UNIVERSITAT DE BARCELONA

Efecto de la calidad de la dieta global en el proceso de envejecimiento utilizando diferentes estrategias de evaluación de la exposición dietética en población de personas mayores

Effect of overall diet quality on the aging process using different strategies for assessing dietary exposure in older population

Nicole Hidalgo Liberona

TESIS DOCTORAL

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Efecto de la calidad de la dieta global en el proceso de envejecimiento utilizando diferentes estrategias de evaluación de la exposición dietética en población de personas mayores.

Memoria presentada por Nicole Hidalgo Liberona para optar al título de doctor por la Universidad de Barcelona,

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RESUMEN

Se entiende por envejecimiento de la población al cambio de distribución en la población de un país hacia edades más avanzadas. En parte, esto viene dado por los avances de la sociedad en aspectos como la ciencia y la medicina, que han permitido aumentar la esperanza de vida en países desarrollados y en vías de desarrollo.
La vejez es un proceso fisiológico normal en el ciclo vital de las personas, sin embargo, este se asocia con una reducción de la productividad y a un aumento de los costes sanitarios dado que este segmento de la población tiene mayores tasas de morbilidad, entre ellas una alta prevalencia de enfermedades crónicas (enfermedades cardiovasculares, neurodegenerativas, y algunos tipos de cáncer).
Por estas razones es que entidades como la Organización Mundial de la Salud, desde hace un par de años vienen promoviendo el envejecimiento saludable y activo desde la edad adulta, en el cual la nutrición desempeña un rol fundamental.

El principal objetivo de esta tesis doctoral consiste en investigar el efecto de la calidad de la dieta en el proceso de envejecimiento, utilizando diferentes estrategias de valoración de la ingesta dietética (métodos convencionales, como los cuestionarios de consumo de alimentos, así como biomarcadores de exposición) para establecer su asociación con el estado de salud en las personas mayores. Este objetivo ha sido desarrollado a través de un estudio de intervención dietética en personas mayores y, en un estudio observacional prospectivo de 20 años de seguimiento para valorar el efecto de la ingesta alimentaria y del seguimiento de un patrón dietético saludable en la salud de las personas mayores.

Del estudio de intervención dietética MaPLE, en el cual se ha proporcionado 3 productos alimenticios ricos en polifenoles con el fin de suplementar la dieta con una cantidad que aumente al doble el consumo habitual que proporcionan los menús de la residencia de personas de edad avanzada, esta intervención se ha planteado el objetivo de valorar el efecto de la intervención en la mejorar de la permeabilidad intestinal (PI) (medida a partir de la zonulina, como marcador sustituto de la PI) así como en otros marcadores asociados a la inflamación sistémica.

En el estudio de cohorte InCHIANTI, se han llevado a cabo trabajos de análisis estadísticos que tienen por objetivo estudiar las asociaciones entre el consumo de los productos lácteos y su posible influencia en la prevención o mejora de la fragilidad, así como también estudiar la asociación del consumo de proteínas (de origen vegetal y animal) y la mortalidad a 20 años. Por último y teniendo en cuenta la importancia del uso de biomarcadores para valorar la exposición dietética en personas mayores, se ha desarrollado un panel de biomarcadores basado en la puntuación de la adherencia a la

Dieta Mediterránea (DM), y se ha valorado su potencial predictivo de la mortalidad a 20 años, comparado con la información reportada a través de cuestionarios dietéticos.

En el estudio de intervención se han llevado a cabo la estimación del aporte de polifenoles tanto de los menús proporcionados como de la ingesta real que han tenido los participantes del estudio, comparando el aporte de polifenoles de la dieta control y de la intervención. También, a partir de análisis metabolómicos se ha estudiado la biodisponibilidad de los polifenoles en personas mayores teniendo en cuenta los niveles de permeabilidad intestinal al tiempo basal de los participantes. Por último, se ha valorado el efecto de la intervención dietética con alimentos ricos en polifenoles y su rol en la mejora y/o mantención de la integridad intestinal de las personas de edad avanzada, así como otros marcadores clínicos incluyendo la presión arterial diastólica y la glicemia.

Los resultados de los análisis dietéticos en el estudio prospectivo InCHIANTI, han mostrado que el efecto del consumo de productos lácteos no tiene mayor influencia en la fragilidad, sin embargo, al estudiar la influencia de la ingesta de proteínas en el estado de salud se ha encontrado que una mayor ingesta de proteína animal se asocia con un menor riesgo de mortalidad en adultos mayores. Analizando más en profundidad el efecto de la alta adherencia a un patrón de dieta mediterránea valorada por métodos de evaluación dietética convencionales, como son los cuestionarios dietéticos, así como por un panel de biomarcadores representativo de la DM, hemos encontrado que una mayor adherencia a la DM evaluada mediante una puntuación de biomarcadores dietéticos se asoció con un menor riesgo de mortalidad en adultos mayores durante un seguimiento de 20 años.

Estos hallazgos son importantes, porque demuestran la utilidad del uso de un método de evaluación de la exposición dietética combinado, es decir que consideren tanto los cuestionarios dietéticos (CFCA, registro de alimentos por pesada, etc.) como los biomarcadores de exposición para la valoración de la ingesta en personas mayores. De la misma manera, el conocer el efecto del seguimiento de un patrón dietético rico en polifenoles, como es la dieta mediterránea, y su efecto en la preservación de la integridad intestinal y otros marcadores de salud, así como en la mortalidad a largo plazo. Nos proporcionan evidencia sólida y de calidad que podría contribuir a mejorar las recomendaciones dietéticas dirigidas a las personas mayores y de esta manera avanzar hacia el asesoramiento dietético individualizado.

ABSTRACT

Population ageing is defined as a shift in the distribution of a country's population towards older ages. In part, this is due to advances in society in areas such as science and medicine, which have increased life expectancy in developed and developing countries.
Old age is a normal physiological process in the life cycle of individuals; however, it is associated with reduced productivity and increased healthcare costs as this segment of the population has higher morbidity rates, including a high prevalence of chronic diseases (cardiovascular diseases, neurodegenerative diseases, and some types of cancer).
For these reasons, organizations such as the World Health Organization have been promoting healthy and active ageing from adulthood onwards, in which nutrition plays a fundamental role.

The main objective of this doctoral thesis is to investigate the effect of diet quality on the ageing process, using different dietary intake assessment strategies (conventional methods, such as food consumption questionnaires, as well as biomarkers of exposure) to establish their association with health status in the elderly. This objective has been developed through a dietary intervention study in older people and a 20-year prospective observational study to assess the effect of dietary intake and adherence to a healthy dietary pattern on the health of older people.

From the MaPLE dietary intervention study, in which 3 polyphenol-rich food products were provided to supplement the diet with an amount that doubles the usual intake provided by the menus of the elderly care home, this intervention aimed to assess the effect of the intervention on improving intestinal permeability (IP) (measured from zonulin, as a surrogate marker for IP) as well as on other markers associated with systemic inflammation.

In the InCHIANTI cohort study, statistical analyses have been carried out to study the associations between the consumption of dairy products and their possible influence on the prevention or improvement of frailty, as well as to study the association between protein consumption (of plant and animal origin) and 20-year mortality. Finally, considering the importance of using biomarkers to assess dietary exposure in the elderly, a biomarker panel based on the Mediterranean Diet (MD) adherence score has been developed and its predictive potential for 20-year mortality has been assessed, compared with information reported through dietary questionnaires.

In the intervention study, we estimated the polyphenol intake of both the menus provided and the actual intake of the study participants, comparing the polyphenol intake of the control and

intervention diets. The bioavailability of polyphenols in elderly people was also studied using metabolomic analysis, taking into account the levels of intestinal permeability at baseline of the participants. Finally, the effect of dietary intervention with polyphenol-rich foods and their role in improving and/or maintaining intestinal integrity in the elderly, as well as other clinical markers including diastolic blood pressure and glycaemia, have been assessed.

The results of dietary analyses in the prospective InCHIANTI study have shown that the role of dairy consumption has no major influence on frailty, however, when studying the influence of protein intake on health status it has been found that a higher intake of animal protein is associated with a lower risk of mortality in older adults. Moreover, we evaluated the adherence to a Mediterranean dietary pattern using conventional dietary assessment methods, such as dietary questionnaires as well as by a biomarker panel representative of Mediterranean Diet. We found that a higher adherence to Mediterranean Diet assessed by a dietary biomarker score was associated with a lower risk of mortality in older adults during a 20-year follow-up.

These findings are important because they show the usefulness of using a combined dietary exposure assessment method, i.e., considering both dietary questionnaires (CFCA, food-for-weighing register, etc.) and biomarkers of exposure for the assessment of intake in older people. In the same way, knowing the effect of following a dietary pattern rich in polyphenols, such as the Mediterranean Diet, and its role on the preservation of intestinal integrity and other health markers, as well as on long-term mortality. They provide us with solid, high-quality evidence that will contribute to improve the dietary recommendations for the older people by providing individualized dietary advice.

ÍNDICE

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

ABREVIATURAS

AGMI: Ácidos grasos mono insaturados

AGPI: Ácidos grasos poli insaturados

BDCA: Base de Datos de Composición de Alimentos

CFCA: Cuestionario de Frecuencia de Consumo de Alimentos

CIE-10 Clasificación Internacional y Estadística de Enfermedades y Problemas Relacionados con la Salud

DM: Dieta Mediterránea

DM2: Diabetes Mellitus tipo 2

DS: Desviación Estándar

ECNT: Enfermedades Crónicas No Transmisibles

ECV: Enfermedades Cardiovasculares

EFSA: European Food Safety Authority "Autoridad Europea de Seguridad Alimentaria"

EII: Enfermedad inflamatoria intestinal

EPIC: Estudio Prospectivo Europeo de Cáncer y Nutrición

EPOC: Enfermedad Pulmonar Obstructiva Crónica

HC: Hidratos de Carbono

IMC: Índice de Masa Corporal

InCHIANTI: Envejecimiento en Chianti

MaPLE:

MMSE: Mini Mental State Examination "Mini-Examen del Estado Mental"

OMS: Organización Mundial de la Salud

PAD: Presión Arterial Diastólica

PAS: Presión Arterial Sistólica

PREDIMED: Prevención con Dieta Mediterránea

PUFAs: Ácidos Grasos Poliinsaturados

RIQ: rango Intercuartílico

VGI: Valoración Geriátrica Integral

AF: Actividad física

IMC: Índice de masa corporal

ABVD: Actividades básicas de la vida diaria.

IC: Intervalo de confianza

IDR: Ingesta diaria recomendada

VCT: Valor calórico total

1. INTRODUCCIÓN

EPIDEMIOLOGÍA DEL ENVEJECIMIENTO

El envejecimiento de la población se refiere a los cambios en la composición por edades de una población que provoca un aumento del promedio de edad, y en consecuencia un incremento de la proporción de personas de edad avanzada. Así, cualquier motivo que contribuya a disminuir la proporción de niños y jóvenes hará que automáticamente aumente la proporción de adultos y personas mayores (1).

La edad escogida para demarcar a la población mayor suele estar relacionada con las instituciones gubernamentales y varía entre los 60 y 65 años. Definiendo así, el envejecimiento demográfico como el fenómeno que ocurre cuando un 10% de la población sobrepasa la edad establecida.

Las personas de 65 años o más constituyen el grupo de edad de más rápido crecimiento a nivel mundial. Casi todos los países prevén un aumento del porcentaje de personas mayores en su población. Según información proporcionada en el informe de las Naciones Unidas *"World Population Ageing 2019"*, el año 2019 había 703 millones de personas mayores de 65 años en el mundo (2), aun así, no es posible afirmar que existe una población envejecida a nivel mundial a partir de este dato debido a que representa un 9.1% de la población total.

En la tabla 1 se presentan los porcentajes de la distribución de población de 65 o más años, a nivel mundial, para países desarrollados como en vías de desarrollo y sus respectivas proyecciones de envejecimiento para los próximos años, en la cual se muestra que actualmente, la mayor parte de la población de personas mayores se concentra en las regiones de Asia oriental y sudoriental, Australia y Nueva Zelanda, Europa y América del Norte (con más de 200 millones de personas mayores), con porcentajes de población mayor de 65 años superiores al 10%. A pesar de este fenómeno casi mundial, en las regiones de África subsahariana y Oceanía (exceptuando a Nueva Zelanda y Australia) se observan cifras de población envejecida bastante bajas (3.0% y 4.2%, respectivamente).

Las proyecciones para el año 2050 prevén que el panorama de envejecimiento cambie completamente a nivel mundial proyectándose un cambio demográfico que será encabezado por la región europea y de América del Norte llegando al 26.1% de su población con 65 años o más (2,3).

Tabla 1. Porcentaje de personas de 65 años o más por región geográfica, 2019 y 2100

Región	2019	2030	2050	2100
Mundo	9.1	11.7	15.9	22.6
África subsahariana	3.0	3.3	4.8	13.0

África del Norte y Asia Occidental	5,7	7,6	12,7	22,4
Asia central y meridional	6,0	8,0	13,1	25,7
Asia oriental y sudoriental	11,2	15,8	23,7	30,4
América Latina y el Caribe	8,7	12,0	19,0	31,3
Australia/Nueva Zelanda	15,9	19,5	22,9	28,6
Oceanía*	4,2	5,3	7,7	15,4
Europa y América del Norte	18,0	22,1	26,1	29,3

Fuente: Adaptado de Perspectivas de la población mundial 2019: aspectos destacados

*Excluye Australia y Nueva Zelanda

En definitiva, se prevé que entre 2019 y 2050, el número de personas de 65 años o más a nivel mundial será más del doble, mientras que el número de niños menores de cinco años se mantendrá relativamente sin cambios. Además, se prevé que en 2050 se llegará a tener unos 1.500 millones de personas de 65 años o más en todo el mundo y que este grupo etario superará también en número a los adolescentes y jóvenes de 15 a 24 años (1.300 millones).

De acuerdo a datos presentados por el informe de las Naciones Unidas, en el año 2019 el 38% de todas las personas de 80 años o más residen en Europa y América del Norte, un porcentaje que se espera que disminuya al 26% en 2050 y al 17% en 2100, ya que se espera que la edad de la población de otras regiones siga aumentando (2).

Dentro de las causas que condicionan el envejecimiento de la población, se puede destacar: la disminución de la mortalidad infantil, la reducción de la fecundidad y en consecuencia de la natalidad, y el aumento de la esperanza de vida y la longevidad. Estos factores permiten que las poblaciones en casi todos los países y zonas estén envejeciendo de edad. De hecho, según datos de las Naciones Unidas, en 2018 por primera vez en la historia de la humanidad, las personas de 65 años o más superaron en número a los niños menores de cinco años en todo el mundo. A esto, se le debe sumar el efecto de las migraciones, otro factor social que experimentan algunas sociedades y que afectan la distribución de sus pirámides poblacionales, con claros efectos a nivel local con el vaciamiento y envejecimiento de las zonas rurales (debido al éxodo de población adulta joven desde zonas rurales hacia las zonas urbanas), situación que ha quedado de manifiesto en gran parte de los países desarrollados.

El envejecimiento del conjunto de la población es un hecho difícilmente reversible, aunque podría llegar a darse a través de dos posibles mecanismos: a) por un aumento del número de nacimientos que aumentaría la población joven o, b) por un crecimiento de la inmigración de población joven y

adulta que sobrepase el número de población mayor. Si bien estos mecanismos, no disminuirán el número de población mayor de 65 años en términos absolutos, lo hará porcentualmente, y como se sabe el envejecimiento de la población es un porcentaje relativo que depende de la distribución total de la población. Planteando la necesidad de ofrecer mejoras y alternativas con visión de edad en los diferentes ámbitos que afectan la vida cotidiana, como pueden ser aspectos psicosociales y de salud incluyendo aspectos relacionados con la alimentación y nutrición de las personas mayores.

i. Impacto de la pandemia por COVID-19 en el envejecimiento demográfico

El 11 de marzo de 2020, la Organización Mundial de la Salud (OMS) declaró una pandemia causada por la enfermedad por coronavirus (COVID-19), originada por el nuevo coronavirus del síndrome respiratorio agudo severo 2 (SARS-CoV-2)(4).

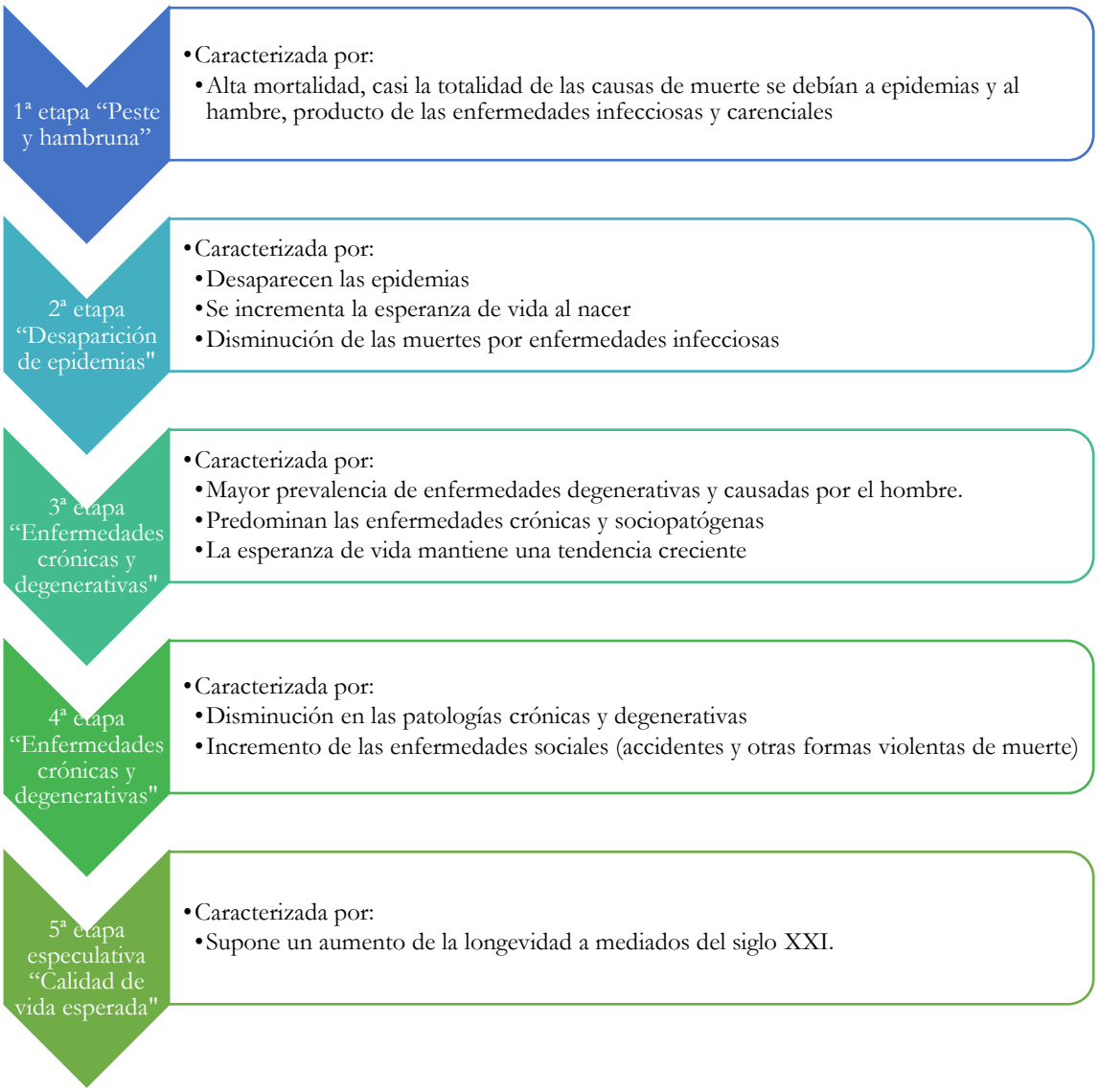
La COVID-19 se ha convertido en una de las principales causas de enfermedad y muerte, provocando un número considerable de muertes adicionales de forma indirecta a nivel mundial, regional y nacional. Estimaciones preliminares de la Organización Mundial de la Salud (OMS) sugieren que el exceso de muertes totales en el mundo atribuibles a la COVID-19, tanto directa como indirectamente, ascienda al menos a 3 millones en el año 2020, situando a la COVID-19 entre las 10 principales causas de muerte a nivel mundial. En la región Europea se ha estimado un exceso de mortalidad entre 1,11-1,21 millones de muertes, el doble de las 590 000 muertes reportadas por COVID-19 (5), asimismo, ha generado un impacto en la morbilidad a largo plazo y que recién se está estudiando (6).

Los datos muestran que los casos y las muertes por COVID-19 también varían entre grupos etarios de población dentro de los países, incluyendo los diferentes grupos de edad. Del total de casos de COVID-19, alrededor del 60% se presentaron entre las edades de 20 a 60 años, tanto para los hombres como para las mujeres, sin embargo, la distribución por edad del número de muertes por COVID-19 muestra un patrón muy diferente, dado que éste aumenta con la edad y es más alto para las personas de 80 años o más (que representan un tercio de todas las muertes, tanto en hombres como en mujeres) (5,7,8), quedando en evidencia que afecta en forma desproporcionada a las personas mayores, especialmente a las que padecen enfermedades no transmisibles o viven en centros de atención geriátrica (9). Sin embargo, durante la pandemia de COVID-19, se ha ido descubriendo una vulnerabilidad asociada a la edad en la carga de la enfermedad (10–12).
Según datos de un estudio multicéntrico realizado en España, la tasa de fatalidad de los pacientes mayores hospitalizados estaba sobre el 49% e incrementaba con la edad (13). En otro estudio realizado en población inglesa en la cohorte UK Biobank, se encontró una asociación exponencial entre la edad y la mortalidad por COVID-19, en la cual más de un tercio del exceso de riesgo de mortalidad de los adultos de mayor edad estaba mediado por una peor función pulmonar, hipertensión, debilidad muscular y multimorbilidad. Entre los participantes de mayor edad, estos factores eran más comunes y estaban más fuertemente asociados con una mayor mortalidad por COVID-19 (11).

ENVEJECIMIENTO Y SALUD

La transición demográfica ha sido posible gracias a la transición epidemiológica ocurrida a lo largo siglo XX. Se han estudiado las causas y consecuencias que producen estos cambios en cuanto a modelos de morbimortalidad, de acuerdo a los diferentes modelos (occidental, acelerado, retardado y de transición) propuestos por Omran en 1971 y 1998 (14,15), definiéndose en diferentes etapas (Figura 1):

Figura 1. Modelo occidental de transición epidemiológica, Omran 1971, 1998.



Fuente: Elaboración propia basada en propuesta de Omran (14,15)

Es así como la transición epidemiológica tiene un efecto del envejecimiento poblacional, no obstante, debe quedar claro que el envejecimiento es un proceso fisiológico normal y no es un proceso patológico, a pesar de que el envejecimiento de las personas lleve asociado a una mayor incidencia y gravedad de enfermedades.

En los seres humanos, la vejez es aceptada convencionalmente como una etapa del ciclo vital que inicia alrededor de los 65 años y que acaba con la muerte, sin embargo, es complejo circunscribir sus límites a nivel fisiológico. La vejez no tiene un punto de inicio determinado y/o evidente, dado que se produce en algún punto después de que cesa el proceso de crecimiento y desarrollo y continúa progresivamente según las características de cada individuo. De tal forma que se entiende el envejecimiento como un proceso heterogéneo que depende de factores ambientales y sociales a los que se ve sometida la persona a lo largo de los años, y por esta misma razón, la tasa de envejecimiento puede ser variable en función de las características genéticas y epigenéticas de cada individuo (16,17).

Además, los cambios acontecidos en el proceso de envejecimiento carecen de linealidad y de uniformidad, tanto entre los individuos de la población como en una misma persona, lo cual quiere decir que, el inicio y la magnitud de los cambios varían según el tipo de célula, tejido, órgano y sistema o parámetro de laboratorio utilizado para medir el cambio (18). Es así como, en algunas funciones las respuestas homeostáticas parecen ser eficientes en edades avanzadas, en cambio otras, parecen disminuir a edades más tempranas. Algunos ejemplos de este tipo de regulación diferenciada por la edad cronológica son la concentración posprandial de glucosa en sangre, la cual permanece estable hasta alrededor de los 70-90 años, por el contrario, la tasa metabólica basal va disminuyendo progresivamente con el paso del tiempo a lo largo de toda la vida (19).

Para el estudio del envejecimiento se han propuesto teorías desde perspectivas moleculares, celulares y orgánicas que han intentado dar explicación a través de la evidencia científica (20). Aunque muchas de estas teorías propuestas han sido abandonadas debido a falta de apoyo experimental o porque los resultados no han sido extrapolables a otros modelos de estudio, por ejemplo, de modelos animales a humanos. Aun así, existen diversas hipótesis que proponen una relación directa entre el envejecimiento, la programación genética, el acortamiento telomérico que ocurre en la división, y en la muerte celular por apoptosis, y aunque es posible que todos estos mecanismos estén implicados en el desarrollo de enfermedades asociadas al envejecimiento, el papel de estos procesos en el envejecimiento normal de los organismos aún está en estudio (21).

En el año 2013, López-Otín et al. (22). propusieron nueve rasgos o marcas distintivas que, en general se considera, contribuyen al proceso de envejecimiento y que, en conjunto, determinan el fenotipo

del envejecimiento. Cada una de estas marcas que responden a cambios moleculares y celulares, debe cumplir idealmente los siguientes criterios para ser considerada como tal:

1. Debe manifestarse durante el envejecimiento normal;
2. Su agravamiento experimental debe acelerar el envejecimiento;
3. Su mejora experimental debería retardar el proceso normal de envejecimiento y, por lo tanto, aumentar la esperanza de vida saludable.

Además, estas 9 marcas del envejecimiento propuestas por López-Otín et al. (22). (Figura 2), se han vinculado con alteraciones metabólicas que modulan el proceso de envejecimiento, dado que las numerosas alteraciones metabólicas acumuladas a través del tiempo producen una reducción del *fitness* biológico, lo cual sugeriría un control metabólico del envejecimiento.

Figura 2. Marcas distintivas del envejecimiento

Fuente: Obtenido de la publicación de López-Otín et al. (22)

Estas marcas están fuertemente interconectadas, tanto es que los autores las han categorizado en 3 grupos. Las **marcas primarias**, son marcas biológicas que incluyen: i) la inestabilidad genómica (daño en el ADN); ii) el acortamiento de los telómeros; iii) las alteraciones epigenéticas; y ix) la pérdida de

la proteostasis. Estas marcas son consideradas como desencadenantes iniciales que tienen consecuencias negativas para el organismo, además de acumularse progresivamente.

Luego están las **marcas antagonistas**, las cuales tienen efectos opuestos dependiendo de su intensidad o gado de alteración, aquí se incluyen las marcas relacionadas a v) la desregulación en la señalización de nutrientes, vi) la disfunción mitocondrial, y vii) la senescencia celular. De acuerdo con la literatura, se ha observado que niveles bajos de estas marcas pueden mediar efectos beneficiosos, mientras que niveles altos serían promotores de eventos perjudiciales. Específicamente este tipo de eventos cumplen una función biológica de protección enfrente de daños o de la escasez de nutrientes, sin embargo, su incremento crónico y exagerado también podría favorecer dicho daño. Por último, las marcas antagonistas, que inicialmente se tornan como protectoras se pueden ver afectadas negativamente de forma progresiva por procesos modulados por las marcas primarias.

La tercera categoría está compuesta por **marcas integrativas**, que afectan la homeostasis del tejido y a su función, aquí se incluyen: viii) el agotamiento de las células madre y, ix) las alteraciones en la comunicación intercelular. Estas marcas ocurren cuando el daño acumulado es causado por las marcas primarias y antagonistas en conjunto, provocando una afectación en la homeostasis del tejido y dicho daño no puede ser compensado.

Debido a que aún no está del todo clara la interconexión de cada una de estas marcas, y que muchos de los estudios de los mecanismos biológicos provienen de estudios realizados en modelos animales son necesarios más estudios que consideren estos mecanismos propuestos de forma individual o colectiva (20,22,23). Sin embargo es necesario conocer los mecanismos implicados en el proceso de envejecimiento, para establecer vínculos entre su posible relación con el desarrollo de enfermedades crónicas, lo cual proporciona evidencia clave para elaborar enfoques de prevención respecto a las enfermedades asociadas a la edad.

i. Comorbilidades

La morbilidad es un término que hace referencia a la proporción de personas que enferman en un lugar y tiempo determinado. La tasa de morbilidad aumenta con la edad, esto quiere decir que las tasas de morbilidad más bajas corresponden al tramo de edad entre 5 a 24 años y las tasas de morbilidad más altas al tramo de edad superior o igual a 85 o más años (24,25).

Desde inicios del siglo XX, ha habido un cambio en los patrones de enfermedad, donde la prevalencia de enfermedades infecciosas ha dado paso a enfermedades crónicas y dependientes de la edad, como las cardiovasculares, neurodegenerativas y el cáncer. Estas enfermedades crónicas son la principal

causa de incremento en los años perdidos por mortalidad prematura y años vividos con discapacidad (*Disability Adjusted Life Years, DALYs*, por sus siglas en inglés) (5).

Aunque no existe una definición consensuada universal para la morbimortalidad, la presencia de dos o más enfermedades crónicas de forma simultánea parece la definición más aceptada. En el caso de las personas mayores, éstas presentan habitualmente una serie de enfermedades crónicas concomitantes como diabetes mellitus, patologías cardiovasculares (hipertensión, insuficiencia cardíaca, isquemia cardíaca, etc.), osteoporosis, enfermedades neurodegenerativas, y algunos tipos de cáncer. De acuerdo a datos de una revisión sistemática realizada por Salive M (26), reportó la prevalencia de multimorbilidad estaba alrededor de un 62% para las personas mayores de 65 años y que esta aumentaba con la edad, llegando hasta el 81,5% para los de mayores de 85 años, afectando negativamente la condición clínica de las personas mayores, aumentando la dependencia y la discapacidad, razón que los convierte además en potenciales consumidores de un elevado porcentaje de recursos sanitarios (27), además de estar asociada a un elevado riesgo de muerte (28).

Tanto es así que, el envejecimiento poblacional representa un desafío para el sistema de salud por el aumento de personas mayores y la mayor incidencia y prevalencia de enfermedades, entre ellos los síndromes geriátricos, los cuales son definidos como condiciones de salud no específicas de causa multifactorial, relacionadas con el envejecimiento y la disminución de la reserva funcional, y que se asocian con eventos graves en salud, requiriendo atención médica especializada.

ii. Fragilidad

El proceso de envejecimiento provoca la pérdida progresiva de algunas reservas homeostáticas en los sistemas fisiológicos ante cualquier agente estresor, lo cual resultará en la descompensación de la persona mayor desencadenando el síndrome de fragilidad. Éste puede desarrollarse debido a factores como influencias sociodemográficas (por ejemplo, pobreza, vivir solo, bajo nivel educativo); factores psicológicos (por ejemplo, depresión); malnutrición (por ejemplo, desnutrición); polimedicación; enfermedades y complicaciones (por ejemplo, estados inflamatorios, cáncer, trastornos endocrinos, demencia); y sedentarismo (29).

Una primera aproximación a la definición de la fragilidad fue hecha por Campbell y Buchner quienes definieron la fragilidad como "una condición o síndrome que resulta de una reducción multisistémica de la capacidad de reserva hasta el punto de que un número de sistemas fisiológicos están cerca, o

más allá, del umbral del fracaso sintomático y como resultado, la persona frágil tiene un mayor riesgo de discapacidad o de muerte a causa de pequeñas tensiones externas"(30).

Posteriormente, Fried et al. (31). definió la fragilidad como: "un síndrome fisiológico, caracterizado por una reducción de las reservas y de la resistencia a agentes estresores, que resulta de la acumulación del pobre rendimiento de diferentes sistemas fisiológicos, lo que a su vez conduce a que el organismo esté más susceptible a consecuencias adversas". Otro enfoque fue propuesto por Rockwood et al. (32), en el cual plantea un modelo basado en la acumulación de déficits que operativiza la fragilidad como una colección de signos y síntomas, comportamientos de salud, diagnósticos y limitaciones funcionales: incluyendo trastornos cognitivos, síntomas depresivos, funcionalidad reducida, múltiples enfermedades, malnutrición, aislamiento social; con una elevada acumulación que acelera el envejecimiento del organismo.

Otras definiciones más recientes, refieren a la fragilidad como un "síndrome multidimensional de pérdida de reservas homeostáticas (energía, capacidades físicas y mentales), que promueve la acumulación de déficits, aumentando la sensibilidad y el riesgo del paciente a consecuencias médicas adversas" (33,34). Una definición consensuada por las sociedades de geriatría actuales la definen como: "un síndrome clínico multicausal, caracterizado por la disminución de la fuerza, la resistencia y la reducción de los procesos fisiológicos, lo que aumenta la susceptibilidad del individuo al desarrollo de la dependencia y/o la muerte" (35). De hecho, desde el año 2013 se recomienda realizar una identificación de la fragilidad en personas mayores de 70 años con el fin de promover un modelo de atención holístico y de mejorar el diseño de los planes de intervención.

En esta tesis doctoral, se trabajará con el enfoque de fragilidad propuesto por Fried et al.(31), quienes propusieron una definición fenotípica de fragilidad, la cual se derivó y validó a partir del estudio longitudinal *Cardiovascular Health Study* basado en 5.317 hombres y mujeres estadounidenses mayores de 65 años, sobre la base de la predicción prospectiva de resultados adversos durante varios años posteriores. Las variables evaluadas se basaban en la propuesta de cinco parámetros representativos que evidenciaban una función fisiológica reducida clínicamente relevante. Las cinco variables estudiadas fueron:

1. Pérdida de peso involuntaria
2. Fatiga auto reportada o agotamiento
3. Disminución de la realización de actividad física
4. Fuerza de agarre* o debilidad muscular
5. Velocidad de la marcha* o lentitud

*Marcador de deterioro (en comparación con los estándares por edad)

Aunque estas características clínicas se asocian a menudo con enfermedades y/o son sintomatología que representan diferentes patologías. La definición de fragilidad se basa en la presencia de tres de estas cinco variables, mientras que el estado intermedio o subclínico de pre-fragilidad se basa en la presencia de una o dos de estas variables. Este enfoque fenotípico está realizado en base a la propuesta de que la fragilidad es un síndrome con cambios fisiológicos y metabólicos subyacentes, que pueden estar interrelacionados y que son responsables de conducir a un deterioro físico y/o cognitivo progresivo hasta la pérdida de la capacidad funcional, a menudo potenciado por enfermedades o lesiones agudas o crónicas(31).

La formulación de una definición única y común de síndrome de fragilidad parece ser importante tanto desde el punto de vista científico como clínico, ya que en la práctica clínica podría ser útil para utilizarlo como métodos de cribado.

En Europa se estima que alrededor del 17% de las personas presentan algún síntoma de fragilidad (36). Es de tener en cuenta que los cambios del síndrome de fragilidad se asemejan y a menudo se solapan con los cambios asociados al proceso de envejecimiento fisiológico del organismo.
De acuerdo con la bibliografía reciente, basada en revisiones sistemáticas y metaanálisis que han evaluado el valor predictivo de la fragilidad en personas mayores, se observa que el estado de fragilidad o pre-fragilidad incrementa de 1.2 a 1.8 veces el riesgo de hospitalización, así como de 1.8 a 2.3 veces el riesgo de mortalidad .

iii. Inflamación de bajo grado asociada al envejecimiento

Se han identificado varios factores posibles que inician y mantienen una respuesta inflamatoria de bajo grado, entre ellos se encuentran el envejecimiento, una dieta desequilibrada, un nivel bajo de hormonas sexuales y el tabaquismo e incluso una alteración de la integridad intestinal. Actualmente existe la hipótesis que asocian la inflamación y la inflamación molecular con el envejecimiento, término conocido como "*Inflammaging*", la cual podría estar desencadenada por un "intestino permeable", es así que la hipótesis de que una alteración de la integridad intestinal (que da paso a un aumento de la permeabilidad intestinal) podría inducir a la translocación de compuestos potencialmente nocivos (bacterias, toxinas, y residuos), llevando a una toxicidad que dará paso a la inducción de señales proinflamatorias en todo el organismo (37). Sugiriendo que la inflamación

crónica en sujetos de edad avanzada aumenta el riesgo de afecciones patológicas y enfermedades relacionadas con la edad. Los sujetos de edad avanzada tienen niveles constantemente elevados de citoquinas inflamatorias, especialmente la interleucina-6 (IL-6) y el factor de necrosis tumoral-α (TNF-α), así lo demuestran los aumentos de 2 a 4 veces en los niveles séricos de varios mediadores inflamatorios en sujetos de este grupo etario (38).

Se ha demostrado una asociación significativa y consistente que demuestran que concentraciones circulantes elevadas de marcadores y mediadores de la inflamación en personas mayores pueden inducir a la atrofia muscular y cáncer a través de daños en el ADN. Así mismo, la obesidad, también favorece que el tejido adiposo visceral produzca tanto IL-6 como TNF-α, afectando al metabolismo sistémico). Debido a que la inflamación crónica está tan amplia y se plantea que está profundamente implicada en muchos trastornos crónicos relacionados con la edad, como la aterosclerosis, la diabetes, la obesidad, la sarcopenia y la enfermedad de Alzheimer, sin embargo es necesario establecer las bases fisiopatológicas para la inflamación crónica en relación con el proceso de envejecimiento (39,40).

Ante la evidencia presente, es de tener en cuenta que muchas de estas patologías crónicas inducidas por la inflamación crónica de bajo grado, pueden prevenirse parcialmente con modificaciones en el estilo de vida, como el seguimiento de una dieta saludable.

iv. Mortalidad

La mortalidad es un indicador del número de personas fallecidas en una población. Está estrechamente relacionada con el concepto de morbilidad, dado que esta última puede desencadenar la mortalidad.
Aunque cada vez hay más evidencia a favor de que la funcionalidad, más que la multimorbilidad, es el principal predictor de mortalidad en personas mayores de 80 años, dado que es el principal factor que modula la asociación del curso de las enfermedades crónicas con resultados adversos.

A nivel mundial, 7 de las 10 causas principales de muerte en el año 2019 fueron enfermedades no transmisibles. Estas 7 causas principales representaron el 44% de todas las muertes. Un 74% de las muertes a nivel mundial durante el año 2019, correspondieron al conjunto de enfermedades no transmisibles.

Figura 3. Principales causas de muerte en el mundo, año 2019.

Fuente: Estimaciones globales OMS(41)

v. Capacidad intrínseca

La capacidad intrínseca es un concepto propuesto e introducido por la Organización Mundial de la Salud (OMS) en el informe de Envejecimiento y salud del año 2015, donde plantea un indicador operacional de salud con enfoque en un envejecimiento saludable.

La capacidad intrínseca tiene como objetivo medir las capacidades -y no los déficits- de múltiples sistemas biológicos humanos basados en las funciones corporales más relevantes. Se define como el conjunto de todas las capacidades físicas y mentales a las que puede recurrir un individuo en cualquier momento, y representa la cantidad de recursos que la persona puede aprovechar durante su vida, en resumen, la capacidad intrínseca presenta las reservas del individuo al interactuar con el entorno (42). Cesari et al.(43) , han propuesto que para su valoración se consideren cinco dominios en el modelo, entre ellos: i) la movilidad, ii) la cognición, iii) lo sensorial (visión y audición), iv) la capacidad psicológica y, v) la vitalidad, cada uno de estos dominios está definido por diferentes atributos característicos medibles (considerados como subdominios) (44–48).

vi. Costes y gastos en salud

El aumento de la esperanza de vida, ligado a una mayor tasa de morbilidad propone retos a la sociedad actual, para los cuales se debe buscar opciones y/o soluciones.
Sin duda, el pago de las pensiones a poblaciones que viven más, la transformación del mercado laboral con menos jóvenes y más seniors, el incremento de gastos sanitarios, dado que las personas mayores con mayores tasas de morbilidad consumirían una mayor cantidad de insumos hospitalarios y farmacéuticos, además de tener la mayor prevalencia de casos de discapacidad y dependencia en la sociedad actual tendrán impacto en la estructura económica y la sostenibilidad financiera. No obstante, más allá de los cuidados sanitarios, el continuo y progresivo envejecimiento de la población, pone de manifiesto la necesidad de otros recursos a tener en cuenta, como lo son los cuidados profesionales e informales y los costos asociados que estos pueden tener para la sociedad.
De hecho, trabajos recientes plantean que la variable clave para explicar el gasto sanitario, reside en el tiempo hasta la muerte (*time to death*) y en el estado de salud, y no tanto en la edad.(49)

En el caso de los cuidados de larga duración (profesionales y familiares/informales) los expertos indican que es necesario realizar un análisis más exhaustivo del impacto económico que puede conllevar este tipo de situaciones a la economía. En cuanto a la influencia de la fragilidad en el gasto sanitario, los datos aún son incipientes, pero sugieren que la fragilidad añade un coste sanitario de alrededor del 15 al 20%

Dentro de las estrategias que la OMS propone para dar solución a estos desafíos que tienen grandes implicaciones económicas, es contribuir al envejecimiento saludable desde la edad adulta, entendiéndose el concepto de Envejecimiento Saludable "como el proceso de desarrollo y mantenimiento de la capacidad funcional que permite el bienestar en la edad avanzada"(24).

ENVEJECIMIENTO Y ALIMENTACION

Los hábitos alimentarios son factores modificables para un envejecimiento saludable (50). A pesar de este conocimiento disponible sobre alimentación saludable, tanto en población adulta y adulta mayor existe una fuerte asociación entre la prevalencia de malnutrición y eventos adversos a la salud. Específicamente, en personas adultas los problemas asociados a la malnutrición son el sobrepeso y la obesidad, mientras que en personas mayores de edades más avanzadas y con múltiples comorbilidades, el principal punto de preocupación en la valoración del estado nutricional es la disminución de la ingesta y la pérdida de peso y finalmente la desnutrición, las cuales tienen una alta prevalencia e impacto en su salud. Se estima que la prevalencia de desnutrición en personas mayores residentes en la comunidad alcanza valores cercanos al 5%, y que este valor asciende a un 20% en personas internadas en residencias geriátricas a un 40% en personas mayores ingresadas en hospitales y alrededor del 50% en quienes están personas mayores inscritas en programas de rehabilitación (51,52).

Entre los varios mecanismos implicados en la pérdida de peso en las personas mayores, unos pueden estar condicionados por el envejecimiento per se y otros por causas clínicas, psicológicas y/o sociales. Durante el envejecimiento pueden ocurrir cambios en la composición corporal del individuo, además de situaciones de salud que se relacionan con una mayor frecuencia con la pérdida de peso, como la anorexia, caquexia, sarcopenia o el síndrome de hipermetabolismo. llevando al individuo a un estado de malnutrición (19,53).

Por estas razones, la detección precoz de la desnutrición y el inicio temprano del tratamiento de la malnutrición por déficit son las dianas para minimizar su prevalencia de desnutrición en este grupo de la población. Además, es relevante su identificación y tratamiento porque la desnutrición y trastornos nutricionales se asocian con un peor pronóstico funcional, se considera que la pérdida de peso agudo es el principal predictor de deterioro funcional. En efecto, la malnutrición y la discapacidad están vinculadas de forma bidireccional, por lo tanto, el conocer y monitorizar el estado nutricional y hábitos alimentarios de las personas mayores se convierte en un aspecto primordial de promoción y estimulación de hábitos alimentarios saludables para contribuir a mantener la funcionalidad y la calidad de vida.

VALORACIÓN NUTRICIONAL

La malnutrición es uno de los aspectos más relevantes y preocupantes en las personas mayores, debido a su asociación con una mayor morbilidad, estancias hospitalarias prolongadas y mortalidad. Así, su identificación y detección se convierte en un punto crítico en la atención de las personas mayores La detección temprana de malnutrición por déficit puede también tener efectos positivos en otros resultados clínicos como la mejora de la función física e inclusive la reducción de la estancia hospitalaria en pacientes hospitalizados, por lo tanto, el primer paso es realizar una valoración del estado nutricional el cual debe formar parte de la valoración geriátrica integral (VGI).
Se debe tener en cuenta que la valoración del estado nutricional completa debe incluir:

i. Anamnesis clínica y alimentaria detallada
ii. Exploración física exhaustiva
iii. Cuestionarios de valoración y cribado nutricional
iv. Determinación de parámetros antropométricos con medición de la composición corporal
v. Parámetros bioquímicos.

La anamnesis, que es la primera fase de la valoración nutricional, consta de una historia clínica que recoge antecedentes personales y factores de riesgo de desnutrición. En este punto es importante detectar síntomas como anorexia, baja ingesta, presencia de disfagia, alteraciones bucales y/o pérdida de peso mayor al 5% durante el último mes. También se obtiene información sobre comorbilidades, hábitos tóxicos, alteraciones sensoriales, factores psicosociales (aislamiento social, depresión, etc.) y el consumo de fármacos. Es importante incluir una valoración funcional, cognitiva afectiva y social, ya que la alteración de cualquiera de estas dimensiones puede conllevar a un cuadro de malnutrición.

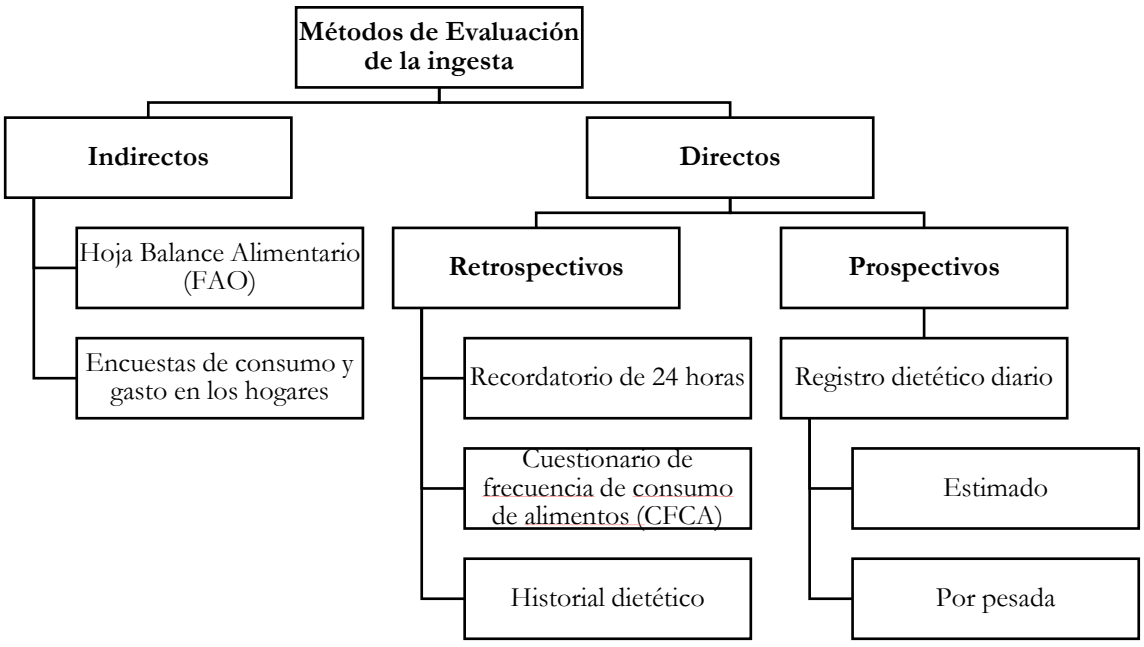
En la anamnesis alimentaria se debe hacer énfasis en la historia alimentaria del individuo, en el caso de las personas mayores se debe procurar recoger toda la información relativa a los hábitos dietéticos y alimentarios teniendo en cuenta quien está a cargo de la alimentación, si se alimenta de forma autónoma o está a cargo de un familiar o cuidador, la variedad de la dieta, incluyendo frecuencia y tipo de alimentos, cantidades, horarios y número de comidas durante el día. También es importante reconocer posibles problemas de masticación o trastornos de la deglución, si la persona mayor come sola o en compañía, gustos y preferencias, realización de dietas restrictivas o estrictas (bajas en grasa, bajas en sal (sodio), bajas en proteína, etc.) que los pueden llevar a tener una alimentación muy rutinaria, organolépticamente poco atractiva y que puede comportar una menor ingesta.

i. Valoración de la ingesta alimentaria

La evaluación dietética corresponde a la valoración de la ingesta de alimentos, nutrientes y del patrón dietético de un individuo o de un grupo de población en un período de tiempo específico, y que, junto con la evaluación de parámetros antropométricos, bioquímicos, examen clínico y de funcionalidad permitirán realizar una evaluación del estado nutricional completo (53,54). Además, conocer la ingesta alimentaria de la población es útil para el desarrollo de políticas públicas en materia alimentaria y para formular recomendaciones nutricionales.

Existen diferentes métodos de evaluación de la ingesta alimentaria y la elección del método más apropiado dependerá de los objetivos de estudio. Los métodos de evaluación dietética se clasifican según su naturaleza como (Figura 4):

Figura 4. Métodos de evaluación dietética para estimar el consumo de alimentos y nutrientes a nivel nacional, familiar e individual



Fuente: Visión general de los métodos de evaluación dietética, basada Guía para la Valoración dietética, FAO (55)

a) **Métodos indirectos** los cuales utilizan datos secundarios para evaluar la ingesta alimentaria.
b) **Métodos directos** los cuales recogen datos dietéticos a partir de información proporcionada directamente por los individuos o cuidadores. Este tipo de métodos evalúa la ingesta de alimentos y

la información proporcionada se utiliza para identificar las tendencias de consumo, la ingesta de alimentos y nutrientes, los patrones de alimentación y para evaluar asociaciones entre dieta-enfermedad. Además, estos últimos se clasifican de acuerdo con el momento en el que se ha registrado el consumo de alimentos y se dividen en métodos prospectivos y retrospectivos.

El **método retrospectivo** recoge la información sobre la ingesta de alimentos y bebidas consumidos en el pasado, dependen de la memoria del encuestado y de su capacidad para recordar todos los alimentos y tamaños de las porciones consumidas durante un período de tiempo de referencia, incluyen herramientas como: **Recordatorio de 24 horas, Cuestionario de frecuencia de consumo de los de alimentos (CFCA) e Historial dietético.**
En cambio, en el **método prospectivo**, se realiza el registro de todos los alimentos y bebidas al momento de consumo, lo que permite un detalle preciso de la ingesta. Estos métodos incluyen el Registro estimado de alimentos y el registro de alimentos por pesada (55).

En la tabla 2 se detalla cada uno de los cuestionarios o registros con sus respectivas ventajas y limitaciones, las cuales además están influenciadas por el método de administración, el cual puede ser realizado por un profesional entrenado a través de una entrevista personal o telefónica o pueden ser auto administrados y enviados vía correo electrónico o postal.

Tabla 2. Métodos de evaluación de la ingesta alimentaria, fortalezas y limitaciones.

Método de evaluación de la ingesta	Fortalezas	Limitaciones
Recordatorio de 24 horas (R24h):	- La realización de múltiples R24h permite evaluar la ingesta habitual. - Capta información sobre el patrón de alimentación, métodos de preparación, lugar de consumo, etc. - No genera cambios en la ingesta alimentaria. - Menor carga para la memoria de los encuestados (mejor precisión e índice de respuesta).	- Se necesitan R24h de varios días para representar la ingesta habitual. - Depende de la memoria del encuestado. - Riesgo de sesgo de recuerdo por parte del encuestado. - Requiere entrevistadores entrenados. - Es costoso, necesita entrevistadores formados y mayor tiempo de dedicación para la introducción y análisis.

	-No depende de las habilidades de lectura y escritura del encuestado.	- En R24h auto administrado, depende de la alfabetización del encuestado y de su capacidad para describir los alimentos y estimar el tamaño de las porciones.
Cuestionario de frecuencia de consumo de los de alimentos (CFCA)	- Evalúan la ingesta habitual durante un largo periodo de tiempo. - Puede utilizarse para captar una serie de alimentos, un nutriente específico (CFCA cuantitativo) o un grupo de alimentos. - Una sección abierta añadida al final del cuestionario puede permitir añadir alimentos consumidos que no están presentes en la lista de alimentos. - No afecta al comportamiento alimentario. - Baja carga para el encuestado. - Relativamente sencillo de administrar - Bajo costo en comparación con otros métodos. - CFCA basado en una entrevista no depende de las habilidades de lectura y escritura del encuestado. - Se puede autoadministrar por correo o Internet. - Apropiado para estudios epidemiológicos	- Listado de alimentos cerrado, puede dar lugar a una infradeclaración. - No ofrece información precisa sobre el tamaño estimado de las porciones consumidas. - CFCA auto reportados, encuestados tiene que saber leer y escribir y tener habilidades numéricas -- CFCA autoadministrados pueden dar lugar a una interpretación errónea de las preguntas y a la omisión de alimentos que el encuestado no entiende. - Deben estar adaptados y validados para reflejar la ingesta de la población y el propósito del estudio. - Depende en gran medida de la memoria; por lo tanto, la disminución de la capacidad cognitiva puede dar lugar a errores al informar sobre la frecuencia y la estimación del tamaño de las porciones - Errores al informar sobre las frecuencias combinadas (reporte de

		alimento consumido solo como en platos mixtos)
Historia dietética.	- Proporciona detalles de los patrones de comida, los alimentos individuales consumidos y la ingesta habitual de alimentos después de completar una sola entrevista - Proporciona estimaciones cuantitativas de la ingesta de energía y nutrientes - Es útil para describir la ingesta habitual de alimentos o nutrientes durante un periodo de tiempo relativamente largo. – Útil para estimar la prevalencia de dietas inadecuadas - No depende de la alfabetización del encuestado - Proporciona información sobre los alimentos que no se consumen habitualmente - No interfiere con hábitos alimentarios normales	- Depende de la memoria del encuestado (sesgo de recuerdo) - Requiere mucho trabajo y tiempo (poco adecuada para niños pequeños y personas mayores) - Para obtener información detallada sobre la ingesta de alimentos, se necesitan más horas de entrevista, lo que supone una gran carga para el encuestado - La estimación del tamaño de las porciones de las comidas anteriores puede ser difícil, incluso con el uso de ayudas - Requiere personal capacitado - Requiere que los encuestados sepan leer y escribir y sean capaces de estimar el tamaño de las porciones (historial dietético autoadministrado) - Es caro de administrar - La introducción de datos y la codificación llevan mucho tiempo y requieren personal capacitado
Registro estimado de alimentos	- Proporciona estimaciones de la dieta real en lugar de la habitual. - Depende del número de días de medición - Proporciona un alto nivel de especificidad y detalles sobre	Requiere encuestados alfabetizados y motivados con habilidades numéricas - Alto coste de administración y análisis de datos - Requiere de mucho tiempo y dando lugar a una mayor carga al encuestado

	los alimentos consumidos y la ocasión - Proporciona información detallada sobre los patrones alimentarios - Permite recoger información de los encuestados con hábitos alimentarios esporádicos - No depende de la memoria del encuestado, la información se registra en el momento del consumo. - Permite estimar el tamaño de las porciones en tiempo real, reduciendo los errores en la estimación de la ingesta	- Riesgo de olvido de registrar alimentos específicos o comidas completas - Dificultad para realizar la estimación de las porciones - Puede interferir con los hábitos alimentarios - La fiabilidad de los registros disminuye con el tiempo, debido al aumento de la carga del encuestado - Es posible que no se registren los alimentos que se consumen con menos frecuencia - Necesidad de personal capacitado para la introducción y codificación de datos
Registro de alimentos por pesada	Evalúa la ingesta real o habitual de los individuos, en función del número de días de medición - Es más preciso que otros métodos de evaluación dietética. Se ha considerado el método de referencia para la evaluación dietética - No depende de la memoria, ya que la información se registra en el momento del consumo - Proporciona tamaños exactos de las porciones, y no se basa en la estimación - Proporciona un alto nivel de especificidad y detalles sobre	- Requiere mucho tiempo y trabajo tanto para el encuestado como para el investigador - Alto nivel de carga para el encuestado. - Encuestados pueden alterar sus hábitos alimenticios. - Costoso (equipamiento y personal necesario para la formación y la supervisión) - Dificultad para pesar alimentos ingeridos fuera de casa - Requiere un entorno para pesar los alimentos Requiere alfabetización, encuestados motivados y con conocimientos de aritmética (si son autodeclarados) para pesar los

	los alimentos consumidos y los patrones de las comidas - Proporciona información sobre los alimentos que se consumen regularmente	alimentos y registrar la ingesta de alimentos

Fuente: Elaboración propia basada Guía para la Valoración dietética, FAO (55)

ii. Biomarcadores

El término "biomarcador" hace referencia a indicadores objetivos del estado de salud que pueden medirse de forma precisa y reproducible. La OMS los ha definido como "casi cualquier medición que refleje una interacción entre un sistema biológico y un peligro potencial, que puede ser químico, físico o biológico. La respuesta medida puede ser funcional y fisiológica, bioquímica a nivel celular o una interacción molecular", de este modo quedan dentro de esta definición de biomarcadores signos clínicos como el pulso y la presión sanguínea, los análisis químicos básicos, hasta pruebas de laboratorio más complejas de sangre, orina y otros tejidos(56).

En el campo de la epidemiología nutricional, un biomarcador de exposición o de ingesta puede ser cualquier compuesto biológico que sea un indicador y que refleje el estado nutricional respecto a la ingesta o metabolismo de los componentes de la dieta, así como de las consecuencias biológicas de la ingesta alimentaria. Un biomarcador de ingesta o exposición dietética debería indicar con precisión el nivel de ingesta alimentaria y debería ser específico, sensible y aplicable a un gran número de poblaciones De este modo, al momento de escoger un biomarcador de ingesta hay que tener en cuenta los criterios de validación biológica y analítica establecidos (57,58).

En el **manuscrito 2,** hay más detalle sobre los tipos de biomarcadores que hasta ahora, se han utilizado biomarcadores individuales de la ingesta alimentaria para evaluar la exposición a alimentos o grupos de alimentos específicos. Las limitaciones más relevantes pueden agruparse en la amplia distribución de los componentes de los alimentos (baja especificidad de los biomarcadores), la elevada variación interindividual y el metabolismo causado por la microbiota, entre otras (59).

ALIMENTACIÓN DE LAS PERSONAS MAYORES

Una dieta adecuada es aquella que permite alcanzar y mantener un nivel óptimo de salud la cual se caracteriza por garantizar el soporte adecuado de energía y nutrientes para mantener el gasto energético. El gasto energético basal (GEB) representa aproximadamente un 60-70% del gasto energético total (GET). El GEB es el gasto de energía mínimo que un individuo necesita para mantener las funciones vitales y varía en función del sexo, la edad, el peso y la estatura y otros condicionantes fisiológicos.

Las personas mayores tienen un requerimiento energético menor, debido a una disminución del gasto metabólico basal, cambios en la composición corporal y menor actividad física según datos reportados por la OMS, se considera que, a partir de los 60 años, cada 10 años se reduce en un 10% el gasto energético. Es así como los valores de ingesta recomendada de energía y nutrientes para la población general y de las personas mayores de 60, se establecen teniendo en cuenta los factores que afectan el GEB y el GET.

La malnutrición es una problemática común en las personas mayores que se puede desencadenar por factores que afectan la ingesta de alimentos, por ejemplo, la falta de piezas dentales o prótesis mal adaptadas que modifican en gran medida los hábitos alimentarios de las personas mayores, muchas imposibilitando el proceso de masticación y deglución de alimentos. Asimismo, la presencia de síndromes y enfermedades crónicas (enfermedad pulmonar obstructiva crónica, insuficiencia cardíaca, úlceras por presión, etc.) pueden tener un impacto sobre el estado nutricional al producir un aumento del gasto energético. Además, pueden coexistir con otras enfermedades (ictus, enfermedad de Parkinson, demencia, depresión o anorexia geriátrica) que provocan una reducción de la ingesta. La polifarmacia, definida como la presencia de cinco o más fármacos de manera concomitante, es otro factor que se asocia a un aumento del riesgo de desnutrición, así como las discapacidades en personas de edad avanzada que a menudo, plantean problemas tanto en la adquisición y la ingestión de los alimentos (19).

El deterioro de los sentidos del gusto y del olfato, se agudiza con el paso de la edad, produciendo una falta de interés por el consumo de alimentos y, en consecuencia, pérdida del apetito

En este contexto, una correcta nutrición podría ayudar a optimizar el estado global de salud de las personas mayores, mejorar la eficacia del tratamiento de las patologías crónicas y síndromes geriátricos y reduce las complicaciones de salud

i. Energía (kcal), macro y micronutrientes

De acuerdo con las recomendaciones de las *Dietary Guidelines for Americans, 2020-2025* (60) y las recomendaciones del *Dietary Reference Values for nutrients Summary report (EFSA)* (61). Las mujeres de 60 años o más necesitan entre 1.600 y 2.200 kilocalorías al día y los hombres de 60 años o más necesitan entre 2.000 y 2.600 kilocalorías al día. Estas necesidades se encuentran supeditadas a las condiciones fisiológicas y el nivel de actividad física de las personas, generalmente la recomendación de energía (kilocalorías) es menor para las mujeres en comparación con los hombres, así como para aquellas personas más mayores y menos activas físicamente.

Respecto a la recomendación de macronutrientes y micronutrientes para personas mayores, la EFSA ha propuesto recomendaciones de ingesta diaria. Para los hidratos de carbono, propuso un rango de referencia entre un 45-60% de la energía total tanto para hombres como para mujeres.
En el caso de las proteínas, la recomendación dietética actual es de 0,8 g/kg/día para todas las personas adultas mayores de 18 años, incluidas las mayores de 65 años. No obstante, los expertos en el campo de las recomendaciones de proteínas alimentarias para los adultos mayores sugieren una ingesta de proteínas más elevada que la de los adultos jóvenes. Por una parte, un grupo de expertos ESPEN sugiere que los adultos mayores sanos deberían consumir entre 1,0 y 1,2 g/kg/día [15,18-20], mientras que el grupo de estudio PROT-AGE recomendó una ingesta de proteínas entre 1,0 - 1,5 g/kg/día para las personas mayores (62).
La recomendación de ingesta de grasa está en un rango entre el 20% y el 35% de la energía total diaria. Para los ácidos grasos saturados y ácidos grasos *trans*, EFSA recomienda que la ingesta debe ser lo más baja posible, en el contexto de una dieta nutricionalmente equilibrada. Las recomendaciones de ingesta de los ácidos grasos ω-3 y ω-6 son para el ácido α-linolénico un 0.5% de la energía total diaria y un 4% de la energía total diaria, respectivamente. La recomendación de ingesta diaria para el conjunto EPA y DHA es de 250 mg al día. La EFSA recomienda una ingesta diaria de fibra dietética de 25 gramos al día para personas mayores y, para los micronutrientes las recomendaciones son similares a las de la población adulta, tanto para hombres como para mujeres, sin embargo, otras organizaciones médicas como la Academia Nacional de Medicina de EE.UU. suelen recomendar ingestas más elevadas de vitamina D en personas mayores de 70 años, elevando su recomendación a 800 UI/día (20 µg de vitamina D/día). En la tabla 3 se presenta un resumen de las recomendaciones de energía, macro y micronutrientes realizadas por EFSA para personas mayores de 60 años.(61)

Tabla 3. Recomendaciones diarias de energía y nutrientes para la población adulta y adulta mayor, EFSA 2017 (61)**.**

	Hombres	Mujeres
Energía (kcal/día)	2006 - 2890*	1624 - 2317*
Hidratos de Carbono (%)	45-60	45-60
Proteínas (g/kg/día) (%)	0.83 (12-15)	0.83 (12-15)
Lípidos totales (%)	20-35	20-35
Ácido α-linolénico (Ácidos grasos ω-3) (%)	0.5	0.5
Ácido linoléico (Ácidos grasos ω-6) (%)	4	4
EPA + DHA (mg/día)	250	250
Fibra (g/día)	25	25
Calcio (mg/día)	950	950
Hierro (mg/día)	11	11
Selenio (µg/día)	70	70
Zinc (mg/día)	16.3[a]	12.7[a]
Vitamina A (µg de RE/día) [b]	750	650
Vitamina C (mg/día)	110	95
Vitamina D (µg /día)	15	15
Vitamina E (mg/día)	13	11
Vitamina B1 (mg/día)	0.1	0.1
Vitamina B6 (mg/día)	1.7	1.6
Folatos (µg DEF /día)	330	330
Vitamina B12 (mg/día)	4.0	4.0

*Las necesidades de energía han sido estimadas considerando niveles de actividad física

[a] Requerimiento de zinc ajustado por una alta ingesta de fitatos correspondiente a 1200 mg/día

[b] RE: equivalente de retinol; 1 µg de RE equivale a 1 µg de retinol, 6 µg de β-caroteno y 12 µg de otros carotenoides provitamina A

[c] DFE de la sigla en inglés "equivalentes de folato alimentario". Para la ingesta combinada de folato alimentario y ácido fólico, se calcula como µg DFE = µg de folato alimentario + (1,7 x µg de ácido fólico)

[d] En condiciones de supuesta síntesis cutánea mínima de vitamina D. En presencia de una síntesis cutánea endógena de vitamina D, la necesidad de vitamina D en la dieta es menor. (1 µg de vitamina D: = 40 UI o 0,025 µg = 1 UI;)

ii. Patrones dietéticos

Es importante que las personas mayores sigan un patrón dietético saludable para cubrir las necesidades alimentarias y prevenir el riesgo de desnutrición. En edades avanzadas, las necesidades calóricas son más bajas debido a diversos factores que la afectan (por ejemplo, disminución de la tasa metabólica, menor actividad física y/o por la pérdida de masa ósea y muscular relacionada a la edad). Además, las necesidades de nutrientes y su absorción en personas mayores se pueden ver alteradas por enfermedades agudas y/o crónicas, el uso de múltiples medicamentos y los cambios en la composición corporal. Por estas razones las necesidades de algunos nutrientes están aumentadas en las personas mayores, respecto a las recomendaciones para personas adultas y, es en este contexto, que un patrón dietético saludable debe contener una densidad global de nutrientes, además de proporcionar una variedad de alimentos y bebidas que permitan a las personas realizar elecciones dentro de cada grupo de alimentos en función de su estilo de vida, tradiciones, cultura y otras necesidades individuales (63–65).

Los patrones dietéticos saludables, han sido definidos en el Informe Científico del Comité Asesor de las Guías Alimentarias de 2015-2020,como la combinación de alimentos y bebidas que se consumen con cierta frecuencia en el contexto de un hábito alimentario (a lo largo de un día, una semana o un año) y por lo tanto deberían relacionan de mejor forma el estado de salud general y el riesgo de enfermedad en comparación con el consumo de alimentos o nutrientes individuales (66).
Un patrón dietético saludable se compone de alimentos y bebidas densos en nutrientes, de todos los grupos de alimentos, en cantidades recomendadas y dentro de los límites de kilocalorías diarias. Se caracterizan por ser dietas con un alto contenido de frutas, verduras, cereales integrales, lácteos bajos en grasa o sin grasa y proteínas magras, además de promover el consumo de productos bajos en grasas saturadas, grasas *trans*, sodio y azúcares añadidos. Dentro de los patrones dietéticos saludables, se han definido el patrón dietético saludable al estilo estadounidense descrito principalmente en las DGAs, el patrón dietético saludable vegetariano, patrón de dieta mediterránea, además, la dieta DASH, entre otros patrones dietéticos saludables regionales (Tabla 4). Muchos de estos cuentan con evidencia científica de calidad que indican que los patrones dietéticos saludables reducen el riesgo de padecer las principales enfermedades crónicas relacionadas con la alimentación, como la diabetes, las enfermedades cardiovasculares y algunos tipos de cáncer, entre otras patologías, así como indicarlos como favorecedores del envejecimiento saludable (67–71) .

Existen varios métodos para evaluar los patrones dietéticos en los estudios poblacionales, los que caracterizan los patrones dietéticos utilizando sistemas de puntuación de índices a priori, como el Índice de Alimentación Saludable, pueden ser los más valiosos porque ofrecen una métrica

consistente que puede aplicarse en múltiples estudios. La coherencia de los métodos permite entonces comparar los resultados entre poblaciones.

Tabla 4: Patrones dietéticos saludables

PATRÓN DIETÉTICO	CARACTERÍSTICAS
Patrón dietético saludable al estilo estadounidense	**Promueve**: ingesta de frutas, verduras, integrales, productos lácteos sin grasa o bajos en grasa, grasas saludables, carnes magras y aves de corral. **Limita**: kilocalorías procedentes de azúcares añadidos y almidones refinados.
Patrón dietético vegetariano saludable	Variación del patrón dietético saludable al estilo estadounidense. No incluye carnes, aves ni mariscos. **Promueve**: la ingesta de productos derivados de soja (tofu y otros productos procesados de soja); legumbres; frutos secos y semillas; y cereales integrales. **Limita**: kilocalorías procedentes de azúcares añadido y grasas saturadas. *Patrón ovo-lacto vegetariano: incluye huevos y productos lácteos (leche, yogur natural y queso reducidos en grasa).
Dieta Mediterránea	Patrón dietético que se basa en la ingesta de alimentos tradicionales que se consumen en los países Mediterráneos. **Promueve**: consumo de alimentos de origen vegetal (frutas, verduras, legumbres; frutos secos y semillas y cereales integrales) incorpora algunos alimentos de origen animal como el pescado y su principal fuente de grasa es el aceite de oliva virgen. **Limita**: la ingesta de dulces, la carne roja y las carnes procesadas.
Dieta DASH (Dietary Approaches to Stop Hypertension)	**Similar al patrón alimentario saludable para la población estadounidense.** **Limita**: la ingesta de sodio, recomendando s menos sodio que la DGA.

Fuente: Elaboración propia

iii. Nutrientes

Como se ha mencionado en otros apartados, existen diversos que afectan tanto la ingesta como la absorción de nutrientes en las personas mayores, razón por la que se hace estrictamente necesario cubrir las necesidades de ciertos nutrientes de interés en este grupo etario:

- **Proteínas**

La ingesta de proteínas es importante para mantener la masa muscular y para prevenir o reducir el riesgo de sarcopenia y osteoporosis. Por esta razón, se recomienda consumir suficiente cantidad proteínas con el objetivo evitar la pérdida de masa muscular magra que se produce de forma natural con la edad. De acuerdo con la literatura, la ingesta de proteínas y/o de alimentos proteicos es menor en estadounidenses de 71 años o más, en comparación con los de 60 a 70 años; además alrededor del 50% de las mujeres y el 30% de los hombres de 71 años o más no cumplen con las recomendaciones de ingesta de proteínas

La ingesta de proteínas se puede mejorar eligiendo entre una mayor variedad de fuentes alimentarias de proteínas, en algunos casos, esto puede implicar el uso de mariscos en lugar de carnes, aves o huevos o el uso de legumbres en platos mixtos o encontrar formas agradables de añadir alimentos proteicos a las preparaciones para garantizar que se cubren los requerimientos de proteínas (72–74).

- **Vitamina B12**

Es un nutriente crítico en la población de edad avanzada, porque la capacidad de absorción de la vitamina B12 puede disminuir con la edad y/o el uso de medicamentos puede reducir su absorción.

La vitamina B12 participa en la formación de glóbulos rojos, contribuye la función neurológica normal y a la síntesis de ADN. El déficit de esta vitamina se asocia con anemia megaloblástica, también se asocia con el desarrollo de enfermedades neurológicas, entre ellas deterioro cognitivo y demencia, dado que el déficit de vitamina B12 afecta la duplicación celular provocando alteraciones en los procesos del sistema nervioso central. Se recomienda a los adultos mayores que cumplan las recomendaciones de alimentos proteicos, una fuente común de vitamina B12, y que incluyan alimentos fortificados con vitamina B12, como los cereales del desayuno. Algunas personas mayores sanas incluso podrían necesitar el uso de complementos alimenticios de vitamina B12 (75).

- **Vitamina D y calcio**

La vitamina D y el calcio son nutrientes reconocidos por su rol en el mantenimiento de la salud ósea. Por su parte, la vitamina D desempeña un papel en la absorción del calcio y el mantenimiento de la homeostasis del calcio y del fosforo. La vitamina D se sintetiza en la piel mediante la acción de la luz ultravioleta B del sol, las personas mayores tienen una menor capacidad de sintetizar vitamina D en la piel tras la exposición al sol. Razón por la cual se convierte en un nutriente crítico para este grupo de la población. El déficit de vitamina D se asocia con la malabsorción del calcio, y por consecuencia un aumento del riesgo padecer fracturas, osteoporosis, osteopenia, también su déficit se asocia con debilidad muscular, enfermedades cardiovasculares mortalidad general. Por otra parte, el déficit de calcio se asocia con mayor pérdida ósea y mayor riesgo de fractura, con consecuencias de mayor predisposición a la osteoporosis en el largo plazo. Estudios observacionales han demostrado que los niveles séricos de 25-hidroxivitamina D de 80 nmol/L (32 ng/mL) y superiores se asocian a un menor riesgo de fracturas óseas, varios tipos de cáncer, esclerosis múltiple y diabetes de tipo 1 (insulinodependiente) (76). La suplementación diaria con 2.000 UI (50 µg) de vitamina D es especialmente importante para personas mayores.

- **Otros micronutrientes**

Si bien, no existen recomendaciones diferentes a la población adulta en general, se recomienda la vigilancia de la ingesta de la vitamina B6 y folatos, dado que su déficit se asocia niveles altos de homocisteína, los cuales podrían estar implicados en el desarrollo de enfermedad de Alzheimer (EA) y otros tipos de demencia en personas mayores, además se asocia con depresión, y otros trastornos neuropsiquiátricos.

Lo mismo sucede con la ingesta de vitaminas A, C y E, las cuales están implicadas en el proceso de neutralización del exceso de radicales libres, confiriendo protección a las células y en consecuencia contribuyendo a la prevención de enfermedades crónicas causadas, en parte, por el daño oxidativo. El déficit las vitaminas A, C y E se asocia con patologías crónicas y degenerativas, como cáncer, patologías autoinmunes, cataratas, enfermedades cardiovasculares y neurodegenerativas. De la misma forma ocurre con la ingesta de selenio dado que su déficit se asocia a un mayor deterioro cognitivo, y en el caso del **zinc,** su déficit en personas mayores se asocia con mayor predisposición a infecciones, diarrea, dermatitis y disminución de la agudeza gustativa, además de que se ha sugerido que el déficit de zinc podría estar relacionado con el daño neuronal que se observa en la enfermedad de Alzheimer.

iv. Compuestos bioactivos

Los compuestos bioactivos de los alimentos (CBA) se refieren a todos los compuestos, en su mayoría son nutrientes no esenciales que están presentes de forma natural en los alimentos, que ejercen un determinado efecto bioactivo en el organismo. Los CBA abarcan una amplia gama de compuestos, producidos en su mayoría por las plantas como metabolitos secundarios, y utilizados en diferentes funciones como defensa, atracción y señalización, Por ejemplo, los flavonoides son agentes protectores contra los radicales libres generados durante la fotosíntesis. A su vez, se ha informado de que algunos terpenoides atraen a los polinizadores o a los dispersores de semillas o inhiben a las plantas competidoras, y diferentes alcaloides se utilizan para repeler a los herbívoros e insectos (77). Los CBA se encuentran esencialmente en las frutas, las verduras, los cereales y las legumbres y, a pesar de su importancia para la salud, no se han definido valores de ingesta diaria recomendados para su ingesta en la mayoría de los CBA, como ocurre con las proteínas, los lípidos o las vitaminas. Dentro de los CBA se incluyen polifenoles, los carotenoides, las vitaminas, los ácidos grasos omega-3, los ácidos orgánicos, los nucleósidos y los nucleótidos, y los fitoesteroles (78).

Tabla 5. Principales fuentes de compuestos bioactivos alimentarios (CBA) y clasificación.

Clasificación/Clase	Compuesto Bioactivo	Fuente de alimentos
Polifenoles	Ácidos clorogénicos	arándanos y frambuesas
Fitosteroles	Estigmasterol	soja
Terpenoides	Limoneno	cítricos
Polisacáridos	Celulosa	semillas de lino
Carotenoides y tocoferoles	β-caroteno/vitamina A	
Glucosinolatos	Sulforafano	brócoli
Triterpenos	Escualeno	aceite de oliva
Alcaloides	Cafeína	granos de café
Capsaicinoides	Capsaicina	pimientos
Péptidos bioactivos	Carnosina	carne roja
Ácidos grasos poliinsaturados (AGPIs)	Ácido docosahexaenoico (DHA)	Nueces, algunos tipos de pescados (salmón, atún, etc.)

Fuente: Elaborado a partir publicación de Câmara et al. (78).

IMPORTANCIA DEL MANTENIMIENTO DE LA INTEGRIDAD INTESTINAL EN EL BINOMIO ALIMENTACIÓN Y SALUD EN PERSONAS DE LA TERCERA EDAD

Cada vez hay más consenso en que la barrera intestinal desempeña un papel importante para la salud y la enfermedad. No solo las enfermedades gastrointestinales crónicas, como la enfermedad inflamatoria intestinal (EII) o el síndrome del intestino irritable, sino también en las enfermedades metabólicas, la enfermedad del hígado graso no alcohólico, la artritis crónica y los trastornos neuropsiquiátricos se han relacionado con la disfunción de la barrera intestinal (79).

La pared intestinal representa una barrera que transporta selectivamente nutrientes, iones y agua desde el lumen al torrente sanguíneo, a través de mecanismos pasivos y activos. En la cual una monocapa de células epiteliales constituye la principal barrera física entre el lumen intestinal y los tejidos de la mucosa. Las uniones estrechas, compuestas por proteínas transmembrana y moléculas de adhesión de unión regulan el flujo de agua, iones y pequeñas moléculas y, sellan los espacios paracelulares. Varias proteínas distintas contribuyen a formar las uniones estrechas, incluyendo principalmente ocludinas y claudinas, dependiendo del tejido y la ubicación que se interrelacionan dentro del espacio paracelular.

Las estructuras de las uniones estrechas actúan de manera dinámica, es decir que pueden "abrirse" y "cerrarse" tras estímulos específicos. Por ejemplo, estímulos fisiológicos pueden contraerlas para impedir la difusión de toxinas, virus o fragmentos bacterianos a la capa de la mucosa, mientras que otros favorecen la apertura del espacio paracelular para permitir la difusión de nutrientes (80). Por otra parte, la estructura fisiológica y el dinamismo de las uniones estrechas podrían verse alterados como resultado de estados patológicos, lo que conduce a una condición de aumento de la IP, también conocida como "intestino permeable". Patologías como la enfermedad celíaca, la enfermedad inflamatoria intestinal y la diabetes de tipo 1 son tres de las principales causas patológicas del intestino permeable, que conduce a la permeación de moléculas, organismos o fragmentos microbianos potencialmente dañinos desde el lumen intestinal hasta la capa de la mucosa, induciendo una cascada de acontecimientos que dan lugar a la activación inmunitaria y a la inflamación local o sistémica (81). Con frecuencia, las personas mayores se ven afectadas por una disminución de la función de la barrera intestinal y, en consecuencia, por un intestino permeable. Entre las causas, se cree que la disminución de la función inmunitaria relacionada con el envejecimiento (es decir, la senescencia inmunitaria), la

remodelación del epitelio intestinal y las alteraciones de la composición de la microbiota intestinal son los factores más importantes. Así pues, la microbiota intestinal puede considerarse un regulador crítico del PI. Los microorganismos intestinales pueden actuar directamente sobre la PI afectando a las propiedades y actividades de las uniones estrechas e indirectamente modulando la inflamación, que es un factor bien reconocido que promueve el deterioro de la PI. Como se ha observado en el aumento de la PI asociado a la enfermedad, la disfunción de la barrera intestinal en los sujetos de edad avanzada facilita la difusión de sustancias o péptidos tóxicos y fragmentos microbianos a la capa de la mucosa y al torrente sanguíneo, desencadenando una respuesta inflamatoria sistémica.

Un estado nutricional adecuado es fundamental para mantener la función normal de la microbiota intestinal (pudiendo afectar a todos los componentes de la microbiota) y, por lo tanto, la desnutrición se asocia a un aumento de la PI. Se ha demostrado que la dieta occidental, caracterizada por una ingesta elevada de energía y grasas o un consumo elevado de fructosa puede alterar la IP al afectar a la composición de la microbiota intestinal. Además, este patrón dietético suele implicar el consumo de componentes alimentarios, como ácidos grasos específicos, alcohol, aditivos, gliadina y quitosano, y métodos de procesamiento de alimentos que se sabe que alteran la homeostasis de la estructura física de la barrera intestinal y/o la homeostasis microbiana comensal. Por otro lado, un patrón dietético dieta saludable, como la dieta mediterránea (DM), rica en frutas, verduras, legumbres y cereales no refinados, se ha sugerido que se ha sugerido que afecta positivamente a la PI y a las afecciones relacionadas. Esto puede estar relacionado con una mayor producción de ácidos grasos de cadena corta (SCFAs), incluyendo acetato, propionato, butirato y valerato, por las bacterias comensales del intestino tras la degradación de la fibra proporcionada por el patrón dietético de la DM. Se ha sugerido que estos metabolitos se ha sugerido que juegan un papel importante como sustratos para un epitelio colónico funcional y el mantenimiento de la IB.
Además, los patrones dietéticos basados en plantas incluyendo la DM, también suelen ser abundantes en compuestos bioactivos, como los polifenoles (PP), que han estado recientemente en el punto de mira de la investigación por su actividad moduladora con respecto a la PI (79,82).

En los manuscritos **4 y 5** se presenta más detalle sobre el link entre la ingesta de nutrientes (compuestos polifenoles) además de la relación con el mantenimiento de la permeabilidad intestinal, que repercuten en el estado de salud general.

2. HIPOTESIS Y OBJETIVOS

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS DE TRABAJO

Esta tesis doctoral tiene como hipótesis:

La adherencia a un patrón dietético saludable, como podría ser seguir un patrón dietético mediterráneo (rico en polifenoles) tendría efectos protectores en el envejecimiento de personas mayores.

En el caso particular del estudio de intervención cruzada MaPLE, nuestra hipótesis se fundamenta en que la eficacia de las intervenciones dietéticas con compuestos fitoquímicos puede estar significativamente influenciada por factores de variabilidad interindividual que afectan a su biodisponibilidad y consecuentemente a su actividad biológica. En dicho estudio nos hemos planteado que un patrón dietético rico en polifenoles en el que se han incorporado 3 porciones de alimentos ricos en polifenoles al día puede disminuir los niveles séricos de zonulina, un marcador sustitutivo de la permeabilidad intestinal (PI), pudiendo llegar a alterar beneficiosamente la microbiota intestinal y los marcadores bioquímicos y clínicos asociados a la PI en sujetos de edad avanzada.

En el caso de la cohorte prospectiva InCHIANTI, nuestra hipótesis se centró en evaluar si la adherencia a un patrón dietético saludable (dieta mediterránea) o alimentos/nutrientes saludables (proteína y productos lácteos) mejoraban marcadores de salud relevantes en población adulta mayor como son la fragilidad y mortalidad total. Específicamente, la adherencia a la dieta Mediterránea se evaluó usando biomarcadores y cuestionaros dietéticos y nuestra hipótesis es que los biomarcadores ofrecerán evaluaciones más precisas ya que son capaces de estimar la exposición dietética de forma más exacta que los cuestionarios, especialmente en poblaciones adultas mayores.

OBJETIVOS GENERALES Y ESPECÍFICOS

OBJETIVO GENERAL

Estudiar el efecto de la calidad de la dieta global en el proceso de envejecimiento saludable utilizando diferentes estrategias de evaluación de la exposición dietética (cuestionarios y biomarcadores nutricionales) y su asociación con el estado de salud en población de personas mayores.

OBJETIVOS ESPECÍFICOS

Objetivo específico	**Trabajos Publicados** *(del 1*-12)*
1. Evaluar la utilización y combinación de cuestionarios dietéticos y de biomarcadores nutricionales para la óptima determinación de la exposición dietética.	*Manuscrito 2* *Trends in Food Science & Technology, 2017, 69, 220-229*
2. Revisar la heterogeneidad de las fuentes de información, así como la contribución específica de las diferentes fuentes dietéticas para valorar la evidencia científica actual sobre el efecto de la ingesta de polifenoles y su impacto en la salud humana.	*Manuscrito 3* *Nutrients, 2019, 11, 1355*
3.a. Revisar la literatura científica reciente sobre el rol de la alimentación en la composición de la microbiota y la permeabilidad intestinal, utilizando un enfoque metabolómico centrándose en las vías moleculares involucradas, a partir de estudios in vivo e in vivo en humanos.	*Manuscrito 4* *J. Agric. Food Chem. 2020, 68, 1780−1789*
3.b. Estudiar la idoneidad de la recomendación de la ingesta de polifenoles para el mantenimiento de la permeabilidad intestinal y fortalecimiento de la salud intestinal.	*Manuscrito 5* *J. Agric. Food Chem. 2020, 68, 1816−1829*

4.a Estimar la ingesta dietética real de polifenoles en población de edad avanzada institucionalizada en residencias de larga estadía. 4.b. Estudiar la eficacia de dicha intervención dietética rica en compuestos polifenólicos mediante el estudio del perfil metabólico en orina valorando el efecto en el mantenimiento de la integridad intestinal y el efecto de la variación interindividual.	*Manuscrito 6* *Nutrients 2020, 12, 2458* *Manuscrito 7* *J. Agric. Food Chem. 2020, 68, 12476−12484*
5. Estudiar el efecto de los polifenoles específicos como moduladores potenciales de la permeabilidad intestinal y sus posibles mecanismos de acción: 5.a. Estudiar el efecto de un patrón dietético rico en polifenoles en el mantenimiento de la integridad de la barrera intestinal mediante la medición de la zonulina sérica, considerado un marcador indirecto de la permeabilidad intestinal 5.b. Valorar el potencial beneficio de una intervención dietética con productos ricos en polifenoles en la salud de la microbiota intestinal, los marcadores bioquímicos y clínicos asociados con el aumento de la permeabilidad intestinal en personas mayores.	*Manuscrito 8* *Antioxidants 2021, 10, 730* *Manuscrito 9* *Clinical Nutrition 2021 40, 3006-3018*
6. Desarrollar un panel de biomarcadores dietéticos basado en grupos de alimentos clave de la Dieta mediterránea en la población del estudio InCHIANTI e investigar su asociación a largo plazo con la mortalidad total y por causas específicas como ECV y cáncer.	*Manuscrito 10* *Aceptado en revista: BMC Medicine*
7. Evaluar las asociaciones a largo plazo de la ingesta de proteínas animales y vegetales con la mortalidad por todas las causas y por causas específicas en la cohorte de personas mayores del estudio InCHIANTI.	*Manuscrito 11* *Enviado*
8. Analizar la asociación entre la ingesta habitual de productos lácteos y el riesgo de fragilidad en adultos mayores de la cohorte InCHIANTI.	*Manuscrito 12* *Enviado*

*El manuscrito 1 corresponde a la publicación del diseño del estudio MAPLE, presentado en el apartado de Metodología.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

The hypothesis of this doctoral thesis is as follows:

Adherence to a healthy dietary pattern, such as following a Mediterranean dietary pattern (rich in polyphenols) would have protective effects on ageing in older people.

In particular case of the MaPLE cross-intervention study, our hypothesis is that the efficacy of dietary interventions with phytochemical compounds may be significantly influenced by inter-individual variability factors that affect their bioavailability and consequently their biological activity. In this study, we proposed that a polyphenol-rich dietary pattern incorporating 3 servings of polyphenol-rich foods per day may decrease serum levels of zonulin, a surrogate marker of intestinal permeability (IP), and may beneficially alter gut microbiota and biochemical and clinical markers associated with IP in elderly subjects.

In the case of the prospective InCHIANTI cohort, our hypothesis focused on assessing whether adherence to a healthy dietary pattern (Mediterranean diet) or healthy foods/nutrients (protein and dairy products) improved relevant health markers in older adults such as frailty and total mortality. Specifically, adherence to the Mediterranean diet was assessed using biomarkers and dietary questionnaires and we hypothesize that biomarkers will provide more accurate assessments as they are able to estimate dietary exposure more accurately than questionnaires, especially in older adult populations.

GENERAL AND SPECIFIC OBJECTIVES

GENERAL OBJECTIVE

To study the effect of overall diet quality on the process of healthy ageing using different dietary exposure assessment strategies (questionnaires and nutritional biomarkers) and their association with health status in an elderly population.

SPECIFIC OBJECTIVES

Specific Objectives	**Publications** *(from 1*-12)*
1. To evaluate the use and combination of dietary questionnaires and nutritional biomarkers for optimal determination of dietary exposure.	*Manuscript 2* *Trends in Food Science & Technology, 2017, 69, 220-229*
2. To review the heterogeneity of information sources as well as the specific contribution of different dietary sources to assess the current scientific evidence on the effect of polyphenol intake and its impact on human health.	*Manuscript 3* *Nutrients, 2019, 11, 1355*
3.a. Review recent scientific literature on the role of diet on microbiota composition and intestinal permeability, using a metabolomic approach focusing on the molecular pathways involved, from in vivo and in vivo studies in humans.	*Manuscript 4* *J. Agric. Food Chem. 2020, 68, 1780−1789*
3.b. To study the suitability of the recommendation of polyphenol intake for the maintenance of intestinal permeability and strengthening of intestinal health.	*Manuscript 5* *J. Agric. Food Chem. 2020, 68, 1816−1829*

4.a. To estimate the actual dietary intake of polyphenols in the elderly population institutionalised in long-stay homes. 4.b. To study the efficacy of this dietary intervention rich in polyphenolic compounds by studying the metabolic profile in urine, assessing the effect on the maintenance of intestinal integrity and the effect of interindividual variation.	
5. To study the effect of specific polyphenols as potential modulators of intestinal permeability and their possible mechanisms of action: 5.a. To study the effect of a polyphenol-rich dietary pattern on the maintenance of intestinal barrier integrity by measuring serum zonulin, considered an indirect marker of intestinal permeability. 5.b. To assess the potential benefit of a dietary intervention with polyphenol-rich products on gut microbiota health, biochemical and clinical markers associated with increased intestinal permeability in the elderly.	*Manuscript 6* *Nutrients 2020, 12, 2458* *Manuscript 7* *J. Agric. Food Chem. 2020, 68, 12476−12484*
6. Develop a panel of dietary biomarkers based on key food groups of the Mediterranean Diet in the InCHIANTI study population and investigate their long-term association with total and cause-specific mortality such as CVD and cancer.	*Manuscript 8* *Antioxidants 2021, 10, 730* *Manuscript 9* *Clinical Nutrition 2021 40, 3006-3018*
7. To assess the long-term associations of animal and plant protein intake with all-cause and cause-specific mortality in the InCHIANTI study cohort of older people.	*Manuscript 10* *Accepted in journal: BMC Medicine*
8. To analyse the association between regular intake of dairy products and the risk of frailty in older adults in the InCHIANTI cohort.	*Manuscript 11* *Enviado*

*Manuscript 1 corresponds to the publication of the MAPLE study design, presented in the Methodology section.

3. METODOLOGIA

METODOLOGIA

Esta tesis doctoral se ha desarrollado en base a dos estudios en población de edad avanzada que relacionan la ingesta alimentaria con resultados en salud:

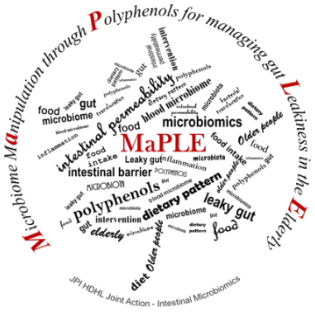
2. **Estudio de intervención dietética: MaPLE**, ***M****icrobiome m****a****nipulation through* ***P****olyphenols for managing gut* ***L****eakiness in the* ***E****lderly, "Gut and blood microbiomics for studying the effect of a polyphenol-rich dietary pattern on intestinal permeability in the elderly".* Microbioma intestinal y sanguíneo para evaluar el efecto de un patrón dietético rico en polifenoles sobre la permeabilidad intestinal en el adulto mayor

2. Estudio prospectivo observacional. Estudio InChianti , *The InCHIANTI Study. Invecchiare nel Chianti*

DISEÑO DE ESTUDIO DE INTERVENCIÓN DIETÉTICA MaPLE, Microbiome manipulation through Polyphenols for managing gut Leakiness in the Elderly

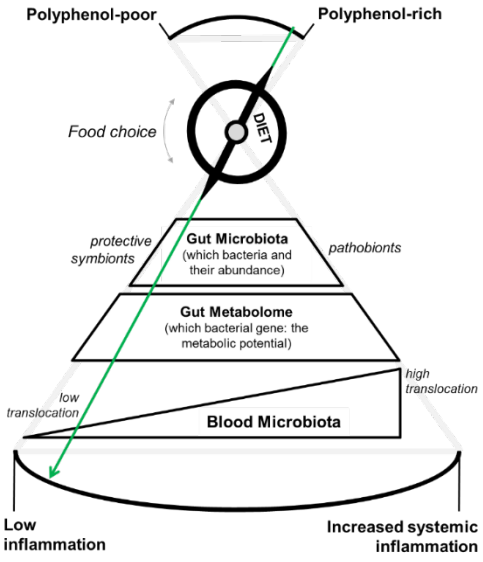
En este apartado se presenta el diseño del estudio de intervención seguido para dar cumplimiento a los 5 primeros objetivos planteados en esta tesis doctoral.

Figura 5: Logotipo del estudio MaPLE



El presente trabajo se diseñó con el propósito de evaluar la **hipótesis** de que una ingesta elevada de alimentos ricos en polifenoles en sujetos de edad avanzada puede reducir los niveles séricos de zonulina como marcador indirecto de la permeabilidad intestinal (PI) y disminuir los factores bacterianos inflamatorios en el torrente sanguíneo, promoviendo un fenotipo metabólico protector en las personas mayores (Figura 6).

Figura 6: Esquema de la hipótesis de trabajo del estudio MaPLE



El estudio MaPLE, se ha realizado en el marco del proyecto internacional *"Gut and blood microbiomics for studying the effect of a polyphenol-rich dietary pattern on intestinal permeability in the elderly*, concedido por la Iniciativa de Programación Conjunta Europea "A Healthy Diet for a Healthy Life" (JPI-HDHL) "Intestinal Microbiomics" (IM2015) (http://www.healthydietforhealthylife.eu/) https://www.healthydietforhealthylife.eu/index.php/projects/research-area-supported-project/report/182 a través del Ministerio de Economía y competitividad (España, PCIN-2015-238).

El estudio MaPLE cuenta con el consorcio:

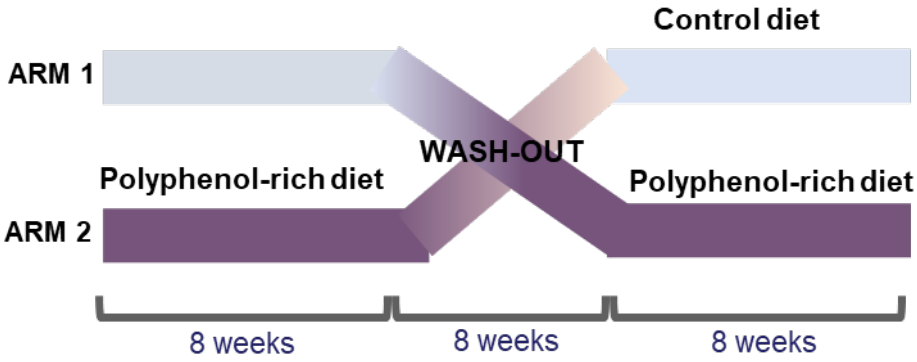
- Universidad de Barcelona
- Universidad de Milan (Italia)
- Quadram Institute (Reino Unido)
- INRCA Istituto Nazionale di Ricovero e Cura per Anziani. Department of Geriatric Medicine, Universidad de Perugia.

MaPLE es un estudio de intervención dietética cruzado, aleatorizado, controlado y simple ciego, que se llevó a cabo en la Fundación *Opera Immacolata Concezione (OIC*) con la aprobación del comité de bioética de la Universidad de Milán y con el consentimiento informado por escrito de los participantes (83).

La Fundación OIC está presente en los territorios italianos de Padua, Vicenza, Treviso, Mantova, Gorizia a partir de 12 centros residenciales con una oferta que incluye la atención domiciliaria, la acogida de personas autosuficientes y no autosuficientes y la gestión de departamentos hospitalarios. El estudio MaPLE se llevó a cabo en el centro de la ciudad de Pádova (Italia)

Figura 7: Fundación OIC, Padua, Italia

Figura 8: Esquema general del diseño de intervención del estudio MaPLE



Los criterios de inclusión se definieron teniendo en cuenta el estado funcional que debían cumplir las y los participantes:

- Edad ≥60 años
- Ser autosuficientes (Índice de Barthel ≥ 60)
- Tener un adecuado estado nutricional (Mini Nutritional Assessment ≥ 24)
- Adecuado estado cognitivo (Mini Mental State Examination ≥ 24)
- Presentar permeabilidad intestinal aumentada (evaluada por nivel de zonulina en suero, >20 ng/ml).

Como criterios de exclusión se consideraron:

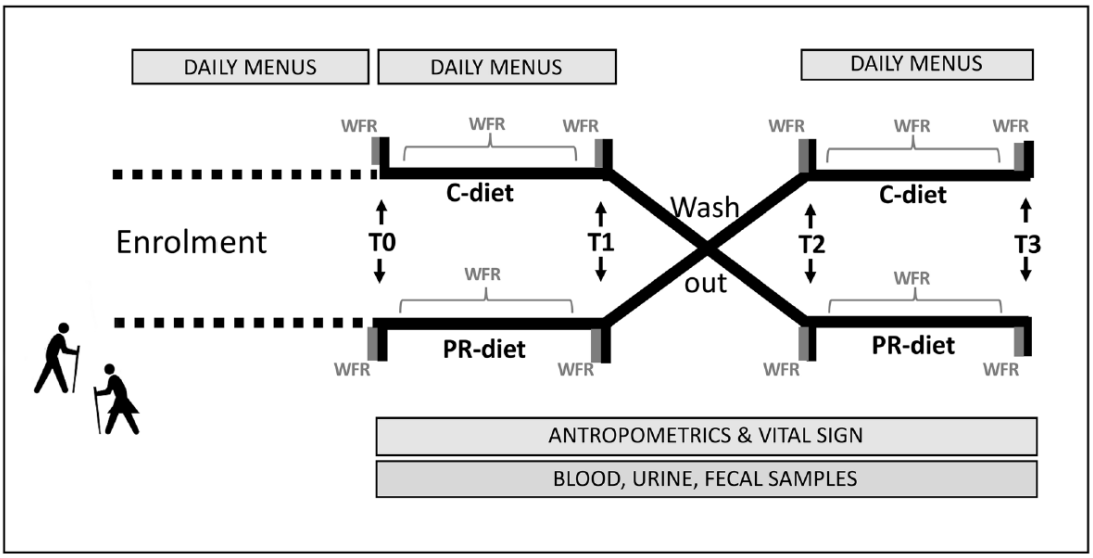
- Presencia de enfermedades graves como:
  - Enfermedad celíaca
  - Enfermedad hepática grave con cirrosis
  - Insuficiencia renal grave (Diálisis)
  - Enfermedad pulmonar obstructiva crónica (EPOC)
  - Enfermedad cardiovascular grave (Insuficiencia cardíaca clase III o IV New York Heart Association),
- Tumor maligno en los últimos dos años
- Tratamiento con antibióticos en el último mes

El tamaño inicial de la muestra fue de 60 personas de 60 años o más. Los participantes fueron divididos en grupo intervención y grupo control (30 personas en cada uno), no obstante, debido a una tasa de abandono del 15%, se obtuvo una muestra final de 51 sujetos.

El diseño del estudio MaPLE es un ensayo cruzado, controlado, aleatorizado en el cual los sujetos fueron asignados al azar para consumir una dieta rica en polifenoles (dieta-PR) o una dieta de control (dieta-C) durante 8 semanas, separadas por un período de lavado de la misma duración. Al inicio, a la mitad y al final del estudio, se tomaron muestras de sangre en ayunas, orina de la mañana (*spot*) y heces posteriores a un ayuno programado, junto con la recogida de los registros de alimentos por pesada que se aplicaron a cada participante a lo largo del ensayo.

En el desarrollo del estudio, se determinaron dos períodos de evaluación (T0-T1; T2-T3) de 8 semanas cada uno, separados por un periodo de lavado (8 semanas), teniendo el ensayo una duración total de 24 semanas para cada participante. El primer periodo de la dieta control (dieta-C) contó con un total de 23 participantes y el segundo con 28 participantes (Figura 8 y 9).

Figura 9: Diseño experimental del estudio MaPLE



Diseño del estudio: Representación esquemática del flujo de trabajo del estudio. WFR: Registros de alimentos pesados; T0, T1, T2, T3: tiempo de intervención; C-diet: dieta de control; PR-diet: dieta rica en polifenoles.
Fuente: Protocolo del estudio MaPLE (84)

Los participantes del estudio vivían en la residencia la geriátrica *Civitas Vitae Nazaret* en Padova, Italia, donde dependiendo de su nivel de discapacidad, residían en edificios de atención residencial o residencias independientes.

El ensayo el proyecto MaPLE ha sido registrado prospectivamente el 28 de abril de 2017; ISRCTN10214981, https://www.cochranelibrary.com/es/central/doi/10.1002/central/CN-01884980/full

- **Diseño de las dietas del estudio MaPLE**

Ambas dietas (dieta-PR y la dieta-C), fueron desarrolladas para proporcionar niveles comparables de energía y nutrientes. La dieta-PR se consiguió sustituyendo tres raciones al día de alimentos o bebidas con bajo contenido en polifenoles por alimentos o bebidas específicos ricos en polifenoles que aportaron adicionalmente una media de 724 mg/día de polifenoles totales, según el análisis de Folin-Ciocalteu (Tabla 6). Este ensayo es un método colorimétrico que analiza la cantidad de polifenoles totales (85).

Tabla 6 Plan diario de productos alimenticios ricos en polifenoles entregados en la intervención del estudio MaPLE.

Días de la semana	**Raciones de productos ricos en polifenoles**		
Lunes	Tableta de chocolate (10g)	ǂPuré de manzana *Reineta* (100g)	Zumo de granada (125 ml)
Martes	Zumo de Naranja sanguina (o roja) (200ml)	Manzana (*Reineta*) (150g)	Puré de Berries (100g)
Miércoles	¥Chocolate en polvo (2g)	Manzana (*Reineta*) (150g)	Arándanos (120g)
Jueves	Té verde§ (200ml)	Tableta de chocolate (10g)	Manzana (*Reineta*) (150g)
Viernes	Zumo de Naranja sanguina (o roja) (200ml)	Zumo de granada (125 ml)	Arándanos (120g)
Sábado	Tableta de chocolate (10g)	Puré de Berries (100g)	ǂPuré de manzana *Reineta* (100g)
Domingo	Té verde§ (200ml)	Naranja sanguina (o roja) (110ml)	Manzana (*Reineta*) (150g)

¥El chocolate en polvo se disolvió en leche o agua caliente; §El té verde se preparó por solubilización de 200 mg de extracto de té verde en 200 ml de agua caliente. ǂEl puré de manzana reineta se preparó en condiciones controladas y se almacenó a - 18 °C.

Fuente: Protocolo del estudio MaPLE (84)

A continuación se adjuntan los manuscritos y material suplementario donde se recoge el protocolo del diseño del estudio MaPLE:

Manuscrito 1: Protocolo del estudio MaPLE

Guglielmetti S, Bernardi S, Del Bo' C, Cherubini A, Porrini M, Gargari G, et al. Effect of a polyphenol-rich dietary pattern on intestinal permeability and gut and blood microbiomics in older subjects: Study protocol of the MaPLE randomised controlled trial. BMC Geriatr . 2020 Dec 26;20(1):77. https://bmcgeriatr.biomedcentral.com/articles/10.1186/s12877-020-1472-9

Factor de Impacto: 3.921 Q1 (6/36); Categoría: GERONTOLOGY – SSCI

Comunicaciones en congresos del protocolo del estudio MaPLE

a) **COST action "Personalized Nutrition in aging society: redox control of major age-related diseases"**

Bernardi, Del Bo', Guglielmetti, Cherubini, Kroon, Kirkup, et al. Role of a Polyphenol-Rich Dietary Pattern in the Modulation of Intestinal Permeability in Olde Subjects: The MaPLE Study. Proceedings 2019;11:8. https://doi.org/10.3390/proceedings2019011008.

b) 13th European Nutrition Conference, FENS 2019, 15–18 October 2019, Malnutrition in an Obese World: European Perspectives , 2020 , E535

Bernardi S, Del Bo' C, Guglielmetti S, Gargari G, Cherubini A, Kroon P, et al. Intestinal permeability modulation through a polyphenol-rich dietary pattern in older subjects: MaPLE project outcomes and perspectives. Proc Nutr Soc 2020;79:E535. https://doi.org/10.1017/S002966512000484X.

MANUSCRITO 1

PROTOCOLO DEL ESTUDIO MaPLE

Antecedentes: Durante el envejecimiento, pueden producirse alteraciones del ecosistema microbiano intestinal que contribuyen a la inmunosenescencia, al envejecimiento inflamatorio y al deterioro de la función de barrera intestinal (aumento de la permeabilidad intestinal; PI). En el contexto de un eje dieta-microbiota-IP en sujetos de edad avanzada, los bioactivos alimentarios como los polifenoles pueden desempeñar un papel modulador beneficioso.

Métodos: MaPLE es un proyecto centrado en un ensayo aleatorio y controlado de intervención dietética cruzada [dieta rica en polifenoles (dieta PR) frente a dieta de control (dieta C)] dirigido a personas mayores (≥ 60 años) que viven en un entorno bien controlado (es decir, una residencia de ancianos). Las intervenciones de 8 semanas están separadas por un período de lavado de 8 semanas. Durante la intervención se consumen tres pequeñas porciones diarias de alimentos ricos en polifenoles seleccionados en sustitución de otros productos comparables dentro de la dieta C. Se recogen muestras biológicas antes y después de cada período de tratamiento para evaluar los marcadores relacionados con la PI, la inflamación, la función vascular, el estrés oxidativo y la microbiómica intestinal y sanguínea, metabolómica. Se definió un tamaño de muestra de 50 sujetos basado en la PI como resultado primario.

Discusión: La evidencia de que el aumento del consumo de productos alimenticios ricos en polifenoles puede afectar positivamente el ecosistema microbiano intestinal, lo que resulta en una reducción de la PI y una menor translocación de factores bacterianos inflamatorios al torrente sanguíneo. inflamatorios en el torrente sanguíneo. La integración de los datos del microbioma intestinal y sanguíneo, de la metabolómica y otros marcadores relacionados con la PI mejorará la comprensión del efecto beneficioso de la intervención en el contexto de las interacciones polifenoles-microbiota-PI. Por último, los resultados obtenidos proporcionarán una prueba de concepto de la fiabilidad de la intervención dietética, contribuyendo también a futuras implementaciones de directrices dietéticas dirigidas a la gestión de la PI en las personas mayores y otros sujetos de riesgo.

Registro del ensayo: El ensayo está registrado en (ISRCTN10214981); 28 de abril de 2017.

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STUDY PROTOCOL Open Access

Effect of a polyphenol-rich dietary pattern on intestinal permeability and gut and blood microbiomics in older subjects: study protocol of the MaPLE randomised controlled trial

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Abstract

Background: During aging, alterations of the intestinal microbial ecosystem can occur contributing to immunosenescence, inflamm-aging and impairment of intestinal barrier function (increased intestinal permeability; IP). In the context of a diet-microbiota-IP axis in older subjects, food bioactives such as polyphenols may play a beneficial modulatory role.

Methods: MaPLE is a project centered on a randomized, controlled cross-over dietary intervention trial [polyphenol-rich diet (PR-diet) versus control diet (C-diet)] targeted to older people (≥ 60 y) living in a well-controlled setting (i.e. nursing home). The 8-week interventions are separated by an 8-week wash-out period. Three small portions per day of selected polyphenol-rich foods are consumed during intervention in substitution of other comparable products within the C-diet. Biological samples are collected before and after each treatment period to evaluate markers related to IP, inflammation, vascular function, oxidative stress, gut and blood microbiomics, metabolomics. A sample size of 50 subjects was defined based on IP as primary outcome.

Discussion: Evidence that increasing the consumption of polyphenol-rich food products can positively affect intestinal microbial ecosystem resulting in reduced IP and decreased translocation of inflammogenic bacterial factors into the bloodstream will be provided. The integration of data from gut and blood microbiomics, metabolomics and other IP-related markers will improve the understanding of the beneficial effect of the intervention in the context of polyphenols–microbiota–IP interactions. Finally, findings obtained will provide a proof of concept of the reliability of the dietary intervention, also contributing to future implementations of dietary guidelines directed to IP management in the older and other at risk subjects.

Trial registration: The trial is registered at (ISRCTN10214981); April 28, 2017.

Keywords: Gut barrier function, Leaky gut, Flavonoids, Phenolics, Inflammation, Aging, Inflamm-aging

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Background

Age-associated changes significantly compromise health status and increase the risk of chronic diseases. Within these modifications, recent research has been focusing on those that specifically occur at the gut epithelium level with impact on intestinal immune homeostasis and related systemic responses [1]. The maintenance of a functional intestinal barrier (the functional entity separating the gut lumen from the inner host) [2], seems to be of utmost importance to facilitate healthy aging. Nevertheless, no conclusive evidence exists for a direct or causal link between the aging process and intestinal mucosa integrity impairment [3, 4].

The intestine acts both as a barrier (to keep harmful substances out of the body) and as a selectively permeable surface that allows the controlled passage of substances from the gut lumen through the gut wall and into the body. This controlled flux across the intestinal wall is known as intestinal permeability (IP) [2]. Inappropriate IP (i.e. loss of control of the influx of substances from the gut) has been associated with several disorders and diseases, such as irritable bowel syndrome, inflammatory bowel disease, allergy, colon cancer, obesity, celiac disease, inflammatory joint diseases and neurologic pathologies (e.g. Parkinson's disease) [5–8]. In this regard the intestinal microbiota is considered an important factor in the regulation of IP, in fact, gut microorganisms may directly affect IP through tight junction modulation [9] and indirectly by contributing to the up/down regulation of inflammatory processes, which is a key factor in causing impaired IP [10]. Consequently, manipulation of the complex intestinal microbial ecosystem (i.e. the microbiota and derived metabolic products) has been proposed as a novel strategy to maintain/improve normal IP function [2].

