Efecto de componentes de la Dieta Mediterránea sobre la cascada del ácido araquidónico y la proliferación de células epiteliales intestinales

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Memoria presentada por Carolina Emilia Storniolo para optar al título de doctor por la Universidad de Barcelona

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Juan José Moreno Aznárez, Catedrático del Departamento de Nutrición, Ciencias de la Alimentación y Gastronomía de la Facultad de Farmacia y Ciencias de la Alimentación de la Universidad de Barcelona,

INFORMA:

Que la memoria titulada "*Efecto de componentes de la Dieta Mediterránea sobre la cascada del ácido araquidónico y la proliferación de células epiteliales intestinales*" presentada por CAROLINA EMILIA STORNIOLO para optar al título de Doctor por la Universidad de Barcelona, ha sido realizada bajo mi dirección en el Departamento de Nutrición, Ciencias de la Alimentación y Gastronomía, y considerándola finalizada, autorizo su presentación para ser juzgada por el tribunal correspondiente.

Y para que así conste, firmo la presente, en Santa Coloma de Gramenet a 16 de junio de 2017.

Dr. Juan José Moreno Aznárez

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Abreviaturas

AA	Ácido araquidónico
AdhDM	Adherencia a la Dieta Mediterránea
AG	Ácido graso
AGMI	Ácido graso monoinsaturado
AGPI	Ácido graso poliinsaturado
AGS	Ácido graso saturado
CCR	Cáncer colorrectal
COX	Ciclooxigenasa
DHA	Ácido docosahexaenoico
DM	Dieta Mediterránea
ECV	Enfermedad cardiovascular
EPA	Ácido eicosapentaenoico
LOX	Lipoxigenasa
LT	Leucotrieno
MAI	Mediterraean adherence index
PG	Prostaglandina
RV	Resveratrol
SFB	Suero fetal bovino
Wnt	Wingless-type MMTV Integration Site Family

Resumen

La adherencia a la Dieta Mediterránea se ha asociado a una disminución de enfermedades de alta prevalencia como el cáncer colorrectal, y este hecho se ha relacionado con el consumo moderado de vino y aceite de oliva, que son dos alimentos característicos de la Dieta Mediterránea. Ambos alimentos son una mezcla compleja de numerosos componentes con estructura y características fisicoquímicas diversas, entre los que destacan los polifenoles. Los estilbenos y los tirosoles son polifenoles que se encuentran presentes en pocos alimentos, entre los que se incluyen el vino y el aceite de oliva. El objetivo de este trabajo ha sido estudiar el papel de componentes bioactivos del vino y el aceite de oliva en el control del crecimiento de células epiteliales intestinales. Para ello, como modelo experimental se utilizó una línea celular procedente de un adenocarcinoma humano denominada Caco-2.

El resveratrol (RV), que es un polifenol representativo del vino, es capaz de regular el crecimiento de las células epiteliales intestinales *in vitro* a concentraciones que no se alcanzan en plasma tras un consumo moderado de vino, dado que esta molécula se metaboliza muy rápidamente. En este sentido, los resultados muestran que los metabolitos sulfatados y glucuronidados del RV mantienen la capacidad antioxidante de este, así como la de inhibir el crecimiento y de inducir apoptosis. El *trans*-piceido, que es el glucósido del RV presente en concentraciones elevadas en el vino, presenta un efecto antioxidante, una actividad antimitogénica y pro-apoptótica sobre las células Caco-2 similar a la del RV. Además se observó que la capacidad antioxidante del RV está relacionada con la presencia de grupos hidroxilo en posición 3, 5 y 4'. Aunque es este último hidroxilo el determinante de dicha actividad antioxidante. La sustitución de estos hidroxilos por grupos cloro o metoxi, a pesar de reducir su actividad antioxidante, conserva sus efectos biológicos sobre el crecimiento de las células Caco-2.

Los ácidos grasos monoinsaturados, y en especial el ácido oleico, que es el ácido graso mayoritario del aceite de oliva, inducen el crecimiento de las Caco-2, probablemente a través de la síntesis de metabolitos por la vía de las lipoxigenasas. Algunos ácidos grasos poliinsaturados, como el eicosapentaenoico y el docosahexaenoico, presentaron un efecto dual, ya que a baja concentración indujeron la proliferación celular, pudiendo estar implicados en este efecto las prostaglandinas y los ácidos hidroxieicosapentaenoicos. Mientras que una concentración alta de estos ácidos grasos poliinsaturados tuvo un efecto opuesto sobre el crecimiento celular e indujo apoptosis. Es

interesante señalar que el efecto mitogénico del ácido oleico fue revertido por diversos componentes minoritarios del aceite de oliva como el hidroxitirosol, la oleuropeina, el escualeno, el ácido maslínico y el pinoresinol. Algunos de los cuales inhibieron la liberación de ácido araquidónico y la síntesis de eicosanoides, elementos implicados en el control del crecimiento de las células epiteliales intestinales.

En conclusión, la cantidad y el tipo de ácidos grasos consumidos junto con los componentes minoritarios del vino y del aceite de oliva, así como sus metabolitos, pueden tener un importante papel en el control de la cascada del ácido araquidónico y de la proliferación de células epiteliales intestinales Caco-2.

I. Introducción

1. Dieta Mediterránea

La Dieta Mediterránea (DM) tradicional se podría definir como el estilo de vida que comparten las poblaciones que viven en la cuenca del Mediterráneo (Willett y col., 1995; Trichopoulou y Lagiou, 1997), zona de contacto entre Europa, África y Asia. Pero ha ido evolucionando con el tiempo, a la vez que se ha ido extendiendo geográficamente a regiones alejadas del Mediterráneo. En el año 2013 la UNESCO inscribió la DM en la Lista Representativa del Patrimonio Cultural Inmaterial de la Humanidad, ya que comprende un conjunto de conocimientos, competencias prácticas, rituales, tradiciones y símbolos relacionados con los cultivos y cosechas agrícolas, la pesca y la cría de animales, y también con la forma de conservar, transformar, cocinar, compartir y consumir los alimentos. Poniendo de relieve los valores de hospitalidad, buena vecindad, diálogo intercultural y creatividad, así como un modo de vida que se guía por el respeto de la diversidad (Disponible en https://ich.unesco.org/es/RL/la-dieta-mediterranea-00884, 8.COM).

1.1. Origen de la Dieta Mediterránea

El olivo, la vid, la cebada, el trigo, las lentejas, las nueces, las avellanas, las almendras y los dátiles (Khoury y col., 2016), junto con otras plantas autóctonas de la cuenca Mediterránea fueron los primeros alimentos consumidos en las culturas que florecieron en este entorno. Históricamente, estas culturas establecieron numerosos contactos culturales/comerciales con otras zonas de los continentes que forman parte de la cuenca Mediterránea, lo que contribuyó a enriquecer la dieta con nuevos alimentos como el arroz, el ajo, la naranja, la berenjena y el café, entre otros (Khoury y col., 2016).

Posteriormente, la DM tradicional se enriqueció con la aportación de nuevos alimentos procedentes del continente Americano como los pimientos, las patatas, los tomates, el aguacate y el maíz, entre otros (Khoury y col., 2016). De manera que la DM tradicional ha ido integrando diferentes alimentos a lo largo del tiempo, como consecuencia de los movimientos migratorios y los intercambios comerciales. Como se observa en la **Figura 1**, los alimentos llegaron a la cuenca Mediterránea desde diversas partes del mundo para conformar la DM tradicional, variada y completa, que se conoce hoy en día.

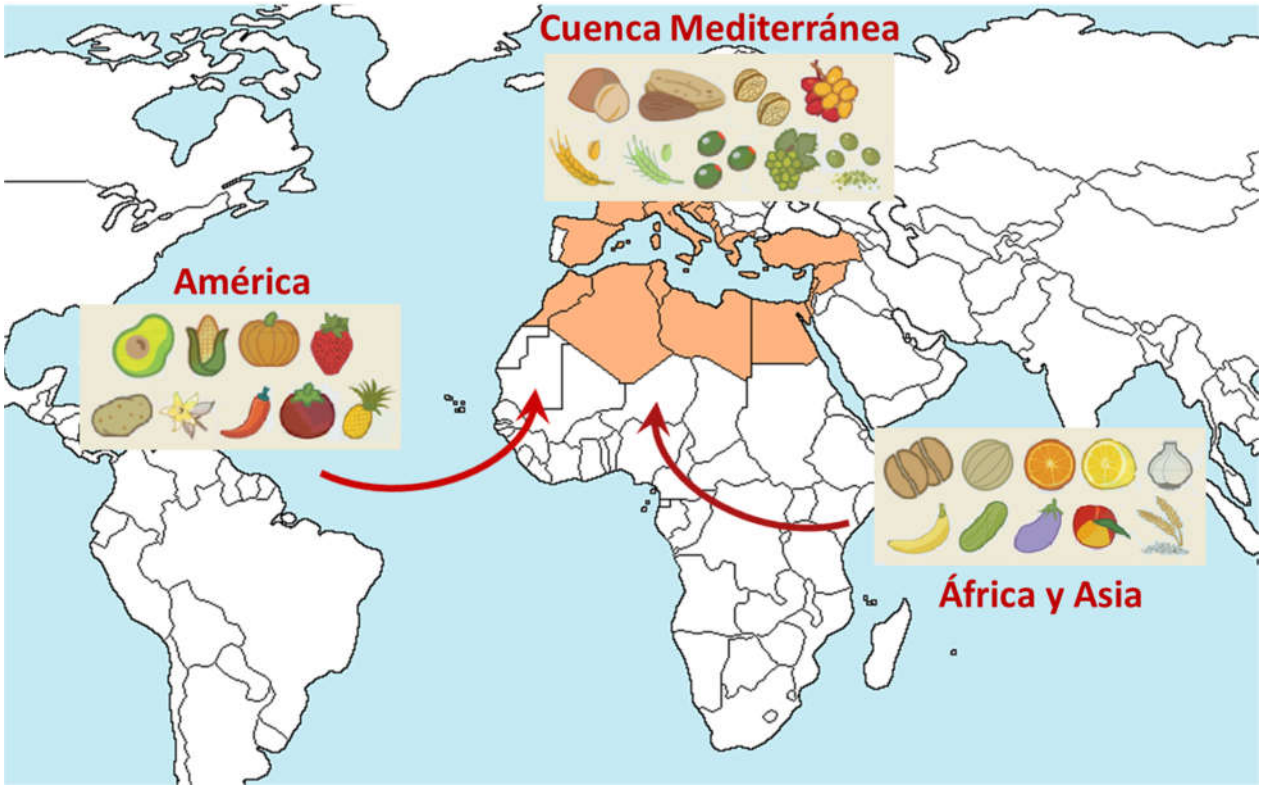

Figura 1. Origen de los alimentos más característicos de la Dieta Mediterránea. Adaptado de Khoury y col. (2016).

Aunque la DM tradicional tiene una dilatada historia, la creación del concepto DM se remonta a los años 60, cuando Ancel Keys demostró los efectos beneficiosos del patrón de dieta de los países Mediterráneos sobre las enfermedades cardiovasculares en comparación con la dieta de los países de Europa Central y Estados Unidos. Estos resultados han sido confirmados con numerosos estudios realizados a lo largo del último siglo, demostrando que la DM incrementa la longevidad (Mitrou y col., 2007; Sofi y col., 2010 y 2013) al estar asociada a una menor incidencia de cáncer (Couto y col., 2011), enfermedades cardiovasculares (Estruch y col., 2013), diabetes mellitus (Salas-Salvadó y col., 2014) y enfermedades neurodegenerativas (Mitrou y col., 2007; Sofi y col., 2013).

1.2. Características principales de la Dieta Mediterránea

A pesar de que con el paso del tiempo las características de la DM han ido evolucionando, actualmente se considera un patrón alimentario saludable (Willett y col., 1995), que se asocia a un mejor estado de salud, debido al efecto protector frente a numerosas enfermedades crónicas de alta prevalencia (Keys y col., 1980; Serra-Majem y col., 2006; Sofi y col., 2008).

Actualmente, a pesar de que resulta difícil encontrar una definición consensuada, se considera la DM tradicional como un patrón alimentario basado en un consumo diario de frutas, vegetales, cereales (mayoritariamente de grano entero o integrales), legumbres, frutos secos, semillas y aceite de oliva (como fuente mayoritaria de grasa); un consumo semanal de lácteos (principalmente queso y yogur), aves, huevos y pescado; un consumo esporádico de carne roja y carnes procesadas, así como de alimentos ricos en azúcares simples y en grasas saturadas; y un consumo moderado de vino durante las comidas (Willett y col., 1995; Serra-Majem y col., 2004; Trichopoulou 2004; Bach-Faig y col., 2006). Las principales características de esta dieta se recogen en la denominada Pirámide de la DM (**Figura 2**). Además, la DM se asocia a otros elementos que no son puramente alimentarios, como la actividad física regular, los factores culturales y sociales asociados al acto de comer así como un descanso oportuno.

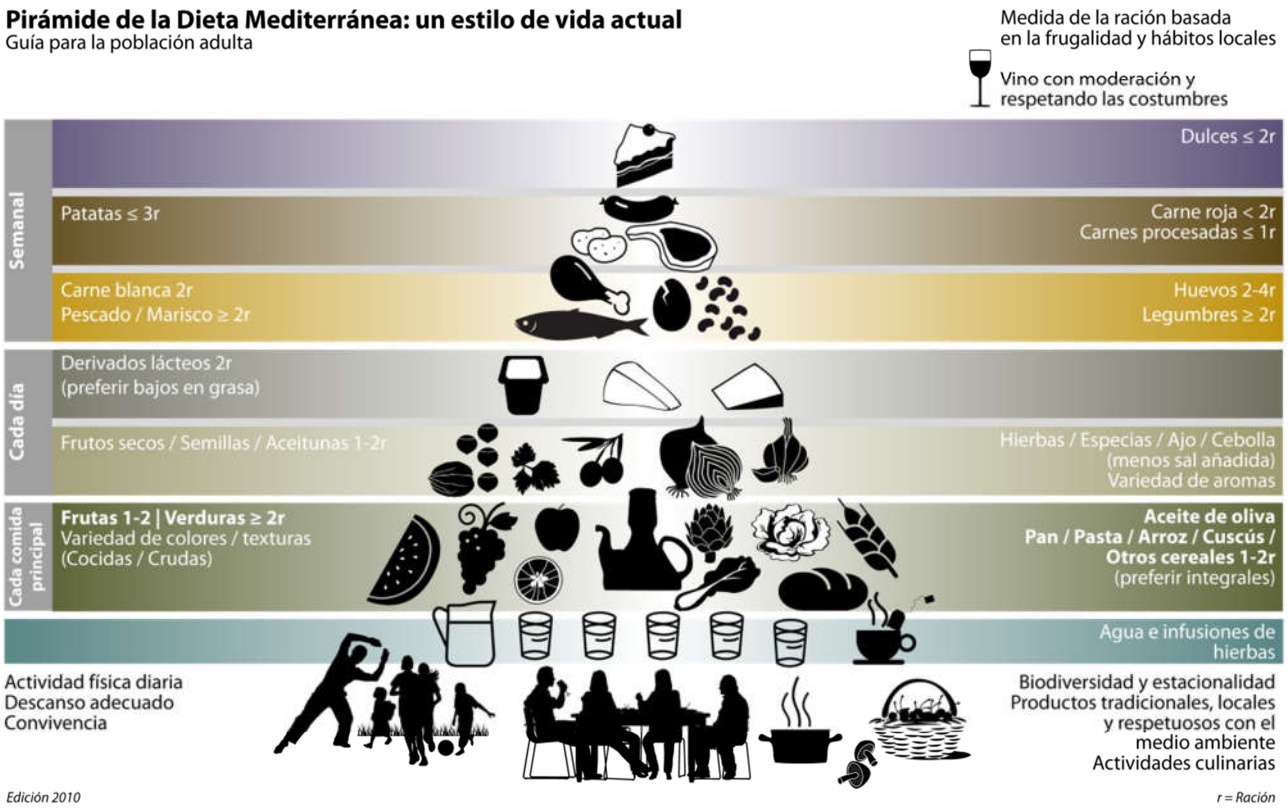

Figura 2. Pirámide de la Dieta Mediterránea. Fuentes: Fundación Dieta Mediterránea (disponible en https://dietamediterranea.com/nutricion-saludable-ejercicio-fisico/#piramide) y Bach-Faig y col. (2011).

1.3. Evolución de la adherencia a la Dieta Mediterránea

Como se ha comentado anteriormente, la definición actual de DM no se haya totalmente consensuada y es en cierto modo teórica, ya que se trata de un patrón dietético que tiene

características diferenciales entre los diferentes países y ciudades Mediterráneos (Noah 2001; Karamanos y col., 2002). Muchos estudios han definido la DM como un patrón alimentario saludable al que se ha ido perdiendo adherencia progresivamente en todos los países del mundo en las últimas décadas.

El índice de adecuación Mediterránea (Mediterranean adequacy index, MAI) mide el grado de adherencia de una población a la DM. El MAI medio mundial entre 1961 y 1965 fue de 2,86, bajando a 2,03 en el período 2000-2003. En 1961-1965 los países Mediterráneos tenían un MAI de 3,44, mientras que los países no Mediterráneos tenían un MAI de 1,55, y en el período 2000-2003 el MAI medio fue de 1,95 y 1,14, respectivamente (Da Silva y col., 2009). La evolución de este parámetro muestra que los países donde más ha disminuido este índice es en los países Mediterráneos, como se puede observar en la **Figura 3**.

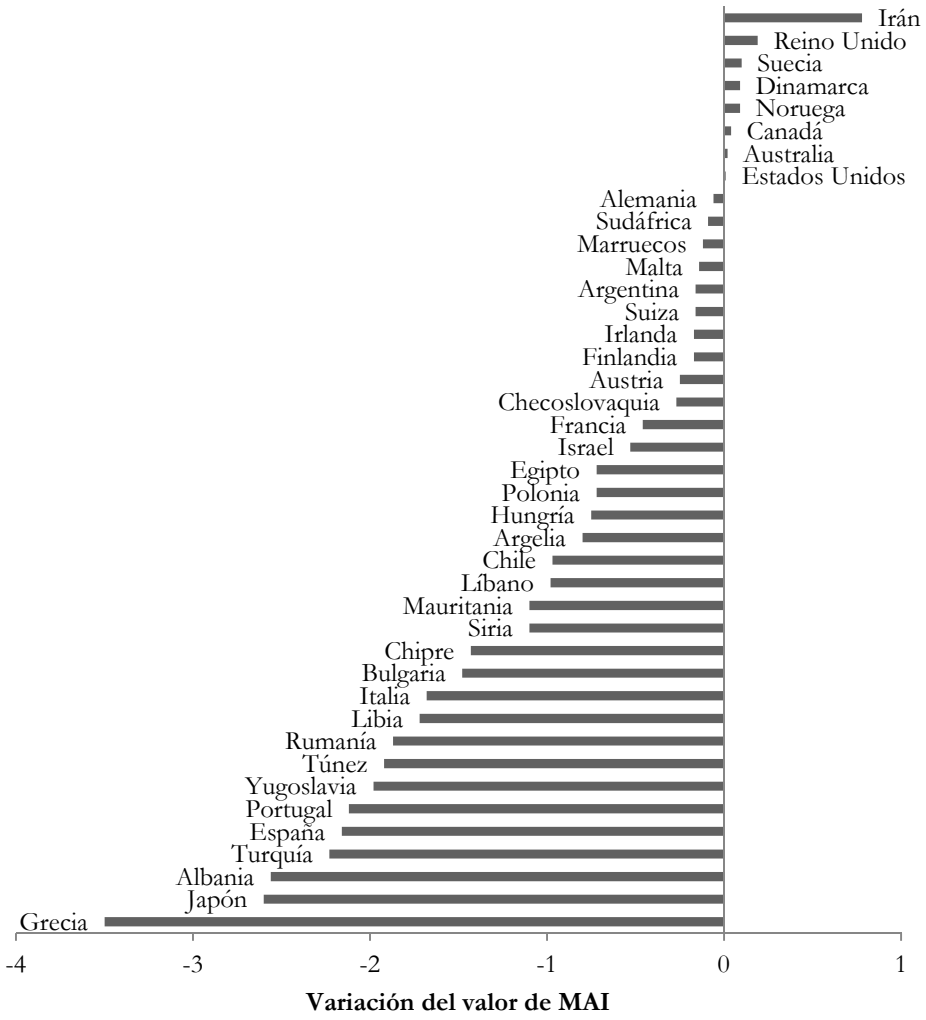

Figura 3. Variación del Índice de Adecuación Mediterránea (MAI) en todos los países entre los períodos 1961–1965 y 2000–2003. Adaptado de Da Silva y col. (2009).

En el caso de España entre el año 1987 al 2005 la adherencia a la DM (adhDM) ha sufrido variaciones, así en el período 1987-1997 se observó una disminución significativa del MAI, entre los años 1998 y 2001 la disminución ya no fue tan marcada, indicando una etapa de estabilización de la adherencia, y finalmente entre los años 2002 y 2005 el MAI mostró un incremento, indicio de una recuperación de la adhDM en España (Bach-Faig y col., 2011).

Todos los cambios citados anteriormente en cuanto a la adhDM se relacionan, entre otros factores, con el consumo de comida preparada, el aumento de las comidas realizadas fuera del hogar (Aranceta, 2001) y la adopción de hábitos culinarios y patrones alimentarios ajenos (Rodríguez-Artalejo, 1996; Popkin y Gordon-Larsen, 2004).

1.4. Cuestionarios de adherencia a la Dieta Mediterránea

Los beneficios de la DM se basan en estudios científicos que en muchos casos relacionan la adhDM con un determinado aspecto de la salud o la enfermedad. Para valorar esta adherencia no hay un método directo único, así como no hay un patrón de DM único a nivel mundial, por tal motivo se suelen utilizar índices o escalas específicas que cuantifican el consumo de alimentos, grupos de alimentos o incluso una combinación de nutrientes y alimentos definidos a priori como característicos de la DM que sigue cada población (Bach y col., 2006; Milà-Villarroel y col., 2011). Muchos de estos estudios reconocen la ingesta diaria de aceite de oliva y el consumo moderado de vino como dos componentes típicos de la DM (Sánchez-Villegas y col., 2006; Buckland y col., 2009; Rumawas y col., 2009; Chrysohoou y col, 2010; Menotti y col., 2012), demostrando que dichos alimentos son distintivos de la DM tradicional. Algunos índices muy utilizados en la investigación clínica son: puntuación de DM de Trichopoulou (Trichopoulou y col., 1995 y 2003), puntuación modificada de DM (Trichopoulou, 2005), puntuación alternativa de DM (Fung y col., 2006), puntuación relativa de DM (Buckland y col., 2009), patrón de DM *a priori* (Martínez-González y col., 2002), valoración de adhDM de PREDIMED (Estruch y col., 2006 y 2013; Schröder y col., 2011) y MAI (Fidanza y col., 2004; Alberti y col., 2009; Menotti y col., 2012). Además, diferentes autores han desarrollado versiones adaptadas a partir de los índices citados anteriormente, como: puntuación de DM relativa adaptada (Buckland y col., 2012a y 2012b), puntuación de DM adaptada por Bamia y col. (2013), puntuación de DM de Knoops y col. (2004) e índice Mediterráneo Italiano (Agnoli y col., 2013) (**Tabla 1**).

Tabla 1. Principales índices de valoración de la adherencia a la Dieta Mediterránea utilizados en estudios de cohorte y caso-control.

Nombre del índice	Autor, año	País/Estudio	Muestra (n)	Rango de puntuación
Puntuación de DM	Trichopoulou y col., 1995 y 2003	Grecia/EPIC	22.043	0-9
Puntuación de DM modificada	Trichopoulou y col., 2005	Europa[1]/EPIC	74.607	0-9
Puntuación de DM alternativa	Fung y col., 2006	Estados Unidos/Nurses' Health Study	71.058	0-9
Puntuación de DM relativa	Buckland y col., 2009	España/EPIC	41.078	0-18
Patrón de DM *a priori*	Martínez-González y col., 2002	España/Caso-control	342	0-8
Valoración de adhDM de 14 puntos	Estruch y col., 2006 y 2013; Schröder y col., 2011	España/ PREDIMED	772	0-14
MAI	Fidanza y col., 2004	16 cohortes[2] /Seven Countries Study	12.763	Relación entre ingesta de alimentos Med vs no Med
Puntuación de DM relativa adaptada	Buckland y col., 2012a y 2012b	España/EPIC	40.622	0-18
Puntuación de DM adaptada	Bamia y col., 2013	Europa[3]/EPIC	5.296.617	0-9
Puntuación de DM de Knoops	Knoops y col., 2004	Europa[4]/HALE	1.507	0-8
Índice Mediterráneo Italiano	Agnoli y col., 2013	Italia/EPIC	45.275	0-11

[1] = Alemania, Dinamarca, España, Francia, Grecia, Holanda, Inglaterra, Italia y Suecia;
[2] = Estados Unidos, ex-Yugoslavia, Finlandia, Grecia, Holanda, Italia, Japón y Serbia;
[3] = Alemania, Dinamarca, España, Francia, Grecia, Holanda, Inglaterra, Italia, Noruega y Suecia;
[4] = Bélgica, Dinamarca, Italia, España, Finlandia, Francia, Grecia, Holanda, Hungría, Portugal y Suiza;
EPIC, European Prospective Investigation into Cancer and Nutrition Study; HALE, The Healthy Ageing: a Longitudinal study in Europe; MAI, Mediterranean adequacy index; Med, Mediterráneos; PREDIMED, Prevención con Dieta Mediterránea Study.

Todos estos índices y sistemas de puntuación resultan de gran ayuda en la cuantificación de parámetros, a veces muy subjetivos, como la adherencia a un patrón alimentario determinado, donde las cantidades y frecuencia de consumo de alimentos pueden diferir de una población a otra. Pero sobre todo, son la esencia de muchos estudios de cohorte y de intervención nutricional como se verá a continuación.

2. Principales estudios clínicos relacionados con la Dieta Mediterránea

En las últimas décadas se han desarrollado diversos estudios clínicos relacionando la DM o alguno de sus componentes en relación con diferentes patologías de elevada incidencia, para explicar su efecto en la prevención de estas enfermedades. Estos estudios se basan, especialmente, en la valoración, mediante índices de adhDM, del consumo de alimentos típicos de la DM en poblaciones de diferentes países. En la **Tabla 2** se presenta un resumen de los estudios más destacados.

Tabla 2. Resumen de los estudios clínicos más destacados sobre Dieta Mediterránea.

Nombre del estudio	Tipo de estudio	Tipo de dieta de los participantes
Seven Countries	Estudio de cohorte prospectivo multicéntrico	DM tradicional
EPIC	Estudio de cohorte prospectivo multicéntrico	Dieta propia de los países de estudio
PREDIMED	Estudio de intervención nutricional controlado, aleatorizado	DM + AOVE (intervención) DM + frutos secos (intervención) Dieta baja en grasa (control)
Lyon Heart	Estudio de intervención nutricional controlado aleatorizado	DM (intervención) Dieta Francesa (control)
Moli-Sani	Estudio de cohorte prospectivo	Dieta tipo "Mediterránea" Dieta tipo "Occidental" Dieta tipo "huevos y dulces"
HALE Project	Incluye participantes de 2 estudios longitudinales: SENECA y FINE	Dieta propia de los países de estudio
Healthy Eating for Colon Cancer Prevention Study	Estudio de intervención nutricional	Dieta de alimentación saludable DM modificada
ATTICA	Estudio de cohorte longitudinal	Dieta propia del país de estudio (DM)
EUROLIVE	Ensayo clínico cruzado, controlado, doble-ciego, aleatorizado	Intervención nutricional con aceite de oliva con concentración variable de polifenoles
NUTRAOLEOUM	Ensayo clínico controlado, doble-ciego, aleatorizado	Intervención nutricional con aceite de oliva
Aceite de oliva, dieta y CCR	Estudio ecológico	Dieta propia del país de estudio
Consumo de alcohol y prevención del CCR en una población Mediterránea	Sub-estudio del ATTICA, caso-control	Dieta propia del país de estudio (DM)
Prevalencia y riesgo de CCR en consumidores de alcohol	Observacional, transversal	Dieta propia del país de estudio
Consumo de alcohol pre-diagnóstico y supervivencia al CCR	Observacional, prospectivo	Dieta propia de los países de estudio

AOVE, Aceite de oliva virgen extra; CCR, Cáncer colorrectal; DM, Dieta Mediterránea; EPIC, European Prospective Investigation into Cancer and Nutrition Study; HALE, The Healthy Ageing: a Longitudinal study in Europe; PREDIMED, Prevención con Dieta Mediterránea Study.

Tabla 2. (Continuación)

Ámbito geográfico	Población de estudio	Tamaño de la muestra (n)	Tiempo de seguimiento/ observación
Estados Unidos, Finlandia, Grecia, Holanda, Italia, Japón, Yugoslavia	Hombres 49-59 años	12.763	25 años
Alemania, Dinamarca, España, Francia, Grecia, Holanda, Italia, Noruega, Reino Unido y Suecia	Hombres y mujeres > 35 años	521.000	15 años
España	Hombres y mujeres 55-80 años	7.500	5 años
Lion, Francia	Hombres y mujeres < 75 años	584	5 años
Región de Molise, Italia	Hombres y mujeres > 35 años	24.325	> 10 años
Bélgica, Dinamarca, España, Finlandia, Francia, Grecia, Holanda, Hungría, Italia, Polonia, Portugal, Serbia y Suiza	Hombres y mujeres 70-90 años	2.339	10 años
Estados Unidos	Hombres y mujeres 22-82 años	108	6 meses
Región de Attica, Grecia	Hombres y mujeres 18-89 años	3.042	10 años
Alemania, Dinamarca, España, Finlandia e Italia	Hombres 30-60 años	200	3 semanas
Granada, España	Hombres y mujeres 20-50 años	57	3 semanas
Alemania, Australia, Austria, Brasil, Canadá, China, Colombia, Dinamarca, España, Estados Unidos, Finlandia, Francia, Grecia, Holanda, Hungría, India, Inglaterra, Irlanda, Israel, Italia, Japón, Noruega, Nueva Zelanda, Polonia, Portugal, República Checa, Suecia, Yugoslavia	Población total de cada país	datos cada 10.000 habitantes	1 año
Región de Attica, Grecia	Hombres y mujeres > 50 años	500	1 año
Nueva York, Estados Unidos	Hombres y mujeres 47-68 años	2.291	10 años
Australia, Canadá, Estados Unidos	Hombres y mujeres 35-75 años	4.996	5 años

2.1. Seven Countries Study: Liderado por Ancel Keys entre 1958 y 1964, comparó los hábitos dietéticos de diferentes cohortes de Estados Unidos, Japón, Finlandia, Holanda, la antigua Yugoslavia, Italia y Grecia (Keys, 1970). El seguimiento de estas poblaciones durante 5-15 años confirmó una menor mortalidad por enfermedad coronaria y una mayor esperanza de vida en los países mediterráneos, particularmente en Grecia (Keys y col., 1986 y 1995; Fung y col., 2009). Además, se observó que el consumo de fibra vegetal está relacionado con la disminución del cáncer colorrectal (CCR) (Jansen y col., 1999).

2.2. EPIC, European Prospective Investigation into Cancer and Nutrition Study: Se trata de un estudio de cohorte, prospectivo y multicéntrico, diseñado para investigar la relación entre la nutrición y el cáncer, con potencial para estudiar otras enfermedades. El objetivo principal es investigar, de manera prospectiva, la etiología de diferentes tipos de cáncer en relación con la dieta y el estilo de vida, intentado identificar interacciones entre factores nutricionales, genéticos y de estilo de vida (Riboli y col., 2002). Hay subestudios que analizan la adherencia a la DM con el riesgo de diversos tipos de cáncer, entre ellos el CCR (Bamia y col., 2013).

2.3. PREDIMED, Prevención con Dieta Mediterránea: Es un estudio de grupos paralelos, aleatorio, multicéntrico y controlado cuyo objetivo es valorar los efectos de la DM en la prevención primaria de enfermedades cardiovasculares. Hasta el momento ha demostrado un asociación inversa entre adhesión a la DM suplementada con aceite de oliva virgen extra o frutos secos y enfermedad cardiovascular (Estruch y col., 2013). Recientemente se ha descrito que un consumo elevado de frutos secos se asocia con una reducción de un 40% del riesgo de mortalidad por cáncer (Guasch-Ferre y col., 2013).

2.4. Lyon Heart Study: Investiga el efecto de una dieta tipo Mediterránea rica en ácido α-linolénico (según las Guías dietéticas de la American Heart Association) sobre diversos factores de riesgo que afectan a la tasa de recurrencia coronaria en pacientes que ya han sufrido un primer infarto de miocardio. Los resultados obtenidos a partir de este estudio muestran que los hábitos alimentarios asociados a la DM disminuyen o mejoran los factores de riesgo asociados a las enfermedades cardiovasculares tanto a nivel de prevención primaria (de Lorgeril y col., 1999; Kris-Etherton y col., 2001) como secundaria (de Lorgeril y col., 1999).

2.5. Moli-Sani Study: Estudio de cohorte prospectivo que tiene por objetivo investigar los factores genéticos y los factores de riesgo/protección de enfermedades cardiovasculares y tumorales (Iacoviello y col., 2007). Los resultados parecen indicar que la dieta de tipo Mediterráneo influye positivamente en diversas enfermedades de alto riesgo en la población occidental, como la diabetes mellitus, la enfermedad cardiovascular o el cáncer entre otras (Iacoviello y col., 2007; Bonaccio y col., 2016).

2.6. HALE Project, Healthy Ageing: a Longitudinal study in Europe: Realizado en una población de ancianos de 13 países europeos, demostró que la DM, junto a un estilo de vida saludable, se asocia a una disminución de más del 50% en la tasa de mortalidad total y por causas específicas, como la enfermedad cardiovascular (64%) y el cáncer (61%) (Knoops y col., 2004 y 2006).

2.7. Healthy Eating for Colon Cancer Prevention Study: Evaluó la interacción entre polimorfismos de los genes ácido graso (AG) desaturasa-1 y -2, y los cambios dietéticos en la concentración de AGs en suero y en el tejido del colon de individuos con elevado riesgo de padecer CCR que siguieron dos tipos de dieta (DM *versus* dieta saludable) durante 6 meses. Los resultados demostraron una interacción significativa entre dieta y genotipo en cuando a la concentración de ácido araquidónico (AA) en el tejido colónico; así, los individuos que presentaban todos los alelos de estos dos genes estudiados y seguían la DM presentaron una concentración de ácido AA en el tejido del colon un 16% menor que los participantes que seguían la dieta de tipo saludable. Indicando que los genotipos de las AG desaturasas podrían modificar el efecto que tiene la dieta sobre las concentraciones de AA en el colon (Djuric y col., 2013; Porenta y col., 2013).

2.8. ATTICA Study: Uno de los objetivos del estudio fue evaluar el nivel de adhDM en relación con la capacidad antioxidante total en plasma. Así observaron que los participantes con una elevada adhDM tenían mayor capacidad antioxidante y menor concentración de LDL oxidada. Además, la capacidad antioxidante plasmática resultó estar positivamente relacionada con el consumo de aceite de oliva, frutas y vegetales, mientras que se asoció negativamente al consumo de carnes rojas. De este modo los autores concluyen que la buena adhDM se asocia a una menor

oxidación plasmática y por lo tanto podría estar relacionada con los efectos beneficiosos de la DM sobre la enfermedad cardiovascular y el cáncer (Pitsavos y col., 2005).

2.9. Estudios clínicos sobre aceite de oliva o vino y cáncer:

Hay diversos estudios clínicos que investigan la relación entre algunos componentes específicos de la DM, como el aceite de oliva y el vino, sobre diferentes enfermedades de elevada prevalencia. A continuación se presentan algunos relacionados específicamente con el CCR:

2.9.1. EUROLIVE Study: Uno de los objetivos del estudio fue evaluar el efecto de los polifenoles del aceite de oliva sobre la capacidad inmunogénica de la LDL oxidada para producir autoanticuerpos. Se demostró que los polifenoles del aceite de oliva promueven la generación autoanticuerpos, siendo este efecto concentración-dependiente (Castañer y col., 2011). Además, observaron que el aceite de oliva previene la oxidación del DNA, muy relacionada con el desarrollo del CCR. Dicho efecto pareció estar relacionado principalmente con el contenido en AGs monoinsaturados (AGMI), y no de polifenoles, del aceite de oliva (Machowetz y col., 2007).

2.9.2. NUTRAOLEOUM Study: El estudio pretende aportar evidencia de primer nivel sobre los beneficios para la salud in vivo en seres humanos de triterpenos del aceite de oliva (ácido oleanólico y maslínico) además de su biodisponibilidad y disposición (Biel y col., 2016), ambos relacionados con la prevención del crecimiento de células tumorales del colon (Li y col., 2015; Reyes-Zurita y col., 2016).

2.9.3. Aceite de oliva, dieta y cáncer colorrectal: El objetivo de este estudio ecológico fue comparar los niveles nacionales de diversos componentes dietéticos, con particular énfasis en el aceite de oliva, con la incidencia de CCR de cada país. Tras analizar los datos de 28 países observaron que el aceite de oliva parece tener un efecto protector sobre el desarrollo del CCR. Incluso proponen como hipótesis que el aceite de oliva puede influir en el patrón de ácidos biliares secundarios en el colon que, a su vez, podría influir en el metabolismo de las poliaminas en los enterocitos del colon de formas que reducirían la progresión de la mucosa normal a adenoma y carcinoma (Stoneham y col., 2000).

2.9.4. Consumo de alcohol y prevención del CCR en una población Mediterránea: El objetivo del estudio fue valorar la relación entre el consumo y tipo de alcohol y la incidencia de CCR. Se observó que la asociación entre la cantidad de alcohol consumida y la presencia de CCR sigue una curva en forma de J. Demostrando, por un lado, el efecto nocivo del consumo elevado de alcohol, y por otro, la capacidad protectora del consumo moderado de alcohol, relacionada posiblemente con los efectos del vino tinto (Kontou y col., 2012a)

2.9.5. Prevalencia y riesgo de cáncer colorrectal en consumidores de alcohol: El objetivo de este estudio transversal fue analizar el impacto del consumo regular de alcohol en la detección de neoplasias colorrectales en un cribado poblacional. Los autores observaron un mayor riesgo de neoplasia en los pacientes que bebían cerveza y bebidas espirituosas y una disminución del mismo en los que bebían vino, en comparación con los pacientes abstemios (Anderson y col., 2005)

2.9.6. Consumo de alcohol pre-diagnóstico y supervivencia al cáncer colorrectal: El objetivo de este estudio fue analizar la relación entre el consumo de alcohol antes del diagnóstico de CCR y la supervivencia posterior de los pacientes. No se observó ninguna asociación significativa entre la supervivencia al CCR y el consumo de bebidas espirituosas y cerveza, mientras que los niveles más altos de consumo de vino se asociaron modestamente con un mejor pronóstico global del CCR (Phipps y col., 2017). Estos resultados concuerdan con un estudio de características muy similares publicado por la misma autora en el año 2016 (Phipps y col., 2016).

3. Efectos de la Dieta Mediterránea sobre la longevidad y la salud

Es indudable que un estilo de vida saludable es primordial para aumentar la esperanza y la calidad de vida. En este caso, la DM cumple con estas características saludables tan apreciadas para una vida larga y sana. Así, se ha demostrado que una elevada adhDM está inversamente relacionada con la mortalidad y la incidencia de enfermedades crónicas (Sofi y col., 2008), y además, es beneficiosa en el proceso de envejecimiento, retrasando la pérdida de capacidad cognitiva, la disminución de la movilidad y la aparición de demencia (Feart y col., 2009; Scarmeas y col., 2009; Milaneschi y col., 2011). En este sentido, los resultados del estudio HALE Project, tras 10 años de seguimiento, hombres y mujeres de entre 70 y 90 años, con elevada adhDM, no fumadores (o ex-fumadores de más de 15 años), activos físicamente y que bebían alcohol moderadamente presentaron una menor

mortalidad, y especialmente una disminución de la mortalidad debida a enfermedad cardiovascular y cáncer (Knoops y col., 2004 y 2006).

3.1. Efectos de la Dieta Mediterránea sobre enfermedades de alta prevalencia

Actualmente, uno de los objetivos principales de la investigación científica en torno a la DM es conocer mejor como afecta a la incidencia de enfermedades metabólicas muy frecuentes en nuestra población, como la obesidad, la dislipemia, el síndrome metabólico y la diabetes mellitus, que son patologías relacionadas con nuestro estilo de vida, y principalmente con los factores dietéticos (Caterson y col., 2004; Kastorini y col., 2011; Perez-Martinez y col., 2011).

Otra de las principales causas de morbilidad y mortalidad en nuestra sociedad son las enfermedades cardiovasculares, la hipertensión y los accidentes cerebrovasculares. En el apartado 2 de esta introducción se pueden consultar algunos estudios clínicos más destacados (Seven Countries, PREDIMED, Lyon Heart Study, Moli-Sani Study, etc.) que muestran una relación positiva entre la adhDM y la disminución de la prevalencia de estas enfermedades y la mejora de su pronóstico. Los resultados de estos estudios se complementan con numerosos trabajos que avalan los beneficios de la DM sobre la salud cardiovascular (de Lorgeril y col., 1999; Fidanza y col., 2004; Misirli y col., 2012; Shen y clo., 2015; Widmer y col., 2015).

Finalmente, el cáncer es otra de las principales causas de muerte en los países desarrollados y uno de los factores que contribuyen a su desarrollo es el estilo de vida, y dentro de este el patrón alimentario.

3.2. Efecto de la Dieta Mediterránea sobre el cáncer

Hay numerosas evidencias epidemiológicas que demuestran que la adhDM se relaciona con la protección frente a diferentes tipos de cáncer (Sofi y col., 2008 y 2010). Desde hace más de una década se han desarrollado estudios observacionales que muestran una disminución del cáncer en los países Mediterráneos en comparación con países del norte de Europa, Inglaterra y Estados Unidos (Trichopoulou y col., 2000). Un meta-análisis recientemente publicado por Sofi y col. (2014) mostró que la adhDM está relacionada con una reducción del 4% de enfermedades neoplásicas en cuanto a incidencia y/o mortalidad, en coincidencia con los resultados aportados por dos meta-análisis publicados por Schwingshackl y Hoffmann (2014a y 2015). Estos autores observaron además que los tipos de cáncer más susceptibles a los beneficios de la DM son el CCR,

el de tracto aerodigestivo, de mama, de estómago, de páncreas, de próstata, de hígado y de cabeza y cuello. Tras un meta-análisis incluyendo 11 estudios de cohorte y más de 1.7 millones de participantes, se estimó una disminución del 13% de la mortalidad por cáncer en las personas con una adhDM elevada (Schwingshackl y Hoffmann, 2015).

Un estudio realizado en la población Griega, demostró que la adhDM se asocia a una disminución de un 12% en la incidencia de cáncer. Más específicamente, los componentes de la DM a los que se les atribuyó este efecto fueron el consumo de lípidos con una elevada proporción monoinsaturado/saturado, y el consumo de vegetales y aceite de oliva (Benetou y col., 2008). En este mismo sentido, otro estudio caso-control que agrupaba diversas sub-poblaciones Italianas encontró una disminución significativa del riesgo de diferentes tipos de cáncer en aquellas personas con una elevada adhDM (Bosetti y col., 2009). A pesar de estos resultados, que demuestran el efecto potencialmente beneficioso de una elevada adhDM sobre la mortalidad por cáncer y la recurrencia del mismo en pacientes que ya han superado un cáncer, otros estudios de cohorte no han encontrado asociaciones significativas (Cottet y col., 2005; Kim y col., 2011; Fung y col., 2014; Kenfield y col., 2014).

3.2.1. Efecto de la Dieta Mediterránea sobre el cáncer colorrectal

La etiología del CCR es multifactorial y está influenciada por una variedad de factores genéticos y ambientales (Quadrilatero y Hoffman-Goetz, 2003), siendo estos últimos la causa de más del 90% de estos tipos de cáncer (D'Alessandro y col., 2016). De este modo, el patrón dietético puede influir en el riesgo de desarrollar CCR (Gingras y Béliveau, 2011; Schwingshackl y Hoffmann 2014a). Además, el desarrollo relativamente lento de las etapas benignas de este tipo de cáncer permite la existencia de ventanas de oportunidad para la prevención del mismo a través de estrategias dietéticas que incluyen componentes bioactivos característicos del patrón dietético Mediterráneo (Donovan y col., 2016).

La DM se asocia con una disminución del riesgo de CCR, en cambio el patrón dietético Anglosajón está claramente asociado a un elevado riesgo (Yusof y col., 2012; Agnoli y col., 2013; Bamia y col., 2013). Según datos de la OMS la prevalencia de CCR en algunos países del norte del Mediterráneo (Italia, Francia y España) presenta una tendencia similar a otros países desarrollados de Europa (Inglaterra y Alemania), del Norte de América (Canadá y Estados Unidos), y de Oceanía (Australia, Nueva Zelanda). Mientras que otros países del Sur de la costa Mediterránea (Marruecos, Argelia y Libia) presentan un riesgo más bajo, y similar a otros países Asiáticos (China e India)

(WHO, 2014). Esto puede ser debido en gran parte a cambios en los patrones dietéticos tradicionales, que actualmente evolucionan de una DM tradicional a un patrón dietético anglo-americano, como consecuencia, principalmente de la globalización.

