fish oil resulted in marked increases in the incorporation of EPA and DHA into plasma phospholipids of patients and controls in substitution of arachidonic acid, in similar extent to previous studies (22,41).

It is important to note that IL-6 and TNF-a had higher initial concentrations in patients than in controls (TNF-a non significant; ns). This fact agrees with the concept that PVD has an underlying inflammatory process (33-36). However, the overall effect of dietary fish-oil supplementation was null on IL-6 or TNF-a production, but resulted in an increase in PGE2 production by PBMCs taken from patients.

Connected to this, IL-6 released by PBMCs correlated positively with eicosanoid production (PGE2 and LTB4), and we have previously demonstrated that the sensitivity of a person to the suppressive effects of n-3 PUFAs on cytokine production might be linked to the inherent level of production of the cytokine by cells from the person before supplementation (22,23). For that reason we split data among those who responded to fish oil treatment by decreasing inflammation (in terms of IL-6 decrease) and those who did not (showing IL-6 increase). The latter effect appears to be a paradoxical effect of fish oil. However, the ability of fish oil to enhance rather than to reduce inflammation was not unexpected and has been reported before in terms of enhanced TNF-a production (22). During inflammation, phospholipase A2 (EC 3.1.1.4) hydrolyzes membrane phospholipids, thus making arachidonic acid available for the production of their eicosanoids prostaglandin E2 (PGE2) and leukotriene B4 (LTB4). Fish oil supplementation may alter pro-inflammatory cytokine production in either direction by the n-3 PUFAs that it contains, replacing arachidonic acid in the cell membrane and competing for the production of their eicosanoids-derived cascade. Thus, the overall effect on IL-6 production (inhibition or stimulation) will depend on the balance among the different stimulatory and inhibitory eicosanoids produced from arachidonic and eicosapentaenoic acids.

Splitting data according to IL-6 behavior, it is observed that whether individuals (patients and controls) responded to fish oil supplementation by decreasing IL-6 production, reductions in TNF-a and LTB4 accompany each other. When individuals responded to fish oil supplementation by increasing IL-6 production, LTB4 increases and non-significant TNF-a increases occur.

There may be other factors influencing an individual's response to fish oil supplementation. The ratio LTB4/PGE2 determines the inflammatory response, and patients had a higher ratio than controls as a reflect of their inherent inflammatory condition. After fish oil supplementation, this ratio was only reduced in patients, not in controls, and positive correlations were found in both groups when comparing changes in inflammation (changes in IL-6 production) with changes in the ratio. This possible role of fish oil in the balance of eicosanoid production suggest a new hypothesis for the mechanism by which fish oil exerts an

anti-inflammatory effect in the context of an inflammatory stress. This possible association, has been evidenced as well different individual's response to fish oil treatment.

Clearly other mechanisms for the variable mechanism(s) of action of dietary fish oil on cytokine production by PBMCs still remain unexplained. It has been postulated that increased n-3 PUFAs concentrations in the cell membrane are associated with both stimulatory and inhibitory effects on cell function, ranging from modulation of intercellular eicosanoid release to alterations in gene transcription (23). The results of this study confirms this postulate suggesting that in diseases in which inflammation plays a part, n-3 PUFAs might exert anti-inflammatory effect by affecting the eicosanoid ratio LTB4/PGE2 and evidencing different behavior, among individuals, in response to fish oil treatment. The mechanisms by which this modulating effect of fish oils could have been exerted needs to be elucidated, and show that the interaction of n-3 PUFAs intake and cytokine biology is complex. The understanding of the precise nature of the determinants of the ability of fish oil to act as an anti-inflammatory agent will enable supplementation with this foodstuff to be used more effectively in suppressing inflammation than is presently the case.

Acknowledgments

JJ Carrero has a PhD educational grant from University of Granada, Spain. We are grateful to the BBSRC for financial support, to Seven Seas Ltd for the gift of fish oil capsules and to Puleva Biotech for supporting the visit of JJ Carrero to Southampton University.

Bibliography.

1. Paoletti R, Gotto AM Jr, Hajjar DP. Inflammation in atherosclerosis and implications for therapy. Circulation. 2004;109(23 Suppl 1):III20-6.
2. Libby P. Inflammation in atherosclerosis. Nature. 2002;420(6917):868-74.
3. Prescott SM, McIntyre TM, Zimmerman GA, Stafforini DM. Sol Sherry lecture in thrombosis: molecular events in acute inflammation. Arterioscler Thromb Vasc Biol. 2002;22(5):727-33.
4. GISSI investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. Lancet. 1999; 354(9177):447-55.
5. Lemaitre RN, King IB, Mozaffarian D, Kuller LH, Tracy RP, Siscovick DS. n-3 Polyunsaturated fatty acids, fatal ischemic heart disease, and nonfatal myocardial infarction in older adults: the Cardiovascular Health Study. Am J Clin Nutr. 2003; 77(2):319-25.
6. Erkkila AT, Lichtenstein AH, Mozaffarian D, Herrington DM. Fish intake is associated with a reduced progression of coronary artery atherosclerosis in postmenopausal women with coronary artery disease. Am J Clin Nutr. 2004; 80(3):626-32.
7. Hino A, Adachi H, Toyomasu K, Yoshida N, Enomoto M, Hiratsuka A, Hirai Y, Satoh A, Imaizumi T. Very long chain N-3 fatty acids intake and carotid atherosclerosis: an epidemiological study evaluated by ultrasonography. Atherosclerosis. 2004; 176(1):145-9.

8. Yaqoob P, Calder PC. N-3 polyunsaturated fatty acids and inflammation in the arterial wall. Eur J Med Res. 2003; 8(8):337-54.
9. Calder PC. n-3 polyunsaturated fatty acids and cytokine production in health and disease. Ann Nutr Metab. 1997; 41(4):203-34.
10. Grimble RF. Dietary lipids and the inflammatory response. Proc Nutr Soc. 1998; 57(4):535-42.
11. Calder PC, Zurier RB. Polyunsaturated fatty acids and rheumatoid arthritis. Curr Opin Clin Nutr Metab Care. 2001; 4(2):115-21.
12. Belluzzi A, Brignola C, Campieri M, Pera A, Boschi S, Miglioli M. Effect of an enteric-coated fish-oil preparation on relapses in Crohn's disease. N Engl J Med. 1996; 334(24):1557-60.
13. Mayser P, Mrowietz U, Arenberger P, Bartak P, Buchvald J, Christophers E, Jablonska S, Salmhofer W, Schill WB, Kramer HJ, Schlotzer E, Mayer K, Seeger W, Grimminger F. Omega-3 fatty acid-based lipid infusion in patients with chronic plaque psoriasis: results of a double-blind, randomized, placebo-controlled, multicenter trial. J Am Acad Dermatol. 1998;38(4):539-47.
14. Trebble T, Arden NK, Stroud MA, Wootton SA, Burdge GC, Miles EA, Ballinger AB, Thompson RL, Calder PC. Inhibition of tumour necrosis factor-alpha and interleukin 6 production by mononuclear cells following dietary fish-oil supplementation in healthy men and response to antioxidant co-supplementation. Br J Nutr. 2003;90(2):405-12.
15. Schmidt EB, Varming K, Moller JM, Bulow Pedersen I, Madsen P, Dyerberg J. No effect of a very low dose of n-3 fatty acids on monocyte function in healthy humans. Scand J Clin Lab Invest. 1996; 56(1):87-92.
16. Mayer K, Meyer S, Reinholz-Muhly M, Maus U, Merfels M, Lohmeyer J, Grimminger F, Seeger W. Short-time infusion of fish oil-based lipid emulsions, approved for parenteral nutrition, reduces monocyte proinflammatory cytokine generation and adhesive interaction with endothelium in humans. J Immunol. 2003;171(9):4837-43.
17. Meydani SN, Lichtenstein AH, Cornwall S, Meydani M, Goldin BR, Rasmussen H, Dinarello CA, Schaefer EJ. Immunologic effects of national cholesterol education panel step-2 diets with and without fish-derived N-3 fatty acid enrichment. J Clin Invest. 1993; 92(1):105-13.
18. Meydani SN, Endres S, Woods MM, Goldin BR, Soo C, Morrill-Labrode A, Dinarello CA, Gorbach SL. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr. 1991; 121(4):547-55.
19. Mori TA, Woodman RJ, Burke V, Puddey IB, Croft KD, Beilin LJ. Effect of eicosapentaenoic acid and docosahexaenoic acid on oxidative stress and inflammatory markers in treated-hypertensive type 2 diabetic subjects. Free Radic Biol Med. 2003; 35(7):772-81.
20. Sundrarjun T, Komindr S, Archararit N, Dahlan W, Puchaiwatananon O, Angthararak S, Udomsuppayakul U, Chuncharunee S. Effects of n-3 fatty acids on serum interleukin-6, tumour necrosis factor-alpha and soluble tumour necrosis factor receptor p55 in active rheumatoid arthritis.J Int Med Res. 2004;32(5):443-54.
21. Vega-Lopez S, Kaul N, Devaraj S, Cai RY, German B, Jialal I. Supplementation with omega3 polyunsaturated fatty acids and all-rac alpha-tocopherol alone and in combination failed to exert an anti-inflammatory effect in human volunteers. Metabolism. 2004;53(2):236-40.
22. Grimble RF, Howell WM, O'Reilly G, Turner SJ, Markovic O, Hirrell S, East JM, Calder PC. The ability of fish oil to suppress tumor necrosis factor alpha production by peripheral blood mononuclear cells in

healthy men is associated with polymorphisms in genes that influence tumor necrosis factor alpha production. Am J Clin Nutr. 2002; 76(2):454-9.

23. Markovic O, O'Reilly G, Fussell HM, Turner SJ, Calder PC, Howell WM, Grimble RF. Role of single nucleotide polymorphisms of pro-inflammatory cytokine genes in the relationship between serum lipids and inflammatory parameters, and the lipid-lowering effect of fish oil in healthy males. Clin Nutr. 2004; 23(5):1084-95.
24. Calder PC, Grimble RF. Polyunsaturated fatty acids, inflammation and immunity. Eur J Clin Nutr. 2002; 56 Suppl 3:S14-9.
25. Blok WL, Katan MB, van der Meer JW. Modulation of inflammation and cytokine production by dietary (n-3) fatty acids.J Nutr. 1996;126(6):1515-33.
26. Calder PC. n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clin Sci (Lond). 2004;107(1):1-11.
27. Rola-Pleszczynski M, Thivierge M, Gagnon N, Lacasse C, Stankova J. Differential regulation of cytokine and cytokine receptor genes by PAF, LTB4 and PGE2. J Lipid Mediat. 1993; 6(1-3):175-81.
28. Vassiliou E, Jing H, Ganea D. Prostaglandin E2 inhibits TNF production in murine bone marrow-derived dendritic cells. Cell Immunol. 2003; 223(2):120-32.
29. Grimminger F, Fuhrer D, Papavassilis C, Schlotzer E, Mayer K, Heuer KU, Kiss L, Walmrath D, Piberhofer S, Lubbecke F, et al. Influence of intravenous n-3 lipid supplementation on fatty acid profiles and lipid mediator generation in a patient with severe ulcerative colitis. Eur J Clin Invest. 1993; 23(11):706-15
30. Henke PK, Blackburn S, Proctor MC, Stevens J, Mukherjee D, Rajagopalin S, Upchurch GR Jr, Stanley JC, Eagle KA. Patients undergoing infrainguinal bypass to treat atherosclerotic vascular disease are underprescribed cardioprotective medications: effect on graft patency, limb salvage, and mortality. J Vasc Surg. 2004; 39(2):357-65.
31. Leng GC, Fowkes FG, Lee AJ, Dunbar J, Housley E, Ruckley CV. Use of ankle brachial pressure index to predict cardiovascular events and death: a cohort study. BMJ. 1996; 313(7070):1440-4.
32. Criqui MH, Langer RD, Fronek A, Feigelson HS, Klauber MR, McCann TJ, Browner D. Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med. 1992; 326(6):381-6.
33. Gokce N, Keaney JF, Hunter LM, et al. Predictive value of noninvasively determined endothelial dysfunction for long-term cardiovascular events in patients with peripheral vascular disease. J Am Coll Cardiol. 2003; 41: 1769–1775
34. Brevetti G, Silvestro A, Di Giacomo S, et al. Endothelial dysfunction in peripheral arterial disease is related to increased plasma markers of inflammation and severity of peripheral circulatory impairment, but not to classic risk factors and atherosclerotic burden. J Vasc Surg. 2003; 38: 374–379
35. Yataco AR, Corretti MC, Gardner AW, et al. Endothelial reactivity and cardiac risk factors in older patients with peripheral arterial disease. Am J Cardiol. 1999; 83: 754–758.
36. Tisi PV, Hulse M, Chulakadabba A, Gosling P, Shearman CP. Exercise training for intermittent claudication: does it adversely affect biochemical markers of the exercise-induced inflammatory response? Eur J Vasc Endovasc Surg. 1997;14(5):344-50.