Increasing evidence suggests that dietary patterns can represent a relevant factor in shaping the intestinal microbiota and modifying the relative abundance of specific bacterial taxa [11–13]. Consequently, modulating the concentrations of health-affecting microbial metabolites in the gut such as butyrate [14, 15], has been suggested to preserve tight junction integrity and inhibit TNF-alpha release, thus maintaining appropriate IP condition [16]. Nutrients are also essential themselves and malnutrition is associated with increased IP [17].

Older subjects are often characterized by alterations of the intestinal microbial ecosystem [18, 19], which may be due to inadequate nutrition, drug treatments and other age-related factors: all of these seem to contribute to immunosenescence and inflamm-aging [18, 20].

In the context of a diet-microbiota-IP axis, food bioactives may have a key role in regulating the numerous interconnected processes involved. Particularly, polyphenols exert antioxidant, anti-inflammatory/immunomodulatory properties at intestinal and systemic levels, and there is increasing mechanistic evidence suggesting their potential to modulate IP [21, 22]. In addition, polyphenols are extensively metabolized by the microbiota and can affect its composition [13, 23]. The combination of the modulation of intestinal ecology by polyphenols and the effect on derived microbial metabolites has been shown to improve inflammatory markers [24]. Taken together, these data support findings obtained from observational studies in older subjects suggesting that a high polyphenol diet is associated with favorable health outcomes [25]. But, well-controlled intervention studies are still lacking [21].

Aim

The aim of the MaPLE project (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) is to evaluate the hypothesis that an increased intake of polyphenol-rich foods can reduce IP and lower inflammogenic bacterial factors in the bloodstream promoting an overall protective/beneficial metabolic phenotype in older subjects. Three approaches have been taken; the main study, a dietary intervention randomized controlled trial described here, combined with preclinical studies in an animal model of aging to test the impact of the polyphenol-rich diet on IP associated markers, and also in cultured human intestinal cells (caco-2) to investigate the capacity of single polyphenols to modulate IP.

Methods/design

MaPLE RCT: protocol and study design

The MaPLE RCT is a single-blind, randomised, controlled, cross-over trial [polyphenol-rich diet (PR-diet) versus control diet (C-diet)] in older people (≥ 60 y) living in a nursing home. Each intervention period consists of 8 weeks and is separated by an 8-week wash-out period in which participants consume their habitual diet to avoid carry-over effects.

The PR-diet and C-diet were developed to provide adequate and comparable levels of energy and nutrients. The PR-diet was achieved by replacing three portions per day of low polyphenol foods/beverages with specific polyphenol-rich foods/beverages (as detailed below). During the study, subjects are asked to fast overnight before each scheduled time-point of blood, urine and feces collection. In addition, daily menus and weighted food records (WFRs) are collected throughout the trial. An overview of the study design is represented in Fig. 1 and Table 1. The study adhered to SPIRIT guidelines.

Trial status

The trial has been prospectively registered (April 28, 2017; ISRCTN10214981).

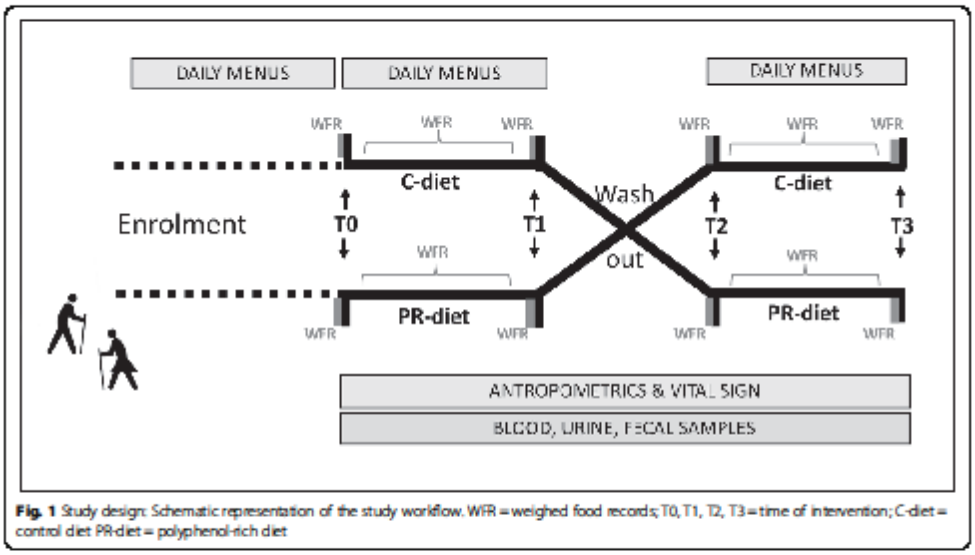

Fig. 1 Study design: Schematic representation of the study workflow. WFR = weighed food records; T0, T1, T2, T3 = time of intervention; C-diet = control diet PR-diet = polyphenol-rich diet

The whole trial has been completed (December 2019); analyses and data elaboration are still ongoing.

Location

The intervention has been performed at Civitas Vitae (OIC Foundation, Padua, Italy) which hosts a large number of older people living in residential care buildings or in independent residences located in the same area, depending on individual willingness and level of disabilities. The OIC Foundation provides several facilities and dedicated area for meal preparation. This allows to collect accurate information with regard the composition of the diets from the recipes used for each of the foods in the meals delivered daily to the participants. We were able to accurately assess food intake using weighed food records in the intervention study.

Participant enrollment

Before recruitment, a meeting with the medical staff and nurses' coordinators at OIC Foundation took place in order to present and widely discuss the aim, methodologies and technical aspects related to the development and the management of the MaPLE RCT. After this meeting, several formal presentations of the project aim and some general information on the intervention planned were organized at OIC Foundation for the hosts and their families. Finally, an accurate evaluation of the host characteristics was performed in collaboration with the physicians/geriatricians and nurses' coordinators to pre-select based on the verification of the main inclusion and exclusion criteria (see below) and to identify plausible candidates for the study. Subjects who were interested in participating in the study signed an informed consent reporting all the information on the dietary intervention, the analysis and protocols that they were asked to undertake/follow.

More specifically, volunteers were selected according to the inclusion and exclusion criteria reported below:

Inclusion criteria

- Age ≥ 60 years
- Adequate nutritional status evaluated with Mini Nutritional Assessment (MNA), score ≥ 24
- Good cognitive status tested with Mini Mental State Examination (MMSE), score ≥ 24
- Self-sufficiency assessed with validated tests (e.g. Barthel index – activities of daily living, score ≥ 60)
- Increased intestinal permeability evaluated by serum zonulin level

Exclusion criteria

- Celiac disease
- Severe liver disease with cirrhosis
- Severe renal insufficiency (dialysis)
- Presence of severe Chronic Obstructive Pulmonary Disease (COPD; oxygen therapy for many hours a day) or severe cardiovascular disease (heart failure

Table 1 Standard protocol items: recommendations for interventional trials (SPIRIT)

	ENROLLMENT	T0	T1	T2	T3
Eligibility screening	X				
Informed consent	X				
Intestinal Permeability test	X				
Daily menu evaluation	X				
Polyphenol intake	X				
PHYSICAL ASSESSMENT AND VITAL SIGN					
Anthropometrics		X	X	X	X
Blood pressure		X	X	X	X
DIETARY ASSESSMENTS					
Daily menu evaluation		X	X	X	X
Weighted Food Record		X	X	X	X
BIOCHEMICAL ASSESSMENTS					
Intestinal Permeability test		X	X	X	X
Inflammatory markers		X	X	X	X
Vascular function markers		X	X	X	X
Oxidative stress markers		X	X	X	X
Metabolomics		X	X	X	X
Microbiota composition		X	X	X	X
Blood microbiomics		X	X	X	X

class III or IV NYHA - New York Heart Association)
- Antibiotic treatment in the last month
- Malignant tumor that required treatment in the previous 2 years

Each subject enrolled has been assigned to an ID number. The encoding of samples is hidden to both the investigators and the participants. All clinical and personal data, including the biological samples, of the subjects involved in the study are collected and stored anonymously.

Polyphenol-rich dietary protocol

In order to define the polyphenol-rich dietary protocol, an initial estimation of nutrient and total polyphenol intake was performed through the analysis of the daily menu provided at the OIC Foundation.

Subsequently, an identification of the specific polyphenol-rich food products to be included in the diet was carried out in order to consider not only the amount and contribution of the different polyphenols but also the food preparation in order to ensure their bioavailability. In addition, an evaluation of conditions to enable optimal texture (e.g. considering the use of purées instead of the whole product) and an assessment of the product acceptability by the target population was also undertaken.

The polyphenol-rich dietary protocol (PR-diet) was finally developed by including in the C-diet 3 portions per day of the following selected polyphenol-rich foods: berries and related products, blood orange, pomegranate, green tea, Renetta apple, and dark chocolate.

A schematic plan of the type and serving sizes of polyphenol-rich products consumed daily in the PR-diet is shown in Table 2. The MaPLE polyphenol-rich foods provided a mean of 724 mg/day of total polyphenols as estimated by Folin-Ciocalteu analysis [26]. In addition, the PR-diet and C-diet were kept comparable in terms of energy intake and nutrient composition, and to achieve this, polyphenol-rich products were substitute for other comparable products (e.g. foods used for snack or breakfast) and this continued across the entire period of intervention.

Table 2 Daily plan of MaPLE polyphenol-rich food products: 3 portions per day are scheduled. Legend: ° Chocolate powder was dissolved in hot milk or water; *Green tea was prepared by solubilization of 200 mg of green tea extract in 200 ml of hot water. †Renetta apple purée was prepared in controlled conditions and stored at − 18 °C.

WEEK	POLYPHENOL-RICH FOOD PRODUCTS (SERVINGS)		
MONDAY	Chocolate callets (10 g)	†Renetta apple purée (100 g)	Pomegranate juice (125 ml)
TUESDAY	Blood Orange (200 ml)	Renetta apple (150 g)	Berry purée (100 g)
WEDNESDAY	Cocoa powder° (2 g)	Renetta apple (150 g)	Blueberry (120 g)
THURSDAY	Green tea* (200 ml)	Chocolate callets (10 g)	Renetta apple (150 g)
FRIDAY	Blood Orange (200 ml)	Pomegranate juice (125 ml)	Blueberry (120 g)
SATURDAY	Chocolate callets (10 g)	Berry purée (100 g)	†Renetta apple purée (100 g)
SUNDAY	Green tea (200 ml)	Blood orange fruit (110 g)	Renetta apple (150 g)

Information on potential adverse effects

Even though no reports of adverse effects due to a polyphenol-rich diet had been registered or reported in the literature, subjects were advised to annotate and communicate any adverse symptom perceived during the intervention period. Since green tea was selected within the polyphenol-rich food sources to be used in the intervention study, there was a comprehensive discussion to define the dose to use. Green tea extract is a rich source of epigallocatechin-3-gallate (EGCG) known for many different protective effects; however, the intake of very high doses of EGCG/green tea extracts as supplements has been reported to cause liver toxicity. Recently, it has been proposed an EGCG upper level (UL) based on human intervention studies of 300 mg EGCG/day in healthy adults [27]. The proposed UL based on an ADI derived from animal toxicity data was 322 mg EGCG/day in a 70 kg adult. These values are applicable to the oral exposure under fed conditions, and consistent with those published by France [28] and Italy [29]. In MaPLE, the dietary intervention provided 200 mg of green tea powder (i.e. 120 mg total polyphenol including about 100 mg EGCG) 2 times per week. This quantity was regarded as very likely to be safe taking into account the target population and the contribution of other food sources containing EGCC.

Assessment of food intake

Food intake before (enrollment phase) and during the intervention periods was recorded through the evaluation of OIC Foundation daily menus and the use of WFRs. The daily menus, covering different seasons, were analyzed to quantify nutrient and polyphenol content. Moreover, the day before each time-point, a WFR was completed and both nutrient and polyphenol intake were estimated. At least 3-WFRs were completed during each intervention period. Daily menus and WFRs were

assessed using MetaDieta® (Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) to estimate energy and nutrient intake. Total polyphenol estimation was performed by using the Phenol Explorer database (phenol-explorer.eu) to provide estimates of polyphenol concentrations in each food, and where there were no useful values, using our proprietary data or values obtained from the literature. Total polyphenol content of the foods was estimated directly using the Folin-Ciocalteau method [30].

Biological sampling

Blood, urine and fecal samples were collected at each time-point as defined in Fig. 1. For blood drawing, a specific vacutainer was used. Urine and fecal samples were collected using specific containers designed for this purpose. An aliquot of each collected blood sample was immediately stored at − 80 °C for microbiomic analyses. The remaining blood was processed by centrifugation and then serum and peripheral blood mononuclear cell (PBMC) fractions were obtained, divided into aliquots and stored at 80 °C. Urine and fecal sample were divided into aliquots, and all human tissue samples were stored at − 80 °C until analysis.

In addition, a brush was used to collect an oral mucosal sample from each participant for further evaluation. The brush with the collected tissue was stored in a cryovial containing a buffered saline solution, which was immediately frozen.

Outcome measurements

The primary selected outcome of the study was zonulin as an IP marker, whereas other IP related markers (e.g. CD14, calprotectin), inflammatory markers (CRP, TNF-α, IL-6), oxidative stress and vascular function markers (DNA damage, VCAM-1, ICAM-1), metabolomics and microbiomics (16S rRNA gene quantification and taxonomic profiling) were included as secondary outcomes to support and validate our study hypothesis.

Anthropometric measurements

Body weight, height and BMI calculation were assessed at the beginning and the end of each intervention period following the international guidelines of Lohman et al. [31].

Blood pressure

Each participant was monitored at the beginning and the end of each intervention period measuring both systolic and diastolic pressure obtained in a resting, seated position following the validated JNC 7 guidelines [32].

Metabolic and functional markers

At enrollment and at each time-point, metabolic and functional parameters (i.e. glucose, insulin, lipid profile, liver and renal function) were assessed by a standardized validated protocol, using an automatic biochemical analyzer (ILAB 650, Instrumentation Laboratory, Lexington, MA). Low density lipoprotein cholesterol (LDL-C) concentration was estimated using the Friedewald formula [33], while non-high density lipoprotein cholesterol (non-high density lipoprotein-cholesterol, HDL-C) was calculated by subtracting HDL-C from total cholesterol (TC). The HOMA-Index and Cockroft-Gault index were calculated according to the relevant formula [34, 35].

Intestinal permeability evaluation

Intestinal permeability was evaluated by quantifying serum zonulin concentrations. Human zonulin is a protein (i.e. prehaptoglobin-2) released by enterocytes able to promote the activation of the signaling transduction pathway that cause tight junction protein disassembly enabling potential bacterial factor translocation [36]. In this study, zonulin serum levels were quantified using the Immunodiagnostik® ELISA kit (Bensheim, Germany) with samples collected in the selection phase and at the beginning and the end of each intervention period. Subjects selection based on IP was performed by considering reference values reported in the manufacturer's instructions and data published on different target groups [37–39]. Other IP related markers, such as serum CD14 and fecal calprotectin, were also quantified to support the primary outcome.

Inflammatory markers

The concentrations of several markers related to inflammatory processes were quantified using specific ELISA kits (R&D Systems, Biotechne, Abingdon, UK). CRP (DCRP00), IL-6 (HS600B), TNF-α (HSTA00E) were quantified in serum at the beginning and the end of each intervention periods.

Vascular function markers

In order to assess vascular function, vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) were quantified in serum samples at each intervention time point using an ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy).

Oxidative stress marker (comet assay)

The levels of endogenous and oxidatively-induced DNA damage, as markers of oxidative stress, were assessed in PBMCs by the comet assay. The samples are collected before and after each intervention period. Levels of endogenous DNA damage were assessed using a specific enzyme (formamidopyrimidine DNA glycosylase, FPG sensitive sites) that can be used to detect 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases. Oxidatively-induced DNA damage was measured by treating the cells with

hydrogen peroxide and by evaluating the capacity of cells to counteract an oxidative insult. Both Comet assay protocols have been previously described by Del Bo' et al. [32].

Blood bacterial load and taxonomic profiling

Bacterial DNA quantification and sequencing reactions were performed by Vaiomer SAS (Labège, France) using optimized blood-specific techniques as described earlier [40–43]. Specifically, DNA was extracted from 100 µl of whole blood and quantified by quantitative PCR targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene with primers EUBF 5′-TCCTACGGGA GGCAGCAGT-3′ and EUBR 5′ -GGACTACCAGGGT ATCTAATCCTGTT-3′ [44]. The results are reported as 16S rRNA gene copies per ng of total DNA and per µl of blood. DNA from whole blood was also used for 16S rRNA gene taxonomic profiling using MiSeq Illumina® technology (2 × 300 paired-end MiSeq kit V3, set to encompass 467-bp amplicon) as previously described [42, 43]. To determine bacterial community profiles, the bar-coded Illumina paired reads were demultiplexed, then single read sequences were trimmed and paired for each sample independently into longer fragments; non-specific amplicons (< 350 bases or > 500 bases) were removed and remaining sequences clustered into operative taxonomic units (OTUs) using FROGS v1.4.0 [45] with default parameters; a taxonomic assignment was finally performed against the Silva 128 Parc database. Bioinformatics analysis of the sequencing data was also performed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline [46].

Fecal microbiota composition

All of the following steps were performed in-house at QIB. Fecal samples were weighed into Lysing Matrix E bead beating tubes (MPBio, Santa Ana, CA, USA) and extraction was completed according to the manufacturer's protocol for the FastDNA™ SPIN Kit for Soil (MPBio) but extending the bead beating time to 3x60s. DNA was quantified using a Qubit® 2.0 fluorometer (Invitrogen, Carlsbad CA, USA), normalized to 5 ng/µl and the V3/V4 region of the 16S rRNA was amplified using the primers detailed below. Sequencing was performed using a 600 cycle MiSeq v3 reagent kit (Illumina, San Diego, CA, USA) giving approximately 100,000 reads per sample.

Bioinformatic analysis was conducted using VSEARCH [47]; reads were merged, and primer sequences trimmed. Reads were dereplicated and singletons removed. Prior to Chimera removal, reads were clustered at 97% similarity, de novo Chimera removal was performed using the UCHIME algorithm [48] and the OTU table and sequences were prepared. Data was subsequently analyzed using the phyloseq package in R [49].

Primers:

16S 341F – TCGTCGGCAGCGTCAGATGTGTATA AGAGACAGCCTACGGGNGGCWGCAG.

16S 806R – GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC.

In addition, taxonomic profiling was carried out through shotgun sequencing. In brief, metagenomic DNA isolated from fecal samples was sequenced using an HiSeq instrument (Illumina, San Diego, CA) by CosmosID (Rockville, MD, USA). Microbial community composition was determined through the analysis of shotgun metagenomic datasets with the CosmosID metagenomic software as previously described [50].

Metabolomics

Urine samples collected prior and after each intervention period were subjected to targeted metabolomics analysis by applying the Quantitative Dietary Fingerprinting approach recently developed by González-Domínguez et al. [51] with the aim of monitoring metabolite alterations derived from the polyphenol-rich diet and to associate these changes with improvements in clinical and biochemical outcome measurements (e.g. IP evaluated through zonulin levels, inflammatory and oxidative stress markers, blood bacterial load). To this end, urine samples were treated by solid phase extraction (SPE) and subsequently analyzed by reversed-phase ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (RP-UHPLC-MS/MS) to obtain a comprehensive assessment of the urinary food metabolome, with the simultaneous quantitative determination of about 350 dietary derived metabolites. Complementarily, plasma samples are also analyzed using a modification of the previously described targeted metabolomics approach, adapted to deal with the chemical complexity of blood samples (high content of proteins and lipids) and to enlarge the metabolomic coverage. This novel method is based on a similar RP-UHPLC-MS/MS instrumental configuration that enables the simultaneous measurement of both food intake biomarkers and endogenous metabolites from multiple chemical classes (ca. 1000 metabolites), including amino acids and derivatives, biogenic amines, carbohydrates, organic and fatty acids, vitamins and various lipid classes (e.g. acylcarnitines, steroid hormones, bile acids), among others. To expand the method coverage towards the high-polarity low molecular weight metabolome, an orthogonal hydrophilic interaction liquid chromatography (HILIC) procedure was also applied, covering a broad range of polar metabolites (ca. 300 metabolites), comprising common and acetylated amino acids and microbiota derivatives, low molecular weight organic acids (including

short chain fatty acids and related compounds) and carbohydrates (e.g. sugars, conjugates and advanced glycation end products).

Sample size, randomisation, and statistics

According to data literature [38, 52] it was estimated that 50 subjects were required to demonstrate an IP reduction of 30% with 80% power and 0.05 significance and considering a 15% drop-out rate. Subjects were randomly divided by using a computer random number generator. The randomisation and allocation were performed by a person not involved in the trial and blinded to the participants, investigators/health care providers and researchers involved in samples analysis. Statistical analyses were performed by means of R statistic software version 3.4.2. Particularly, the following statistical elaborations will be performed to identify significant differences between treatments: (i) the analysis of variance (ANOVA) with repeated measures, (ii) Wilcoxon paired data test, (iii) Linear Mixed Model (LMM) analysis. In addition, regression and correlation analyses (Spearman and Kendal test) are carried out to highlight associations between blood microbiomic data, fecal bacterial profiling data, and physiological and biochemical data. When appropriate, a post-hoc *p*-value adjustment is performed using the Hochberg-Benjamin correction. Significance is set at $P \leq 0.05$; significance in the range $0.05 < P < 0.10$ is accepted as trend. Potential gender differences will be also considered in all the analyses.

Discussion

There is growing evidence of a link between IP impairment and increased inflammation [2]. Since aging is characterized by low grade systemic inflammation it is possible that an increase in IP may induce the activation of inflammatory pathways and the immune system caused by the translocation of intestinal microbes, toxins, and/or nutritional components from the gut lumen through the epithelium and into the bloodstream [52]. While there is preliminary mechanistic evidence obtained in animal models on the complex interaction between age-associate microbial dysbiosis, IP and inflammation [5], the properties of the human intestinal barrier, in the context of the ageing process, has not been fully investigated [4]. The dietary pattern and the intestinal microbial ecosystem homeostasis have been addressed as potential key points for the development of strategies to enable healthy aging. The manipulation and/or improvement of the diet by increasing the consumption of food bioactives (e.g. polyphenols) or specific nutrients is recognized as a potential powerful tool to be explored also in the context of IP. However, human intervention studies are still very scarce, and most of these performed using probiotics, prebiotic fibers and dietary supplements [21].

By considering this premise, the MaPLE RCT here described aimed to investigate whether a PR rich-diet can improve the intestinal microbial ecosystem of older subjects characterised by an increased IP. In addition, it is hypothesized that such modulation could promote an overall beneficial impact on IB function, a decreased IP and translocation of inflammogenic bacterial factors in the blood.

The development and management of well-controlled and adequately balanced dietary intervention studies is not an easy task and it becomes even more difficult when the target population is older subjects. Consequently, the first task of the project was dedicated to the optimization of the trial in order to overcome the possible problems related to compliance with the dietary instructions and to other relevant potential confounding factors (e.g. periods of illness or the use of drugs that may be relevant in this target group). For this reason, the MaPLE RCT was planned in a residential area for older people, since it provided a favourable and controlled environment in which it was possible to optimize and standardize most of the important experimental conditions. For example, since outcome data from dietary intervention studies are prone to being affected by individual differences in diets and lifestyle behaviour over time (e.g. during the two eight week periods of dietary intervention), we were able to ensure both strict compliance with the dietary intervention and a consistent dietary pattern among participants by including the polyphenol-rich products in their usual meals provided by the residential home. In addition, the selection of polyphenol-rich foods was based on three important considerations: (i) That the types of foods selected were largely universally liked, (ii) that the texture of the selected products was suitable for older subjects (e.g. with dentition challenges), and (iii) that the portion of food would reliably provide a high dose of polyphenols. In addition, weighed food intake was also assessed to provide us with data to allow accurate estimates of actual nutrient and polyphenol intake in the two periods of treatment (PR- and C- diet). This allowed a high degree of control and substantially reduced between treatments differences.

As regard the primary outcome, serum zonulin concentrations were used as the marker of IP because of the low reliability and applicability of the multi-sugar test in the older population (i.e. due to a high rate of incontinence amongst the elderly participants and the need for adherence to a strict dietary protocol before the test) [52].

It is also noteworthy that the MaPLE RCT is testing, for the first time, the hypothesis that a dietary intervention may modulate quantitatively the bacterial DNA in bloodstream and qualitatively the blood microbiota

composition. This should provide further evidence of the impact of the dietary intervention on IP being potentially associated with a reduction in translocation of bacterial factors. Other objectives of the MaPLE RCT are to integrate microbiota profiling data with inflammation and metabolomics data to improve understanding on the impact of the dietary intervention. In addition, the inter-individual response to the treatment will be investigated and food metabolite profiling data will be exploited for the identification of a set of potential biomarkers with relevance in the context of preventing or treating impaired IP.

Finally, results will be pivotal for the development of new dietary approaches and guidelines for managing IP related conditions in the complex context of healthy aging.

Abbreviations

IP: Intestinal permeability; MaPLE: Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly; PR-diet: Polyphenol-rich diet; C-diet: Control diet; WFRs: Weighted food records; MNA: Mini nutritional assessment; MMSE: Mini mental state examination; COPD: Chronic obstructive pulmonary disease; NYHA: New York heart association; EGCG: Epigallocatechin-3-gallate; UL: Upper level; PBMCs: Peripheral blood mononuclear cells; LDL-C: Lipoprotein cholesterol; HDL-C: Non-high density lipoprotein-cholesterol; TC: Total cholesterol; VCAM-1: Vascular cell adhesion molecule-1; ICAM-1: Intercellular adhesion molecule-1; 8-oxodG: 8-oxo-7,8-dihydro-2′-deoxiguanosine; QIIME: Quantitative Insights Into microbial ecology; SPE: Solid phase extraction; HILIC: Orthogonal hydrophilic interaction liquid chromatography; ANOVA: Analysis of variance; LMM: Linear mixed model

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Authors' contributions

MaPLE project includes three research units: (I) University of Milan, Italy, coordinated by Patrizia Riso (PR) and Simone Guglielmetti (SG), who are the principal investigators of the project, (II) University of Barcelona, Spain, coordinated by Cristina Andres-Lacueva (CAL), and (III) Quadram Institute, UK, coordinated by Paul A Kroon (PAK).
All listed authors meet the ICMJE criteria for authorship. In particular, PR and SG are responsible for the trial conception and design. AC contributed to the development of study protocol for clinical and ethical aspects and to the selection of biochemical markers under study from a clinical perspective. CAL and PAK contributed to the definition of polyphenol-rich dietary intervention and markers selection. CDB contributed to the development of the study protocol and with PR, SG and SB drafted the first version of the manuscript. SG and GG contributed to the definition of the trial protocol enabling the study of blood microbiomic and metagenomic. RGD and GP coordinated by CAL implemented the study protocol to enable metabolomics approach. MP, NHL and RZR contributed to the definition of the design needed to enable accurate dietary intake assessment. BK and MSW contributed to the development of protocol aspects defined for microbiota composition evaluation and inflammation coordinated by PAK who also set the markers for polyphenol metabolism evaluation. All the authors critically revised the draft and approved the final version.

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The sponsors of this work have no role in the development of study protocol, methods, data collections and analyses, and the preparation of the manuscript.

Availability of data and materials

At the end of the project, after the final elaborations, the datasets generated during the study will be made freely available in the Dataverse repository, https://dataverse.unimi.it/dataverse/IPIMaPLE.

Ethics approval and consent to participate

The Ethics Committee of the Università degli Studi di Milano approved the study protocol (15/02/2016; ref: 6/16/CE_15.02.16_Verbale_All-7). All participants gave their written informed consent to participate in the MaPLE RTC allowing also the use of samples and data collected within the trial.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

A) COMUNICACIÓN A CONGRESO DEL PROTOCOLO DEL ESTUDIO MaPLE

Resumen

En los últimos años, la investigación se ha centrado en estrategias para contrarrestar los procesos inflamatorios y las enfermedades relacionadas con la edad. Durante el envejecimiento, una inflamación sistémica de bajo grado suele estar asociada a una permeabilidad intestinal (PI) alterada, una condición que se ha demostrado que promueve la inflamación, posiblemente a través de la translocación de factores dietéticos y bacterianos hacia el torrente sanguíneo que, en este sentido, el patrón dietético y los factores ambientales podrían desempeñar un papel fundamental debido a su capacidad potencial para modular la inflamación, la PI y el ecosistema microbiano intestinal (EMI). Además, se ha planteado la hipótesis de que los compuestos bioactivos, como los polifenoles, pueden afectar a la PI y al EMI. El proyecto MaPLE (*Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly*) tenía como objetivo investigar la hipótesis de que una dieta rica en polifenoles puede mejorar el estado de IP en una población objetivo con cambios beneficiosos a nivel intestinal y sistémico. Para ello, se realizó un estudio de intervención dietética aleatorio, controlado y cruzado (dieta rica en polifenoles de 8 semanas frente a una dieta de control de 8 semanas, separada por un período de lavado) en un grupo de personas mayores (> 60 años) que vivían en un entorno bien controlado (es decir, una residencia de ancianos). Se investigaron en muestras de suero, orina y/o heces los marcadores relacionados con la PI, la inflamación el estrés oxidativo, la función vascular y el ecosistema microbiano intestinal. Además, se ha evaluado la DNAemia de las bacterias de la sangre y la metabolómica del suero y la orina. Asimismo, se realizó una evaluación nutricional consistente del menú estándar (proporcionado por la residencia geriátrica) y de los registros de alimentos pesados, proporcionando también datos sobre la ingesta real de polifenoles durante la intervención. Los resultados muestran que había niveles más altos de PI en los sujetos de mayor edad, y que la dieta enriquecida con polifenoles modificó los niveles de zonulina sérica, un marcador de PI. Además, se demostró una asociación entre la zonulina y la carga bacteriana en sangre. Los experimentos in vitro e in vivo que se están llevando a cabo exploran los efectos potenciales de diferentes polifenoles sobre la PI y los mecanismos implicados. El proyecto MaPLE generará nuevos datos para mejorar la comprensión del papel de los polifenoles en la modulación del microbioma intestinal y sus interacciones con el huésped.

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Role of a Polyphenol-Rich Dietary Pattern in the Modulation of Intestinal Permeability in Older Subjects: The MaPLE Study †

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1. Introduction

The inevitable rise of the proportion of people aged >65 years worldwide is paralleled by an increased burden of chronic diseases often associated with low-grade systemic inflammation. Recent findings suggest a link between inflammation and intestinal permeability (IP), a condition characterized by an impairment of intestinal barrier function which enables the translocation of dietary and bacterial factors into the blood activating the host immune system [1,2]. Dietary components can be significant modulators of inflammation and IP, and can also affect the intestinal microbial ecosystem. In the context of a diet-microbiota-IP axis in older subjects, dietary bioactives such as polyphenols may play a significant protective role due to their widely reported antioxidant and immunomodulatory properties and potential to regulate IP [3–6].

2. Material and Methods

The MaPLE project involves a multidisciplinary approach developed to ascertain the impact of a polyphenol-rich dietary pattern on a large number of markers in a target group of older subjects living in a controlled setting (i.e., nursing home).

A controlled, randomized cross-over dietary intervention study (8-week polyphenol-rich diet versus 8-week control diet) was undertaken. Markers of IP, inflammation, oxidative stress and vascular function and assessments of gut microbiota structure and function were quantified in serum,

urine and/or fecal samples. In addition, bacterial DNAemia, and serum/urine metabolomics were assessed. In vivo with a dietary mixture similar to the human study and in vitro studies with isolated polyphenols were carried out to investigate mechanisms of action.

3. Results & Discussion

The dietary intervention has been completed and as expected, IP was relatively high in this cohort of older participants, as assessed by serum levels of zonulin at baseline. Quantification of changes in various markers in response to the high polyphenol diet compared to the normal polyphenol diet are being completed and will provide evidence of the putative beneficial effect of increased polyphenol consumption in this target population.

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B) COMUNICACIÓN A CONGRESO DEL PROTOCOLO DEL ESTUDIO MaPLE

1. Introducción

El inevitable aumento de la proporción de personas de más de 65 años en todo el mundo va acompañado de un incremento de la carga de enfermedades crónicas a menudo asociadas a la inflamación sistémica de bajo grado. Los últimos descubrimientos sugieren una relación entre la inflamación y la permeabilidad intestinal (PI), una condición caracterizada por una alteración de la función de barrera intestinal que permite la translocación de factores dietéticos y bacterianos a la sangre, activando el sistema inmunitario del huésped. Los componentes de la dieta pueden ser importantes moduladores de la inflamación y la PI, y también pueden afectar al ecosistema microbiano intestinal. En el contexto de un eje dieta-microbiota-IP en sujetos de edad avanzada, los bioactivos de la dieta, como los polifenoles, pueden desempeñar un papel protector importante debido a sus propiedades antioxidantes y sus propiedades inmunomoduladoras y su potencial para regular la PI].

Material y Métodos: El proyecto MaPLE implica un enfoque multidisciplinar desarrollado para determinar el impacto de un patrón dietético rico en polifenoles sobre un gran número de marcadores en un grupo objetivo de personas mayores que viven en un entorno controlado (es decir, una residencia de ancianos).

Se llevó a cabo un estudio controlado y aleatorizado de intervención dietética cruzada (dieta rica en polifenoles de 8 semanas versus dieta de control de 8 semanas). Se cuantificaron los marcadores de PI, inflamación, estrés oxidativo y función vascular y las evaluaciones de la estructura y función de la microbiota intestinal en muestras de suero, orina y/o heces. Además, se evaluó la *DNAemia* bacteriana y la metabolómica de suero y orina. suero/urina. Se realizaron estudios in vivo con una mezcla dietética similar a la del estudio en humanos y estudios in vitro con polifenoles aislados se realizaron estudios in vitro con polifenoles aislados para investigar los mecanismos de acción.

Resultados y discusión: La intervención dietética se ha completado y, como se esperaba, la PI fue relativamente alta en esta cohorte de participantes de edad avanzada, como se evaluó por los niveles séricos de zonulina al inicio del estudio. La cuantificación de cambios en varios marcadores en

respuesta a la dieta alta en polifenoles en comparación con la dieta normal en polifenoles. de polifenoles, en comparación con la dieta normal de polifenoles, y proporcionará pruebas del supuesto efecto beneficioso del aumento del consumo de polifenoles en esta población. de un mayor consumo de polifenoles en esta población.

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Intestinal permeability modulation through a polyphenol-rich dietary pattern in older subjects: MaPLE project outcomes and perspectives

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Abstract

In recent years, research has been focusing on strategies to counteract inflammatory processes and age-related diseases[(1)]. During ageing, a low-grade systemic inflammation is often associated to an altered intestinal permeability (IP) a condition that has been shown to promote inflammation possibly through the translocation of dietary and bacterial factors into the blood stream that activates the immune system[(2)].In this regard, dietary pattern and environmental factors could play a fundamental role because of their potential ability to modulate inflammation, IP and the gut microbial ecosystem (GME). Moreover, it has been hypothesized that bioactive compounds such as polyphenols may affect IP and GME[(3)].The MaPLE project (Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly) aimed to investigate the hypothesis that a polyphenol-rich diet can improve IP condition in a target population with beneficial changes at intestine and systemic level. To this aim, a randomised, controlled, cross-over dietary intervention study (8-week polyphenol-rich diet versus 8-week control diet, separated by a wash-out period) was carried out in a group of older subjects (> 60 years) living in a well-controlled setting (i.e. nursing home). Markers related with IP, inflammation, oxidative stress, vascular function and intestinal microbial ecosystem were investigated in serum, urine and/or fecal samples. Moreover, blood bacteria DNAemia, and serum/urine metabolomics has been assessed. Moreover, a consistent nutritional evaluation of the standard menu (provided by the nursing home) and of weighed food diaries was performed, providing also data on actual polyphenol intake during the intervention. The results show there were higher levels of IP in the older subjects, and that the polyphenol-enriched diet changed the levels of serum zonulin, a marker of IP. In addition, an association between zonulin and blood bacterial load was demonstrated. Ongoing in vitro and in vivo experiments are exploring the potential effects of different polyphenols on IP and the mechanisms involved. The MaPLE project will generate new data to improve the understanding on the role of polyphenols in the modulation of intestinal microbiome and its interactions with the host.

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Conflict of Interest

There is no conflict of interest

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i. Análisis de la dieta estudio MaPLE

En el estudio MaPLE, se ha evaluado el aporte de los menús, el aporte de los alimentos ricos en polifenoles y la ingesta de polifenoles de los participantes.

La ingesta alimentaria ha sido evaluada mediante registros de alimentos por pesada (3-5 registros por cada participante), los cuales fueron recogidos por personal entrenado. Los registros por pesada se obtuvieron durante el transcurso del estudio en los correspondientes períodos de evaluación (T0-T1; T2-T3).

Una vez obtenidos los registros de cada participante, se procedió con el análisis de los registros de alimentos por pesada para obtener la estimación del contenido de energía y de nutrientes, la cual fue realizada por investigadores de la Universidad de Milán, utilizando el programa informático MetaDieta® (Me.Te.Da S.r.l., San Benedetto del Tronto, Italia). Por otra parte, la estimación del aporte de polifenoles ha sido estimada en la Universidad de Barcelona, siguiendo la metodología que se explica a continuación:

- **Evaluación del contenido de polifenoles de los menús ofertados:**

Se analizaron los menús semanales proporcionados en la residencia de personas mayores. Se consideraron 3 tipos de menús que abarcaban la ingesta de distintas estaciones del año implicadas en la duración del estudio, específicamente se consideró el menú de invierno, de media estación y de verano. Además de cada uno de los tiempos de comida que componían el menú diario (desayuno, comida, cena y colaciones de media mañana, de tarde y de noche)

- **Evaluación del contenido de polifenoles de los productos ricos en polifenoles.**

Se estimó el aporte de polifenoles de los productos incluidos en la dieta rica en polifenoles: tableta de chocolate (10g); puré de manzana *Reineta* (100g); zumo de granada (125 ml); zumo de naranja sanguina (o roja) (200ml); manzana *Reineta* (150g); puré de berries (100g); chocolate en polvo (2g), arándanos (120g); té verde (200 mg de extracto de té verde); naranja sanguina (o roja) (110ml).

- **Evaluación de la ingesta de polifenoles de los participantes del estudio:**

Respecto a la estimación de la ingesta de los 51 particpantes, se analizaron los registros de alimentos disponibles para cada sujeto en cada período del estudio (T0-T1; T2-T3)

A través de un software denominado "Cálculo Nutricional" desarrollado en el grupo de investigación de Biomarcadores y Metabolómica Nutricional y de los alimentos de la Universidad de Barcelona, se ha estimado el contenido de polifenoles de los menús ofertados y de la ingesta de los participantes.
En el software se incluyen las bases de datos de composición de alimentos (BDCA) de USDA sobre flavonoides (86), proantocianidinas (87) e isoflavonas (88) expresada como agliconas, y la BDCA de Phenol Explorer 3.6 (89) con su respectiva clasificación (flavonoides, ácidos fenólicos, estilbenos, lignanos y otros polifenoles) expresados en forma de agliconas o cono se encuentran en la naturaleza (glucósidos, ésteres, agliconas), los valores incluídos incluidos en las BDCA han sido previamente determinados a partir de métodos cromatográficos y, también los polifenoles totales determinados por el método de Folin-Ciocalteau (90). Además, se añadieron valores propios obtenidos de laboratorio del análisis de los productos de los alimentos ricos en polifenoles del estudio MaPLE o de la literatura científica.

Se ha incorporado al software las dos bases de datos de referencia mencionadas, para estimar el contenido de polifenoles en los alimentos. En ambas, la estimación de compuestos fenólicos se realiza por cada 100 gramos de alimentos. La primera BDCA ha sido desarrollada por el Departamento de Agricultura de los Estados Unidos [United States Department of Agriculture (USDA)], y actualmente se encuentra conformada por tres tablas de composición que proporcionan información sobre el contenido de flavonoides, proantocianidinas e isoflavonas, expresadas en forma de agliconas. La tabla de composición de flavonoides contiene valores para 506 alimentos y 26 flavonoides predominantes en la dieta (86). Para las proantocianidinas, existen valores para 283 flavonoides individuales presentes en los alimentos (87); y 560 valores en alimentos para isoflavonas (88).
La siguiente base de datos incorporada en el software es la BDCA de Phenol-Explorer (www.phenol-explorer.eu) la cual contiene datos con información de nomenclatura química, datos taxonómicos y analíticos de 501 polifenoles, que han sido clasificadas en 5 clases (flavonoides, ácidos fenólicos, estilbenos, lignanos y otros polifenoles) y 31 subclases, expresados como agliconas o glucósidos. Estos datos, se encuentran disponibles para 458 alimentos y fueron extraídos a partir de una recopilación sistemática de 1300 publicaciones científicas. Además, esta BDCA tiene incorporado 4.296 datos sobre factores de retención (que considera los efectos que causan el procesado y la transformación de los alimentos) en 139 polifenoles, 155 alimentos y 35 procesos. Actualmente, está disponible la versión 3.6 donde se han añadido 1451 nuevos valores para lignanos (89).

ii. Metodología de análisis y del cálculo del aporte/ingesta de polifenoles con la aplicación "Calculo Nutricional" en el estudio MaPLE

Para la estimación del aporte de los menús y de las ingestas se ha realizado los siguientes pasos (Figura 10):

1. Una vez obtenidos los registros de alimentos por pesada y/o los menús (gramos de alimento), se analizaron y se identificaron 243 ítems (incluyendo alimentos y recetas).
    a. Se elaboraron las recetas de las preparaciones correspondientes, asignado los correspondientes ítems de alimentos.

2. Se identificaron los alimentos que contienen polifenoles (n=130)
    a. A los alimentos sin polifenoles se les asignó el código "unknown=99999" que son normalmente alimentos de origen animal: lácteos, carnes, pescados, etc. reportados en los registros.

3. Emparejamiento de datos de la ingesta alimentaria o de los menús (gramos de alimento/día) del estudio MaPLE (llamados "alimentos MaPLE") con los ítems de alimentos (g/100g) de cada una de las bases de datos utilizadas en el software Cálculo Nutricional:
    a. Base de datos de composición de alimentos de Phenol Explorer 3.6
        i. Polifenoles totales (Método Folin Ciocalteau)*
        ii. Ácidos fenólicos, lignanos, estilbenos y otros polifenoles (Agliconas)
    b. Bases de datos de composición de alimentos de USDA
        i. Flavonoides (USDA 2018)
        ii. Isoflavonas (USDA 2015)
        iii. Proantocianidinas (USDA 2018)

Los enlaces (links) entre los alimentos MaPLE y los ítems de alimentos de cada base de datos de composición de alimentos (BDCAs), se realizaron a través del sistema SQL Manager Lite for MySQL, que es un sistema de gestión de base de datos relacional, que define relaciones entre las diferentes bases de datos que se incluyen.

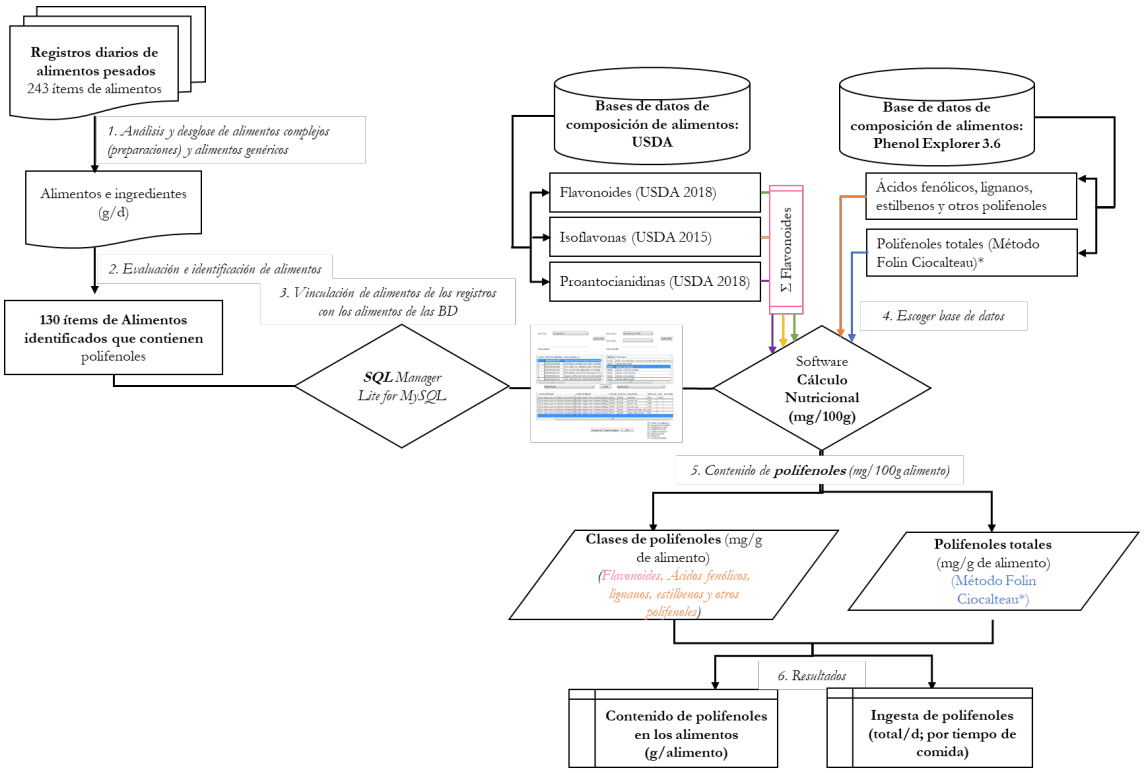
Cabe destacar que, para el emparejamiento con las diferentes BDCAs, se tuvo en cuenta la forma en la que los alimentos fueron consumidos (por ejemplo, manzana con o sin piel, o cruda o cocida, etc.), dado que las bases de datos consideran estas características de los alimentos, variando el contenido del o los compuestos estudiados en dicho alimento.

Cuando un alimento de MaPLE no coincidía con los alimentos disponibles en las BDCAs se marcó con el código "unknown=99999" para la correspondiente BDCA.

4. Cálculo del contenido de polifenoles:

   En el estudio MaPLE tanto para la estimación del aporte de los menús, de los alimentos utilizados en la dieta-RP y de la estimación de la ingesta dietética de los participantes, se calcularon los polifenoles totales por el método de Folin-Ciocalteau y, se estimó el contenido de polifenoles totales a partir de la suma de 191 polifenoles individuales provenientes de las 4 clases siguientes: flavonoides, ácidos fenólicos, lignanos, estilbenos y otros polifenoles.

Figura 10. Procedimiento la estimación del aporte de los menús y de la ingesta de polifenoles del estudio MaPLE



Fuente: Elaboración propia

DISEÑO DE ESTUDIO PROSPECTIVO OBSERVACIONAL. ESTUDIO INCHIANTI, *THE INCHIANTI STUDY: INVECCHIARE NEL CHIANTI*

Se presenta el diseño del estudio Prospectivo Observacional, InCHIANTI a partir del cual se ha trabajado los objetivos 6,7 y 8 planteados en esta tesis doctoral:

Figura 11: Logotipo The InCHIANTI Study

El estudio InCHIANTI se ha realizado en colaboración con el Dr. Luigi Ferrucci, director científico del National Institute of Aging (NIA) del National Institute of Health (NIH) en Baltimore, Estados Unidos, y el Dr. Antonio Cherubini, profesor titular de la Universidad de Perugia y director del INRCA-IRCCS en Ancona, Italia. La colaboración entre la Universidad de Barcelona, el NIA-NIH y el ARS (Proyecto InCHIANTI) corresponde a un convenio de colaboración internacional firmado previamente por dichas instituciones y diversas convocatorias competitivas.

El estudio InCHIANTI (*Invecchiare in Chianti, Envejecimiento en la región de Chianti*) es un estudio de cohorte prospectivo que tiene por objetivo evaluar los factores de riesgo que afectan la perdida de movilidad y las enfermedades relacionadas con el envejecimiento de personas mayores de la región de la Toscana, específicamente en las localidades de Greve in Chianti y Bagno a Ripoli. El reclutamiento del estudio comenzó en el año 1998 y se completó en el año 2000. Hasta la fecha se han realizado diversos seguimientos a los 3, 6, 9, 12, 15 años, los que corresponden a los años 2001-2003, 2004-2006, 2007-2009, 2010-2012, 2013-2015, 2016-2018, respectivamente.
De la última evaluación actualmente, sólo se disponen de datos de mortalidad.
En esta tesis doctoral se han tenido en cuenta datos del tiempo basal y de los seguimientos a los 3, 6 y 9 años para la fragilidad y de los 12, 15 hasta los 20 años para la mortalidad.

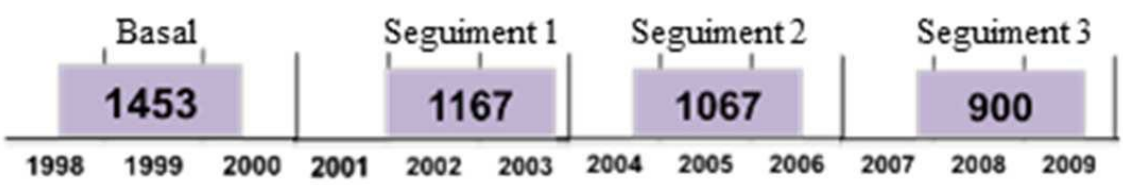
Figura 12: Municipios fuente de estudio en The InCHIANTI Study

Se ha realizado seguimiento de los participantes hasta el año 2018 (Figura 13 y 14).

Para ser incluidos en el estudio los participantes debían residir en una de las localidades (Greve o Bagno a Ripoli). Además de se debía contar con la firma del consentimiento informado de cada participante, el cual fue aprobado por El Comité Ético del Instituto Nacional Italiano de Investigación y Cuidado del Envejecimiento.

El diseño y los métodos de recolección de datos han sido descritos detalladamente por Ferrucci et al.(91) y se encuentra registrado en ClinicalTrials.gov: NCT01331512

Figura 13. Diseño del estudio InCHIANTI



Fuente: http://inchiantistudy.net

Figura 14. Diseño del estudio InCHIANTI, seguimientos

Fuente: Elaboración propia en base a información de la web: http://inchiantistudy.net

A la totalidad de los participantes reclutados se les realizó mediciones antropométricas y una evaluación gerontológica que incluía evaluaciones clínicas para valorar el estado de salud general y el estado funcional, entre ellas, se hicieron entrevistas en el hogar que permitían recopilar información sociodemográfica, de hábitos de vida, uso de medicamentos y patologías prevalentes, preguntas relativas a la salud física y mental, historia familiar, actividades diarias y las condiciones de vida relacionadas a la movilidad continua, además se aplicó un cuestionario para valorar el nivel de actividad física y un cuestionario de frecuencia de consumo de alimentos (CFCA) desarrollado y validado en el estudio EPIC-Italia.

Se tomaron muestras de sangre y orina sólo en el basal, para esta última, los participantes recolectaron orina de 24 horas previas a la visita inicial y posteriormente las muestras fueron alicuotadas y almacenadas a -80ºC hasta el momento de los respectivos análisis.

Todos los participantes que acordaron participar en el estudio InCHIANTI, entregaron el correspondiente consentimiento informado al momento de su inclusión en el estudio.

Los participantes del estudio InCHIANTI eran hombres y mujeres de 65 años o más. La muestra final de la cual se ha iniciado los estudios en esta tesis doctoral fue de 1155 participantes con un rango de edad comprendido entre 65 y 102 años.

Con la finalidad de dar cumplimiento a los objetivos planteados, se ha valorado la exposición dietética de alimentos, nutrientes y de un patrón de dieta mediterránea en personas mayores que viven en la comunidad en dos ciudades de la Toscana en Italia y se ha asociado su ingesta alimentaria reportada a través de un cuestionario de frecuencia de consumo de alimentos con fragilidad a los 9 años y mortalidad a los 20 años.

i. Valoración de la dieta en el estudio InCHIANTI

- **Cuestionario, número de ítems de alimentos, nutrientes**

Para la valoración de la dieta de los participantes del estudio InCHIANTI, se ha utilizado un cuestionario de frecuencia de consumo de alimentos (CFCA) semicuantitativo, desarrollado y validado en centros italianos del estudio EPIC (92), este fue aplicado a los participantes por entrevista telefónica o presencial por entrevistadores entrenados con el objetivo de estimar el consumo habitual en frecuencia y cantidad de ciertos alimentos y bebidas y/o grupos de alimentos. Específicamente en este estudio, el análisis del CFCA permitió conocer con qué frecuencia (semanal, mensual o anual) ocurría el consumo de un listado cerrado de alimentos y bebidas que incluía alrededor de 198 ítems. Además, para facilitar la respuesta del CFCA y también como forma de reducir los errores derivados de la interpretación personal del tamaño de las raciones, se incluyó un atlas con fotografías en color con diferentes tamaños de porciones para las preparaciones principales, el cual ayudaba a los entrevistados a responder con mayor precisión al momento de indicar tamaño de la ración consumida. Posteriormente a la información obtenida y teniendo en cuenta el tamaño de la ración y la cantidad reportada, se calculó el consumo diario de cada ítem de alimento o bebida, obteniendo de esta manera gramos de alimento al día (g/día).

Luego de obtener la ingesta en gramos al día de cada uno de los alimentos y bebidas que representan la dieta habitual de los participantes, se procedió con la estimación de la ingesta de energía y nutrientes de cada participante. Para esto se utilizó un software específico creado para el estudio EPIC (93), el cual transformó los datos del consumo de gramos de alimentos y/o bebidas en ingesta diaria de energía, macronutrientes y micronutrientes.

Desde aquí ha obtenido la información sobre la ingesta alimentaria diaria en gramos al día, relevante para el desarrollo de los objetivos 6, 7 y 8 y resultados de las publicaciones que serán presentadas más adelante, concretamente para cada manuscrito se ha hecho uso de la siguiente información dietética:

- **Patrón de dieta mediterránea**

- **Score de adherencia a la dieta mediterránea a partir de CFCA**

El cálculo de la adherencia a la dieta mediterránea se ha realizado con base la información dietética de grupos de alimentos y nutrientes obtenida a partir de los CFCA. Para la estimación del score, se

utilizó una escala lineal de 0 a 18 puntos, donde 0 a la adherencia más baja y un total de 18 puntos es considerado la mayor adherencia, de acuerdo con la metodología propuesta por Trichopoulou, A et al. (94). en la cual se incluyen 9 componentes clave de este patrón dietético (Tabla 7).

Los componentes han sido divididos en tertiles de ingesta, entonces, para los 6 componentes que se adecuan al patrón dietético mediterráneo se ha asignado una puntuación de 0 al primer tertil de ingesta, 1 al segundo y 2 al tercero e inversamente, para los dos componentes que no se adecuan al patrón dietético mediterráneo, la puntuación ha sido invertida en 2, 0, 1 para cada tertil respectivamente. El alcohol se puntuó como una variable dicotómica, asignando 2 puntos a los consumidores moderados (rango: 5-25 g/día para las mujeres y 10-50 g/día para los hombres) y 0 a los sujetos por encima o por debajo del rango específico de cada sexo, incluidos los abstemios.

Tabla 7. Componentes dietéticos considerados para la estimación del Score de adherencia a la dieta mediterránea (MDS) a partir de CFCA en el estudio InCHIANTI.

Grupos de alimentos:	**% de contribución de los ítems de alimentos del CFCA**	**Score (0-18)**
Vegetales	46,3% hortalizas de fruto	Tertil 0,1,2
	19,5% patatas	
	12,2% hortalizas de hoja	
	8,6 hortalizas de tallo y brotes	
	4,0 hortalizas de col	
	3,8% hortalizas de raíz	
	3,2% cebollas y ajos	
	1,5% setas	
	0,9% de hortalizas mixtas	
Frutas	99,7% de frutas	Tertil 0,1,2
Legumbres y frutos secos	(0,3% de frutos secos	Tertil 0,1,2
Cereales	51,0% pan	Tertil 0,1,2
	35,9% pasta	
	6,2% pan crujiente	
	4,1 % pastelería	
	2,8% arroz	
Pescados y frutos del mar	91,4% pescado	Tertil 0,1,2

	8,6% crustáceos y moluscos	
Carne y derivados cárnicos	29,4% carne de vacuno	Tertil 2,1,0
	20,3% carne procesada	
	11,7% pollo	
	11,7% pavo	
	8,8% conejo	
	8,7% carne de cerdo	
	4,0% ternera	
	3,9% despojos y resto de carnes rojas	
Leche y productos lácteos	53,3% leche	Tertil 2,1,0
	40,3% quesos	
	6,4% yogur	
Energía y Nutrientes:		
AGMI	Ratio AGMI/AGS	Tertil 0,1,2
AGS		
Alcohol	Alcohol total	Tertil 0,2,0

Fuente: Elaboración propia

- **<u>Panel de biomarcadores de ingesta</u>**

Para la definición del panel de biomarcadores de ingesta que representa la adherencia al patrón de dieta mediterránea, se ha tenido en cuenta:

i) La disponibilidad en la base de datos del estudio InCHIANTI de los biomarcadores en suero, plasma u orina al tiempo basal.
ii) La selección de los biomarcadores de ingesta que se asocian con los componentes del patrón de dieta, según la información reportada previamente en la literatura científica. Se consideraron los siguientes biomarcadores dietéticos, teniendo en cuenta también el primer punto (tabla 7).
iii) Por último, se ha evaluado la correlación de los biomarcadores con los componentes de la adherencia a este patrón de dieta en la población InCHIANTI y se escogieron los que tenían una asociación significativa.

Los biomarcadores de ingesta han sido obtenidos a partir de muestras de plasma, suero u orina de la cohorte InCHIANTI que luego de su obtención al inicio del estudio, han sido almacenadas a -80ºC.

Para cada biomarcador de ingesta se ha seguido el método analítico y de identificación validado correspondiente, los cuales han sido previamente publicados.
Tanto los biomarcadores dietéticos plasmáticos como los urinarios han sido validados frente a las mediciones de la ingesta dietética en estudios previos de la cohorte InCHIANTI (95–102).

Para calcular la adherencia a la dieta mediterránea a partir de los biomarcadores, se ha utilizado una metodología de cálculo similar escala lineal de 0 a 18 puntos, lo que indica una adherencia de baja a alta. En este caso, se clasificaron los biomarcadores candidatos de ingesta de verduras, legumbres, frutas y los frutos secos, cereales, pescado y aceite de oliva y se dividieron por tertiles (Tabla 8) y se les asignó una puntuación de 0 a 2 al primer, segundo y tercer tertil de biomarcadores de ingesta respectivamente. La puntuación se invirtió para los terciles de AGS y vitamina B12, que biomarcadores que representan el consumo de carne y productos lácteos, respectivamente. En el caso de los metabolitos de resveratrol, representativos de consumo de vino, se puntuaron como una variable dicotómica, asignando 2 a los consumidores moderados (rango de valores correspondiente al consumo de vino; 125-375 g/día para los hombres y 50-250 g/d para las mujeres; en la presente población: 589-14.557 nmol/24h para los hombres y 1-11.125 nmol/24h para las mujeres) y 0 para los sujetos por encima o por debajo del rango específico del sexo. En la cohorte InCHIANTI, el vino fue el principal contribuyente a la ingesta de alcohol (88%).

Tabla 8. Score de adherencia a la dieta mediterránea (MDS) mediante cuestionarios dietéticos (CFCA) y biomarcadores dietéticos (dBMK) utilizados en el estudio InCHIANTI.

Grupos de alimentos	Biomarcador candidato	Score (0-18)
Vegetales	Polifenoles Totales	Tertil 0,1,2
Frutas y frutos secos	Carotenoides	Tertil 0,1,2
Legumbres	Ácido linolénico	Tertil 0,1,2
Cereales	Selenio	Tertil 0,1,2
Pescados y frutos del mar	EPA+DHA	Tertil 0,1,2
Carne y derivados cárnicos	AGS, Ácidos Grasos saturados calculados como la suma de C14: 0 (ácido mirístico), C16:0 (ácido palmítico), C18:0 (ácido esteárico), C20:0 (ácido eicosanoico), C22:0 (ácido docosanoico) y C24:0 (ácido tetracosanoico)]	Tertil 2,1,0
Leche y productos lácteos	Vitamina B12	Tertil 2,1,0
Energía y Nutrientes:		
AGMI	Ratio AGMI/AGS	Tertil 0,1,2

AGS	Ácidos Grasos Mono Insaturados (AGMI), calculados a partir de la suma de los siguientes ácidos grasos C14: 1 n-9 cis (ácido miristoleico), C16:1 n-7 cis (ácido palmitoleico), C18: 1 n-9 cis (ácido oleico), C18:1 n-7 trans (ácido octadecenoico), C20:1 n-9 cis (ácido 11-eicosenoico), C22:1 n-9 cis (ácido docosenoico) y C24:1 n-9 cis (ácido tetracosenoico)	
Alcohol	Resveratrol	Tertil 0,2,0

Fuente: Elaboración propia

- **Análisis de la ingesta de proteínas**

Para la elaboración y desarrollo de este trabajo se utilizaron variables del consumo de nutrientes, específicamente el consumo de proteínas, teniendo en cuenta la ingesta diaria de proteína total (g/día), y de su tipología dependiendo si proviene de productos de origen vegetal o animal.

- **Análisis de la ingesta de productos lácteos**

Para la realización de este trabajo se tuvo en cuenta la ingesta de productos lácteos reportada en el CFCA. Para el cálculo de lácteos totales, se realizó el sumatorio de los 4 ítems de alimentos detallados en la tabla 8. De aquí además se establecieron dos subgrupos de productos lácteos diferenciando los lácteos no fermentados que consideraban la leche y el helado y, los lácteos fermentados que incluían el yogurt y el queso.

Tabla 9: Ítems de alimentos del CFCA considerados como productos lácteos consumidos por la población del estudio InCHIANTI.

Grupos de alimentos:	**Ítems de alimentos del CFCA**
Productos lácteos	Leche total (5 ítems, entera, semi-desnatada y la añadida al café)
	Yogurt total (3 ítems, entero, desnatado y con fruta)
	Queso total (15 ítems, incluyendo quesos maduros y frescos)
	Helado total (2 ítems)

Fuente: Elaboración propia

ii. Evaluación de indicadores de salud en el estudio InCHIANTI

- **Valoración de la fragilidad**

Para valorar la fragilidad en el estudio InCHIANTI, se ha utilizado el fenotipo de fragilidad propuesto por Fried et al. (31). el cual ha sido levemente adaptado a este estudio (91). Esta valoración fue realizada por personal médico en cada visita clínica de los participantes al inicio y en el seguimiento del estudio, midiendo los siguientes parámetros:

i) Pérdida de peso involuntaria: Definida como la pérdida de 4.5 kg de peso o más en el último año por razones diferentes de la realización de una dieta.

ii) Fatiga auto reportada o agotamiento: Información obtenida a partir de preguntas de la escala CES-D, donde los participantes auto reportaban la sensación de que "todo lo que hacía era un esfuerzo" y "no podía ponerme en marcha".

iii) Disminución de la realización de actividad física o sedentarismo durante el último año, valorado mediante un cuestionario.

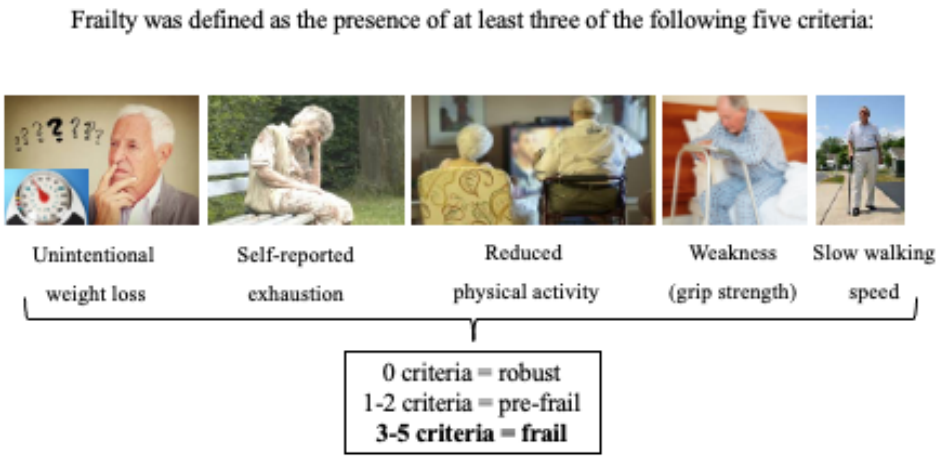
iv) Debilidad o pérdida de la fuerza muscular medida como la fuerza de agarre en el quintil más bajo, estratificado por género y cuartiles de índice de masa corporal (IMC).

v) Velocidad de la marcha* o lentitud, valorada como el tiempo transcurrido para caminar 4.57 m o 15 pies.

**Marcador de deterioro (en comparación con las normas estandarizadas por edad)*

Los participantes que reportaron no tener ninguno de estos criterios se consideraron no afectados por el síndrome de fragilidad y se consideraron robustos, mientras que los participantes que presentaban uno o dos de estos signos o síntomas se consideraron pre-frágiles y quienes presentaban entre tres y cinco criterios se consideraron frágiles (31).

Figura 15. Evaluación del Fenotipo de fragilidad



- **Valoración de la mortalidad**

Los datos sobre mortalidad fueron recopilados mediante el cruce de información entre los datos de los participantes y los del Registro de Mortalidad General de la región de la Toscana y los certificados de defunción obtenidos desde el registro civil de los correspondientes municipios de residencia en Greve in Chinati o Bagmo a Ripoli. Para la codificación de las causas de muerte se utilizó la décima revisión de la Clasificación Internacional y Estadística de Enfermedades y Problemas Relacionados con la Salud (CIE-10) publicada por la OMS.

4. RESULTADOS

MANUSCRITO 2

Novel strategies for improving dietary exposure assessment: Multiple data fusion is a more accurate measure than the traditional single Biomarker approach

Mar Garcia-Aloy, Montserrat Rabassa, Patricia Casas-Agustench, **Nicole Hidalgo-Liberona**, Rafael Llorach, Cristina Andres-Lacueva

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MANUSCRITO 2

Objetivo: El objetivo de este artículo fue analizar los diferentes enfoques utilizados para la evaluación de la exposición dietética y proponer una medida novedosa que combina datos de los cuestionarios dietéticos con las mediciones de los biomarcadores de exposición.

Metodología: Se analizó la bibliografía disponible en cuanto a los diferentes métodos de evaluación de la dieta, considerando: 1) Métodos tradicionales de recogida información dietética (registros de alimentos, recordatorios de 24 horas o cuestionarios de frecuencia de consumo de alimentos), los cuales además requieren de información complementaria de las Tablas de composición de alimentos para su respectivo análisis; 2) Biomarcadores dietéticos que permiten medir en una muestra biológica, la exposición a ciertos alimentos y/o grupos de alimentos; 2.1) Modelos de biomarcadores multi-metabolitos, que se fundamenta en el uso una gama más amplia de metabolitos la cual mejoraría la medición de la ingesta dietética, capturando la dieta desde una perspectiva más amplia y, por lo tanto, dando una cobertura más completa de la exposición dietética; 3) Método combinado de cuestionarios dietéticos y biomarcadores, teniendo en cuenta los aspectos abordados en los puntos anteriores y con perspectiva de valorar la relación entre dieta y enfermedad. Se discute acerca de los enfoques existentes entre los métodos de evaluación dietética y los biomarcadores dietéticos hasta llegar al método combinado y si esta medida podría ayudar a mejorar la precisión en la medición de la ingesta dietética y a reforzar la capacidad de detectar los efectos de la dieta en la salud.

Resultados y conclusiones: De acuerdo con la bibliografía revisada, los métodos tradicionales de recogida de información dietética, biomarcadores de exposición y modelos multi-metabolitos, tienen limitaciones al utilizarlos cada uno por sí solo. Es por esto que las investigaciones futuras deberían centrarse en la exploración de aproximaciones novedosas para el descubrimiento y la validación de biomarcadores de exposición dietética, dado que aún es necesario superar una serie de limitaciones que incluyan la perspectiva analítica y biológica de los biomarcadores, para su completa validación. Entre los diferentes métodos de evaluación de la dieta revisados, los paneles de biomarcadores multi-metabolitos podrían favorecer una estimación más fiable de la exposición dietética que el enfoque en un solo biomarcador. En este artículo se propone la aplicación de un enfoque combinado que utilice datos de cuestionarios dietéticos junto con las mediciones de los biomarcadores dietéticos como una buena estrategia para mejorar la evaluación de la exposición dietética.

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Commentary

Novel strategies for improving dietary exposure assessment: Multiple-data fusion is a more accurate measure than the traditional single-biomarker approach

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ABSTRACT

Background: Accurate measurement of food intake is the cornerstone of understanding the links between diet and optimal health status or risk of disease. The utilization of metabolomics approaches is revolutionizing the field of dietary assessment by associating metabolic profiles with intake of specific foods or dietary patterns and/or investigating human health status in nutritional trials. Combining dietary biomarkers with conventional dietary assessment methods is considered a potential strategy for tackling the complexity of dietary exposure fingerprinting.

Scope and approach: We discuss existing approaches among dietary assessment methods and dietary biomarkers. A combined approach taking into consideration data from dietary questionnaires with measurements of dietary biomarkers is emphasized.

Key findings and conclusions: Trends in novel strategies for improving dietary exposure assessment will be influenced by the discovery and validation of dietary exposure biomarkers. Among different strategies, multi-metabolite biomarker panels enable more reliable estimation of dietary exposure than does the traditional single-biomarker approach. Therefore, a combined approach using data from dietary questionnaires along with measurements of dietary biomarkers is considered an excellent strategy for improving dietary exposure assessment.

1. Introduction

Diet and nutrition are major determinants of human health. Accurate measurement of food intake is the cornerstone of understanding the links between diet and optimal health status or risk of disease. Dietary assessment has been traditionally performed using conventional methodologies of food surveys such as food frequency questionnaires (FFQs), 24-h dietary recalls or food records. However, the accuracy of the dietary intake and nutritional status is frequently challenged due to the subjective nature of these dietary instruments. This limitation can be improved by the application of metabolomics to characterize dietary exposure.

Metabolomics approaches are revolutionizing the field of dietary assessment by associating metabolic profiles with intake of specific foods or dietary patterns, and/or investigating human health status in nutritional trials. Recently, exploring the food metabolome has been defined as a data-driven strategy for identifying novel biomarkers and improving the accuracy of measurement of dietary exposures by traditional dietary assessment instruments (Scalbert et al., 2014). In this way, the use of metabolomics has enabled the identification of novel and robust biomarkers of food or nutrient intake, which provide an objective measure of exposure that is devoid of many of the biases and errors associated with self-reported methods (Scalbert et al., 2014).

However, there are some factors not present in the traditional dietary assessment instruments that could misrepresent biomarker measures of dietary intake. These factors include genetic variability (e.g. biological variation in nutrient absorption and metabolism,

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0924-2244/

epigenetic variation or gene-gene interactions), lifestyle/physiological factors (e.g. smoking, alcohol consumption, physical exercise or influence of microbiota), dietary factors (e.g. nutrient bioavailability or nutrient-nutrient interactions), biological samples and analytical methodology (Scalbert et al., 2014). Further research will address this issue and identify the best emerging dietary biomarkers. Therefore, as biomarkers cannot replace conventional dietary assessment methods, the use of conventional dietary instruments together with dietary biomarkers is considered the best strategy for tackling the complexity of dietary exposure fingerprinting. Fig. 1 represents a schematic diagram for combining dietary questionnaires with biomarkers.

The main aim of this paper is to highlight existing approaches aimed at improving dietary exposure assessment and the novel combined measure considering data from dietary questionnaires with measurements of dietary biomarkers.

2. Dietary assessment methods

2.1. Dietary questionnaires

In nutritional studies, the traditional method of collecting data on foods and beverages consumed over a prescribed period of time relies on information gathered using dietary assessment questionnaires. As previously mentioned, the most commonly used dietary assessment methods are open-ended questionnaires such as food records or 24-h dietary recalls, or closed-ended questionnaires including FFQs (Tucker, 2007). Generally, these instruments require a systematic estimation of the frequency and usual serving size of foods, as well as detailed information about the ingredients of a meal recipe, combinations of foods consumed together, and sometimes cooking processes, which may influence the estimation of exposure to a particular dietary constituent. Moreover, the estimation of nutrient/compound intakes depends largely on the existence of appropriate, complete, reliable and up-to-date food composition tables or databases. Many users are often not conscious of the high composition variability, particularly in terms of micronutrients (e.g. vitamins) and phytochemicals, between similar foods, and even in the same type of food (e.g. raw, cooked or processed foods). For this reason, a great dependency on professionals' knowledge is needed to generate, compile, update and use the food composition data adequately. Indeed, the Food and Agriculture Organization of the United Nations (FAO) and the International Network of Food Data Systems (INFOODS) have recently published three comprehensive guidelines on conversions, data evaluation and food matching in order to improve and harmonize the compilation of food composition data, which could also lead to more accurate nutrient intake estimations (Charrondiere et al., 2016).

The consumption data obtained through these methods are used to compute the intake of whole diets/dietary patterns, food groups, foods, energy, nutrients, bioactive compounds and other food components. Recently, dietary pattern analysis has emerged as a useful approach for investigating diet-disease associations. Thus, eating patterns may be more predictive of disease risk than isolated analysis on foods or nutrients (Hu, 2002). The 2015 Dietary Guidelines Advisory Committee recognized the advantages that dietary patterns offer as an approach for informing public health recommendations (U.S. Department of Health and Human Services & U.S. Department of Agriculture, 2015, p. 2). Different approaches for developing dietary patterns exist (Lassale et al., 2016). The most prominent methods are, a priori, numerical indexes, scores that measure adherence to disease-specific dietary and lifestyle guidelines (e.g. Dietary Approaches to Stop Hypertension (DASH)), scores that measure adherence to a regional diet (e.g. Mediterranean Diet Score) and scores based on nutritional guidelines (e.g. Diet Quality Index International (DQI-I). Other approaches have been proposed to derive patterns by using all food groups available, such as principal component analysis (PCA), reduced rank regression, partial least-squares regression (PLS), confirmatory factor analysis (CFA) and treelet transform (Hu, 2002; Imamura & Jacques, 2011; Varraso et al., 2012).

The strengths of these methods are the lower relative cost, the ease with which the questionnaires can be completed with the help of a trained interviewer (dietician) or by participants themselves (self-reported questionnaires), and the chance to gather a large amount of dietary data. However, the use of questionnaires is also subject to some limitations (Tucker, 2007). The main one is that they are mostly self-reported, wherein the estimation of food portion size is an important source of errors (perception, conceptualization and memory), which could be inappropriate for some populations (children, obese people, and elderly people with cognitive impairment, among others). Such systematic errors inherent in self-reported data plus random errors (e.g. the accuracy of the food composition tables) can bias the estimation of dietary intake. However, the FFQ is the most common dietary assessment method used to estimate habitual dietary intake (e.g. the previous 12 months) of specific nutrients, dietary exposures related to a certain disease or various dietary components in large-population studies, due to its self- or interviewer-administered and economical machine-readable features. In this context, the use of a previously validated FFQ is an essential requirement for improving the measurement of errors previously mentioned. FFQs should be developed specifically for the research objective because diet may be influenced by participant's characteristics such as ethnicity, culture, dietary habits and lifestyle, among others. Nevertheless, more precise measurements are obtained when using multiple 24-h dietary recalls or dietary records, but only short-term intake (actual intake information over the previous 24 hours) is estimated. In this context, long-term intake can also be estimated if repeated during the year. Recently, it has been suggested that a combination of two different dietary assessment instruments, such as four to six 24-h dietary recalls with a FFQ, could improve estimates of dietary intakes with regard to the methods separately. In this study, the association between diet and disease was statistically significant with food records but not with a FFQ (Carroll et al., 2012). Therefore, if new nutritional studies are designed to include FFQs plus repeat 24-h evaluations, further improvements to minimize their measurement errors might be seen by combining data from the two methods. Our group has recently observed that the highest tertile of total dietary polyphenols, which were estimated using a validated FFQ and an ad hoc database on polyphenol content in foods, was not associated with the risk of cognitive and physical decline, frailty and total mortality, in comparison with the lowest tertile (Rabassa, Cherubini, et al., 2015; Rabassa, Zamora-Ros, et al., 2015, 2016; Zamora-Ros et al., 2013). However, an association with total urinary polyphenols was observed. Moreover these studies have demonstrated the importance of assessing dietary polyphenol exposure whenever possible, using dietary biomarkers and not only through dietary questionnaires.

Despite these strengths, more refined and improved techniques of dietary assessment intake are essential to reduce the limitations of traditional dietary questionnaires and also to reduce the cost associated with the collection and processing of dietary data (Illner et al., 2012; Stumbo, 2013). This is being met with intense methodological research and innovative technologies. Many applications of information and communication technologies are currently under development and validation, and great strides have been made. An example of interactive computer-based techniques is a menu-driven standardized 24-h dietary recall program (called EPIC-

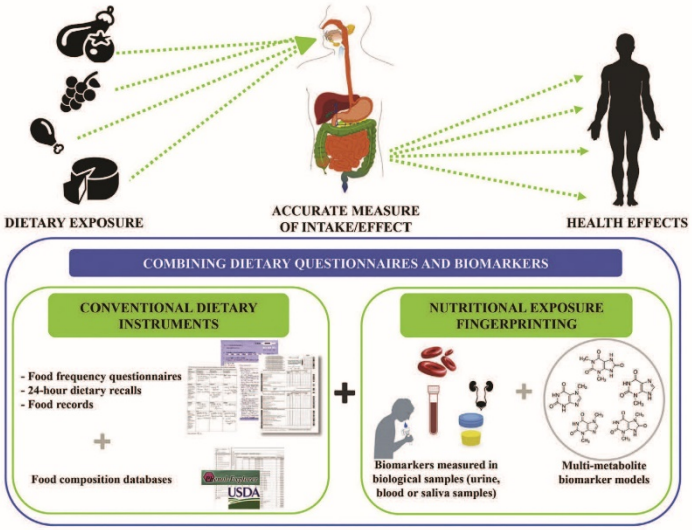

Fig. 1. Schematic diagram for combining dietary questionnaires with biomarkers.

SOFT) developed by the European Prospective Investigation into Cancer and Nutrition study (Slimani et al., 2011). The National Cancer Institute in the US has also developed an interactive computer-based approach but with an Internet-based technology, called the Automated Self-Administered 24-h Dietary Recall, which is based on the Automated Multiple-Pass Method approach (Schatzkin et al., 2009). In addition, mobile phone applications have been released such as Nutricam, which allows users to capture images of foods and verbally describe their items before intake, and then to upload both the image and voice file onto a website for analysis (Rollo, Ash, Lyons-Wall, & Russell, 2011). Another example is a wearable electronic device that resembles a necklace and includes a microphone, camera and other sensors. In this case, dietary intakes are collected from the video recording and are calculated automatically (Sun et al., 2010). However, these methods have not yet been widely implemented in large-population studies due to their related technical issues (data transfer, storage, battery life and others) and methodological difficulties such as self-reporting and higher costs. In addition, certain users are not familiar with innovative technologies or new devices. Despite these limitations, automated versions promise to overcome the labour-intensive and costly coding of the 24-h dietary recalls (Shim, Oh, & Kim, 2014).

2.2. Dietary biomarkers

Thus far, individual biomarkers of dietary intake have been used to assess exposure to specific foods or food groups. However, this strategy has important limitations and only in some cases it has been successful. The more relevant limitations can be grouped into a wide distribution of food components (low specificity of biomarkers), high interindividual variation and the microbiota metabolism, among others.

A common approach when the research community looks for a biomarker of intake is first to study the food composition, then try to identify the possible modifications caused by the host metabolism and finally look for metabolites (e.g. biomarkers) in the biofluid (mostly blood and/or urine). Usually, the food composition is very complex (from the quantitative and qualitative point of view) and many of the compounds are widely distributed in different foods. For instance, most polyphenols are present in a wide range of plant foods, such as chlorogenic acids (e.g. coffee and apple) and flavan-3-ols (e.g. cocoa and tea) (Clifford, 2000; Monagas et al., 2010). Another example of a compound widely distributed in many plant foods is vitamin C. This compound has been used as a biomarker of consumption of fruits and vegetables, although differences in concentration between different foods reduces their ability to be a good biomarker (Scalbert et al., 2014). Therefore using the single-biomarker strategy compromises its usefulness because there are a number of factors that limit the prediction of dietary exposure.

It is worth noting that in some cases, similar compounds from different food sources could produce the same biomarker. One of the most relevant cases is ellagitannins. This class of polyphenols (with some differences) is present in foods such as pomegranate, strawberries and walnuts (Espín, Larrosa, García-Conesa, & Tomás-Barberán, 2013). They are poorly absorbed and when they reach the gut they are largely metabolized by the microbiota, producing urolithin derivatives (Espín et al., 2013). Selma, Beltrán, García-Villalba, Espín, & Tomás-Barberán (2014) showed the ability of the bacteria *Gordinobacter* to produce urolithins from ellagitannins. This means that urolithins are biomarkers of ellagitannin intake instead of particular foods. This relevance of the microbial effect is crucial because a significant number of food components are degraded by the colonic microbiota and after absorption and distribution are excreted in urine. Therefore, the real biomarker of intake is provided by the microbiota instead of the host metabolism.

Procyanidins are a good example of this behaviour. This class of

polyphenols is present in many dietary sources, such as cocoa, tea, wine and apples (Monagas et al., 2010). These polyphenols show low bioavailability. However, gut microbiota have the capacity to degrade these metabolites and produce other compounds called hydroxyphenylvalerolactones and hyxdroyphenylvaleric acids (Monagas et al., 2010). In fact, these metabolites have been used as biomarkers of procyanidin-rich foods. Both urolithins and hydrophenylvalerolactones have been used as biomarkers of intake of single foods (e.g. walnuts and tea, respectively). However, taking into account the variety of dietary sources that could provide these parent compounds (e.g. ellagitannins and procyanidins), these compounds are not suitable for use as single accurate biomarkers.

In relation to the interindividual variation, there are some interesting examples. According to Lars O. Dragsted (2010), creatinine can be considered a potential marker of meat intake (even cooked). However, endogenous levels connected with creatine turnover showed important variations between subjects (Dragsted, 2010). Another example is a recent study about polyphenols and their urinary quantification (Achaintre et al., 2016). In this paper the authors showed that in urine from 475 EPIC participants a total of 34 polyphenols were evaluated and in general these compounds showed large interindividual variations (Achaintre et al., 2016).

There are several examples where a single metabolite could be a potential biomarker of a particular food intake. Some metabolites have been proposed as a biomarker of intake of a particular group of plant foods. However, in these groups there are particular foods that could represent a very important part of the dietary source (e.g. citrus fruits and oranges, or cruciferous vegetables and broccoli). The compound termed proline betaine has been identified in both intervention and cohort studies (Pujos-Guillot et al., 2013) as a candidate biomarker of citrus intake and, in particular, a powerful biomarker of orange intake (Lloyd, Beckmann, Favé, Mathers, & Draper, 2011). This biomarker was detected and its urinary excretion kinetics reported after an intervention study with orange juice consumption (Heinzmann et al., 2010). However, increased urinary excretions of proline betaine have also been observed after diets enriched with rye bran, bringing its specificity under the spotlight (Pekkinen et al., 2015). With regard to cruciferous intake, and in particular that of broccoli, sulforaphane (mainly its mercapturic acid derivative) was proposed as a potential biomarker of intake of this particular food (Dominguez-Perles et al., 2014; Vermeulen, van Rooijen, & Vaes, 2003).

2.2.1. Multi-metabolite biomarker models

Given the already mentioned inconsistencies that can limit the usefulness of single biomarkers for dietary intake evaluation, the question emerges of whether a combination of food-derived metabolites, namely a multi-metabolite biomarker panel (MBP), would be more likely to capture dietary exposure and improve the accuracy and precision of dietary assessment. The rationale behind the use of MBPs is that a wider range of metabolites would improve the measurement of dietary intake, capturing a broader perspective of the diet and thereby giving a more complete coverage of dietary exposure. It opens a new framework in the research area of nutritional biomarkers. However, while almost all studies investigating dietary biomarkers have focused on single candidate biomarkers, MBPs have remained practically unexplored and only a few research groups have addressed this question during the last few years. These studies are summarized in Tables 1 and 2.

Over the last few years, our research group has made great efforts to instigate a novel approach for improving dietary exposure assessment through MBPs. This concept has been used in a number of recent studies where we suggested MBPs of walnuts (García-Aloy et al., 2014), wine (Vázquez-Fresno et al., 2015), cocoa (García-Aloy, Llorach, Urpi-Sarda, Jáuregui, et al., 2015) and bread (García-Aloy, Llorach, Urpi-Sarda, Tulipani, et al., 2015) using urine samples analysed by an untargeted metabolomics approach, and dietary data from FFQs, in studies with different designs. The results showed that MBPs perform better in terms of predicting dietary exposure. Our first study in this field identified an MBP that was highly predictive of walnut intake with an area under the curve [AUC (95% CI)] of 93.4% (90.1–96.8%) and 90.2% (85.9–94.6%) in the training and validation sets, respectively (García-Aloy et al., 2014). In line with these results, a "tartrate-ethyl glucuronide" model showed an AUC of 90.7% (84.5–96.4%) in the training set composed of samples from volunteers that participated in a controlled clinical trial with a nutritional intervention with wine, and an AUC of 92.4% (84.1–100%) in the validation set composed of samples assessed at baseline from a subcohort of volunteers included in the PREDIMED study with a reported wine intake of ≥180 mL/day (Vázquez-Fresno et al., 2015). Additionally, this model showed promising performance in terms of its sensitivity, which enabled discernment of an intake of one glass of wine 3 days after consumption in an observational study. Another MBP was highly predictive for cocoa consumption [AUC = 95.7% (89.8–100%) in the training set, and 92.6% (81.9–100%) in the validation set]. It was built with one component of theobromine metabolism (7-methylxanthine) together with another from microbial metabolism of polyphenols (5-(3′,4′-dihydroxyphenyl)-valerolactone glucuronide). Both metabolites have been proposed as biomarkers of cocoa intake in studies with different designs (i.e., acute interventions, long-term intervention trials and observational studies) and provided the model with complementary information about habitual cocoa intake (García-Aloy et al., 2015). Finally, an additional MBP was highly predictive for wholegrain bread intake [AUC = 93.1% (88.7–97.4%) and 93.7% (89.4–98.1%) for data from positive and negative ionization mode, respectively], while the MBP designed to evaluate white bread consumption had a reasonably good predictive ability [AUC = 80.6% (72.1–89.0%) and 77.8% (69.1–86.4%) for data from positive and negative ionization mode, respectively] (García-Aloy et al., 2015).

Previously, Campbell et al. (1994) published one of the first studies suggesting a combination of biomarkers of intake. They used a stepwise logistic regression analysis to assess fruit and vegetable consumption. The resultant prediction model included compounds measured in biological samples (three carotenoids determined in plasma) and data from dietary questionnaires (energy intake) (Gross et al., 1994). Later, Nielsen, Freese, Kleemola, and Mutanen (2002) proposed measuring the sum of different flavonoids determined in urine for examining the intake of fruits and vegetables (Nielsen et al., 2002), an approach also suggested in other studies (Brevik, Rasmussen, Drevon, & Andersen, 2004; Krogholm, Haraldsdóttir, Knuthsen, & Rasmussen, 2004). In the same vein, summing individual resveratrol or alkylresorcinol metabolites has also been attempted for assessing wine (Queipo-Ortuño et al., 2012; Rotches-Ribalta et al., 2012; Zamora-Ros et al., 2009) and wholegrain wheat and rye consumptions (Kristensen et al., 2012; Landberg et al., 2009; Linko-Parvinen, Landberg, Tikkanen, Adlercreutz, & Peñalvo, 2007), respectively. In parallel, the ratio between to alkylresorcinols, C17:0/C21:0, has shown the ability to discern between wholegrain wheat and rye intakes (Landberg et al., 2009; Linko-Parvinen et al., 2007). However, although these later examples exhibited a good predictive capacity, these statistical approaches could not give the real weight of each metabolite within the biomarker panel, and therefore more sophisticated approaches could be required for the assessment of dietary exposures for more complex foods, food groups or dietary patterns. At the same time, it is important to bear in mind that using metabolites from the same class could not deal with the problem of specificity previously highlighted.

As mentioned above, recent work from our laboratory applied a

Table 1
Summary of multi-metabolite biomarker panels identified using untargeted metabolomics approaches.

Food item	Statistical test	Metabolites in the panel	Study design	TS & VS	Panel-diet associations	Was the panel better than single biomarkers?	Reference
Walnuts	Stepwise logistic regression	3-Indolecarboxylic acid glucuronide 10-Hydroxy-decene-4,6-diynoic acid sulphate Urolithin A glucuronide Tridecadienoic/tridecynoic acid glucuronide Urolithin A sulphate	Observational (cross-sectional)	Yes	AUC (95% CI): • TS – 93.4% (90.1–96.8%) • VS – 90.2% (85.9–94.6%)	Yes	Garcia-Aloy et al., 2014
Cocoa	Stepwise logistic regression	7-Methylxanthine 5-(3′,4′-dihydroxyphenyl)-valerolactone GlcA	Observational (cross-sectional)	Yes	AUC (95% CI): • TS – 95.7% (89.8–100%) • VS – 92.6% (81.9–100%)	Yes	Garcia-Aloy, Llorach, Urpi-Sarda, Jáuregui, et al., 2015
Bread	Stepwise logistic regression	White Bread: HPAA GlcA HMBOA Riboflavin Wholegrain Bread: HHPAA HPPA HMBOA Enterolactone GlcA Pyrraline 3-Indolecarboxylic acid GlcA Riboflavin	Observational (cross-sectional)	No	AUC (95% CI): from 77.8% (69.1–86.4%) to 93.7% (89.4–98.1%).	Yes	Garcia-Aloy, Llorach, Urpi-Sarda, Tulipani, et al., 2015
Wine	Stepwise logistic regression	Tartrate Ethyl glucuronide	Sustained intervention [TS] Observational (cross-sectional) [VS]	Yes	AUC (95% CI): • TS – 90.7% (84.5–96.4%) • VS – 92.4% (84.1–100%)	Yes	Vázquez-Fresno et al., 2015
Orange juice	Random forest	Stachydrine Methyl glucopyranoside (α+β) Dihydroferulic acid Galactonate	Sustained intervention	Yes	AUC (95% CI): 99.6% (96–100%) Accuracy: • Entire data set – 93% • Hold-out data set – 87.5% • Permutation test: Entire data set: p-value – 0.006 • Hold-out dataset: p-value – 0.004	Yes for some metabolites	Rangel-Huerta et al., 2017
Coffee	Support vector machines	Cyclo(isoleucylprolyl) 1-Methylxanthine Trigonelline	Observational (cross-sectional)	Yes	AUC (95% CI): 98% (93–100%)	Yes	Rothwell et al., 2014
Sugar-sweetened beverages	PLS regression	Formate Citrulline Taurine Isocitrate	Observational [TS] Acute intervention [VS]	Yes	AUC [TS]: 80% Specificity [TS]: 80% Sensitivity [TS]: 70%	Yes	Gibbons et al., 2015
Nordic diet	PLS-DA	(2-Oxo-2,3-dihydro-1H-indol-3-yl)acetic acid 6-Amino-5-[N-methylformylamino]-1- methyluracil Hydroquinone GlcA 3,4,5,6-Tetrahydrohippurate 3-Indoleacetic acid GlcA Limonele-1,2-diol GlcA Limonele-8,9-diol-GlcA *p*-Menth-1-2ne-6,8,9-triol Trimethylamine *N*-oxide Cyclo(Pro-Val) Hippuric acid Octanoyl GlcA Proline betaine 7-Methyluric acid 3,7-Dimethyluric acid 7-Methylxanthine	Sustained intervention	Yes[a]	Misclassified samples: • TS: 35% • VS: 19%	NR	Andersen et al., 2014

		Perillic acid GlcA Perillic acid-8,9-diol-GlcA Dihydroperillic acid GlcA Pyroglutamyl proline	Pyrraline Theobromine Unknowns				
Diet in general	PCA	LP1: PCaeC36:2 PCaeC38:3 PEaaC22:2 PEaaC34:0 PEaeC40:4 SMC15:0 SMC19:0 SMC20:2 SMC21:0 SMC21:1 LPCaC18:2 LPEeC18:0	LP6: PEaaC36:1 PSaaC36 PEaaC38:4;2	Observational (cross-sectional)	No	Correlations • LP1 : – SFA: p-value = 0.015 – MUFA: p-value = 0.011 – PUFA: p-value = 0.018 • LP6: – Meat: p-value = 0.024 – Fish: p-value = 0.026 – Vegetable intake: p = 0.036 AUCs: • LP1: Fat = 82% • LP6: – Meat and vegetable <70% – Fish = 76%	NR	O'Gorman et al., 2014

AUC, area under the curve; CI, confidence interval; DA, discriminant analysis; GlcA, glucuronide; HHPAA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; HMBOA, 2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3-one; HPAA, N-(2-hydroxyphenyl) acetamide; HPPA, 2-hydroxy-N-(2-hydroxyphenyl) acetamide; LP, lipid pattern; NR, not reported; PCA, principal component analysis; PLS, partial-least squares; TS, training set; VS, validation set.

[a] Some samples of the training and validation sets were from the same subjects.

multivariate statistical approach to link dietary data with both targeted and untargeted metabolomics data to identify a series of MBPs (García-Aloy et al., 2014, 2015, 2015; Urpi-Sarda et al., 2015; Vázquez-Fresno et al., 2015). In this approach, stepwise logistic regression analysis was used to include more than one metabolite in biomarker panels and regressed against dietary data to identify MBPs. Other research groups also applied regression analysis for addressing this issue (Jin et al., 2014; McGrath et al., 2016; Neuhouser et al., 2003), whereas, more recently, other multivariate statistical strategies such as PCA, PLS, random forest and support vector machine algorithms have also been used to build MBPs for assessing the consumption of different foods or dietary patterns (Andersen et al., 2014; Gibbons et al., 2015; Marklund et al., 2014; O'Gorman et al., 2014; Rangel-Huerta et al., 2017; Rothwell et al., 2014). These multivariate methods may be of interest in terms of the further discovery of dietary exposure biomarkers. For example, Marklund et al. (2014) created a novel dietary biomarker score based on a combination of several individual biomarker concentrations, using rank scores or principal component analysis to assess compliance in a dietary randomized controlled trial, Systems Biology in Controlled Dietary Interventions and Cohort Studies (SYSDIET), and to investigate how a healthy Nordic diet influences cardiometabolic risk factors (Marklund et al., 2014). Their results suggest that this dietary biomarker score provides a better reflection of the dietary intake and thereby increases the strength of detecting potential cardiometabolic health effects. In addition, they proposed that future studies should evaluate the combined use of dietary biomarkers and reported dietary intake methods for assessing compliance.

Nevertheless, since until now MBPs have been proposed for a limited number of foods, food groups and dietary patterns, further work in this line is required. This will enable a broad coverage of dietary exposure, including the assessment of exposure to bioactive compounds, foods, food groups or complex dietary patterns reflective of habitual dietary intake. The challenge that lies ahead will be finding the simplest combination of metabolites that is able to properly evaluate dietary exposure. In the same vein, the capacity of metabolomics to measure simultaneously a high number of metabolites offers a great opportunity to propose new MBPs clustering in the same model metabolites of different classes, providing the panels with complementary information about dietary intake.

However, before translating MBPs into nutritional epidemiology, appropriate validation steps in the post-discovery phase are essential in order to assess their robustness. Firstly, it is necessary to develop analytical methods for accurate and specific quantification of the metabolites included in each MBP, as recently suggested for simultaneous quantification of multiple biomarkers associated with alcohol intake (Monošík & Dragsted, 2016). Additionally, the specificity, sensibility, kinetics and dose-response relationships of proposed MBPs should be investigated in separate studies with different designs and populations, including people with different genetic and dietary backgrounds.

2.3. Methods of combining dietary questionnaires and biomarkers

Taking into account all of the above, and with a view to evaluating the diet-disease relationship, the use of validated dietary biomarkers helps to improve precision in the measure of dietary intake of different foods, nutrients and other components, and to then strengthen the ability to detect dietary effects. In addition, a combined approach using data from dietary questionnaires with measurements of dietary biomarkers has emerged as a good strategy, especially when the extent to which the biomarker mediates the dietary effect is unknown. Freedman, Kipnis, et al.

Table 2
Summary of multi-metabolite biomarker panels identified using targeted approaches.

Food item	Statistical test	Metabolites in the panel	Study design	TS & VS	Panel-diet associations	Was the panel better than single biomarkers?	Reference
Wholegrain wheat and rye	Sum Ratio	Sum of AR homologues (AR C17:0-C25:0) AR C17:0/C21:0 ratio	Sustained intervention	–	Increase: p-value < 0.05	NR	Linko-Parvinen et al., 2007
Rye wholegrain/bran	Sum Ratio	Sum of AR homologues (C17:0, C19:0, C21:0, C23:0, C25:0) AR C17:0/C21:0 ratio	Sustained intervention	–	Increase: p-value < 0.0001	NR	Landberg et al., 2009
Wholegrain wheat	Sum	Sum of AR homologues (C17:0, C19:0, C21:0, C23:0, C25:0)	Sustained intervention	–	Increase: p-value < 0.001	NR	Kristensen M, Toubro S, Jensen MG, Ross AB, Riboldi G, Petronio M, Bügel S, & Tetens I, 2012
Wine	Sum	*cis*-Resveratrol-3-*O*-GlcA *trans*-Resveratrol-3-*O*-GlcA *cis*-Resveratrol-4′-*O*-GlcA *trans*-Resveratrol-3-*O*-sulphate *cis*-Resveratrol-4′-*O*-sulphate *trans*-Resveratrol-4′-*O*-sulphate	Observational (cross-sectional)	–	Correlation: r – 0.895 (p-value < 0.001) AUC (95% CI) – 98.3% (97.3–99.0%) Sensitivity (95% CI) – 93.3% (91.5–94.7%) Specificity (95% CI) – 92.1% (90.2–93.7%)	NR	Zamora-Ros et al., 2009
	Sum	Resveratrol Metabolites: *cis*-Resveratrol-3-*O*-GlcA *trans*-Resveratrol-3-*O*-GlcA *cis*-Resveratrol-4′-*O*-GlcA *cis*-Resveratrol-3-*O*-sulphate *trans*-Resveratrol-3-*O*-sulphate *cis*-Resveratrol-4′-*O*-sulphate *trans*-Resveratrol-4′-*O*-sulphate Dihydroresveratrol Metabolites Total Metabolites (Resveratrol and Dihydroresveratrol)	Sustained intervention	–	Increase: p-value < 0.05	NR	Queipo-Ortuño et al., 2012
	Sum	Resveratrol Phase II Metabolites *cis*-Resveratrol-3-*O*-GlcA *trans*-Resveratrol-3-*O*-GlcA *cis*-Resveratrol-4′-*O*-GlcA *trans*-Resveratrol-4′-*O*-GlcA *cis*-Resveratrol-3-*O*-sulphate *trans*-Resveratrol-3-*O*-sulphate *cis*-Resveratrol-4′-*O*-sulphate *trans*-Resveratrol-4′-*O*-sulphate *trans*-Resveratrol-3,4′-*O*-disulphate Resveratrol sulphoglucuronide Resveratrol Glucosides *cis*-Piceid *trans*-Piceid Piceid-GlcA Piceid sulphate Gut microbial metabolism of Resveratrol Dihydroresveratrol Dihydroresveratrol-GlcA Dihydroresveratrol-sulphate Dihydroresveratrol-sulphoglucuronide	Sustained intervention	–	Increase: p-value < 0.05	NR	Rotches-Ribalta et al., 2012
		Urine - hydrolysed samples: 2,4-Dihydroxybenzoic acid Gallic acid Ethylgallate	Sustained intervention	Yes	AUCs (95% CI):	Yes for most MBP	Urpi-Sarda et al., 2015

	Stepwise logistic regression	Urine - non-hydrolysed samples: Methylgallic acid sulphate Ethylgallate sulphate Plasma - hydrolysed samples: 3-Hydroxyphenylacetic acid Gallic acid *p*-Coumaric acid				• Urine: from 96.00% (89.24–100%) to 98.68% (97.13–100%) • Plasma: from 80.13% (71.75–88.51%) to 91.07% (80.22–100%)		
Fruit & vegetables	Sum	Eriodictyol Naringenin Hesperetin Quercetin	Kaempferol Isorhamnetin Tamarixetin Phloretin	Sustained intervention	–	Increase: p-value < 0.001	NR	Brevik et al., 2004
		Naringenin Hesperetin Quercetin Kaempferol Isorhamnetin		Acute intervention	–	Correlation: • 24 h urine: r – 0.86 (p-value < 0.000001) • morning urine: r – 0.43 (p-value < 0.01)	Yes for 24 h urine No for morning urine	Krogholm et al., 2004
		Tamarixetin Phloretin		Sustained intervention	–	Correlation: r – 0.35 (p-value – 0.0007) Increase: p-value < 0.0001	Yes for most single biomarkers	Nielsen et al., 2002
		Vitamin C b-Carotene Lutein		Observational (cross-sectional)	–	Correlation: r – 0.42	Yes	Cooper et al., 2015
	Stepwise logistic regression	α-Carotene Energy intake Lutein β-Cryptoxanthin		Observational (cross-sectional)	No	Explained variability: 53%	NR	Gross et al., 1994
	Regression model	Vitamin C Carotenoids (cholesterol-adjusted) Ferric-reducing antioxidant power		Sustained intervention	Yes[a]	Correlation • TS: r – 0.47 (p-value < 0.001) • VS: r – 0.18–0.36 (p-value < 0.05)	Yes	Jin et al., 2014
	Logistic regression	Vitamin C Carotenoids		Sustained intervention	No	Correct allocation: 45–86%	Yes for most studies	McGrath et al., 2016
Diet Quality Index Score	Stepwise linear regressions	Vitamin C α-Tocopherol α-Carotene	β-Cryptoxanthin Oleic acid Stearic acid	Observational (cross-sectional)	No	NR	NR	Neuhouser, Patterson, King, Horner, & Lampe, 2003
Mediterranean diet	NR	Carotenes βVitamin E	EPA DHA	Observational (cross-sectional)	No	Correlation: r – –0.52 (p-value – 0.03)	NR	Gerber, 2006
Nordic diet	Rank scores PCA	α-Linolenic acid β-Carotene Alkylresorcinols	EPA DHA	Sustained intervention	No	NR	NR	Marklund et al., 2014

AR, alkylresorcinol; AUC, area under the curve; CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GlcA, glucuronide; MBP, multi-metabolite biomarker panel; NR, not reported; PCA, principal component analysis; TS, training set; VS, validation set.

[a] Samples of the training and validation sets were from the same subjects.

(2010) and Freedman, Tasevska, et al. (2010) proposed two main direct approaches, Howe's method with ranks or PCA, for combining questionnaires and biomarkers related to whole diet, food group, food, nutrient or other food components (Freedman, Kipnis, et al., 2010; Freedman, Tasevska, et al., 2010). Comparing the results obtained from the two approaches, Freedman, Kipnis, et al. (2010) and Freedman, Tasevska, et al. (2010) found that Howe's method gives results close to those from the PCA approach (Freedman, Kipnis, et al., 2010; Freedman, Tasevska, et al., 2010). It can be said that Howe's method is more appropriate due to its simplicity than the approach based on PCA. Later, these authors proposed a more complex modelling-based approach, namely the regression calibration method, for combining dietary biomarkers and reports that recovers lost power and gives unbiased relative risk estimates (Freedman et al., 2011). However, this method is not applicable to food patterns or foods that have no known specific biomarkers. Unlike this, PCA and Howe's method do not require knowledge of the quantitative relationship between biomarker level and true usual intake. Freedman, Kipnis, et al. (2010) and Freedman, Tasevska, et al. (2010) applied Howe's method in an analysis of a diet-disease association in a real example from the Carotenoids and Age-Related Eye Disease Study (CAREDS) (Freedman, Kipnis, et al., 2010; Freedman, Tasevska, et al., 2010). In their study, the estimated odds ratios [OR (95% CI)] for the primary nuclear cataract outcome using the reported dietary intake (FFQ-lutein plus zeaxanthin), the biomarker level (serum lutein plus zeaxanthin) and the combined FFQ-biomarker were 0.77 (0.57–1.02), 0.69 (0.51–0.94) and 0.66 (0.48–0.91), respectively. As can be seen from the statistical significance, the combination of exposure was higher than that for the FFQ and biomarker alone. In addition, an increase in statistical power was also observed in detecting a diet-disease association. Recently, Rabassa, Cherubini, et al. (2015) and Rabassa, Zamora-Ros, et al. (2015) applied this approach to study the association between habitual dietary resveratrol exposure and the development of frailty syndrome in older adults from the InCHIANTI study (Rabassa et al., 2015). Inverse associations between resveratrol exposure and frailty syndrome risk were observed for FFQ-total resveratrol [OR for comparison of extreme tertiles = 0.17 (0.05–0.63)], biomarker-total urinary resveratrol [0.32 (0.09–1.11)] and FFQ&biomarker-total resveratrol [0.11 (0.03–0.45)]. The most successful results from the combined exposure measure will emerge when the strength of the associations for each separate exposure is similar, as occurs in the examples described in this section.

3. Future trends

Future research trends should focus on exploring more novel approaches for the discovery and validation of dietary exposure biomarkers. While considerable progress has been made in demonstrating how dietary biomarkers can be used as dietary assessment tools, a number of challenges have still to be overcome before they can achieve their complete validation, including both analytical and biological perspectives. Recent studies have suggested different strategies for identifying panels of biomarkers. They have also demonstrated that MBPs offer a more reliable estimation of dietary exposure than the traditional single-biomarker approach used until now. Therefore, future studies will face up to the complexity of evaluating the use of MBPs in combination with conventional dietary questionnaires to assess dietary exposure fingerprinting. These advances will enable more detailed information to be obtained about the associations between diet and health, providing better evidence of the development of health claims and dietary advice for both public health institutions and the food industry.

Conflict of interest

The authors have declared no conflict of interest.

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MANUSCRITO 3

Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?

Cristian Del Bo', Stefano Bernardi, Mirko Marino, Marisa Porrini, Massimiliano Tucci, Simone Guglielmetti, Antonio Cherubini, Barbara Carrieri, Benjamin Kirkup, Paul Kroon, Raul Zamora-Ros, **Nicole Hidalgo Liberona,** Cristina Andres-Lacueva and Patrizia Riso

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MANUSCRITO 3

Objetivo: Los objetivos de esta revisión sistemática fueron:

- Realizar una revisión de la evidencia científica de forma exhaustiva y actualizada sobre la heterogeneidad de las fuentes de información y los métodos para estudiar el efecto de la ingesta de polifenoles
- Revisar el efecto de la ingesta de polifenoles considerando las ingestas totales y de subclases según de los factores relacionados con los hábitos dietéticos.
- Valorar la contribución especifica de las diferentes fuentes dietéticas de polifenoles.

Metodología: Para llevar a cabo esta revisión sistemática se valoró la literatura científica en estudios en humanos disponible en las bases de datos PubMed y EMBASE. Para la estrategia de búsqueda se utilizaron los términos: "polyphenols OR flavonoids OR anthocyanins OR flavanols OR flavanones OR flavones OR flavonols OR isoflavones OR proanthocyanidins OR phenolic acids OR hydroxycinnamic acids OR hydroxybenzoic acids OR lignans OR stilbenes AND intake" y para la selección de los estudios se consideraron como criterios de inclusión: i) estudios prospectivos, de cohortes y de casos y controles que analizaran/estimaran la ingesta de polifenoles totales/clases/individuales en la dieta; ii) estudios que informaran de la asociación entre la ingesta de polifenoles totales/clases/individuales en la dieta y los criterios de valoración del riesgo de enfermedad y la mortalidad; iii) estudios publicados desde enero de 2008 hasta diciembre de 2018. Los criterios de exclusión fueron: i) estudios de intervención dietética; ii) estudios que miden la ingesta de polifenoles a través de la excreción de orina; iii) estudios realizados in vitro o en modelos animales; iv) estudios que informan de datos sobre la ingesta de polifenoles a partir de suplementos (no relacionados con los alimentos); v) estudios que evalúan la asociación entre la ingesta de polifenoles y el riesgo de cáncer/mortalidad (recientemente se han realizado numerosas revisiones sistemáticas y meta-análisis); vi)-artículos publicados en un idioma diferente al inglés

Resultados: El proceso de selección de estudios se realizó de acuerdo con las directrices PRISMA y a partir de la búsqueda realizada en las bases de datos (PubMed y EMBASE), se identificaron un total de 3004 registros, de los cuales 91 estudios fueron seleccionados, considerando los criterios de inclusión y exclusión. De éstos (n=91), se analizaron por separado estudios que se centraron únicamente en la ingesta de polifenoles en poblaciones específicas (n=45), los que evaluaron la

asociación entre ingesta de polifenoles y el riesgo cardiovascular (CV)/diabetes (n=24), los que se centraron en la asociación con la mortalidad (por todas las causas y por eventos CV) (n=9) y los que evaluaron la asociación entre la ingesta de polifenoles con otros resultados en salud (por ejemplo, fragilidad, fractura ósea, demencia, etc.) (n=13).

Entre los métodos utilizados para valorar la ingesta dietética de polifenoles, generalmente se utilizaron recordatorios dietéticos de 24 horas (R24h; 56%) y cuestionarios de frecuencia de consumo de alimentos (CFCA; 31%). Para la estimación de la ingesta de polifenoles se utilizaron principalmente las bases de datos científicas de USDA (*United States Department of Agriculture;* 22%) y Phenol-Explorer (PE; 20%), también se utilizaron datos combinados desde USDA con PE y otras bases de datos y/o literatura científica (24%).