En diversos estudios de cohorte y caso-control se ha encontrado relación inversa significativa entre la adhDM y el riesgo de CCR (Fung y col., 2005; Dixon y col., 2007; Kontou y col., 2012b; Bamia y col., 2013; Grosso y col., 2014), mientras que otros estudios observan esta tendencia pero sin llegar a ser significativa (Reedy y col., 2008; Tognon y col., 2012; Whalen y col., 2014). Según resultados de un estudio de cohorte en una población Italiana, una elevada adhDM se asocia a una disminución significativa del riesgo de CCR (Agnoli y col., 2013). Estos datos concuerdan con otros observados en otras poblaciones Europeas, e incluso en una cohorte de Estados Unidos, según describió en un meta-análisis Sofi y col. (2008).

Según los resultados de un meta-análisis que incluyó la mayoría de los estudios citados anteriormente, y que englobó cuatro estudios de cohorte y tres de caso-control, la adhDM redujo un 15% la incidencia de CCR (Schwingshackl y Hoffmann, 2015). Por ello, a partir de las evidencias aportadas en los últimos años, la OMS promueve es estilo dietético Mediterráneo como un patrón de prevención de las enfermedades crónicas, entre las que se encuentra el CCR (Zappia y col., 1995; World Health Organization Study Group, 2003).

3.2.1.1. Cáncer colorrectal

El CCR es el tercero más frecuente y el cuarto por su tasa de mortalidad a nivel mundial. El 55% de los casos se dan en países desarrollados, pero la mayoría de las muertes asociadas al CCR (52%) se dan en país subdesarrollados (Ferlay y col., 2015).

En España en el año 2012 la incidencia de CCR fue del 29%, sobre el total de casos de cáncer diagnosticados, siendo el segundo más frecuente en mujeres y el tercero en hombres; y el segundo por tasa de mortalidad por cáncer (Sociedad Española de Oncología Médica, 2016).

La incidencia del CCR ha aumentado de forma progresiva en los últimos años, y se estima que lo continuará haciendo, lo cual es consecuencia no sólo del aumento de la población, sino del envejecimiento de la misma y de la mejora de las técnicas de detección precoz, que son capaces de detectar la enfermedad en estadios iniciales. Además, los cambios en el patrón dietético pueden estar influyendo en este aumento de la incidencia del CCR. Dado que se considera que el 70-80%

de los factores de riego del CCR son de origen ambiental, como es el caso de las características culturales, sociales y de estilo de vida, que incluirían los factores dietéticos (Giovannucci, 2002). Por otro lado encontramos los factores no modificables, como la edad, la historia personal o familiar de CCR o de pólipos adenomatosos y la historia personal de enfermedad inflamatoria intestinal (Jussila y col., 2013). Así, hay que destacar, que a diferencia de otros tipos de cáncer muy relacionados con factores genéticos, la etiología del CCR se relaciona sobre todo con factores modificables, como es el caso de la alimentación.

3.2.1.1.1. Definición y clasificación del cáncer colorrectal

El epitelio del colon está formado por pequeñas invaginaciones, o criptas de Lieberkühn, que aumentan la superficie epitelial y contribuyen a la funcionalidad del mismo. Las células epiteliales diferenciadas que conforman el epitelio se alinean en la parte superior de la cripta y median la función secretora y absortiva de la mucosa colónica. Esta población celular tiene una vida media muy corta y, tras sufrir apoptosis, se desprenden hacia la luz intestinal (van der Wath y col., 2013). Esta renovación celular, que dura unos 3 – 4 días, está mediada por células madre pluripotentes situadas en la base de las criptas, que dan lugar a células amplificadoras temporales, que migran por la pared de la cripta hacia el exterior de la misma y se diferencian, finalmente, en células epiteliales funcionales o colonocitos (Tany col., 2013). El mantenimiento normal de la mucosa del colon depende de la regulación homeostática precisa del equilibrio entre la apoptosis de las células epiteliales diferenciadas y la proliferación y diferenciación de células madre y células amplificadoras temporales (Donovan y col., 2016) (**Figura 4**).

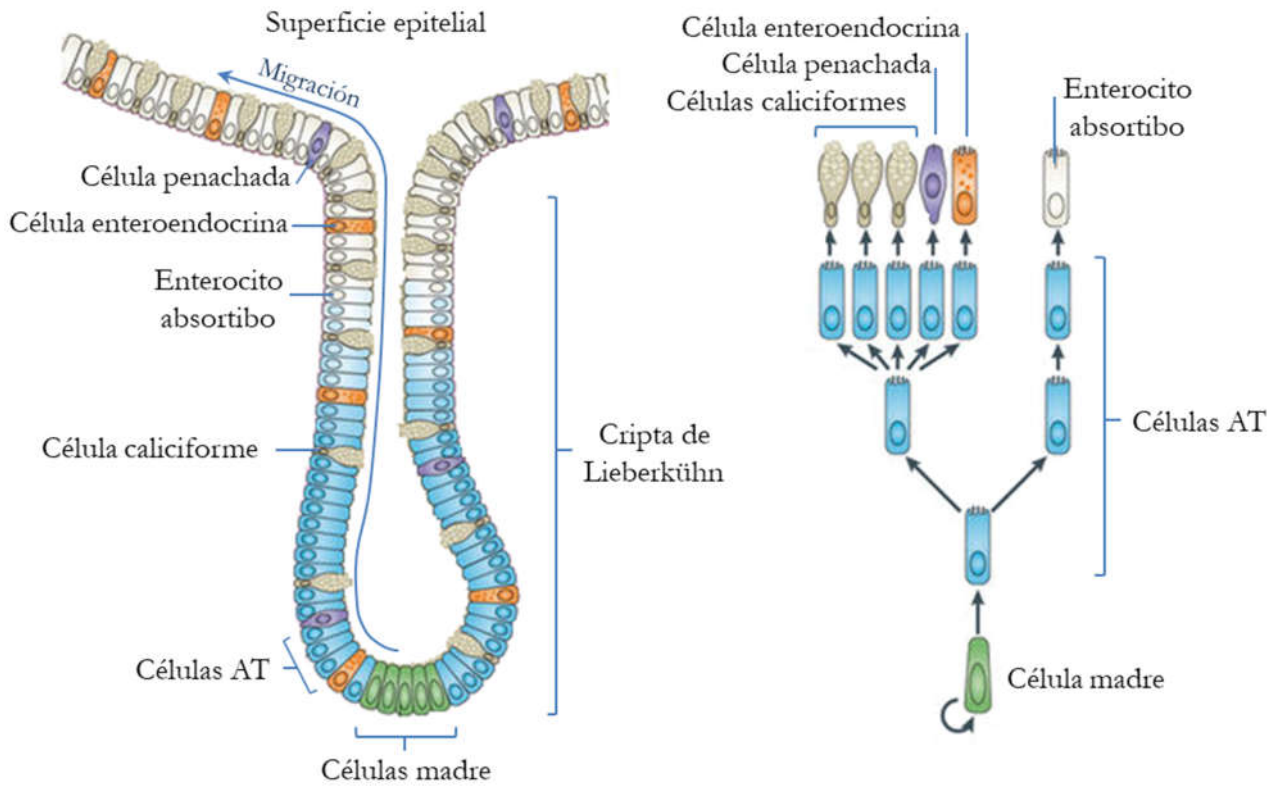

Figura 4. Proceso de renovación celular en el tejido colónico. Adaptado de (Barker 2014). Células AT, células amplificadoras temporales.

El inicio del CCR se asocia a una pérdida de las funciones del gen APC (Adenomatous polyposis coli) que se traduce en la formación de microadenomas precancerosos (Berrino y Muti, 1989). La progresión de éstos últimos hacia adenomas de mayor tamaño se asocia con la regulación negativa de la vía supresora de tumores CDC4/CIN y la sobre-regulación de la vía oncogénica KRAS/BRAF. Finalmente, la progresión de tumores benignos a malignos está relacionada con la regulación negativa de las vías p53/BAX y SMAD4/TGFβ, y con la regulación positiva de la vía PIK3CA/PTEN (**Figura 5**). Esta transición, de un microadenoma a un carcinoma avanzado, se desarrolla durante 15 o 20 años, en cambio la última fase, de carcinoma a tumor metastásico, puede darse en apenas 2 o 3 años (Donovan y col., 2016).

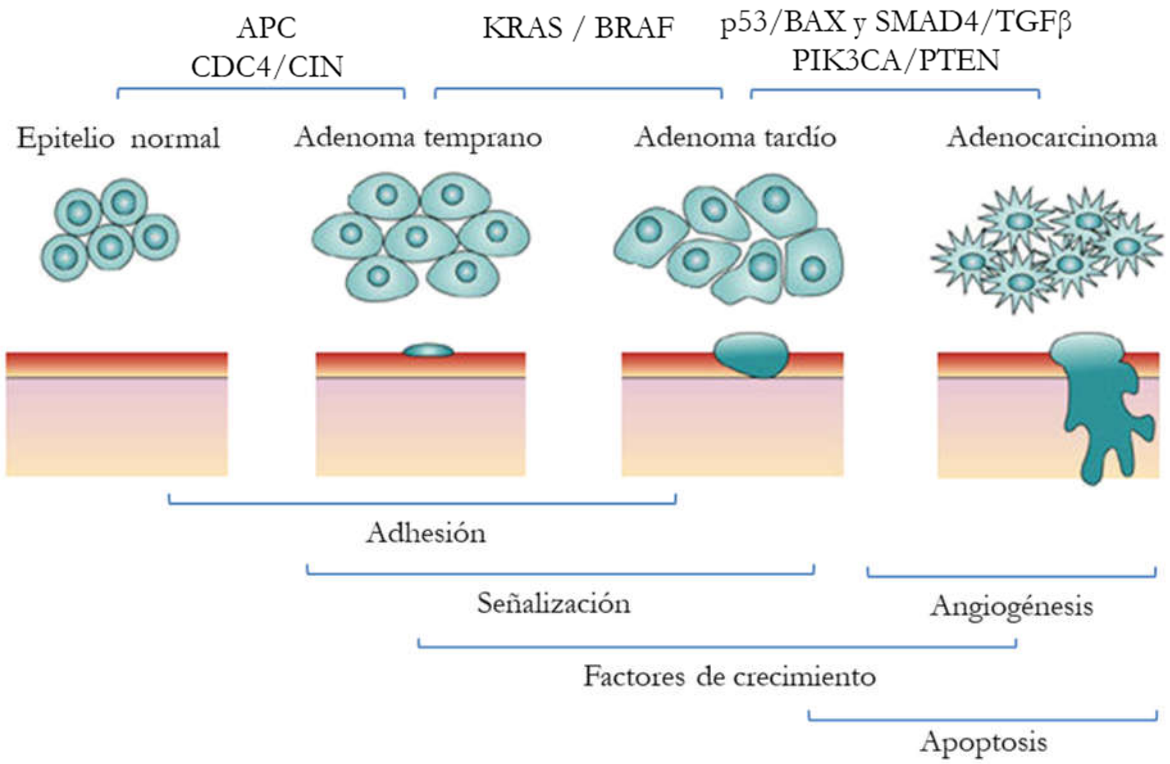

Figura 5. Proceso de desarrollo y progresión del adenocarcinoma. Inicialmente se produce una pérdida de funciones del gen APC, que codifica una proteína implicada en la adhesión celular y la transcripción. Después, se ven alteradas las vías KRAS/BRAF, implicadas en la producción de factores de crecimiento y en el control de la proliferación que promueven el crecimiento anormal de las células tumorales. Posteriormente se desregulan las vías p53/BAX, SMAD4/TGFβ y PIK3CA/PTE, que promueven la angiogénesis y la resistencia de las células cancerosas a la apoptosis. Adaptado de Kerr y col. (2003) y de Subramaniam y col. (2016).

Por su forma de originarse y expresarse se distinguen tres tipos de CCR: el esporádico, el familiar y el hereditario. El CCR esporádico supone un 60-80% de los casos y no se asocia a ninguna mutación genética ni tiene vinculación familiar. De todos modos, el factor genético parece influir en la probabilidad de la aparición del CCR, aún en ausencia de una mutación específica (Watson y Collins, 2011), ya que se origina por la agregación secuencial de aberraciones en vías oncogénicas y de supresión de tumores (Mundade y col., 2014). El cáncer de origen familiar supone un 20-40% de los casos y tampoco se asocia a una mutación genética específica. El riesgo de desarrollar este tipo de CCR puede verse aumentado cuando los familiares de primera consanguinidad ya presentan CCR de tipo esporádico y también por algunos factores de tipo ambiental (Lin, 2012). El CCR hereditario presenta varios subtipos o síndromes asociados, que varían dependiendo de la presencia, o no, de pólipos adenomatosos. Los dos más frecuentes son la poliposis adenomatosa familiar y el cáncer hereditario no asociado a pólipos. El primero, es el más frecuente de los síndromes hereditarios y representa menos de 1% de los casos de CCR (Markowitz y Bertagnolli, 2009). Se relaciona con mutaciones en el gen supresor de tumores APC (Bertario y col., 1999) y se

caracteriza por la presencia de múltiples pólipos difusos que pueden malignizarse a edad temprana en caso de no ser extraídos a tiempo (de Campos y col., 2010). El segundo, también denominado Síndrome de Lynch, constituye un 3% del total de los casos de CCR y se trata de una variante autosómica y dominante. Se trata de tumores que pueden malignizar en un breve período de tiempo, con elevado riego de desencadenar metástasis en otros órganos fuera del aparato digestivo (Hassen y col., 2012). Se definen dos subtipos: los de tipo I, ubicados exclusivamente en el colon; y los de tipo II, ubicados en otros órganos externos, como el estómago, el páncreas, el sistema nervioso central, los ovarios, etc. (Boland y col., 1998).

3.2.1.1.2. Cáncer colorrectal y la cascada del ácido araquidónico

La inflamación, consecuencia de un daño en la mucosa del colon causado por diferentes agentes ambientales, juega un papel muy importante en los primeros estadios de la carcinogénesis del CCR. La lesión del tejido colónico desencadena una respuesta inmune que da lugar a la proliferación células y a la regeneración tisular. Pero si esta respuesta no es capaz de contrarrestar la agresión, y tarda más de lo normal en resolverse, se crea un ambiente rico en citoquinas, factores de crecimiento y especies oxigenadas reactivas que podrían dar lugar a la aparición de errores genéticos y a una disfunción de la proliferación celular (Mariani y col., 2014).

La cascada del AA comprende la metabolización de diferentes AGs poliinsaturados (AGPI, como el AA, el ácido eicosapentaenoico, EPA, el docosaexaenoico, DHA, linoleico, γ-linolénico) que por medio de enzimas generan numerosos metabolitos. La primera etapa de la cascada está protagonizada por la liberación de AGs a partir de los fosfolípidos de la membrana citoplasmática por la acción de fosfolipasas. Estos enzimas, junto con la aciltransferasa, hidrolizan y esterifican, respectivamente los AGs y contribuyen a mantener una concentración basal muy baja de AGs en estado libre. En una segunda etapa, estos AGs continúan su metabolización a través de tres vías enzimáticas: vía de las ciclooxigenasas (COXs); vía de las lipoxigenasas (LOXs) y vía del Citocromo P450 (CYP450) (Kalish y col., 2012) (**Figura 6**). Dando lugar a los eicosanoides, moléculas biológicamente activas de 20 carbonos, con acción pro- y antiinflamatoria, que actúan como segundos mensajeros (Funk, 2001). Éstos actúan regulando activamente diversas funciones biológicas incluyendo la el sistema inmunitario, la función vascular y la reproducción. Los eicosanoides tienen receptores en diversos tipos celulares del organismo de modo que son capaces de ejercer sus efectos sobre una gran variedad de funciones fisiológicas.

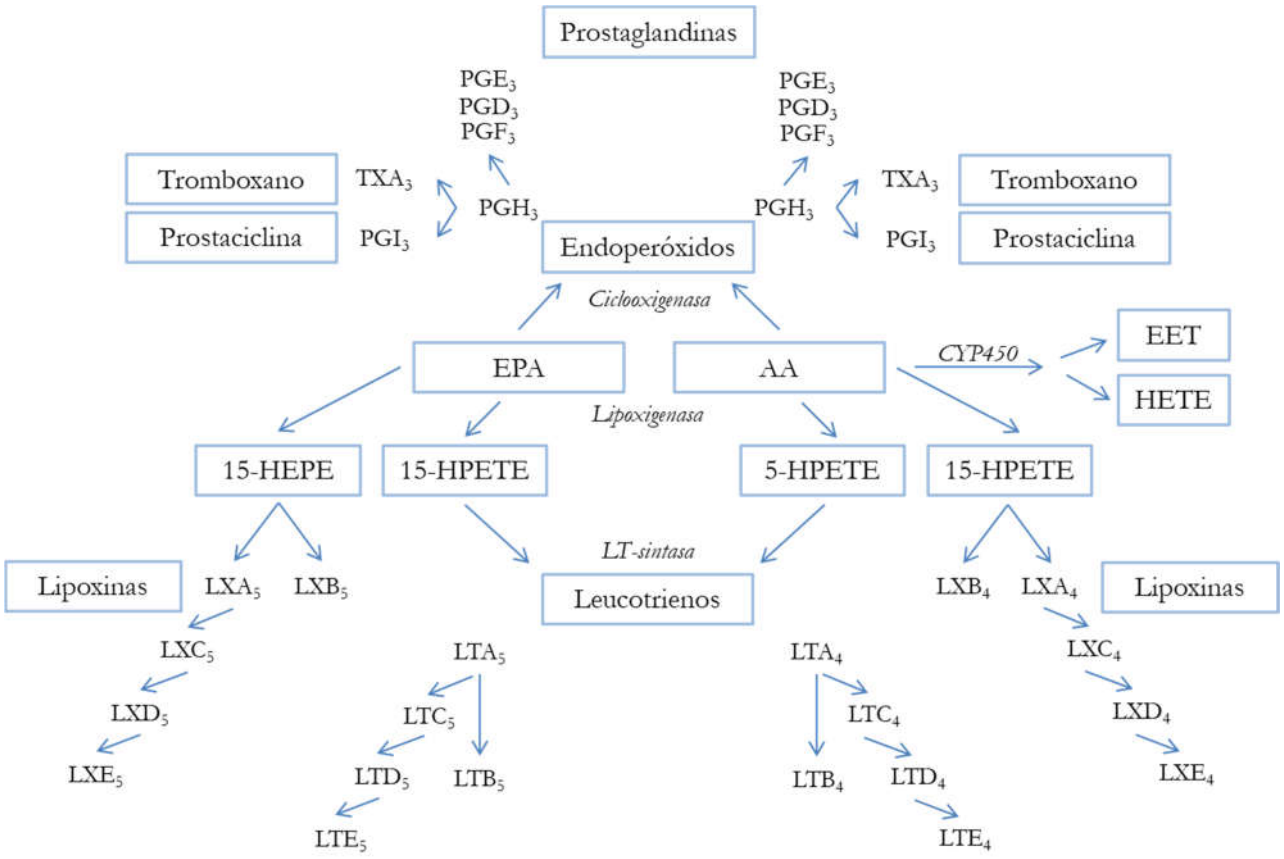

Figura 6. Principales eicosanoides producidos a partir de la metabolización de los ácidos araquidónico y eicosapentaenoico por las tres vías de la cascada del ácido araquidónico. Adaptado de Kalish y col. (2012). AA, ácido araquidónico; CYP450, Citocromo P450; EET, ácido epoxieicosatrienoico; EPA, ácido eciosapentaenoico; HEPE, ácido hidroxieicosapentaenoico; HETE, ácido hidroxieicosatetraenoico; HPEPE, ácido hidroperoxieicosatetraenoico; LT, leucotrieno; LX, lipoxina; PG, prostaglandina; TX, tromboxano.

Hay cuatro tipos principales de eicosanoides, las prostaglandinas, las prostaciclinas, los tromboxanos y los leucotrienos. Las primeras regulan el crecimiento celular y el tono de la musculatura lisa, la sensibilización al dolor y la formación de trombos (Harizi y col., 2008). Las prostaciclinas inhiben la agregación plaquetaria y promueven la vasodilatación, mientras que los tromboxanos promueven la homeostasis mediada por la agregación plaquetaria. Los leucotrienos actúan como agentes quimiotácticos de las células inflamatorias, inducen broncoconstricción y aumentan la permeabilidad vascular (Haeggstrom y Funk, 2011). Los eicosanoides no son almacenados en las células, sino que son sintetizados en respuesta a estímulos extracelulares, como los factores de crecimiento y las hormonas.

La vía de las COXs da lugar a la producción de prostaglandinas, prostaciclinas y tromboxanos, conjuntamente denominados prostanoides. Hay dos enzimas principales en esta vía: la COX-1, de expresión constitutiva en todo en organismo, genera eicosanoides que participan en funciones fisiológicas tales como la agregación plaquetaria, la regulación del flujo sanguíneo renal y la

citoprotección de la mucosa gástrica (Kraemer y col., 1992); y la COX-2, cuya expresión está inducida por estímulos proinflamatorios como citoquinas, factores de crecimiento y promotores tumorales, está implicada en la patogénesis de enfermedades inflamatorias y en el desarrollo del cáncer (Herschman, 1994). Recientemente se ha sugerido que ambas enzimas se expresan por igual en condiciones fisiológicas y patológicas (Zidar y col., 2009). En la mayoría de los casos de CCR existe una sobrexpresión de COX-2 (Gupta y DuBois, 2001; Wang y DuBois, 2010; Cathcart y col., 2011), por eso a partir de los años 90 se estableció que inhibidores selectivos de la COX-2 podían reducir el riesgo de CCR y promover la regresión del tumor tanto en humanos como en modelos animales (Narko y col., 1997; Muller-Deker y col., 2002; Wang y DuBois, 2010).

La vía de las LOXs está conformada por al menos 6 enzimas diferentes de las cuales solo se han detectado tres en humanos (5-, 12- y 15-LOX), cuyo producto final son los leucotrienos y las lipoxinas, siendo estas últimas potentes mediadores antiinflamatorios y moduladoras del tráfico leucocitario (Serhan, 2005). Se ha descrito que generalmente la 5- y la 12-LOX están ausentes en el tejido sano y se expresan en el CCR (Chen y col., 1994; Gao y col., 1995). En este sentido, se ha demostrado la relación entre la expresión de estas enzimas y el aumento del tamaño de los pólipos, siendo más elevada la expresión de la 5-LOX en estadios iniciales y de la 12-LOX en estadios más avanzados del CCR (Melstrom y col., 2008; Wasilewicz y col., 2010; Cathart y col., 2011). Además, se ha demostrado la eficacia de inhibidores de las LOXs en el tratamiento de neoplasias de colon en modelos experimentales (Galfi y col., 2005; Cianchi y col., 2006; Melstrom y col., 2008).

La vía del CYP450 comprende numerosas enzimas que metabolizan el AA. Aunque la mayoría de estos genes se expresan de forma constitutiva, la transcripción de alguno de ellos está regulada por numerosos factores como hormonas, citoquinas, fármacos y compuestos químicos (Waxman, 1999; Capdevila y col., 2000). El producto final de esta vía son los ácidos epoxieicosatrienoicos e hidroxieicosatetraenoicos, los primeros son antiinflamatorios, vasodilatadores y proangiogénicos, mientras que los segundos son proinflamatorios, proangiogénicos y regulan el tono vascular (Imig, 2012; Panigrahy y col., 2012).

3.2.1.1.3. Cáncer colorrectal y factores dietéticos

La dieta puede influir en el riesgo de CCR alterando la expresión de genes involucrados en la inflamación y microbiota intestinales (Berrino y Muti, 1989; Moghaddam y col., 2007; Okabayashi y col., 2012; Agnoli y col., 2013; Bamia y col., 2013; Hullar y Fu, 2014) y en el metabolismo de

xenobióticos (Modan, 1977). En general un pequeño adenoma de colon tarda años en evolucionar a un tumor metastásico (Jones y col., 2008), por tal motivo la dieta y el estilo de vida pueden jugar un papel destacado en la prevención a largo plazo del CCR.

El principal beneficio de consumir cereales integrales o de grano entero sobre la prevención del CCR reside básicamente en el aporte de fibra y compuestos bioactivos presentes en el germen y el salvado del cereal que se pierden durante el refinado del mismo (Fardet 2010). Hay numerosos estudios que han observado una relación inversa el consumo de cereales integrales y el riego de desarrollar CCR (Chatenoud y col., 1998; Jacobs y col., 1998; Bingham y col., 2005; Haas y col., 2009; Aune y col., 2011). El efecto biológico de la fibra como agente protector se basa en su capacidad para aumentar el volumen de las heces y disminuir su tiempo de tránsito, reduciendo así el contacto de los carcinogenes con la mucosa colorrectal (Lipkin y col., 1999). Además la fibra puede secuestrar los ácidos biliares secundarios, que podrían tener un efector pro-tumoral, promoviendo la proliferación celular y favoreciendo las mutaciones y una mayor replicación de células anormales según se ha demostrado experimentalmente (Nagengast y col., 1995; Slavin, 2000). Por último, la fermentación de la fibra da lugar a ácidos grasos de cadena corta, como butirato, acetato y propionato, que podrían tener efectos protectores frente al CCR según Slavin y col. (2000). Estos autores mostraron que los ácidos grasos de cadena corta disminuyen el pH intestinal, y esto inhibe la conversión de ácidos biliares primarios en ácidos biliares secundarios, así se reduce la solubilidad de los ácidos biliares libres, disminuyendo su actividad carcinogénica. Además, la fibra dietética reduce el índice glicémico y mejora la sensibilidad a la insulina, influenciando favorablemente algunos factores de crecimiento relacionados con la promoción de la carcinogénesis (Yu y Rohan, 2000).

Otra fuente de fibra son los vegetales y las frutas, que también parecen ayudar a proteger al colon del cáncer, ya que se ha observado que los individuos que consumen poca cantidad de los mismos podría tener más riesgo de padecer CCR (Benetou y col., 2008; Boffetta y col., 2010; Aune y col., 2011). Cabe destacar que hay otros estudios de cohorte que no coinciden con estos resultados (George y col., 2009). Sin embargo, un meta-análisis realizado en 16 cohortes mostró una disminución de la mortalidad en personas que consumían elevadas cantidades de frutas y verduras, aunque esta relación no resultó significativa en el caso de la mortalidad asociada a cáncer (Arts y Hollman, 2005). Este posible efecto anticancerígeno podría estar relacionado con el elevado contenido en flavonoides de las frutas y los vegetales, que se ha propuesto que podrían actuar como antioxidantes, antiinflamatorios, antimutagénicos o incluso como agentes antiproliferativos (Arts y Hollman, 2005).

La carne roja, descrita como un alimento poco recomendado según el patrón de DM, ha sido asociado con un mayor riesgo de cánceres de tipo digestivo, sobre todo de estómago, colon y recto (Norat y col., 2005). En este sentido un meta-análisis, que incluyó 21 estudios (16 caso-control y 5 de cohorte), encontró la misma relación entre el consumo elevado de carne roja y carnes procesadas y un riesgo elevado de padecer CCR (Xu y col., 2013).

Un elevado consumo de AGs ω-3 e, inversamente, un bajo consumo de AGs ω-6 ha sido asociado con una disminución del CCR tanto en modelos experimentales como en estudios clínicos, ya que estos AGs pueden actuar como supresores de la carcinogénesis y como protectores frente al inicio y progresión de tumores (Murff y col., 2009; Cockbain y col., 2012). Estos resultados van en línea con la recomendación de consumir pescado por su aporte de AGs ω-3, aunque no hay estudios concluyentes que relacionen el consumo de pescado con la incidencia o mortalidad por cáncer (Han y col., 2013; Zheng y col., 2013).

Las vitaminas B2, B6 y D también podrían tener un papel protector frente al CCR (Eussen y col., 2010; Jenab y col., 2010), ya que las vitaminas del grupo B estarían implicadas en el "metabolismo de un carbono", implicado en la metilación del DNA, muy relacionada con el desarrollo de aberraciones cancerígenas; y la vitamina D actuaría a nivel de crecimiento celular y apoptosis mediante la reducción de la angiogénesis.

Dos alimentos característicos de la DM que pueden influir en la incidencia del CCR, son el vino, que aporta polifenoles, y el aceite de oliva, que contiene AGMI y diversos compuestos beneficiosos como los polifenoles también.

4. El vino: un componente tradicional de la Dieta Mediterránea

El vino forma parte de la dieta desde hace más de 7000 años, cuando ya se consumía en la cuenca Mediterránea (Willett y col., 1995). Hay hallazgos que sitúan las primeras vasijas de vino en el territorio que actualmente ocupa Irán entre los años 5400-5000 a.C. (Guasch-Jané y col., 2004). Por tanto, se trata de un alimento que forma parte de las culturas y tradiciones de numerosos países Mediterráneos que actualmente extendido a otras regiones situadas en el paralelo 40 (tanto del hemisferio norte como del sur) que comparten características climáticas y medio ambientales similares a las Mediterráneas, y por tanto muy idóneas para el cultivo de la vid y por ende para la producción de vino. España es el tercer productor mundial de vino, detrás de Italia y Francia. Es una bebida que ha estado presente a lo largo de los siglos, pero no siempre ha sido un alimento

apreciado. La publicación en el año 1992 de la denominada "Paradoja Francesa" (Renaud y de Lorgeril, 1992) mostró que el consumo de vino es el responsable de una menor incidencia de las enfermedades cardiovasculares a pesar de que la población Francesa consumía elevadas cantidades de AGs saturados (AGS). De este modo se impulsó el estudio científico de los efectos del vino para entender los mecanismos implicados en estas enfermedades.

4.1. Tipos y composición del vino

El terreno, el clima, la variedad de la uva, la técnica de producción y el envejecimiento harán variar la composición del vino. Pero el agua, alcohol, glicerol, polisacáridos y diferentes ácidos y compuestos fenólicos siempre forman parte de su composición (**Tabla 3**).

Tabla 3. Composición del vino excluyendo los compuestos fenólicos. Adaptado de German y Walzem (2000).

Componente	Concentración (g/mL
Agua	80-90
Carbohidratos	
Glucosa	0,05-0,1
Fructosa	0,05-0,1
Pentosas	
Arabinosa, ramnosa, xilosa	0,08-0,2
Pectina	Trazas
Inositol	0,03-0,05
Fucosa	0,0005
Alcoholes	
Etilo	8-15
Otros	
Metilo, 2,3-butilen glicol	0,3-1,9
Glicerol	0,3-1,4
Aldehído	0,001-0,05
Ácidos orgánicos	0,3-1,1
Compuestos nitrogenados	0,01-0,09
Minerales	0,15-0,4

El vino tinto es el que más compuestos fenólicos contiene, ya que dichos componentes se pierden durante la elaboración del vino blanco (Vinson y col., 1995). En la **Tabla 4** se muestra un resumen de los principales grupos de compuestos fenólicos presentes en el vino tinto, rosado y blanco, señalando algunos os componentes más representativos.

Tabla 4. Principales compuestos fenólicos del vino. Valores expresados en mg/100 mL. Adaptado de Neveu y col. (2010) y Rothwell y col. (2012 y 2013).

Compuesto	Vino blanco	Vino rosado	Vino tinto
Flavonoides			
Antocianinas: malvidina, delfinidina, petunidina	0,04	0	22,34
Dihidroflavonoles	0,57	0,38	5,44
Flavanoles: catequinas, procianidinas	2,62	2,37	47,02
Flavononas: naringina, naringenina	0,23	0	0,85
Flavonoles: quercetina, miricetina, kaemferol	0,94	0	6,86
Ácidos fenólicos			
Ácidos hidroxibenzoicos: ácido gálico, ácido genístico	2,47	1,55	7,01
Ácidos hidroxicianímicos: ácido cafeoilquínico, ácido cafeoil	2,82	0,53	9,97
Estilbenos			
Piceatanol, viniferina, RV	1,23	0,49	4,35
Otros polifenoles			
Hidroxibenzaldehidos	0,1	0	0,71
Hidroxicumarinas	0,31	0	0
Tirosoles: tirosol, hidroxitirosol	0,42	1,01	3,65

4.1.1. Estilbenos, los polifenoles característicos del vino

Hay una gran variedad de compuestos fenólicos presentes en el vino, pero un grupo característico es el de los estilbenos, cuya estructura se basa en dos anillos aromáticos unidos por un puente etileno. Y dentro de esta gran familia destaca el RV (3,5,4'-trihidroxiestilbeno), uno de los estilbenos más estudiados por su múltiple propiedades biológicas y presente en concentraciones moderadas en el vino y la uva (Garrido y Borges, 2013). Además, el aporte dietético de RV, así como el de otros estilbenos, se ve relativamente limitado al consumo de vino, ya que su concentración en otros alimentos de consumo habitual, como las frutas y verduras, es muy baja (Goldberg y col., 1995). Se puede encontrar también en bayas, cacahuetes, cacao en polvo y plantas medicinales como *Polygonum cuspidatum* (Smoliga y col., 2011). El RV existe en dos formas isoméricas: *cis*- y *trans*-, siendo la primera la más frecuente en el vino (Brown y col., 2009).

Estudios previos indican que a pesar de que aproximadamente un 70% del RV ingerido es rápidamente absorbido, solo se detectan trazas de RV en plasma (Walle y col., 2004; Wenzel y Somoza, 2005; Zamora-Ros y col., 2006). La biodisponibilidad del RV parece no estar afectada por la ingesta conjunta con otros alimentos, incluso los que contienen mucha grasa. Por otro lado la solubilidad del RV mejora en presencia de alcoholes o disolventes orgánicos, haciendo que se absorba más fácilmente (Gambini y col., 2015). A nivel intestinal el RV se absorbe por difusión

pasiva o formando complejos con transportadores de membrana, como las integrinas; una vez dentro del enterocito es rápidamente metabolizado a las formas glucuronidada y sulfatada (de Santi y col., 2000a y 2000b; Yu y col., 2002; Vitrac y col., 2003; Burkon y Somoza, 2008). En el torrente sanguíneo, el RV se encuentra mayoritariamente glucuronidado, sufatado y glucosilado, y en menor media en estado libre, pero unido a albúmina y lipoproteínas, que serán disociados al interactuar con receptores específicos en la membrana de las células (Delmas y col., 2011) (**Figura 7**). Así, hay estudios que muestran que más del 90% del RV se encuentra unido a lipoproteínas, actuando estas últimas como un reservorio y demostrando una interacción entre el RV y los AGs dietéticos (Urpi-Sarda y col., 2005 y 2007).

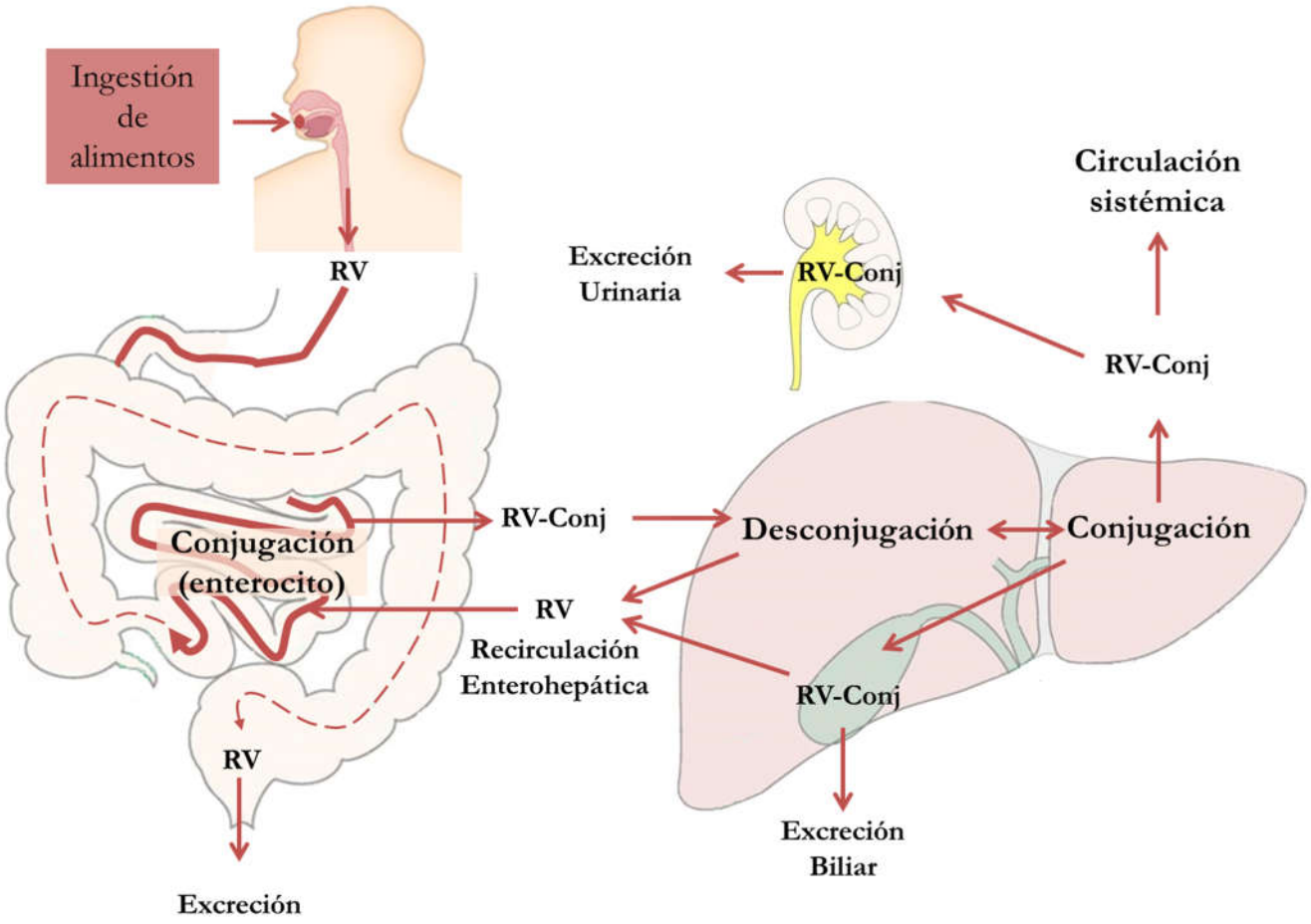

Figura 7. Metabolización del resveratrol tras su ingesta. Adaptada de Ruivo y col. (2015). RV, Resveratrol; RV-Conj, resveratrol conjugado.

La fase II del metabolismo del RV y sus metabolitos ocurre en el hígado, donde se da un transporte enterohepático que retorna pequeñas cantidades de RV al intestino (Bertelli y col., 1996; Crozier y col., 2009). A partir de aquí el RV es metabolizado a formas conjugadas, facilitando así su

posterior excreción biliar y urinaria. Los metabolitos del RV pueden presentarse en diversas formas, además de glucuronidados y sulfatados en diferente posición, pueden estar hidroxilados e incluso se han descrito isómeros *cis*- de la mayoría de ellos (Walle y col., 2004; Boocock y col., 2007; Urpi-Sarda y col., 2007).

A pesar de que la biodisponibilidad del RV es muy baja sus efectos biológicos in vivo e in vitro son destacables (Schneider y col., 2000; Wolter y col., 2001; Lancon y col., 2004; Torres y col., 2010), esto podría ser debido a la conversión de los metabolitos a su forma original en los órganos diana, como el hígado (Vitrac y col., 2003; Wenzel y Somoza, 2005), y a la recirculación enterohepática seguida de la desconjugación y reabsorción en el intestino (Marier y col., 2002).

Entre las propiedades biológicas del RV destaca su actividad antioxidante (Frankel y col., 1993; Miller y Rice-Evans, 1995), antiinflamatoria (Martinez y Moreno, 2000), antiisquémica (Ray y col., 1999; Shigematsu y col., 2003), neuroprotectiva (Bureau y col., 2008), antienvejecimiento (Valenzano y col., 2006), antiobesidad (Rayalam y col., 2008), antiviral (Kapadia y col., 2002), antifúngica (Schouten y col., 2002), fitoestrogénica (Kopp, 1998), cardioprotectiva (Bertelli y col., 1995; Pace-Asciak y col., 1996; Frémont, 2000), antiplaquetaria (Pace-Asciak y col., 199 y 1996) y antiproliferativa/quimiopreventiva (Moreno, 2000). Este último efecto ha hecho que el RV pueda ser utilizado como agente preventivo y/o terapéutico contra el cáncer (Kuhnle y col., 2000; Roupe y col., 2006; Buiarelli y col., 2007; Zamora-Ros y col., 2009).

4.2. Efectos del consumo de vino sobre el cáncer colorrectal

Hasta hace unos años el consumo de alcohol parecía tener un efecto perjudicial sobre el cáncer, dado que se ha asociado a un aumento del 3,2-3,7% del riesgo de muerte por cáncer (Nelson y col., 2013) y del riesgo de desarrollar CCR (Doll y Peto, 1981; Ferrari y col., 2007). Pero, recientemente diversos estudios muestran un cambio de dirección respecto al efecto del consumo de bebidas alcohólicas en relación al riesgo de CCR, sobretodo en cuanto al consumo de vino, muy característico de la DM. En este sentido, el estudio EPIC demostró que el consumo total de alcohol no se asocia significativamente con el CCR y tampoco el consumo de cerveza, jerez o bebidas espirituosas; por el contrario, un consumo moderado de vino redujo el riesgo de CCR (Park y col., 2009). En este sentido, otros dos estudios de cohorte demostraron un aumento significativo del riesgo de CCR en personas que bebían cerveza y bebidas espirituosas, mientras que las personas que consumían vino presentaron una disminución de dicho riesgo (Pedersen y col., 2003; Anderson y col., 2005). Anderson y col. (2005) demostraron que el consumo moderado

de vino se asocia a una disminución del riesgo de padecer CCR, incluso a nivel de pronostico y supervivencia se observaron mejorías (Zell y col., 2007). En relación con estos resultados, Kontou y col. (2012a) sugieren que incluso el consumo moderado de alcohol previene el CCR en hombres y mujeres; manteniendo este efecto cuando se ajustaron los resultados por tipo de bebida alcohólica, mostrando que el vino se asocia a una mayor reducción del riesgo. Sin embargo, cabe destacar que este estudio observó que el consumo elevado de alcohol se relacionó directamente con el riesgo de CCR. Estos resultados también se han reproducido utilizando modelos experimentales de CCR; así, la administración de extractos de vino en ratones previno la aparición de focos aberrantes en las criptas del colon (Mazue y col., 2014).

Dado que como se ha comentado anteriormente no todas las bebidas alcohólicas tienen efecto beneficioso sobre el CCR, y como además se ha observado que el vino desalcoholizado mantiene la mayoría de las propiedades biológicas de este (Clifford y col., 1996), se ha planteado que parte, al menos, de los efectos beneficiosos del vino deberían atribuirse a sus compuestos fenólicos y especialmente a aquellos que son característicos del vino como los estilbenos.

4.2.1. Efecto del resveratrol sobre el cáncer colorrectal

El RV es capaz de ejercer diversos efectos sobre el crecimiento y proliferación de células cancerígenas. Así, en estudios realizados con modelos animales se observó que el tratamiento con RV (a dosis variables) induce una disminución del número de adenomas en ratas y ratones (Tessitore y col., 2000; Sale y col., 2005; Sengottuvelan y Nalini, 2006; Kineman y col., 2007; Huderson y col., 2013) y de la incidencia de CCR en ratones (Tessitore y cpet al. 2000; Cui y col., 2010).

Hay pocos estudios clínicos utilizando el RV como tratamiento del CCR. En 2009 se publicó un estudio clínico de fase I sobre el efecto del RV como tratamiento para pacientes con cáncer (Nguyen y col., 2009). Al final del estudio se observó que el RV, en combinación con otros componentes presentes en la uva, podría disminuir el riesgo de desarrollo de CCR al disminuir la vía de señalización Wnt, muy relacionada con la formación del tumor. En otro estudio clínico en pacientes con CCR y metástasis hepática, se observó que el porcentaje de células apoptóticas en el tumor aumentó en los pacientes tratados con RVl durante 21 días (Howells y col., 2011). Hay que destacar que dicho estudio fue realizado con una población muy pequeña y, por lo tanto, hay que considerar sus resultados con cautela.

Se han realizado algunos estudios clínicos sobre el efecto de la suplementación con RV en la prevención del CCR en sujetos sanos. Uno de estos estudios demostró una disminución en los niveles de IGF1, parámetro muy relacionado con el desarrollo tumoral y la metástasis, tras la administración de suplementos de RV (Brown y col., 2010). En otro ensayo, Chow y col. (2010) observaron que el RV afecta la actividad de enzimas implicados en la carcinogénesis y en el metabolismo de fármacos.

