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La importancia del balance en ácidos grasos omega-3 y omega-6 en la esteatohepatitits no alcohólica asociada a la obesidad

Cristina López Vicario

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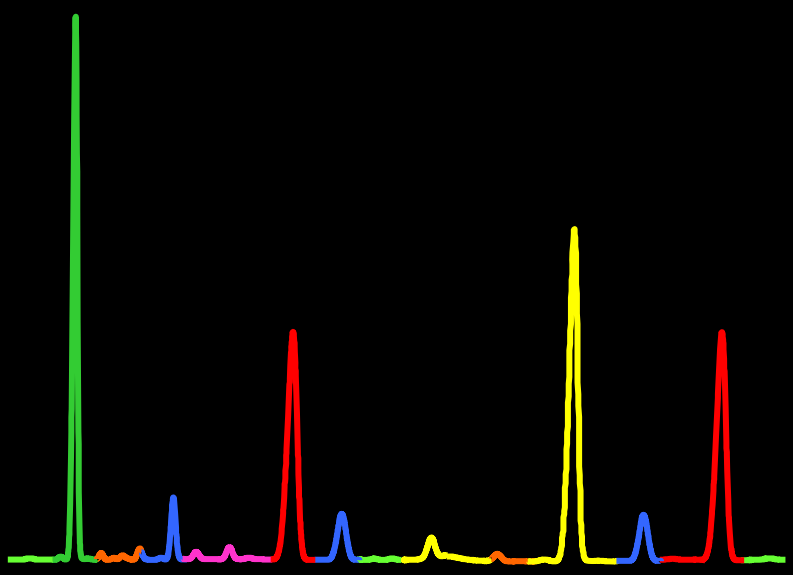

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LA IMPORTANCIA DEL BALANCE EN ÁCIDOS GRASOS OMEGA-3 Y OMEGA-6 EN LA ESTEATOHEPATITIS NO ALCOHÓLICA ASOCIADA A LA OBESIDAD

Memoria presentada por

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para optar al título de Doctora en Biomedicina

Trabajo realizado bajo la dirección del **Dr. Joan Clària Enrich**

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Tesis inscrita en el programa de doctorado de Biomedicina
Facultad de Medicina

Ne quid nimis

AA	Ácido araquidónico
AGL	Ácidos grasos libres
ALA	Ácido alfa-linolénico
ALT	Alanina aminotransferasa
AST	Aspartato aminotransferasa
C.elegans	*Caenorhabditis elegans*
COX	Ciclooxigenasa
CYP	Citocromo P450
DGLA	Ácido dihomo-gamma-linolénico
DHA	Ácido docosahexaenoico
DHET	Ácido dihidroxieicosatrienoico
DiHDPA	Ácido dihidroxidocosapentaenoico
DiHETE	Ácido dihidroxieicosatetraenoico
DPA	Ácido docosapentaenoico
EDP	Ácido epoxidocosapentaenoico
EET	Ácido epoxieicosatrienoico
EEQ	Ácido epoxieicosatetraenoico
EHGNA	Enfermedad del hígado graso de origen no alcohólico
EPA	Ácido eicosapentaenoico
IKK	Quinasa K
IMC	Índice de masa corporal
JNK	C-jun-N-terminal
IFN	Interferón
IL	Interleuquina
LA	Ácido linoleico
LOX	Lipoxigenasa
LPL	Lipoproteína lipasa
LPS	Lipopolisacárido
LSH	Lipasa sensible a hormona
LT	Leucotrienos

MCP-1	Proteína quimioatrayente de monocitos 1
MDA	Malondialdehído
MUFA	Ácidos grasos monoinsaturados
n-3	Omega-3
n-6	Omega-6
PG	Prostaglandinas
PL	Fosfolípidos
PLA2	Fosfolipasa A2
PUFA	Ácidos grasos poliinsaturados
RE	Retículo endoplasmático
sEH	Epóxido hidrolasa soluble
SFA	Ácidos grasos saturados
TG	Triglicéridos
TNFα	Factor de necrosis tumoral alfa
VLDL	Proteínas de muy baja densidad
Δ5D	Delta-5 desaturasa
Δ6D	Delta-6 desaturasa

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

Introducción

I. ÁCIDOS GRASOS POLIINSATURADOS (PUFA)

Los ácidos grasos son principalmente cadenas hidrocarbonadas que contienen un grupo carboxílico (-COOH). Desde una perspectiva evolutiva, los ácidos grasos son elementos clave para la vida, más debido a su implicación como componentes estructurales y funcionales de membrana que como fuente de energía (Hulbert AJ, 2014). Los ácidos grasos se encuentran presentes en una gran variedad de sustancias lipídicas, incluyendo aceites, ceras, esteroles, triglicéridos (TG) y fosfolípidos (PL), siendo estos últimos partes estructurales de las membranas celulares (Figura 1) (Guillou H, 2010; Schmitz G y Ecker J, 2008). Los PL están compuestos de 2 ácidos grasos esterificados a un glicerol en la posición sn-1 y sn-2 y de un grupo polar fosforilado (fosfocolina o fosfoetanolamina) esterificado en la posición sn-3 (Vance DE y Vance JE, 2008).

Los ácidos grasos se clasifican en tres clases en función del número de dobles enlaces o insaturaciones de la cadena hidrocarbonada. Los ácidos grasos saturados (SFA) contienen únicamente uniones carbono-carbono simples (C-C), los ácidos grasos monoinsaturados (MUFA) contienen un único doble enlace carbono-carbono (C=C), mientras que los ácidos grasos poliinsaturados (nombrados PUFA por su terminología inglesa "*polyunsaturated fatty acids*") contienen de 2 a 6 dobles enlaces (Wallis JG, 2002).

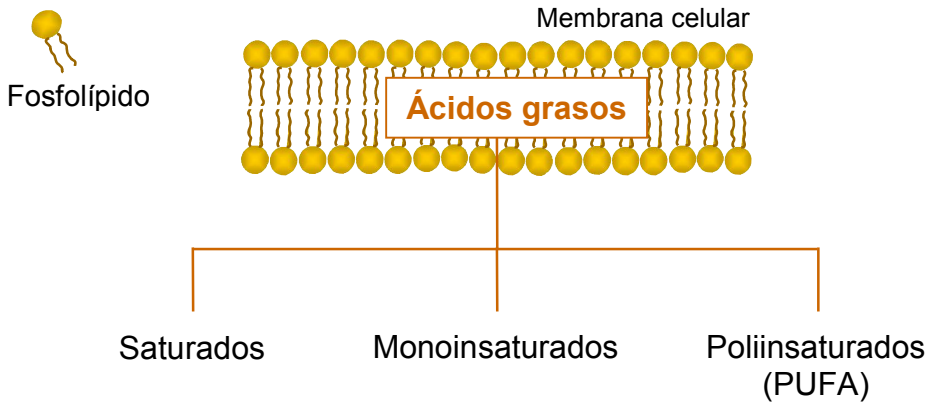

FIGURA 1. Clasificación de los ácidos grasos en función del grado de insaturación. Los ejemplos representativos de cada clase son los ácidos palmítico (C16:0), oleico (C18:1n-9), araquidónico (C20:4n-6) y docosahexaenoico (C22:6n-3). La abreviación indica el número de carbonos, el número de dobles enlaces y la posición del primer doble enlace desde el extremo metilo de la cadena.

El grado de insaturación de los ácidos grasos que constituyen los PL de membrana es importante debido a la dominante influencia sobre las propiedades físicas de la estructura de la bicapa lipídica (Hulbert AJ, 2014). De hecho, un mayor grado de insaturación confiere fluidez y flexibilidad a las membranas celulares (Wallis JG, 2002). Los PUFA no sólo proporcionan a nivel estructural un estado de mayor fluidez en comparación con SFA y MUFA, sino que también permiten una mayor curvatura y un rápido movimiento lateral de la bicapa lipídica facilitando la interacción con proteínas de anclaje y la permeabilidad celular (Hulbert, 2014; Antonny B, 2015). Un ejemplo de la contribución de los PUFA sobre la fluidez de membrana es la correcta unión hormona-receptor, como es el caso de los receptores de la insulina. De hecho, la resistencia a la insulina se asocia a una membrana rígida, la cual limita el número de receptores de insulina. Sin embargo, aumentando la fluidez de la membrana mediante la incorporación de PUFA puede resultar en un aumento de la afinidad de la insulina por su receptor, mejorando así, la sensibilidad a la insulina (Das UN, 2006).

Las características biofísicas de los PUFA también influyen en procesos celulares y fisiológicos importantes como la adaptación al frío y la supervivencia, la modulación de canales iónicos, los procesos de endocitosis/exocitosis y la defensa de patógenos (Wallis JG, 2002). Además, los PUFA actúan como moléculas de señalización que participan en funciones biológicas clave como la división celular y la regulación del metabolismo lipídico, la regulación del tono vascular, la función renal, la coagulación sanguínea, la cicatrización y, notablemente, la inflamación (Guillou H, 2010; Hulbert AJ, 2014).

1. Ácidos grasos omega-6 y omega-3

Existen dos familias principales de PUFA, los omega-3 y los omega-6, llamados así por la posición del primer doble enlace en su cadena hidrocarbonada desde el extremo metilo (Figura 2). Los dobles enlaces de la mayoría de los PUFA se encuentran en configuración *cis* y contienen enlaces dobles a intervalos de tres carbonos por lo que los dobles enlaces están separados por un grupo metileno ($-CH_2-$). Debido a esta característica de regularidad, la mayoría de los PUFA pueden ser descritos simplemente mediante la indicación del número total de carbonos de la cadena hidrocarbonada, el número de dobles enlaces y la posición de los dobles enlaces más cercana al grupo metilo terminal (el carbono omega, ω ó n) (Wallis JG, 2002).