37. Mori TA, Vandongen R, Mahanian F, Douglas A. Plasma lipid levels and platelet and neutrophil function in patients with vascular disease following fish oil and olive oil supplementation. Metabolism. 1992;41(10):1059-67
38. Sommerfield T, Hiatt WR. Omega-3 fatty acids for intermittent claudication. Cochrane Database Syst Rev. 2004;(3):CD003833.
39. Jacob CO, Fronek Z, Lewis GD et al. Heritable major histocompatibility complex class II- associated difference in production of tumor necrosis factor: Relevance to genetic predisposition to systemic lupus erythematosus. Proc natl Acad Sci USA. 1990; 87: 1233-1237.
40. Burdge GC, Wright P, Jones AE, Wootton SA. A method for separation of phosphatidylcholine, triacylglycerol, non-esterified fatty acids and cholesterol esters from plasma by solid-phase extraction. Br J Nutr. 2000; 84(5):781-7.
41. Trebble TM, Arden NK, Wootton SA, Mullee MA, Calder PC, Burdge GC, Fine DR, Stroud MA. Peripheral blood mononuclear cell fatty acid composition and inflammatory mediator production in adult Crohn's disease. Clin Nutr. 2004;23(4):647-55

9

Discusión general, conclusiones y perspectivas

La aterosclerosis es una enfermedad multifactorial, que presenta diversas manifestaciones clínicas, como la Enfermedad Cardiovascular (ECV) o la Enfermedad Vascular Periférica (EVP). EVP y ECV guardan una relación causal, de manera que los pacientes con EVP tienen un riesgo 5 veces mayor a padecer un evento coronario. Algunos de los factores de riesgo asociados a la aparición de la lesión aterosclerótica común a ambas enfermedades, son susceptibles de ser modificados y corregidos mediante una dieta equilibrada.

La evidencia científica avala el papel positivo que una alimentación saludable tiene en la reducción de la incidencia de eventos cardiovasculares. Sin embargo la sociedad moderna, incluyendo en ella a países como el nuestro considerados típicamente mediterráneos, parece alejarse del patrón de alimentación saludable ya que: 1) Aumenta el consumo energético y disminuye su gasto; 2) Aumenta el consumo de grasas saturadas, ácidos poliinsaturados omega-6 y ácidos grasos *trans*, disminuyendo el consumo de ácidos poliinsaturados omega-3, carbohidratos y fibra; 3) Disminuye el consumo de frutos y verduras, aumentando el de cereales; 4) Aumenta el consumo de proteínas y disminuir el de antioxidantes.

El presente proyecto de tesis tuvo como objetivo evaluar la eficacia del consumo continuado de una mezcla de nutrientes considerados saludables (ácidos grasos omega-3, ácido oleico, ácido fólico y vitaminas A, D, E, B_6 y B_{12}) administrados de manera conjunta en una matriz láctea, en la reducción de diversos factores de riesgo de la enfermedad cardiovascular y de la enfermedad vascular periférica.

Biomarcadores de ingesta

Los biomarcadores nutricionales han ido cobrando especial relevancia en los últimos años en lo que se refiere a la ingesta o al metabolismo de nutrientes procedentes de la dieta, pues dan una idea mas fiel de la relación causa-efecto entre la ingesta o el perfil dietético seguido y el efecto biológico producido (1). El uso de biomarcadores nutricionales puede ser en ocasiones, una estimación más exacta del estatus nutricional que la elaboración de un cuestionario de ingesta, puesto que soluciona posibles limitaciones de éstas como:

1. La variabilidad con que cada individuo cumple el protocolo de ingesta o cumplimenta las encuestas dietéticas (grado de compromiso, formación cultural, incapacidad por enfermedad…).

2. Aquellos casos en que la combinación de ciertos alimentos ingeridos al mismo tiempo o cocinados de determinada manera pueda influenciar sobre la absorción y disponibilidad del contenido del nutriente objeto de estudio (2).

Mientras que la mayoría de ácidos grasos existentes en el tejido humano proviene o bien de la dieta, o bien de la síntesis endógena, los ácidos grasos poliinsaturados son esenciales para el ser humano y proceden exclusivamente de la ingesta dietética. Por ello, la medida de la proporción relativa de los ácidos grasos poliinsaturados en el plasma de los sujetos intervenidos nos proporciona una información fidedigna acerca de la toma del producto.

Los estudios descritos en los capítulos 4 y 5 plantearon la hipótesis de una ingesta continuada y conjunta de dosis dietéticas de ácidos grasos poliinsaturados omega-3, ácido oleico, ácido fólico y vitaminas E, B_6 y B_{12}, como herramienta efectiva en la reducción del riesgo cardiovascular en una población sana o afectada de hiperlipemia moderada. Durante las 4 semanas en que estas poblaciones tomaron leche semidesnatada no se produjo ningún cambio significativo en el perfil de ácidos grasos, aunque se observó cierto descenso no significativo en los niveles de EPA y DHA. La exclusión del pescado de la dieta durante el período de intervención pudo ser la responsable de este descenso, pues los sujetos del estudio, tradicionalmente adscritos al perfil mediterráneo, consumían pescado con una frecuencia moderada-alta. El posterior consumo durante dos meses del producto lácteo funcional enriquecido con la mezcla de nutrientes arriba descrita, permitió aumentar de forma significativa las proporciones plasmáticas de EPA y DHA en más de un 20%. Este incremento es similar al de otros estudios precedentes que usan una matriz láctea como vehículo de administración (3). Sin embargo, la proporción de ácido oleico no siguió la misma pauta creciente, y la adhesión de los individuos al modelo de dieta mediterránea puede ser de nuevo la responsable de este hecho, pues la cantidad de ácido oleico que proporcionaban otros alimentos de la dieta distintos del producto funcional es relativamente mucho mayor, en torno a los 23 g/día.

El estudio descrito en el capítulo 7 planteó la hipótesis de una ingesta continuada y conjunta de dosis dietéticas de ácidos grasos poliinsaturados omega-3, ácido oleico, ácido fólico y vitaminas E, B_6 y B_{12}, como herramienta coadyuvante en la reducción de la sintomatología y de los factores de riesgo asociados a la enfermedad vascular periférica (EVP). En esta población, los nutrientes fueron suministrados como complemento a su dieta habitual (sin restricciones de ningún tipo), y en el marco del programa de fomento de hábitos saludables en el que estaban incluidos los pacientes desarrollado por el Servicio Hospitalario. Nuevamente, los pacientes que consumieron el producto enriquecido, mostraron proporciones crecientes de EPA y DHA en plasma a lo largo de la intervención, hecho que no ocurrió en el grupo control. Este aumento consigue también variar la proporción de poliinsaturados totales. De manera similar, la ingesta continuada de bajas dosis de ácido oleico durante un año también consigue aumentar gradualmente las proporciones de este ácido graso en plasma y las de los monoinsaturados totales.

Estos biomarcadores plasmáticos reflejan la absorción efectiva o biodisponibilidad de la grasa adicionada en el producto lácteo y hacen presuponer la consiguiente incorporación de los ácidos grasos poliinsaturados omega-3 en las membranas celulares en detrimento de los omega-6. El Capítulo 8 muestra cómo los ácidos omega-3 de la dieta son capaces de incorporarse de manera significativa a los fosfolípidos de membrana tras 12 semanas de intervención. La literatura precedente asocia esta incorporación efectiva a la composición de la membrana con una serie de implicaciones biológicas que pueden ser parcialmente responsables del descenso de algunos factores de riesgo lipídicos y de la mejora clínica que se observó en los pacientes.

El producto lácteo enriquecido aportó a los sujetos estudiados, 150 µg diarios de ácido fólico, 1.5 mg de vitamina B_6 y 7.5 mg de vitamina E. Estas cantidades representan aproximadamente el 75% de las RDA (4). Actualmente, la deficiencia de folato es la deficiencia vitamínica más común en los países desarrollados. El contenido de folato en plasma es un indicador del estatus de folato válido para intervenciones a corto plazo, pues refleja de manera directa la ingesta dietética dando valores fiables a partir de la cuarta semana de intervención (6). Todas las poblaciones estudiadas presentaron un estatus de folato sérico deficiente (<15 nmol/L, 5; Capítulos 4, 5 y 7) que consiguió subsanarse tras la intervención con el producto enriquecido. Para intervenciones a largo plazo (mínimo 3 meses), el contenido de folatos en los glóbulos rojos supone un mejor índice de su estatus en el organismo, pues refleja su almacén en el mismo (6). La concentración de folato eritrocitario sólo aumentó de manera significativa a partir del tercer mes de intervención en la población de enfermos afectados de EVP (Capítulo 7).

La concentración plasmática de vitamina B_6 sólo se midió en la población afectada de EVP, en la que un aumento gradual, que consigue diferenciarse del grupo control, confirma la absorción y disponibilidad de dicha vitamina tras la intervención. Por último, los niveles plasmáticos de vitamina E no aumentaron en las intervenciones a corto plazo. Otros estudios de duración similar en los que se usaron dosis mucho mayores tampoco consiguieron un aumento en la concentración plasmática de esta vitamina (7,8). Una posible explicación a estos resultados pudiera ser que el tiempo de intervención no fue el suficiente para observar cambios, ya que la concentración plasmática de vitamina E de los enfermos de EVP que consumieron el producto enriquecido consigue diferenciarse del grupo placebo tras 12 meses de intervención. Por otro lado, el descenso en el perfil lipídico puede haber limitado la concentración plasmática de vitamina E. En este sentido, al expresar los niveles de vitamina E en función de los lípidos plasmáticos (ratio Vit E/colesterol >5.2 µmol/L; 9), se evidenció la existencia de un estatus deficiente de vitamina E en los pacientes afectados de EVP. Este ratio refleja en mayor medida el estatus nutricional de vitamina E (9) y supone un mejor marcador de estrés oxidativo que la propia concentración de vitamina E (10). Al igual que se

observó en la población hiperlipémica (Capítulo 5), la intervención con el alimento funcional permitió aumentar este ratio en los enfermos con EVP (Capítulo 7).

A modo de resumen, el consumo diario de cantidades dietéticas de ácidos grasos omega 3, ácido oleico, ácido fólico y vitaminas B_6 y E, aumentó de manera positiva sus niveles plasmáticos en las poblaciones estudiadas. El aumento de estos biomarcadores de ingesta muestra el cumplimiento de los protocolos de toma del producto y debe interpretarse como una incorporación efectiva de los nutrientes al organismo. Esta incorporación al torrente sanguíneo y a los tejidos se asocia a una serie de implicaciones biológicas que pudieran contribuir a explicar las mejoras bioquímicas y clínicas descritas.

Es importante recalcar que las cantidades de los nutrientes suministrados con el producto son reducidas y en el contexto de una dosis alcanzable mediante una dieta equilibrada. Aun así, parecen afectar favorablemente a la reducción de ciertos factores de riesgo cardiovascular. Otros estudios anteriores usando dosis mayores en forma de cápsulas o dosificaciones mas "farmacológicas" no han logrado el mismo grado de incorporación plasmática (7, 11). El uso de la leche como vehículo transportador de estos nutrientes, puede haber contribuido a este hecho, puesto que: 1) la leche es un alimento de uso diario, lo que facilita su acceso y consumo, y puede facilitar el cumplimiento del protocolo ; 2) la leche se consume en contextos espaciados a lo largo del día (en el café del desayuno, la merienda, la cena…) y se ha demostrado que la absorción en el intestino de los ácidos grasos omega-3 mejora cuando se éstos ingieren asociados a otras grasas y en pequeñas dosis distribuidas a lo largo del día (12); 3) la caseína de la leche favorece la dispersión de la grasa y productos liposolubles en micelas, incrementando de esta manera la superficie de absorción y por consiguiente la biodisponibilidad potencial de las grasas y vitaminas adicionadas (3).

Riesgo cardiovascular lipídico

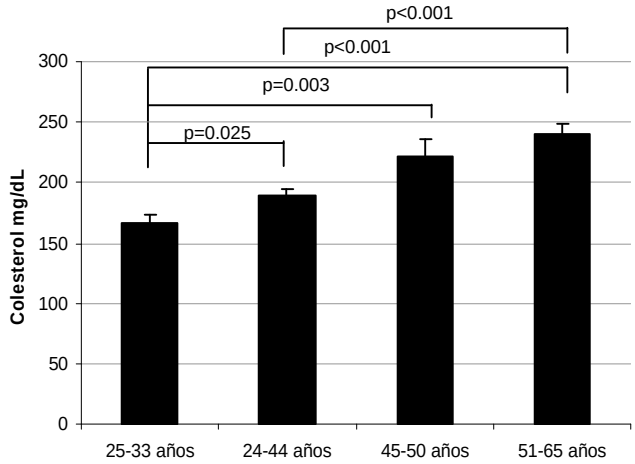
La hipercolesterolemia es uno de los principales factores de riesgo modificables que intervienen en el proceso aterosclerótico. El estudio *Multiple Risk Factor Intervention Trial* (MRFIT) demostró la existencia de una relación continua y gradual (sin umbral para el comienzo de esa relación) entre colesterolemia y mortalidad por enfermedad coronaria (13). La reducción de la colesterolemia produce una disminución de la incidencia y mortalidad por ECV (14) y EVP (15).

La prevalencia de hipercolesterolemia en la población española es alta. Un 18% (18,6% en varones y 17,6% en mujeres) de la población española de 35-64 años presenta una colesterolemia = 250 mg/dL y un 57,8% (56,7% en varones y el 58,6% en mujeres) posee valores de colesterol = 200 mg/dL (16). El estudio "*Dieta y Riesgo de Enfermedad Cardiovascular en España II*" (DRECE II) obtiene unos valores medios para la población

española de 35-64 años de edad de 221 mg/dL para el colesterol total, 53 mg/dL para el HDL, 141 mg/dL para el LDL y 135 mg/dL para los triglicéridos (17).