5. El aceite de oliva: un componente indispensable de la Dieta Mediterránea

Según algunos autores el cultivo del olivo se inició hace aproximadamente 6000 años en Oriente Medio, ya que hay estudios genéticos que han demostrado con claridad que el origen de los olivos se sitúa en Palestina, Líbano, Siria, Chipre y Creta (Zohary y Spiegel-Roy, 1975; Kaniewski y col., 2012; Besnard y col., 2013). El aceite de oliva es un elemento característico de la DM, que junto con otros alimentos típicos como el vino y los cereales, se cultiva alrededor de toda la cuenca Mediterránea, pero también a lo largo del paralelo 40º, que incluye las regiones situadas al Norte y al Sur de este paralelo, donde se da un clima templado-cálido con una estación seca. Por todo esto, en el patrón de DM el consumo de aceite de oliva es el aporte lipídico de origen vegetal predominante. Aunque el cultivo del olivo se ha ido extendiendo a numeroso países situados a lo largo del paralelo 40ª como Chile, Argentina, Australia, China, etc., los países Mediterráneos continúan siendo los principales productores, siendo España el principal productor y exportador mundial de aceite de oliva y olivas (Disponible en: http://www.internationaloliveoil.org/estaticos/view/136-country-profiles; Vossen, 2007).

5.1. Tipos y composición del aceite de oliva

La composición del aceite de oliva varía en función de diversos factores como la variedad de aceituna, el grado de maduración, las condiciones agronómicas, y las características tecnológicas de producción. Pero se pueden describir dos grandes grupos de componentes: la fracción saponificable y la insaponificable. La primera representa el 98-99% del peso del aceite y está compuesta por AGs, en su mayoría AGMI como el ácido oleico (70%), AGPI (10-15%) y AGS (5%), y una pequeña cantidad de diglicéridos, ésteres de esteroles, ceras y fosfátidos (Boskou, 1996 y 2000; Civantos y col., 1999; Harwood y Aparicio, 2000). En la **Tabla 5** se resumen los principales componentes de la fracción saponificable en un aceite de oliva estándar.

Tabla 5. Principales ácidos grasos de la fracción saponificable del aceite de oliva. Adapatada de Boskou (1996) y Montedoro y col., (2007).

Nombre común y símbolo numérico	Porcentaje
Mirístico (14:0)	0,0-0,05
Palmítico (16:0)	7,5-20
Palmitoleico (16:1)	0,3-3,5
Margárico (17:0)	0-0,3
Heptadecenoico (17:1)	0-0,3
Eteárico (18:0)	0,5-5
Oleico (18:1, ω9)	55-83
Linoleico (18:2, ω6)	3,5-21
α-linolénico (18:3, ω3)	0-0,9
Araquídico (20:0)	0-0,6
Eicosenoico (20:1, ω9)	0-0,4
Behenico (22:0)	0-0,2
Lignocérico (24:0)	0-0,2

La fracción insaponificable supone un 2% del peso del aceite de oliva y se compone principalmente de elementos que aportan al aceite sus características organolépticas, le dan estabilidad y a muchos de ellos se les atribuyen diversas propiedades bioactivas (Kitts, 1994) entre las que se encuentran las anticancerígenas (Hashim y col., 2005). Se compone de pigmentos, compuestos volátiles, esteroles, tocoferoles y polifenoles (Ramírez-Tortosa y col., 2006). En la **Tabla 6** se describen los principales polifenoles del aceite de oliva, de oliva virgen y de oliva virgen extra. Más del 70% de estos polifenoles son tirosol, hidroxitirosol y sus derivados (Allouche y col., 2007; Raederstorff, 2009; Romero y Brenes, 2012; Enache y col., 2013; Gosetti y col., 2015). Como se puede observar en la tabla 1.5, muchos de estos componentes se van perdiendo a medida que el aceite se va refinando; por ello, el aceite de oliva virgen extra es el que tiene una composición más compleja (Jiménez y col., 2001). Así, el aceite de oliva es una fuente de numerosos compuestos activos cuya composición y concentración varía dependiendo de las características propias de cada aceite de oliva.

Tabla 6. Principales compuestos fenólicos del aceite de oliva. Valores expresados en mg/100 mL. Adaptado de Neveu y col. (2010) y Rothwell y col. (2012 y 2013).

Compuesto	Aceite de oliva virgen extra	Aceite de oliva virgen	Aceite de oliva
Flavonoides			
Flavononas: apigenina y lutoenina	1,53	0,23	0,15
Ácidos fenólicos			
Ácidos hidroxibenzoicos: ácido siríngico, ácido vanílico	0,08	0,39	0
Ácidos hidroxicianímicos: ácido cafeico, ácido ferúlico	0,06	0,07	0
Ácido hidroxifenilacético	0,141	0,06	0
Lignans: pinoresinol	1,08	2,81	3,16
Otros polifenoles			
Hidroxibenzaldehidos: vanilina	0	0,02	0
Tirosoles: tirosol, derivados del hidroxitirosol, oleuropeina, ligstrósido	59,49	53,82	33,34
Otros: hidroxifenilglicol	0	0,28	0,25

5.2. Efectos del consumo de aceite de oliva sobre el cáncer colorrectal

La DM se caracteriza por un elevado aporte calórico proveniente de las grasas, cuya fuente mayoritaria es el aceite de oliva virgen extra (Trichopoulou y Lagiou, 1997). Estudios epidemiológicos, y principalmente experimentales han demostrado la relación entre los lípidos dietéticos y cierto tipo de cáncer, como el de mama, colorrectal y de próstata (Escrich y col., 2006; Llor y col., 2003; World Cancer Research Fund / American Institute for Cancer Research, 2011). La DM, que aporta aproximadamente un 30-40% de las calorías en forma de grasa, se asocia a un bajo riesgo de desarrollar CCR en contraposición a otras dietas con mayor aporte de grasas y que se relacionan con una elevada incidencia de CCR. Esta diferencia se puede atribuir, también, al tipo de grasas que forman parte de la dieta, siendo determinantes en el desarrollo de enfermedades como el CCR (Willett y col., 1995; Slattery y col., 1997; Austin y col., 2011). Así, el aceite de oliva se puede considerar un factor determinante en el efecto preventivo de la DM frente al CCR y puede contribuir al mantenimiento de la salud, mediante sus efectos antiinflamatorios y antioxidantes (Tripoli y col., 2005; Lau y col., 2008).

Un estudio ecológico que examinó los niveles de consumo nacional de diferentes alimentos, haciendo énfasis en el consumo de aceite de oliva, demostró que el 76% de la variación en la incidencia de CCR entre países se debía a tres factores dietéticos muy concretos: la carne, el pescado y el aceite de oliva. Mientras que el consumo de carne y pescado aumentó la incidencia del CCR, el de aceite de oliva tuvo el efecto contrario (Stoneham y col., 2000).

En un estudio de cohorte realizado en Italia con pacientes con CCR versus pacientes sanos se observó que el riesgo de CCR no se relacionó significativamente con el consumo de aceite de oliva, aunque si observaron una reducción no significativa (Braga y col., 1998). En este sentido, Galeone y col. (2007) tampoco observaron un papel relevante del consumo de alimentos fritos respecto al riego de CCR en Italia, aunque sugieren un posible efecto favorable del aceite de oliva utilizado como grasa de fritura en la prevención de este cáncer.

El efecto beneficioso del aceite de oliva podría estar relacionado con su efecto sobre el estado redox, debido a su elevado contenido en ácido oleico, un ácido graso poco susceptible de ser oxidado, y también a la presencia de polifenoles, moléculas con actividad antioxidante (Owen y col., 2000). Sin embargo, es plausible pensar que el efecto beneficioso del aceite de oliva sobre el CCR no sería debido a un único elemento, sino al conjunto de todos sus componentes, y al patrón dietético y al estilo de vida asociados al consumo de este tipo de aceite, que están ampliamente relacionados con la prevención del CCR. En este sentido, recientemente Barone y col. (2014) en un estudio in vivo observaron que el aceite de oliva de la dieta administrada redujo el número y volumen de pólipos colónicos.

5.2.1. Fracción saponificable

El aceite de oliva es único en cuanto a su elevado contenido en ácido oleico (Newmark, 1997), que al ser monoinsaturado es mucho más resistente a la oxidación, lo que da al aceite de oliva una menor susceptibilidad a la oxidación, y por ello, una elevada estabilidad y una vida útil larga (Owen y col., 2000).

El efecto del ácido oleico sobre la incidencia y desarrollo de CCR no está claro. Se ha descrito un posible papel en la prevención del CCR, pero no se ha determinado aún si es debido a un efecto anticancerígeno directo o a una modulación del estrés oxidativo (Visioli y col., 2002). Así, las dietas de tipo Americano en las que se consumen alimentos con poca cantidad de ácido oleico y mayor proporción de otro tipo de ácidos grasos (Newmark, 1997) están relacionados una elevada incidencia de enfermedades cardiovasculares y cáncer. Un estudio caso-control llevado a cabo por Theodoratou y col. (2007) no encontró ninguna asociación significativa entre la ingesta de ácido oleico y el riesgo de CCR.

En un modelo experimental de CCR, Llor y col. (2003) estudiaron el efecto del aceite de oliva y del ácido oleico sobre la neoplasia colorrectal, observando que el aceite de oliva induce apoptosis,

diferenciación celular y una disminución de la expresión de COX-2 y de la proteína Bcl-2, ambas implicadas en el desarrollo del cáncer y en el control de la apoptosis, respectivamente. En cambio, el ácido oleico solo indujo apoptosis en una de las dos líneas celulares estudiadas (HT-29 y Caco-2) y no afectó a la expresión de COX-2 y Bcl-2. Además, el aceite de oliva no tuvo efectos sobre la proliferación celular. Los autores concluyeron que el ácido oleico tiene poco efecto, o ninguno, sobre la quimioprevención y estos autores proponen que son otros los componentes del aceite de oliva los que estarían involucrados en este efecto protector (Llor y col., 2003). En este sentido, en un estudio in vivo con ratones $Apc^{min/+}$ alimentados con ácido oleico se observe un aumento en el número de tumores intestinales de los animales tratados, según un estudio de Hansen y col. (Hansen Petrik y col., 2000).

5.2.2. Fracción insaponificable

Algunos polifenoles, como el tirosol, el hidroxitirosol y sus derivados son consumidos casi exclusivamente con el aceite de oliva, además de ser los polifenoles mayoritarios del mismo. El tirosol y el hidroxitirosol se forman a partir de la hidrólisis de las agliconas de los secoiridoides ligstrosido y oleuropeina (Romero y col., 2007). Se ha especulado que los polifenoles del aceite de oliva virgen y del aceite de oliva virgen extra son capaces de modular oncogenes implicados en el cáncer (Sotiroudis y Kyrtopoulos, 2008; Schwingshackl y Hoffmann 2014a y 2014b). Además, pueden ejercer un fuerte efecto quimiopreventivo actuando como antioxidantes y afectando la señalización celular y la progresión del ciclo celular (Corona y col., 2009). Los compuestos fenólicos presentes en el aceite de oliva pueden interactuar con la cascada inflamatoria mediante su acción antioxidante, principalmente actuando como secuestrador de radicales libres y previniendo así el daño celular (Biesalski, 2007). Los polifenoles ejercen sus efectos como componentes naturales del aceite de oliva, pero se ha observado que su potencia se pierde cuando son aislados y aplicados en forma de moléculas puras. Así, hay estudios que han demostrado que los polifenoles del aceite de oliva refuerzan sus efectos biológicos cuando son consumidos dentro de una comida, junto con más alimentos (Covas y col., 2006).

II. Objetivos

Desde hace siglos en la cuenca Mediterránea la vid y el olivo son parte de nuestra cultura y tradiciones, por ello el vino y el aceite de son dos de los componentes más característicos de la DM. Dieta que se asocia desde hace décadas a un estilo de vida saludable y a la prevención de diversas enfermedades de elevada incidencia como el CCR, aunque se desconocen, en parte, los mecanismos moleculares implicados.

Por todo ello, el **objetivo general** de esta tesis es *estudiar el papel de componentes biactivos del vino y el aceite de oliva en el control del crecimiento de células epiteliales intestinales*.

Para cumplir con el objetivo principal de esta tesis hemos utilizado como modelo experimental la línea celular Caco-2. Estas células se caracterizan porque en preconfluencia presentan un crecimiento celular propio de un adenocarcinoma, mientras que a partir de la confluencia se diferencian para dar lugar a una monocapa de células que presentan características morfológicas, bioquímicas y funcionales similares a las de los enterocitos del intestino delgado y grueso (Martín-Venegas y col., 2006). Los trabajos de esta tesis se han desarrollado con cultivos preconfluentes que presentan características similares a las células de adenocarcinoma.

Las células Caco-2 expresan la mayor parte de enzimas de la cascada del AA, y por tanto, son capaces de generar gran cantidad de diferentes eicosanoides, como PGE2, LTB4, 5-, 12 y 15-HETE (Martin-Venegas y col., 2011 2014), representativos de las diferentes vías de la cascada del AA. Además, también expresan los receptores de estos eicosanoides; todos estos elementos tienen gran relevancia en el control del crecimiento de estas células (Cabral y col., 2013, 2014 y 2015). Los niveles plasmáticos de *trans*-RV después de un consumo moderado de vino son muy bajos o no pueden cuantificarse (Walle y col., 2004; Wenzel y Somoza, 2005; Zamora-Ros y col., 2006), mientras que se alcanzan concentraciones apreciables (25-130 ng/mL) de sus metabolitos sulfatados y/o glucuronidados (Bosetti y col., 2009; Capitani y col., 2009; Kapetanovic y col., 2011). Esta metabolización se inicia en el epitelio intestinal y se completa en el hígado (Wenzel y Somoza, 2005). El *trans*-RV se ha descrito que regula el crecimiento de las células epiteliales intestinales (Martinez y Moreno, 2000). Sin embargo, se ha cuestionado la relevancia fisiológica de los efectos biológicos del *trans*-RV descritos in vitro, ya que la concentración de trans-RV en plasma o tejidos es muy baja tras su absorción y metabolización.

Como **objetivo 1** nos planteamos *analizar el papel de los principales metabolitos del trans-RV sobre el ciclo celular y la proliferación de células epiteliales intestinales*.

El *trans*-RV es uno de los componentes más característicos del vino y previamente se ha observado que modula el estrés oxidativo, la cascada del ácido araquidónico y tiene efecto sobre el control del crecimiento celular (Martinez y Moreno, 2000; Moreno, 2000; Mitjavila y Moreno, 2012). Recientemente, Zamora-Ros y col. (2008) observaron que el *trans*-RV, y especialmente el *trans*-piceido, son componentes característicos de la DM, siendo el vino su principal fuente con un consumo estimado de 500 mg/dia, que es entre 5 y 10 veces superior al consumo medio de *trans*-RV. Sin embargo, no hay información sobre su efecto sobre el crecimiento de células epiteliales intestinales.

Considerando estos antecedentes el **objetivo 2** ha sido *estudiar el papel del trans-piceido en el control del crecimiento de las células epiteliales intestinales y analizar el mecanismo de acción*.

Los efectos biológicos del *trans*-RV han sido asociados, al menos en parte, a su actividad antioxidante que está relacionada con la estructura de sus dos anillos fenólicos y los tres grupos hidroxilo (Capitani y col., 2009). Por ello, la modificación de su estructura química podría afectar a su actividad antioxidante y a sus efectos biológicos.

El **objetivo 3** de esta tesis ha sido *analizar el efecto de diversos cambios en la estructura del trans-RV sobre su actividad antioxidante y la capacidad de regular el ciclo celular y el crecimiento de las células epiteliales intestinales*.

Aunque el consumo de grasa está relacionado con la incidencia del CCR (Bosetti y col., 2009), no se ha podido establecer claramente la relación entre el consumo de determinados tipos de AGs de la dieta y la incidencia de este tipo de cáncer. Sin embargo, se sabe que los AGs de cadena corta producidos como resultados de la fermentación de carbohidratos no digeridos tienen un papel importante en la regulación de la proliferación y diferenciación de las células epiteliales intestinales (Barnard y Warwick, 1993; Hass y col., 1997).

Por ello el **objetivo 4** ha sido *analizar el efecto de diferentes AGs de cadena larga con número variable de insaturaciones sobre el control del crecimiento celular y la apoptosis de células epiteliales intestinales*.

Hansen Petrik y col. (2000) observaron que los ratones $APC^{min/+}$ alimentados con una dieta rica en ácido oleico presentaban un mayor número de tumores intestinales, sin embargo, cuando la dieta fue preparada con aceite de oliva, Barone y col. (2014), observaron que disminuía el número y el tamaño de los pólipos en este modelo experimental de CCR. Estos resultados sugieren que el consumo de ácido oleico o de aceite de oliva podría tener diferente efecto, o incluso un efecto contrario, sobre la incidencia y el desarrollo del CCR.

Por ello, nos planteamos como **objetivo 5** *analizar el efecto del ácido oleico sobre el crecimiento de las células epiteliales intestinales en presencia, o no, de algunos de los componentes minoritarios característicos del aceite de oliva.*

Y como **objetivo 6** *valorar la acción de estos compuestos sobre el estrés oxidativo y la cascada del AA, mecanismos implicados en el control de la proliferación de las células Caco-2.*

III. Resultados

Artículo 1

Resveratrol metabolites have an antiproliferative effect on intestinal epitelial cancer cells

Carolina E. Storniolo and Juan J. Moreno

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Los resultados de esta publicación se han presentado en los siguientes congresos:

Biological effect of resveratrol metabolites on intestinal epithelial cancer cell growth. CE Storniolo, JJ Moreno. 22nd Biennal Congress of the European Association for Cancer Research Participación: Barcelona, España. 07-10/07/2012. **Póster**

Estos resultados fueron publicados en:

CE Storniolo, JJ Moreno. Biological effect of resveratrol metabolites on intestinal epithelial cancer cell growth. European Journal of Cancer 2012;48:S236.

Resumen Artículo 1

Objetivo: Estudiar el efecto de los metabolitos del RV sobre la inhibición del crecimiento, el ciclo celular y la apoptosis de células epiteliales intestinales.

Material y métodos: Las células de adenocarcinoma Caco-2 en estado preconfluente se incubaron con los tres metabolitos del RV (RV 3-*O*-sulfato, RV 3-*O*-glucurónido y RV 4'-*O*-glucurónido) en presencia de 10 % de SFB. Después de 48h se determinó el crecimiento celular mediante marcaje con bromuro de etidio/naranja de acridina, la síntesis de DNA mediante la incorporación de [^{3}H]thimidina, el ciclo celular por citometría de flujo y la fragmentación del DNA por TUNEL.

Resultados: Los metabolitos del RV inhibieron el crecimiento celular y la síntesis de DNA de forma concentración-dependiente, de forma similar al RV. Observamos que inducen un aumento del porcentaje de células en la fase G_0/G_1 del ciclo celular así como de la apoptosis.

Conclusiones: A partir de nuestros resultados se propone por primera vez que los metabolitos del RV son activos después de su biosíntesis, contribuyendo a los beneficios sobre la salud previamente atribuidos al RV. Estos metabolitos podrían ser un objetivo de estudio en la prevención y tratamiento del CCR.

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Resveratrol metabolites have an antiproliferative effect on intestinal epithelial cancer cells

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ABSTRACT

Trans-resveratrol (RV) is an active polyphenol with numerous physiological properties including antitumour activity, especially in colon cancer. RV is metabolized in the intestine and then in the liver to sulphated and glucuronidated forms that are exported to target organs. After exerting their effects, they are eliminated in the urine and stools. There are few and contradictory findings on the biological effects of RV metabolites. On the basis of RV metabolism, we selected three metabolites RV 3-*O*-sulphate, RV 3-*O*-glucuronide and RV 4′-*O*-glucuronide, and studied their effects on cell growth inhibition, the cell cycle and apoptosis using human adenocarcinoma cell line (Caco-2 cell) cultures.

Our results show that RV metabolites have an antioxidant activity similar to that RV. Moreover, all metabolites inhibited cell growth in a concentration-dependent manner as well as [^{3}H] thymidine incorporation. Furthermore, we observed an increase in the percentage of cell in G_0/G_1 phase induced by RV metabolite treatments, as well as the induction of apoptosis.

On the basis of our results we propose, for the first time, that RV metabolites remain active after their biosynthesis, contributing to the health benefits attributed previously only to RV. These metabolites are a potential target for more research into the prevention and treatment of colon cancer.

1. Introduction

Resveratrol (3,4′,5-trihydroxy-trans-stilbene) (RV) is present in at least 72 species of the plant kingdom, but only grapes (Romero-Perez, Lamuela-Raventos, Andres-Lacueva, & de La Torre-Boronat, 2001; Soleas, Diamandis, & Goldberg, 2001; Sovak, 2001), peanuts (Chukwumah et al., 2007), pistachios (Gentile et al., 2007) and various berries (Rimando, Kalt, Magee, Dewey, & Ballington, 2004) are part of our regular diet.

RV crosses the apical membrane of the enterocyte by passive transcellular transport, although MRP2 (protein associated with drug resistance 2) also seems to be involved in regulating transmembrane efflux (Henry et al., 2005; Kaldas, Walle, & Walle, 2003). RV is rapidly metabolized by the enterocyte, mainly being transformed into its corresponding sulphated and glucuronidated forms (Burkon & Somoza, 2008; De Santi, Pietrabissa, Mosca, & Pacifici, 2000a, 2000b; Vitrac et al., 2003; Yu et al., 2002). In Caco-2 cells and enterocytes the sulphated forms predominate at low concentration of RV (De Santi et al., 2000a; Henry et al., 2005), while the glucuronidated forms predominate when the RV concentration is high (Kuhnle et al., 2000; Maier-Salamon et al., 2006; Maier-Salamon et al., 2008). However, in recent studies *in vivo*, sulphates were the main metabolites after administering a high dose of RV (Boocock et al., 2007; Urpi-Sarda et al., 2007). Moreover, at low concentrations (<10 uM) RV is almost totally conjugated (84%) in Caco-2 cells, while at high concentrations (>200 mM) metabolization decreases to 7.6% (Maier-Salamon et al., 2006); this could be due to the saturation or inhibition of enzymatic pathways.

Once absorbed, RV is transported to the liver (Bertelli et al., 1996) where it is rapidly metabolized to sulphates and glucuronides, which facilitates urinary and bile excretion. In human liver biopsies, De Santi et al. (2000a) observed that RV is a better substrate for sulphatases than for UDP glucuronosyltransferases. In contrast, studies carried out on liver microsomes showed that glucuronidated forms predominate after incubation with *trans*- and *cis*-RV (Aumont et al., 2001). Glucuronidation is regio-selective, leading to the formation of 3-*O* and 4′-*O*-glucuronide, but not 5-*O*-glucuronide (Aumont et al., 2001; Wang et al., 2004).

When RV is administered like a compound of wine or juices, the free form is not detectable or is only present at a very low concentration in human plasma (Walle, Hsieh, DeLegge, Oatis, & Walle, 2004), but when it is administered at a dose of 25 mg, the plasma concentration of the free form reaches 1–5 ng/ml, and at higher doses (up to 5 g) it can reach 530 ng/ml (Boocock et al., 2007).

The maximum concentrations of RV and its metabolites generally appear within 30–60 min after ingestion (Cottart, Nivet-Antoine, Laguillier-Morizot, & Beaudeux, 2010; Soleas et al.,

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2001; Walle et al., 2004), although for some metabolites the peak may appear after 3 or even 6–8 h (Boocock et al., 2007; Burkon & Somoza, 2008; Walle et al., 2004; Yu et al., 2002). Yu et al. (2002) and Kapetanovic, Muzzio, Huang, Thompson, and McCormick (2011) found that conjugated RV rather than its free form predominated in the circulation, but other authors detected low plasma concentrations of free RV and its metabolites after dietary intake (Vitaglione et al., 2005), and yet others detected no metabolites in plasma or serum at similar doses (Meng, Maliakal, Lu, Lee, & Yang, 2004).

RV 3-*O*-sulphate seems to be the most abundant metabolite, followed by two glucuronidated forms of RV (Boocock et al., 2007). A recent study added diglucuronides and bisulphates to this list. Thus, Burkon and Somoza (2008) identified seven metabolites: 3-sulphate, 3,4-disulphate, 3,5-disulphate, 3-glucuronide, 4-glucuronide, and two diglucuronides. The main tissues in which RV and RV metabolites are found, in addition to the gastrointestinal tract (small intestine and colon), are the kidneys, liver and lungs.

RV plays numerous biological roles, possessing antioxidant (Frankel, Waterhouse, & Kinsella, 1993; Miller & Rice-Evans, 1995), anti-inflammatory (Martinez & Moreno, 2000), anti-ischemic (Ray et al., 1999; Shigematsu et al., 2003), neuroprotective (Bureau, Longpre, & Martinoli, 2008), anti-ageing (Valenzano et al., 2006), anti-obesity (Rayalam, Yang, Ambati, Della-Fera, & Baile, 2008), antiviral (Kapadia et al., 2002), antifungal (Schouten, Wagemakers, Stefanato, van der Kaaij, & van Kan, 2002), phytoestrogenic (Kopp, 1998), cardioprotective (Bertelli et al., 1995; Fremont, 2000; Pace-Asciak, Rounova, Hahn, Diamandis, & Goldberg 1996), antiplatelet (Pace-Asciak, Hahn, Diamandis, Soleas, & Goldberg, 1995; Pace-Asciak et al., 1996) and antiproliferative/chemopreventive activitity (Moreno, 2000). The antitumourigenic activity of RV means that it can be used as a preventive and therapeutic agent against cancer (Buiarelli, Coccioli, Jasionowska, Merolle, & Terracciano, 2007; Kuhnle et al., 2000; Roupe, Remsberg, Yanez, & Davies, 2006; Zamora-Ros, Lamuela-Raventos, Estruch, & Andres-Lacueva, 2009). RV affects cell cycle arrest, suppresses tumour proliferation, induces apoptosis and differentiation, reduces inflammation and angiogenesis, and inhibits adhesion, invasion and metastasis, as well as having an antioxidant effect. However, there are few and contradictory findings about the effect of RV metabolites on these parameters. Here, we studied the action of RV and its main metabolites on the cell cycle and growth of cancer colon cell.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Nonessential amino acids, FBS, Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, potassium ferricyanide, ferric chloride and quercetin were supplied by Sigma–Aldrich (St. Louis, MO, USA). Tissue culture supplies and sterile material were obtained from Corning (NY, USA), Nirco S.L. (Barcelona, Spain), NORM-JECT (PA, USA) and Biosigma S.R.L. (Venice, Italy). The scintillation cocktail Biogreeen 3 and the [Methyl-^{3}H] thymidine (20 Ci/mmol) were supplied by Scharlau CEIME (Barcelona, Spain) and American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), respectively. The MebStain Apoptosis Kit was obtained from MBL International (Woburn, MA, USA) and paraformaldehyde extra pure from Merck Chemicals (Barcelona, Spain). Trans-RV (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol was supplied by Cayman Chemical (MI, USA) and RV 3-*O*-sulphate, RV 3-*O*-glucuronide and RV 4′-*O*-glucuronide were obtained from Bertin Pharma (Montigny-le-Bretonneux, France).

2.2. Cell culture

Caco-2 cells (HTB-37TM) were kindly provided by Dr. David Thwaites of the School of Cell and Molecular Biosciences, University of Newcastle-upon-Tyne (UK). The cells (passages 19–40) were routinely grown in 25 or 75 cm^2 plastic flasks, at a density of 2–2.5 $\times$ 10^4 cells/cm^2 and cultured in DMEM with 4.5 g/l D-glucose and 2 mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 in air. We used a second culture medium as described above, but without FBS as a negative control of cell growth. Cells grown to ~80% confluence were released by trypsinization and subcultured at a density of 1.5–2.5 $\times$ 10^4 cells/cm^2 in 12 mm diameter plastic dishes, or of 1.5 $\times$ 10^4 cells/cm^2 in 60 mm diameter plastic dishes. The growth medium was replaced twice a week. The experiments were performed on cells maintained in culture for 3 days (preconfluent cells) and consequently with non-differentiated Caco-2 cells. All experimentation products were diluted in DMSO (final concentration of DMSO was less than 0.1%).

2.3. Cell growth assay

The effect of the treatments on Caco-2 was assessed in 24-well plates. The cells were cultured for 96 h in DMEM medium supplemented with 10% FBS. They were then incubated for 48 h in the presence of various compounds. Finally, cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to enable counting of cells and assessment of viability (Parks, Bryan, Oi, & Herzenberg, 1979).

2.4. [^{3}H]-thymidine incorporation assay

DNA synthesis in Caco-2 cells was assessed by measuring the incorporation of [^{3}H]-thymidine. Caco-2 cells were seeded in 24-well culture plates (1.5–2 $\times$ 10^4 cell/cm^2) and cultured for 96 h in DMEM medium supplemented with 10% FBS. The cells were then incubated for 48 h in the presence of RV metabolites at different concentrations and 0.1 μCi/ml of [^{3}H]-thymidine. After 48 h [^{3}H]-thymidine-containing media were aspirated and the cells were washed twice with 0.5% BSA in PBS to remove the unincorporated [^{3}H]-thymidine. Washed cells were lysed with 300 μl of 1% Triton X-100 in PBS and added to a vial containing 3 ml of scintillation cocktail. Radioactivity present in the cell fraction was measured using a liquid scintillation counter (Packard Tri-Carb 1500, GMI Inc., MN, USA).

2.5. Flow cytometry cell cycle analysis

Caco-2 cells were seeded in 60 mm dishes, and after 96 h in culture, they were then incubated for 48 h in 10% FBS DMEM containing the treatments. Thereafter, cells were trypsinized, fixed with 70% ethanol, and stored at 4 °C for at least 2 h. Next, low molecular weight DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS, containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analysed on an Epics XL flow cytometer (Coulter Corporation, Philadelphia, USA). DNA was analysed (ploidy analysis) via single fluorescence histograms using Multicycle software (Phoenix Flow Systems, CA, USA).

2.6. TUNEL assay

The degradation of chromosomal DNA was evaluated via the TUNEL method using a MebStain Apoptosis Kit. Caco-2 cells were cultivated in media containing 10% FBS with treatments for 48 h. Next, cells were fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labelled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and analysed on an Epics XL flow cytometer.

2.7. Total antioxidant activity of the products

We selected different concentrations of products and applied an Antioxidant Assay Kit (Cayman Chemical, MI, USA). This method was based on the ability of antioxidants in the sample (products diluted in PBS) to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^+$ by methmyoglobin (Miller & Rice-Evans, 1997). The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and was quantified as molar Trolox equivalents.

2.8. Determination of reducing power

Total reducing power was determined according to the method of Oyaizu (1986). The first step was to mix 40 μl of the sample solution with 200 μl of phosphate buffer (0.2 M, pH 6.6) and 200 μl of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 minutes, after which 200 μl of 10% trichloroacetic acid was added to the mixture. Finally, 60 μl of 0.167% ferric chloride was added and incubated at room temperature for 50 min. Increased absorbance of the reaction mixture at 690 nm indicated increased reducing power. The results were compared with a standard quercetin curve.

2.9. Data analysis

The results are expressed as the mean ± standard error of the mean. Differences between non-treated and treated cells were tested using Student's *t*-test. Differences of $P < 0.05$ were considered significant.

3. Results

During the metabolism of RV by sulphatases and glucuronidases, the polyphenol loses one or more of its hydroxyl groups. The question is whether these changes affect its antioxidant activity and other biological activities. Our results show that RV has higher antioxidant and reducing power than Trolox and quercetin, respectively (Table 1). More interestingly, we observed that RV 3-*O*-sulphate, RV 3-*O*-glucuronide and RV 4′-*O*-glucuronide show antioxidant activity similar to that of RV, although with lower reducing power (Table 1). Given these findings, our aim was to examine the antiproliferative action of these RV metabolites on human colorectal cancer cell line cultures.

Table 1
Total antioxidant activity and reducing power of resveratrol and resveratrol metabolites. Data are the mean ± of three experiments performed in duplicate. Total antioxidant activity is expressed as equivalents of Trolox (mM). Reducing power is expressed as equivalents of quercetin (mg/ml).

	Total antioxidant activity	Reducing power
Resveratrol (50 μM)	0.183 ± 0.021	7.33 ± 0.04
Resveratrol 3-*O*-sulphate (50 μM)	0.114 ± 0.017	0.04 ± 0.01
Resveratrol 3-*O*-glucuronide (50 μM)	0.115 ± 0.034	0.05 ± 0.02
Resveratrol 4′-*O*-glucuronide (50 μM)	0.140 ± 0.028	0.03 ± 0.02

To determine the effect of RV metabolites on intestinal epithelial cell growth induced by FBS 10%, non-differentiated Caco-2 cells were incubated with RV 3-*O*-sulphate, RV 3-*O*-glucuronide and RV 4′-*O*-glucuronide (1–50 μM). As shown in Fig. 1, the three RV metabolites significantly inhibited Caco-2 cell growth in a concentration-dependent manner. Interestingly, the three RV metabolites had a similar antiproliferative effect as RV on Caco-2 cells, inhibiting Caco-2 growth by 50% at around 10 μM.

The effect of RV metabolites on non-differentiated Caco-2 cell DNA synthesis was also examined by [^{3}H]-thymidine incorporation in Caco-2 cultures. The three metabolites induced significant inhibition of [^{3}H]-thymidine uptake by Caco-2 cells after 48 h exposure to RV metabolites (25 μM) (Fig. 2).

To better analyse the above-described cell cycle imbalance induced by RV and RV metabolites, the distribution of cells in each phase of the cell cycle was quantified by determining DNA content via flow cytometry, after 48 h exposure in the presence of FBS 10%. Cell cycle analysis of RV-treated cells showed that this polyphenol induced an appreciable accumulation of cells in the S phase with a consequent reduction in the percentage of cells in the G_0/G_1 phase, with respect to the cell cycle distribution of control Caco-2 cells cultured in 10% FBS (Fig. 3). Interestingly, under the same conditions, the three RV metabolites led to an accumulation of cells in the G_0/G_1 phase and reduced the percentage of cells in the S phase at concentrations between 10 and 25 μM (Fig. 3B). These results suggest, for the first time, that RV metabolites inhibit non-differentiated Caco-2 cell growth by the induction of a G_0/G_1 phase arrest.

Ethidium bromide/acridine orange staining and morphological examination revealed that these treatments did not affect cell structure or cell viability at concentration up to 25 μM. Moreover, we observed no hypodiploid DNA peak, characteristic of apoptosis, in these experimental conditions (Fig. 3A). However, RV metabolites induced appreciable cell detachment at 50 μM, unlike RV (data not shown). Considering this data we measured DNA fragmentation induced by the treatments as an index of their apoptotic capability. Fig. 4 shows that the three RV metabolites induced marked DNA fragmentation at 50 μM, whereas RV had a similar effect at higher concentrations (100 μM).

4. Discussion

RV has many different health-promoting properties, including antioxidant and antitumour activities. However, despite its reported beneficial effects, usually assayed *in vitro*, the bioavailability of RV is poor because it is rapidly absorbed, metabolized and excreted (Boocock et al., 2007; Walle et al., 2004). Consequently, considerable controversy exits as to whether RV is an active molecule *in vivo* (Corder, Crozier, & Kroon, 2003). The controversy stems from the fact that RV is rapidly metabolized to its 3-*O*-sulphate and 3, 4′-*O*-glucuronide conjugates (Boocock et al., 2007; Walle et al., 2004). Thus, it has been observed that following a 25 mg oral dose of RV, the plasma concentration of this polyphenol is only in the nanomolar range compared with the micromolar range of its metabolites (Kaldas et al., 2003; Walle et al., 2004). These observations suggest that *in vivo* RV concentrations may be too low to elicit an effective biological response. Therefore, the question has been raised as to whether or not RV metabolites are capable of eliciting biological responses to concentrations reached in plasma or tissues. To help resolve this controversy, we investigated the antioxidant action of RV metabolites, as well as their effects on intes-

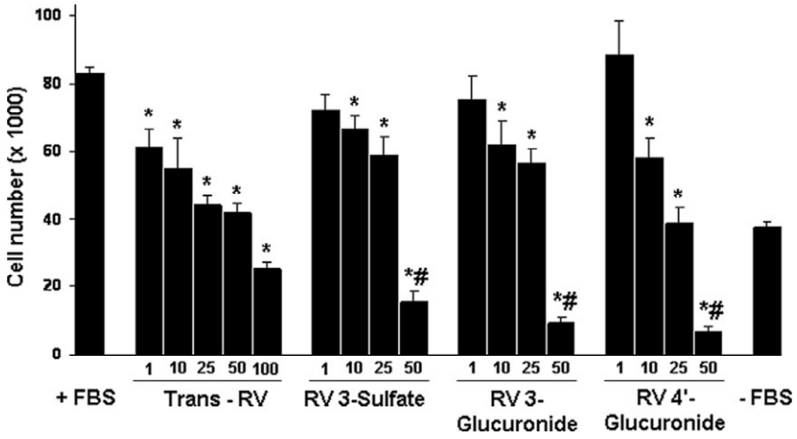

Fig. 1. Effect of resveratrol, resveratrol 3-*O*-sulphate, resveratrol 3-*O*-glucuronide and resveratrol 4′-*O*-glucuronide on Caco-2 cell growth induced by FBS 10%. Cells were treated for 48 h with treatments and then counted. Values are the mean ± SEM of three independent experiments performed in triplicate. **P* < 0.05 versus control group (cells cultured in the presence of FBS 10%), #*P* < 0.05 versus cell cultured without FBS.

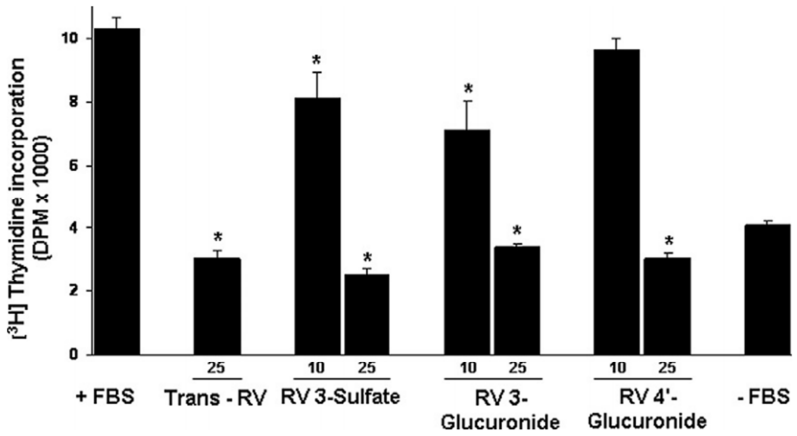

Fig. 2. Effect of resveratrol, resveratrol 3-*O*-sulphate, resveratrol 3-*O*-glucuronide and resveratrol 4′-*O*-glucuronide on Caco-2 DNA synthesis induced by FBS 10%. Cells were treated for 48 h with treatments in the presence of [^{3}H]-thymidine and [^{3}H]-thymidine uptake was assayed. Values are the mean ± SEM of three independent experiments performed in triplicate. *P < 0.05 versus control group (cells cultured in the presence of FBS 10%).

tinal adenocarcinoma cell growth, cell cycle progression and apoptosis using non-differentiated Caco-2 cell cultures as a experimental model.

The hydroxyl groups of RV play an important role in its antioxidant activity (Wang, Jin, & Ho, 1999). Thus, recently, Shang et al. (2009) observed that the radical-scavenging activity of RV increases with the introduction of electron-donating groups, such as hydroxyls, and decreases in the presence of electron-withdrawing groups. Moreover, the 4′-OH group has been considered a fundamental structure for the radical scavenger activity of RV (Caruso, Tanski, Villegas-Estrada, & Rossi, 2004). However, our results demonstrated that the formation of sulphate or glucuronide conjugates from RV in 3 or 4′ position did not markedly modify its antioxidant activity whether the metabolite conserve two hydroxyl groups. Interestingly, we observed that RV metabolized in 3 position conserve a higher antioxidant activity than 4′ metabolite, in agreement with Torres, Poveda, Jimenez-Barbero, Ballesteros, and Plou (2010) who observed that an acyl chain in the position 3 caused a higher loss of antioxidant activity compared to the acetyl derivative at 4′-OH. Furthermore, these findings suggest that RV metabolites might be conserved other biological activities previously attributed to RV.

Recently, the distribution of RV and some of its metabolites has been reported in the colorectal tissue of patients who consumed daily doses of RV (Patel et al., 2010). The authors observed similar tissue levels of RV and RV 4′-*O*-glucuronide in colon mucosa and higher concentrations of RV 3-*O*-glucuronide and RV 3-*O*-sulphate (4-fold and 2-fold versus the RV concentration, respectively). These clinical findings indicate the importance of studying the effect of RV metabolites on intestinal epithelial cell growth/apoptosis in order to understand the antitumourigenic action of RV consumption (Cui et al., 2010; Sengottuvelan, Viswanathan, & Nalini, 2006; Tessitore, Davit, Sarotto, & Caderni, 2000).

Schneider et al. (2000) and Wolter, Akoglu, Clausnitzer, and Stein (2001) reported that RV (up to 50 μM) inhibits Caco-2 cell growth and leads to the accumulation of Caco-2 cells in the S phase of the cycle, without signs of apoptosis. Our results confirm this action of RV and clearly demonstrate that RV metabolites, such as RV 3-*O*-sulphate, RV 3-*O*-glucuronide and RV 4′-*O*-glucuronide, are able to inhibit non-differentiated Caco-2 cell proliferation, as well as Caco-2 DNA synthesis in a similar way to RV. Considering that sulphate and glucuronide metabolites of resveratrol reached concentrations between 1 and 30 μM and 1 and 50 μM in human colon after receiving resveratrol daily at 0.5 g (Patel et al., 2010). Our findings demonstrate that resveratrol metabolites concentrations reached in colon after resveratrol intake might have anti-proliferative action.

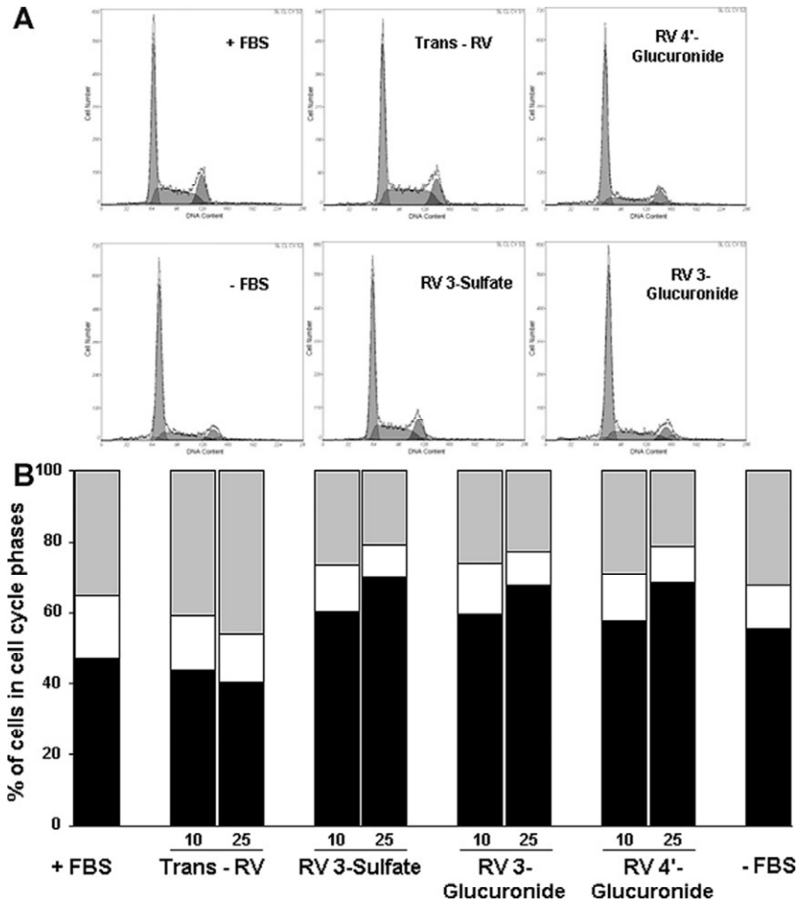

Fig. 3. Effect of resveratrol, resveratrol 3-*O*-sulphate, resveratrol 3-*O*-glucuronide and resveratrol 4'-*O*-glucuronide on Caco-2 cell cycle. Cells were incubated with resveratrol or resveratrol metabolites at 10 and 25 μM for 48 h and flow cytometric analysis of the cell cycle was conducted. (A) Cell cycle distribution of a representative experiment. (B) Grey, white and black bars represent the percentage of cells in the S, G_2/M and G_0/G_1 phase, respectively. Values are the mean ± SEM of three independent experiments performed in duplicate. *$P < 0.05$ versus control group (cells cultured in the presence of FBS 10%).

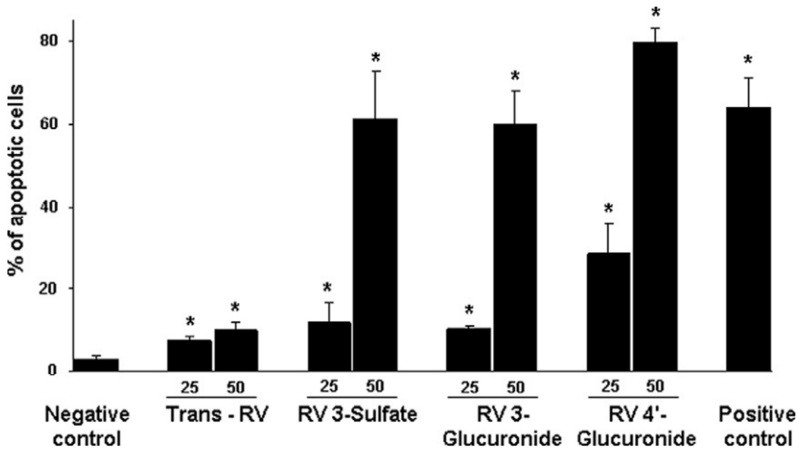

Fig. 4. Effect of resveratrol, resveratrol 3-*O*-sulphate, resveratrol 3-*O*-glucuronide and resveratrol 4′-*O*-glucuronide on apoptosis. Cells were incubated with resveratrol metabolites for 48 h and DNA fragmentation was measured. Values are the mean ± SEM of three independent experiments performed in triplicate. *$P < 0.05$ versus control group (cells cultured in the presence of FBS 10%).