La ingesta total de polifenoles para la población en general se estimó en alrededor de 900 mg/día; valor que variaba según las diferencias en los grupos de sujetos evaluados. Los flavonoides totales y subclases específicas se asociaron aparentemente con un bajo riesgo de diabetes, eventos CV y mortalidad por todas las causas, no así los polifenoles totales. Además, la ingesta de flavonoides se asoció con otros resultados en salud como una mayor función endotelial, mayor densidad mineral ósea, menor riesgo de fracturas óseas y de degeneración macular, así mismo, las proantocianidinas se asociaron inversamente con el riesgo de eventos de insuficiencia renal y falla renal, mientras que las isoflavonas se asociaron con un mejor desarrollo puberal.

Entre las principales fuentes alimentarias de polifenoles se encontraron alimentos y bebidas como el té, el café, el vino tinto, las frutas y las verduras.

Conclusiones: Tal como lo respalda la evidencia científica en estudios in vivo e in vitro, los polifenoles ejercen numerosas actividades biológicas. En este estudio se ha encontrado una asociación inversa entre la ingesta de polifenoles y los eventos de riesgo CV y la mortalidad, así como en otros resultados de salud, además estos resultados están en línea con los encontrados en anteriores revisiones sistemáticas y metaanálisis. La mayoría de las asociaciones entre ingesta de polifenoles y salud se encontraron para clases/subclases específicas de éstos, por lo cual sigue siendo difícil establecer una ingesta de referencia y/o prudente de polifenoles totales, aunque en este estudio encontramos una ingesta media aproximada de unos 900 mg/día. Sin embargo, este valor debe considerarse tentativo debido a la elevada heterogeneidad de los estudios y a las numerosas limitaciones asociadas a la evaluación y estimación de la ingesta de polifenoles. No obstante, la cantidad de datos disponibles, parece sugerir que existe un efecto protector al seguir un patrón dietético rico en polifenoles, aunque debería fomentarse una investigación más focalizada y metodológicamente sólida para definir recomendaciones específicas.

nutrients

MDPI

Review

Systematic Review on Polyphenol Intake and Health Outcomes: Is there Sufficient Evidence to Define a Health-Promoting Polyphenol-Rich Dietary Pattern?

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Abstract: Growing evidence support association between polyphenol intake and reduced risk for chronic diseases, even if there is a broad debate about the effective amount of polyphenols able to exert such protective effect. The present systematic review provides an overview of the last 10-year literature on the evaluation of polyphenol intake and its association with specific disease markers and/or endpoints. An estimation of the mean total polyphenol intake has been performed despite the large heterogeneity of data reviewed. In addition, the contribution of dietary sources was considered, suggesting tea, coffee, red wine, fruit and vegetables as the main products providing polyphenols. Total flavonoids and specific subclasses, but not total polyphenols, have been apparently associated with a low risk of diabetes, cardiovascular events and all-cause mortality. However, large variability in terms of methods for the evaluation and quantification of polyphenol intake, markers and endpoints considered, makes it still difficult to establish an evidence-based reference intake for the whole class and subclass of compounds. Nevertheless, the critical mass of data available seem to strongly suggest the protective effect of a polyphenol-rich dietary pattern even if further well targeted and methodologically sound research should be encouraged in order to define specific recommendations.

Keywords: polyphenol intake; polyphenol databases; dietary pattern; disease risk; cardiovascular and all-cause mortality

1. Introduction

The possibility to develop dietary guidelines for the intake of food bioactives with health promoting effects can be of utmost importance to try to evolve the concept of adequate nutrition to that of optimal nutrition. Clearly, this implies at least 2 levels of knowledge: (1) the availability of reliable data of food composition and food intake to estimate exposure to food bioactives and (2) the capacity to assess the amount needed to exert the protective activity.

Polyphenols have been suggested to exert a plethora of biological activities including antioxidant, anti-inflammatory, anti-microbial, anti-proliferative, pro-apoptotic activity and hormonal regulation capacity [1]. There is also increasing evidence that long-term intake can have favorable effects on the incidence of several cancers and other chronic diseases, including cardiovascular disease (CVD), type II diabetes, and neurodegenerative diseases [2]. More recently research has been focused on the impact of polyphenols on healthy aging and/or age-related diseases [3].

The emerging evidence, obtained through both animal models and human studies, on the direct and indirect role of polyphenols in the modulation of metabolic and functional features of the host, has enhanced the interest for an estimation of polyphenol intake in the general population or in at risk target groups. In addition, the assessment of specificity in the protective properties of the single polyphenol classes/compounds (Figure 1) has been increased in the last years favored by the improvement of dedicated food databases (i.e., Phenol-Explorer, USDA database) reporting more accurate and detailed polyphenols composition and considering factors affecting the intake such as the "retention factors" (i.e., the loss or gain of a compound during food processing). Despite the transformation of food intake data into polyphenol intake remains still a critical, even if improved, step of the process, the accuracy of self-reported methods to evaluate dietary patterns is often limited. In particular, it has been suggested that the notion that fruit and vegetables intake represents the main dietary sources of polyphenols could be over-reported [4]. Finally, as far as polyphenols are concerned, the low bioavailability and extensive metabolism demonstrated in numerous studies makes it difficult to clearly state recommendations on intake.

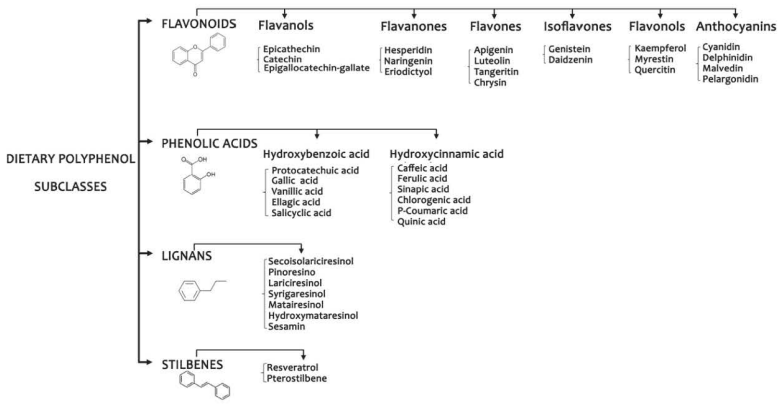

Figure 1. Polyphenol subclasses.

Nevertheless, the analysis of polyphenol intake data registered in several target population with different dietary patterns and lifestyle/exposure may help better understanding whether it is possible to identify a range of intake apparently associated to an overall reduced risk.

To this aim a comprehensive updated review on data and tools/methods used for the estimation of polyphenol intake was performed by considering differences in total and subclasses intake depending

on factors related to dietary habits. In addition, main results on the association among polyphenol intake and specific endpoints of disease risk have been taken into account, when available, to suggest possible recommendation.

1.1. Search Strategy and Study Selection

A literature search of all English language studies published was performed using PubMed (http://www.ncbi.nlm.nih.gov/pubmed), and EMBASE (http://www.embase.com/) databases (updated December 2018) with the addition of other scientific papers of relevance fount in web sources or in previously published reviews. The search terms and strategy used for the study selection were: polyphenols *OR* flavonoids *OR* anthocyanins *OR* flavanols *OR* flavanones *OR* flavones *OR* flavonols *OR* isoflavones *OR* proanthocyanidins *OR* phenolic acids *OR* hydroxycinnamic acids *OR* hydroxybenzoic acids *OR* lignans *OR* stilbenes *AND* intake. Human studies were used as further criteria of literature search. The search was limited to the last 10 years of publication. Three independent reviewers (S.B., M.M. and M.T.) conducted the literature search in the scientific databases and assessed and verified the eligibility of the studies based on the title and abstract. Disagreement between reviewers was resolved through consultation with a third reviewer (P.R. or C.D.B.) to reach a consensus. Inclusion criteria: (i) prospective, cohort and case-control studies analysing/estimating dietary total/classes/individual polyphenol intake; (ii) studies reporting association between dietary total/classes/individual polyphenol intake and endpoints of disease risk and mortality; (iii) studies published from January 2008 to December 2018. The exclusion criteria were: (i)-dietary intervention studies; (ii)-studies measuring polyphenols intake through urine excretion; (iii)-studies performed in in-vitro or in animal models; (iv)-studies reporting data on polyphenol intake from supplements (not food related); (v)-studies evaluating the association between polyphenol intake and cancer risk/mortality (numerous systematic reviews and meta-analysis have been recently performed); (vi)-published articles in a language different from English and with no accessible translation.

1.2. Data Extraction

For the papers meeting the inclusion criteria, the full text was retrieved, analysed and summarized in Tables. Data extraction was performed by three independent reviewers (S.B., M.M., M.T., P.R. and C.D.B.). The following information was collected: (i) first author name and year of publication; (ii) study design; (iii) number and subjects' characteristics; (iv) country; (v) tools used for estimating dietary polyphenols intake; (vi) polyphenol database source; (vii) overall results. For the studies evaluating the association with disease risk or mortality this information was included in the table. Additional revisions of contents have been performed by other reviewers (N.H.L., B.K. and B.C.).

2. Results

2.1. Study Selection

The study selection process according to PRISMA guidelines is reported in Figure 2. A total of 3004 records were identified from the database search (PubMed and EMBASE) and other sources. After removing 48 duplicate articles, 2956 studies were screened and 2566 were excluded based on title and/or abstract. The full text of eligible studies (n = 390) was read; 299 studies were excluded because not meeting the inclusion criteria (n = 282) or not of interest/pertinent (n = 17). At the end of the selection process, 91 papers were included.

2.2. Study Characteristics

The main characteristics of the 91 included studies are reported in Tables 1–4; 45 studies focused only on polyphenol intake in specific target populations, 24 studies assessed the association between polyphenols and cardiovascular/diabetes risk (1 study included also data on CV mortality), 9 studies focused specifically on the association with mortality for cardiovascular and all other events,

while 13 studies evaluated the association between polyphenol intake with others outcomes (e.g., frailty, bone fractures).

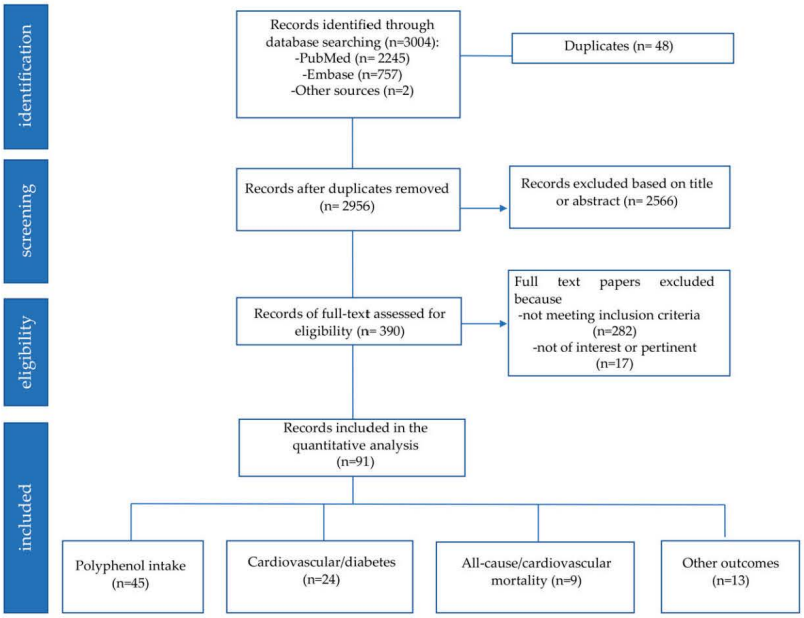

Figure 2. PRISMA Diagram.

2.3. Dietary Intake of Polyphenols

Table 1 shows reported data from literature focused on polyphenols intake. A total of 45 studies were found and analyzed [3–47]. Most of the studies were performed in Europe, North America and Asia (Figure 3A). The researches (Figure 3B) were carried out in the adult + older population (63%) or only adults (20%), while few studied reported data specifically in older subjects (7%), in children and adolescents (7%); the dietary intake of polyphenols was assessed generally through 24-h dietary records (24-h DR; 56%) and food frequency questionnaire (FFQ; 31%) as reported in Figure 3C. The main scientific databases (Figure 3D) used for the estimation of polyphenol intake were USDA (22%) and Phenol-Explorer (PE; 20%). However, most of the studies combined USDA with PE and other databases and/or scientific sources (24%). Total polyphenol intake for the overall population was estimated to be about 900 mg/day; this value varied according to differences in target groups of subjects. The main food sources of polyphenols were represented by tea, coffee, red wine, fruit and vegetables.

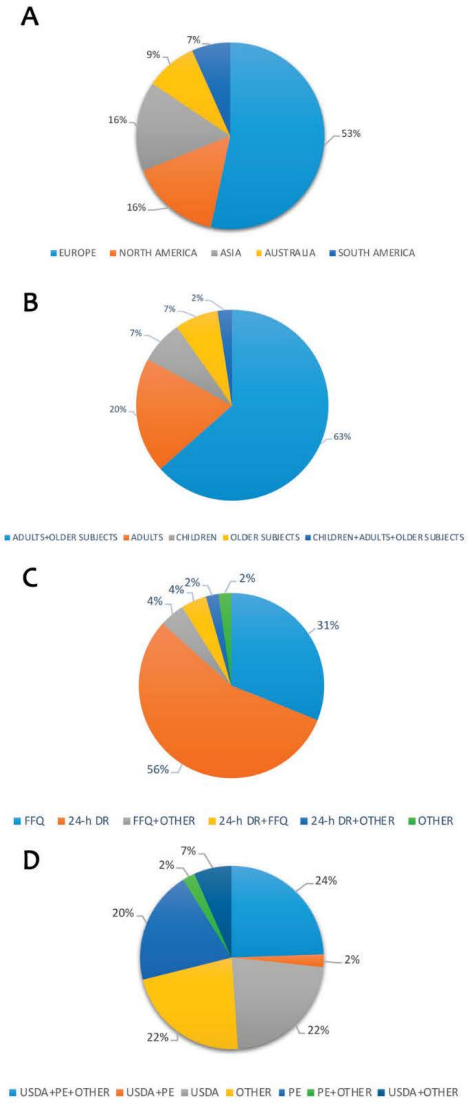

Figure 3. Estimation of polyphenols intake among countries. Legend: (**A**) Target population considered; (**B**) Distribution of published data by country; (**C**) Questionnaires used to evaluate food intake; (**D**) Polyphenol database used for evaluation of intake. FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer.

Table 1. Polyphenol intake registered in adults.

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Song et al. [5]	8809 subjects (NHANES 1999–2000 and 2001–2002) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (1,2)	Total flavonoids Mean intake = 189.7 ± 11.6	Flavan3-ols Mean intake = 156.5 ± 11.3 Flavanones Mean intake = 14.4 ± 0.6 Flavanols Mean intake = 12.9 ± 0.4 Anthocyanidins Mean intake = 3.1 ± 0.5 Flavones Mean intake = 1.6 ± 0.2 Isoflavones Mean intake = 1.2 ± 0.2	Tea (82.8%) Citrus juices (4.3%) Wine (2.1%) Citrus fruits (1.8%)	Different total flavonoids intake was observed between tea consumers (21% of the population) and tea non-consumers (697.9 vs. 32.6 mg/day respectively) with flavonols and flavan-3-ols as main compounds
Ilow et al. [6]	203 subjects W = 121 M = 82 Age = 50 year	Poland	FFQs 48 food items	USDA database (1)	Total flavonoids (median) M+F = 610.8 M = 612.0 F = 609.2	n.a.	Tea Fruit Vegetable	The flavonoid intake in tea was the same in women as in men. Tea flavonoids constituted about 96% of all the consumed flavonoids in this population
Otaki et al. [7]	514 subjects W = all M = 0 Age = 58 ± 10 year	Japan	1 24-h WDR	FFF (functional food factor) database	Total polyphenols-	* Total flavanols Mean = 1277 ± 1403 * Total isoflavones Mean = 215.7 ± 147.3 * Total flavonols Mean = 58.4 ± 62.7 * Total flavanones Mean = 30.5 ± 145.8 * Total flavones Mean = 15 ± 51.6 * data expressed in μmol/day	Green tea Onion Soy processed food (tofu, natto and miso)	The study showed higher total flavonoid intake compared to previous studies performed in the Japanese population. The sources of flavonoids differed from those of Western countries. Green tea, soy foods and onion constituted the main sources of flavan-3-ols, isoflavones and flavonols, respectively. Grapefruits and citrus fruits were the main sources of flavanones, while Malabar spinach, green peppers and grapefruits the main sources of flavones
Chun et al. [8]	8809 subjects NHANES 1999–2000 (n = 4175) and 2001–2002 (n = 4634) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (1,2)	Total flavonoids (1999–2000) Mean intake = 209.8 ± 18.9 Total flavonoids (2001–2002) Mean intake = 204.5 ± 14.5	n.a.	Tea (76.8%) Citrus fruit juice (3.7%) Beers and ales (2.9%) Wine (2.4%) Citrus fruit (1.7%) Melon and berries (1.4%) Other vegetables (1.4%)	Daily intake of flavonoids was dependent on sociodemographic characteristics and lifestyle behaviors. Daily flavonoid intake was provided mainly by teas (i.e., catechins)

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Yang et al. [9]	128 subjects W = n.a. M = n.a. Age = 20–28 year	China	2 sFFQs 126 food items 2 7-day 24-h DRs (used to validate FFQs data)	Specifically developed database *	**Total flavonoids (FFQ1)** Mean intake = 45.39 ± 25.52 **Total flavonoids (FFQ2)** Mean intake = 46.94 ± 27.72 **Total flavonoids (24-h DRs)** Mean intake = 50.15 ± 35.83	FFQ 1 data: **Total flavonol** Mean intake = 34.74 ± 18.80 **Total flavone** Mean intake = 10.65 ± 7.02 FFQ 2 data: **Total flavonol** Mean intake = 35.75 ± 20.45 **Total flavone** Mean intake = 11.19 ± 7.57 24-h DRs data: **Total flavonol** Mean intake = 38.37 ± 28.59 **Total flavone** Mean intake = 11.78 ± 8.45	n.a.	The FFQ used had reasonable reproducibility (measured 1 year apart) and validity to estimate dietary intake of flavonols (quercetin, kaempferol, isorhamnetin) and flavones (apigenin, luteolin) in the Chinese population, as compared to the other type of assessment methods
Zhang et al. [10]	5046 subjects W = 2910 M = 2136 Age = 18–72 year	China	2 sFFQs 126 food items 2 7-day 24-h DRs (used to validate FFQs data)	Specifically developed database *	**Total flavonols-Flavones** Mean intake = 19.13 ± 8.28	**Flavonols** Mean intake Quercetin = 5.96 ± 3.09 Kaempferol = 4.14 ±1.95 Myricetin = 1.81 ± 1.24 Isorhamnetin = 2.34 ± 1.48 **Flavones** Mean intake Apigenin = 1.06 ± 0.56 Luteolin = 3.82 ± 1.88	Apple (12%) Potato (8%) Celery (7%) Eggplant (7%) Actinidia (5%)	The total intake of flavonols and flavones was higher in men than in women. Gender and above all age were independent predictors for total flavonols and flavones intake. Main food sources were vegetables (61%) and fruits (36%) while tea was only a minor source
Hanna et al. [11]	551 subjects W = 551 M = 0 Age = 40–79 year	Australia	Phytoestrogen frequency questionnaire 112-item	USDA and specific literature	**Total isoflavones-lignans** Mean = 8.44 ± 17.03 Median intake = 2.2 Min and max = 0.44–174	**Total isoflavones** Mean = 4.5 ± 10.07 Median = 0.03 Min and max = 0–98 Total Lignans Mean = 2.71 ± 3.04 Median intake = 1.83 Min and max = 0.16–33	Soy and soy product (tofu, miso, soy grits or cereal)	Isoflavone intake was significantly different depending on age, i.e., 40–49 years and 50–59 years age groups introduced higher isoflavone amount compared to 60–69 years and 70–79 years age groups. There was no significant difference in lignan intake among age groups
Péréz-Jimenéz et al. [12]	4942 subjects (SU.VI.MAX cohort 1994.1995) W = 2346 M = 2596 Age = 45–60 year	France	6 24-h DRs 736 food items	Phenol Explorer	**Total polyphenols** Mean intake = 1193 ± 510 Median intake = 1123	**Flavonoids** Mean intake = 506 ± 219 **Phenolic acids** Mean intake = 639 ± 273	Non-alcoholic beverages (55.2%) Fruit (17.3%) Alcoholic beverages (8.3%) Cocoa products (7.5%) Vegetables (6.8%) Cereals (3.9%)	Total polyphenol intake was higher in men than in women. Age had no significant influence on intake. Three beverages Coffee, tea, and red wine accounted for 44%, 9%, and 6% of the total polyphenol intake while fruit, cocoa products, vegetables, and cereals for 17%, 8%, 7%, and 4% of the total polyphenol intake confirming data from other Western populations

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Zamora-Ros et al. [13]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-SOFT)	USDA database expanded with Phenol Explorer 1877 food items	Total flavonols-flavanones-flavones Mean intake ± SEM = 66.76 ± 0.89 W = 70.32 ± 0.65 Min W = 37.2 mg/day (Sweden) Min M = 36.7 mg/day (Sweden) Max W = 97.0 mg/day (UK) Max M = 130.9 mg/day (UK)	Flavonols Min = 38.5% (South) Max = 47.4% (North) Flavanones Min = 46.6% (UK) Max = 52.9% (South) Flavones Min = 5.8% (North) Max = 8.6%. (South)	Citrus fruits Citrus-based juices Tea Wine Fruits Vegetables	A large variation in flavanols, flavanones and flavones intake across European regions was registered Overall, flavanones were the main compounds introduced and UK health-conscious group the highest consumers. The total intake was higher in women and dependent on sociodemographic and lifestyle factors. Main food sources differed being juices and tea intake higher in the north while citrus fruit, juices, vegetables and wine in the south
Wang et al. [14]	8809 subjects NHANES 1999–2000 (n = 4175) and 2001–2002 (n = 4634) W = 4348 M = 4461 Age = >19 year	US	1 24-h DR	USDA Database (3)	Total proanthocyanidins (1999–2000) Mean intake (PI) = 88.8 ± 6.3 Total proanthocyanidins (2001–2002) Mean intake (PII) = 100.0 ± 4.2	Monomers Mean intakePI = 20.9 ± 1.5 PII = 20.7 ± 1.4 Dimers Mean intake PI = 15.0 ± 1.0 PII = 15.9 ± 1.1 Trimers Mean intake PI = 4.7 ± 0.3 PII = 5.3 ± 0.2 4–6mers Mean intake PI = 13.5 ± 1.2 PII = 15.7 ± 0.5 7–10mers Mean intake PI = 9.4 ± 0.9 PII = 11.2 ± 0.5 Polymers Mean intake PI = 25.4 ± 2.8 PII = 31.4 ± 1.9	Tea Legumes Wines	A south to north gradient intake was observed. In general, a mean intake of 95 mg/day was found represented by polymers (30%), monomers (22%), dimers (16%), 4–6 mers (15%), 7–10 mers (11%), and trimers (5%). After adjustment for energy intake, the PA intake increased with age, in women and in alcohol consumer. Tea, legumes, and wines, contributed to about 48% of daily PA intake

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Knaze et al. [15]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-SOFT)	USDA database Phenol Explorer 1877 food items	**Total flavan-3-ols** Mean intake ± SE MED countries = 268.8 ± 2.6 Non-MED countries = 274.7 ± 1.9 UK = 406.6 ± 7.6 **Total monomers** Mean intake ± SE MED countries = 90.2 ± 0.7 UK = 182.4 ± 3.0 **Total proanthocyanidins** (Mean intake ± SE MED countries = 217.2 ± 2.2 Non-MED countries = 177.9 ± 1.5 UK = 198.4 ± 6.3 **Total theaflavins** (Mean intake ± SE) MED countries = 1.6 ± 0.1 Non-MED countries = 6.5 ± 0.1 UK = 25.9 ± 0.3	**Flavan-3-ols subclasses:** **Flavan-3-ol monomers** MED 18.6% non-MED 32.9% UK 44.9% **PA or condensed tannins** MED 80.8% non-MED 64.8% UK 48.8%; **Theaflavins** MED 0.6% non-MED 2.4% UK 6.4%	Tea Wine Fruits Pulses (UK)	Socio-demographic, anthropometric and lifestyle factors were associated with consumption of flavan-3-ols, PA and theaflavins. Differences among different countries were observed. Flavan-3-ol intake in the UK (health-conscious) was about 2-fold that of the MED countries and mainly due to tea providing theaflavins and epigallocatechins. Overall PA intake was higher in the MED countries, even if with large differences, and non-citrus fruit (i.e., apples and pears) and wine the main sources
Zamora-Ros et al. [16]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries of EPIC cohort	1 24-h DR (EPIC-SOFT)	USDA database expanded with Phenol Explorer 1877 food items	**Total anthocyanidin** W: Mean ± SE = 33.52 ± 0.39 Max intake = 44.08 (Turin, Italy) Min intake = 18.73 (Granada, Spain) M: Mean ± SE = 29.44 ± 0.53 Max intake = 64.88 (Turin, Italy) Min intake = 19.83 (Bilthoven, The Netherlands)	**Cyanidin** Mean intake W = 15.09 ± 0.23 M = 12.01 ± 0.31 **Delphinidin** Mean intake W = 2.71 ± 0.09 M = 2.26 ± 0.13 **Malvidin** Mean intake W = 9.94 ± 0.18 M = 10.27 ± 0.25 **Pelargonidin** Mean intake W = 3.02 ± 0.09 M = 2.19 ± 0.12 **Peonidin** Mean intake W = 1.64 ± 0.04 M = 1.49 ± 0.05 **Petunidin** Mean intake W = 1.13 ± 0.02 M = 1.23 ± 0.03	Fruits, nuts and seeds (38.1–61.2%) Wines (14.4–24.5%) Non-alcoholic beverages (15.8%) Vegetables (4.8–9.7%)	The highest total anthocyanidins (mainly cyanidins and malvidins). intake was recorded in the south European region. Women (central-southern regions) were the highest consumers. Main food sources were different depending on countries. Central and northern countries: non-citrus fruits (berries, apples and pears, and grapes), wine and non-alcoholic beverages (juices and soft drinks of anthocyanidin-rich fruits). Southern countries: wine, non-citrus fruits (grapes, stone fruits, apples and pears, and olives) and leafy vegetable. A possible underestimation of anthocyanidin intake have been hypothesized due to missing food composition data

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Beking et al. [17]	Subjects = n.a.	UK Ireland	FAO Food Balance Sheets	USDA Database (1) Lacking data from literature	**Total flavonoids** Mean intake Ireland = 176.8 UK = 182.2	Ireland (mean intake): **Anthocyanidins** = 60.3 **Flavanols** = 47.4 **Flavanones** = 29.0 **Flavones** = 5.8 **Flavonols** = 34.2 UK (mean intake): **Anthocyanidins** = 69.2 **Flavanols** = 52.4 **Flavanones** = 26.0 **Flavones** = 4.0 **Flavonols** = 30.3	Grapes and oranges (41.6% UK, 34.9% Ireland) Beer and wine (8.8% UK, 12.8% Ireland) Apples and onions (6.8% UK, 6.5% Ireland) Tea (4.0% UK, 5.3% Ireland).	Estimated dietary intake of anthocyanidins, flavanones, flavanols, flavonols, flavones, and all five combined is similar in the UK and Ireland. Anthocyanidins and flavanols were about 65% of total intake. Data on flavones and flavonols were in line with those obtained in food intake surveys in UK and US. In general, as more types of food flavonoids are analyzed and included in food composition databases, intake estimates are expected to rise and to be more accurate
Ilow et al. [18]	1520 subjects Cardiovascular Disease Prevention Program) W = 879 M = 641 Age = 49–50 year	Poland	FFQs 1 24-h DR	USDA Database (1)	**Total flavonoids** Mean intake W = 622.6 M = 616.9	**Flavan-3-ols** W = 93.6% of total flavonoid M = 94.2% **Flavonols** W = 4.0% M = 4.2% **Anthocyanidins** W = 0.9% M = 1.1% **Flavanones** W = 0.9% M = 0.9% **Flavones** W = 0.1% M = 0.1%	Tea (93.6%, 94.2%) Fruits (2.2%, 1.6%) Vegetables (1.4%, 1.1%) Fruit juices (0.7%, 0.8%) Chocolate (0.1%, 0.1%)	A higher flavonoid intake was reported in comparison with other studies. Tea was the main food source of total flavonoids and mainly of flavan-3-ols intake (from tea, fruits, fruit juices, chocolate)
Zujko et al. [19]	6661 subjects (Polish National Multicenter Health Survey, WOBASZ) W = 3529 M = 3132 Age = 20–74 year	Poland	1 24-h DR	Database of polyphenol contents in food products (developed by the authors) 118 items	**Total polyphenols** Mean intake W = 10,311,054 (20–40 years) 1089 (41–60 years) 947 (61–74 years) M = 1172 1251 (20–40 years) 1183 (41–60 years) 1076 (61–74 years)	n.a.	Beverages (tea, coffee) Vegetables (potato) Fruits (apples) Cereals (white bread)	Polyphenol intake was about 1 g independently from gender and age and apparently similar to that of other countries. However, patterns of consumption were different depending on gender and age groups

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Lee et al. [20]	8502 subjects W = n.a. M = n.a. Age = >2 year	Korea	1 24-h DR	Phytonutrient database (Korea National Academy of Agricultural Science)	Total polyphenols-	Subjects meeting the recommendations Anthocyanidins = 73 ± 4.8 Hesperitin = 25.4 ± 3.2 Catechin = 24.8 ± 1.4 Quercetin = 9.1 ± 0.3 Isoflavones = 25.8 ± 2.8 Gallic Acid = 18.9 ± 2.6 Subjects not meeting the recommendations: Anthocyanidins = 8.7 ± 0.3 Hesperitin = 3.5 ± 0.5 Catechin = 2.2 ± 0.2 Quercetin = 2.9 ± 0.1 Isoflavones = 5.4 ± 0.5 Gallic acid = 4.3 ± 0.7	Fruits Onions Soybeans Nuts	Flavonoids (anthocyanidins, hesperitin, quercetin, catechin, and isoflavones), and one phenolic compound (gallic acid) were significantly higher among subjects who met the recommendations for fruit and vegetable consumption compared with those who did not
Zamora-Ros et al. [21]	36,037 subjects (EPIC cohort) W = 23,009 M = 13,028 Age = 35–74 year	10 European countries	1 24-h DR (EPIC-Soft)	USDA database (1)	Total polyphenols-	Total thearubigins M: Min = 0.9 Max = 532.5 W: Min = 1.2 Max = 455.6	Tea	Large differences in dietary thearubigins (TR) estimations intake across European countries; TR intake is low in Spanish men and high in men from UK; TR contributed < 5% to the total flavonoid intake in Greece, Spain and Italy while contributed 48% to the total flavonoids intake in UK
Tresserra-Rimbau et al. [22]	7200 subjects (PREDIMED) W = n.a. M = n.a. Age = 55–80 year	Spain	FFQs	Phenol Explorer 137 foods item	Total polyphenols Mean intake = 820 ± 323	Flavonoids = 443 ± 218 Phenolic acids = 304 ± 156 Other polyphenols = 71.2 ± 46.7	Fruits (44%) non-alcoholic beverages i.e., coffee (55%), vegetables (12%) alcoholic beverages (10%) Olive oil (11%)	Coffee and fruits resulted the main sources of polyphenols even if olives and olive oil represented significant and peculiar Mediterranean dietary sources of polyphenols (i.e., hydroxycinnamic acids, other phenolic acids, lignans and other polyphenols) with respect to other countries

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Vogiatzoglou et al. [23]	15,371 subjects W = 8278 M = 7093 Age = 14–80 year	Germany	1 24-h DR (EPIC-SOFT)	FLAVIOLA Database	Total polyphenols-	Total flavanols Mean intake = 385.9 Min = 195.8 Max = 840.7 Proanthocyanidins Mean intake = 196.4 Min = 138.7 Max = 300.3 Flavan-3-ol monomers Mean intake = 119.8 Min = 18.3 Max = 414.3 Theaflavins Mean intake = 69.7 Min = 38.8 Max = 126.1	Data are referred to total flavanols: Pome fruits (27%) Black tea (25%) Non-alcoholic beverages (46%) Green/fruit herbal tea (10–16%) Berries (6%)	Women had slightly higher intakes of total flavanols than men in all age groups, except for the elderly. There was a steep age gradient with an increase in total flavanols, flavan-3-ol monomers, and theaflavins across the age groups. Proanthocyanidins were the main contributor of total flavanols in both men and women
Grosso et al. [24]	10,477 subjects (HAPIEE study) W = 5340 M = 5137 Age = 45–69 year	Poland	FFQs 148 items	Phenol Explorer	Total polyphenols Mean intake = 1740.7 ± 630.2 Median intake = 1662.5	Total flavonoids Mean intake = 897.6 ± 423.4 Total phenolic acids Mean intake = 800.2 ± 345.8	Coffee (40%) Tea (27%) Chocolate (8%)	Intakes were slightly higher in men than in women, but when adjusted for energy intake, women had a higher intake of polyphenols than men. Age had significant influence on total and energy-adjusted polyphenol intake, being higher among younger participants
Witkowska et al. [25]	6661 subjects W = 3529 M = 3132 Age = 20–74 year	Poland	24-h DR	Phenol Explorer USDA database (1–3)	Total polyphenols Mean Intake = 989.3 ± 360	Total flavonoids Mean Intake USDA = 524.6 ± 155 PE = 403.5 ± 150 Total phenolic acids Mean Intake USDA = n.a. PE = 556.3 ± 204	Total polyphenols (PE): Non-alcoholic beverages (75%) Total flavonoids: Non-alcoholic beverages: (PE 78.5%) (USDA 90%)	Flavonoids estimated through various databases might substantially differ. The use of several databases can truly reflect the real intake but it will be difficult to comparison for which only one method has been used for calculations

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Kim et al. [26]	11,474 Subjects W = n.a. M = n.a. Age = ≥ 19 year	Korea	1 24-h DR	USDA database (1) Korean-targeted flavonoid database	**Total flavonoids** Mean Intake ± SE = 96.6 ± 1.34 Median = 70.4 P10 – P90 = 22.8 – 192	**Total anthocyanidins** Mean Intake ± SE = 26.4 ± 0.9 Median = 6.36 P10 – P90 = 0 – 68.1 **Total flavanols** Mean Intake ± SE = 25.5 ± 1.8 Median = 1.08 P10 – P90 = 0 – 43.2 **Total flavanones** Mean Intake ± SE = 8.15 ± 0.39 Median = 0P10 – P90 = 0 – 25.1 **Total flavones** Mean Intake ± SE = 0.87 ± 0.03 Median = 0.45 P10 – P90 = 0.13 – 1.86 **Total flavonols** Mean Intake ± SE = 24.6 ± 0.42 Median = 16.8 P10 – P90 = 4.88 – 50.2 **Total isoflavones** Mean Intake ± SE = 21.9 ± 0.39 Median = 12.1 P10 – P90 = 0.27 – 53.9	Kimchi (traditional fermented vegetable product) (12%) Green tea (9%) Persimmon (7%) Soybean (7%) Onion (7%) Tofu (6%) Radish (5%) Tangerine (5%) Apple (4%) Pear (3%)	Total Flavonoid intake was lower in Korea than in western countries. A major difference came from tea intake and also by the lower flavonoid density of major sources (kimchi, persimmon, tangerine, onion, radish etc.) in Korea than those (tea, citrus fruit, apples, pears, wine, etc.) in western countries. Contrast the isoflavone intake was much higher than the estimates for western countries due to high intakes of soybeans, tofu, and fermented soy pastes
Zamora-Ros et al. [27]	36,037 Subjects W = 23,009 M = 13,028 Age = 35–74 year	10 European countries of EPIC cohort	1 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake ± SEW = 1192 ± 6 M = 1177 ± 8 highest in Denmark M = 1786 W = 1626 lowest in Greece M = 744 W = 584	**Total flavonoids:** Mean intake ± SEW = 546 ± 4 M = 492 ± 5 **Total phenolic acids** Mean intake ± SEW = 625 ± 6 M = 593 ± 5 **Total lignans** Mean intake ± SEW = 3.6 ± 0.1 M = 2.5 ± 0.2 **Total stilbenes** Mean intake ± SEW = 2.4 ± 0.0 M = 3.0 ± 0.1	MED countries: Coffee (36%) Fruits (25%) Wine (10%) Non-MED countries: Coffee (41%) Tea (17%) Fruits (13%)	Mean intake of polyphenols was three times higher in men from Denmark than in women from Greece. Stratifying by region, mean of total polyphenols intake was in non-MED countries due to the higher intake of phenolic acids. The study showed a large heterogeneity in both the nature of polyphenols and levels of intake across the countries due to different habits and socio-demographics status

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Vogiatzoglou et al. [28]	30,000 subjects W = n.a M = n.a Age = 18–64 year	14 Countries	2–7 24-h DR	FLAVIOLA Database	**Total flavonoids:** Mean Intake = 428 ± 49 central region = 506 ± 75 northern region = 348 ± 20 southern region = 301 ± 27 Median Intake = 164 ± 55 central region = 249 ± 87 northern region = 56 ± 22 southern region = 47 ± 7	**Theaflavins and thearubigins** Mean intake = 168 ± 39 Median intake = 89 ± 38 **Proanthocyanidins:** Mean intake = 124 ± 7 Median intake = 27 ± 5 **(Epi)catechin** Mean intake = 24 ± 2Median intake = 7 ± 2 **Gallated compounds** Mean intake = 53 ± 12 Median intake = 28 ± 12 **Anthocyanidins** Mean intake = 19 ± 2 Median intake = 3 ± 1 **Flavonols** Mean intake = 23 ± 2 Median intake = 8 ± 2 **Flavanones** Mean intake = 14 ± 2 Median intake = 1 ± 0 **Flavones** Mean intake = 4 ± 1 Median intake = 1 ± 0 **Flavonoids (monomeric)** Mean intake = 136 ± 14 Median intake = 49 ± 15	Non-alcoholic beverages Fruits	Large regional differences, both in the type of flavonoids consumed and the distribution of intake. Intakes of anthocyanidins (in particular cyanidin) and flavanones (in particular hesperetin) were highest in the Northern Region, in particular in Finland. Within the Central Region, there was also a large variability of intake between countries. While overall flavonoid intake in Ireland was the highest in Europe, the intake of anthocyanidins was the lowest overall, and intake of flavanones was also very low. France was included in the Southern Region as dietary intake was more comparable with intake in Italy and Spain. However, there are some important differences, and the intake of flavan-3-ols and anthocyanidins in France is considerably higher than in the other countries of the Southern Region
Sebastian et al. [29]	5420 subjects W = 2758 M = 2662 Age = >20 year	USA	1 24-h DR	USDA database (1)	**Total flavonoids** Mean intake = 251 ± 16.8 IQR = 18.8–272 W Mean intake = 241 ± 15.2 IQR = 16.3–272 M Mean intake = 263 ± 20.4 IQR = 20.4–271	Mean intake: **Total flavonols** = 19.4 ± 0.91 IQR = 6.05–25.4 **Total flavones** = 0.9 ± 0.1 IQR = 0.1–1.1 **Total flavanones** = 13.1 ± 0.88 IQR = 0.00–5.15 **Total isoflavones** = 1.7 ± 0.3 IQR = 0–0 **Total flavanols** = 204 ± 15.6 IQR = 3.07–189 **Total anthocyanidins** = 11.6 ± 1.07 IQR = 0–9.92	Tea (80%) Fruit Vegetables	A positive association between flavonoid intake and dietary quality suggest that a diet high in flavonoids is synonymous with greater compliance with national guidance. Individuals with higher flavonoids intake not only consume more fruit and vegetables but also eat more healthfully

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Kozłowska et al. [30]	151 subjects Polish = 91 Spanish = 60 Polish W = 74 M = 17 Spanish W = 36 M = 24 Total: W = 110 M = 41 Age = n.a.	PolandSpain	FFQs	USDA Database (1)	Total flavonoids Mean intake Polish students = 801 Spanish students = 297	n.a.	Polish Students: Black and green tea Oranges Orange juice Spanish Students: Oranges Green tea Orange Juice	Flavonoid consumption in Polish students was more than two times higher than in the Spanish students. The main sources of flavonoids in Spanish and Polish diets were different as black tea in the Spanish group provided weekly about 236 mg of flavonoids, over 12 times less than in the Polish group. On the other hand, the Spanish diet was richer than the polish diet in sources of flavonoids such as oranges, chickpeas, dried parsley, onions, strawberries, almonds or pomelo
Zujko et al. [31]	6661 subjects M = 3132 W = 3529 Age = 20–74 year	Poland	1 24-h DR	Database developed by the authors	Total flavonoids Mean intake = 276 W (20–40 year) = 278 CI95% = 266–290 M (20–40 year) = 304CI95% = 291–317 W (41–60 year) = 275 CI95% = 264–286 M (41–60 year) = 291 CI95% = 279–311 W (61–74 year) = 238 CI95% = 227–249 M (61–74 year) = 268 CI95% = 256–280	n.a.	Beverages (47%) Fruit and fruit jams (27%) Tea (22%) Vegetables (18%) Apples (12%) Coffee (8%)	The consumption of tea, coffee, and apples was associated with the largest contributions to the flavonoid content. In comparison to the young and middle age participants, the elderly consumed less beverages and vegetables with a lower level of flavonoids
Taguchi et al. [32]	610 subjects M = 569 W = 41 Age = 52–89 year	Japan	FFQs	Database developed by the author	Total polyphenols Mean intake = 1492 ± 665	n.a.	Coffee (43.2%) Green tea (26.6%)	The present study showed that a population of elderly Japanese (mostly men) consumed higher amounts of polyphenols than previous data in Japanese adults, and coffee and green tea were the largest sources of polyphenols in their daily life

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Sun et al. [33]	887 subjects W = 887 Age = 12–18 year	China	FFQs 4 24-h DR	Flavonoids database developed by the authors	**Total flavonoids** Mean intake = 20.60 ± 14.12	**Total flavonol** = 16.29 ± 11.91 **Quercetin** = 5.51 ± 4.00 **Kaempferol** = 5.49 ± 3.68 **Myrucetin** = 2.29 ± 1.84 **Isorhamntin** = 3.00 ± 2.37 **Total flavones** = 4.31 ± 2.21 **Luteolin** = 3.27 ± 1.63 **Apigenin** = 1.03 ± 0.58	Apple (11.7%) Potatoes (9.9%) Lettuce (7.3%) Oranges (7.0%) Chinese Cabbage (4.7%) Tomatoes (4.2%) Celery (4.2%) Soyabean Sprouts (4.2%) Leeks (3.9%) Aubergine (3.9%)	The dietary flavonoid intakes among female adolescents in the Suihua area were similar to those reported in previous studies. In the present study, apples, potatoes, lettuce, oranges, soyabean sprouts and leeks were the main food sources of flavonols, whereas tomatoes, aubergine, white radishes, celery and sweet potatoes were the main sources of flavones
Kim et al. [34]	9801 subjects W = 5032 M = 4769 Age = >19 year	US	2 24-h DR	USDA databases (1,2)	**Total flavonoids** Mean intake = 200.1 ± 8.9	**Total flavonols** Mean intake = 15.9 ± 0.4 **Total flavones** Mean intake = 1.2 ± 0.1 **Total flavanones** Mean intake = 12.2 ± 0.5 **Total flavanols** Mean intake = 158.4 ± 8.5 **Total anthocyanidins** Mean intake = 11.5 ± 0.7 **Total isoflavones** Mean intake = 0.9 ± 0.1	Tea Citrus fruit juices Berries Citrus fruit Wine Apples	Flavonoid intake increased with age from 19 to 30 years until 50–70 years in both men and women. After adjusting for energy intake, flavonoid density of women was greater than those of men ($p < 0.0001$). The difference of flavonoid density among ethnicity was reduced after adjusting for energy intake. Flavonoid density of alcohol non-consumer was greater than that of alcohol consumer ($p < 0.05$)
Burkholder-Cooley et al. [35]	77,441 subjects W = 50,336 M = 27,105 Age = 57 year	USA Canada	FFQs	Phenol Explorer USDA database (1-2)	**Total polyphenols** Mean intake coffee consumers = 1370 ± 1069 non-coffee consumers = 541 ± 368	**Total flavonoids** Mean intake non-coffee consumer = 305 ± 238 coffee consumer = 273 ± 213 **Total phenolic acids** Mean intake non-coffee consumers = 125 ± 106 coffee consumers = 986 ± 1030	Coffee Fruit Vegetables Fruit juice Legumes (including soya)	Significant differences in mean adjusted total polyphenol intakes were observed between dietary patterns. 34% of the participants reported coffee consumption in the FFQ. In the group of non-coffee consumers vegans reported the highest intake of total polyphenols followed by pesco-vegetarians, lacto-ovo vegetarians, semi-vegetarians and non-vegetarians. In the group of coffee consumers non- vegetarians reporting the highest intakes, followed by vegans, semi-vegetarians, pesco-vegetarians and lacto-ovo-vegetarians

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Pounis et al. [36]	14,029 subjects W = 7048 M = 6981 Age = n.a.	Italy	EPIC-FFQs specifically adapted for the Italian population 164 food items	Eurofir-eBASIS USDA database	Total polyphenols -	Median intake: Total flavonols = 17.0 Total flavones = 0.7 Total flavanones = 32.4 Total flavanols = 51.2 Total anthocyanidins = 144 Total isoflavones = 23.5 Total lignans = 80	Seasonal fruits Citrus fruits Leafy vegetable Grain Root vegetables Onions Garlic	Total energy intake was positively associated with the consumption of all polyphenol classes and sub-classes in both genders. Men or older participants seemed to have higher intakes of most of the polyphenols compared with women or younger participants. No significant sex difference was observed for lignans. Educational level did not account for differences in most of flavonoid and lignan intake among participants. No/former smokers presented higher intake of polyphenols. Participants with higher physical activity level consumed greater quantities of all classes of polyphenols
Ivey et al. [37]	1063 subjects W = 1063 M = 0 Age = >75 year Mean age = 80 ± 3 year	Australia	sFFQs	Phenol Explorer USDA database [(1–3)]	Total flavonoids USDA database [(1–3)] Mean intake = 834 ± 394 PE Mean intake = 487 ± 243	Total flavonols USDA = 30 ± 17 PE = 104 ± 61 Total flavanols USDA = 666 ± 345 PE = 327 ± 179 Total flavones USDA = 4 ± 3 PE = 13 ± 7 Total flavanones USDA = 40 ± 36 PE = 33 ± 31 Total anthocyanidins USDA = 88 ± 77 PE = 11 ± 11	n.a.	The mean flavonol PE intake of the cohort was nearly 350% greater than the flavonol USDA estimate. This difference may be, in part, due to the fact that the PE database provides data for five additional groups of flavonol compounds which were not expressed in USDA. Furthermore, the USDA database does not include the flavonol content data of chocolate

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Godos et al. [38]	1937 subjects W = n.a. M = n.a. Age = >18 year	Italy	FFQs 110 food items	Phenol Explorer	Total polyphenols Mean intake = 663.7 ± 608.1	Total flavonoids Mean intake = 258.7 ± 199.1 Total flavonols Mean intake = 57 ± 45.6 Total flavanols Mean intake = 93.9 ± 118.2 Total flavanones Mean intake = 37.9 ± 42.0 Total flavones Mean intake = 8.4 ± 10.2 Total anthocyanins Mean intake = 55.4 ± 55.3 Total isoflavones Mean intake = 4.0 ± 14.4 Total phenolic acids Mean intake = 362.7 ± 516.0 Total stilbenes Mean intake = 1.9 ± 3.5 Total lignans Mean intake = 2.8 ± 2.6	Nuts (29%) Non-alcoholic beverages (23%) Fruits (20%) Vegetables (15%) Alcoholic beverages (7%)	Compared to other Mediterranean cohorts the main differences with all the other cohorts was the contribution of nuts. In this population nuts were among the main contributors of hydroxybenzoic acids, which in other cohorts were generally provided by tea and red wine.
Miranda et al. [39]	1103 subjects W = 678 M = 425 Age = >20 year	Brazil	1 24-h DR	Phenol Explorer	Total polyphenols Mean intake ± SE = 377.5 ± 15.3 Median intake = 300.3 IQR = 154.1–486.9	Mean ± SE Phenolic acids = 284 ± 15.9 Hydroxycinnamic acids = 281.2 ± 15.9 Hydroxybenzoic acids = 3.4 ± 0.4 Flavonoids = 54.6 ± 3.5 Flavanones = 16.1 ± 1.9 Flavonols = 14.6 ± 0.9 Flavanols = 11.4 ± 0.8 Anthocyanins = 6.8 ± 1.1 Flavones = 3.6 ± 0.3	Coffee (70.5%) Citrus fruit (4.6%) Tropical fruit (3.4%)	The polyphenol intake was three times lower than the estimated value compared with other countries probably due to sociodemographic differences and food choices. Older subjects (>60 y) consumed more flavonoids and tyrosol than adults (20–59 y) and also more fruits.
Burkholder-Cooley et al. [40]	899 subjects W = 602 M = 297 Age = 58 ± 13.2 year	USA Canada	24-h DR FFQs	Phenol Explorer USDA database (1,2)	Total polyphenols FFQs Mean intake = 717 ± 646 24-h DR Mean intake = 402 ± 345	n.a.	Coffee Fruit juice	Beverages and fruit were key contributors to total daily polyphenol intake. Subjects could over-report the frequency of intake of fruit and fruit juice in the FFQ even if a positive correlation with 24-h DR is observed.

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Bawaked et al. [41]	3534 subjects W = 2015 M = 1509 Age = 2–24 year	Spain	1 24-h DR	USDA database [(1)] Phenol Explorer	**Total Flavonoids** Mean intake = 70.7 ± 84.1 Median intake = 48.1 25th–75th percentile = 19.3–93.1	**Total flavonols** Mean intake = 15.6 ± 30.6 Median intake = 5.9 25th–75th percentile = 1.8–17.2 **Total flavones** Mean intake = 2.2 ± 9.1 Median intake = 0.3 25th–75th percentile = 0.0–1.1 **Total flavanones** Mean intake = 19.7 ± 34.1 Median intake = 0.1 25th–75th percentile = 0.0–28.1 **Total flavan-3-ols** Mean intake = 25.2 ± 47.1 Median intake = 14.1 25th–75th percentile = 4.7–28.1 **Total anthocyanins** Mean intake = 7.7 ± 27.1 Median intake = 0.3 25th–75th percentile = 0.0–4.2 **Total isoflavones** Mean intake = 0.1 ± 1.4 Median intake = 0.0 25th–75th percentile = 0.0–0.0	Fruit (42.8%) Cocoa powder and chocolate (23.5%) Vegetables (spinach, onions, artichokes and lettuce) (22%)	Higher adherence to the Mediterranean diet was correlated with higher flavonoids intake. Fruits were the main source of dietary flavonoids
Zamora-Ros et al. [42]	115,315 subjects W = 115,315 M = 0 Age = >25 year	Mexico	sFFQs 140 food items	Phenol Explorer	**Total polyphenols** Median intake = 694 Min and max = 536 and 750 25th–75th percentile = 413–1103	**Total flavonoids** Median intake = 235 Min and max = 188–270 25th–75th percentile = 141–367 **Total phenolic acid** Median intake = 361 Min and max = 243 and 439 25th–75th percentile = 166–690	Total polyphenol: Coffee (29%) Decaffeinated coffee (19%) Total flavonoids: Apple (19%) Orange and mandarins (13%) Orange juice (12%)	Large heterogeneity in intakes of individual polyphenols among Mexican women, but a moderate heterogeneity across Mexican states. Main food sources were also similar in the different states

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Ziauddeen et al. [43]	9374 subjects W = 5075 M = 4299 Children (age < 18 year) = 4636 Adults or older (age > 18 year) = 4738 Age > 1.5 year	UK	4D-FR	Phenol Explorer	Total polyphenols Mean intake by age ranges = (1.5–3 year) = 266.6 ± 166.1 (4–10 year) = 388.8 ± 188.8 (11–18 year) = 455.0 ± 263.2 (19–34 year) = 635.9 ± 448.9 (35–49 year) = 846.1 ± 514.1 (50–64 year) = 1053.2 ± 545.3 (65+ year) = 1035.1 ± 544.3	Total flavonoids (1.5–3 year) = 212.2 ± 151.7 (4–10 year) = 312.1 ± 170.3 (11–18 year) = 355.4 ± 230.9 (19–34 year) = 433.8 ± 335.1 (35–49 year) = 568.3 ± 398.2 (50–64 year) = 714.5 ± 415.2 (65+ year) = 716.2 ± 404.9 Phenolic acids (1.5–3 year) = 54.3 ± 24.8 (4–10 year) = 76.5 ± 43.2 (11–18 year) = 99.6 ± 63.4 (19–34 year) = 201.3 ± 228.5 (35–49 year) = 276.2 ± 232.6 (50–64 year) = 336.7 ± 292.0 (65+ year) = 317.6 ± 297.0 Stilbenes (1.5–3 year) = 0.1 ± 0.2 (4–10 year) = 0.1 ± 0.1 (11–18 year) = 0.1 ± 0.4 (19–34 year) = 0.8 ± 2.4 (35–49 year) = 1.6 ± 3.8 (50–64 year) = 1.9 ± 4.1 (65+ year) = 1.3 ± 3	Non-alcoholic beverages Fruits	Polyphenol intake increased with age ($p < 0.001$) and was higher in males with exception of adults aged between 19–34 and 50–64 that showed higher levels in females
Karam et al. [44]	211 subjects W = 112 M = 99 Age = 55–80 year	Spain	2 24-h DR	Phenol Explorer USDA databases pecific literature. (449 food items; 245 polyphenol containing products considered)	Total polyphenols Mean intake = 332.7 ± 197.4 Median intake = 299.5 IQR = 250.4 Energy adjusted Mean intake = 187.5 ± 100.5 Median intake = 172.9 IQR = 140.3	Flavonoids = 170.3 ± 144.4 Flavanols = 46.0 ± 57.7 Flavonols = 22.7 ± 29.9 Flavanones = 30.7 ± 50.6 Flavones = 10.7 ± 20.3 Anthocyanin = 36.7 ± 61.9 Dihydrochalcones = 0.3 ± 1.8 Isoflavonoids = 19.3 ± 71.1 Phenolic acids = 100.0 ± 130.0 Lignans = 7.2 ± 15.6 Stilbenes = 2.6 ± 4.4	Total polyphenol: Red wine 17.7% Artichoke 6.2% Soy milk 5.4% Total flavonoids: Red wine 26.8% Soy milk 10.8% Orange 9.5%	Flavonoids were the highest ingested polyphenols in the older population under analysis. Polyphenol intake was generally higher in female (adjusted for energy intake), in subjects aged 64–67 y, in physically active and alcoholic product drinkers
Rossi et al. [45]	241 subjects W = n.a M = n.a Age = 6–12 year	Argentine	sFFQs	Phenol Explorer Lacking data from literature	Total polyphenols Mean intake = 412	Phenolic acid Mean intake = 310 Flavonoids Mean intake = 94.1	Mate (60%) Tea (19%) Coffee (5%) Onion (3%)	Low intake of polyphenols was found in this scholar population of high region of the northwest Argentine due to the very low consumption of fruits and vegetables

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Wisnuwardani et al. [46]	2428 subjects (HELENA study) W = 1289 M = 1139 Age = 12.5–17.5 year	Different European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria, Spain)	2 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake = 329 Median intake = 326 Q1 = 167 Q4 = 564 Mean intake by age ranges (12.5–13.99 year) = 346 ± 0.1 (14–14.99 year) = 345 ± 0.2 (15–15.99 year) = 356 ± 0.2 (16–17.49 year) = 396 ± 0.2	**Total flavonoids** (12.5–13.99 year) = 267 ± 0.1 (14–14.99 year) = 256 ± 0.1 (15–15.99 year) = 253 ± 0.1 (16–17.49 year) = 271 ± 0.1 **Phenolic acids** (12.5–13.99 year) = 75 ± 0.1 (14–14.99 year) = 75 ± 0.1 (15–15.99 year) = 85 ± 0.1 (16–17.49 year) = 104 ± 0.1 **Stilbenes** (12.5–13.99 year) = 0.038 ± 0.0 (14–14.99 year) = 0.048 ± 0.0 (15–15.99 year) = 0.046 ± 0.0 (16–17.49 year) = 0.060 ± 0.0 **Lignans** (12.5–13.99 year) = 1.0 ± 0.0 (14–14.99 year) = 1.0 ± 0.0 (15–15.99 year) = 1.1 ± 0.0 (16–17.49 year) = 1.1 ± 0.0	Fruit (apple and pear 16%) (23%) Chocolate products (19.2%) Fruit and vegetable juices (16%)	Total polyphenol intake was lower compared to intake of adults reported in previous studies. Polyphenol intake differed largely among countries. Overall, intake for flavonoids was = 75–76% of total polyphenol, for phenolic acids was = 17–19% of total polyphenol and for stilbenes and lignans was = <1% of total polyphenol.
Kent et al. [47]	79 subjects (The Blue Mountains Eye Study) W = 45 M = 34 Age mean = 70.1 year Age = 60–80 year	Australia	12 24-h DR (weighed)	USDA database (1)	**Total flavonoids** Mean intake = 678.69 ± 498.53 Median intake = 581.84 IQR = 619.58	**Anthocyanins** Mean intake = 6.73 ± 12.7 Median intake = 1.05 IQR = 7.88 **Flavonols** Mean intake = 28.04 ± 33.29 Median intake = 24.06 IQR = 21.21 **Flavones** Mean intake = 1.87 ± 4.78 Median intake = 0.55 IQR = 2.11 **Flavan 3-ols** Mean intake = 596.17 ± 494.95 Median intake = 499.72 IQR = 622.95 **Flavanones** Mean intake = 21.43 ± 61.46 Median intake = 2.15 IQR = 12.14	n.a.	Substantial within-individual variation and between individual variation was documented for both total flavonoid intake and intake of flavonoid subclasses. The within-individual variation was in the range 80–140% while the between individual variation was in the range 60–117%. It is speculated that a minimum of 6-day weighed food records is necessary to obtain a reliable estimate of flavonoid intake.

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Vitale et al. [48]	2573 subjects (TOSCA.IT Study) W = n. a. M = n. a. Age = 50–75 year Mean = 62.2 ± 0.1 year	Italy	FFQs (Epic)	USDA [1] Phenol Explorer Lacking data from literature	Total polyphenols Mean intake = 683.3 ± 5.8 Mean intake (mg/1000 Kcal/day) Mean = 376.6 ± 3.2 W = 374.0 ± 4.9 M = 378.7 ± 4.1 Mean intake by geographical area North = 387.4 ± 6.0 Center = 355.2 ± 6.1 South = 381.9 ± 4.5 Mean intake by age <60 year = 367.9 ± 4.7 60–65 year = 376.1 ± 5.8 >65 year = 388.4 ± 6.1	Total flavonoids Mean intake = 324.7 ± 4.1 Phenolic acids Mean intake = 324.2 ± 3.0 Lignans Mean intake = 4.1 ± 0.06 Stilbenes Mean intake = 3.5 ± 0.11 Other polyphenols Mean intake = 27.0 ± 0.27	Non-alcoholic beverages (coffee 54%, tea 27%), fruits (apple 37%, orange 13%), alcoholic beverages (red wine 93%) and vegetables (artichokes 40%, spinach 20%, onions 18%)	A lower intake of polyphenols has been registered in diabetic subjects compared with other groups, showing a different dietary pattern in this type of Italian population.
Nascimento-Souza et al. [49]	620 subjects W = 330 M = 290 Age = 60–98 years	Brazil	Multiple 24-h DR	Phenol Explorer	Total polyphenols Mean intake = 1198.6 ± 693.8 Median = 1052.7 IQR = 740.5–1477.9 Mean intake by sex W Mean intake = 1097.6 ± 616 Median = 949.4 IQR = 692.4–1407.9 M Mean intake = 1313.5 ± 757.3 Median = 1169.2 IQR = 844.7–1610.3 Mean intake by age 60–74 years Mean intake = 1197.8 ± 619.3 Median = 1092.4 IQR = 806.9–1502.9 >75 years Mean intake = 1310.2 ± 699.4 Median = 1186.9 IQR = 818.3–1582.2	Total flavonoids Mean intake = 444.7 ± 345.1 Phenolic acids Mean intake = 729.5 ± 545.4 Lignans Mean intake = 13.6 ± 25.5	Non-alcoholic beverages (coffee 45.8%), beans (32.8%), polenta (1.3%)	The intake of polyphenols was in a range similar to that reported for other populations, in particular European countries, but it differs for the main food contributors (high in beans and polenta, low in fruits and vegetables)

Table 1. *Cont.*

Reference by Year	Population Characteristics	Country	Dietary Assessment n° Food Containing Items	Polyphenol Database n° Food Items	Estimated Intake (mg/day) mean/median/min-max	Polyphenol Main Subclasses Intake (mg/day or percentage) mean ± ds/median/min-max	Main Dietary Sources (Based on % Contribution)	Overall Results
Nascimento-Souza et al. [49]	620 subjects W = 330 M = 290 Age = 60–98 years	Brazil	Multiple 24-h DR	Phenol Explorer	Mean intake energy-adjusted Mean = 1198.6 ± 591.1 Median = 1102.8 IQR = 817.3–1504.8 Mean intake by sex W Mean intake = 1183.8 ± 545.4 Median = 1097.6 IQR = 816.7–1494.8 M Mean intake = 1215.4 ± 639.8 Median = 1116.0 IQR = 829.5–1537.2 Mean intake by age 60–74 years Mean intake = 1197.8 ± 619.1 Median = 1092.4 IQR = 806.9–1502.9 >75 years Mean intake = 1200.7 ± 522.1 Median = 1143.9 IQR = 858.5–1508.6	Total flavonoids Mean intake = 444.7 ± 345.1 Phenolic acids Mean intake = 729.5 ± 545.4 Lignans Mean intake = 13.6 ± 25.5	Non-alcoholic beverages (coffee 45.8%), beans (32.8%), polenta (1.3%)	The intake of polyphenols was in a range similar to that reported for other populations, in particular European countries, but it differs for the main food contributors (high in beans and polenta, low in fruits and vegetables)

Legend: * Cao J, Zhao XJ, Wu K, Zhang Y, and Zhang YQ: Simultaneous determination of five flavonoid compounds in vegetables and fruits by high performance liquid chromatography. Chinese J Prev Med Inf 7, 525–527,2008. n.a. = not available; 24-h DR = 24 h dietary recall; M = men. W = women; FR = food record; FFQ = food frequency questionnaire. (1) USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation.

2.4. Polyphenol Intake and Cardiovascular Diseases/Diabetes Risk

In Table 2 the results of studies that examined the association between polyphenol intake and cardiovascular diseases risk are reported [50–73]. Seven out of 24 studies were conducted in United States (US), 2 in South America, 12 in Europe, 3 in Asia (Figure 4A). Most of the studies were carried out in the adult population—including older subjects (63%) while the remaining studies were performed in adult population (37%) i.e., aged < 65 years (Figure 4B).

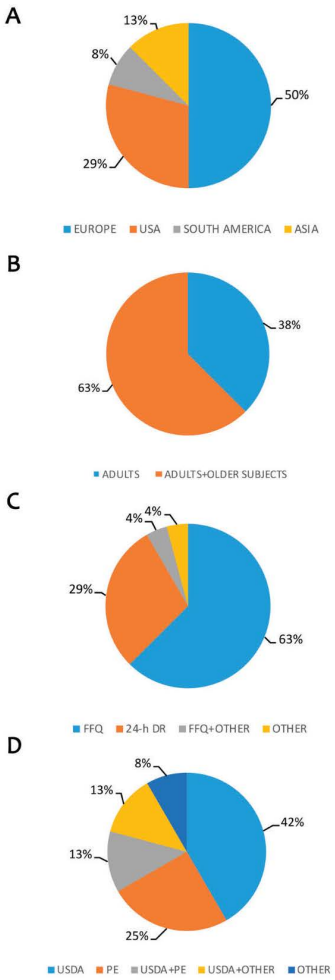

Figure 4. Estimation of polyphenols intake and risk for cardiovascular diseases and diabetes. Legend: (**A**) Distribution of published data by country; (**B**) Target population considered; (**C**) Questionnaires used to evaluate food intake; (**D**) Polyphenol database used for evaluation of intake. FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer.

Table 2. Polyphenol intake and CVD/Diabetes risk.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Huffman et al. [50]	Cohort study	507 subjects W = 263 M = 244 Age = 43–65 year	USA	FFQs	USDA database (1)	**Total flavonoids** Median intake: without diabetes = 280 (387 IQR) with diabetes = 222 (260 IQR)	↓ LDL associated with higher flavanones intake in the group with diabetes ↓ LDL associated with higher flavan-3-ols, and flavanones intake in the group without diabetes ↓ LDL associated with lower polyflavonoids intake in the group without diabetes ↑ HDL associated with higher anthocyanidins and flavan-3-ols intake in the group without diabetes ↓ HDL associated with lower polyflavonoids intake in the group without diabetes There was no relationship between HDL and flavonoids for the group with diabetes.
Pellegrini et al. [51]	Cross-sectional study	242 subjects W = 91 M = 151 Age = 60 year	Italy	3D-WR	Information provided by specific literature [a]	**Total lignans** Mean (95%CI) Q1 = 382 (332–433) Q2 = 586 (537–636) Q3 = 788 (739–837) Q4 = 1101 (1051–1152)	Total lignans intake are not associated with vascular inflammation and endothelial dysfunction
Cassidy et al. [52]	Cohort study (from NHS I, NHS II, and from HPFS)	156,957 subjects W = 133,914 M = 23,043 Age = 25–75 year	USA	FFQs	USDA database (1–3) EuroFIR	**Total flavonoids** NHS I Mean = 358 Q1 = 93 Q5 = 944 NHS II Mean = 413 Q1 = 103 Q5 = 1122 HPFS Mean = 376 Q1 = 115 Q5 = 933	↓ 6% hypertension incidence risk associated with higher total flavonoids' intake (Q5 vs. Q1; RR = 0.94; 95% CI: 0.90–0.99) in NHS I Total flavonoids' intake was not significantly associated with the risk of hypertension incidence in NHS II (RR = 1.01; 95% CI: 0.95–1.07) e HPFS (RR = 1.06; 95% CI: 0.97–1.16)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Wedick et al. [53]	Cohort study (from NHS I, NHS II, and from HPFS)	200,894 subjects W = 159,560 M = 41,334 Age = 25–75 year	USA	FFQs 118–131-item	USDA database (1)	**Total flavonoids** NHS I Q1 = 105.2 Q2 = 174.8 Q3 = 249.2 Q4 = 369.1 Q5 = 718.1 NHS II Q1 = 112.1 Q2 = 182.5 Q3 = 256.1 Q4 = 378.4 Q5 = 770.3 HPFS Q1 = 112.5 Q2 = 182.2 Q3 = 251.7 Q4 = 352.9 Q5 = 624.3	↓15% type 2 diabetes risk associated with higher total flavonoids' intake (Q5 vs. Q1; HR = 0.85; 95% CI: 0.79–0.92) in NHS I Total flavonoids' intake was not significantly associated with the risk of hypertension incidence in NHS II (HR = 0.99; 95% CI: 0.89–1.11) e HPFS (HR = 0.92; 95% CI: 0.81–1.04)
Zamora-Ros et al. [54]	Center stratified subcohort from Cohort study (EPIC-InterAct sub-cohort)	12,403 subjects W = 11,067 M = 5768 Age = 52.4 ± 9.1 year	8 European countries	24-h DR	Phenol Explorer USDA database (1–3)	**Flavanols** Mean = 334 ± 286 Median = 246 5th–95th percentile = 60.9–938 **Flavonols** Mean = 24.8 ± 16.0 Median = 20.4 5th–95th percentile = 7.8–57.4 Proanthocyanidins Mean = 183 ± 140 Median = 15 15th–95th percentile = 41.7–423	↓8% type 2 diabetes risk associated with higher consumption of myricetin (Q5 = >5.38 vs. Q1 = <0.37; cut off for each quintile) (P-trend = 0.001; HR = 0.92; 95% CI: 0.88, 0.96). ↓14% type 2 diabetes risk associated with higher consumption of proanthocyanidin dimers (Q5 = >49.5 vs. Q1 = <14.1; cut off for each quintile) (P-trend = <0.003; HR = 0.94; 95% CI: 0.90, 0.99). ↓7% type 2 diabetes risk associated with higher consumption of (-)-Epicatechin (Q5 = >28.75 vs. Q1 = <6.76; cut off for each quintile) (P-trend = <0.040; HR = 0.93; 95% CI: 0.89, 0.98). ↓6% type 2 diabetes risk associated with higher consumption of (+)-Catechin (Q5 = >20.08 vs. Q1 = <5.50; cut off for each quintile) (P-trend = <0.005; HR = 0.94; 95% CI: 0.91, 0.98). ↓2% type 2 diabetes risk associated with higher consumption of (+)-Gallocatechin (Q5 = >3.45 vs. Q1 = <0.04; cut off for each quintile) (P-trend = <0.027; HR = 0.98; 95% CI: 0.97, 0.99).

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Zamora-Ros et al. [55]	Cohort study (EPIC cohort)	15,258 subjects W = 9484 M = 5774 Age = 52.4 ± 9.1 year	Denmark, France, Germany, Greece, Italy, Netherlands, Norway, Spain, Sweden, and the United Kingdom	FFQs (98–266-item) Diet histories Food record	EPIC Nutrient Database based on: Phenol Explorer USDA database (1)	**Total flavonoids** Mean intake = 414.9 ± 311.7 median intake = 326.7 5th percentile = 93.2 95th percentile = 1050.4 Median intake Q1 = 126.8 Q2 = 223.7 Q3 = 326.7 Q4 = 478.4 Q5 = 817.5	↓10% type 2 diabetes risk associated with higher consumption of total flavonoids (HR 0.90 [95% CI 0.72–1.07; P value trend = 0.040) ↓18% type 2 diabetes risk associated with higher consumption of flavanols (HR 0.82 [95% CI 0.68–0.99; P value trend = 0.012) ↓27% type 2 diabetes risk associated with higher consumption of flavan-3-ol monomers (HR 0.73 [95% CI 0.57–0.93; P value trend = 0.029) ↓19% type 2 diabetes risk associated with higher consumption of flavonols (HR 0.81 [95% CI 0.69–0.95; P value trend = 0.020) Conversely lignans did not show any association (HR 0.88 [95% CI 0.72–1.07] P value trend = 0.119)
Jacques et al. [56]	Cohort study (Framingham Heart Study Offspring cohort)	2915 subjects W = 1341 M = 1574 Age = 54 y CL = 53.8–54.5 year	USA	FFQs	USDA database (1–3)	**Total flavonoids** Median = 210 Min = 2 Max = 1963 Median intake Q1 = 85 Q2 = 165 Q3 = 272 Q4 = 537	Total flavonoids' intake was not significantly associated with the risk of diabetes incidence (HR = 0.89; 95% CI: 0.75–1.05) ↓ risk of diabetes incidence associated with flavanols (HR = 0.68; 95% CI: 0.54–0.86) P-trend = 0.001
Tresserra-Rimbau et al. [57]	Cohort study (PREDIMED cohort)	7172 subjects W = 3923 M = 3249 Age = 67 ± 6 year	Spain	FFQs	Phenol Explorer	**Total polyphenols** Median intake Q1 = 562 Q2 = 701 Q3 = 800 Q4 = 917 Q5 = 1170	↓ 46% CV events risk associated with higher total polyphenol intake (Q5 vs. Q1; HR = 0.54; 95%CI: 0.33–0.91) ↓ CV events risk associated with several polyphenols' subclasses:Lignans (HR = 0.51; 95% CI: 0.30–0.86) Flavanols (HR = 0.40; 95% CI: 0.23–0.72) Hydroxybenzoic acids (HR = 0.47; 95% CI: 0.26–0.86)
Jennings et al. [58]	Cross-sectional-study	1997 subjects W = 1997 M = 0 Age = 18–76 year	UK	FFQs (131-item)	USDA database (1–3)	**Total flavonoids** Mean intake = 1170 ± 639 IQR = 617–1700	Total flavonoids were not significant associated with cardiovascular outcomesTotal flavonoids inversely associated with biomarkers of insulin resistance and inflammation: ↓ HOMA-IR, insulin, hs-CRP associated with anthocyanins intake (Q5 vs. Q1) ↓ HOMA-IR, insulin, adiponectin associated with flavones intake (Q5 vs. Q1)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Ponzo et al. [59]	Cohort study	1658 subjects W = 878 M = 780 Age = 45–64 year	Italy	FFQs	USDA Database [1-2-3] extended with information from a European database	**Total flavonoids** Median intake T1 = 89 T2 = 251.4 T3 = 532.3	↓ 54% non-fatal CV events risk associated with higher flavonoid intake (T3 vs. T1; HR = 0.46; 95% CI: 0.28–0.75) ↓ non-fatal CV events risk associated with several flavonoids' subclasses: Proanthocyanids (HR = 0.43; 95% CI: 0.27–0.70) Flavan-3-ols (HR = 0.42; 95% CI: 0.26–0.68) Anthocyanidins (HR = 0.56; 95% CI: 0.36–0.89) Flavanones (HR = 0.48; 95% CI: 0.29–0.77) Flavonols (HR = 0.53; 95% CI: 0.34–0.83) Total and subclasses of flavonoids were not significantly associated with the risk of CV mortality ↓ all-cause mortality associated with the T3 of several flavonoid subclasses: Flavan-3-ols (HR = 0.68; 95% CI 0.48–0.96) Anthocyanidins (HR = 0.66; 95% CI 0.46–0.95) Flavanones (HR = 0.59; 95% CI 0.40–0.85)
Jacques et al. [60]	Cohort study (Framingham Heart Study Offspring cohort)	2880 subjects W = 1302 M = 1578 Age = 54 year CL = 53.8–54.5	USA	FFQs	USDA database [1–3]	**Total flavonoids** **Exam 5 (1991–1995)** Median = 212 25th = 124 75th = 372 **Exam 8 (2005–2008)** Median = 259 25th = 157 75th = 436	Total flavonoids' intake was not significantly associated with the risk of incidence of CVD events (RR = 0.93; 95% CI: 0.82–1.06)
Yeon et al. [61]	Cohort study	4186 subjects W = 2575 M = 1611 Age = 40–59 year	Korea	24-h DR	USDA Database [1]	**Flavanones** W = 29.24 ± 4.17 M = 21.26 ± 4.37 **Flavones** W = 0.48 ± 0.04 M = 0.36 ± 0.02 **Flavonols** W = 17.06 ± 0.55 M = 15.72 ± 0.59	↓ insulin (β-coefficient = −0.0067; p for trend = 0.0092) and HOMA (β-coefficient = −0.0016; p for trend = 0.0239) associated with flavonols intake in men ↓ insulin (β-coefficient = −0.0008; p for trend = 0.0063) and HOMA (β-coefficient = −0.0002; p for trend = 0.0119) associated with flavanones intake in women
Oh et al. [62]	Cohort study	7963 subjects W = 7963 M = 0 Age = >30 years	Korea	24-h DR	Flavonoid Korean Database	**Total flavonoids** Mean Intake: Normal fasting glucose group = 107.40 ± 1.69 Type 2 diabetes mellitus group = 97.81 ± 8.11	↓prevalence of type 2 diabetes associated with intake of flavones above the 25th percentile (≥0.25 mg/day) compared with intake below the 25th percentile (OR = 0.593, 95% CI: 0.414–0.847)

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Goetz et al. [63]	cohort study	20,024 subjects W = 11,253 M = 8771 Age = >45 years	US	FFQs (107-item)	USDA database (1–3)	**Total flavonoids** Median intake (range) Q1 = 34.3 (<50.8) Q2 = 66.6 (50.9–83.4) Q3 = 102.9 (83.5–127.0) Q4 = 156.9 (127.1–208.3) Q5 = 296.8 (≥ 208.4)	↓risk of incident acute ischemic stroke (HR = 0.72; 95% CI: 0.55, 0.95; P-trend = 0.03) was associated with flavanone intake, but not total or other flavonoid subclasses. Associations did not differ by sex race, or region for any flavonoid measure.
Goetz et al. [64]	Cohort study	16,678 subjects W = 9798 M = 6880 Age = >45 years	US	FFQs (107-item)	USDA database (1–3)	**Total flavonoids** W: Mean intake = 234 Median intake = 131 M: Mean intake = 227 Median intake = 131	↓incident CHD associated with consumption of anthocyanidin and proanthocyanidin. Anthocyanidins Q1 vs. Q5; HR = 0.71; 95% CI: 0.52–0.98; P-trend = 0.04; proanthocyanidins Q1 vs. Q5; HR = 0.63; 95% CI: 0.47–0.84; P-trend = 0.02). There was no significant effect modification by age, sex, race, or region of residence
Miranda et al. [65]	Cohort study	550 subjects W = 346 M = 204 Age = 20–59 years Age older adults = >60 years	Brazil	2 24-h DR	Phenol Explorer	**Total polyphenols** Mean intake = 392.6 Median intake = 360.6	↓hypertension associated with highest tertiles of some classes of polyphenols: tyrosols (OR = 0.33; 95% CI 0.18–0.64), alkylphenols (OR = 0.45; 95% CI 0.23–0.87), lignans (OR = 0.49; 95% CI 0.25–0.98), as well as stilbenes (OR = 0.60; 95% CI 0.36–0.98), and other polyphenols (OR = 0.33; 95% CI 0.14–0.74). ↓hypertension associated with middle tertiles of total polyphenols and phenolic acids.There was no significant association for total flavonoids
Cassidy et al. [66]	Cohort study (HPFS cohort)	43,880 subjects M = 43,880 W = 0 Age = 32–81 years	UK	FFQs	USDA database (1)	**Anthocyanins** Q1 = 1.9 Q2 = 4.5 Q3 = 7.8 Q4 = 13.7 Q5 = 26.3 intake range = 0–613 IQR = 3.9–15.7 **Flavanones** Q1 = 7.5 Q2 = 23.6 Q3 = 43.5 Q4 = 64.5 Q5 = 103.9 intake range = 0–728 IQR = 18.8–70.9	↓total or fatal MI risk associated with higher anthocyanin intake (HR = 0.87; 95% CI: 0.75–1.00; P = 0.04; P-trend = 0.098); this association was stronger in normotensive participants (HR = 0.81; 95% CI: 0.69–0.96; P-interaction = 0.03). Anthocyanin intake was not associated with stroke risk. ↓ischemic stroke associated with higher flavanone intake (HR = 0.78; 95% CI: 0.62–0.97; P = 0.03, P-trend = 0.059); with the greatest magnitude in participants aged > 65 years (P-interaction = 0.04). Flavanone intake was not associated with MI or total stroke risk

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Kim et al. [67]	Cohort study	4042 subjects W = 1970 M = 2072 Age = >19 years	US	2 24-h DR	USDA Database (1-2-3)	**Total flavonoids** Mean intake Q1 = 12.5 Q2 = 59 Q3 = 197.6 Q4 = 585.5	Changes in percentages of cardiovascular risk factors with a 100% increase in flavonoid intake: ↑ 0.54% HDL-cholesterol associated with higher total flavonoid intake ↓ 1.25% TAG and ↓ 1.60% TAG:HDL-cholesterol ratio associated with anthocyanidin intake ↓ 1.31% TAG and ↓ 1.83% TAG:HDL-cholesterol ratio associated with total flavonoid intake ↓ 3.18% insulin and ↓ 3.10% HOMA-IR were associated with flavone intake ↓ 3.11% insulin and ↓ 4.01% HOMA-IR were associated with isoflavone intake ↓ 0.60% BMI associated with anthocyanidin intake
Rizzi et al. [68]	Cohort study	443 subjects W = 175 M = 268 Age = 20–85 years	Italy	24-h DR	USDA Database (1) Phenol Explorer EIO Database	**Total polyphenols** range intake T1 = 99.4–804.5 T3 = 1288.0–4342.2	High polyphenols intake was not associated with significant differences in the lipid profile compared with low polyphenols intake
Grosso et al. [69]	Cohort study (HAPIEE study)	5806 subjects W = 3075 M = 2731 Age = 45–69 years	Poland	FFQs (148-item)	Phenol Explorer	**Total polyphenols** Mean intake Q1 = 1026.7 ± 212 Q2 = 1469.6 ± 102.2 Q3 = 1872.6 ± 136.7 Q4 = 2632.1 ± 608	↓ 32% of risk of type 2 diabetes in the whole population associated with highest intake of total polyphenol (Q4 vs. Q1)
Witkowska et al. [70]	Cohort study	2599 subjects W = 2599 M = 0 Age = 20–74 years	Poland	24-h DR (367-item)	Phenol Explorer	**Total polyphenols** Mean intake Q1 = 948.2 ± 236 Q2 = 1523.2 ± 142 Q3 = 2016.3 ± 154 Q4 = 2975.8 ± 724	↓ 1.1% odds ratio of CVD in postmenopausal women with higher dietary polyphenol intake (per 100 mg/day)
Grosso et al. [71]	Cohort study (HAPIEE study)	8821 subjects W = 4530 M = 4291 Age = 50–65 years	Poland	FFQs (148-item)	Phenol Explorer	**Total polyphenols** n.a.	↓ metabolic syndrome associated with the highest quartile of polyphenol intake (OR = 0.80; 95% CI: 0.64–0.98 and OR = 0.70; 95% CI: 0.56–0.86 for both men and women, respectively). ↓ blood pressure, waist circumference, high lipoprotein cholesterol, and triglycerides associated with high total polyphenol intake in women. ↓ fasting plasma glucose associated with high total polyphenol intake in both genders.

Table 2. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° food-containing items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± sd/quantile/min-max/IQR	Overall Results/Association with Outcome
Sohrab et al. [72]	Cohort study	1265 Subjects W = 711 M = 554 Age = 19–74 years	Iran	FFQs	Phenol Explorer	**Total polyphenols** Median Intake (range) T1 = 827 (≤1128) T2 = 1425 (1129–1819) T3 = 2459 (≥1820) **Total flavonoids** Median intake (range) T1 = 38.4 (≤52.8) T2 = 69.5 (52.9–88.4) T3 = 115.1 (≥88.5)	Total polyphenols were not significant associated with metabolic syndrome ↓ 31% metabolic syndrome risk (OR = 0.69; 95% CI: 0.48–0.98, P-trend: 0.04) associated with total flavonoid intake (T3 vs. T1)
Mendonça et al. [73]	Cohort study (SUN cohort)	17,065 Subjects W = 10,358 M = 6707 Age = 20–89 years	Spain	FFQs 136-item	Phenol Explorer USDA database	**Total polyphenols** Mean Intake Q1 = 396 (±134) Q2 = 526 (±149) Q3 = 653 (±149) Q4 = 812 (±156) Q5 = 1248 (±405) **Total flavonoids** Mean Intake Q1 = 186 (±72) Q2 = 234 (±86) Q3 = 302 (±97) Q4 = 424 (±105) Q5 = 772 (±330)	Total polyphenols were not significant associated with cardiovascular events (HR = 0.61; 95% CI: 0.33–1.13 P for trend 0.28) Total flavonoids were not significant associated with cardiovascular events (HR = 0.53; 95% CI: 0.29–0.98 P for trend 0.09)

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire. (1) = USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. (2) = USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. (3) = USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation. a = Milder et al. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matair- esinol. Br J Nutr 2005.; Valsta et al. Phyto-estrogen database of foods and average intake in Finland. Br J Nutr 2003.; Mazur et al.Adlercreutz H. Lignan and isoflavonoid concentrations in tea and coffee. Br J Nutr 1998.; Mazur et al. Natural and anthropogenic environ- mental oestrogens: the scientific basis for the risk assessment. Naturally occurring oestrogen in food. Pure Appl Chem 1998.

Food intake was mainly assessed through FFQs (63%) or with 24-h DR (29%); 1 study adopted the FFQ in combination with other tools, while 1 study used other assessment methods (Figure 4C).

The main databases used were USDA (42%) and PE (25%). Three studied combined USDA and PE, while the rest of the studies evaluated polyphenol intake with different databases alone or in combination such as Epic Nutrient database, EuroFIR, U.K. Food Standard Agency, Flavonoid Korean Database (Figure 4D).

The association between polyphenol intake and cardiovascular disease risk and diabetes was evaluated by considering several outcomes such as: HDL-cholesterol, triacylglycerols (TAGs), TAG: HDL-cholesterol ratio, HOMA-IR (Homeostatic Model Assessment of Insuline Resistence), Body Mass Index (BMI), cardiovascular events (CV events), stroke events, hypertension and type 2 diabetes (T2D).

On the whole, 12 studies reported an inverse association between polyphenol intake and CV events. In some studies a significant decreased CV risk was observed at the highest quartile of total polyphenol intake (1170 mg/day for Spain and 2632 mg/day for Poland) [57,69] while no effect was demonstrated in other studies performed in Spain and Iran (1248 mg/day and 2459 mg/day respectively) [72,73]. Ten studies evaluated the association with polyphenol subclasses, mainly total flavonoids but only 3 found a significant inverse association with CV events [52,67,72] with intake ranging from 115 to 944 mg/day.

As regard T2D, 1 study performed in Poland showed an increased protection for total polyphenol intake higher than 2632 mg/day while mixed results were found in the other studies focused on total flavonoids and/or subclasses only in some cases able to demonstrate significant T2D risk reduction [53,55,61,62]. Finally, 1 study [67] reported an inverse association for both CV and T2D with the highest quartile of total flavonoids (585 mg/day).

2.5. *Polyphenols Intake and all-Cause/Cardiovascular Mortality*

In Table 3 the association between polyphenol intake and all-cause mortality is reported with a specific focus on cardiovascular mortality. A total of 10 studies [59,74–82] (Figure 5A) were found; most of the them (50%; 5 out of 10) were performed in Europe (Spain, Italy and The Netherland), 2 in USA, 2 in Australia and 1 was performed including USA, Canada and Australia. Five out of 10 trials (50%) involved older subjects (> 65 years), 3 studies were performed in adults while 2 trails included both adult and older subjects (Figure 5B). The food intake was assessed mainly by FFQ (60%; 6 out of 10 studies); however, some studies (30%) associated FFQs with other tools for the evaluation of food intake (i.e., computerized dietary history questionnaire). One study combined FFQ with EPIC questionnaire (Figure 5C). The evaluation of polyphenol intake was estimated by USDA database (30%; 3 out of 10 studies), or a combination of USDA with others database (40%), or USDA with PE (20%; 2 out of 10 studies). When polyphenol content of specific food-products was missing in available databases, data were obtained from the literature. One study estimated polyphenol intake, in particular monomeric flavan-3-ol, by considering their content in 120 commonly consumed plant foods and beverages obtained by combining results from reverse-phase HPLC and data from literature (Figure 5D).

Overall, one study that investigated the association with total polyphenol intake and all-cause mortality failed to demonstrate a significant effect [75]. Similar findings were also reported by considering the association between total flavonoids and CV mortality [59]. On the contrary, a reduction of mortality risk for cardiovascular events and all–cause mortality was associated with total flavonoid intake in the highest quintiles ranging from 360 mg/day [78] to about 800 mg/day [80]. The impact of the single subclasses has been evaluated in some of the studies, but the effects were conflicting depending on the subject's characteristics (i.e., age, sex) and cause of mortality. Generally, the models adjusted for the age, as confounding factor, reported a protection also for specific flavonoid subclasses such as isoflavones, flavan-3-ols, flavones. The effects in some cases were found both in women and men. However, generally adjustments for the different confounding factors (i.e., BMI, smoking and alcohol habits, energy intake, physical activity, medications, etc. affected the significance of the associations.

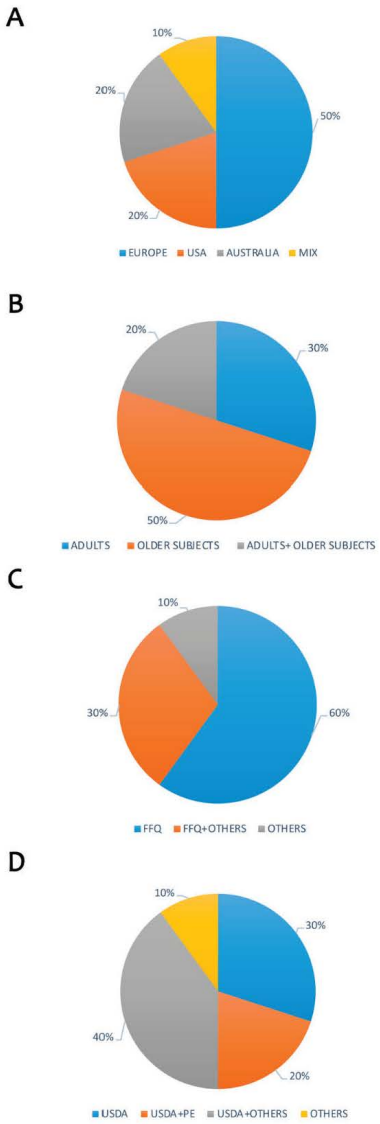

Figure 5. Estimation of polyphenols intake, all-cause and cardiovascular mortality risk. Legend: (**A**) Distribution of published data by country; (**B**) Target population considered; (**C**) Questionnaires used to evaluate food intake; (**D**) Polyphenol database used for evaluation of intake. FFQ: Food Frequency Questionnaire; USDA: United States Department of Agriculture; PE: Phenol-Explorer.

Table 3. Association between polyphenol intake and all-cause/cardiovascular mortality.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
McCullough et al. [74]	Cohort study (American Cancer Society's CPS-II Nutrition Cohort Study)	98,469 subjects W = 60,289 M = 38,180 Mean Age W = 70 years Mean Age M = 69 years	USA	FFQs (152 food items)	USDA database [1-2-3] other research publications	**Total flavonoids** Mean intake (energy-adjusted) **Men** Mean intake = 268 Median intake = 203 10th–90th percentile = 99–498 **Women** Mean intake = 268 Median intake = 201 10th–90th percentile = 92–522	**Cardiovascular mortality** **Age-adjusted model:** Inverse association were observed for high total flavonoid, anthocyanidins (median 22.2 (≥16.7) mg/day), flavan-3-ols (median 63.7 (≥37.2) mg/day), flavones (median 3.0 (≥2.1) mg/day), flavonols (median 27.2 (≥20.6) mg/day), proanthocyanidins (median 379.4 (≥253.6) mg/day) and isoflavones (median 0.713 (≥0.142) mg/day) in both the sex. Inverse association for flavanones (median 49.9 (≥35.4) mg/day) in women. **Multivariable-adjusted model** [3]: No association in men. Inverse association for high total flavonoid, anthocyanidin, flavan-3-ol intake in women. Subjects with high total flavonoid consumption (median 512.5 (≥359.7) mg/day) showed a low risk of death (−18%) in both the sex.Inverse association for high anthocyanidin, flavan-3-ol, flavones, flavanol and proanthocyanidin intake by considering women + men. **Ischemic heart disease mortality** **Age-adjusted model**: Inverse association for high anthocyanidin and flavone intake in both the sex. Inverse association for high total flavonoid intake in men and women + men; high flavanone intake in women + men; high flavanol intake in women and women + men; high proanthocyanidin intake in women + men; high isoflavone intake in men and women + men. **Multivariable-adjusted model** [3]: Inverse association for high flavone intake in women and women + men **Stroke mortality** **Age-adjusted model**: Inverse association for high total flavonoid intake in men, and high flavones intake in men and women + men. **Multivariable-adjusted model** [3]: Inverse association for high total flavonoid intake in men

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Zamora-Ros et al. [75]	Cohort study(Invecchiare in Chianti study)	807 subjects W = 447 M = 360 Age = 74.3 ± 6.9 years Survived W = 313 M = 240 Age = 71.8 ± 5.3 years Died W = 134 M = 140 Age = 79.2 ± 7.2 years	Italy	FFQs (Italian version) Urinary polyphenol assessment	Phenol Explorer USDA database	**Total polyphenols** Mean intake = 594 ± 196 **Survived** Mean intake = 600 ± 201 Died Mean intake = 584 ± 185	No association between total dietary polyphenols and all-cause mortality
Zamora-Ros et al. [76]	Cohort study (EPIC Spain Study)	40,622 subjects W = 25,298 M = 15,324 Age = 29–69 years	Spain	FFQs computerized diet history questionnaire developed and validated specifically for the EPIC study in Spain	USDA database Phenol-explorer UK Food Standards Agency (10% missing values in 1877 food items)	**Total flavonoids** Mean intake = 387.3 ± 280.2 Median intake = 329.8 25th percentile = 218.4 75th percentile = 489.6 **Total lignans** Mean intake = 1.0 ± 0.5 Median intake = 0.9 25th percentile = 0.7 75th percentile = 1.2	**Multivariable-adjusted model** [2]: subjects with high flavanones (>51.3 mg/day), flavanols (>28.0 mg/day) and total flavonoids intake (>447.8 mg/day) showed a low risk of all-cause mortality (flavanones: 0.60 (95% CI = 0.38–0.94) and flavonols: 0.59 (95% CI = 0.40–0.88). This reduction was due entirely to a decrease in mortality from CVD. Proanthocyanidins were the most important contributor (66%) to total flavonoid intake, followed by flavanones (11%), flavan-3-ol monomers (9%), anthocyanidins (7%), and flavonols (6%), flavones (1%), isoflavones (0.1%), and theaflavins (<0.1%). No evidence of an association between dietary flavonoid or lignan intake and mortality from cancer or other causes.
Ivey et al. [77]	Cohort study	1063 Subjects W = 1063 Age > 75 years	Australia	FFQs developed by the AntiCancer Council of Victoria	USDA	**Total flavonoids** **Flavonol**: 31 ± 14 **Flavan-3-ol**: 431 ± 279 **Proanthocyanidin**: 215 ± 147 Flavone: 3 ± 2 **Flavanone**: 53 ± 38 **Anthocyanidin**: 37 ± 26 **Isoflavone**: 5 ± 6	**Unadjusted model**: Subjects with high intake of total flflavonol (>35 mg/day), flflavan3-ol (>563 mg/day), flavone (>3 mg/day) and flavanone (>61 mg/day) showed a reduced risk of atherosclerotic vascular disease mortality. **Age- and energy-adjusted model and multivariate-adjusted model** [4]: Subjects with high intake of total flflavonol (>35 mg/day), flflavan3-ol (>563 mg/day) showed a reduced risk of atherosclerotic vascular disease mortality No association was observed for the other flavonoid subclasses Tea contributed 59% of total flavonoid intake; the major contributors were flavonols (65%) and flavan-3-ols (93%). **Multivariate-adjusted model** [4]: Subjects with high intake of flavonols derived from tea and non-tea sources (≥12 mg/day and ≥27 mg/d, respectively) showed a low risk of atherosclerotic vascular disease mortality.

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Ivey et al. [78]	Cohort study	1063 subjects W = 1063 M = 0 Age > 75 years	Australia	FFQs (93 items) Beverage questionnaire (to assess tea and coffee consumption)	Phenol Explorer (47 foods recorded as not containing flavonoids or not present in the database) USDA database [1-2-3] (19 foods recorded as not containing flavonoids or not present in the database)	**Total flavonoids (PE)** Mean intake = 674 ± 326 Median intake = 648 IQR = 449–872 **Total flavonoids (USDA)** Mean intake = 696 ± 322 Median intake = 668 IQR = 468–889	Subjects with high total flavonoid intake (≥813 mg/day USDA; ≥788 mg/day PE) showed a low risk of all-cause mortality and cardiovascular mortality
Ponzo et al. [59]	Cohort study	1658 subjects W = 878 M = 780 Age = 45–64 year	Italy	FFQs	USDA Database [1-2-3] extended with information from an European database	**Total flavonoids** Median intake T1 = 89 T2 = 251.4 T3 = 532.3	Total and subclasses of flavonoids were not significantlyassociated with the risk of CV mortality The third tertile of flavan-3-ols (HR = 0.68; 95% CI 0.48–0.96), anthocyanidins (HR = 0.66; 95% CI 0.46–0.95) and flavanones (HR = 0.59; 95% CI 0.40–0.85) was inversely associated with all-cause mortality
Dower et al. [79]	Cohort study (Zutphen Elderly Study)	774 subjects W = 0 M = 774 Age = 65–84 years	The Netherlands	Cross-check dietary history (adapted for the Dutch setting) [5]	Monomeric flavan-3-ol contents of 120 commonly consumed plant foods and beverages were determined with the use of reverse-phase HPLC with ultraviolet and fluorescence detection. (−)-epicatechin, (+)-catechin, (−)-epigallocatechin, (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg), and (+)-gallocatechin concentrations were determined as reported in previous published papers [6]	**Total epicatechins** Mean intake = 15.2 ± 7.7 Range intake = 0.01–60.6	**Coronary heart disease mortality** Subjects with high epicatechin intake (>18 mg/day) showed a low (−38%) risk of CHD mortality **Cardiovascular disease mortality** Subjects with high epicatechin intake (>18 mg/day) showed a low (−46%) risk of CVD mortality in men with prevalent CVD but not in men who were free of CVD The major dietary sources of epicatechin intake were tea (7.8 mg/day; 51% of total epicatechin intake), apples (4.3 mg/day; 28% of total epicatechin intake), cocoa (1.1 mg/day; 7% of total epicatechin intake), and other sources (2.0 mg/day; 13% of total epicatechin intake)

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Ivey et al. [80]	Cohort study (Nurses' Health Study II)	93,145 subjects W = 93.145 M = 0 Age = 25–42 years	USA	FFQs	USDA database	**Total flavonoids** Mean intake = 379 ± 374	**Age-adjusted model**: subjects with high total flavonoids intake (≥518 mg/day) showed a 19% reduction of overall mortality in the 18-year follow-up period. Subjects with high flavan-3-ols (≥86 mg/day), proanthocyanidins (≥356 mg/day) and anthocyanin intake (≥17 mg/day) showed a low risk of mortality for CVD and other causes. **Multivariable adjusted model** [1]: no association High consumption (more than once per week) of red wine, tea, peppers, blueberries and strawberries was associated with reduced risk of total and cause-specific mortality.
Zhang et al. [81]	Cohort study	6235 subjects with breast cancer W = 6235 M = 0 Age = 51.8 ± 10.6 years	USA, Canada, Australia	FFQs	USDA database	**Total isoflavones** Mean intake: 1.8 ± 3.9 Median intake: 0.7 IQR intake: 1.2	Quartile 4 (≥1494 mg/day) associated with a 21% decrease in all-cause mortality. This result was limited to women with negative tumor hormone receptors and those not treated with hormonal therapy for breast cancer

Table 3. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean± sd/quantile/ min-max/IQR	Overall Results/Association with Outcome
Pounis et al. [82]	Cohort study (Moli-sani cohort Study)	21,302 subjects W = 10,980 M = 10,322 Age = 54–56 years	Italy	FFQs	Eurofir-eBASIS FCTs USDA database [(1-2-3)]	Data reported as polyphenol antioxidant content (PAC)-score [6] (−28 to 28)	**Risk for all-cause mortality** Women: low risk for high intake of flavones (>1.12 mg/day), flavanones (>46.5 mg/day), isoflavones (>32.7 mg/day), and lignans (>116.1 mg/day) had a low risk. **After adjustments for potential confounders (model 4)** [7]: the effects remained significant for Q4 (4–13) and Q5 (>13) of PAC-score Men: low risk for some quintile of intake; flavonols (Q2: 11.2–15.1 mg/day and Q5: >25.8 mg/day), flavones (Q3: 0.61–0.81 mg/day), flavanones (Q2: 22–29 mg/day), isoflavones (Q2: 16.5–21 mg/day, Q3: 21–25.7 mg/day and Q5: >32.7 mg/day), lignans (Q3: 72.8–90.3 mg/day). **After adjustments for potential confounders (model 4)** [8]: the effects remained significant for Q2 (−13 to −4), Q3 (−4 to 4) and Q4 (4–13) of PAC-score **Vascular causes** Women: no association Men: low risk of mortality for Q2 (−13 to −4) and Q3 (−4 to 4) **Other causes** Women and men at Q2 (−13 to −4) and Q4 (4–13) of PAC-score showed a low mortality risk from other causes

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire. [1] BMI, smoking status, menopausal status, family history of diabetes/cancer/myocardial infarction, multivitamin supplement use, aspirin use, race, type 2 diabetes, hypercholesterolaemia, hypertension, physical activity, alcohol consumption and energy intake and the Alternative Health Eating Index (minus alcohol) score. [2] Cox proportional hazards regression models were stratified by center, age (1 year) and sex and adjusted for BMI, education level, physical activity, tobacco smoking, alcohol lifetime, total energy, vitamin C and fiber intake. [3] Age, smoking, beer and liquor intake, history of hypertension, history of cholesterol, family history of myocardial infarction, BMI, physical activity, energy intake, aspirin use, hormone replacement therapy (in women only), and sex (in combined model only) by using Cox proportional hazards regression. [4] age, previous CVD, previous diabetes, energy expended in physical activity and history of smoking. [5] Keys A et al., Acta Med Scand Suppl 1966;460:1–392. [6] Arts ICW et al., J Agric Food Chem 2000;48:1752–7; Arts ICW et al., J Agric Food Chem 2000;48:1746–51. [7] Pounis et al., European Journal of Clinical Nutrition 2016; 70;338–345. [8] Age, energy intake, smoking habits, social status, physical activity level and INFLA-score. Eurofir-eBASIS: European Food Information Resource—Bioactive Substances in Food Information Systems; FCTs: Italian Food Composition Tables; [(1)] USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. [(2)] USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. [(3)] USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation.

2.6. Polyphenols Intake and other Outcomes

Table 4 shows the associations between polyphenol intake and other outcomes in a total of 13 studies [83–95]. The associations were evaluated for endothelial function (1 study), kidney function (1 study), bone health (i.e., bone mineral density, frailty and fractures; 3 studies), eyes health (i.e., cataract and macular degeneration; 2 studies), physical performance decline (1 study), dementia (1 study), cognitive decline (1 study) and pubertal development (1 study).

Six out of 13 studies (46%) were performed in Europe, 3 in Australia, 2 in the USA and in Asia Figure 6A). Over than a half of the studies (58%) were carried out in the older population while 33% included adult and older subjects. 1 study was performed only in adults and 1 in adolescents (Figure 6B). The most frequent tools used for the evaluation of the diet were the FFQs (77%; 10 studies), 1 study used a 24-h DR while 2 studies combined FFQs with other tools (Figure 6C). Half of the studies (50%) used USDA database, or a combination of USDA with PE (3 studies) or USDA with other databases (2 studies). Only one study performed the estimation using PE, while one study used a different specific database for the calculation of polyphenol intake (Figure 6D). An overall association between high intake of polyphenols and subclasses, and different outcomes was observed. Conversely, in the InCHIANTI study urinary total polyphenols, but not total dietary polyphenols, were associated with a lower probability of frailty or pre-frailty [86] and cognitive decline [95]. Flavonoids have been associated with a higher endothelial function (>640 mg/day) [83], a lower risk of reduced forced vital capacity and spirometric restriction of the lung (≈290 mg/day) [90], a higher bone mineral density (≈490 mg/day). In addition, flavonoids have been inversely associated with bone fractures (≈1500 mg/day) [85,87] and macular degeneration (≈875 mg/day) [91]. Proanthocyanidins (≥229 mg/day) were inversely associated with risk of renal failure events and kidney insufficiency, while isoflavones (>3 mg/day) with a better pubertal development [84,94].

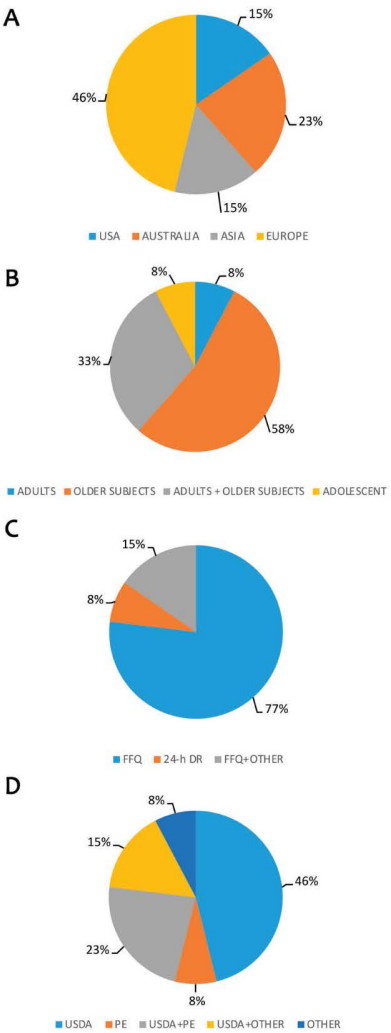

Figure 6. Estimation of polyphenols intake and other outcomes. Legend: (**A**) Distribution of published data by country; (**B**) Target population considered; (**C**) Questionnaires used to evaluate food intake; (**D**) Polyphenol database used for evaluation of intake. Legend: FFQ: Food Frequency Questionnaire; 24-h DR: 24-h Dietary Recall; USDA: United States Department of Agriculture; PE: Phenol-Explorer.

Table 4. Polyphenol intake and other outcomes.

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Fisher et al. [83]	Analytical	19 subjects W = 11 M = 8 Age = 72 ± 7 years	US	FFQs	USDA database [1–3] (22 food item)	Total flavonoids Median intake = 2428 mg/week Median = 347 Q1–Q4 = 1242–4789 mg/week	Habitual dietary intake of flavonoids was associated with higher endothelial function evaluated as reactive hyperemia (RH)-PAT response. Subjects with habitual flavonoid intake (>4500 mg/week) had significantly higher (RH)-PAT response
Ivey et al. [84]	Prospective	948 subjects W = 948 M = 0 Age = ≥75 years	Australia	FFQs	USDA database [1-2-3]	Total Proanthocyanidins Mean intake = 215 ± 147 Min-max = 18–1728	Over 50% of total proanthocyanidin intake were from fruit (89 ± 63 mg/day), chocolate (43 ± 75 mg/day), and alcoholic beverages (32 ± 86 mg/day). Subjects with habitual proanthocyanidin intake (≥229 mg/day) had lower risk of moderate chronic kidney insufficiency and renal failure events
Zhang et al. [85]	Cross-sectional	3317 subjects W = 2239 M = 1078 Age = 60.2 years	China	FFQs (79-item)	USDA database [1–3] Hong Kong database of isoflavones [1]	Total flavonoids Median intake **W**(Q1) = 53.3 IQR = 40.5–66.3 W(Q2) = 110.0 IQR = 92.2–132.1 W(Q3) = 232.4 IQR = 194.8–274.4 **W**(Q4) = 486.9 IQR = 402.2–584.4 **M**(Q1) = 63.1 IQR = 44.9–94.6 M(Q2) = 207.9 IQR = 174.4–237.2 M(Q3) = 351.8 IQR = 297.6–392.2 **M**(Q4) = 555.3 IQR = 479.6–618.2	High total flavonoid intake (Q4 vs. Q1) was associated with higher bone mineral density (BMD) in women, but not in men. A dose dependent positive relationship was found for all BMD measured sites. In addition, a significant association was found also for flavonoid subclasses (flavonols, flavan-3-ols, flavones, and proanthocyanidins)
Urpi- Sarda et al. [86]	Cross-sectional (Invecchiare CHIANTI Study)	811 subjects W = 446 M = 364 Age = >65 years	Italy	FFQs	Phenol explorer USDA database	Total polyphenols Mean intakeAll (N = 811) = 595.2 ± 195.6 Non-frail (n = 418) = 608.5 ± 199.8 Prefrail (n = 321) = 587.3 ± 195.9 Frail (n = 72) = 550.5 ± 158.7 1T < 509.2 2T = 509.2–645.2 3T < 645.2	No association between total dietary polyphenols and frailty and pre-frailty in older subjects

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Rabassa et al. [95]	Cross-sectional (Invecchiare CHIANTI Study)	652 subjects W = 361 M = 291 Mean Age = 73	Italy	FFQs	Phenol explorer USDA database	**Total polyphenols** Median intake All (n = 652) = 574 IQR = 472–701 1T = 430 IQR = 354–470 2T = 574 IQR = 543–610 3T = 766 IQR = 701–855	No association between total dietary polyphenols and any cognitive test in older subjects
Myers et al. [87]	Prospective	1188 subjects W = 1188 Age = >70 years	Australia	FFQs Beverage questionnaire	USDA database [1-2-3]	**Total flavonoids** Median intake **Tea Low consumer** = 266 IQR = 191–361 **Tea Moderate consumer** = 845 IQR = 672–959 **Tea High consumer** = 1570 IQR = 1325–1915	Higher intake of black tea and flavonoids was associated with lower hospitalization (30–40% reduction) for fractures in older women at high risk
Ma et al. [88]	Case-control	249 subjects (cases) 66 subjects (controls) W = 182 M = 133 Age = 50–70 years	China	FFQs 3 24-h DR	USDA database	**Total flavonoids** **Cases** Median intake = 51.13 IQR = 38.06–64.21 **Controls** Median intake = 64.92 IQR = 53.66–75.61	Total dietary anthocyanidin, flavan-3-ol, flavanone, flavone, and flavonol intake was not associated with age related cataract risk. Only quercetin and isorhamnetin intake appeared to be associated with the risk in this population
Rabassa et al. [89]	Cross-sectional (Invecchiare CHIANTI Study)	368 subjects W = 199 M = 169 Age = >65 years	Italy	FFQs	USDA database [1–3] Phenol explorer 236 food items	**Total polyphenols** **Baseline** Median intake = 556 IQR = 462–682 **3-year follow-up** Median = 539 IQR = 429–656 **6-years** Median = 513 IQR = 415–619 **9-years** Median = 500 IQR = 407–595	Total dietary polyphenol (TDP) intake was higher in older subjects and women with higher physical activity level. No association between TDP and physical performance decline was found

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Pounis et al. [92]	Cross-sectional (Moli-sani study)	9659 subjects W = 4551 M = 5108 Age = ≥35 years	Italy	FFQs (164-item)	Eurofir–e BASIS USDA database [1-2-3]	Flavanols Median intake (Q1–Q3) W = 41.6 (24.4–73.0) M = 66.1 (36.3–108.8) Anthocyanidins Median intake (Q1–Q3) W = 145.3 (99.8–209.3) M = 148.0 (101.9–216.3) Lignans Median intake (Q1–Q3) W = 82.7 (61.1–109.8) M = 81.2 (61.1–107.2)	Higher polyphenol intake was associated with better pulmonary function (forced vital capacity, and forced expiratory volume in the first second) in the population under study. A potential anti-inflammatory activity of polyphenols was hypothesized in men where a reduction in C-reactive protein and white blood cells was observed
Lefevre-Arbogast et al. [93]	Cohort study (The 3C Bordeaux cohort)	1329 subjects W = 824 M = 505 Mean Age = 75.8 years	France	24-h DR	Phenol Explorer	Total polyphenols Mean intake All subjects = 1071 ± 570 Incident dementia = 1029 ± 542 (n = 256) No dementia = 1081 ± 576 (n = 1073)	Polyphenol intake was associated with a decreased risk of all-cause dementia and of Alzheimer disease (AD) over 12 years. Subjects in the higher quintile of intake had a ≈ 50% lower risk of both dementia and AD. The pattern of polyphenol intake associated with the reduced risk was characterized by flavonoids (e.g., dihydroflavonols, anthocyanins, isoflavonoids, and flavanones), stilbenes (including resveratrol), lignans, and additional isolated polyphenols (hydroxybenzaldehydes, naphthoquinones, and furanocoumarins)
Segovia-Siapco et al. [94]	Cross-sectional (The Teen Food and Development Study)	248 subjects W = 0 M = 248 Age = 12–18 years	USA	Web-FFQs (151-item)	Nutrition Data Systems for Research (NDS-R) Specific database [2]	Total Isoflavones Mean intake = 22.1 Min and max = 18.3–26.0	Moderate (3–20 mg/day) and high (>20 mg/day) consumers of soy isoflavones nearly follow the same pattern for pubertal development. Whether soy isoflavones play a role in the rate of maturation and sequencing of pubertal development in boys cannot be determined based on our study findings

Legend: n.a. = not available; 24-h DR = 24 h dietary recall; M = men; W = women; FR = food record; FFQ = food frequency questionnaire; sFFQs = semi-quantitative FFQ. [(1)] USDA database (Flavonoids) USDA Database for the Flavonoid Content of Selected Foods, Release 2.1. Internet. 2007 Ref Type: Electronic Citation. [(2)] USDA database (isoflavones) U. S. Department of Agriculture. Beltsville: MD: USDA; 2008. Database for the Isoflavone Content of Selected foods. Ref Type: Electronic Citation. [(3)] USDA database (proanthocyanidins) USDA Database for the Proanthocyanidin Content of Selected Foods. Internet. 2004 Ref Type: Electronic Citation. [1] Chan SG, Murphy PA, Ho SC, Kreiger N, Darlington G, So EK, Chong PY (2009) Isoflavonoid content of Hong Kong soy foods. J Agric Food Chem 57:5386–5390. [2] Jaceldo-Siegl K, Fraser GE, Chan J, Franke A, Sabaté J (2008).