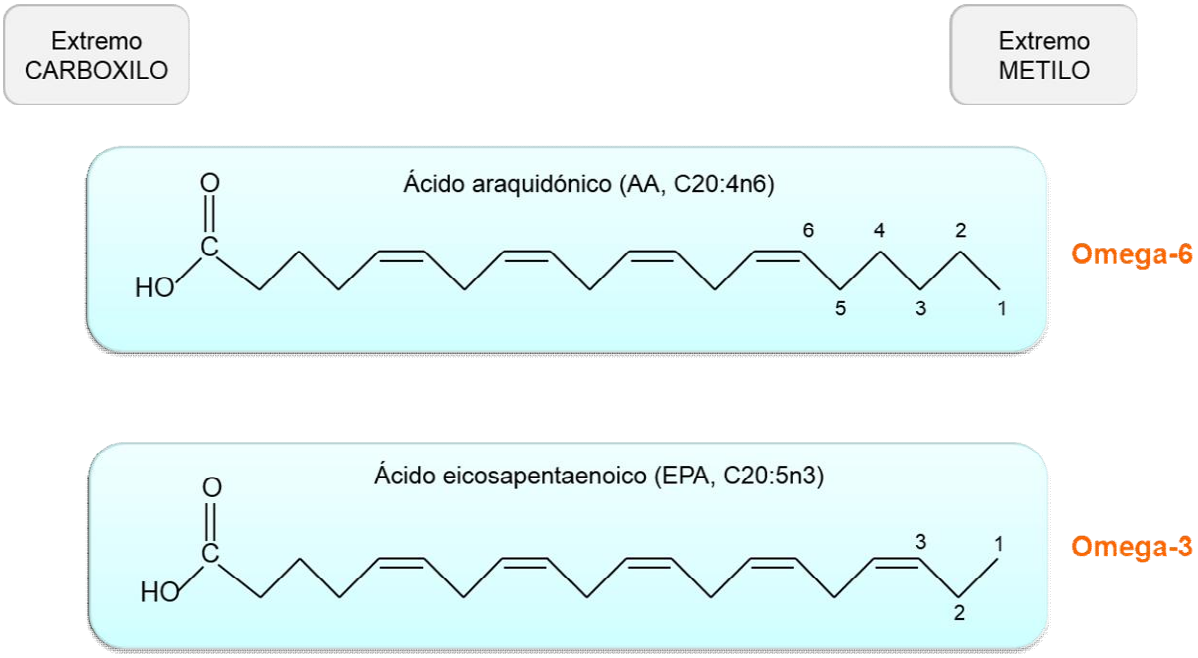

FIGURA 2. Estructura del PUFA omega-6 ácido araquidónico y del PUFA omega-3 ácido eicosapentaenoico. La nomenclatura simplificada especifica la cadena larga, el número de dobles enlaces y la posición de dobles enlaces.

Los PUFA precursores son el ácido linoleico (LA; C18:2n-6) para los omega-6 y el ácido alfa-linolénico (ALA; C18:3n-3) para los omega-3 y ambos se consumen a través de la dieta. Las fuentes de omega-6 son los aceites vegetales tales como el maíz, el cártamo y la soja mientras que la principal fuente de omega-3 es el pescado azul como el salmón, la trucha y el atún (Tabla 1) (Marszalek JR, 2005). El LA y el ALA son considerados ácidos grasos esenciales en mamíferos porque se cumplen los dos criterios: los animales son incapaces de su síntesis “de novo” a partir de otros ácidos grasos y son componentes importantes para las funciones fisiológicas. Por ello, deben ser provistos a través de la dieta y sirven de precursores para la síntesis de sus derivados PUFA de cadena más larga (Simopoulos AP, 2006; Guillou H, 2010). Sin embargo, se ha demostrado recientemente, en un modelo murino, que la administración directa de los derivados AA, EPA y DHA revierte los parámetros bioquímicos y los efectos fisiopatológicos producidos por una dieta deficiente en ácidos grasos esenciales, sugiriendo que AA, EPA y DHA también pueden ser alternativamente considerados “verdaderos” ácidos grasos esenciales (Le HD, 2013).

Producto	LA	ALA	AA	EPA/DHA
Fuentes de omega-6				
Aceite de maíz	50000	900		
Aceite de cacahuete	23900			
Aceite de soja	53400	7600		
Aceite de girasol	60200	500		
Aceite de cártamo	74000	470		
Margarina	17600	1900		
Manteca de cerdo	8600	1000	1070	
Huevos de gallina	3800	220		
Tocino	6080	250	250	
Fuentes de omega-3				
Aceite de colza	19100	8600		
Aceite de linaza	13400	55300		
Arenque	150	61,66	36,66	1700
Salmón	440	550	300	1200
Trucha	74		30	500
Atún	260	270	280	400
Bacalao	4	2	3	300

TABLA 1. Fuentes nutricionales de omega-6 y omega-3. Datos expresados como mg/100g. Datos elaborados en la publicación de Russo GL, Biochemical pharmacology, 2009.

Los PUFA, una vez consumidos, son incorporados en las membranas celulares mediante esterificación de los grupos carboxílicos con el grupo hidroxilo del glicerol de los PL. Ante un estímulo hormonal o inducido por factores de crecimiento sobre los receptores de membrana, se activa la enzima fosfolipasa A2 (PLA2) y libera los PUFA esterificados desde la posición sn-2 de los PL. Estas moléculas una vez liberadas al citoplasma celular, actúan como sustratos para la biosíntesis de moléculas de señalización o eicosanoides que presentan actividad biológica (Schmitz G y Ecker J, 2008).

2. Mediadores lipídicos derivados de los ácidos grasos omega-6 y omega-3

Los PUFA pueden ser oxigenados para formar numerosos mediadores lipídicos bioactivos. La formación biológica de mediadores y moléculas de señalización se caracteriza por la síntesis de un hidroperóxido en la estructura de los PUFA (Brash AR, 1999). Una de las clases mejor estudiadas de mediadores oxigenados son los eicosanoides. Técnicamente, el término de eicosanoide se refiere a los ácidos grasos que contienen 20 átomos de carbono (del griego “eikosa”), pero en el campo, generalmente, puede utilizarse esta terminología de forma más amplia para aquellos metabolitos similares a los PUFA (Buczynski MW, 2009; Astarita G, 2015). Los eicosanoides pueden ser oxigenados mediante reacciones enzimáticas o no enzimáticas. Las tres vías enzimáticas predominantes involucradas en la generación de mediadores oxigenados son catalizadas por la ciclooxigenasa (COX), la lipoxigenasa (LOX) y el citocromo P450 (CYP) (Buczynski MW, 2009; Astarita G, 2015).

2.1 Vía de las ciclooxigenasas y las lipoxigenasas

La oxigenación de los PUFA a través de la COX y la LOX es el paso inicial en la formación de una amplia colección de mediadores lipídicos (Schneider C, 2007). Los productos de la COX y la LOX comúnmente actúan por la señalización mediada por receptores acoplados a proteínas G y están involucrados en procesos de homeostasis celular, proliferación, diferenciación y también en procesos fisiopatológicos como la inflamación. A pesar de que tanto la COX como la LOX catalizan una reacción de oxigenación de un sustrato de ácido graso, las dos enzimas no mantienen relación en cuanto a estructura, expresión tisular y localización celular (Schneider C, 2007).

2.1.1 Vía de las ciclooxigenasas

Las enzimas COX son hemoproteínas que actúan como homodímeros insertados en la membrana del retículo endoplasmático (RE) o en la membrana nuclear a través de cuatro hélices alfa alifáticas (Schneider C, 2007). Cada monómero de la COX contiene un lugar de actividad peroxidasa y un lugar de actividad COX independientes en los cuales se cataliza la oxigenación de los PUFA (Schneider C, 2007).

Existen dos isoformas diferentes de la COX, la COX-1 y la COX-2. Estas dos isoformas exhiben una similitud del 60% y los residuos activos son casi idénticos pero presentan diferente patrón de expresión (Buczynski MW, 2009). La COX-1 se expresa de forma constitutiva en la mayoría de las células y está implicada en la mayoría de procesos, incluyendo el control de acidez del estómago, el ciclo endometrial y la función renal. Por el contrario, la COX-2 se expresa bajo el control del factor de transcripción proinflamatorio NF-κB y se sobreexpresa como respuesta a una infección o ante enfermedades como aterosclerosis y cáncer (Buczynski MW, 2009). Además, existe una diferencia estructural importante entre ambas isoformas y es la sustitución de isoleucina a valina en el lugar de unión. Esto confiere a la COX-2 una unión de sustratos mayor y ser más permisiva en la selección del sustrato. A diferencia de la COX-1, la COX-2 puede metabolizar los precursores de eicosanoides del omega-3, EPA y DHA, además de los del AA (Buczynski MW, 2009).

Las prostaglandinas (PG) son las moléculas de señalización derivadas de la COX a partir mayormente del AA, pero también acogen a su predecesor sintético ácido dihomo-γ-linolénico (DGLA). El lugar activo COX incorpora oxígeno molecular en el AA/DGLA para formar PGG_2, y el lugar peroxidasa reduce un peróxido a hidroxilo para formar el PGH_2, el sustrato de las enzimas PG sintasas (Buczynski MW, 2009). A través de las PG sintasas se pueden formar un gran número de productos bioactivos como PGI_2 (prostaciclina), tromboxano A_2 (TXA_2), tromboxano B_2 (TXB_2), PGE_2 (dinoprostona), $PGF_{2\alpha}$, 6-ketoPGF$_{2\alpha}$ y PGD_2. La PGD_2 puede padecer deshidratación e isomerización consecutivas hasta su transformación a 15d-PGJ_2. Además de formar PGH_2, la COX puede oxigenar alternativamente el AA para formar trazas de 11-HETE o 15-HETE (Buczynski MW, 2009).

Los lugares de acción de la COX pueden ser sometidos a acetilaciones por parte de la aspirina, y en estado acetilado la enzima genera los intermediarios hidroxilados 18-HEPE y 17-HDHA a partir del EPA y del DHA, respectivamente (Serhan CN, 2002). En este punto, las enzimas LOX juegan un papel importante ya que transformarán estos intermediarios lipídicos en resolvinas de la serie E si son generados a partir del EPA y resolvinas de la serie D si provienen del DHA. Estos mediadores son actualmente mejor conocidos como SPM, de la terminología inglesa “specialized pro-resolving mediators”, y están en boga en el campo de investigación de los omega-3 por su función de pro-resolución de la inflamación (Serhan CN, 2002).