Previous studies have shown that RV induces apoptosis at concentration of 100 μM in colorectal cancer cells (Trincheri, Nicotra, Follo, Castino, & Isidoro, 2007). It should be noted that RV metabolites at 50 μM decreased cell number respect to control conditions without FBS, suggesting a possible cytotoxic or pro-apoptotic effect of these metabolites at this concentration. This hypothesis was

confirmed by the observation an apoptotic effect of RV metabolites at 50 μM, whereas RV had no apoptotic effect at this concentration, as was reported previously by Schneider et al. (2000).

The flow cytometry results indicated a significant reduction in the number of cells in the G_0/G_1 phase of the cell cycle by RV, whereas the S phase population increased, which is consistent with previously published results (Schneider et al., 2000; Wolter et al., 2001). However, these effects were specific for RV because RV metabolites led to the accumulation of Caco-2 cells in the G_0/G_1 phase. These findings suggest different mechanism of action of RV and its metabolites on the cell cycle, with a similar final effect on Caco-2 cell growth. In this way, Roberti et al. (2006) reported that terphenyl analogue of stilbene-based, structurally related with our RV metabolites, block cell cycle in the G_0/G_1 phase, while RV as well as other stilbenes induced a recruitment of cells in S phase. Thus, RV and RV metabolites may act to distinct and redundant molecular targets and pathways important for the cell cycle. Additional studies are necessary to identify these targets to RV and RV metabolites.

To our knowledge this is the first time that an antiproliferative effect of the main RV metabolites, through the control of cell cycle and apoptosis, has been described. These findings suggest that these RV metabolites effects may mediate or contribute to the health benefits previously attributed to RV in intestinal epithelial cell growth and colorectal cancer models. These data prompted us to investigate in further detail the mechanism of action of RV and its metabolites on the cell cycle machinery in the future.

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JJM designed the study; CS conducted the research; JJM and CS analysed the data and wrote the paper. Both authors read and approved the final manuscript.

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Artículo 2

Piceid presents antiproliferative effects in intestinal epithelial Caco-2 cells, effects unrelated to resveratrol release

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Los resultados de esta publicación se han presentado en los siguientes congresos:

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Resumen Artículo 2

Objetivo: Estudiar el efecto del piceido sobre el crecimiento, el ciclo celular y la apoptosis de células epiteliales intestinales.

Material y métodos: Las células de adenocarcinoma Caco-2 en estado preconfluente se incubaron con los piceido en presencia de 10 % de SFB. Después de 48h se determinó el crecimiento celular mediante marcaje con bromuro de etidio/naranja de acridina, la síntesis de DNA mediante la incorporación de [^{3}H]thimidina, el ciclo celular por citometría de flujo y la fragmentación del DNA por TUNEL. La actividad antioxidante y la actividad enzimática α y β-glucosidasa se determinaron mediante técnicas espectrofotométricas y la presencia de piceido en el medio de cultivo mediante cromatografía líquida acoplada espectrometría de masas.

Resultados: El piceido presentó una actividad antioxidante similar al RV y superior al Trolox. Además, el piceido inhibió el crecimiento celular y la síntesis de DNA de forma concentración-dependiente, incrementó el porcentaje de células en la fase G0/G1 del ciclo celular e indujo apoptosis. También, observamos que las células Caco-2 no tienen actividad β-glucosidasa y no son capaces de deglicosilar el piceido

Conclusiones: A partir de estos resultados se propone por primera vez que el piceido no debe ser considerado una mera fuente de RV, ya que presenta actividad antiproliferativa mediante la modulación del ciclo celular y la apoptosis por sí solo. Además, se debería considerar el potencial efecto sinérgico del piceido junto con el RV.

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Piceid presents antiproliferative effects in intestinal epithelial Caco-2 cells, effects unrelated to resveratrol release

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Trans-piceid (*T*-Pc) is abundant in *Polygonum cuspidatum* and in grapes and grape products such as wine. Piceid reaches high levels in the stomach and intestine with rapid oral absorption. Tissues, such as liver tissue, can deglycosylate piceid to release resveratrol, so piceid can be considered a source of resveratrol, which has numerous biological activities such as antiproliferative effects. Therefore, the aim of this work was to analyze the action of *T*-Pc on intestinal epithelial cell growth. Our results show that *T*-Pc has antioxidant activity similar to that of *trans*-resveratrol (*T*-Rv) and higher than that of Trolox. Moreover, *T*-Pc (1–50 μM) inhibited Caco-2 cell growth and DNA synthesis in a concentration-dependent manner. We observed an increase in the percentage of cells in G_0/G_1 phase induced by *T*-Pc and the induction of apoptosis. Furthermore, we observed that Caco-2 cells did not have β-glucosidase activity and that Caco-2 cell cultures did not significantly deglycosylate *T*-Pc in our experimental conditions. On the basis of our results we propose, for the first time, that *T*-Pc must not be considered exclusively as a *T*-Rv source, and presents antiproliferative effects on intestinal epithelial cells through the modulation of the cell cycle and apoptosis by itself. Moreover, a synergistic action of *T*-Pc and *T*-Rv can be considered.

Introduction

Piceid (*trans*-resveratrol-3-*O*-β-glucoside), also named polydatin, is the main metabolite/precursor of resveratrol in plants. It is relatively abundant in *Polygonum cuspidatum*, a weed that is used in traditional Chinese and Japanese medicines. The presence of *trans*-resveratrol (*T*-Rv), *trans*-piceid (*T*-Pc) and their respective *cis*-isomers in the human diet is limited. They are principally found in grapes and grape derivatives such as grape juices and wines,[1–4] where the level of the *T*-Pc in red wine may be 10 times greater than the level of *T*-Rv.[5–7] Although there are other sources like peanuts,[8] pistachios[9] and some berries,[10] their total resveratrol levels are from 10 to 100 times lower than those in grape products.[11] Recently, Zamora-Ros *et al.*[4] reported that *T*-Rv, and especially *T*-Pc, are common components of the Mediterranean diet, and wine was found to be the main source with an estimated consumption mean of *T*-Pc of 500 μg per day.

The numerous biological effects of resveratrol are well known,[12] but there is little information about the biological activity of *T*-Pc, the predominant form of *T*-Rv in foods. The aglycone *T*-Rv seems to have a greater biological effect than the *T*-Pc.[13,14] However, it has been found that *T*-Pc can inhibit platelet aggregation, improve circulation, recover tissue damage induced by ischemia reperfusion, lower blood cholesterol and suppress lipid peroxide formation.[15,16] *T*-Rv plays numerous biological roles, possessing anti-inflammatory[17] and anti-proliferative/chemopreventive[18] activities, but the action and mechanisms of the antiproliferative effects of *T*-Pc in human tumor cells remain vague.

Actually, there are some references that support the hypothesis that deglycosylation by the β-glycosidases of the intestine is the first step in the absorption and metabolism of dietary polyphenol glycosides.[19–22] Thus, the deglycosylation of *T*-Pc could increase the levels of *T*-Rv available from diet.[20,23] However, we cannot exclude the possibility that *T*-Pc may be active on intestinal epithelium. Considering all together, the aim of this study was to clarify the action of *T*-Pc on intestinal epithelial cell growth and propose a mechanism for its biological effects.

Experimental

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO. Nonessential amino acids, fetal bovine serum (FBS), Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine

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pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, potassium ferricyanide, ferric chloride, gluconolactone, α-glucosidase, β-glucosidase, 4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-β-D-glucopyranoside and quercetin were supplied by Sigma-Aldrich. Tissue culture supplies and sterile material were obtained from Corning, Nirco S.L., NORM-JECT and Biosigma S.R.L. [Methyl-^{3}H] thymidine (20 Ci mmol^{-1}) were supplied by American Radiolabeled Chemicals Inc. Paraformaldehyde extra pure was from Merck Chemicals. The BioRad Protein Assay was obtained from Bio-Rad Laboratories, Inc. *T*-Rv and *T*-Pc were supplied by Cayman Chemical.

Cell and bacterial cultures

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37) (Manassas, VA, USA). The cells (passages 19–40) were routinely grown in 25 or 75 cm^2 plastic flasks at a density of 2–2.5 × 10^4 cells per cm^2 and cultured in DMEM with 4.5 g L^{-1} D-glucose and 2 mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10% (v/v) heat-inactivated FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. Cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 in air. Cells grown to ~80% confluence were released by trypsinization and subcultured at a density of 1.5–2 × 10^4 cells per cm^2 in 12 mm diameter plastic clusters and of 1.5 × 10^4 cells per cm^2 in 60 mm diameter plastic dishes. Growth medium was replaced twice per week. The experiments were performed on cells maintained for 3 days in culture (preconfluent cells). All experimentation products were diluted in DMSO (final concentration of DMSO was less than 0.1%).

Lactobacillus plantarum (ATCC® number 8014™) was provided by the Department of Microbiology of the University of Barcelona and grown in deMan, Rogosa and Sharpe (MRS) agar at 37 °C and 5% CO_2. After 24 h in culture, the bacteria were scraped for lysis and protein determination.

Cell growth and [^{3}H]-thymidine incorporation assays

The effect of the treatments was assessed on Caco-2 cell clusters in 24-well plates (1.5–2 × 10^4 cells per cm^2). Cells were cultured for 96 h in DMEM medium supplemented with 10% FBS. Then, the cells were incubated for 48 h in the presence of both compounds. Finally, the cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to count cells and to assess viability.[24]

DNA synthesis in Caco-2 was assessed by measuring the incorporation of [^{3}H]-thymidine. The cells were seeded in 24-well culture plates (1.5–2 × 10^4 cells per cm^2) and cultured for 96 h in DMEM medium supplemented with 10% FBS. Then, cells were incubated for 48 h in the presence of *T*-Rv or *T*-Pc at different concentrations and 0.1 μCi mL^{-1} of [^{3}H]-thymidine. After 48 h, the [^{3}H]-thymidine-containing media were aspirated and the cells were washed twice with 0.5% BSA in PBS for [^{3}H]-thymidine elimination. The washed cells were lysed with 300 μL of 1% Triton X-100 in PBS and added to a vial containing 3 mL of scintillation cocktail. Radioactivity present in the cell fraction was measured with a liquid scintillation counter (Packard Tri-Carb 1500, GMI Inc.).

Flow cytometry cell cycle analysis

Caco-2 cells were seeded in 60 mm dishes (1.5 × 10^4 cells per cm^2), and 96 h after culturing the cells were then incubated for 48 h in 10% FBS DMEM containing the treatments. Thereafter, the cells were trypsinized, fixed with 70% ethanol, and stored at 4 °C for at least 2 h. Next, low molecular weight DNA was extracted from the cells, which were stained for 1 h at room temperature with a 20 μg mL^{-1} propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg mL^{-1} DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corporation). DNA was analyzed (ploidy analysis) using single fluorescence histograms using Multicycle software (Phoenix Flow Systems).

TUNEL assay

Degradation of chromosomal DNA was evaluated with the TUNEL method using a MebStain Apoptosis Kit (MBL Int.). After 96 h in culture, Caco-2 cells were cultivated in media containing 10% FBS DMEM with treatments for 48 h. Next, the cells were fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analyzed on an Epics XL flow cytometer (Coulter Corporation).

Total antioxidant activity and reducing power

We selected different concentrations of each product and applied an Antioxidant Assay Kit (Cayman Chemical). This method is based on the ability of antioxidants in the sample (products diluted in PBS) to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS$^{\cdot+}$ by metmyoglobin.[25] The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as molar Trolox equivalents.

Total reducing power was determined according to the method by Oyaizu.[26] The first step was to mix 40 μL of the sample solution with 200 μL of phosphate buffer (0.2 M, pH 6.6) and 200 μL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 minutes. Afterwards, 200 μL of 10% trichloroacetic acid was added to the mixture. Finally, 60 μL of 0.167% ferric chloride was added and incubated at room temperature for 50 minutes. Increased absorbance at 690 nm of the reaction mixture indicated an increase in reducing power. Results were compared with a quercetin standard curve.

α-Glucosidase and β-glucosidase activity assays

For enzymatic activity determination it was necessary to extract proteins from the cell and bacteria cultures. Caco-2 cells were scraped with EDTA, centrifuged and resuspended in PBS, and bacteria were scraped only with a scraper and resuspended in

PBS. Afterwards, the cells and bacteria were lysed mechanically and sonicated (Ultrasonic processor UP200S, Hielscher Ultrasonic, Germany) to obtain a protein suspension without affecting the enzymatic activity of the sample. The protein concentration was determined in the homogenate by the Bradford method,[27] using the Bio-Rad protein assay kit with BSA as standard.

Enzymatic activity was measured using a spectrophotometric assay that measured the release of 4-nitrophenol from 4-nitrophenyl-α-D-glucopyranoside or 4-nitrophenyl-β-D-glucopyranoside (*p*-NPG, 5 mM) in citrate buffer (5 mM sodic citrate, 5 mM citric acid, pH 4.8). Samples (cellular and bacterial lysates), and α-glucosidase and β-glucosidase standards with *p*-NPG were incubated for 1 to 2 h at 37 °C, and the reaction was stopped with 1 M sodium carbonate. All tubes were analyzed at 405 nm using a Shimadzu UV-160A spectrophotometer.

Liquid chromatography-mass spectrometry analysis

Culture medium samples were filtered using 0.45 μm polytetrafluoroethylene filters, and then 15 μL volumes of culture medium were injected. The analysis was performed based on the method previously described by Urpi-Sarda *et al.*[28] with some modifications. Briefly, a Phenomenex Luna C_{18} 50 mm × 2.0 mm 5 μm column was used for the HPLC analysis, coupled to a single quadrupole mass spectrometer equipped with a turbo ion spray source working in negative mode. The compounds were separated using 0.5 mL L^{-1} acetic acid as mobile phase A and 700 mL L^{-1} acetone, 300 mL L^{-1} acetonitrile with 0.4 mL L^{-1} acetic acid as mobile phase B. A non-linear gradient was employed: 0 min, 90% A; 0–1 min, 70% A; 1–5 min, 0% A; 5–5, 6 min, 0% A; 5, 6–8 min, 90% A; 11 min, 90% A. The flow rate was 0.5 mL min^{-1} and the column was maintained at 40 °C to improve the chromatographic separation. The MS parameters were: Capillary voltage −3500 V, curtain gas (N_2) 12 (arbitrary units), nebulizer gas (N_2) 10 (arbitrary units), focusing potential −200 V, entrance potential −10 V, declustering potential −50 V, dry gas (N_2) heated at 400 °C and introduced at a flow rate of 6000 cm^3 min^{-1}. SIM (Single Ion Monitoring) mode was used to quantify *cis*-Rv (*C*-Rv), *T*-Rv, *cis*-Pc (*C*-Pc) and *T*-Pc. The dwell time applied for piceid (*m*/*z* 389) was 500 msec and for taxifolin and resveratrol (*m*/*z* 227 303 185 143) was 250 msec. Quantification of *T*-Rv and *T*-Pc was carried out using a six-point weighted calibration curve of the standards, with taxifolin as internal standard. *C*-Rv and *C*-Pc were quantified using an isomerized calibration curve, since *T*-Rv and *T*-Pc were isomerized by UV exposure.

Data analysis

Results are expressed as the mean ± standard error of the mean (SEM). Differences between non-treated and treated cells were tested by using Student's *t*-test. Differences of $P < 0.05$ were considered significant.

Results

Our results show that *T*-Rv has higher antioxidant and reducing power than Trolox and quercetin, respectively. Interestingly, we observed that *T*-Pc also shows antioxidant activity similar to that of *T*-Rv, although without the reducing power. Moreover, we observed a combined effect with both together (Table 1). Biological free radicals are among the underlying pathophysiological causes of the development of cancer.[29,30] Because *T*-Pc presents direct antioxidant activity, our next aim was to examine its antiproliferative action on a human colon cancer cell line.

To determine the effect of *T*-Pc and *T*-Rv on cell growth induced by FBS 10%, non-differentiated Caco-2 cells were incubated with these polyphenols. As shown in Fig. 1A, *T*-Pc and *T*-Rv significantly inhibited Caco-2 cell growth in a concentration-dependent manner. Considering that Henry *et al.*[31] reported that intestinal epithelial cells can hydrolyze *T*-Pc and release *T*-Rv, it is important to study whether the antiproliferative effect induced by *T*-Pc is consequence of *T*-Rv release from *T*-Pc. When cells were treated with *T*-Pc combined with a β-glucosidase inhibitor, gluconolactone,[32] it was observed that the presence of the inhibitor did not reverse the effect induced by *T*-Pc in a significant manner at 2 mM (Fig. 1B). The effect of *T*-Pc and *T*-Rv on non-differentiated Caco-2 cell DNA synthesis was also examined by [^{3}H]-thymidine incorporation in the cell cultures. At 10 μM, *T*-Rv inhibited DNA synthesis more than *T*-Pc at the same concentration, but at 25 μM both induce around 50% inhibition of [^{3}H]-thymidine uptake by Caco-2 cells after 48 h exposure (Fig. 2A). When the experiment was carried out in the presence of gluconolactone at 2 mM, we did not observe a significant reversal of the effects induced by *T*-Pc on cellular [^{3}H]-thymidine incorporation (Fig. 2B). Moreover, we observed that *T*-Rv and *T*-Pc added their effects in Caco-2 cell growth inhibition when combined (Fig. 1A and 2A).

The lack of an effect of the β-glucosidase inhibitor on the inhibition of Caco-2 cell growth and DNA synthesis induced by *T*-Pc suggests that *T*-Pc could have a direct action on these events. This hypothesis is supported by the low β-glucosidase activity present in non-differentiated and differentiated Caco-2 cells compared to *Lactobacillus plantarum* β-glucosidase activity (Table 2).[33] To corroborate the lack of *T*-Pc deglycosylation under our experimental conditions, Caco-2 cell cultures were incubated with *T*-Pc and we observed a constant concentration of the polyphenol over time (0–24 h), and a small concentration of *C*-Pc, probably because the commercial product has a purity of 95% (Fig. 3A). When cell cultures were treated with *T*-Rv, we found 95% of *T*-Rv at 0 time and this concentration decreased to 9% over the next 6 h, probably as consequence of its metabolization by intestinal epithelial cells (Fig. 3B).

To analyze the effect of *T*-Pc and *T*-Rv on the cell cycle, the distribution of cells in each phase of the cell cycle was quantified by determining the DNA content *via* flow cytometry, after 48 h exposure in the presence of FBS 10% and polyphenols. Cell cycle analysis of *T*-Rv treated cells showed that this polyphenol induced a significant accumulation of cells in the S phase with a consequent reduction in the percentage of cells in the G_0/G_1 phase at 25 μM. Interestingly, under the same conditions, *T*-Pc led to a significant accumulation of cells in the G_0/G_1 phase and reduced the percentage of cells in the S phase at concentrations between 10 and 25 μM (Fig. 4A).

Table 1 Total antioxidant activity and reducing power of *T*-Rv and *T*-Pc[a]

	Total antioxidant activity (mM)	Reducing power (mg mL^{-1})
T-Rv (50 μM)	0.183 ± 0.01	7.385 ± 0.07
T-Rv (100 μM)	0.225 ± 0.03	ND
T-Pc (50 μM)	0.205 ± 0.03	0.02 ± 0.01
T-Pc (100 μM)	0.230 ± 0.01	ND
T-Rv (100 μM) + *T*-Pc (100 μM)	0.462 ± 0.1	ND

[a] Data are the mean ± SEM of three experiments performed in duplicate. Total antioxidant activity is expressed as equivalents of Trolox (mM). Reducing power is expressed as equivalents of quercetin (mg mL^{-1}). ND, not determined.

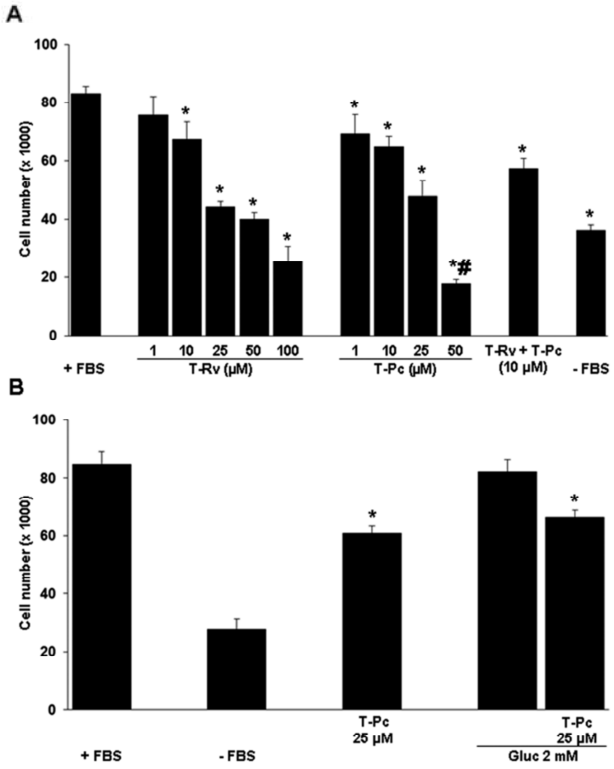

Fig. 1 Effect of *T*-Rv and *T*-Pc on Caco-2 cell growth induced by FBS 10%. (A) Cells were treated with individual (1–100 μM) and mixed (10 + 10 μM) *T*-Rv and *T*-Pc for 48 h and counted. Values are mean ± SEM ($n = 12–20$). (B) Effect of *T*-Pc (25 μM) with and without the inhibitor gluconolactone (Gluc) at 2 mM on Caco-2 cell growth induced by FBS 10%. Cells were treated for 48 h with treatments and counted. Negative control of proliferation was performed in the absence of FBS (-FBS). Values are mean ± SEM ($n = 14–15$). *$P < 0.05$ *versus* control group (cells cultured in the presence of FBS). #$P < 0.05$ *versus* cells cultured with *T*-Rv.

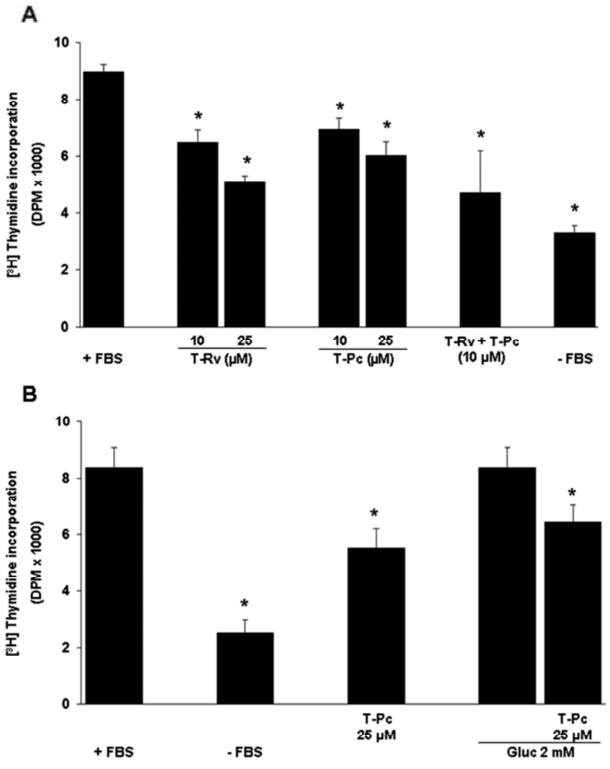

Fig. 2 Effect of *T*-Rv and *T*-Pc on Caco-2 DNA synthesis induced by FBS 10%. (A) Cells were treated with individual (10–25 μM) and mixed (10 + 10 μM) *T*-Rv and *T*-Pc for 48 h in the presence of [^{3}H]-thymidine and afterwards the [^{3}H]-thymidine uptake was assayed. Values are mean ± SEM ($n = 7–14$). (B) Effect of *T*-Pc (25 μM) with and without the inhibitor gluconolactone (Gluc) at 2 mM on Caco-2 DNA synthesis induced by FBS. Cells were treated for 48 h in the presence of [^{3}H]-thymidine and the [^{3}H]-thymidine uptake was assayed. Values are mean ± SEM ($n = 8–10$). Negative control of proliferation was performed in the absence of FBS (-FBS). *$P < 0.05$ *versus* control group (cells cultured in the presence of FBS).

We did not observe a hypodiploid DNA peak, characteristic of apoptosis, in these experimental conditions (Fig. 4B). However, we measured DNA fragmentation induced by *T*-Pc and *T*-Rv as an index of their apoptotic activity. Fig. 5 shows that the *T*-Pc induced marked DNA fragmentation (50%) at 50 μM, whereas *T*-Rv had a lesser effect at different concentrations (25–100 μM).

Discussion

T-Pc has diverse biological effects[15,16] that could be attributed to *T*-Rv released as a consequence of deglycosylation of *T*-Pc by the intestine and/or liver. In this way, Kineman *et al.*[34] reported that *T*-Rv incorporated into the diet reduced the number of aberrant crypt foci formed in experimental colon cancer, whereas a diet

Table 2 Glucosidase activities of non-differentiated (preconfluent) and differentiated Caco-2 cells cultured in the presence of FBS 10%[a]

	(μ units of enzyme/μg of protein/minute)	
	α-Glucosidase	β-Glucosidase
Non-differentiated cells	2.13 ± 0.3	0.11 ± 0.02
Differentiated cells	6.52 ± 0.4*	0.11 ± 0.01
Lactobacillus plantarum	ND	1.17 ± 0.09

[a] Data are the mean ± SEM of three experiments performed in duplicate. *$P < 0.05$ *vs.* non-differentiated cells. ND, not determined.

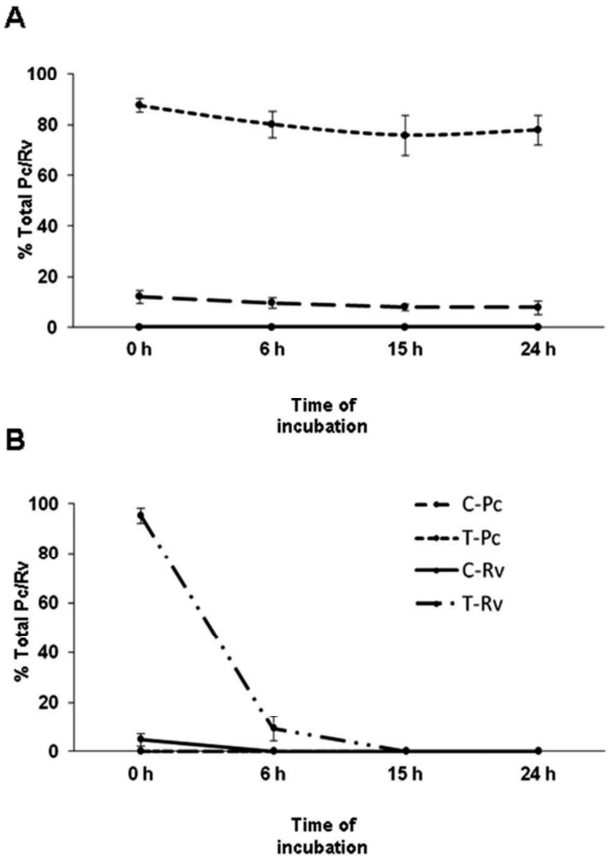

Fig. 3 *T*-Rv and *T*-Pc levels in Caco-2 supernatant cultures. Non-differentiated Caco-2 cells were incubated with *T*-Pc (A) or *T*-Rv (B) at 25 μM. After 0, 6, 15 and 24 h we took samples of culture supernatant and *T*-Pc, *T*-Rv and their *cis*-isomers were quantified by HPLC. Results are expressed as percentage of variation, with respect to the total content of *T*-Pc or *T*-Rv and their *cis*-isomers at baseline (0 h). Values are mean ± SEM of two experiments performed in duplicate.

prepared with transgenic alfalfa rich in *T*-Pc was not effective. However, diets containing *T*-Pc with exogenous β-glucosidase did significantly reduce aberrant crypt foci formation, suggesting that *T*-Rv released from *T*-Pc was effective in this murine model of carcinogenesis.[34] Our results show that *T*-Pc has direct antioxidant activity *in vitro* similar in extent to *T*-Rv, as was recently reported by Medina *et al.*[35] The hydroxyl group at the 4′-position is relevant for the antioxidant efficiency of *T*-Rv and it is maintained in *T*-Pc, which consequently keeps the antiradical activity. Moreover, our results demonstrated that *T*-Pc is antiproliferative towards non-differentiated Caco-2 cells, similar to *T*-Rv, in agreement with Ha do *et al.*[36] who reported the inhibition of L1210 (mouse lymphocytic leukemia cell line) and K562 (human erythromyeloblastoid leukemia cell line) cells by *T*-Pc.

The Caco-2 cell line spontaneously differentiates into polarized enterocytes, expressing brush border enzymes typical of small intestine epithelial cells such as disaccharidases. Under our experimental conditions, we observed very low β-glucosidase activity in these cells, whereas the non-differentiated intestinal epithelial cells presented high α-glucosidase activity. Interestingly, we did not observe appreciable deglycosylation of *T*-Pc by non-differentiated Caco-2 cell cultures. Altogether, this suggests that *T*-Pc is not appreciably deglycosylated under our experimental conditions and might have direct antiproliferative action in Caco-2 cell cultures without *T*-Rv involvement in these events. Moreover, our findings show that the *T*-Pc effect was added to the *T*-Rv action in Caco-2 cell growth inhibition.

The flow cytometry results indicated a significant reduction in the number of cells in the G_0/G_1 phase of the cell cycle by *T*-Rv, whereas the S phase population increased in agreement with previous reports.[37] However, these effects were specific for *T*-Rv, because *T*-Pc led to the accumulation of Caco-2 cells in the G_0/G_1 phase. These findings suggest different mechanisms of action of *T*-Rv and *T*-Pc in the cell cycle, with a similar final effect on Caco-2 cell growth. Interestingly, the action of *T*-Pc on the Caco-2 cell cycle was similar to the effects induced by *T*-Rv metabolites (sulphates and glucuronides).[37] Thus, *T*-Rv and *T*-Pc may act on different and redundant molecular targets and pathways involved in the regulation of the cell cycle. Additional studies are necessary to identify the targets of *T*-Pc.

Previous studies have shown that *T*-Rv induces apoptosis at a concentration of 100 μM in colorectal cancer cells.[11] It should be noted that *T*-Pc at 50 μM decreased the cell number, with respect to control conditions without FBS, to a greater extent than *T*-Rv under our experimental conditions. These findings suggest a possible cytotoxic or pro-apoptotic effect of *T*-Pc. This hypothesis was confirmed by the observation that *T*-Pc at 50 μM induced DNA fragmentation, whereas *T*-Rv had a minor effect at this concentration.

T-Pc diffuses to most tissues immediately after oral administration, with a peak level at 10 min. The maximum level of *T*-Pc was detected in the stomach (169 μg g^{-1}) and intestine (109 μg g^{-1}) after an oral dose of 50 mg kg^{-1} in Wistar rats.[38] Recently, Rotches-Ribalta reported approximately μM concentrations of total human plasma resveratrol/piceid (free and metabolites) after moderate red wine consumption.[39] Considering these pharmacokinetic data, our findings suggest that the *T*-Pc concentrations reached in colon mucosa after oral *T*-Pc intake might have appreciable anti-proliferative/pro-apoptotic actions. However, additional investigation into the biological activity of piceid and piceid plus resveratrol on intestinal epithelial proliferation in *in vivo* models is needed.

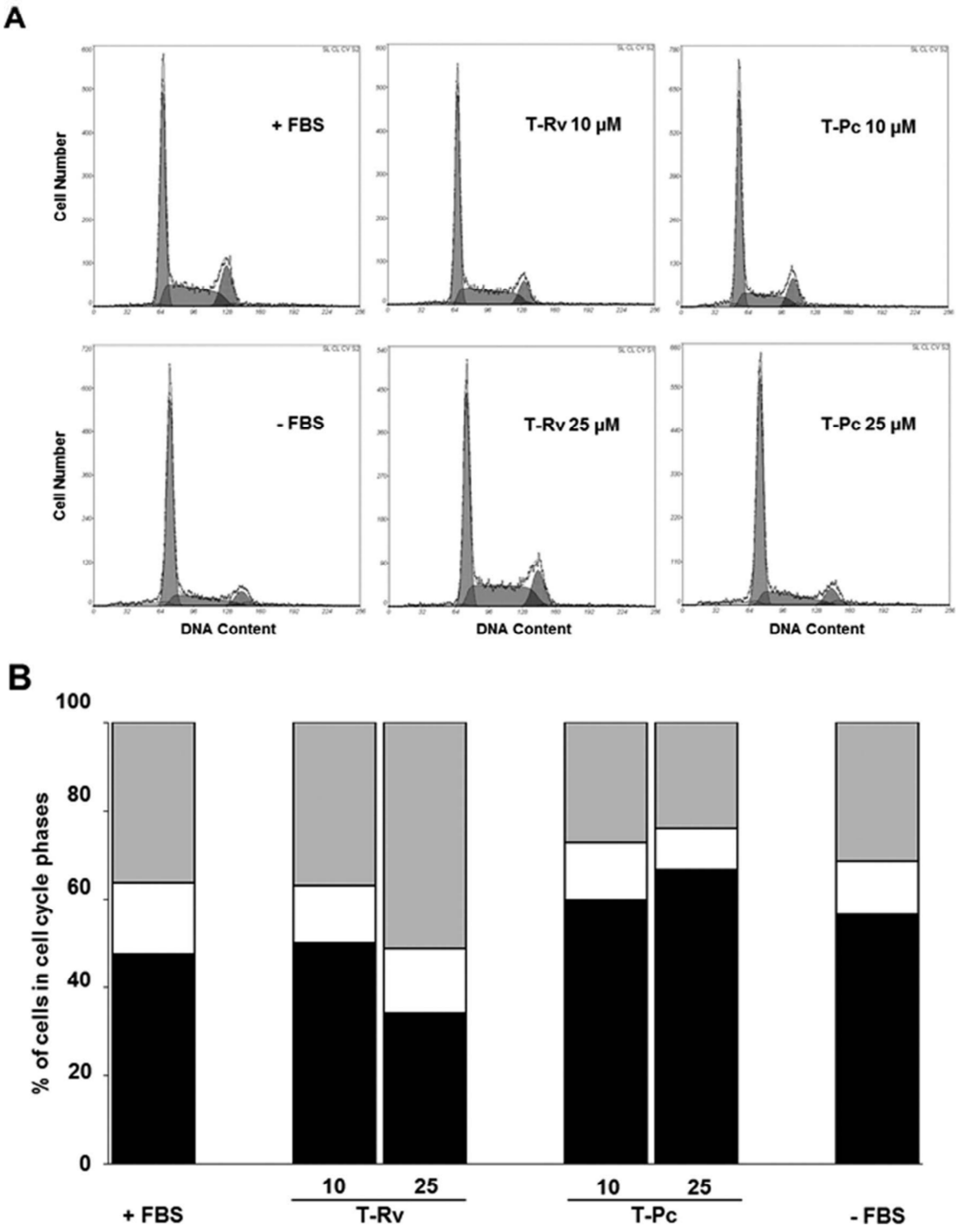

Fig. 4 Effect of *T*-Rv and *T*-Pc on the Caco-2 cell cycle. Non-differentiated cell cultures were incubated with *T*-Rv or *T*-Pc (10–25 μM) for 48 h and flow cytometric analysis of the cell cycle was conducted; we show a cell cycle distribution of a representative experiment (A). Grey, white and black bars represent the percentage of cells in S, G_2/M and G_0/G_1 phases, respectively (B). Negative control of proliferation was performed in the absence of FBS (−FBS).

Conclusion

To our knowledge, this is the first time that antiproliferative effects of *T*-Pc, through the control of cell cycle and apoptosis, have been described in intestinal epithelial cells. The results are in agreement with recent studies that reported an anti-proliferative effect of *T*-Pc on human liver and breast cancer cells[40] and lung cancer cells.[41] Moreover, these findings suggest that *T*-Pc must not be considered exclusively as a *T*-Rv source and can raise biological effects by itself. Furthermore, a synergistic

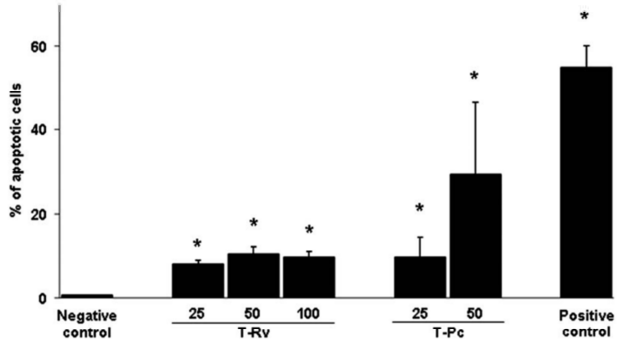

Fig. 5 Effect of *T*-Rv and *T*-Pc on apoptosis. Non-differentiated Caco-2 cells were incubated with polyphenols (25–100 μM) for 48 h and DNA fragmentation was measured. Values are mean ± SEM ($n = 7–14$). *$P < 0.05$ *versus* the negative control group (cells cultured in the presence of FBS 10%). As positive controls we used staurosporine (1 μM).

action of *T*-Rv and *T*-Pc can be considered. These data have prompted us to investigate in further detail the mechanism of action of *T*-Pc on the cell cycle machinery in the future.

Conflict of interest

Authors have no conflict of interest affecting the conduct or reporting of the work submitted.

Acknowledgements

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Resultados no publicados 1

Resveratrol analogues present antiproliferative effect on intestinal epithelial cancer cell

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Preparada para enviar a revista científica

Los resultados de esta publicación se han presentado en los siguientes congresos:

Biological effect of resveratrol analogues on intestinal epithelial cancer cell growth. CE Storniolo, RM Lamuela-Raventós, MT Mitjavila, JJ Moreno. Resveratrol2010, 1st International conference of resveratrol and health. Elsinore, Dinamarca. 13-16/09/2010. **Póster**

Biological effect of resveratrol analogues on intestinal epithelial cancer cell growth. CE Storniolo, JJ Moreno. 5th International Conference on Polyphenols and Health (ICPH2011). Sitges, España. 17-20/10/2011. **Póster**

Resultados no publicados 1

(Editados para enviar a revista científica)

RESVERATROL ANALOGUES PRESENT ANTIPROLIFERATIVE EFFECT ON INTESTINAL EPITHELIAL CANCER CELL

Introduction

Resveratrol (3,4',5-trihydroxy-trans-stilbene, Res) is a polyphenol and has been classified as a phytoalexin as it is synthesized in spermatophytes in response to injury, UV irradiation and fungal attack (1). It was first isolated in 1940 as an ingredient of the roots of white hellebore (Veratrum grandiflorum O. Loes) and has since been found in a wide variety of ~70 plant species, including grapes, mulberries and peanuts (2,3). Res is formed by a condensation reaction between 3 molecules of malonyl CoA and a molecule of 4-coumaroyl coacatalyzed by Res synthase (4). It is classified as a polyphenol compound with more than one phenol group and antioxidant activity, which by reacting with free radicals makes them less toxic and suppresses tumor development through the removal of reactive oxidant species.

Several *in vivo* studies have been conducted. Oral administration of Res on tumorigenesis in Min mice (which are genetically predisposed to develop intestinal tumors as a result of mutation of APC gene) starting from 5 wk of age prevented the development of colon cancer and reduced the formation of intestinal tumors by 70% in comparison with the control group (5). A comparison of the gene expression profile showed that Res downregulated the genes directly involved in cell cycle progression and proliferation (e.g., Cyclin D1 and D2) and upregulated the genes involved in recruitment and activation of immune response (5). Studies on the effects of Res on azoxymethane-induced (AOM) carcinogenesis revealed a significant reduction in the number and multiplicity of ACF in the colorectal mucosa (6). Also, Res was found to differentially regulate the expression of BAX and p21 in mucosa with ACF and non-ACF peripheral mucosa (6). In the xenograft gastric tumor model, 6 injections of high doses of Res at an interval of 2 days near the tumor site inhibited tumor progression (7). Studies performed on in Wistar rats found a significant reduction in tumor incidence and the occurrence of histological lesions following administration of Res (8 mg/kg body weight for 30 wk) (8).

Even though the exact mechanism responsible for the chemopreventive property of Res is not clear, various studies have implicated its involvement in modulating a variety of pathways or processes leading to tumor development, resulting in inhibition of cellular events associated with all 3 stages of cancer development, namely, initiation, promotion, and progression (9). The number of studies toward understanding the role of Res in preventing or reversing colon cancer progression has been growing. A significant number of these studies have shown that induction of apoptotic cell death and inhibition of cell cycle progression are the two major pathways responsible for the chemopreventive role of Res in colon cancer (10). Res induces apoptosis by clustering FAS and forming a death inducing signaling complex in SW480 human colon cancer cell line (11). Res induced dose dependent apoptotic cell death in a colon cancer cell line (HT 27), and this is caused by induction of endoplasmic reticulum stress response as indicated by the induction of ER stress markers such as eIF-2 (eukaryotic initiation factor 2a) (12). Studies using the human colonic adenocarcinoma cell line Caco-2 and the colon carcinoma cell line HCT-116 showed that the chemopreventive effects on colonic cancer cells is by cell cycle arrest as a consequense of decrease on cyclin D1 and CDK4 levels decrease and by induction of apoptosis as indicated by increased caspase activity (13). A study using multiple colon cancer cell lines showed that Res has direct dose dependent antiproliferative activity (14).

The aim of this study was to determine how different molecular changes, like hydroxylation, methylation, chlorination and isomerization, in Res structure can modify their effect on antioxidant activity, DNA synthesis and cellular growth, cell cycle and induction of apoptosis in Caco-2 cells, a human adenocarcinoma cell line.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO (Paisley, Scotland). Nonessential amino acids, FBS, Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, potassium ferricyanide, ferric chloride and quercetin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Tissue culture supplies and sterile material were obtained from Corning (NY, USA), Nirco S.L. (Barcelona, Spain), NORM-JECT (PA, USA) and Biosigma S.R.L. (Venice, Italy). The scintillation cocktail Biogreeen 3 and the [Methyl-3H] thymidine (20 Ci/mmol) were supplied by Scharlau CEIME (Barcelona, Spain) and American Radiolabeled Chemicals Inc. (St. Louis, MO, USA), respectively. The MebStain

Apoptosis Kit was obtained from MBL International (Woburn, MA, USA) and paraformaldehyde extra pure from Merck Chemicals (Barcelona, Spain). *Trans*-Res (5-[(1E)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol, tRes) cis-Res (5-[(1Z)-2-(4-hydroxyphenyl)ethenyl]-1,3-benzenediol, cRes), *trans*-trimethoxy-Res (1,3-dimethoxy-5-[(1E)-2-(4-methoxyphenyl)ethenyl]-benzene), Piceatannol (4-[(1E)-2-(3,5-dihydroxyphenyl) ethenyl]-1,2-benzenediol), Pterostilbene (4-[(1E)-2-(3,5-dimethoxyphenyl)ethenyl)-phenol), CAY10464 (1,3-dichloro-5-[(1E)-2-(4-methoxyphenyl)ethenyl]-benzene), CAY10465 (1,3-dichloro-5-[(1E)-2-[4-(trifluoromethyl)phenyl]ethenyl]-benzene), CAY10616 (5-[(1Z)-2-(3,5-dimethoxyphenyl)ethenyl]-2-methoxy-phenol), pinostilbene (3-[(1E)-2-(4-hydroxyphenyl)ethenyl]-5-methoxy-phenol), PDM2 (1,3-dichloro-5-[(1E)-2-(4-chlorophenyl)ethenyl]-benzene) and PDM11 ((E)-5-[2-(4-chlorophenyl)ethenyl]-1,3-dimethoxyphenyl) were supplied by Cayman Chemical (MI, USA).

Cell culture. Caco-2 cells (HTB-37TM) were kindly provided by Dr. David Thwaites at the School of Cell and Molecular Biosciences, University of Newcastle-upon-Tyne (UK). The cells (passages 19-40) were routinely grown in 25 or 75 cm2 plastic flasks at a density of 2-2.5x104 cells/cm2 and cultured in DMEM with 4.5 g/L D-glucose and 2mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were incubated at 37 ºC under a humidified atmosphere of 5% CO2 in air. We use another culture medium like the described above but without FBS for obtain a negative control group of cell growth. Cells grown to □ 80% confluence were released by trypsinization and subcultured at a density of 1.5-2.5 x 104 cells/cm2 in 12 mm diameter plastic clusters and of 1.5 x 104 cells/cm2 in 60 mm diameter plastic dishes. Growth medium was replaced twice per week. The experiments were performed in cells maintained for 3 days in culture (preconfluent cells). All experimentation products were diluted in DMSO (final concentration of DMSO was lesser than 0.1%).