Table 4. *Cont.*

References	Type of Study	Population Characteristics	Country	Dietary Assessment - n° Food-Containing Items	Polyphenol Database Source n° Food Items	Estimated Polyphenol Intake (mg/day) mean ± SD/quantile/min-max/IQR	Overall Results/Association with Outcome
Garcia-Larsen et al. [90]	Cross-sectional (GA^{2}LEN study)	2599 subjects W = 1516 M = 1083 Age = 47.2 ± 14.5 years	Denmark Finland Sweden UK Portugal Belgium Germany Netherlands Poland	FFQs (250-item)	USDA database [1–3]	**Total flavonoids** Median intake = 291.2 IQR = 126.8–569.4	Total flavonoid intake and pro-anthocyanidins was positively associated with a good ventilatory function (forced vital capacity), while a negative association with spirometric restriction was found in the cohort. In particular, subjects with total flavonoid intake at the highest quintile had a 42% lower risk of reduced forced vital capacity
Gopinath et al. [91]	Cohort study (Blue Mountains Eye Study)	2856 subjects W = 1597 M = 1259 Age = ≥ 49 years	Australia	FFQs (145-item)	USDA database [1-2-3]	**Total flavonoids** Median intake = 875 Q1 ≤ 410.6 Q2 = 412.4–881.5 Q3 = 881.6–1232.3 Q4 ≥ 1232.4	Total flavonoids and subclasses (*e.g.*, flavonols and flavanones), were associated with age-related macular degeneration (AMD) among older adults. The consumption of oranges and orange juice, contributing to total flavanone intake, was found to significantly affect AMD risk
Pounis et al. [92]	Cross-sectional (Moli-sani study)	9659 subjects W = 4551 M = 5108 Age = ≥35 years	Italy	FFQs (164-item)	Eurofir–e BASIS USDA database [1-2-3]	**Flavonols** Median intake (Q1–Q3) W = 15.4 (11.1–21.2) M = 19.1 (14.1–26.0) **Isoflavones** Median intake (Q1–Q3) W = 23.3 (17.9–31.0) M = 23.7 (18.1–31.1) **Flavones** Median intake (Q1–Q3) W = 0.77 (0.53–1.10) M = 0.65 (0.44–0.95) **Flavanones** Median intake (Q1–Q3) W = 31.1 (22.9–42.1) M = 35.0 (26.1–45.9)	Higher polyphenol intake was associated with better pulmonary function (forced vital capacity, and forced expiratory volume in the first second) in the population under study. A potential anti-inflammatory activity of polyphenols was hypothesized in men where a reduction in C-reactive protein and white blood cells was observed

3. Discussion

The great interest for the protective role of polyphenols is demonstrated by the rapid increase of publications evaluating the mechanisms of action of these heterogeneous/complex and multi-target compounds, and also by the studies focused on association between polyphenol intake and different diseases or mortality. In particular, the association of both total or polyphenol subclasses with different types of cancer has been largely addressed in recent reviews and meta-analyses even if the effects are often nulls [96–101].

The present study analyzed the literature on polyphenol intake assessment *per se* or in relation to CVD, diabetes, other health outcomes or mortality.

As expected, the review of data obtained from different studies underlines a consistent difference in the estimated polyphenol intake which may be attributed to different methodological issues such as the type of tool administered to assess the intake, the database used for the calculation of polyphenol intake and the type of polyphenols under evaluation.

It is well known that dietary intake is difficult to measure, and single methods (i.e., questionnaires) cannot perfectly estimate dietary exposure. This is particularly critical especially for micronutrients and bioactive compounds. FFQs, and sometimes 24-h DR, represent the main tools used within the epidemiological studies to assess dietary intake. They have different characteristics; for example, FFQs consist in a pre-finite list of foods and beverages (the number of items queried typically ranges from 80 to 120) with response categories to indicate usual frequency of consumption over the time period queried. Conversely, the 24-h DR consists of an open-ended questionnaire administered by a trained interviewer able to collect detailed information about all foods and beverages consumed by the subjects in the previous 24 h. Both questionnaires present several limitations; for example, FFQs lack of detailed information about food preparation, specific food and beverages consumed, as well as different brands. Moreover, the pre-specified food list does not necessarily reflect the eating behavior of the population under study and the presence of systematic errors must be partially mitigated through appropriate statistical modeling that take into consideration the adjustments for cofounding factors such, as an example, age and energy intake. Regarding 24-h DR it requires multiple days to assess usual intake. In addition, multiple administrations are also recommended when 24-h DRs are used to examine diet impact on health outcomes or other parameters. On the other hand, it has been reported that the assessment of total flavonoid intake requires at least 6 days of weighed food records, and between 6 and 10 days to determine intake of specific flavonoid subclasses with an acceptable degree of accuracy [47]. Most of the studies analyzed in the present review did not perform a multiple evaluation of food intake as highly recommended thus, an under or overestimation of total polyphenols and their classes/subclasses intake cannot be excluded.

Another important critical point for the estimation of polyphenol intake is the choice of the databases. The most commonly used are USDA and Phenol-Explorer. USDA database focuses predominantly on flavonoids as aglycones (anthocyanins, flavanols, flavanones, flavones, flavonols and isoflavones), while Phenol-Explorer, in addition to the above mentioned flavonoids (mainly as glycosides), provides data also of the precursors (chalcones, dihydrochalcones and dihydroflavonols) and information on total polyphenols measured by Folin-Ciocalteu [25]. Despite both data sources are systematically extended to reflect most accurately phenolic contents in food, it is clear that they show several limitations. First of all, since they provide information on different classes of polyphenols, the comparison of the results obtained on the basis of the various data sources may differ. For example, some studies reported that the intake of flavonoids are generally higher when calculated using the USDA databases in relation to the Phenol-Explorer database [102]. In addition, despite they provide information on a wide range of foods, the list does not include all food and polyphenol sources; this represents a critical aspect since missing data have to be found by using different databases and/or by consulting the scientific literature with an increase of risk of bias. Moreover, the effect of seasonality, storage and cooking process is not always considered but certainly, it could represent a critical point. Finally, in view of these issues, it should be remarked that all databases allow only

an estimation of dietary polyphenols intake. In this regard, it is noteworthy that databases do not consider non-extractable polyphenols thus contributing to an overall under estimation of intake [103]. This is relevant since these compounds seem to have potential protective properties exerted through gut microbiota metabolites production [104].

In the present review, we found that most of the studies used USDA and Phenol-Explorer databases alone, in combination, or together with other databases and/or data sources (i.e., specific scientific publications). An estimation of polyphenol intake data obtained from reviewed studies using FFQs and from those using 24-h DR, seem to provide comparable results in terms of total polyphenol intake (FFQs 910 mg; 24-h DR 890 mg), total flavonoids (FFQs 360 mg; 24-h DR 380 mg) and total phenolic acids (FFQs 410 mg; 24-h DR 450 mg). In addition, it is noticeable that generally data come from single evaluations instead of multiple evaluations of food intake as recommendable, thus an under or overestimation of polyphenols and/or specific subclasses cannot be excluded.

Polyphenol intake is also affected by intrinsic factors such as the geographical area, the population characteristics in term of age, gender and socio-cultural factors and above all the dietary habits. In this regard, we have found that the intake of total polyphenols is higher in Japan (about 1500 mg/day) compared to European countries and North and South America (about 900 mg/day and 800 mg/day respectively). Within Europe, we found a large variability of intake between countries; Poland and France had the highest intake of total polyphenols (above 1000 mg/day), followed by Italy (about 650 mg/day) and Spain (about 300 mg/day). Conversely, within the EPIC study, Denmark showed the highest intake of total polyphenols (1786 mg/day) while Greece the lowest (584 mg/day) [27].

Regarding total flavonoids, Poland and Australia had the highest intake (about 600 mg/day) while USA and South America the lowest (about 200 and 400 mg/day, respectively) followed by Asia (China and Korea, at about 60 mg/day). Finally, regarding total phenolic acids, France, Poland and Brazil had the highest intake (above 600 mg/day), while USA, Italy and Spain the lowest (about 300 mg/day). These data were also in accordance with the results obtained within EPIC study, which showed a high flavonoid and phenolic acid intake in non-Mediterranean countries [15] associated to different dietary habits. For example, in the North and Central Europe, non-alcoholic beverages, in particular tea and coffee, are the main polyphenol contributors, while in South Europe the main contributors are fruits alcoholic beverages (e.g., red wine). In Asia, such as China and Korea, apples and vegetables seem to be the main polyphenol sources, while green tea in the Japanese population. Finally, tea, citrus and legumes seem to be the main polyphenol contributors in the USA.

As far as gender differences in polyphenol intake are concerned, data in literature are not univocal even if more studies suggest a higher intake in females compared to males, above all when standardization for energy intake is taken into account. In addition, differences in polyphenol sources selected seem to be dependent on gender (e.g., higher contribution of fruit and vegetables in females compared to males who are higher consumers of alcoholic beverages and coffee).

Notwithstanding, most of the data available have been assessing polyphenol intake in adults, a large number of studies considered also the intake in older subjects. Nine studies specifically reported results on total polyphenol and/or subclasses in target of older populations (2 Australia, 2 Spain, 1 Brazil, 1 Italy, 1 Poland, 1 UK and 1 Japan). Total polyphenol intake ranged from about 333 mg/day in Spain [44] to 1492 mg/day in Japan [32]. In addition, those considering total flavonoid intake registered values from about 170 mg/day in Spain [44] to about 834 mg/day in Australia [102]. When available the contribution of phenolic acids was approximately 30–40% of the total polyphenol intake. Studies considering different age classes found controversial results, even if generally, all studies reported differences in food habits affecting polyphenol intake. For example, Vitale et al. [48] showed that flavonoid and stilbene increased with age in the TOSCA.IT study, being higher in over 65 years subjects compared to those with age lower than 65 years. Accordingly, Miranda et al. [39] reported that older subjects (>60 years) from a Brazil cohort consumed more flavonoids and tyrosol than adults (20–59 years) and also more fruits. Moreover, Zamora-Ros et al. [27], showed an increased intake of flavonoids, stilbenes, lignans and other polyphenols with age, while no effect on total

polyphenol intake in the EPIC cohort. Other studies reported no differences in polyphenol intake depending on age, or a slight increase after energy adjustment [43,49]. Others (Zujko et al. [19]) showed lower levels of flavonoid intake in older Brazilian subjects who generally consumed less beverages and vegetables. Finally, Karam et al. [44] found an increased energy adjusted polyphenol intake by age classes in older adults from Mallorca island showing also the impact of factors such as gender, educational level and lifestyle significantly affecting eating habits. Large differences in food selection depending on region/country have been underlined reflecting a different pattern of polyphenol intake.

Only 3 studies reported data on children and adolescents showing a low polyphenol intake associated to the overall dietary pattern generally poor in fruit and vegetables even if direct comparison among results is difficult due to the lack of energy adjustment of data in the different age subclasses. The main sources of polyphenols identified depending on the country were non-alcoholic beverages (UK, Argentine), fruit (apple, pear), juices, chocolate (in Helena European study [46]).

Extensive research on polyphenols in human studies has shown a potential role of these compounds in the modulation of CVD markers [105]. In the present systematic review, we found an overall inverse association between total polyphenol intake (highest quantile, above 1170 mg/day) and CV risk events and mortality. In addition, an increased protection against T2D events was observed for total polyphenol intake (mean intake of the 4th quartile) higher than 2632 mg/day [69]. However, the results are not univocal and 4 out of 9 papers reported no association at doses of polyphenols higher than 1200 mg/day or above (>2400 mg/day). These conflicting results could be attributed to the high heterogeneity of the studies in term of selected population characteristic, markers/endpoints measured (i.e., marker of CV risk analyzed), dietary habits (very different between countries), and polyphenol food sources (i.e., tea, coffee, fruits, alcoholic beverages).

Recent evidence from systematic reviews and meta-analyses of cross-sectional and prospective cohort studies seem to suggest that the intake of certain polyphenol classes and subclasses, more than total polyphenols, may reduce the incidence of T2D, CVD events and CVD mortality. However, most of the effects were found when comparing the highest quantiles *versus* the lowest. In fact, we reported a lower risk of CV events for an intake of total flavonoids ranging from 115 to 944 mg/day, an inverse association for T2D with the highest quartile of total flavonoids (585 mg/day), and a low risk of mortality for cardiovascular events and all–cause mortality for the highest quintile of total flavonoid intake (range 360–800 mg/day) [78,80]. These results are in line with observations reported by other authors. For example, McCullough et al. [74], showed that a total flavonoid intake above 512 mg/day was inversely associated with fatal events for CVD in men and women. Feliciano and coworkers [106], reported that high consumers (>788 mg/day of total flavonoids) showed an inverse association with CVD events and CVD mortality. Wang and colleagues [107] found a reduced risk of CVD events for doses of flavonoids (including flavonols, anthocyanidins, proanthocyanidins, flavones, flavanones and flavan-3-ols) between 139 and 604 mg/day. Finally, Grosso et al. [108] showed that increasing by 100-mg/day flavonoid intake led to a linear decreased risk of 6% and 4% of all-cause and CVD mortality.

As regard the diverse subclasses of polyphenols, several studies have reported a positive effect for flavonols, flavones, flavanones, isoflavones, anthocyanidins and proanthocyanidins. For example, Wedick and coworkers [53], have shown that the highest quintile of anthocyanins (about 22.3 mg/day) and anthocyanin-rich fruit intake (≥5 times/week) was associated with a lower risk of T2D. Conversely, limited evidence is available for lignans. One study performed by Rienks and colleagues [109] showed that high levels of plasma enterolactones (lignan precursors) were associated with a 30% and 45% reduction of all-cause and CVD mortality risk.

Interestingly, in the last years, a growing attention has been devoted to the impact of polyphenols on different health outcomes including for instance renal insufficiency, respiratory function, immune function, and vascular activity. For these outcomes, flavonoids and proantocyanidins have shown an apparent promising beneficial effect. Very recently, another research path has focused on the contribution of polyphenols in the older subject health outcomes. Specifically, the effect on retardation/prevention

of some age-related complications such as cognitive decline, frailty and bone fractures has been investigated. On the whole, we have found an overall positive association between high intake of polyphenols and classes/subclasses, and a modulation of different outcomes associated with aging. In particular, total flavonoids and subclasses have been apparently associated with a higher bone mineral density, low risk of bone fractures and macular degeneration, while only total urinary polyphenols, but not dietary polyphenols, have been associated with a low risk of pre-frailty and frailty in older subjects. However, this type of investigation is at early stages thus, further studies have to be performed in order to strength the evidence on the associations found. In addition, since the preliminary observations on protective effects have been found mainly for specific compounds, future studies should be focused on the contribution of subclasses or individual polyphenolic compounds, and even metabolites, instead of total polyphenols.

4. Conclusions

Undoubtedly, polyphenols exert numerous biological activities as reported in a plethora of in vitro and in vivo studies. In addition, several systematic reviews and meta-analyses of observational and intervention studies have found a reduced risk for numerous chronic diseases. We documented an overall inverse association between polyphenol intake and CV risk events and mortality, as well as, between polyphenols and other outcomes of health status. However, most of the associations were found for specific polyphenol classes/subclasses as well as markers/endpoints. At present, few and conflicting results are available for total polyphenols thus, as also reported more than 10 years ago [110], it is still difficult to establish a reference and/or prudent intake of total polyphenols, even if we found an approximate mean intake of about 900 mg/day. Some studies suggest an inverse association between high total flavonoid intake (generally higher 500 mg/day) and CV events and/or mortality. However, this value should be considered as a temptative level due to the elevated heterogeneity of the studies and the numerous limitations associated with the evaluation and estimation of polyphenol intake. It is then fundamental to consider that polyphenol intake correspond to differences in dietary behavior and selection of diverse food sources of the same compounds could affect the overall impact differently. Therefore, it is reasonable to argue in terms of dietary patterns more than focusing on single contributions. In this context, polyphenol-rich dietary pattern seems to exert health benefits and should be considered a valid tool for the prevention of numerous chronic diseases.

At the same time, further investigation is highly recommended in order to address the need for: (1) improved dietary assessment methods; (2) standardized and validated analytical procedures for the analysis of polyphenols and related subclasses in foods; (3) implementation of food databases increasing food items and information available on the different polyphenol subclasses; (4) validation of specific polyphenol intake biomarkers. Nevertheless, despite information from observational studies are necessary to identify potential role of diet-related compounds, the availability of well controlled and specifically targeted dietary intervention studies (addressing also dose-response effects) seems to be mandatory to allow the identification of a reference or prudent intake (e.g., in term of health-promoting properties) for food bioactives such as polyphenols, directed to the general population or specific vulnerable groups (e.g., older subjects).

Author Contributions: S.B., M.M. and M.T. performed independently the literature search through scientific databases, reviewed the abstracts, assessed and verified the eligibility of the studies. B.K., B.C. and N.H.L. acted as additional independent reviewers, fixed any bias or controversial in the study selection and contents. M.M. and S.B. prepared the tables. M.T. prepared the figures. C.D.B. revised the tables, the figures and wrote the first draft of the manuscript. M.P. and S.G. improved the manuscript. P.R., designed the study, interpreted the results, improved and critically revised the entire manuscript. A.C., R.Z.-R., C.A.-L., P.K. and A.C. participated to the critical discussion of the results and final revision of the manuscript.

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MANUSCRITO 4

Exploring the Molecular Pathways Behind the Effects of Nutrients and Dietary Polyphenols on Gut Microbiota and Intestinal Permeability: A Perspective on the Potential of Metabolomics and Future Clinical Applications

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MANUSCRITO 4

Objetivo: Este trabajo científico se ha llevado a cabo con dos objetivos a cumplir:

- Revisar la literatura actual sobre el rol y la aplicación de la metabolómica en el estudio de las interacciones entre los componentes de los alimentos y la microbiota intestinal y sus efectos sobre la permeabilidad intestinal (PI), con especial atención a la elucidación de las vías moleculares implicadas.
- Ofrecer una visión sobre la futura investigación de los polifenoles de a dieta y su potencial uso en el manejo de la PI aumentada.

Metodología: Se analizó la bibliografía científica disponible respecto papel de la microbiota y de los metabolitos dietéticos derivados de ésta en la regulación de la permeabilidad intestinal y la aplicación de la metabolómica a la función barrera del intestino y la permeabilidad intestinal.

Resultados: A la fecha, los catabolitos microbianos más estudiados respecto a su efecto en la regulación de la PI son los ácidos grasos de cadena corta (AGCC), producidos por varias bacterias intestinales (entre ellas *Clostridium, Eubacterium y Butyrivibrio*) a partir de la degradación de las fibras alimentarias, siendo el butirato un marcador de los efectos positivos del consumo de fibra dietética no digerible en la composición de la microbiota y la PI. Se ha encontrado en modelos de ratones envejecidos que, tras el consumo de altas dosis de fibra soluble, aumenta la producción de butirato y ocurre una expresión inducida de las proteínas TJ (claudina-1 y claudina-2), TJP2 y FFAR2, además de una compensación de la disbiosis de la microbiota relacionada con la edad, mejorando significativamente la PI. A partir de un enfoque metabolómico, se han obtenido aclaraciones preliminares sobre el papel del triptófano y sus derivados microbianos y endógenos en la regulación de la función barrera y la actividad de la microbiota intestinal. U estudio demostró que el ácido indol-3-propiónico (IPA), producido por el firmicute *Clostridium sporogenes*, regula la integridad de la mucosa y la función de la barrera intestinal mediante la activación del receptor X de pregnano (PXR) y la regulación al alza de los ARNm que codifican las proteínas de unión. También, numerosos estudios in vitro y en animales han mostrado que el consumo de alimentos ricos en polifenoles podría afectar positivamente la PI, reforzando las propiedades de barrera del epitelio intestinal por influencia directa en la síntesis y expresión de las proteínas TJ o por interacción con la microbiota intestinal, que además está implicada en la transformación metabólica de los polifenoles y en la producción de derivados de bajo peso molecular, que, a su vez, contribuyen al mantenimiento de la función de barrera e impulsan cambios en los componentes del microbioma intestinal con importantes efectos para la salud del huésped..

Conclusión: La dieta desempeña un papel importante en el mantenimiento de la integridad de la barrera intestinal y es, por tanto, determinante para la PI. Aunque el estudio de los efectos de las intervenciones dietéticas sobre la microbiota intestinal y la PI y las investigaciones de los mecanismos de acción han comenzado recientemente, parece claro que los hábitos dietéticos adecuados y el seguir patrones dietéticos saludables ricos en fibra y polifenoles desempeñan un papel importante en el mantenimiento de las funciones intestinales adecuadas. La aplicación de enfoques multiómicos integrados, está dilucidando gradualmente la participación interrelacionada de los componentes de los dieteticos, microbiota y compuestos derivados del microbioma, en el mantenimiento de la función de la barrera intestinal y las vías moleculares implicadas.

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Review

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Exploring the Molecular Pathways Behind the Effects of Nutrients and Dietary Polyphenols on Gut Microbiota and Intestinal Permeability: A Perspective on the Potential of Metabolomics and Future Clinical Applications

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ABSTRACT: The gut microbiota is involved in the regulation of the intestinal permeability (IP), whose disruption is a frequent condition in older people and is associated with the development of several diseases. The diet can affect the gut microbiota and IP, although the molecular mechanisms involved are unclear. Metabolomics is one of the suitable approaches to study the effects of diet on gut microbiota and IP, although, up to now, the research has focused only on a few dietary components. The aim here was to review the most recent literature concerning the application of metabolomics to the study of the diet-induced alterations of gut microbiota and the effects on IP, with a particular focus on the molecular pathways involved. An additional aim was to give a perspective on the future research involving dietary polyphenols, because despite their potential use in the management of increased IP, few studies have been reported to date.

KEYWORDS: *metabolomics, gut microbiota, intestinal permeability, nutrients, polyphenols, aging*

INTRODUCTION

The gastrointestinal tract (GI) is responsible for a wide range of functions, including digestion and absorption of nutrients, water, and ions, regulation of host immunity, protection against the ingress of pathogenic microorganisms, and metabolism and detoxification of xenobiotics. The GI also hosts the largest microbial population of the human body, which works in symbiosis with the host to accomplish these various intestinal functions. Gut bacteria are particularly important for host health, being involved in the synthesis of vitamins, secondary bile acids, and neurotransmitters and playing a direct role in the metabolism and degradation of dietary components and drugs that can affect their bioavailability and absorption.[1] It has been estimated that over than 1000 different bacterial species populate the intestinal environment, with a genome comprising 100-fold more genes than those found in the human genome.[2] The physiological variations in the small intestine and colon, such as the presence of distinct chemical environments, nutrients, and host immune activity, allow for distinct groups of bacterial species to populate the different regions of the lower gastrointestinal tract,[3,4] and this variability becomes even more complex considering the interindividual variations and the influence of host genetics.[5−7] Nevertheless, most human gut microbiota share a core set of resident bacteria and related microbial genes.[8,9] Firmicutes, Bacteroidetes, and Actinobacteria are the three most abundant phyla, among the over 50 that have been identified by metagenomic approaches.[10,11] A synergistic equilibrium among the different species and the maintenance of a microbial diversity are of crucial importance for health, because the microbiota plays a central role on the proper functioning of the intestinal barrier and maintaining appropriate intestinal permeability (IP), which is directly involved in the development of numerous disorders. In this vein, a low diversity and a scarce abundance of species, such as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii*, have been associated with gut disease states, e.g., Crohn's disease,[12] type 1, type 2, and gestational diabetes,[13−15] celiac disease,[16] and obesity.[17]

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Diet, as a source of macro- and micronutrients and other bioactive components, is one of the factors that can most affect the microbiota. Among the dietary constituents, polyphenols have been in the spotlight in recent years as a result of their particular physicochemical properties and their potential to directly affect microbiota activity and host health. Polyphenols are secondary metabolites of plants, fruits, and vegetables, and major components of commonly consumed foods and beverages, such as chocolate, tea, and coffee,[18−20] which, as a result of their characteristic (poly)hydroxylated phenyl moieties and the presence of ionizable functional groups on their scaffolds, have a low bioavailability and are scarcely absorbed by the intestine.[21,22] Consequently, they are prone to catabolism by the gut microbiota, which leads to the production of smaller molecular weight (MW) compounds that can be absorbed across the intestinal wall, enter the bloodstream, and eventually undergo further transformation and conjugation in the liver.[23,24] It has been estimated that total polyphenol absorption in the small intestine is around 5−10%, while the remaining 90−95% transits to the large intestinal lumen and accumulates in the millimolar range.[25] Hence, microbial polyphenol derivatives could be responsible for the biological effects attributed to their parent compounds or at least contribute to the overall activity. Catechins from green tea, for example, have been reported to exert antioxidant, anti-inflammatory, and antitumorigenic activities.[26−28] However, the most representative green tea catechin, (−)-epigallocatechin gallate, is scarcely absorbed from the intestine and is extensively metabolized by gut microbiota[29] to form smaller MW derivatives that not only contribute to the observed bioactivities of green tea but can also exert higher activity than the parent compound.[30] Polyphenols and their microbial metabolites could also exert antimicrobial and bacteriostatic activities, hence regulating the overgrowth of harmful bacteria on the intestinal and urinary tract epithelia.[20,31] As an example, cranberry (*Vaccinium macrocarpon* Ait.) fruits, rich sources of type A procyanidins (PAC-A), are known to exert antiadhesive activity against the uropathogenic bacteria responsible for most of the lower urinary tract infections, although the mechanisms of action are still unknown and the outcomes of *in vitro* assays and *in vivo* clinical trials aimed at reducing urinary tract infections are frequently inconsistent.[32] Recent studies conducted in both rats and human volunteers show that, after supplementation with dry cranberry extracts, urine samples exert effective antiadhesive activity against uropathogenic *Escherichia coli*, despite their negligible contents of intact PAC-A.[33,34] However, the same urine samples were characterized by high amounts of hydroxyphenyl-valeric acid and hydroxyphenyl-valerolactone derivatives, previously reported as end products of microbial degradation of flavan-3-ols,[35] indicating the important contribution of the microbial metabolites of procyanidins to the observed bioactivity.[33,34] Finally, the effects of polyphenols on microbiota, inflammation, and oxidative stress and their capacity to regulate the synthesis and expression of specific proteins on the intestinal epithelium seem to be part of the mechanisms by which these compounds can regulate the permeability of the intestinal barrier,[36] whose alterations are related to the development of several diseases, especially in older subjects.

Many efforts have been made to characterize the microbial community colonizing the human intestine, for which the widespread use of metataxonomics based on 16S rRNA gene profiling and metagenomics (microbiomics) has been particularly important. However, although representing powerful tools for bacterial identification and classification, microbiomics does not allow for the acquisition of information about fluctuations in metabolic activities.[1] To this purpose, metabolomics is the most suitable approach, and numerous reports based on metabolomic analysis have been reported over the past decade.[37] Focusing on the application of metabolomics in the study of diet−microbiota interactions and searching for the keywords "metabolomics AND diet AND microbiota" in PubMed, we found that the number of publications almost doubled from 2014 to 2018, as an index of the popularity that metabolomics gained during the recent years (Figure 1). Metabolomic approaches have been widely

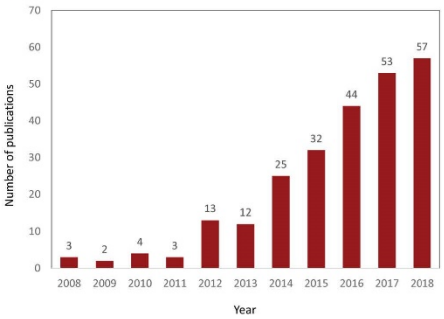

Figure 1. Increase of the scientific literature regarding the use of metabolomics in the study of the interactions between diet and gut microbiota during the last 11 years. Source: PubMed (https://www.ncbi.nlm.nih.gov/pubmed/).

used to study the transformation of nutrients and xenobiotics by intestinal microbiota,[38−43] thus allowing for the characterization of hundreds of metabolites derived from macro- and micronutrients and polyphenols coming from fruits and vegetables. In 2009, Jacobs et al. published a first review regarding the role of colonic microbiota in the degradation of non-digestible food ingredients and their impact on gut health and immunity.[44] For the first time, the importance of metabolomics in the study of the links between the bioconversion of non-digestible food ingredients, their bioavailability, and their downstream effects on microbiota composition and host metabolism was recognized.[44] More recently, the use of integrated multiomics approaches has facilitated the study of the molecular interactions between diet and microbiota and has led to the identification of several metabolites that are produced as a result of microbial metabolism of various dietary constituents. Nevertheless, considering the challenges to study the mutual relationship between gut microbiota and the host, its tight connection with diet, environment, and lifestyle, and the still incomplete characterization of the huge microbial metabolome, the path to assess precise and validated metabolites to link the microbial activity to specific effects on health is just starting. In a way to find a clinical relevance of metabolomics data and offer to clinicians a robust tool to predict, prevent, and treat several diseases, further progress is necessary.

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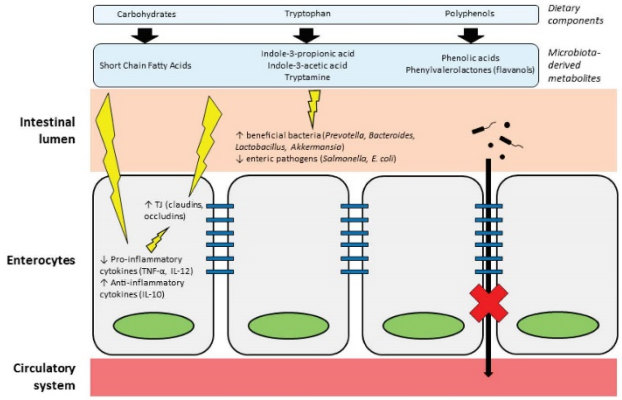

Figure 2. Schematic representation of the mechanisms of action responsible for the effects of microbiota-derived dietary metabolites on intestinal permeability.

The aim of this work was to review the most recent literature regarding the application of metabolomics in the study of the interactions between food components and gut microbiota and the effects on IP, with a particular focus on the elucidation of the molecular pathways involved. Since to date the research has mainly focused on the degradation of non-digestible fibers and tryptophan and the bioactivity of their metabolites, a major part of the work will be dedicated to these important dietary components. Additionally, a perspective on the future research involving the role of dietary polyphenols in modulating the activity and composition of gut microbiota and the effects on IP will be discussed, given that, despite their potential implication in the prevention and treatment of several diseases, few clinical studies have been performed up to now.

ROLE OF MICROBIOTA AND MICROBIOTA-DERIVED DIETARY METABOLITES IN REGULATING INTESTINAL PERMEABILITY: THE APPLICATION OF METABOLOMICS FOR THE DISCOVERY OF NEW BIOMARKERS

The intestinal wall represents a barrier that selectively transports nutrients, ions, and water from the lumen to the bloodstream, via passive and active mechanisms. A layer of epithelial cells constitutes the main physical barrier between the intestinal lumen and the mucosal tissues.[45] Tight junction (TJ), composed of transmembrane proteins and junctional adhesion molecules that regulate the flow of water, ions, and small molecules, seals the paracellular spaces.[46] Several distinct proteins contribute to form the TJ, including mainly occludins and claudins, depending upon the tissue and location that interlink within the paracellular space.[47] Although highly cross-linked, the structure of TJ is dynamic, so that it can be "opened" and "closed" following specific stimuli.[48] Physiological stimuli could shrink the TJ to prevent the diffusion of toxins, viruses, or bacterial fragments to the mucosal layer, while they can open the paracellular space to allow for the diffusion of nutrients.[49] For instance, the activation of the sodium-dependent glucose transporter led to the opening of TJ and allowed for the diffusion of small molecules and peptides with a MW of <40 000 Da.[50] On the other hand, the physiological structure and dynamism of TJ could be altered as a result of pathological states,[51] leading to a condition of increased IP, also known as "leaky gut". Celiac disease, inflammatory bowel disease, and type 1 diabetes are three of the principal pathological causes of leaky gut,[52] which leads to the permeation of potentially harmful molecules, organisms, or microbial fragments from the intestinal lumen to the mucosal layer, inducing a cascade of events that result in immune activation and local or systemic inflammation. Older people are frequently affected by decreased intestinal barrier function and, consequently, leaky gut.[53] Among the causes, the aging-related decline of immune function (namely, immune senescence), the remodeling of intestinal epithelium, and the alterations of gut microbiota composition are thought to be the most important causes.[53−55] As observed in disease-associated increased IP, the dysfunction of the intestinal barrier in older subjects facilitates the diffusion of toxic substances or peptides and microbial fragments to the mucosal layer and the bloodstream and the triggering of a systemic inflammatory response.[56]

As previously stated, diet plays an important role in the maintenance of the gut barrier integrity and is, hence, determinant for IP. The short-chain fatty acids (SCFAs), produced by the degradation of dietary fibers by several bacteria in the gut (including *Clostridium*, *Eubacterium*, and *Butyrivibrio*), have been the most studied microbial catabolites involved in the regulation of IP to date. Among them, butyrate has been identified as a marker of the positive effects of non-digestible dietary fiber consumption on microbiota composition and intestinal permeability. It exerts several activities on the intestinal wall, such as controlling inflammation by altering the expression of pro-inflammatory cytokines,[57] preserving the intestinal barrier function by inducing the expression of TJ proteins claudin-1 and claudin-2,[58] and modulating composition of gut microbiota by inhibiting the growth of pathogenic bacteria[59] (Figure 2). Food is the only source of non-digestible carbohydrates, and alterations in diet lead to variations in the production of intestinal butyrate. In aged mice, the increased butyrate production after the consumption of high doses of

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Table 1. Summary of the Studies Involving the Application of Metabolomics to the Study of the Effects of Diet–Gut Microbiota Interactions on Intestinal Permeability *in Vivo*

	intervention/condition	source, dose, and length of treatment	model	biofluid/biomatrix analyzed	metabolomic approach[42]	gut-derived metabolites correlated to effects on IP	main outcomes of the study[6]	reference
dietary fibers	high-fiber diet	laboratory diet composed of 30% barley and 70% standard AIN-93 for 28 days	mouse, healthy	feces	targeted GC–MS	butyrate, propionate, and acetate	butyrate from fiber ↓ pro-inflammatory cytokines ((IL-17A, IL-6, and Cxcl-1) ↑ IL-10 and TGF-β mRNA expression ↓ intestinal tract lesions ↑ claudin-1, occludin, and ZO-1 ↓ bacterial translocation	Hu et al.[61]
	inulin-enriched diet	laboratory diet supplemented with 5% cellulose and 25% inulin for 7 days	mouse, healthy and colonized with 1×10^8 CFU *Clostridium difficile*	feces	targeted GC–MS	butyrate, propionate, and acetate	butyrate from fiber ↓ pro-inflammatory cytokines (IL-6, IL-1β, and Cxcl-1) ↑ anti-inflammatory cytokine IL-10 ↓ intestinal tract lesions ↑ claudin-1 and occludin ↓ bacterial translocation ↑ intestinal barrier integrity	Fachi et al.[62]
	tryptophan	gavage with *Clostridium sporogenes* and standard chow diet	standard chow (LabDiet 5k67) containing 0.25% tryptophan for 4 weeks	mouse, germ-free colonized with *C. sporogenes* by oral gavage ($\sim 1 \times 10^7$ CFU)	serum	targeted LC–MS	indole 3-propionic acid (IPA)	IPA produced by *C. sporogenes* colonization with *C. sporogenes* ↓ intestinal permeability IPA signals through PXR to fortify the intestinal barrier	Dodd et al.[65]
	gavage with probiotics (mice)/irritable bowel disease (IBD) (human)	oral gavage with $0.6–2 \times 10^8$ CFU *Peptostreptococcus* species every other day, for 2 weeks (mice)	mouse, dextran sodium sulfate-induced colitis/human, ulcerative colitis and Crohn's disease	feces	untargeted LC–MS	IPA and indoleacrylic acid (IA)	*Peptostreptococcus* species ↑ barrier function through production of IPA and IA IA ↓ pro-inflammatory cytokine production IA ↑ intestinal epithelial barrier function microbes of IBD patients have reduced ability to cleave mucins and metabolize tryptophan	Wlodarska et al.[69]

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Table 1. continued

	intervention/condition	source, dose, and length of treatment	model	biofluid/biomatrix analyzed	metabolomic approach[a]	gut-derived metabolites correlated to effects on IP	main outcomes of the study[b]	reference
	high-fat diet (mice) supplemented with IPA; obese T2D subjects before and after RYGB (human)	daily oral gavage with 20 mg/kg IPA for 4 consecutive days (mice)	mouse, diet-induced obese (DIO)/human, obese with type 2 diabetes	plasma	targeted and untargeted LC–MS and GC–MS	IPA, indoxyl 3-sulfuric acid (ISA), and indole 3-acetic acid (IAA)	↓ mucin utilization by gut bacteria in IBD ↓ colonization of microbes that metabolize tryptophan in the intestine of IBD IPA ↓ IP in DIO mice ↓ IPA, IAA, and ISA in obese subjects ↑ IPA, IAA, and ISA 3 months after RYGB IPA ↓ IP in obese subjects	Jennis et al.[66]
	dietary polyphenols	oral bilberry anthocyanin (BA) consumption in aging model	old and young animals treated with three different BA doses (six animal groups) for 10 weeks: LBA group, 10 mg kg^{-1} day^{-1}; MBA group, 20 mg kg^{-1} day^{-1}; and HBA group, 40 mg kg^{-1} day^{-1}	rat, young (4 months) and old (12 months), healthy	cecal content	targeted GC–FID	butyrate, propionate, and acetate	BA ↑ starch-utilizing and butyrate-producing bacteria BA ↓ inflammatory factors (TNF-α and IL-6) and mucosa damages in the colon	Li et al.[80]
		combination of flavonoid supplementation and moderate physical exercise (45 min walking and 2.5 h running)	capsule containing 329 mg of total flavonoids: bilberry fruit extract (64 mg of anthocyanins), green tea leaf extract (184 mg of total flavan-3-ols), 104 mg of quercetin aglycone; 1 capsule/day for "walking" group; 2 capsules/day for "running" group; supplementation time: 2 weeks	human, healthy	plasma	targeted LC–MS	hippuric acid, 3-hydroxyhippuric acid, quercetin-3-O-glucuronide, delphinidin-3-O-glucoside, 4-hydroxycinnamic acid, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, 3-(3-hydroxy-4-methoxyphenyl)propanoic acid-3-O-glucuronide, methoxybenzoic acid derivatives, and benzaldehyde derivatives	physical exercise ↑ absorption of gut-derived flavonoid metabolites flavonoid consumption associated with physical exercise ↓ IP flavonoids and their gut-transformed metabolites ↑ intestinal barrier integrity	Nieman et al.[81]

[a]LC–MS, liquid chromatography coupled to mass spectrometry; GC–MS, gas chromatography coupled to mass spectrometry; and GC–FID, gas chromatography coupled to a flame ionization detector. [b]↓ indicates "decrease", and ↑ indicates "increase".

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J. Agric. Food Chem. 2020, 68, 1780−1789

soluble fiber was associated with an induced expression of the TJ proteins Tjp2 and Ffar2 and to a counterbalance of the age-related microbiota dysbiosis, with a significant amelioration of the increased IP condition typical of older individuals.[60] Similar effects of a high fiber diet were also observed in mice affected by autoimmune hepatitis, characterized by an imbalance of Treg/Th17 cells and increased IP.[61] Metabolomics analysis of feces showed increased levels of butyrate after dietary intervention, and the expression of TJ proteins ZO-1, occludin, and claudin-1 was induced in the ileum, with consequent increased intestinal barrier function and decreased translocation of bacterial components through the intestinal wall[61] (Table 1). The same effects were also observed in mice treated with sodium butyrate, indicating a direct involvement of this bacterial metabolite in the regulation of IP.[61] Similar results were recently reported by Fachi and colleagues, who showed that an inulin-enriched diet protects mice from *Clostridium difficile*-induced colitis through the production of SCFAs.[62] Metabolomics analysis of feces showed the increased production of butyrate, propionate, and acetate after dietary intervention (Table 1). Butyrate reduced the levels of pro-inflammatory cytokines and increased the anti-inflammatory cytokine IL-10 in the colon at the peak of infection, leading to an overall attenuation of the intestinal inflammation.[62] Butyrate induced the expression of genes associated with claudin-1 and occludin, leading to a reduction of the IP and, consequently, a reduction of the microbial translocation in the liver and spleen.[62]

Microbial tryptophan metabolites also play an important role in regulating barrier functions and gut microbiota activity. A metabolomic approach allowed for the acquisition of preliminary elucidations about the role of tryptophan and its microbial and endogenous derivatives in the regulation of immune tolerance toward intestinal microbiota.[63] Starting from these findings, further research has elucidated the role of other microbial-derived tryptophan metabolites in the regulation of gut permeability by direct effects on epithelial cells. Venkatesh et al. showed that indole-3-propionic acid (IPA), produced by the firmicute *Clostridium sporogenes*, regulates mucosal integrity and intestinal barrier function by activating the pregnane X receptor (PXR) and upregulating junctional protein-coding mRNAs.[64] More recently, Dodd et al. used an integrated targeted−untargeted approach to identify 12 microbial metabolites derived from the reductive activity of *C. sporogenes* on aromatic amino acids (phenylalanine, tyrosyne, and tryptophan), of which 9 (lactate, acrylate, and propionate derivatives) were reported to accumulate in host plasma.[65] The authors particularly focused on IPA and its effects on gut barrier and the mucosal immune system, and their results supported the findings of Venkatesh and colleagues about the PXR-mediated effect on gut permeability[64,65] (Table 1). A treatment with 20 mg kg^{-1} IPA for 4 consecutive days was shown to significantly decrease the IP in high-fat diet (HFD)-fed obese type 2 diabetes (T2D) mice,[66] which, prior to treatment, were characterized by higher IP and lower circulating IPA levels compared to lean animals. Plasma IPA amounts were also reported to increase in obese subjects 3 months after Roux-en-Y gastric bypass (RYGB) surgery,[66] indicating, once again, the direct involvement of gut microbiota in the maintenance of the intestinal barrier functions. Furthermore, results from *in vitro* assays reported by the same authors showed that IPA could reduce the permeability of the T84 cell monolayer compromised by pro-inflammatory cytokines.[66] Other metabolites derived from the same degradation pathway of tryptophan, i.e., indole (produced by *Escherichia coli, Clostridium bifermentans, Proteus vulgaris, Paracolobactrum coliforme, Achromobacter liquefaciens,* and *Bacteroides* spp.),[67] indole-3-acetic acid (produced by *C. sporogenes*), and tryptamine (produced by *C. sporogenes* and *Ruminococcus gnavus*),[68] were also reported to exert anti-inflammatory activity in both the intestinal lumen and the liver[68,69] and to upregulate the expression of several proteins involved in the transepithelial cell linkage on the intestinal wall, such as TJ proteins TJP1, TJP3, and TJP4 and gap junction proteins GJE1, GJB3, GJB4, and GJA8, among others.[67] A schematic resume of these results is reported in Figure 2.

In recent years, polyphenols have been widely considered for their beneficial effects on health and polyphenol-rich diets have been evaluated for the prevention of several chronic diseases, ranging from metabolic disorders to inflammation and cancer. Some studies have also evaluated the consumption of polyphenol-rich food for the prevention of diseases associated with aging, such as cognitive impairment[70] and depression,[71] although, up to now, the reported effects have been inconsistent. However, numerous *in vitro* and animal studies show that the consumption of polyphenol-rich food could positively affect IP, reinforcing the barrier properties of the intestinal epithelium by direct influence on the synthesis and expression of TJ proteins[72,73] or interaction with gut microbiota. As previously described, this latter is directly involved in the metabolic transformation of plant polyphenols and the production of smaller MW derivatives,[74] which, in turn, contribute to the maintenance of barrier function and drive changes in gut microbiome constituents,[75,76] with important effects for host health. However, although several molecular targets of dietary polyphenols and their metabolites on the intestinal epithelium have been elucidated,[77] it is unclear how the interaction of the same compounds with gut microbiota leads to beneficial effects on the intestinal barrier. In recent studies, through integrated metagenomics−metabolomics analyses of feces and plasma, some authors correlated the variations of the amounts of specific gut-derived metabolites to the effects of polyphenol ingestion on IP (Table 1). It was observed that a HFD supplemented with 4% (w/w) powdered green tea leaves rich in flavanols leads to an increased intestinal population of *Akkermansia* spp. after 22 weeks,[78] a bacterium that has been implied in the maintenance of a functional intestinal barrier through the preservation of mucus layer thickness.[79] Li et al. reported that the consumption of a medium-dose (20 mg kg^{-1} day^{-1}) of bilberry anthocyanin extract (BAE) promoted the generation of SCFAs (acetic, propionic, and butyric acids) in aging rats, through the regulation of the intestinal microbiota.[80] Specifically, several starch-utilizing and butyrate-producing bacteria (among whom *Lactobacillus* and *Bacteroides*) were induced by BAE, while harmful species, such as Verrucomicrobia and Euryarchaeota where inhibited. These variations, associated with decreased levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) in the colon induced by BAE consumption, contributed to the restoring of the intestinal barrier function typically altered in an older individual.[80] In a more recent work by Nieman and colleagues, the authors observed the effects of the association of acute moderate physical activity (sustained walking for 45 min and moderate-intensity running for 2.5 h) and a 2 week flavonoid supplementation on the IP in healthy volunteers.[81] The results,

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obtained using a targeted metabolomics approach, showed that acute moderate exercise leads to higher circulating amounts of 15 metabolites derived from flavonoid metabolism by gut microbiota (mainly hippuric acid, methoxybenzoic acid, and benzaldehyde derivatives; Table 1). The increased levels of these compounds were correlated to the significant decrease of IP observed in both "walking" and "running" groups of volunteers, although information about the mechanism(s) of action involved are lacking.[81]

Overall, the data published up to now indicate that the effects of polyphenols on IP are related to both direct activity on the expression of TJ proteins and changes induced to the intestinal microbiota, with an increase in the prevalence of species that can preserve barrier functions through the production of active metabolites or by direct action on the mucous layer (Figure 2). On the other hand, the data supporting these observations are still scarce, and up to now, only few compounds (e.g., butyrate and some gut-derived polyphenol metabolites) correlating the polyphenol-induced modifications of gut microbiota to the effects on the intestinal integrity and permeability have been discovered. Nevertheless, as demonstrated by the works of Li et al.[80] and Nieman et al.,[81] the integration of metagenomics and metabolomics approaches for the study of the bacterial and metabolic composition of feces and biological fluids represents one of the most suitable approaches for the identification of the pathways leading to the effects of polyphenols on gut microbiota and IP as well as the assessment of the key metabolites involved.

■ CONCLUSION AND FUTURE PERSPECTIVE

Although the study of the effects of dietary interventions on gut microbiota and IP and investigations of the mechanisms of action have begun only recently, it appears clear that appropriate dietary habits and the regular consumption of vegetables and fruits rich in fibers and polyphenols play an important role in the maintenance of proper intestinal functions. The precursors of SCFAs and several indole or phenolic derivatives produced by bacterial catabolism in the intestinal lumen, for example, are abundant constituents of both plant-derived foods, such as cereals, nuts, fruits, and vegetables, rich in non-digestible fibers[82] and animal-based foods, such as dairy products, eggs, and meat, which are rich sources of tryptophan.[83] Thanks to the employment of integrated multiomics approaches, the involvement of several partners (food components, microbiota, and microbial-derived compounds) in the maintenance of the intestinal barrier function and the molecular pathways behind this activity are being gradually elucidated, although further efforts are required to link specific food components and their metabolites to specific mechanisms of action. Nevertheless, the increasing amounts of data regarding specific metabolites (e.g., physicochemical properties, spectroscopic properties, location in biofluids, and involvement in metabolic pathways) stored in freely available databases and the affordability of even more sensitive and robust instrumentations will allow, in the near future, for the acquisition of further biological information to better understand the molecular mechanisms behind the effects of diet on gut microbiota and IP. Once both metabolites and molecular pathways are assessed and validated for clinical relevance, they will represent novel instruments available to clinicians for the assessment of the "intestinal health" and the development of dietary plans aimed at managing and preventing diseases directly linked to increased IP, such as chronic inflammation and immunological disorders, which are a determinant for the gradual decline of health in older subjects.

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Notes

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MANUSCRITO 5

Polyphenols and Intestinal Permeability: Rationale and Future Perspectives

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MANUSCRITO 5

Objetivo: Esta revisión tiene como objetivo ofrecer una visión general resumida de las principales evidencias científica procedentes de estudios in vitro e in vivo que apoyan el papel de los polifenoles en la modulación de la permeabilidad intestinal (PI) y discutir las perspectivas futuras en este ámbito de investigación.

Metodología: Se analizó la literatura científica disponible respecto a la función barrera del intestino y la permeabilidad intestinal, el efecto de la ingesta alimentaria y de un patrón dietético rico en polifenoles en el mantenimiento de la PI; además de analizar la evidencia respecto de los mecanismos de regulación de la PI por parte de los polifenoles en estudios in vitro e in vivo.

Resultados: Se llevó a cabo la búsqueda bibliográfica, utilizando palabras claves como: subclases y/o el nombre polifenoles individuales y "permeabilidad intestinal", la cual proporcionó un mayor número de estudios in vitro y en animales que estudios de intervención en humanos.

La mayoría de los estudios que analizaron los efectos de los polifenoles en la modulación de los posibles mediadores y vías reguladoras implicadas en el PI *in vitro*, se han realizado la línea celular Caco-2 (modelo celular de la barrera intestinal). Entre los polifenoles que ejercieron un efecto protector, se encontró la berberina, quercetina y catequina, que fueron testeadas concentraciones fisiológicas hasta farmacológicas (concentraciones entre 10 y 200 μM). Otros polifenoles evaluados fueron la genisteína, antocianinas, resveratrol, teaflavina y una mezcla de polifenoles. La mayoría de los polifenoles han demostrado aumentar la expresión y/o la producción de numerosas proteínas de las uniónes estrechas (*tigh junctions*), como la ZO-1, la ocludina y la familia de las claudinas, cuya alteración puede dar lugar a un aumento de la permeabilidad paracelular y, algunos estudios han informado de la capacidad de los polifenoles para contrarrestar el proceso inflamatorio inducido por el TNF-α y el interferón γ (IFN-γ) disminuyendo la expresión de varias interleucinas como la IL-8 y la IL-6. En modelos animales (ratones), los resultados encontrados fueron similares, éstos apoyan una mejora de la PI tras la intervención con polifenoles o con extractos ricos en polifenoles.

Conclusiones: Cada vez hay más pruebas que relacionan el aumento de la permeabilidad intestinal (PI), una característica de la barrera intestinal, con varias condiciones patológicas o disfuncionales. Varios factores del huésped y del entorno, incluidos los dietéticos, pueden afectar al mantenimiento de la PI normal. En este sentido, los compuestos bioactivos de los alimentos, como los polifenoles, se han propuesto como potenciales moduladores de la PI, aunque los mecanismos implicados aún no se han dilucidado del todo.

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Perspective

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Polyphenols and Intestinal Permeability: Rationale and Future Perspectives

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ABSTRACT: Increasing evidence links intestinal permeability (IP), a feature of the intestinal barrier, to several pathological or dysfunctional conditions. Several host and environmental factors, including dietary factors, can affect the maintenance of normal IP. In this regard, food bioactives, such as polyphenols, have been proposed as potential IP modulators, even if the mechanisms involved are not yet fully elucidated. The aim of the present paper is to provide a short overview of the main evidence from *in vitro* and *in vivo* studies supporting the role of polyphenols in modulating IP and briefly discuss future perspectives in this research area.

KEYWORDS: *polyphenols, intestinal permeability, in vitro studies, animal studies, human studies*

INTRODUCTION

Over the last 10 years, there has been significant research effort to investigate the central role of gut function and properties in the promotion of human health and/or the development of several pathological conditions.

The intestine is the main organ involved in the absorption of nutrients and water, and it is the largest area of contact with environmental factors. It contains a large number of specialized immune cells that can coordinate with defensive responses that prevent or counteract exposure of the host and its immune system to luminal antigens of different origins (e.g., microbial and dietary origin).[1]

The definition and specific ontology related to the gut as a complex anatomical and functional system has been widely debated. Bischoff et al.[2] defined the intestinal barrier (IB) as a functional entity separating the gut lumen from the inner host and consisting of mechanical elements (mucus and epithelial layer), humoral elements [defensines and immunoglobulin A (IgA)], immunological elements (lymphocytes and innate immune cells), and muscular and neurological elements. Differently, intestinal permeability (IP), which contributes to the regulation of solute and fluid exchange between the lumen and tissues, should refer to a key feature of IB that is measurable as a whole or at a given site (e.g., evaluating specific molecule/factor flux rates). IP evaluation can be used to address a normal/stable or disturbed/compromised permeability related with IB function.[2] In this context, it is fundamental to underline that IB integrity and functionality can be affected also by the characteristics of the intestinal microbial ecosystem and mucosal immune system.

From an anatomical point of view, a well-organized monolayer of epithelial cells is required to form a selective permeability system mainly controlled by the transcellular and paracellular pathways.[3]

While the absorption and/or transport of nutrients (i.e., sugars, amino acids, vitamins, fatty acids, and minerals) occur through specific transporters or membrane channels (transcellular path),[3] a complex system of junctions crucial for the transport between adjacent cells [i.e., tight junction (TJ), gap junction (GJ), adherens junction (AJ), and desmosomes] constitutes the paracellular path.[4]

TJs have a composite molecular structure consisting of multiple protein complexes (with more than 50 proteins identified) that include a series of transmembrane tetraspan proteins, named occludin, claudins, and tricellulin, able to develop fibrils crossing the membranes and creating a connection with adjacent cell proteins. In addition, single-span transmembrane proteins are included and are mostly

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represented by junctional adhesion molecules (JAMs, belonging to the immunoglobulin superfamily). The claudin proteins are considered to be the structural pillar of TJs.[5] Specifically, TJ sealing, fundamental to avoid paracellular permeability, is provided by claudin-1, -3, -4, -5, and -8, while claudin-2 can form charge-selective pores. Less information is available for the specific activities of claudins-7, -12, and -15 and occludin.[6]

The transmembrane proteins strictly interact with the intracellular scaffold proteins, such as zonula occludens (ZO-1, ZO-2, and ZO-3) and cingulin tight fitting the actin cytoskeleton. In particular, increased paracellular permeability is activated by perijunctional actomyosin ring contraction induced by myosin light-chain kinase (MLCK). In addition, other signaling proteins, including protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), together with phosphorylation are involved in the regulation pathways of assembly, disassembly, and maintenance of TJ-specific properties.[7] Finally, AJs, together with desmosomes and GJs located beneath the TJs, are involved in the cell-to-cell adhesion and intracellular signaling but do not seem to contribute to paracellular permeability.[8]

By considering the complex interplay of functions and activities of TJ proteins and signals regulating the fluxes/exchanges of molecules between the lumen and the environment, it is clear that TJ barrier integrity is essential for human health and metabolic homeostasis.

In fact, an impairment or defect in IB function can lead to modest (i.e., subclinical) but chronic immune system activation that might contribute to the pathogenesis of intestinal diseases, such as inflammatory bowel disease,[4] celiac disease,[9] intestinal bowel syndrome,[10] and colon cancer.[11] In addition, recent research showed a possible correlation of IB dysfunction with several clinical conditions, such as metabolic syndrome, obesity, non-alcoholic fatty liver disease (NAFLD),[12] diabetes,[13] and inflammatory joint diseases,[14] but also neurological conditions, such as major depression and degenerative disorders, such as Parkinson's disease[15] and multiple sclerosis (MS), involving the central nervous system (CNS).[16]

It is noteworthy that emerging experimental evidence suggests that an alteration of IB function and/or increased IP can actually occur also during aging, thus potentially representing a further mechanism underpinning the activation of the low-grade systemic inflammation process (also named inflammaging) identified in older subjects.[17] The alterations can take place at different levels of the IB, for example, induced by impairment of the epithelium (physical barrier) and/or the immune cell/function or by an alteration of the chemical barrier consisting of the thick mucus layer able to reduce the passage of bacteria through the epithelium (i.e., mucin secretion) or as a result of an inefficient/inadequate microbial barrier (represented by the commensal "protective" bacteria). In this regard, it has been demonstrated that age-associated microbial dysbiosis can increase gut microbiota lipopolysaccharide (LPS) production and promote IP with an increased risk of systemic endotoxemia and inflammation. In particular, bacteria LPS has been demonstrated to activate nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) by triggering the toll-like receptor 4 (TLR4) inflammatory cascade in immune cells (e.g., macrophages and monocytes).[18]

In addition, dysbiosis is not only an age-associated characteristic but can also be found in different clinical conditions associated with inflammation (e.g., obesity, diabetes, and NAFLD).

Thus, intestinal microbiota can be considered a critical regulator of the IP. Gut microorganisms may act directly on IP by affecting TJ properties and activities and indirectly by modulating inflammation, which is a well-recognized factor promoting IP impairment.[19] Consequently, the manipulation of the complex intestinal microbial ecosystem has been proposed as a novel strategy to restore IP.[2]

DIET AND IP

An adequate nutritional status is fundamental to maintain normal IB function (being able to affect all of the components of IB), and accordingly, malnutrition is associated with increased IP.[20] For example, Guerriero et al.[21] showed that a depletion of glutamine, tryptophan, and zinc could lead to increased IP.

Overall, it has been demonstrated that dietary patterns are a dominant factor in shaping the intestinal microbiota.[22] Hence, strategies to modify the relative abundance of specific bacterial groups by means of dietary interventions have been proposed with the aim also to modulate the concentrations of microbial metabolites in the gut, affecting inflammation.[23]

It has been demonstrated that the Western diet, characterized by high-energy and high-fat intake or high-fructose consumption, can alter IP by affecting the gut microbiota composition.[21] In addition, this dietary pattern often involves the consumption of food components, such as specific fatty acids, alcohol, additives, gliadin, and chitosan, and food-processing methods that are known to alter IB physical structure homeostasis and/or commensal microbial homeostasis. On the other hand, a healthy dietary pattern, such as the Mediterranean diet (MD), rich in fruit, vegetables, legumes, and unrefined cereals, has been suggested to positively affect IP and related conditions.[21] This may be related to an increased production of short-chain fatty acids (SCFAs), including acetate, propionate, butyrate, and valerate,[2] by gut commensal bacteria following fiber degradation provided by the MD dietary pattern. These metabolites have been suggested to play an important role as substrates for a functional colonic epithelium and the maintenance of the IB. For example, butyrate was shown to affect TJ integrity but also inhibit tumor necrosis factor α (TNF-α) release and inflammation.[23] In addition, butyrate has shown to increase expression of claudin-1 and ZO-1, to reverse the aberrant expression of ZO-1 and decrease LPS translocation, leading to inhibition of macrophage activation and pro-inflammatory cytokine production.[24] Moreover, plant-based dietary patterns, including MD, are also commonly abundant in bioactive compounds, such as polyphenols (PPs), that have been recently in the spotlight of research for their potential modulatory properties with respect to IP.[25]

RATIONALE FOR PP CONTRIBUTION TO A PROTECTIVE DIETARY PATTERN IN THE CONTEXT OF IP

PPs are secondary metabolites of plants, widely distributed in fruits, vegetables, and plant-derived foods. A diet rich in fruits, vegetables, and plant-based beverages has been estimated to provide about 1 g of PPs/day,[26] with significant variations depending also upon the extent of consumption of beverages rich in PPs (tea, wine, coffee, and fruit juices). The basic monomer in PPs is the phenolic ring. Phenols can be mainly classified into phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (flavons, flavanones, flavanols, flavonols, isoflavones, and anthocyanidins), stilbenes (i.e.,

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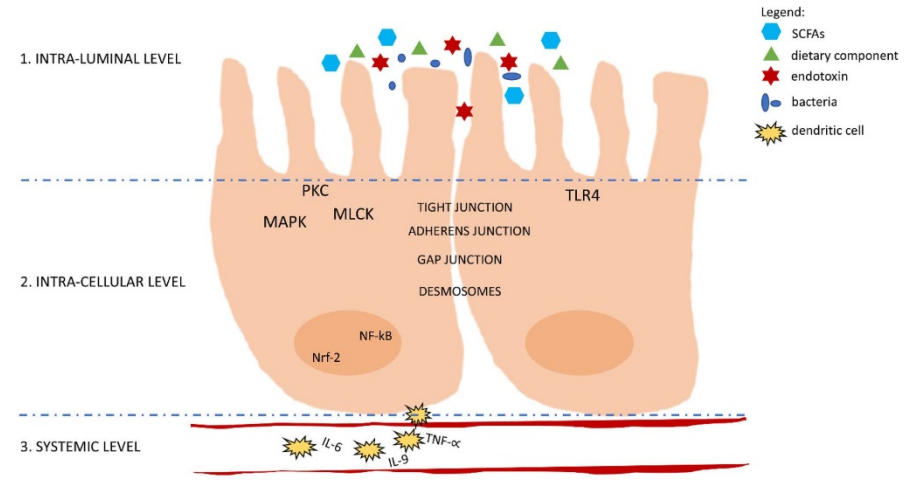

Figure 1. Putative effects of PPs on IP at different physiological levels: (1) intraluminal level, modulation of microbiota composition, endotoxin and/or SCFA production, redox status, and dietary component absorption and/or activity; (2) intracellular level, regulation of expression of TJ, AJ, GJ, and desmosome proteins, upregulation of kinases and Nrf-2, and downregulation of NF-κB and TLR4; and (3) systemic level, maintenance of the functional immune system and regulation of inflammatory processes (toward a reduced pro-inflammatory status).

resveratrol), and lignans. PPs are recognized to be poorly bioavailable, rapidly absorbed, and extensively metabolized by gut microbiota.[27] Additional biotransformation can occur in the liver and kidneys through methylation, glucuronidation, and sulfation reactions of phenolic hydroxyl groups;[28] for these reasons, the concentration of the native compounds in the blood is low compared to their metabolic derivates (from nanomoles up to micromoles per liter).

PPs and their metabolites are widely studied for their numerous biological activities, including antimicrobial, antiproliferative, antioxidant, and anti-inflammatory functions.[29] These effects are exerted at both intestinal and systemic levels. In particular, PPs may exert their effects by downregulating inflammatory genes (i.e., NF-κB) and upregulating cytoprotective and antioxidant genes [i.e., nuclear factor erythroid 2-related factor 2 (Nrf-2)]. This modulation may bring a reduction of cytokine production [e.g., interleukin (IL)-8, IL-1β, and TNF-α] and boost the bodies' own antioxidant status [heme oxygenase 1 (HO-1), superoxide dismutase (SOD), and glutathione peroxidase (GPx)].[30] Furthermore, recent reviews[31,32] have shown that PPs may affect, in either a positive or negative way, pattern recognition receptors, such as toll-like receptors and nucleotide-binding oligomerization domain proteins, whose activation in epithelial cells may lead to intestinal inflammation. Moreover, PPs seem to be involved in the regulation of epigenetic factors through interaction with the enzymes responsible for DNA methylation and acetylation by reducing intestinal inflammation.[32]

Several studies documented the effects of PPs in the modulation of the intestinal microbial ecosystem. However, the mechanisms by which these compounds modulate the gut microbiota remain unclear. Some studies report that the interaction between PPs and microbiota may involve interference with enzymatic expression and activity and modulation of specific pathways related to antioxidant and anti-inflammatory activity.[33] In addition, PPs has been proposed to exert a prebiotic effect potentially inhibiting the pathogenic bacteria and stimulating the growth of beneficial microbes.[34−36] In fact, the microbiota can extensively metabolize PPs in numerous derivatives that could affect not only the composition of microbiota but also specific signaling pathways.[33] Another important aspect regards the possible involvement of PPs in the metabolism of colonic products, such as SCFAs, sterols (cholesterol and bile acids), and microbial products of non-absorbed proteins, which may directly or indirectly counteract or suppress pro-oxidant and/or pro-inflammatory responses with an overall improvement of gut health.[37]

To unravel the complex scenario related with PP−microbiota interaction *in vivo*, a combination of metabolomic, microbiome, and metagenomic approaches is strongly demanded.[33]

Finally, in the last few decades, specific research has been devoted to the evaluation of PPs as promising protective factors and regulators of epithelial homeostasis and the IB function. In particular, a direct/indirect effect of the regulation of TJ proteins has been investigated.

■ MECHANISMS OF PP REGULATION OF IP

At present, the exact mechanisms linking PPs with the intestinal epithelial barrier function have not yet been established (Figure 1). Some studies hypothesized a direct/indirect involvement of NF-κB signaling in the onset of IP. This pathway is recognized as one of the most important mediators of the inflammation; cytokines and interleukins have been shown to activate NF-κB and impair the epithelial barrier function by TJ disassembly. Conversely, PPs have documented to block NF-κB activation by inhibiting IκB kinase (IKK) phosphorylation and/or preventing proteasomal degradation of IκB.[38]

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Table 1. Summary of the Main *In Vitro* Studies Highlighting the Mechanisms of Action of PP Compounds in the Modulation of Barrier Integrity and Function

reference	cells	stimulation	PP source and dose	signaling pathway	response/marker	effect
Atkinson and Rao[60]	Caco-2	acetaldehyde	genistein (30–300 μM)	↓ tyrosine kinase	TEER,[a] occludin, and ZO-1[b]	↑ TEER ↑ occludin ↑ ZO-1
Watson et al.[59]	T84	IFN-γ[c]	epigallocatechin gallate (100 μM)	↓ STAT-1[d] ↓ MAPK[e]	TEER	↑ TEER
Amasheh et al.[51]	Caco-2		quercetin (0–200 μM)	↓ MLCK[f] and PKCβ[g]	TEER, occludin, claudin-1, claudin-3, claudin-4, and claudin-7	↑ TEER ↑ claudin-4 = claudin-1 = claudin-3 = claudin-7 = occludin
Suzuki and Hara[52]	Caco-2		quercetin (0–100 μM)	↓ PKCδ	ZO-2, occludin, claudin-1, and claudin-4	↑ ZO-2 ↑ occludin ↑ claudin-1 ↑ claudin-4
Amasheh et al.[60]	HT29/B6	TNF-α[h]	berberine (50 μM)	↓ NF-κB,[i] PI3K/Akt,[j] and tyrosine kinase	claudin-1 and claudin-2	↑ claudin-1 ↓ claudin-2
Chuenkityanon et al.[64]	ECV304	H_2O_2[k]	quercetin (10 μM)	↓ p38[l]	ZO-1 and occludin	↑ ZO-1 ↑ occludin
Rogoll et al.[61]	T84		(+)-catechin (10 μM) (−)-epicatechin (10 μM) quercetins (10 μM) phloretins (20 μM) D-(−)-quinic acids (10–50 μM) *p*-coumaric acids (10 μM) caffeic acids (20 μM)	↓ TJ permeability	TEER, ZO-1, occludin, and claudin-4	↑ TEER ↑ ZO-1 ↑ occludin ↑ claudin-4
Shin et al.[66]	HCT-116		anthocyanin mixture (45 μg/mL; delphinidin, cyanidin, petunidin, delphinidin, malvidin, peonidin-3,5-diglucoside, cyanidin, petunidin, peonidin, and malvidin-3-glucoside)	↑ p38	TEER, claudin-1, claudin-3, and claudin-4	↑ TEER ↓ claudin-1 ↓ claudin-3 ↓ claudin-4
Suzuki et al.[53]	Caco-2		kaempferol (100 μM)	↓ TJ permeability	TEER, ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4	↑ TEER ↑ occludin ↑ claudin-1 ↑ claudin-3 ↑ claudin-4 ↑ ZO-1 ↑ ZO-2
Noda et al.[54]	Caco-2		chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin (100 μM)	↓ TJ permeability	TEER, ZO-1, ZO-2, JAM1, claudin-1, claudin-3, and claudin-4	↑ TEER (negative effect for chrysin) effect on TJ proteins was compound-dependent
Amasheh et al.[62]	HT-29/B6	IFN-γ and TNF-α	quercetin (200 μM)	↓ TJ permeability	TEER, claudin-1, claudin-2, claudin-3, claudin-4, claudin-7, and occludin	↑ TEER ↓ claudin-2 ↓ claudin-3 = claudin-1

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Table 1. continued

reference	cells	stimulation	PP source and dose	signaling pathway	response/marker	effect
						= claudin-4
						= claudin-7
						= occludin
Noda et al.[55]	Caco-2		naringenin (100 μM)	↑ Sp1[m]-dependent transcriptional regulation	TEER, ZO-1, ZO-2, occludin, JAM-A,[n] claudin-1, claudin-3, and claudin-4	↑ TEER
				↓ TJ permeability		
						↑ claudin-1
						↑ claudin-4
						↑ occludin
						= ZO-1
						= JAM-A
Cao et al.[56]	Caco-2	IFN-γ and TNF-α	berberine (100 μM)	↓ MLCK	occludin, claudin-1, ZO-1, and IP	↑ occludin
						↑ claudin-1
						↑ ZO-1
						↓ IP
Carrasco-Pozo et al.[57]	Caco-2	indomethacin	mix of quercetin (33 μM), resveratrol (438 μM)), rutin (164 μM), and epigallocatechin gallate (218 μM)	↑ epithelial barrier function	TEER, FD4,[o] ZO-1, and occludin	↑ TEER (no effect with rutin)
						↓ FD4 (no effect with rutin)
						↑ ZO-1 after quercetin
						↑ occludin after quercetin
Piegholdt et al.[58]	Caco-2	TNF-α	biochanin A (50 μM) and prunetin (50 μM)	↓ NF-κB, ERK,[p] and tyrosine kinase	TEER, claudin-1, occludin, ZO-1, and E-cadherin	↑ TEER
						= claudin-1
						= ZO-1
						= E-cadherin
Park et al.[41]	Caco-2		theaflavin 3′-O-gallate (20 μM)	↓ MLCK	occludin, claudin-1, and ZO-1	↑ occludin
						↑ claudin-1
						↑ ZO-1
Contreras et al.[42]	Caco-2	TNF-α	(−)-epicatechin (0.5–5 μM)	↓ NF-κB, p-IKK-α,[q] p-IkBα, and MLCK	occludin, ZO-1, and claudin-2	↑ ZO-1
						= occludin
						= claudin-2
Valenzano et al.[43]	Caco-2		berberine (50–200 μM) quercetin (100–400 μM)	↑ epithelial barrier function	TEER, claudin-1, claudin-2, claudin-3, claudin-4, claudin-5, claudin-7, occludin, tricellulin, and D-mannitol	↑ TEER (only berberine)
						quercetin (↑ claudin-2, claudin-4, and claudin-5 and ↓ tricellulin)
						berberin (↓ claudin-2 and D-mannitol)
Ling et al.[65]	IPEC-J2	deoxynivalenol	resveratrol (0–200 μM)	↓ p38, ERK, and p-JNK[r]	TEER, FD4, claudin-1, claudin-3, claudin-4, claudin-7, occludin, and ZO-1	↑ TEER
						↑ occludin
						↑ claudin-3
						↑ claudin-4
						↓ FD4
						= claudin-1
						= claudin-7
Wang et al.[44]	Caco-2		PP-rich propolis extract (25 and 50 $\mu g/mL$)	↑ AMPK-α,[s] ERK1/2, Akt, and p38	ZO-1 and occludin	↑ TEER
						↑ occludin
						↑ ZO-1

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Table 1. continued

reference	cells	stimulation	PP source and dose	signaling pathway	response/marker	effect
Azzini et al.[45]	Caco-2		three different PP-rich extracts from chicory (0.2, 1.3, 10, 17, 34, and 70 μM)	↑ epithelial barrier function	TEER	↑ TEER
Luescher et al.[38]	Caco-2	TNF-α	xanthohumol (chalcone, 10 μM) and isoxanthohumol (prenylflavone, 10 μM)	↓ TJ permeability	TEER	↑ TEER
Cremonini et al.[46]	Caco-2	TNF-α	cyanidin, delphinidin, malvidin, petunidin, or peonidin-3-*O*-glucoside (0.25−1 μM)	↓ IKK and p65 phosphorylation	TEER	↑ TEER (only cyanidin, delphinidin, and ACN-rich plant extracts)
		IFN-γ	crowberry extract (1−10 μg/mL)			
			anthocyanin-rich plant extracts [black chokeberry, black kernel rice, wild blueberry, bilberry, crowberry, domesticated blueberry, and red grape (5 μg/mL)]			
Rybakovsky et al.[47]	Caco-2	^{14}C-D-mannitol	theaflavins (5−20 μg/mL)	↑ membrane permeability	claudin-1, claudin-2, claudin-4, and claudin-5	↑ TEER (quercetin)
			quercetin (100−400 μM)			↓ transepithelial mannitol permeability (quercetin)
			berberine (50−200 μM)			↑ claudin-2
						= claudin-1
						= claudin-4
						= claudin-5
Van Buiten et al.[48]	Caco-2		decaffeinated green tea PPs (0−100 μg/mL)	↓ paracellular permeability	TEER, IL-6,[z] and IL-8	↑ TEER
						↓ IL-6
						↓ IL-8
Li et al.[67]	MODE-K	LPS[aa]	naringin (50−200 μM)	↓ NF-κB and MLCK/MLC	TNF-α, IL-10, IL-6, MLCK, p-MLC/MLC, p-p65/p65, and p-IkBα/IkBα	↓ TNF-α
						↓ IL-10
						↓ IL-6
						↓ MLCK
						↓ p-MLC/MLC
						↓ p-p65/p65
						↓ p-IkBα/IkBα
Cremonini et al.[49]	Caco-2	TNF-α	(−)-epicatechin	↑ ERK1/2, and AMPK and ↓ NF-κB	NOX1/NOX4,[bb] FITC[cc]−dextran transport, and TEER	↑ TEER
						↓ FITC
						↓ NOX1/NOX4
Vazquez-Olivo et al.[50]	Caco-2		four PP-rich mango extracts (100 μg/mL)	↑ membrane permeability	Papp	↑ improvement of apparent membrane permeability
			gallic acid (100 μg/mL)			
Nunes et al.[53]	HT-29	TNF-α, IL-1, and IFN-γ	non-alcoholic polyphenolic red wine extract (catechin, oligomeric procyanidins, anthocyanin, phenolic acids, ethyl cinnamate, and condensed tannin), 200, 400, and 600 μg/mL	↓ paracellular permeability	occludin, claudin-5, and ZO-1	↑ occludin
						↑ claudin-5
						↑ ZO-1

[a]TEER = transepithelial eletrical resistance. [b]ZO-1 = zonula occludens 1. [c]IFN-γ = interferon γ. [d]STAT-1 = signal transducer and activator of transcription 1. [e]MAPK = mitogen-activated protein kinase. [f]MLCK/MLC = myosin light-chain kinase. [g]PKC = protein kinase C. [h]TNF-α = tumor necrosis factor α. [i]NF-κB = nuclear factor κB. [j]PI3K/Akt = phosphoinositide 3-kinase. [k]H_2O_2 = hydrogen peroxide. [l]p38 = p38 pathway. [m]SP-1 = specific protein transcription factor 1. [n]JAM-A = junctional adhesion molecule A. [o]FD4 = fluorescein isothiocyanate-labeled dextran. [p]ERK1/2 = extracellular signal-regulated kinases 1 and 2. [q]p-IKKα = phosphorylated IκB kinase α. [r]JNK = c-Jun N-terminal kinase. [s]IL-6, IL-8, and IL-10 = interleukins 6, 8, and 10. [t]AMPK = AMP-activated protein kinase. [u]LPS = lipopolysaccharide. [v]NOX = nicotinamide adenine dinucleotide oxidase. [w]FITC = fluorescein isothiocyanate.

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Other important factors potentially involved in increasing IP are the multiple protein kinases, such as MAPK, phosphoinositide-3-kinase (PI3K)/Akt, PKC, tyrosine kinase, MLCK, and adenosine monophosphate (AMP)-activated protein kinase (AMPK). Most of them are regulators of fundamental biological processes in epithelial cells, including barrier function, primarily through regulating TJ expression. Some PPs (e.g., quercetin, curcumin, epigallocathechin 3-gallate, and myricetine) have shown to improve the epithelial barrier function through the inhibition of different kinases (PKC and MLCK) involved in phosphorylation of target proteins controlling IP.[3,30,39]

To ascertain the availability of data supporting the role of PPs on IP, a literature search has been performed using the following terms "intestinal permeability" OR "intestinal barrier" AND "polyphenols" OR "bioactives" OR "phenolics" as keywords in PubMed. The use of the word "polyphenols" as a specific keyword consistently reduced the number of results. On the contrary, a more appropriate search with single PP subclasses AND "intestinal permeability" provided a larger number of *in vitro* and animal studies mainly summarized in Table 1 and 2 and an apparent lack of human intervention studies.

***In Vitro* Studies.** The main lines of evidence on the *in vitro* effects of PPs in the modulation of the potential mediators and regulatory pathways involved in the IP are reported in Table 1. Most of the studies are performed on the Caco-2 cell line,[38,40−58] as a model of the IB, followed by T84 and HT29/B6 cells (colonic adenocarcinoma cell line),[59−63] IPEC-J2 cells (intestinal porcine enterocytes), and ECV304 cells (human endothelial cell line).[64,65] The main evidence of protection is available for berberine, quercetin, and catechin tested in a range of concentrations between 10 and 200 μM (from physiological to pharmacological concentrations). Other PPs tested included genistein, anthocyanins, resveratrol, theaflavin, and a mix of PPs. Most the studies have shown an increase in transepithelial electrical resistance (TEER) across a cellular monolayer, confirming the integrity and functional permeability of the membranes.[38,43−49,53−55,57,58,62,65,66] In addition, most the PPs tested have shown to increase the expression and/or production of numerous TJ proteins, including ZO-1, occludin, and the family of claudins, whose alteration may result in increased paracellular permeability.[41,42,44,53,55−57,63,65] Finally, some studies have reported the capacity of PP to counteract the inflammatory process induced by TNF-α and interferon γ (IFN-γ) downregulating the expression of several interleukins, such as IL-8 and IL-6.[48,67]

Animal Studies. Table 2 reported the effects of PPs and PP-rich extracts in the modulation of IP in animal models.[44,49,67−74] Most of the studies were performed in healthy rat models (i.e., Wistar rats and Sprague Dawley rats), and IP was induced by stimuli, such high-fat diets, mannitol, inflammatory cytokines, or chemicals.[44,72,74] Two studies used mice with IL-10 deficiency to test the effect on IP.[69,70]

The main PPs used were obtained from grape seed extracts (1% GSE; grams of GSE per gram of dry food weight)[69,70] and grape seed proanthocyanidin extracts (5−50 mg/kg).[74] Other studies included berberine (200 mg/kg),[68] (−)-epicatechin (2−20 mg/kg),[49] and epigallocatechin-3-gallate (about 3 mg/mL).[73] Some studies were performed by testing anthocyanin-rich raspberry extract, PP-rich propolis extract, and oregano essential oil.[44,72] The doses administered ranged from nearly physiological (epicatechin) up to supraphysiological (i.e., berberine). The duration of the intervention varied from a few days (3−10 days) up to several weeks (15−16 weeks).

On the whole, the results obtained support an improvement of IP following the intervention with PPs and PP-rich extracts. In particular, the studies showed the capacity of PPs to upregulate some important genes, such as AMPK and ERK, and downregulate NF-κB as pathways involved in the inflammation process. In line with the observations reported in the *in vitro* studies, the compounds tested have shown to increase the expression of ZO-1, occludin, and several claudins involved in the functioning of TJs.

Human Studies. The number of human intervention studies with IP as primary or secondary outcome increased in the last few years, as also documented by the number of trials made available and reported in public registers (i.e., ISRCTN and ClinicalTrial.gov).

Most of these studies were performed or are ongoing using probiotics, prebiotic fibers, dietary supplements, and sugars. Only four studies seem to have explored the potential beneficial effects of PPs/PP-rich foods on IP in humans (Table 3).[75−78] The studies differ in terms of population (overweight/obese, cyclists, and older subjects), foods administered (green tea, flavonoid-rich beverage, and mix of PP-rich foods), dose of bioactives (650 mg of flavonoids and 750 mg of PPs), duration of intervention (from 2 weeks up to 8 weeks), and marker of IP selected (endotoxin, lactulose/mannitol ratio, and zonulin levels). The trials are still ongoing, and the results will be useful to increase the understanding on the actual role of PPs and PP-rich foods in humans, where a large number of factors can interact, affecting IP. For example, it is well-recognized that PPs are poorly bioavailable and are biotransformed by gut microbiota into metabolites that can be absorbed in the colon. At the same time, PPs may modulate the composition of the gut microbial community, shaping toward protective symbionts and reducing pathobionts. The complex and not fully elucidated two-way interaction between PPs and gut microbiota is postulated to play a potential direct/indirect role on IP regulation.

In this context, the MaPLE project (Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly) has been developed with the aim to test the hypothesis that changing the diet of older subjects with established enhanced IP by increasing their PP consumption can alter the intestinal microbial ecosystem (IME) in a way that is beneficial for IB function, resulting in reduced IP and decreased translocation of inflammogenic bacterial factors from the digestive tract into the bloodstream.[78] To test this hypothesis, a multidisciplinary approach has been used (i) to evaluate the impact of a PP-rich dietary pattern on IB, IP, and IME in a target group of older subjects and (ii) to investigate the possible mechanisms involved in the PP−microbiota−IP interactions through *in vitro* and animal models.

Findings obtained from our and other studies will be "pivotal" for the development of new and advanced hypothesis and experimental approaches in this complex area of research.

■ SOME CONSIDERATIONS ON IP ASSESSMENTS IN DIFFERENT CONTEXTS

IP can be evaluated through numerous methodologies, and consequently, data obtained can differ among studies. The techniques vary depending upon the setting (*in vitro*, *ex vivo*, or *in vivo* models), the models (cells, animals, and humans), and the markers (i.e., ions, macromolecules, bacteria, and bacterial products) but also the compartments (i.e., tissues, blood, and urine). The measurement of IP can be performed through *ex*

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Table 2. Summary of the Main Evidence from Animal Models Reporting the Effects of PPs and PP-Rich Extracts in the Modulation of Barrier Integrity and Function

reference	animal model	diet	PP source and dose	signaling pathway	response/marker	main findings
Gu et al.[68]	male C57BL/6 mice	BBR versus C	BBR, berberine (200 mg/kg)	↓ MLCK[a]	IP	↑ ZO-1
		LPS stimulation	C, control diet		claudin-1	↑ occludin
			7 days		claudin-4	↑ claudin-1
					occludin	↑ claudin-4
					ZO-1[b]	↓ IP
Yang et al.[69]	C57BL/6 (WT) and IL-10$^{-/-}$-deficient (IL-10$^{-/-}$; IL10KO) female mice	GSE versus C	GSE, grape seed extract (0 or 1% GSE)[d]	↓ NF-κB[e]	claudin-1	↑ claudin-1
		dextran sulfate sodium stimulation	C, standard rodent diet		claudin-2	↓ claudin-2
			16 weeks			
Wang et al.[70]	IL10-deficient mice (IL10KO)	GSE versus C	GSE, grape seed extract (0 or 1% GSE)[d]	↓ AMPK[f]	claudin-1	↑ claudin-1
		dextran sulfate sodium stimulation	C, standard rodent diet		claudin-2	↓ claudin-2
			16 weeks			
Li et al.[71]	BALB/c mice	ARF versus C	ARF, anthocyanin-rich raspberry extract (20 mg/kg)	↓ NF-κB	colonic histological architecture	↑ colonic histological architecture
		dextran sulfate sodium stimulation	C, saline solution as control treatment	↓ MAPKs[g]		
			10 days			
Wei et al.[72]	male Wistar rats	OEO versus C	OEO, oregano essential oil (5 or 20 mg/kg of BW)	↓ SOD[h]	ZO-1	↑ ZO-1
		diquat stimulation	C, saline solution as control treatment	↓ GSH-Px[i]	occludin	↑ occludin
			14 days			
Wang et al.[44]	male Sprague Dawley rats	PPE versus C	PPE, PP-rich propolis extract (0.3%, w/w)[d]	↑ AMPK	ZO-1	↑ ZO-1
		2,4,6-trinitrobenzenesulfonic acid stimulation	C, control diet	↑ ERK[j]	occludin	↑ occludin
			14 days			
Bitzer et al.[73]	male CF-1 mice	DSS[k] treatment + D (0.5% citric acid)	EGCG, epigallocatechin-3-gallate (3.2 mg/mL)		GLP-2[l]	↓ GLP-2
		DE (DDS + EGCG) + D (0.5% citric acid)	C, control diet		LAC/RHA[m]	↓ LAC/RHA
		C diet	3 days		SUC/ERY[m]	↓ SUC/ERY
Gil-Cardoso et al.[74]	female Wistar rats	CAF	CAF, cafeteria diet[d]		ZO-1	↑ ZO-1
		CAF + GSPE	CAF + GSPE, (cafeteria diet + grape seed proanthocyanidin extract, 5–50 mg/kg)		occludin	
		C group	C, control diet		claudin-1	
			15 weeks CAF		JAM-A[o]	
			3 weeks CAF + GSPE			
Cremonini et al.[49]	C57BL/6J mice	HF versus C	CE, (−)-epicatechin (2–20 mg/kg)	↑ ERK1/2	p65[p]	↑ p65 (HF)
		HFE20 versus CE	C, control diet	↑ NF-κB (p65)	GLP-2	↑ GLP-2 (CE and HFE20)
			HF, high-fat diet (60% total calories from fat); HFE20, high-fat diet + 20 mg/kg of epicatechin	↑ AMPK	NOX1/NOX4[q]	↑ NOX1/NOX4 (HF)
			15 weeks			
Li et al.[67]	male Kunming mice	CLP[r] + vehicle	NG, naringin (30 and 60 mg/kg)		TEM[s]	↑ survival CLP + NG (30–60)
		CLP + NG (30)	C, no control diet		FITC[t]–dextrane	↑ IM impairment CLP + vehicle
		CLP + NG (60)	24–72 h		D-lactate	CLP ↑ FITC–dextrane and D-lactate
						CLP + NG ↓ FITC–dextrane (dose-dependent)

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J. Agric. Food Chem. 2020, 68, 1816−1829

Table 2. continued

[a]MLCK/MLC = myosin light-chain kinase. [b]ZO-1 = zonula occludens 1. [c]IL = interleukin. [d]Data on PP characterization were not provided. [e]NF-κB = nuclear factor κB. [f]AMPK = AMP-activated protein kinase. [g]MAPK = mitogen-activated protein kinase. [h]SOD = superoxide dismutase. [i]GSH-Px = glutathione peroxidase. [j]ERK1/2 = extracellular signal-regulated kinases 1 and 2. [k]DSS = dextran sulfate sodium. [l]GLP-2 = glucagon-like peptide 2. [m]LAC/RHA = lactulose/rhamnose ratio. [n]SUC/ERY = sucralose/erythritol ratio. [o]JAM = junctional adhesion molecule. [p]p65 = transcription factor p65. [q]NOX1/NOX 4 = NADPH oxidases. [r]CLP = cecal ligation and puncture. [s]TEM = transmission electron microscopy. [t]FITC = fluorescein isothiocyanate.

vivo and *in vivo* approaches.[79] An example of an *ex vivo* approach includes the use of an Ussing chamber able to measure the transport of ions and molecules (i.e., nutrients and drugs) across various epithelial tissues using fresh intestinal tissue. *In vivo*, the assessment of IP can be performed through permeability assays (i.e., evaluation of the ratio of lactulose/mannitol, sucralose, sucrose, polyethylene glycols, or ^{52}Cr-EDTA in urine), analysis of bacterial-related markers (i.e., endotoxin test, EndoCAb, D-lactate, and butyrate production), markers of epithelial damage (i.e., citrullin, fatty-acid-binding protein, and claudin-3), and/or other related markers (i.e., fecal calprotectin). Finally, histological approaches measuring, for example, globet cell analysis, shedding of the epithelium, or Paneth cell loss, can be performed.[2]

On the whole, on the basis of revised literature, it can be assumed that current *in vitro* permeability models are still far from reflecting an *in vivo* situation. This limits the relevance of data obtained within the cell culture and the possibility to transfer the results to humans. In fact, the comparison between *in vitro* and *in vivo* permeability data is difficult and dependent upon numerous factors, including the type of cells used, the molecule under study, the transport route evaluated, and the method used for the assessment of IB function and permeability [i.e., mainly transepithelial electrical resistance (TEER) and biomarkers of epithelial integrity], which can significantly affect the results obtained, making it difficult to identify the best approach.

A novel biomarker of IP *in vivo* is zonulin, a protein secreted by enterocytes but also from other type of cells (i.e., epithelial cells), known to be a physiological modulator and, thus, to control IP reversibly via intercellular TJs.[80] Increased zonulin serum levels have been observed in many gut-related diseases, and emerging evidence suggests an increased zonulin level in specific subjects (e.g., older persons)[81] and in different diseases or conditions (e.g., diabetes and obesity).[82,83] The reliability and accuracy of the different markers to assess IP is clearly a fundamental part of the recent discussion and a hot topic considering the increasing demand for non-invasive diagnosis tools.[84] In this regard, the concurrent evaluation of different markers of IP seems highly recommendable to improve reliability of findings on IB function.

■ CONCLUSIONS AND FUTURE PERSPECTIVES

There is increasing demand for non-invasive strategies able to modulate critical regulatory functions for human health, such as IP, which can play a role in the pathogenesis of intestinal and systemic diseases. The improvement or manipulation of the diet, for example, increasing or reducing specific nutrients and/or including food bioactives, such as PPs, is recognized as a potential powerful tool to be explored also in the context of IP. From data available, PP activity seems to be plausibly a consequence of multiple mechanisms, which may also depend upon the type and amount of compounds considered. The results from *in vitro* studies have shown the capacity of PPs to increase the expression and/or production of numerous TJ proteins and to reduce the release of several interleukins/cytokines. These results are partially in line with the findings obtained in the animal models, showing the capacity of PPs to up-/downregulate some important genes involved in the inflammatory process. With regard to human studies, recent literature suggests that PPs may modulate IP through a number of direct and indirect effects, including the impact on the intestinal ecosystem and immune system. This type of research

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Table 3. Summary of the Ongoing Human Studies Evaluating the Effect of PPs and PP-Rich Foods on IP

title	source	subject number/characteristics and inclusion criteria	study design	intervention	duration of intervention	markers under study
dietary green tea confection for resolving gut permeability-induced metabolic endotoxemia in obese adults	ClinicalTrials.gov	40 overweight/obese (BMI = 28–40 kg/m^2)	randomized parallel design	test group, green tea extract (GTE)-rich confection	4 weeks	primary outcome: Endotoxin
	NCT03413735	fasting glucose of <126 mg/dL		placebo group, no GTE-rich confection		secondary outcome, gut permeability (lactulose/mannitol ratio and sucralose/erythritol ratio)
	75	normotensive (blood pressure of <140/90 mmHg)		dose, daily (no information about the amount provided in terms of PPs)		microbiota Firmicutes/Bacteroidetes ratio)
		non-dietary supplement user				calprotectin
		non-smoker				green tea PP bioavailability
effect of flavonoids on gut permeability in cyclists	ClinicalTrials.gov	22 male or female of any race or ethnicity between 18 and 49 years of age	randomized crossover design	test group, a high-flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin (8.6%), blueberry powder (2.4%), cocoa powder (1.6%), green tea extract (0.1%), and whey protein isolate (0.6%) containing approximately 620 mg of flavonoids per serving	2 weeks	primary outcome, urinary lactulose/mannitol ratio
	NCT03427879	competed in a road race or triathlon in past 12 months		placebo group, a low-flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin (8.6%), placebo blueberry powder (2.4%), alkalized cocoa powder (1.6%), and whey protein isolate (0.6%), containing approximately 5 mg of flavonoids per serving		plasma intestinal fatty-acid-binding protein
	76	free of chronic disease and gut inflammation conditions		dose, 330 mL/day		secondary outcome, fecal calprotectin
		train at least 3 times per week, 1 h at a time on average				urinary sucralose/mannitol ratio
		willing to prepare and consume provided pre-workout beverage daily				inflammatory markers ($TNF\text{-}\alpha$[b] and IL-10)[c]
		maintain weight (no more/less than 5 kg change)				endotoxin
		willing to avoid consumption of high flavonoid foods/supplements, large dose vitamin and mineral supplements, and NSAIDs[a] or other medication known to affect inflammation during the study period				other variables related to exercise performance
effect of dietary flavonoids on intestinal microbiota, intestinal inflammation and metabolic syndrome	ClinicalTrials.gov	30 overweight/obese (BMI[d] = 25–35 kg/m^2)	randomized crossover design	test group, prepared diet with high levels of dietary flavonoids (340 mg of flavonoids/1000 kcal) with a macronutrient composition of 17% energy from protein, 30% energy from fat, and 53% energy from carbohydrate	6 weeks	primary outcome, fecal calprotectin
	NCT02728570			control group, prepared diet with high levels of dietary flavonoids (10 mg of flavonoids/1000 kcal) with a macronutrient composition of 17% energy from protein, 30% energy from fat, and 53% energy from carbohydrate		serum PCR[e]
	77					serum TNF-α
						serum insulin
						secondary outcome, fecal microbiome composition, SCFAs, eosinophil protein X, and myeloperoxidase
						IP by four sugar differential absorption tests
						serum endotoxin, IL-6, soluble $TNFr\text{-}2$,[f] and fasting glucose

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J. Agric. Food Chem. 2020, 68, 1816−1829

Table 3. continued

title	source	subject number/characteristics and inclusion criteria	study design	intervention	duration of intervention	markers under study
						calculated homeostatic model assessment for insulin resistance
						serum C peptide
						plasma lipid profile
						blood pressure
						other outcome measures, serum resistin, visfatin, adiponectin, and leptin
						body weight
effect of a polyphenol-rich diet on leaky gut in the elderly	ISRCTN registry	60 healthy older subjects	randomized crossover design	test group, habitual diet + PP-rich products (berries and derived products, blood oranges and derived products, pomegranate juice, Renetta apple and purée, green tea, and dark chocolate products)	8 weeks	primary outcome, zonulin serum levels
	ISRCTN10214981	age of >60 years old		control group, comparable diet without the PP-rich products		secondary outcome, total blood bacterial load
	78	IP evaluated by zonulin serum level		dose, three portions of PP-rich food products daily (about 750 mg of PPs)		fecal microbiota composition and metabolism
		adequate nutritional status evaluated with a mini nutritional assessment (MNA) score of ≥24				SCFAs and PP derived metabolites
		good cognitive status tested with mini mental state evaluation (MMSE) score of ≥24				inflammatory, oxidative stress, and related markers
		self-sufficiency assessed with validated tests (e.g., Barthel index for activities of daily living and Tinetti balance assessment)				endotoxin
						LPS-BP[g]
						metabolomic markers
						metabolic and anthropometric markers

[a]NSAID = non-steroidal anti-inflammatory drug. [b]TNF-α = tumor necrosis factor α. [c]IL-10 = interleukin 10. [d]BMI = body mass index. [e]PCR = C-reactive protein. [f]TNFr-2 = tumor necrosis factor receptor 2. [g]LPS-BP = lipopolysaccharide-binding protein.

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is still in its infancy by considering the few human studies available. Future research should be targeted to identify the PPs and/or their metabolites eventually involved in the modulation of IP while also demonstrating their specific dose-dependent mechanisms of action. Meanwhile, *in vivo* studies should be performed to increase understanding of the diet−microbiota−IP axis possibly through the development of well-controlled dietary intervention studies. Finally, by considering the wide discussion in the literature on IP evaluation, further effort is needed to better define the reliability of the already available IP biomarkers and the potential exploitation of new and/or improved candidate biomarkers.

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ABBREVIATIONS USED

IP, intestinal permeability; IB, intestinal barrier; IME, intestinal microbial ecosystem; TJ, tight junction; GJ, gap junction; AJ, adherens junction; JAM, junctional adhesion molecule; ZO, zonula occludens; MLCK, myosin light-chain kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TLR4, toll-like receptor 4; NAFLD, non-alcoholic fatty liver disease; MS, multiple sclerosis; CNS, central nervous system; TNF-α, tumor necrosis factor α; MD, Mediterranean diet; SCFA, short-chain fatty acid; PP, polyphenol; NF-κB, nuclear factor κB; Nrf-2, nuclear factor erythroid 2-related factor 2; IL, interleukin; HO-1, heme oxygenase 1; SOD, superoxide dismutase; GPx, glutathione peroxidase; DNA, deoxyribonucleic acid; IKK, IκB kinase; PI3K, phosphoinositide-3-kinase; AMPK, AMP-activated protein kinase; TEER, transepithelial electrical resistance; INF-γ, interferon γ; ERK, extracellular regulated kinase; MAPLE, microbiome manipulation through polyphenols for managing gut leakiness in the elderly

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MANUSCRITO 6

Estimated Intakes of Nutrients and Polyphenols in Participants Completing the MaPLE Randomized Controlled Trial and Its Relevance for the Future Development of Dietary Guidelines for the Older Subjects

Daniela Martini, Stefano Bernardi, Cristian Del Bo', **Nicole Hidalgo Liberona**, Raul Zamora-Ros, Massimiliano Tucci, Antonio Cherubini, Marisa Porrini, Giorgio Gargari, Raúl González-Domínguez, Gregorio Peron, Benjamin Kirkup, Paul A. Kroon, Cristina Andres-Lacueva, Simone Guglielmetti, and Patrizia Riso

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MANUSCRITO 6

Objetivo: Los objetivos del presente estudio fueron:

- Evaluar la composición nutricional de los menús dietéticos proporcionados en un entorno de atención residencial a personas mayores
- Estimar la ingesta real de nutrientes y polifenoles al tiempo basal en los voluntarios que participan en el estudio MaPLE
- Investigar el impacto de un patrón dietético rico en polifenoles de ocho semanas, en comparación con una dieta de control de ocho semanas, en la ingesta general de nutrientes y polifenoles en los participantes mayores

Metodología: Este estudio se diseñó para investigar el efecto de un patrón de dieta rico en polifeneoles sobre permeabilidad intestinal y otros marcadores inflamatorios. 51 hombres y mujeres mayores de 60 años residentes de Civitas Vitae, Fundación OIC en Padova, Italia, completaron este ensayo controlado aleatorizado cruzado en el que los voluntarios fueron asignados al azar para consumir una dieta rica en polifenoles (dieta PR) o una dieta control (dieta C) durante 8 semanas separado por un período de lavado de 8 semanas. Durante el estudio se tomaron muestras de orina, sangre y heces y, registros de alimentos por pesada.

Durante la intervención, los sujetos recibieron tres porciones diarias de alimentos ricos en polifenoles como sustitutos de los alimentos con menor contenido en polifenoles incluidos en la dieta C. Se estimó la composición energética, de nutrientes y polifenoles de las comidas planificadas que se ofrecen regularmente en el centro, se evaluaron los menús semanales durante las diferentes estaciones (verano, media estación e invierno) incluyendo todas las recetas.

El contenido total de polifenoles (TPC) de los productos ricos en polifenoles fue cuantificado mediante el análisis de su composición, utilizando las bases de datos de Phenol-Explorer (PE) con datos del método Folin-Ciocalteau, incluyendo datos propios (de los productos caracterizados para la intervención) y otras fuentes bibliográficas para aquellos ingredientes que no estaban disponibles en dicha base de datos. La estimación de los polifenoles totales (TP) se calculó como la suma de flavonoides, ácidos fenólicos, lignanos, estilbenos y otras clases de polifenoles expresados en mg (aglicona/100 g). Dichas estimaciones se realizaron utilizando una base de datos propia de composición de alimentos sobre polifenoles, que recopila información de las bases de datos de USDA (para flavonoides, isoflavonas y proantocianidinas) y Phenol-Explorer (para compuestos fenólicos lignanos, estilbenos y otras clases de polifenoles menores) mediante una aplicación informática que

utiliza el sistema de bases de datos relacionales. Los polifenoles se expresaron como mg de agliconas por 100 g.

De los registros de alimentos por pesada, en concreto, se analizaron hasta seis diarios detallados (en los que se registraba la cantidad de alimentos suministrados y la cantidad realmente consumida pesando las sobras) para cada voluntario durante los dos periodos de intervención.

Resultados. Los menús servidos a los participantes proporcionaron en promedio ~770 mg por día de polifenoles totales (TPC), con pequeñas variaciones entre estaciones.

La ingesta real de energía, nutrientes y polifenoles estimada al inicio del estudio fue inferior a la calculada para las estimaciones basadas en los alimentos consumidos de los menús, en consonancia con el hecho de que no se consumieron todos los alimentos en ninguna comida concreta. No hubo diferencias significativas entre mujeres y hombres en ninguna de las variables dietéticas que se evaluaron en la línea de base. En cuanto a los polifenoles, los flavonoides y los ácidos fenólicos fueron las clases más consumidas.

El consumo de productos PR aumentó significativamente ($p < 0,0001$) la ingesta de TPC en unos 600 mg/día en comparación con la dieta C y fue comparable tanto en hombres como en mujeres. Los flavonoides fueron la principal subclase que aumentó en la dieta rica en PR y representaron el 74,6%, seguidos de los ácidos fenólicos (23,3%), mientras que los lignanos y otros polifenoles representaron el resto.

Conclusión: La evaluación de la ingesta de alimentos en sujetos de edad avanzada es crucial para poder verificar la adherencia a las recomendaciones nutricionales. En este contexto, la estimación de la ingesta de compuestos bioactivos dietéticos específicos, como los polifenoles, aunque es especialmente difícil, es necesaria para planificar posibles estrategias de intervención para aumentar su ingesta.

nutrients

MDPI

Article

Estimated Intakes of Nutrients and Polyphenols in Participants Completing the MaPLE Randomised Controlled Trial and Its Relevance for the Future Development of Dietary Guidelines for the Older Subjects

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Abstract: The evaluation of food intake in older subjects is crucial in order to be able to verify adherence to nutritional recommendations. In this context, estimation of the intake of specific dietary bioactives, such as polyphenols, although particularly challenging, is necessary to plan possible intervention strategies to increase their intake. The aims of the present study were to: (i) evaluate the nutritional composition of dietary menus provided in a residential care setting; (ii) estimate the actual intake of nutrients and polyphenols in a group of older subjects participating in the MaPLE study; and (iii) investigate the impact of an eight-week polyphenol-rich dietary pattern, compared to an eight-week control diet, on overall nutrient and polyphenol intake in older participants. The menus served to the participants provided ~770 mg per day of total polyphenols on average with small variations between seasons. The analysis of real consumption, measured using weighed food diaries, demonstrated a lower nutrient (~20%) and polyphenol intake (~15%) compared to that provided by the menus. The feasibility of dietary patterns that enable an increase in polyphenol intake with putative health benefits for age-related conditions is discussed, with a perspective to developing dietary guidelines for this target population.

Keywords: nursing home; residential care; aging; menu; flavonoids; phenolic acids

1. Introduction

It is well recognized that nutrition plays an important role in health status, with increasing evidence of associations between intake of specific dietary components and risk of many non-communicable diseases (NCDs), such as cardiovascular diseases (CVDs), type 2 diabetes, and some types of cancer. For instance, the Global Burden of Diseases has recently indicated that high intake of sodium, low intake of whole grains, and low intake of fruits are the leading dietary risk factors for deaths and disability-adjusted life-years (DALYs) worldwide [1]. These findings have been widely used to prepare national and international dietary guidelines aimed both at recommending the adequate intake of energy and nutrients for different targets of population and possibly at reducing the risk for the most common NCDs [2].

The ageing process affects the nutrient needs of older subjects, whose requirements for some nutrients may be reduced or increased with respect to younger adults. In this life-stage, a variety of factors such as sensory losses, chewing and swallowing problems, and medications may compromise dietary intake and lead to nutritional deficiencies and malnutrition, which has been contributing to the progression of many diseases and common syndromes in older people [3].

For this reason, specific recommendations have been proposed to meet the nutritional requirements of this target group; for instance, energy, protein and fibre intake should be individually adjusted by considering their nutritional status and physical condition and accounting for the presence of specific disease [4]. In addition to macronutrients, micronutrients also play a fundamental role in promoting health and preventing NCDs and their deficiencies are often common in aged people for a number of reasons including reduced food intake or lack of a varied diet, but they are also associated with the vicious cycle promoted by diseases and pharmacological treatments.

It is noteworthy that these factors may also affect the intake, absorption and/or metabolism of bioactive compounds such as polyphenols. In this regard, data on polyphenol intake in different older target groups are not univocal, possibly due to differences in geographical area considered, and in the individual characteristics in terms of health/disease status, and living conditions, as previously evidenced [5]. The interest in the assessment of polyphenol intake and the study of their potential impact on older subjects has been growing by considering several findings suggesting the protective role they can play against age-related diseases and in the promotion of healthy aging [6]. Regarding the changes on polyphenol intake with age, conflicting results have been reported so far, with some studies showing an increased intake [7,8] while others reporting no differences depending on age [9,10].

For the above-mentioned reasons, the nutritional assessment of older people represents a critical issue, which may be particularly true for those living in residential care settings where the prevalence of malnutrition has been reported to be extremely variable, ranging from 1.5 to 66.5% [11]. This represents a current clinical and public health concern at both the individual and population level [12,13]. Several methods have been developed for the assessment of energy and nutrient intake, including food-frequency questionnaires, food diaries and 24-h dietary recalls, all having pros and cons to be considered when choosing the best method to use in each specific context [14]. The estimation of micronutrients and bioactives like polyphenols is particularly challenging, mainly due to methodological issues, including the tool and the database used for the evaluation, as well as the type of polyphenol under consideration (e.g., total polyphenols versus single classes and subclasses of polyphenols) [5]. Being able to make accurate estimates of actual polyphenol intake is a fundamental requirement of developing a better understand of the role of these compounds and their relationship with health or disease conditions. In addition, this information is crucial to define potential polyphenol exploitation for the development of dietary strategies to prevent against age-associated diseases.

Based on these premises, the aim of this research was to evaluate the nutritional composition of nursing home dietary menus and to estimate the actual intake of nutrients and polyphenols in a group of older subjects living in a residential care setting. The assessments were performed as part of the MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) project, funded within the European Joint Programming Initiative "A Healthy Diet for a Healthy

Life" (JPI HDHL), with the aim to investigate benefits of a polyphenol-enriched diet on intestinal permeability in older subjects. An increased gut permeability, often associated with dysbiosis and inflammation, could play a role in the development of some age-related conditions. In this regard, it has been suggested that the intake of polyphenols may represent a promising strategy to improve intestinal permeability (IP) as demonstrated mainly in experimental studies suggesting the involvement of these bioactives in both direct and indirect modulatory mechanisms [15]. In this context, a more accurate estimation of the intake of polyphenols in a vulnerable target such as older subjects, in terms of amount, sources and distribution across the day and even in different seasons, can be of relevance. This could enable a better understanding of their potential benefits and the development of specific recommendations based on findings from dietary intervention studies.