Las poblaciones estudiadas en las intervenciones a corto plazo (capítulos 4 y 5) participaron en los estudios como respuesta a un anuncio y se consideraban a sí mismas como sanas. Sin embargo, sus niveles de colesterol total en estas poblaciones se encuadran dentro de las observaciones descritas en el estudio DRECE II y pueden reflejarse como un alejamiento de patrón dietético Mediterráneo, donde la prevalencia de la hipercolesterolemia es baja. La unión de ambas cohortes permite apreciar que el colesterol en la población estudiada es un factor de riesgo que aumenta con la edad, y que las personas incluidas en el tercer y cuarto cuartil de edad (aproximadamente el 50% de los individuos estudiados) mostraban al inicio del estudio, niveles de colesterol superiores a los 200 mg/dL (18; **Tabla 1**).

Tabla 1. Distribución de los niveles plasmáticos de colesterol por cuartiles de edad en la población de los estudios de intervención a corto plazo.



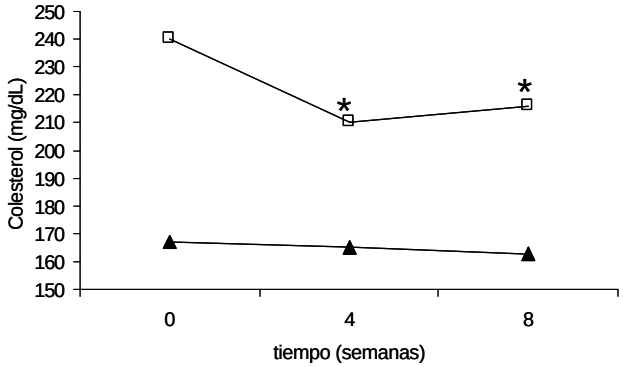
Voluntarios incluidos en los estudios descritos en los capítulos 4 y 5 (n=62). Primer cuartil: 25-33 años (n=16); Segundo cuartil: 24-44 años (n=13); Tercer cuartil: 45-50 años (n=16); Cuarto cuartil: 61-65 años (n=16). Comparaciones por *t de student* para muestras independientes.

El consumo continuado de los nutrientes contenidos en el alimento funcional contribuyó a reducir los niveles de colesterol total tanto en las intervenciones a corto plazo como en la población con EVP. A pesar de encontrar reducciones significativas, es interesante destacar que las mayores reducciones se consiguen en aquellas personas con elevados niveles de partida (**Figura 2**). Corroborando esta hipótesis, la selección de los enfermos de EVP con niveles de colesterol >200 mg/dL, permitió observar un descenso significativo de los niveles de colesterol a partir del tercer mes de intervención que no se llegó a observarse en los pacientes inicialmente normolipidémicos.

Esta idea que parece evidenciarse en el análisis global de los estudios que constituyen el presente proyecto de tesis, sugiere la existencia de una regulación fisiológica de la acción de

los nutrientes, en función del grado de alteración de la variable fisiológica objeto. Asimismo, apoya la necesidad de una dieta equilibrada como estrategia coadyuvante al tratamiento farmacológico, en aquellas enfermedades crónicas donde los nutrientes tengan un papel potencial. El uso de una nutrición adecuada contribuye a la estabilización del perfil lipídico cuando este perfil se encuentra desequilibrado.

Figura 2: Reducción de la concentración de colesterol total tras 4 y 8 semanas de intervención (Capítulos 4 y 5).



Voluntarios incluidos en los estudios de intervención descritos en los capítulos 4 y 5 (n=62). (?) Colesterol plasmático <200 mg/dL (n=28); (?) Colesterol plasmático >200 mg/dL (n=34). *p<0.05 mediante ANOVA de medidas repetidas.

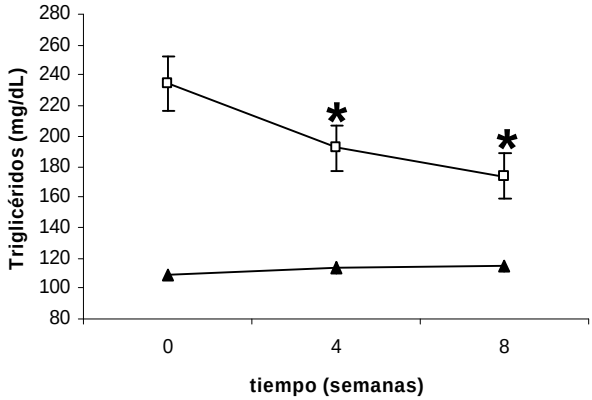
La fracción de colesterol-LDL también disminuyó de manera significativa y similar en las intervenciones realizadas a corto plazo. En aquellos pacientes afectados de EVP no pareció observarse este efecto. Sin embargo, el estudio de los niveles de ApoB en éstos reflejó una disminución importante. Se ha sugerido que la medida de ApoB supone un mejor índice de riesgo cardiovascular que la fracción de colesterol-LDL en sí (19), ya que denota una disminución del riesgo aterogénico mediante la reducción del número de partículas densas y pequeñas de LDL que no se hubiera apreciado de sólo contemplarse los niveles de colesterol-LDL de este grupo.

Diversos estudios han corroborado los efectos positivos que el consumo de ácido oleico o de ácidos grasos omega-3 tiene en la mejora del perfil lipídico mediante su implicación en una gran variedad de efectos fisiológicos (20,21). Estos nutrientes pueden haber sido responsables potenciales de los efectos aquí descritos.

Un estudio anterior llevado a cabo en voluntarios normolipidémicos que consumieron durante 6 semanas una leche enriquecida en ácidos grasos omega 3 (400 mg/día), describió un aumento en los niveles de colesterol-HDL de aproximadamente un 19% junto con una reducción similar en los niveles de triglicéridos (3). Es interesante destacar que sólo se consiguió un grado de reducción similar cuando se suministraron cápsulas de aceite de

pescado (4.5 g/día) durante 5 semanas en personas con hiperlipemia moderada (22). Los autores sugirieron que el uso de la leche como vehículo transportador de grasas pudiera contribuir a la efectiva incorporación a plasma y por consiguiente, a un mayor efecto a pesar de usar dosis reducidas. En las intervenciones a corto plazo que constituyen la presente memoria, los niveles de colesterol HDL aumentaron modestamente sin llegar a la significación estadística.

Figura 3: Reducción de la concentración de triglicéridos tras 4 y 8 semanas de intervención (Capítulos 4 y 5).



Voluntarios incluidos en los estudios de intervención descritos en los capítulos 4 y 5 (n=62). (?) Triglicéridos plasmáticos <150 mg/dL (n=36); (?) Triglicéridos plasmáticos >150 mg/dL (n=25). *p<0.05 mediante ANOVA de medidas repetidas

El efecto más destacado que la literatura científica atribuye al consumo de ácidos grasos omega-3 está relacionado con la reducción de los niveles de triglicéridos. Tras la intervención nutricional llevada a cabo en la población sana (Capítulo 4), que partía de valores basales de triglicéridos dentro de la normalidad, no se vio efecto alguno a este nivel. Sin embargo, el estudio descrito en el capítulo 5, partiendo de voluntarios con hipertrigliceridemia, encuentra reducciones notables en los niveles de triglicéridos tras las 8 semanas de intervención. Tal efecto se muestra más exactamente en el análisis conjunto de las poblaciones incluidas en las intervenciones a corto plazo (**Figura 3**). Cuando los sujetos fueron separados en función de si sus niveles plasmáticos de triglicéridos estaban elevados o no (considerado como triglicéridos plasmáticos <150 mg/dL; 20), éstos sólo disminuyeron tras el período de intervención en aquellos sujetos que presentaban valores elevados al inicio del estudio, y puede contribuir a confirmar la hipótesis antes expuesta de que los nutrientes son capaces de regular alteraciones bioquímicas en aquellos casos en que se presenten valores alterados. El estudio aleatorio realizado en enfermos de EVP no encontró diferencias en los niveles de triglicéridos después de la intervención, pues los valores medios de partida estaban por debajo de los límites de riesgo.

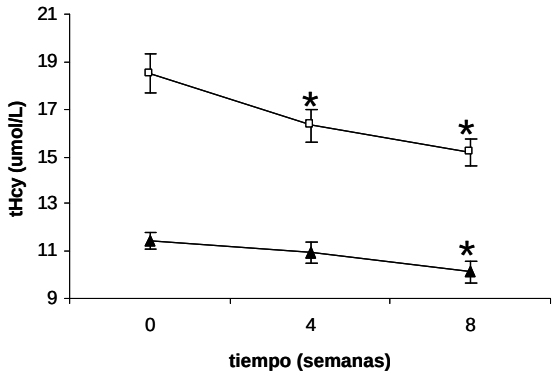
La enfermedad cardiovascular es de origen multifactorial, y aunque la dislipemia es un factor de riesgo muy importante de la ECV, debe ser considerada en el contexto de otros factores de riesgo (23,24). En cualquier caso, las diferentes intervenciones nutricionales realizadas sugieren que los nutrientes aportados a la dieta han ejercido un efecto positivo en la regulación fisiológica del perfil lipídico. Esta regulación puede ser efectiva en la prevención del riesgo cardiovascular, así como en el tratamiento de la EVP, ya que podría contribuir disminuir el uso de fármacos.

La Homocisteína y el riesgo cardiovascular

La Hcy es un aminoácido azufrado procedente del metabolismo de la metionina. Unos niveles plasmáticos de Hcy elevados (Hcy>15 µmol/L) se asocian a una mayor agregación plaquetaria, mayor estrés oxidativo y proliferación de células del músculo liso. Al mismo tiempo, una Hcy plasmática elevada disminuye la producción de óxido nítrico y reduce la función endotelial (Capítulo 3). Por todo ello, los niveles plasmáticos de Hcy constituyen un factor de riesgo independiente para el desarrollo de enfermedades con un componente aterosclerótico, como son la ECV (25) y la EVP (26).

Abundan los estudios epidemiológicos que relacionan la ingesta de folatos con la reducción de los niveles de Hcy. La razón de esta asociación se debe a que la molécula de folato actúa como coenzima en la regulación metabólica de la concentración de homocisteína (Hcy) a través de una reacción de remetilación en que también están involucradas las vitaminas B_6 y B_{12}.

Figura 4: Concentración plasmática de Homocisteína total tras 0, 4 y 8 semanas de intervención (Capítulos 4 y 5).



Voluntarios incluidos en los estudios de intervención descritos en los capítulos 4 y 5 (n=62). (?) tHcy <15 µmol/L (n=32); (?) tHcy >15 µmol/L (n=30). *p<0.05 mediante ANOVA de medidas repetidas.

En los estudios de intervención a corto plazo (Capítulos 4 y 5), la concentración de tHcy disminuyó de manera significativa tras los dos meses de ingesta de la mezcla de nutrientes. El análisis conjunto de ambas cohortes (**Figura 4**), refleja cómo los individuos con niveles de

tHcy elevados (tHcy >15 µmol/L), disminuyeron sus niveles en aproximadamente un 18%, mientras que aquellos individuos con tHcy <15 µmol/L lo hicieron en un 10%. Este hecho puede apoyar de nuevo la idea antes propuesta de una regulación fisiológica de la acción de los nutrientes, en función del grado de alteración de la variable fisiológica.

Este mismo comportamiento fue observado en la intervención realizada sobre los enfermos de EVP: sólo en aquellos pacientes con una hiperhomocisteinemia de partida, redujeron sus niveles de tHcy de forma significativa tras la intervención. Sin embargo, esta reducción no fue tan prominente como en las intervenciones anteriores. Quizás sea debido a que estos pacientes presentaban una enfermedad ya establecida.

Los efectos que la ingesta de ácido fólico tiene sobre la reducción de la tHcy han sido recogidos en numerosos estudios (27,28). Aunque el ácido fólico parece ser el mayor responsable en esta reducción, el efecto parece potenciarse considerablemente cuando se ingiere en combinación con vitaminas B_6 y B_{12} (29). El destacable aumento del estatus nutricional de folatos descrito anteriormente, seguido del aumento de la concentración de vitamina B_6 plasmática observado en la población con EVP, puede ser responsable, al menos en parte, de estos descensos.

Es interesante destacar, que las intervenciones resliazas disminuyeron los niveles de tHcy hasta valores normales (tHcy <15 µmol/L) en todos los individuos hiperhomocisteinémicos, tanto en enfermos de EVP como en individuos hiperlipémicos. Un reciente meta-análisis estimó que la reducción de los niveles de Hcy en aproximadamente 3 µmol/L e independientemente del valor de partida, puede reducir el riesgo de enfermedad isquémica del corazón en un 16%, el riesgo de trombosis en un 25% y el riesgo de infarto en un 24% (30).

Oxidación plasmática y lipoproteica

Se ha sugerido que una dieta rica en ácidos grasos monoinsaturados reduce la susceptibilidad de las LDL a la oxidación, mientras que un dieta rica en poliinsaturados de la serie omega-3 y omega-6 la aumenta (31). Los ácidos grasos poliinsaturados omega-3 son muy sensibles a la oxidación, debido a la presencia de numerosos dobles enlaces alternos en su estructura. Unas lipoproteínas LDL con alto contenido en poliinsaturados omega-3 debieran ser por tanto más vulnerables al ataque oxidativo y, en teoría, debieran ser más aterogénicas que unas lipoproteínas ricas en monoinsaturados. Nos preguntamos si una ingesta continuada de dosis dietéticas de ácidos grasos omega-3 en combinación con el resto de grasas, vitaminas y antioxidantes contenidos en el producto lácteo, podrían alterar el estado de oxidación en estos estudios de intervención.