Cell growth assay. The effect of the treatments was assessed on Caco-2 12mm clusters in 24-well plates. Cells were cultured for 96 h in DMEM medium supplemented with 10% FBS. Then, cells were incubated for 48 h in the presence of both compounds. Finally, cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to count cells and to assess viability.

Flow cytometry cell cycle analysis. Caco-2 cells were seeded in 60 mm dishes, and 96 h after culture cells were then incubated by 48 h in 10% FBS DMEM containing the treatments. Thereafter, cells were trypsinized, fixed with 70% ethanol, and stored at 4°C for at least 2 h. Next, low molecular weight

DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corporation, Philadelphia, USA). DNA was analyzed (ploidy analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems, CA, USA).

Tunnel assay. Degradation of chromosomal DNA was evaluated with TUNEL method using a MebStain Apoptosis Kit. Caco-2 cells were cultivated in media containing 10% FBS with treatments for 48 h. Next, cells were fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3'-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analyzed on an Epics XL flow cytometer.

Total Antioxidant Activity of the products. We selected different concentration of any product and applied an Antioxidant Assay Kit (Cayman Chemical, MI, USA). This method is based on the ability of antioxidants in the sample (products diluted in PBS) to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS•+ by metmyoglobin(31). The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as molar Trolox equivalents.

DNA synthesis. Was assessed by a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell proliferation ELISA, BrdU Kit, from Roche). Caco-2 cells were cultured at 1000-1500 cell/well in 96 well plates for 96 h in DMEM supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of compounds. Thereafter, cells were treated following the manufacturer instructions. Absorbance was measured at 450 nM in a plate reader (TECAN, Sunrise, Grödig, Austria).

Mitochondrial membrane potential determination. Mitochondrial membrane depolarization (MMP) was measured through tetramethylrhodamine ethyl ester perclorate (TMRE) incorporation into active preconfluent Caco-2 cell mitochondria. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used like a positive control for MMP. Cell cultures were treated with experimental compounds for 20 minutes and, after tripsinization, cell suspension was labelled with TMRE for 30 minutes. Finally samples were analysed in FC500 flow cytometer (Coulter Corporation) to measure mitochondrial depolarization or hyperpolarization.

Data analysis. Results are expressed as mean ± standard error of the mean. Differences between non-treated and treated cells were tested by using Student's t-test. Differences of $P < 0.05$ were considered significant.

Results

Figure 1 shows the chemical structure of the tRes and several analogues used in this study. Piceatannol presents an additional hydroxyl respect to tRes. Pinostilbene, pterostilbene and trans-trimethoxy tRes replace one, two or three hydroxyls by methoxyls, respectively. PDM11, CAY10464 and PDM2 replace one, two or three hydroxyls by chloro, respectively. CAY10465 presents two chloro and one trifluoromethyl and finally CAY10616 has a cis-piceatannol structure but with and additional hydroxyl.

TRes and piceatannol have the highest antioxidant activity. These both compounds together with cis-Res and pinostilbene have an antioxidant capacity higher than Trolox. CAY10616 and pterostilbene present a equivalent antioxidant activity to Trolox (**Figure 2**). Thus, the impairment of the number of hydroxyls replaced by methoxyls or chloros decrease the antioxidant capacity of the molecules.

TRes inhibited Caco-2 cell growth in concetration dependent manner, reached a completed inhibition of Caco-2 cell proliferation around 10 μM (**Figure 3**). The cis- isomer presents a similar effect but with a low potency. All structural analogues assayed were able to induce Caco-2 cell growth. Interentingly, the addition of an hydroxyl group to tRes structure did not significantly enhance the antiproliferative activity. In a similar way, the replace of hydroxyls by methoxyls did not improve the inhibition of Caco-2 cell growth. The replace of methoxyls by chloros induced a slight decrease of antiproliferative capacity of these structures. The replace of one chloro by a trifluoromethyl did not change the antimitogenic effect of PDM2 (trans-trichloro Res). Finally, CAY10616 that has a *cis*-trimethoxy Res structure with a additional hydroxyl, presents a higher antiproliferative action than trans- and cis-Res or trans-trimethoxy Res (**Figure 3**). These fundings were confirmated by DNA synthesis assay (**Figure 4**). Thus, all compunds at concentrations that induced Caco-2 cell growth inhibition were able to induce DNA synthesis.

tRes and their isomers/analogues assayed increased the percentage of Caco-2 cells in S phase and decreased the percentage of cells in G_0/G_1 phase (**Figure 5**). CAY10616 induced an additional increase in the percentage of cells in G_2/M phase.

In **Figure 3** we can observe that trans- and cRes, piceatannol, pinostilbene, pterostilbene, CAY10464, CAY10465 and CAY10616 at high concentration decreased the cell number respect to Caco-2 cell cultured without FBS, foundings that suggest an cytotoxic or apoptotic effect. When we analyzed the effect of these compounds on apoptosis through DNA fragmentation assay, we observed that all of them presents apoptotic capacity being tRes, pinostilbene, CAY10464, CAY10465 and CAY10616 the most active in our experimental conditions (**Figure 6**). To study the mechanism of this effect we analyzed the action of these compounds on mitochondrial membrane potential variation, that is involved in the intrinsic pathway of the apoptosis. Our results shown that tRes and pinostilbene induced mitochondrial membrane despolarization at short incubation period, whereas at long time incubation all assayed compounds induce a mitochondrial membrane hiperpolarization (**Figure 7**).

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Figures

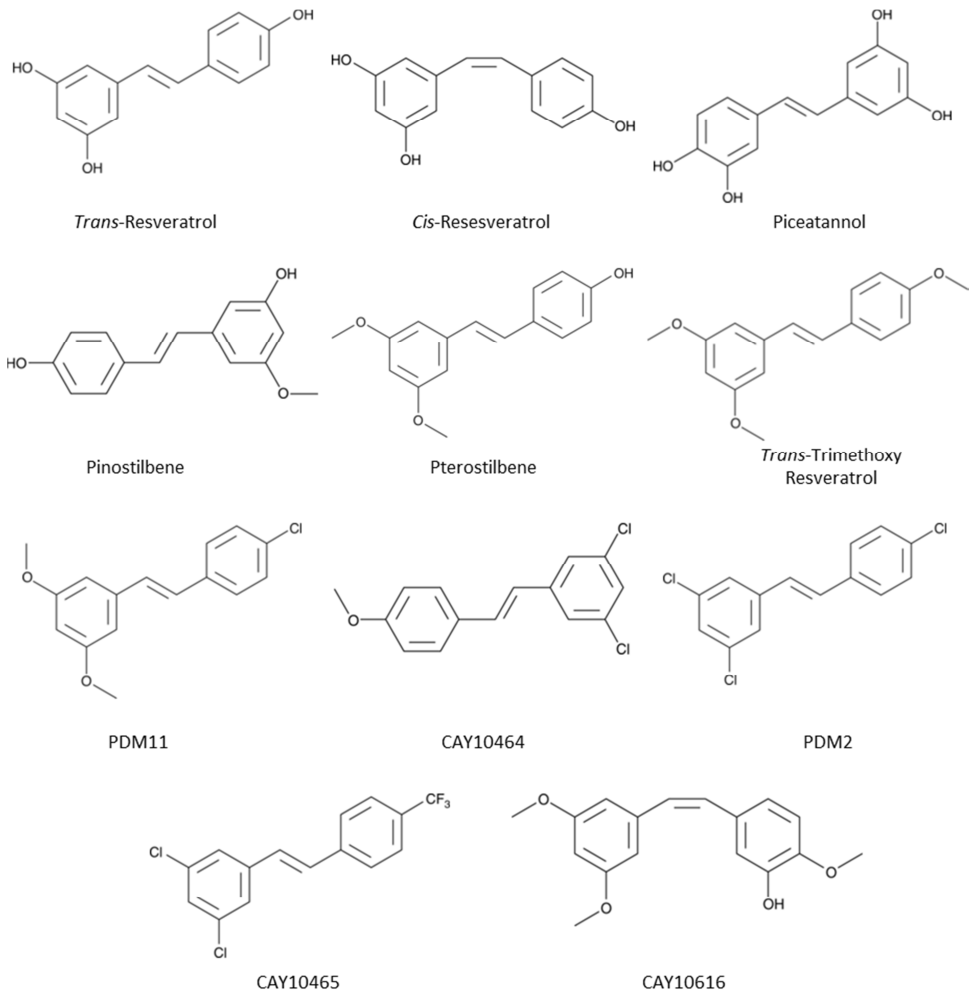

Figure 1. Chemical structure of trans-resveratrol and analogues used in this study.

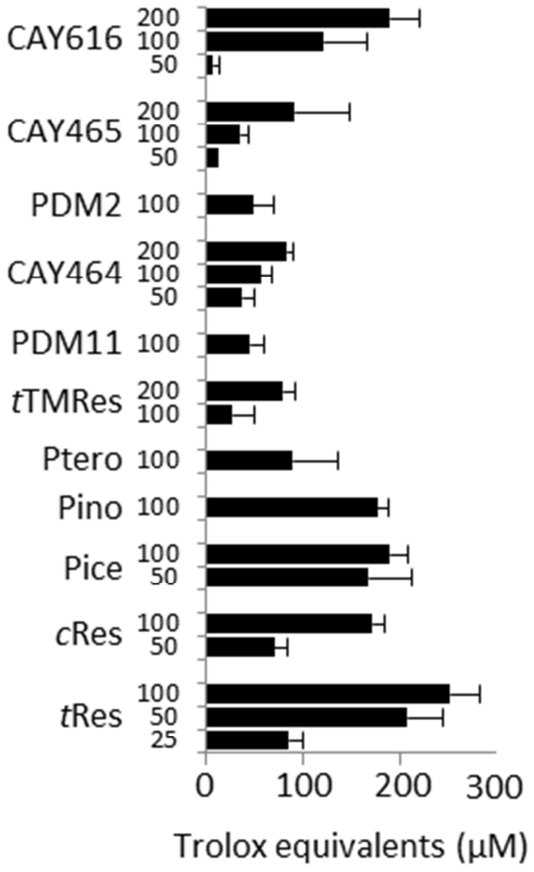

Figure 2. Total antioxidant activity of resveratrol and analogues. *Trans*-resveratrol (tRes, 25, 50 and 100 μM), *cis*-resveratrol (cRes, 50 and 100 μM), piceatannol (Pice, 50 and 100 μM), pinostilbene (Pino, 100 μM), pterostilbene (Ptero, 100 μM), *trans*-trimethoxyresveratrol (tTMRes, 100 and 200 μM), PDM11 (100 μM), CAY10464 (CAY464, 50, 100 and 200 μM), PDM2 (100 μM), CAY10465 (CAY465, 50, 100 and 200 μM) and CAY10616 (CAY616, 50, 100 and 200 μM) were analyzed as described in the material and methods section. Total antioxidant activity is expressed as equivalents of Trolox (μM). Data are the mean ± SEM of 2 experiments performed in duplicate.

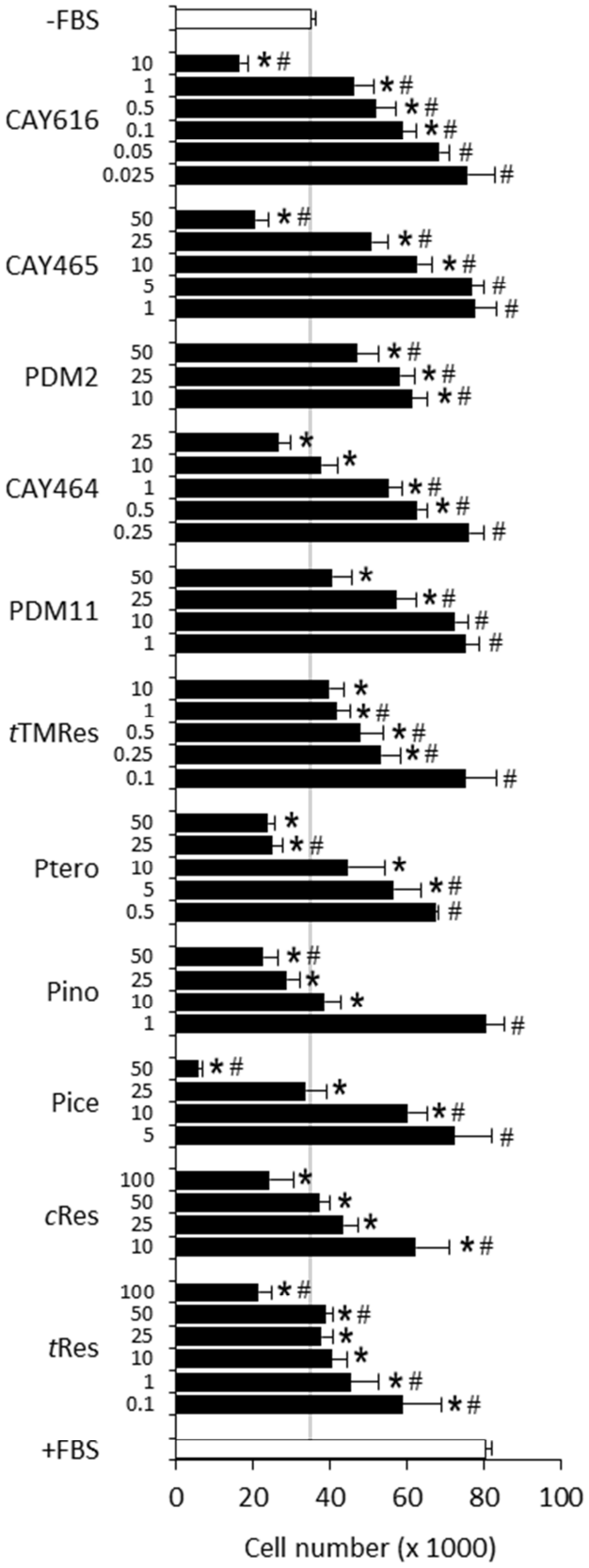

Figure 3. Effect of resveratrol and analogues on Caco-2 cell growth in presense of FBS. *Trans*-resveratrol (tRes, 0.1, 1, 10, 25, 50 and 100 μM), *cis*-resveratrol (cRes, 10, 25 ,50 and 100 μM), piceatannol (Pice, 5, 10, 25 and 50 μM), pinostilbene (Pino, 1, 10, 25 and 50 μM), pterostilbene (Ptero, 0.5, 5, 10, 25 and 50 μM), *trans*-trimethoxyresveratrol (tTMRes, 0.1, 0.25, 0.5, 1 and 10 μM), PDM11 (1, 10, 25 and 50 μM), CAY10464 (CAY464, 0.25, 0.5, 1 and 10 μM), PDM2 (10, 25 and 50 μM), CAY10465 (CAY465, 1, 5, 10, 25 and 50 μM) and CAY10616 (CAY616, 0.025, 0.05, 0.1, 0.5, 1 and 10 μM) were incubated with Caco-2 cells with 10% FBS for 48 hours and were then counted. Results are expressed as mean ± SEM (n = 8-30). * $P < 0.05$ vs control group with FBS (+ FBS). # $P < 0.05$ vs control group whitout FBS (- FBS).

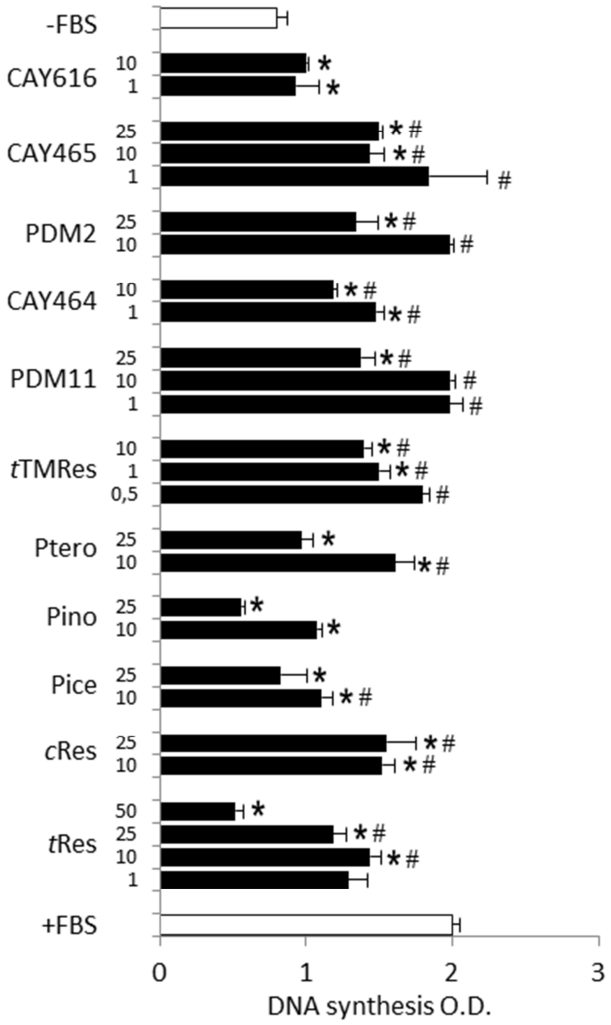

Figure 4. Effect of resveratrol and analogues on Caco-2 DNA synthesis in presence of growth factors. . *Trans*-resveratrol (tRes, 1, 10, 25, 50 and 100 μM), *cis*-resveratrol (cRes, 10 and 25 μM), piceatannol (Pice, 10 and 25 μM), pinostilbene (Pino, 10 and 25 μM), pterostilbene (Ptero, 10 and 25 μM), *trans*-trimethoxyresveratrol (tTMRes, 0.5, 1 and 10 μM), PDM11 (1, 10 and 25 μM), CAY10464 (CAY464, 1 and 10 μM), PDM2 (10 and 25 μM), CAY10465 (CAY465, 1, 10 and 25 μM) and CAY10616 (CAY616, 1 and 10 μM) were incubated with Caco-2 cells with 10% FBS for 48 hours and DNA synthesis measured by cell BrdU incorporation. Results are expressed as mean ± SEM (n = 3-12). * $P < 0.05$ vs control group with FBS (+ FBS). # $P < 0.05$ vs control group whitout FBS (- FBS).

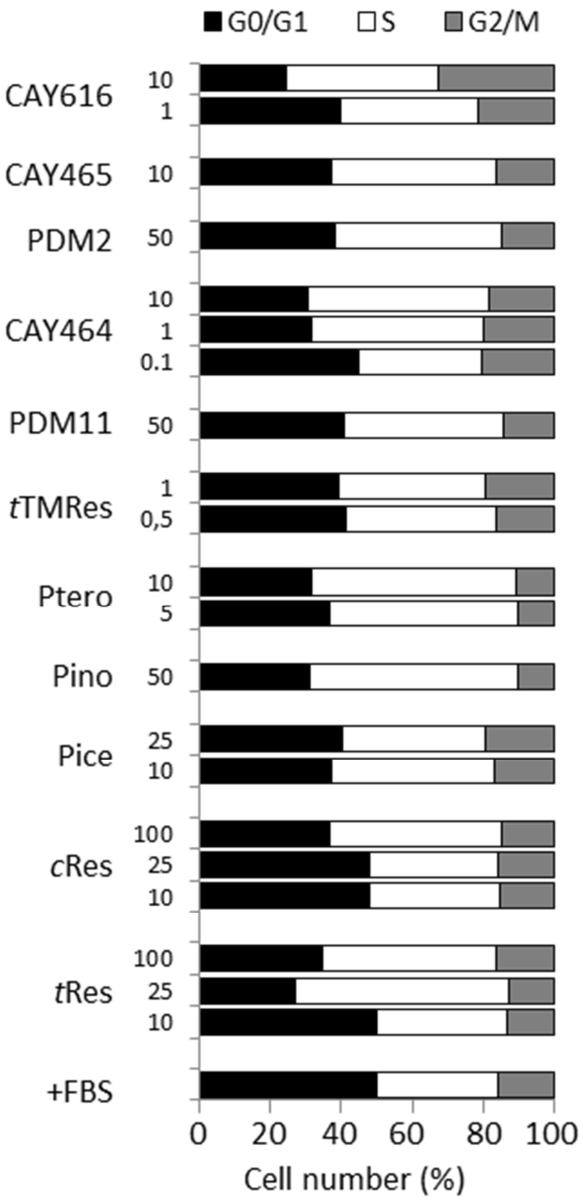

Figure 5. Effect of resveratrol and analogues on Caco-2 cell cycle. Cells were incubated with *trans*-resveratrol (tRes, 10, 25 and 100 μM), *cis*-resveratrol (cRes, 10, 25 and 100 μM), piceatannol (Pice, 10 and 25 μM), pinostilbene (Pino, 50 μM), pterostilbene (Ptero, 5 and 10 μM), *trans*-trimethoxyresveratrol (tTMRes, 0.5 and 1 μM), PDM11 (50 μM), CAY10464 (CAY464, 0.1, 1 and 10 μM), PDM2 (50 μM), CAY10465 (CAY465, 10 μM) and CAY10616 (CAY616, 1 and 10 μM) for 48 h and the flow cytometry assay of cell cycle was performed. Grey, white and black bars represent the percentage of cells in G0/G1, S and G2/M phase, respectively. Values are mean ± SEM of three independent experiments (n = 3-6).

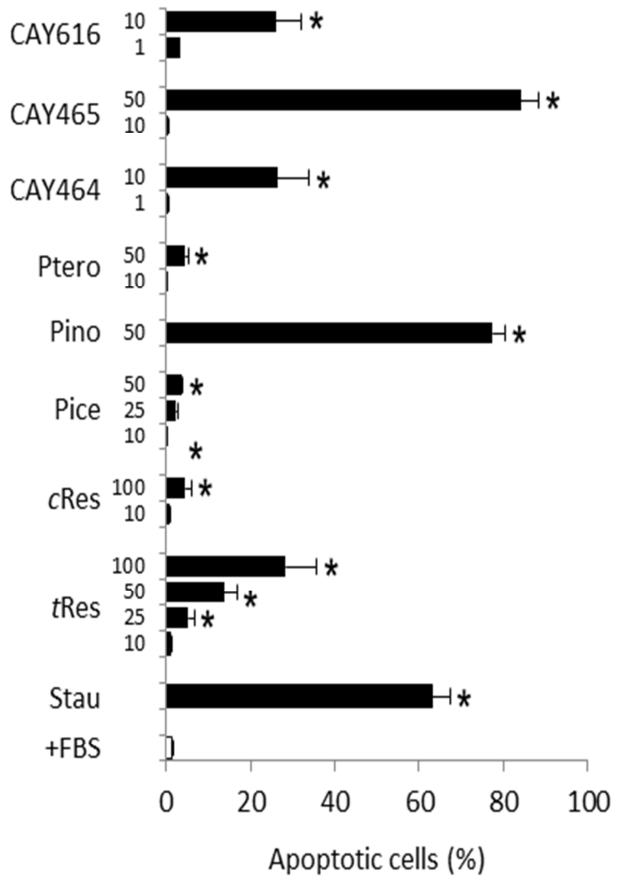

Figure 6. Effect of resveratrol and analogues on Caco-2 cell apoptosis. Caco-2 cells were incubated with *trans*-resveratrol (tRes, 10, 25, 50 and 100 μM), *cis*-resveratrol (cRes, 10 and 100 μM), piceatannol (Pice, 10, 25 and 50 μM), pinostilbene (Pino, 50 μM), pterostilbene (Ptero, 10 and 50 μM), *trans*-trimethoxyresveratrol (tTMRes, 1 and 10 μM), PDM11 (50 μM), CAY10464 (CAY464, 1 and 10 μM), PDM2 (50 μM), CAY10465 (CAY465, 10 and 50 μM) and CAY10616 (CAY616, 1 and 10 μM)) in presence of 10% FBS for 48 h and DNA fragmentation was measured. Values are mean ± SEM (n = 3-8). * $P < 0.05$ vs control group (+ FBS, cells cultured in presence of FBS, white bar). As positive control we used staurosporine (Stau, 1 μM) in presence of 10 % FBS).

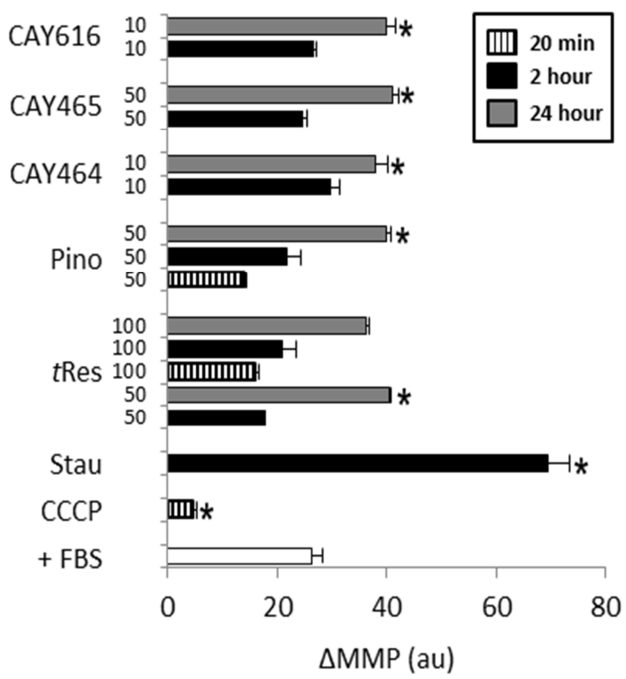

Figure 7. Effect of trans-resveratrol and analogues on mitochondrial membrane potential variation. *Trans*-resveratrol (tRes, 50 and 100 μM), pinostilbene (Pino, 50 μM), *trans*-trimethoxyresveratrol (tTMRes, 10 μM), PDM11 (50 μM), CAY10464 (CAY464, 10 μM), CAY10465 (CAY465, 50 μM) and CAY10616 (CAY616, 10 μM) were incubated 20 minutes, 2 and 24 hours (line, black and grey bars respectively) and mitochondrial membrane potential variation (ΔMMP) was measured by flow cytometry as material and methods section described and was expressed as arbitrary units (au). Results are expressed as mean ± SEM (n = 3-5). As positive control we used Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in presence of 10% FBS. * $P < 0.05$ vs s control group +FBS (cells cultured in presence of FBS, white bar).

Artículo 3

Effect of long chain fatty acids and long chain fatty acid metabolites on human intestinal epithelial cancer cell growth

Carolina E. Storniolo, Marisol Cabral, María A. Busquets, Raquel Martín-Venegas and Juan J. Moreno

Preparada para enviar a Journal of Lipid Research

Los resultados de esta publicación se han presentado en los siguientes congresos:

Effect of MUFAs and PUFAs on Caco-2 cell growth and prostaglandin synthesis. I Corral, M Pequera, CE Storniolo, JJ Moreno. 11th FENS European Nutrition Conference. Madrid, España. 26-29/10/2011. **Póster**

Estos resultados fueron publicados en:

I Corral, M Pequera, CE Storniolo, JJ Moreno. Effect of MUFAs and PUFAs on Caco-2 cell growth and prostaglandin synthesis. Ann Nutr Metab 2011;58:158.

Effect of oleic acid on intestinal epithelial cancer cell growth. CE Storniolo, M Pijuan, JJ Moreno. World Forum for Nutrition Research Conference. Reus, España. 20-21/05/2013. **Póster**

Estos resultados fueron publicados en:

CE Storniolo, M Pijuan, JJ Moreno. Effect of oleic acid on intestinal epithelial cancer cell growth Ann Nutr Metab 2013;62:16-7

Resumen Artículo 3

Objetivo: Estudiar el efecto de diferentes AGs grasos de cadena larga saturados e insaturados en el crecimiento de una línea celular de CCR y los posibles mecanismos de acción.

Material y métodos: Las células de adenocarcinoma Caco-2 en estado preconfluente se incubaron con los diferentes AGs de cadena larga en ausencia de factores de crecimiento. Después de 48h se determinó el crecimiento celular mediante marcaje con bromuro de etidio/naranja de acridina, la síntesis de DNA mediante la incorporación de bromodesoxiuridina, el ciclo celular y el potencial de membrana mitocondrial por citometría de flujo y la fragmentación del DNA por TUNEL. Las vías de señalización células se valoraron mediante ELISA. La unión al ligando PPARγ se determinó utilizando una técnica de fluorescencia polarizada.

Resultados y conclusiones: El ácido oleico resultó ser un potente agente mitogénico sobre las células Caco-2, probablemente a través de metabolitos de la vía de las lipooxigenasas. Mientras que el EPA mostró un efecto dual dependiendo de su concentración. A elevada concentración el EPA indujo apoptosis, mientras que a baja concentración indujo la proliferación celular a través, posiblemente, de la síntesis de eicosanoides como la PGE_3 y el ácido 12-hidroxieicosapentaenoico y la consiguiente inducción de las vías de señalización celular mitogénicas. Obviamente, estas conclusiones no excluyen un posible efecto directo de los ácidos grasos de cadena larga en el crecimiento de las células Caco-2 mediante su unión a GPR40 y la consecuente activación de la señalización celular implicada en la proliferació

Effect of long chain fatty acids and long chain fatty acid metabolites on human intestinal epithelial cancer cell growth

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Abbreviations: AA, arachidonic acid; CCCP, carbonyl cyanide 3-chlorophenylhydrazine; COX, cyclooxygenase; CRC, Colorectal cancer; HEPE, hydroxyeicosapentaenoic acid; LCFA, long chain fatty acids; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin; SCFA, short-chain fatty acids; TMRE, tetramethylrhodamine ethyl ester.

Abstract

Ethiology of colorectal cancer (CRC) is related, at least in part, with nutritional profile and epidemiological data indicated a key role of dietary fat on CRC pathogenesis. Moreover, inflammation and eicosanoids produced from arachidonic acid might have a pivotal role in CRC development. However, the effect of FAs on intestinal epithelial cell growth is not completely studied at the moment. By this reason, the aim of this work is determinate the effect of different saturated and unsaturated long chain fatty acids (LCFA) and some LCFA metabolites on CRC cell line growth and their possible mechanisms of action. Our results demonstrated that oleic acid is a potent mitogenic factor to Caco-2 cells probably through lipoxigenase pathway metabolite, whereas EPA has a dual behavior effect depending on the FA concentration. A high concentration, EPA induced apoptosis, whereas a low concentration induced cell proliferation that could be related to the synthesis of eicosanoids such as prostaglandin E_3 and 12-hydroxyeicosapentaenoic acid and the subsequent induction of mitogenic cell signaling pathways. Obviously, these conclusions did not exclude a direct effect of LCFA on Caco-2 cell growth through bind to GPR40 and the subsequent cell signaling activation involved in cell proliferation.

Supplementary key words apoptosis ● cell proliferation ● colorectal cancer ●eicosanoids ● eicosapentaenoic acid ● hydroxyeicosapentaenoic acids ● oleic acid ● polyunsaturated fatty acids ● prostaglandin E

INTRODUCTION

Cancer causes around 7 million of deaths annually, becoming 12.5 % in the entire world (1) and colorectal cancer (CRC) is the third leading cause of cancer-related death in developed countries (2). According to recent rates, the lifetime risk of developing CRC is 4.3% (3). Although a great effort has been made toward developing detection and surgical strategies, there has been little improvement in the outcome for patients with advanced disease. Carcinogenesis is a multistage process consequence of the breakdown or dysfunction of finely controlled processes such as cell differentiation, proliferation and apoptosis (4).

CRC does not appear to be a consequence of aging. However, it is linked with environmental factors, being lifestyle and nutritional profile the major but controllable implicated factors (5–7). In fact, around 90% of the CRC cases appear to relate to lifestyle, with the highest incidence in economically developed countries (8). Refined carbohydrates, alcoholic beverages, red and processed meat, saturated fat, and a high energy intake, specially associated with abdominal body fatness, would favor

CRC development. In contrast, high consumption of dietary fiber, fruits and vegetables, calcium, antioxidants and vitamins would have the opposite effect (5, 6, 8, 9).

The data indicated that not only the amount of dietary fat but also fat diet composition could be determinant in the pathogenesis of different neoplasms as CRC (10). Thus, some experimental studies demonstrated that ω-3 PUFA protect against CRC, while ω-6 PUFA promote this cancer development, proposing mechanisms like modulation of inflammation, cellular oxidative stress, membrane dynamics and cellular receptors function (11, 12). Thus, eicosanoids produced from arachidonic acid (AA) might have a pivotal role in these events. AA biosynthesized from linoleic acid is the substrate of cyclooxygenase (COX) and lipoxygenase (LOX) for the production of eicosanoids such as 2-series prostaglandins (PGs), 4-series leukotrienes (LTs) and HETEs that may facilitate CRC progression by stimulating cell proliferation and survival, tumor cell invasiveness, and angiogenesis (13, 14).

In epidemiological studies there are many different results, and data seems to be inconclusive. Some authors demonstrated an inversely relation between ω-3 or ω-6 PUFA consumption and CRC risk (15–18), while in others there are no association (19–22), or there are a positive relationship (23–27). In the other hand, studies including saturated fat consumption and CRC, demonstrated that there are a direct relation between these two factors (28–30) but other authors have not found any significant relation (31, 32). Oleic acid, ω-9 FA, is considered one of the healthier sources of fat in the diet. Also, oleic acid was attracted much attention as characteristic component of Mediterranean diet and has been linked to a protector effects against cancer (33).

Thus, the effect of fatty acids on intestinal epithelial cell growth is not completely understood at the moment. By this reason, the aim of this work is determinate the effect of different saturated and unsaturated long chain FAs (LCFA) and some fatty acid metabolites on CRC cell line growth and their possible mechanisms of action.

MATERIAL AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO. Nonessential amino acids, fetal bovine serum (FBS), Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, SC19220, paraformaldehyde extra pure, tetramethylrhodamine ethyl ester, perclorate (TMRE) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were supplied by Sigma-Aldrich (St- Louis, MO, USA). Tissue culture supplies and sterile material were obtained from Corning, Nirco S.L., NORM-JECT and Biosigma S.R.L. The BioRad Protein Assay was obtained from Bio-Rad Laboratories, Inc. Myristoleic, palmitoleic, stearic, oleic, elaidic, linoleic, α-linolenic, γ-linolenic, mead,

arachidonic (AA), EPA, erucic DHA acids, prostaglandin (PG) E_2, PGE_3, leukotriene (LT) B_4, LTB_5, 12-S-HETE, 12-S-HEPE, ketoprofen, baicalein, MK571, MK886 LY171883, zileuton, U75302 and the PPARγ ligand screening assay were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). Cell proliferation ELISA - BrdU (Colorimetric) Kit, from Roche (Basel, Switzerland). LY255283 was from Tocris Biosc. AH23848 and ONO-329 were kindly provided by Glaxo-Wellcome (Stevenage, UK) and Ono Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. The MebStain Apoptosis Kit was supplied by MBL International (Woburn, MD, USA) and the Multi Kinase Array (MKA) EIA by Symansis.

Cell Culture

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37) (Manassas, VA, USA). The cells were routinely grown in 25 or 75 cm^2 plastic flasks at a density of 1 x 10^4 cells/cm^2 and cultured in DMEM with 4.5 g/L D-glucose and 2 mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10 % (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 ºC under a humidified atmosphere of 5 % CO_2 in air. Cells grown to ~80 % confluence were released by trypsinization and subcultured at a density of 1.5-2 x 10^4 cells/cm^2 in 12 mm diameter plastic clusters and of 1 x 10^4 cells/cm^2 in 60 mm diameter plastic dishes. Growth medium was replaced twice per week. The experiments were performed in cells maintained for 3 days in culture (preconfluent cells). All experimentation products were diluted in DMSO (final concentration of DMSO was lesser than 0.1 %).

Cell Growth and DNA synthesis Assays

The effect of the treatments was assessed on Caco-2 cells clusters in 24-well plates (5 -10 x 10^3 cells/cm^2). Cells were cultured for 96 h in DMEM supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of treatments. Finally, cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to count viable cells (34).

DNA synthesis in Caco-2 cells was assessed by a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell proliferation ELISA, BrdU Kit, from Roche). Caco-2 cells were cultured at 1000-1500 cell/well in 96 well plates for 96 h in DMEM supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of compounds. Thereafter, cells were treated following the manufacturer instructions. Absorbance was measured at 450 nM in a plate reader (TECAN, Sunrise, Grödig, Austria).

Tunnel assay

Degradation of chromosomal DNA was evaluated with TUNNEL method using a MebStain Apoptosis Kit (MBL Int.). After 96 h in culture, Caco-2 cells were cultivated in media containing treatments for 48 h. Next, cells were fixed with 4 % paraformaldehyde and permeabilized with 70 % ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analyzed on an Epics XL flow cytometer (Coulter Corporation, San Francisco, CA).

Measurement of cell signalling pathways

Cells were seeded in 60 mm plastic clusters (10^4 cells/cm^2). After 4 days, the cultures were incubated with the treatments Maximal phosphorylation was observed after 5 min incubation for ERK1/2, Akt and p38, and after 15 min for GSKβ and CREB. To measure the kinase activation with total cellular lysates, Caco-2 cells were lysed using a denaturing cell lysis buffer containing 6 M urea and protease (leupeptin 2 µg/ml, pepstatin 10 µM, aprotinin 3 µg/ml) and phosphatase (NaF 5 mM, $Na_4P_2O_7$ 2 mM, Na_3VO_4 1 mM) inhibitors. The resulting solutions containing 80-100 µg of proteins were then added to a kinase ELISA plate and the assay was performed following the manufacturer's recommendations (Symansis, Auckland, New Zealand). Finally, optical density was measured at 450 nm. Thus, we studied the effect of PGE_2/PGE_3 on the phosphorylation of AKT1 (pS473), AKT2 (pS474), ERK1/2 (pT202/Y204;pT185/Y187), GSK3β (pS9), p38α (pT180/Y182) and CREB (pS133).

PPARγ ligand assay.

FAs binding to PPARγ were studied with a fluorescence polarization-based single-step PPARγ ligand screening assay (Cayman). The assay was adapted to be performed in a microcuvette with a luminescence spectrometer (AMINCO-Bowman Series 2, Spectronic Unicam, Leeds, UK).

Mitochondrial membrane potential determination

Mitochondrial membrane depolarization (MMP) was measured through tetramethylrhodamine ethyl ester perclorate (TMRE) incorporation into active preconfluent Caco-2 cell mitochondria. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used like a positive control for MMP. Cell cultures were treated with experimental compounds for 20 minutes and, after tripsinization, cell suspension was labelled with TMRE for 30 minutes. Finally samples were analysed in FC500 flow cytometer (Coulter Corporation) to measure mitochondrial depolarization or hyperpolarization.

Data Analysis

The results are expressed as mean ± SEM. All data were compared by one-way ANOVA and the Student's t-test, using SPSS software (SPSS Inc., Chicago, IL). Significance was taken as $p < 0.05$.

RESULTS

Long chain fatty acids are mitogenic on intestinal epithelial Caco-2 cell

When Caco-2 cells were incubated with different LCFA in absence of FBS we observed that myristoleic, palmitoleic, oleic, elaidic, EPA and DHA induced cell proliferation, at low concentrations (1-10 μM) by EPA and DHA, at high concentrations (10-100 μM) in the case of myristoleic, palmitoleic and elaidic acids and at all concentrations assayed by oleic acid (**Fig. 1A**). These mitogenic effects of the above mentioned FAs are confirmed in DNA synthesis analysis except for palmitoleic (**Fig. 1B**). In **Fig. 1A** it can be also seen that some FAs like linoleic, α and γ-linolenic, mead, AA and DHA at high concentration promotes a decrease in cell number below control cell group. By this reason, cells were incubated with FBS to assess the antiproliferative capacity of these FAs in the presence of growth factors. **Fig. 2** shows how these LCFAs such as linoleic, α- and γ-linolenic, AA, EPA and DHA decreased cell number whereas we observed that oleic acid enhanced the mitogenic effect induced by FBS. Thus, oleic acid was the most mitogenic saturated/monounsaturated FA and EPA/DHA the PUFAs with higher effect, presenting a dual effect on Caco-2 cell growth depending of concentration.

The mitogenic effect of oleic acid and EPA can be related with cyclooxygenase and lipoxygenase metabolite release

In **Fig. 3** we observed that cell proliferation induce by oleic acid or EPA at 10 μM was reverted by MK886 (5-LOX inhibitor), U75302 (BLT_1 antagonist) and LY255283 (BLT_2 antagonist). The experiments also showed that ketoprofen (COX inhibitor), AH23838 (EP_4 antagonist), SC19220 (EP_1 antagonist) and baicalein (12-LOX inhibitor) also reverted the mitogenic effect induced by EPA (**Fig. 3**) whereas the mitogenic effect induced by oleic acid was also reverted by zileuton (5-LOX inhibitor), MK571 (cysteinyl leukotriene antagonist) and LY171883 (cysteinyl LT receptor antagonist) (**Fig. 3**). This reversion of oleic/EPA mitogenic effects by AA cascade inhibitors and eicosanoid receptor antagonists suggest a role of oleic/EPA metabolites in the mitogenic effects induced by both FAs.

Some eicosanoids from EPA such as PGE_3 and 12-S-HEPE are mitogenic whereas LTB_5 did not induce Caco-2 cell growth

EPA can be incorporated into cell membrane phospholipids (35) and it is substrate for COX and LOX, giving rice to the 3-series prostanoids and 5-series leukotrienes and HEPEs (36). In **Fig. 4A/B** we observed that PGE_3 progressively increased Caco-2 cell growth and DNA synthesis up to 10 nM. **Fig. 4A/B** also shows that Caco-2 cell growth and DNA synthesis induced by PGE_3 was totally inhibited by an EP_1 antagonist (SC19220) and by an EP_4 antagonist (AH23838). However, an EP_3 antagonist (ONO-AE3-240, 2nM) did not have any effect. Therefore, these results indicate that PGE_3 acts through EP_1 and EP_4 receptors, but not through the EP_3 receptor.

Our results also show that 12-S-HEPE (100-1000 nM) induced significant cell growth and DNA synthesis in Caco-2 cell cultures in the absence of growth factors, in a similar way to 12-S-HETE (100 nM). The mitogenic action of 12-S-HEPE was blocked by a COX inhibitor (ketoprofen) and by a BLT_1 antagonist (U75302) and a BLT_1 and BLT_2 antagonist (LY255283) (**Fig. 4C/D**).

Previously we observed that LTB_4 has a mitogenic effect on Caco-2 cells (35). However, our findings show that LTB_5 derived from EPA did not induce proliferation in the range of 1-100 nM (**Fig. 5**).

Finally, we studied the capacity of PGE_3 (10 nM) to phosphorylate pivotal elements in the cell signalling pathways implicated in the regulation of cell growth. Our findings show that PGE_3 presents a similar pattern to PGE_2. Thus, PGE_3 (10 nM) could increase the phosphorylation of ERK 1/2, CREB, GSKβ and p38 (**Fig. 6**), cell signaling pathways involved in cell growth.

Polyunsaturated long chain fatty acids are anti-mitogenic and apoptotic in Caco-2 cell cultures

Considering that some LCFAs studied have antiproliferative effect, we analyzed their apoptotic activity by flow cytometry. Our results show that LCFAs such as α- and γ-linolenic, AA, EPA and DHA decreased cell number and induced apoptotic at high concentrations whereas oleic acid has no apoptotic activity (**Fig. 7**). In additional assays we measured the capacity of LCFAs to bind to PPARγ and to alter MMP (**Table 1**). We found that apoptotic LCFAs (α- and γ-linolenic, AA, EPA and DHA) have high affinity by PPARγ ligand. Furthermore, AA, EPA and DHA were capable to induce the improvement of MMP, both parameters implicated in the denominated intrinsic or mitochondrial pathway of apoptosis.

DISCUSSION

Undigested carbohydrates are fermented in the large intestine to form short-chain fatty acids (SCFA) such as acetate, propionate and butyrate. SCFAs and specially butyrate induce cellular

differentiation and decrease proliferation of CRC-derived cell lines (36, 37). These effects may related with the inhibition of histone deacetylase by DNA hypermethylation to promote cell differentiation (38) and the inhibition of cell proliferation via p21, cyclin D1 and β-catenin pathway (39). Thus, SCFAs have been reported to have important effects on intestinal epithelial cell growth/differentiation and consequently on CRC (40). However, there is a great debate about the role of saturated, monounsaturated and polyunsaturated (ω-3 and ω-6) LCFAs in CRC, but few studies have been conducted on the potency of LCFAs and/or specific LCFA metabolites in modulating CRC cell line growth. Interestingly, our study shows that LCFAs were mitogenic at physiological concentrations being oleic acid the most effective, whereas PUFA such as EPA and DHA at high concentrations might have the opposite effects.

Monounsaturated LCFAs such as myristoleic, palmitoleic and oleic acids are mitogenic in Caco-2 cell cultures being oleic acid the most active. Our results clearly demonstrated that oleic acid (1-100 μM) has a mitogenic effect in Caco-2 cell cultures, action that was also observed by the $C_{18:1}$ *trans*, elaidic acid. These findings are in agreement with Hansen et al. (41), that reported that $Apc^{min/+}$ mice feed with oleic acid present a high number of intestinal tumors.