2. Materials and Methods

2.1. Study Design and Population

The study design of the MaPLE randomized controlled trial (RCT) has been previously reported [16]. Briefly, the central hypothesis that this study sought to address was that a polyphenol-enriched dietary pattern would reduce IP and systemic inflammation and cause beneficial changes in various biomarkers of cardiometabolic health, and that this would be associated with changes in the gut microbiota in these older subjects. To this aim, volunteers were randomized to consume a polyphenol-rich diet (PR-diet) or a control diet (C-diet) for 8 weeks following a cross-over design separated by an 8-week wash-out. The development of the PR-diet and C-diet has been reported previously [16]. During the intervention, subjects were given three small portions of polyphenol-rich foods daily as substitutes for foods with lower polyphenol contents that were part of the C-diet (developed by analyzing the regular menus provided to the study participants and specifically assessing the nutrient and polyphenol composition). The characteristics and polyphenol content of the servings provided in the PR-diet for each product are reported in Table 1. The amount of polyphenols provided was more than double that deriving from the replaced products. Data shown include total polyphenol content (i.e., TPC) quantified by analysing products through the Folin–Ciocalteau method [17] and estimates of total polyphenols (i.e., TP). The estimation of TP was calculated as the sum of flavonoids, phenolic acids, lignans, stilbenes and other polyphenol classes expressed in mg (aglycone/100 g). The estimations were performed using an in-house ad hoc database of food composition on polyphenols, compiled from the USDA (fdc.nal.usda.gov/) for databases (for flavonoids, isoflavones and proanthocyanidins) and the Phenol-Explorer (PE; www.phenol-explorer.eu) database (for phenolic compounds lignans, stilbenes and other minor polyphenol classes) through a computer application developed that uses the relational database system. This methodology has been used and previously described [18–21]. Polyphenols were expressed as mg of aglycones per 100 g.

For the intervention study, all the participants were recruited from residents at Civitas Vitae, a large residential care setting (OIC Foundation including both nursing homes and independent residencies for older subjects, Padua, Italy) according to specific inclusion and exclusion criteria. Among inclusion criteria, subjects had to be aged 60 years and to have increased intestinal permeability evaluated by serum zonulin level as previously reported [16].

All the participants recruited into the study were self-sufficient and were in good cognitive health. The Ethics Committee of the Università degli Studi di Milano approved the study protocol (15/02/2016; ref.: 6/16/CE_15.02.16_Verbale_All-7). All the participants were provided with detailed information about their involvement in the study and gave their informed consent before beginning the intervention. The trial was registered in the ISRCTN Registry on 28 April 2017; ISRCTN10214981.

Table 1. Polyphenol content and composition of each serving of MaPLE products included in the dietary intervention, expressed as mg per serving.

	TPC	TP	Flavonoids	Phenolic Acids	Stilbenes	Lignans	Other
Blood orange juice	178	63.4	42.0	21.4	-	-	-
Blood orange fruit	178	34.8	23.1	11.8	-	-	-
Renetta apple	296	225.9	201.2	24.7	-	0.01	-
Renetta apple purée [+]	167	150.6	134.1	16.5	-	0.00	-
Whole blueberry [§]	291	259.5	165.1	94.5	-	-	-
Blueberry purée [❖]	259	199.0	163.6	35.4	-	0.0	0.02
Pomegranate juice	189	135.5	55.1	80.3	-	-	-
Green tea [*]	146	129.2	116.2	13.0	-	0.08	-
Cocoa powder [°]	234	92.2	90.5	1.7	-	0.00	0.01
Chocolate callets	337	167.8	165.4	2.4	0.01	-	-

[§] Frozen whole blueberry product was thawed and prepared before consumption; [❖] Blueberry purée was a ready-to-eat product; [°] Cocoa powder was dissolved in hot milk or water; [*] Green tea was prepared by solubilization of 200 mg of green tea extract in 200 mL of hot water; [+] Renetta apple purée was prepared in controlled conditions and stored at −18 °C until consumption. TPC, total polyphenol content by Folin–Ciocalteau assay; TP, total polyphenols determined by USDA and Phenol Explorer databases.

2.2. Nutritional and Polyphenol Composition of the Menus

To estimate the energy and nutrient composition of the planned meals regularly provided, the weekly menus during different seasons (summer, mid-season and winter) were evaluated (i.e., covering the whole intervention study). To this aim, Metadieta ® software (Me.te.da srl, S. Benedetto del Tronto, Italy) was used to include all the recipes and to estimate the nutritional composition of the different menus.

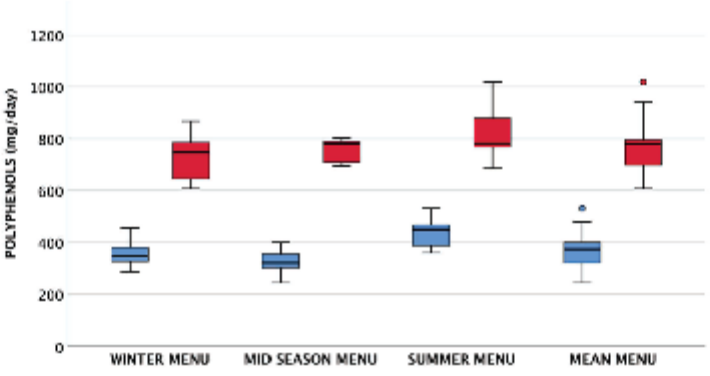
In addition, the TPC content of the menus was estimated by PE databases with the addition of our own data (characterized products in Table 1 used for the intervention) and other literature sources for those ingredients that were not available in those databases [22–24]. TP was instead estimated through the PE/USDA database, as also described in Section 2.1.

2.3. Evaluation of Actual Energy, Nutrient and Polyphenol Intake

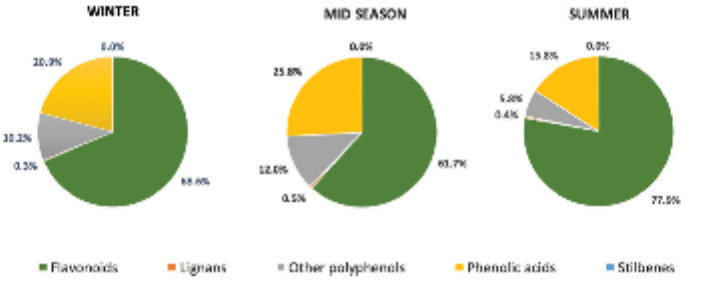
During both intervention periods, weighed food records (WFR) were used to estimate food, energy, nutrient and polyphenol intake as reported in Section 2.2. In particular, up to six detailed daily diaries (recording the amount of foods provided and the amount actually consumed by weighing the leftovers) were analysed for each subject during the two intervention periods. In addition, one diary was filled in by participants at baseline and scheduled the day of blood drawings and sampling according to what was previously reported [16].

2.4. Statistical Analysis

Statistical analysis was conducted using the Statistical Package for Social Sciences software (IBM SPSS Statistics, Version 26.0, IBM corp., Chicago, IL, USA) and R statistical software (version 3.6.). One-way ANOVA was applied to analyse differences between the winter, mid-season and summer menus provided during the intervention in terms of nutrients and polyphenol composition. The nonparametric Wilcoxon–Mann–Whitney test with Benjamini–Hochberg correction pairing the data when possible was performed to ascertain differences at baseline between men and women in terms of actual intake and to verify the impact of treatment (PR vs. C-diet) and gender (men vs. women) on both nutrient and polyphenol intake in participants. The level of significance was set at $p \leq 0.05$. All results were expressed as mean ± standard deviation (SD).



(A)



(B)

Figure 1. Box plot (panel **A**) showing polyphenol content in the seasonal menus, estimated through PE/USDA databases and other published data (TP in light blue) and by Folin–Ciocalteau data as reported in the PE database and other sources (TPC in red); percentage distribution of polyphenol classes (panel **B**) in the seasonal menus. Dots represent mild outliers that are more extreme than Q1 − 1.5 * IQR or Q3 + 1.5 * IQR but are not extreme data (where Q1=quartile 1; Q3=quartile 3; IQR=interquartile range).

3.2. *Actual Energy, Nutrient and Polyphenol Intake at Baseline and during the Intervention*

The actual energy, nutrient and polyphenol intake estimated at baseline for women, men and the whole group of participants is shown in Table 3. Overall, energy intakes, and accordingly nutrient intakes, were lower than calculated for the estimates based on the foods consumed from the menus, in keeping with the fact that not all the food was consumed for any particular meal. There were no significant differences between women and men for any of the dietary variables that were assessed at baseline. This was also confirmed by analysing the data obtained during the intervention study (Supplementary Materials Figure S1), except for simple carbohydrates in women and for total lipids and PUFA in men when comparing intake measured during the PR-diet and the C-diet ($p < 0.05$).

Finally, differences were observed in ω-6 fatty acids, iron and calcium intake following the PR-diet in both women and men.

Table 3. Daily mean energy, nutrient and polyphenol intake at baseline in the whole group of subjects, in women and men.

Variables	All (*n* = 51)	Women (*n* = 29)	Men (*n* = 22)	*p*-Value †
Energy (kcal)	1582 ± 108	1569 ± 110	1599 ± 105	0.318
Total CHO (% of energy)	50.0 ± 2.7	50.0 ± 2.7	49.8 ± 2.7	0.641
Simple CHO (% of energy)	20.4 ± 3.1	20.3 ± 3.3	20.5 ± 3.0	0.939
Proteins (% of energy)	17.8 ± 0.8	18.0 ± 0.8	17.7 ± 0.9	0.216
Animal proteins (% of energy)	12.1± 1.1	12.2 ± 1.0	11.8 ± 1.1	0.262
Plant proteins (% of energy)	5.7 ± 0.6	5.7 ± 0.6	5.7 ± 0.6	0.864
Total lipids (% of energy)	32.1 ± 2.3	31.9 ± 2.2	32.4 ± 2.5	0.441
SFA (% of energy)	8.6 ± 1.5	8.6 ± 1.4	8.7 ± 1.7	0.655
MUFA (% of energy)	16.3 ± 1.3	16.3 ± 1.1	16.4 ± 1.6	0.834
PUFA (% of energy)	3.2 ± 0.5	3.3 ± 0.6	3.2 ± 0.4	0.435
ω-3 (% of energy)	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.753
ω-6 (% of energy)	2.5 ± 0.4	2.6 ± 0.5	2.5 ± 0.2	0.341
Total Fibre (g/1000 kcal)	11.2 ± 1.2	11.3 ± 1.1	11.1 ± 1.3	0.458
Cholesterol (mg)	207.7 ± 30.3	204.3 ± 29.9	212.2 ± 30.9	0.682
Total CHO (g)	210.7 ± 21.5	209.6 ± 22.8	212.2 ± 20.1	0.864
Simple CHO (g)	81.0 ± 15.2	80.8 ± 15.4	81.3 ± 15.2	0.954
Proteins (g)	70.3 ± 4.0	70.2 ± 4.0	70.4 ± 4.0	0.849
Animal proteins (g)	47.6 ± 3.9	47.9 ± 3.7	47.2 ± 4.2	0.536
Plant proteins (g)	22.4 ± 2.7	22.2 ± 2.7	22.7 ± 2.8	0.601
Total lipids (g)	56.2 ± 4.9	55.2 ± 3.8	57.5 ± 5.8	0.192
SFA (g)	15.2 ± 3.0	14.9 ± 2.7	15.5 ± 3.3	0.447
MUFA (g)	28.7 ± 2.4	28.3 ± 1.2	29.1 ± 3.4	0.575
PUFA (g)	5.7 ± 0.7	5.7 ± 0.7	5.7 ± 0.8	0.371
Total ω-3 (g)	1.1 ± 0.3	1.1 ± 0.1	1.1 ± 0.4	0.600
Total ω-6 (g)	4.5 ± 0.6	4.5 ± 0.6	4.4 ± 0.5	0.274
Fibre (g/day)	17.8 ± 2.4	17.8 ± 2.2	17.8 ± 2.6	0.932
Calcium (mg)	804 ± 136	808 ± 128	799 ± 147	0.761
Iron (mg)	9.4 ± 0.9	9.4 ± 0.7	9.4 ± 1.0	0.879
Vitamin B12 (µg)	4.2 ± 1.0	4.2 ± 0.4	4.3 ± 1.5	0.394
Vitamin C (mg)	111.8 ± 56.1	115.1 ± 45.2	107.4 ± 68.8	0.464
Vitamin E (mg)	11.4 ± 2.9	11.5 ± 1.3	11.2 ± 4.1	0.327
Vitamin B1 (mg)	0.8 ± 0.2	0.8 ± 0.1	0.7 ± 0.2	0.156
Folates (µg)	302 ± 73	311 ± 54	289 ± 93	0.536
Vitamin B6 (mg)	1.5 ± 0.3	1.5 ± 0.2	1.4 ± 0.3	0.588
Flavonoids (mg)	181.1 ± 137.5	174.4 ± 123.7	190.8 ± 157.8	0.984
Phenolic acids (mg)	130.9 ± 36.0	126.6 ± 28.6	137.1 ± 44.5	0.598
Stilbenes (mg)	0.04 ± 0.06	0.04 ± 0.06	0.04 ± 0.07	0.542
Lignans (mg)	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.737
Other polyphenols (mg)	27.8 ± 4.3	28.0 ± 3.7	27.6 ± 5.0	0.723

All data are presented as mean ± standard deviation (SD); Data with $p < 0.05$ are significantly different. CHO, carbohydrates; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids. † Comparison between women and men using Wilcoxon–Mann–Whitney test.

Regarding polyphenols, flavonoids and phenolic acids were the most consumed classes and were comparable between women and men.

3.3. *Polyphenol Intake at Baseline and during Intervention*

Figure 2 shows the polyphenol intake at baseline and in the two intervention periods. At baseline, the intake of TPC was 663.4 ± 147.5 mg/d and comparable between women (669.2 ± 160.1 mg/d) and

men (655.2 ± 130.8 mg/d). The consumption of PR-products significantly ($p < 0.0001$) increased the intake of TPC by about 600 mg/d compared to the C-diet and was comparable in both men and women.

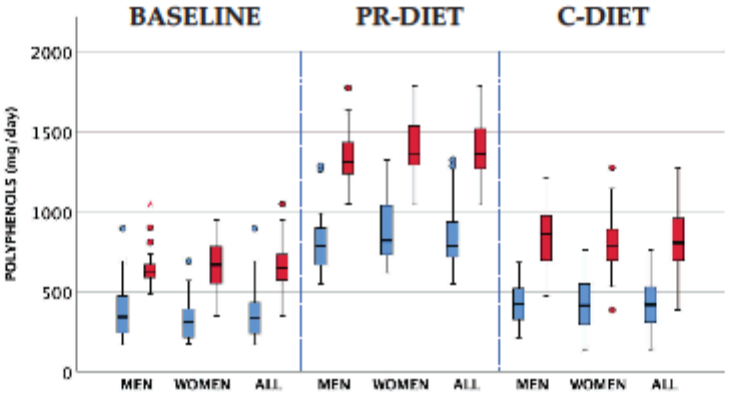


Figure 2. Polyphenol intake at baseline and in polyphenol (PR)-diet and control (C)-diet in the whole group of subjects and stratified by gender. The intake has been estimated using the PE and USDA databases and other published data (TP in light blue) and by Folin–Ciocalteau data as reported in the PE database and other sources (TPC in red). Dots represent mild outliers that are more extreme than Q1 − 1.5 * IQR or Q3 + 1.5 * IQR but are not extreme data. Asterisks are extreme data that are more extreme than Q1 − 3 * IQR or Q3 + 3 * IQR (where Q1=quartile 1; Q3=quartile 3; IQR=interquartile range).

Table 4 shows the contribution of different polyphenol classes to the total polyphenol intake during the PR and C-diet. Flavonoids were the main subclass increased in the PR-rich diet and accounted for 74.6%, followed by phenolic acids (23.3%), while lignans and other polyphenols accounted for the remainder. A treatment effect ($p < 0.0001$) for total flavonoids and phenolic acids was observed (Table 4), while a gender effect was observed for stilbenes showing a higher intake in men compared to women ($p = 0.033$).

Table 4. Intake of total polyphenols and classes (according to PE/USDA databases) during the PR-diet and the C-diet.

Title 1	Flavonoids	Phenolic Acids	Stilbenes	Lignans	Other Polyphenols
PR-diet					
All	634.3 ± 171.8	198.1 ± 52.2	0.2 ± 0.4	0.8 ± 0.3	16.4 ± 5.3
Men	594.6 ± 152.2	201.1 ± 74.3	0.4 ± 0.6	0.7 ± 0.3	16.7 ± 6.3
Women	662.1 ± 163.5	195.9 ± 42.0	0.1 ± 0.2	0.8 ± 0.3	16.2 ± 5.3
p value #	0.098	0.810	0.108	0.206	0.827
C-diet					
All	273.8 ± 119.8	128.2 ± 60.9	0.3 ± 0.5	0.9 ± 0.4	17.0 ± 5.4
Men	260.9 ± 109.6	128.8 ± 57.8	0.4 ± 0.4	0.9 ± 0.4	15.9 ± 5.7
Women	282.9 ± 125.6	127.8 ± 63.0	0.2 ± 0.5	0.9 ± 0.4	17.8 ± 4.9
p value #	0.453	0.271	0.033	0.745	0.271
p value †	<0.0001	<0.0001	0.386	0.303	0.164
p value ¥	<0.0001	0.001	0.575	0.068	0.807
p value §	<0.0001	<0.0001	0.348	0.060	0.331

All data are expressed as mean ± standard deviation (SD); PR, polyphenol-rich diet; C, control diet. † Comparison between PR-diet vs. C-diet in women. ¥ Comparison between PR-diet vs. C-diet in men. # Comparison between women and men in PR-diet and C-diet. § Comparison between PR-diet vs. C-diet in all subjects. Comparisons have been performed using the Wilcoxon–Mann–Whitney test.

Considering the total polyphenol (TP) contribution from the different meals, in the PR-diet, ~50% of polyphenol intake derives from snacks and the remaining ~50% from breakfast, lunch and dinner (Figure 3). In particular, there is a significant contribution to mid-morning and afternoon snacks from the intake of PR-products. Conversely, during the C-diet, only ~15% of the total polyphenols consumed were derived from snacks.

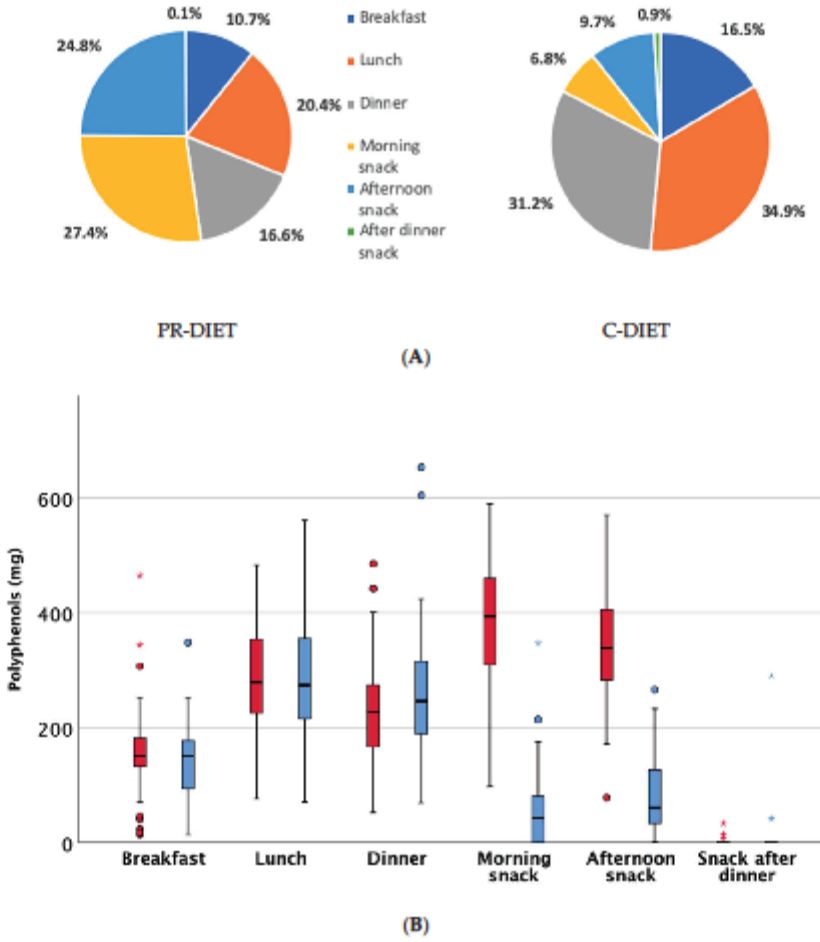


Figure 3. Total phenolic contribution from different meals during the polyphenol (PR)-diet and the control (C)-diet expressed as percentage (Panel **A**), or expressed as amount in mg during the PR-diet (in red) and the C-diet (in blue) as estimated through PE/USDA databases and other published data (Panel **B**). Dots represent mild outliers that are more extreme than Q1 − 1.5 * IQR or Q3 + 1.5 * IQR but are not extreme data. Asterisks are extreme data that are more extreme than Q1 − 3 * IQR or Q3 + 3 * IQR (where Q1=quartile 1; Q3=quartile 3; IQR=interquartile range).

Overall, through the analysis of the menu items provided to the volunteers and recorded in the WFRs during the two intervention periods and by considering the frequencies of consumption of the single ingredients, we estimated the main polyphenol sources contributing to the different meal times.

During the PR-diet, the main foods providing polyphenols at breakfast were fruit and fruit-derived products (e.g., orange, grape, orange juice, apricot jam, etc.), followed by barley coffee and minor contributions from coffee and tea. Polyphenol-rich products on the PR-rich diet were occasionally consumed at breakfast, where green tea, pomegranate juice, chocolate callets and blood orange juice were the most commonly consumed. For lunch and dinner, the main sources during the PR-diet were vegetables (e.g., chard, asparagus, broccoli, carrots), extra virgin olive oil, legumes and spices. A few participants occasionally consumed white wine in small portions (usually 1 glass), which also made a contribution to the polyphenol intake. PR-rich products were mainly consumed as mid-morning and mid-afternoon snacks, as reported in Figure 3. During the C-diet, we found similar foods providing polyphenols at breakfast, lunch and dinner compared to the PR-diet, except for the introduced PR-rich products. Major differences between the two treatments were largely due to the snack foods because only fruits and fruit-based products (i.e., juices), cakes (including sometime chocolate-based cakes) or yogurt were available during the C-diet, whereas a more extensive range of PR-foods were available as snacks on the PR-rich diet.

4. Discussion

The evaluation of the adequacy of diets in older subjects is of utmost importance not only to identify possible deviations from desirable nutritional targets but also to contribute to the development of new recommendations that address gaps in the current guidance. In this context, the MaPLE project has given us the unique opportunity to assess dietary intake in a well-controlled setting where it is also possible to analyse the daily menus provided to the residents, while considering all the recipes and ingredients used for the preparation of the meals. At the same time, long-term residences often have facilities enabling the measurement of food intake (e.g., by collecting multiple weighed food records) and this represents the best procedure to estimate actual consumption. Menu planning in residential care involves modifications of recipes during the year to take account of seasonal changes in ingredient availability and this may partially affect not only nutritional characteristics in terms of macro- and micro- nutrients but also food sources of bioactive compounds with potential impact on host metabolism and other functions.

In the present study, the evaluation of three different menus showed that overall they were comparable in terms of nutritional composition, and also that they were in line with the dietary recommendations for older subjects in Italy (i.e., Italian Reference Intake) [2], with some dissimilarities that are worth highlighting. In regards to total energy, menus provided suitable amounts for the target population, at least in consideration of the main Italian guidelines developed for dietary management in residential care [25]. Some studies carried out in nursing homes showed lower energy provided by menus [26,27], while others reported data higher or similar to our observation [28–30]. The distribution in macronutrients was consistent with the recommendations: carbohydrates accounted for ~47% of total energy intake on average (reference intake range: 45–60% energy (E)), although we found there was a higher intake of simple carbohydrate in comparison with the recommendations (20% E vs. < 15% E) due to the wide use of fruit juices and hot beverages with added sugars as has been commonly reported in this target population. Protein intake derived mainly from animal sources (about two-thirds) and was higher in comparison with the suggested dietary target (1.1 g/kg/day), while total lipid intake was within the reference intake range (20–35% E). Specifically, SFAs were in accordance with the national/international recommendation (<10% E), while total PUFAs were slightly lower than 5% E due to the low intake of ω-6 in favour of higher MUFAs, as can often be found in the Mediterranean areas. The amount of fibre provided by the menus was slightly lower than the suggested dietary target of 25 g per day defined by Italian and international guidelines [2,31]. Regarding micronutrients, iron contribution was adequate while, as also reported in the literature, calcium content in the three menus was lower than the population reference intake (PRI, 1200 mg for both women and men ≥ 60 years) [2]. However, it is worth noting that these data included only calcium derived from recipes and did not consider contributions from other sources such as water and

supplements. Vitamin B1, B6 and B12 provided by menus were higher than reference values, while folates were slightly lower than the established population reference intake of 400 µg per day. With regard to antioxidants, vitamins E and C were both adequate, in particular vitamin C largely exceeded the PRI levels (i.e., 85 mg and 105 mg per day for women and men respectively). Overall, the results on the nutritional composition of the menus suggest that, although they are generally developed following specific guidelines, it is still possible to improve the content of critical nutrients such as fibre, specific micronutrients and bioactives, above all in institutionalised subjects as also reported in the literature [29,30].

Notably, actual food intake in older subjects can be significantly lower with respect to that provided by the menus. For these reasons, we also estimated the actual food consumption through the analysis of detailed and repeated weighed food records. Measured energy and nutrient intake were indeed lower than that provided through the menus (by about 20%), with no differences between women and men. In this regard, it is underlined that the subjects enrolled in the present study generally had a good nutritional status, evidenced also by their anthropometric characteristics (BMI = 26.8 ± 5.5 kg/m^2).

The energy intakes we have reported here (mean approximately 1580 kcal) were slightly lower than those found in the InCHIANTI study, performed on about 1200 free-living older subjects (>65 years) in Tuscany, in which mean energy intakes ranged from 1764 to 2260 kcal/d and from 1521 to 1793 kcal/d in men and women, respectively [32]. However, despite the higher energy intake, in the InCHIANTI study, a large group of subjects reported inadequate intakes of protein, calcium and other nutrients, which have been independently associated with frailty [33]. In our assessments, the lower food intake was associated with reduced protein intake (about 0.9 g/kg day on average), increasing the rate of inadequate intake above all in male subjects (about 22% with intake ≤ 0.71 g/kg per day and only 18% with intake ≥ 1.1 g/kg per day as defined by the suggested dietary target). The consumption of simple carbohydrates in older subjects was confirmed to be higher than the suggested values, while the fat intake appeared to be within the suggested intake range, although the amount of ω-6 fatty acids remained lower than recommended values, as did the intake of calcium, vitamins B1, B6 and folates. These results confirmed previous observations of a potential risk of long-term inadequate intake of nutrients that are fundamental for maintenance of functional and metabolic integrity in older subjects, and that these inadequate intakes are likely due to the actual food intake being significantly less than the amount of food provided to the care home residents in each meal (i.e., incomplete meal consumption is likely a major cause). Moreover, there is not only a problem related to overall food intake but also to specific classes of products that appear to be consumed in lower amounts with respect to others, for example justifying a low intake of fibre that has been found for most, if not all, the subjects under study. This is an underestimated consideration that should be a target for future multidisciplinary research that is able to finally implement guidelines for the achievement of nutritional targets through traditional or possibly alternative strategies.

A major focus in this study was polyphenols because these compounds have the potential to provide further specific benefits to the target population under study. It has been reported that there is a large variation in the polyphenol content of foods available in different periods of the year [34–36], and for this reason we specifically analysed recipes and ingredients used to develop seasonal menus and the results obtained showed a relatively comparable amount of these bioactive compounds (about 770 mg per day on average as TPC) among the different seasons. We could not find other data on the impact of seasonality on polyphenol content of dietary plans provided in long-term residences for older people, while more literature is available in free-living older subjects. In this regard, in the Blue Mountains Eye Study, a longitudinal study performed in Australia [35], the authors found that season did not affect the overall total flavonoid intake in a group of adult and older subjects; however, it was relatively higher in spring and lower in autumn in line with our results. Conversely, Tatsumi et al. [37] showed that total antioxidant intake in a Japanese population (39–77 years) was highest in winter and lowest in summer. The authors attributed this difference to the participants' selection of food (in particular fruits and vegetables) but also beverages across seasons.

In our study, the assessment of actual food consumption at baseline indicated a mean TPC intake of ~660 mg/d (i.e., evaluated by Folin–Ciocalteau through the PE database and specific literature), about 15% less than the amount estimated in the menus served to the study participants. Although a thorough comparison with other published data must be done cautiously because of the differences in the populations under study and the methods and databases used for estimating the intakes of total polyphenols and polyphenol classes, the overall actual intake estimated in the present study seems to be comparable with mean intake observed in the InChianti study [20], but lower with respect to others previously reported.

In fact, assessments in older subjects estimated polyphenol intakes from 333 mg/day up to 1492 mg/day, as reported previously [5]. For example, in the PREDIMED study evaluating a big cohort of Spanish older subjects aged 55–80 years, a mean polyphenol intake of 820 ± 323 mg/day expressed as glycosides was estimated through the PE database, by analysis of food consumption data obtained from FFQs [38]. With regard to the contribution of the classes, total flavonoid intake is generally the larger part of the intake, while data available in some studies suggest that up to 30–40% of the total polyphenol intake can be represented by phenolic acids [5]. Results from the EPIC cohort showed that older subjects tended to have increased intake of flavonoids, stilbenes, lignans, and other polyphenols with respect to younger individuals, while no differences were found for total polyphenol intake [7], and similar findings were reported by Karam and colleagues [8], also showing an impact of gender. In our study in a controlled setting, the data confirmed that the flavonoid subclass was the greatest contributor to total polyphenol intake followed by phenolic acids, while no differences were detected between men and women. Some studies have suggested a higher total and subclass polyphenol intake in females compared to males [8,10], above all when standardized by energy intake, and this may also be the reason for the lack of differences in our study. In addition, it is relevant that the overall lower availability of food alternatives for selection in controlled, with respect to a free-living condition, may have affected eating behaviour, increasing the comparability of the dietary intake.

With regard to polyphenol food sources, tea and coffee have been underlined as the main polyphenol contributors in northern European older subjects, while red wine, extra virgin olive oil and fruit are the main sources in Southern Europe [7,39]. In our evaluation, fruit and fruit juices, vegetable and extra virgin olive oil represent the main food categories providing polyphenols. In addition, we could not demonstrate a different selection of polyphenol sources depending on gender, despite some studies having reported a higher contribution from fruit and vegetables in females compared to males [8,34]. It is noteworthy that in the nursing home, the intake of coffee and wine was strongly limited, if not denied, to limit risks associated with caffeine and alcohol consumption and this may represent an important behavioural difference with respect to what may be observed in free-living older subjects.

The evaluation of habitual polyphenol intake in the older target group was a fundamental step in the process of developing a reliable and evidence based polyphenol rich dietary pattern to use for the intervention trial. In particular, the aim was to approximately double the habitual polyphenol intake of the nursing home residents when on the PR-rich diet in order to reach amounts in the highest quantile of intake identified in previous observational studies, where older subjects were included or specifically considered [7,21,40].

Indeed, the main objective of the MaPLE study was to investigate whether the increased intake of polyphenols might cause a reduction in intestinal permeability (IP) and inflammation associated with an improved intestinal microbial ecosystem, also affecting metabolic and functional activities in the older subjects [16]. In particular, the intervention was developed by replacing three portions per day of low polyphenol foods/beverages with specific products rich in polyphenols. The selection of the products was performed by considering different aspects: (i) the total amount of polyphenols provided, (ii) the contribution of the different polyphenol classes, (iii) the adequate portion of food able to provide a reliable high dose of polyphenols, and (iv) the possible food preparation in order to ensure polyphenol bioavailability. Additionally, foods selection was carried out by considering the

characteristics of the target group and their specific needs in terms of acceptability and suitability in the context of residential care settings. Through the administration of the selected foods, we provided mainly flavonoids (approximately four times higher compared to the amount introduced through the C-diet) and phenolic acids. These bioactives have been suggested as potential modulators of critical factors and specific targets regulating IP, including the impact on microbiota composition and activities [15,41]. Overall, our results demonstrate that it is possible to obtain a significant increase in polyphenol intake in older subjects, through the use of small amounts of well-accepted polyphenol-rich food products. Moreover, it has been demonstrated that the intake is well tolerated and without undesirable effects. Participants appreciated the products and were interested in continuing with the dietary protocol after the end of the trial, suggesting that older people can change their diet if it does not dramatically modify their eating habits.

An interesting observation highlighted was that older subjects preferred the consumption of PR-products during the intervention as mid-morning and afternoon snacks. In fact, the protocol adopted did not fix the timing for the PR-food intake, but the products should have been consumed within the day according to preferences and/or habits. For this reason, our results give an important contribution to the development of dietary guidelines for this target population. At the same time, the analysis of the pattern of consumption of polyphenol-rich foods may also contribute to a better understanding of chronobiological aspects related to the effect of bioactive compounds. In this regard, it has been suggested that the inclusion of polyphenols within the meals may have an impact on related metabolic responses, e.g., through reduction of glucose and lipid levels, inflammation, oxidative stress, and blood pressure, associated with food intake [42–44]. Consuming most of the polyphenols outside of the main meals could also affect their bioavailability for direct absorption and their use as substrates for microbial transformation.

This work has several strengths mainly related to the well-controlled setting of the intervention, enabling both the evaluation of the nutrient and bioactive content of the menus and the actual intake during the whole intervention, ensuring high adherence to dietary instructions. Conversely, possible study limitations include the small sample size and the partial generalizability to free-living community dwelling older subjects. Finally, the limited food choices available in the main standard menus provided could have reduced the possibility of showing gender differences.

5. Conclusions and Perspectives

In conclusion, the assessments performed within the MaPLE project have further underlined the need for a careful revision of dietary menus addressed for older subjects not only to optimize the intake of essential nutrients, but also of bioactive compounds, such as polyphenols, in order to lower the risk of chronic diseases and improve specific metabolic and functional activities during aging. In this context, we have shown that there is a possibility to develop feasible and reliable polyphenol-rich dietary patterns that can be appreciated and consumed by the older population with excellent compliance, while assuring a significant increase in the intake of these bioactive compounds. Moreover, the products and preparations included in the dietary menu have been easily managed in the residential care setting and this is a practical aspect of relevance for the success of new recommendations.

Further studies are needed to: (i) improve tools available to better estimate polyphenol intake and enable comparison of different data in the literature, as previously reported [5]; and (ii) improve dietary recommendations by defining the amount of polyphenol needed in order to obtain, if confirmed, the postulated health benefits in the older subjects. This is not an easy task and imply a strong research effort that needs to consider the potential impact of these results for the development of evidence-based dietary guidelines for the management of age-related conditions.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/8/2458/s1, Figure S1: comparison of percentage energy and nutrient intake during 8-week polyphenol-rich diet (PR-diet) and control diet (C-diet) in older women and men.

Author Contributions: P.R. and S.G. designed the trial and in collaboration with A.C., C.A.-L. and P.A.K. optimised the study protocol including the development of the polyphenol rich dietary pattern. D.M., S.B., C.D.B. and P.R. drafted the manuscript. S.B., M.T., supervised by M.P. and P.R., evaluated the nutritional composition of dietary plans and estimated the actual nutrient intake. N.H.L., R.Z.-R. and C.A.-L. estimated the actual dietary polyphenol intake in collaboration with R.G.-D. and G.P., D.M., C.D.B., B.K. and G.G. performed the statistical analysis. All authors have read and agreed to the published version of the manuscript.

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MANUSCRITO 7

Increased Intestinal Permeability in Older Subjects Impacts the Beneficial Effects of Dietary Polyphenols by Modulating Their Bioavailability

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Aquñi si quieres añadir el doi de la corrección, páginas, ok, pero es al final.

MANUSCRITO 7

Objetivo: El objetivo de este trabajo fue investigar por primera vez el impacto del aumento de la permeabilidad intestinal (PI) en personas mayores sobre la biodisponibilidad de los polifenoles de la dieta y, por tanto, sobre su bioactividad y capacidad para modular la integridad de la barrera intestinal.

Metodología: 51 sujetos de edad avanzada (≥60 años) completaron el ensayo cruzado consistente en una dieta rica en polifenoles (dieta PR) y una dieta de control (dieta C), cada uno de los brazos con una duración de 8 semanas y separados por un período de lavado de 8 semanas. Se midieron los niveles de zonulina en suero como marcador de PI (kit ELISA Immunodiagnostik, Bensheim, Alemania) al tiempo basal, y se estratificó a los sujetos en dos subgrupos: el grupo de zonulina sérica basal más baja (LSZ) (zonulina sérica basal ≤ al valor mediano) y el grupo de zonulina sérica basal más alta (HSZ) (zonulina sérica basal > el valor mediano).
Al inicio del estudio y después de cada período de intervención, se pidió a los sujetos que ayunaran durante la noche para recoger muestras de suero y de orina del primer vacío de la mañana. Las muestras de orina recogidas fueron sometidas a un análisis metabolómico para investigar el impacto del aumento de la PI en la biodisponibilidad de los polifenoles.

Resultados y conclusiones: Los niveles urinarios de metabolitos de fase II y derivados de la microbiota fueron significativamente diferentes entre los sujetos con una integridad de la barrera intestinal más saludable (es decir, el grupo LSZ) y aquellos con una mayor alteración de la PI (grupo HSZ). Los resultados apoyan que este deterioro de la biodisponibilidad de los polifenoles dependiente de la PI podría atribuirse a las alteraciones del metabolismo microbiano intestinal y a los procesos de metilación de fase II. Además, también observamos que los metabolitos derivados de la microbiota podrían ser responsables en gran medida de la actividad biológica provocada por los polifenoles de la dieta contra la alteración de la PI relacionada con la edad.

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Article

Increased Intestinal Permeability in Older Subjects Impacts the Beneficial Effects of Dietary Polyphenols by Modulating Their Bioavailability

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ABSTRACT: Polyphenols have great potential in regulating intestinal health and ameliorating pathological conditions related to increased intestinal permeability (IP). However, the efficacy of dietary interventions with these phytochemicals may significantly be influenced by interindividual variability factors affecting their bioavailability and consequent biological activity. In the present study, urine samples collected from older subjects undergoing a crossover intervention trial with polyphenol-rich foods were subjected to metabolomics analysis for investigating the impact of increased IP on the bioavailability of polyphenols. Interestingly, urinary levels of phase II and microbiota-derived metabolites were significantly different between subjects with healthier intestinal barrier integrity and those with increased IP disruption. Our results support that this IP-dependent impaired bioavailability of polyphenols could be attributed to disturbances in the gut microbial metabolism and phase II methylation processes. Furthermore, we also observed that microbiota-derived metabolites could be largely responsible for the biological activity elicited by dietary polyphenols against age-related disrupted IP.

KEYWORDS: *polyphenols, intestinal permeability, aging, metabolomics, microbiota, bioavailability*

INTRODUCTION

The intestinal barrier is a complex functional structure that separates the gut luminal environment from the inner host, which is composed of not only a physical wall comprising epithelial cells and mucus layers but also other elements such as the gut microbiota, immunological elements (e.g., immunoglobulin A, cytokines), as well as the intestinal endocrine, neurenteric, and vascular systems.[1] The integrity of this barrier is crucial in human health for maintaining normal intestinal permeability (IP), which regulates the transport and absorption of nutrients (e.g., sugars, vitamins, amino acids, fatty acids, and other lipids) and other food-related compounds (e.g., polyphenols), and the translocation of bacterial components from the lumen to the bloodstream. The IP is controlled by a complex system of junctions, namely, tight junctions (TJ), gap junctions, and adherens junctions, comprising a myriad of transmembrane proteins (e.g., occludins, claudins) and junctional adhesion molecules that rule the flux between adjacent enterocytes.[2] However, the disruption of these intestinal junctions leads to increased IP, a pathological condition also known as leaky gut. This results in the diffusion of toxins, viruses, and bacterial fragments from the intestinal environment to the circulating stream, which consequently activates the immune function and provokes systemic inflammation.[3] Increased IP has been proposed as a major contributor to multiple diseases, including gastro-intestinal (e.g., irritable bowel syndrome, celiac disease),[4] metabolic (e.g., obesity, type II diabetes),[5] cardiovascular (e.g., atherosclerosis, chronic heart failure),[6] psychiatric (e.g., depression, autism),[7] and neurodegenerative (e.g., Parkinson's disease, Alzheimer's disease) disorders.[8] Furthermore, it is also noteworthy that leaky gut can frequently be observed during aging, contributing to the characteristic low-grade systemic inflammation detected in older adults, i.e., the inflamm-aging process.[9] The most common causes behind this age-related increase of the IP include impairments in the intestinal epithelial and mucus barriers,[10] declined immune function (i.e., immune senescence),[9] and changes in the gut microbiota composition.[11]

Adequate nutritional status is crucial for maintaining normal gut barrier function. Adherence to the Western diet, characterized by high fat and sugar intake, is associated with increased IP,[12,13] whereas the Mediterranean diet, rich in fruits, vegetables, and fiber, prevents the leaky gut.[13] In this vein, numerous studies have been conducted over the past years aimed to test the efficacy of dietary interventions for improving the IP and related conditions, with special focus on polyphenols.[14,15] These bioactive compounds are secondary metabolites widely distributed in plant-derived foods, including

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fruits, vegetables, legumes, cereals, beverages (e.g., tea, coffee), and many other foods, with recognized antioxidant and anti-inflammatory properties. Thus, it has previously been reported that polyphenols can ameliorate the leaky gut by directly regulating the TJ function, enhancing the synthesis and redistribution of TJ proteins, such as occludin, claudins, and zonula occludens,[16,17] and by inhibiting different kinases involved in TJ expression.[2] Polyphenolic compounds are also able to block the production of inflammatory cytokines (e.g., necrosis factors, interleukins) and oxidative stress, thus protecting the intestinal barrier integrity.[2] Furthermore, polyphenols and the gut microbiota are interconnected through a bidirectional network, which plays a pivotal role in intestinal health.[18] On one hand, the gut microbiota is involved in the biotransformation processes needed for the absorption and biological activity of these compounds. Indeed, various studies have described that microbiota-derived metabolites could be responsible, at least in part, for the intrinsic biological effects traditionally attributed to polyphenols, especially taking into consideration the usual low bioavailability of the parent compounds.[19] Complementarily, the prebiotic activity of polyphenols and microbiota derivatives is also well known,[20] being the consumption of polyphenol-rich foods able to shape the microbiota composition toward the preservation of the intestinal barrier health by means of different mechanisms. For instance, the gut microbiota may directly influence the IP by contributing to the intestinal barrier integrity (e.g., affecting the turnover of intestinal epithelial cells, organization of TJs), but it is also involved in the modulation of inflammation.[21,22] Accordingly, the dietary-driven manipulation of the intestinal microbial ecosystem with polyphenols has previously demonstrated great efficacy for improving the IP and related inflammatory processes.[23−25] However, to the best of our knowledge, there is currently a total lack of studies focused on determining how increased IP and associated pathological conditions occurring during aging, such as inflammation and microbial dysbiosis, may affect the bioavailability of polyphenols and consequently impact their biological activity.

The aim of the present work is to investigate for the first time the impact of increased IP in older subjects on the bioavailability of dietary polyphenols and, therefore, on their bioactivity and capacity to modulate the intestinal barrier integrity. To this end, a crossover intervention trial with a polyphenol-rich diet was conducted in older adults, and serum zonulin was measured as a marker of the intestinal barrier integrity for stratifying the population in two subgroups according to their IP (i.e., increased IP dysfunction and healthier subjects). Then, comprehensive quantitative metabolomics analyses were performed to characterize the urinary food-related metabolome, comprising polyphenolic and other compounds of food origin, metabolites derived from phase I/II metabolism, and microbial-transformed derivatives.[26,27]

MATERIALS AND METHODS

Study Design. A randomized, controlled, crossover intervention trial with polyphenol-rich foods was conducted in older people living in a residential care setting (i.e., the MaPLE study, Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly), as described elsewhere.[28] The study was performed in accordance with the principles contained in the Declaration of Helsinki. The Ethics Committee of the University of Milan approved the study protocol, and all of the participants provided written informed consent. The trial was registered under ISRCTN.com (ISRCTN10214981).

Briefly, 51 older subjects (≥60 yr) completed a crossover trial consisting of a polyphenol-rich diet (PR-diet) and a control diet (C-diet), each one of the arms lasting for 8 weeks and being separated by an 8 week wash-out period. Serum zonulin levels were measured as a marker of IP (Immunodiagnostik ELISA kit, Bensheim, Germany),[29] and the median value within the study population (median = 40 ng/mL) was employed to stratify subjects in two subgroups: the lower serum zonulin at baseline (LSZ) group (serum zonulin at baseline ≤ the median value) and the higher serum zonulin at baseline (HSZ) group (serum zonulin at baseline > the median value). Accordingly, zonulin levels were 33.2 ± 5.6 and 51.5 ± 8.9 ng/mL (expressed as the mean ± standard deviation) for the LSZ and HSZ individuals, respectively. Subjects in these two groups were matched for sex (men/women: 11/15 vs 11/14), age (79.2 ± 10.4 vs 76.4 ± 10.2 yr), and BMI (26.4 ± 6.4 vs 27.2 ± 4.5 kg/m^2). During the C-diet period, subjects consumed the regular menu provided by the nursing home, whereas the PR-diet was designed by substituting three portions per day of low-polyphenol products from the C-diet with selected food items with a higher polyphenol content but maintaining comparable levels of energy and nutrients. Specifically, PR foods employed in this intervention study were berries (raw fruits and puree), blood orange (raw fruits and juice), pomegranate juice, green tea, Renetta apple (raw fruits and purée), and cocoa (chocolate callets and cocoa powder drink). At baseline and after each intervention period, subjects were asked to fast overnight for collecting serum and first morning void urine samples. A detailed description of the inclusion and exclusion criteria, the intervention trial, and the collection of biological samples has been previously reported by Guglielmetti et al.[28]

Metabolomics Analysis of Urine Samples. Multitargeted quantitative metabolomics analysis of the urinary food metabolome was accomplished by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), following the methodology optimized by González-Domínguez et al.[26,27] To this end, urine samples were subjected to solid-phase extraction (SPE) using Oasis HLB extraction plates (Waters, Milford, MA) with the aim of simultaneously extracting and preconcentrating polyphenols and other food-related compounds and their biotransformed metabolites (i.e., phase I/II and microbiota derivatives). Complementarily, urine samples were also analyzed after 10-fold dilution to determine highly concentrated metabolites and polar compounds, these latter not extracted when using SPE. A set of internal standards (taxifolin and caffeine-$^{13}C_3$, 100 μg/L) was added to all of the samples for quantification and quality control (QC) assessment, as previously described.[26,27] Subsequent UHPLC-MS/MS metabolomics fingerprinting was performed using the chromatographic and MS conditions described elsewhere for the simultaneous detection and quantitation of almost 350 dietary compounds and their host and microbial metabolites.[26] Metabolomics results were normalized with reference to the urinary refractive index (OPTi Digital Handheld Refractometer, Bellingham + Stanley, UK) to account for interindividual differences in the hydration status and micturition frequency.

Quality Control Assessment. Quality control (QC) assessment of the metabolomics data was carried out using a standardized protocol developed in-house. For this purpose, data were first preprocessed for removing metabolites with more than 20% missing values in all of the study groups.[30] The remaining missing values were imputed using the root square of the limit of detection for each metabolite,[26] and data were then log-transformed and Pareto-scaled. Afterward, distances to the group centroid were computed based on Euclidean distances to remove outliers from the data matrix. Metabolites known to be influenced by preanalytical factors (e.g., hippurate) were checked for the absence of abnormal values (±1.5 × IQR), which could be indicative of improper handling/storage of urine samples.[31] Finally, the coefficient of variation was computed for areas, retention times, and peak widths of the internal standards added to samples with the aim of evaluating the analytical reproducibility along the sequence run.

Statistical Analysis. Metabolomics data were preprocessed as detailed in the previous section and were then subjected to statistical

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Table 1. Urinary Food and Microbiota-Related Metabolites Significantly Altered after the PR-Diet for the Entire MaPLE Population, the LSZ and HSZ Subgroups[a]

metabolite	MaPLE ($N = 51$)	LSZ ($N = 26$)	HSZ ($N = 25$)
phenolic acids, hydroxybenzenes & hydroxybenzaldehydes (microbiota)			
2-hydoxybenzoic acid glucuronide	186.7 (3.3×10^{-5})	317.5 (1.1×10^{-4})	44.4 (NS)
3-hydoxybenzoic acid glucuronide	208.5 (1.8×10^{-5})	87.6 (1.1×10^{-2})	81.7 (NS)
4-hydoxybenzoic acid glucuronide	85.1 (4.5×10^{-2})	92.9 (NS)	76.1 (NS)
3-hydoxybenzoic acid sulfate	634.9 (1.1×10^{-4})	332.6 (8.4×10^{-4})	454.4 (NS)
3,4-dihydoxybenzoic acid 3-glucuronide	217.6 (2.3×10^{-4})	329.9 (9.1×10^{-4})	95.0 (NS)
3,4-dihydoxybenzoic acid 4-glucuronide	99.0 (2.3×10^{-4})	118.1 (2.0×10^{-4})	74.8 (3.2×10^{-1})
3,4-dihydoxybenzoic acid 3-sulfate	134.0 (4.3×10^{-5})	156.0 (NS)	111.0 (NS)
hippuric acid	864.6 (3.7×10^{-2})	1062.3 (NS)	658.8 (NS)
3-hydroxyhippuric acid	1715.4 (4.3×10^{-5})	1009.7 (NS)	2450.5 (NS)
vanillic acid glucuronide	174.2 (6.8×10^{-4})	129.3 (2.8×10^{-4})	221.1 (NS)
isovanillic acid glucuronide	193.9 (2.0×10^{-5})	281.9 (2.3×10^{-4})	94.0 (NS)
syringic acid	155.1 (2.2×10^{-5})	104.5 (1.0×10^{-4})	61.7 (NS)
4-methylgallic acid	548.8 (2.6×10^{-2})	824.4 (1.4×10^{-4})	287.1 (NS)
methylgallic acid glucuronide	75.3 (5.5×10^{-4})	85.6 (1.8×10^{-2})	64.5 (NS)
methylgallic acid sulfate	235.3 (2.5×10^{-2})	248.9 (NS)	221.1 (NS)
3-hydoxyphenylacetic acid	187.3 (2.3×10^{-4})	150.7 (4.0×10^{-3})	185.7 (NS)
4-hydoxyphenylacetic acid glucuronide	91.6 (4.1×10^{-2})	77.4 (NS)	78.2 (NS)
3,4-dihydoxyphenylacetic acid glucuronide	870.3 (1.8×10^{-5})	107.3 (1.1×10^{-4})	56.9 (NS)
homovanillic acid glucuronide	200.2 (1.9×10^{-5})	263.3 (NS)	131.6 (NS)
homovanillyl alcohol	104.5 (1.7×10^{-2})	65.8 (NS)	77.9 (NS)
o-coumaric acid	133.6 (9.9×10^{-4})	215.2 (1.3×10^{-4})	76.4 (NS)
o-coumaric acid glucuronide	158.9 (NS)	223.6 (4.5×10^{-4})	88.3 (NS)
m-coumaric acid glucuronide	222.5 (1.2×10^{-5})	292.3 (2.8×10^{-4})	169.3 (2.4×10^{-1})
p-coumaric acid glucuronide	224.9 (1.2×10^{-5})	303.3 (4.7×10^{-4})	143.2 (3.2×10^{-1})
m-coumaric acid sulfate	257.3 (2.7×10^{-5})	293.8 (1.5×10^{-4})	219.2 (NS)
caffeic acid 3-glucuronide	84.4 (2.2×10^{-5})	118.9 (2.4×10^{-4})	48.5 (3.2×10^{-1})
caffeic acid 4-glucuronide	167.4 (7.3×10^{-4})	206.8 (NS)	126.3 (2.2×10^{-2})
ferulic acid glucuronide	582.2 (2.1×10^{-5})	57.5 (NS)	100.2 (3.2×10^{-2})
isoferulic acid glucuronide	1158.7 (9.4×10^{-5})	168.5 (NS)	80.9 (NS)
ferulic acid sulfate	44.9 (4.8×10^{-2})	55.3 (NS)	34.0 (NS)
isoferulic acid sulfate	109.1 (3.6×10^{-2})	157.1 (NS)	57.0 (NS)
methylpyrogallol sulfate	312.0 (2.6×10^{-5})	492.4 (1.8×10^{-4})	124.1 (NS)
4-methylcatechol glucuronide (isomer 1)	166.7 (1.3×10^{-5})	190.1 (1.7×10^{-4})	141.0 (NS)
4-methylcatechol glucuronide (isomer 2)	333.5 (3.8×10^{-2})	495.2 (3.5×10^{-4})	157.1 (NS)
vanillin	119.0 (3.3×10^{-2})	183.8 (3.3×10^{-4})	51.4 (NS)
flavan-3-ols			
(epi)catechin glucuronide (isomer 1)	169.7 (1.2×10^{-4})	281.4 (7.6×10^{-4})	72.9 (NS)
(epi)catechin glucuronide (isomer 2)	433.2 (1.9×10^{-5})	602.2 (6.1×10^{-5})	348.7 (1.1×10^{-2})
(epi)catechin glucuronide (isomer 3)	679.6 (5.5×10^{-4})	1053.2 (2.1×10^{-4})	341.6 (NS)
(epi)catechin glucuronide (isomer 4)	4462.2 (6.1×10^{-5})	7009.2 (2.9×10^{-5})	2157.8 (1.1×10^{-2})
(epi)catechin sulfate (isomer 1)	1678.6 (2.0×10^{-5})	3790.6 (3.2×10^{-4})	1150.9 (NS)
(epi)catechin sulfate (isomer 2)	4687.8 (5.5×10^{-5})	5271.0 (1.7×10^{-4})	2157.8 (NS)
methyl(epi)catechin glucuronide (isomer 1)	294.7 (1.3×10^{-5})	324.1 (NS)	181.4 (NS)
methyl(epi)catechin glucuronide (isomer 2)	334.7 (2.0×10^{-5})	428.9 (NS)	168.6 (NS)
methyl(epi)catechin glucuronide (isomer 3)	1034.0 (2.6×10^{-6})	1232.1 (3.5×10^{-4})	813.9 (2.7×10^{-5})
methyl(epi)catechin glucuronide (isomer 4)	391.6 (8.1×10^{-6})	556.0 (6.5×10^{-4})	207.7 (2.7×10^{-5})
methyl(epi)catechin sulfate (isomer 1)	711.0 (2.5×10^{-5})	700.6 (NS)	721.8 (NS)
methyl(epi)catechin sulfate (isomer 2)	1194.0 (1.9×10^{-5})	725.3 (1.9×10^{-4})	1662.6 (6.4×10^{-5})
methyl(epi)catechin sulfate (isomer 3)	4601.4 (1.2×10^{-6})	1833.2 (8.9×10^{-4})	7485.0 (4.6×10^{-5})
methyl(epi)catechin sulfate (isomer 4)	1619.9 (3.5×10^{-6})	1291.8 (1.3×10^{-4})	1962.2 (4.6×10^{-5})
methyl(epi)catechin sulfate (isomer 5)	2701.9 (6.0×10^{-6})	1964.3 (2.3×10^{-4})	3471.5 (1.1×10^{-2})
methyl(epi)catechin sulfate (isomer 6)	817.5 (1.2×10^{-6})	861.1 (1.6×10^{-4})	767.7 (4.4×10^{-5})
hydroxyphenyl-γ-valeric acids & hydroxyphenyl-γ-valerolactones (microbiota)			
5-(3′,4′-dihydroxyphenyl)−4-hydroxyvaleric acid 3′-glucuronide	367.7 (9.5×10^{-4})	681.5 (2.3×10^{-4})	116.6 (NS)
5-(3′,4′-dihydroxyphenyl)−4-hydroxyvaleric acid 4′-glucuronide	884.5 (8.5×10^{-4})	1038.5 (NS)	723.5 (NS)
5-(3′,4′-dihydroxyphenyl)−4-hydroxyvaleric acid 3′-sulfate	2579.2 (7.9×10^{-4})	4018.7 (2.8×10^{-4})	1079.7 (NS)
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 3′-glucuronide	6782.4 (1.2×10^{-5})	10020.4 (3.1×10^{-4})	3544.4 (3.2×10^{-1})
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 4′-glucuronide	1415.3 (1.0×10^{-4})	2534.3 (4.8×10^{-3})	245.4 (NS)
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 3′-sulfate	948.5 (4.1×10^{-6})	605.6 (4.2×10^{-4})	195.6 (NS)

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J. Agric. Food Chem. 2020, 68, 12476−12484

Table 1. continued

metabolite	MaPLE (N = 51)	LSZ (N = 26)	HSZ (N = 25)
5-(3′,4′-dihydroxyphenyl)-γ-valerolactone 4′-sulfate	7772.8 (1.2×10^{-3})	13594.6 (3.5×10^{-4})	1673.8 (NS)
5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone 3′-sulfate	2393.4 (7.6×10^{-4})	331.6 (8.9×10^{-4})	320.6 (NS)
5-(3′,4′,5′-trihydroxyphenyl)-γ-valerolactone 4′-sulfate	12 184.4 (2.0×10^{-2})	22 850.4 (NS)	548.7 (NS)
5-(4′-hydroxy-3′-methoxyphenyl)-γ-valerolactone	377.3 (9.3×10^{-3})	507.2 (3.2×10^{-2})	247.5 (NS)
5-(4′-hydroxy-3′-methoxyphenyl)-γ-valerolactone glucuronide	4957.0 (7.2×10^{-3})	7987.1 (4.5×10^{-3})	1800.6 (NS)
5-(4′-hydroxy-3′-methoxyphenyl)-γ-valerolactone sulfate	1342.5 (1.1×10^{-3})	2065.2 (4.5×10^{-3})	589.8 (NS)
Urolithins (microbiota)			
urolithin A glucuronide	23 040.5 (7.6×10^{-4})	38 347.0 (3.5×10^{-4})	10 649.5 (NS)
urolithin A sulfate	998.1 (8.2×10^{-4})	1397.2 (1.3×10^{-3})	660.4 (NS)
Enterolignans (microbiota)			
enterolactone glucuronide	2882.1 (3.2×10^{-3})	495.5 (2.3×10^{-2})	83.9 (NS)
Anthocyanins			
cyanidin 3-glucoside	523.6 (8.5×10^{-4})	649.3 (1.0×10^{-2})	421.4 (NS)
xanthine alkaloids			
theobromine	2138.1 (3.2×10^{-3})	3983.6 (2.1×10^{-4})	215.7 (NS)
other flavonoids			
naringenin glucuronide	520.7 (NS)	804.3 (2.6×10^{-2})	237.1 (NS)
luteolin 3′-glucuronide	19.6 (4.3×10^{-2})	34.6 (9.2×10^{-3})	3.3 (NS)

[a]Results are expressed as the percentage of change, with FDR-corrected p-values in brackets (NS, non-significant).

analysis using R 3.6.2 software packages (http://www.r-project.org) to look for altered metabolites because of the intervention trial and to associate these metabolic alterations with changes in the IP. For this purpose, data normality was first checked by inspecting probability plots. Then, a linear mixed model was built to evaluate the impact of the PR-dietary intervention on urinary metabolites compared with the C-diet, taking into account the repeated measures by subject, the period (pre- and postintervention), and the arm within the crossover design (i.e., first C-diet and then PR-diet or vice versa). For each arm of the crossover trial, the effect of the intervention was estimated as the difference between the final and baseline metabolite concentrations. Finally, Pearson's correlations were computed between serum zonulin levels and significant urinary metabolites according to the previous linear model. All of these analyses were conducted in the entire study population (i.e., the MaPLE study) as well as separately in participants stratified according to their baseline zonulin levels (i.e., the LSZ and HSZ subgroups), as reported in Section 2.1. All of the statistical analyses were adjusted for the age, sex, BMI, and the allocation order in the crossover trial as covariates and were adjusted for multiple comparisons using the Benjamini−Hochberg false discovery rate (FDR). FDR-corrected p-values below 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Differential Bioavailability of Dietary Polyphenols Depending on the IP Status. Metabolomics analysis of urine samples was accomplished to investigate the metabolism and bioavailability of polyphenols supplied through a PR-dietary intervention in older adults. For evaluating the impact of increased IP on metabolomics results, serum zonulin was measured as a surrogate marker of the intestinal barrier integrity because the high rate of incontinence among the elderly participants participating in the intervention trial impeded the lactulose−mannitol urinary test to be performed. In this vein, although there is a growing debate about the reliability of using zonulin as a marker of IP,[32] a high correlation between serum zonulin and the urinary lactulose/mannitol ratio has been previously demonstrated.[33] On this basis, we stratified the study population according to the baseline zonulin levels with the aim of separately assessing the effect of the PR intervention in subjects with healthier intestinal barrier integrity (i.e., the LSZ group) and in those with increased IP dysfunction (i.e., the HSZ group). This is in line with previous works reporting that serum zonulin concentrations are normally raised during aging[34] but especially in older adults with gastrointestinal symptoms compared to the general older population.[35]

The PR-diet supplied an average of 724 mg of total polyphenols per day, thus almost doubling the estimated polyphenol intake compared with the C-diet.[28] We observed that this PR-dietary intervention induced a slight decrease in serum zonulin levels in the MaPLE population.[36] This finding is supported by a large amount of scientific evidence that highlights the great potential of polyphenols in regulating the intestinal barrier function and preventing leaky gut, both in vitro and in vivo.[2,16] However, different behaviors were interestingly observed when stratifying subjects according to the serum zonulin levels at baseline, since only the subjects with higher IP (i.e., HSZ group) experienced a significant decrease of serum zonulin, whereas those with LSZ were unaffected. Overall, these results underline the potential existence of different phenotypic groups in the older subjects characterized by the degree of IP, which significantly influences the efficacy of the PR-dietary intervention. This therefore demonstrates the crucial need for investigating the interindividual variability in the bioavailability of polyphenols driving these discrepancies.

To this end, we employed a multitargeted metabolomics platform with integrated QC assessment, which provided a comprehensive, accurate, and quantitative characterization of the urinary food metabolome based on the simultaneous analysis of around 350 diet-related metabolites, including polyphenols and other compounds of food origin, metabolites derived from the host metabolism (i.e., phase I and II transformation processes), and microbiota derivatives.[26,27] Among all of the metabolites measured, the intervention with PR foods in the MaPLE trial induced a significant increase in the urinary levels of numerous food and microbiota-related metabolites compared with the C-diet (ca 70), as shown in Table 1. The concentrations within the four study groups (i.e., C-diet baseline, C-diet postintervention, PR-diet baseline, and PR-diet postintervention) for the metabolites significantly

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altered because of the PR-dietary intervention are listed in Tables S1-S3, for the entire MaPLE population and the LSZ and HSZ subgroups, respectively. Many of these metabolites are well-known food-intake markers, as defined in the Food Biomarker Ontology,[37] thus accurately mirroring the consumption of the specific PR foods employed in this intervention study. The most remarkable finding was the increased urinary content of phase II metabolites of flavan-3-ols (i.e., (epi)catechins and methyl(epi)catechins) and their microbiota-derived hydroxyphenyl-valeric acids and hydroxyphenyl-γ-valerolactones, associated with the consumption of procyanidin-rich foods (e.g., tea, berries, apple, cocoa). The intake of tea, cocoa, and berries during the PR period was also reflected in the urinary excretion of methylgallic acid derivatives, theobromine, and cyanidin 3-glucoside, respectively. The production of urolithins, derived from the microbial transformation of ellagitannins, could be attributed to pomegranate and berries. Furthermore, other numerous nonspecific metabolites derived from the microbial metabolism of a wide range of polyphenol classes were also accumulated in urine samples, including phenolic acids (e.g., hydroxybenzoic acids, hydroxycinnamic acids) and enterolignans (e.g., enterolactone). Nonetheless, the most remarkable results were obtained when subjects were stratified according to the baseline zonulin levels. For LSZ individuals, the PR-dietary intervention induced similar metabolomics changes to those previously described for the entire MaPLE population (Table 1). However, the number of metabolites that were significantly increased as a consequence of the PR-diet in HSZ subjects was considerably lower with respect to the LSZ group, especially regarding microbiota derivatives. The HSZ group of subjects showed urinary alterations only in the levels of flavan-3-ol phase II metabolites, hydroxycinnamic acids, and a few other microbiota compounds compared with the C-diet. Interestingly, the fold of increase after the PR-diet for most of these metabolites was more pronounced in LSZ subjects compared with HSZ ones, except for methyl(epi)catechin derivatives that were excreted in larger amounts in this latter group. All of these findings therefore suggest that the baseline IP status could be an important factor affecting the bioavailability of dietary polyphenols, considering that only subjects with a healthier intestinal integrity were able to properly metabolize them. Particularly, metabolic discrepancies between the LSZ and HSZ groups were mainly observed in microbial metabolites, as shown in Table 1, which could support that alterations in the gut microbiota composition might play a central role in this hypothesized IP-driven reduced bioavailability.

In this context, the gut microbiota has been proposed as one of the most important factors influencing the bioavailability of polyphenols and, consequently, their bioactivity.[19] The microbial metabolism of polyphenols usually comprises an initial hydrolysis step of the conjugated species present in foods to release the corresponding aglycones, which can subsequently be transformed by a range of reactions, including ring fissions, dehydroxylations, decarboxylations, demethylations, reductions, and many others.[18,38] While numerous enterobacterial species from the four most abundant phyla can be involved in the deconjugation of polyphenols (i.e., Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria), only two phyla have been associated with further metabolism of the aglycones (Firmicutes and Actinobacteria), as illustrated in Figure 1. Among them, *Clostridium* and *Eubacterium* species from the

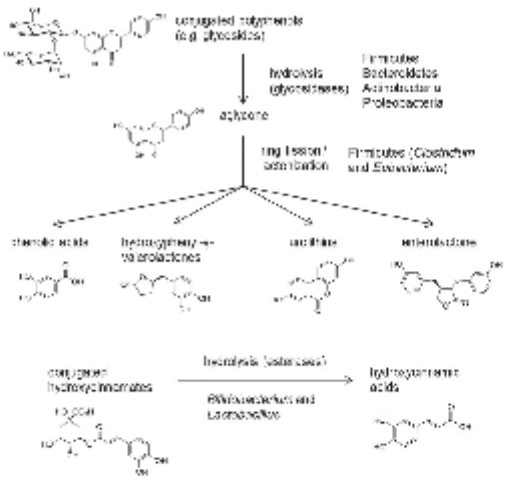

Figure 1. Interplay between the gut microbiota and the metabolism of polyphenols.

Firmicutes phylum are essential for the bioavailability of most polyphenols by driving C-ring cleavage reactions, which lead to the production of simpler phenolic acids and other intermediates that may undergo subsequent conversions to generate more complex microbiota derivatives (e.g., hydroxyphenyl-γ-valerolactones, urolithins, enterolignans). In contrast, hydroxycinnamic acids are mainly released in the colon by the action of microbial species from the *Bifidobacterium* and *Lactobacillus* genera (Figure 1).[38] Within this complex interplay between the gut microbiota and dietary polyphenols, it should also be noted that aging-related impairments in the intestinal health have closely been associated with significant gut dysbiosis. In general, the microbiota composition in older adults is characterized by an overall decrease of the bacterial diversity and stability, with a shift in the proportion of Bacteroidetes (increased) and Firmicutes (decreased) species[39,40] and increased abundance of potentially pathogenic and pro-inflammatory bacteria.[40−42] Among the Firmicutes, numerous studies have demonstrated that older subjects with impaired intestinal health have decreased the content of *Clostridium* and *Eubacterium* species,[42−44] which are directly involved in the microbial biotransformations of polyphenols as described above. On the other hand, various authors have recently described that aging does not have a significant impact on the *Bifidobacterium* genus,[41,44] refuting earlier studies,[45,46] whereas contradictory results have been published regarding the influence of aging in *Lactobacillus* bacteria.[47,48] Therefore, these previous metagenomics findings totally support the metabolomic discrepancies observed in the present study between the LSZ and HSZ groups, since older subjects with increased IP (i.e., HSZ) are expected to have lower Firmicutes diversity, thus negatively affecting the bioavailability of most polyphenols and consequently reducing the urinary excretion of their microbiota derivatives, while showing only a minor impact on the content of hydroxycinnamates produced by *Bifidobacterium* and *Lactobacillus* species. On the other hand, increased methylation of dietary (epi)catechins was also observed in the HSZ group, which was paralleled by a

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J. Agric. Food Chem. 2020, 68, 12476−12484

decreased rate of glucuronidation and sulfation processes (Table 1). In this vein, it has been previously described that the in vitro bioavailability and intestinal absorption of methylated polyphenols are considerably higher than that elicited by the corresponding glucuronide and sulfate species.[49] These results could therefore suggest that a shift toward increased methylation is induced in HSZ individuals to partially compensate for the impairments in the microbial metabolism of polyphenols described above. This sharpened excretion of phase II methyl(epi)catechin metabolites in the HSZ group could be attributed to an altered expression of catechol-O-methyltransferase, the enzyme responsible for the conversion of dietary polyphenols into their methylated analogues.[50] The proper regulation of this catechol-metabolizing system has been demonstrated to be crucial in human health due to its potential pathophysiological and pathogenic roles in neurodegenerative diseases, cancers, and cardiovascular disorders.[51] However, this is the first time to our knowledge that an IP-dependent regulation of this methylation system is described in older adults.

Association between Dietary Polyphenols, Microbial Metabolites, and Intestinal Barrier Health. To further investigate the possible impact of the hypothesized IP-driven reduced bioavailability on the beneficial effects of polyphenols supplied through the PR-diet, linear correlations were computed between urinary metabolite concentrations and serum zonulin levels. For the LSZ subgroup, two conjugated phenolic acids were strongly and negatively correlated with zonulin levels, namely, 3,4-dihydroxybenzoic acid 3-glucuronide ($r = -0.47$, FDR-corrected $p = 0.042$) and *m*-coumaric acid glucuronide ($r = -0.50$, FDR-corrected $p = 0.061$), but no significant associations were found with parent polyphenol compounds (Table S4). In contrast, no statistically significant correlations were observed between zonulin and food-derived metabolites when considering the HSZ group (FDR-corrected $p > 0.2$, Table S4). Phenolic acids are common microbial metabolites derived from the intestinal degradation of multiple polyphenol classes, although they can also be present in original foods.[18] Thus, these results reinforce that the gut microbiota is responsible to a large extent for the bioavailability and subsequent biological activity elicited by dietary polyphenols. In this context, multiple in vitro and in vivo studies have previously reported that polyphenols (e.g., quercetin, kaempferol, myricetin, genistein, catechin, curcumin) can modulate the intestinal barrier function by promoting TJ integrity, protecting against inflammatory and oxidative disruptions, and consequently decreasing intestinal permeability.[16] However, the results presented here allow hypothesizing that (i) microbial phenolic acids could be the major contributors to the IP improvement induced by the PR-dietary intervention in older subjects and (ii) that the efficacy of dietary polyphenols is considerably impaired in subjects with increased IP dysfunction.

In conclusion, we have demonstrated, in the present study, a connection between the degree of IP at baseline and the bioavailability of dietary polyphenols in older adults. On the basis of our findings and the previous literature, we hypothesize that disturbances in the gut microbiota composition and IP-associated regulation of phase II methylation of polyphenols could explain, at least in part, the metabolomics results presented here. Furthermore, we also found that microbial metabolites could be the major contributors to the biological activity elicited by dietary polyphenols, this bioactivity being significantly impaired in older subjects with increased IP. To validate these hypotheses, future metagenomic studies are needed to associate polyphenol-driven changes in the IP (i.e., serum zonulin) and the food metabolome with the gut microbiota composition. Therefore, this work highlights the crucial need for developing personalized nutritional strategies for managing the IP in older adults and the pivotal role of gut microbiota in modulating the beneficial effects of the diet on human health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c04976.

Concentrations within the four study groups (C-diet baseline, C-diet postintervention, PR-diet baseline, PR-diet postintervention) of the metabolites significantly altered because of the PR-dietary intervention, considering the entire MaPLE population and the LSZ and HSZ subgroups; and Pearson's correlation coefficients between these metabolites and serum zonulin levels (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

C, control; HSZ, higher serum zonulin at baseline; IP, intestinal permeability; LSZ, lower serum zonulin at baseline; PR, polyphenol-rich; TJ, tight junction

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Addition/Correction

Correction to "Increased Intestinal Permeability in Older Subjects Impacts the Beneficial Effects of Dietary Polyphenols by Modulating Their Bioavailability"

Nicole Hidalgo-Liberona, Raúl González-Domínguez,* Esteban Vegas, Patrizia Riso, Cristian Del Bo', Stefano Bernardi, Gregorio Peron, Simone Guglielmetti, Giorgio Gargari, Paul Antony Kroon, Antonio Cherubini, and Cristina Andrés-Lacueva*

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MANUSCRITO 8

Association between Food Intake, Clinical and Metabolic Markers and DNA Damage in Older Subjects

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MANUSCRITO 8

Objetivo: Evaluar el nivel de daño en el ADN (evaluado como roturas de la cadena de ADN, daño endógeno y daño en el ADN inducido por la oxidación) en un grupo de sujetos de edad avanzada con permeabilidad intestinal inscritos en el ensayo de intervención MaPLE (Gut and Blood Microbiomics for Studying the Effect of a Polyphenol-Rich Dietary Pattern on Intestinal Permeability in the Elderly), para valorar su asociación con marcadores clínicos, metabólicos y dietéticos.

Metodología: El daño en el ADN de las células mononucleares de la sangre periférica se evaluó mediante el ensayo cometa en 49 sujetos de edad avanzada que participaron en el estudio. Se determinaron en suero marcadores clínicos y metabólicos, marcadores de inflamación, función vascular y permeabilidad intestinal. La ingesta de alimentos se estimó mediante diarios alimentarios ponderados.

Resultados. En general, se observó una tendencia a niveles más altos de daño en el ADN en los hombres en comparación con las mujeres ($p = 0,071$). Se halló una asociación positiva entre el daño del ADN y los marcadores clínicos/metabólicos (por ejemplo, ácido úrico, perfil lipídico) y una asociación inversa con los marcadores dietéticos (por ejemplo, vitamina C, E, B6, folatos), que diferían en función del sexo.

Conclusión: Teniendo en cuenta la importancia de la estabilidad del ADN durante el envejecimiento, los resultados obtenidos sobre las diferencias de sexo y el posible papel de los factores dietéticos y metabólicos en el daño del ADN subrayan la necesidad de realizar más investigaciones en un grupo más amplio de adultos mayores para confirmar las asociaciones encontradas y promover estrategias preventivas.

antioxidants

Artide

Association between Food Intake, Clinical and Metabolic Markers and DNA Damage in Older Subjects

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Abstract: The use of DNA damage as marker of oxidative stress, metabolic dysfunction and age-related diseases is debated. The present study aimed at assessing the level of DNA damage (evaluated as DNA strand-breaks, endogenous and oxidatively-induced DNA damage) in a group of older subjects with intestinal permeability enrolled within the MaPLE (Gut and Blood Microbiomics for Studying the Effect of a Polyphenol-Rich Dietary Pattern on Intestinal Permeability in the Elderly) intervention trial, to evaluate its association with clinical, metabolic and dietary markers. DNA damage in peripheral blood mononuclear cells was assessed by the comet assay in 49 older subjects participating in the study. Clinical and metabolic markers, markers of inflammation, vascular function and intestinal permeability were determined in serum. Food intake was estimated by weighted food diaries. On the whole, a trend towards higher levels of DNA damage was observed in men compared to women ($p = 0.071$). A positive association between DNA damage and clinical/metabolic markers (e.g., uric acid, lipid profile) and an inverse association with dietary markers (e.g., vitamin C, E, B_6, folates) were found and differed based on sex. By considering the importance of DNA stability during aging, the results obtained on sex differences and the potential role of dietary and metabolic factors on DNA damage underline the need for further investigations in a larger group of older adults to confirm the associations found and to promote preventive strategies.

Keywords: aging; DNA damage; diet; metabolic markers; older subjects

1. Introduction

DNA represents one of the most biologically significant targets of oxidative stress, and it is widely recognised that continuous oxidative damage to DNA may contribute to the development of numerous age-related diseases [1]. Different markers of oxidative DNA damage have been proposed and utilized in numerous studies present in literature [2]. One of the most widely used markers to assess DNA damage is represented by 8-hydroxy-2-deoxy-guanosine (8-OHdG) [3,4]. This marker can derive from oxidative damage and excision-repair, oxidation of free bases or nucleotides, but also from other nucleic acids. Free 8-OHdG can be determined in different biological samples such as blood, urine and tissues; however, urinary 8-OHdG does not directly reflect DNA oxidation

within cells [5]. Another measurement of DNA damage can be performed through the use of comet assay [6,7]. The comet assay represents one of the most popular, versatile, simple, sensitive and non-invasive electrophoretic techniques able to detect, in individual cells, different types of damage (e.g., single and double-strand DNA breaks, alkali-labile lesions, DNA–DNA/DNA–protein cross-links) [8]. The comet assay is widely used in numerous in vitro and in vivo studies including observational and dietary intervention studies [9]. Measures of DNA damage using the traditional comet assay, which detect DNA strand breaks by the appearance of tailing, are not specific for oxidative damage. A direct measurement of oxidative damage to DNA (i.e., oxidised DNA bases) can be obtained in vivo by using modifications of the comet assay [10]. The most common modifications include the use of specific enzymes such as endonuclease III for the evaluation of oxidised pyrimidines, and formamidopyrimidine DNA glycosylase (FPG) for the detection and removal of the oxidatively damaged purines (e.g., 8-OHdG). This procedure allows a more direct measurement of DNA damage within the cells and a quantitative comparison with appropriate control [10,11]. A further comet protocol often utilised enables the evaluation of DNA susceptibility to oxidative damage generally induced by using H_2O_2. This assay can give further information on cell capacity to counteract an oxidative stress [9].

It has been suggested that there is an association between age and DNA damage [12]. Specifically, several factors can contribute in increasing the levels of DNA damage, from both intrinsic and extrinsic sources [13]. Aging is considered as a progressive and biological phenomenon that leads to loss of physiological integrity and to an impairment of numerous functions, at the molecular, cellular, tissue and organ level [12,13]. Several causes of aging have been hypothesized including an exacerbation of oxidative stress attributed to an imbalance between oxidant molecules and the antioxidant defence mechanisms [13]. This condition brings an accumulation of damage to macromolecules such as DNA but also carbohydrates, lipids, and proteins [14]. In this regard, one important aspect of the ageing process is the progressive accumulation of DNA damage over time [12]. A phenomenon strictly correlated with the aging process is inflammation. A greater presence of pro-inflammatory factors, such as interleukins (e.g., interleukin-6, IL-6; interleukin-1beta, IL-1β) and cytokines (tumour necrosis factor alpha, TNF-α) has been observed in older subjects contributing to generate a persistent and prolonged low-grade inflammation phenomenon called "inflamm-aging" [15]. Additionally, during inflammatory response, reactive oxygen and nitrogen species are produced to combat pathogens and to stimulate tissue repair and regeneration; however, these substances can also damage DNA and induce mutations.

Among the external factors that may contribute to the onset and progression of oxidative stress, and thus on DNA damage, diet and its components can play a pivotal role [16]. Several essential nutrients such as vitamins (e.g., folate, vitamin B12, niacin, vitamin C and E), minerals (e.g., magnesium, zinc, iron, manganese, selenium), and non-essential nutrients such as phytochemicals (e.g., polyphenols, carotenoids, and other bioactives) constitute a fundamental pillar for the preservation of DNA integrity due to their numerous important biological activities [17–23]. In fact, they are required for numerous functions including nucleotide synthesis, DNA replication, maintenance of DNA methylation, chromosome stability, prevention of DNA oxidation, DNA damage repair [24,25]. On the other hand, excessive energy intake, mainly from fats and in particular from saturated fatty acids, may bring to overweight/obesity and to increased levels of DNA damage also related to an alteration of the repair mechanisms [26]. This is quite common in older subjects in which physiological-social and environmental factors, oral-dental-deglutition disorders, dementia, long drug treatments can compromise the nutritional status thus potentially affecting DNA stability. In particular, several studies have reported that older subjects have higher levels of DNA damage compared to younger individuals [27,28].

To date it is not possible to identify a predominant factor responsible for the levels of DNA damage in specific target groups, but it can be assumed that the damage may derive from a combination of multiple factors that directly or indirectly affect DNA stability. In this regard, it has been suggested that increased intestinal permeability (IP), promoting the

translocation of inflammogenic factors could contribute to increase oxidative stress and inflammation, thus representing a further potential factor to control [29].

The main aim of the present study was to assess the levels of DNA damage in a group of older subjects with increased intestinal permeability enrolled in the MaPLE (Gut and Blood Microbiomics for Studying the Effect of a Polyphenol-Rich Dietary Pattern on Intestinal Permeability in the Elderly) study. The association of DNA damage with clinical, metabolic and dietary markers has been also investigated in order to identify the potential relationships in this target group.

2. Materials and Methods

2.1. Setting and Subjects' Recruitment

Fifty-one subjects completed the MaPLE trial carried out at Civitas Vitae (OIC Foundation, Padua, Italy), an institution including residential care and independent residences for older subjects [30]. Subjects were selected based on different inclusion and exclusion criteria as previously reported. Briefly, subjects had to be ≥60 years old, with a good cognitive and nutritional status, and a general good health condition while presenting an increased IP evaluated as serum zonulin level [30]. Celiac disease, major renal, liver or respiratory dysfunctions were considered as exclusion criteria together with antibiotic treatment or malignant tumour in the near previous period. Volunteers recruited were non-smokers and information on past smoking habits was not collected. The MaPLE study protocol was approved by the Ethics Committee of the Università degli Studi di Milano (15 February 2016; ref.: 6/16/CE_15.02.16_Verbale_All-7) while the trial was registered in a public register (28 April 2017; ISRCTN10214981; http://www.isrctn.com/ISRCTN10214981, accessed on 15 April 2021). Each participant received detailed information about the study purpose and procedures. Specifically, the MaPLE trial was aimed at demonstrating the role of a polyphenol-rich dietary pattern in the reduction of intestinal permeability and improvement of metabolic phenotypes in older adults. A written consent was obtained by each volunteer after acceptance. The full experimental design and inclusion and exclusion criteria were previously reported [30]. For the present study, the characteristics of subjects at baseline have been considered.

2.2. Food Intake

Food intake was collected through the analysis of weighed food diaries. In particular, subjects were asked to provide a food diary the day before the blood drawing. Data obtained were compared with those collected along the whole study in order to verify reproducibility of food habits of the volunteers. This was ensured thanks also to the moderate variety of the meals available within the standard menu provided at the nursing home [31]. The full protocol has been reported in a previous manuscript [30]. Energy, nutrient and polyphenol (single classes and total polyphenols assessed by Folin-Ciocalteu method) intake were estimated through the Metadieta software (Me.Te.Da S.r.l., Rome, Italy) and Phenol-explorer (phenol-explorer.eu).

2.3. Anthropometric, Clinical and Metabolic Makers Analysis

Weight, height, body mass index (BMI) and blood pressure were analysed according to international guidelines of Lohman et al. [32] and the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure guidelines (JNC 7) [33], as previously described. Blood samples were collected after an overnight fasting for the analysis of a plethora of markers [30]. Plasma and serum were obtained as previously reported [30] and stored at −80 °C until analysis. Metabolic markers (i.e., glycaemia, insulin, lipid profile, liver and renal function) were evaluated in serum through a validated protocol, using an automatic biochemical analyser (ILAB 650, Instrumentation Laboratory, Lexington, MA, USA). Inflammatory (e.g., IL-6, TNF-α, C-reactive protein (CRP) and vascular function markers (i.e., vascular cell adhesion molecule 1, VCAM1; intercellular adhesion molecule 1, ICAM1) were analysed in serum through ELISA kits (R&D System,

Minneapolis, MN, USA). Finally, serum zonulin levels, as marker of IP, were quantified using the Immunodiagnostik ELISA kit (Bensheim, Germany) [34].

2.4. Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) were separated by using Histopaque 1077 density gradient, according to the procedure reported by Del Bo' et al. [35]. Isolated PBMCs were then diluted into a medium constituted by fetal bovine serum, RPMI 1640 and dimethyl sulfoxide (50:40:10) and stored at −80 °C until analysis. The analysis of DNA damage was performed on PBMCs after a rapid thawing at 37 °C followed by a washing step with fresh RPMI medium and cold phosphate buffer saline. For the analysis, both endogenous and oxidatively-induced DNA damage were evaluated by comet assay [35]. The analysis was performed on samples at baseline.

2.5. Analysis of DNA Damage by the Comet Assay

The evaluation of endogenous DNA damage as formamidopyrimidine DNA glycosylase (FPG) sensitive sites was carried out enzymatically by the use of FPG able to detect the oxidized purines (mainly 8-oxo-7,8-hydroxyguanine). A description of the specific steps and conditions of the protocol used has been previously reported [35]. In brief, a solution of low melting point agarose was added to PBMCs suspension, mixed and rapidly spotted onto GelBond Film (VWR International U.R.L., Monroeville, PA, USA) precoated with normal melting point agarose. Coverslips were added on top of the slides and left to solidify for a few minutes at 4 °C. After solidification, coverslips were removed and slides were transferred into a lysis buffer for 1 h at 4 °C in the dark. Then, slides were washed 3 times in a cold buffer and further processed. One slide was treated with a buffer solution containing the FPG enzyme, while the other slide (control) with the same buffer without FPG. Samples were then incubated at 37 °C for 45 min. Successively, the slides were transferred in a horizontal electrophoresis tank containing an alkaline electrophoresis buffer for 40 min at 4 °C in order to favour DNA unwinding, followed by an electrophoresis step (25 V, 300 mA, 20 min). Finally, samples were washed in a neutralizing buffer for 15 min at 4 °C in the dark and dried in ethanol for 2 h.

Oxidatively induced DNA damage was assessed according to the procedure previously reported [35]. Briefly, two GelBonds Film containing cell suspensions were prepared for each subject: one was treated with hydrogen peroxide (H_2O_2 500 µM, 5 min) in a buffer solution at room temperature in the dark; the other was treated for 5 min only with the buffer solution (control slide). Following the oxidative treatment, slides were immersed in a lysis buffer for 1 h at 4 °C in the dark, and then transferred in a horizontal electrophoresis tank and treated as previously reported for the evaluation of endogenous DNA damage.

Ethidium bromide was used for the staining process. At least fifty comets per gel (i.e., 100 comets per condition) were scored using an epifluorescence microscope and analysed with an image analysis system (Comet 1.5; Immagini e Computer, Bareggio, Milan, Italy). The levels of DNA damage were calculated as tail intensity (% DNA in tail).

2.6. Statistical Analysis

Values were reported as mean ± standard deviation (SD), median and interquartile range (IQR, 25–75° percentile). Statistical analyses were performed by means of STATISTICA software (Statsoft Inc., Tulsa, OK, USA). Normality was assessed by the Shapiro–Wilk test. Significant differences at baseline based on sex were determined by unpaired Student's t test. The regression and correlation analyses (Spearman and Kendall test) were carried out to highlight associations between the levels of DNA damage and dietary, clinical and metabolic parameters in the whole group of subjects and in women and men. In addition, volunteers were stratified according to clinical variables in quartiles, and significant differences in DNA damage biomarkers in each quartile were assessed by unpaired Student's *t* test. Significance was set at $p \leq 0.05$; significance in the range $0.05 < p < 0.10$ was considered as a trend.

3. Results

3.1. Subject Characteristics

Supplementary Table S1 reports the characteristics of 49 subjects (22 men, 27 women) enrolled within the MaPLE intervention study. The full data set has been already reported [34]. Briefly, subjects analysed ranged between 60 and 98 years old with a median value of 77 years. A high inter-individual variability was shown for BMI (IQR: 22.7–30.7), glucose levels (IQR: 87–113), total cholesterol (IQR: 167–237) (Table S1). Similar findings were observed for the levels of FPG-sensitive sites (IQR: 21.4–34.9) and H_2O_2-induced DNA damage (IQR: 9.0–24.0) also after stratification based on sex (Table 1). On the whole, data on DNA damage were comparable between groups. Only a trend towards higher, but not significant ($p = 0.071$), levels of FPG-sensitive sites was documented in men compared to women.

Table 1. Levels of DNA damage markers evaluated at baseline.

Marker of DNA Damage	All (n = 49)	Men (n = 22)	Women (n = 27)	*p*-Value
FPG-sensitive sites (% DNA in tail)	16.5 ± 9.0	18.6 ± 10.4	14.8 ± 6.8	0.071
H_2O_2-induced DNA damage (% DNA in tail)	28.7 ± 11.4	28.5 ± 11.6	29.0 ± 2.4	0.438

Data are reported as mean ± SD (standard deviation); $p < 0.05$ are significantly different between groups. FPG, formamidopyrimidine DNA glycosylase.

3.2. Correlation between DNA Damage and Dietary Markers

Supplementary Table S2 reports the data obtained for dietary markers in the 49 out of 51 subjects enrolled within the MaPLE intervention study. The full data set has been already reported [34]. In Table 2 are shown the correlations between the levels of DNA damage and the dietary markers (energy, macro and micronutrients, and polyphenols).

Regarding the levels of FPG-sensitive sites, an overall negative correlation was found in the whole group of subjects for the intake of vitamin C ($p = 0.03$), vitamin E ($p = 0.008$), vitamin B_6 ($p = 0.002$) and folates ($p = 0.008$), while no significant correlations were evidenced for the rest of macro and micronutrients analysed. In addition, no association was also found when considering polyphenols and their subclasses.

As regards the levels of H_2O_2-induced DNA damage, an overall positive correlation was reported in the whole group of subjects with the intake of cholesterol ($p = 0.003$), omega 3 fatty acids ($p = 0.021$) and vitamin B_6 ($p = 0.032$), while no association emerged for the other dietary markers including polyphenols and the different subclasses analysed.

The analysis performed stratifying subjects based on sex (Table 3) did not show any significant correlation between the levels of DNA damage (both FPG-sensitive sites and H_2O_2-induced DNA damage) and the majority of the dietary markers. Regarding the levels of FPG-sensitive sites, a negative correlation was found with the intake of folates and vitamin B_6 ($p = 0.004$; $p = 0.046$, respectively) in women, and the intake of vitamin E ($p = 0.042$) in men. In addition, a negative correlation with the intake of monounsaturated fatty acids (MUFA; $p = 0.048$) was observed. No association was observed for polyphenols and their subclasses, apart from an apparent, but not significant ($p = 0.052$) inverse correlation found in men. Regarding the levels of H_2O_2-induced DNA damage, a positive correlation with dietary cholesterol ($p = 0.010$) and a negative correlation with total fibre intake ($p = 0.049$) was found in women, while in men no correlation was evidenced with the dietary markers considered including with the intake of polyphenols and their subclasses.

Table 2. Correlation analysis between dietary markers and DNA damage in the whole group of subjects.

Dietary Markers	FPG-Sensitive Sites			H_2O_2-Induced Damage		
	Tau	Z	*p*-Level	Tau	Z	*p*-Level
Energy (kcal)	0.064	0.650	0.516	0.023	0.234	0.815
Total Carbohydrates (% of energy)	0.021	0.217	0.828	−0.032	−0.322	0.748
Simple Carbohydrates (% of energy)	−0.026	−0.261	0.794	−0.019	−0.191	0.849
Proteins (% of energy)	−0.016	−0.167	0.867	−0.032	−0.325	0.745
Animal Proteins (% of energy)	−0.067	−0.675	0.500	0.051	0.517	0.605
Vegetal Proteins (% of energy)	0.013	0.135	0.893	0.038	0.386	0.700
Total Lipids (% of energy)	−0.048	−0.488	0.626	0.082	0.827	0.408
SFA (% of energy)	−0.069	−0.699	0.485	0.113	1.143	0.253
MUFA (% of energy)	−0.137	−1.387	0.165	0.099	1.000	0.317
PUFA (%of energy)	−0.056	−0.571	0.568	0.061	0.616	0.538
ω-6 (%of energy)	−0.112	−1.131	0.258	0.082	0.835	0.404
ω-3 (%of energy)	−0.101	−1.027	0.305	0.114	1.159	0.247
Total Fibre (g/1000 kcal)	−0.084	−0.849	0.396	−0.108	−1.093	0.274
Cholesterol (mg)	0.006	0.061	0.951	0.289	2.929	**0.003**
Calcium (mg)	−0.022	−0.225	0.822	0.038	0.381	0.703
Iron (mg)	0.157	1.596	0.110	0.016	0.158	0.875
Vitamin B_{12} (mcg)	−0.002	−0.019	0.985	0.161	1.632	0.103
Vitamin C (mg)	−0.294	−2.978	**0.003**	0.123	1.245	0.213
Vitamin E (mg)	−0.262	−2.653	**0.008**	0.083	0.843	0.399
Vitamin B_1 (mg)	−0.100	−1.012	0.312	0.008	0.076	0.939
Folates (mcg)	−0.310	−3.138	**0.002**	0.153	1.546	0.122
Vitamin B_6 (mg)	−0.263	−2.670	**0.008**	0.212	2.147	**0.032**
Flavonoids (mg)	0.167	1.692	0.091	0.005	0.052	0.959
Lignans (mg)	−0.031	−0.315	0.753	0.149	1.508	0.132
Other polyphenols (mg)	−0.135	−1.370	0.171	0.124	1.255	0.210
Phenolic acids (mg)	−0.005	−0.052	0.958	0.012	0.122	0.903
Stilbenes (mg)	0.012	0.122	0.903	−0.039	−0.395	0.693
Total Polyphenols (mg)	0.138	1.401	0.161	−0.032	−0.328	0.743
TPC Folin (mg)	−0.024	−0.242	0.809	−0.055	−0.553	0.580

Legend: Regression and correlation analyses obtained by Spearman and Kendall test. Data reported in bold are statistically significant ($p < 0.05$); FPG, formamidopyrimidine DNA glycosylase; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids; TPC, total polyphenol content evaluated by Folin-Ciocalteu method.

3.3. Correlation between the Levels of DNA Damage and Anthropometric, Metabolic and Clinical Markers

In Table 4 the correlations between the levels of DNA damage and anthropometric, metabolic and clinical markers are reported. Despite not being significant ($p = 0.086$), an inverse association between the levels of FPG-sensitive sites and age was observed. On the whole, no correlation was found between FPG-sensitive sites and metabolic and clinical markers neither in the whole group of subjects nor following sex stratification (Table 5).

Table 3. Correlation analysis between dietary markers and DNA damage in women and men.

	Women						Men					
	FPG-Sensitive Sites			H_2O_2-Induced Damage			FPG-Sensitive Sites			H_2O_2-Induced Damage		
Dietary Markers	**Tau**	**Z**	***p*-Level**	**Tau**	**Z**	***p*-Level**	**Tau**	**Z**	***p*-Level**	**Tau**	**Z**	***p*-Level**
Energy (kcal)	0.054	0.398	0.690	−0.060	−0.440	0.660	0.004	0.028	0.977	0.135	0.878	0.380
Total Carbohydrates (% of energy)	−0.052	−0.377	0.706	0.080	0.586	0.558	0.083	0.538	0.591	−0.135	−0.878	0.380
Simple Carbohydrates (% of energy)	0.040	0.293	0.769	−0.034	−0.251	0.802	−0.113	−0.735	0.462	−0.035	−0.226	0.821
Proteins (% of energy)	−0.123	−0.902	0.367	−0.014	−0.105	0.917	0.130	0.848	0.397	−0.052	−0.339	0.735
Animal Proteins (% of energy)	−0.057	−0.419	0.675	−0.040	−0.293	0.769	0.009	0.057	0.955	0.087	0.565	0.572
Vegetable Proteins (% of energy)	−0.046	−0.335	0.738	−0.006	−0.042	0.967	0.009	0.057	0.955	0.052	0.339	0.735
Total Lipids (% of energy)	0.100	0.734	0.463	−0.054	−0.398	0.690	−0.191	−1.243	0.214	0.234	1.526	0.127
SFA (% of energy)	0.109	0.796	0.426	0.046	0.335	0.738	−0.182	−1.187	0.235	0.139	0.904	0.366
MUFA (% of energy)	0.080	0.586	0.558	−0.006	−0.042	0.967	−0.304	−1.978	**0.048**	0.191	1.243	0.214
PUFA (%of energy)	0.052	0.377	0.706	0.000	0.000	1.000	−0.174	−1.130	0.258	0.148	0.961	0.337
ω-6 (%of energy)	0.052	0.379	0.705	−0.017	−0.126	0.899	−0.253	−1.646	0.100	0.148	0.965	0.335
ω-3 (%of energy)	−0.020	−0.149	0.881	0.020	0.149	0.881	−0.181	−1.177	0.239	0.181	1.177	0.239
Total Fibre (g/1000 kcal)	−0.023	−0.167	0.867	−0.269	−1.968	**0.049**	−0.161	−1.048	0.295	0.022	0.142	0.887
Cholesterol (mg)	0.029	0.212	0.832	0.353	2.584	**0.010**	−0.052	−0.341	0.733	0.200	1.306	0.192
Calcium (mg)	0.063	0.461	0.645	−0.137	−1.005	0.315	−0.087	−0.565	0.572	0.148	0.961	0.337
Iron (mg)	0.114	0.837	0.402	−0.097	−0.712	0.477	0.200	1.300	0.194	0.052	0.339	0.735
Vitamin B_{12} (mcg)	−0.036	−0.264	0.791	0.181	1.322	0.186	0.113	0.738	0.461	0.131	0.856	0.392
Vitamin C (mg)	−0.263	−1.926	0.054	0.057	0.419	0.675	−0.300	−1.954	0.051	0.170	1.105	0.269
Vitamin E (mg)	−0.172	−1.260	0.208	−0.046	−0.336	0.737	−0.312	−2.035	**0.042**	0.200	1.300	0.194
Vitamin B_1 (mg)	−0.035	−0.253	0.801	−0.109	−0.800	0.424	−0.083	−0.540	0.589	0.031	0.199	0.842
Folates (mcg)	−0.391	−2.864	**0.004**	0.197	1.443	0.149	−0.229	−1.494	0.135	0.100	0.649	0.517
Vitamin B_6 (mg)	−0.273	−1.998	**0.046**	0.043	0.315	0.752	−0.244	−1.589	0.112	0.288	1.873	0.061
Flavonoids (mg)	0.126	0.919	0.358	0.023	0.167	0.867	0.152	0.987	0.324	−0.004	−0.028	0.978
Lignans (mg)	0.009	0.064	0.949	0.155	1.138	0.255	−0.115	−0.751	0.453	0.177	1.156	0.248
Phenolic acids (mg)	−0.011	−0.084	0.933	0.114	0.835	0.404	−0.056	−0.367	0.714	−0.091	−0.592	0.554
Stilbenes (mg)	−0.060	−0.442	0.659	0.226	1.657	0.098	−0.233	−1.519	0.129	0.069	0.447	0.655
Other polyphenols (mg)	0.006	0.042	0.967	0.074	0.544	0.586	−0.299	−1.946	0.052	0.152	0.987	0.324
Total_Polyphenols (mg)	0.060	0.438	0.662	0.048	0.354	0.723	0.143	0.931	0.352	−0.100	−0.649	0.517
TPC_Folin (mg)	0.157	1.147	0.252	−0.003	−0.021	0.983	−0.195	−1.269	0.204	−0.126	−0.818	0.414

Legend: Regression and correlation analyses obtained by Spearman and Kendall test. Data reported in bold are statistically significant ($p < 0.05$); FPG, formamidopyrimidine DNA glycosylase; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids, ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids; TPC, total polyphenol content.

Table 4. Correlation analysis between anthropometric, metabolic and clinical markers and DNA damage in the whole group of subjects.

Metabolic and Clinical Markers	FPG-Sensitive Sites			H_2O_2-Induced Damage		
	Tau	Z	*p*-Level	Tau	Z	*p*-Level
Age (y)	−0.169	−1.715	0.086	0.119	1.210	0.226
Weight (kg)	0.104	1.053	0.292	−0.210	−2.124	**0.034**
BMI (kg/m^2)	0.063	0.638	0.524	−0.201	−2.034	**0.042**
SBP (mm Hg)	0.011	0.107	0.915	−0.163	−1.653	0.098
DBP (mm Hg)	0.142	1.435	0.151	−0.225	−2.283	**0.022**
Glucose (mg/dL)	0.039	0.399	0.690	−0.060	−0.608	0.543
Creatinine (mg/dL)	0.009	0.086	0.931	−0.044	−0.450	0.653
Uric Acid (mg/dL)	0.013	0.131	0.896	0.083	0.844	0.398
TC (mg/dL)	−0.130	−1.314	0.189	0.087	0.881	0.378
HDL-C (mg/dL)	−0.048	−0.487	0.626	−0.045	−0.452	0.651
TC/HDL (ratio)	−0.083	−0.845	0.398	0.133	1.345	0.179
LDL-C (mg/dL)	−0.123	−1.245	0.213	0.135	1.367	0.172
LDL/HDL (ratio)	−0.128	−1.302	0.193	0.166	1.682	0.093
TG (mg/dL)	−0.001	−0.009	0.993	0.037	0.372	0.710
AST (U/L)	0.029	0.292	0.770	−0.212	−2.151	**0.031**
ALT (U/L)	0.111	1.122	0.262	−0.252	−2.554	**0.011**
GGT (U/L)	0.113	1.149	0.251	−0.137	−1.392	0.164
Insulin (uU/mL)	−0.043	−0.432	0.665	−0.128	−1.297	0.194
HOMA Index	0.009	0.086	0.931	−0.119	−1.207	0.228
C-G index	0.119	1.207	0.228	−0.087	−0.879	0.379
Zonulin (ng/mL)	0.017	0.172	0.863	−0.063	−0.638	0.524
sICAM-1 (ng/mL)	0.027	0.276	0.783	0.022	0.224	0.823
sVCAM-1 (ng/mL)	−0.097	−0.983	0.326	0.133	1.345	0.179
CRP (mg/L)	0.085	0.862	0.389	0.172	1.741	0.082
TNF-α (pg/mL)	−0.107	−1.086	0.277	0.065	0.655	0.512
IL-6 (pg/mL)	−0.050	−0.509	0.611	0.076	0.767	0.443

Legend: Regression and correlation analyses obtained by Spearman and Kendall test. Data reported in bold are statistically significant ($p < 0.05$); BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft–Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6.

As regards the levels of H_2O_2-induced DNA damage (Table 4), a negative correlation was found with weight ($p = 0.034$), BMI ($p = 0.042$), diastolic blood pressure (DBP: $p = 0.022$), aspartate transaminase (AST: $p = 0.031$) and alanine transaminase (ALT: $p = 0.011$). Interestingly, the distribution of data in quartiles showed a lower level of DNA damage in subjects with the highest BMI, compared to those with the lowest BMI. Conversely, the subjects with the highest low density lipoprotein/high density lipoprotein ratio (LDL/HDL ratio) showed the highest DNA damage (Supplementary Figure S1). Stratification on the basis of HOMA index and CRP showed only a trend towards an increase of H_2O_2-induced DNA damage between the first and fourth quartile. No correlation was found for any of the other markers analysed.

After sex stratification (Table 5), a positive correlation between the levels of H_2O_2-induced DNA damage and both uric acid ($p = 0.042$) and LDL/HDL ratio ($p = 0.035$) were found for women, while a negative correlation was observed with BMI ($p = 0.032$). Regarding men, statistical analysis revealed a negative correlation between the levels of H_2O_2-induced DNA damage and DBP ($p = 0.039$) and ALT ($p = 0.035$). No correlation was shown for the rest of the markers under study both in men and women.

Table 5. Correlation analysis between anthropometric, metabolic and clinical markers and DNA damage in women and men.

Markers	Women						Men					
	FPG-Sensitive Sites			H_2O_2-Induced Damage			FPG-Sensitive Sites			H_2O_2-Induced Damage		
	Tau	Z	*p*-Level	Tau	Z	*p*-Level	Tau	Z	*p*-Level	Tau	Z	*p*-Level
Age (y)	−0.095	−0.696	0.486	0.176	1.286	0.198	−0.205	−1.337	0.181	0.048	0.313	0.754
Weight (kg)	0.114	0.835	0.404	−0.171	−1.253	0.210	0.057	0.368	0.713	−0.143	−0.935	0.350
BMI (kg/m2)	0.048	0.354	0.723	−0.293	−2.147	**0.032**	0.108	0.705	0.481	−0.117	−0.761	0.446
SBP (mm Hg)	−0.178	−1.302	0.193	−0.136	−0.998	0.318	0.149	0.974	0.330	−0.202	−1.317	0.188
DBP (mm Hg)	0.081	0.590	0.555	−0.158	−1.159	0.246	0.156	1.018	0.309	−0.317	−2.066	**0.039**
Glucose (mg/dL)	0.069	0.504	0.614	0.040	0.294	0.769	0.013	0.085	0.932	−0.135	−0.882	0.378
Creatinine (mg/dL)	−0.080	−0.586	0.558	−0.029	−0.209	0.834	−0.066	−0.427	0.670	−0.118	−0.768	0.442
Uric Acid (mg/dL)	−0.020	−0.147	0.883	0.278	2.034	**0.042**	0.031	0.199	0.842	−0.100	−0.654	0.513
TC (mg/dL)	−0.120	−0.877	0.381	0.000	0.000	1.000	−0.121	−0.791	0.429	0.130	0.848	0.397
HDL-C (mg/dL)	0.000	0.000	1.000	−0.184	−1.348	0.178	−0.061	−0.399	0.690	0.079	0.513	0.608
TC/HDL (ratio)	−0.094	−0.688	0.491	0.236	1.730	0.084	−0.082	−0.536	0.592	0.108	0.705	0.481
LDL-C (mg/dL)	−0.160	−1.169	0.242	0.114	0.835	0.404	−0.061	−0.396	0.692	0.121	0.791	0.429
LDL/HDL (ratio)	−0.134	−0.980	0.327	0.288	2.106	**0.035**	−0.134	−0.874	0.382	0.126	0.818	0.414
TG (mg/dL)	0.003	0.021	0.983	0.060	0.440	0.660	−0.004	−0.028	0.977	0.004	0.028	0.977
AST (U/L)	0.070	0.514	0.607	−0.170	−1.243	0.214	−0.089	−0.581	0.562	−0.223	−1.451	0.147
ALT (U/L)	0.230	1.686	0.092	−0.190	−1.387	0.165	−0.058	−0.376	0.707	−0.325	−2.114	**0.035**
GGT (U/L)	0.124	0.909	0.363	0.043	0.317	0.751	0.035	0.227	0.820	−0.244	−1.589	0.112
Insulin (uU/mL)	0.009	0.063	0.950	−0.117	−0.855	0.393	−0.083	−0.538	0.591	−0.152	−0.991	0.322
HOMA Index	0.060	0.438	0.662	−0.054	−0.396	0.692	−0.013	−0.085	0.933	−0.169	−1.100	0.271
C-G index	0.094	0.688	0.491	−0.123	−0.896	0.370	0.091	0.592	0.554	−0.030	−0.197	0.844
Zonulin (ng/mL)	−0.020	−0.146	0.884	−0.088	−0.646	0.518	0.048	0.310	0.756	−0.022	−0.141	0.888
sICAM-1 (ng/mL)	0.048	0.354	0.723	0.151	1.105	0.269	−0.013	−0.085	0.933	−0.100	−0.649	0.517
sVCAM-1 (ng/mL)	−0.037	−0.271	0.786	0.077	0.563	0.574	−0.108	−0.705	0.481	0.255	1.664	0.096
CRP (mg/L)	0.014	0.104	0.917	0.174	1.272	0.203	0.160	1.043	0.297	0.229	1.494	0.135
TNF-α (pg/mL)	−0.014	−0.104	0.917	0.111	0.813	0.416	−0.212	−1.382	0.167	−0.022	−0.141	0.888
IL-6 (pg/mL)	−0.066	−0.479	0.632	0.105	0.771	0.441	0.056	0.367	0.714	0.039	0.254	0.800

Legend: Regression and correlation analyses obtained by Spearman and Kendall test. Data reported in bold are statistically significant ($p < 0.05$); BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft–Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor alpha; IL-6, interleukin-6; FPG, formamidopyrimidine DNA glycosylase.

4. Discussion

The aging process can be considered a significant determinant of DNA damage levels due to increased oxidative stress, inflammation and other age-related conditions. However, available studies do not always report univocal results, in particular when DNA damage is evaluated by using the comet assay [9,36–38].

In the present study, we could not demonstrate a significant correlation between age or sex on DNA damage. However, it was observed a trend towards an inverse association between the levels of FPG-sensitive sites (endogenous DNA damage) and age, and a higher (but still not significant) level of damage in men compared to women, we may attribute to the limited sample size analysed.

As regards the discrepancy among the results found in literature on the topic, it could be related to many potential confounding factors. In this regard, the findings of a recent review showed that lifestyle factors, including diet, and other external exposures may contribute to the damage during aging more than age and sex itself [39].

Diet and dietary components have been largely studied for their modulatory effects on numerous biological functions including the protection against oxidative stress. These factors can contribute to the positive/negative modulation of DNA damage during the aging process. In our study, one of the main results observed regards the inverse association between dietary fibre and vitamins and the levels of DNA damage, and the positive association found with dietary lipids and cholesterol. A plethora of research has reported a positive and/or inverse association between DNA damage and specific dietary patterns [40,41] and/or different macro/micronutrients intake [9,16]. For example, the amount of dietary fats and calories has been reported to play a role in the modulation of oxidative DNA damage levels. In fact, high dietary fat consumption contributes to free radical-induced lipid peroxidation causing damage to macromolecules, including enzymes and DNA [16]. In particular, the intake of saturated fatty acids (SFAs) appeared to be an important determinant of basal DNA damage, on the contrary monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), specifically omega-3, have been associated with a reduced level of damage [16,42].