Kratz *et al.* (32) demostraron que la ingesta de cantidades moderadas de ácidos grasos omega-3 en el contexto de una dieta rica en grasa monoinsaturada (como el ácido oleico), no

incrementa la oxidabilidad de las LDL. Schnell *et al.* (33) sugirieron que la vitamina E, administrada junto a ácidos grasos poliinsaturados, es capaz de reducir *in vivo* la susceptibilidad de las LDL a la oxidación.

En nuestros estudios de intervención, los ácidos grasos omega-3 se consumieron junto con pequeñas dosis de vitamina E (7.5 mg/día), 5.3 g/día de ácido oleico y en el contexto de una dieta Mediterránea. Trascurridas las diferentes intervenciones, ninguno de los parámetros de oxidación contemplados tanto en plasma (hidroperóxidos basales, capacidad antioxidante, TBARS, malondialdehído) como en la propia fracción LDL (Lag time, unidades de LDL oxidada) sufrió cambios significativos. Ello pudo ser debido a que, o bien la cantidad suministrada de ácidos grasos omega-3 fue demasiado pequeña para inducir cambios en la oxidación plasmática o de las LDL, o bien la administración conjunta de vitamina E pudo compensar los posibles efectos oxidantes.

La concentración plasmática de vitamina E sólo se incrementó en la intervención a largo plazo sobre enfermos de EVP. Sin embargo, tanto en ésta como en la intervención realizada sobre los voluntarios hiperlipémicos, el ratio vitamina E / lípidos sanguíneos aumentó notablemente, sugiriendo que la vitamina E suministrada pudo haber ejercido su efecto antioxidante y que el descenso en el perfil lipídico pudo limitar la concentración plasmática de la misma.

Moléculas de adhesión y activación endotelial

El endotelio vascular desempeña un papel esencial en los procesos hemostáticos de adhesión y migración celular, trombosis y fibrinolisis, que desencadenan el proceso aterosclerótico. Cuando el endotelio vascular se enfrenta a diferentes estímulos (como una molécula de LDL oxidada, radicales libres, homocisteína, o alteraciones fisiológicas como la hipertensión), desencadena una serie de cambios, conocidos como “activación endotelial”, que comprende la expresión de moléculas de adhesión y citocinas inflamatorias (34). Estas moléculas de adhesión, como la Selectinas P y E, la molécula de adhesión intercelular (ICAM-1) o la molécula de adhesión a la célula vascular (VCAM-1), participan de manera activa en los procesos de reclutamiento de monocitos y leucocitos, adhesión de plaquetas durante la trombosis e inflamación.

La activación endotelial es crucial en el desarrollo de la aterosclerosis, y es responsable de la atracción de monocitos a la pared del vaso por acción de las quimiocinas. Una vez allí, mediante el proceso de diapédesis penetrarán en la matriz subendotelial, donde posteriormente se diferenciarán en macrófagos. Estos macrófagos van a interaccionar con las LDL oxidadas, para luego transformarse en células espumosas, y contribuir de esa manera al inicio del desarrollo de la estría grasa que marca el inicio del proceso de ateroclerosis (35). Por

ello, la medida de las moléculas de adhesión y marcadores de inflamación nos puede proporcionar información acerca del grado de progresión de la lesión aterosclerótica.

Recientemente se han identificado ciertos nutrientes que juegan un papel importante en la regulación de la actividad endotelial, como son los ácidos grasos omega-3, las vitaminas antioxidantes (especialmente la vitamina E y C) y el ácido fólico, lo que planteó la medida de estas moléculas de adhesión en los estudios de intervención presentados.

En las intervenciones a corto plazo se observó un descenso significativo de los niveles de VCAM-1 soluble. Sin embargo, no se observó este efecto en los niveles de ICAM-1 soluble, lo que puede sugerir la existencia de un comportamiento distinto en la expresión de VCAM-1 e ICAM-1. Se ha sugerido que estas moléculas pudieran estar implicadas en diferentes etapas del proceso aterosclerótico, describiéndose un mayor protagonismo de la VCAM-1 y no de la ICAM-1 en las etapas iniciales del proceso aterosclerótico (36). Ello podría explicar la ausencia de efecto sobre las VCAM-1 en los enfermos de EVP, cuyo proceso aterosclerótico esta ya avanzado. Sin embargo, el consumo de la mezcla de nutrientes no consiguió alterar de manera significativa los niveles de selectina-P o ICAM-1, si bien es interesante destacar que el grupo control mostró un incremento significativo de esta molécula tras la intervención, lo que puede sugerir cierta acción protectora del endotelio.

Estudios previos de intervención, usando una dieta rica en ácidos grasos monoinsaturados durante dos meses, describieron un descenso en los niveles de moléculas de adhesión (37). También la ingesta de ácidos grasos omega-3 contribuye a reducir la síntesis de moléculas de adhesión, si bien disminuyen principalmente los niveles de VCAM-1 y E-selectina (38-40), mientras que los niveles de ICAM-1 pueden no verse afectados (41). La ingesta de ácido fólico también se ha asociado con la disminución de la activación endotelial, por su papel en la reducción de los niveles de Hcy. La Hcy es una agente oxidante capaz de promover la producción de especies reactivas de oxígeno mediante una serie de mecanismos que incluyen la inhibición de la glutation peroxidasa, una de las principales defensas que el organismo tiene frente a la oxidación (42). Las especies reactivas de oxígeno y el estrés oxidativo en general, promueven la trascripción del factor nuclear ?ß (NF- ?ß), que origina una respuesta pleiotrópica, incrementando la expresión de moléculas de adhesión como la VCAM-1. Además, recientemente se ha descrito que la Hcy en sí también es capaz de estimular la trascripción de este NF- ?ß (43). La reducción de los niveles de Hcy que se aprecia en estas intervenciones a corto plazo, puede haber influido también en la disminución antes descrita de los niveles plasmáticos de VCAM-1.

La activación endotelial que caracteriza al proceso aterosclerótico disminuye el proceso fibrinolítico. Este efecto es producido por un aumento en los niveles plasmáticos del activador inhibidor-1 del plasminógeno (PAI-1). Existe cierta evidencia que señala que la relación entre

fibrinólisis deprimida y aterosclerosis es debida a niveles altos de (PAI-1). Por ello, la disminución de PAI-1 puede ser beneficiosa para el organismo, ya que disminuye también la adherencia de las plaquetas (44). La ingesta de ácidos grasos monoinsaturados se ha asociado a un descenso del PAI-1 (45), pero no hemos podido observar efecto a este nivel en los enfermos de EVP intervenidos. Quizás puede deberse a la pequeña cantidad consumida de monoinsaturados en el contexto de un estilo de vida mediterráneo.

Sintomatología clínica en EVP

A pesar de que el tratamiento farmacológico y las recomendaciones dietéticas y de hábitos de vida resultaron en una mejora de la distancia de claudicación de los dos grupos considerados, la mejora observada en aquellos enfermos de EVP que consumieron el producto enriquecido con la mezcla de nutrientes fue importante, pasando casi a cuadruplicar esta distancia de claudicación con respecto a los metros recorridos al inicio del estudio. Se observaron correlaciones positivas entre la mejora de la distancia de claudicación y el aumento de la proporción de EPA ($p=0.006$) y ácido oleico ($p=0.08$) en plasma, y el aumento de la concentración de folato eritrocitario ($p=0.04$). Estos resultados sugieren que los nutrientes administrados pueden haber contribuido esta mejoría. Estos resultados van en consonancia con el aumento en el índice brazo-tobillo que se observó en el grupo intervenido, y que sugieren una mejora del flujo sanguíneo en las extremidades inferiores de estos pacientes.

Tal y como se sugirió en el Capítulo 6, una nutrición saludable puede ejercer un papel beneficioso en el desarrollo de la EVP. La malnutrición es común en este tipo de pacientes, y se asocia a cambios en otros factores de riesgo que predicen una mayor complicación en la progresión de la enfermedad (46). Si bien es cierto que existen muchos factores (como la enfermedad vascular crónica, diabetes, estilo de vida sedentario, deficiencias nutricionales y envejecimiento) que pueden contribuir a la progresión de la EVP y de la debilidad muscular que la isquemia provoca, únicamente la malnutrición y la falta de ejercicio muscular pueden ser potencialmente reversibles con intervenciones como la que presentamos en esta memoria.

El efecto que ciertos nutrientes de manera aislada produce en la EVP ha sido abordado anteriormente, pero muy pocos de éstos estudios abordaron el uso de alimentos enriquecidos como estrategia de intervención. Recientemente, se publicó un trabajo que utilizaba una barrita energética enriquecida con L-arginina. Tras dos semanas de intervención, el consumo de dos barritas energéticas se asoció a un incremento significativo de la distancia de claudicación frente al placebo. Los autores observaron una vasodilatación endotelial en estos pacientes, que se tradujo en un aumento del flujo. El consumo de L-arginina pudo haber estimulado la actividad del óxido nítrico endotelial (47,48). Por otro lado, otro trabajo usó una leche en polvo como vehículo para administrar 15 g diarios de aceite de oliva en enfermos de

EVP durante 4 meses. Los autores no encontraron cambios a nivel clínico (quizás debido a la corta duración del estudio), pero describieron una menor susceptibilidad a la oxidación en las lipoproteínas LDL en comparación con el grupo control, que había consumido aceite de girasol (49).

La limitación de nuestro estudio es que, al administrar una mezcla de nutrientes en el producto lácteo, no podemos explicar de una manera causal a qué nutrientes se debe esta mejoría clínica. Sí podemos, sin embargo, basarnos en la evidencia científica precedente para proponer cómo cada uno los nutrientes administrados ha podido influir en esta mejora.

Tanto el EPA como el DHA poseen propiedades antiinflamatorias. EPA y DHA compiten con el AA por la inserción en los fosfolípidos de membrana que se traducirá en la producción de unos eicosanoides menos potentes. La ingesta de cápsulas de aceite de pescado disminuyó la producción de leucotrieno B4 (procedente del AA) en pacientes con PVD, mientras que los niveles de leucotrieno B5 (procedente del EPA) aumentaron de manera significativa (50). En consonancia con esta idea, el **capítulo 8** sugiere cómo la ingesta de aceite de pescado puede influir sobre el proceso de inflamación crónica que sufren estos enfermos de EVP, mediante una alteración en la producción de leucotrieno B4 (LTB4) y prostaglandina E2 (PGE2). El LTB4 estimula la producción de citocinas inflamatorias, mientras que la PGE2 la disminuye. Por ello, una disminución del ratio LTB4:PGE2 conllevaría un descenso en la producción de citocinas. Además, el ratio AA:EPA disminuyó en aquellos enfermos de EVP intervenidos con la mezcla de nutrientes, al tiempo que aumentaron las proporciones de EPA y DHA en plasma. La prostaglandina I3 (PGI3) producida en el endotelio a partir del EPA es un vasodilatador más activo y un mejor inhibidor de la agregación plaquetaria que la prostaglandina I2 (PGI2) producida a partir del AA. Un descenso en el ratio AA:EPA puede relacionarse con vasodilatación e inhibición de la agregación plaquetaria, y una agregación plaquetaria disminuida conlleva, de manera potencial, un aumento del flujo sanguíneo (51). Por último, la ingesta de EPA y DHA provoca una mayor estabilidad en la placa fibrosa y reduce la infiltración de macrófagos, ralentizando de esta manera la progresión de la lesión vascular (52) y quizás así la aparición de los síntomas clínicos. Mediante todos estos mecanismos, la ingesta de EPA+DHA podría potenciar el efecto vasodilatador y antiagregante de la medicación pautada, como se puede deducir de la mayor mejoría clínica observada en los pacientes suplementados respecto de los controles.

Los estudios epidemiológicos sugieren que el consumo de folato y vitaminas del complejo B pueden prevenir la aparición de EVP. El estudio de la población del "*Health Professionals Follow-up Study*" reveló que los hombres que consumían la mayor cantidad de folato (aprox. 840 µg/día) tenían un riesgo a padecer EVP un 33% menor que aquellos hombres que consumían menos folato (aprox. 244 µg/día). Además, describió una asociación inversa entre la ingesta de vitamina B6 y el riesgo a padecer EVP (p=0.06) (53). El considerable incremento

de los niveles de folato sérico y eritrocitario que se observó en la población intervenida ha podido ser también responsable de la mejoría clínica descrita. Por otro lado, la hiperhomocisteinemia (HHcy) supone un factor de riesgo independiente a padecer EVP (54). El riesgo relativo de desarrollar EVP varía de 2.0 a 11.0 cuando el individuo posee HHcy (55, 56), que suele estar presente en aproximadamente el 30% de los pacientes con EVP (57). En aquellos sujetos con HHcy, la intervención nutricional consiguió aproximar estos niveles a valores normales, reduciendo posiblemente este riesgo.

Se desconocen los mecanismos específicos por los que la mejora del estatus de folato y B6 o la reducción de los niveles de Hcy que se observa en nuestro estudio pueda haber influido sobre la distancia de claudicación o el índice brazo-tobillo. Sin embargo, pueden estar relacionados con cambios en el proceso de coagulación (58,59) o una mayor vasodilatación, ya que la reducción de los niveles de Hcy influye sobre la síntesis de NO (60), la producción de prostaciclina (61) y el factor hiperpolarizante derivado del endotelio (endothelium-derived hyperpolarizing factor), que determina el tono vascular en los vasos de pequeño calibre (62).