2.1.2 Vía de las lipoxigenasas

Las enzimas LOX pueden iniciar la síntesis de las moléculas de señalización o bien estar involucradas en la inducción de cambios metabólicos o estructurales en la célula (Brash AR, 1999). Las reacciones de la LOX catalizan la inserción estereoselectiva de oxígeno molecular en los PUFA con la formación de hidroperóxidos de ácidos grasos (HpETE, HpEPE y HpDHA a partir de AA, EPA y DHA, respectivamente) que subsecuentemente serán reducidos a los correspondientes hidróxidos de ácidos grasos (HETE, HEPE y HDHA) (Astarita G, 2015; Brash AR, 1999). Las isoformas de la LOX reciben una nomenclatura convencional en función de su especificidad sobre el sustrato, es decir, se basa en la indicación del carbono estereoespecífico que oxigena de la cadena hidrocarbonada de los PUFA (Brash AR, 2012). De esta manera, se describen mayormente la 5-LOX, la 12-LOX y la 15-LOX (Astarita G, 2015; Brash AR, 1999).

La 5-LOX reordena de forma catalítica la 5-HpETE dando lugar a los leucotrienos de la serie A (LTA). Estos compuestos pueden ser hidrolizados por la LTA hidrolasa para generar LT de la serie B (LTB), o, alternativamente, pueden padecer una conjugación catalizada por la LTC_4 sintasa para formar el cisteinil-LT denominado LTC_4, que se transforma consecuentemente en LTD_4 y en LTE_4 (Astarita G, 2015). Por otro lado, la 5-LOX convierte el 18-HEPE en resolvina E1 (RvE1) y, tras una peroxidación, en RvE2 (Serhan CN, 2000; Haas-Stapleton EJ, 2007). Recientemente, se ha encontrado un nuevo miembro en esta serie de resolvinas, la RvE3, generado a partir de la vía 12/15-LOX (Isobe Y, 2012). La 15-LOX transforma el DHA en el intermediario 17-HDHA. Este intermediario sirve de sustrato de la 5-LOX para la biosíntesis de las resolvinas de la serie D (RvD1-6) (Serhan CN, 2014). Actualmente, las moléculas mejor caracterizadas de esta serie son la RvD1 y la RvD2. La 15-LOX, además, puede también transformar el DHA en la protectina D1 (PD1), conocida también como neuroprotectina o (N)PD1 cuando se forma en las células neuronales (Bazan NG, 2009). Por último, la 12-LOX oxigena el DHA y lo transforma en el intermediario 14-HDHA. Este intermediario tras reacciones de hidrólisis y peroxidación dará lugar al conjunto de maresinas 1 y 2 (Deng B, 2014).

2.2 Vía del citocromo P450

El citocromo P450 (CYP) constituye una superfamilia de enzimas crucial para el metabolismo oxidativo, peroxidativo y reductivo de una gran gama de compuestos incluyendo endobióticos tales como ácidos grasos, PG, LT, esteroides y ácidos biliares, así como también de xenobióticos como fármacos y contaminantes ambientales (Tabla 2) (Nelson DR, 1996). Las enzimas CYP se encuentran ampliamente presentes en bacterias, hongos, protozoos, plantas y animales (Buczynski MW, 2009). Su denominación fue acuñada en 1962 cuando se caracterizó por primera vez por espectrofotometría con un único pico de absorbancia máxima a la longitud de onda de 450 nm (Buczynski MW, 2009). La notable diversidad de las enzimas CYP ha generado una lista de familias y subfamilias categorizadas que crece constantemente. En 2004, Nelson realizó un análisis extensivo del genoma humano y de ratón, identificando 57 CYPs funcionales en humano y 102 en ratón. En la actualidad la lista actualizada de los CYPs existentes están bien definidos por David R. Nelson (http://drnelson.uthsc.edu/P450db.html) (Buczynski MW, 2009).

Família	Función principal
CYP1	Metabolismo de fármacos
CYP2	Metabolismo de fármacos y ácidos grasos / PUFA
CYP3	Metabolismo de fármacos y esteroides
CYP4	Metabolismo de ácidos grasos / PUFA
CYP5	Síntesis de tromboxanos
CYP7	7a-hidroxilación del colesterol
CYP8	Síntesis de prostaciclinas

TABLA 2. Ejemplificación de principales funciones descritas de familias de CYP en humanos. Datos adaptados de Danielson PB, 2002.

Las vías metabólicas de la COX y la LOX están bien establecidas como rutas biosintéticas de los mediadores lipídicos bioactivos a partir de los PUFA (Deng Y, 2010). Los últimos avances en la bioquímica del campo del CYP indican que varias isoformas de esta enzima también participan en la

formación de mediadores bioactivos, siendo conocida ahora también como “la tercera vía del metabolismo de los PUFA” (Capdevila JH y Falck JR, 2002).

Las enzimas CYP presentan actividad catalítica de hidroxilación y de epoxidación del AA (Buczynski MW, 2009). Utilizando NADPH como cofactor, CYP cataliza la reducción del hierro y el oxígeno molecular y forma especies altamente reactivas. Dependiendo de la posición del AA en el centro activo de la enzima, el oxígeno reacciona con hidrógeno o con carbono y se transfiere al intermediario de AA inestable formando una serie de isómeros del HETE, siendo el 20-HETE el mejor caracterizado, como producto de la actividad omega-hidrolasa, o isómeros del ácido epoxieicosatrienoico (EET) como productos de la actividad epoxigenasa (Buczynski MW, 2009).

2.2.1 Actividad epoxigenasa de las enzimas CYP

Las isoformas del CYP que presentan actividad epoxigenasa sobre los PUFA son principalmente las CYP2C8, CYP2C9, CYP2J2 y CYP1A2 (Lucas D, 2010). La epoxidación del AA tiene como resultado la formación de mediadores lipídicos bioactivos únicos denominados EETs. Cada doble enlace muestra susceptibilidad a la oxidación, resultando en 5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET. En mamíferos, las CYP-epoxigenasas mejor documentadas son las familias 2C y 2J, aunque actualmente muchas otras están siendo descritas por su implicación en la biosíntesis de los EETs. De hecho, la promiscuidad de las CYP-epoxigenasas genera la formación de más de un tipo de EET, pero además, retienen también una significante actividad omega-hidrolasa (Lucas D, 2010). Los PUFA epoxidados del AA están implicados en importantes procesos biológicos, incluyendo tono vascular, función renal, adhesión leucocitaria, señalización neuronal y angiogénesis. Sin embargo, el efecto de todos los isómeros específicos permanece siendo incierto. Varias evidencias sugieren que la señalización de estos mediadores ocurre a través del receptor putativo GPCR, a pesar que hasta la fecha no se le conoce ninguno (Fidelis P, 2010).

Actualmente se ha demostrado que las CYP epoxigenasas, previamente caracterizadas como enzimas metabólicas del AA, también aceptan de forma eficiente como sustrato a los omega-3 PUFA, el DHA y el EPA (Westphal C, 2011). Por lo tanto, las CYP también producen seis isoformas específicas del DHA denominadas ácido epoxidocosapentaenoico o EDP (4,5-EDP, 7,8-EDP, 10,11-EDP, 13,14-EDP, 16,17-EDP y 19,20-EDP), así como también cinco derivados del EPA conocidos como ácido epoxieicosatetraenoico o EEQ (5,6-EEQ, 8,9-EEQ, 11,12-EEQ, 14,15-EEQ y 17,18-EEQ).

(Lucas D, 2010). Recientemente se ha demostrado que algunos isómeros de los EEQs y EDPs presentan una actividad más potente que incluso los EETs sobre la vasodilatación y la inhibición de la agregación plaquetaria (Westphal C, 2011; Lucas D, 2010; Morisseau C, 2010).

2.2.2 La epóxido hidrolasa soluble

La epóxido hidrolasa soluble (sEH) es la enzima que cataliza la hidrólisis del grupo epóxido de los metabolitos del CYP para formar dos grupos dioles (Schmelzer KR, 2005). En humanos, la sEH es codificada por el gen EPXH-2 que se expresa de forma distribuida en los tejidos pero mayormente en hígado, riñón, intestino y vasos (Spector AA y Kim HY, 2015). Debido a la pérdida de actividad de los PUFA epoxidados, de hecho presentan una vida media predecible del orden de segundos, la formación de los correspondientes dioles mediante la sEH se considera el paso de inactivación metabólica para la señalización de estos mediadores lipídicos (Schmelzer KR, 2005; Shen HC, 2010).

Concretamente, la sEH convierte los derivados epóxidos del AA (5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET) en las respectivas formas enantioméricas del ácido dihidroxieicosatrienoico (DHET). Asimismo, la sEH también presenta afinidad por los omega-3 epoxidados, metabolizando los derivados del DHA y del EPA (los enantiómeros de los EDP y los EEQ) en los correspondientes dioles,DiHDPA y DiHETE, respectivamente (Morisseau C, 2010). Los DHET, DiHDPA y DiHETE tienen menor actividad biológica que la observada en los PUFA epoxidados (Spector AA y Kim HY, 2015). Además, los dioles son mucho más polares que los epóxidos y por ello se acumulan en el fluido extracelular y son rápidamente removidos del lugar de producción y excretados (Spector AA y Kim HY, 2015). Sin embargo, la actividad de la sEH puede ser inhibida, sugiriendo una estrategia directa para la regulación del metabolismo de los metabolitos epóxidos (Spector AA y Kim HY, 2015). La inhibición de la sEH estabiliza los mediadores epóxidos, aumenta su incorporación en los PL de membrana y potencia sus efectos biológicos por lo que actualmente se está estudiando su actividad con fines terapéuticos (Spector AA y Kim HY, 2015).