In our experimental conditions, the subjects enrolled had an overall good nutritional status [34]; in addition, there were no significant differences between women and men for any of the dietary variables evaluated [31]. This is in part due to the homogenous food choices related to the availability of meals provided through the daily menu at the nursing home. However, we found a positive association between the intake of dietary cholesterol and the levels of oxidatively induced-DNA damage. This positive association was also confirmed when considering differences according to sex, in particular a positive association with total cholesterol was observed in women while an inverse association with MUFA in men.

As regards dietary fibre, it plays a key role in human health and its intake is associated with a reduction in weight gain, the incidence of diabetes, obesity prevalence, hypercholesterolemia, inflammation and indirectly also reduced oxidative stress [43,44]. Overall, the intake of fibre in our population was relatively low (i.e., about 17–18 g/day) [31] with respect to dietary recommendation (at least 25 g/day) defined by Italian and international guidelines. Statistical analysis did not show any inverse association between total fibre intake (adjusted for energy intake) and the levels of DNA damage. However, when considering data stratified by sex, an inverse association was found in women.

Also vitamins and minerals can contribute in the modulation of activities against DNA damage [25]. For example, the intake of vitamin C and vitamin E has been found inversely associated with the levels of 8-oxodG and DNA strand breaks [45]. Similarly, folates, vitamin B_6 and B_{12} have been associated with lower risk of single and double DNA strand breaks [45–48]. Among minerals, magnesium, calcium, iron, copper, zinc and selenium have been found inversely associated with DNA damage [49,50] probably due to their role in the functioning of numerous antioxidant enzymes [25]. However, other studies have found a positive correlation between high calcium and iron intake and DNA

damage [51,52]. In our trial, we did not underline critical vitamin and mineral intakes [31]. However, interestingly, we have found an inverse association between the levels of FPG-sensitive sites and the intake of vitamin C, vitamin E, folates and vitamin B_6, while no correlations were found with the intake of minerals. The inverse associations were also confirmed when stratifying subjects by sex further underlying the importance of adequate vitamin intake in this target population.

Besides macro and micronutrients, also the protective effect of bioactives such as polyphenols against oxidative stress has been largely investigated [18]. A number of studies have shown the capacity of these compounds to neutralize the harmful effect of reactive oxygen species as well as to disrupt their propagation [53,54]. Furthermore, several studies have reported their ability to increase the activity of numerous antioxidant enzymes (e.g., catalase, superoxide dismutase, glutathione peroxidase) and to upregulate nuclear factor erythroid-related factor and antioxidant-responsive elements, crucial for the modulation of oxidative stress and thus DNA damage [53,54]. In this regard, a recent review has documented the capacity of polyphenols to protect cells from DNA damage suggesting it could be dependent on the type and amount of polyphenols tested, especially in in vitro studies where the doses administered can be very high with respect to an in vivo situation [48]. In fact, several in vitro studies reported an apparent increase in the levels of DNA damage after supraphysiological concentrations of polyphenols.

The contribution of polyphenols in the modulation of DNA damage has been documented also in numerous human trials [55,56] showing a decrease in endogenous and oxidatively induced-DNA damage after administration of dietary doses of polyphenols and/or polyphenol-rich foods in different target group of population [57–60].

In the present study, we estimated a total dietary polyphenol intake of about 660 mg/day (data based on Folin–Ciocalteau analysis as reported in the Phenol-Explorer database and other literature) as previously reported [31], with flavonoids and phenolic acids as the most consumed polyphenol classes. The intake was comparable between women and men and in line with other studies performed in the Italian population (In Chianti study) [61], but apparently lower if compared to the intake of other target populations [57]. Moreover, we could not find any significant association between total polyphenols or subclasses intake, and the levels of DNA damage, apart from an apparent, but not significant, inverse correlation in men. It is also noteworthy that we could not demonstrate in our group of older subjects the protective effect of these bioactive compounds against DNA damage even following a polyphenol rich dietary pattern [34].

In our study, we also tried to identify the possible relationship between anthropometric, clinical and metabolic markers, and the levels of DNA damage. It is widely recognized that metabolic and clinical markers such as glycaemia, insulin, lipid profile, blood pressure together with inflammatory markers (e.g., CRP, IL-6, TNF-α), as well as body weight and BMI, may represent important determinants in the onset and/or progression of oxidative stress [9]. As previously reported, these variables were in the normal range in our target group apart from an overall mild overweight (mean BMI 27 kg/m^2) [34].

We did not evidence associations between endogenous DNA damage, as FPG-sensitive sites, and the markers under evaluation, even if an apparent trend for age ($p = 0.086$) was underlined. On the contrary we have found an unexplained inverse association between the levels of H_2O_2-induced DNA damage (as marker of cell resistance to oxidative stress) and BMI, body weight and DBP while only a trend towards a positive association with SBP, LDL/HDL ratio and CRP. After sex stratification, the associations seemed to be sex-specific (e.g., the inverse correlation with renal/liver function enzymes, DBP, body weight and BMI). Despite some cross-sectional studies that have reported results in line with our findings, a large number of human trials documented a positive association between overweight/obesity and DNA strand breaks or oxidative DNA damage [45,62,63]. A possible explanation for these conflicting results could be related to the cryopreservation process that could have activated a further metabolic activity in the frozen cells that make them more resistant when exposed to an oxidative stress condition, as already reported [35].

In addition, we may hypothesize that the inverse association found between the levels of H_2O_2-induced DNA damage and body weight and BMI could be related to catalase activity. This enzyme is involved in the conversion of H_2O_2 to hydrogen and water. Some studies have reported higher catalase activity in overweight/obese subjects compared to lean individuals as a compensatory mechanism to counterbalance an oxidative stress condition and an increase in the metabolic production of H_2O_2 in these subjects [64,65]. Although this could partially explain the inverse correlation found between DNA damage and BMI, the lack of data on enzymatic activity and the mild overweight of the subjects makes this consideration only a speculation.

Regarding the other metabolic markers under study, a positive association was depicted with LDL/HDL ratio (significant) and total cholesterol/HDL ratio (not significant) in women, supporting the contribution of dietary and circulating lipids in the increase of DNA damage as also reported in literature in different target groups [42,66,67]. Another interesting positive correlation was observed between the levels of uric acid and DNA damage evaluated following the ex vivo induced oxidative stress. Uric acid is the major endogenous end-product of purine metabolism. Experimental and clinical evidence suggests that uric acid could play an important role as antioxidant and could be involved in DNA repair mechanisms [68,69]. However, at the same time, high levels of uric acid were recognized as a marker of acute, severe and chronic inflammatory states [70,71]. In addition, evidence exists, mainly based on epidemiological studies, that high uric acid levels can be considered as an important risk factor for oxidative stress and may contribute to an early onset of cardiovascular, renal and metabolic diseases [72]. It is worth noting that in our population the levels of this marker were within the normal range, thus these findings should be further investigated to support the significance of the association in the older subjects also in view of recent published results [73].

Finally, in the present investigation we could not evidence any relationship between serum zonulin levels, evaluated as a marker of IP, and the different DNA damage markers analysed. The selection of subjects based on their increased IP and small sample size could justify the lack of significant relationships. This result cannot be considered conclusive since no other data have been previously published, although it may be considered plausible that an increased IP, often linked to age related dysbiosis, could promote higher oxidative stress [74,75].

The results obtained in the present study are preliminary and the overall approach has some strengths related to the type of population under study and the specific setting enabling the control of numerous variables, including diet. However, some limitations are worth to be highlighted. First of all, the number of subjects enrolled does not allow a definitive conclusion above all by considering the high inter-individual variability in DNA damage levels found in our study and reported also in the literature. In addition, the lack of an actual analysis of nutritional status through adequate biomarker of intake could represent a further limitation.

5. Conclusions

In conclusion, the results obtained support the association among some dietary and clinical/metabolic markers and the levels of DNA damage in older subjects. However, further investigations are needed to confirm the association found in a larger group of older individuals in which also the impact of other confounding factors should be considered.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10050730/s1, Figure S1: Levels of H_2O_2-induced DNA damage stratified by quartiles of body mass index (**A**), LDL/HDL ratio (**B**), triglycerides (**C**), HOMA-index (**D**) and C-reactive protein (**E**). Table S1: Baseline characteristics of subjects selected for the study, Table S2: Nutrient and polyphenol intake at baseline.

Author Contributions: P.R. and S.G. are responsible for the trial conception and design, and funding acquisition, and in collaboration with A.C., C.A.-L. and P.A.K. contributed to the development of

study protocol for clinical and ethical aspects and to the selection of biochemical markers under study from a clinical perspective. C.D.B., D.M. and S.B. drafted the manuscript; C.D.B. and S.B. collected the samples and performed the analysis of clinical and metabolic markers; S.B., L.G., M.M. performed the analysis of comet assay under C.D.B., P.R., and M.P. supervision and contributed to the draft of the manuscript; T.M., N.H.-L. performed the analysis on polyphenol intake; L.G. and G.G. performed the statistical analysis. Finally, P.R., S.G., M.P., T.M.; N.H.-L., C.A.-L., P.A.K., critically reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee) of the University of Milan (15 February 2016, ref: 6/16 CE_15.02.16_Verbale_All-7). The trial was prospectively registered (28 April 2017; ISRCTN10214981).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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MANUSCRITO 9

A polyphenol-rich dietary pattern improves intestinal permeability, evaluated as serum zonulin levels, in older subjects: The MaPLE randomised controlled trial

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MANUSCRITO 9

Antecedentes y objetivo: El aumento de la permeabilidad intestinal (PI) puede darse en personas mayores y contribuir a la activación del sistema inmunitario y la inflamación. Las intervenciones dietéticas pueden representar una posible estrategia para reducir la PI. En este sentido, se han propuesto bioactivos alimentarios específicos, como los polifenoles, como potenciales moduladores de la PI debido a su capacidad para afectar a varias dianas y vías críticas que controlan la PI.
El ensayo pretendía probar la hipótesis de que un patrón dietético rico en polifenoles puede disminuir los niveles séricos de zonulina suero, un marcador sustitutivo de la PI que interviene en la modulación de la unión hermética, y puede alterar de forma beneficiosa la microbiota intestinal microbiota intestinal y los marcadores bioquímicos y clínicos asociados a la IP en sujetos de edad avanzada.

Métodos: Se realizó un ensayo de intervención aleatorio, controlado y cruzado. Sesenta y seis sujetos (de 60 años de edad) con un aumento de la PI basado en los niveles de zonulina en suero, fueron asignados aleatoriamente a uno de los dos brazos de la intervención, consistente en una dieta de control (dieta C) frente a una dieta rica en polifenoles (dieta PR). Cada intervención de intervención duró 8 semanas y estuvo separada por un periodo de lavado de 8 semanas. Al principio y al final de cada Al principio y al final de cada período de intervención, se recogieron muestras de suero para la cuantificación de la zonulina y otros marcadores biológicos. También se recogieron muestras fecales para investigar el ecosistema microbiano intestinal. intestinal. Además, se evaluaron los parámetros antropométricos/físicos/bioquímicos y la ingesta de alimentos. alimentos.

Resultados: Cincuenta y un sujetos completaron con éxito la intervención y se demostró un alto cumplimiento de los protocolos dietéticos. de los protocolos dietéticos. En general, la ingesta de polifenoles aumentó significativamente, pasando de una media de 812 mg/ día en la dieta C a 1391 mg/día en la dieta PR. El análisis de varianza de dos vías mostró un efecto significativo de la interacción entre el tratamiento (p ¼ 0,008) y el tiempo de tratamiento (p ¼ 0,025) sobre los niveles de zonulina en suero, que disminuyeron después de las 8 semanas de dieta PR. Además, se observó una interacción tratamiento-tiempo que mostró una de la presión arterial diastólica (p ¼ 0,028) tras la dieta de RP, que fue mayor en los que no que no utilizaban fármacos antihipertensivos. Una disminución de la presión arterial diastólica (p ¼ 0,043) y sistólica (p ¼ 0,042) en las mujeres. Curiosamente, se observó un aumento significativo de las bacterias fermentadoras de fibra y productoras de butirato, como la familia Ruminococcaceae y miembros del género Faecalibacterium. de la intervención.

La eficacia de esta intervención dietética fue mayor en los sujetos con una zonulina sérica más elevada al inicio, que mostraron alteraciones más pronunciadas en los marcadores en estudio. Además, la reducción de la zonulina fue también mayor entre los sujetos con mayor índice de masa corporal y con resistencia a la insulina al inicio, lo que demuestra la estrecha interacción entre la PI y las características metabólicas.

Conclusiones: Estos datos muestran, por primera vez, que una dieta de PR puede reducir los niveles de zonulina en suero, un marcador marcador indirecto de PI. Además, la dieta PR redujo la presión arterial y aumentó las bacterias fermentadoras de fibra y bacterias productoras de butirato. Estos resultados pueden representar un avance inicial para otros estudios de intervención de intervención que evalúen posibles tratamientos dietéticos para la gestión de la PI, la inflamación y la función intestinal en diferentes poblaciones. intestinal en diferentes poblaciones objetivo.

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Randomized Control Trials

A polyphenol-rich dietary pattern improves intestinal permeability, evaluated as serum zonulin levels, in older subjects: The MaPLE randomised controlled trial

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SUMMARY

Background & aim: Increased intestinal permeability (IP) can occur in older people and contribute to the activation of the immune system and inflammation. Dietary interventions may represent a potential strategy to reduce IP. In this regard, specific food bioactives such as polyphenols have been proposed as potential IP modulator due to their ability to affect several critical targets and pathways that control IP. The trial aimed to test the hypothesis that a polyphenol-rich dietary pattern can decrease serum zonulin levels, an IP surrogate marker involved in tight junction modulation, and can beneficially alter the intestinal microbiota, and IP-associated biochemical and clinical markers in older subjects.

Methods: A randomised, controlled, cross-over intervention trial was performed. Sixty-six subjects (aged ≥ 60 y) with increased IP based on serum zonulin levels, were randomly allocated to one of the two arms of the intervention consisting of a control diet (C-diet) *vs.* a polyphenol-rich diet (PR-diet). Each intervention was 8-week long and separated by an 8-week wash out period. At the beginning and at the end of each intervention period, serum samples were collected for the quantification of zonulin and other biological markers. Faecal samples were also collected to investigate the intestinal microbial ecosystem. In addition, anthropometrical/physical/biochemical parameters and food intake were evaluated.

Results: Fifty-one subjects successfully completed the intervention and a high compliance to the dietary protocols was demonstrated. Overall, polyphenol intake significantly increased from a mean of 812 mg/day in the C diet to 1391 mg/day in the PR-diet. Two-way analysis of variance showed a significant effect of treatment ($p = 0.008$) and treatment × time interaction ($p = 0.025$) on serum zonulin levels, which decreased after the 8-week PR-diet. In addition, a treatment × time interaction was observed showing a reduction of diastolic blood pressure ($p = 0.028$) following the PR-diet, which was strongest in those not using antihypertensive drugs. A decrease in both diastolic ($p = 0.043$) and systolic blood pressure ($p = 0.042$) was observed in women.

Interestingly, a significant increase in fibre-fermenting and butyrate-producing bacteria such as the family Ruminococcaceae and members of the genus *Faecalibacterium* was observed following the PR intervention.

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The efficacy of this dietary intervention was greater in subjects with higher serum zonulin at baseline, who showed more pronounced alterations in the markers under study. Furthermore, zonulin reduction was also stronger among subjects with higher body mass index and with insulin resistance at baseline, thus demonstrating the close interplay between IP and metabolic features.

Conclusions: These data show, for the first time, that a PR-diet can reduce serum zonulin levels, an indirect marker of IP. In addition, PR-diet reduced blood pressure and increased fibre-fermenting and butyrate-producing bacteria. These findings may represent an initial breakthrough for further intervention studies evaluating possible dietary treatments for the management of IP, inflammation and gut function in different target populations.

This study was registered at www.isrctn.org as: ISRCTN10214981.

1. Introduction

The integrity of the intestinal barrier is fundamental for gut and human health. This barrier is maintained thanks to the active involvement of "tight junctions", in which multiprotein complexes serve to seal the junctions between epithelial cells. Tight junctions control mucosal permeability and act as intermediates/transducers in cell signalling cascades [1]. The layer of epithelial cells represents a physical barrier against external factors, including microbial factors, while maintaining a controlled crosstalk with commensal bacteria [2]. The disruption of the junctions between epithelial cells results in increased intestinal permeability (IP), also known as "leaky gut". It enables the translocation of microorganisms and/or microbial factors from the intestinal lumen to the blood stream, leading to the activation of immune function and inflammation [3]. Increased IP has been proposed as a potential contributor to a wide range of intestinal disorders such as irritable bowel syndrome, and inflammatory bowel and coeliac diseases. More recently, increased IP has also been proposed as a potential cause of age-related conditions [4]. In fact, age has reported as an independent risk factor for altered IP [5], and some studies have shown an increased IP over the age of 50 y due to a potential progressive process of deterioration in the functions and integrity of the intestinal barrier [5]. During ageing, an increased IP may contribute to the onset of chronic low-grade inflammation, also known as inflamm-ageing [6,7], responsible for the higher risk of several age-related diseases including metabolic syndrome, obesity, diabetes and cardiovascular diseases. Gut microbiota seems to play a central role in driving inflamm-ageing, as it can release several inflammatory factors, and contribute to IP (dys)regulation [8,9]. For example, gut microorganisms may act directly on IP by affecting tight junction functionality and/or indirectly by modulating inflammation [4]. Consequently, the manipulation of gut microbiota has been proposed as a potential novel strategy to improve IP. Dietary patterns and specific food bioactives are considered important factors capable to manipulate and shape gut microbiota, which can positively or negatively affect IP. Recent studies discussed the role of several macro and micronutrients in the modulation of IP. The results highlighted that an excessive energy intake, high-fat, high-sugar and high-animal protein consumption, as well as alcohol intake are associated with an alteration of the intestinal microbial ecosystem and an increased IP [10–13]. Moreover, inadequate nutrient intake (e.g. low protein intake) that often occurs in older subjects can contribute to increase IP [4]. Conversely, diets rich in low-energy dense foods (e.g. fruits and vegetables) and fibres have been associated with a healthier gut microbiota and a reduced IP [14]. In the context of a diet-microbiota-IP axis, several food bioactives, including polyphenols, may represent a potential strategy to positively affect microbiota composition and to improve IP and related conditions [15]. Polyphenol biological functions include antioxidant and anti-inflammatory properties, and immunomodulatory activity at both intestinal and systemic levels [2]. Although the exact molecular mechanisms are not completely understood, polyphenols may directly and/or indirectly act at different levels of the intestinal barrier by regulating tight junction function, the production of numerous inflammatory cytokines and the activation of antioxidant genes [2]. Furthermore, polyphenols undergo extensive modifications by the gut microbiota and, consequently, affect the intestinal microbial ecosystem. For such reasons, polyphenols are promising candidates for developing dietary intervention strategies to counteract the detrimental effects of elevated IP.

The evaluation of IP in human subjects is challenging. Zonulin, also known as prehaptoglobin-2, has been suggested as a candidate marker; it is a 47-kDa protein produced mainly by epithelial cells (e.g. in the gut) that is able to reversibly modulate paracellular permeability [16]. In fact, zonulin is a fundamental regulator of intercellular junctions since it can bind the epidermal growth factor receptor through the activation of protease-activated receptor 2. The derived complex induces the signalling pathway causing tight junction disassembly (induced by the phosphorylation of zonula occludens proteins) thus enabling the paracellular passage of factors between the luminal environment and the inner part of the mucosa. For this reason, zonulin has been considered as a good surrogate marker of impaired intestinal barrier function and increased IP, and has been shown to be involved in different physiological and pathological conditions [17]. Moreover, several studies have reported correlations between the results obtained from the most common and validated IP test (based on lactulose/mannitol urine excretion evaluation following standardised sugar intake; the 'multi-sugar assay') and serum zonulin levels [18–20]; while such correlation was not reported considering lactulose/rhamnose urine excretion in healthy adults [21].

To the best of our knowledge, human intervention studies aimed at investigating the role of polyphenols in the modulation of IP are still lacking. Within this context, the MaPLE (Microbiome mAnipulation through Polyphenols for managing Leakiness in the Elderly) randomised, controlled, crossover trial was designed to assess whether a high intake of polyphenol-rich foods in older subjects would modulate the intestinal microbial ecosystem, reduce serum zonulin levels as indirect marker of IP and improve markers of inflammation, oxidative stress and vascular function.

2. Materials and methods

2.1. Setting and subjects' recruitment

The MaPLE trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy), an institution including residential care and independent residences for older subjects. The setting was selected in

order to enable a significant control of most of the experimental variables affecting dietary intervention studies as previously described [22]. Subjects selection was performed in collaboration with physicians and staff at OIC Foundation, based on medical examination and the evaluation of drug therapies. The final eligibility was defined according to the inclusion and exclusion criteria reported below.

To be included in the trial, the subjects had to be ≥ 60 years old, with an adequate nutritional status, a good cognitive status, good functional autonomy, and with an increased IP evaluated as serum zonulin level concentrations by considering reference values and other literature as previously detailed [17,18,22,23]. Exclusion criteria included: having Coeliac disease, advanced stage of chronic diseases such as cirrhosis, renal insufficiency (dialysis), severe Chronic Obstructive Pulmonary Disease (COPD) or severe cardiovascular disease (heart failure class III or IV NYHA - New York Heart Association). Moreover, subjects with malignant tumours that required treatment in the previous 2 years were excluded as well as those treated with antibiotics in the last month before the intervention period.

The entire process of subject selection and randomization within the clinical trial is reported in Fig. 1. The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the University of Milan, Italy (ref: 6/16/CE_15.02.16_Verbale_All-7). All participants were informed about the study protocol and they signed an informed consent before the enrolment. The trial was registered under ISRCTN.com (ISRCTN10214981).

2.2. Definition and set up of the dietary intervention

The dietary intervention protocol was developed following an initial evaluation of the nutrient composition (through MetaDieta® software by Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) and total polyphenol content (mainly through Phenol-Explorer.eu database) of the daily menu provided by OIC Foundation to the host. The development of the polyphenol-rich (PR) dietary pattern was designed by the substitution of some low-polyphenol products in the control diet (C-diet) with other comparable PR-products (e.g. foods used for snack or breakfast) and maintaining as much as possible the overall energy and nutrient composition. Specifically, subjects consumed three small portions per day of the following selected PR-foods: berries and related products, blood orange and juice, pomegranate juice, green tea, Renetta apple and purée, and dark chocolate (callets and cocoa powder-based drink), which provided a mean of 724 mg/day of total polyphenols estimated by Folin-Ciocalteu analysis [24]. Thus, the total polyphenol intake in the intervention diet, i.e. including the menu plus the PR-foods, was roughly doubled compared to the C-diet.

A schematic plan of the type and serving sizes of PR-foods consumed daily within the intervention has been reported previously [22].

2.3. Experimental design

The trial consisted of an 8-week, randomised, repeated measure cross-over intervention study (i.e. PR-diet vs C-diet). Volunteers were randomly allocated in one of the two arms of the intervention starting with PR-diet or C-diet according to a computerized randomization protocol [22]. Subjects assigned to the PR-diet received the 3-daily portions of selected PR-products described before. During the C-diet period, subjects followed the regular menus provided by the nursing home that were previously evaluated for their nutritional composition. After a wash-out period (8 weeks) performed to avoid any carry-over effect, the groups were switched to the other treatment.

At the beginning and at the end of each intervention periods all participants underwent to physical and general condition examinations (i.e. height, weight, blood pressure and clinical signs). In addition, biological samples (blood and faeces) were collected for the analysis of metabolic and functional markers and microbial ecosystem.

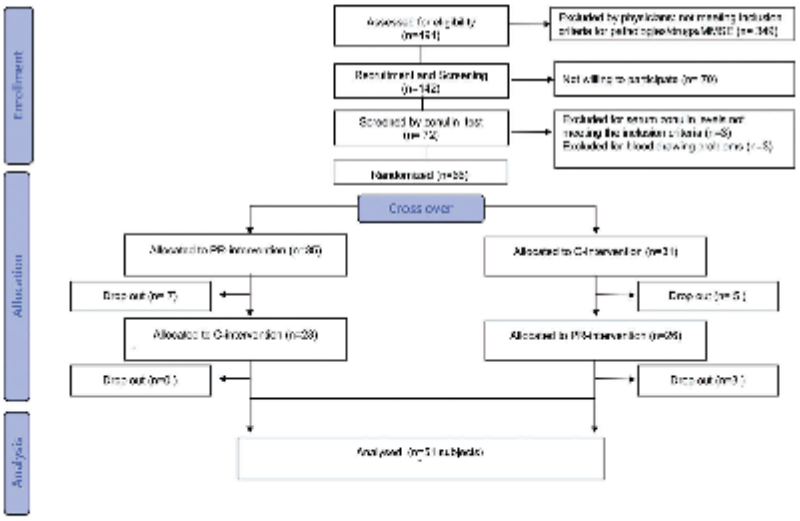

Fig. 1. Consort flow diagram.

2.4. Compliance

To ensure adequate compliance to the dietary intervention protocol PR-rich foods, that were in part or completely not consumed, were registered at the end of each day. In addition, weighted food diaries were filled in during the trial to assess the adherence to both dietary treatments (PR- and C- diet) [21].

2.5. Anthropometrical and physical evaluations

Height and weight were measured according to Lohman et al. international guidelines [25]; body mass index (BMI) was calculated according to the formula – weight (kg)/height (m^2). Reference scores were defined according to international guidelines [25]. Blood pressure was obtained in resting, seated position following the JNC 7 guidelines [26].

2.6. Blood sampling and analysis

After an overnight fast, blood samples were drawn in Vacutainer tubes containing silicon gel for serum and maintained at room temperature for at least 30 min. Serum was then obtained by tube centrifugation (1400 g × 15 min, 4 °C), splitted in small aliquots into specific vials and stored at −80 °C until analysis. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood by density gradient centrifugation with Histopaque 1077 [27] and cryopreserved at −80 °C in a media (50% fetal bovin serum, 40% RPMI-1640, 10% DMSO) until analysis. Samples were used for the evaluation of several metabolic and functional parameters [22].

In particular, glucose, insulin, lipid profile (total cholesterol, triglycerides), liver and renal function (i.e. aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, creatinine) were analysed using a standardized routine-use automatic biochemical analyser (ILAB 650, Instrumentation Laboratory, Lexington, MA). Serum concentration of low-density lipoprotein cholesterol (LDL-C) and non-high-density lipoprotein cholesterol (HDL-C) were estimated by using the Friedewald formula [28] and by subtracting HDL-C from total cholesterol (TC), respectively. In addition, the homeostasis model assessment of insulin resistance (HOMA-IR) was performed, and values > 3 were considered as a criterion for insulin resistance [29]. The Cockroft-Gault (C-G) index based on creatinine clearance was calculated according to the formula previously defined in literature [30,31].

2.7. Evaluation of IP

Serum samples for IP evaluation (at recruitment and at each time point of intervention) were defrosted at room temperature and the serum zonulin levels were quantified using the Immunodiagnostik® ELISA kit (Bensheim, Germany). The assay, based on a competitive Elisa method, entails the addition to each sample (including standard and control samples) of a biotinylate zonulin tracer and the subsequent use of a pre-coated 96-well plate with polyclonal anti-zonulin antibody. Peroxidase-labelled streptavidin addition was used to bind the biotinylate zonulin tracer and after the reaction, a plate reader (TECAN Infinite F200, Tecan Group Ltd. Mannedorf, Switzerland) was used to read the fluorescence at 450 nm. Serum zonulin concentrations were quantified using a standard curve calculated by a 4-parameter algorithm as reported by the manufacturer.

2.8. Evaluation of inflammatory markers

C-reactive protein (CRP), tumour necrosis factor α (TNF-α) and interleukin-6 (IL-6) levels were quantified in serum at the beginning and the end of each intervention period using specific ELISA kits (DCRP00, HSTA00E, and HS600B, respectively; R&D Systems, Biotechne, Abingdon, UK).

2.9. Evaluation of vascular markers

Serum samples at each time point were used to quantify vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) using an ELISA kit (Booster® from Vinci Biochem S.r.l., Vinci, Italy). After competitive treatment with antibodies and fluorophore, fluorescence was quantified using a TECAN Infinite F200 plate reader. A 4-parameter algorithm was used to create the standard curve and to calculate serum concentrations.

2.10. Evaluation of oxidative stress markers

The levels of endogenous and oxidatively-induced DNA damage were evaluated in PBMCs using the comet assay. Endogenous DNA damage was determined by using a specific endonuclease (formamidopyrimidine DNA glycosylase, FPG sensitive sites) able to detect 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) and ring-opened formamidopyrimidine nucleobases, while the levels of oxidatively-induced DNA damage were measured by exposing cells to hydrogen peroxide and measuring their capacity to counteract an oxidative insult. The full protocols of comet assay have been previously published [27].

2.11. Evaluation of faecal bacterial community structure

The intestinal microbiota of volunteers was assessed by 16S rRNA gene profiling. In brief, DNA isolation and amplicon sequencing were carried out as previously described [22]. In brief, DNA was isolated from faeces resuspended in Lysing Matrix E bead beating tubes (MPBio, Santa Ana, CA, USA) through the FastDNA™ SPIN Kit for Soil (MPBio) according to the manufacturer's protocol. Then, the V3–V4 region of the 16S rRNA gene was amplified with panbacterial primers 16S 341F (5′– TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3′) and 16S 806R (5′– GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3′). Finally, amplicons were sequenced using a 600 cycle MiSeq v3 reagent kit (Illumina, San Diego, CA, USA).

Subsequently, sequencing reads were subjected to pairing, filtering, taxonomic assignment, and biodiversity analyses by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) 2 version 2020.6 [32] through the Devisive Amplicon Denoising Algorithm (DADA2) using the Greengenes database (version 13_5). Illumina sequencing generated 4,030,722 filtered paired-end reads (median of 19,328 reads per sample). After merging and denoising by DADA2 the final sequences were 1,076,356 (mean – 5,021, SD – 3306). The sequence length statistics in bp showed: min – 240, max – 457, median – 433, standard deviation – 25. Overall, 7729 unique amplicon sequence variants (ASVs) were identified.

2.12. Statistical analysis

Sample size was calculated based on previous published data [26,33]. It was estimated that 50 subjects were needed to detect a 30% decrease in plasma zonulin with 80% power and alpha – 0.05 with an estimated drop-out rate of 15%.

Differences between treatments were computed by ANOVA for repeated measures design (using the least significant difference (LSD) test as a post hoc analysis to evaluate differences among

means). In addition, although a relatively high zonulin level was used as an inclusion criterion [22], we were interested to determine whether the response to dietary treatments would be different in subjects stratified with respect to median serum zonulin levels at baseline, as it was also reported in recent publications [9,10]. Specifically, subjects were stratified in two groups: LSZ group (lower serum zonulin levels; i.e. ≤ median value) and HSZ group (higher serum zonulin levels; i.e. > median level). The regression and correlation analyses (Spearman and Kendal test) were carried out to highlight associations between zonulin levels (HSZ *vs* LSZ) and physiological and biochemical parameters. In addition, a further statistical analysis in which subjects were stratified in two groups based on median values for BMI and HOMA-index was performed in order to investigate the contribution of metabolic characteristics on IP and markers under study. Potential gender differences were also considered in the analyses.

To identify the bacterial taxa that significantly changed over the trial, taxonomic abundance data were normalized with the negative binomial distribution method (R/Bioconductor DESeq2 package) and statistically analysed using ANOVA for repeated measures design.

Significance was set at $p \leq 0.05$. P values in the range $0.05 < p < 0.10$ were considered as trends. All analyses were performed using the R statistic software version 3.4.2.

3. Results

3.1. Recruitment phase workflow

Of the initial 491 older subjects considered, 349 were excluded after evaluation by OIC physicians since they did not meet the inclusion criteria and 70 subjects declared not to be interested into participate for personal reasons. A total of 72 subjects were further screened and 3 subjects were excluded for low serum zonulin levels. Difficulty in drawing blood was the reason for excluding others 3 subjects.

Finally, 66 subjects (27 men, 39 women) were enrolled in the trial, but only 51 subjects completed the entire intervention study. A schematic flowchart of the protocol, reporting all the information from the recruitment until the end of the study, is shown in Fig. 1.

3.2. Baseline characteristics of the participants

The main characteristics at baseline of the 51 subjects who completed the study protocol are provided in Table 1. Participant ages ranged between 60 and 98 years with a median value of 77 years. Overall, a high inter-individual variability was observed for several markers and in particular BMI (IQR: 22.5; 30.7), glucose (IQR: 86; 113) and total cholesterol levels (IQR 167; 242). Age distribution and data obtained for most of the variables under evaluation were comparable in men and women. HDL-C and insulin levels were significantly higher in women with respect to men ($p = 0.03$ and $p = 0.007$ respectively).

3.3. Correlation analysis of subjects' characteristics based on HSZ or LSZ at baseline

Serum zonulin levels were positively correlated with creatinine ($p = 0.033$) and triglycerides ($p = 0.004$) considering all participants (Fig. 2A). However, the correlation of zonulin with creatinine clearance (C-G index) was not significant. A positive correlation was observed among inflammatory markers (i.e. IL-6, TNF-α, CRP); in addition, a positive correlation emerged between CRP levels and BMI ($p = 0.021$), and between TNF-α and TG ($p = 0.0009$) (Fig. 2A).

Table 1
Baseline characteristics of subjects selected for the study.

Variables	Median (IQR)	Mean ± SD
Age (y)	77 (70; 87)	78.0 ± 10.3
Body weight (kg)	73.6 (62; 83)	73.1 ± 14.0
BMI (kg/m^2)	25.7 (22.5; 30.7)	26.8 ± 5.5
SBP (mm Hg)	125 (120; 130)	125.6 ± 10.8
DBP (mm Hg)	75 (70; 80)	74.5 ± 8.2
Glucose (mg/dL)	95 (86; 113)	113.5 ± 67.2
Creatinine (mg/dL)	0.87 (0.62; 1.05)	0.9 ± 0.29
Uric Acid (mg/dl)	5.10 (4.20; 6.60)	5.5 ± 1.76
TC (mg/dL)	194 (167; 242)	196.3 ± 50.1
HDL-C (mg/dL)	45 (37; 55)	46.5 ± 14.9
LDL-C (mg/dL)	120 (85; 146)	120.5 ± 36.7
TC/HDL-C (ratio)	4.18 (3.54; 5.43)	4.45 ± 1.17
LDL/HDL-C (ratio)	2.57 (2.08; 3.45)	2.72 ± 0.76
TG (mg/dL)	117 (89; 169)	146.1 ± 98.4
AST (U/L)	17 (13; 22)	17.8 ± 5.7
ALT (U/L)	11 (8; 19)	13.4 ± 7.2
GGT (U/L)	23 (17; 46)	38.1 ± 39.0
Insulin uU/mL	6.20 (4.70; 9.20)	8.4 ± 6.4
HOMA index	1.55 (1.15; 2.50)	2.9 ± 5.4
C-G index	69.4 (53.7; 82.5)	74.8 ± 40.5
Zonulin (ng/mL)	40 (34.5; 49.2)	42.2 ± 11.8
sVCAM-1 (ng/mL)	967.9 (628.0; 1327.1)	1239 ± 1683
sICAM-1 (ng/mL)	51.4 (43.9; 65.4)	55.6 ± 20.5
CRP (mg/L)	3.5 (1.6; 9.8)	7.02 ± 8.0
TNF-α (pg/mL)	1.2 (1.0; 1.8)	1.6 ± 1.2
IL-6 (pg/mL)	3.1 (1.9; 5.4)	4.5 ± 4.1

All data are presented as median and interquartile range (IQR) and as mean ± standard deviation (SD).
BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor-alpha; IL-6, interleukin-6.

When subjects were stratified according to high *versus* low serum zonulin levels at baseline, the HSZ group showed a positive correlation between zonulin and HOMA index ($p = 0.037$), and creatinine ($p = 0.025$) (Fig. 2B). This last correlation was not confirmed when C-G index was used. Regarding LSZ subjects, no significant correlation was observed between serum zonulin levels and the other markers under study (Fig. 2C).

3.4. Compliance to the dietary intervention

The nutrient composition of the diet consumed by participants during both treatment periods is reported in Table 2. A comparable pattern of food consumption was evidenced, except for the PR-products provided in the PR-diet. Energy and overall composition of the diet did not differ in the two periods of intervention (PR-diet *vs* C-diet). Following the PR-diet a small decrease in animal proteins and lipids and an increase in carbohydrates and fibre intake (less than 1 g as a mean) was observed with respect to the C-diet. Overall, a high adherence to the dietary protocol was registered: the subjects accepted and easily consumed all the PR-products provided daily and no adverse effects were reported. On average, subjects increased their total polyphenol intake by approximately 70% during the PR-diet compared to the C-diet (Table 2).

3.5. Effect of dietary interventions on markers under study

Table 3 shows the results concerning anthropometrical and physical characteristics, biochemical, inflammatory, vascular and oxidative stress markers evaluated before and after each treatment.

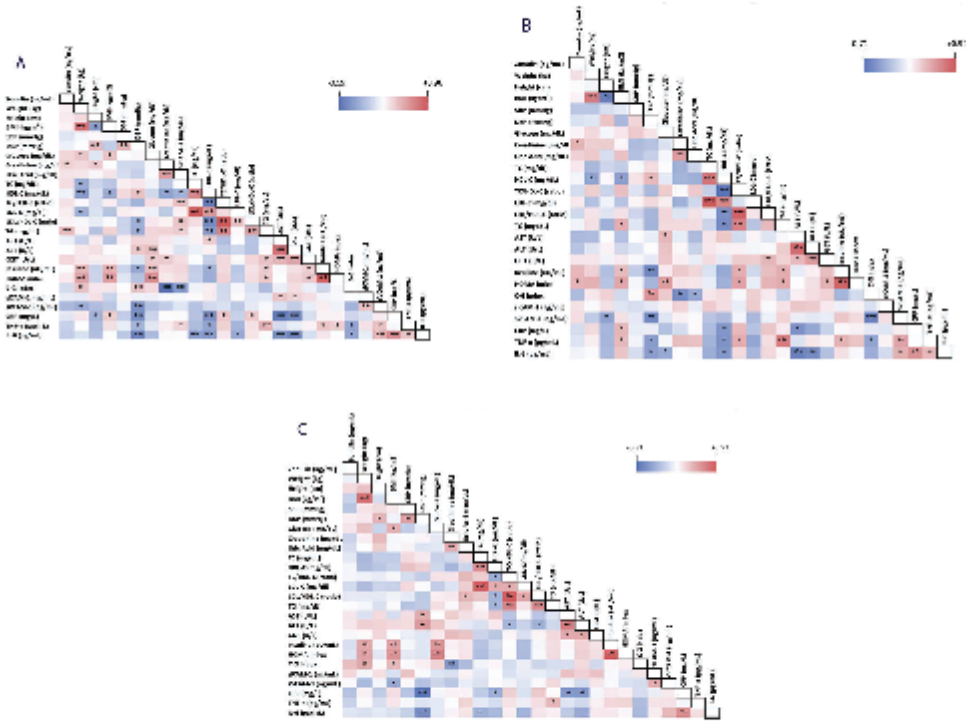

Fig 2. Correlations between the different markers at baseline in the whole group of older subjects (A), in HSZ subjects (serum zonulin levels > median) (B) and LSZ subjects (serum zonulin levels ≤ median) (C). The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation: *P < 0.05; **P < 0.01; ***P < 0.001. Legend: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor –α; IL-6, interleukin-6.

A treatment × time interaction was observed for diastolic blood pressure ($p = 0.024$) and uric acid levels ($p = 0.034$). Post hoc analysis highlighted a decrease of uric acid by 5.2% following the C-diet and a significant reduction in DBP of 3.8% (−2.9 mmHg) following the PR-diet intervention. It is noteworthy that the analysis of data stratified based on anti-hypertensive treatment showed a significant effect of the PR-diet only in the group not taking drugs ($p < 0.05$).

Overall, body weight and BMI measured along the study resulted different in the two treatment periods ($p = 0.023$ and $p = 0.017$, respectively) being slightly lower (−0.4%) during C-diet intervention. Finally, a time effect ($p = 0.039$) was observed for TC with a trend towards reduction following both interventions (−2.8% and −1.8% respectively; −5.4 and −3.5 mg/dL). No significant effect was found for all the remaining variables.

Considering sex (Table 3A and 3B, supplementary material), a significant time effect was observed within men for TC ($p = 0.003$), LDL-C ($p = 0.020$) and the ratio TC/HDL-C ($p = 0.039$), LSD test showed a significant reduction after the PR-diet (by about −7.5%, −7.6% and −6% respectively; i.e. −14, −8.9 and −0.26 mg/dL) but not the control diet. A significant treatment effect was found for AST ($p = 0.042$) and CRP ($p = 0.032$) that showed a trend towards a reduction following both interventions.

Regarding women, a treatment × time interaction was evidenced for SBP ($p = 0.042$) and DBP ($p = 0.043$) showing a reduction after the PR-diet (−3.8% and −3.9% i.e. −4.8 and −2.9 mmHg, respectively), but not after the C-diet. A significant effect of treatment was observed for triglycerides ($p = 0.030$).

3.6. Effect of intervention on IP and other markers

In Table 3 are reported the results on serum zonulin levels before and after each treatment. A significant treatment ($p = 0.008$) and treatment × time interaction ($p = 0.025$) was observed showing a decrease in serum zonulin levels (−6.9%) after the PR-diet. After stratifying by gender (Table 3A and 3B, supplementary material), significant treatment and treatment × time interaction ($p = 0.004$ and $p = 0.010$ respectively) were detected for women but not for men.

The analysis of data based on HSZ or LSZ highlighted the importance of baseline zonulin level as a significant contributor to the impact of the dietary intervention. In fact, HSZ subjects were those with the higher IP reduction ($p = 0.026$) following PR-diet (i.e. −14%) and a significant decrease of DBP ($p = 0.01$; i.e. −4.6%), glucose levels ($p = 0.049$; i.e. −10.9%) and a trend towards a reduction of IL-6 ($p = 0.097$; i.e. −19%); conversely a significant

Table 2
Effect of intervention on nutrient and polyphenol intake.

Variables	PR- diet	C diet	P value
Energy (Kcal)	1537 ± 183	1559 ± 153	0.271
Total carbohydrates (% of energy)	47.2 ± 5.4	45.2 ± 5.2	0.024
Protein (% of energy)	17.7 ± 1.8	18.0 ± 1.9	0.191
Animal proteins (% of energy)	11.7 ± 2.2	12.3 ± 2.2	0.019
Vegetable proteins (% of energy)	4.6 ± 0.9	4.9 ± 0.9	0.005
Total lipids (% of energy)	34.9 ± 4.7	36.9 ± 4.7	0.023
SFA (% of energy)	11.3 ± 2.3	11.8 ± 2.5	0.079
MUFA (% of energy)	15.2 ± 2.8	16.4 ± 2.7	0.020
PUFA (% of energy)	3.2 ± 0.8	4.0 ± 1.5	<0.001
ω-3 (% of energy)	0.6 ± 0.2	0.6 ± 0.2	0.341
ω-6 (% of energy)	2.6 ± 0.7	3.4 ± 1.3	<0.001
Total Fibre (g/1000 kcal)	11.4 ± 1.8	10.5 ± 1.8	0.001
Cholesterol (mg)	216.3 ± 62.2	210.8 ± 67.0	0.468
Total carbohydrates (g)	188.6 ± 24.2	184.2 ± 27.0	0.263
Proteins (g)	66.7 ± 10.5	68.9 ± 8.7	0.040
Animal proteins (g)	45.0 ± 9.8	48.0 ± 8.7	0.002
Vegetable proteins (g)	17.7 ± 3.8	19.3 ± 3.7	0.001
Total lipids (g)	59.1 ± 13.3	63.1 ± 11.3	0.024
SFA (g)	19.2 ± 5.5	20.3 ± 5.3	0.043
MUFA (g)	26.0 ± 5.5	28.6 ± 6.0	0.008
PUFA (g)	5.6 ± 2.0	6.9 ± 2.6	<0.001
Total ω-3 (g)	1.0 ± 0.4	1.1 ± 0.4	0.296
Total ω-6 (g)	4.5 ± 1.7	5.7 ± 2.3	<0.001
Fibre (g/day)	17.4 ± 3.3	16.4 ± 3.2	0.004
Calcium (mg)	736.9 ± 207.7	875.0 ± 233.2	<0.001
Iron (mg)	8.5 ± 1.7	9.2 ± 1.6	0.008
Vitamin B_{12} (μg)	6.2 ± 6.5	5.4 ± 6.3	0.537
Vitamin C (mg)	128.8 ± 47.2	111.7 ± 40.1	0.009
Vitamin E (mg)	8.5 ± 2.2	8.9 ± 2.3	0.341
Vitamin B_1 (mg)	0.9 ± 0.2	0.9 ± 0.2	0.171
Folates (μg)	233.3 ± 66.0	250.8 ± 72.7	0.091
Vitamin B_6 (mg)	1.4 ± 0.3	1.5 ± 0.3	0.130
Total Polyphenols (mg/day)	1391.2 ± 188.1	812.3 ± 198.1	<0.001

All data are expressed as mean ± standard deviation (SD); Data with $P < 0.05$ are significantly different. PR, polyphenol-rich diet; C, control diet; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ω-3, omega-3 fatty acids; ω-6, omega-6 fatty acids.

reduction (p = 0.03; i.e. −8.1%) in uric acid levels was found after C-diet (data not shown).

After stratifying subjects by BMI, a significant reduction of zonulin levels (p = 0.007) and DBP (p = 0.024) was observed after PR diet in the group with BMI higher than the median value. Additionally, a significant increase in IL-6 serum levels (p = 0.049) and decrease in uric acid levels (p = 0.027) was found during the C-diet (data not shown).

Similarly, by considering HOMA-index (i.e. higher *vs.* lower depending on median basal values) as stratification factor, a significant reduction of serum zonulin levels (p = 0.027) and DBP (p = 0.013) following the PR-diet and a decrease (p = 0.027) in uric acid after C-diet was observed (data not shown).

3.7. Effect of intervention on faecal bacterial community structure

The dietary interventions did not significantly affect the α- and β-diversity of the bacterial community structure within the faecal samples (data not shown). On the contrary, repeated measure ANOVA revealed a significant treatment × time interaction for 12 taxonomic units. In specific, 8 taxa belonging to the order Clostridiales (1 family, 3 genera, and 4 ASVs) and two Bacteroidales (1 genus and 1 ASV) were found to be significantly increased after the PR-diet, whereas only two ASVs, ascribed to *Bacteroides uniformis* and *Streptococcus agalactiae* (p = 0.026 and p = 0.034, respectively) were reduced (Table 4). Notably, the most abundant bacterial significantly changed during the trial was the Clostridiales family Ruminococcaceae (mean relative abundance of 21%; p = 0.049). Within this family, the genus of butyrate producing *Butyricicocci* (P = 0.049) and *Faecalibacterium prausnitzii* (3 ASVs; p = 0.022, p = 0.035 and p = 0.049, respectively) were found to be significantly increased (Table 4). Finally, the relative abundance of two members of the Clostridiales family Lachnospiraceae was found to be significantly increased: the genus *Lactonifactor* (p = 0.040) and an ASV ascribed to the species Anaerobutyricum hallii (p = 0.018). The members of the Ruminococcaceae and Lachnospiraceae families were the most influenced by the dietary intervention also when subjects were stratified according to zonulin levels in both the LSZ and HSZ group (data not shown). On the contrary, after stratification, the Bacteroidetes taxa did not show any significant change with the only exception of the same ASV ascribed to *Bacteroides umilis* mentioned above, which was confirmed to be significantly decreased after the PR-diet in the LSZ group (p = 0.037).

An additional correlation analysis was performed between the bacterial taxa significantly modified by the PR-diet and the different markers analysed in the older subjects at baseline (Fig. 3). The results reported a significant inverse correlation between two *F. prausnitzii* taxonomic units and TNF-α serum levels. The same correlation was also found when the relative abundance of the entire *Faecalibacterium* genus was considered. In addition, a significant inverse correlation of the genus *Butyricicoccus* to CRP and IL-6 was reported.

4. Discussion

In this study, we have shown that modifying the diet of older subjects by including small portions of PR-products can reduce serum zonulin concentrations, a widely recognised surrogate marker of IP. Interestingly, greater reductions in serum zonulin concentrations following the PR-diet were observed in the HSZ sub-group, which was accompanied by decreases in diastolic blood pressure, glucose and IL-6 levels (even if the latter was not statistically significant). This supports the notion that the efficacy of PR-diet could depend on the baseline IP condition.

Increased serum zonulin levels and impaired IP condition have been previously found in individuals with metabolic disorders, such as diabetes and obesity [19]. In this regard, we documented a significant association between serum zonulin levels and HOMA index at baseline in subjects classified in the HSZ group but not in the LSZ group suggesting an important contribution of zonulin in discriminating subjects suffering metabolic dysregulation [19].

Similarly, we observed a more pronounced IP reduction after the PR-diet in subjects with higher BMI and HOMA index at baseline, which supports the hypothesis of a link between IP and metabolic disorders.

Previous studies have also reported that leaky gut can play a significant role in age-related inflammation and frailty. Interestingly, Qi et al. [34] found, in a preliminary exploratory study, higher serum zonulin levels in older subjects with respect to young ones. Moreover, a positive association between zonulin levels and markers of inflammation (TNF-α, IL-6) was shown, and an inverse one with physical performance (muscle strength and steps/day). In another study, higher levels of zonulin were associated with gastrointestinal symptoms and psychological distress suggesting the contribution of IP to these signs that are frequently found in the older population [35].

It has been suggested that increased serum zonulin levels also reflect the host response to an inflammatory process, suggesting that a two-way interaction can be present between inflammation and IP [36]. This is also supported by the observation of increased IP in most of the inflammation-related diseases both at intestinal (e.g. inflammatory bowel disease, irritable bowel syndrome, coeliac

Table 3
Effect of 8-week intervention with PR-diet and C-diet on anthropometrical, physical, biochemical, functional characteristics, oxidative stress markers and serum zonulin levels in the whole group of subjects.

Variables (n = 51)	Before PR-diet	After PR-diet	Before C diet	After C diet	P for T	P for t	P for T × t
Body weight (kg)	73.4 ± 14.5	73.7 ± 14.6	72.8 ± 13.7	72.6 ± 13.9	0.023	0.779	0.126
BMI (kg/m^2)	26.9 ± 5.7	27.0 ± 5.7	26.7 ± 5.4	26.6 ± 5.6	0.017	0.677	0.090
SBP (mmHg)	127.2 ± 12.7	124.5 ± 14.6	126.5 ± 9.8	126.2 ± 10.4	0.749	0.107	0.234
DBP (mmHg)	76.7 ± 8.6	73.8 ± 9.4	75.5 ± 6.8	76.9 ± 7.5	0.345	0.285	0.024
Glucose (mg/dL)	114.4 ± 68.2	107.4 ± 42.8	108.6 ± 42.3	105.7 ± 38.2	0.163	0.096	0.360
Creatinine (mg/dL)	0.89 ± 0.29	0.89 ± 0.32	0.89 ± 0.35	0.87 ± 0.31	0.386	0.220	0.422
Uric Acid (mg/dL)	5.6 ± 1.8	5.7 ± 1.7	5.8 ± 1.9	5.5 ± 1.7	0.793	0.361	0.034
TC (mg/dL)	194.9 ± 51.1	189.5 ± 49.7	191.6 ± 49.2	188.1 ± 50.9	0.411	0.039	0.700
HDL (mg/dL)	47.1 ± 14.6	46.6 ± 14.0	47.0 ± 14.9	46.9 ± 15.6	0.876	0.607	0.695
LDL (mg/dL)	119.3 ± 36.6	115.4 ± 33.9	116.4 ± 35.3	114.1 ± 36.9	0.321	0.054	0.646
TC/HDL (ratio)	4.3 ± 1.2	4.2 ± 1.0	4.3 ± 1.1	4.2 ± 1.1	0.610	0.107	0.511
LDL/HDL-C (ratio)	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.7	2.6 ± 0.7	0.426	0.238	0.775
TG (mg/dL)	140.2 ± 86.9	136.9 ± 76.3	141.6 ± 91.7	135.6 ± 92.9	0.992	0.285	0.781
AST (U/L)	17.7 ± 5.4	17.4 ± 5.2	17.7 ± 5.3	17.9 ± 5.3	0.632	0.840	0.509
ALT (U/L)	13.7 ± 7.2	13.2 ± 6.6	13.5 ± 6.8	13.9 ± 6.5	0.656	0.831	0.382
GGT (U/L)	38.7 ± 31.9	37.1 ± 30.7	38.8 ± 39.6	36.8 ± 29.0	0.954	0.354	0.903
Insulin (uU/mL)	8.3 ± 6.6	7.2 ± 3.6	8.4 ± 6.7	7.3 ± 4.4	0.467	0.068	0.639
HOMA index	2.9 ± 5.5	2.0 ± 1.9	2.7 ± 4.6	2.1 ± 2.2	0.153	0.145	0.810
C-G index	72.8 ± 36.0	74.8 ± 40.5	74.3 ± 40.8	74.6 ± 38.7	0.494	0.189	0.449
sVCAM-1 (ng/mL)	980.4 ± 527.8	1037.4 ± 683.9	1319.9 ± 1713.2	1094.4 ± 703.0	0.095	0.462	0.197
sICAM-1 (ng/mL)	54.9 ± 20.5	59.9 ± 28.8	57.9 ± 23.8	55.7 ± 22.8	0.665	0.352	0.600
CRP (mg/L)	6.8 ± 8.7	5.9 ± 7.6	5.0 ± 5.6	6.3 ± 7.7	0.364	0.846	0.158
TNF-α (pg/mL)	1.5 ± 1.1	1.4 ± 0.6	1.4 ± 0.7	1.4 ± 0.6	0.148	0.376	0.562
IL-6 (pg/mL)	4.5 ± 3.7	4.3 ± 5.1	4.2 ± 3.8	5.3 ± 9.3	0.500	0.628	0.189
net–H_2O_2–induced DNA damage (% DNA in tail)	29.1 ± 11.5	30.2 ± 9.7	28.2 ± 9.0	28.5 ± 9.5	0.235	0.507	0.650
net-FPG sensitive sites (% DNA in tail)	20.2 ± 11.3	21.5 ± 10.6	19.9 ± 10.4	22.4 ± 10.8	0.765	0.030	0.577
Zonulin (ng/mL)	41.9 ± 10.4	39.0 ± 8.9	42.8 ± 10.9	44.3 ± 12.5	0.008	0.462	0.025

All data are expressed as mean ± standard deviation (SD). Data with $P < 0.05$ are significantly different. T: treatment effect; t: time effect; T × t: treatment × time interaction. PR, polyphenol-rich diet; C, control diet; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor –α; IL-6, interleukin-6; H_2O_2, hydrogen peroxide; FPG, formamidopyrimidine DNA glycosilase.

Table 4
Bacterial taxa in faecal samples that significantly changed their relative abundance during the MaPLE dietary intervention trial.

ASV nr.	Taxonomy	p	PR-diet		C diet	
			Before	After	Before	After
Dada_235	*p_Bacteroidetes;c_Bacteroidiqa_Bacteroidales;f_Bacteroidaceae;g_Bacteroides;s_uniformis**	0.026	0.16	0.09	0.04	0.11
	p_Bacteroidetes;c_Bacteroidiqa_Bacteroidales;f_Rikenellaceae;g_Alistipes	0.048	0.93	1.31	1.35	1.17
Dada_247	*p_Bacteroidetes;c_Bacteroidiqa_Bacteroidales;f_Rikenellaceae;g_Alistipes;s_onderdonkii*	0.043	0.04	0.16	0.15	0.04
Dada_199	*p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus;s_agalactiae*	0.034	0.18	0.08	0.08	0.12
Dada_184	*p_Firmicutes;c_Clostridiqa_Clostridiales;f_Lachnospiraceae;g_Anaerobutyricum;s_hallii**	0.018	0.03	0.16	0.18	0.06
	p_Firmicutes;c_Clostridiqa_Clostridiales;f_Lachnospiraceae;g_Lactonifactor	0.040	0.22	0.30	0.41	0.21
	p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae	0.049	20.2	22.0	20.8	20.1
	*p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae;g_*	0.048	1.89	2.36	2.09	1.76
	p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae;g_Butyricicoccus	0.049	0.47	0.73	0.45	0.44
Dada_128	*p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.049	0.07	0.16	0.15	0.09
Dada_212	*p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.022	0.03	0.13	0.08	0.07
Dada_89	*p_Firmicutes;c_Clostridiqa_Clostridiales;f_Ruminococcaceae;g_Faecalibacterium;s_prausnitzii**	0.035	0.09	0.22	0.17	0.13

Significant differences were determined according to repeated measure ANOVA (p). The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. ASV, amplicon sequence variant. C, control diet; PR, polyphenol-rich diet. Data with $P < 0.05$ are significantly different. *, the taxonomic identification of the species level has been carried out manually by means of a BLAST search within the 16S ribosomal RNA database of the GenBank, setting a similarity limit of 98% for the taxonomic assignment.

disease) and systemic levels (e.g. obesity, type 2-diabetes) including the age-related low-grade systemic inflammation [37].

The study of the inflammatory state is complex, because each of the available inflammatory markers provide different information on a multifaceted process that is dependent on the triggers and is modulated by both the host and environmental conditions. One of the most used markers is the C-reactive protein (CRP) which is considered a hallmark for inflammation and a sensitive risk factor for cardiovascular diseases. CRP is one of the major acute proteins phase reactants secreted in response to increased levels of inflammatory cytokines such as IL-6, interleukin-1β and TNF-α. High levels of serum CRP, IL-6 and TNF-α have been reported in smokers, obese subjects, diabetics and older adults [38]. In our experimental conditions, we documented that the 8-week intervention with the PR-diet failed to modulate inflammatory markers, in line with other intervention studies with polyphenol-rich foods both in adults and older individuals [39–44]. The lack of effect we observed could be for a number of reasons: the large inter-individual variability in the concentrations of the inflammatory markers reducing the possibility to demonstrate a significant reduction; the high baseline levels registered for inflammatory markers, or an insufficient modulatory effect of the diet. As far as the latter is concerned, it is

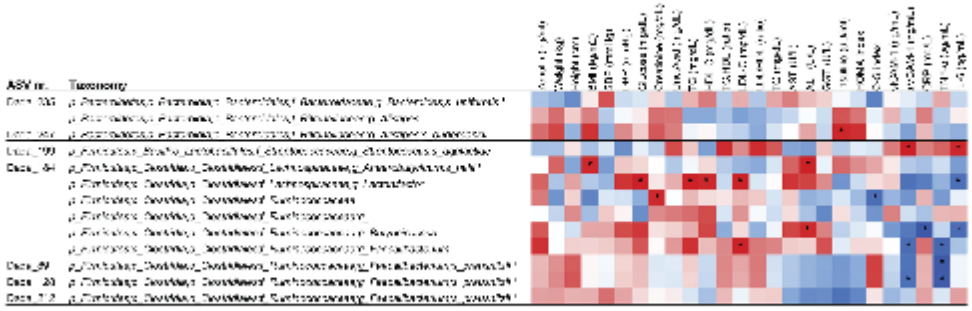

Fig. 3. Correlations between the relative abundance of bacterial taxa that resulted significantly modified by the intervention with the polyphenol-rich diet (+ the genus *Faecalibacterium*) and the different markers in the whole group of older subjects at baseline. The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation: *P < 0.05; **P < 0.01; ***P < 0.001. The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. ASV, amplicon sequence variant. +, the taxonomic identification of the species level has been carried out manually by means of a BLAST search within the 16S ribosomal RNA database of the GenBank, setting a similarity limit of 98% for the taxonomic assignment. Legend: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, Total cholesterol, HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA index, homeostasis model assessment index; C-G index, Cockcroft-Gault index; sVCAM-1, vascular cells adhesion molecules-1; ICAM-1, intercellular cells adhesion molecules-1; CRP, C-reactive protein; TNF-α, tumour necrosis factor –α; IL-6, interleukin-6.

not excluded that higher daily intakes of polyphenols and/or longer dietary intervention periods could cause a bigger effect on these biomarkers. In fact, several clinical trials providing tart cherry juice, supplements of resveratrol, freeze-dried strawberries, purée and dried bilberries or juice for different time periods (from 4 to 26 weeks of intervention) observed an effect on inflammation strictly dependent on the markers analysed, the trial characteristics and the target subjects considered [45–50].

It is well known that oxidative stress increases with age and some reported meta-analyses have shown an association between age and DNA damage in humans [51]. High polyphenol intake has been inversely associated with a reduced risk of oxidative stress, cardiovascular events and mortality [52], possibly by decreasing the levels of reactive oxygen species, adhesion molecules or by inducing the production of vasodilators [53]. In the present study, we could not demonstrate an effect of the PR dietary pattern on DNA damage evaluated both as FPG sensitive sites and protection from H_2O_2-induced strand breaks. These results are in contrast with our previous observations [39] and with the findings reported in other human trials, and summarized in a recent systematic review [54], showing a reduction of endogenous and oxidatively-induced DNA damage following polyphenols and polyphenol-rich food intervention. We may speculate that the different results could be ascribable to the type of target population under study i.e. the older subjects and/or the sample size and the extent and duration of the intervention. The same could be hypothesized for the lack of effect of the dietary intervention on ICAM-1 and VCAM-1 levels and similar results are present in literature [39–41]. As previously reported for inflammatory markers, we found a large inter-individual variability for the vascular markers and this may have precluded the observation of significant effects of the treatment versus control diet. Furthermore, it is noteworthy that consistent with the data reported here, previously studies have described elevated levels of both VCAM-1 and ICAM-1 in older compared to younger individuals [55,56].

The ageing process is not only associated with a physiological alteration of blood vessels and vascular function but also with increasing systolic blood pressure. Therefore, hypertension, in particular systolic hypertension is very common in older subjects representing a major risk factor for cardiovascular disease and strokes [57]. Data from the literature suggest a potential role of polyphenols and polyphenol-rich foods in the modulation of blood pressure [58]. In the present study, most of the subjects showed normal blood pressure levels or a mild hypertension treated with drugs [59]. The PR-diet intervention significantly reduced diastolic blood pressure in both men and women and this effect was strongest in the group of subjects not taking anti-hypertensive drugs (mainly women) but not in those taking anti-hypertensive drugs. However, it is possible that the relatively small number of subjects taking hypertensive drugs (n = 19) prevented us from observing a significant treatment effect. In addition, we found a significant reduction in systolic blood pressure in women, but not in men. The impact of gender on the response of blood pressure to treatments has been recently reviewed, and the role of the kidneys, the renin-angiotensin system, relaxin, and developmental programming highlighted as potential contributors to the differences observed [60].

Ageing is associated with numerous physiological dysfunctions at cellular and tissue levels, including deregulation of lipids and glucose metabolism. Dietary polyphenols seem to play a role in the regulation of glucose homeostasis, insulin sensitivity and lipid metabolism [52,61,62]. In our study the PR-diet did not modify glucose and lipid parameters, apart from a reduction trend in total and LDL-C. Similar findings were observed for tea and tea extracts [63–65], orange juice/hesperidin [66], pomegranate [61,62] and different fruit juices [67]. On the contrary, beneficial effects were documented following the consumption of cocoa products, dark chocolate, and flavan-3-ols [68–70], berries [71,72] and black cumin [73,74]. Nevertheless, despite the overall lack of significant effect of the PR-diet on metabolic features of the host, the degree of IP at baseline was found to affect the impact of the treatment on glucose levels, which was significantly reduced only in the HSZ group, as previously discussed. It is also interesting that a decrease in TC and LDL-CHOL was only found in men together with a significant decrease in CRP levels. However, the small sample size may represent a limitation not enabling a strong emphasis on a potential gender specific response to the dietary treatment.

This study also suggested that the intestinal bacterial community structure, which has been reported to change with ageing [75] and may influence IP [4], can be positively affected by the PR-diet. In fact, we observed a significant increase of *Anaerobutyricum hallii*, *Butyricicoccus* spp. and *F. prausnitzii*, which are fibre-fermenting human gut commensal bacteria that produce as the main end-product of their catabolism, butyrate, a short chain fatty acid of

pivotal importance for intestinal homeostasis, and whose production by the gut microbiota was shown to be lower in older adults [76]. Notably, we found an increase in three taxonomic units belonging to *F. prausnitzii*, a dominant member of the healthy human large intestine, which (a) has been negatively correlated to inflammatory bowel disease and colorectal cancer in numerous reported studies [77], (b) possesses the ability to counteract inflammatory processes [78], and (c) has been proposed as a live biotherapeutic agent to promote intestinal health [79]. In the present study, we found an inverse correlation between two *F. prausnitzii* taxonomic units, the relative abundance of the entire *Faecalibacterium* genus and serum TNF-α levels as marker of inflammation. In addition, the genus *Butyricicoccus*, including butyrate-producing bacteria of the Ruminococcaceae family, was found to be inversely correlated with CRP and IL-6. In this regard, it was reported that patients with inflammatory bowel disease have lower relative abundance of *Butyricicoccus* bacteria in their feces [80]. It is also noteworthy that after the intervention with the PR-diet, we also observed a significant increase of the genus *Lactonifactor*, a member of the Lachnospiraceae family that includes bacteria reported to be involved in the conversion of dietary phytoestrogens such as the plant lignan secoisolariciresinol diglucoside into bioactive forms within the human gut [81,82]. Finally, we also observed an increase of *Alistipes*, a relatively new genus of the phylum Bacteroidetes that includes succinate producing bacteria whose role in human health is unclear due to contradictory outcomes from previously reported studies [83].

The only taxonomic units that were significantly reduced after the PR-diet were ascribed to *B. uniformis* and *S. agalactiae*. These changes may be considered potentially beneficial for the intestinal microbiota of the older subjects. In fact, *S. agalactiae* (Group B streptococci) is a common constituent of the intestinal microbiota that possesses highly invasive and inflammatory potential, often reported to cause sepsis in infants and in older people [84]. On the other hand, *B. uniformis* has been reported to be increased in the gut of breast-fed infants compared to non-breast-fed [85] and one strain of this species (named CECT 7771) has been recently proposed as a potential next generation probiotic [86].

Overall, the main outcome of the MaPLE RCT was to generate evidence that supports the notion that IP reduction can be achieved by increasing the daily intake of polyphenol-rich food sources, and that dietary interventions have real potential as strategies to improve IP status in older populations. It is noteworthy that only limited research has been carried out to investigate the efficacy of dietary treatments in the management of IP [2], and only in one recently published report were both healthy adults and older subjects considered as target populations. In this study, Wilms et al. [87] reported no significant effects of a dietary fibre (sugar beet pectins) on multiple IP parameters, it is tempting to speculate that the effects of the MaPLE treatment diet were due, as hypothesized, to the polyphenols. However, the various other differences between the trials must also be considered, for example the MaPLE trial PR products contained several different sources/types of fibre (not just a single-source pectin), the trial durations and control of diets were different, and there were differences in subject characteristics. Nevertheless, since older subjects are generally low consumers of dietary fibre, and there are frequently reasons why it is difficult to increase their intakes, the evidence reported here that supports the idea that non-fibre dietary interventions can be effective has real potential to be exploited in the development of foods, diets and dietary advice that has beneficial effects on the maintenance of host functional and metabolic homeostasis.

The MaPLE study has several strengths including the well-controlled protocol of intervention including the setting, the daily preparation of products and the continuous interaction with the participants. On the other hand, it has also some limitations related mainly to the relatively small sample size. Furthermore, the evaluation of IP using also the gold standard method (i.e. multi-sugar test, difficult to apply in the population under study) or multiple IP markers could have provided a better insight on the impact of the diet on this condition. This is an important aspect that deserves future investigation in order to support our observation obtained through the analysis of serum zonulin levels.

5. Conclusions

In conclusion, the MaPLE RCT has demonstrated the feasibility and efficacy of a PR dietary pattern, providing approximately 700 mg of total polyphenols daily for 8 weeks, in the reduction of serum zonulin levels, as a marker of IP. Moreover, the intervention caused a reduction in blood pressure and a beneficial effect on fibre-fermenting and butyrate-producing bacteria such as *F. prausnitzii*, an anti-inflammatory commensal bacterium. These results are novel and have potentially important clinical implications. Further intervention studies should be performed aimed at investigating the role of non-pharmacological dietary treatments in the management of IP and markers linking inflammation, metabolic features of the host and gut barrier alteration.

Authors' contributions

PR and SG designed the trial and in collaboration with AC, CAL and PAK optimised the study protocol including the selection of clinical and biochemical markers and the development of the polyphenol-rich diet. CDB contributed to the development of the study protocol and with PR, SG and SB drafted the first version of the manuscript. CDB and SB performed the analysis of zonulin, VCAM-1 and ICAM-1. BK and MSW performed the evaluation of inflammatory markers. BK, GG, SG and PAK carried out the analysis of the faecal microbiota, while MM and LG analysed and elaborated DNA damage supervised by CDB and PR. GG performed the statistical analysis in collaboration with RGD and GP. MP, NHL and RZR contributed to the elaboration of dietary polyphenol intake. All the authors critically revised the draft and approved the final version.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.12.014.

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MANUSCRITO 10

Adherence to Mediterranean diet assessed by a novel dietary biomarker score and mortality in older adults: the InCHIANTI cohort study

Nicole Hidalgo-Liberona*, Tomás Meroño*, Raul Zamora-Ros, Montserrat Rabassa, Richard Semba, Toshiko Tanaka, Stefania Bandinelli, Luigi Ferrucci, Cristina Andrés-Lacueva, Antonio Cherubini

**Igual Contribución*

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MANUSCRITO 10

Objetivo: El presente trabajo tuvo como objetivos desarrollar un *score* de biomarcadores dietéticos a-posteriori basado en los grupos de alimentos de la dieta mediterránea (MDS), evaluar su asociación con la mortalidad la población de la cohorte InCHIANTI y, comparar la predicción de la mortalidad utilizando el biomarcador dietético-MDS y el CFCA-MDS

Métodos: 642 participantes (56% mujeres), de edad ≥65 años, con datos completos sobre biomarcadores dietéticos fueron seguidos durante 20 años en el estudio de cohorte InCHIANTI (Toscana, Italia). Se evaluó la mortalidad por todas las causas, mortalidad cardiovascular y por cáncer. Los biomarcadores dietéticos se seleccionaron a partir de la literatura y de los análisis de correlación con la ingesta dietética de los grupos de alimentos de la dieta mediterránea en el estudio. Se eligieron los niveles de referencia de los siguientes biomarcadores dietéticos: polifenoles totales en orina y metabolitos del resveratrol, y carotenoides en el plasma, selenio, vitamina B12, ácidos linolénico, eicosapentanoico y docosahexanoico, y la proporción de ácidos grasos monoinsaturados/saturados. Se evaluaron las asociaciones de la puntuación de la dieta mediterránea mediante biomarcadores dietéticos y un cuestionario de frecuencia de alimentos (FFQ) validado (en forma de terciles) con la mortalidad mediante regresión de Cox.

Resultados: Durante los 20 años de seguimiento [mediana (Q1-Q3), 14 (8-18) años], se produjeron 435 muertes (139 por enfermedades cardiovasculares y 89 por causas relacionadas con el cáncer). En los modelos totalmente ajustados, la puntuación del biomarcador dietético Dieta Mediterránea se asoció de forma inversa con la mortalidad por todas las causas (HRT3vs.T1 0,72; IC95% 0,56-0,91) y cardiovascular (HRT3vs.T1 0,60; IC95% 0,38-0,93), pero no con la mortalidad por cáncer. Las asociaciones entre la puntuación de la dieta mediterránea del FFQ y la mortalidad no fueron estadísticamente significativas.

Conclusiones: Una mayor adherencia al inicio a una dieta mediterránea evaluada por una puntuación de biomarcadores dietéticos se asoció con un menor riesgo de mortalidad en adultos mayores durante un seguimiento de 20 años. La medición de los biomarcadores dietéticos puede contribuir a orientar el asesoramiento dietético individualizado a las personas mayores.

BMC Medicine

Adherence to Mediterranean diet assessed by a novel dietary biomarker score and mortality in older adults: the InCHIANTI cohort study

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Abstract:

Background

Dietary biomarkers may complement dietary intake assessment made by dietary questionnaires. We developed an a-posteriori dietary biomarkers score based on Mediterranean diet food groups, and evaluated its association with mortality.

Methods

642 participants (56% female), aged ≥65y, with complete data on dietary biomarkers were followed during 20 years in the InCHIANTI cohort study (Tuscany, Italy). Main outcomes were all-cause, cardiovascular and cancer mortality. Dietary biomarkers were selected from literature and from correlation analyses with dietary intakes of Mediterranean diet food groups in the study. The baseline levels of the following dietary biomarkers were chosen: urinary total polyphenols and resveratrol metabolites, and plasma carotenoids, selenium, vitamin B12, linolenic, eicosapentanoic and docosahexanoic acids, and the mono-unsaturated/saturated fatty acids ratio. Associations of the Mediterranean diet score using dietary biomarkers and a validated food frequency questionnaire (FFQ) (as tertiles) with mortality were assessed through Cox regression.

Results

During the 20y follow-up [median (Q1-Q3), 14 (8-18)years], 435 deaths occurred (139 from cardiovascular diseases and 89 from cancer-related causes). In the fully-adjusted models, the dietary biomarker-Mediterranean diet score was inversely associated with all-cause (HR T3vs.T1 0.72; 95%CI 0.56-0.91) and cardiovascular (HR T3vs.T1 0.60; 95%CI 0.38-0.93), but not with cancer mortality. Associations between the FFQ-

	Mediterranean diet score and mortality were not statistically significant. Conclusions A greater adherence at baseline to a Mediterranean diet assessed by a dietary biomarker score was associated with lower risk of mortality in older adults during a 20-y follow-up. The measurement of dietary biomarkers may contribute to guide individualized dietary counseling to older people. Trial registration NCT01331512
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Response to Reviewers:	3rd October 2021 Ming Yang, PhD Associate Editor BMC Medicine RE: BMED-D-21-01164R2 Dear Dr. Yang, Please find attached the revised version of our manuscript entitled "Adherence to Mediterranean diet assessed by a novel dietary biomarker score and mortality in older adults: the InCHIANTI cohort study". We thank the Editors and Reviewers for giving us the opportunity to improve our manuscript. We provide below point-by-point responses and details of the changes made in the manuscript as a result of these comments. We now hope you find this revised version of our manuscript acceptable for publication in the BMC Medicine.

	Kind regards, Raul Zamora-Ros, PhD Editor: 1.We require confirmation of authorship from all co-authors. Still to confirm co-authorship •Nicole Hidalgo-Liberona •Richard Semba •Toshiko Tanaka •Stefania Bandinelli •Luigi Ferrucci •Cristina Andres-Lacueva •Antonio Cherubini All co-authors have now confirmed their authorship. 2.Please make the following change to your section headings: change 'Keyworkds' to 'Keywords'. Done. 3.Please make the following change to your section headings: change 'Introduction' to 'Background'. Done. 4.Please place the Abbreviations list just before the Declarations section. Done. 5.Please make the following change to your section headings: change 'Appendix' to 'Declarations". Done. 6.BMC requires that all publicly available datasets be fully referenced in the reference list with an accession number or unique identifier such as a digital object identifier (DOI). For previously published datasets, we ask authors to cite both the related research articles and the datasets themselves. No publicly available datasets have been used in our study. 7.We have noted that the authors, Toshiko Tanaka [TT] and Stefania Bandinelli [SB] are missing in the listed authors' contributions. Done. Reviewer #3: Thank you for the revision. I have no further comments. We would like to give thanks to the reviewer for their suggestions. Reviewer #4: The authors have provided necessary information and have responded well to my comments. We would like to give thanks to the reviewer for their comments.

Adherence to Mediterranean diet assessed by a novel dietary biomarker score and mortality in older adults: the InCHIANTI cohort study

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Short title: A dietary biomarker score for Mediterranean diet and mortality in older adults.

Abbreviations: BMI, body mass index; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; dietary biomarker MDS, dietary Biomarker Mediterranean Diet Score; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPIC, European Prospective study into Cancer and Nutrition; FFQ, food frequency questionnaire; FFQ-MDS, Food Frequency Questionnaire-Mediterranean Diet Score; HALE, Healthy aging: a Longitudinal study in Europe; HR, hazard ratio; HT, hypertension; ICC, intraclass correlation coefficient; ICD, International Classification of Diseases; InCHIANTI, Invecchiare nel Chianti; IRF, impaired renal function; MD, Mediterranean diet; MDS, Mediterranean diet score; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; STROBE-NUT, Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology.

Abstract

Background: Dietary biomarkers may complement dietary intake assessment made by dietary questionnaires. We developed an a-posteriori dietary biomarkers score based on Mediterranean diet food groups, and evaluated its association with mortality.

Methods: 642 participants (56% female), aged ≥65y, with complete data on dietary biomarkers were followed during 20 years in the InCHIANTI cohort study (Tuscany, Italy). Main outcomes were all-cause, cardiovascular and cancer mortality. Dietary biomarkers were selected from literature and from correlation analyses with dietary intakes of Mediterranean diet food groups in the study. The baseline levels of the following dietary biomarkers were chosen: urinary total polyphenols and resveratrol metabolites, and plasma carotenoids, selenium, vitamin B12, linolenic, eicosapentanoic and docosahexanoic acids, and the mono-unsaturated/saturated fatty acids ratio. Associations of the Mediterranean diet score using dietary biomarkers and a validated food frequency questionnaire (FFQ) (as tertiles) with mortality were assessed through Cox regression.

Results During the 20y follow-up [median (Q1-Q3), 14 (8-18)years], 435 deaths occurred (139 from cardiovascular diseases and 89 from cancer-related causes). In the fully-adjusted models, the dietary biomarker-Mediterranean diet score was inversely associated with all-cause ($HR_{T3vs.T1}$ 0.72; 95%CI 0.56-0.91) and cardiovascular ($HR_{T3vs.T1}$ 0.60; 95%CI 0.38-0.93), but not with cancer mortality. Associations between the FFQ-Mediterranean diet score and mortality were not statistically significant.

Conclusions A greater adherence at baseline to a Mediterranean diet assessed by a dietary biomarker score was associated with lower risk of mortality in older adults during a 20-y follow-up. The measurement of dietary biomarkers may contribute to guide individualized dietary counseling to older people.

Trial registration NCT01331512

Keywords: Dietary biomarkers, older adults, Mediterranean diet, mortality, polyphenols, carotenoids, dietary questionnaires.

~~Introduction~~Background

In 2018, there were 101.1 million persons aged >65 years (19.7%) living in Europe. In 2050, estimations predict an increase up to 149.2 million of older adults, which will represent almost 30% of the overall population [1]. Strategies to promote healthy aging are one of the pillars to minimize the health care and socio-economic impact of the increasing proportion of older adults in Europe [2]. Healthy aging can help to reduce the burden of chronic diseases, disability and increasing health expenditure related to longer life expectancy of older adults [3, 4].

A healthy diet is considered one of the fundamental factors to achieve healthy aging [5]. Indeed, a growing body of epidemiological evidence shows that the Mediterranean Diet (MD) may delay or prevent frailty, cognitive decline, and the onset of many chronic diseases in older subjects [6–9]. Furthermore, several observational studies, including the European Prospective study into Cancer and Nutrition (EPIC)-elderly study, a cohort of 74,607 men and women aged ≥60 years, have shown inverse associations between a greater adherence to different MD scores (MDS), in both Mediterranean and non-Mediterranean countries, and total mortality [8].

Diverse modifications or adaptations of the original MDS, inititallly developed by Tricophoulou et al. [10], have been applied to evaluate relationships between MD and health outcomes [11]. However, to date, adherence to MD has been almost exclusively assessed using dietary questionnaires, such as 24-h recalls and food frequency questionnaires (FFQ), which are susceptible to random and systematic errors in estimating dietary intake [12]. In addition, age-related changes in the digestion and absorption of foods and nutrients could introduce further bias into the accurate assessment of the relationships between dietary intakes and health outcomes in older adults. In our previous analyses from the *Invecchiare nel Chianti* (InCHIANTI) study, no association was observed between either dietary total polyphenol or polyunsaturated fatty acid (PUFA) intakes and all-cause mortality. However, statistically significant inverse associations were found with their dietary biomarkers: total urinary polyphenols [13], and serum PUFA concentrations [14], respectively. Both dietary biomarker are directly related to key features of a MD pattern. Total urinary polyphenol concentrations positively correlate with plant-based foods, such as vegetables, fruits and nuts, among others [15], while plasma PUFA levels positively correlate with fish and seafood consumption [16]. Thus, the use of dietary biomarker may improve the estimations of MD exposure during a long-term follow-up [17]. Other relevant candidates to be included as dietary biomarker in a panel correlated with MD are plasma levels of carotenoids and selenium [18, 19]. In

particular, total carotenoids have been shown as relevant dietary biomarker for the consumption of vegetables, fruits, cereals, and nuts significantly associated with their health promoting effects [19]. Recently, Li *et al.* captured a metabolomics signature related with dietary MDS (based on 67 endogenous metabolites) that was inversely associated with incident cases of cardiovascular disease (CVD) in a Spanish and 3 US cohorts, even after adjustment for the dietary MDS from it was developed [20]. These findings give further support to the hypothesis that biomarkers are better correlated with the overall health-promoting effects of MD.

The current research aims at developing a dietary biomarker panel based on key MD food groups in the population from the InCHIANTI study and investigating its long-term association with all-cause, CVD and cancer mortality. We also compared mortality prediction using dietary biomarker-MDS and FFQ-MDS.

Methods

Study design

The InCHIANTI study is an ongoing prospective cohort of a representative sample of older adults living in the Chianti geographic area (Tuscany, Italy). It was designed to evaluate factors that influence mobility and disability in late adulthood [21]. Details of the InCHIANTI study have been previously published [21]. Participants were recruited in 1998–2000 and were invited every three years to a follow-up visit. The Italian National Institute of Research and Care of Aging Ethical Committee approved the study protocol, and all participants signed an informed participation consent.

The current study was conducted and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology (STROBE-NUT) guidelines (Additional file 1. Supplementary Table S1, [22]).

Study population

At baseline, 1155 subjects aged ≥65 years agreed to participate, with a participation rate of 91.7%. Out of these, participants who had missing data in the FFQ (n=16), in any of the selected dietary biomarkers (n=472) or covariates of interest (n=21) were excluded. The major cause of missing data in dietary biomarkers was the failure to complete the baseline 24h urine collection.

Dietary assessment

Habitual dietary intake was assessed at baseline by trained interviewers using the Italian version of the FFQ developed and validated in the EPIC-Italy study [23]. This questionnaire asked how often (daily, week, monthly) the consumption of 198 food and beverages items in the past year, considering its respective portions sizes. Daily intake of energy, macronutrients and micronutrients were estimated from the dietary questionnaire using a specific software developed for the EPIC study [24]. For the current analysis, dietary data were available at baseline, 3-, 6-, and 9-years of follow-up.

Dietary score of Mediterranean diet

Adherence to a dietary MDS was computed using an 18-point linear scale that incorporated 9 key components of the diet. Each component was divided into tertiles of intakes, and a score of 0, 1 and 2 was assigned to the first, second and third tertiles of intake for the 6 components presumed to fit the MD: vegetables, legumes, fruits and nuts, cereals, fish, ratio monounsaturated fatty acids (MUFA)/ saturated fatty acids (SFA). Alcohol was scored as a dichotomous variable, assigning 2 for moderate consumers (range: 5-25 g/d for women and 10-50 g/d for men) and 0 for subjects above or below the sex-specific range, including teetotallers. The scoring was inverted for the 2 components presumed to not fit the MD: total meat and dairy products. The overall adherence to MDS from dietary intakes (FFQ-MDS) was calculated for each subject as the sum of the values from each component, which resulted in a score between 0 (lowest adherence) and 18 (highest adherence) [25].

Nutritional biomarkers assessment

For this study, the measurement of dietary biomarkers was only available at baseline.

Plasma carotenoids were measured using high-performance liquid chromatography (HPLC). Total carotenoids were calculated as the sum of α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene in micromoles per liter (μmol/L). Within-run and between- run coefficients of variation, respectively, were 7.3% and 9.6% for α-carotene, 4.5% and 5.4% for β-carotene, 2.7% and 3.5% for β-cryptoxanthin, 2.6% and 7.1% for lutein, 6.2% and 6.8% for zeaxanthin, and 7.5% and 7.8% for lycopene [26, 27].

Selenium concentration (μmol/L) at baseline was measured by graphite furnace atomic absorption spectrometry with an Analyst 600 with Zeeman background correction (Perkin Elmer, Norwalk, CT). For baseline measurements, the instrument was calibrated daily by using known plasma selenium standards

(UTAK Laboratories Inc, Valencia, CA). Within-run and between-run CVs were 3.1% and 7.1%, respectively [28].

Plasma fatty acids (FAs) were measured by gas chromatography (HP-6890, Hewlett-Packard, Palo Alto, CA, USA) with a fused silica capillary column (30 m x 0.25 mm internal diameter, HP-225 from Hewlett-Packard, Palo Alto, CA, USA). Total lipids were extracted from 0.15 mL of plasma by using the procedure of Folch (1957). A known amount (50 µg) of heptadecanoic acid (C17:0, Sigma Chemical Co., St. Louis, MO, USA) was added to each sample before extraction as an internal standard. Fatty acid methyl esters (FAMEs) were prepared through transesterification using Lepage and Roy's method, modified according to Rodriguez-Palmero et al (1998). FAMEs were identified by comparison with pure standards (Nu-Chek Prep, Inc., Elysian, MN, USA), and peaks were identified by comparison with standard mixtures of fatty acids. For quantitative and qualitative analysis of fatty acids as methyl esters, calibration curves for FAME (ranging from C14:0 to C24:1) were prepared by adding six increasing amounts of individual FAME standards to the same amount of internal standard (C17:0; 50 µg). The correlation coefficients for the calibration curves of 20 fatty acids were in all cases higher than 0.998 in the range of concentrations studied. The amount of plasma fatty acids (ranging from C14:0 to C24:1) was quantified based on the amount of FAME internal standard (C17:0) that was recovered. The coefficient of variation for all fatty acids was on average 1.6% for intraassay and 3.3% for interassay.[29]. The percentage of values below the limit of detection were: 33% for C24:0 (tetracosanoic acid), 14% for C20:0 (eicosanoic acid), 5% for C22:1 n-9 cis (docosenoic acid), and 3% for C22:0 (docosanoic acid). In these cases, samples were assigned with the minimum detectable value (0.15 µM)

Serum Vitamin B12 was measured at baseline using by radioligand-binding assay (SimulTrac-SNB Radio- assay; ICN Pharmaceuticals). The minimum detectable concentrations was 75 ng/L for vitamin B12 and the intraassay and interassay CVs were 11% and 12%, respectively [30].

In 24h urine samples, total polyphenol concentration was measured by the Folin-Ciocalteau assay after a solid-phase clean-up which allows the elimination of interfering substances that could react with the F-C assay, as described previously [31]. Total polyphenol concentrations were expressed as mg of gallic acid equivalents (GAE) per 24-h urine. Phase II resveratrol metabolites were measured by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described [32]. Briefly, 1mL of urine with the internal standard was loaded into a previously equilibrated Oasis (Waters) HLB (hydrophilic-lipophilic-balanced) solid-phase extraction 96-well plate (30 mg). Urinary resveratrol

metabolites were eluted with acidified methanol solution and ethylacetate. After evaporation, samples were re-constituted with 100 μL of the mobile phase and then analyzed by liquid chromatography (PerkinElmer S200) coupled to a triple-quadrupole mass spectrometer (API3000; AppliedBio- systems). Intra batch and inter- batch coefficients of variation were less than 10.5% and less than 10.7%, respectively [32]. Both plasma and urinary dietary biomarkers were already validated against dietary intake measurements in the InCHIANTI study [28–31, 33, 34]. In the case of urinary resveratrol 31% of the samples had values below the limit of detection. These belonged mostly to teetotallers (56%) and participants who did not consume wine (26%). A zero value was assigned to all these samples.

Biomarker score of Mediterranean diet

The following dietary biomarkers were considered: total carotenoids (calculated as the sum of α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene), selenium, linoleic acid, eicosapentanoic (EPA) and docosahexaenoic acids (DHA), MUFAs [calculated by summing of the following fatty acids: C14:1 n-9 cis (myristoleic acid), C16:1 n-7 cis (palmitoleic acid), C18:1 n-9 cis (oleic acid), C18:1 n-7 trans (octadecenoic acid), C20:1 n-9 cis (11-eicosenoic), C22:1 n-9 cis (docosenoic acid), and C24:1 n-9 cis (tetracosenoic acid)], SFAs [calculated as the sum of C14:0 (myristic acid), C16:0 (palmitic acid), C18:0 (stearic acid), C20:0 (eicosanoic acid), C22:0 (docosanoic acid), and C24:0 (tetracosanoic acid)], and vitamin B12.

Similarly to the FFQ-MDS, the dietary biomarker-MDS was computed using an 18-point linear scale that incorporated dietary biomarker from 9 key components of the diet. From the available measurements in the InCHIANTI database, we selected those that were suggested in previous literature as dietary biomarker of the key MD food groups [16, 18, 35–40] and in addition were significantly associated with dietary intake data in the present study (shown in Table 1). Dietary biomarker for vegetables, legumes, fruits and nuts, cereals, fish, and olive oil were ranked and divided by tertiles. A score of 0,1 and 2 was assigned to the first, second, and third tertiles of dietary biomarker, respectively. Resveratrol metabolites as dietary biomarker of alcohol consumption was scored as a dichotomous variable, assigning 2 for moderate consumers (range of values corresponding to wine consumption; 125-375 g/day for men and 50-250 g/d for women; in the present population: 589-14,557 nmol/24h for men and 1-11,125 nmol/24h for women) [39] and 0 for subjects above or below the sex-specific range, including teetotallers. Wine was the major contributor to alcohol intake (88%) in this older Mediterranean population. The scoring

was inverted for the SFA and Vitamin B12 tertiles, representing meat and dairy products, respectively. dietary biomarker-MDS ranged from 0 to 18, indicating low to high adherence.

Genetic factors related with mortality and parental longevity score

Overnight fasted blood samples were used for genomic DNA extraction as previously described [41]. Illumina Infinium HumanHap 550K SNP arrays were used for genotyping of the following single nucleotide polymorphism (SNP)s: *APOE* ε4 (using the rs429358 and rs7412 SNPs), rs1421783 *MAT2B*, rs6997892 *WRN*, rs10817931 *TRIM32*, rs2684766 *IGF1R*, rs11630259 *IGF1R* [42].The parental longevity score was created from the parental age at death or current age (if alive) as described previously [43]. Briefly, a normal curve using a non-linear least square regression was used to determine the modal age (M) of death for each parent. They were then categorized as short lived if *M* was less than M- 1 standard deviation (mothers: 61–76 years and fathers: 46–74 years), intermediate as M± 1 standard deviation (mothers: 77–91 years and fathers: 75–87 years), and long lived (mothers: older than 91 years and fathers: older than 87 years).

Outcome assessment

Data on 20-year mortality were collected using the Mortality General Registry maintained by the Tuscany Region, as well as death certificates delivered after participants' decease to the registry office of the municipality of residence [13]. Cardiovascular mortality, based on underlying cause of death, was defined as any cardiovascular mortality coded by 9th Revision of the International Classification of Diseases (ICD-9, codes: 390-459). Cancer mortality was defined as any mortality related to known cancer (coded: 140 to 239 by the ICD-9). Other mortality causes (also coded by ICD-9) included respiratory system diseases; unknown causes; injury and poisoning; nervous system and sense organs diseases; endocrine, nutritional and metabolic diseases, and immunity disorders; mental disorders; digestive system diseases; symptoms, signs, and ill-defined conditions; infectious and parasitic diseases; blood and blood-forming organs diseases; and musculoskeletal system and connective tissue diseases. Cases lost during follow-up (i.e., emigration, or refuse to participate) were censored using the date of last contact.

Other main baseline covariates assessment

Covariates were selected *a priori* on the basis of previously reported associations with both MD and mortality [12,15,37]. Trained interviewers administered standardized questionnaires on sociodemographic and lifestyle variables including age, sex and years of education. Smoking habits were self-reported, and participants were classified into never smokers, former smokers, and current smokers. Physical activity was evaluated using a structured questionnaire specifically developed and validated for the InCHIANTI study. The questionnaire required that the participant provide data on past and current physical activity. The details of the questionnaire have been previously reported [45]. Physical activity was coded into the following categories: inactive or sedentary (physical activity <2 h/wk; i.e., walking); light physical activity (2–4 h/wk), and moderate-high physical activity (light-intensity activity >4 h/wk or moderate-intensity activity 1–2 h/wk; i.e., swimming) [45]. Height and weight were measured, and body mass index (BMI) was computed into kg/m^2. Comorbidities included in this analysis were diabetes mellitus (type 1 or type 2), hypertension (HT), chronic obstructive pulmonary disease (COPD), cardiovascular disease (CVD, including angina, myocardial infarction, congestive heart failure, and stroke), impaired renal function (glomerular filtration rate <60 ml/min), Parkinson's disease, dementia, and cancer. They were defined using standard clinical definitions by combining information from self-reported physician diagnoses, pharmacological treatments, medical history, clinical examinations and blood tests [46].Statistical analysis

Descriptive analysis of baseline characteristics was presented as mean (standard deviation) for normally distributed variables or median (25^{th} and 75^{th} percentiles) for variables that deviated from normal ditribution. Spearman rank correlation analyses were performed to examine the relations between proposed dietary biomarkers and MD diet food groups and between FFQ-MDS and dietary biomarker-MDS. The final sum of both scores was divided into population tertiles to achieve categories with similar number of participants in each group. Cut-offs for FFQ-MDS tertiles were: ≤7, 8-10, and ≥11; and for dietary biomarker-MDS tertiles: ≤8, 9-10, and ≥11. Squared-weighted Kappa coefficient was calculated as a measure of the agreement between FFQ-MDS and dietary biomarker-MDS tertiles. Baseline characteristic comparisons across the FFQ-MDS and dietary biomarker-MDS tertiles were assessed using generalized linear models adjusted for age and sex.

Cox proportional hazard models were used to evaluate the associations between tertiles of FFQ-MDS or baseline dietary biomarker-MDS and all-cause, cardiovascular and cancer mortality. The base model was adjusted for age (continuous) and sex. The final model was additionally adjusted for BMI

(continuous), years of education (continuous), smoking status (3 categories), physical activity (3 categories), impaired renal function, diabetes mellitus, HT, COPD, CVD, cancer, dementia, and Parkinson's disease (dichotomous), and energy intake (continuous). Similarly, each component of the dietary biomarker-MDS (as tertiles) were individually tested in the fully-adjusted model. Tests for linear trend were performed by assigning ordinal scores to the tertiles. For linear dose-response plots, Cox regression models were carried out with dietary biomarker-MDS or FFQ-MDS as continuous variables using the 'rms' R package developed by Frank Harrell [47].

Interactions between FFQ-MDS and dietary biomarker-MDS (as tertiles) and age (< or ≥80y), sex, BMI categories (< 25 kg/m^2, 25-30 kg/m^2 and >30 kg/m^2), smoking status (never, former, and current smokers), HT, CVD, impaired renal function, diabetes mellitus, COPD, and cancer in relation to total, cardiovascular and cancer mortality were evaluated in the fully adjusted model using the likelihood ratio test. Sensitivity analyses were run after exclusion of participants who died in the first 2 year of the follow-up, or participants using dietary supplements or lipid-lowering medications. In all Cox models, proportional hazard assumption was tested by visual inspection of the plots based on the Schoenfeld residuals and they were satisfied.

In addition, to better understand genetic predisposed mortality risks we further adjusted the Cox regression models for SNPs with previously reported associations with mortality [42]: *APOE* ε4 (using the rs429358 and rs7412 SNPs), rs1421783 *MAT2B*, rs6997892 *WRN*, rs10817931 *TRIM32*, rs2684766 *IGF1R*, rs11630259 *IGF1R*, and a parental longevity score.

Linear mixed models were used to check for differences in the FFQ-MDS during the repeated measures of the study using individual-specific random effects. Fixed categorical factors were interview number (4 levels: baseline, 3-, 6-, and 9-years of follow-up) and sex; and continuous covariates were age and energy intake. Mixed effect Cox regression models with time-dependent covariates were used to test the FFQ-MDS relationship with all-cause, cardiovascular and cancer mortality including the dietary data collected at baseline, 3, 6, and 9 years of follow-up in the base and the fully adjusted models.

SPSS statistical software 25.0 (IBM, USA) and R version 3.2.3 (R Foundation for Statistical Computing, Austria) were used for all statistical analyses. *P*-values (two-tailed) <0.05 were considered statistically significant.

Results

Descriptive analysis

Out of the 1,155 participants surveyed at baseline, 642 [357 women and 285 men, with a mean (SD) age of 74±7 years] were included in the study (Fig 1). The main cause of exclusion from the study was not collecting 24h urine specimens (472 out of 513). These 513 participants were slightly older (77 *vs.* 74 years), with less years of education (5.1 *vs.* 5.4) and showed a higher prevalence of low physical activity (33% *vs.* 17%), dementia (11.3% *vs.* 3.7%), and Parkinson's disease (2.2% *vs.* 0.8%), as well as lower prevalence of HT (48% *vs.* 63%) than the 642 participants included in this study (all $p<0.05$). Among the 642 selected participants, HT and impaired renal function were the most common comorbidities at baseline with a prevalence of 63% and 39%, respectively (Table 2), followed by CVD (23%) and diabetes mellitus (14%).

The correlations among dietary components of FFQ-MDs and concentrations of dietary biomarker in the population are presented in Table 1. For the dietary biomarker-MDS, we grouped the categories of vegetables, fruits and nuts, legumes, and cereals because the selected dietary biomarker (i.e., total polyphenols and carotenoids) were ubiquitously distributed among these food groups. Alcohol intake in the FFQ-MDS and urine resveratrol in the dietary biomarker-MDS were highly correlated. The total FFQ-MDS (0-18) was moderately correlated with the dietary biomarker-MDS (r=0.26) and the level of agreement between the classifications of FFQ-MDS and dietary biomarker-MDS tertiles was relatively low [squared-weighted Kappa coefficient (95% CI) = 0.218 (0.164-0.272)].

Table 1. Mediterranean diet adherence score (MDS) by dietary components and biomarkers.

Score	MDS		Spearman's rank correlation coefficient
	Dietary components (FFQ)	Dietary biomarkers (dBMK)	
Tertiles (0,1,2)	Vegetables Legumes Fruits and nuts Cereals	Total polyphenols Carotenoids Linolenic acid Selenium	0.170 (P<0.001)
Tertiles (0,1,2)	Fish	EPA+DHA	0.177 (P<0.001)

Tertiles (0,1,2)	MUFA/SFA	MUFA/SFA	0.229 (P<0.001)
Tertiles (0,2,0)	Alcohol	Resveratrol	0.668 (P<0.001)
Tertiles (2,1,0)	Meat	SFA	0.109 (P=0.005)
Tertiles (2,1,0)	Dairy products	Vitamin B12	0.135 (P=0.001)
Total score (0-18)	All dietary components	All biomarkers	0.263 (P<0.001)

MDS, Mediterranean diet adherence score; FFQ, food-frequency questionnaire; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acid.

The characteristics of the study population categorized by dietary biomarker-MDS and FFQ-MDS tertiles are shown in Table 2. Participants in the highest tertile of both dietary biomarker-MDS and FFQ-MDS were younger and more likely to have higher energy intake and being more physically active than those in the lowest tertile. In addition, participants in the highest dietary biomarker-MDS tertile showed a lower proportion of current smokers and diabetes mellitus at baseline; while subjects in the highest FFQ-MDS tertile were predominantly men compared to those at the lowest tertile. Dietary intakes of food groups and concentrations of dietary biomarker according to dietary biomarker-MDS and FFQ-MDS tertiles are shown in Additional file 1, Supplementary Tables S2 and S3, respectively.

Association between Mediterranean diet exposure and mortality

During the 20 years of follow-up (median: 14 years, Q1-Q3: 8 - 18 years), 435 deaths occurred (139 attributed to CVD and 85 to cancer-related causes). In the base models, a greater adherence to dietary biomarker-MDS at baseline was significantly associated with a lower all-cause mortality ($HR_{T3vs.T1}$ 0.66; 95%CI 0.52, 0.83) and this association remained statistically significant in the fully-adjusted model ($HR_{T3vs.T1}$ 0.72; 95%CI 0.56, 0.91) (Fig 2, and Additional file 1, Supplementary Table S4). Moreover, the dietary biomarker-MDS showed a linear dose-response relationship with overall mortality [(HR per unit increase 0.96; 95%CI 0.83, 0.99); Additional file 1, Supplementary Table S4 and Supplementary Fig S1]. The FFQ-MDS was inversely, but not significantly, associated with all-cause mortality either in the base model ($HR_{T3vs.T1}$ 0.91; 95%CI 0.70, 1.19) or in the fully adjusted model ($HR_{T3vs.T1}$ 0.90; 95%CI 0.69, 1.19) (Fig 2, and Additional file 1, Supplementary Table S4). Similarly, no

linear association was observed between FFQ-MDS and overall mortality [(HR per unit increase 1.01; 95%CI 0.97, 1.05); Additional file 1, Supplementary Table S4 and Supplementary Fig S1].

Each component of the dietary biomarker-MDS at baseline was individually tested for its relationship with overall mortality (Fig 2, and Additional file 1, Supplementary Table S4). Baseline urinary total polyphenols were significantly and inversely associated with all-cause mortality in the fully adjusted model ($HR_{T3vs.T1}$ 0.77; 95%CI 0.60, 0.98, p=0.036). Plasma concentrations of carotenoids (p=0.076), selenium (p=0.068), and plasma SFA levels (p=0.059) were negatively associated with overall mortality, but without achieving statistical significance. Moreover, a linear inverse association with all-cause mortality was observed for linolenic acid (HR per log-unit increase 0.62; 95%CI 0.40, 0.95) and EPA+DHA (HR per log-unit increase 0.54; 95%CI 0.30, 0.99) (Supplementary Table S4).

Similar results were obtained when CVD mortality was defined as outcome (Additional file 1, Supplementary Table S5). While the dietary biomarker-MDS was inversely associated with CVD mortality in the fully-adjusted model ($HR_{T3vs.T1}$ 0.60; 95%CI 0.38, 0.93), the FFQ-MDS was not ($HR_{T3vs.T1}$ 1.05; 95%CI 0.64, 1.72, Fig 2). Likewise, the dietary biomarker-MDS showed a statistically significant linear association with CVD mortality (HR per unit increase 0.93; 95%CI 0.87, 0.99), while the FFQ-MDS did not (HR per unit increase 0.99; 95%CI 0.94, 1.07) (Additional file 1, Supplementary Table S5). Among the individual components of the dietary biomarker-MDS, baseline total plasma carotenoid concentrations were significantly associated with CVD mortality ($HR_{T3vs.T1}$ 0.60; 95%CI 0.39, 0.93), while linolenic acid showed an inverse marginal association (p=0.064, Fig 2, and Additional file 1, Supplementary Table S5). Linear inverse associations with CVD mortality were observed for linolenic acid (HR per log-unit increase 0.31; 95%CI 0.15, 0.66), selenium (HR per log-unit increase 0.09; 95%CI 0.01, 0.76) and SFA (HR per log-unit increase 0.17; 95%CI 0.03, 0.96) (Additional file 1, Supplementary Table S5). No significant association was observed between either any MDS or dietary biomarker individual component and cancer mortality (all p>0.05, Fig 2, and Additional file 1, Supplementary Table S6).

Interactions between age, sex, BMI, smoking status, HT, CVD, diabetes mellitus, cancer and both the FFQ-MDS and the dietary biomarker-MDS in relation to all-cause, CVD or cancer mortality were mostly not significant. There was a significant interaction between FFQ-MDS and COPD (p=0.017) by which its association with all-cause mortality was only significant in patients with COPD (n 53, deaths 40; $HR_{T3vs.T1}$ FFQ-MDS 0.24; 95%CI 0.06, 0.93). On the other hand, a significant interaction was noticed

between the dietary biomarker-MDS and impaired renal function for its association with all cause-mortality (p for interaction=0.031). The association between the dietary biomarker-MDS and all-cause mortality remained significant only among the participants without impaired renal function at baseline (n 390, deaths 226; $HR_{T3vs.T1}$ dietary biomarker-MDS 0.56; 95%CI 0.39, 0.80). In those with impaired renal function, this association was not statistically significant (n 253, deaths 212; $HR_{T3vs.T1}$ dietary biomarker-MDS 0.94; 95%CI 0.67, 1.33). For CVD mortality, the only statistically significant interaction detected was between dietary biomarker-MDS and BMI (P=0.022). The inverse association between dietary biomarker-MDS and CVD mortality was stronger among participants with BMI>30 kg/m^2 (n 161, CVD deaths 36; $HR_{T3vs.T1}$ dietary biomarker-MDS 0.28; 95%CI 0.09, 0.90).

The inverse associations between dietary biomarker-MDS and all-cause and CVD mortality were confirmed in the sensitivity analyses after exclusion of participants who died in the first two years of follow-up ($HR_{T3vs.T1}$ dietary biomarker-MDS 0.71; 95%CI 0.55, 0.90; and $HR_{T3vs.T1}$ dietary biomarker-MDS 0.59; 95%CI 0.37, 0.94; for all-cause and CVD mortality, respectively). Further sensitivity analyses after the exclusion of participants using dietary supplements (3.3%, n=21) and lipid-lowering medications (3.9%, n=25) were computed and the results remained similar.

In addition, we further adjusted the Cox regression models for a genetic score (including APOE ε4, among other SNPs) and a parental longevity score to account for genetically predisposed mortality risk. In these models the association between dietary biomarker-MDS and all-cause mortality was $HR_{T3vs.T1}$ 0.70; 95%CI 0.54, 0.90; and between the dietary biomarker-MDS and CVD mortality was $HR_{T3vs.T1}$ 0.57; 95%CI 0.35, 0.91.

The intraclass correlation coefficient (ICC) of the FFQ-MDS between follow-ups (0, 3, 6, and 9 years) was 0.49 (95% CI: 0.44, 0.52). A statistically significant difference of the FFQ-MDS between the baseline and the 9-year examination (β 0.26; 95%CI 0.20, 0.50) was observed, but not among the other follow-up times. After including data from all follow-up dietary assessments in the analysis, we observed a significant association between the FFQ-MDS and all-cause mortality in the base model [$HR_{T3vs.T1}$ 0.77; 95%CI 0.60, 0.99), but not in the fully adjusted model ($HR_{T3vs.T1}$ 0.81; 95%CI 0.63, 1.04). The FFQ-MDS, including the repeated measures, was inversely associated with CVD mortality in both the base ($HR_{T3vs.T1}$ 0.59; 95%CI 0.37, 0.93) and the fully-adjusted models ($HR_{T3vs.T1}$ 0.62; 95%CI 0.39, 0.99). For cancer mortality, no significant associations were observed with any model.

Discussion

In the present study a baseline dietary biomarker score based on key MD food groups but not a MDS based on the FFQ was inversely associated with long-term all-cause and CVD mortality in a cohort of older adults (median follow-up 14 years). These findings strongly suggest that a panel of dietary biomarkers may provide a more objective and accurate assessment of the health benefits associated with diet quality in older adults than self-reported questionnaires. This dietary biomarker panel can be used in both epidemiological and clinical research to further investigate the relationships between the adherence to MD and health outcomes.

Our results showing a non-significant association between FFQ-MDS and all-cause mortality somewhat contrast with previous findings from the EPIC [8], MOLI-SANI [7], Healthy aging: a Longitudinal study in Europe (HALE) [48] and Women's Health Initiative (WHI) [49] studies. These differences could be due to the older mean age of our population, the lower number of participants included, the longer follow-up [14y *vs.* 8.1y (in the EPIC and MOLI-SANI studies)], the higher proportion of deaths [68% *vs.* 10-17% (in the EPIC and MOLI-SANI)], differences on dietary backgrounds when comparing studies from Mediterranean *vs.* non-Mediterranean regions, or on the relatively higher presence of chronic conditions like CVD at baseline, among other factors. Older age might affect the ability to report food intake using FFQ, which depends on memory, and this could hamper the accurate estimation of the associations between dietary intakes and health outcomes [50]. Moreover, dietary intakes can change over time, and therefore, the association between FFQ-MDS, measured at baseline, and long-term mortality could be inaccurate. However, the intraclass correlation coefficient of FFQ-MDS was acceptable across the consecutive examinations (0.49). Moreover, although the participants were older over the consecutive interviews, we observed minor differences in the adherence to FFQ-MDS, which was only statistically significant when comparing the first and the last evaluation. The consideration of data from the dietary assessments of the follow-ups, showed similar results for overall mortality; but for CVD mortality, including the dietary data from the follow-ups did show a statistically significant inverse association between FFQ-MDS and CVD mortality.

Recently, two metabolomics studies discovered a plasma metabolite panel based on the MD adherence including more than 60 metabolites of which > 60% were lipids (such as phospholipids, glycerolipids, carnitines and acylcarnitines) [20, 51]. Both studies used an *a posteriori* approach to explore metabolite fingerprints, which were significantly correlated with the MDS adherence from dietary

questionnaires. In the present study, we included metabolites derived from the dietary sources, i.e., total polyphenols, resveratrol or carotenoids, which were not considered in the above-mentioned metabolomics analyses. In the study of Li *et al.* [20], fruits and legumes were only slightly correlated with 7 out of the 67 metabolites that constituted the total score. Therefore, these metabolites may track the biological changes induced by a MD (biomarkers of effect) but may not correlate with the intake of certain major food groups of the MD. Indeed, in both metabolomics studies, high correlations with the intake of fish and seafood, and olive oil were expected, as they were mostly based on lipid metabolites [20, 51]. Future studies with a more comprehensive metabolomic analysis combining endogenous and exogenous metabolites are still warranted. We expect that the inclusion of more dietary biomarkers with higher specificity would improve the assessment of MD adherence and would reflect better its potential health benefits [52]. In our score, total polyphenols, selenium, linolenic acid and carotenoids were grouped as dietary biomarkers of vegetables, fruits and nuts, legumes and cereals altogether because these dietary biomarkers are present at different concentrations across these highly-heterogenous food groups and one-to-one relationships can not be established. The analysis of interactions allowed us to detect that impaired renal function affected the association between the dietary biomarker-MDS and all-cause mortality, probably through its influence in the excretion of urinary dietary biomarkers. Further studies are needed to develop more robust adherence scores from dietary biomarker concentrations that may not be affected by impaired renal function.