En los últimos años, diversos estudios han evidenciado la falta acciones específicas para la reducción de los factores de riesgo de la EVP (63-65). Este estudio controlado aporta nuevas evidencias sobre el papel de los nutrientes en el desarrollo y progresión de la EVP.

Perspectivas

Los estudios descritos en esta memoria de tesis, describen el papel que determinados nutrientes tienen en la disminución del riesgo cardiovascular y en el tratamiento de la enfermedad vascular periférica. La evidencia que sugieren plantea una serie de preguntas que ya han comenzado a ser abordadas en nuestro laboratorio:

Evaluación del posible papel de estos nutrientes en cohortes mayores y en otro tipo de patologías o manifestaciones clínicas de la aterosclerosis. Los resultados descritos en esta memoria sugieren que la acción de los nutrientes en la prevención cardiovascular es mayor en sujetos con elevado riesgo cardiovascular (entendiendo con ello sujetos que presenten hipercolesterolemia, hipertrigliceridemia, hiperhomocisteinemia …etc., o una combinación de factores de riesgo elevados). Por ello, sería interesante abordar el papel del consumo de estos nutrientes de manera específica en poblaciones mayores sobre los marcadores bioquímicos de riesgo cardiovascular elevado. Hemos comenzado una intervención de este tipo en colaboración con la Consulta de Endocrinología del CPE Zaidín del Hospital Universitario “San Cecilio” de Granada, incluyendo a pacientes dislipémicos sin indicación de tratamiento farmacológico. Asimismo, también se ha iniciado la evaluación de una alimentación saludable mediante el producto lácteo enriquecido en individuos con Síndrome Metabólico, en colaboración con el Servicio de Medicina Interna del Hospital Reina Sofía de Córdoba.

La aterosclerosis presenta diversas manifestaciones clínicas y está asociada a otras patologías. Por ello, los resultados de esta memoria plantean la pregunta de si el consumo continuado de cantidades dietéticas de ácidos grasos omega-3, ácido oleico, vitaminas B6, B12, E y ácido fólico, puede contribuir a la prevención y/o la paliación de estas manifestaciones. Así, hemos comenzado la evaluación del consumo de los nutrientes contenidos en el producto lácteo como herramienta que acompañe a un programa de rehabilitación cardiaca en sujetos que hayan sufrido al menos un episodio de infarto de miocardio, en colaboración con la Unidad de Rehabilitación Cardiaca del Hospital Universitario “San Cecilio” de Granada. El estudio del consumo de nutrientes saludables como herramienta de acompañamiento en el tratamiento de enfermedades como la diabetes también resultaría de interés.

Estudio de los mecanismos de acción por los que cada nutriente suministrado ha podido contribuir a la mejora clínica observada en la enfermedad vascular periférica. Los nutrientes suministrados en el producto lácteo participan en los procesos de inflamación, fibrinolisis, vasodilatación, tensión arterial…. Todos ellos causas potenciales de esta mejora clínica. El estudio *ex vivo* contenido en esta memoria de tesis inicia esta línea de investigación que abre nuevas perspectivas encaminadas por ejemplo, a la evaluación del consumo de

aceite de oliva a largo plazo en enfermos de EVP de países con dieta no Mediterránea, y a la evaluación del consumo de folatos y vitamina B6 y B12 en pacientes con EVP que presenten una hiperhomocisteinemia combinada.

Estudio de nuevas combinaciones de nutrientes en la consecución de una nutrición "a medida". Por último, el uso de alimentos funcionales como herramienta de alimentación saludable, abre un campo interesante en la prevención a largo plazo de este tipo de enfermedades. Un campo fascinante que incluye el estudio de nuevas combinaciones de nutrientes más efectivas en estas patologías y la identificación de nuevos nutrientes que puedan contribuir a dotar a la diete de un rigor científico que proporcione una nutrición óptima, saludable y personalizada para cada uno de nosotros. De seguro, este tipo de estudios e intervenciones constituirán un punto fuerte en la ciencia de la nutrición de los próximos años.

Conclusiones

1. El consumo continuado durante dos meses, de dosis dietéticas de ácidos grasos omega-3, ácido oleico, vitaminas B_6, B_{12}, E y ácido fólico administrados de manera conjunta en una matriz láctea, contribuyó a la reducción de diversos factores de riesgo de la enfermedad cardiovascular en población sana y con moderada hiperlipemia, pues redujo las concentraciones sanguíneas de colesterol total y LDL, homocisteína y formas solubles de moléculas de adhesión. La magnitud de estas reducciones es mayor cuando los valores de partida son elevados. Así, los niveles de triglicéridos se redujeron únicamente en los sujetos hiperlipémicos.

2. El consumo continuado durante un año, de pequeñas cantidades de ácidos grasos omega-3, ácido oleico, vitaminas B_6, B_{12}, E y ácido fólico administrados de manera conjunta en una matriz láctea, estabilizó el perfil lipídico y produjo un aumento sostenido en la distancia de claudicación y en el índice brazo-tobillo en un grupo de individuos afectados de enfermedad vascular periférica que presentaron claudicación intermitente. Esta mejoría clínica se correlacionó de manera positiva con la incorporación plasmática de los nutrientes suministrados.

3. El consumo de ácidos grasos poliinsaturados omega-3 produjo una alteración en el balance de producción de leucotrienos y tromboxanos, en monocitos estimulados con LPS procedentes de enfermos afectados de enfermedad vascular periférica. Esta alteración no se observó en los controles sanos.

Bibliografía

1. Potischman N, Freudenheim JL. Biomarkers of nutritional exposure and nutritional status: an overview. J Nutr. 2003;133 Suppl 3:873S-874S
2. Potischman N. Biologic and methodologic issues for nutritional biomarkers. J Nutr. 2003;133 Suppl 3:875S-880S.
3. Visioli, F, Rise P, Plasmati E, Pazzucconi F, Sirtori C, and Galli C. Very low intakes of n-3 fatty acids incorporated into bovine milk reduce plasma triacylglycerols and increase HDL-cholesterol concentrations in healthy subjects. Pharmacol. Res 2000; 41: 571-576
4. Expert group on vitamins and minerals (EVN). Safe upper levels for vitamins and minerals: report of the expert group on vitamins and minerals. ISBN 1-904026-11-7. Food Standards Agency Publications. May 2003.
5. Rader JI. Folic acid fortification, folate status and plasma homocysteine. J Nutr 2002; 132(8):2466S-2470S
6. Stanger O. (2002) Physiology of folic acid in health and disease. Curr Drug Metab. 3(2):211-23.
7. Hayes KC, Pronckuk A and Perlman D. Vitamin E in fortified cow milk uniquely enriches human plasma lipoproteins. Am J Clin Nutr 2001; 74: 211-218.
8. Meydani SN et al. Assessment of the safety of supplementation with different amounts of vitamin E in healthy older adults. Am J Clin Nutr 1998: 68(2):311-318
9. Morrissey, P.A & Sheehy, P.J. (1999) Optimal nutrition: vitamin E. Proc Nutr Soc. 58:459-468.
10. Kadiiska MB et al. Biomarkers of oxidative stress study: are plasma antioxidants markers of CCl(4) poisoning? Free Radic Biol Med 2000; 28: 838-845.
11. Cobiac L, Clifton PM, Abbey M, Belling GB and Nestel PJ. Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n-3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr 1991; 53: 1210-1216
12. Lawson LD, Hughes BG. Absorption of eicosapentaenoic acid and docosahexaenoic acid from fish oil triacylglycerols or fish oil ethyl esters co-ingested with a high-fat meal. Biochem Biophys Res Commun 1988; 156: 960-963.
13. Multiple Risk Factor Intervention Trial Group. Multiple risk factor intervention trial. Risk factor changes and mortality results. JAMA 1982; 248: 1465-1477.
14. Shepherd J, Cobe S, Ford I, Isles C, Ross A, Macfarlane P et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N Engl J Med 1995; 333: 1301-1307.
15. Hutter CM, Austin MA, Humphries SE. Familial hypercholesterolemia, peripheral arterial disease, and stroke: a HuGE minireview. Am J Epidemiol. 2004;160(5):430-5.
16. Banegas Banegas JR, Villar Álvarez F, Pérez de Andrés C, Jiménez García-Pascual R, Gil López E, Muñiz García J et al. Estudio epidemiológico de los factores de riesgo cardiovascular en la población española de 35 a 64 años. Rev San Hig Pub 1993; 67: 419-445.
17. Gutiérrez Fuentes JA, Gómez Gerique J, Gómez de la Cámara A, Rubio MA, García Hernández A, Arístegui I. Dieta y riesgo cardiovascular en España (DRECE II). Descripción de la evolución del perfil cardiovascular. Med Clin (Barc) 2000; 115: 726-729.
18. Third Report of the National Cholesterol Education Programme Expert Panel on “Detection, evaluation and treatment of high blood cholesterol in adults (ATP III)”. (2002) National Institutes of Health (NIH), publication number 02-5215.

19. Grundy, S.M. (2002) Low-density lipoprotein, non-high-density lipoprotein, and apolipoprotein B as targets of lipid-lowering therapy. Circulation. 106:2526-2529.
20. Feldman EB. Assorted monounsaturated fatty acids promote healthy hearts. Am J Clin Nutr 1999; 70: 953-4.;
21. Connor WE. Importance of n-3 fatty acids in health and disease. Am J Clin Nutr 2000; 71: 171S-5S.
22. Cobiac L, Clifton PM, Abbey M, Belling GB and Nestel PJ. Lipid, lipoprotein, and hemostatic effects of fish vs fish-oil n-3 fatty acids in mildly hyperlipidemic males. Am J Clin Nutr 1991; 53: 1210-16.
23. Kannel WB, Castelli W, Gordon T. Cholesterol in the prediction of coronary heart disease: new perspectives based on the Framingham Study. Ann Intern Med 1979; 90: 1985-1991.
24. Rose G. La Estrategia de la Medicina Preventiva. Barcelona: Masson-Salvat, 1994.
25. Cleophas TJ, Hornstra N, van Hoogstraten B, van der Meulen J. Homocysteine, a risk factor for coronary artery disease or not? A meta-analysis. Am J Cardiol. 2000; 86(9):1005-9, A8.
26. Cheng SW, Ting AC, Wong J. Fasting total plasma homocysteine and atherosclerotic peripheral vascular disease. Ann Vasc Surg. 1997;11(3):217-23.
27. Homocysteine Lowering Trialists' Collaboration. Lowering blood homocysteine with folic acid based supplements: meta-analisys of randomised trials. BMJ 1998; 316: 894-98.
28. van den Berg M, Franken DG, Boers GH, et al. Combined vitamon B6 plus folic acid in young patients with atherosclerosis and hyperhomocysteinaemia. J Vasc Surg 1994; 20: 933-40.
29. Bronstrup A, Hages M, Prinz-Langenohl R, Pietrzik K. Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women. Am J Clin Nutr 1998; 68(5):1104-10.
30. Wald DS, Law M, Morris JK. Homocysteine and cardiovascular disease: evidence on causality from a meta-analysis. BMJ 2002; 325(7374):1202.
31. Visioli F, Bellomo G, Montedoro G, Galli C. Low density lipoprotein oxidation is inhibited in vitro by olive oil constituents. Atherosclerosis. 1995;117(1):25-32.
32. Kratz M et al. Effects of dietary fatty acids on the composition and oxidizability of low-density lipoprotein. Eur J Clin Nutr 2002; 56(1):72-81.
33. Schnell JW, Anderson RA, Stegner JE, Schindler SP, Weinberg RB. Effects of a high polyunsaturated fat diet and vitamin E supplementation on high-density lipoprotein oxidation in humans. Atherosclerosis 2001; 159: 459-466.
34. Brown AA, Hu FB. Dietary modulation of endothelial function: implications for cardiovascular disease. Am J Clin Nutr. 2001;73(4):673-86.
35. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999; 340(2):115-26.
36. Cybulsky MI et al. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Invest 2001; 107: 1255-1262.
37. Yaqoob P, Knapper JA, Webb DH, Williams CM, Newsholme EA and Calder P. Effect of olive oil on immune function in middle-aged men. Am J Clin Nutr 1998; 67: 129-35.
38. Abe Y, El-Masri B, Kimball KT, Pownall H, Reilly CF, Osmundsen K, Smith CW, Ballantyne CM. Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion. Arterioscler Thromb Vasc Biol. 1998;18(5):723-31.