Cancer cells are characterized by higher rates of lipid biosynthesis in addition to increased glycolysis and lactate production than those of normal cells (42) as well as high fatty acid uptake that require the expression of CD36, over-expressed in the majority of tumor tissues (43). Recently, stearoyl-CoA desaturases expression and activity and the subsequent conversion of saturated LCFA to monounsaturated LCFA were linked to CRC pathogenesis (44, 45). Interestingly, our study reports data that suggest an alternative mechanism to explain the mitogenic action of oleic acid. Thus, our findings suggest that this effect of oleic acid appears dependent on 5-LOX pathway metabolism and the subsequent production of oleic acid metabolite. Clapp et al. (46) reported that lipoxygenation of oleic acid gives allylic hydroperoxides followed by conversion to enones. In this sense, Cabral et al. (35, 47) reported that LTs from AA and hydroxyoctadecanoid acids from linoleic acid induced Caco-2 cell growth. Thus, we can consider the possible implication of an LOX pathway oleic-derived metabolite in these events, but more research is need to identify the olive oil metabolites involved in their mitogenic action. Recently, Barone and co-workers (48) observed a decrease in polyp number and polyp volume by olive oil diet. These apparent discrepancies put into consideration the fact that oleic acid and olive oil can exert different effect on CRC cell line growth considering that olive oil is a complex mix content bioactive compounds that can modulate oleic acid action.

Dommels et al. (49) reported that AA and EPA (10-100 μM) induced Caco-2 cell growth inhibition and cytotoxicity through peroxidation products generated during lipid peroxidation and COX activity. In agreement with these authors, we observed that other PUFAs such as α- and γ-linolenic and DHA were able to reduce cell number and that these findings were related with their capacity to induced apoptosis and binding to PPARγ. Interestingly, our findings indicate that although EPA as well as linoleic, α- and γ-linolenic, AA and DHA are apoptotic at the highest concentration (around 100 μM),

EPA and DHA have a mitogenic effect at 1-10 μM. Furthermore, we observed that this mitogenic effect of EPA is COX- and LOX-pathway dependent, suggesting that EPA metabolites could be involved in this event.

Experimental studies have shown that diets rich in fish oil significantly reduce the amount of AA present in membrane phospholipids (50) and consequently the synthesis of AA metabolites such as PGE_2 (51), but increase the release of EPA metabolites. Even though the theory of formation of the 3-series PGs by EPA has been studied for decades, we still do not fully understand the role of PGE_3 in cancer cells (52). Here, we observed, for the first time, that PGE_3 increased cell growth and DNA synthesis in non-differentiated intestinal epithelial cells, in a similar form to PGE_2. These findings are, apparently, in disagreement with Fan et al. (53) who reported that PGE_3 diminished the ability to support colonic stem cell expansion, but using a non-physiological concentration (10 μM). Furthermore, we demonstrated that this PGE_3 proliferative effect was a consequence of interaction with the PGE_2 receptor EP_1 and EP_4, in agreement with their affinities, and with a recent report showing similar effects of both PGs on the disruption of the intestinal epithelial barrier function (54). Moreover, we observed that cell signalling pathways involved in the mitogenic action of PGE_3 are similar to those involved in PGE_2 action (35), being p38α, CREB and ERK 1/2 pathways involved in the mitogenic action of PGE_3.

HETEs have mitogenic effect on different types of cells and are also involved in the pathogenesis of cancer (13). Recently, 12-S-HETE was reported to have a proliferative effect on Caco-2 cells (55). To our knowledge, this is the first study to show that 12-S-HEPE has similar effect on intestinal epithelial cell growth. No specific cellular receptors for HETEs/HEPEs have been identified to date. However, it has been reported that the binding of 12-HETE to the BLT_2 receptor may be involved in its mitogenic action (35). Here, we demonstrate that Caco-2 cell growth induced by 12-S-HEPE can be reverted by BLT_1 and BLT_2 antagonists and a COX inhibitor, which suggests that the 12-S-HEPE mitogenic action is at least partly due to PGs synthesis after 12-S-HEPE interaction with both BLT receptors; a mechanism previously described for LTB_4 and 12-HETE (35) and 13-R-hydroxyoctadecadienoic acid (55).

EPA administered to patients with Crohn's disease (56) or ulcerative colitis (57) increases the generation of LTB_5 and the LTB_5-LTB_4 ratio, which were related with an improvement in these patients. In our study, LTB_5 had no proliferative effect, while LTB_4 significantly induced Caco-2 cell growth, findings in agreement with Bortuzzo et al. (58) who found a lower affinity of LTB_5 to the receptor of LTB_4. Furthermore, since the treatment with 5-LOX inhibitor or cysteinyl LT receptor antagonist reduced the mitogenic effect of EPA, these results indirectly suggest that 5-serie cysteinyl LTs could also be involved, at least in part, in the mitogenic EPA effects, in our experimental conditions. There are little literature about the affinity of EPA derived LTs and cycteinyl LT receptors but Wallace and McKnight (59) reported that LTC_5 or LTD_5 have biological activity although less potent than LTs derived from AA. Furthermore, although LTB_5 did not have the mitogenic effect of its AA-derived

partners, PGE_3 and 12-S-HEPE have considerable mitogenic effects on intestinal epithelial Caco-2 cells, and may be involved in cell proliferation induced by EPA. To our knowledge, is the first time that it is reported the effect of PGE_3, LTB_5 and 12-S-HEPE derived from EPA on epithelial cell growth. However, we believe that future research should analyse the role of EPA and EPA eicosanoids on non-transformed intestinal epithelial cells. Furthermore, it will be interesting to study the role of DHA metabolites to explain the proliferative effect of this PUFA at low concentrations.

In conclusion, the results obtained herein demonstrated that oleic acid is a potent mitogenic factor to Caco-2 cells probably through LOX pathway metabolite whereas EPA/DHA has a dual behavior effect on Caco-2 cell growth depending on the FA concentration (**Fig. 8**). A high concentration EPA induced apoptosis, a process related with its binding to PPARγ. Meanwhile, low EPA concentration induced Caco-2 cell proliferation that could be related to the synthesis of mitogenic eicosanoids such as PGE_3 and 12-HEPE and the subsequent induction of mitogenic cell signaling pathways. Obviously, these conclusions did not exclude a direct PGE_2/PGE_3 effect of LCFA such as oleic acid and EPA on Caco-2 cell growth through bind to GPR40 and the subsequent cell signaling activation involved in cell proliferation (60).

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Table 1. Effect of LCFAs on PPARγ ligand assay and mitochondrial membrane potential variation.

Treatment	PPARγ bind (mP)	ΔMMP (au)
Vehicle	108 ± 9	21 ± 1.9
Rosiglitazone (10 μM)	43 ± 2*	ND
CCCP (5 μM)	ND	6 ± 0.5*
Staurosporine (1 μM)	ND	70 ± 4*
Oleic (100 μM)	109 ± 12	21 ± 1.6
Linoleic (100 μM)	98 ± 10	22 ± 0.7
α-Linolenic (100 μM)	76 ± 8*	24 ± 0.2
γ-Linolenic (100 μM)	71 ± 16*	22 ± 0.9
AA (100 μM)	70 ± 8*	28 ± 1.9*
EPA (100 μM)	63 ± 15*	34 ± 1.3*
DHA (100 μM)	56 ± 3*	38 ± 0.7*

PPARγ binding was measured by fluorescence polarization and mitochondrial membrane potential variation (ΔMMP) was measured by flow cytometry as material and methods section described and was expressed as mP and arbitrary units (au), respectively. Results are expressed as mean ± SEM (n = 3-5). * $P < 0.05$ versus control group (vehicle).

FIGURE LEGENDS

Figure 1. Effect of different LCFA on Caco-2 cell growth (A) and DNA synthesis (B) in absence of growth factors. Myristoleic, palmitoleic, stearic, oleic, elaidic, linoleic, α-linolenic, γ-linolenic, mead, AA, EPA, erucic and DHA were incubated with Caco-2 cells at 1, 10, 50 and 100 μM (dot, grey, line and black bars, respectively) without FBS for 48 hours and were then counted or DNA synthesis measured

by cell BrdU incorporation. Results are expressed like mean ± SEM (n = 8-30). * P < 0.05 versus control group (white bar, cells cultured without FBS).

Figure 2. Effect of different LCFA on Caco-2 cell growth in presence of 10 % FBS. Oleic, linoleic, α-linolenic, γ-linolenic, AA, EPA and DHA were incubated with Caco-2 cells at 1, 10 and 100 µM (dot, grey and black bars, respectively) in presence of 10 % FBS for 48 hours and were then counted. Results are expressed like mean ± SEM (n = 4-12). * P < 0.05 vs. control group (white bar, cells cultured with 10 % FBS).

Figure 3. Effect of pharmacological modulation of AA cascade on Caco-2 cell growth induced by oleic and EPA. Caco-2 cells were incubated without FBS for 48 hours with oleic or EPA at 10 µM in presence or absence of ketoprofen (Kp, 5 µM), AH23848 (AH23, 20 nM), SC19220 (SC19, 60 nM), baicalein (Bai, 25 µM), MK886 (MK8, 10 µM), zileuton (Zil, 5 µM), U75302 (U75, 5 µM), LY25283 (LY25, 25 µM), MK571 (MK5, 25 µM) or LY171883 (LY17, 25 µM). After cells were counted. Results are expressed as mean ± SEM (n = 8-16). * P < 0,05 vs. control group (white bar, cells cultured without FBS), # P < 0,05 versus oleic acid (10 µM) and § P < 0,05 versus EPA (10 µM).

Figure 4. Effect of PGE_3 or 12-S-HEPE on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with PGE_3 (0.1-10 nM, line, dot and grey bars, respectively) or PGE_3 (10 nM) plus SC19220 (SC, 60 nM) or AH 23848 (AH, 20 nM) or ONO-AE3-240 (ON, 2 nM) or with 12-S-HEPE (10, 100 and 1000 nM, line, grey and dot bars, respectively) or with 12-S-HEPE (100 nM, grey bar) plus U 75302 (U, 5 µM) or LY 255283 (LY, 25 µM) or ketoprofen (Kp, 5 µM). Cells were then counted (A, C) and DNA synthesis was measured (B, D). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * P < 0.05 vs. Caco-2 cell cultures in the absence of FBS, ≠ P < 0.05 vs. cells incubated with 10 nM PGE_3.

Figure 5. Effect of LTB_5 on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with LTB_5 (1-100 nM, dot, grey and black bars, respectively) or LTB_4 (10 nM, grey bar) in absence of FBS. Cells were then counted. Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * P < 0.05 vs. Caco-2 cell cultures in the absence of FBS.

Figure 6. Effect of PGE_3 on cell signalling. Caco-2 cells were incubated with PGE_2 or PGE_3 (10 nM, dot and grey bars, respectively) for 5 or 15 min, cells were then collected and finally phosphorylated. ERK 1/2, CREB, GSKβ, p38α, Akt1 and Akt2 were measured as described in the Material and methods section. Data are expressed as means ± SEM of 2-4 experiments performed in triplicate. * P < 0.05 vs. Caco-2 cell cultures in the absence of FBS.

Figure 7. Effect of different LCFA on Caco-2 cell apoptosis. Caco-2 cells were incubated with stearic, oleic, linoleic, α-linolenic, γ-linolenic, AA, EPA and DHA at 100 µM (black bars) in absence (A) or presence of FBS (B) for 48 h and DNA fragmentation was measured. Values are mean ± SEM (n = 3-

8). * P < 0.05 versus negative control group (cells cultured in absence of FBS, white bar). As positive control we used staurosporine (1 μM) in presence of 10 % FBS (white bar).

Figure 8. Effect of oleic acid and EPA/DHA on Caco-2 cell growth and apoptosis. Oleic acid is mitogenic probably through LOX pathway metabolite synthesis whereas EPA/DHA present a dual effect on Caco-2 cell proliferation. Low EPA concentrations (up to 10 μM) induce Caco-2 cell growth as consequence, at least in part, of PGE_3 and 12-HEPE release whereas high EPA concentrations inhibit Caco-2 cell growth and induce apoptosis through binding to PPARγ.

Figure 1

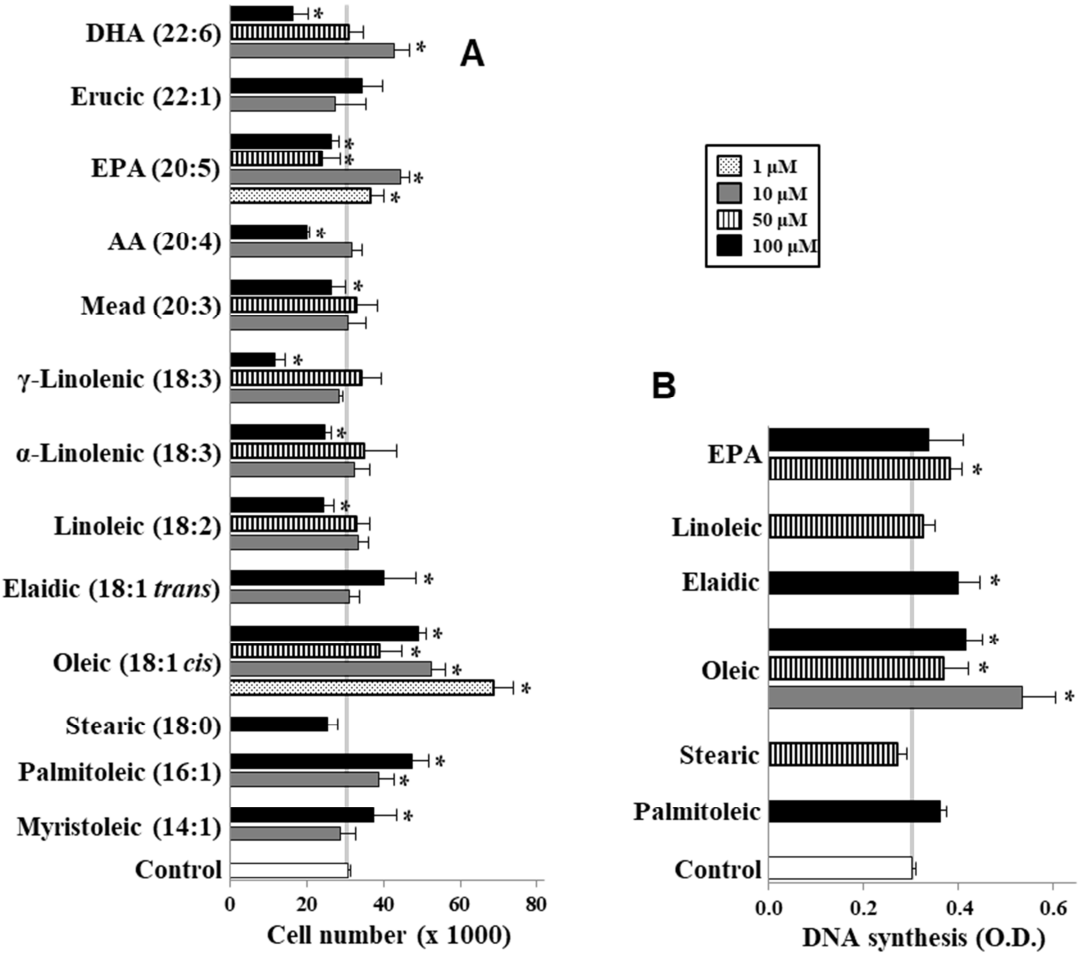


Figure 2

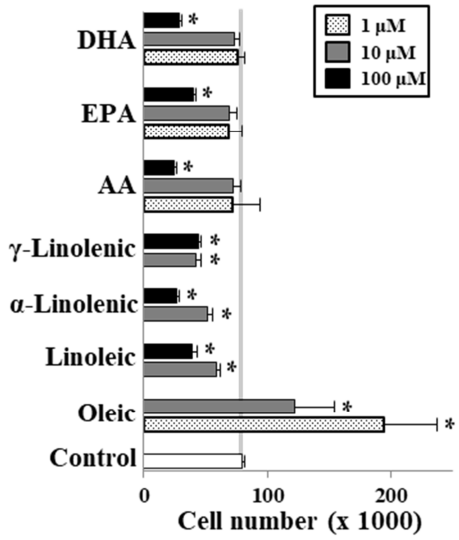


Figure 3

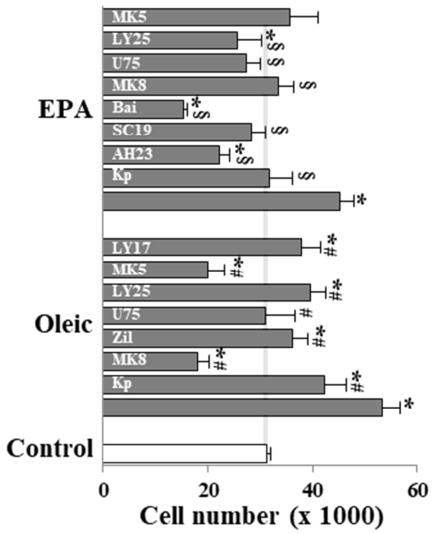


Figure 4

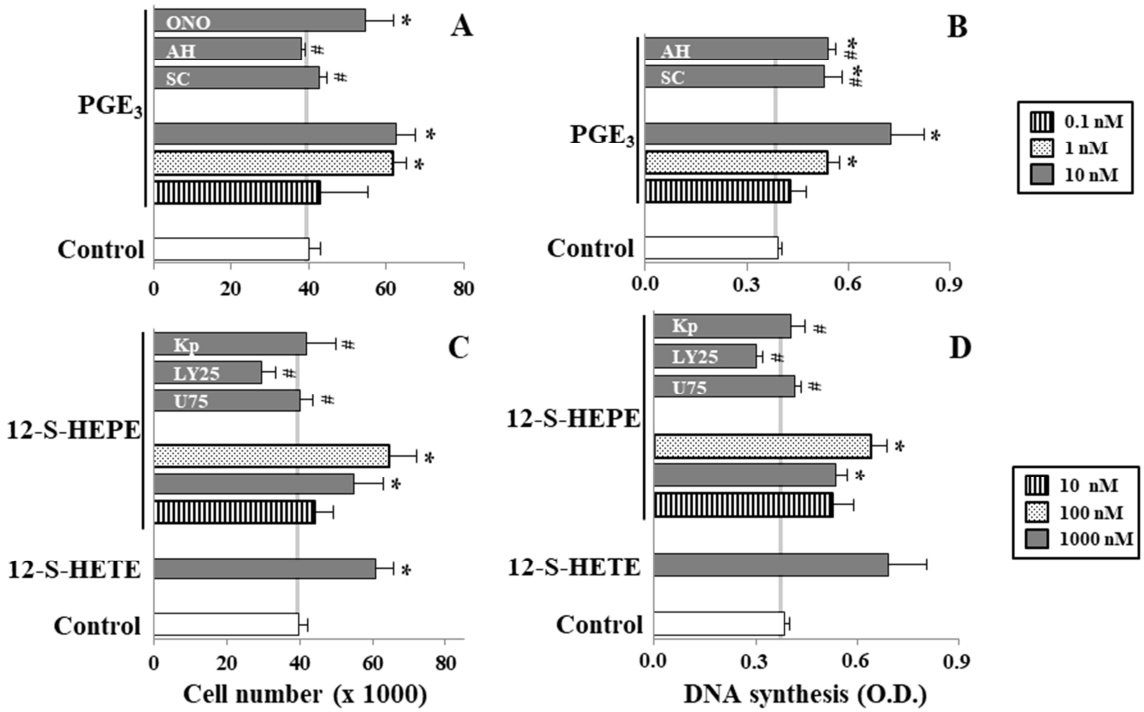


Figure 5

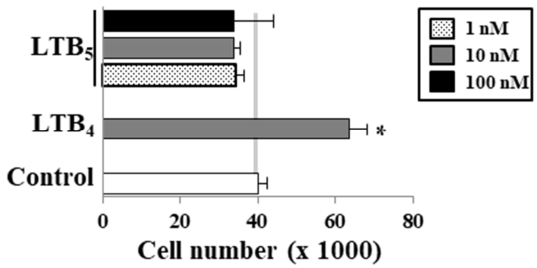


Figure 6

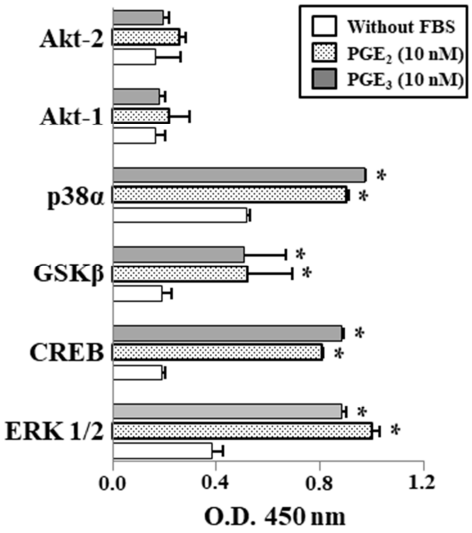


Figure 7

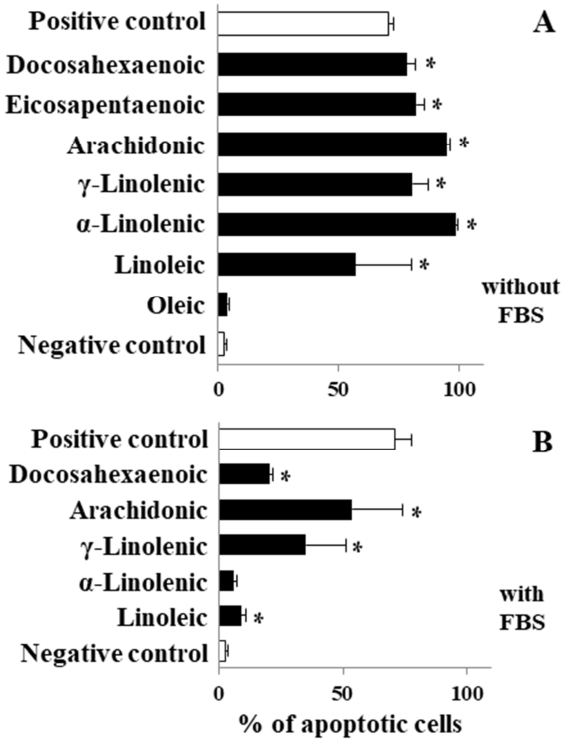
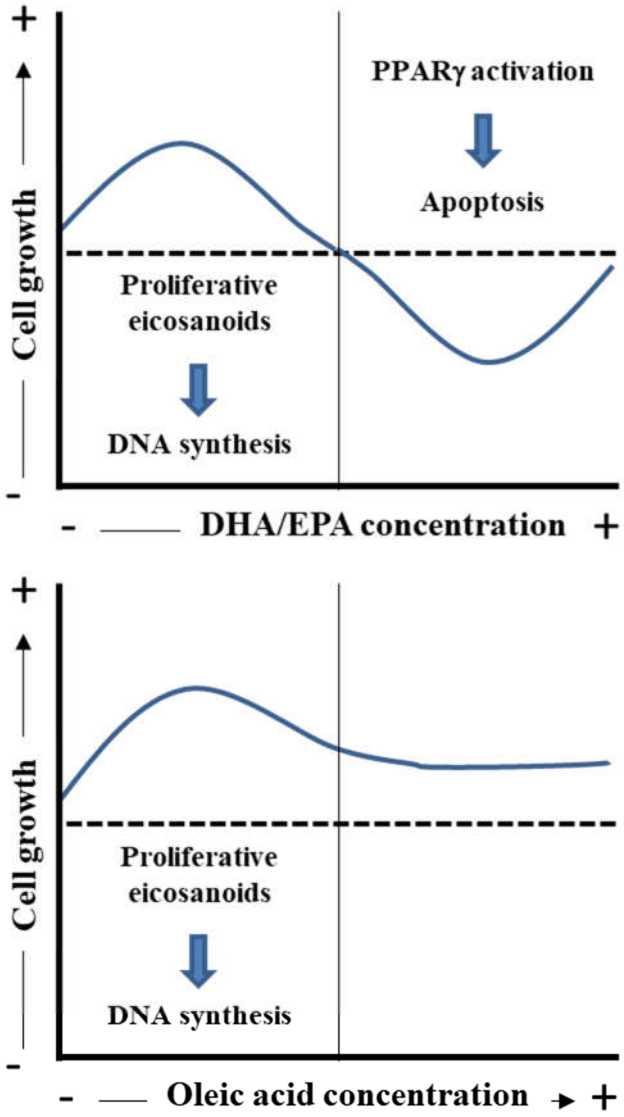


Figure 8


+
Cell growth
-
PPARγ activation
Apoptosis
Proliferative
eicosanoids
DNA synthesis
- DHA/EPA concentration +
+
Cell growth
-
Proliferative
eicosanoids
DNA synthesis
- Oleic acid concentration +

Artículo 4

Extra virgin olive oil minor compounds modulate mitogenic action of oleic acid on colon cancer cell line

Carolina E. Storniolo, Natalia Martínez-Hovelman, Miriam Martínez-Huélamo, Rosa M. Lamuela-Raventos and Juan J. Moreno

Preparada para enviar a Journal of Nutritional Biochemistry

Los resultados de esta publicación se han presentado en los siguientes congresos:

Effects of olive oil minor components on intestinal epithelial cancer cell growth induced by oleic acid. N Martínez-Hovelman, CE Storniolo, JJ Moreno. World Forum for Nutrition Research Conference. Reus, España. 20-21/05/2013. **Póster**

Estos resultados fueron publicados en:

N Martínez-Hovelman, CE Storniolo, JJ Moreno. Effects of olive oil minor components on intestinal epithelial cancer cell growth induced by oleic acid. Ann Nutr Metab 2013;62:34.

Effect of extra virgin olive oil components on colon cancer cell line proliferation. CE Storniolo, N Martínez-Hovelman, M Martínez-Huélamo, RM Lamuela, J J Moreno. I Workshop Anual INSA-UB - El Universo del Aceite de Oliva. Santa Coloma de Gramenet, España. 11/11/2015. **Póster**

Resumen Artículo 4

Objetivo: Estudiar el efecto del ácido oleico y de algunos componentes minoritarios presentes en el aceite de oliva, sobre el crecimiento de células epiteliales intestinales y su posible mecanismo de acción.

Material y métodos: Las células de adenocarcinoma Caco-2 en estado preconfluente se incubaron con los componentes del aceite de oliva (oleuropeina, pinoresinol, ácido maslínico, escualeno e hidroxitirosol) con o sin ácido oleico. Tras 48h se determinó el crecimiento celular mediante marcaje con bromuro de etidio/naranja de acridina, la síntesis de DNA mediante la incorporación de bromodesoxiuridina, y la fragmentación del DNA por TUNEL. La actividad antioxidante se analizó con un Kit específico.

Resultados: Observamos que el oleico, pero no su isómero trans-, indujo el crecimiento celular y la síntesis de DNA de forma concentración-dependiente en ausencia de factores de crecimiento. Este efecto fue revertido por inhibidores de la 5-lipoxigenasa así como por antagonistas de leucotrienos, sugiriendo la implicación de estos metabolitos en la acción mitogénica del ácido oleico. Todos los componentes minoritarios del aceite de oliva estudiados inhibieron el crecimiento celular y la síntesis de DNA inducida por el ácido oleico. Además, mostramos que estos resultados no fueron consecuencia de un arresto del ciclo celular ni del deterioro de la viabilidad celular, con excepción del hidroxitirosol y el ácido maslínico que provocaron que las células se despegaran y entraran en apoptosis.

Conclusiones: El ácido oleico es mitogénico mientras que el ácido oleico en presencia de los componentes minoritarios del aceite de oliva tiene un efecto opuesto sobre el crecimiento de las células epiteliales intestinales.

Extra virgin olive oil minor compounds modulate mitogenic action of oleic acid on colon cancer cell line

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Running title: Olive oil compounds and Caco-2 cell growth

ABSTRACT

Several epidemiological studies reports that Mediterranean diet (MD) adherence was associated with reduced risk of colorectal cancer (CRC). Olive oil, the primary source of fat in MD has been also found to have a protective effect in clinical and experimental studies, whereas animals feed with oleic acid diet present a high number of intestinal tumors. Findings that suggest that oleic acid and olive oil consumption can exert different antitumor effects. Considering that olive oil is a complex mix of fatty acids, mainly oleic acid and minor compounds such as phenolic compounds, lignans, hydrocarbons and triterpenes. We study the effect of oleic acid on intestinal epithelial cell growth in absence and in presence of representative olive oil minor components. Our results show that oleic acid (1-100 μM) but not elaidic acid induced DNA synthesis and Caco-2 cell growth in absence of growth factors. This mitogenic effect induced by oleic acid was reverted by 5-lipoxygenase inhibitors as well as leukotriene antagonist suggesting the implication of these metabolites in this mitogenic action. Hydroxytyrosol, oleuropein, pinoresinol, squalene and maslinic acid (0.1-10 μM) inhibited DNA synthesis and Caco-2 cell growth induced by oleic acid. Our results also show that these effects were not consequence of cell cycle arrest or the impairment of cell viability with the exception of hydroxytyrosol and maslinic acid that induced cell detachment and apoptosis. In conclusion, oleic acid and oleic acid in presence of olive oil representative minor components have opposite effect on intestinal epithelial cell growth.

Keywords: oleuropein; hydroxytyrosol; pinoresinol; squalene; maslinic acid

1. Introduction

The traditional Mediterranean diet (MD) is widely recognized as one of the healthiest in the world and it is likely that the adoption of this diet would lead to a significant reduction in the incidence of many chronic diseases [1]. This recent meta-analysis of prospective cohort studies concluded that the MD is responsible of a 6% reduction from death and/or the incidence of neoplastic diseases. Updated reports from a large cohort such as the European Prospective Investigation into Cancer and Nutrition including 335,873 individuals have found a lower overall cancer risk among those with greater adherence to MD [2].

Regarding colorectal cancer (CRC), MD adherence was inversely associated with reduced risk of colon cancer and rectal cancer in a study conducted on 45,275 participants of the Italian section of the EPIC study followed for a mean of 11.28 years [3, 4]. Similar results were obtained in the large cohort

of 492,382 subjects belonging to the National Institutes of Health [5]. Is important remark that it is the consumption of olive oil, more than any other single factor, which distinguishes the traditional MD from other dietary pattern [6].

Dietary fat uptake has been implicated to cancer development and olive oil is the primary source of dietary lipids in MD. Examining the association of olive oil and monounsaturated fat intake with CRC, olive oil has been found to have a slight protective effect [7, 8], while monounsaturated fat intake appeared uninfluential [9]. Interestingly, Hansen Petrik et al., [10] reported that ApcMin/+ mice feed with oleic acid diet present a high number of intestinal tumors. However, when diet was prepared with olive oil, Barone and co-workers [11] observed that olive oil diet decreases polyp number and polyp volume, respect to soybean oil diet (PUFA rich), in ApcMin/+ mice, an important discrepancy upon consideration of the fact that oleic acid and olive oil can exert different effect on CRC development. Considering that olive oil is a complex mix contains phenolic compounds such as simple phenols (tyrosol and hydroxytyrosol), aldehydic secoiridoids (oleuropein), flavonoids and lignans (pinoresinol) as well as hydrocarbons (squalene) and triterpenes (maslinic acid), and that some of these compounds may modulate intestinal epithelial cell growth [12-14]. We hypothesize that oleic acid and oleic acid in presence of minor compounds could have differential effect of intestinal epithelial cell growth. Here, we study the effect of oleic acid on Caco-2 cells growth in absence and in the presence of representative olive oil minor compounds, opening the way to a more precise understanding of the molecular basis of olive oil action on CRC.

2. Material and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO (Thermo Fisher Scientific, Walthan, MA, USA). Nonessential amino acids, fetal bovine serum (FBS), Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, potassium ferricyanide, ferric chloride, oleuropein, pinoresinol, squalene, maslinic acid and quercetin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Oleic acid, elaidic, hydroxytyrosol prostaglandin (PG) E2, PGE2-d4, leukotriene (LT) B4, LTB4-d4, 5-hydroxyeicosatetraenoic acid (HETE), 5-HETE-d8, 12-HETE, 12-HETE-d8, 15-HETE, 15-HETE-d8, and 13-hydroxyoctadecadienoic acid (HODE) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Tissue culture supplies and sterile materials were obtained from Corning, Nirco S.L., NORM-JECT and Biosigma S.R.L. [5,6,8,9,11,12,14,15-3H] arachidonic acid (AA) (200-240 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

2.2. Cell Culture

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37) (Manassas, VA, USA). The cells (passages 19-40) were routinely grown in 25 or 75 cm2 plastic flasks at a density of 2-2.5 x 104 cells/cm2 and cultured in DMEM with 4.5 g/L D-glucose and 2 mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10 % (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 ºC under a humidified atmosphere of 5 % CO2 in air. Cells grown to around 80 % confluence were released by trypsinization and subcultured at a density of 1.5-2 x 104 cells/cm2 in 12 mm diameter plastic clusters and in 60 mm diameter plastic dishes. Growth medium was replaced twice per week. The experiments were performed in cells maintained for 3 days in culture (preconfluent cells). All experimentation products were diluted in DMSO (final concentration of DMSO was lesser than 0.1 %).

2.3. Cell Growth and DNA synthesis assays

The effect of the treatments was assessed on Caco-2 cells clusters in 24-well plates (1.5-2 x 104 cells/cm2). Cells were cultured for 96 h in DMEM medium supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of both compounds. Finally, cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to count cells and to assess viability.

DNA synthesis was assayed using a colorimetric immunoassay based on the measurement of BrdU incorporation during DNA synthesis (Cell proliferation ELISA, BrdU Kit, from Roche, Basel, Swizerland). Caco-2 cells were cultured at 1000-1500 cell/well in 96 well plates for 4 days in DMEM medium supplemented with 10% FBS. Then, cells were washed and incubated for 48 h in DMEM without FBS but with the test compounds. Thereafter, cell cultures were treated following the manufacturer's instructions. Final absorbance was measured at 450 nM in a plate reader.

2.4. Flow Cytometry Cell Cycle Analysis

Caco-2 cells were seeded in 60 mm dishes (1.5-2 x 104 cells/cm2), and 96 h after culture cells were then incubated by 48 h in 10 % FBS DMEM containing the treatments. Thereafter, cells were trypsinized, fixed with 70 % ethanol, and stored at 4 °C for at least 2 h. Next, low molecular weight DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 µg/mL propidium iodide solution in PBS containing 0.1 % Triton X-100 and 0.2 mg/mL DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corporation, San Francisco, CA, USA).

DNA was analyzed (ploidy analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems).

2.5. Tunnel Assay

Degradation of chromosomal DNA was evaluated with TUNNEL method using a MebStain Apoptosis Kit (MBL Int., Woburn, MD, USA). After 96 h in culture, Caco-2 cells were cultivated in media containing 10 % FBS DMEM with treatments for 48 h. Next, cells were fixed with 4 % paraformaldehyde and permeabilized with 70 % ethanol. Thereafter, 3'-OH DNA ends generated by DNA fragmentation was labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analyzed on an Epics XL flow cytometer (Coulter Corporation).

2.6. Total Antioxidant Activity and Reducing Power

We selected different concentration of any product and applied an Antioxidant Assay Kit (Cayman Chemical). This method is based on the ability of antioxidants in the sample (products diluted in PBS) to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS•+ by metmyoglobin [15]. The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as molar Trolox equivalents.

Total reducing power was determined according to the method of Oyaizu [16]. The first step was mix 40 µL of the sample solution with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 200 µL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 minutes. Afterwards, 200 µL of 10 % trichloroacetic acid was added to mixture. Finally, 60 µL of 0.167 % ferric chloride was added and incubated at room temperature for 50 minutes. Increased absorbance at 690 nm of the reaction mixture indicated increase in reducing power. Results were compared with a quercetin standard curve.

2.7. HPLC-UV analysis of oleuropein and hydroxytyrosol

Culture medium samples were filtered using 0.45 mm polytetrafluoroethylene filters, and then 20 µL of the samples filtered were injected in the HPLC-UV system. Hydroxityrosol and oleuropein were quantified by HPLC-UV, following the method described by Carrasco-Pancorbo et al. [17] with minor modifications. Chromatographic analysis was carried out in an HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany), consisting of a quaternary pump and an auto sampler coupled to a diode array detector DAD G1315B. Chromatographic separation was achieved on a Luna C18 50x2.0

mm (5 µm) column from Phenomenex (Torrance, CA, USA) using a pre-column Phenomenex securityguard C18 (4 x 3 mm i.d.). System was controlled by software Agilent ChemStation Software (Santa Clara, CA, USA). The HPLC-UV chromatograms were acquired at 280 nm wavelength. The mobile phases used were (A) H2O / acetic acid (99.8 / 0.2; v / v) and (B) MeOH. An increasing linear gradient (v/v) of [B] was used (t(min), %B), as follows: (0,5), (2,5), (10,5), (12,100) (15,100) (17,5) (22,5) at a constant flow-rate of 350 µL/min.

2.8. Incorporation and release of [3H]AA

Cells were harvested with trypsin/EDTA and passed to 24-well plates at a density of 104 cells/cm2. After 4 days, cells were FBS starvated during 24 h and then the medium was replaced by 0.5 ml DMEM containing 0.1% fatty acid free BSA and 0.1 µCi [3H]AA (1 nM) for a period of 6 h. Cells were then washed three times with 0.5% BSA-containing medium to remove any unincorporated [3H]AA. After the study period (1 or 3 h), the medium was removed to determine the amount of [3H] radioactivity release. The amount of [3H]AA released into the medium was expressed as a percentage of cell-incorporated [3H]AA, which was determined in solubilized cells, as previously described [18].

2.9. Liquid chromatography/tandem mass spectrometry measurements of eicosanoids

Cells were seeded in 12 mm plastic clusters (104 cells/cm2) and, after 4 days, the cultures were incubated with the treatments. Finally, eicosanoids in the cell culture medium were extracted using a solid phase method. To simultaneously separate eicosanoids and deuterated internal standards we used a liquid chromatograph Perkin Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and thermostated auto-sampler. To obtain the MS/MS data we used a triple quadrupole mass spectrometer API3000 (ABSciex, Concord, Ontario, Canada) equipped with a TurboIonspray source operating in negative ion mode as described previously [19, 20].

2.10. Data analysis

The results are expressed as the mean ± standard error of the mean. Differences between groups were tested using Student’s t-test. Differences of $P < 0.05$ were considered significant.

3. Results

3.1. Oleic acid has a proliferative effect that is mainly dependent on its metabolism by lipoxygenase pathway

Oleic acid (1-100 μM), a C18:1 cis fatty acid, induced Caco-2 cell growth in absence of growth factors, whereas this effect disappeared when Caco-2 cell were incubated in presence of C18:1 trans, elaidic acid (Fig. 1A). Interestingly, this oleic acid-induced mitogenic effect was reverted by MK886, a 5-lipoxygenase inhibitor, by U75302 (BLT1 antagonist) and by MK571 (cysteinyl leukotriene antagonist). In contrast, ketoprofen (cyclooxygenase inhibitor) did not modify the mitogenic action of oleic acid (Fig. 1B). Oleic acid also induced DNA synthesis whereas elaidic acid did not have any effect (Fig. 2A) and we also observed that the mitogenic effect of oleic acid was reverted when Caco-2 cells were incubated with a 5-LOX inhibitor (MK886) or a cysteinyl leukotriene antagonist (MK571) (Fig. 2B).

3.2. Minor compounds of olive oil modulate Caco-2 cell growth induced by oleic acid or FBS

Our results show that characteristic olive oil minor compounds such as hydroxytyrosol (0.1-10 μM), oleuropein (10 μM), pinoresinol (0.1 μM), squalene (0.1-10 μM) and maslinic acid (0.1-10 μM) significantly inhibited Caco-2 cell growth induced by oleic acid (10 μM) (Fig. 3A). A similar effect was observed when Caco-2 cells growth was stimulated with 10% FBS (Fig. 3B). The effect of these compounds on DNA synthesis induced by oleic acid was also examined in Caco-2 cultures. Figure 3C shows that hydroxytyrosol, oleuropein, pinoresinol, squalene and maslinic acid were able to induce a significant inhibition of DNA synthesis after 48 h exposure to these compounds in presence of oleic acid (10 μM).

3.3. Effect of extra virgin olive oil minor compounds on cell cycle and apoptosis

Ethidium bromide/acridine orange staining and morphological examination revealed that these studied olive oil minor compounds did not affect cell structure or cell viability with the exception of hydroxytyrosol (10 μM) and maslinic acid (10 μM) that induced appreciable cell detachment (data not shown), suggesting an apoptotic capacity to both compounds that was demonstrated by a TUNEL assay. Thus, hydroxytyrosol and maslinic acid (10 μM) increased the percentage of apoptotic cells up to 35 ± 3% and 43 ± 2.5%, respectively). Also we observed that olive oil minor compounds did not induce any significant change in cell cycle phases (data not shown).

3.4. Oleuropein is hydrolyzed to release hydroxytyrosol in Caco-2 cell cultures

Oleuropein contents hydroxytyrosol that could be released whether cell cultures present the suitable biochemical activity. Corona et al. (2006) reported that oleuropein was rapidly degraded by the colonic microflora resulting in the formation of hydroxytyrosol. Obviously, it is important to analysis whether there is oleuropein hydrolysis in our experimental conditions. To study this possibility, Caco-2 cell cultures were incubated with oleuropein and we observed the disappearance of oleuropein and the apparition of hydroxytyrosol. When oleuropein was incubated to 1 μM after 24 h only detected hydroxytyrosol, when oleuropein was incubated to 10 μM we observed the hydrolysis of around the 25 % of oleuropein (Table 1).

3.5. Hydroxytyrosol, oleuropein and pinoresinol present antioxidant activity

Numerous natural compounds with antioxidant activity also present antiproliferative actions, and both activities have been related. However, our findings shown that only hydroxytyrosol, oleuropein, pinoresinol and squalene have higher antioxidant activity than trolox and that exclusively hydroxytyrosol presents higher reducing power than quercetin used as control (Fig. 4).

3.6. Effect of hydroxytyrosol on AA release and eicosanoid synthesis

Caco-2 cells synthesize PGE2 by COX pathway, LTB4 and 5-HETE as representative metabolites of 5-LOX pathway, 12-HETE by 12-LOX pathway and 15-HETE and 13-HODE by 15 LOX pathway from AA and linoleic acid, respectively (Table 2). However, we were not able to detect the presence of HETEs and EETs produced by cytochrome P-450 pathway as we previously described [20]. Considering that hydroxytyrosol is the compound with higher antioxidant and antiproliferative activities, it was chosen to determine its effects on AA cascade. Table 2 shown that hydroxytyrosol were able to inhibit AA release as well as the synthesis of all AA cascade metabolites measured in our experimental conditions. We must consider that the percentage of inhibition of eicosanoid synthesis induced by hydroxytyrosol was higher than the effect on AA release (Table 2). Findings that suggest that hydroxytyrosol can modulate enzymes involved in AA cascade. This hypothesis was confirmed when hydroxytyrosol reverted the synthesis of AA metabolites induced by the exogenous addition of AA to Caco-2 cell cultures (Table 2). Thus, our results show a profound modulation of AA release as well as COX, 5-LOX, 12-LOX and 15-LOX pathways by hydroxytyrosol.

4. Discussion

Epidemiological data have been obtained in the recent decades indicating a key role from the amount of dietary fat in the pathogenesis of different neoplasms in humans such as CRC [21, 22]. Several experimental models and clinical trials have also provided evidence incriminating the fatty acid composition of the diet as an important determinant of risk of CRC development [23, 24]. However, the precise mechanisms underlying their action on inflammation, carcinogenesis or immunomodulation remain largely unknown.

Enhancement of cell proliferation is widely understood to be an important factor determining carcinogenesis. In the colon, increased numbers of cycling cells or mitoses, leading to expansion of the cell proliferation zone and increased crypt height, as well as depressed apoptosis, are considered risk factors for tumor development [25].

Here, our results shown that oleic acid induce cellular proliferation in Caco-2 cells. But, in the literature there are contradictory results in reference to the oleic acid effect among cell growth, depending on cell line, their tumorogenesis (or not) capacity and the determination techniques used [26-28]. Regarding to these effects, our findings suggest that oleic acid promotes Caco-2 cell growth, but its effect is significantly reverted by minor components from olive oil. Hydroxytyrosol was the most potent inhibitor of cellular proliferation, followed by maslinic acid, squalene, pinoresinol and oleuropein that only have effect at 10 μM. Interestingly, these olive oil components were also able to inhibit Caco-2 cell growth induced by FBS. These results are in concordance with other authors that demonstrate that the phenolic fraction of olive oil can reduce cell proliferation or tumorogenesis in experimental models of colon cancer [12, 29-32]. Is important to note that in the current literature are not available experimental conditions where cells are cultured in presence of oleic acid and olive oil minor components individually. Consequently, our results demonstrated, for the first time, that olive oil minor components are capable to reverse the proliferative effect induced by oleic acid on intestinal epithelial cell cultures. Llor and Pons [33] concluded that olive oil induces apoptosis and cell differentiation and down-regulates the expression cyclooxygenase-2. Our results suggest that hydroxytyrosol and maslinic acid could be related with the capacity to induced apoptosis.

Corona et al. [34] reported a hydroxytyrosol release from oleuropein induced by colonic microflora, findings in agreement with our results that also demonstrated a complete oleuropein hydrolysis at 1 μM and partial hydrolysis at 10 μM. Furthermore, our results show that hydroxytyrosol was not metabolized in our experimental conditions by Caco-2 cell cultures as was reported previously [34]. Thus, considering these results we cannot exclude that the anti-mitogenic effects of oleuropein was consequence of the hydroxytyrosol release.