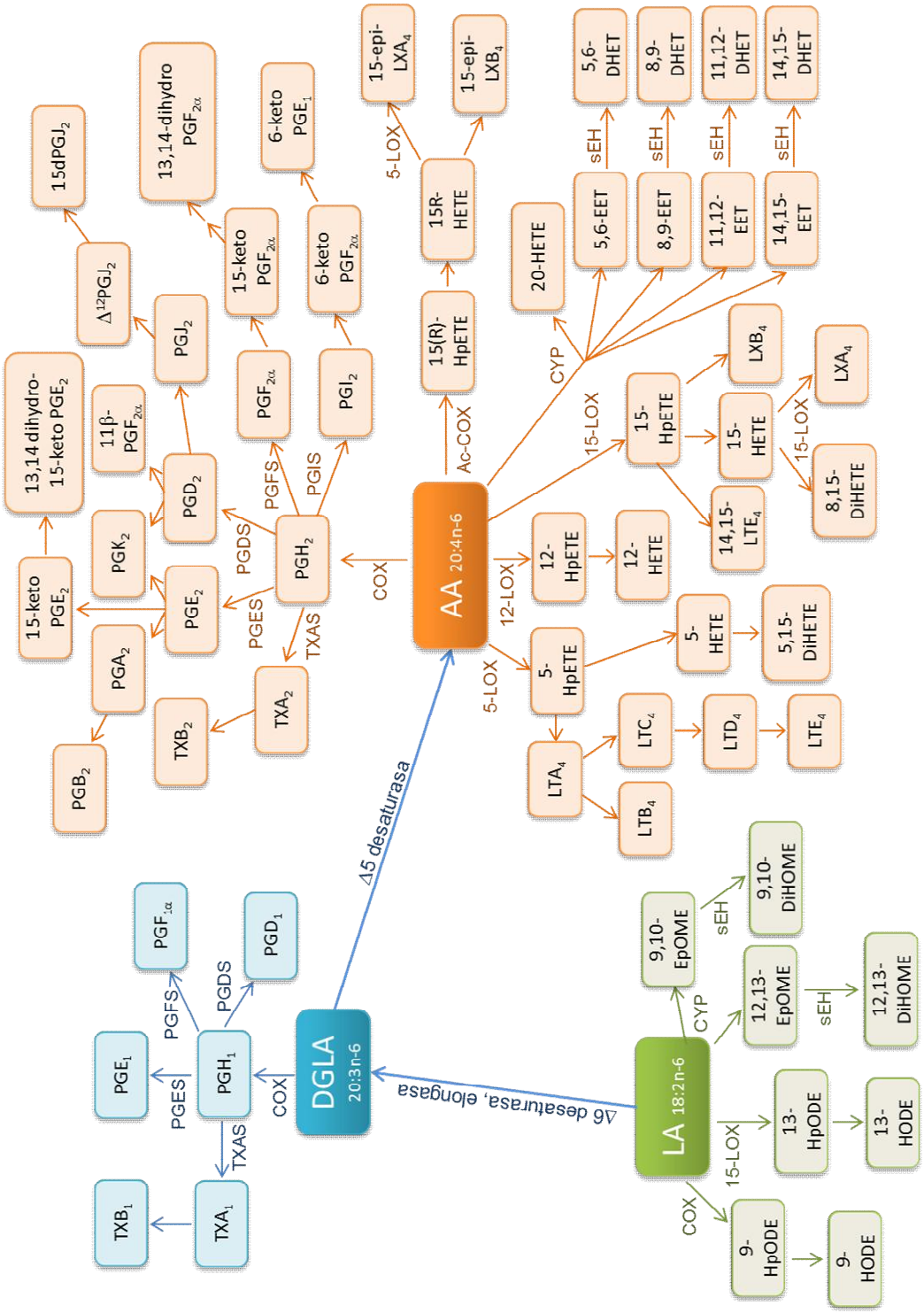

FIGURA 3. Recopilación esquemática del metabolismo enzimático de los PUFA omega-6, ácido linoleico (LA), ácido dihomo-γ-linolénico (DGLA) y AA (adaptación de Astarita G, 2015).

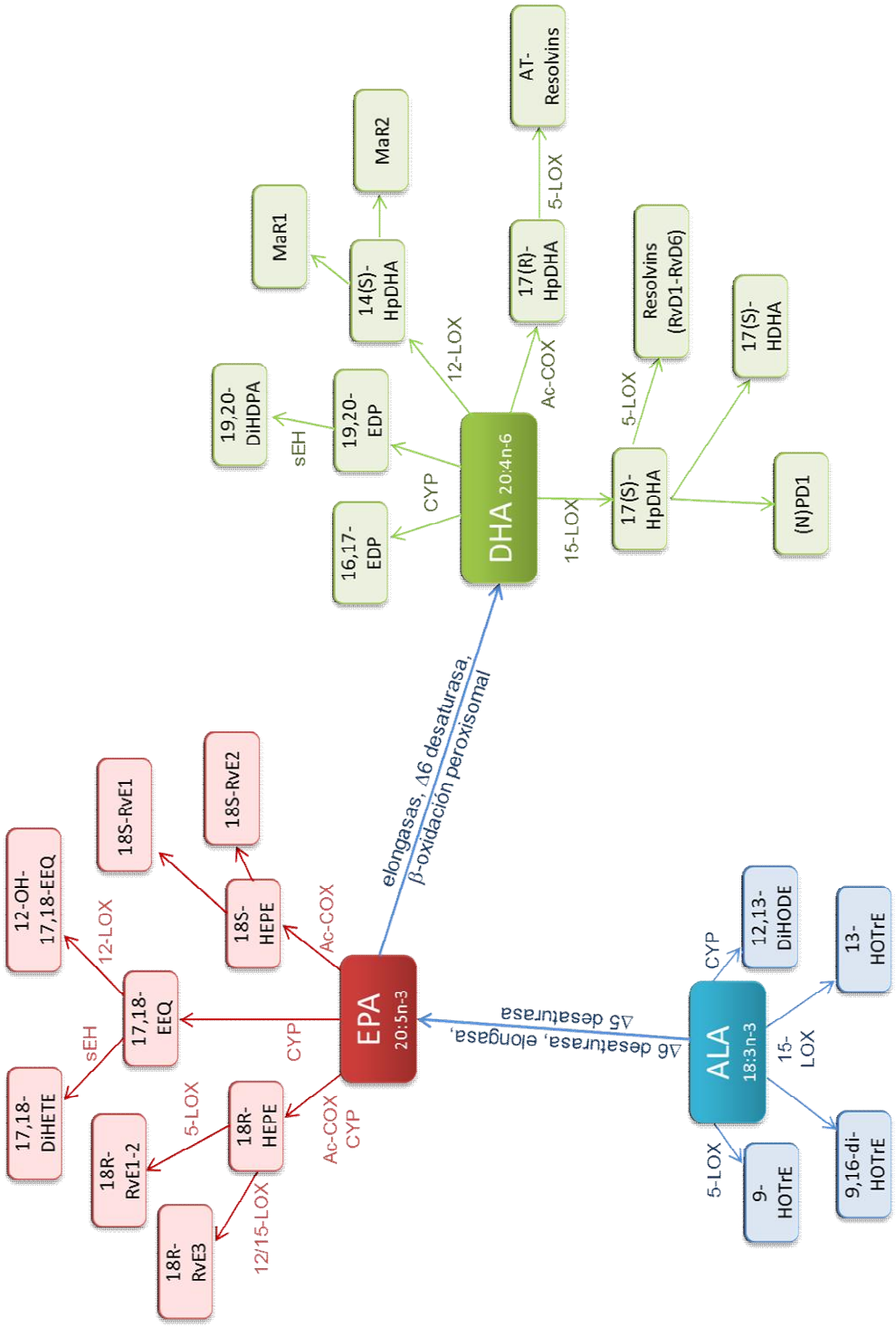

FIGURA 4. Recopilación esquemática del metabolismo enzimático de los PUFA omega-3, ácido linolénico (ALA), EPA y DHA. Se indican en color aquellos metabolitos con demostrada actividad biológica (adaptación de Astarita G, 2015).

II. BALANCE ENTRE LOS PUFA OMEGA-3 Y OMEGA-6

Los PUFA consumidos a través de la dieta son absorbidos por las células intestinales. Una vez ingeridos, por una parte son incorporados en todas las membranas de bicapa lipídica como componentes importantes de la mismas, especialmente en plaquetas, eritrocitos, neutrófilos, monocitos y células hepáticas (Simopoulos AP, 2006). Dependiendo de la proporción de PUFA presente en las membranas, éstas se someten a los cambios en su fluidez y como consecuencia, en su capacidad de contener receptores, enzimas, canales y poros, permitiendo así la mejora de las funciones fisiológicas de la célula (Arterburn LM, 2006). Por otra parte, tras ser liberados por la PLA2 al citoplasma celular, los PUFA son metabolizados por las vías de COX, LOX y CYP y se generan sus derivados lipídicos bioactivos. Los mediadores lipídicos juegan un papel crucial en los síntomas de la inflamación (vasoconstricción, vasodilatación, coagulación, dolor y fiebre), y además, la inflamación constituye la base de muchas enfermedades crónicas tales como enfermedades cardiovasculares, obesidad, diabetes, artritis, enfermedad mental, cáncer y condiciones autoinmunes. Por este motivo, la relación que existe entre los diferentes mediadores lipídicos es muy importante para el mantenimiento de la homeostasis (Simopoulos AP, 2006).

A causa del aumento en el consumo de omega-6 en las dietas occidentales, los productos derivados del AA, el mayoritario omega-6, se forman en mayores cantidades que aquellos formados de los ácidos grasos omega-3, específicamente del EPA y del DHA. Los ácidos grasos esenciales omega-3 y omega-6 no son interconvertibles, son metabólicamente y funcionalmente distintos. La biosíntesis de los PUFA omega-3 y omega-6 ocurre mediante reacciones de desaturación y elongación secuenciales. En base a la competición por el sustrato, se ha propuesto que las vías biosintéticas de ambos ácidos grasos están formadas por las mismas desaturasas y elongasas (Guillou H, 2010; Brenner RR y Peluffo RO, 1966). Los mamíferos, a diferencia de otras especies como plantas y nematodos, carecen de la maquinaria (Δ12 y Δ15 desaturasas) involucrada en la síntesis de precursores de omega-6 y omega-3 (Guillou H, 2010), así como también carecen de la enzima convertidora de un tipo a otro, la enzima omega-3 desaturasa (Figura 5) (Simopoulos AP, 2006). Por lo tanto, los mamíferos no pueden insertar un doble enlace en las posiciones omega-3 de la cadena hidrocarbonada de los omega-6 y deben confiar enteramente en la proporción de la ingesta dietética de los PUFA omega-3 (Figura 5) (Astarita G, 2015).