The present findings on dietary biomarker-MDS are in accordance with previous InCHIANTI results showing that PUFA and total polyphenols inverse associations with overall mortality were only significant using dietary biomarkers but not using dietary questionnaires [13, 14]. Moreover, the metabolite score developed by Li *et al* [20] was associated with CVD events independently of the MDS based on the FFQ. The explanation of why dietary biomarker-MDS was significantly associated with all-cause mortality, while the FFQ-MDS was not, might be related to the ability of dietary biomarkers to better address the complex diet-health relationship [52] . Furthermore, dietary biomarker may better capture dietary exposure accounting for interindividual variations in different age-related changes.

The main strengths of this study is its longitudinal design, long follow-up, and the use of dietary biomarkers that reduces the potential dietary assessment errors of FFQ-based data. We also included repeated measures of the FFQ-MDS in the analysis as older adults are susceptible to change their dietary habits due to various conditions influenced by physiologic, pathologic, and/or psychologic factors [53]. In

addition, we used a genetic score and a parental longevity score to better understand the predisposed mortality risks. Last, we used one of the common definitions of MDS [10], facilitating the comparison with results from other studies [54]. However, this investigation also have some limitations. Firstly, we only had baseline measurements of the dietary biomarkers, and their stability over time in this cohort is uncertain. However, in other longitudinal studies dietary biomarker like plasma carotenoids, total SFA, MUFA and PUFA were reported to be stable, with an intraclass correlation coefficient ranging between 0.50-0.68 over 3 to 15 years apart [55–57]. Taking into consideration that FFQ-MDS slightly changed across follow-ups, we may assume similar changes for the dietary biomarker-MDS. Secondly, there are more specific dietary biomarker for some MD food groups as described in the literature [16, 18, 58–60], but they were not available in our cohort. In the present study, the panel of dietary biomarkers was selected based on a literature search and an *a posteriori* validation through correlation analyses. However, the existence of multiple food sources affecting the levels of these dietary biomarkers may have reduced the specificity of the present score for Mediterranean diet. Indeed, the correlation coefficient and level of agreement between the FFQ- and dietary biomarker-MDS was low. Thirdly, although we adjusted our model by several potential confounders, residual confounding cannot be ruled out. Last, our results require confirmation in other populations from different geographical regions.

Conclusions

Adherence to MD assessed by a dietary biomarker panel based on key MD food groups, but not using a traditional FFQ, was inversely associated with long-term mortality in older adults. The linear dose-response between the dietary biomarker-MDS and mortality further supports its use in long follow-up evaluations to monitor the potential health benefits associated with MD. Finally, we would like to highlight the use of dietary biomarker to improve nutritional assessment and to guide individualized dietary counseling to older people.

Abbreviations:

BMI, body mass index; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; dietary biomarkers -MDS, dietary Biomarker-Mediterranean Diet Score; DHA, docosahexaenoic acid; EPA, eicosapentanoic acid; EPIC, European Prospective study into Cancer and

Nutrition; FFQ, food frequency questionnaires; FFQ-MDS, Food Frequency Questionnaire-Mediterranean Diet Score; HALE, Healthy aging: a Longitudinal study in Europe; HR, hazard ratio; HT, hypertension; ICC, intraclass correlation coefficient; ICD, International Classification of Diseases; InCHIANTI, *Invecchiare nel Chianti*; IRF, impaired renal function; MD, Mediterranean diet; MDS, Mediterranean diet score; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; STROBE-NUT, Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology.

~~Appendix~~Declarations

Ethics approval and consent to participate

The Italian National Institute of Research and Care of Aging Ethical Committee approved the study protocol, and all participants signed an informed participation consent.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the responsible of the InCHIANTI study (Dr. Luigi Ferrucci) on reasonable request. Data of the InCHIANTI study is available to all researchers upon justified request using the proposal form available in the InChianti website (http://inchiantistudy.net/wp/how-to-submit-a-proposal/).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

R.Z.-R., C.A.-L. and A.C. designed the research. N.H., T.M., R.Z.-R. and M.R. conducted the research; T.M., N.H. M.R., performed statistical analysis; N.H., T.M., R.Z.-R and M.R., wrote the paper; R.S., T.T., S.B., L.F., C.A.-L., and A.C. provided critical revision; R.Z.-R., CA-L and A.C. had primary responsibility for the final content. All the authors revised and approved the final version of manuscript.

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Figures

Fig 1. Flowchart of participants of the study.

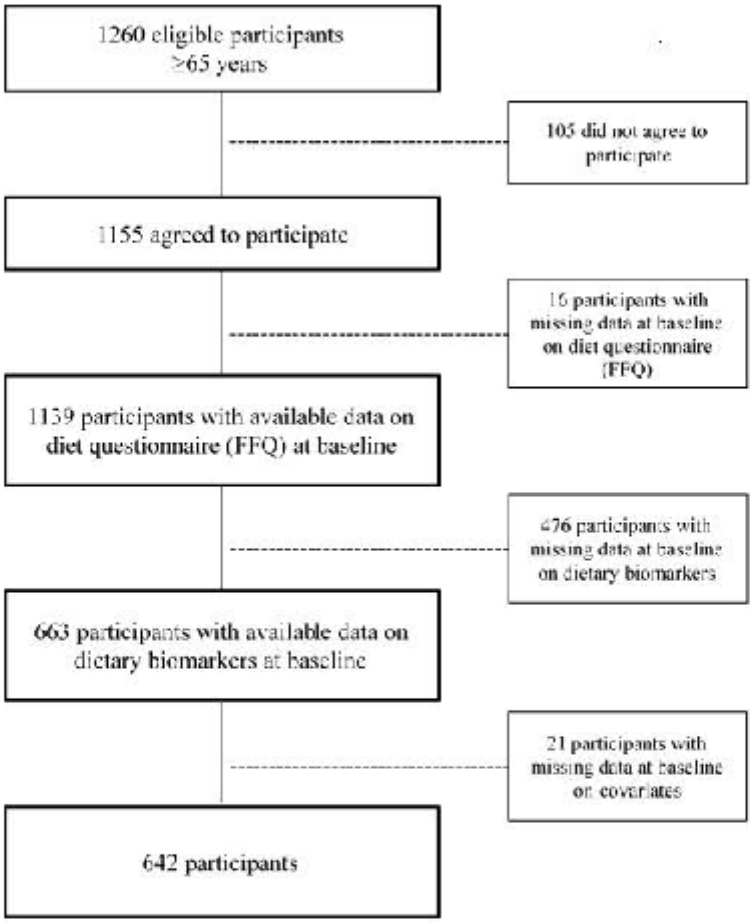
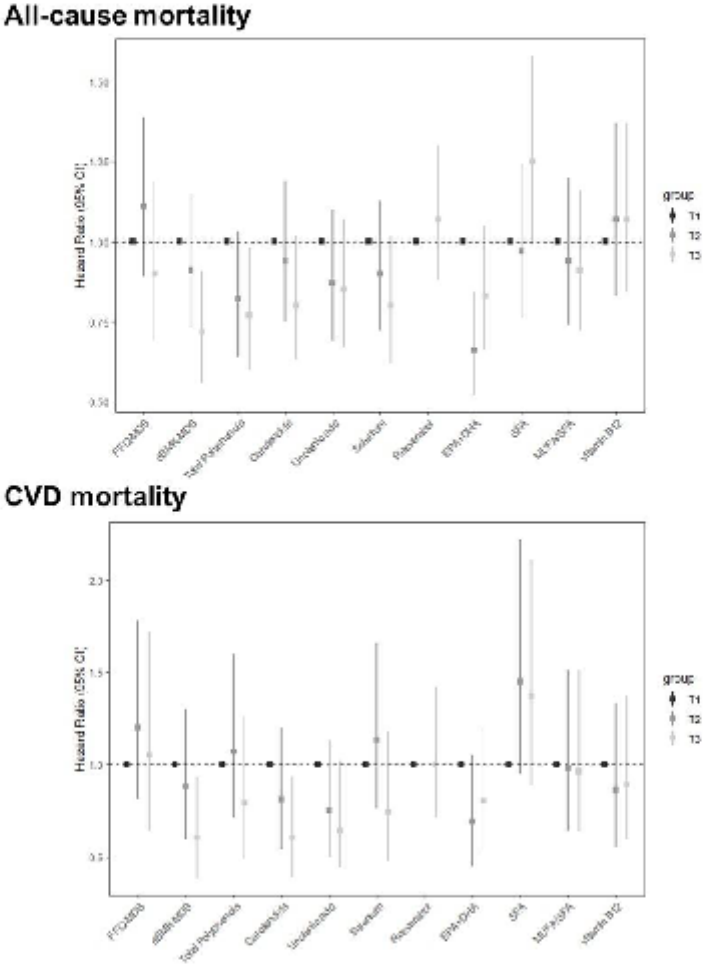


Fig 2. Association between FFQ- and dietary biomarker-MDS and individual dietary biomarkers (as tertiles), and all-cause, CVD and Cancer mortality in the InCHIANTI Study. Cox regression model included sex, age, BMI, education, smoking status, physical activity, impaired renal function, diabetes mellitus, chronic obstructive pulmonary disease, hypertension, cardiovascular disease, cancer, dementia, Parkinson's disease and energy intake. FFQ, food frequency questionnaire; dBMK, dietary

biomarker; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. Total number of deaths, 435; CVD deaths, 139; Cancer deaths, 85. Resveratrol was categorized in two groups: moderate *vs.* no or high consumers.



Tables

Table 2. Baseline characteristics of the study population by dietary biomarker-MDS and FFQ-MDS tertiles

Characteristics	All (n=642)	Dietary biomarker-MDS			P*	FFQ-MDS			P*
		Tertile 1 (n=251)	Tertile 2 (n=193)	Tertile 3 (n=198)		Tertile 1 (n=191)	Tertile 2 (n=265)	Tertile 3 (n=186)	
Age at Baseline (years) *	74 (7)	75 (7)	75 (7)	73 (6)	0.013	76 (7)	75 (7)	72 (5)	<0.001
Female sex (n,%)	357 (56)	145 (58)	105 (54)	107 (54)	0.82	130 (68)	155 (58)	72 (39)	<0.001
BMI (kg/m²) *	27.4 (3.9)	27.4 (4.3)	27.9 (3.7)	27.0 (3.4)	0.19	27.4 (4.3)	27.4 (3.7)	27.6 (3.6)	0.67
Education (years) *	5.4 (3.3)	5.1 (2.6)	5.6 (3.6)	5.7 (3.6)	0.19	5.1 (3.1)	5.3 (3.2)	6.0 (3.4)	0.58
Smoking (n,%)					0.001				0.12
Never	382 (60)	1147 (59)	115 (60)	120 (61)		122 (64)	167 (63)	93 (50)	
Former	174 (27)	58 (23)	54 (28)	62 (31)		44 (23)	65 (25)	65 (35)	
Current	86 (13)	46 (18)	24 (12)	16 (8)		25 (13)	33 (12)	28 (15)	
Physical activity (n,%)					0.034				0.044

Sedentary	110 (17)	52 (21)	437 (19)	21 (11)		49 (26)	44 (17)	17 (9)	
Light	281 (44)	115 (46)	78 (40)	88 (44)		87 (46)	117 (44)	77 (41)	
Moderate-High	251 (39)	84 (33)	78 (40)	89 (45)		55 (29)	104 (39)	92 (49)	
Energy intake (kcal/day)*	1928 (543)	1833 (511)	1979 (546)	1998 (564)	0.003	1751 (539)	1905 (515)	2141 (515)	<0.001
HT (n,%)	402 (63)	157 (62)	124 (64)	121 (61)	0.96	131 (66)	165 (62)	106 (56)	0.19
IRF (n,%)	253 (39)	104 (41)	71 (37)	78 (39)	0.31	92 (48)	116 (44)	45 (24)	0.16
DM (n,%)	89 (14)	44 (18)	24 (12)	21 (11)	0.023	29 (15)	39 (15)	21 (11)	0.38
COPD (n,%)	49 (8)	24 (10)	14 (7)	11 (6)	0.05	15 (8)	18 (7)	16 (9)	0.25
CVD (n,%)	147 (23)	66 (26)	45 (23)	36 (18)	0.06	44 (23)	63 (24)	40 (22)	0.76
Cancer (n,%)	39 (6)	19 (8)	9 (5)	11 (6)	0.44	13 (7)	18 (7)	8 (4)	0.55
Dementia (n,%)	24 (4)	12 (5)	8 (4)	4 (2)	0.22	10 (5)	8 (3)	6 (3)	0.52
Parkinson's disease (n,%)	5 (0.8)	1 (0.4)	1 (0.5)	3 (1.5)	0.24	-	3 (1.1)	2 (1.0)	0.93

BMI, body mass index; IRF, impaired renal function; DM, diabetes mellitus; COPD, chronic obstructive pulmonary disease; HT, hypertension; CVD, cardiovascular disease.

Cut-offs for FFQ-MDS tertiles were: ≤7, 8-10, and ≥11; and for dietary biomarker-MDS tertiles: ≤8, 9-10, and ≥11. These cutoffs were chosen to achieve 3 categories with similar number of participants in each group.

* P-values calculated using generalized linear models adjusted for age and sex.

743 * Data reported as mean (SD).

Additional files

Additional file 1 – .doc file including

Supplementary Table S1. STROBE-nut checklist.

Supplementary Table S2. Baseline data on dietary intake and dietary biomarkers by dietary biomarkers-MDS tertiles. Data shown as median (p25, p75).

Supplementary Table S3. Baseline data on dietary intake and dietary biomarkers by FFQ-MDS tertiles. Data shown as median (p25, p75).

Supplementary Table S4. Association between MDS and individual components of dietary biomarker-MDS (as tertiles), and all-cause mortality in the InCHIANTI Study. *Resveratrol was categorized in two groups: moderate *vs.* no or high consumers. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. Total number of deaths, 435. Base model was adjusted for age and sex. The fully-adjusted model included sex, age, BMI, education, smoking status, physical activity, impaired renal function, diabetes mellitus, chronic obstructive pulmonary disease, hypertension, cardiovascular disease, cancer, dementia, Parkinson disease, and energy intake.

Supplementary Table S5. Association between MDS and individual components of dietary biomarker-MDS (as tertiles), and CVD mortality in the InCHIANTI Study. *Resveratrol was categorized in two groups: moderate *vs.* no or high consumers. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. Total number of cardiovascular deaths, 139. Base model was adjusted for age and sex. The fully-adjusted model included sex, age, BMI, education, smoking status, physical activity, impaired renal function, diabetes mellitus, chronic obstructive pulmonary disease, hypertension, cardiovascular disease, cancer, dementia, Parkinson disease, and energy intake.

Supplementary Table S6. Association between MDS and individual components of dietary biomarkers-MDS (as tertiles), and cancer mortality in the InCHIANTI Study. *Resveratrol was categorized in two groups: moderate *vs.* no or high consumers. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids. Total number of Cancer deaths, 85. Base model was adjusted for age and sex. The fully-adjusted model included sex, age, BMI, education, smoking status, physical activity, impaired renal function, diabetes mellitus, chronic

obstructive pulmonary disease, hypertension, cardiovascular disease, cancer, dementia, Parkinson disease, and energy intake.

Supplementary Fig S1. Dose-response relationship between Mediterranean Diet Score (MDS) and all-cause mortality. Panel A, FFQ-MDS; Panel B, dietary biomarker-MDS. Cox regression models included sex, age, BMI, education, smoking status, physical activity, impaired renal function, diabetes mellitus, chronic obstructive pulmonary disease, hypertension, cardiovascular disease, cancer, dementia, Parkinson disease, and energy intake.

Figure 1

Click here to access/download;Figure;Fig 1. Hidalgo et al.jpg

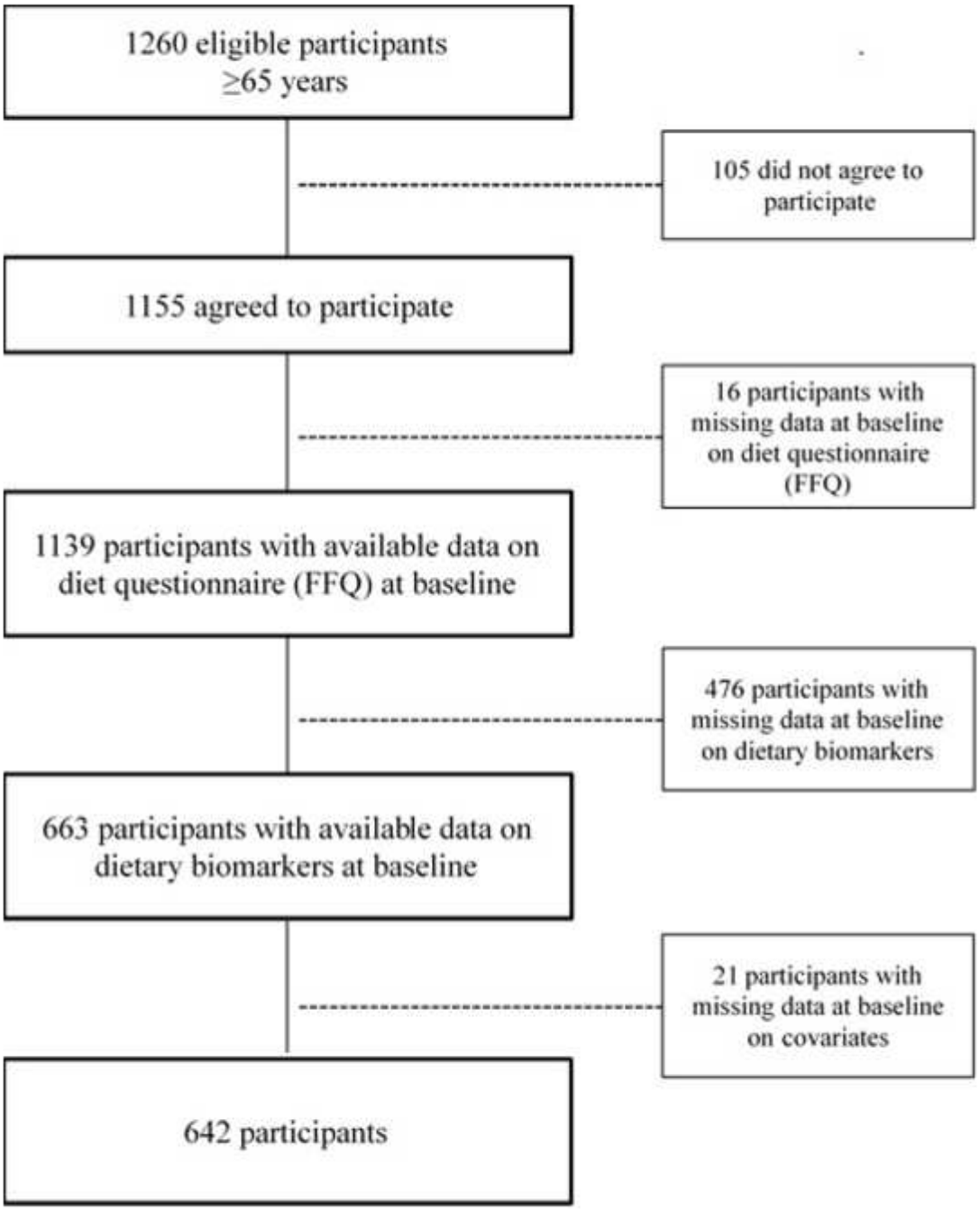


Figure 2

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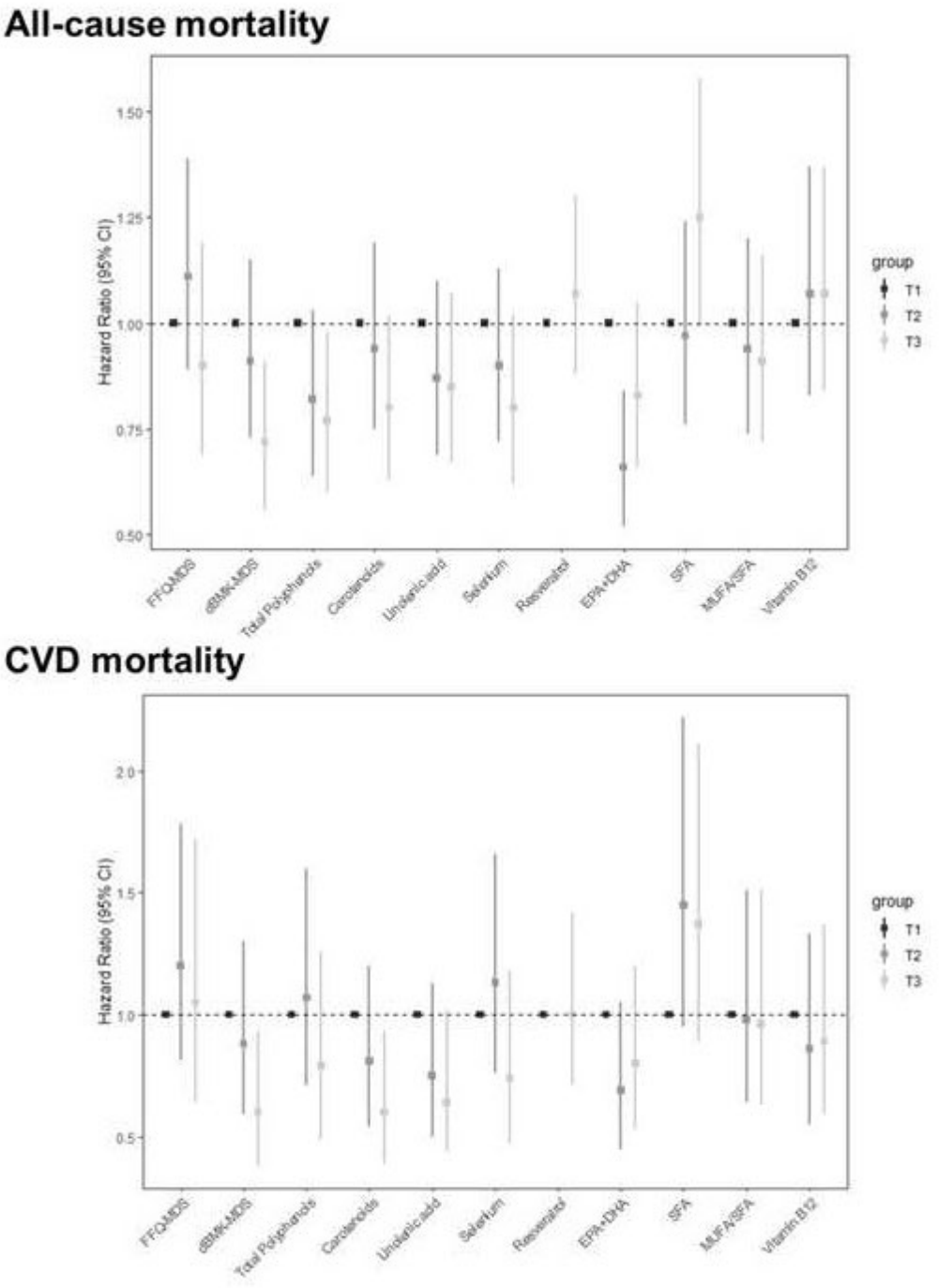

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Dear Dr. Lin Lee

Chief Editor

BMC MEDICINE

I am pleased to send the manuscript entitled "Adherence to Mediterranean diet assessed by a novel dietary biomarker score and mortality in older adults: the InCHIANTI cohort study" by Nicole Hidalgo-Liberona *et al.* for its consideration to be published in BMC MEDICINE.

The present study is suitable for publication in BMC MEDICINE because it is of interest for general practitioners, medical specialists and policymakers, as well as researchers in the Areas of Nutrition, Food science and Gerontology. Promoting the adherence to a healthy diet is nowadays one of the most cost-effective strategies to prevent non-communicable diseases and to minimize health care and socio-economic impact associated with an aging world population. Dietary assessments are commonly done through dietary questionnaires which provide raw estimates on intake levels and rely heavily in self-reported dietary recall. In addition, dietary questionnaires do not consider the age-related decline in memory and/or in the digestion and absorption of nutrients commonly found in older adults. As an alternative, the use of dietary biomarkers has been proposed to conduct an objective and relatively unbiased dietary assessment. However, the prospective association of dietary biomarkers with all-cause and cause-specific mortality in older adults has been scarcely studied. In the present study, we developed a novel dietary biomarker panel to assess the adherence to Mediterranean diet and compared its association with mortality versus a Mediterranean diet adherence score based on data from dietary questionnaires in a cohort of 642 older adults (>65 years, mean age: 74±7years) living in Italy, during a follow-up of up to 20 years. The *Invecchiare nel Chianti* (InCHIANTI) study is a population-based cohort of in-habitants of the Chianti geographic area (Tuscany, Italy) stablished by 1998-2000 aimed to evaluate factors that influence mobility and disability in late adulthood. It is a well-characterized population, with follow-up evaluation every three years, which has several publications and well-stablished algorithms, combining medical, laboratory and imaging tests with medication use, to define the presence of comorbidities. The complete characterization of baseline socio-demographic and clinical characteristics, and the long follow-up of this analysis carried out in older adults are some of the strengths of the present study.

The novel dietary biomarker panel developed showed a linear inverse association with all-cause mortality confirming the health benefits of following a Mediterranean diet, and therefore, it would be included in all Public Health guidelines for healthy ageing prevention, at least in Mediterranean countries. Moreover, it may inspire further research aiming to its implementation as a tool for follow-up and to objectively assess the impact of nutritional advice in older adults. Last, it may promote the shift from the single biomarker paradigm to the multiple biomarker one, which is more suitable for the complexity of dietary patterns.

This study is multidisciplinary and involved specialist in gerontology, nutrition, biochemistry and nutritional epidemiology. The present submission includes two tables and two figures and contains original data, not been under consideration for publication elsewhere, and if accepted in BMC MEDICINE, will not be republished in any other journal in the same or similar form. The final version of the manuscript has been read and approved by

all the authors who contributed substantially to the article. The authors have no conflict of interest to declare. Data is available upon justified request to the responsible of the InCHIANTI study (Dr. Luigi Ferrucci).

Thanking in advance for your attention and waiting for a prompt answer, yours sincerely

Prof. Dr. Cristina Andrés-Lacueva

Supplementary Material

MANUSCRITO 11

Animal protein intake is inversely associated with mortality in community-dwelling older adults: the InCHIANTI study

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EN PROCESO DE PUBLICACIÓN

En segunda revisión en la revista Journal of Gerontology

MANUSCRITO 11

Objetivo: el objetivo de este artículo fue evaluar las asociaciones a largo plazo de la ingesta de proteínas animales y vegetales con la mortalidad por todas las causas y por causas específicas.

Diseño, entorno y participantes: Se llevó a cabo un estudio de cohorte prospectivo que incluyó a 1.139 adultos mayores residentes en la comunidad (edad media de 75 años, 56% mujeres) que vivían en la Toscana, Italia, con un seguimiento durante 20 años (estudio InCHIANTI). La ingesta dietética mediante cuestionarios de frecuencia de alimentos y la información clínica se evaluaron cinco veces durante el seguimiento (al inicio, 3, 6, 9 y 15 años). La ingesta de proteínas se expresó como porcentaje de la energía total.
Se estimaron los cocientes de riesgo (CRI) y los intervalos de confianza (IC) del 95% para la mortalidad por todas las causas y por causas específicas mediante modelos de regresión de Cox dependientes del tiempo y ajustados por factores de confusión, incluida la calidad de la dieta.

Resultados: La ingesta de proteínas animales se asoció de forma inversa con la mortalidad por todas las causas (HR por aumento del 1%, IC del 95%: 0,96, 0,93-0,99) y con la mortalidad cardiovascular (HR por aumento del 1%, IC del 95%: 0,93, 0,87-0,98). La ingesta de proteínas vegetales no mostró asociación con ninguno de los resultados de mortalidad. Entre los participantes sin hipertensión, hubo una asociación inversa marginalmente significativa entre la proteína vegetal y la mortalidad por ECV (HR por aumento del 1%, IC del 95%: 0,79, 0,62-1,01).

Conclusiones y relevancia: Una mayor ingesta de proteína animal se asoció con un menor riesgo de mortalidad por todas las causas y cardiovascular en adultos mayores. Las diferencias en las asociaciones a largo plazo entre la ingesta de proteínas animales o vegetales y la mortalidad en los adultos mayores deberían tenerse en cuenta en las futuras recomendaciones dietéticas.

Animal protein intake is inversely associated with mortality in community-dwelling older adults: the InCHIANTI study

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Supplementary material= Two Figures and four tables

Key points

Question: What is the long-term association between both animal and plant protein intakes and all-cause and cause-specific mortality in a prospective cohort of community-dwelling older adults?

Findings: In the InCHIANTI cohort of 1,139 older adults (mean age: 75 years) followed up to 20 years, animal protein intake showed an inverse association with all-cause and cardiovascular mortality independently of confounders and diet quality. However, plant protein intake was not related to any of the mortality outcomes.

Meaning: In older adults, differences in long-term associations between animal or plant protein intake and mortality should be considered in future dietary recommendations.

Abstract

Importance. The long-term association between animal or plant protein intake and all-cause and cause-specific mortality among older adults is not clear.

Objective. To evaluate the long-term associations of animal and plant protein intake with all-cause and cause-specific mortality.

Design, settings, and participants. A prospective cohort study including 1,139 community-dwelling older adults (mean age 75 years, 56% women) living in Tuscany, Italy, followed for 20 years (InCHIANTI study) was conducted. Dietary intake by food frequency questionnaires and clinical information were assessed five times during the follow-up (baseline, 3, 6, 9 and 15 years). Protein intakes were expressed as percentages of total energy.

Main outcomes and measures. Hazard ratios (HRs) and 95% confidence intervals (CIs) for all-cause and cause-specific mortality were estimated using time-dependent Cox regression models adjusted for confounders, including diet quality.

Results. Animal protein intake was inversely associated with all-cause (HR per 1% increase, 95%CI: 0.96, 0.93-0.99) and cardiovascular mortality (HR per 1% increase, 95%CI: 0.93, 0.87-0.98). Plant protein intake showed no association with any of the mortality outcomes. Among participants without hypertension, there was a marginally significant, inverse association between plant protein and CVD mortality (HR per 1% increase, 95%CI: 0.79, 0.62-1.01).

Conclusions and relevance. Higher intake of animal protein was associated with lower risk for all-cause and cardiovascular mortality in older adults. The differences in long-term associations between animal or plant protein intake and mortality in older adults should be considered in future dietary recommendations.

Keywords: protein, diet, mortality, longevity, older adults, cohort study.

Introduction

Total protein requirements in older adults are higher than in middle-aged adults.[1] However, debate still exists due to the heterogeneity of the older adult population.[2] According to the current recommendation of protein intake in older adults of 1.2 g/kg per day, inadequately low protein intake is frequent among older adults.[3] In general, the relationship between protein intake and health outcomes, such as all-cause or cause-specific mortality, depends on the source of dietary protein.[4–9] In middle-age adults higher animal protein intake was associated with an increased cardiovascular mortality, while plant protein exhibited an inverse relationship.[4,5,7–11] Studies carried out in older adults (≥65 years) observed an inverse association between total protein intake and both all-cause and cardiovascular disease (CVD) mortality, but they did not evaluate differences between protein sources.[12,13] Only Chan *et al.*[14] studied a cohort of community-dwelling older adults in Hong Kong and observed an inverse association between both total and plant protein intake and all-cause mortality among women but not in men. Among men, animal protein intake was associated with lower mortality[14], contrary to US and European studies carried out in middle-age adults.[4–6]

To date, no study has evaluated the association between plant and animal protein and all-cause and cause-specific mortality in a cohort of individuals aged ≥65 years in a Mediterranean country. The main aim of the present study was to evaluate the association of animal and plant protein intake as well as of total protein intake with all-cause, CVD, and cancer mortality after 20-years of follow-up in the InCHIANTI study. Our hypothesis is that total and plant protein, but not animal protein, would be a protective factor against mortality.

Methods

Study design

The InCHIANTI (Invecchiare in Chianti, aging in the Chianti area) cohort study included community-dwelling older adults living in the Chianti geographic area (Tuscany, Italy) and study details have been previously reported.[15]

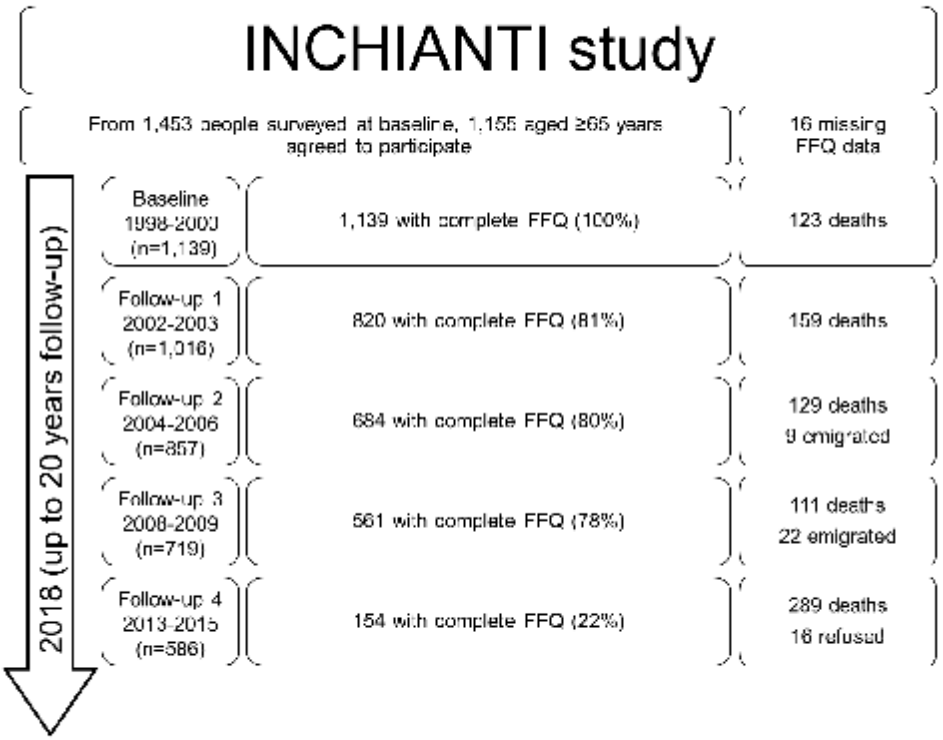
The Italian National Institute of Research and Care of Aging Institutional Review and Medstar Research Institute (Baltimore, MD, USA) approved the study protocol, and all participants signed an informed consent.

The current study was conducted and reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology (STROBE-NUT) guidelines (Supplementary Table 1).[16]

Study population

1,453 community-dwelling adults were randomly selected from the population registries of two Italian communities: *Greve in Chianti* and *Bagno a Ripoli*, with the use of a multistage, stratified sampling method. Baseline participation rate was 91.7%, and 1,155 participants aged ≥ 65 years agreed to participate. Sixteen participants had missing data on dietary questionnaires and the final studied population included 1,139 participants. Participants and data included in the analysis are shown in Figure 1. Clinical data was available in >95% of the cases during all the follow-up evaluations, except for the Follow-up 4 in which 80% had available data. In total, 4,317 observations from 1,139 participants were included in the analysis.

Figure 1. InCHIANTI study flowchart of participants across follow-up assessments



Dietary intake

Habitual dietary intake was assessed by trained interviewers using the Italian version of the food frequency questionnaire (FFQ) developed and validated in the European Prospective Investigation into Cancer and Nutrition (EPIC)-Italy study.[17] Daily intake of energy, macronutrients and micronutrients were estimated from food intake by a specific software developed for the EPIC-Italy study.[18] For this analysis, dietary data at baseline, 3-, 6-, 9- and 15-years of follow-up were considered (Figure 1). In the 15-years follow-up, the reason for the low number of complete FFQ was that only participants from one site, *Bagno a Ripoli*, underwent the dietary assessment.

Mediterranean diet adherence score

Adherence to Mediterranean diet was computed using an 9-point linear scale as described by Trichopoulou *et al.* [19]. The overall adherence to Mediterranean diet was calculated as the sum of each component resulting in a score between 0 (no adherence) and 9 (high adherence).

Covariates

Covariates were selected on the basis of existent literature and previous associations with mortality in the InCHIANTI study.[4,11,20,21] Age, sex, years of education, disability in activities of daily life (disabled ADL ≥1 or not), and physical activity (categorized in 3 levels) were assessed through questionnaires. Smoking habits were self-reported, and participants were classified into never smokers, former smokers or current smokers. Height and weight were measured, and BMI was calculated in kg/m^2. Global cognitive performance was assessed with the Mini-Mental State Examination (MMSE). Comorbidities considered were hypertension, diabetes, impaired renal function [three categories, normal: glomerular filtration rate (GFR) >60 ml/min, mild: GFR<60 ml/min but ≥30 ml/min and severe: GFR<30 ml/min], chronic obstructive pulmonary disease (COPD), ischemic heart disease (IHD, angina pectoris or myocardial infarction), cerebrovascular disease (stroke or transient ischemic attack), peripheral artery disease, congestive heart failure (CHF), Parkinson's disease, dementia, cognitive impairment (MMSE<24 points), and cancer. They were defined using standard clinical definitions by algorithms combining information from self-reported physician diagnoses, pharmacological treatments, medical history, clinical examinations and blood tests. The presence of comorbidities was updated in every follow-up evaluation. In addition, cognitive decline was defined as ≥3 points decline in MMSE between follow-up evaluations.[22]

Outcome assessment

Mortality data on 20-years was collected using the Mortality General Registry from the Tuscany Region, as well as death certificates delivered after the decease of participants to the municipal registry office. Mortality records were coded by the 9th and 10th Revision of the International Classification of Diseases (ICD-9 or -10).

Statistical analysis

Data preparation. Energy equivalents of 9, 4 and 7 kcal/g were used for fat (and fat subtypes), for carbohydrates and protein, and for alcohol, respectively, to express macronutrients as percentage of energy. Total, animal, and plant protein as percentage of energy were categorized into quintiles for descriptive statistics. Missing data at the baseline evaluation were found in BMI and renal function of 166 participants (15%) and values were imputed by the median. During the follow-up evaluations, missing values in clinical information (n=206 observations, 4.7%) and in dietary data (n=959 observations, 22.2%, Figure 1) were imputed by Last Observation Carried Forward (LOCF). Dummy variables were created to identify participants with imputed values at baseline and observations with LOCF imputation.

Descriptive analysis. Data for continuous variables are shown as means (SD) or medians (interquartile range). Categorical variables are expressed as percentages. Differences in general characteristics across quintiles of total, animal, or plant protein intake and in participants with and without hypertension were assessed using generalized linear models adjusted for age and sex. To evaluate differences in dietary data across the follow-up assessments linear mixed models using site- and individual-specific random effects were used, and intraclass correlation coefficients were calculated.

Survival analysis. A multivariable nutrient density model was used for Cox Regression models with adjustment for total energy and percentage of energy from subtypes of fats, total or animal and plant protein, and alcohol as described previously.[4,11,23] For the main analyses, we used two time-dependent Cox models stratified by site: model 1 included total or animal and plant protein (continuous) adjusted for age, sex, total energy and percentage of energy from SFA, MUFA, PUFA, and alcohol. Model 2 was further adjusted for BMI, years of education, smoking, ADL disability, physical activity, impaired renal function, diabetes, COPD, hypertension, IHD, cerebrovascular disease, peripheral artery disease, CHF, Parkinson's disease, dementia, cognitive impairment, cognitive decline, cancer, and Mediterranean diet score (continuous). In all Cox models, visual inspection of scaled Schoenfeld residuals were used to check for the proportional hazard assumption and they were mostly satisfied. Presence of hypertension was the only variable that showed a major violation of the proportional hazard assumption. In

consequence, Cox regression models also included hypertension as stratification variable.

We compared the models with and without restricted cubic splines with 3 knots by the likelihood ratio test to assess for non-linear relationships between exposure and outcomes. Cox models were centered to the median intake of total, animal, or plant protein for better visualization.

Interactions between total, animal or plant protein intake and age, sex, physical activity, BMI categories, diabetes, smoking, ADL disability, hypertension, IHD, cerebrovascular disease, CHF, cognitive impairment, and cancer were tested using likelihood ratio tests.

Sensitivity analysis were done after the exclusion of participants with imputed values, subjects who died within the first 2 years of follow-up and subjects with baseline cancer, severe impaired kidney function or cognitive impairment. Last, we created a dummy variable to identify the different periods between follow-up evaluations (from 0 to 4) and included an interaction terms between it and total or animal and plant protein in Cox regression models. Significant interactions in these analyses represent a difference of effect across the different periods of the follow-up. The same approach was used to test for difference of effect between study sites in participants with and without imputations, considering that FFQs of the last follow-up were only available in one site of the study.

For the statistical analyses SPSS version 25.0 (IBM, USA) and R 3.6.2 (R foundation, Vienna, Austria) were used.

Results

Baseline characteristics

The studied population consisted in 1,139 participants (56% women) with a mean age of 75±8 years at baseline. Mean (SD) intake of total protein was 74 (21) g/day, and the normalized value by weight was 1.1 (0.3) g/kg of body weight per day. Overall, a 63 (1) % of total protein intake was animal protein. Sources of animal protein were: 26% for dairy products, 26% for processed meat products, 20% for red meat, 7.7% for fish and seafood, 6.3% for chicken, 2.7% for eggs, and the rest from rabbit, game and offals. Mean contribution from different sources of plant protein were: 73% for cereals, 11.4% for vegetables, 9.0% for fruits and nuts, and 5.3% for legumes.

According to quintiles of total protein intake, participants in the highest compared to the lowest quintile were more likely to be women, less educated, and to present diabetes at baseline (Table 1). There were no differences in plant protein as percentage of energy across the quintiles of total protein intake. Participants in the highest quintile of total protein intake tended to consume more meat and dairy products, fish and seafood, as well as less fruits, cereals, and alcohol, and showed a lower Mediterranean diet score compared to those in the lowest quintile (Supplementary Table 2).

Across quintiles of animal protein intake, sociodemographic, clinical characteristics and dietary intake data are similar to the results across total protein intake quintiles (Table 1 and Supplementary Table 2). Participants in the highest compared to the lowest quintile of animal protein intake were older and showed a lower plant protein intake.

Participants in the highest quintile of plant protein intake were more likely to be men, less educated, and to present higher prevalence of hypertension and diabetes than those in the lowest quintile (Table 1). Total energy and total protein intakes were not different across plant protein quintiles. Moreover, the consumption of vegetables and cereals was higher and the consumption of meat and dairy products, fish and seafood, and alcohol were lower in the highest compared to the lowest plant protein quintile (Supplementary Table 2).

Table 1. Baseline characteristics of the population according to quintiles of intake of total, animal or plant protein.

(n= 1,139)	*Total protein*			*Animal protein*			*Plant protein*		
	Q1 (227)	Q3 (228)	Q5 (227)	Q1 (228)	Q3 (229)	Q5 (228)	Q1 (227)	Q3 (227)	Q5 (227)
Clinical characteristics									
Age (years)	75±7	75±8	76±7	74±7	75±8	76±8†	77±8	76±8	75±7
Women (%)	42	52	70*	38	55	72*	64	59	51*
BMI (kg/m²)#	27±4	27±4	27±4	27±4	27±4	27±4	27±4	27±4	27±4
Education (years)	6±3	5±3	5±3†	6±3	5±3	5±3	6±4	5±3	5±3*
Current smoking (%)	33	27	21	35	25	24	31	24	31
ADL disability (%)	6	11	12	3	11	15†	13	12	6‡
Physical activity (n,%)									
Sedentary	21	23	30	20	20	31	27	29	20
Light	37	42	44	37	45	43	41	40	45
Mod- High	41	35	26	43	35	25	30	31	35
Hypertension (%)	55	56	56	56	59	56	52	56	57‡
Diabetes (%)	7	15	22*	6	13	18*	8	16	16†
IRF (%)#									
Mild	27	32	33	25	33	33	34	33	27
Severe	3	1	2	3	3	0	2	1	3
IHD (%)	6	7	11‡	5	7	9	5	8	8

Cerebrovascular disease (%)	5	8	6	6	8	7	5	7	8
PAD (%)	11	11	8	10	10	9	10	12	12
CHF (%)	4	4	7‡	4	6	6	2	8	6
COPD (%)	11	7	7	11	7	7	7	11	6
Cancer (%)	5	3	7	4	7	8	5	5	3
Dementia (%)	5	7	7	4	4	10	7	9	5
Cognitive impairment (%)	23	31	38†	21	31	40†	35	32	31
Parkinson's disease (%)	1	1	1	1	2	2	1	2	2
Dietary characteristics									
Energy (10^3 kcal/d)	2.1±0.6	1.9±0.5	1.6±0.4*	2.1±0.7	1.9±0.5	1.7±0.5*	1.8±0.6	1.8±0.6	1.9±0.6
Total protein (% E)	13±1	16±1	19±1*	13±1	16±1	19±1*	16±2	16±2	16±2
Animal protein (% E)	7±1	10±1	13±2*	7±1	10±1	14±1*	12±2	10±2	8±2*
Plant protein (% E)	5.6±1.0	5.9±1.0	5.7±1.0	6.4±1.0	5.8±0.8	5.1±0.9*	4.3±0.5	5.8±0.1	7.2±0.5*
SFA (% E)	10±2	10±2	11±2*	9±2	10±2	12±2*	12±2	11±2	8±2*
MUFA (% E)	14±3	15±3	16±3*	14±3	15±3	17±3*	16±3	15±3	14±3*
PUFA (% E)	3.1±0.7	3.3±0.7	3.6±0.6*	3.0±0.7	3.3±0.6	3.7±0.6*	3.4±0.6	3.4±0.7	3.3±0.7
Carbohydrates	52±7	52±6	47±6*	54±7	51±5	46±6*	46±7	50±5	56±5*

(% E)									
Alcohol (% E)	6 (10)	3 (5)	0 (3)*	5 (10)	3 (7)	0 (5)*	5 (10)	2 (7)	1 (4)*

BMI, body mass index; ADL, activities of daily life; PA, physical activity; IHD, ischemic heart disease; PAD, peripheral artery disease; CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Data for continuous variables are shown as mean ± S.D. or median (interquartile range). Cut-off points of intake category (percentage of total energy): for total protein were: quintile 1, <14.1%; quintile 2, 14.1% to <15.2%; quintile 3, 15.2% to <16.3%; quintile 4, 16.3% to <17.6%; and quintile 5, ≥17.6%. For animal protein were: quintile 1, <8.1%; quintile 2, 8.1% to <9.3%; quintile 3, 9.3% to <10.5%; quintile 4, 10.5% to <12.0%; and quintile 5, ≥12.0%. For plant protein were: quintile 1, <4.9%; quintile 2, 4.9% to <5.5%); quintile 3, 5.5% to <6.0%; quintile 4, 6.0% to < 6.6%; and quintile 5, ≥6.6%.

#Data available in 973 subjects.

*p for trend<0.001, † p for trend<0.01, ‡ p for trend<0.05 using age- and sex-adjusted generalized linear models.

The prevalence of chronic diseases increased across the follow-up assessments being hypertension, mild impaired renal function (both around 80%) and cognitive impairment (67%) the most frequent conditions at the last evaluation (Supplementary Figure 1). Small changes were observed in dietary intake data across follow-up assessments (within the ±8% of baseline values for almost all the macronutrients except for PUFA intake which varied within ±12%). Intraclass correlation coefficients were within the range of 0.23-0.50.

Association between dietary protein sources and mortality

During the 20-years of follow up (mean: 12±6 years), 811 deaths occurred (292 of CVD and 151 of cancer-related causes). Associations between total, animal, and plant protein intake and mortality outcomes are shown in Figure 2 and Supplementary Table 3. In the fully adjusted model, a statistically significant inverse association was observed between total protein and CVD mortality. Animal protein, but not plant protein intake, was inversely associated with both all-cause and CVD mortality. Neither total, nor animal, nor plant protein intakes were associated with cancer mortality (Figure 2). The associations between protein intake and mortality were linear in all cases, and non-linear terms did not improve the models fit.

Figure 2. Association between total, animal and plant protein intake and all-cause, cardiovascular and cancer mortality

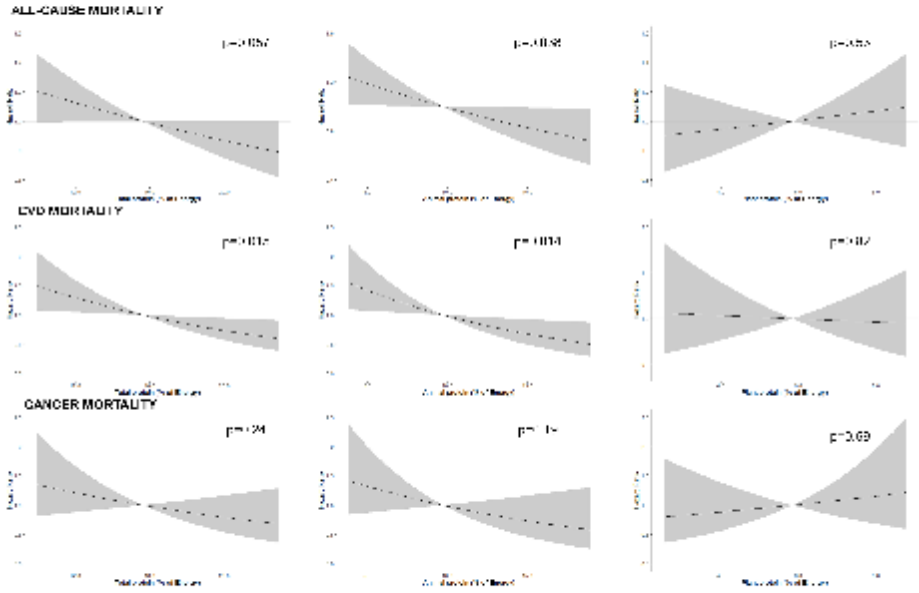


Figure 2. Cox regression stratified by site and baseline hypertension adjusted for age, sex, total energy, percentage of energy from saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA), alcohol, total or plant and animal protein, BMI, years of education, smoking status (current *vs.* former and non-smoker), ADL disability, physical activity, impaired renal function, diabetes, ischemic heart disease, cerebrovascular disease, peripheral artery disease, congestive heart failure, chronic obstructive pulmonary disease, cancer, dementia, cognitive impairment, cognitive decline, Parkinson's disease and Mediterranean diet adherence score. Number of participants: 1,139. Number of observations = 4,317. Shaded areas are the 95% confidence intervals.

For all-cause mortality, there was a statistically significant interaction between plant protein and hypertension (Supplementary Figure 2.); for CVD mortality, between animal protein and IHD, and between plant protein and hypertension, and IHD; and for cancer mortality, between plant protein and ADL disability, and cognitive impairment. No other interaction was statistically significant for any of the mortality outcomes.

Hypertension had a major effect on the association between plant protein and all-cause and CVD mortality. Indeed, plant protein displayed an inverse, marginally significant association with CVD mortality among participants without baseline hypertension (HR, 95%CI: 0.79, 0.62-1.01, Supplementary Figure 2). Comparison of participants with and without baseline hypertension (n=644 *vs.* n=495), showed that subjects with hypertension were more likely to be women and showed higher age and sex-adjusted prevalence of obesity, impaired renal function, IHD, PAD, and CHF (Supplementary Table 4). Moreover, they showed a lower intake of SFA and a trend towards a higher plant protein intake than those without hypertension (p=0.057, Supplementary Table 4).

In sensitivity analyses, results were similar after the exclusion of subjects with imputed values, participants who died within the first 2 years of the follow-up, subjects with severe impaired renal function at baseline, baseline cancer, or with cognitive impairment, who may be inaccurate in reporting food intake (Supplementary Table 3). Across the follow-up period, the association between plant protein and CVD mortality was significantly reduced during the last follow-up periods of the study (p= 0.032). No other difference of effects was observed during the follow-up assessments with neither total protein nor animal protein intake for any mortality outcome. Likewise, there was no difference of effect between sites whether using the data with or without imputations for participants and observations (p>0.05).

Discussion

Higher intakes of animal protein were associated with lower risk for all-cause and CVD mortality in this study of community-dwelling older adults after a 20-years follow-up.

The present study is the first showing an inverse association between animal protein and mortality in older women and men from a Mediterranean country. Chan *et al.*[14] reported a similar association in older men, but not in women. The borderline statistically significant inverse association between total protein intake and overall mortality is also in agreement with other studies carried out in older adults.[12–14] In this study, total and animal protein intake displayed similar relationships with all-cause and CVD mortality, opposite to results from a recent meta-analysis.[7]

Increased animal protein intake may be inversely associated with mortality in older adults through its protective effects on muscle strength, frailty, sarcopenia or immune responses, all of which merit further studies.[24] Increased intake of total or animal protein was positively associated with muscle strength,[25] which in turn was inversely associated with all-cause mortality in a recent meta-analysis of studies in older adults.[26] Moreover, chronic or acute inflammatory conditions may impair the direct relationship between protein intake and muscle strength in older adults, increasing dietary protein requirements.[1,27] Further studies should determine if intrinsic characteristics of animal protein (i.e. its amino acid profile) and/or the overall levels of protein intake are behind its inverse association with mortality.

Recently, an analysis from the Rotterdam study showed a positive association between animal protein and all-cause mortality.[6] Besides the differences in age and comorbidities at baseline between their study and ours, there were also large differences in dietary intake. For instance, total fat (as percentage of total energy) was lower in our study (27% *vs.* 35%) and qualitatively different with a higher MUFA intake (14% *vs.* 12%) and lower SFA and PUFA intakes (10% *vs.* 14%, and 3.2% *vs.* 7.0%, respectively). In line with an effect of fatty acid composition of the consumed foods, they found that substitution of dairy or meat protein (correlated with SFA intake) for carbohydrates were the largest contributors to the positive association between animal protein and mortality.[6] These dietary particularities may have contributed to the differences between studies.

Importantly, we adjusted for overall diet quality, which has major implications shaping the relationship between intake of protein from different sources and mortality.

In the present cohort, plant protein was mostly coming from cereals, and this fact could be related to the lack of association between plant protein and mortality. Indeed, in Asian studies where an inverse association between plant protein and all-cause mortality was observed, legumes and pulses contributed to approximately 25% of total plant protein (*vs.* 5% in the present study).[11,14] Recently, Huang *et al.* reported an inverse association between plant protein and mortality in a US prospective cohort study.[8] In their analysis they observed that the inverse association between plant protein intake and mortality was decreased in the groups of participants aged ≥65y and with a follow-up>10 years. Thus, our results are not in disagreement with these results, but rather complimentary by extending the analysis to an older age segment of the population.

The strengths of the current study include a long follow-up in a well-established cohort of older adults, and the inclusion of repeated dietary assessments to reduce bias from measurement errors in dietary questionnaires. Our study also has limitations. The relatively small sample size and low incidence of cancer-related deaths could have compromised the statistical power. However, protein intake was not associated with cancer mortality in a meta-analysis.[7] Medical advice could have promoted a lower intake of carbohydrates and higher intake of proteins (from plant or animal sources to a different extent) potentially affecting the dietary choices. Indeed, higher plant protein intakes are encouraged within a DASH diet compared to a western diet.[28] Last, residual confounding may still remain, even though we adjusted the analyses by a Mediterranean diet adherence score.

In conclusion, refuting our initial hypothesis, higher intake of animal protein was associated with lower risk for all-cause and CVD mortality in older adults. Nonetheless, plant protein showed a borderline statistically significant association with CVD mortality, at least in participants without hypertension. In older adults, differences in long-term associations between animal or plant protein intake and mortality should be considered in future dietary recommendations to promote longevity.

AUTHOR CONTRIBUTIONS

TM, RZR and CAL designed the research; NHL, MR, SB, LF, AC and CAL, conducted the research; TM, RZR, NHL and MR performed statistical analysis; TM, NHL and MR wrote the paper; RZR, SB, LF, MF, AC and CAL provided critical revision; and CAL had primary responsibility for the final content. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors are not aware of any conflict of interest.

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MANUSCRITO 12

Association between dairy products intake and frailty phenotype in older adults: the InCHIANTI Cohort study.

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EN PROCESO DE PUBLICACIÓN

MANUSCRITO 12

Introducción: La fragilidad es una condición de deterioro de la función física que aumenta el riesgo de eventos adversos para la salud en las personas mayores. Los productos lácteos son una buena fuente de proteínas y otros nutrientes esenciales, asequibles y fáciles de consumir por los adultos mayores. Se evaluó la asociación entre el consumo de productos lácteos y el riesgo de fragilidad en adultos mayores que viven en la comunidad.

Materiales y métodos: El estudio InCHIANTI es una cohorte prospectiva realizada en la región de Chianti (Italia). Este estudio incluyó al inicio 920 hombres y mujeres de 65 años o más. El estado de fragilidad se definió como tener tres o más y la pre-fragilidad como tener uno o dos de los siguientes cinco criterios: agotamiento, debilidad, baja actividad física, velocidad de marcha lenta y pérdida de peso involuntaria. La ingesta se evaluó mediante un cuestionario validado de frecuencia de alimentos y la ingesta habitual de productos lácteos como el yogur, el queso, la leche y el helado se informó en porciones por día al inicio, a los 3, 6 y 9 años de seguimiento. Se obtuvieron odds ratios (OR) ajustadas para potenciales factores de confusión utilizando regresiones multinomiales y logísticas de efectos mixtos para evaluar la relación entre los productos lácteos y el riesgo de fragilidad y sus componentes.

Resultados: Durante 9 años de seguimiento se identificaron 62 casos incidentes de fragilidad y 200 casos de pre-fragilidad. Después de ajustar por potenciales factores de confusión, no se observó ninguna asociación entre el consumo total de lácteos (OR por ración al día= 0.94, 95%IC 0.86-1.03 y OR por ración al día= 1.10, 95%IC 0.92-1.30, para pre-frágiles y frágiles, respectivamente) o los tipos de productos lácteos (incluyendo los fermentados y no fermentados) y la fragilidad y sus componentes.

Conclusiones:

Es poco probable que los productos lácteos como la leche, el yogur, el queso y el helado jueguen un papel predominante en el desarrollo del síndrome de fragilidad en una población italiana de edad avanzada que vive en la comunidad. Sin embargo, es aconsejable mantener un consumo moderado de productos lácteos siguiendo las recomendaciones de un patrón dietético saludable.

Association between dairy products intake and frailty phenotype in older adults: the InCHIANTI Cohort study.

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Abstract

Purpose

To evaluate the association between the consumption of dairy products and frailty risk in community-dwelling older adults from Tuscany region.

Methods

The InCHIANTI study is a prospective cohort conducted in the Chianti region (Italy). This study included at baseline 920 men and women aged 65 years and older. Frailty state was defined as having three or more and pre-frailty as having one or two of the following five criteria: exhaustion, weakness, low physical activity, slow walking speed and unintentional weight loss. Intake was assessed using a validated food frequency questionnaire and the habitual intake of dairy products such as yogurt, cheese, milk, and ice cream was reported in servings per day at baseline, 3-, 6- and 9-years of follow-up. Adjusted odds ratios (OR) for potential confounders were obtained using generalized mixed effect models to assess the relationship between dairy products and frailty risk and its components.

Results

Frailty prevalence increased from 8.5 to 15.7%, and pre-frailty from 39.0 to 50.6% from baseline to the ninth year of follow-up. After adjusting for potential confounders, no associations were observed between either total dairy ($OR_{\text{per serving per day}}$= 0.94, 95%IC 0.86-1.03 and $OR_{\text{per serving per day}}$= 1.10, 95%IC 0.92-1.30, for pre-frail and frail, respectively) or types of dairy product (fermented and non-fermented) consumption and frailty risk, or its components.

Conclusion

Dairy products such as milk, yogurt, cheese, and ice cream are unlikely to play a predominant role in frailty syndrome development in an Italian community-dwelling old population. However, it is advisable to maintain a moderate consumption of dairy products.

Keywords: Frailty; nutritional status; protein; yogurt; dairy products

Abbreviations

BMI Body mass index
CI Confidence interval
EPIC European prospective investigation into cancer and nutrition
FFQ food-frequency questionnaire
InCHIANTI Invechiare in Chianti
OR Odd ratio
SD Standard deviation

Introduction

According to current estimates, the number of people over the age of 65 years is expected to double by 2050 [1]. Nowadays, people are living longer due to advances in medical services, food availability, lifestyle, safety, and education. Although frailty can be observed across all adult ages, it is closely linked to ageing, and therefore, its prevalence increases as the population ages [2]. Hence, the increasing number of older people is becoming a public health challenge, with implications for the planning and delivery of both health and social care [1].

Frailty is a multidimensional geriatric condition characterized by a functional decline, which is accompanied of a high risk of illness, falls, hospitalizations, disability, and high vulnerability to death, however, it is a condition that can be reversed [3–5]. Several methods to assess frailty has been proposed, being the frailty phenotype proposed by Fried et al. [3] one of the most common definitions in terms of physical characteristics. It considers five components: shrinking or unintentional weight loss, weakness (low grip strength), poor endurance or exhaustion, slowness (slow walking speed) and low physical activity [3].

Different health conditions and lifestyle habits, including deficient nutritional status, contribute to the development of frailty,[6–8]. Inadequate energy intake may cause undernutrition and micronutrient deficiencies, leading to frailty in older adults [5,7]. Indeed, previous cross-sectional studies have already shown that a low caloric diet was associated with frailty in the InCHIANTI [Invecchiare in Chianti (Aging in Chianti)] and in an US-based study [9,10]. Likewise, high protein intake was inversely associated with frailty status in older adults [11]. Thus, dietary habits seem to have a relevant role in frailty development. Several studies have found that a high intake of orange juice [12], milk and dairy products [13], fruits and vegetables [14–16], fish [16] as well as healthy dietary patterns [17–25] were related with lower frailty risk.

Dairy products are a complex source of nutrients and there is an increasing interest on their potential health effects. They are a good source of protein and essential micronutrients, such as calcium, phosphorus, vitamin A, vitamin D, riboflavin, vitamin B12, potassium, zinc, choline, magnesium, and selenium. The 2020-2025 Dietary Guideline for Americans (DGA) recommends an intake of 3 daily servings of dairy products, particularly fat-free or low-fat alternatives, such as milk, cheese and/or yogurts for those 9 years and older [26]. Moreover, recent studies have shown that higher intakes of dairy foods were associated with weight loss, and lower risk for type 2 diabetes [27], colorectal cancer [28], cardiovascular disease and overall mortality [29]. Nonetheless, to our knowledge, only few studies have evaluated the relationship between dairy products and frailty in older adults [30,31], and they showed contradictory results.

In the current prospective study, we examined the association between the habitual intake of dairy products and the risk of frailty in older adults from the InCHIANTI study. Our hypothesis is that a higher intake of dairy products will be associated with lower frailty risk.

Methods

This study is developed and reported according to the Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology (STROBE-NUT) checklist (see Supplementary Table 1: STROBE-nut statement) [32].

Population

The InCHIANTI study is a prospective cohort of randomly selected older adults conducted in two towns in the Tuscany countryside (Greve in Chianti and Bagno a Ripoli) in Italy. Further details of the study design have been described elsewhere [33]. The study population includes 1,155 women and men who agreed to participate. Participants who had missing data in the food frequency questionnaire (FFQ) (n=16) and frailty (n=219) at baseline were excluded. A total of 920, 647, 548 and 395 participants had completed frailty and dairy intake information at baseline, 3-, 6-, and 9-years follow-up visits, respectively (Figure 1). The study protocol was examined and approved by the Italian National Institute of Research and Council of Aging ethical committee. All participants provided written informed consent.

Habitual dairy intake assessment

Habitual food intake was assessed at baseline, and 3, 6 and 9 years after enrollment, using the Italian version of the FFQ developed and validated in the European Prospective Investigation into Cancer and Nutrition (EPIC-Italy) study [34]. Participants reported the frequency of consumption and usual portion size of a list of 198 food items. Total dairy products intake (in servings per day) was estimated as the sum of four food items: yogurt, cheese, milk, and ice cream. Fermented dairy products were calculated as the sum of cheese and yogurt, while non-fermented dairy products were the sum of milk and ice cream. Serving size of dairy products was established according to FFQ, for instance hard and semi hard cheeses was 65 grams, 100 grams for soft and fresh cheeses, 120 milliliters for milk, 125 grams for yogurt and 75 grams for ice cream[34]. Total energy (kcal/day), alcohol (g/day) and total protein (g/day) intakes were calculated using an Italian food composition database [33]. Missing dietary data during the follow-up (3.4%) were imputed by the Last Observation Carried Forward (LOCF).

Assessment of Frailty Syndrome

We used the operational definition of frailty phenotype developed by Fried et al. [3]. It was developed based on 5 components: unintentional weight loss, exhaustion, low physical activity, slow walking speed and muscle weakness. Briefly, shrinking or unintentional weight loss was self-reported defined as involuntary weight loss (not due to diet or exercise) >4.5 kg in the prior year. Weakness was defined as grip strength measured using a handheld dynamometer (Nicholas Muscle Tester, Sammon Preston, Inc., Chicago, IL) in the lowest sex-specific quintile at baseline and at follow-up visits. Low physical activity was defined as either complete inactivity or spending <1 hour/week of low-intensity activities in a physical activity questionnaire developed and validated for the InCHIANTI study. Self-reported exhaustion or poor endurance was assessed using the statement "I felt that everything was an effort" from the Center for Epidemiological Studies–Depression scale (CES–D), a validated version for Italian population [35]. Slowness was defined as the time to walk 4.57 m or 15 feet (average of two repetitions) in the lowest sex-specific quintile. Frail, pre-frail, and robust subjects were defined as those having three or more, at least one, and none of these criteria, respectively [3]. Frailty and its components were evaluated at baseline, and at 3-, 6-, and 9-year visits.

Covariate assessment

A standardized interview and clinical examination were conducted by trained physicians. Personal information as age, sex and lifestyle data were collected using standardized questionnaires. Education level was assessed as years of education, and self-reported smoking habits were categorized as never, former, and current smokers. Height and weight were also measured using standard procedures and body mass index (BMI) was calculated. Physical activity data was collected with a structured questionnaire specifically developed and validated for the InCHIANTI study and participants were categorized as sedentary-light physical activity (none or light-intensity physical activity <2–4 h/wk), or moderate-high physical activity (light-intensity activity >4 h/wk or moderate- intensity activity 1–2 h/wk; *i.e.*, swimming) [36]. Cognitive function was assessed by the Mini-Mental State Examination (MMSE) and participants with an MMSE score <24 were considered cognitively impaired. Depression was evaluated using the Centre for Epidemiologic Studies Depression Scale (CES-D) and subjects with a CES-D score ≥16 was defined as a depressed mood. Functional status was evaluated using the Katz's Activities of Daily Living (ADL), and ADL disability was defined as disabled ADL ≥ 1. Chronic diseases were ascertained by combining information from self-reported medical diagnoses, pharmacological treatments, medical history, clinical examination and blood tests. Comorbidities considered in this analysis were hypertension, cardiovascular diseases (including acute myocardial infarction, stroke, angina pectoris and peripheral arterial disease) and diabetes mellitus.

Statistical analysis

Participants were classified into tertiles according to usual daily intake of dairy products (servings per day) for descriptive analysis. Baseline characteristics of subjects were reported as means and standard deviations (SD) for normal continuous variables, medians and 10^{th} and 90^{th} percentiles for non-normal continuous variables, and numbers of participants (n) and percentages for categorical variables. Baseline characteristics between subjects by tertiles of dairy consumption or frailty phenotypes were compared using parametric ANOVA, non-parametric Kruskall-Wallis, and Chi-square tests, as appropriate.

The estimation of odds ratios (ORs) and the corresponding 95% confidence intervals (95% CI) of the associations between the consumption of both total and individual dairy products (continuous variable; in servings per day) with frailty risk and its components were tested using generalized linear mixed models (GLMM) including subject as random effect. A multinomial mixed effect logistic model was used for modelling frailty as outcome (using Robust as the reference category) and a mixed effect logistic model for the individual frailty components. All associations were investigated using four additive models. Model 1 was adjusted for age, sex and follow-up visit; Model 2 was additionally adjusted for physical activity, years of education, smoking status, BMI, and total energy, protein and alcohol intakes; Model 3 was further adjusted for baseline cardiovascular disease (including acute myocardial infarction, stroke, angina pectoris and peripheral artery disease), depression, hypertension, diabetes mellitus and cognitive impairment; and finally Model 4 was further adjusted for the consumption of fruit and vegetables. Interactions between the frequency of dairy products consumption and age (<75 *vs.* ≥75years), sex, smoking status, physical activity, and obesity (BMI<30 *vs.* ≥30kg/m^2) in relation to frailty or its components were assessed using the likelihood ratio test based on the GLMM models with and without interaction terms. Sensitivity analyses were performed after the exclusion of participants that died during the first 2 years of the follow-up, or with baseline diagnosis of cognitive impairment, dementia, depression, ADL disability, cardiovascular diseases, cancer, or with imputations in dietary data during the 9-year follow-up period. All analyses were computed using the IBM SPSS 26.0 (SPSS Inc., Chicago, IL).

Results

The baseline characteristics for the 920 participants are presented in Table 1. The mean age of participants was 74±6.6 years 44.6% of them were men. Regarding, socio-demographics and lifestyle habits, a 13.7% of the sample were current smokers, a 39.6% reported to have intense physical

activity, and had in average 5.5±3.3 years of education. A 47.5% of the sample were diagnosed with hypertension, 21.3% cardiovascular diseases, and 13.4% diabetes mellitus. At baseline, participants were mostly robust (52.5%) and pre-frail (39%), while few cases were frail (8.5%). The main prevalent frailty symptom was slowness (21.2%), followed by muscle weakness (19.5%), exhaustion (17.9%), low physical activity (16%), and unintentional weight loss (4.8%).

The median intake (p10th- p90th) of total dairy products was 2.7 (1.2–3.9) servings per day, and the main contributors were cheese 1.7 (0.4–2.6) and milk 1.0 (0–1.0) servings per day, followed by ice cream 0.1 (0–0.4) and yogurt 0 (0–0.3) servings per day. Participants in the highest tertile of total dairy product consumption (>3.12 servings per day) were more likely to have a higher intake of total energy, total protein, calcium, phosphorous, potassium and vitamin D than those in the lowest tertile (<2.14 servings per day) ($p<0.001$). There were no differences in other lifestyle factors and comorbidities between tertiles of dairy product consumption. (Table 1).

Frail individuals had a lower median energy intake (p10th- p90th) compared to robust ones [1,736 (1,030-2,306) *vs.* 1,923 (1,356-2,721) kcal/day; $p<0.001$)]. Statistically significant differences were found in the intake of total protein, phosphorus, potassium, vitamin D, and vegetables comparing frail versus robust individuals ($p<0.001$). No difference in total dairy products intake according to frailty status was observed. Robust participants were more physically active (52.1% *vs.* 5.1%) and had lower prevalence of cognitive impairment (17.6% *vs.* 59%), ADL disability (0% *vs.* 26.9%), and depressive mood (16.6% *vs.* 61.5%) than frail subjects. (Supplementary Table 2).

Using the fully adjusted GLMM, no associations were observed between total dairy products intake and frailty risk (pre-frail or frail status) during the study: pre-frail ($OR_{\text{per serving per day}}$= 0.94, 95% CI: 0.86-1.03), and frail ($OR_{\text{per serving per day}}$= 1.10, 95% CI: 0.92-1.30) compared to robust status. Similar results were found for the consumption of both fermented (pre-frail $OR_{\text{per serving per day}}$= 0.96, 95% CI: 0.83-1.10 and frail $OR_{\text{per serving per day}}$= 1.01, 95% CI: 0.78-1.32, respectively) and non-fermented dairy products (pre-frail $OR_{\text{per serving per day}}$= 0.94, 95% CI: 0.83-1.05, and frail $OR_{\text{per serving per day}}$= 1.14, 95% CI: 0.93-1.39) (Table 2). The associations between consumption of dairy products (fermented, non-fermented and total dairy products) and the individual frailty components during 9 years of follow up are shown in Supplementary Table 3. No statistically significant association was observed with frailty components.

No interactions between age, sex, smoking status, physical activity, and obesity and dairy product consumption in relation to frailty were observed (all $p>0.05$). Sensitivity analyses were performed excluding participants who died in the first two years of follow-up (n=30); who had baseline diagnosis of cognitive impairment (n=231), dementia (n=18), depression (n=279), ADL disability (n=33), cardiovascular diseases (n=196) and cancer (n=53); and finally the participants with imputed dietary

data (n=912) and the results remained similar to those obtained when analyzing the entire cohort (Supplementary Tables 4 and 5).

Discussion

The current study investigated the association between dairy products intake and frailty in a cohort of Italian community-dwelling adults older than 65 years enrolled in the InCHIANTI study. We found that the habitual consumption of dairy products was not significantly associated with the risk of either frailty or frailty components (unintentional weight loss, exhaustion, low physical activity, slowness and weakness).

According to our knowledge, only few studies have assessed the association between dairy products and frailty obtaining mixed results. Similar null results were also found in a recent analysis conducted by Rahi et al. in a French cohort of older adults, in which they evaluated the dairy intake (including total dairy, milk, fresh dairy and cheese) and its cross-sectional and prospective associations with frailty risk after 10 years of follow-up. The authors observed non-significant associations between consumption of total dairy products and frailty prevalence (OR = 1.40, 95% CI: 0.65–3.01 for high consumption *vs.* low) or incidence after 10 years (OR = 0.75, 95% CI: 0.42–1.32 when they compared the lowest frequency to the highest frequency of consumption) and with dairy products sub-types in fully adjusted models [31]. O'Connell et al. observed no association between milk and milk products consumption and physical frailty risk in an Irish Community-Dwelling Older Adults cohort [16]. Moreover, another study carried out in older adults from the Korean Frailty and Aging Cohort Study showed that the "milk" pattern was not associated with the frailty status [37].

On the contrary, Lana et al. found that participants consuming ≥7 servings per week of low-fat milk and yogurt were at lower frailty risk at 3.5 years of follow up (OR = 0.52, 95% CI: 0.29-0.90) compared to those consuming <1 serving per week in an ≥60 years Spanish population [30]. Similarly, a study based in older Japanese adults observed that an increasing dairy product intake was negatively associated with frailty development (OR=0.73, 95% CI: 0.55-0.96) after 2 years of follow-up [38]. Furthermore, in a cross-sectional Australian study in older community-dwelling women; a high intake of dairy products (≥2.2 servings per day) was positively associated with hand grip strength [39].

According to the literature, a link has been found between reduced frailty risk and adherence to healthy dietary patterns such as Mediterranean diet [40]. Struijk et al. evaluated the association of alternate Mediterranean diet (AMED), the Dietary Approaches to Stop Hypertension (DASH) diet, and the alternate Healthy Eating Index-2010 (AHEI-2010) with frailty risk among participating in the

Nurses' Health Study aged ≥60 years showing that all diet quality scores were inversely associated with the risk the individual frailty components [24]. In the same line, Tanaka et al. found a protective association between the adherence to the Mediterranean diet and a better frailty index over a 10 year follow-up period in the InCHIANTI cohort [22]. It is noteworthy that Mediterranean diet score is an algorithm developed by Trichopoulou et al [41] and consider the intake of dairy products as a "detrimental" food item. Although this dietary pattern recommends a moderate dairy products consumption, the Mediterranean diet pattern has proven to be protective for frailty and functional disability in adults and older adults.

At last, a recent systematic review [13] suggests that the consumption of dairy products in older people could reduce the risk of frailty, considering a high intake of low-fat milk and yogurt. Thus, the addition of nutrient-rich dairy proteins to the usual diet (e.g., ricotta cheese) may also reduce the risk of sarcopenia by improving skeletal muscle mass. Although the literature addressing the topic is scarce, this review shows that dairy consumption may have positive effects on frailty and sarcopenia [13]. Dairy products could decrease the risk of frailty through different mechanisms, such as delaying sarcopenia and bone mass loss (related conditions to frailty syndrome) through an adequate supply of energy, proteins, calcium, phosphorus, others bioactives and improving the bioavailability of these nutrients [42,43]. Indeed, dairy products is one of the main sources of protein intake in older people [44–47]. Recent studies have proposed that nutrients, such as vitamin K, from fermented dairy products could act as a cofactor in osteocalcin synthesis favoring the bone mineralization process [48]. Furthermore, dairy fermented products are an important source of prebiotics and probiotics which may regulate the gut microbiota, resulting in an increase in the mineral absorption [49,50].

Strengths of this research are the size of the cohort studied with a follow-up over 9 years, the use of a validated FFQ to estimate the habitual intake of foods and nutrients and the availability of repeated measures in the analysis for dairy products consumption and frailty and its components. This is very relevant since older people are susceptible to change their dietary habits over time due to different factors that affected their health status (physiologic, pathologic, and/or psychologic factors) [51]. One of the constraints is that despite the instrument is validated, data on the type of dairy products (high-fat *vs.* low-fat) was not collected which did not allow realize analyses with the types of dairy products that promotes the healthy dietary recommendations.

In conclusion, results obtained in this study suggest that the consumption of dairy products does not play an important role in the prevention of either frailty or its components in older persons from a Mediterranean country after 9 years of follow up. Despite these null results, we consider that dairy products are adequate for older people because they are a nutrient-dense food providing high-quality protein, micronutrients and bioactive compounds. Moreover, inclusion of dairy products into an older person's diet is practical and convenient.

Nevertheless, further studies are necessary to enhance the scientific evidence about this topic, differentiating between low and high fat dairy products, and investigating populations from different geographical areas. Maybe identification of specific biomarkers of dairy intake will provide a better understanding between dairy product exposure and disease outcomes.

Data availability

Data is available to all researchers upon justified request using the proposal form available in the InCHIANTI website (http://inchiantistudy.net/wp/how-to submit-a-proposal/).

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Author contributions

RZ-R and CA-L designed the research; NHL and TM performed the statistical analyses; NHL wrote the draft; TM, RZ-R, AC, MF, SB, LF and CA-L provided critical revision; AC, MF, SB and LF provided data of the study; CA-L had primary responsibility for final content. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interests.

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Table 1. Baseline characteristics of the InCHIANTI population according to tertiles of dairy product consumption.

Demographics	All (N=920)	Tertil 1 (N=309) < 2.14 servings per day	Tertil 2 (N=307) 2.14 - 3.12 servings per day	Tertil 3 (N=304) > 3.12 servings per day	*P-value*
Age, mean ± SD, y	74.0±6.6	73.2±6.4	74.1±6.7	74.4±6.7	0.16
Male, n (%)	410.0(44.6)	136.0(44.0)	145.0(47.2)	129.0(42.4)	0.48
Education, mean ± SD, y	5.5±3.3	5.6±3.4	5.5±3.3	5.4±3.1	0.93
Behaviour - related variables					
Body Mass Index, mean ± SD, kg/m²	27.6±4.1	27.7±4.3	27.8±4.0	27.2±4.1	0.07
Current smoker, n (%)	126.0(13.7)	37.0(12.0)	48.0(15.6)	41.0(13.5)	0.76
Physical activity, n (%)					0.39
Sedentary-Moderate	556.0(60.6)	189.0(61.2)	176.0(57.7)	191.0(63.0)	
Intense	361.0(39.4)	120.0(38.8)	129.0(42.3)	112.0(37.0)	
Cognitive impairment, MMSE, n (%)	231.0(25.1)	86.0(27.8)	72.0(23.5)	73.0(24.0)	0.39
ADL disability, n (%)	33.0(3.6)	14.0(4.5)	13.0(4.2)	6.0(2.0)	0.18
Depressed mood, CES-D ≥16, n (%)	279.0(30.3)	92.0(29.8)	88.0(28.7)	99.0(32.6)	0.56
Dietary intake, median (p10-90th)					
Energy, (kcal/d)	1875.7(1298.0–2656.7)	1665.0(1101.4–2479.8)	1874.6(1344.9–2670.2)	2064.1(1474.6–2809.7)	0.000
Protein, (g/day)	74.3(51.7–103.2)	63.7(43.4–91.8)	74.5(53.2–101.1)	83.0(61.1–110.4)	0.000
Alcohol, (g/day)	7.6(0.0–40.7)	6.1(0.0–41.4)	9.6(0.0–49.6)	6.0(0.0–32.9)	0.05
Calcium, (mg/day)	801.0(494.0–1191.9)	567.1(392.9–832.6)	814.5(598.9–1082.3)	985.4(768.2–1429.5)	0.000
Phosphorous, (mg/day)	1165.1(816.8–1615.9)	989.7(690.7–1389.1)	1164.8(867.8–1547.4)	1327.0(1015.0–1806.3)	0.000
Vitamin D, (mcg/day)	1.7(1.0–2.8)	1.6(0.9–2.8)	1.7(1.0–2.8)	1.8(1.1–3.0)	0.001
Potassium (mg/day)	2867.5(2021.6–3940.5)	2600.3(1788.3–3994.9)	2887.8(2060.5–3998.3)	3028.7(2217.7–4064.2)	0.000
Fruits, (g/day)	275.3(143.6–467.4)	260.2(132.3–477.3)	282.7(146.8–457.0)	279.1(153.4–478.8)	0.21
Vegetables, (g/day)	137.5(66.7–301.3)	134.4(65.5–304.3)	138.3(67.5–286.1)	138.9(68.3–305.6)	0.67

Dairy products intake (servings per day)					
Milk	1.0(0.0–1.0)	0.3(0.0–1.0)	1.0(0.0–1.0)	1.0(1.0–2.0)	0.000
Yogurt	0.0(0.0–0.3)	0.0(0.0–0.1)	0.0(0.0–0.3)	0.0(0.0–0.7)	0.000
Cheese	1.7(0.4–2.6)	0.6(0.2–1.6)	1.7(1–2.5)	2.3(1.7–3.0)	0.000
Ice cream	0.1(0.0–0.4)	0.1(0.0–0.3)	0.1(0.0–0.5)	0.1(0.0–0.5)	0.000
Fermented dairy products	1.8(0.5–2.7)	0.7(0.2–1.6)	1.8(1.1–2.5)	2.4(1.9–3.2)	0.000
Non-fermented dairy products	1.0(0.1–1.5)	0.5(0.2–1.1)	1.0(0.7–1.4)	1.1(1.0–2.1)	0.000
Total dairy products	2.7(1.2–3.9)	1.5(0.6–2.0)	2.7(2.2–3.0)	3.6(3.2–4.6)	0.000
Frailty Phenotype, n (%)					0.88
Robust	483.0(52.5)	158.0(51.1)	168.0(54.7)	157.0(51.6)	
Pre-Frail	359.0(39.0)	123.0(39.8)	116.0(37.8)	120.0(39.5)	
Frail	78.0(8.5)	28.0(9.1)	23.0(7.5)	27.0(8.9)	
Frailty Symptoms, n (%)					
Weight loss	44.0(4.8)	14.0(4.5)	14.0(4.6)	16.0(5.3)	0.89
Exhaustion	165.0(17.9)	58.0(18.8)	57.0(18.6)	50.0(16.4)	0.71
Low physical activity	147.0(16.0)	48.0(15.5)	51.0(16.6)	48.0(15.8)	0.93
Slowness	195.0(21.2)	65.0(21.0)	57.0(18.6)	73.0(24.0)	0.26
Muscle weakness	179.0(19.5)	64.0(20.7)	52.0(16.9)	63.0(20.7)	0.39
Diseases and conditions, n (%)					
Hypertension	438.0(47.6)	151.0(48.9)	152.0(49.5)	135.0(44.4)	0.39
Cardiovascular diseases	196.0(21.3)	69.0(22.3)	69.0(22.1)	59.0(19.4)	0.61
Diabetes mellitus	123.0(13.4)	44.0(14.2)	38.0(12.4)	41.0(13.5)	0.79
Diagnosis of dementia	18.0(2.0)	6.0(1.9)	5.0(1.6)	7.0(2.3)	0.83

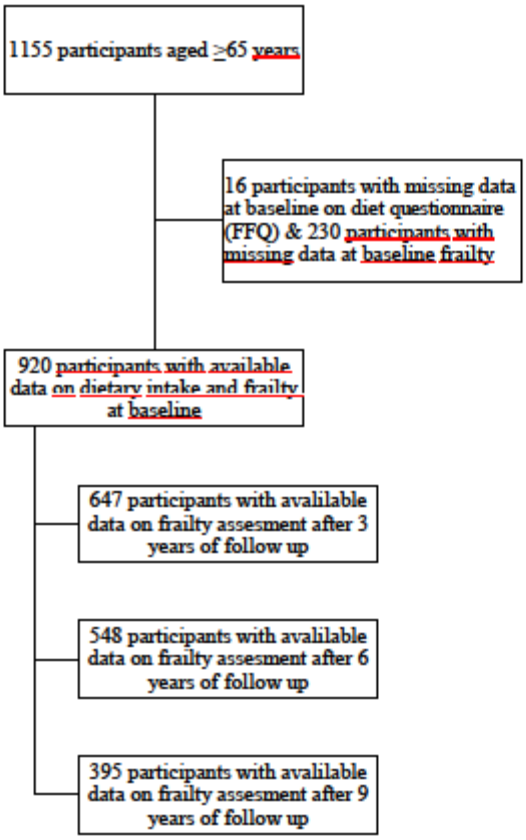
Notes: Statistical comparisons are from Kruskall-Wallis or Chi-Square tests as appropriate. ADL= Activities of Daily Living; CES-D= Center for Epidemiologic Studies Depression Scale; IADL= Instrumental Activities of Daily Living; MMSE= Mini-Mental State Examination

Table 2. Association between habitual intake of dairy products (per serving increase /day) and Pre-frailty and Frailty status in the InCHIANTI study.

	Pre-frailty (cases*= 1,069)			Frailty (cases*= 284)		
Total dairy products	**OR**	**95%CI**	***P-value***	**OR**	**95%CI**	***P-value***
Model 1	0.99	(0.91-1.07)	0.75	1.05	(0.91-1.21)	0.52
Model 2	0.94	(0.86-1.03)	0.17	1.10	(0.93-1.29)	0.28
Model 3	0.94	(0.86-1.03)	0.21	1.10	(0.93-1.31)	0.26
Model 4	0.94	(0.86-1.03)	0.19	1.10	(0.92-.1.30)	0.29
Fermented dairy products						
Model 1	1.01	(0.89-1.13)	0.93	0.94	(0.75-1.17)	0.56
Model 2	0.96	(0.84-1.09)	0.51	0.98	(0.76-1.27)	0.87
Model 3	0.96	(0.84-1.10)	0.53	1.02	(0.78-1.33)	0.89
Model 4	0.96	(0.83-1.10)	0.51	1.01	(0.78-1.32)	0.92
Non-Fermented dairy products						
Model 1	0.97	(0.87-1.09)	0.59	1.13	(0.95-1.35)	0.18
Model 2	0.93	(0.83-1.05)	0.23	1.15	(0.95-1.40)	0.14
Model 3	0.94	(0.83-1.06)	0.29	1.14	(0.93-1.40)	0.20
Model 4	0.94	(0.83-1.05)	0.27	1.14	(0.93-1.39)	0.22

[1]Multinomial mixed effects logistic regression analyses were done in additive models. Model 1: adjusted for age, sex, and follow-up wave (categorical, 4 levels); Model 2: Model 1 additionally adjusted for physical activity, years of education, smoking habits, BMI and energy, protein and alcohol intake; Model 3: Model 2 plus cardiovascular disease (including acute myocardial infarction, cerebrovascular disease, angina pectoris and peripheral artery disease), depression, hypertension, diabetes mellitus and cognitive impairment; Model 4: Model 3 plus fruit and vegetable intake. *Cases accumulated across the four follow-up evaluations. Serving sizes were the following: 60 grams per hard and semi hard cheeses, 40 grams per soft and fresh cheeses, 120 grams per milk, 125 grams per yogurt and 75 grams per ice cream.

Figure 1. Flowchart of participants at period of the study.



Supplementary material

Association between dairy products intake and frailty phenotype in older adults: the InCHIANTI Cohort study.

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Supplementary Table 1: STROBE-nut statement

Supplementary Table 2. Baseline characteristics InCHIANTI participants according to Frailty status.

Supplementary Table 3. Association between habitual intake of dairy products (per serving increase /day) and individual frailty components in the InCHIANTI cohort.

[1]Mixed effects logistic regression models were using a subject-specific random effect were done in additive models. Model 1: adjusted for age, sex, and follow-up wave (categorical, 4 levels); Model 2: Model 1 + physical activity, years of education, smoking habits, BMI and energy, protein and alcohol intake; Model 3: Model 2 + cardiovascular disease (including acute myocardial infarction, cerebrovascular disease, angina pectoris and peripheral artery disease), depression, hypertension, diabetes mellitus and cognitive impairment; Model 4: Model 3 + fruit and vegetable intake. *Cases accumulated across the four follow-up evaluations. Serving sizes were the following: 60 grams per hard and semi hard cheeses, 40 grams per soft and fresh cheeses, 120 grams per milk, 125 grams per yogurt and 75 grams per ice cream.

Supplementary Table 4. Sensitivity analysis between habitual intake of dairy products (per serving increase /day) and Pre-frailty and Frailty status in the InCHIANTI study.

[1]Multinomial mixed effects logistic regression models using a subject-specific random effect were adjusted for age, sex, follow-up wave(categorical, 4 levels), physical activity, years of education, smoking habits, BMI, cardiovascular disease (CVD, including acute myocardial infarction, cerebrovascular disease, angina pectoris and peripheral artery disease), depression, hypertension, diabetes mellitus, cognitive impairment (CI), energy, protein, alcohol, and fruit and vegetable intake. *Total number of cases accumulated across the four follow-up evaluations. Numbers in brackets are n= number of subjects, k= number of observations. Serving sizes were the following: 60 grams per hard and semi hard cheeses, 40 grams per soft and fresh cheeses, 120 grams per milk, 125 grams per yogurt and 75 grams per ice cream.

Supplementary Table 5. Sensitivity analysis between habitual intake of dairy products (per serving increase /day) and individual frailty criteria in the InCHIANTI cohort.

[1]Mixed effects logistic regression using frailty components as dependent variables, a subject-specific random effect and the following variables as fixed factors age, sex, follow-up wave (categorical, 4 levels), physical activity, years of education, smoking habits, BMI, energy, protein, alcohol, fruit and vegetable intake, cardiovascular disease (including acute myocardial infarction, cerebrovascular disease, angina pectoris and peripheral artery disease), depression, hypertension, diabetes mellitus and cognitive impairment. *Cases accumulated across the four follow-up evaluations. Serving sizes were the following: 60 grams per hard and semi hard cheeses, 40 grams per soft and fresh cheeses, 120 grams per milk, 125 grams per yogurt and 75 grams per ice cream.

5. DISCUSIÓN

DISCUSIÓN

Según los datos proporcionados por la OMS, gran parte de los países tendrán un aumento del porcentaje de personas mayores de 65 años en su población.

Si bien, es un hecho que la vejez es una etapa del ciclo vital y un proceso fisiológico normal, éste debe ser entendido como un proceso heterogéneo que depende de las características genéticas y epigenéticas del individuo, así como de los factores ambientales y psicosociales, económicos, nutricionales, entre otros; que lo afectan con el transcurrir de los años.

Diversas investigaciones proponen una relación directa entre el envejecimiento y diversas alteraciones metabólicas acumuladas con el tiempo, que provocan una reducción de las respuestas fisiológicas para el mantenimiento de la homeostasis, relacionando el envejecimiento con mayores tasas de morbilidad, dando paso a la aparición de enfermedades crónicas como las cardiovasculares, neurodegenerativas, algunos tipos de cáncer y otras condiciones clínicas que aumentan la dependencia, el deterioro funcional y la discapacidad. En este ámbito, existe suficiente evidencia que sugiere que el seguimiento de hábitos saludables optimiza el estado de salud global de las personas adultas y personas de edad avanzada, por lo cual una nutrición adecuada de las personas mayores tiene un impacto positivo en su estado de salud, contribuyendo a mantener la funcionalidad y a reducir las complicaciones de salud.

La OMS propone diversas estrategias para abordar el reto del envejecimiento poblacional con el fin de minimizar el impacto sanitario y socioeconómico de la creciente proporción de población de adultos mayores. Uno de los pilares fundamentales de esta estrategia es promover el envejecimiento saludable desde la edad adulta con el objetivo de reducir la carga de enfermedades crónicas y la discapacidad y en el cual la nutrición juega un papel fundamental, potenciando la prevención de enfermedades crónicas y favoreciendo el mantenimiento de la capacidad funcional que permita el bienestar en edades avanzadas.

Considerando este contexto, el objetivo general de esta tesis doctoral se focaliza en el estudio del efecto de la calidad de la dieta global en el proceso de envejecimiento saludable utilizando diferentes aproximaciones para la valoración de la exposición dietética (cuestionarios y biomarcadores nutricionales) y su asociación con el estado de salud en población de personas de edad avanzada. Este objetivo ha sido abordado a través de dos tipos de estudios en personas mayores; un estudio de intervención dietética y un estudio observacional prospectivo con un seguimiento de 20 años.

El primero ha sido el estudio de intervención dietética MaPLE, investigación que tiene un diseño cruzado, aleatorizado, controlado y simple ciego en el que han participado 51 personas mayores de 60 años. Éste se llevó a cabo con el propósito de evaluar la hipótesis de que una ingesta elevada de alimentos ricos en polifenoles puede reducir los niveles séricos de zonulina (marcador indirecto de la

permeabilidad intestinal (PI)) así como disminuir los factores bacterianos inflamatorios en el torrente sanguíneo, favoreciendo un fenotipo metabólico beneficioso en los participantes.
El segundo ha sido un estudio observacional prospectivo de la cohorte InCHIANTI, donde se ha valorado la exposición dietética de alimentos y nutrientes, con especial foco de atención en productos lácteos y proteínas, y la adherencia a un patrón de dieta mediterránea (DM) en personas mayores de 65 años y su asociación con la fragilidad a los 9 años y mortalidad a los 20 años.

El proyecto MaPLE nos ha permitido realizar la valoración de la composición nutricional y de polifenoles de los menús ofertados y de ingesta alimentaria de los 51 participantes del estudio (**manuscrito 6**), así como estudiar la eficacia de la intervención dietética mediante el estudio del perfil metabólico en orina (**manuscrito 7)**, para posteriormente estudiar el efecto de la intervención dietética con alimentos ricos en polifenoles y su potencial modulador de la PI junto a sus posibles mecanismos de acción, así como en otros resultados en salud (**manuscritos 8 y 9**).

Es importante valorar la adecuación nutricional de las dietas proporcionadas a sujetos de edad avanzada en residencias de larga estancia para gente mayor, con el fin de identificar posibles desviaciones de los objetivos nutricionales planteados y tomar medidas correctivas al respecto. Después de realizar la evaluación de la composición nutricional de los menús dietéticos proporcionados a lo largo del estudio MaPLE (**manuscrito 6**), encontramos que los menús servidos a los participantes eran comparables durante todo el año en términos de energía aportando en promedio (menú de invierno, verano y primavera) 1976 ± 133 kcal/día, la cual está en línea con las recomendaciones nutricionales italianas (103) y con otros análisis que mostraron resultados similares o incluso mayores de lo observado en nuestro análisis (104–106), aunque también se ha encontrado otros estudios que han reportado menores aportes de energía diaria (107,108). En cuanto a aspectos cualitativos de la dieta, al comparar nuestros resultados con las referencias nutricionales notamos que el aporte de hidratos de carbono en los menús fue de un 47% del valor calórico total y el de lípidos de un 34%, estando ambos macronutrientes dentro del rango recomendado (61,103), mientras que el aporte de proteínas era mayor respecto de las recomendaciones (19% del VCT). La cantidad promedio de fibra aportada por los menús fue ligeramente menor a los 25 g/día recomendados. En cuanto al aporte de micronutrientes aportados por los menús fueron superiores a los valores de referencia, a excepción del contenido de calcio que fue inferior a la ingesta de referencia (103). En lo que respecta al aporte de polifenoles totales de los menús, su estimación media fue de ~770 mg al día, aporte que tuvo pequeñas variaciones cuando se consideraba la estacionalidad, en concordancia con datos previos donde se ha informado sobre dicha variación estacional en el contenido de polifenoles en los alimentos (109) y también se ha encontrado una variabilidad de ingesta de polifenoles en sujetos mayores no institucionalizados la que también se ve influenciada por la estacionalidad (110,111). Aun así, no existe evidencia del impacto de la estacionalidad en el contenido de polifenoles en los planes dietéticos proporcionados en las residencias de larga duración para

personas mayores. De los resultados encontrados en el análisis de la valoración del consumo real medido mediante la recogida de múltiples registros de alimentos por pesada, el cual se ha definido como el método de referencia más preciso para estimar un consumo de alimentos (55), encontramos que tanto la ingesta de nutrientes y de polifenoles fue inferior en comparación con la proporcionada por los menús. La ingesta media de energía de los participantes fue aproximadamente de 1580 kcal/día, ligeramente inferior a las encontradas en el estudio observacional InCHIANTI, realizado en unos 1200 sujetos mayores de 65 años en condiciones de dieta libre (112). La menor ingesta de energía se asoció con una menor ingesta de proteínas (~0,9 g/kg al día de media), de ácidos grasos ω-6, calcio, vitaminas B1, B6 y folatos por parte de los participantes. Estas ingestas inadecuadas se deben a que la ingesta real de alimentos es significativamente menor que la cantidad de comida proporcionada a los residentes en cada comida, estos resultados deben ser considerados debido al riesgo potencial que supone una inadecuada ingesta de nutrientes a largo plazo, teniendo una influencia negativa en las funciones metabólicas y en la capacidad funcional de los sujetos de edad avanzada. En el caso particular de los polifenoles (con una ingesta media de ~660 mg/día), las comparaciones con datos publicados previamente deben ser cautelosas, debido a las diferencias de las características de las poblaciones estudiadas, los métodos de análisis de la dieta y de las bases de datos de composición de alimentos utilizados para estimar la ingesta total de polifenoles y de sus clases. Previamente realizamos una revisión sistemática para evaluar la ingesta de polifenoles en personas de edad avanzada (**manuscrito 2**), los resultados revelaron un rango de ingesta de polifenoles entre 333 mg/día hasta 1.492 mg/día (113). No obstante, la ingesta total real estimada en el presente estudio parece ser comparable con la ingesta media observada en el estudio observacional InCHIANTI (114), aunque inferior respecto a resultados en personas mayores del estudio PREDIMED (115). Mediante la administración de los alimentos seleccionados para la dieta rica en polifenoles, los flavonoides y los ácidos fenólicos fueron las principales clases de polifenoles aportadas por la intervención. Tal como hemos evidenciado en las revisiones de los **manuscritos 4 y 5**, estas clases de compuestos bioactivos (polifenoles) han sido sugeridos como moduladores potenciales de factores específicos que regulan la PI, incluyendo el impacto en la composición de microbiota, así como de su actividad (116,117).

En el contexto del mismo estudio de intervención, nos propusimos investigar el impacto del aumento de la PI en personas mayores sobre la biodisponibilidad de los polifenoles de la dieta, los resultados de este trabajo mostrados en la **manuscrito 6** mostraron diferencias significativas en los niveles urinarios de metabolitos de fase II y derivados de la microbiota entre los sujetos que al iniciar el estudio tenían una la barrera intestinal mejor preservada (zonulina basal ≤ a la media) respecto de aquellos participantes con una mayor alteración de la PI (zonulina basal > a la media). Teniendo en cuenta que la dieta rica en polifenoles duplicó la ingesta de polifenoles estimada en comparación con la dieta control, observamos que la intervención indujo una ligera disminución de los niveles de zonulina en suero, siendo esta diferencia significativa en los sujetos con una mayor PI (es decir, el grupo con zonulina basal alta), mientras que los que tenían menor zonulina basal no se vieron

afectados. Estos resultados quedan avalados por la evidencia que demuestra el potencial rol de los polifenoles en la regulación de la función de la barrera intestinal y la prevención del aumento de la PI, sugiriendo además la posible existencia de dos grupos fenotípicos caracterizados por el grado de PI. A partir de nuestros resultados y de la literatura consultada, es posible sostener que la biodisponibilidad de los polifenoles dependiente de la PI se podría atribuir a las alteraciones del metabolismo microbiano intestinal (118–120) y a los procesos de metilación de fase II (121–123), también según lo reportado por Suzuki (124) observamos que ciertos metabolitos derivados de la microbiota podrían ser en gran medida responsables de la actividad biológica provocada por los polifenoles de la dieta contra la alteración de la PI relacionada con el envejecimiento.

Posteriormente, estudiamos el efecto de la intervención con el patrón dietético rico en polifenoles en el mantenimiento de la integridad de la barrera del intestino mediante la medición de un marcador indirecto de PI (zonulina sérica) y otros marcadores relacionados a la salud de las personas mayores que participaron en el estudio. De acuerdo a los resultados informados en la **manuscrito 8,** estudio que se realizó a raíz de la reciente evidencia científica en la que se observó que los factores de estilo de vida como la dieta y otras exposiciones externas pueden contribuir al daño del ADN durante el envejecimiento (125), se evaluó el nivel de daño en el ADN y su asociación con marcadores clínicos, metabólicos y dietéticos y se observó que si bien no fue posible demostrar una correlación significativa entre la edad o el sexo con el daño del ADN, los resultados mostraron una tendencia inversa entre los niveles de sitios sensibles a la Fpg (daño endógeno del ADN) y la edad, y un nivel de daño mayor (pero no significativo) en los hombres en comparación con las mujeres. La dieta y sus componentes se han investigado en gran medida como factores moduladores sobre las funciones biológicas, incluida la protección contra el estrés oxidativo, éstos pueden contribuir a la modulación positiva/negativa del daño en el ADN durante el proceso de envejecimiento (22). Los análisis realizados en nuestro estudio mostraron que hay una asociación negativa entre la ingesta de fibra y las vitaminas de la dieta y los niveles de daño del ADN, y una asociación positiva con los lípidos y el colesterol de la dieta, en particular, la ingesta de ácidos grasos saturados (AGS) que parece ser un determinante del daño basal del ADN. En cuanto a la fibra dietética, ésta desempeña un papel fundamental en la salud humana, relacionándose su ingesta con una reducción del aumento de peso, la incidencia de la diabetes, la prevalencia de la obesidad, manejo de la hipercolesterolemia, con la inflamación e indirectamente también la con la reducción del estrés oxidativo (126,127), aunque la ingesta de fibra en nuestra población de estudio fue relativamente baja (entre 17-18 g/día) respecto a la recomendación dietética (al menos 25 g/día) (61,103). Nuestros análisis mostraron que cuando estratificábamos la población por sexo, era posible encontrar una asociación inversa entre la ingesta total de fibra (ajustada a la ingesta de energía) y los niveles de daño en el ADN en las mujeres del estudio. Respecto a la ingesta de vitaminas y minerales, hemos encontrado una asociación inversa entre los niveles de Fpg sensibles (formamidopirimidina ADN glicosilasa, marcador del daño endógeno del ADN) y la ingesta de vitamina C, vitamina E, folatos y vitamina B6, mientras que no se encontraron correlaciones con la ingesta de minerales. Las asociaciones inversas también se

confirmaron al estratificar los sujetos por sexo, lo que subraya aún más la importancia de la ingesta adecuada de vitaminas en esta población. De acuerdo con la literatura, tanto las vitaminas y los minerales pueden contribuir a la modulación de las actividades contra el daño del ADN (73,128–132).

En cuanto a la ingesta de polifenoles no se encontró ninguna asociación significativa entre la ingesta total de polifenoles o subclases de polifenoles y los niveles de daño en el ADN, no pudiendo demostrar el efecto protector de estos compuestos bioactivos contra el daño del ADN, a pesar de que ha sido ampliamente investigado el efecto protector de algunos compuestos bioactivos como los polifenoles contra el estrés oxidativo (133), así como su capacidad para neutralizar el efecto nocivo de las especies reactivas de oxígeno (134,135). Los ácidos grasos monoinsaturados (AGMI) y los ácidos grasos poliinsaturados (AGPI), específicamente los omega-3, también se han asociado con un nivel reducido de daño oxidativo (136,137). Así mismo, existe respaldo desde varias investigaciones que previamente han informado sobre una asociación positiva y/o inversa entre el daño del ADN y patrones dietéticos específicos (138,139) y/o la ingesta de diferentes macro/micronutrientes (136). En parte nuestros resultados pueden estar supeditados a la homogeneidad de las elecciones alimentarias relacionadas con la disponibilidad de las comidas proporcionadas a través del menú diario en la residencia de gente mayor.

Así mismo en los resultados del **manuscrito 9**, hemos demostrado que la modificación de la dieta de los participantes mediante la inclusión de pequeñas porciones de productos ricos en polifenoles puede reducir las concentraciones de zonulina en suero, de hecho las mayores reducciones de este marcador sustitutivo de la PI fueron observados tras la dieta de rica en polifenoles en el subgrupo de altos niveles de zonulina sérica, resultados que se acompañaron de reducciones en la presión arterial diastólica, glucosa y niveles de IL-6 (aunque este último no fue estadísticamente significativo). Estos resultados están en consonancia con estudios anteriores que han mostrado que el intestino permeable puede desempeñar un papel importante en la inflamación y en la fragilidad relacionadas con la edad, por ejemplo, en un estudio exploratorio preliminar Qi et al. (140) encontraron altos niveles de zonulina sérica en sujetos de edad avanzada en comparación con los más jóvenes, demostrando además una asociación positiva entre los niveles de zonulina y marcadores de inflamación (TNF-a, IL-6), y una asociación inversa con el rendimiento físico (fuerza muscular y pasos/día). También se ha sugerido que los niveles aumentados de zonulina sérica reflejan la respuesta del huésped a un proceso inflamatorio, proponiendo una posible interacción bidireccional entre la inflamación y la PI (141), de hecho en la mayoría de las enfermedades relacionadas con la inflamación se ha observado una mayor PI, tanto a nivel intestinal (por ejemplo, la enfermedad inflamatoria intestinal, el síndrome del intestino irritable, la enfermedad celíaca) como sistémico (por ejemplo, la obesidad, la diabetes mellitus de tipo 2), incluida la inflamación sistémica de bajo grado relacionada con la edad (142). En nuestro estudio, después de la intervención de 8 semanas con la dieta que incluía alimentos ricos en polifenoles, no se observaron cambios en los marcadores inflamatorios, estos resultados están en línea con otros estudios de intervención con alimentos ricos en polifenoles tanto en adultos como en

personas mayores (143–148). El envejecimiento está asociado a numerosas disfunciones fisiológicas, incluidas la desregulación del metabolismo de los lípidos y el metabolismo de la glucosa, de ahí la importancia que sugiere la literatura revisada respecto del potencial rol de los polifenoles y los alimentos ricos en polifenoles incluidos en la intervención en la modulación de la de la presión arterial (149), así como en la regulación de la homeostasis de la glucosa (113,150). Por último, los resultados de este estudio sugieren que a pesar de que la composición del ecosistema microbiano intestinal cambia con el envejecimiento (151) y que este puede influir en la modulación PI (152), la PI puede verse afectada positivamente por un patrón de dieta rica en polifenoles.

Previamente, para la preparación de esta temática y conocer a fondo los antecedentes bibliográficos establecidos en el consorcio del proyecto MAPLE, la presente tesis doctoral colaboró en las 4 revisiones del consorcio a tal finalidad.

Como punto de partida y teniendo en cuenta que una medición precisa de la ingesta de alimentos es un punto crítico para comprender los vínculos entre la dieta y el estado de salud o riesgo de enfermedad, en el **manuscrito 2**, nos planteamos el objetivo de analizar y destacar los enfoques destinados a mejorar la evaluación de la exposición dietética. Después de analizar la bibliografía disponible del uso de diversas estrategias para valorar la exposición dietética como los cuestionarios dietéticos, los biomarcadores de exposición incluidos los paneles multimetabolitos, proponemos un enfoque combinado, que utilice datos de cuestionarios dietéticos junto con las mediciones de los biomarcadores de exposición, por considerarla una estrategia ventajosa para mejorar la evaluación de la exposición alimentaria, especialmente cuando se desconoce hasta qué punto el biomarcador media en el efecto dietético. El método planteado ha sido utilizado en un estudio sobre carotenoides y enfermedades oculares relacionadas con la edad (CAREDS), proporcionando resultados en los que se apreciaba la mayor potencia estadística al utilizar la combinación de CFCA junto al biomarcador de exposición era mayor que la del CFCA y el biomarcador por separado, así como, para detectar una asociación dieta-enfermedad (153,154). En la misma línea estuvieron los resultados de Rabassa et al. en la cual aplicaba el enfoque combinado entre la exposición habitual al resveratrol y el desarrollo del síndrome de fragilidad en adultos mayores del estudio InCHIANTI, encontrando que había asociaciones inversas entre la exposición al resveratrol y el síndrome de fragilidad evaluado por CFCA, por biomarcador urinario y por el método combinado (155,156). Los aspectos abordados en estas revisiones son claves para el desarrollo de los trabajos realizados en el estudio de intervención, así como en el estudio observacional.