39. Seljeflot I, Arnesen H, Brude IR, Nenseter MS, Drevon CA, Hjermann I. Effects of omega-3 fatty acids and/or antioxidants on endothelial cell markers. Eur J Clin Invest. 1998;28(8):629-35.
40. Johansen O, Seljeflot I, Hostmark AT, Arnesen H. The effect of supplementation with omega-3 fatty acids on soluble markers of endothelial function in patients with coronary heart disease. Arterioscler Thromb Vasc Biol. 1999;19(7):1681-6.
41. Miles EA et al. Influence of age and dietary fish oil on plasma soluble adhesion molecule concentrations. Clin Sci 2001; 100: 91-100.
42. Upchurch GR, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF and Loscalzo J. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 1997; 272: 17012-17.
43. De Caterina R. Endothelial dysfunctions: common denominators in vascular disease. Curr Opin Clin Nutr Metab Care 2000; 3: 453-67.
44. Alessi MC, Juhan-Vague I. Contribution of PAI-1 in cardiovascular pathology. Arch Mal Coeur Vaiss. 2004 Jun;97(6):673-8.
45. Lopez-Segura F, Velasco F, Lopez-Miranda J, Castro P, Lopez-Pedrera R, Blanco A, Jimenez-Pereperez J, Torres A, Trujillo J, Ordovas JM, Perez-Jimenez F. Monounsaturated fatty acid-enriched diet decreases plasma plasminogen activator inhibitor type 1. Arterioscler Thromb Vasc Biol. 1996;16(1):82-8.
46. Spark JI, Robinson JM, Gallavin L, Gough MJ, Homer-Vanniasinkam S, Kester RC, Scott DJ. Patients with chronic critical limb ischemia have reduced total antioxidant capacity and impaired nutritional status. Eur J Vasc Endovasc Surg. 2002;24(6):535-9.
47. Maxwell AJ, Anderson B, Zapien MP, Cooke JP. Endothelial dysfunction in hypercholesterolemia is reversed by a nutritional product designed to enhance nitric oxide activity. Cardiovasc Drugs Ther. 2000;14(3):309-316.
48. Maxwell AJ, Anderson BE, Cooke JP. Nutritional therapy for peripheral arterial disease: a double-blind, placebo-controlled, randomized trial of HeartBar. Vasc Med. 2000;5(1):11-19.
49. Aguilera CM, Mesa MD, Ramirez-Tortosa MC, Nestares MT, Ros E, Gil A. Sunflower oil does not protect against LDL oxidation as virgin olive oil does in patients with peripheral vascular disease. Clin Nutr. 2004;23(4):673-81.
50. Mori TA, Vandongen R, Mahanian F, Douglas A. Plasma lipid levels and platelet and neutrophil function in patients with vascular disease following fish oil and olive oil supplementation. Metabolism. 1992;41(10):1059-67.
51. Vicaut, E. (1995) Opposite effects of red blood cell aggregation on resistance to blood flow. J. Cardiovasc. Surg. 36:361-368.
52. Thies, F., Garry, J.M., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C.P., Gallagher, P.J., Calder, P.C. & Grimble, R.F. (2003) Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial. Lancet. 361:477-485.
53. Merchant AT, Hu FB, Spiegelman D, Willett WC, Rimm EB & Ascherio A. (2003) The use of B vitamin supplements and peripheral arterial disease risk in men are inversely related. J Nutr. 133, 2863-2867.
54. Kuan YM, E Dear A & Grigg MJ. (2002) Homocysteine: an aetiological contributor to peripheral vascular arterial disease. ANZ J Surg 72, 668-671.

55. Boers GH. (1997) Hyperhomocysteinemia as a risk factor for arterial and venous disease. A review of evidence and relevance. Thromb Haemost 78, 520-522.

56. de Jong SC, Stehouwer CD, van den Berg M, Geurts TW, Bouter LM & Rauwerda JA. (1999) Normohomocysteinaemia and vitamin-treated hyperhomocysteinaemia are associated with similar risks of cardiovascular events in patients with premature peripheral arterial occlusive disease. A prospective cohort study. J Intern Med 246, 87-96.

57. Taylor LM Jr, Moneta GL, Sexton GJ, Schuff RA & Porter JM. (1999) Prospective blinded study of the relationship between plasma homocysteine and progression of symptomatic peripheral arterial disease. J Vasc Surg 29, 8-21.

58. Lentz SR & Sadler JE. (1991) Inhibition of thrombomodulin surface expression and protein C activation by the thrombogenic agent homocysteine. J Clin Invest 88, 1906-1914.

59. Mujumdar VS, Aru GM & Tyagi SC. (2001) Induction of oxidative stress by homocyst(e)ine impairs endothelial function. J Cell Biochem 82, 491-500.

60. Stamler JS, Osborne JA, Jaraki O, Rabbani LE, Mullins M, Singel D & Loscalzo J. (1993) Adverse vascular effects of homocysteine are modulated by endothelium-derived relaxing factor and related oxides of nitrogen. J Clin Invest 91, 308-318.

61. Wang J, Dudman NP & Wilcken DE. (1993) Effect of homocysteine and related compounds on prostacyclin production by cultured human vascular endothelial cells. Thromb. Haemost 70, 1047–1052.

62. de Vriese AS, Blom HJ, Heil SG, Mortier S, Kluijtmans LA, Van de Voorde J & Lameire NH. (2004) Endothelium-derived hyperpolarizing factor-mediated renal vasodilatory response is impaired during acute and chronic hyperhomocysteinemia. Circulation 109, 2331-2336.

63. Henke PK, Blackburn S, Proctor MC, Stevens J, Mukherjee D, Rajagopalin S, Upchurch GR Jr, Stanley JC & Eagle KA. (2004) Patients undergoing infrainguinal bypass to treat atherosclerotic vascular disease are underprescribed cardioprotective medications: effect on graft patency, limb salvage, and mortality. J Vasc Surg 39, 357-365.

64. Mukherjee D, Lingam P, Chetcuti S, et al. (2002) Missed opportunities to treat atherosclerosis in patients undergoing peripheral vascular interventions: insights from the University of Michigan Peripheral Vascular Disease Quality Improvement Initiative (PVD-QI2). Circulation 106, 1909-1912.

65. Cassar K, Coull R, Bachoo P, Macaulay E & Brittenden J. (2003) Management of secondary risk factors in patients with intermittent claudication. Eur J Vasc Endovasc Surg 26, 262-266..

Resumen

La aterosclerosis es una enfermedad progresiva caracterizada por la acumulación de lípidos en las capas íntima y media arterial que terminan por invadir la luz de las arterias dificultando la llegada de la sangre a los tejidos irrigados. La localización de este acúmulo de lípidos determina diversas manifestaciones clínicas, como la Enfermedad Cardiovascular (ECV) o la Enfermedad Vascular Periférica (EVP). Algunos de los factores de riesgo asociados a la aparición de la lesión aterosclerótica, son susceptibles de ser modificados y corregidos mediante una dieta equilibrada.

La evidencia científica avala el papel positivo que una alimentación saludable tiene en la reducción de alguno de los factores que desencadenan esta patología. El consumo frecuente de pescado (rico en ácidos grasos poliinsaturados omega-3), de aceite de oliva (rico en ácido oleico y antioxidantes naturales) y de frutas y verduras (ricas en vitaminas y antioxidantes) se asocia a una variedad de efectos beneficiosos que contribuyen a la prevención y paliación del proceso aterosclerótico. Diversos organismos internacionales establecen recomendaciones y estrategias para promover un modelo de alimentación saludable. Sin embargo, la sociedad moderna, tiende a alejarse de estas recomendaciones: 1) Aumentando el consumo energético y disminuyendo su gasto; 2) Aumentando el consumo de grasas saturadas, ácidos poliinsaturados omega-6 y ácidos grasos *trans*, disminuyendo el consumo de ácidos poliinsaturados omega-3, carbohidratos y fibra; 3) Disminuyendo el consumo de frutos y verduras, aumentando el de cereales; 4) Aumentando el consumo de proteínas y disminuyendo el de antioxidantes.

El presente proyecto de tesis tiene como objetivo evaluar la eficacia del consumo continuado de una mezcla de nutrientes considerados saludables (ácidos grasos omega 3, ácido oleico, ácido fólico y vitaminas A, D, E, B_6 y B_{12}) administrados de manera conjunta en un producto lácteo, en la reducción de diversos factores de riesgo de aterosclerosis en dos manifestaciones: la enfermedad cardiovascular y la enfermedad vascular periférica.

Se presenta un ensayo de intervención longitudinal, donde 30 voluntarios sanos normolipidémicos, consumieron durante 8 semanas 500 mL diarios del producto lácteo enriquecido con la mezcla de nutrientes antes descrito. La ingesta continuada de estos nutrientes se asoció a un incremento en las proporciones plasmáticas de ácido eicosapentanoico (EPA), ácido docosahexanoico (DHA) y de la concentración sérica de folatos. Las posibles implicaciones fisiológicas de estos incrementos pudieron ser responsables del descenso observado en la concentración de colesterol total, colesterol LDL, homocisteína total (tHcy) y en niveles circulantes de las moléculas de adhesión vascular (VCAM-1).

En el segundo ensayo presentado consistió una intervención similar sobre una cohorte de 30 voluntarios que presentaron al inicio del ensayo una hiperlipemia combinada moderada. El consumo continuado de la mezcla de nutrientes provocó similares incorporaciones al torrente sanguíneo que aproximaron el perfil lipídico de los sujetos a valores más saludables, observando disminuciones en las concentraciones plasmáticas de colesterol total, colesterol LDL y en este caso, también triglicéridos. Esto resultados llevan a sugerir la existencia de una regulación fisiológica de la acción de los nutrientes consumidos, en función del grado de alteración de la variable fisiológica.

La EVP es una patología frecuente que, de manera asintomática o manifestando dolor claudicante en las extremidades inferiores, se cree afecta a un 20% de la población mayor de 70 años. EVP y ECV guardan entre sí una relación causal, de manera que los pacientes con EVP tienen un riesgo 5 veces mayor de padecer un evento coronario agudo.

Existen muchas estrategias encaminadas a la prevención cardiovascular que involucran la adopción de una dieta saludable. Sin embargo, numerosos estudios publicados en los últimos años han puesto de manifiesto la falta de acciones específicas encaminadas tanto a la paliación de los síntomas propios de la EVP, como a la prevención de los eventos coronarios que generalmente acaban desarrollando estos enfermos.

Intervenciones nutricionales previas han descrito efectos beneficiosos sobre diversos factores de riesgo y sobre los síntomas presentes en los enfermos de EVP. Por ello, se realizó un estudio longitudinal, aleatorio y controlado de un año de duración. 60 enfermos de EVP sintomáticos (con una distancia de claudicación <200 m y un índice brazo-tobillo <0.70), fueron asignados a dos grupos que consumieron, como complemento a su prescripción médica y de manera diaria, bien 500 mL del producto enriquecido con la mezcla de nutrientes o bien idéntica cantidad de leche semidesnatada como grupo control. La ingesta continuada de cantidades reducidas de EPA, DHA, ácido oleico, ácido fólico y vitaminas E y B_6 durante 12 meses se tradujo en un incremento de sus niveles plasmáticos. Estos incrementos se correlacionaron positivamente con el aumento de la distancia de claudicación de los enfermos, que cuadriplicó sus valores tras la intervención. El índice brazo-tobillo también aumentó en este grupo, no observándose cambio alguno en el control. Para poder explicar la mejora clínica observada en el grupo intervenido, se observa un descenso gradual en los niveles de colesterol total y tHcy de aquellos pacientes que presentaban una hipercolesterolemia o una hiperhomocisteinemia al comienzo de la intervención. Asimismo, se observó una mejora de las defensas antioxidantes (ratio vitamina E / colesterol), una reducción en la concentración de apolipoproteina B y una reducción en proporción plasmática ácido araquidónico / EPA.

Esta mejora clínica lleva a plantear la búsqueda de los posibles mecanismos de acción por los que los nutrientes aportados en el producto lácteo pueden haber contribuido. Sin

embargo, al administrar una mezcla de nutrientes, no podemos explicar de una manera causal a cual o cuáles de estos nutrientes es debida.

Esto abrió una serie de perspectivas en nuestro grupo de investigación que comienzan por el diseño de un estudio *ex vivo* sobre monocitos estimulados con LPS procedentes de 30 pacientes afectados de enfermedad vascular periférica y 70 controles sanos, tras consumo de ácidos grasos omega-3 en forma de cápsulas. En este ensayo se intentó profundizar acerca de los posibles mecanismos de acción por los que los ácidos grasos omega-3 incluidos en la mezcla de nutrientes han podido contribuir a la mejora de los síntomas. Se observó que la población enferma presentaba un estado inflamatorio mayor que sus controles sanos. La intervención con cápsulas de aceite de pescado en dosis farmacológicas (6 g/día) durante 12 semanas consiguió incorporar de manera efectiva el EPA y el DHA a la composición estructural de los fosfolípidos de membrana en detrimento del ácido araquidónico. Esta incorporación a los tejidos se cree determina y dirige la respuesta inflamatoria hacia la síntesis de citocinas antiinflamatorias. Así, se observó que la intervención con cápsulas de omega-3 disminuyó la proporción de leucotrieno B4 frente a prostaglandina E2 en los enfermos de EVP pero no en los controles sanos. La alteración de la producción de eicosanoides hacia una respuesta anttinflamatoria podría haber participado en la mejora de los síntomas descritos en el estudio controlado.

Los estudios que conforman esta memoria de tesis, ofrecen nuevas evidencias acerca del papel que los nutrientes podrían desempeñar en la prevención y tratamiento de la aterosclerosis, y sugieren una herramienta de sencillo cumplimiento para el consumo constante y continuado de estos nutrientes saludables.

Summary

Atherosclerosis is the common form of arteriosclerosis in which deposits of yellowish plaques (atheromas) containing cholesterol, lipoid material and lipophages are formed within the intima and inner media of large and medium sized arteries, impairing tissues blood flow. The location of the atheroma plaque determines several clinical manifestations, such as cardiovascular disease (CVD) or peripheral vascular disease (PVD). There are some risk factors associated to the initiation and progression of the atherosclerotic lesion, that are modifiable ad preventable through healthy dietary habits.