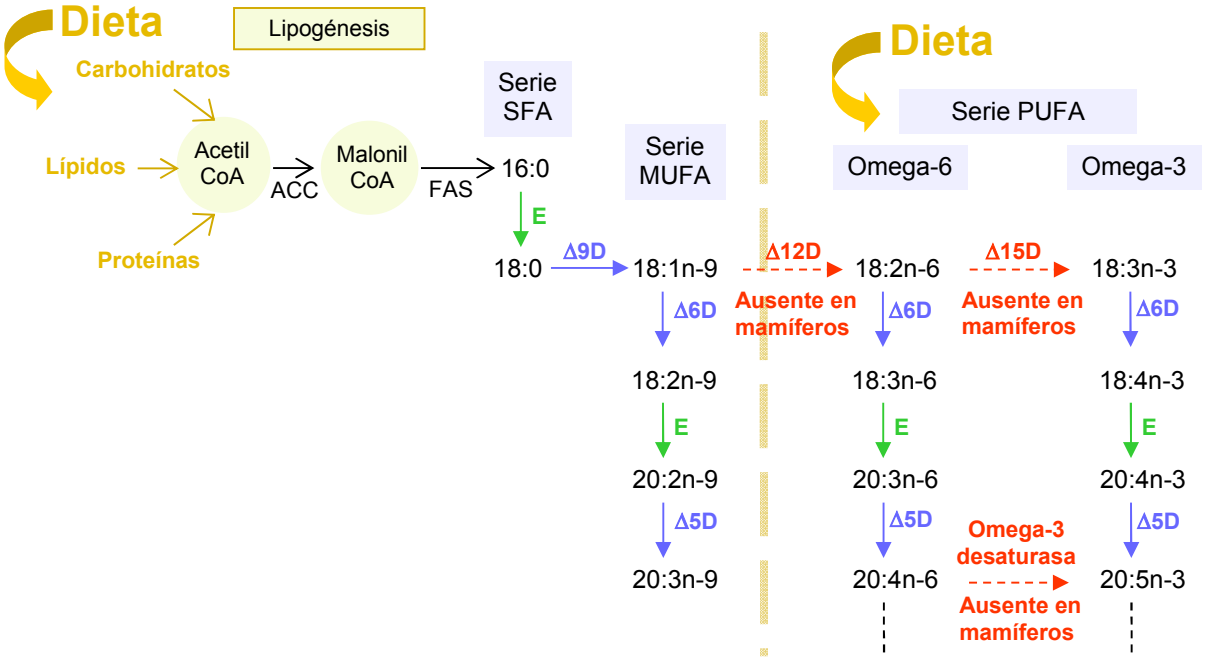

FIGURA 5. Esquema sintético de los ácidos grasos en mamíferos. E se refiere a las enzimas elongasas mientras que D se refiere a las enzimas desaturasas (Δ12, Δ15, Δ9, Δ5 y Δ6). La ausencia de las desaturasas Δ12, Δ15 y omega-3 convierte a los PUFA omega-6 y omega-3 en ácidos grasos esenciales y deben proporcionarse directamente a través de la dieta.

Estudios epidemiológicos demuestran que existe una relación entre la ingesta de los PUFA, establecida como el valor del balance omega-6/omega-3 y los efectos sobre la salud. Un aspecto importante y controvertido es la influencia de la ingesta de los PUFA omega-3 y omega-6 en las diferentes patologías como enfermedades cardiovasculares, obesidad, diabetes, artritis y enfermedades degenerativas. Simopoulos describe que un adecuado balance entre ellos es importante para la prevención y el tratamiento de las enfermedades cardiovasculares. Sin embargo, mientras unos autores señalan la necesidad de reducir el consumo de omega-6 para mejorar el balance (Hibbeln JR, 2006), otros apoyan la estrategia de aumentar el consumo de omega-3, particularmente de EPA y DHA, y buscan alternativas para compensar tales deficiencias nutricionales (Harris WS, 1997, 2008).

Dada la importancia de la relación entre los ácidos grasos omega-6 y omega-3 en la dieta, se están sumando a estudio nuevas estrategias dirigidas a la prevención de enfermedades metabólicas debido a los cambios nutricionales.

1. Evolución de los hábitos nutricionales

Estudios antropológicos, nutricionales y genéticos indican que la dieta humana, incluyendo la ingesta de energía y gasto energético, ha cambiado a lo largo de los pasados miles de años, con un pico de cambio durante los últimos 150 años (Simopoulos AP, 2006). De entre los muchos cambios que ha sufrido la dieta en función de la proporción de nutrientes, los cambios más destacables han sido en el tipo y en la cantidad de ácidos grasos esenciales (Simopoulos AP, 2006; Leaf A, 1987; Cordain L, 2005).

En el periodo Paleolítico, la dieta humana se caracterizaba por una ingesta baja en calorías de origen graso (20-25%), de las cuales la ingesta de SFA era muy reducida (<6%) y la ingesta de ácidos grasos *trans* era inapreciable (Eaton SB, 1996; Simopoulos AP, 2006). Además, la dieta de la población cazadora de ese periodo presentaba un balance omega-6/omega-3 equilibrado (de 1/1 a 2/1) como resultado del consumo de ácidos grasos omega-3, los cuales se encontraban en grandes cantidades en la mayoría de alimentos que consumían: carne, plantas, huevos, pescado y frutos. Esta situación contribuyó en la evolución humana, influyendo en el desarrollo cognitivo y cerebral (Crawford MA, 1999). Actualmente, las dietas occidentales presentan un alto índice calórico de origen graso por encima de las recomendaciones nutricionales (30-35%). Específicamente, la dieta occidental se caracteriza por una elevada proporción de SFA (>10%), rica en omega-6 y una proporción muy baja de omega-3, resultando en un balance omega-6/omega-3 en el rango de 20/1 a 30/1 (Figura 6) (Eaton SB, 1996; Simopoulos AP, 2006).

Estos cambios nutricionales han sido la consecuencia de cambios socioeconómicos, estimulados en parte por los avances tecnológicos y agrícolas, durante los pasados 100-150 años, donde cabe destacar la aparición de la revolución industrial (Simopoulos AP, 2006). Desde entonces, el consumo de SFA comenzó a aumentar exponencialmente, así como también el de ácidos grasos *trans*. Paralelamente, el consumo de omega-3 fue disminuyendo mientras que la ingesta de omega-6 fue aumentando progresivamente alcanzando niveles muy diferentes a los originales.

	-4x10^6 a	-10000 a	1900	2000
%Grasa total	25%		35%	40%
PUFA/SFA	1/1		0.75/1	0.5/1
n-6/n-3	1/1		10/1	25/1
%n-6	25%		50%	75%

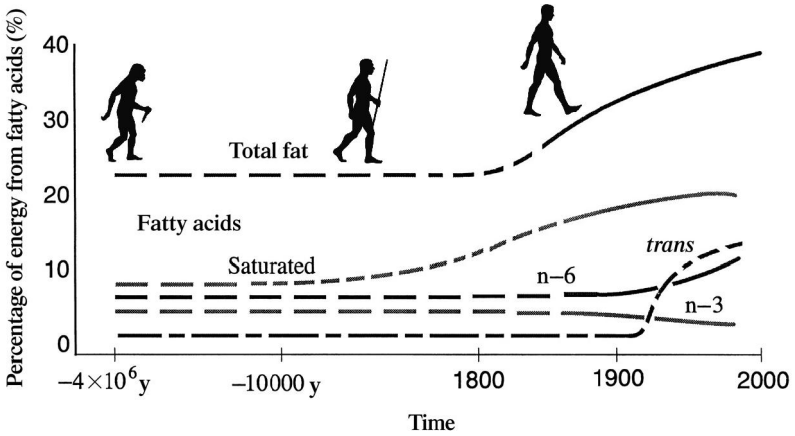

FIGURA 6. Esquema hipotético de la ingesta de grasa y ácidos grasos (PUFA n-3 y n-6, trans y SFA) expresado como % de energía calórica procedente de los lípidos con el paso del tiempo. Los datos han sido extrapolados de un análisis transversal de la población cazadora contemporánea y los cambios producidos en los últimos 100 años (Figura adaptada de Simopoulos, 1999).

Esto es causado, por un lado, por el interés en aumentar la producción industrial de alimentos de origen animal causando la pérdida de gran parte del contenido en omega-3 debido al cambio en su composición nutricional (Simopoulos AP, 2006). En general, el porcentaje de saturados aumentó debido al confinamiento y al excesivo contenido energético de la alimentación de los animales de granja, así como también hubo un ascenso en el porcentaje de omega-6 por el pienso y aceites vegetales ricos en este tipo de PUFA (Kang JX, 2005). Por otro lado, los omega-3 han ido desapareciendo prácticamente, desde que disminuyó el consumo de alimentos de “hoja verde” y de pescado azul (Kang JX, 2005).

También ha contribuido a esta pérdida la sustitución indiscriminada de SFA en productos alimentarios con omega-6 diseñados para reducir las concentraciones séricas de colesterol (según el Report of the National Cholesterol Education Program, 1988). Otro factor relevante es el aumento del consumo del pescado de granja, el cual basa su alimentación en una muy baja cantidad de omega-3 (van Vliet T y Katan MB, 1990). Lo mismo ocurre con los huevos, procedentes de gallinas con

elevado consumo de omega-6 a través del pienso (Simopoulos AP y Salem N Jr, 1992). Incluso las verduras cultivadas contienen menos omega-3 que los vegetales salvajes (Simopoulos AP, 1999). En general, la agricultura moderna pone especial énfasis en la producción a gran escala, generando un detrimento en el contenido de omega-3 en la mayoría de productos alimentarios: verdura de hoja verde, carne roja y blanca, huevos y pescado (Simopoulos AP, 1999).

El balance omega-6/omega-3 equilibrado existió durante millones de años a lo largo de la historia evolutiva (Simopoulos AP, 2006). Sin embargo, la información genética se ha mantenido sin grandes variaciones y el patrón genético humano actual está adaptado a la dieta del hombre cazador/recolector de hace 40.000 años. El pico de cambio nutricional producido durante los últimos 100-150 años ha sido demasiado brusco para aceptarlo metabólicamente. Por lo tanto, la sociedad occidental vive hoy en día en un ambiente nutricional que difiere de aquél por el cual su patrón genético fue establecido. Estos cambios han sido los que han provocado importantes consecuencias sobre la salud (Simopoulos AP, 2006).