Compounds with catechol group, like hydroxytyrosol and oleuropein, can stabilize free radicals, giving antioxidant activity to these compounds. Some author support that, of all the phenols present in

olive oil, only catechols are important [35]. But pinoresinol seems to have antioxidant activity even though it have not catechol group in their structure. Our results demonstrate that all minor components studied have appreciable antioxidant activity, except squalene and maslinic acid in agreement with Owen et al. [36] and Hasim et al. [37], being hydroxytyrosol the olive oil component with highest antioxidant activity and reducing power. Despite the fact that antioxidant activity is commonly related to anticancer properties, we cannot establish a direct relationship between antioxidant activity and antiproliferative action given that minor components without antioxidant activity such as maslinic acid have a significant antiproliferative action.

Previously, we observed a relationship between oxidative stress and AA release and AA cascade activation [18] and that natural compounds that regulate redox state can have an important effect on AA cascade [38]. Furthermore, eicosanoids from AA such as prostaglandins and leukotrienes [39, 40] as well as HETEs [39] and linoleic acid metabolites such as HODEs [41] have a pivotal role in the control of intestinal epithelial Caco-2 cells growth. Taking into account that oleic acid seem to exert their effect through 5-LOX pathway metabolite biosynthesis and the subsequent binding to BLT1 and CysLT receptors, we hypothesized that minor components could act reverting the biosynthesis of these ω-9 metabolites from oleic acid and consequently Caco-2 cell proliferation. Hydroxytyrosol was able to inhibit LTB4 synthesis [42] whereas squalene [43] and pinoresinol [44] did not have a significant action on LOX pathway. Considering these antecedents, we aim to study hydroxytyrosol action on AA cascade. Our findings demonstrate that hydroxytyrosol is able to inhibit AA release and AA metabolism by COX and 5-, 12- and 15-LOX pathways. Thus, the presence of hydroxytyrosol could modulate oleic acid metabolism. This mechanism can be explained, at least in part, the reversion of Caco-2 cell growth induced by oleic acid in the presence of hydroxytyrosol or oleuropein. Obviously, this hypothesis should be confirmed with additional research to identify oleic acid metabolites involved in these events.

An interesting and innovative aspect of our study is represented by the fact that although oleic acid consumption might contribute to mitogenesis, the beneficial effect of olive oil on colon cancer may be mediated by olive oil minor components that modulate the proliferative effect of oleic acid on intestinal epithelial cells. In these sense, is important emphasize that the difference between olive oil and other oleic sources (like seed oils…..) is the presence of these minor bioactive components that provided added value and modulate the response to tumor cells. Thus, we explored new possible molecular mechanisms that could mediate the effects of olive oil which are alternative to those reported in the literature that reported that olive oil phenolic compounds may inhibit carcinogenesis at the initiation, promotion and progression stages of colorectal cancer [45-47].

In conclusion, our findings suggest that minor components from olive oil are mayor contributors to the protective properties of olive oil, although representing only 2% of the olive oil could be enough to exert their chemoprotective effect on CRC. Traditionally, the beneficial effects of olive oil have been mainly ascribed to its oleic acid content. However, a wide range of evidence indicates that the

beneficial effects of olive oil intake are due to the minor bioactive compounds present in the unsaponificable fraction with numerous effects on oxidative stress, AA cascade and intestinal epithelial cell growth as we reported in this study. Consequently, we must consider that the consumption of seed oils, seed oils with high oleic content or olive oil will probably have different effects on CRC development. Furthermore, a profound clinical and experimental study on the effects of olive oil minor components regarding CRC is also necessary to elucidate the mechanisms involved.

Acknowledgments

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Table 1. Oleuropein hydrolysis in Caco-2 cells.

	Oleuropein (μM)	Hydroxytyrosol (μM)
24h control	0	0
Oleuropein 1 μM (24h)	0	1.29 ± 0,01
Oleuropein 10 μM (24h)	8.01 ± 0,08	2.25 ± 0,03
48h control	0	0
Oleuropein 1 μM (48h)	0	1.98 ± 0,02
Oleuropein 10 μM (48h)	7.25 ± 0,04	2.49 ± 0,02

Non-differentiated Caco-2 cells were incubated with oleuropein 1 and 10 μM. After 24 and 48 hours we take samples of culture supernatant and oleuropein and hydroxytyrosol were quantified by HPLC. Results are expressed as μM concentration of product. Values are mean ± SEM of two experiments performed in duplicate.

Table 2. Effect of hydroxytyrosol on AA release and the synthesis of AA cascade metabolites by Caco-2 cell cultures.

		Metabolites					
	[^{3}HAA] release (%)	PGE_2	LTB_4	5-HETE	12-HETE	15-HETE	13-HODE
Treatments							
Control	6.1 ± 0.3	0.39 ± 0.06	0.32 ± 0.08	3.12 ± 0.09	2.14 ± 0.11	3.89 ± 0.19	0.87 ± 0.06
FBS	22.4 ± 2.3*	1.79 ± 0.43*	1.96 ± 0.11*	8.22 ± 1.05*	4.38 ± 0.37*	9.19 ± 1.15*	3.12 ± 0.12*
FBS + HT	10.6 ± 1.2$^{\neq}$	0.48 ± 0.05$^{\neq}$ (94)	0.58 ± 0.06$^{\neq}$ (84)	4.67 ± 0.21$^{\neq}$ (69)	2.89 ± 0.09$^{\neq}$ (66)	5.76 ± 0.07$^{\neq}$ (65)	1.92 ± 0.04$^{\neq}$ (53)
AA		3.37 ± 0.22*	2.11 ± 0.07*	9.05 ± 0.32*	5.17 ± 0.29*	10.02 ± 0.52*	
AA + HT		1.09 ± 0.04$^{\neq}$ (76)	0.89 ± 0.10$^{\neq}$ (68)	4.85 ± 0.07$^{\neq}$ (70)	3.21 ± 0.11$^{\neq}$ (65)	5.97 ± 0.12$^{\neq}$ (66)	

[^{3}H]AA was incorporated to Caco-2 cells (104 cells/cm^2) to study [^{3}H]AA release and finally cells were cultured with or without FBS (10%) or AA (10 μM) in absence or presence of hydroxytyrosol (HT, 1 μM) for 3 or 1 h, respectively. [^{3}H]AA release or eicosanoids present in culture supernatant were extracted and measured. AA release is expressed as % respect to AA incorporated to cell cultures and eicosanoid concentrations are presented as ng/ml. Results are shown as means ± SEM of 3 experiments performed in triplicate. (% inhibition respect to cells stimulated in absence of polyphenols)
* $P < 0.05$ versus control values. ≠ $P < 0.05$ versus cells cultured with FBS or AA.

Figure Legends

Figure 1. Effect of Oleic acid on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with oleic and elaidic acid (1-100 μM) in absence of FBS (A), and with oleic acid with and without Ketoprofen (Kp, 5 μM), MK886 (MK8, 10 μM), U75302 (U75, 5 μM), LY255283 (LY25, 25 μM) and MK571 (MK5, 25 μM) in absence of FBS (B). Cells were then counted. Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ versus control group (cells cultured in absence of FBS). # $P < 0.05$ versus oleic acid 10 μM in absence of FBS.

Figure 2. Effect of Oleic acid on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with oleic acid (1-100 μM) in absence of FBS (A), and with oleic acid with and without MK886 (MK8, 10 μM), U75302 (U75, 5 μM), and MK571 (MK5, 25 μM) in absence of FBS (B). DNA synthesis was measured. Data are expressed as means ± SEM of 2-3 experiments performed in triplicate. * $P < 0.05$ versus control group (cells cultured in absence of FBS). # $P < 0.05$ versus oleic acid 10 μM in absence of FBS.

Figure 3. Effect of Oleic acid and minor components on Caco-2 cell growth. Caco-2 cells were incubated for 48 h with oleic acid (10 μM) with and without hydroxytyrosol (HTyr), oleuropein (Oleu), pinoresinol (Pino), squalene (Squa) and maslinic acid (Masl) at 0.01-10 μM in absence of FBS (A). Cells were incubated with minor components (10 μM) in presence of FBS (B). Finally cells were incubated with oleic acid (10 μM) with and without minor components (10 μM) in absence of FBS. Cells were then counted (A and B) and the uptake of BrdU incorporation was also measured (C). Data are expressed as means ± SEM of 3-4 experiments performed in triplicate. * $P < 0.05$ versus control group (cells cultured in absence or presence of FBS, A/C and B figure, respectively). # $P < 0.05$ versus oleic acid 10 μM in absence of FBS.

Figure 4. Total antioxidant activity and reducing power of minor components of olive oil. Different components were analyzed at 10 μM (white bars) and 100 μM (black bars) as described in the Material and methods section. Total antioxidant activity is expressed as equivalents of Trolox (μM). Reducing power is expressed as equivalents of quercetin (μM). Data are the mean ± SEM of 2 experiments performed in duplicate.

Figure 1

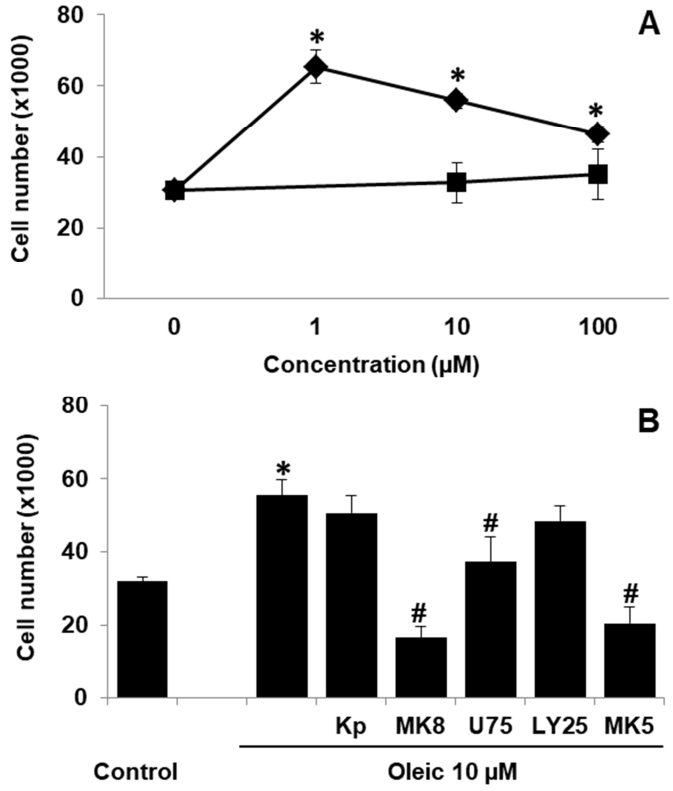


Figure 2

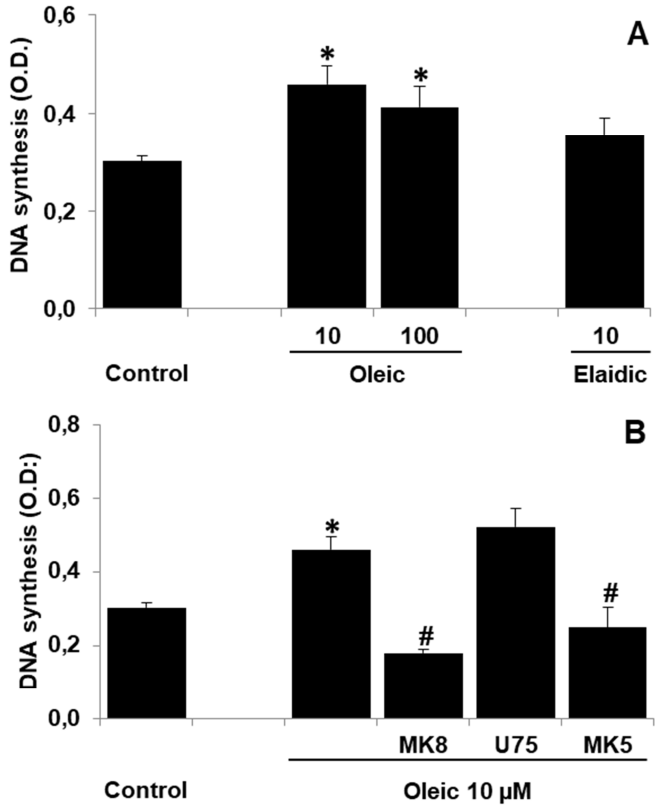


Figure 3

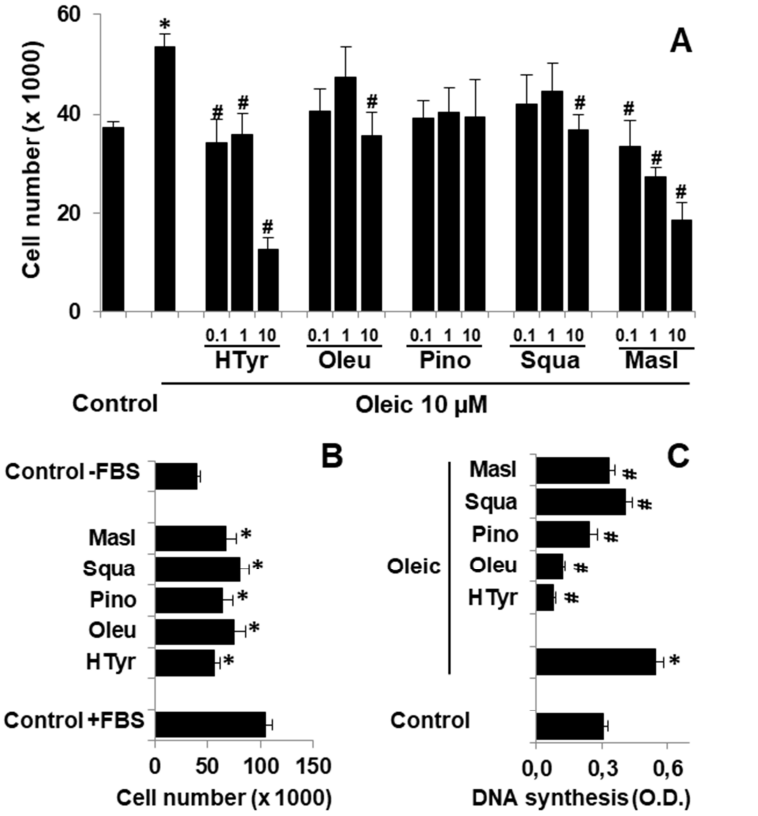
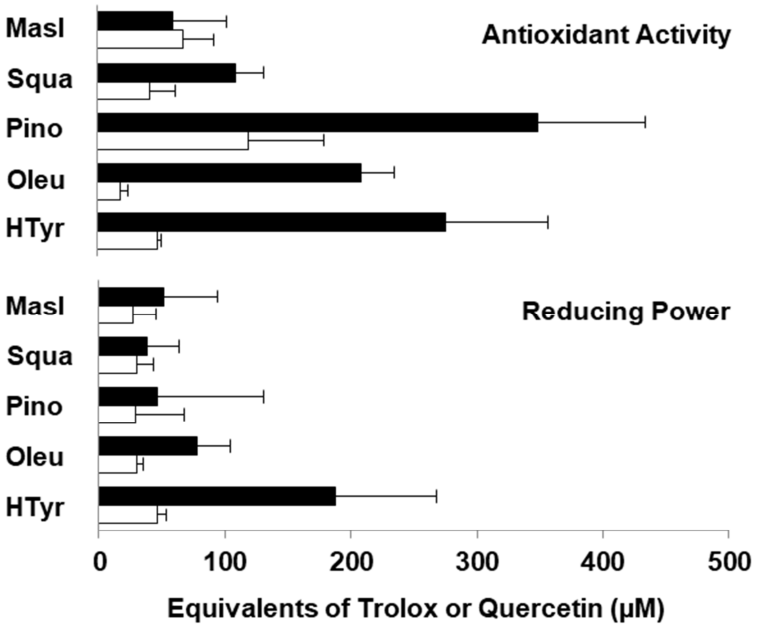


Figure 4



Artículo 5

Effect of extra virgin olive oil components on the arachidonic acid cascade, colorectal cancer and colon cancer cell proliferation

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Categoria (posición, cuartil): *Food Science and Technology* (85/129, Q3)

Resumen Artículo 5

Objetivo: Analizar la información existente sobre el efecto de los componentes del aceite de oliva virgen extra sobre la cascada del AA y los mecanismos implicados en el CCR como el crecimiento de las células epiteliales intestinales/apoptosis.

Resultados y conclusiones: El ácido oleico solo y en presencia de componentes minoritarios del aceite de oliva virgen extra tiene diferentes efectos sobre el estrés oxidativo, la cascada del AA y el crecimiento de las células epiteliales intestinales; y consecuentemente el consumo de aceites de semillas, aceites de semillas con alto contenido en ácido oleico o del aceite de oliva virgen extra probablemente tendrán diferentes efectos en el desarrollo del CCR.

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Effect of extra virgin olive oil components on the arachidonic acid cascade, colorectal cancer and colon cancer cell proliferation*

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SUMMARY: The mediterranean diet (MD) reduced the risk of colorectal cancer (CRC), and olive oil, the primary source of fat in the MD, has also been found to have a protective effect. However, animals fed with oleic acid present a high number of intestinal tumours, suggesting that oleic acid and olive oil consumption can exert different effects on CRC. Considering that extra virgin olive oil (EVOO) is a complex mix of fatty acids and minor compounds such as polyphenols, hydrocarbons, phytosterols and triterpenes; and that these compounds have antioxidant activity and consequently they can modulate the arachidonic acid (AA) cascade and eicosanoid synthesis. This review analyzes the state of the art of olive oil components on the AA cascade and cellular mechanism involved in CRC such as intestinal epithelial cell growth/apoptosis, to understand the fact that the consumption of seed oils with high oleic content or EVOO will probably have different effects on CRC development.

***KEYWORDS*:** *Cell growth; Colorectal cancer; Eicosanoid; Oleic acid; Oxidative stress; Polyphenol*

RESUMEN: ***Efecto de los componentes del aceite de oliva virgen extra en la cascada del ácido araquidónico, el cáncer colorrectal y la proliferación de células de cáncer de colon.*** La dieta Mediterranea (DM) y el aceite de oliva reducen el riesgo de cáncer colorrectal (CCR). Sin embargo, animales alimentados con dietas ricas en ácido oleico presentan un elevado número de tumores intestinales, lo que sugiere que el consumo de ácido oleico y aceite de oliva pueden tener efectos diferentes sobre el desarrollo de CCR. Considerando que el aceite de oliva extra virgen (AOEV) es una compleja mezcla de ácidos grasos y compuestos minoritarios como polifenoles, lignanos, hidrocarburos, fitoesteroles y triterpenos; y que algunos de estos compuestos son antioxidantes y modulan la cascada del ácido araquidónico (AA) y la producción de eicosanoides. Analizamos la información existente sobre el efecto de los componentes del AOEV sobre la cascada del AA y los mecanismos implicados en el CCR como el crecimiento de las células epiteliales intestinales/apoptosis, lo que nos permitirá entender por qué el consumo de aceites de semillas altos en oleico o AOEV probablemente tendrán diferentes efectos sobre el desarrollo del CCR.

***PALABRAS CLAVE*:** *Ácido oleico; Cáncer colorrectal; Crecimiento celular; Eicosanoide; Estrés oxidativo; Polifenol*

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1. ROLE OF THE ARACHIDONIC ACID CASCADE ON INTESTINAL EPITHELIAL CELL GROWTH AND COLORECTAL CANCER

Considerable amounts of arachidonic acid (AA) are found esterified at the sn-2 position of the phospholipid biomembranes. Under physiological conditions, the amount of free intracellular AA available is quite small, but numerous stimuli can induce AA release through phospholipase A_2(PLA_2) activation. Then, AA can be metabolized by cyclooxygenases (COXs) to produce prostaglandins (PGs), by lipoxygenases (LOXs) to synthesize leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs), and by cytochrome P-450 pathway to produce HETEs and epoxieicosatetraenoic acids (EETs) (Figure 1). These bioactive lipids have pleitropic effects even though they were classically considered inflammatory mediators.

Today, the causal relationship between inflammation, innate immunity and cancer is more widely accepted; nevertheless, many of the molecular and cellular mechanisms mediating this relationship remain unresolved. However, there is now evidence that inflammatory mediators have a powerful effect on tumor development. Early in the neoplastic process, eicosanoids produced by the AA cascade could be powerful tumor promoters, producing an attractive environment for tumor growth and promoting angiogenesis in the intestinal mucosa (Ferrer and Moreno, 2010).

Studies in the early 1980s indicated that nonsteroidal anti-inflammatory drugs (NSAIDs) were chemo preventive in animal models of colorectal cancer (CRC). In 1991, Thun *et al.* reported that aspirin reduces the relative risk and mortality of CRC. Even more relevant for the clinician were subsequent studies that demonstrated that NSAID therapy can cause the regression of adenoma in patients with familial adenomatous polyposis (Koehne and Dubois, 2004). Furthermore, COX-2 is elevated in CRC, with the subsequent increase of PGE_2 and 6-keto $PGF_{1\alpha}$ levels (Moran *et al.,* 2004). Using an elegant experimental model of CRC induced by genetic manipulation, Oshima *et al.* (1996) reported that COX-2 is located in the stromal component and may promote tumor growth by producing bioactive PGs that affect tumor growth in a paracrine fashion.

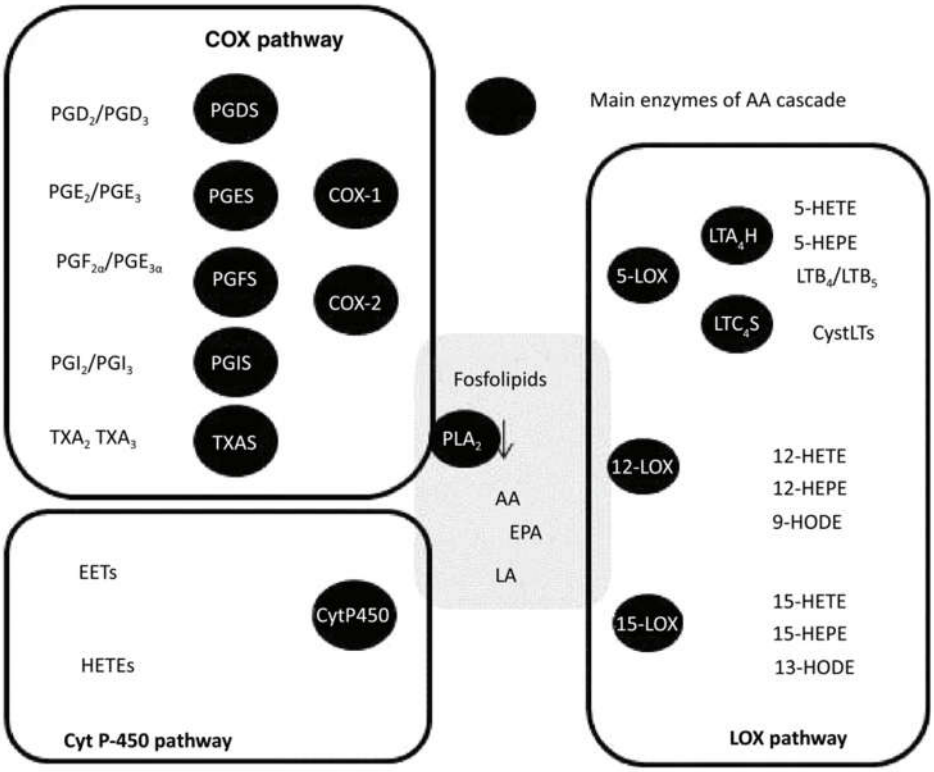

FIGURE 1. Arachidonic acid cascade. This scheme illustrates the main elements of the AA cascade including the main enzymes and metabolites of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P-450 (Cyt P-450) pathways: PGES, prost-aglandin E synthase; PGDS, prostaglandin D synthase; PGFS, prostaglandin F synthase; PGIS, prostaglandin I synthase; TXAS, thromboxane A synthase; LTA_4H, leukotriene A_4 hydrolase; LTC_4S, leukotriene C_4 synthase.

The beneficial effects of dietary manipulation of the AA content in the bio-membranes of animals that spontaneously induce polyps (APC^{min} mice) suggest that AA is a key element involved in tumorigenesis (Petrik *et al.,* 2000). Inducing a cytosolic PLA_2 deletion in APC^{min} mice, Hong *et al.* (2001) demonstrated the pivotal role of these enzymes involved in the AA release in small intestine polyp formation. These findings support the hypothesis that the anti-tumorigenic effects of NSAIDs are related to the impairment of PG production. However, these explanations have lacked molecular details, in largely as a consequence of a poor understanding of the role of PG receptors. In the last decade, important findings were obtained with respect to this point. Targeted deletion of the EP_1 receptor of PGE_2 reduced the number of colonic lesions in APC^{min}-induced polyp formation that was also reduced by a specific EP_1 antagonist (Watanabe *et al.,* 1999), whereas EP_4 deletion reduced the tumor size. Considering all of this together, we have a complete picture of the main elements involved in the effects of the AA cascade on epithelial cell growth in physiological and patho-physiological conditions: AA is released by PLA_2, then it is metabolized by COX-1/COX-2 to produce bioactive eicosanoids such as PGE_2, which interact with specific receptors (EP_1 and EP_4) and activate cell signaling pathways involved in the control of intestinal epithelial cell growth (Sanchez and Moreno, 2002; Cabral *et al.,* 2013; Cabral *et al.,* 2014).

Cianchi *et al.* (2006) reported that the two major metabolic pathways of the AA cascade, COX and 5-LOX, are simultaneously up-regulated in CRC. Interestingly, an inhibition of either COX or 5-LOX alone resulted in the activation of the other pathway, and consequently, combined treatment with COX and 5-LOX inhibitors produced greater inhibition of tumor cell proliferation. A high expression of the BLT_1 receptor of LTB_4 (Ihara *et al.,* 2007) and the $CystLT_1$ receptor of $LTD_4$was detected in human colon cancer tissues, whereas $CysLT_2$ receptor expression was reduced in colon cancer and was associated with poor prognosis, due to its capacity to induce differentiation and growth inhibition (Magnusson *et al.,* 2007). Recently, we observed that several LOX metabolites from AA and linoleic acid such as LTB_4, LTD_4, 5-HETE, 12-HETE, 15-HETE and 13-hydroxyoctadecanoic acid are involved in the control of CRC cell line proliferation (Cabral *et al.,* 2013; Cabral *et al.,* 2014; Martín-Venegas *et al*., 2014; Cabral *et al.,* 2015).

2. EFFECT OF THE MEDITERRANEAN DIET AND OLIVE OIL ON COLORECTAL CANCER

CRC is the third most commonly diagnosed cancers in developed countries, and is the second cause of cancer-related deaths. Although a great effort has been made toward developing detection and surgical strategies, there has been little improvement in the outcome for patients with advanced disease. Obviously, many elements such as the dysregulation of intestinal epithelial cell growth/apoptosis, angiogenesis and metastasis are involved in the development of CRC. Enhancement of cell proliferation is widely understood to be an important factor determining carcinogenesis. In the colon, an increased numbers of cycling cells or mitoses leading to expansion of the cell proliferation zone and increased crypt height as well as depressed apoptosis are considered risk factors for tumor development.

The traditional Mediterranean diet (MD) is widely recognized as one of the healthiest in the world and it is likely that the adoption of this diet would lead to a significant reduction in the incidence of many chronic diseases (Sofi *et al.,* 2011). This recent meta-analysis of prospective cohort studies concluded that the MD is responsible of a significant reduction in death that included the impairment in the incidence of neo-plastic diseases. In this sense, updated reports from a large cohort such as the European Prospective Investigation into Cancer and Nutrition (EPIC) have found a lower overall cancer risk among individuals with greater adherence to the MD (Couto *et al.,* 2011). Similar results were obtained in the large cohort belonging to the National Institutes of Health (Reedy *et al.,* 2008).

It is the consumption of olive oil, more than any other single factor that distinguishes the traditional MD from other dietary patterns, and it has been related to the reduced risk of various neoplasms including CRC (Pelucchi *et al.,* 2011). It is important to consider that extra virgin olive oil (EVOO) is a complex mix containing fatty acids such as oleic acid and minor compounds such as simple phenols (tyrosol and hydroxytyrosol), adhehydic secoiridoids (oleuropein), flavonoids and lignans (pinoresinol) as well as hydrocarbons (scualene), phytosterols (β-sitosterol) and triterpenes (maslinic acid) (Table 1), and that some of these compounds may modulate the mechanism

TABLE 1. Main components of extra virgin olive oil and their daily consumption by humans

Compounds	Content	Daily consumption
Oleic acid	550–850 g/Kg	25–40 g
Linoleic acid	50–200 g/Kg	2–10 g
Hydrocarbons	1200–7500 mg/kg	50–300 mg
Phytosterols	1800–2500 mg/kg	100–150 mg
Polyphenols	up to 1000 mg/kg	50 mg
Tocopherols	100–300 mg/kg	5–15 mg
Triterpens	150–1000 mg/kg	10–50 mg
Lignans	up to 100 mg/kg	5 mg

involved in the pathogenesis of CRC such as intestinal epithelial cell growth as well as apoptosis, angiogenesis and metastasis. Thus, flavonoid and lignan intakes have been inversely associated with CRC risk in several case-control studies, and The Polyp Prevention Trial Study reported that high intakes of flavonols and isoflavones were related to a decreased risk in advanced CRC. Here, we analyze the effect of oleic acid and representative EVOO minor bioactive compounds on oxidative stress, AA cascade and colon cancer cell line growth, opening the way to a more precise understanding of the molecular basis of the action of EVOO on CRC.

3. EFFECT OF OLIVE OIL COMPONENTS ON OXIDATIVE STRESS AND AA CASCADE

Olive oil contains a high amount of oleic acid and only a small amount of linoleic acid, the precursor of AA in mammals. Thus, olive oil provides monounsaturated fatty acids, which are not as readily oxidized as the polyunsaturated fatty acids, and consequently reduces membrane susceptibility to lipid peroxidation. Furthermore, Bartoli *et al*. (2000) reported that a diet rich in olive oil significantly reduced AA concentration in tissues, changes that might be responsible for the impairment of oxidative stress and the synthesis of AA metabolites (Moreno *et al.,* 2001). There is a well-established inverse relationship between the dietary intake of antioxidant-rich foods and the incidence of human diseases. EVOO contains numerous minor components with antioxidant activity such as polyphenols. These facts are consistent with the observation that the minor components of olive oil protect against DNA oxidation and lipid peroxidation (Mitjavila *et al.,* 2013). Among the minor components of EVOO, phenolic compounds are those most extensively studied. However, it should not be overlooked that EVOO contains other components that are quantitatively more significant such as hydrocarbons and phytosterols. Thus, β-sitosterol inhibits ROS production (Moreno, 2003) through the enhancement of antioxidant enzymes such as Mn superoxide dismutase and glutathione peroxidase (Vivancos and Moreno, 2005). Interestingly, polyphenols and phytosterols can modulate oxidative stress through distinct and complementary mechanisms that induce synergistic effects (Vivancos and Moreno, 2005).

We must consider that the cellular redox state may act as a molecular switch that regulates the activity of many enzymes and genes. In this way, ROS are involved in the PLA_2 activation, AA release and eicosanoid synthesis (Martínez and Moreno, 2001). Consequently, foods rich in antioxidants such as EVOO modulate cellular oxidative stress, the AA cascade and reduce eicosanoid synthesis (Moreno *et al.,* 2001), events that may be specifically modulated by bioactive components of EVOO such as polyphenols and phytosterols (Moreno, 2003; Vivancos and Moreno, 2008).

4. EFFECT OF OLEIC ACID ON COLORECTAL CANCER AND COLON CANCER CELL LINE PROLIFERATION

In recent decades, epidemiological data indicated a key role from the amount of dietary fat in the pathogenesis of different neoplasms as CRC (Bartsch *et al.,* 1999). Several experimental models have also provided evidence that the fatty acid composition in the diet is a major determinant in the risk of tumor development. However, the precise mechanisms underlying their inflammatory/anti-inflammatory, tumorigenic/anti-tumorigenic or immune-modulating effects remain largely unknown. Interestingly, dietary olive oil modulates the lipid membrane composition and the production of inflammatory mediators including PGs and nitric oxide (Moreno *et al.,* 2001) as we mentioned above.

Olive oil has been found to have a slight protective effect on CRC development (Braga *et al.,* 1998), while monounsaturated fat intake appeared uninfluential (Franceschi *et al.,* 1998). Interestingly, Hansen Petrik *et al*., (2000) reported that $Apc^{Min/+}$ mice fed with an oleic acid diet presented a high number of intestinal tumors, whereas, when the diet was prepared with olive oil, Barone and co-workers (2014) observed a decrease in polyp number and polyp volume, with respect to a soybean oil diet (PUFA rich). These important discrepancies put into consideration the fact that oleic acid and olive oil can exert different effects on CRC. In this sense, we recently observed that oleic acid induces intestinal epithelial cell growth whereas oleic acid in the presence of EVOO components such as hydroxytyrosol, oleuropein, pinoresinol or maslinic acid did not have this mitogenic action (Martínez-Hovelman *et al.,* 2013).

5. EFFECTS OF EXTRA VIRGIN OLIVE OIL MINOR COMPONENTS ON ARACHIDONIC ACID CASCADE AND COLON CANCER CELL LINE PROLIFERATION

Traditionally the beneficial effects of olive oil have been ascribed to its oleic acid content. However, a wide range of evidence indicates that the beneficial effects of EVOO intake are due to the minor bioactive compounds present in the unsaponifiable fraction. Thus, EVOO polyphenols such as tyrosol and hydroxytyrosol are potent antioxidants and radical scavengers (Visioli *et al*., 1998) that can inhibit COX pathway and the synthesis of PGs (Moreno 2003; Vivancos and Moreno 2008) as well as the synthesis

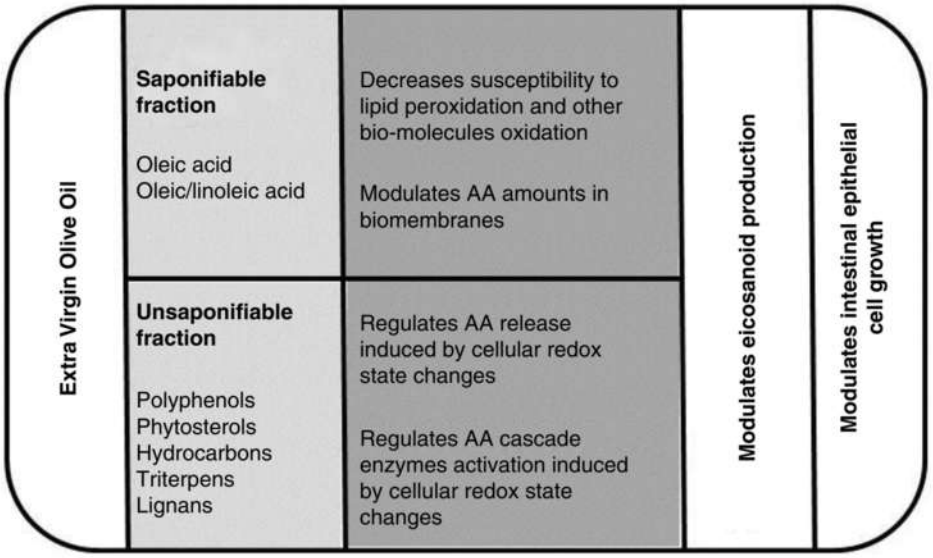

FIGURE 2. Scheme illustrating the main effects of extra virgin olive oil components on oxidative stress, AA cascade and intestinal epithelial cancer cell line growth.

of LTs by the LOX pathway (De la Puerta *et al.*, 1999; Moreno 2003).The main phenolic compounds present in EVOO, oleuropein and hydroxytyrosol, induce a reduction in proliferation and an increase in apoptosis in human colorectal cancer cell lines by down regulating FAS activity (Notarnicola *et al.*, 2011). In this way, we recently reported that EVOO lignans such as pinoresinol, EVOO triterpenes such as maslinic acid and EVOO hydrocarbons such as squalene inhibited cell proliferation and DNA synthesis induced by oleic acid in adenocarcinoma cell cultures (Martínez-Hovelman *et al.*, 2013).

6. CONCLUSIONS AND FUTURE PERPECTIVES

In conclusion, oleic acid and oleic acid in the presence of the representative minor components of EVOO have different effects on oxidative stress, the AA cascade and intestinal epithelial cell growth (Figure 2) and consequently the consumption of seed oils, seed oils with high oleic content or EVOO will probably have different effects on CRC development. Additional research is necessary to clarify this point with important consequences for nutrition, health and economy. Furthermore, a profound clinical and experimental study on the effect of EVOO's minor components regarding CRC is also necessary to elucidate the mechanisms involved.

ACKNOWLEDGMENTS

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Artículo 6

Effect of extra virgin olive oil polyphenols on arachidonic acid cascade in human intestinal epithelial cells

Carolina E. Storniolo, Olga Jáuregui and Juan J. Moreno

Preparado para enviar a Prostaglandins and Other Lipid Mediators

Los resultados de esta publicación se han presentado en el siguiente congreso:

Effect of extra virgin olive oil polyphenols on arachidonic acid cascade in human intestinal epithelial cells. CE Storniolo, JJ Moreno. 6th European Workshop on Lipid Mediators. Frankfurt, Alemania. 27-30/09/2016. **Póster**

Resumen Artículo 6

Objetivo: Examinar el efecto de los polifenoles del aceite de oliva virgen extra más representativos, como el tirosol y el hidroxitirosol, en la liberación de AA y la síntesis de los principales metabolitos de la cascada del AA.

Material y métodos: Utilizamos cultivos de células Caco-2 como modelo experimental y una metodología analítica de cromatografía líquida acoplada a espectrometría de masas para el análisis de múltiples metabolitos de cascada de AA.

Resultados: Nuestros resultados muestran que ambos polifenoles inhiben la liberación de AA inducida por los factores de crecimiento, el TNFα + IFNα y el H_2O_2 de concentración-dependiente. El tirosol y el hidroxitirosol también redujeron la síntesis de los metabolitos del AA, tales como la PGE_2, el LTB_4, los ácidos 5-, 12-, 15-hidroxieicosatetraenoicos, así como la producción de ácido 13- hidroxioctadecanoico.

Conclusiones: Estos resultados demostraron que los polifenoles del aceite de oliva virgen extra regulan la liberación del AA y la síntesis de eicosanoides a través de las vías de las COXs y las LOXs, efectos involucrados en la patogénesis de enfermedades con un importante componente inflamatorio.

Effect of extra virgin olive oil polyphenols on arachidonic acid cascade in human intestinal epithelial cells

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ABSTRACT

Extra virgin olive oil (EVOO) consumption is associated with protection from cardiovascular diseases, inflammatory chronic processes and cancer. Oxidative stress and arachidonic acid cascade have a pivotal role in these processes and beneficial effects of polyphenols can be related with its actions on redox state and eicosanoid production. The aim of this study was to examine the effect of representative EVOO polyphenols, tyrosol and hydroxytyrosol, on arachidonic acid (AA) release and the synthesis of the main AA cascade metabolites. We use Caco-2 cell cultures as experimental model and an analytical LC-MS/MS methodology for the analysis of multiple AA cascade metabolites. Our results show that both polyphenols (0.1-10 μM) inhibit AA release induced by FBS (10 %), H_2O_2 (1 mM) and TNFα + IFNα (50 ng/ml) in a concentration manner. Tyrosol and hydroxytyrosol (1 μM) also reduced the synthesis of AA metabolites such as prostaglandin E_2, leukotriene B_4, 5-, 12-, 15-hydroxyeicosatetraenoic acids induced by these stimuli or AA (10 μM) as well as the production of 13-hydroxyoctadecanoic acid. Our findings demonstrated that EVOO polyphenols regulate AA release and eicosanoid synthesis by cyclooxygenases and lipoxygenases, events involved in the pathogenesis of diseases with an important inflammatory component.

1 Introduction

Virgin olive oil, the most representative food of the traditional Mediterranean diet, is associated with protection from high prevalence diseases with high mortality such as cardiovascular disease [1], inflammatory chronic processes [2,3] and cancer [4,5]. Oxidative stress and arachidonic acid (AA) cascade activation have a pivotal role in these pathological processes and beneficial effects of olive oil can be related with its actions on redox state and eicosanoid production [6]. In this sense, dietary olive oil modulates the production of inflammatory mediators including prostaglandins and reactive oxygen species [7]. Extra virgin olive oil is a complex mix of fatty acids, mainly oleic acid, and a plethora of minor compounds of different chemical nature such as hydrocarbons, phytosterols, triterpenes and polyphenols [8]. Many of the beneficial effects of extra virgin olive oil (EVOO) can be attributed to

virgin olive oil minor components [8,9], including the antioxidant effect of polyphenols and the effects of these compounds on AA cascade that are involved in the eicosanoids synthesis. Important components of polyphenol fractions of EVOO are tyrosol and hydroxytyrosol, natural antioxidants [10] derived from a chemical or enzymatic hydrolysis of glycoside oleuropein, and consequently present in large amounts in all parts of olive trees (*Olea europaea*) including olives and olive oil. These compounds were reported to inhibit prostaglandin E_2 synthesis [11-14], leukotriene B_4 synthesis [15] or the synthesis of both eicosanoids [16] in different experimental models. Unfortunately, there is not a complete and holistic picture of the effect of these polyphenols on AA cascade, a metabolic route with several pathways such as cyclooxygenase (COX) pathway, 5-, 12-, and 15-lipoxygenase (LOX) pathway and cytochrome P-450 pathways with cross-talk inter-connections.

The aim of the present study was to examine the effect of tyrosol and hydroxytyrosol on the synthesis of the representative AA cascade metabolites by human intestinal epithelial cells using a versatile and reliable analytical LC-MS/MS methodology for the simultaneous analysis of multiple AA cascade metabolites.

2 Materials and Methods

2.1 Materials

Leukotriene (LT) D_4, prostaglandin (PG) E_2, PGE_2-d_4, LTB_4, LTB_4-d_4, 14,15-dihydroxyeicosatrienoic acid (DHET), 11,12-DHET, 5-hydroxyeicosatetraenoic acid (HETE), 5-HETE-d_8, 12-HETE, 12-HETE-d_8, 12-HETE, 15-HETE, 15-HETE-d_8, 20-HETE, 20-HETE-d_6, 5,6-epoxyeicosatrienoic acid (EET) and 5,6-EET-d_{11}, 8,9-EET, 11,12-EET, 14,15-EET, 14,15-EET-d_{11} and 13-hydroxyoctadecadienoic acid (HODE) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Tumor necrosis factor-α (TNFα), interferon-γ (IFNγ), tyrosol, hydroxytyrosol, ethidium bromide and acridine orange were purchased from Sigma Chemical (St. Louis, MO). Bromoenol lactone (BEL) and arachidonyl trifluoromethylketone (ATK) were acquired from Alexis Corp. (San Diego, CA, USA). [5,6,8,9,11,12,14,15-^{3}H]AA (200-240 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA).

2.2 Cell culture and cell growth assay

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37, Manassas, VA, USA). The cells were routinely grown in 75 or 150 cm^2 plastic flasks at a density of 10^4 cells/cm^2 and cultured in DMEM supplemented with 4.5 g/l D-glucose, 1% (v/v) nonessential amino acids, 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37ºC in a modified atmosphere of 5% CO_2 in air. The growth medium was replaced twice per week and the day before the experiment. All the experiments were performed in pre-confluent cultures and consequently, in non-differentiated cells [17].

2.3 Incorporation and release of [^{3}H]AA

Cells were harvested with trypsin/EDTA and passed to 24-well plates at a density of 10^4 cells/cm^2. After 4 days, cells were FBS starvated during 24 h and then the medium was replaced by 0.5 ml DMEM containing 0.1% fatty acid free BSA and 0.1 µCi [^{3}H]AA (1 nM) for a period of 6 h. Cells were then washed three times with 0.5% BSA-containing medium to remove any unincorporated [^{3}H]AA. After the study period (1 or 3 h), the medium was removed to determine the amount of [^{3}H] radioactivity release. The amount of [^{3}H]AA released into the medium was expressed as a percentage of cell-incorporated [^{3}H]AA, which was determined in solubilized cells, as previously described [17]. Background release from non-stimulated cells (5 ± 1.3 % of ^{3}H incorporated) was subtracted from all data.

2.4 Liquid chromatography/tandem mass spectrometry measurements of eicosanoids

Cells were seeded in 12 mm plastic clusters (10^4 cells/cm^2) and, after 4 days, the cultures were incubated with the treatments. Finally, eicosanoids in the cell culture medium were extracted using a solid phase method. To simultaneously separate 13 eicosanoids and deuterated internal standards we used a liquid chromatograph Perkin Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and thermostated auto-sampler. To obtain the MS/MS data we used a triple quadrupole mass spectrometer API3000 (ABSciex, Concord, Ontario, Canada) equipped with a TurboIonspray source operating in negative ion mode as described previously [18].