There is plenty of scientific evidence to support the relationship between a healthy dietary habit and a lower atherosclerotic risk factors. A moderate-frequent intake of fish (rich in n-3 polyunsaturated fatty acids), olive oil (rich in oleic acid and natural antioxidants), fruits and vegetables (rich in vitamins and antioxidants) is associated to a variety of beneficial effects that contribute to the prevention and reduction of atherosclerosis. Several International Health Societies establish nutritional recommendations and related strategies to promote the adherence to a healthy diet. However, modern western societies do not meet these recommendations by: 1) Increasing energy consumption and decreasing its expenditure; 2) Increasing saturated fat intake, n-6 polyunsaturated fatty acid and trans fatty acids, and decreasing n-3 polyunsaturated fatty acids intake, carbohydrates and fiber; 3) Decreasing fruit and vegetable, and increasing cereal intake; 4) Increasing protein and reducing antioxidants intake.

This PhD Project evaluates the biochemical and clinical effects after a long-term consumption of small doses of n-3 polyunsaturated fatty acids, oleic acid, folic acid and B vitamins administered together in a functional dairy product, as a valid strategy for reducing atherosclerosis risk factors in two manifestations: CVD and PVD.

A longitudinal nutritional intervention is presented. 30 normolipidaemic healthy volunteers consumed daily 500 mL of the abovementioned enriched dairy product during 8 weeks. This daily intake of nutrients was associated with an increase in the plasma proportions of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and the plasma concentration of folate. The physiological implications of these plasma increases might have been responsible for the decreases in total and LDL cholesterol plasma concentrations, total homocysteine (tHcy) and soluble vascular cell adhesion molecule 1 (VCAM-1).

Another similar intervention trial is presented with 30 mildly hiperlipidemic volunteers. The daily intake of the fortified dairy product was responsible for similar incorporations to plasma of the nutrients supplemented. The intervention brought down the subject's lipid-profile to a healthier status, observing reductions in total and LDL cholesterol plasma concentrations, and

also triacylglicerols. These results suggest the possibility of a physiological regulation of the actions of nutrients depending on the degree of alteration of the variable they affect.

PVD is a common disease that, either asymptomatic or presenting with intermittent claudication in the lower limbs, affects approximately 20% of the population older than 70 years. PVD and CVD are closely related and connected, and PVD patients have a 5 fold increased risk to die from a acute coronary event.

There are many implemented strategies for cardiovascular prevention that involve the adoption of a healthy diet and a healthy lifestyle. However, numerous studies in the last years have evidenced the lack of specific actions for the reduction in PVD symptoms and for the prevention of coronary events in this kind of patients.

Previous nutritional interventions on PVD patients describe beneficial effects in the reduction of PVD symptoms and risk factors. For this reason, we performed a longitudinal, randomised controlled trial during one year. 60 symptomatic PVD patients (presenting a claudication distance <200 m and an ankle-brachial index <0.70), were randomly assigned to two nutritional intervention groups together with their usual drug prescription. One group consumed 500 ml/day of the dairy product enriched with the abovementioned nutritional mix. The other group consumed the same amount of semi-skimmed milk (control group). The long-term supplementation of dietetic amounts of EPA, DHA, oleic acid, folic acid and vitamins E, B_6 and B_{12}, incremented the plasma levels of all these nutrients in the supplemented group. These increases were positively associated with the increase in the claudication distance, that was more than tripled after the intervention. The ankle-brachial index was also augmented in this group, but no change in clinical symptoms was observed in the controlled group. In order to justify the clinical improvement found in the intervened group, a gradual decrease in total cholesterol and tHcy plasma concentration was observed in those patients presenting initial hypercholesterolaemia or hyperhomocysteinaemia. Moreover, antioxidant defences were improved (measured as ratio vitamin E / cholesterol), and apolipoprotein B or the ratio arachidonic acid / EPA, were also decreased.

The improvements in PVD symptoms led us to search for posible mechanisms of action by which nutrients in the fortified product might have been responsible for. However, by administering a nutrient mix, it is not possible to attribute the effects to an isolated nutrient. This study opened new research perspectives in our group, starting with the design of an *ex vivo* study performed in PBMCs stimulated with LPS from 30 PVD patients and 70 healthy matched controls, after fish oil capsules consumption. This study aims to contribute to elucidate possible mechanisms of action by which the n-3 polyunsaturated fatty acids included in the fortified dairy product might have contributed in the clinical improvement. It was observed that patients were undergoing a major inflammatory stress than controls. The 12-

week intervention with capsules containing pharmacological doses of fish oil (6 g/d) was able to effectively incorporate EPA and DHA to the membrane phospholipids in substitution of arachidonic acid. This tissue incorporation is believed to determine and address the inflammatory response towards the production of anti-inflammatory citokines. Thus, it was observed that after the fish oil intervention, the ratio leucotriene B4 to prostaglandin E2 was reduced in PVD patients but not in healthy controls. The change in eicosanoids production towards an antiinflammatory response might have contributed to the symptoms improvements earlier described.

The studies included in this Thesis, offer new evidences about the role of nutrients in the prevention and management of atherosclerosis-related diseases, and suggest an nutritional tool of easy compliance, to assure constant and long-term healthy nutrients intake.

Agradecimientos

Curriculum vitae y lista de publicaciones

Juan J Carrero es natural de Jerez de la Frontera (Cádiz). Obtuvo la Licenciatura de Farmacia en la Universidad de Granada (2000). En el año 2000 colaboró en el Departamento de Fisiología Vegetal de la Facultad de Ciencias bajo la supervisión de la Dra. Carmen Lluch Plá. En el año 2001 obtuvo una beca predoctoral para la realización de una Tesis Doctoral en el departamento de Bioquímica y Biología Molecular en colaboración con Puleva Biotech S.A. Obtuvo la Suficiencia investigadora con el tema: "La homocisteína y el riesgo cardiovascular. Revisión y puesta a punto de la determinación de Homocisteína total en plasma por HPLC". En el año 2003 obtuvo el grado Máster en Dirección y Administración de Empresas (Escuela de Negocios de Andalucía). En el año 2004 realizó una estancia predoctoral en la Universidad de Southampton (Reino Unido), Institute of Human Nutrition, bajo la dirección del Prof. RF Grimble.

1. Lluch C, **Carrero JJ**, Tejera N, Ocaña O, "Ecofisiología Vegetal", en "Interacción Planta-Microorganismos del Suelo: Simbiosis Fijadoras de Nitrógeno" Ed. Paraninfo, pags. 331-360, 2002. ISBN 84-9732-267-3.

2. **Carrero JJ**, Campos R, Tejera N, Ocaña O, Lluch C, "Efecto del estrés salino en la fijación de nitrógeno en el metabolismo oxidativo en nódulos de *Phaseolus vulgaris*", en "Avances en el Metabolismo del Nitrógeno: De la Biología Molecular a la Agronomía" Ed. Lankopi S.A, pags 361-368, 2002. ISBN 84-95075-88-1.

3. López-Huertas E, Baró L, **Carrero JJ**, Fonollá J, Jiménez J, Boza JJ. "n-3 fatty acids-enriched foods. Cardiovascular disease as a target". *Nutrafoods* (2003). 2(1) 13-19.

4. López-Huertas E, Baró L, **Carrero JJ,** Fonollá J, Jiménez J, Boza JJ. "n3-fatty acids: health effects and opportunities to increase intake". *AGROfood Industry Hi-tech* (2003). May/June 18-21.

5. **Carrero JJ,** J Fonollá, M González-Santiago, L Baró, Jiménez J, Boza JJ, López-Huertas E. "Health effects due to folic acid supplementation. Food fortification strategies to achieve adequate folate status" *Nutrafoods* (2004). 3(1) 5-13.

6. **Carrero JJ,** Baró L, Fonollá J, González-Santiago M, Martínez-Férez A, Castillo R, Jiménez J, Boza JJ, López-Huertas E. "Cardiovascular effects of milk enriched with omega-3 polyunsaturated fatty acids, oleic acid, folic acid, and vitamins E and B6 in volunteers with mild hyperlipidemia". *Nutrition*. (2004) 20(6): 521-527

7. **Carrero JJ,** Martín-Bautista E, Baró L, Fonollá J, Jiménez J, Boza JJ, López-Huertas E. "Efectos cardiovasculares de los ácidos grasos omega-3 y alternativas para incrementar su ingesta". *Nutr Hosp* (2005) 20(1): 56-62

8. **Carrero JJ,** López-Huertas E, Salmerón LM, Baró L, Ros E. "Daily supplementation with (n-3) PUFAs, oleic acid, folic acid and vitamins B-6 and E increases pain-free walking distance and improves risk factors in men with peripheral vascular disease" *J Nutr*, *In press*.

9. **Carrero JJ,** Salmerón LM, Ramos VE, Baró L, Ros E, López-Huertas E. "Combined strategy of simvastatin plus a nutritional supplementation with n-3 fatty acids, oleic acid, folic acid and B vitamins in the management of intermittent claudicants" *Submitted for publication*.

10. **Carrero JJ,** Madden J, Hadley J, Dastur N, Luu NT, Rainger R, Nash G, Shearman GP, Howell WM, Calder PC, Lopez-Huertas E, Grimble RF. "Fish oil alters the balance between leukotriene B4 and prostaglandin E2 production by LPS stimulated PBMCs in patients with peripheral vascular disease but not in healthy controls". *Submitted for publication*.

11. **Carrero JJ,** Grimble RF. "Does nutrition have a role in peripheral vascular disease? Potential implications of nutrient interactions" *Submitted for publication*.

Nutrición Hospitalaria

Nutr. Hosp. (2004) XIX (6) 364-370
ISSN 0212-1611 • CODEN NUHOEQ
S.V.R. 318

Original

La expresión de IL-10 interviene en la regulación de la respuesta inflamatoria por los ácidos grasos omega 3

S. Sierra, F. Lara-Villoslada, M. Olivares, J. Jiménez, J. Boza y J. Xaus

Departamento de Inmunología y Estudios Preclínicos. Puleva Biotech. Granada.

Resumen

Objetivo o antecedente: **Los ácidos grasos poliinsaturados son importantes para el organismo humano debido a su implicación en numerosas funciones biológicas. Las dietas occidentales se caracterizan por ser excesivamente ricas en ácidos grasos w-6 y pobres en ácidos grasos w-3.**

Los ácidos grasos de la serie w-3 son necesarios para el normal crecimiento y desarrollo del individuo así como para la regulación de la respuesta inmunológica. El objetivo de este estudio es analizar el efecto de una dieta enriquecida en ácidos grasos w-3 frente a un proceso inflamatorio así como el estudio de los mecanismos implicados en dicho efecto.

Intervenciones: **Para ello, ratones Balb/c fueron alimentados durante un mes con una dieta cuya fuente lipídica era 100% aceite de girasol (control), o con la misma dieta en la que el 12% de la grasa era aceite de pescado y el resto aceite de girasol (W-3). Doce horas antes de su sacrificio se indujo en una de las orejas de cada animal una dermatitis de contacto que cursó con inflamación y edema. Como agente inflamatorio se utilizó 2,4 dinitrofluorobenceno. Tras el sacrificio se tomaron diversas muestras y se analizaron.**

Resultados: **La inflamación, medida como peso y contenido de agua de las orejas, disminuyó significativamente en los ratones alimentados con w-3. La medida de la infiltración leucocitaria y los parámetros de oxidación revelaron también la mejora en el proceso inflamatorio de dichos ratones. Para explicar estos hechos se analizó la expresión de diversas citocinas, observándose un incremento de IL-10 y una disminución de citocinas tanto Th1 como Th2.**

Conclusiones: **Los ácidos grasos w-3 poseen un efecto inmunomodulador al actuar como antiinflamatorios y antialérgicos, al tiempo que aumentan algunas defensas del organismo. La citocina reguladora IL-10 podría ser la responsable del efecto antiinflamatorio ejercido por los ácidos grasos w-3.**

(*Nutr Hosp* 2004, 19:364-370)

Palabras clave: *Ácidos grasos poliinsaturados. Inflamación. Organismo.*

Correspondencia: J. Xaus.
Departamento de Inmunología y Estudios Preclínicos.
Puleva Biotech.
Camino de Purchil, 66. 18004 Granada.
E-mail: jxaus@pulevabiotech.es

Recibido: 21-I-2004.
Aceptado: 13-II-2004.

IL-10 EXPRESSION IS INVOLVED IN THE REGULATION OF THE IMMUNE RESPONSE BY OMEGA 3 FATTY ACIDS

Abstract

Introduction: **Polyunsaturated fatty acids play a key role in a huge number of biological functions. Western diets are highly rich in w-6 fatty acids. However the content of w-3 fatty acids is not suitable in those diets, despite of their importance in normal development of the human body and regulation of immune response.**

The aim of this work is to examine the effect of w-3 fatty acids enriched diet in the regulation of inflammatory response.

Material and methods: **Balb/c mice were fed either w-6 fatty acids rich diet (100% sunflower oil) or w-3 fatty acids fortified diet (12% fish oil plus 88% sunflower oil) during 28 days. Twelve hours prior to sacrifice, the mice were treated with 2,4-ninitro-1-fluorobezene on the left ear to induce the inflammatory reaction. Afterwards the mice were sacrificed and the different samples collected were analized.**

Results: **Ear inflammation of mice fed the w-3 diet was significantly lower. Leukocyte infiltration and oxidative stress were also lower in those mice. To explain these results, cytokine expression and plasma eicosanoid concentration were measured. An increase in IL-10 levels and a down regulation of Th1 and Th2 responses were observed in mice fed the w-3 diet.**

Conclusion: **Not only n-3 fatty acids exerts an antiinflammatory and an antialergical role but also they enhance some of the organism defenses.**

Our data suggest that w-3 fatty acids downregulate the inflammatory response by enhancing IL10 expression.