2. Implicaciones sobre la salud

El estudio del balance omega-6/omega-3 se ha extendido al estudio de la patogénesis de las enfermedades cardiovasculares, cáncer, enfermedades inmunológicas e inflamatorias. A grandes rasgos, la principal diferencia entre los mediadores lipídicos derivados de los PUFA omega-3 y los omega-6 es que la mayoría de los mediadores formados a partir del EPA y el DHA presentan acciones antiinflamatorias y pro-resolutivas, mientras que la gran parte de aquellos formados a partir del AA son proinflamatorios o muestran otros efectos desencadenantes de una enfermedad (Schmitz G y Ecker J, 2008; Bagga D, 2003; Robinson JG y Stone NJ, 2006). La investigación sobre el papel que ejercen los omega-3 en el organismo se encuentra en auge y está atrayendo especial interés a nivel nutricional y terapéutico (Connor WE, 2000; Simopoulos AP 1999)

La síntesis de los PUFA se encuentra bajo una fuerte regulación por retroalimentación. Esto indica la importancia del suplemento equilibrado de omega-3 y omega-6 para el requerimiento necesario del organismo. Los precursores de omega-3 y omega-6 obtenidos a través de la dieta se almacenan en grandes cantidades en los TG del tejido adiposo. Sin embargo, el análisis de la composición de los TG del tejido adiposo realizados previamente en la población estadounidense resulta en unos niveles muy bajos del precursor de omega-3 (18:3n-3) generando la preocupación de

que el suplemento dietético en la población es deficiente (Garland M, 1998; Phinney SD, 1990). Diversos estudios han demostrado que los niveles de AA en los tejidos se mantienen constante bajo una suficiente ingesta dietética del precursor (18:2n-6) (Nakamura MT, 2004). Δ5D y Δ6D son completamente inducidas en condiciones de deficiencia de ácidos grasos, y son suprimidas ante una adecuada incorporación de los PUFA a través de la dieta, indicando que la capacidad de la vía sintética endógena es suficiente para el requerimiento necesario de PUFAs (Nakamura MT, 2004).

Debido al aumento del balance omega-6/omega-3 en las dietas occidentales, los eicosanoides procedentes del AA, específicamente PG, TX y LT se generan en grandes cantidades en comparación con los derivados del EPA y del DHA (Simopoulos AP, 1991; Schmitz G y Ecker J, 2008). Los eicosanoides que proceden del AA son biológicamente activos a bajas concentraciones y su formación masiva contribuye a la síntesis de trombos y ateromas, desórdenes inflamatorios y alérgicos, y a la proliferación celular incontrolada (Schmitz G y Ecker J, 2008). De esta forma, una dieta rica en omega-6 desplaza el estado fisiológico a un estado que es protrombótico y proagregatorio con aumento de la viscosidad sanguínea, vasoespasmos y vasocontricción y disminución del tiempo de sangrado. Además, la prevalencia de enfermedades metabólicas tales como diabetes tipo II, hiperlipidemia, hipertensión y enfermedades cardiovasculares está estrechamente relacionada con el incremento de la relación omega-6/omega-3 (Simopoulos AP, 1999, 2006; Schrör K, 1990; Bagga D, 2003; Levy BD, 2001; Calder PC, 2006).

Varios estudios indican que un suplemento equilibrado en omega-3 mejora el crecimiento y el desarrollo cognitivo en recién nacidos prematuros. Por otro lado, también existen estudios epidemiológicos que sugieren que el consumo de aceite de pescado es beneficioso en la prevención de la demencia senil (Nakamura MT, 2004). De hecho, existe toda una serie de enfermedades metabólicas en las cuales se ha reportado la existencia de una deficiencia en el contenido tisular de PUFAs como en los desórdenes peroxisomales y el alcoholismo (Nakamura MT, 2004). La suplementación de omega-3 a través de la dieta, por ejemplo en forma de aceite de pescado rico en EPA y DHA, muestra un efecto hipotrigliceridémico por supresión de la secreción de las lipoproteínas de baja densidad (LDL). Se ha demostrado también que un aporte dietético de omega-3 exhibe efectos antiinflamatorios debido a la acción de contrarrestar las acciones de los eicosanoides derivados del AA (Nakamura MT, 2004).

Los efectos cardioprotectores de los omega-3 están siendo ampliamente reconocidos. La primera observación fue publicada por Sinclair donde se demostraban los efectos negativos de una deficiencia en PUFA omega-3 sobre las enfermedades cardiovasculares. Su hipótesis fue reforzada cuando se observó una disminución en la tasa de mortalidad por enfermedad cardiovascular en la población esquimal de Groenlandia, una población caracterizada por un alto consumo de omega-3 (Sinclair HM, 1956; Dyerberg J, 1975). Recientemente, von Shacky y Harris propusieron "el índice omega-3" como un nuevo factor de riesgo de padecer parada cardíaca. Se define como el porcentaje de EPA + DHA del total de ácidos grasos determinado en la membrana de los eritrocitos y refleja los niveles de PUFA omega-3 en el organismo (von Schacky C y Harris WS, 2007).

3. Competencia metabólica

Las dos clases de PUFA omega-6 y omega-3 están bien diferenciadas ya que presentan funcionalmente efectos fisiológicos opuestos, y su balance es importante para la homeostasis y el desarrollo apropiado del organismo (Simopoulos AP, 1999). El precursor de la vía metabólica de los ácidos grasos omega-6, el LA (18:2n-6), se convierte a ALA (18:3n-6) y a ácido dihomo-γ-linolénico (20:3n-6) para formar el intermediario clave, el AA (20:4n-6) por una serie de enzimas desaturasas y elongasas (Figura 7). El AA es después metabolizado a ácido docosapentaenoico (DPA, 22:5n-6) o a eicosanoides (Schmitz G y Ecker J, 2008). El precursor de omega-3, el ALA (18:3n-3) se convierte a ácido estearidónico (18:4n-3) y a ácido eicosatetraenoico (20:4n-3) para formar EPA (20:5n-3) utilizando la misma serie de enzimas que participan en la síntesis del AA. El EPA es metabolizado a DHA (22:6n-3) o a eicosanoides (Schmitz G y Ecker J, 2008).

Cho y colaboradores demostraron mediante la clonación de los genes Δ5D/Δ6D de mamíferos que las mismas enzimas catalizan la síntesis de ambos omega-6 y omega-3. Además, la conversión de omega-3 y omega-6 comparten las mismas enzimas en los procesos de esterificación de los PL en las membranas y de transformación a sus respectivos derivados causando una competencia metabólica entre ambas familias de ácidos grasos (Cho HP, 1999). Esto produce que el exceso de un tipo de ácido graso provoque un decremento significativo en la conversión del otro, reduciendo su incorporación en la membrana celular y alterando sus efectos biológicos (Schmitz G y Ecker J, 2008).

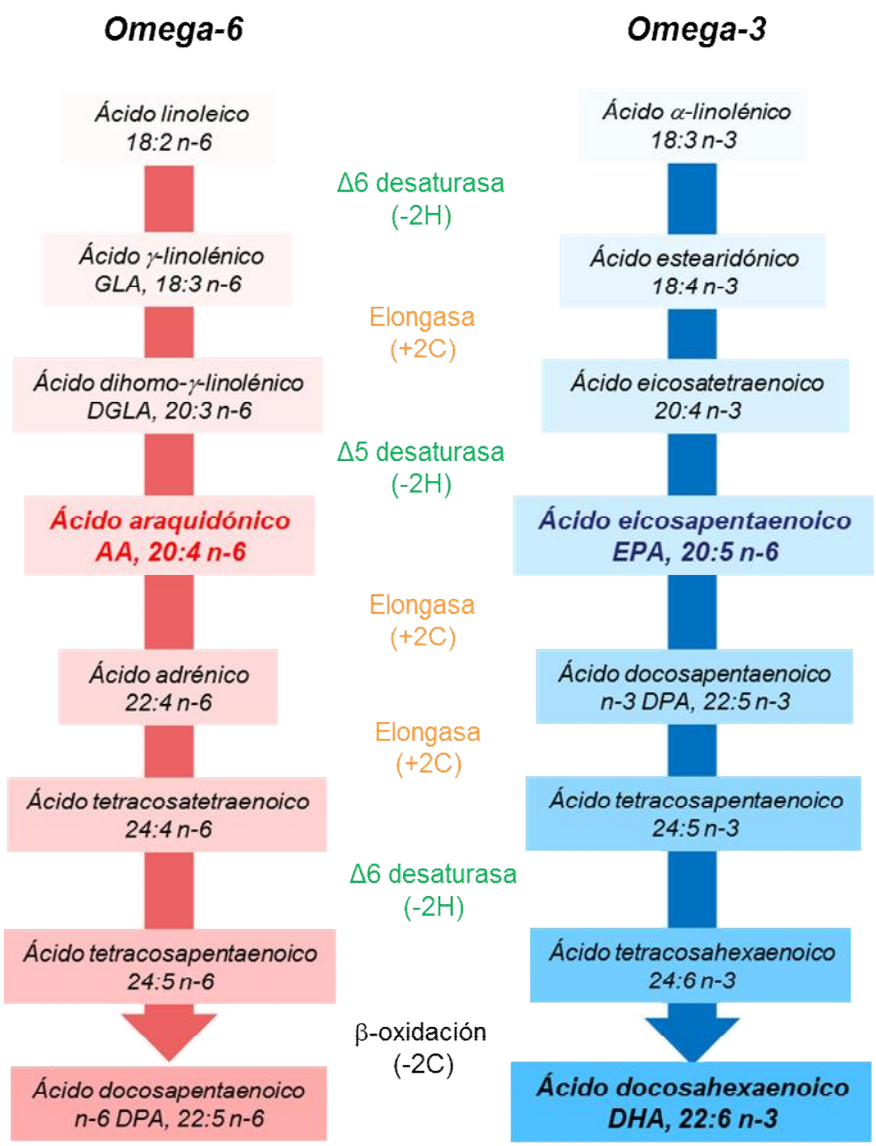

FIGURA 7. Metabolismo compartido entre los PUFA omega-6 y omega-3.

La interferencia en la ruta sintética de los PUFA produce un desplazamiento metabólico que explica el desequilibrio omega-6/omega-3. Este desequilibrio puede exacerbarse debido a que el sistema enzimático es extremadamente lento y de bajo rendimiento para la síntesis de EPA y DHA a partir de su precursor (18:3n-3), viéndose afectado además, por el alto aporte de omega-6 en la dieta actual (Pawlosky RJ, 2001; Horrobin DF, 1993).