El **manuscrito 3** realizada en el contexto del estudio MaPLE con el objetivo de evaluar la evidencia científica disponible en los últimos 10 años, sobre la evaluación de la ingesta de polifenoles y su asociación con enfermedades como la cardiovascular, diabetes, o la mortalidad en personas adultas y adultos mayores. En esta revisión se realizó una estimación de la ingesta media total de polifenoles, teniendo en cuenta la heterogeneidad de los datos analizados, de la misma manera se consideró la contribución de las fuentes dietéticas, quedando en evidencia que el té, el café, el vino tinto, la fruta

y las verduras son las principales fuentes alimentarias que contribuyen a la ingesta de polifenoles. También se encontró una gran variabilidad en cuanto a los métodos de evaluación y cuantificación de la ingesta de polifenoles, así como en los marcadores y los criterios de valoración de la ingesta de polifenoles, de hecho la evidencia disponible ha informado de que la evaluación de la ingesta total de flavonoides requiere al menos de 6 días de registros de alimentos por pesada, y entre 6 y 10 días para determinar la ingesta de subclases específicas de flavonoides con un grado aceptable de precisión (111), aunque tal como se recomienda, la mayoría de los estudios analizados en la presente revisión no realizaron una evaluación múltiple de la ingesta de alimentos, con el fin de evitar subestimación o sobreestimación de la ingesta total de polifenoles y de sus clases/subclases. Otro punto crítico importante para la estimación de la ingesta de polifenoles es la elección de las bases de datos, si bien las más utilizadas son las del USDA que se centra predominantemente en los flavonoides como agliconas (flavonoides, proantocianidinas e isoflavonas) , y Phenol-Explorer (89) que además de proporcionar valores de flavonoides mencionados (como glicósidos o como agliconas), proporciona datos de sus precursores (chalconas, dihidrochalconas y dihidroflavonoles), otras clases (ácidos fenólicos, estilbenos, lignanos, etc.) e información sobre los polifenoles totales medidos por Folin-Ciocalteu (157). Sin embargo, estas bases de datos tienen varias limitaciones, en primer lugar, se dificulta la comparación de los resultados obtenidos a partir de las distintas fuentes de datos dado que proporcionan información sobre diferentes clases de polifenoles, de hecho, algunos estudios han reportado que la ingesta de flavonoides es generalmente mayor cuando se calcula utilizando las bases de datos de USDA en relación con la base de datos Phenol-Explorer (157). Por último, a pesar de que la evidencia disponible sugiere firmemente que un patrón de dieta rico en polifenoles tiene un efecto protector sobre la salud, recientes resultados de revisiones y metaanálisis que han evaluado la asociación de las subclases de polifenoles con diferentes tipos de cáncer han mostrado que los efectos a menudo son nulos (158–163), razón por la que continua siendo difícil establecer una ingesta de referencia basada en la evidencia para los polifenoles y sus clases sobre todo en población de personas de edad avanzada.

Dentro del proyecto MaPLE también ha sido necesario valorar el rol de la dieta, específicamente el efecto de los polifenoles y otros compuestos bioactivos en la modulación de la microbiota y sus implicancias en la mantención de la integridad intestinal, en este contexto se ha trabajado en dos revisiones, en el **manuscrito 4** nos enfocamos en revisar la literatura reciente sobre la aplicación de la metabolómica en el estudio de las alteraciones de la microbiota intestinal inducidas por la dieta y de los efectos sobre la PI, mientras que en el **manuscrito 5** realizamos una revisión general de las principales evidencias obtenidas en estudios in vitro e in vivo que apoyan el papel de los polifenoles en la modulación de la PI. En concreto, encontramos que el mantenimiento de la integridad intestinal puede verse afectada tanto por factores ambientales como por factores alimentarios, y en este sentido, los compuestos bioactivos obtenidos de los alimentos, como los polifenoles, se han propuesto como moduladores potenciales de la PI, también se ha evidenciado que la dieta puede afectar a la microbiota

y ésta puede modular la PI, así mismo la metabolómica ha sido uno de los enfoques más adecuados para estudiar los efectos de la dieta en la microbiota intestinal y la PI. Es así que en algunas publicaciones recientes se ha planteado que los metabolitos microbianos derivados del triptófano identificados a través de métodos metabolómicos, desempeñan un papel importante en la regulación de las funciones de barrera y de la actividad de la microbiota intestinal (164,165). Sin embargo, los manuscritos **4 y 5** concuerdan en que aún no se ha dilucidado totalmente la interacción entre los polifenoles, la microbiota intestinal y los mecanismos implicados en la modulación de la PI, siendo necesario para ello una combinación de enfoques metabolómicos, metagenómica y del estudio del microbioma (166).

En esta tesis doctoral, se ha trabajado con datos de ingesta alimentaria del estudio observacional prospectivo de la cohorte de personas mayores de 65 años del estudio InCHIANTI de la cual se ha obtenido 3 publicaciones. De la misma manera, que se planteó en el **manuscrito 2**, sobre la importancia de aplicar un método combinado que considere la información obtenida desde los cuestionarios donde se reporta la ingesta, como el CFCA, y los datos de los biomarcadores de exposición dietética (obtenidos a partir de muestras biológicas de sangre, orina, etc.). El **manuscrito 10** tuvo como objetivo desarrollar un panel de biomarcadores dietéticos basado en grupos de alimentos clave de la DM en la población del estudio InCHIANTI e investigar su asociación a largo plazo con la mortalidad por todas las causas, la ECV y el cáncer. Comparando las asociaciones con la mortalidad utilizandon el biomarcador dietético y el *score* de DM estimado a partir del CFCA. De acuerdo con nuestros análisis hemos mostrado que una puntuación de biomarcadores dietéticos de referencia basada en grupos de alimentos clave de la DM medida al tiempo basal, se asociaron significativamente de forma inversa con la mortalidad total, así como por ECV considerando un seguimiento medio de 14 años. Sin embargo, no se han visto las mismas asociaciones al valorar el score de DM basado en el CFCA, tal como sí se ha evidenciado en estudios previos en cohortes de personas mayores como los estudios EPIC-elderly prospective cohort study (167), MOLI-SANI (168), *Healthy aging*: a Longitudinal study in Europe (HALE) (169) y Women's Health Initiative (WHI) (170), diferencias que podrían deberse a que los participantes de los estudios referidos tenían en promedio una menor edad que los sujetos de la cohorte InCHIANTI incluidos en el análisis, así como al tiempo de seguimiento, dado que nuestro estudio ha considerado un seguimiento medio de 14 años versus el seguimiento medio 8,1 años (en los estudios EPIC y MOLI-SANI), otra causa de estas diferencias podría ser la mayor proporción de muertes encontradas en nuestro estudio 68% frente a 10-17% (en los estudios EPIC y MOLI-SANI). Nuestros resultados sugieren que un panel de biomarcadores dietéticos proporciona una evaluación más objetiva y precisa de los beneficios para la salud asociados a la calidad de la dieta en los adultos mayores a diferencia de los cuestionarios auto reportados. Estudios metabolómicos publicados recientemente crearon un panel de biomarcadores que incluía más de 60 de metabolitos plasmáticos basado en la adherencia a la DM, de los cuales > 60% eran lípidos (como fosfolípidos, glicerolípidos, carnitinas y acilcarnitinas) (171,172), utilizando

un enfoque a posteriori para explorar las huellas digitales de los metabolitos, que se correlacionaron significativamente con la adherencia al score de DMa partir de cuestionarios dietéticos.

En nuestros análisis incluimos metabolitos derivados de fuentes alimenticias como polifenoles totales, resveratrol o carotenoides, que no han sido considerados en los análisis metabolómicos mencionados anteriormente. De hecho, en el estudio de Li et al. (171), las frutas y legumbres estaban ligeramente correlacionadas con 7 de los 67 metabolitos que constituían la puntuación total, quedando en evidencia que estos metabolitos pueden rastrear los cambios biológicos inducidos por la adherencia a la DM (biomarcadores del efecto) pero pueden no correlacionarse con la ingesta de ciertos grupos de alimentos principales de la DM. Nuestros hallazgos sobre el score de adherencia a DM basado en biomarcadores dietéticos están en consonancia con resultados anteriores del estudio InCHIANTI que muestran que las asociaciones inversas de los AGPI y los polifenoles totales con la mortalidad general sólo fueron significativas utilizando biomarcadores dietéticos, pero no utilizando cuestionarios dietéticos (173,174). Considerando nuestros resultados, se debe tener en cuenta que el biomarcador dietético puede captar mejor la exposición dietética teniendo en cuenta las variaciones interindividuales relacionadas a la edad, así como la capacidad de informar sobre la ingesta de alimentos mediante el CFCA o de cualquier método de valoración de la dieta que sea altamente dependiente de la memoria de las personas mayores, lo cual podría dificultar la estimación precisa de las asociaciones entre las ingestas dietéticas y los resultados de salud (175).

El **manuscrito 11** se ha realizado con el objetivo de estudiar la exposición dietética de las proteínas totales y su asociación con la mortalidad a los 20 años. En los resultados presentados en dicho manuscrito, se encontró que la ingesta media de proteínas totales en la población de estudio fue de 74 g/día (1,1 g/kg de peso corporal al día) la cual se adecua a las recomendaciones diarias para esta población (61,103). Alrededor de un 63 % de la ingesta total de proteínas era de origen animal y entre sus principales fuentes dietéticas encontramos productos lácteos, cárnicos procesados, carne roja, pescados y mariscos, pollo, huevos y otras carnes de caza, mientras que la ingesta de proteínas vegetales venía mayoritariamente de fuentes dietéticas como los cereales, verduras, frutas y los frutos secos, y legumbres. De acuerdo a nuestros resultados, la ingesta de proteínas animales se asoció de forma inversa con la mortalidad por todas las causas así como con la mortalidad por enfermedades cardiovasculares tras un seguimiento de 20 años, resultados que coinciden con otros estudios realizados en población de personas mayores (170,176,177). El aumento de la ingesta de proteína de origen animal puede estar inversamente asociado a la mortalidad en los adultos mayores por sus efectos protectores sobre la fuerza muscular (178,179), así como con la fragilidad, la sarcopenia o las respuestas inmunitarias, aunque es necesario que estas asociaciones sean investigadas con mayor profundidad (180). No obstante, un reciente meta-análisis ha mostrado resultados contrarios a los anteriormente expuestos (181). Por otra parte, la ingesta de proteínas vegetales, que procedían principalmente de fuentes alimentarias derivadas de cereales, no mostró asociación con ninguno de los resultados de mortalidad. Sin embargo, en un reciente estudio realizado por Huang et al. hallaron una asociación inversa entre la ingesta de proteína vegetal y la mortalidad en un estudio de cohortes

prospectivo de EE.UU. (182), a pesar de que las fuentes alimentarias de la proteína vegetal son similares a las de nuestra cohorte.

El último trabajo incluido en los resultados de esta tesis doctoral, se presenta en el **manuscrito 12** en la que se realizó la valoración de la exposición dietética de los productos lácteos reportados en un CFCA y su asociación con la fragilidad en personas mayores de 65 años después de 9 años de seguimiento. Conforme a los análisis realizados en este estudio encontramos que la mediana de la ingesta de productos lácteos totales fue de 2,7 raciones al día, y los principales alimentos que con contribuyeron a este consumo fueron el queso, seguido de la leche, y a mucha distancia el helado y el yogurt. En cuanto a las asociaciones entre el consumo habitual de productos lácteos y el riesgo de fragilidad o sus componentes individuales (pérdida de peso involuntaria, agotamiento, baja actividad física, lentitud y debilidad), no encontramos resultados estadísticamente significativos. Nuestros hallazgos están en concordancia con los resultados de los pocos estudios que se han realizado respecto a esta temática. Rahi et al.(183) evaluaron la asociación de la ingesta de lácteos de una cohorte francesa de adultos mayores con la fragilidad tras 10 años de seguimiento de la cual también han reportados resultados nulos. De la misma forma, O'Connell et al. no observaron ninguna asociación entre el consumo de leche y productos lácteos y la fragilidad física en una cohorte irlandesa de adultos mayores (184). A pesar de que previamente, Lana et al. (185) en su estudio realizado en población de personas mayores de 60 años en España había reportado que los participantes que consumían ≥7 raciones a la semana de leche y yogur bajos en grasa, tenían un menor riesgo de fragilidad a los 3,5 años de seguimiento. Del mismo modo, un estudio basado en adultos mayores japoneses encontró que la ingesta de productos lácteos se asoció negativamente con el desarrollo de la fragilidad después de 2 años de seguimiento (186). Sobre la base de la literatura publicada, el patrón de DM ha demostrado ser protector de la fragilidad y la discapacidad funcional en adultos y personas de edad avanzada de diferentes poblaciones (187,188), incluida la cohorte InCHIANTI, analizada en el estudio de Tanaka et al. (189), sin embargo, la DM considera la alta ingesta de productos lácteos como un alimento "perjudicial", recomendando un consumo moderado de este tipo de alimentos (2-3 raciones/día) (94). Por último, una reciente revisión sistemática sugiere que el consumo de productos lácteos en personas mayores podría reducir el riesgo de fragilidad y, sarcopenia (190) , y que la adición de proteínas lácteas ricas en nutrientes a la dieta habitual, por ejemplo, el queso ricotta, también puede reducir el riesgo de sarcopenia al mejorar la masa muscular esquelética, contribuyendo con un adecuado aporte de energía, proteínas, calcio, fósforo, otros bioactivos, además de contribuir a una mejor biodisponibilidad de estos nutrientes (191,192).

6. CONCLUSIONES

CONCLUSIONES

Se presentan las principales conclusiones de esta tesis doctoral, para cada uno de los objetivos planteados:

Objetivos	**Conclusiones**
1. Evaluar la utilización y combinación de cuestionarios dietéticos y de biomarcadores nutricionales para la óptima determinación de la exposición dietética	1) La combinación de biomarcadores nutricionales con métodos tradicionales de valoración dietética mediante cuestionarios se puede considerar una óptima estrategia para afrontar la complejidad del estudio de la huella dietética en la asociación con la salud.
2. Revisar la heterogeneidad de las fuentes de información, así como la contribución específica de las diferentes fuentes dietéticas para valorar la evidencia científica actual sobre el efecto de la ingesta de polifenoles y su impacto en la salud humana.	2.a) La gran variabilidad en términos de metodología para la evaluación y cuantificación de la ingesta de polifenoles, dificulta el establecimiento de recomendaciones dietéticas para polifenoles totales. No obstante, los flavonoides se han asociado con un menor riesgo de diabetes, eventos cardiovasculares y mortalidad total. 2.b. Alimentos como el té, café, vino tinto, frutas y verduras son las principales fuentes dietéticas de polifenoles en nuestra dieta.
3.a. Revisar la literatura científica reciente sobre el rol de la alimentación en la composición de la microbiota y la permeabilidad intestinal, utilizando un enfoque metabolómico centrándose en las vías moleculares involucradas, a partir de estudios in	3.a) El mantenimiento de hábitos dietéticos saludables basados en un consumo regular de alimentos ricos en polifenoles y fibra dietética que provienen principalmente de fuentes alimentarias de origen vegetal como las cereales, frutos secos, frutas y verduras, así como los alimentos de origen animal, productos lácteos, huevos y carne (alimentos ricos en triptófano) son precursores de ácidos grasos de cadena corta, índoles y derivados fenólicos que favorecen el óptimo mantenimiento de la salud intestinal. La integración entre la composición de alimentos, la microbiota y los compuestos derivados de la microbiota intestinal con origen en los alimentos, permite el estudio de la funcionalidad de la barrera intestinal y

vivo e in vivo en humanos.

de las vías metabólicas involucradas en dicha actividad, aunque todavía se necesitan más esfuerzos para asociar estos tres conceptos.

3.b. Estudiar la idoneidad de la recomendación de la ingesta de polifenoles para el mantenimiento de la permeabilidad intestinal y fortalecimiento de la salud intestinal.

3.b) El conocimiento de los metabolitos y de las vías moleculares implicadas en el mantenimiento de la integridad intestinal representarán nuevos instrumentos para la evaluación de la salud intestinal y el desarrollo de planes dietéticos dirigidos a la gestión y prevención de enfermedades directamente relacionadas con un aumento de la permeabilidad intestinal, como inflamación crónica y trastornos inmunológicos, que son un factor determinante para el deterioro gradual de la salud en las personas mayores.

4.a Estimar la ingesta dietética real de polifenoles en población de edad avanzada institucionalizada en residencias de larga estadía.

4.a.i) El análisis del consumo real, medido utilizando diarios de alimentos por pesada, permitió demostrar una menor ingesta de nutrientes (~ 20%) y polifenoles (~ 15%) en comparación con la proporcionada por los menús.

4.a.ii) Una dieta rica en compuestos polifenólicos se puede conseguir con una ingesta real de 770 mg al día de dichos compuestos teniendo unas mínimas variaciones estacionales.

4.a.iii) El estudio del consumo real de polifenoles mediate técnicas precias que así lo permitan es una herramienta indispensable para valorar el impacto de este tipo de intervenciones en la salud de las personas mayores.

4.b. Estudiar la eficacia de dicha intervención dietética rica en compuestos polifenólicos mediante el estudio del perfil metabólico en orina valorando el efecto en el mantenimiento de la integridad intestinal y el efecto de la variación interindividual.

4.b.i) Los metabolitos de fase II y aquellos derivados de la microbiota intestinal son los que varían significativamente entre sujetos con una barrera intestinal integra y sujetos con una mayor alteración de la IP.

4.b.ii) La modulación de la biodisponibilidad de los compuestos polifenólicos dependiente de la permeabilidad intestinal podría atribuirse a alteraciones en el metabolismo microbiano intestinal y en los procesos de metilación de fase II.

4.b.iii) Los metabolitos derivados de la microbiota intestinal podrían ser en gran parte responsables de la actividad biológica provocada por los polifenoles de la dieta contra la alteración de la permeabilidad intestinal relacionada con la edad.

5. Estudiar el efecto de los polifenoles específicos como moduladores potenciales de la permeabilidad intestinal y sus posibles mecanismos de acción: *5.a. Estudiar el efecto de un patrón dietético rico en polifenoles en el mantenimiento de la integridad de la barrera intestinal mediante la medición de la zonulina sérica, considerado un marcador indirecto de la permeabilidad intestinal* *5.b. Valorar el potencial beneficio de una intervención dietética con productos ricos en polifenoles en la salud de la microbiota intestinal, los marcadores bioquímicos y clínicos asociados con el aumento de la permeabilidad intestinal en personas mayores.*	5.a) Los datos obtenidos como resultado del estudio de intervención dietética, muestran por primera vez la viabilidad y eficacia de que un patrón dietético rico en polifenoles durante 8 semanas, aporte que puede reducir los niveles séricos de zonulina (marcador indirecto de permeabilidad intestinal). 5.b) La intervención con un patrón de dieta rico en polifenoles produjo efectos beneficiosos a los voluntarios, provocando una reducción de la presión arterial, así como un aumento de las bacterias fermentadoras de fibra y productoras de butirato. Estos hallazgos son novedosos y pueden representar un avance inicial para estudios de intervención adicionales que evalúen posibles tratamientos dietéticos para el manejo de la PI, la inflamación y la función intestinal en diferentes poblaciones diana.
6. Desarrollar un panel de biomarcadores dietéticos basado en grupos de alimentos clave de la Dieta mediterránea en la población del estudio InCHIANTI e investigar su asociación a	6.a) Una mayor adherencia a un patrón de dieta mediterráneo al tiempo basal evaluada por una puntuación de biomarcadores dietéticos se asoció con un menor riesgo de mortalidad en adultos mayores durante un seguimiento de 20 años. 6.b) La medición de los biomarcadores dietéticos pueden complementar la evaluación de la ingesta dietética realizada mediante cuestionarios dietéticos y de esta manera podrían contribuir a orientar el asesoramiento dietético individualizado en personas mayores.

largo plazo con la mortalidad total y por causas específicas como ECV y cáncer.	
7. Evaluar las asociaciones a largo plazo de la ingesta de proteínas animales y vegetales con la mortalidad por todas las causas y por causas específicas en la cohorte de personas mayores del estudio InCHIANTI.	7. Una mayor ingesta de proteína animal en adultos mayores se asoció con un menor riesgo de mortalidad por todas las causas y mortalidad cardiovascular. Las diferencias en las asociaciones a largo plazo entre la ingesta de proteínas animales o vegetales y la mortalidad deberían ser consideradas en las futuras recomendaciones dietéticas dirigidas a las personas mayores.
8. Analizar la asociación entre la ingesta habitual de productos lácteos y el riesgo de fragilidad en adultos mayores de la cohorte InCHIANTI.	8. Es poco probable que los productos lácteos jueguen un papel predominante en el desarrollo de la fragilidad en una población italiana de edad avanzada. No obstante, los productos lácteos son una buena opción alimentaria práctica y conveniente. para las personas mayores. Por lo tanto, es aconsejable mantener un consumo moderado siguiendo las recomendaciones de un patrón dietético saludable.

CONCLUSIONS

The main conclusions of this doctoral thesis are presented for each of the objectives set:

Objectives	**Conclusions**
1. To evaluate the use and combination of dietary questionnaires and nutritional biomarkers for optimal determination of dietary exposure.	1. The combination of nutritional biomarkers with traditional methods of dietary assessment using questionnaires can be considered an optimal strategy to address the complexity of the study of the dietary footprint in association with health.
2. To review the heterogeneity of information sources and the specific contribution of different dietary sources in assessing the current scientific evidence on the effect of polyphenol intake and its impact on human health.	2.a) The great variability in terms of methodology for the assessment and quantification of polyphenol intake makes it difficult to establish dietary recommendations for total polyphenols. Nevertheless, flavonoids have been associated with a lower risk of diabetes, cardiovascular events and total mortality. 2.b) Foods such as tea, coffee, red wine, fruits and vegetables are the main dietary sources of polyphenols in our diet.
3.a To review the latest scientific literature on the role of diet on microbiota composition and intestinal permeability, using a metabolomic approach focusing on the molecular pathways involved, from in vivo and in vivo studies in humans.	3.a) The maintenance of healthy dietary habits based on regular consumption of foods rich in polyphenols and dietary fibre that come mainly from plant-based food sources such as cereals, nuts, fruits and vegetables, as well as foods of animal origin, dairy products, eggs and meat (foods rich in tryptophan) are precursors of short-chain fatty acids, indoles and phenolic derivatives that favour the optimal maintenance of intestinal health. The integration between food composition, microbiota and gut microbiota-derived compounds of food origin allows the study of the functionality of the gut barrier and the metabolic pathways involved in this activity, although further efforts are still needed to link these three concepts.
3.b. To study the suitability of the recommendation of polyphenol intake for the maintenance of intestinal permeability and the associated strengthening of intestinal health.	3.b) Knowledge of the metabolites and molecular pathways involved in the maintenance of gut integrity will represent new tools for the assessment of gut health and the development of dietary plans aimed at the management and prevention of diseases directly related to increased intestinal permeability,

such as chronic inflammation and immune disorders, which are a determining factor in the gradual deterioration of health in the elderly.

4.a To estimate the actual dietary intake of polyphenols in the elderly population institutionalized in long-stay homes.

4.a.i) Analysis of actual intake, measured using food diaries per weighing, demonstrated a lower intake of nutrients (~20%) and polyphenols (~15%) compared to that provided by the menus.
4.a.ii) A diet rich in polyphenolic compounds can be achieved with an actual intake of 770 mg per day of polyphenolic compounds with minimal seasonal variations.
4.a.iii) The study of actual polyphenol intake using appropriate techniques is an essential tool for assessing the impact of this type of intervention on the health of older people.

4.b. To study the efficacy of such a dietary intervention rich in polyphenolic compounds by studying the metabolic profile in urine to assess the effect on the maintenance of intestinal integrity and the effect of inter-individual variation.

4.b.i) Phase II metabolites and those derived from the gut microbiota are the ones that vary significantly between subjects with an integrated gut barrier and subjects with a more impaired IP.
4.b.ii) Intestinal permeability-dependent modulation of the bioavailability of polyphenolic compounds could be attributed to alterations in intestinal microbial metabolism and phase II methylation processes.
4.b.iii) Metabolites derived from the gut microbiota could be largely responsible for the biological activity of dietary polyphenols against age-related alterations in intestinal permeability.

5. To study the effect of specific polyphenols as potential modulators of intestinal permeability and their possible mechanisms of action:
5.a. To study the effect of a polyphenol-rich dietary pattern on the maintenance of intestinal barrier integrity by measuring serum zonulin, considered an indirect marker of intestinal permeability.

5.a) The data obtained as a result of the dietary intervention study show for the first time the feasibility and efficacy of a polyphenol-rich dietary pattern for 8 weeks, which can reduce serum levels of zonulin (indirect marker of intestinal permeability).
5.b) Intervention with a polyphenol-rich dietary pattern produced beneficial effects in volunteers, leading to a reduction in blood pressure, as well as an increase in fibre-fermenting and butyrate-producing bacteria. These findings are novel and may represent an initial step forward for further intervention studies

5.b. To assess the potential benefit of a dietary intervention with polyphenol-rich products on gut microbiota health, biochemical and clinical markers associated with increased intestinal permeability in the elderly.	to evaluate potential dietary treatments for the management of IP, inflammation, and gut function in different target populations.
6. To develop a panel of dietary biomarkers based on key food groups of the Mediterranean Diet in the InCHIANTI study population and investigate their long-term association with total and cause-specific mortality such as CVD and cancer.	6.a) Greater adherence to a Mediterranean dietary pattern at baseline as assessed by a dietary biomarker score was associated with a lower risk of mortality in older adults during a 20-year follow-up. 6.b) Measurement of dietary biomarkers may complement the assessment of dietary intake by dietary questionnaires and thus could help to guide individualised dietary advice in older people.
7. To assess the long-term associations of animal and plant protein intake with all-cause and cause-specific mortality in the InCHIANTI study cohort of older people.	7) Higher animal protein intake in older adults was associated with a lower risk of all-cause mortality and cardiovascular mortality. Differences in long-term associations between animal or plant protein intake and mortality should be considered in future dietary recommendations for older people.
8. To analyse the association between regular dairy intake and frailty risk in older adults in the InCHIANTI cohort.	8) Dairy products are unlikely to play a predominant role in the development of frailty in an elderly Italian population. Nevertheless, dairy products are a good, practical and convenient food option for older people. Therefore, it is advisable to maintain a moderate consumption following the recommendations of a healthy dietary pattern.

7. REFERENCIAS

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8. ANEXOS

ANEXOS

Publicaciones relacionadas al proyecto MaPLE

MANUSCRITO Anexo 1

Crosstalk among intestinal barrier, gut microbiota and serum metabolome after a polyphenol-rich diet in older subjects with "leaky gut": The MaPLE trial.

Gregorio Peron, Giorgio Gargari, Tomás Meroño, Antonio Miñarro, Pol Castellano Escuder, Raúl González-Domínguez, **Nicole Hidalgo Liberona,** Cristian Del Bo', Stefano Bernardi, Paul Antony Kroon, Barbara Carrieri, Antonio Cherubini, Patrizia Riso, Simone Guglielmetti, and Cristina Andres-Lacueva

Clinical Nutrition

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MANUSCRITO Anexo 1

Objetivo: Estudiar los cambios en el metaboloma del suero en el ensayo MaPLE, como un paso más necesario para describir la compleja diafonía entre los polifenoles de la dieta, el GM y la barrera intestinal.

Métodos: El metaboloma del suero fue monitoreado usando un análisis UHPLC-MS/MS semi-dirigido. Se realizó un análisis metatexonómico (perfil del gen 16S rRNA) de los MG en muestras fecales. Las características clínicas y los niveles séricos del marcador IP zonulina se relacionaron con los datos de la MG y la metabolómica en una red multiómica.

Resultados

En comparación con la dieta de control, la dieta PR aumentó los metabolitos séricos relacionados con los polifenoles y la ingesta de metilxantina. La teobromina y las metilxantinas, derivadas del cacao y/o del té verde, se correlacionaron positivamente con las bacterias productoras de butirato (el orden Clostridiales y los géneros Roseburia, Butyricicoccus y Faecalibacterium) e inversamente con la zonulina. También se observó una correlación directa entre los metabolitos de polifenoles ácido-sulfato hidroxifenilpropiónico, sulfato de 2-metilpirogalol y sulfato de catecol con Butyricicoccus, mientras que el ácido-sulfato hidroxifenilpropiónico y el sulfato de 2-metilpirogalol se correlacionaron negativamente con Methanobrevibacter. La red multiómica indicó que la edad de los participantes, los niveles basales de zonulina y los cambios en la abundancia de Porphyromonadaceae fueron los principales factores que impulsaron los efectos de una dieta de PR sobre la zonulina.

Conclusión

En general, estos resultados revelan las complejas relaciones entre el consumo de polifenoles, la permeabilidad intestinal y la composición GM en los adultos mayores, y pueden ser importantes a la hora de establecer intervenciones dietéticas personalizadas para los adultos mayores.

Número de registro del ensayo: ISRCTN10214981.

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Original article

Crosstalk among intestinal barrier, gut microbiota and serum metabolome after a polyphenol-rich diet in older subjects with "leaky gut": The MaPLE trial

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SUMMARY

Background & aim: The MaPLE study was a randomized, controlled, crossover trial involving adults ≥60 y.o. (n = 51) living in a residential care facility during an 8-week polyphenol-rich (PR)-diet. Results from the MaPLE trial showed that the PR-diet reduced the intestinal permeability (IP) in older adults by inducing changes to gut microbiota (GM). The present work aimed at studying the changes in serum metabolome in the MaPLE trial, as a further necessary step to depict the complex crosstalk between dietary polyphenols, GM, and intestinal barrier.

Methods: Serum metabolome was monitored using a semi-targeted UHPLC-MS/MS analysis. Metataxonomic analysis (16S rRNA gene profiling) of GM was performed on faecal samples. Clinical characteristics and serum levels of the IP marker zonulin were linked to GM and metabolomics data in a multi-omics network.

Results: Compared to the control diet, the PR-diet increased serum metabolites related to polyphenols and methylxanthine intake. Theobromine and methylxanthines, derived from cocoa and/or green tea, were positively correlated with butyrate-producing bacteria (the order Clostridiales and the genera *Roseburia*, *Butyricicoccus* and *Faecalibacterium*) and inversely with zonulin. A direct correlation between polyphenol metabolites hydroxyphenylpropionic acid-sulfate, 2-methylpyrogallol-sulfate and catechol-sulfate with *Butyricicoccus* was also observed, while hydroxyphenylpropionic acid-sulfate and 2-methylpyrogallol-sulfate negatively correlated with *Methanobrevibacter*. The multi-omics network indicated that participant's age, baseline zonulin levels, and changes in Porphyromonadaceae abundance were the main factors driving the effects of a PR-diet on zonulin.

Conclusion: Overall, these results reveal the complex relationships among polyphenols consumption, intestinal permeability, and GM composition in older adults, and they may be important when setting personalized dietary interventions for older adults.

Trial registration number: ISRCTN10214981.

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1. Introduction

Increased intestinal permeability (IP), a condition also known as "leaky gut", has been proposed as a potential contributor to inflamm-aging and a wide range of intestinal disorders such as inflammatory bowel disease, coeliac disease, and Crohn's disease, as well as several chronic diseases such as cardio-renal-metabolic diseases [1,2]. Increased IP is characterized by a low-grade systemic inflammation triggered by the diffusion of toxins or bacterial factors to the bloodstream [3].

Age has been reported as an independent risk factor for altered IP, and some studies have shown an increased IP over the age of 50 [4]. Moreover, gut microbiota (GM) is another regulator of IP implicated in the renovation of the intestinal epithelial cells and in maintaining the integrity of tight junctions [5]. Indeed, a detrimental modification of the microbial community structure in the gut (dysbiosis) can lead to a loss of immune tolerance and to the development of a gut inflammatory environment coupled with increased IP [6]. In consequence, dysbiosis and/or altered IP may not only lead to the overproduction and absorption of toxic metabolites with potential deleterious effects on host-health [7–9], but also compromise the bioavailability of nutrients and beneficial food components, such as polyphenols [10].

Among the strategies to prevent "leaky gut" and to decrease IP associated with aging or chronic diseases, changes of lifestyle factors, including diet, should be the most feasible [11]. Higher consumption of fruits, vegetables and other plant-based foods provides dietary fibre and polyphenols which might help to counteract an impairment of IP through aging [12,13]. In addition, GM activity may improve the structure of the tight junctions and modulate the inflammatory environment in the gut through lower molecular weight compounds derived from food components [14,15].

In the MaPLE trial, we found that an 8-week polyphenol-rich (PR) diet comprising 3 daily portions of PR-foods such as cocoa, green tea and berries (1391 mg/day of dietary polyphenols *vs.* 812 mg/day registered in the control diet) led to a significant reduction of the IP marker, zonulin, in older subjects affected by "leaky gut" [16]. Thus, our primary hypothesis is that serum metabolome changes will be associated with the improvement of IP in older adults through GM-dependent and GM-independent pathways. Here, we also investigated the crosstalk between intestinal barrier and the changes of GM composition and serum metabolome linking clinical characteristics to metataxonomics and metabolomics data through a multi-omics network using Mixed Graphical Models.

2. Materials and methods

2.1. Recruitment of the volunteers and assessment of the dietary protocol

The trial was carried out at Civitas Vitae (OIC Foundation, Padua, Italy) in residential care and independent residences for older subjects, as previously described [16]. To be included in the trial, the subjects had to be ≥ 60 years old and with increased IP, evaluated by means of serum zonulin level. Other inclusion and exclusion criteria were already reported in [16] and the dietary intervention protocol has been previously published [17]. Briefly, the PR dietary pattern was designed by the substitution of some low-polyphenol products in the control diet with other comparable products but high in polyphenols, i.e. PR-products (e.g. foods used for snack or breakfast), while maintaining as much as possible the overall energy and nutrient composition. Specifically, subjects consumed 3 portions/day of selected PR-products including berries and related products, blood orange and juice, pomegranate juice, green tea, Renetta apple and purée, and dark chocolate (callets and cocoa powder-based drink), providing a mean of 724 mg/day of total polyphenols as estimated by Folin-Ciocalteu analysis. Mean total polyphenol intake was 1391 mg/day in the PR-diet *vs.* 812 mg/day in the control diet.

The study protocol complied with the principles of the Declaration of Helsinki, and was approved by the Ethics Committee of the University of Milan, Italy (ref: 6/16/CE_15.02.16_Verbale_All-7). All subjects and their relatives were informed about the study protocol and they signed an informed consent before the enrolment. The trial was registered under the code: ISRCTN10214981 [17].

2.2. Experimental design

The study consisted of an 8-week, randomized, cross-over intervention trial (PR-diet *vs.* control diet). Volunteers were randomly allocated in one of the two arms of the trial, starting with PR-diet or control diet according to a computerized randomization protocol. Subjects assigned to the PR-diet received the three daily portions of selected PR-foods described before. During the control diet period, subjects followed the regular menu provided by the nursing home, which was previously evaluated for the nutritional and polyphenol composition. After the wash-out period (8 weeks) performed to avoid any carry-over effect, the groups were switched to the other diet.

At four time-points during the trial (visits 1–4), corresponding to the beginning and the end of each intervention period, all participants underwent to physical and general condition examinations (e.g. weight, blood pressure and clinical signs), as already described [16], and blood and faecal samples were collected.

2.3. Dietary assessment

The dietary assessment was performed by weighed food records. Three records per subject were obtained during each intervention period. Energy, macro and micronutrients intakes were estimated with MetaDieta® software (Me.Te.Da S.r.l., San Benedetto del Tronto, Italy) [16]. Total polyphenol estimation was performed using the Phenol Explorer database (phenol-explorer.eu) to provide estimates of polyphenol concentrations in each food. In the cases that no useful values, using our proprietary data or values obtained from the literature, total polyphenol content of the foods was estimated directly using the Folin-Ciocalteau method as described in [17].

2.4. Blood sampling and biomarkers analysis

After an overnight fast, blood samples were drawn in Vacutainer tubes containing silica gel for serum separation. Serum was obtained by tube centrifugation at 1400 g and 4 °C for 15 min, divided in small aliquots into labelled vials and stored at −80 °C until analysis.

Anthropometric, metabolic and functional parameters were measured as previously described [17]. Serum zonulin levels were quantified using a specific ELISA kit as previously reported [17].

2.5. Sample preparation and UHPLC-MS/MS metabolomics analysis of serum

Serum samples were prepared for UHPLC-MS/MS analysis by a simple protein precipitation protocol. After thawing on ice, 100 µL of serum were added of 500 µL ACN containing 1.5% v/v formic acid and 10 mM of ammonium formiate. Samples were mixed and kept at −20 °C for 10 min, then centrifuged at 10,000 rpm and 4 °C for 10 min. After protein precipitation, 500 µL of supernatant were dried on vacuum and the residue was recovered with 100 µL of an 80/20 v/

v mixture of water/ACN, containing 0.5% v/v formic acid, 10 mM of ammonium formiate and 100 ppb of a mixture of 13 internal standards (glutamic acid ^{15}N, phenylalanine ^{15}N, urea d_4, acetyl-L-carnitine d_3, myristoyl-L-carnitine d_9, caffeine $^{13}C_3$, glucose d_2, palmitic acid d_{31}, succinic acid d_4, glycocholic acid ^{13}C, ferulic acid, $^{13}C_3$, epicatechin $^{13}C_3$, and taxifolin, Sigma–Aldrich, Steinheim, Germany). Finally, after centrifugation at 10,000 rpm and 4 °C for 5 min, samples were transferred to a 96-well plate and analysed using the targeted UHPLC-MS/MS method described in [18]. Briefly, an Agilent 1290 Infinity UHPLC system coupled to a Sciex QTRAP 6500 mass spectrometer was used for the analysis. A Phenomenex Luna Omega Polar C18 column (100 mm × 2.1 mm, 1.6 μm) equipped with a fully porous polar C18 security guard cartridge was used as stationary phase. Chromatographic conditions were as follows: column temperature, 40 °C; autosampler temperature, 4 °C; injection volume, 2 μL; flow rate, 0.5 mL/min. In the negative ion mode, the mobile phase consisted in a gradient of 0.1% formic acid and 10 mM ammonium formate in water (A) and pure ACN (B). The gradient program was: 0–8 min, 5–20% B; 8–10 min, 20–100% B; 10–12 min, 100% B; 12–12.1 min, 100-5% B; 12.1–14 min, 5% B. On the other hand, in positive ion mode water and ACN, both containing 0.5% formic acid, were used as mobile phases A and B, respectively. In this case, the gradient profile was: 0–5 min, 5–50% B; 5–8 min, 50–100% B; 8–10 min, 100% B; 10–10.1 min, 100-5% B; 10.1–12 min, 5% B. MS detection was performed by using the scheduled multiple reaction monitoring (sMRM) mode. The mass spectrometer operated in positive and negative ionization modes in separate runs, using the following parameters: ion spray voltage, +4500/-3500 V; source temperature, 600 °C; curtain gas, 30 psi; ion source gas 1 and gas 2, 50 psi each; collision-activated dissociation gas, 3 psi; entrance potential, ±10 V; target scan time, 0.05 s.

Analyst 1.6.2 and Sciex OS software by Sciex were used for data acquisition and data processing, respectively.

The quality control of metabolomics data was performed using the POMA R/Bioconductor package (https://github.com/pcastellanoescuder/POMA) [48]. Data pre-processing included the removal of metabolites with more than 80% missing values in all the study groups [19], the imputation of the remaining missing values using the KNN algorithm, and finally data normalization by means of *log* transformation and Pareto scaling. Afterwards, distances to the group centroid were computed based on Euclidean distances to remove outliers from the data matrix (±1.5 × IQR). Finally, the coefficients of variation for areas, retention times and peak widths of the internal standards added to samples were calculated for analytical reproducibility assessment.

2.6. Metataxonomics of faecal samples

The bacterial community structure of faecal samples was assessed as described [17]. In brief, DNA was isolated from faces resuspended in Lysing Matrix E bead beating tubes (MP Biomedicals, Santa Ana, CA, USA) through the FastDNA™ SPIN Kit for Soil (MP Biomedicals) according to the manufacturer's protocol. Then, the V3–V4 region of the 16S rRNA gene was amplified with panbacterial primers 16S 341F (5′– TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3′) and 16S 806R (5′–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3′). Finally, amplicons were sequenced using an Illumina MiSeq sequencer (Illumina Inc, San Diego, CA, USA) using a 600 cycle MiSeq v3 reagent kit. Pairing, filtering, taxonomic assignment, and biodiversity analyses of sequencing reads were carried out by means of the bioinformatic pipeline Quantitative Insights Into Microbial Ecology (QIIME) 2 [20] through the Divisive Amplicon Denoising Algorithm (DADA2) using the Greengenes database (version 13_5). Illumina sequencing generated 4,030,722 filtered paired-end reads (median of 19,328 reads per sample). Bioinformatic analysis was conducted using DADA2; reads were merged, and denoised. Reads were dereplicated and singletons removed. After merging and denoising by DADA2 the final sequences were 1,076,356 (mean = 5,021, SD = 3,306). The sequence length statistics in bp showed: min = 240, max = 457, median = 433, standard deviation = 25. Overall, 7,729 unique amplicon sequence variants (ASVs) were identified [16]. Sequencing data have been deposited as FASTQ files in the European Nucleotide Archive (ENA) of the European Bioinformatics Institute under accession code PRJEB46689.

2.7. Statistical analyses

For each period, the diet effect on clinical and metabolomics data were estimated as the change between the "end of dietary intervention − baseline" measurements. For metabolomics data, after exclusion of the metabolites with more than 80% of missing values, they were imputed using the K-nearest neighbour method, log-transformed and Pareto scaled. Afterwards, dietary intervention effects on normalized metabolite concentrations were compared using a subject-specific random effect linear mixed model using baseline metabolite concentrations (at visit 1 and 3), period, diet, period × diet interaction, age, sex and body mass index (BMI) as fixed factors or covariates. P-values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR).

Correlations between the changes of zonulin levels and plasma metabolites and macro and micronutrients significantly altered by the PR-diet were performed using partial correlation tests accounting for baseline zonulin during the PR-diet intervention. Next, stepwise linear multiple regression was used to assess for significant predictors in a model that included age, sex, period, and metabolites and macro and micronutrients significantly correlated with zonulin changes during PR-diet intervention. Residuals were checked for normality using the Shapiro–Wilk test.

Correlation analyses (Spearman and Kendal test) were carried out between the normalized relative abundance of bacterial taxa and metabolites significantly affected by the PR-diet using median data from the trial time-points.

For the integration of metataxonomics and metabolomics data we used a Mixed Graphical Model (MGM). MGMs are undirected probabilistic graphical models, where each node corresponds to one variable, and the edges between two nodes represent a conditional dependency between them given all other variables in the graphical model [21]. We have used 'mgm' R-package to estimate the network of dependencies [22]. Specifications were set to allow the maximum number of interactions in the network. Variables in the model were changes in zonulin levels during each intervention period, baseline zonulin, age, sex, BMI and randomized allocation sequence, metabolomic set of variables (change in metabolites affected by the PR intervention), and metataxonomic variables (changes in relative abundances of all the analysed gut bacteria at the taxonomic level of family). A subsequent model included also data on macro and micronutrients intake that differed between the PR- and control diets.

All statistical analyses were performed using IBM SPSS Statistics 25 (IBM, USA) and R version 4.0.5 (R foundation, Austria).

3. Results

3.1. Trial outcomes

The baseline clinical parameters of the participants, the intake of macro and micronutrients during the PR- and control diets, changes in GM composition, and the main results of the trial have

already been published [16]. Briefly, fifty-one participants (22 men and 29 women) with a mean age of 78 ± 10 years completed the two, 8-weeks, dietary intervention periods. The intake of energy, total protein, saturated fatty acids, total ω-3 fatty acids, cholesterol, B vitamins (B1, B6, B12), vitamin E, and folates was similar between the PR- and control diet. During the PR-diet participants showed a statistically significant lower intake of animal and plant protein (−6% and −7%, respectively), total lipids (−5%), monounsaturated fatty acids (MUFA, −7%), polyunsaturated fatty acids (PUFA, −20%), total ω-6 fatty acids (−23%), calcium (−16%), and iron (−7%) than during the control diet. Conversely, significantly higher intakes of total carbohydrates (+5%), total dietary fibre (+6%), vitamin C (+15%), and total polyphenols (+71%) were reported during the PR-*vs.* the control diet. The overall bacterial community structure (α- and β-diversity) of faecal microbiota did not change significantly during the PR-diet in comparison to the control one (Supplementary Fig. S2); nonetheless, the following shifts in specific bacterial taxa were observed: i) relative reduction in: *Bacteroides uniformis* (−63%), and *Streptococcus agalactiae* (−83%); and ii) relative increase in: *Alistipes onderdonkii* (+300%), *Anaerobutyricum hallii* (+216%), *Faecalibacterium prausnitzii* (+100%), and bacterial members of the genera *Lactonifactor* (+38%) and *Butyricicoccus* (+59%). Zonulin levels were significantly reduced (−9%, Supplementary Fig. S1) by the PR-diet in comparison to the control diet.

3.2. Variation of serum metabolome induced by the PR-diet intervention

Using a targeted UHPLC-MS/MS approach, the effects of the PR-diet intervention on the serum metabolome of the 51 participants was evaluated. As shown in Fig. 1 and Table 1, ten metabolites were significantly associated to the effects of the PR-diet intervention on serum composition. Among these, catechol sulfate (CAT-S), hippuric acid (HA), 2-methylpyrogallol sulfate (2-MePyr-S), and hydroxyphenylpropionic acid sulfate (HPPA-S) were increased in serum following PR-diet and could be considered as markers of polyphenols intake and subsequent degradation by gut microbiota [23–26], followed by phase II metabolism in the liver. A brief description of these metabolites is reported in the Supplementary Material. Theobromine (TB), also increased in serum after the PR-diet, derived from the consumption of cocoa during the intervention period or from the metabolism of theophylline from green tea [27], as well as 3-methylxanthine (3-MX) and 7-methylxanthine (7-MX) [28].

The serum levels of asparagine, deoxycarnitine and hydroxyhexanoylcarnitine decreased after the PR-diet intervention. Notably, the lowered levels of deoxycarnitine could be associated with the effects of the PR-diet on the IP, considering that a positive correlation between circulating deoxycarnitine and IP has been previously reported [29].

As shown in the heatmap reported in Fig. 2, a positive correlation was observed between the changes in the two methylxanthine metabolites and its parent metabolite, TB. Several other markers of PR-food ingestion were positively correlated with each other, as an indication of the relationship between the ingestion of PR-foods and the production of lower molecular weight derivatives by GM [23]. Most importantly, the two methylxanthine metabolites of TB, 3-MX and 7-MX, were negatively correlated to serum levels of zonulin, expressed as the difference between the levels at the end and at the beginning of the PR-intervention period, respectively (Fig. 2). Correlation analysis using micro and macronutrients data showed an inverse correlation between changes in zonulin levels and the intake of PUFA, iron, and animal protein. Other correlations were observed between dietary data and the changes in the metabolites altered by the PR-diet, specially between calcium intake and hippuric acid and the two methylxanthines (Fig. 2). Multiple regression analysis showed that the increase of 7-MX ($\beta = -0.15$, $p = 0.003$), baseline zonulin levels ($\beta = -0.72$, $p < 0.001$), age ($\beta = 0.008$, $p = 0.024$), and iron intake ($\beta = -0.44$, $p = 0.040$) were significant predictors of the decrease of serum zonulin induced by the PR-diet ($R^2 = 0.52$).

3.3. Correlations between serum metabolome and gut microbiota composition

Significant correlations between plasma metabolites associated to the PR-diet and specific changes of bacterial taxa of the GM were observed (Fig. 3). Among the other metabolites, TB was the one showing more statistically significant correlations. Most importantly, plasma TB was positively correlated with dominant SCFAs-producing bacteria, such as the members of Clostridiales order, *Roseburia*, *Butyricicoccus* and *Faecalibacterium*, and with *Lactonifactor* (all $p < 0.0001$), a bacterial genus involved in the transformation of dietary plant lignin to enterolactone [30]. Statistically significant negative correlations were also observed between TB and potentially pathogenic bacterial genera, such as the *Methanobrevibacter* [31,32], and the members of Proteobacteria phylum (*Desulfovibrio* and Enterobacteriaceae) (all $p < 0.01$). Similarly, methylxanthine metabolites 3-MX and 7-MX showed positive correlations with *Butyricicoccus* and *Lactonifactor*, and negative correlations with the Bacteroidales order (Fig. 3).

Other statistically significant correlations were observed between the polyphenol metabolites HPPA-S, 2-MePyr-S and CAT-S with *Butyricicoccus* (positive correlations), and between HPPA-S and 2-MePyr-S with *Methanobrevibacter* (negative correlations) (Fig. 3).

3.4. Interdependent associations between changes in zonulin, serum metabolome and gut microbiota composition

The changes in zonulin levels during the PR-diet were directly related to age, to changes in serum levels of HA and CAT-S, and to the changes in the relative abundance of the bacterial families Porphyromonadaceae and Coriobacteriaceae in the gut (Fig. 4). In line with previous reports [16], changes in zonulin levels were inversely correlated with baseline zonulin, showing that the effects of the intervention were more evident among participants with an overt alteration in IP. Baseline zonulin was directly related to age, to changes in deoxycarnitine levels and to the relative abundance of Lachnospiraceae family. Conversely, inverse relationships were observed with changes in 7-MX, TB and CAT-S (Fig. 4). Altogether, the multi-omics network shows that changes in zonulin depended on three main factors: a) participant's age, which was also associated to different responses in GM composition (positively associated with changes in members of Lachnospiraceae, Enterobacteriaceae, Porphyromonadaceae and Coriobacteriaceae, while negatively with changes in members of the family Verrucomicrobiaceae), b) baseline zonulin levels, which was one of the main factors affecting serum metabolome changes, and c) changes in Porphyromonadaceae family abundance. Interdependency between these three factors illustrates the complexity of IP regulation, and it shows that changes in serum metabolome were related to changes in zonulin through its association with baseline zonulin and changes in GM composition (Lachnospiraceae, Coriobacteriaceae and Enterobacteriaceae). Several of these relationships were specific for the PR-diet period and were not present during the control diet

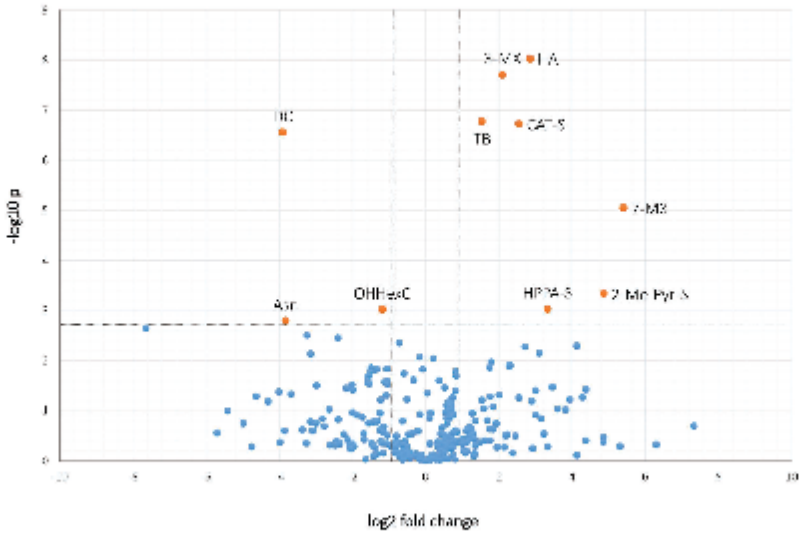

Fig 1. Volcano plot of log_2 (fold-change) (x-axis) *vs.* $-log_{10}$(FDR-corrected p-value) (y-axis), showing the metabolites significantly associated to the changes of serum metabolome induced by the PR-diet intervention in the whole MaPLE cohort (n = 51). The Fold Change value of each metabolite, reported as log_2(Fold Change), corresponds to the ratio between Δconcentration during the intervention period and Δconcentration during the control period. P-values were calculated by comparison of ΔControl *vs.* ΔPR values using linear mixed models with subject-specific random effects adjusted for age, sex, body mass index, baseline metabolite concentration, period and the period × diet interaction, followed by Benjamini-Hochberg correction for multiple comparisons. HA: hippuric acid; CAT-S: catechol sulfate; 3-MX: 3-methylxanthine; TB: theobromine; DC: deoxycarnitine; 7-MX: 7-methylxanthine; 2-MePyr-S: 2-methylpyrogallol sulfate; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; OHHexC: hydroxyhexanoylcarnitine; Asn: asparagine.

Table 1
Variables significantly (FDR-adjusted p-value <0.05) associated to the changes of serum metabolome induced by the PR-diet intervention in the whole MaPLE cohort (n = 51).

Name	Fold Change[a]	FDR-corrected p value	Origin (Classification)
Hippuric acid	+2.86	<0.0001	Exogenous (microbial polyphenol metabolite)
3-Methylxanthine	+2.40	<0.0001	Exogenous (theophylline metabolite)
Theobromine	+1.97	<0.0001	Exogenous (methylxanthine, from diet)
Catechol sulfate	+2.56	<0.0001	Exogenous (microbial polyphenol metabolite)
Deoxycarnitine	−1.05	<0.0001	Endogenous (carnitine)
7-Methylxanthine	+5.44	0.0004	Exogenous (theophylline metabolite)
2-Methylpyrogallol sulfate	+4.92	0.019	Exogenous (microbial polyphenol metabolite)
Hydroxyhexanoylcarnitine	−1.48	0.032	Endogenous (acylcarnitine)
HPPA-S	+3.47	0.032	Exogenous (microbial polyphenol metabolite)
Asparagine	−0.95	0.048	Endogenous (amino acid)

HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate.
[a] Fold Change corresponds to the ratio between Δ concentration during the PR-diet intervention period and Δconcentration during the control period. It is reported as log_2(Fold Change). Statistical analysis was carried out using linear mixed models with subject-specific random effects adjusted by age, sex, body mass index, baseline metabolite concentration, intervention period and the period × diet interaction, followed by Benjamini-Hochberg correction for multiple comparisons.

(Fig. 4), as for example the direct correlation between baseline zonulin and Lachnospiraceae, or the negative correlation between the changes in zonulin and Clostridiaceae. On the other hand, opposite correlations between nodes during the control and PR-diet periods could be observed, as in the case of the relationship between baseline zonulin and Enterobacterioceae, that in control diet was characterized by a direct association. Including the dietary data in the multi-omics network showed inverse associations between total dietary fibre and baseline zonulin, as well as between iron intake and changes in zonulin levels during the PR-dietary intervention (Supplementary Fig. S3). Furthermore, calcium intake showed a positive association with Porphyromonadaceae, only during the PR-diet. The association between total dietary fibre and baseline zonulin was common to both dietary intervention periods (Supplementary Fig. S3).

4. Discussion

The present study shows a determinant role of IP in the metabolomic changes related to the PR-diet intervention and the interdependent associations between changes in zonulin, serum metabolome and gut microbiota composition. These relationships were visualized in a multi-omics network integrating clinical

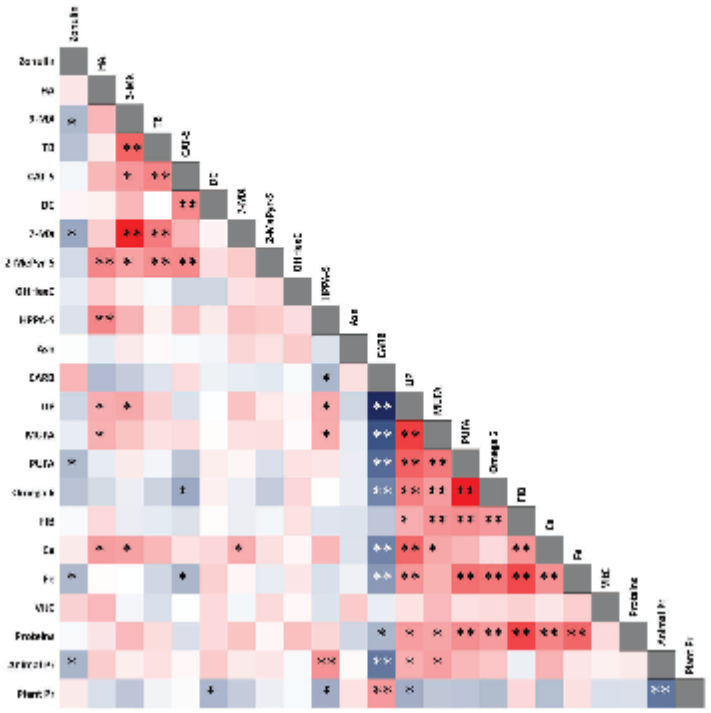

Fig. 2. Heatmap showing the correlations among serum zonulin, serum metabolites significantly altered by the PR-diet intervention, and the amounts of nutrients provided by the MaPLE diet during the trial, specifically: carbohydrates (CARB), lipids (LIP), mono-unsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA), omega-6 fatty acids, fibers (FIB), calcium (Ca), iron (Fe), vitamin C (VitC) and total, animal and plant proteins (Pr). HA: hippuric acid; 3-MX: 3-methylxanthine; TB: theobromine; CAT-S: catechol sulfate; DC: deoxycarnitine; 7-MX: 7-methylxanthine; 2-MePyr-S: 2-methylpyrogallol sulfate; OHHexC: hydroxyhexanoylcarnitine; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; Asn: asparagine. *: P-value < 0.05; **: P-value < 0.01.

characteristics, baseline zonulin levels, and metabolomic and metataxonomic data.

Metabolomics analysis of serum samples revealed that an 8-week PR-diet significantly altered the levels of 10 metabolites. Seven of these were exogenous, hence derived from the consumption of specific PR-foods or from the metabolism of their constituents by endogenous metabolic pathways or by the intestinal microbiota. Specifically, these were methylxanthine metabolites (TB, 3-MX, and 7-MX) and phenolic compounds derived from the degradation of dietary polyphenols by the GM (HA, CAT-S, HPPA-S and 2-MePyr-S). Among the endogenous metabolites, deoxycarnitine decreased after the PR-diet intervention. In the MGM network, the increase of CAT-S, 7-MX and TB and the decrease of deoxycarnitine levels were correlated with baseline zonulin, and not with its changes, showing that the effects of the intervention on these metabolites depended on higher extent on baseline IP. TB is the most abundant methylxanthine of cocoa, but it could be produced also by the gut microbiota upon the intake of food products containing caffeine, such as coffee or tea [27]. Recent studies show that TB could exert anti-tumour, anti-inflammatory and antioxidant activities, and act as a cardiovascular protector through the inhibition of phosphodiesterases and the blockade of adenosine receptors [33]. In healthy rats, a two-week administration of TB (from cocoa) was shown to induce significant alterations of the GM composition, reducing the proportion of the colitogenic IgA-coated bacteria, and to enhance butyric acid production [34]. The increase of butyrate-producing bacteria belonging to the families Lachnospiraceae and Ruminococcaceae has been already reported in the MaPLE trial, and here, positive correlations were observed between *Butyricicoccus*, *Roseburia*, and *Faecalibacterium* and TB levels. Methylxanthines are metabolites of theophylline and TB mainly formed by endogenous metabolism in the liver, and to lower extent in small intestine and colonic mucosa cells [28]. In the MaPLE trial, they could also be associated to the consumption of green tea and cocoa included in the dietary intervention. 7-MX was negatively correlated with the PR diet-induced variation of serum zonulin in correlation analysis, and with baseline zonulin in the multi-omics network. A possible mechanism behind this relationship could be the inhibition of the enzyme poly (ADP-ribose)polymerase-1 (PARP1) [35,36]. In previous studies on the depletion of this protein in mice, it has been observed that the PARP1 deficiency was associated with a modulation of the colonic microbiota, with increased relative abundance of clostridial clusters IV and XIVa, and a concomitant increase in the frequency of several mucosal regulatory T cells [37]. In another study, it was observed that the inhibition of PARP1, using 3-aminobenzamide, leaded to a decrease of the IP and a local anti-inflammatory effect in an animal model of colitis

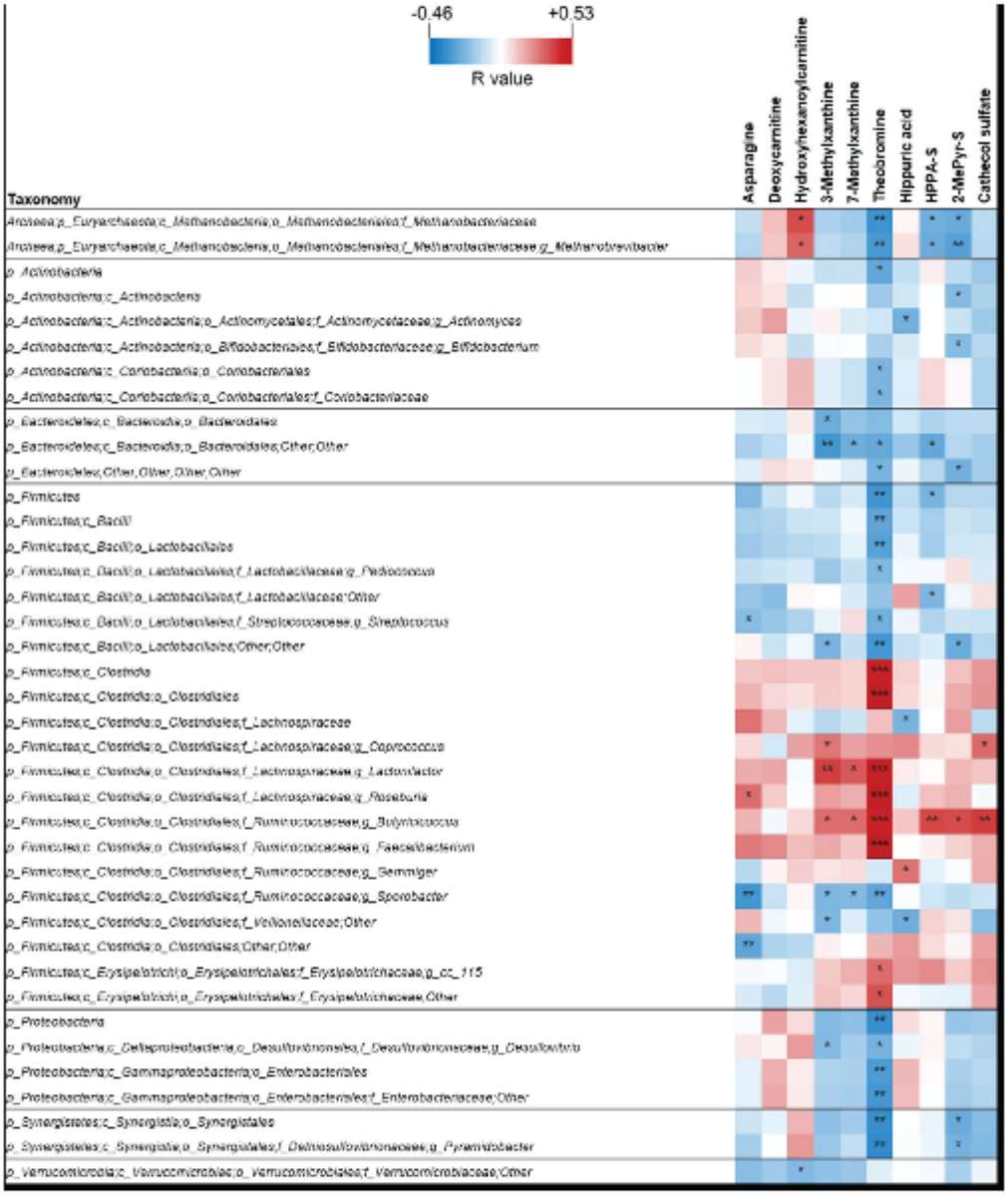

Fig. 3. Heatmap showing correlations between the median relative abundance bacterial taxa in the gut and median concentration of the metabolites that were altered in serum after the polyphenol-rich diet. The heatmap represents the R value of Spearman's correlation. Asterisks indicate the Kendall rank correlation. *p < 0.05; **p < 0.01; ***p < 0.001. The taxonomic lineage of each taxon is p: phylum; c: class; o: order; f: family; g: genus; s: species. HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate; 2-MePyr-S: 2-methylpyrogallol sulfate.

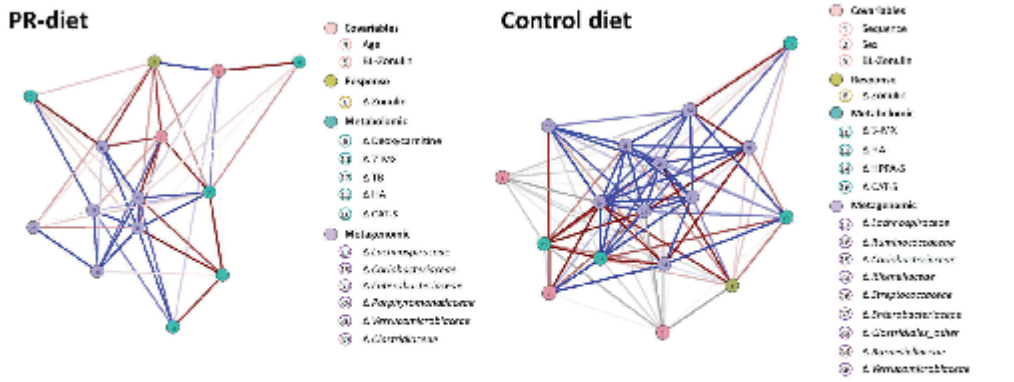

Fig. 4. Neighbourhood of the nodes "Δ Zonulin" and "Baseline Zonulin" during the polyphenol-rich (PR) and control diet. Edge intensity reflects the strength of an association from strong positive (dark red) to strong negative association (dark blue). The node colour indicates the type of data. Variables in the mixed graphical model were changes in zonulin levels (Δ Zonulin), baseline zonulin (BL-Zonulin), age, sex, BMI, and randomized allocation sequence, change in metabolites affected by the PR intervention, and metataxonomic variables (changes in relative abundances of all the analyzed gut bacteria at the taxonomic level of family). HA: hippuric acid; CAT-S: catechol sulfate; 3-MX: 3-methylxanthine; 7-MX: 7-methylxanthine; TB: theobromine; HPPA-S: 3-(3-hydroxyphenyl)propanoic acid sulfate. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[38]. Considering these preliminary results in literature, it could be hypothesized that 7-MX could have a role modulating the IP through PARP1 inhibition.

In the multi-omics network, while changes in 7-MX levels showed an inverse association with changes in Coriobacteriaceae and Enterobacteriaceae, changes in TB exhibited direct associations. Members of the Coriobacteriaceae family are considered as pathobionts, and *Collinsella* is its dominant taxon [39]. Gnotobiotic approaches have shown that administration of *Collinsella* reduces the expression of tight junction proteins in enterocytes and stimulates gut leakage [40]. On the other hand, Enterobacteriaceae are Gram-negative bacteria that produce the endotoxin lipopolysaccharide, hence they can induce inflammatory reactions in the gut promoting gut barrier disruption and chronic inflammation [41]. Thus, the negative association between 7-MX and these bacterial families could be implicated in the positive effects of cocoa or green tea consumption on GM. However, the inverse associations of TB and of 7-MX with these bacterial members in the multi-omics network are intriguing. One possible explanation could be that GM may modulate the metabolism of TB in colonic cells [42], hence limiting the absorption and posterior metabolism to 7-MX on one side, while promoting the degradation of TB to other metabolites (such as 5-acetylamino-6-amino-3-methyluracil and 3,7-dimethylurate) on the other side [42]. The latter would only be evidenced by determining faecal metabolomics.

Finally, older age, the low levels of zonulin at baseline, and the increase in the relative abundance of Porphyomonadaceae were the main factors limiting the reduction of IP during the PR-diet. The link between ageing and Porphyromonadaceae has been already reported in a cross-sectional study comparing centenarian *vs.* non-centenarian adults [43]. Regarding the relationship between Porphyromonadaceae and health outcomes, it was previously shown that, in a case–control study on patients with liver cirrhosis, poor cognitive performance in older adults was associated with increases of Porphyromonadaceae [44]. However, increased abundance of Porphyromonadaceae was part of a microbe signature in older adults with a better metabolic profile [45]. In animal models, a link between Porphyromonadaceae, gut inflammation and Th17 lymphocytes in the gut has been observed, where mice with colitis exhibited higher Porphyromonodaceae abundance [46]. Therefore, a contribution of Porphyromonadaceae to gut inflammation and other health outcomes can be hypothesized, but needs further experimental confirmation.

Other changes in bacterial taxa during the PR-diet (Lachnospiraceae, Coriobacteriaceae, and Enterobacterioceae) were interrelated in a complex pattern. However, these changes in GM were not directly associated with changes in zonulin. How the PR-diet specifically modulated the GM ecosystem leading to a reduced IP requires further studies. The inclusion of macro and micronutrients data in the analyses allowed the confirmation up to some extent of the positive effect of dietary fibre on intestinal barrier [12,13]. Furthermore, it suggested an association between iron intake and zonulin levels during a PR-dietary pattern, which also merit further studies.

5. Conclusions

Understanding the impact of IP on host responses to dietary interventions is mandatory for personalized dietary counselling. For this purpose, for the first time we present a metabolomics study on the effect of a PR-diet on IP in older subjects with "leaky gut", also evaluating the impact of changes in GM composition on the main outcome of this intervention. Metabolomics affords important data about the molecular effectors that link the consumption of certain foods to their biological activity, and these should be used as pieces of the complex puzzle that represents the different molecular pathways involved. In light of the close interrelations among IP, food constituents and GM composition, such analyses could reveal the complex interplay between GM, the bioavailability of specific dietary constituents, and their effects on the intestinal barrier.

In conclusion, IP was a main factor modulating the serum metabolome changes during a PR dietary intervention in older adults with "leaky gut". The effects of the PR-diet on IP were mainly related with age, baseline IP and relative abundance of Porphyromonadaceae. Possible relationships between cocoa-derived methylxanthines and IP merit further studies.

6. Limitation of the study

It is worth noting that the MaPLE dietary intervention comprised other foods that provide a high amount of biologically active compounds. Thus, other metabolites from these foods could have contributed to generate lower molecular weight derivatives exerting their own activity or playing a synergic role [47]. Therefore, we cannot exclude that other components of the diet or the metabolites deriving from their degradation in the gut could exert a beneficial effect on the IP of the older volunteers.

Authors' contributions

PR and SG designed the trial and in collaboration with AC, CAL and PAK optimised the study protocol including the selection of clinical and biochemical markers and the development of the polyphenol-rich diet. NHL contributed to the elaboration of the dietary polyphenol intake. CDB and SB performed the analysis of zonulin and other clinical and biochemical markers. RGD developed the UHPLC-MS/MS method. GP performed the metabolomics analysis and the statistical elaboration of the data, in collaboration with GG, TM, PCE, AM and EVL. SG and GG performed metataxonomics analysis of faecal samples. AC and BC helped interpreting the clinical outcomes. GP and TM drafted the first version of the manuscript. All the authors critically revised the draft and approved the final version.

Data availability

The data that support the findings of this study are available from the corresponding authors, T. M. and S. G., upon reasonable request.

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Conflict of Interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2021.08.027.

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MaPLE project

European Joint Programming Initiative "A Healthy Diet for a Healthy Life" (JPI HDHL) - http://www.healthydietforhealthylife.eu/

GUT AND BLOOD MICROBIOMICS FOR STUDYING THE EFFECT OF A POLYPHENOL-RICH DIETARY PATTERN ON INTESTINAL PERMEABILITY IN THE ELDERLY

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SCIENTIFIC BACKGROUND AND RATIONALE

The intestinal barrier (IB) is the "functional entity separating the gut lumen from the inner host". IB comprises the flux regulation across the intestinal wall, known as intestinal permeability (IP). Increased IP (also named "leaky gut") has been associated with a number of disorders and diseases **(1)**. The intestinal microbiota is a major IP regulator that can act directly on IP by affecting tight junctions **(2)**, and indirectly by modulating inflammation, which is a key promoter of impaired IP **(3)**. Consequently, manipulation of the intestinal microbial ecosystem (IME) can be a novel strategy to improve IP. Dietary patterns are a dominant factor in shaping intestinal microbiota **(4,5)**. Therefore, dietary intervention strategies could modify the relative abundance of specific bacterial groups and consequently affect the maintenance of normal intestinal barrier function. Older subjects, who have often inadequate nutrition, are characterized by alterations of the IME **(5,6)**, which seem to contribute to immunosenescence and inflamm-aging **(5,7)**. In the context of a diet-microbiota-IP axis, food bioactives such as polyphenols can have a regulatory role through their antioxidant and immunomodulatory properties at intestinal and systemic levels. Polyphenols are metabolized by the microbiota and can affect its composition **(8-10)**; moreover, observational studies suggest that a high polyphenol diet is associated with favourable outcomes in older subjects. However, well-controlled intervention studies are still lacking.

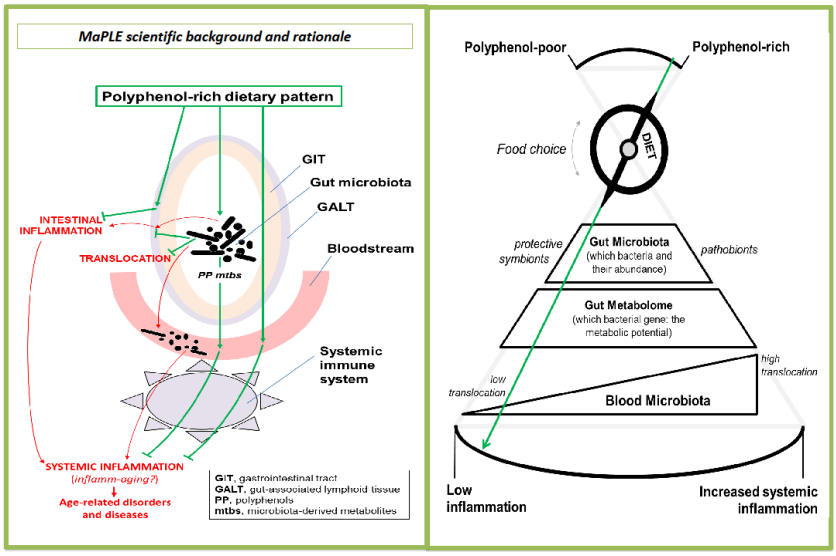
OBJECTIVE OF MAPLE PROJECT

The aim of this project is to test the hypothesis that an increased intake of polyphenol-rich foods reduces intestinal permeability and lowers inflammogenic bacterial factors in the bloodstream promoting a protective metabolic phenotype in the elderly (**Figure 1**). Possible mechanisms of actions are also investigated. Research activities have been divided into several interconnected work-packages (WPs).

WORK PACKAGES (WPS) AND ACTIVITIES

WP1: PROJECT COORDINATION AND MANAGEMENT

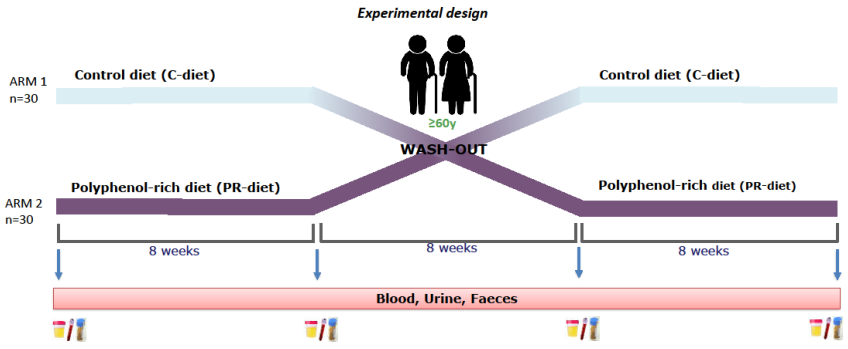
WP1 objective is the optimization of the work schedule and overall regulation of technical /financial aspects including data management.



WP2: INTERVENTION STUDY

Setting of the intervention study

Civitas Vitae - OIC Foundation, Padua, Italy: about 2200 older people living in nursing homes or in independent residences (85% not self-sufficient). Specific care is given to global wellness of hosts through high standard accomodations, activities for social inclusion and attention to diet with the availability of a dedicated area for meal preparations and complete control of intervention studies.



WP3: BIOMARKER EVALUATION

WP3 is focused on the evaluation of the effect of the polyphenol-rich dietary intervention on:
1- Markers related to IB, IP, and IME (LAL test, total bacterial load, taxonomic profiling, inflammatory markers)
2- A panel of metabolomic biomarker in blood and urine using targeted and/or untargeted metabolomic approaches
3- Oxidative stress and endothelial function markers

WP4: MECHANISTIC STUDY

The aim for this WP is to deepen the comprehension of mechanisms through which microbiome, polyphenol consumption, and microbiota-mediated polyphenol metabolism may impact on IB function.
Specific objectives and activities are:
1- Evaluation of mechanisms of IP modulation through mouse feeding studies
2- Analysis of polyphenols and derived metabolites effects on IB using in vitro models

WP5: DATA ELABORATION

Bioinformatics and statistics will be used to evidence significant correlations between data from different analytical activities on samples from intervention trial (WP2-WP3), and to properly interpret them in light of the experimental results generated by mechanistic studies (WP4).

WP6: DISSEMINATION

Results will be communicated to reach a wide national and international audience (i.e. researchers in the different area of expertise involved, nursing homes and community settings, health professionals, communicators, food and drink industry, consumers, policy makers and elderly associations)

MAIN EXPECTED OUTCOMES

Results obtained through MaPLE project will provide evidence to support:
- The development and testing of improved diets, functional foods, and dietary advices for the older subjects with IP or related conditions
- The hypothesis that a dietary intervention may modulate quantitatively the bacterial DNA in bloodstream and qualitatively the blood microbiota composition
- The integration of microbiota profiling data in association with low-grade inflammation and metabolomics to facilitate the study of inter-individual response using a phenotype approach
- The identification of a set of biomarkers with relevance in the context of preventing or treating impaired IP
- The understanding of the involvement of microbial metabolites originated from polyphenols and endogenous microbial metabolites on improvement of IP by using CaCo2 cell models
- The preventive effect of the polyphenol-rich diet on progression of leaky-gut and ability to specifically select a favourable microbiota using an aging mouse model and identifying potential mechanisms of action involved.

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Otras publicaciones realizadas durante el doctorado

MANUSCRITO Anexo 2

Wholegrain Consumption and Risk Factors for Cardiorenal Metabolic Diseases in Chile: A Cross-Sectional Analysis of 2016–2017 Health National Survey

Fabian Lanuza, Raul Zamora-Ros, Nicole Hidalgo-Liberona, Cristina Andrés Lacueva and Tomás Meroño

Nutrients

Factor de Impacto: 5.717 Q1 (17/88)

Categoría: NUTRITION & DIETETICS

Indexado Web of Science

MANUSCRITO Anexo 2

Objetivo: Evaluar la asociación entre el consumo de GT y los factores de riesgo para CRMD en la Encuesta Nacional de Salud de Chile 2016-2017.

Metodología: En este estudio de corte transversal se incluyeron 3110 participantes representativos de una población total de 11.810.647 sujetos > 18 años, que no tomaban insulina y con datos completos sobre los factores de riesgo de CRMD. Los resultados fueron el síndrome metabólico y sus componentes, la albuminuria y el deterioro de la tasa de filtración glomerular (TFG). El consumo de GT se clasificó en regular (cada dos días), esporádico (una vez al mes) y no consumidor.
Las asociaciones se analizaron mediante regresiones logísticas multivariables ajustadas por factores de confusión teniendo teniendo en cuenta el complejo diseño muestral de la encuesta.

Resultados: Los consumidores habituales de GT mostraron un menor riesgo de hipertensión arterial (OR: 0,61; IC del 95%: 0,41-0,91) en comparación con los no consumidores en modelos totalmente ajustados. ajustados. Aunque se observaron asociaciones inversas con otros componentes del síndrome metabólico y el deterioro de la TFG, ninguna fue estadísticamente significativa. La asociación entre el GT y la PA siguió siendo La asociación entre el GT y la PA siguió siendo sólida en el análisis de sensibilidad.

Conclusión: El consumo regular de GT se asoció con un 39% menos de riesgo de hipertensión arterial en adultos chilenos. menor riesgo de hipertensión arterial en adultos chilenos.

Sería positivo introducir los pósteres o presentaciones en congresos relacionadas tanto las que van dentro de la tesis, como las de los trabajo en Anexos.

nutrients

MDPI

Article

Wholegrain Consumption and Risk Factors for Cardiorenal Metabolic Diseases in Chile: A Cross-Sectional Analysis of 2016–2017 Health National Survey

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Abstract: Wholegrain (WG) consumption has been associated with reduced risk factors for cardiorenal metabolic diseases (CRMD). In Latin-America. WG intake is low and scarce studies on this subject have been found. We aimed to evaluate the association between WG consumption and risk factors for CRMD in the 2016–2017 Chilean-National Health Survey. This cross-sectional study included 3110 participants representative of a total population of 11,810,647 subjects > 18 y, not taking insulin and with complete data on CRMD risk factors. Outcomes were metabolic syndrome and its components, albuminuria, and impaired glomerular filtration rate (GFR). WG consumption was categorized as regular (≥every two days), sporadic (≥once a month), and non-consumers. Associations were analyzed by multivariable logistic regressions adjusted for confounders taking into account the complex sample design of the survey. Regular WG consumers showed a lower risk of high blood pressure (OR: 0.61, 95%CI: 0.41–0.91) compared to non-consumers in fully-adjusted models. Although inverse associations were noticed with other metabolic syndrome components and impaired GFR, none was statistically significant. The association between WG and BP remained robust in the sensitivity analysis. In conclusion regular WG consumption was associated with a 39% lower risk of high blood pressure in Chilean adults.

Keywords: wholegrain; cardiovascular disease; metabolic syndrome; chronic kidney disease; Latin America

1. Introduction

Non-communicable diseases are one of the major causes of overall mortality, from which cardiovascular diseases are the leading cause of death worldwide [1]. Cardiovascular, renal, and metabolic diseases (CRMD) share several risk factors and simulation studies have showed that policies and

interventions promoting a healthy lifestyle, including a healthy diet, may significantly reduce the incidence rates of CRMD in the years to come [2].

Wholegrain (WG) consumption has been associated with a lower incidence of CRMD in a recent meta-analysis of cohort studies and clinical trials [3]. In randomized clinical trials, WG interventions reduced several risk factors for CRMD, such as blood pressure (BP) and total cholesterol levels [3]. However, to our knowledge, no studies have evaluated these associations in a Latin-American population.

The Latin American Study of Nutrition and Health (ELANS) showed that WG consumption is scarce and represents approximately 1% of total energy intake [4]. Moreover, in the whole ELANS cohort only 36.3% of the participants consumed WG foods; while in the Chilean sub-cohort, this was even lower, at 27.9% [5]. Among the participants who consumed WG in the ELANS-Chile, dietary WG intake was relatively low with an estimated median of 40 g/day (p25–p75: 20–80 g/day) [5]. Similarly, the previous Chile National Health Survey (2009–2010) showed that only 30.3% of the Chilean population regularly consumes WG foods, while 61.3% never consumed them [6].

The National Survey of Food Consumption in Chile (ENCA) showed that only 5% of the Chilean population followed a healthy diet as defined by the Food Based Dietary Guideline for the Chilean population [7,8]. Briefly, this guideline defines a healthy diet, encouraging the consumption of fruits and vegetables five times/day, of dairy products low in fat and sugar three times/day and of legumes and fish at least two times/week, as well as lowering added-salt consumption and avoiding sugar, candy, sweetened drinks and juices, and fried foods [7]. Previous results from the 2016–2017 Chilean National Health Survey showed that the risk for metabolic syndrome was inversely associated with higher consumption of fruits and vegetables or with moderate-vigorous physical activity among participants with normal weight or obesity, respectively [9]. In their analysis, the authors only analyzed the consumption of fruits/vegetables and of fish/seafood, but, similarly to the national dietary guidelines, they omitted WG consumption. Reynolds et al. [3] identified a linear dose-response relationship showing that a 15 g/day increase of WG intake was associated with a reduction of 7% and 12% of relative risk for incident coronary heart disease and type 2 diabetes, respectively. Therefore, the impact of WG consumption on CRMD risk factors has yet to be determined in a Latin-American population characterized by low levels of intake. The aim of the study was to evaluate the association between WG consumption and risk factors for CRMD in the 2016–2017 Chilean National Health Survey.

2. Materials and Methods

2.1. Study Sample

The 2016–2017 Chilean National Health Survey was a survey with a stratified multistage sample of non-institutionalized participants over 14 years old from urban and rural regions in Chile. Detailed information about the survey has been described elsewhere [10]. In the present study, from the 6233 participants recruited, 3110 subjects with age ≥ 18 years, with complete data on CRMD risk factors and covariates, and not taking insulin were included in this analysis (Figure 1). All participants signed the written consent form. The Ethics Committee from the Pontificia Universidad Católica (PUC) de Chile approved the study protocol (Number: 16–019).

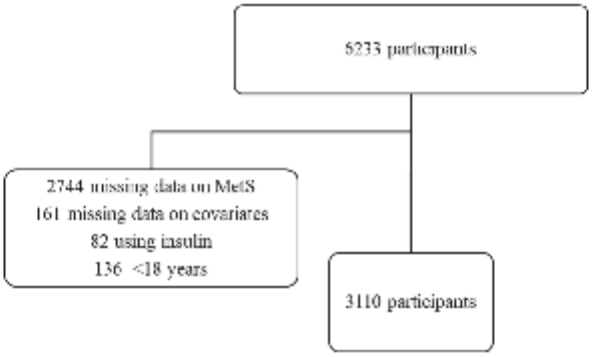

Figure 1. Flowchart of participants in the 2016–2017 Chilean National Health Survey.

2.2. *Dietary Exposure*

Dietary assessment was carried out by trained interviewers collecting information on the frequency of consumption of fish and seafood, dairy products, WG products, fruits, vegetables, legumes, sugar-sweetened beverages and culinary fat. These questions were designed by experts based on the Food Based Dietary Guideline (FBDG) for the Chilean population as described in the 2009–2010 Chilean National Health Survey [6,7].

WG consumption was classified in three categories according to the question: how often do you consume any WG cereal, such as WG bread, WG cereal or any other food that contains WG flour? Regular WG consumers were participants who answered, "more than once a day", "once a day" or "every two days". Sporadic WG consumers were those who answered, "at least once a week" or "once a month". Non-consumers were those that reported that they "never consume WG foods".

The consumption of fruits, vegetables and legumes were selected as dietary covariates because they are also important sources of dietary fiber. Consumption of fruits and vegetables was estimated through the questions: "Typically, how many days a week do you eat fruits?" and "Typically, how many days a week do you eat vegetables or vegetable salad? (do not include legumes or potatoes)", respectively. The answers in days per week were used as continuous variables. Consumption of legumes was assessed through the question: "How often do you consume any type of legumes, such as beans, lentils, green peas or chickpeas?" They were grouped into three categories: (i) "two or more times per week", (ii) between "at least once a week" and "between one and three times a month", and (iii) "less than once a month or never".

2.3. *Assessment of Risk Factors for Cardiorenal Metabolic Disease*

Waist circumference was measured at the midpoint between the costal margin and the iliac crest. Medication use for diabetes and BP was self-reported. BP was measured three times and the mean was used in the study. Fasting blood samples (at least 8 h fast) were drawn for the evaluation of glucose, hemoglobin A1c (HbA1c), creatinine and plasma lipids by standardized methods in a centralized laboratory as described previously. A morning urine sample was collected at the moment of the visit (7–10 am) to measure the urinary albumin-creatinine ratio (uACR) [10]. Urinary albumin values below the lowest reportable value (<0.003 g/L) were replaced by 0.003 g/L and included in the analysis. Very low-density lipoprotein-cholesterol (VLDL-C) and non-high-density lipoprotein-cholesterol (non-HDL-C) and the triglycerides/HDL-C ratio were calculated. The Chronic Kidney Disease-Epidemiology Collaboration (CKD-EPI) formula was used to estimate the glomerular filtration rate (GFR) [11].

2.4. Outcomes

Metabolic syndrome was defined according to the Chilean National Guidelines as having at least three of the following five components: high waist circumference (>90 cm for men and >80 cm for women), low HDL (HDL-C <1.03 mmol/L for men and <1.29 mmol/L for women), hypertriglyceridemia (triglycerides ≥ 1.7 mmol/L), high BP (systolic/diastolic BP > 130/85 mmHG or under BP-lowering treatment) and impaired fasting glucose (IFG, glucose > 5.6 mmol/L or under treatment with antidiabetic drugs) [10]. A GFR < 60 mL/min.1.73 m^2 was used to define chronic kidney disease (CKD) according to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines [12]. uACR was available in 2432 participants. Albuminuria was categorized as microalbuminuria: uACR between 0.34–3.39 mg/mmol, and macroalbuminuria: uACR > 3.39 mg/mmol according to guidelines [12].

2.5. Socio-Demographic and Clinical Covariates

Covariates to be included in the study were selected a priori and were based on previous reports of the 2016–2017 Chilean National Health Survey and in a pathophysiological basis [13,14]. Trained interviewers collected socio-demographic data from all participants including geographical area (urban vs. rural), age (years), sex, education level (<8, 8–12, >13 years of education), body mass index (BMI, <25, 25–30, >30 kg/m^2), physical activity using the Global Physical Activity Questionnaire (GPAQ, low, moderate, high), tobacco use (current smokers vs. non-smokers and former smokers), frequency of alcohol consumption (never, ≤1 time/month, 2–4 times/month, 2–3 times/week, >3 times/week) and number of glasses of alcohol typically consumed (continuous, 0 for non-drinkers), hypertension (self-reported, medication use or systolic/diastolic BP > 140/90 mmHg), diabetes (self-reported or fasting blood glucose > 7 mmol/L) and previous cardiovascular disease (self-reported acute myocardial infarction, stroke or peripheral artery disease). Less than 1% of the participants showed a BMI < 18.5 kg/m^2 and they were included within the BMI category < 25.0 kg/m^2. Alcohol dependence was 0.3% in the Survey and these participants were not excluded from the study.

2.6. Statistical Analyses

The complex sampling design of the Chilean National Health Survey including strata, cluster and weights were considered for all statistical analyses. Sampling weights and their instructions for use were provided in the database. Categorical data are presented as percentage (standard error, SE), unless otherwise stated. Continuous variables are shown as mean (SE). Generalized linear models adjusted for age (18–24, 25–44, 45–64, >65 years) and sex were used to compare the general characteristics of the population and the levels of risk factors for CRMD across categories of WG consumption. Among these biochemical variables, the skewed-distributed variables were log-transformed before being entered in the analysis. The association between WG consumption and each metabolic syndrome component, metabolic syndrome and CKD was tested using a multivariable logistic regression. The association between WG consumption and albuminuria was tested by multivariable ordinal regression. Odds Ratios (OR) and 95% CI were calculated using the following modeling strategy. Model 1 was adjusted by age (18–24, 25–44, 45–64, >65 years), sex and geographical area. Model 2 was additionally adjusted for BMI (<25, 25–30, >30 kg/m^2), education level (<8, 8–12, >13 years of education), tobacco use (current smokers vs. former and non-smokers), frequency of alcohol consumption (never, ≤1 time/month, 2–4 times/month, 2–3 times/week, >3 times/week) and number of glasses of alcohol typically consumed (continuous, 0 for non-drinkers), physical activity (GPAQ, low, moderate, high), diabetes (self-reported or fasting blood glucose >126 mg/dl) and previous cardiovascular disease (self-reported acute myocardial infarction, stroke or peripheral artery disease). For renal outcomes (CKD and albuminuria) the model also included hypertension (self-reported, medication use or systolic/diastolic BP > 140/90 mmHg). Model 3 was further adjusted for consumption of fruits, vegetables (times per week) and legumes (categorical, three levels). Interactions between

WG consumption and age (categorical), sex, BMI (categorical), education level (categorical), physical activity (categorical) and tobacco use in relation to the risk factors for CRMD were tested by using a likelihood ratio test based on the models with and without an interaction term. If it was significant, the analyses were divided into subgroups. Sensitivity analysis excluding participants from rural areas, having a BMI < 18.5 kg/m^2, participants with alcohol dependence, patients with diabetes and previous cardiovascular disease was also conducted. SPSS version 25 (IBM, Armonk, NY, USA) was used for all statistical analyses.

3. Results

3.1. General Characteristics of the Population According to the Frequency of WG Consumption

The studied population consisted of 3110 participants and after applying sampling weights this population was representative of a total *n* (95%CI) of 11,810,647 (10,982,016–12,639,278) subjects. General characteristics are shown in Table 1. In general, the Chilean population featured an elevated prevalence of overweight/obesity (77.5%), metabolic syndrome (41.7%), and physical inactivity (34.7%). The prevalence of hypertension, diabetes and cardiovascular disease was 27.1%, 12.8% and 9.6%, respectively.

Regular WG consumers were younger, more likely to reside in urban areas, to be women, non-smokers or former smokers and to have a higher education level and physical activity than sporadic and non-WG consumers (Table 1). The consumption of fruits and vegetables per week was higher in regular WG consumers and they typically consumed fewer glasses of alcohol (although they did not consume alcohol more frequently). There were no differences in the prevalence of diabetes or previous cardiovascular disease across categories of WG consumption (Table 1).

Table 1. General characteristics of the participants according to the frequency of wholegrain (WG) consumption.

	All	Non–Consumers	Sporadic WG Consumers	Regular WG Consumers	*p* for Trend *
Interviewed participants (*n*)	3110	1739	613	758	
Weighted population (*n*,%)	11,810,647 (100)	6,369,362 (54)	2,316,451 (20)	3,124,834 (26)	
Age (years)	44.2 (0.5)	47.1 (0.8)	42.2 (1.1)	40.0 (0.9)	<0.001
Urban residents (%)	88.9 (0.7)	86.0 (1.1)	90.1 (1.5)	94.1 (1.1)	<0.001
Female sex (%)	51.5 (1.4)	48.3 (1.8)	54.9 (3.3)	55.5 (3.5)	0.025
Education level (%)					
<8 years	17.1 (1.1)	22.6 (1.7)	10.3 (1.5)	11.0 (2.0)	<0.001
8–12 years	54.5 (1.9)	58.9 (2.3)	54.8 (3.3)	45.4 (3.4)	
>12 years	28.4 (1.8)	18.5 (1.9)	35.0 (3.4)	43.6 (3.6)	
BMI category (%)					
<25 kg/m^2	22.5 (1.4)	21.2 (1.7)	19.8 (2.7)	27.0 (3.0)	0.320
25–30 kg/m^2	42.7 (1.8)	42.5 (2.3)	46.2 (3.5)	40.5 (3.2)	
>30 kg/m^2	34.8 (1.5)	36.3 (2.0)	33.9 (3.1)	32.5 (3.1)	
PA (%)					
Low	34.7 (1.5)	37.3 (2.1)	31.4 (3.2)	32.0 (3.0)	0.044
Moderate	23.8 (1.4)	24.5 (1.8)	23.8 (2.8)	22.2 (2.8)	
High	41.5 (1.8)	38.2 (2.3)	44.8 (3.5)	45.7 (3.4)	
Tobacco use (%)	36.1 (1.5)	38.3 (2.0)	39.6 (3.3)	28.9 (3.2)	0.002
Frequency of alcohol consumption (%)					
Never	27.1 (1.3)	25.4 (1.5)	25.4 (2.7)	31.6 (3.0)	0.125
≤1 time/month	37.6 (1.5)	39.0 (2.0)	34.1 (3.2)	37.1 (3.0)	
2–4 times/month	24.8 (1.4)	25.2 (2.0)	29.6 (3.4)	20.4 (2.3)	
2–3 times/week	7.9 (1.1)	7.5 (1.4)	9.1 (2.2)	8.0 (2.0)	
>3 times/week	2.6 (0.5)	2.9 (0.7)	1.8 (0.8)	2.8 (1.1)	

Table 1. *Cont.*

	All	Non–Consumers	Sporadic WG Consumers	Regular WG Consumers	*p* for Trend *
Alcohol consumption (glasses)	2.2 (0.1)	2.4 (0.1)	2.2 (0.1)	1.9 (0.1)	<0.001
Fruits (times/week)	4.3 (0.1)	4..0 (0.1)	4.4 (0.1)	5.1 (0.2)	<0.001
Vegetables (times/week)	5.5 (0.1)	5.4 (0.1)	5.6 (0.1)	5.8 (0.1)	0.008
Legumes (%)					
≤1 time/month	12.1 (1.1)	11.7 (1.3)	12.4 (2.4)	12.5 (2.4)	0.503
>1 time/month	62.8 (1.8)	62.8 (2.3)	65.9 (3.3)	60.7 (3.1)	
≥2 times/week	25.1 (1.4)	25.5 (1.8)	21.7 (2.8)	26.8 (2.9)	
HT (%)	27.1 (1.3)	31.5 (1.9)	27.9 (3.1)	17.7 (2.1)	0.164
Diabetes (%)	12.8 (1.0)	13.0 (1.4)	12.4 (1.8)	12.6 (2.1)	0.368
CVD (%)	9.6 (0.9)	11.0 (1.3)	9.4 (1.8)	6.8 (1.2)	0.396
MetS (%)	41.7 (1.6)	45.2 (2.1)	42.3 (3.3)	34.1 (3.3)	0.323
CKD (%)	2.9 (0.5)	3.6 (0.7)	3.5 (1.1)	1.1 (0.3)	0.181
uACR (%)					
0.34–3.39 mg/mmol	8.3 (0.8)	7.4 (0.9)	10.1 (2.0)	8.7 (2.1)	0.962
>3.39 mg/mmol	1.4 (0.3)	1.7 (0.5)	1.5 (0.7)	0.9 (0.4)	

BMI, body mass index; PA, physical activity; HT, hypertension; CVD, cardiovascular disease; MetS, metabolic syndrome; CKD, chronic kidney disease; uACR, urinary albumin-creatinine ratio. * Generalized linear models adjusted for age and sex. Available data in uACR (2432 interviewed participants representing a population of 8,385,402).

3.2. *Risk Factors for CRMD According to the Frequency of WG Consumption*

The prevalence of hypertension, metabolic syndrome and CKD was similar across categories of WG consumption (Table 1).

However, WG consumers showed lower levels of several risk factors for CRMD than sporadic WG consumers and non-consumers in age- and sex-adjusted models (Table 2). In particular, regular WG consumers showed lower systolic/diastolic BP and a less atherogenic lipid profile characterized by higher HDL-C and lower triglycerides and VLDL-C (Table 2). Markers of renal function (creatinine, GFR and uACR) were similar across categories of WG consumption.

Table 2. Risk factors for cardiorenal metabolic diseases according to the frequency of WG consumption.

	Non–Consumers (*n*: 6,368,362) ‡	Sporadic WG Consumers (*n*: 2,316,451) ‡	Regular WG Consumers (*n*: 3,124,834) ‡	*p* for Trend *
WC (cm)	94.5 (0.5)	93.6 (0.9)	91.7 (0.8)	0.156
SBP (mmHg)	126.1 (0.8)	124.1 (1.4)	118.7 (0.9)	0.006
DBP (mmHg)	75.6 (0.4)	74.9 (0.7)	72.2 (0.6)	0.007
Glucose (mmol/L)	5.36 (0.04)	5.31 (0.07)	5.11 (0.05)	0.389
HbA1c (mmol/mol)	43.2 (0.8)	45.9 (1.8)	41.6 (1.2)	0.993
TG (mmol/L)	1.72 (0.05)	1.60 (0.09)	1.48 (0.07)	0.007
TC (mmol/L)	4.67 (0.04)	4.66 (0.07)	4.50 (0.06)	0.319
HDL-C (mmol/L)	1.18 (0.01)	1.24 (0.02)	1.25 (0.02)	0.005
LDL-C (mmol/L)	2.70 (0.03)	2.69 (0.06)	2.59 (0.05)	0.459
VLDL-C (mmol/L)	0.78 (0.02)	0.71 (0.04)	0.66 (0.25)	0.008
Non-HDL-C (mmol/L)	3.50 (0.04)	3.42 (0.07)	3.25 (0.06)	0.071
TG/HDL-C	1.7 (0.07)	1.5 (0.1)	1.4 (0.1)	0.003
Creatinine (µmol/L)	70.7 (0.8)	69.5 (1.1)	68.4 (1.1)	0.251
GFR (mL/min.1.73 m^2)	101 (1)	104 (1)	106 (1)	0.343
uACR (mg/mmol)	0.28 (0.05)	0.26 (0.04)	0.21 (0.04)	0.990

WC, waist circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1c, hemoglobin A1c; TG, triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; GFR, glomerular filtration rate; uACR, urinary albumin-creatinine ratio. Values are mean (SE). * Generalized linear models adjusted for age and sex. Available data in HbA1c (1106 interviewed participants representing a population of 3,335,061) and uACR (2432 interviewed participants representing a population of 8,385,402). ‡ *n* after applying sampling weights.

3.3. *Association of WG Consumption with Cardiorenal Metabolic Outcomes*

The prevalence of individual metabolic syndrome components in the participants was 76.6% for high waist circumference, 52.2% for low HDL, 37.6% for high BP, 34.8% for hypertriglyceridemia, and 24.9% for IFG.

Age-, sex- and geographical area-adjusted models showed an inverse association of WG consumption with high BP and CKD (Table S1). Results from the fully adjusted model are shown in Table S1 and Figure 2. Regular WG consumption was significantly associated with a 39% lower risk of high BP than non-consumers.

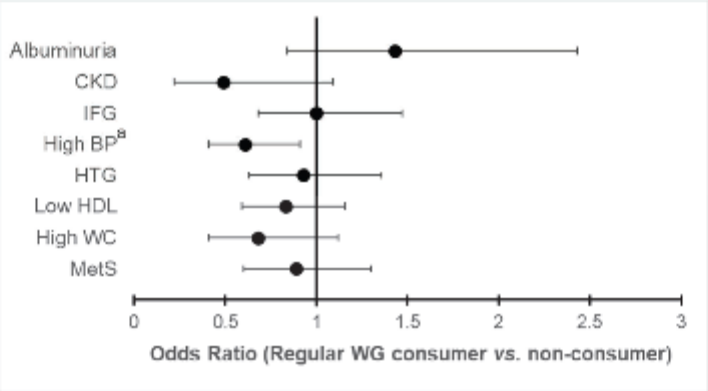

Figure 2. Odds ratio for regular wholegrain consumers (*n*: 3,124,834) vs. non-consumers (*n*: 6,369,362) on cardiorenal metabolic outcomes. MetS, metabolic syndrome; WC, waist circumference; HDL, high density lipoprotein; HTG, hypertriglyceridemia; BP, blood pressure; IFG, impaired fasting glucose; CKD, chronic kidney disease. [a] $p < 0.05$. Model was adjusted for geographical area, age, sex, BMI, education level, tobacco use, frequency of alcohol consumption and quantity of glasses of alcohol typically consumed, physical activity, diabetes, previous cardiovascular disease and consumption of fruits, vegetables and legumes. For renal outcomes (CKD and albuminuria) the model also included hypertension. Albuminuria was available in 2,159,174 regular WG consumers (70%) and in 4,499,396 non-consumers (71%) and ordinal regression instead of logistic regression was used.

The interaction between WG consumption and age for association with MetS was the only significant interaction among the other risk factors for CRMD. In consequence, MetS analyses were repeated in each of the age groups. An inverse association was observed in the 18–24 y ($OR_{regularWG\ vs.\ non\text{-}consumer}$: 0.30, 95%CI: 0.06–1.47), 45–64 y ($OR_{regularWG\ vs.\ non\text{-}consumer}$: 0.76, 95%CI: 0.43–1.34) and >65 y ($OR_{regularWG\ vs.\ non\text{-}consumer}$: 0.71, 95%CI: 0.29–1.72) age groups, but not in the group of 25–44 y ($OR_{regularWG\ vs.\ non\text{-}consumer}$: 1.16, 95%CI: 0.62–2.17). There were no significant interactions between WG consumption and the other investigated covariates (sex, education level, BMI, physical activity, and tobacco use) of the study for all outcomes.

3.4. *Sensitivity Analysis*

Excluding participants from rural areas (*n*: 1,305,651), with underweight (BMI < 18.5 kg/m^2, *n*: 107,656), with alcohol dependence (*n*: 10,770), with diabetes (*n*: 1,513,041) or previous cardiovascular disease (*n*: 1,137,769) showed similar results for the association between WG consumption and high BP (Table S2). The rest of the results remained non-significant.

4. Discussion

This is the first report to show that regular WG consumption is associated with lower levels of risk factors for CRMD, and especially lower risk of high BP, in a Latin American study. Indeed, regular WG consumers showed approximately a 39% lower risk of presenting high BP than non-consumers in this nationwide study representative of almost 12,000,000 Chilean adults.

The inverse association between regular WG consumption and high BP was robust and was in agreement with other studies [14]. Elevated BP is a known-causal risk factor for cardiovascular disease. In randomized clinical trials, a 20% reduction in cardiovascular events was observed for each 10 mmHg of lower systolic BP using medical therapy [15]. Indeed, patients with slight elevations of BP (normal-high BP: 130–139/80–85 mmHg) showed an increased risk of cardiovascular disease [16], and it was shown that patients with other cardiovascular risk factors may benefit more from lower BP at any given value [17]. Therefore, the effect of regular WG consumption on BP may contribute to the prevention of CRMD. It is important to bear in mind that the inverse association between WG consumption and hypertension was confirmed in prospective studies in the US [18] and Asian populations [19]. Thus, a great deal of evidence suggests that low WG consumption may be a causal risk factor for hypertension.

The inverse association between regular WG consumption and high BP may rely, to some extent, on the anti-inflammatory effects attributed to WG and to one of its major nutrients, dietary fiber [20]. These effects could be mediated by beneficial modification of gut microbiota composition and/or the enhancement of short-chain fatty acid production [21–23]. Chronic inflammation, typical of obesity and its metabolic derangements, is a key player in the development and progression of endothelial dysfunction, and consequently hypertension [24]. Metagenomic and metabolomic studies are needed to clarify the exact role of gut microbiota composition or its fermentation products in the BP lowering activities attributed to WG.

In our study, we did not observe a significant association between WG consumption and MetS. In a study of 827 Tehranian adults [14], participants in the highest quartile of WG intake showed a 32% lower risk for MetS than those in the lowest quartile. The population characteristics, which included older participants with a higher prevalence of overweight/obesity, hypertension and diabetes, and the lack of a quantitative analysis of WG consumption might explain the different results. Moreover, in the Tehran study, the mean WG consumption was 93 ± 29 g/day, much higher than the estimated mean consumed in the whole sample of Chilean participants in the ELANS, i.e., ±26 g/day [5]. In fact, in a US study on dietary fiber and its association with MetS, risk estimates showed an inverse linear trend across quintiles of intake [25]. Therefore, the low intake level of WG foods in Chile might explain why we did not observe an association between WG consumption and other CRMD outcomes.

The Nutrition and Chronic Diseases Expert Group (NutriCoDE) [26] showed causal protective cardiorenal metabolic effects of WG foods and established that an intake of 50 g/day of WG was associated with a 12% lower risk of coronary heart disease events due to its effects on BP and LDL-C [26].The optimal WG intake range is still under debate and although a range of 100 to 125 g/day has been proposed [27], another study suggested that a WG intake of 210–225 g/day was needed to reduce the risk of chronic diseases and mortality [28]. The HELGA study, which combined samples from three Scandinavian cohorts with a median WG intake of 121 g/day, showed a 44% and 15% lower cardiovascular mortality for women and men, respectively, in the highest quartile of WG intake compared to the lowest one [29]. However, WG foods can provide several health benefits even at low intake doses. In two large prospective cohort studies from the US with relatively low WG intakes (from quintile 1 to quintile 5 of 4.3 g/day to 35.6 g/day, and of 5.8 g/day to 52.6 g/day, respectively) the highest quintiles of WG intake were associated with a 9% lower cardiovascular mortality independent of other dietary and lifestyle factors [30]. Thus, efforts to promote WG consumption in Chile disregarding the level of intake are expected to impact the incidence of CRMD. The Food Based Dietary Guideline for the Chilean population promulgates 11 main statements to define a healthy diet [7]. However, no message directly alludes to WG. Some probable restraints in the intake of WG foods in the Chilean

population have already been mentioned such as lower palatability, unawareness of health benefits, and higher prices compared to refined grain foods [31].

Consumption of WG changes considerably between populations. WG rye products are commonly consumed in Scandinavian countries, while WG bread and breakfast cereals are common in the US, and brown rice in Asian countries [28]. Thus, the type of grain may also have some influence when comparing our results with studies from other geographical regions. In particular, WG oats showed a potent cholesterol-lowering effect compared to other grains [32]. As shown in the National Survey of Food Consumption in Chile (ENCA) Survey, the cereals most consumed in Chile were bread and pasta, while breakfast cereals were seldom consumed. Although, it was not explicitly addressed, WG wheat was the most likely consumed WG among Chilean participants.

This was a cross-sectional study and causal relationships cannot be ascertained. This study was based on the frequency of WG consumption and, therefore, future studies quantifying WG intake and its association with CRMD risk factors in Latin America are warranted. Moreover, energy intake was not available for adjustment. However, the use of a national representative sample with sampling weights to adjust for overrepresentation, selection probability, non-response rates, and the poststratification adjustment, carried out by trained personnel, with centralized laboratory results, are major strengths of the study. Although the statistical analyses were adjusted by sociodemographic covariates, some residual confounding cannot be ruled out. In particular, CKD prevalence was somewhat lower than expected in the sample (2.9%). Indeed, a Chilean study found a prevalence of CKD of 12.1% in primary care health centers [33]. Finally, the Chilean population has one of the highest prevalence of overweight and obesity compared to other countries from the region and the results might not be generalized to the whole region [34]. Nonetheless, this is the first study on the association between WG consumption and CRMD risk factors carried out in a Latin American country and our results may contribute to promote research on the cardiorenal metabolic effects of WG foods in the Region.

5. Conclusions

Regular WG consumption was associated with lower levels of risk factors for CRMD and especially lower risk of high BP. The benefits of regular WG consumption on BP were independent of socio-demographic characteristics and other dietary sources of fiber. The adjustment for other dietary fiber sources suggests that additional components within WG may also contribute to its BP-lowering effects [35]. Considering its health benefits, access to and consumption of WG foods should be included and promoted by national dietary guidelines and food policies in order to prevent CRMD in Latin America.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/9/2815/s1, Table S1: Association between WG consumption and cardiorenal metabolic outcomes. Table S2: Sensitivity analysis of the association between WG consumption and cardiorenal metabolic outcomes.

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Abbreviations

BP	Blood pressure
CKD	Chronic kidney disease
CRMD	Cardiorenal metabolic diseases
ENCA	Encuesta Nacional de Consumo Alimentario
ELANS	Latin American Study of Nutrition and Health
GFR	Glomerular filtration rate
HbA1c	Hemoglobin A1c
HDL	High-density lipoprotein
IFG	Impaired fasting glucose
LDL	Low-density lipoprotein
VLDL	Very low-density lipoprotein
WG	Wholegrains

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