(*Nutr Hosp* 2004, 19:364-370)

Key words: *Polyunsaturated fatty acids. Antiinflammatory. Organism.*

NUTRAfoods
2004 3(1) 5-13

HEALTH EFFECTS OF FOLIC ACID SUPPLEMENTATION
Strategies to achieve adequate folate status

Juan Jesùs Carrero, Juristo Fonollá, Marìa Gonzàlez-Santiago, Luis Baró, Jesus Jimenez, Julio J Boza, Eduardo López-Huertas
Puleva Biotech, Granada, Spain

Key words

Folic acid
Folate
Homocysteine
Food fortification
Neural tube defects
Cancer

INTRODUCTION

Folate, a member of the vitamin B family, is a collective term for a number of structurally-related folic acid coenzymes. It has gained considerable attention in the last decades because of its presumed role in the pathogenesis of birth defects (**1**), cardiovascular diseases (**2**), cancer (**3**) and neuropsychiatric disorders (**4**).

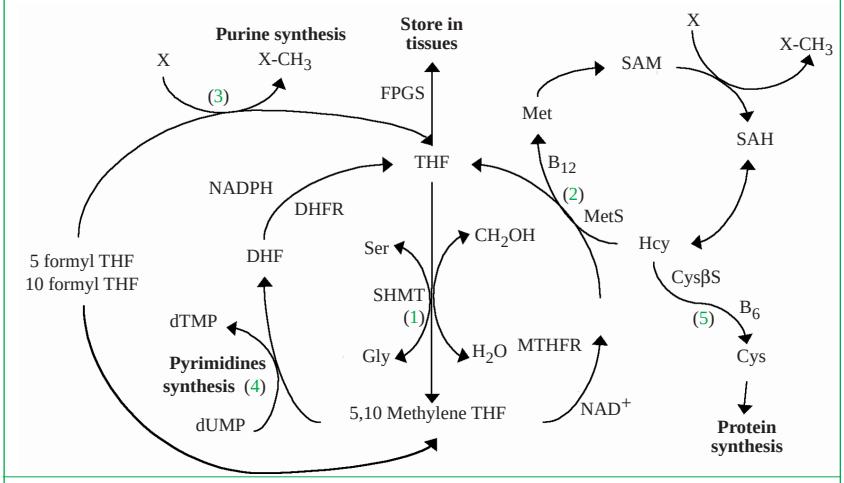
The various enzymes of the folate cycle, facilitate methylation reactions as well as the transfer of 'one-carbon units' from donor molecules needed for the methylation of homocysteine to generate methionine, the formation of purines and pyrimidines in the biosynthesis of DNA, and many other biological methylation reactions.

They also mediate the interconversion of serine and glycine, and play a role in histidine catabolism (***Fig 1***).

Although folate is present in a variety of foods, deficiency in folate is the most common vitamin deficiency in developed countries (**5**).

In this article, we will review the health effects derived from the consumption of folic acid, focusing on fortification strategies in order to enhance the daily intake of the population so as to meet Dietary Reference Intake (DRI) levels.

Figure 1 *Folate cycle metabolism, representing the major pathways in which folate facilitates methylation reactions as well as the transfer of 'one-carbon units' from several donors. Folate participates in the methylation process needed to regenerate serine from glycine (1) methionine from homocysteine (2), the formation of purines (3) and pyrimidines (4) in the biosynthesis of DNA, and many other biological methylation reactions (5)*



Abbreviations: THF: Tetrahydrofolic acid; DHF: Dihydrofolate; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; FPGS: Folylpolyglutamate synthase; MetS: Methionine synthase; CysβS: Cystathionine synthase; THFR: 5,10-Methylenetetrahydrofolate reductase; SHMT: Serinehydroxymethyl transferase; DHF: hydrofolate reductase

Eduardo López-Huertas
Puleva Biotech
66, Camino de Purchil,
Granada, 18004 Spain
tel 34.958.240 152
fax 34.958.240 160
email elopezhuertas@puleva.es

CEC Milan, Italy
tel +39.02.4152 943
fax +39.02.416 737
email info.nutrafoods@cec-editore.com
website www.cec-editore.com

VfCI Augsburg, Germany
tel +49.0821.325 830
fax +49.0821.3258 323
emai fischer@sofw.com
website www.sofw.com

Clinical Nutrition (2003) **22(2)**: 175–182

doi:10.1054/clnu.2002.0620

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ORIGINAL ARTICLE

n-3 Fatty acids plus oleic acid and vitamin supplemented milk consumption reduces total and LDL cholesterol, homocysteine and levels of endothelial adhesion molecules in healthy humans.

L. BARÓ,* J. FONOLLÁ,* J. L. PEÑA,* A. MARTÍNEZ-FÉREZ,† A. LUCENA,‡ J. JIMÉNEZ,* J. J. BOZA,* E. LÓPEZ-HUERTAS*

**Puleva Biotech S.A. 66, Camino de Purchil, Granada 18004, Spain; †Departamento de Ingeniería Química, Universidad de Granada, Granada 18071, Spain, ‡Hospital Universitario 'San Cecilio' 16, Av Dr Oloriz, Granada 18012, Spain. (Correspondence to: ELH, Puleva Biotech S.A. 66, Camino de Purchil, Granada 18004, Spain)*

Abstract—Background and aims: Numerous studies suggest *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) and oleic acid intake have beneficial effects on health including risk reduction of coronary heart disease. The purpose of this study was to evaluate the effect of a commercially available skimmed milk supplemented with *n*-3 PUFA, oleic acid, and vitamins E, B_6, and folic acid (Puleva Omega3®) on risk factors for cardiovascular disease. (CVD).

Methods: Thirty volunteers were given 500 ml/day of semi-skimmed milk for 4 weeks and then 500 ml/day of the *n*-3 enriched milk for 8 further weeks. Plasma and LDL lipoproteins were obtained from volunteers at the beginning of the study (T_{pre}), and at 4, 8 and 12 weeks.

Results: The consumption of *n*-3 enriched milk produced a significant decrease in plasma concentration of total and LDL cholesterol accompanied by a reduction in plasma levels of homocysteine. Plasma and LDL oxidability and vitamin E concentration remained unchanged throughout the study. A significant reduction in plasma levels of vascular cell adhesion molecule 1, and an increase in plasma concentration of folic acid were also observed.

Conclusion: Daily intake of *n*-3 PUFA and oleic acid supplemented skimmed milk plus folic acid and B-type vitamins has favourable effects on risk factors for CVD. © 2003 Elsevier Science Ltd. All rights reserved.

Key words: functional foods; supplemented milk; PUFAs; cardiovascular disease

Introduction

Cardiovascular disease (CVD) is the leading cause of death in Europe, the US and a major part of Asia. A variety of risk factors are associated with CVD, including high cholesterol levels, high plasma levels of homocysteine, hypertension, diabetes, low HDL-cholesterol levels and low levels of antioxidants, most of them influenced by diet (1). Beneficial effects of the Mediterranean diet on CVD are related to reduced saturated fat and high olive oil consumption (rich in oleic acid) and also to a high intake of fruit and vegetables, all of them rich in antioxidants. The consumption of monounsaturated fatty acids (MUFAs), especially oleic acid, has been shown to decrease plasma triacylglycerol and cholesterol concentrations, without affecting plasma HDL-cholesterol levels in healthy normolipidaemic subjects (2, 3).

A considerable number of research studies focus on CVD prevention by *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). *n*-3 PUFAs favourably affect atherosclerosis, coronary heart disease, inflammatory disease, and perhaps even behavioural disorders (4). Dietary *n*-3 PUFA have been reported to prevent CVD through a variety of actions. As they are incorporated into the cellular phospholipids, they produce less active forms of eicosanoids precursors with important physiological implications. Although the exact mechanism by which *n*-3 fatty acids exert an atheroprotective effect is still unclear, they present anti-inflammatory properties, prevent arrythmia, inhibit the synthesis of cytokines and mitogens, stimulate endothelial-derived nitric oxide, lower blood lipids and also inhibit atherosclerosis and thrombosis (5–8).

Atherosclerosis and inflammation share similar mechanisms in their early phases, involving increased interactions between vascular endothelia and circulating leukocytes, where vascular cell adhesion molecule 1 (VCAM-1) and intercellular cell adhesion molecule 1 (ICAM-1) play major roles (9).

Several studies suggest plasma levels of these adhesion molecules constitute a good marker for long-term prediction of cardiovascular events (10, 11). In this

Cardiovascular Effects of Milk Enriched With ω-3 Polyunsaturated Fatty Acids, Oleic Acid, Folic Acid, and Vitamins E and B6 in Volunteers With Mild Hyperlipidemia

Juan J. Carrero, MSc, Luis Baró, PhD, Juristo Fonollá, PhD, María González-Santiago, MSc, Antonio Martínez-Férez, PhD, Rafael Castillo, MD, Jesús Jiménez, PhD, Julio J. Boza, PhD, and Eduardo López-Huertas, PhD

From Puleva Biotech SA, Departamento de Ingeniería Química, Universidad de Granada, and Hospital Universitario "San Cecilio," Granada, Spain

OBJECTIVE: Results from epidemiologic studies and clinical trials have indicated that consumption of ω-3 fatty acids, oleic acid, and folic acid have beneficial effects on health, including decreased risk of cardiovascular disease. We evaluated the combined effects of these nutrients through the consumption of milk enriched with ω-3 polyunsaturated fatty acids, oleic acid, vitamins E and B6, and folic acid on risk factors for cardiovascular disease in volunteers with mild hyperlipidemia.

METHODS: Thirty subjects ages 45 to 65 y (51.3 ± 5.3 y) were given 500 mL/d of semi-skimmed milk for 4 wk and then 500 mL/d of the enriched milk for 8 wk. Plasma and low-density lipoproteins were obtained at the beginning of the study and at 4, 8, and 12 wk.

RESULTS: Consumption of enriched milk for 8 wk increased plasma concentrations of docosahexaenoic acid and eicosapentaenoic acid and significantly ($P < 0.05$) decreased plasma concentrations of triacylglycerol (24%), total cholesterol (9%), and low-density lipoprotein cholesterol (13%). Plasma and low-density lipoprotein oxidation and vitamin E concentration remained unchanged throughout the study. Significant decreases in plasma concentrations of vascular cell adhesion molecule-1 (9%) and homocysteine (17%) were found, accompanied by a 98% increase in plasma concentration of folic acid.

CONCLUSIONS: Dairy supplementation strategies with ω-3 polyunsaturated fatty acids, oleic acid, and vitamins may be useful for decreasing risk factors for cardiovascular disease. *Nutrition* 2004;20: 521–527. ©Elsevier Inc. 2004

KEY WORDS: enriched milk, ω-3 fatty acids, folic acid, homocysteine, cardiovascular disease

INTRODUCTION

There is a wealth of evidence from epidemiologic and clinical studies suggesting that modifications of dietary fat composition affect the risk of cardiovascular disease (CVD).[1] Consumption of ω-3 polyunsaturated fatty acids (ω-3 PUFAs), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has several beneficial properties that prevent CVD, including antiinflammatory, antiarrhythmic, and antihypertensive effects, and are especially valued for their capacity to decrease blood lipids, inhibit the synthesis of cytokines and mitogens, modulate endothelial function, stimulate endothelial-derived nitric oxide, and inhibit atherosclerosis and thrombosis.[2–5] Olive oil also is considered a healthy source of fat, and international nutritional guidelines recommend its consumption due to the cardiovascular beneficial effects reported.

Supplementation with certain nutrients such as folic acid and vitamins B6 and B12 also has come to be regarded as potentially protective against CVD. For instance, plasma homocysteine concentration, a novel risk factor for CVD, is decreased when the intake of these vitamins is increased.[6]

Health authorities have recommended increased consumption of PUFAs,[7] in which fish oil is especially rich. The most recent report by the World Health Organization[8] recommends regular fish consumption to provide approximately 200 to 500 mg/wk of EPA and DHA, replacement of saturated fat by monounsaturated fat, and increased consumption of fruits and vegetables to achieve proper antioxidant and vitamin status. However, modern Western societies tend to include very little fish, vegetables, and fruits in their diets, so ways to increase consumption of PUFAs and folic acid have to be explored and assessed at a community or clinical level.

An oil blend containing ω-3 PUFAs, olive oil, vitamins B6 and E, and folic acid was produced and included in skimmed milk to create a dairy product with the palatability of semi-skimmed milk but with a healthier fatty acid and vitamin profile. Milk, an everyday drink, is a very efficient vehicle for absorption of fat and lipid-soluble compounds because of its dispersion in micelles. In this 8-wk study, we tested the hypothesis that the substitution of regular milk (approximately 70% saturated fat) with this dairy product would have the potential to decrease cardiovascular risk factors in free-living, mildly hyperlipidemic subjects.

MATERIALS AND METHODS

To ensure analytical consistency, samples at the beginning of the study (T_{-4}) and at 8 wk (T_8) from the same volunteers were processed at the same time and analyzed in one batch when techniques involving high-performance liquid chromatography, gas-liquid chromatography, or spectrophotometry were used. For

Correspondence to: Eduardo López-Huertas, PhD, Puleva Biotech SA, 66, Camino de Purchil, Granada 18004, Spain. E-mail: elopezhuertas@pulevabiotech.es

0899-9007/04/$30.00
doi:10.1016/j.nut.2004.03.017