3.1 Desaturasas y elongasas

En las células de mamífero, las desaturasas y las elongasas son enzimas que juegan un papel importante en la regulación de la longitud y del patrón de saturación/desaturación de los ácidos grasos (Guillou H, 2010). La desaturación y la elongación de los ácidos grasos constituyen procesos críticos en el mantenimiento de la homeostasis lipídica (Guillou H, 2010). La actividad de estas enzimas se regula a nivel transcripcional pero simultáneamente también a niveles superiores como la ingesta a través de la dieta (Guillou H, 2010).

3.1.1 Desaturasas

Debido a que los PUFA son esenciales para el mantenimiento de las funciones celulares, todos los organismos vivos poseen maquinarias de insaturación de ácidos grasos (Nakamura MT, 2004). El grado de insaturación de la cadena de los ácidos grasos es un determinante importante de la temperatura de fusión de los TG así como también de las propiedades físicas de las membranas biológicas. Por este motivo, las desaturasas son enzimas muy bien conservadas y extendidas en las diferentes especies (Nakamura MT, 2004). Las desaturasas son una única clase de oxigenasas que eliminan dos hidrógenos para introducir un doble enlace o una insaturación en una posición específica de la cadena hidrocarbonada de los ácidos grasos (Meesapyodsuk D, 2012). En general, la reacción de las delta (Δ) desaturasas requiere oxígeno molecular, NADPH, un sistema de transporte de electrones (por ejemplo citocromo b5) y un dominio de desaturación (Nakamura MT, 2004). Debido a la regioselectividad, las desaturasas son típicamente categorizadas como Δ desaturasas, si introducen un doble enlace en la posición Δ referida a la posición del extremo carboxílico del ácido graso; o bien como omega-desaturasas, si introducen un doble enlace en la posición omega referida a la del extremo metilo (Meesapyodsuk D, 2012). En función de la posición de inserción de la insaturación relativa a un doble enlace preexistente en la cadena hidrocarbonada, las desaturasas son consideradas *front-end* o *methyl-end*. Una *front-end* desaturasa introduce un doble enlace entre el doble enlace preexistente y el extremo carboxílico mientras que una *methyl-end* desaturasa genera una insaturación entre el doble enlace preexistente y el extremo metilo del ácido graso (Meesapyodsuk D, 2012). Los humanos carecen de las *methyl-end* desaturasas como las Δ12, Δ15 y las omega-3 desaturasas mientras que sí disponen de las *front-end* Δ5, Δ6 y Δ9 desaturasas (Meesapyodsuk D, 2012).

Sin embargo, hay *front-end* desaturasas que tampoco se encuentran en humanos, como es el caso de la Δ4, cuya función es la de desaturar directamente el DPA para dar lugar al DHA en la vía de los omega-3. En su lugar, la biosíntesis del DHA se produce mediante una "vía de retroconversión" en la cual 2 elongaciones del EPA preceden a una desaturación por parte de la Δ6 desaturasa, y el producto de 24 carbonos resultante es seguidamente transportado a los peroxisomas para convertirse en DHA mediante un paso de β-oxidación (Voss A, 1992; Sprecher H, 1995). Varios estudios han demostrado el papel que ejercen los peroxisomas en la oxidación de los ácidos grasos de cadena larga y muy larga (20 o más carbonos) y, por lo tanto, están involucrados tanto en la síntesis como en la degradación del DHA. A pesar de que se desconoce cómo estos procesos pueden llevarse a cabo en un mismo orgánulo celular, es plausible que la cantidad de DHA necesaria para las funciones celulares sea rápidamente incorporada en los PL de membrana mientras que el exceso de este omega-3 sea degradado en los peroxisomas (Nakamura MT, 2004; Neschen S, 2002).

3.1.1.1 Delta-9 desaturasas (Δ9D)

Los mamíferos presentan todas las enzimas para la síntesis de los MUFA desde el acetil coenzima A (-CoA). Las Δ9D son *front-end* desaturasas que catalizan el último paso de esta síntesis. Estas enzimas introducen el primer doble enlace *cis* en la posición 9 desde el terminal carboxilo de los ácidos grasos (Nakamura MT, 2004). Las Δ9D en mamíferos, normalmente también conocidas como estearoil-CoA desaturasas (SCDs), se purificaron por primera vez en el hígado de la rata y catalizan la desaturación Δ9 de cadenas de acil-CoA de 12 a 19 carbonos. Se han identificado en ratón cuatro isoformas de las SCD (SCD-1, SCD-2, SCD-3 y SCD-4), mientras que solamente una SCD es altamente homóloga en humanos, la SCD-1 (Nakamura MT, 2004). La SCD-1 exhibe una expresión específica de tejido, por ejemplo, la SCD-1 es constitutiva en tejido adiposo y es marcadamente inducible en hígado en respuesta a una dieta con elevados niveles de carbohidratos (Brener RR, 2003).

El principal producto que sintetizan las Δ9D (o SCD-1) es el ácido oleico (18:1n-9) y se encuentra presente de forma ubicua en todos los tejidos. En mamíferos, los TGs del tejido adiposo principalmente consisten en ácidos grasos de cadena larga de 16 y 18 carbonos. Por ejemplo 18:1-n9 es la mayor especie en los TGs del tejido adiposo comprendiendo cerca de la mitad de los ácidos

grasos totales. Además, la actividad de la SCD-1 es clave para la lipogénesis *de novo* para almacenar el exceso energético en forma de TGs (Nakamura MT, 2004).

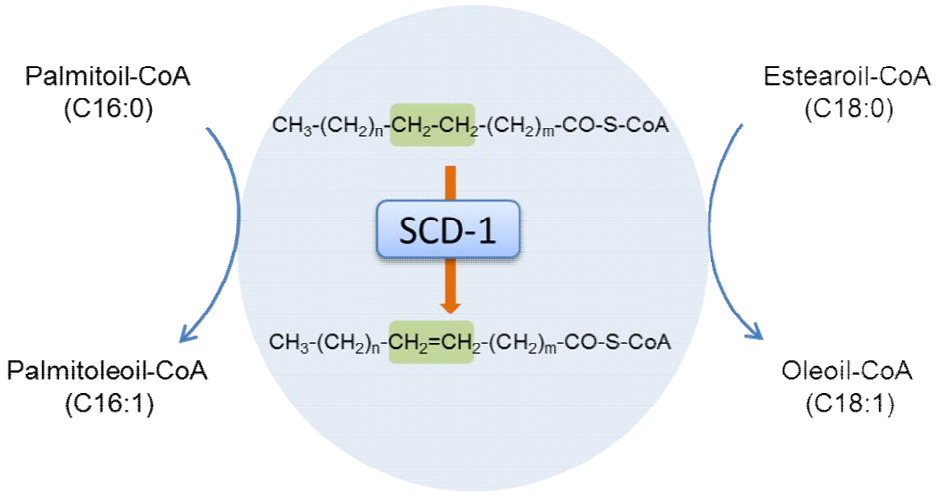

FIGURA 8. Reacción enzimática de la SCD-1. La enzima SCD-1 cataliza la desaturación de productos de 16 y 18 carbonos saturados (palmitoil-CoA y estearoil-CoA) para formar sus correspondientes productos monoinsaturados (palmitoleioil-CoA y oleoil-CoA).

3.1.1.2 Delta-6 desaturasas (Δ6D) y delta-5 desaturasas (Δ5D)

Los genes Δ5D y Δ6D se colocalizan en el cromosoma 11 (11q12-q13.1). La proximidad de sus promotores sugiere la posibilidad de que la transcripción de ambos genes sea coordinada y controlada por secuencias reguladoras comunes (Nakamura MT, 2004). Ambas se encuentran ampliamente expresadas en todos los tejidos, pero especialmente se encuentran niveles elevados en el hígado (Nakamura MT, 2004).

Las Δ6D son desaturasas que concretamente catalizan la reacción inicial limitante de desaturación de los precursores C18:2n-6 y C18:3n-3 para la síntesis de sus derivados PUFA de cadena más larga omega-6 y omega-3, respectivamente (Guillou H, 2010). La Δ6D se caracteriza porque es la única isoforma que puede actuar sobre precursores de los PUFA de 18 carbonos (Stoffel W, 2008). La primera Δ6D fue clonada en 1993 en la cianobacteria *Synechocystis* utilizando la aproximación “ganancia de función” y más tarde fue clonada en nematodo *C. elegans*, humanos, rata y ratón. La Δ6D se clasifica como una *front-end* desaturasa debido a que introduce un doble enlace entre el doble enlace existente y un terminal carboxílico de la cadena del ácido graso. La Δ6D

contiene un citocromo b5 en N-terminal y dominios característicos (motivos ricos en histidina) de anclaje a la membrana celular esenciales para la actividad de la enzima (Nakamura MT, 2004; Meesapyodsuk D, 2012).

Las Δ5D son también *front-end* desaturasas presente en mamíferos que catalizan la síntesis de los PUFA. Tras la desaturación por la Δ6D y la elongación, las Δ5D introducen otro doble enlace a la posición Δ5 de los ácidos grasos de veinte carbonos 20:3n-6 y 20:4n-3. El gen que codifica para la Δ5D fue clonado por primera vez en 1998 del hongo *M. alpina*. Desde entonces, ha sido clonado en varias especies incluyendo humanos, rata, ratón y *C. elegans*. El gen Δ5D humano codifica 444 aminoácidos (el mismo número que para Δ6D) y posee un 75% de similitud con la enzima Δ6D, presentando las mismas características estructurales de la misma (Nakamura MT, 2004).

3.1.2 Elongasas

Las elongasas son enzimas implicadas en el proceso de elongación de los ácidos grasos mediante la incorporación de dos unidades de carbono a la cadena hidrocarbonada. Como las desaturasas, las elongasas contienen dominios que son altamente conservados en ratón, rata y humano. Las elongasas de estas especies son denominadas ELOVLs por la terminología inglesa "Elongation of very-long-chain fatty acids" y se han identificado 7 isoformas con diferente especificidad para el sustrato y tasa de elongación. Mientras las isoformas ELOVL1, ELOVL3 y ELOVL6 elongan SFA y MUFA, las isoformas ELOVL2, ELOVL4 y ELOVL5 son selectivas para el metabolismo de los PUFA, alternándose con la actividad de las Δ5D y Δ6D (Wang Y, 2006). Sin embargo, no se han descrito los sustratos para la ELOVL7 (Leonard AE, 2004; Jakobsson A, 2006).