2.5 Western blot analysis

Caco-2 cells were washed twice with ice-cold PBS, scraped off into PBS containing 2 mM sodium EDTA and pelleted. These pellets were sonicated in PBS containing 4 mM sodium EDTA, 500 µg/ml aprotinin, 500 µg/ml leupeptin, 500 µg/ml PMSF, and 400 µg/ml diethyldithiocarbamic acid and re-suspended in lysis buffer containing 200 mM Tris-HCl, 200 mM NaCl, 2% Igepal CA-630 and 200 µM DTT. Finally, immunoblot analysis for COX-2 was performed as we previously described [17] using a polyclonal antiserum direct against COX-2 (1:1000) from Cayman Chem. For β-actin immunobloting was used monoclonal actin antibody (1:500) (Santa Cruz).

Proteins in cell samples were measured using Bio-Rad protocol with Coomassie brilliant blue G-250 as a dye reagent. The protein amount in each sample was calculated using a standard curve of bovine serum albumin.

2.6 Statistics

Results are expressed as mean ± SEM. All data were compared by one-way ANOVA and Student's t-test using SPSS software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered to denote significance.

3 Results

AA, or other fatty acid, release from phospholipids of membranes by phospholipase A_2s (PLA_2) is the first step in the AA cascade. Several stimuli such as FBS (10%), H_2O_2 (1 mM) or TNF + IFN (50 ng/ml) can induce AA release in Caco-2 cell cultures (Fig. 1). Interestingly, these events were inhibited by ATK, a reversible cytosolic PLA_2 and calcium independent PLA_2 inhibitor [19], and BEL, a selective calcium independent PLA_2 inhibitor [20]. Furthermore, tyrosol and hydroxytyrosol (1-10 µM) reduced AA release induced by the above mentioned stimuli in a concentration dependent manner (Fig. 2).

Caco-2 cells synthesize PGE_2 by COX pathway, LTB_4 and 5-HETE as representative metabolites of 5-LOX pathway, 12-HETE by 12-LOX pathway and 15-HETE and 13-HODE by 15 LOX pathway from AA and linoleic acid, respectively (Table 1). However, we were not able to detect the presence of

HETEs and EETs produced by cytochrome P-450 pathway as we previously described [21]. Table 1 also shown that tyrosol and hydroxytyrosol were able to inhibit the synthesis of all AA cascade metabolites measured in our experimental conditions. We must consider that the percentage of inhibition of eicosanoid synthesis induced by both polyphenols was higher than the effect of both polyphenols on AA release induced in the same experimental conditions. Findings that suggest that these EVOO components can modulate AA cascade enzymes (activity and/or expression) in addition to the inhibitory effect on AA release. This hypothesis was confirmed when both polyphenols reverted the synthesis of AA metabolites induced by the exogenous addition of AA to Caco-2 cell cultures (Table 1). On the other hand, Table 1 shows that tyrosol and hydroxytyrosol inhibited more profoundly COX pathway and consequently PGE_2 synthesis than LTs and HETEs production as biomarkers of LOX pathways. These findings could be related with the inhibition of COX-2 expression induced by tyrosol and hydroxytyrosol (Fig. 3). Thus, our results show a profound modulation of AA release as well as COX, 5-LOX, 12-LOX and 15-LOX pathways by tyrosol and hydroxytyrosol. Finally, we observed that both polyphenols have an additive effect on the inhibition of the synthesis of the above mentioned eicosanoids (Table 2).

4 Discussion

Intestinal epithelial Caco-2 cells express a lot of AA cascade enzymes such as COXs [17] and LOXs [22] and consequently synthetized numerous eicosanoids such as PGs, LTs, HETEs and HODEs after stimulation by FBS, findings in agreement with previous in vitro studies [21]. Here, we demonstrated that H_2O_2 or cytokines (TNFα plus IFNγ) are also able to stimulate COX and LOX pathways in Caco-2 cell cultures. Interestingly, these AA cascade metabolites are also detected in the intestine [23,24] and they control multiple important cellular processes such as cell proliferation and cell survival and different modalities of cell death as well as the state of intercellular junctions. Events which are intricately linked to the intestinal epithelial homeostasis as well as homeostasis disruption in inflammatory bowel diseases (IBD) and colorectal cancer (CRC) [25]. The etiology of IBD is not fully understood, but a dramatic increase in PGs and LTs synthesis is found in the intestine [26,27]. In a

similar form, CRC tissues show an increase in PGs, LTs and HETEs [28,29] that are related with tumor size and bad prognosis. Thus, the chronic production of eicosanoids and related compounds could be involved, at least in part, in the enhancement of the risk of developing IBD and/or CRC. Recently, we reported that eicosanoids are mitogenic stimuli on intestinal epithelial cells [30-32] and that the enhancement of intestinal eicosanoid concentrations could disrupt the barrier function of the intestinal epithelium [33,34]. Considering that EVOO is a complex mix contains mainly fatty acids and phenolic compounds such as tyrosol and hydroxytyrosol that may modulate intestinal epithelial cell growth [35,36]. Here, we demonstrated that these bioactive polyphenols have profound and systemic modulator effects on AA release and the subsequent AA metabolism by COX and LOXs pathways at concentrations that could be reached in the intestinal lumen and plasma after a habitual EVOO consumption in the context of a traditional Mediterranean diet.

Sanchez and Moreno [37] reported that $iPLA_2$ may be involved in AA release and the subsequent prostaglandin production induced by serum in Caco-2 cell cultures. Now, we also demonstrated the role of $iPLA_2$ inhibited by BEL in AA release induced by H_2O_2 and TNFα + IFNγ. Considering that $iPLA_2$ is regulated by changes in the cellular redox state [38], we can propose that the modulation of cellular redox state by tyrosol and hydroxytyrosol [10] could be, at least in part, involved in their inhibitory effects on AA release and the subsequent eicosanoid synthesis. Interestingly, we observed that the percentage of inhibition of eicosanoid synthesis induced by both polyphenols is higher than the percentage of inhibition of AA release, events that suggest an additional effect of these polyphenols on AA cascade enzymes of COXs/LOXs pathways involved in AA metabolism. This hypothesis was confirmed when we observed that tyrosol/hydroxytyrosol were able to inhibit eicosanoids production induced by an exogenous addition of AA to Caco-2 cell cultures. It is important to consider that the effect of both polyphenols on PGE_2 synthesis was higher than on LTB_4/HETEs production, findings that suggest a modulation of COX activity and/or expression. Our findings demonstrated that both EVOO polyphenols were able to revers COX-2 over-expression induced in intestinal epithelial cultures. These findings were recently observed in monocytes/macrophages [13,39] and endothelial cells [40], but it is the first time described in epithelial cells. Furthermore, although the modulation of PGs and LTs synthesis by tyrosol or hydroxytyrosol have been reported [11-16], it is the first time that both polyphenols were able to inhibit the synthesis of HETEs, AA

metabolites that are involved in the control of intestinal barrier function [34] and colorectal development [29].

Considering that natural and synthetic COX inhibitors direct free AA to LOX pathway with the consequent LTs/HETEs over-production whereas LOX inhibitors potentiated PG synthesis, these polyphenols have a more integral modulation of AA cascade. Finally, we observed that tyrosol and hydroxytyrosol did not have a synergistic action on AA cascade but both polyphenols presented an additive effect on the inhibition of AA release and eicosanoid synthesis at concentrations (0.3 μM) that could be reached in a plasma or tissue such as intestinal mucosa after a conventional EVOO consumption [41]. Considering that EVOO contains hundreds of micronutrients, it is important to study the potential synergistic/additive action of these polyphenols with other bioactive minor compounds of EVOO as well as with compounds presented in other characteristics foods of traditional Mediterranean diet to understand the mechanisms to explain the beneficial effects of EVOO consumption. In this way, we previously observed that β-sitosterol also presents in EVOO has important actions on AA cascade and eicosanoid production [42] and that polyphenols of olive oil and wine, tyrosol and resveratrol, together with β-sitosterol have synergistic actions [11].

In conclusion, our findings show for the first time an overall picture of the effects of representative EVOO polyphenols such as tyrosol and hydroxytyrosol, on AA release induced by PLA_2 and on AA metabolism involved in the production of a great family of eicosanoids, events implicated in the pathogenesis of several diseases of high prevalence and with an important inflammatory component [43].

Competing interests

The authors declared that there are no conflict of interest

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Table 1. Effect of tyrosol and hydroxytyrosol on the synthesis of arachidonic acid cascade metabolites by Caco-2 cell cultures.

	Metabolites						
	PGE_2	LTB_4	5-HETE	12-HETE	15-HETE	13-HODE	Total
Treatments							
Control	0.39 ± 0.06	0.32 ± 0.08	3.12 ± 0.09	2.14 ± 0.11	3.89 ± 0.19	0.87 ± 0.06	10.73 ± 0.43
FBS	1.79 ± 0.43*	1.96 ± 0.11*	8.22 ± 1.05*	4.38 ± 0.37*	9.19 ± 1.15*	3.12 ± 0.12*	28.66 ± 2.45*
FBS + T	0.62 ± 0.11$^{\neq}$ (84)	0.74 ± 0.09$^{\neq}$ (74)	4.78 ± 0.21$^{\neq}$ (67)	2.96 ± 0.07$^{\neq}$ (63)	5.28 ± 0.21$^{\neq}$ (74)	2.26 ± 0.06$^{\neq}$ (49)	16.64 ± 1.56$^{\neq}$(67)
FBS +HT	0.48 ± 0.05$^{\neq}$ (94)	0.58 ± 0.06$^{\neq}$ (84)	3.67 ± 0.21$^{\neq}$ (89)	2.59 ± 0.09$^{\neq}$ (79)	4.76 ± 0.07$^{\neq}$ (83)	1.92 ± 0.04$^{\neq}$ (53)	14.03 ± 1.22$^{\neq}$(82)
H_2O_2	2.23 ± 0.26*	1.76 ± 0.14*	7.05 ± 0.38*	3.87 ± 0.26*	7.65 ± 0.87*	2.74 ± 0.17*	25.32 ± 2.34*
H_2O_2 + T	0.58 ± 0.06$^{\neq}$ (89)	0.89 ± 0.21$^{\neq}$ (61)	4.94 ± 0.22$^{\neq}$ (54)	3.07 ± 0.10$^{\neq}$ (46)	5.47 ± 0.12$^{\neq}$(56)	1.65 ± 0.04$^{\neq}$(58)	16.62 ± 1.56$^{\neq}$(60)
H_2O_2 + HT	0.52 ± 0.02$^{\neq}$ (93)	0.56 ± 0.16$^{\neq}$ (83)	3.91 ± 0.08$^{\neq}$ (79)	2.92 ± 0.07$^{\neq}$ (55)	5.09 ± 0.13$^{\neq}$ (68)	1.46 ± 0.05$^{\neq}$ (68)	14.46 ± 1.07$^{\neq}$(74)
TNF+IFN	2.04 ± 0.13*	1.52 ± 0.08*	5.97 ± 0.26*	4.25 ± 0.18*	6.83 ± 0.31*	2.34 ± 0.07*	22.95 ± 2.16*
TNF+IFN + T	0.61 ± 0.08$^{\neq}$ (86)	0.65 ± 0.06$^{\neq}$ (72)	4.54 ± 0.11$^{\neq}$ (50)	2.84 ± 0.07$^{\neq}$ (66)	5.05 ± 0.13$^{\neq}$ (60)	1.62 ± 0.04$^{\neq}$ (49)	15.30 ± 1.09$^{\neq}$(62)
TNF+IFN +HT	0.48 ± 0.04$^{\neq}$ (93)	0.48 ± 0.03$^{\neq}$ (86)	3.85 ± 0.09$^{\neq}$ (74)	2.53 ± 0.08$^{\neq}$ (85)	4.72 ± 0.11$^{\neq}$ (71)	1.38 ± 0.02$^{\neq}$ (65)	13.44 ± 0.89$^{\neq}$(77)
AA	3.37 ± 0.22*	2.11 ± 0.07*	9.05 ± 0.32*	5.17 ± 0.29*	10.02 ± 0.52*		29.72 ± 2.15*
AA + T	1.67 ± 0.11$^{\neq}$ (57)	1.46 ± 0.03$^{\neq}$ (36)	5.56 ± 0.21$^{\neq}$ (34)	3.73 ± 0.13$^{\neq}$ (47)	6.26 ± 0.17$^{\neq}$ (61)		18.68 ± 1.79$^{\neq}$(58)
AA + HT	1.29 ± 0.04$^{\neq}$ (69)	0.89 ± 0.10$^{\neq}$ (68)	4.85 ± 0.07$^{\neq}$ (70)	3.21 ± 0.11$^{\neq}$ (65)	4.97 ± 0.12$^{\neq}$ (82)		15.21 ± 1.02$^{\neq}$(74)

Non-differentiated Caco-2 cells (10^4 cells/cm^2) were cultured with or without FBS (10%), H_2O_2 (1 mM), TNFα (50 ng/ml) + IFNγ (50 ng/ml) or AA (10 μM) in absence or presence of tyrosol (T, 1 μM) or hydroxytyrosol (HT, 1 μM) for 3, 1, 3 or 1 h, respectively. Finally, eicosanoids present in culture supernatant were extracted and measured. Eicosanoid concentrations are presented as ng/ml. Results are shown as means ± SEM of 3 experiments performed in triplicate. (% inhibition respect to cells stimulated in absence of polyphenols) * $P < 0.05$ versus control values. $^{\neq}$ $P < 0.05$ versus cells cultured with FBS, H_2O_2, TNF+IFN or AA, respectively.

Table 2. Effect of low tyrosol and hydroxytyrosol concentrations on the synthesis of arachidonic acid cascade metabolites stimulated by TNF + IFN in Caco-2 cell cultures

	[^{3}H]AA release	AA Metabolites				
	PGE_2	LTB_4	5-HETE	12-HETE	15-HETE	13-HODE
Treatments						
Control	4.5 ± 0.3	0.35 ± 0.02	0.28 ± 0.22	3.21 ± 0.07	2.23 ± 0.11	4.01 ± 0.23	1.02 ± 0.04
TNF + IFN	17.8 ± 1.7*	2.31 ± 0.11*	1.37 ± 0.05*	6.78 ± 0.19*	4.76 ± 0.21*	7.05 ± 0.29*	3.25 ± 0.16*
TNF + IFN + T	13.2 ± 0.8	1.87 ± 0.08	1.24 ± 0.04	6.41 ± 0.18	4.32 ± 0.18	6.71 ± 0.28	3.08 ± 0.14
TNF + IFN + HT	12.1 ± 0.5	1.63 ± 0.05$^{\neq}$	1.12 ± 0.03	6.02 ± 0.16	4.07 ± 0.17	6.32 ± 0.26	2.83 ± 0.12
TNF + IFN + T + HT	9.7 ± 0.6$^{\neq}$	1.19 ± 0.03$^{\neq}$	0.98 ± 0.03$^{\neq}$	5.63 ± 0.15$^{\neq}$	3.68 ± 0.15$^{\neq}$	5.97 ± 0.24$^{\neq}$	2.67 ± 0.13$^{\neq}$

Non-differentiated Caco-2 cells (10^4 cells/cm^2) were cultured with or without TNFα (50 ng/ml) + IFNγ (50 ng/ml) in absence or presence of tyrosol (T, 0.3 μM) or hydroxytyrosol (HT, 0.3 μM) for 3. Finally, eicosanoids present in culture supernatant were extracted and measured. Eicosanoid concentrations are presented as ng/ml. Results are shown as means ± SEM of 3 experiments performed in triplicate. (% inhibition respect to cells stimulated in absence of polyphenols) * $P < 0.05$ versus control values. $^{\neq}$ $P < 0.05$ versus cells cultured with TNF+IFN.

Fig. 1 Effect of PLA_2 inhibitors on [^{3}H]AA release induced by FBS, H_2O_2 or TNF + IFN. [^{3}H] AA was incorporated to Caco-2 cell cultures. Then, cultures were pre-incubated with ATK (3 μM) or BEL (3 μM) for 30 min before cultures were stimulated with FBS (10%), H_2O_2 (1 mM) or TNFα (50 ng/ml) + IFNγ (50 ng/ml) for 3 h, 1 h and 3 h, respectively. Data are means ± SEM of 4-5 determinations performed in duplicate. * $P < 0.05$ significantly different from non-treated cells.

Fig. 2 Effect of tyrosol and hydroxityrosol on [^{3}H]AA release induced by FBS, H_2O_2 or TNFα + IFNγ. [^{3}H] AA was incorporated to Caco-2 cell cultures. Then, cultures were pre-incubated with tyrosol (0.1-10 μM) or hydroxityrosol (0.1-10 μM) for 30 min and cultures were stimulated by FBS (10%), H_2O_2 (1 mM), or TNFα (50 ng/ml) + IFNγ (50 ng/ml) for 3, 1 or 3 h, respectively. Data are means ± SEM of 4-5 determinations performed in duplicate. * $P < 0.05$ significantly different from non-treated cells.

Fig. 3 Western blot analysis of cyclooxygenase-2 (COX-2) expression. Culture Caco-2 cells were incubated with 10% FBS in presence of tyrosol or hydroxytyrosol (1 μM) for 1 h. Results are representative of three separate experiments (A). Values were normalized to the β-actin expression and are expressed as relative units with FBS expression set as 100 (B).

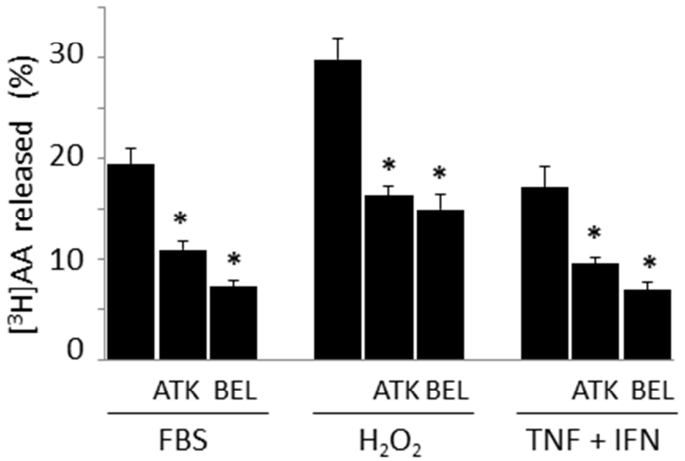
[3H]AA released (%)
30
20
10
0
ATK BEL
FBS
ATK BEL
H2O2
ATK BEL
TNF + IFN

Figure 1

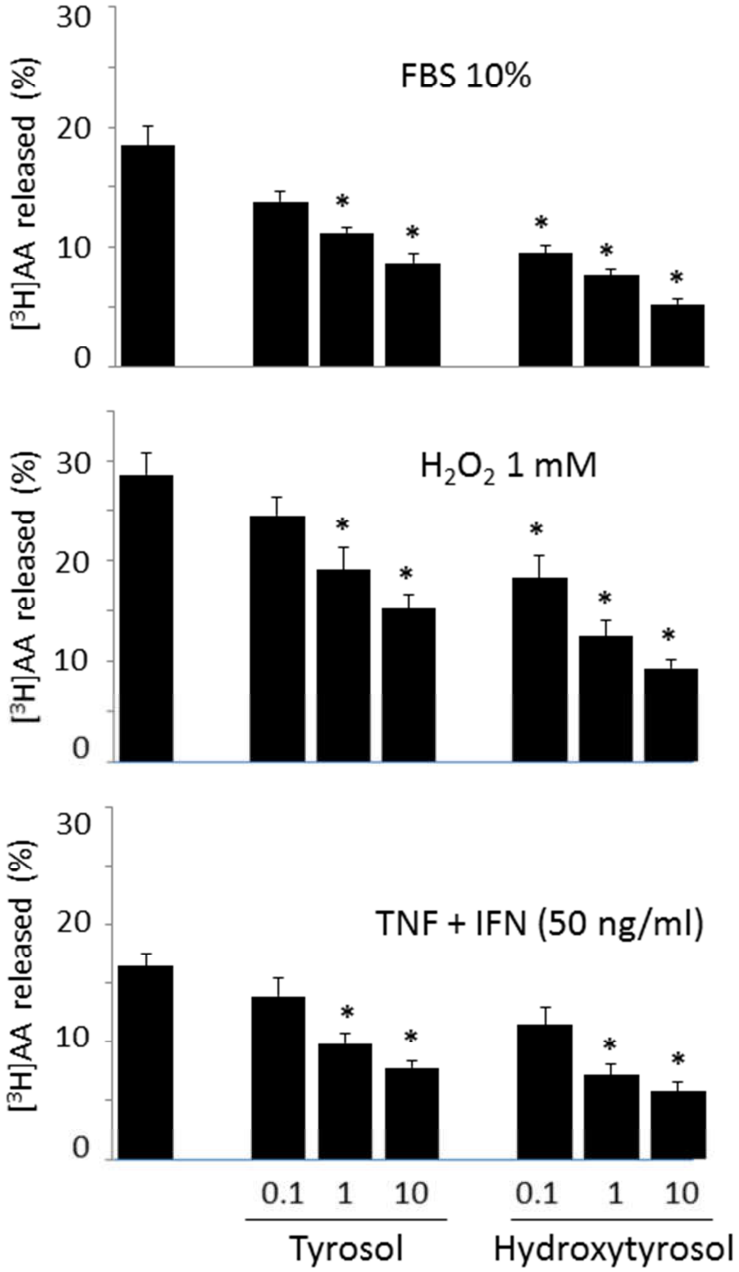
FBS 10%
H2O2 1 mM
TNF + IFN (50 ng/ml)
[3H]AA released (%)
30
20
10
0
0.1
1
10
Tyrosol
Hydroxytyrosol

Figure 2

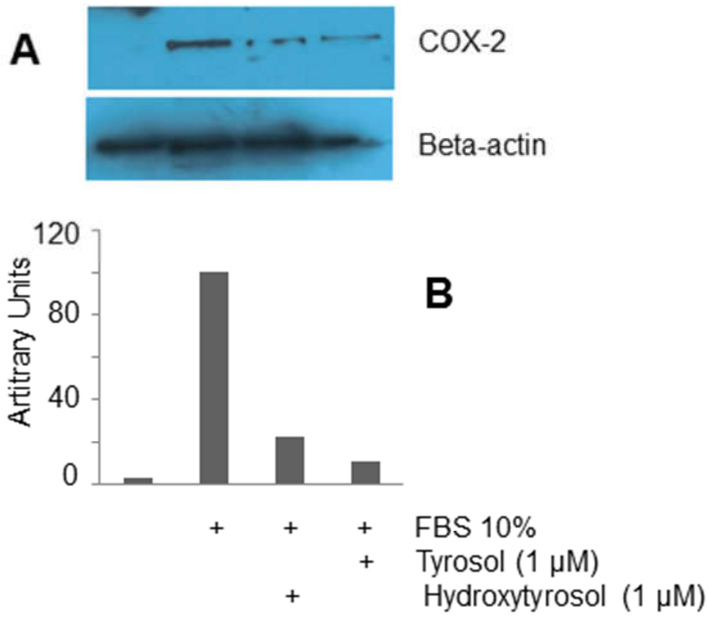
A
COX-2
Beta-actin
B
Artitrary Units
120
80
40
0
+ + + FBS 10%
+ Tyrosol (1 µM)
+ Hydroxytyrosol (1 µM)

Figure 3

IV. Discusión

El vino y el aceite de oliva son dos alimentos característicos de la DM, y ambos son una mezcla compleja de numerosos componentes con estructura y características fisicoquímicas diversas. Así por ejemplo el vino tiene más de 1000 componentes y el aceite de oliva puede contener más de 8000 polifenoles en la fracción no saponificable (Rezaei-Sadabady y Akbarzadeh, 2015). Además estamos ante un alimento, como el vino, que es una solución acuosa mientras que el aceite de oliva es de carácter oleoso.

Dada la enorme diversidad de compuestos que constituyen estos dos alimentos, es difícil centrarse en alguno en particular a la hora de analizar los mecanismos implicados en el efecto beneficioso del vino y del aceite de oliva sobre la incidencia y el desarrollo del CCR. De todos los componentes del vino, los estilbenos constituyen un grupo de compuestos presente en un número muy reducido de alimentos, al menos a concentraciones apreciables. Estos hidrocarburos aromáticos pueden presentarse en forma simple o formando polímeros o metabolitos glucosidados. Actúan como una defensa natural ante situaciones de estrés o ante el ataque a la vid de agentes externos (Langcake y Pryce, 1976). En el caso del aceite de oliva, contiene una gran cantidad de ácido oleico (un AGMI) que lo diferencia claramente del resto de aceites que contienen mayoritariamente AGPI o AGS. Y en la fracción insaponificable encontramos diversas familias de compuestos como fitoesteroles, hidrocarburos, lignanos, compuestos terpénicos y polifenoles. En este último grupo encontramos los tirosoles, que al igual que ocurre con los estilbenos, se encuentran presentes en muy pocos alimentos. Así, los estilbenos y los tirosoles, que son dos familias de polifenoles poco representadas en los alimentos, se encuentran presentes en el vino.

Entre los estilbenos presentes en el vino, los más abundantes son el piceatanol, la viniferina, el piceido y el RV. Sin embargo, este último es uno de los más estudiados en relación a sus efectos biológicos. Así, el RV, tras su ingesta, es rápidamente absorbido por las células intestinales, metabolizado a compuestos glucuronidados y sulfatados, y posteriormente excretado con las sales biliares o la orina; o devuelto a la circulación, en una pequeña proporción, a través de la circulación enterohepática (Walle y col., 2004; Boocock y col., 2007a y 2007b). De manera que las concentraciones de RV en plasma tras su administración oral, en cantidades que podrían ser aportadas por la dieta, resultan muy bajas (Yu y col., 2002; Walle y col., 2004; Cottart y col., 2010), y por tanto, alejadas de concentraciones entre 0,1 y 10 μM que son las concentraciones de RV que habitualmente se han utilizado para el estudio de sus efectos biológicos *in vitro*. Por los que se ha cuestionado la relevancia fisiológica de los resultados obtenidos en estos estudios.

Nuestros resultados muestran que el *trans*-RV es antiproliferativo a partir de 1 μM en cultivos de células Caco-2. Este efecto es concentración-dependiente y está relacionado con una acumulación de células en la fase S del ciclo celular; además, observamos que el *trans*-RV, a partir de 25 μM, induce apoptosis, de acuerdo con Schneider y col.(2000) y con Wolter y col. (2001) que observaron un efecto similar en otras líneas de células epiteliales intestinales. Los principales metabolitos del *trans*-RV, el RV-3-*O*-sulfato, RV-3-*O*-glucurónido y RV-4'-*O*-glucurónido, observamos que inducen una inhibición del crecimiento de las células epiteliales intestinales similar al *trans*-RV, aunque el efecto sobre el ciclo celular fue diferente, ya que estos metabolitos aumentaron la proporción de células en la fase G_0/G_1 del ciclo celular. Estos tres metabolitos del *trans*-RV indujeron la fragmentación del DNA celular de forma más marcada que el *trans*-RV, indicando un efecto apoptótico. Esta es la primera vez que se describe el efecto de los metabolitos sulfatados y glucuronidados del RV sobre el crecimiento celular/apoptosis de células epiteliales intestinales. Posteriormente Aires y col. (2013) publicaron un estudio donde trataron células de adenocarcinoma de colon (SW480 y SW620) con *trans*-RV, RV-3-*O*-sulfato, RV-3-*O*-glucurónido y RV-4'-*O*-glucurónido; y observaron que solo el metabolito sulfatado conserva el efecto antimitogénico o apoptótico del *trans*-RV, aunque en sus condiciones experimentales indujo la acumulación de la células en la fase S del ciclo celular.

Patel y col. (2010) mostraron en un estudio clínico de fase I/II que tras una administración repetida de RV a pacientes con CCR, se detectan RV y metabolitos glucuronidados y sulfatados en el tejido colónico a una concentración de entre 0,2 y 12 nmol/g dependiendo de la zona del intestino afectada. Además, observaron una disminución del 5 % en la proliferación de células tumorales de dichos pacientes, sugiriendo un efecto protector tanto del RV como de sus metabolitos sobre el CCR.

Probablemente el RV es el estilbeno mejor estudiado hasta el momento, pero el piceido, precursor glucosidado del RV, se encuentra a concentraciones mucho más elevadas en la uva y el vino. Por ello es importante conocer el efecto de esta molécula sobre el crecimiento de células epiteliales intestinales. Nuestros resultados han demostrado que el piceido inhibe el crecimiento de las células Caco-2 aumentando el porcentaje de células en la fase S del ciclo celular e induciendo apoptosis de forma similar al RV. A pesar de que observamos que las células Caco-2 tienen la capacidad de metabolizar el RV, no fueron capaces de hidrolizar el piceido para liberar RV, y esto es debido a que no observamos actividad β-glucosidasa en estas células. Por todo ello, la actividad biológica que presenta el piceido no es debida a la liberación de RV. Esta es la primera vez que se observa el efecto antimitogénico del piceido en células epiteliales intestinales, aunque sí que se observó un

efecto similar en otras líneas celulares, como las L1210, propias de leucemia linfocítica murina, y las K562, propias de leucemia eritroblastoide humana (Ha do y col., 2009).

El RV es una molécula a la que se le ha atribuido actividad antioxidante utilizando numerosos modelos experimentales y muchas de sus actividades biológicas *in vitro* se han relacionado con dicha capacidad (Gambini y col., 2015). Nuestros resultados muestran que los metabolitos sulfatados y glucuronidados, así como el piceido, conservan una actividad antioxidante similar a la del RV. Y esta actividad está íntimamente relacionada con la presencia de grupos hidroxilo en determinadas posiciones de los anillos aromáticos, que en el caso del RV se sitúan en posición 3, 5 y 4'. De manera que cuando sustituimos progresivamente estos hidroxilos por grupos metoxi observamos que la capacidad antioxidante de dichos compuestos se mantiene hasta que el grupo hidroxilo es sustituido en posición 4 o 4'. Sin embargo, estos cambios estructurales no modificaron la capacidad de estos compuestos de inhibir la síntesis de DNA y el crecimiento de las células Caco-2 al modificar su ciclo celular de forma similar al RV. Al mismo tiempo observamos que la sustitución del grupo hidroxilo en posición 4' por un grupo metoxi parece aumentar la capacidad apoptótica del compuesto a concentraciones altas. Por otro lado, la sustitución progresiva de los grupos hidroxilo por grupos cloro disminuye la actividad antioxidante y al mismo tiempo la capacidad de inhibir el crecimiento de las células Caco-2, siendo el cloro ubicado en posición 4 o 4' el determinante de dichos efectos. Estos resultados concuerdan con los descritos por Fulda (2010), que destacan las modificaciones en la posición 4 o 4' del RV como determinantes de los efectos biológicos de sus análogos estructurales.

La movilización del AA esterificado en los fosfolípidos de las membranas celulares por acción de la fosfolipasa A2 es el primer paso de la cascada del AA. Posteriormente el AA es liberado y oxidado por COXs, LOXs o monooxigenasas del citocromo P-450, dando lugar a productos biológicamente activos, los eicosanoides (Needleman y col., 1986), que modulan diversas respuestas fisiológicas y patológicas, incluyendo el crecimiento y diferenciación celular (Herschman y Hall, 1994). La sobreexpresión de COX-2 se ha asociado con diversas enfermedades proliferativas como el CCR (DuBois y col., 1998). Así, se ha observado que la síntesis de PGE_2 aumenta en el tejido de CCR (Rigas y col., 1993), incrementando así el potencial tumorigénico de las células epiteliales del colon (Sheng y col., 1998). En este sentido, se ha demostrado que las células Caco-2 sobre-expresan COX-2 y tienen una tendencia elevada a la metabolización de AA para dar lugar a PGE_2, proceso que se mostró implicado en la proliferación y diferenciación de dichas células (Martín-Venegas y col., 2006).

Se ha descrito que el RV es capaz de actuar sobre la cascada del AA a diversos niveles. Así, se ha observado que puede reducir la movilización del AA en diferentes líneas celulares (Martinez y Moreno, 2000; Moreno, 2000) y modelos animales (Vivancos y Moreno, 2008). Además el RV puede modular la expresión de los enzimas implicados en dicha cascada, como la COX -2 (Yoon y Baek, 2005) y la 5-LOX (Werz, 2007). Así, el RV es capaz de regular la síntesis de PGs y LTs, metabolitos implicados en los procesos inflamatorios y en la proliferación tumoral (Werz, 2007; Oi y col., 2010). A través de estos efectos sobre la cascada del AA y la síntesis de eicosanoides se podría explicar, en parte, el efecto del RV sobre la regulación de la proliferación de las células Caco-2.

Además del AA, también pueden actuar como sustrato de la cascada del AA diferentes AGs de cadena larga, entre ellos el EPA, el DHA y el linoleico, que serán metabolizados por las enzimas de la cascada pero dando lugar a la síntesis de otros eicosanoides que pueden tener diferentes efectos fisiológicos o fisiopatológicos a los que tienen los eicosanoides derivados del AA (Cabral y col., 2013). Por ello nos propusimos investigar el efecto de diferentes AGs de cadena larga y con diferente número de insaturaciones sobre la proliferación de las células epiteliales intestinales. Observamos que los AGMIs de cadena larga, como el miristoleico, el palmitoleico y, en especial, el oleico son proliferativos, ya que claramente indujo un efecto mitogénico en las células Caco-2 por sí mismos y potenciando el efecto de los factores de crecimiento presentes en el SFB. El isómero *trans*- del ácido oleico, el ácido elaídico (C18:1 *trans*) también presenta efecto mitogénico en las células epiteliales intestinales. Nuestros resultados sugieren que el efecto proliferativo del oleico depende de su metabolización por la vía 5-LOX y la consecuente producción de metabolitos, similares a los cisteinil LTs. En este sentido Clapp y col. (2006) observaron que la lipoxigenación del ácido oleico da lugar a metabolitos hidroperóxidos que posteriormente se convierten en enonas. Además, Cabral y col. (Cabral y col., 2013 y 2015) demostraron que los LTs y los ácidos hidroxioctadecanoicos sintetizados a partir de AA y linoleico, respectivamente, inducen la proliferación de las células Caco-2. Por estos motivos se puede considerar que los metabolitos producidos por la vía de las LOXs a partir del ácido oleico podrían estar implicados en el efecto mitogénico de este AG.

Por otro lado, hemos observado que los AGs de cadena larga, como el EPA y el DHA, a concentración alrededor de 10 μM, también tuvieron efecto mitogénico, y en el caso del EPA, este efecto parece estar mediado, al menos en parte, por la producción de eicosanoides producidos por las vías de las COXs y de las LOXs. Por el contrario, Dommels y col. (2002), mostraron que el EPA y el AA inhiben la proliferación de células Caco-2 e inducen citotoxicidad mediante la

producción de peróxidos generados por la peroxidación lipídica y la actividad de la COX. De acuerdo con estos resultados observamos que el linoleico, el α- y γ-linolénico, el AA, el EPA y el DHA inhiben el crecimiento celular e inducen apoptosis en torno a 100 μM. Estos efectos están mediados por la unión de estos AGs al PPARγ, y en el caso del EPA, el AA y el DHA, también observamos que son capaces de inducir la hiperpolarización de la membrana mitocondrial, lo que indica la activación de la vía intrínseca de la apoptosis. De manera que los AGMI son mitogénicos sobre las células Caco-2, mientras que los AGPIs tienen un efecto dual dependiendo de la concentración; así, a concentraciones altas inducen apoptosis, y a concentraciones bajas estimular la proliferación de las células epiteliales intestinales. Este último efecto mitogénico podría ser debido a la síntesis de eicosanoides. Así, hemos observado que la PGE_3 y el ácido 12-hidroxieicosapentaenoico, derivados del EPA tienen un efecto mitogénico similar al previamente descrito para la PGE_2 y el ácido 12- hidroxieicosatetraenoico (Cabral y col., 2013). Mientras que el LTB_5 no fue mitogénico a diferencia de lo observado previamente con el LTB_4 en cultivo de células epiteliales intestinales (Cabral y col., 2013). El hecho de que algunos metabolitos del EPA no sean metabólicamente activos podría estar relacionado con el efecto beneficioso del consumo de pescado suplementos de EPA sobre la enfermedad inflamatoria intestinal o el CCR.

El efecto mitogénico que induce el ácido oleico en cultivos de células Caco-2 está de acuerdo con los resultados de Hansen-Petrik y col. (2000) que observaron que una dieta rica en ácido oleico incrementaba el número de tumores intestinales en ratones $Apc^{min/+}$. Sin embargo, recientemente Barone y col. (2014) mostraron que una dieta con aceite de oliva disminuía el número y el volumen de los pólipos en el mismo modelo experimental. Considerando estos resultados y el hecho de que la DM reduce la incidencia de CCR (Schwingshackl y Hoffmann, 2015), nos planteamos analizar el efecto del ácido oleico sobre el crecimiento de las células Caco-2 en presencia de compuestos minoritarios característicos del aceite de oliva.

Dado que la fracción insaponificable del aceite de oliva contiene numerosos componentes bioactivos que pueden estar implicados en sus propiedades beneficiosas, para realizar esta parte del trabajo se seleccionaron componentes representativos de los diferentes grupos de moléculas que encontramos en dicha fracción. Así, se escogieron el hidroxitirosol, que es un fenol simple; la oleuripeina, un secoiridoide aldehído; el pinoresinol y el escualeno, como principales lignanos e hidrocarburos, respectivamente; y finalmente el ácido maslínico, triterpeno característicos del aceite de oliva. Aunque se ha descrito que todos estos compuestos pueden inhibir el crecimiento de células epiteliales intestinales (Rao y col., 1998; Fabiani y col., 2002; Reyes-Zurita y col., 2011 y 2016; Cardeno y col., 2013; Pereira-Caro y col.,2013; Sepporta y col., 2013), nuestros resultados

muestran, por primera vez, que todos los compuestos estudiados fueron capaces de revertir el efecto proliferativo inducido por el ácido oleico, y que este efecto antimitogénico también se observó cuando las células Caco-2 fueron estimuladas con factores de crecimiento. De los cinco compuestos ensayados, el hidroxitirosol y el ácido maslínico fueron los que tuvieron un efecto más marcado sobre el crecimiento de las células Caco-2 a concentraciones que pueden encontrarse en el lumen intestinal consumiendo una dieta de tipo Mediterráneo o aceite de oliva. Además, ambos compuestos a concentraciones altas indujeron apoptosis en un porcentaje elevado de estas células. De estos dos componentes, solo el hidroxitirosol presentó una actividad antioxidante muy superior al Trolox. Además, observamos que este polifenol fue capaz de inhibir la liberación de AA y la producción de PGE_2, LTB_4, ácido 5-, 12- y 15- hidroxieicosatetraenoico y ácido 13-hidroxioctadecadienoico en cultivos de células Caco-2 estimulados con SFB. Es interesante señalar, también, que el hidroxitirosol fue capaz de inhibir la síntesis de PGE_2, LTB_4, ácido 5-, 12- y 15-hidroxieicosatetraenoico estimulada por la adición exógena de AA al medio de cultivo. Resultados que están de acuerdo con de la Puerta y col. (1999) que mostraron que el hidroxitirosol es capaz de inhibir la síntesis de PGE_2 y LTB_4 en leucocitos. Nuestros resultados demuestran que el hidroxitirosol, además de inhibir la liberación de AA, es capaz de modular la actividad de la vía de las COXs y las LOXs. Estos efectos podrían explicar, al menos en parte, la reversión inducida por este compuesto sobre la proliferación de las células Caco-2 tratadas con ácido oleico.

El efecto inhibitorio de la oleuropeina que observamos sobre el crecimiento de las células Caco-2 podría deberse al hecho de que estas células son capaces de hidrolizar la oleuropeina liberando hidroxitirosol al medio, tal como determinamos en nuestras condiciones experimentales. Dado que el escualeno y el pinoresinol también inhibieron el crecimiento de las células Caco-2, sería interesante analizar su efecto sobre la cascada del AA ya que hay antecedentes de que estas moléculas pueden modular la síntesis de eicosanoides en otras líneas celulares (Moreno, 2003) (Lajter y col., 2015). El aceite de oliva contiene además del hidroxitirosol que procede de la hidrólisis de la oleuropeina, tirosol, que procede de la hidrólisis del ligstrósido, llegando a ser su concentración mayor que la de hidroxitirosol en algunos tipos de aceite de oliva (Krichene y col., 2009; Romero y col., 2016). El tirosol, al igual que el hidroxitirosol, fue capaz de inhibir la liberación de AA y la producción de PGs, LTs, y ácidos hidroxihexatetraenoicos, al mismo tiempo que inhibió el crecimiento de las células Caco-2. De manera que diversos tirosoles pueden tener un papel destacado en el control del crecimiento de las células epiteliales intestinales.

Tradicionalmente, el efecto beneficioso del aceite de oliva se ha atribuido a su contenido en ácido oleico. No obstante, nuestros resultados muestran que el ácido oleico y otros AGMIs pueden

estimular el crecimiento de las células epiteliales intestinales, un factor importante en el desarrollo del CCR. Por otro lado, nuestros resultados también muestran que los componentes minoritarios del aceite de oliva pueden tener un papel importante en el efecto preventivo sobre el CCR, al modular el estrés oxidativo, la cascada del AA y el control del crecimiento de las células epiteliales intestinales. De manera que el consumo de ácido oleico en forma de aceites de semillas altos en oleico o en forma de aceite de oliva, característico de la DM, podría tener diferentes efectos sobre la incidencia y el desarrollo del CCR. Por ello, es necesario profundizar en el estudio clínico y experimental de los efectos de los componentes minoritarios del aceite de oliva para entender mejor su papel sobre el CCR.

Probablemente los efectos beneficiosos de la DM no pueden atribuirse a uno o varios componentes de la misma, sino al conjunto de todos ellos; de la misma manera que el efecto beneficioso de un alimento característico de la DM se debe al conjunto de sus componentes y no a la acción aislada de uno de ellos. Esta tesis se ha centrado en el estudio del papel de los estilbenos y tirosoles, presentes en el vino y el aceite de oliva, en el control de la cascada del AA y el crecimiento de las células epiteliales intestinales. Planteando mecanismos que podrían explicar el efecto de la DM sobre el CCR. Previamente, se ha descrito el efecto sinérgico de varios componentes de estos alimentos potenciando sus efectos biológicos (Vivancos y Moreno, 2008). Dado que esta tesis muestra por primera vez que los metabolitos de un estilbeno pueden conservar la actividad biológica del mismo, debemos considerar que el efecto beneficioso de un alimento característico de la DM, como el aceite de oliva o el vino, puede deberse no solo al efecto sinérgico de sus componentes sino también al de sus metabolitos. Lo que contribuiría a entender la relevancia fisiológica de muchos de los efectos biológicos de los compuestos bioactivos del vino y del aceite de oliva que han sido descritos *in vitro*.

V. Conclusiones

Las principales conclusiones de esta tesis son:

- Los metabolitos sulfatados y glucuronidados del RV mantienen la actividad antioxidante de este polifenol así como la capacidad de inhibir el crecimiento de las células epiteliales intestinales y de inducir su apoptosis. Sin embargo, estos metabolitos acumulan las células Caco-2 en la fase G_0/G_1 del ciclo celular, mientras que el RV lo hace en la fase S.

- El trans¬-piceido presenta un efecto antioxidante, una actividad antimitogénica y pro-apoptótica similar al RV en cultivos de células epiteliales intestinales Caco-2, aunque incrementó el porcentaje de células en la fase G_0/G_1 del ciclo celular.

- La actividad antioxidante del RV está relacionada con la presencia de grupos hidroxilo en posición 3, 5 y 4', pero principalmente en esta última. Cuando este hidroxilo es sustituido por un grupo metoxi o cloro la molécula pierde actividad antioxidante.

- La sustitución del hidroxilo en posición 4 o 4' del RV por un grupo metoxi conserva su capacidad de modular el ciclo celular e inhibir el crecimiento de células epiteliales intestinales y parece aumentar su capacidad apoptótica. Mientras que la sustitución de este grupo hidroxilo por un cloro disminuye la actividad antioxidante y al mismo tiempo la capacidad de inhibir el crecimiento de las células Caco-2.

- Los AGMIs, y en especial el ácido oleico, inducen el crecimiento de las células Caco-2, probablemente a través de la síntesis de metabolitos por la vía de las LOXs.

- Algunos AGPIs, como el EPA y el DHA, a concentraciones bajas inducen la proliferación de las células epiteliales intestinales pudiendo estar implicadas en este efecto PGs o ácidos hidroxieicosapentaenoicos sintetizados a partir de ellos. Mientras que a concentraciones más elevadas tienen un efecto opuesto e inducen apoptosis.

- El efecto mitogénico del ácido oleico fue revertido por diversos componentes minoritarios del aceite de oliva, como el hidroxitirosol, la oleuropeina, el escualeno, el pinoresinol y el ácido maslínico.

- El hidroxitirosol y el tirosol inhiben la liberación de AA y la síntesis de PGs, LTs, ácidos hidroxieicosatetraenoics y hidroxioctadecaenoicos, que son elementos importantes implicados en el control del crecimiento de las células Caco-2.

Teniendo en cuenta el conjunto de los resultados que conforman esta tesis doctoral proponemos que los componentes minoritarios del vino y del aceite de oliva, así como sus metabolitos, pueden tener un importante papel en el control de la cascada del AA y de la proliferación de células epiteliales intestinales Caco-2. Eventos que pueden estar también modulados por la cantidad y el tipo de AGs consumidos. Y todo ello podría explicar, al menos en parte, el efecto beneficioso de la DM sobre la incidencia y desarrollo del CCR.

VI. Bibliografía

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