La elongación de los ácidos grasos ocurre en tres compartimentos celulares: citoplasma, mitocondria y RE (microsomas). En el citoplasma, la elongación es parte de la lipogénesis *de novo* y las enzimas involucradas son la acetil-CoA carboxilasa y la ácidos graso sintasa (FAS). La FAS utiliza acetil-CoA y malonil-CoA para elongar ácidos grasos con 2 carbonos. El producto final primario de la lipogénesis *de novo* es el palmitato (C16:0) y en menor proporción, el miristato (14:0) y el estearato (C18:0). En las mitocondrias, la vía minoritaria en las células eucariotas, las elongasas necesitan la enoil-CoA reductasa y acetil-CoA como sustratos (Leonard AE, 2004; Jakobsson A, 2006). Por último, la elongación microsomal es la vía predominante para la elongación de ácidos grasos de 12 carbonos y más (Leonard AE, 2004; Jakobsson A, 2006). Esta vía utiliza tanto ácidos grasos derivados de vías

endógenas como la lipogénesis *de novo*, como también vías exógenas como ácidos grasos derivados de la dieta. La mayoría de las vías de elongación microsomal envuelve 4 enzimas y acil-CoA, malonil-CoA y NADPH como sustratos. Primero, la acil-CoA sintasa cataliza la condensación del malonil-CoA con un precursor acil-CoA. Después se produce una reducción por la acil-CoA reductasa, seguida de una deshidratación y por último, se produce una última reducción. El producto final elongado puede ser utilizado para posteriores pasos del metabolismo, para ejercer sus funciones biológicas o puede ser sometido a un ciclo de elongación adicional en función de las necesidades específicas en las que se encuentra la célula (Cinti DL, 1992).

4. Modelo del nematodo *C.elegans*

La señalización de los ácidos grasos es fundamental para los organismos multicelulares, y los invertebrados simples emplean rutas biosintéticas similares a nivel funcional. Los nematodos están emergiendo como sistemas clave para el estudio de la biología de los lípidos. El desarrollo de métodos espectroscópicos y cromatográficos de elevada sensibilidad permite el análisis exhaustivo de los lípidos en estos sistemas. La anatomía sencilla y la amplia gama de herramientas genéticas disponibles en *Caenorhabditis elegans (C.elegans)* hacen ideal a este modelo para la investigación del metabolismo lipídico y de los efectos biológicos de lípidos específicos en un contexto de organismo entero. La mayoría de los estudios en estos modelos se ha centrado en la adiposidad y en el síndrome metabólico. Diversas revisiones han publicado una recogida de estudios sobre la regulación del almacenaje de grasa en *C. elegans* (Watts JL, 2002; Zheng J y Greenway FL, 2012).

Las plantas producen los PUFA esenciales omega-3 y omega-6 mediante la desaturación del ácido oleico (C18:1n-9) en las posiciones C12 y C15 para producir LA y ALA, pero rara vez poseen las enzimas para desaturar y elongar estas moléculas a PUFA de 20 carbonos. El nematodo *C. elegans* sintetiza un amplio rango de PUFA incluyendo AA y EPA a partir de SFA obtenidos de su dieta basada de *Escherichia coli* (Watts JL, 2002). La ruta biosintética de los PUFA en *C.elegans* desde 18:1n-9 hasta 20:5n-3 difiere de la ruta en mamíferos en que los nematodos pueden convertir 18:1n-9 en PUFA gracias a la presencia de la actividad de las Δ12 desaturasas (gen *fat-2*), ausente en mamíferos. Además, *C. elegans* puede convertir los PUFA omega-6 de 18 y 20 carbonos en PUFA omega-3 a través de la actividad omega-3 desaturasa (gen *fat-1*), mientras que en mamíferos, la ausencia de la omega-3 desaturasa resulta en rutas paralelas en la síntesis de los omega-6 y los

omega-3 (Watts JL, 2002). Con esta finalidad, *C. elegans* expresa un amplio rango de actividades desaturasas encontradas en plantas (Δ12 y omega-3 desaturasas) y en mamíferos (Δ5 y Δ6 desaturasas), así como también, sus actividades elongasas (Figura 9) (Watts JL, 2002).

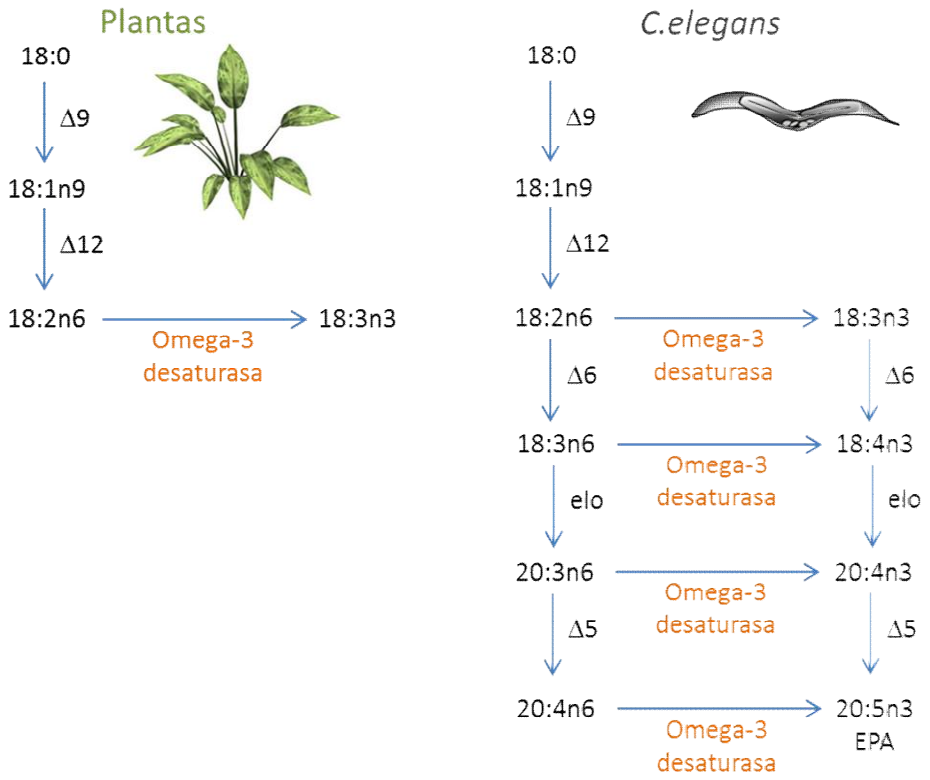

FIGURA 9. Vía metabólica de los PUFA omega-6 y omega-3. Las plantas producen los precursores de los PUFA (18:2n6 y 18:3n3) utilizando las desaturasas Δ12 y omega-3. Sin embargo, el nematodo C.elegans posee las enzimas requeridas hasta la síntesis de AA y EPA (imagen adaptada de Watts JL, 2002).

5. Papel de la enzima omega-3 desaturasa

Las omega-3 desaturasas son esenciales para la desaturación de los ácidos grasos omega-6 y convertirlos a ácidos grasos omega-3. Estas desaturasas están clasificadas como *methyl-end* en función de la posición de inserción del doble enlace en relación con el doble enlace pre-existente en la cadena hidrocarbonada (Aitzetmüller K y Tsevegsüren N, 1994). En función de la solubilidad, las omega-3 desaturasas están clasificadas como desaturasas de membrana y se distinguen de las *front-end* desaturasas y de las delta-9 desaturasas por la ausencia de un dominio citocromo b5 fusionado al N-terminal (Pereira SL, 2003). Este dominio es esencial para estas enzimas y las omega-3 desaturasas utilizan el citocromo b5 libre como dador de electrones. Las omega-3 desaturasas

también comparten los tres motivos típicos ricos en histidina que están probablemente involucrados en la catálisis de la reacción de desaturación (Mitchell AG y Martin CE, 1995).

Desde un punto de vista funcional, los microorganismos responden a la disminución de la temperatura ambiental mediante la desaturación de ácidos grasos en los PL de membrana para compensar la pérdida de fluidez de la membrana. De forma evolutiva, la razón por la cual aparecieron los genes de las omega-3 desaturasas podría ser por la necesidad de aumentar la tolerancia de las células a temperaturas bajas, las cuales podrían también determinar la elección del hábitat natural de los microorganismos (Sakamoto T, 1994).

Existen estudios genéticos recientes con aplicaciones que incluyen las omega-3 desaturasas procedentes del nematodo *C. elegans*. El doctor Kang y sus colaboradores desarrollaron un modelo transgénico de ratón con un metabolismo lipídico excepcional de entre los organismos mamíferos (Kang JX, 2004).

6. Modelo de ratones transgénicos *fat-1*

Los ratones transgénicos *fat-1* presentan características distintas al resto de mamíferos: sintetizan sus propios niveles de omega-3 de forma endógena. Estos ratones llevan un transgen llamado *fat-1*, procedente del nematodo *C.elegans*, cuya función es la de convertir los omega-6 a omega-3. Debido a esta nueva capacidad, los ratones *fat-1* tienen niveles aumentados de omega-3 y una relación omega-6/omega-3 equilibrada en todos sus tejidos y órganos, independientemente de la dieta (Kang JX, 2004).

La idea original fue introducir en células de mamífero un gen que codificara para una enzima convertidora de ácidos grasos, encontrada en el nematodo. Esta enzima tendría la capacidad de convertir ácidos grasos omega-6, que se encuentran de forma excesiva en las dietas y en los tejidos, a ácidos grasos omega-3, los cuales son escasos. Este concepto fue probado por primera vez en cultivo celular mediante la transferencia del gen mediada por adenovirus en miocitos cardíacos de rata (Figura 10) (Kang ZB, 2001). A partir de entonces, se generaron por microinyección del gen en oocitos fertilizados las primeras líneas de ratones transgénicos con actividad omega-3 desaturasa en todo su organismo (Kang JX, 2004). Esta expresión heteróloga de la enzima se produjo gracias a una modificación del gen por optimización del codón para células mamíferas en presencia de un promotor de β-actina, permitiendo una elevada y amplia expresión del transgen (Kang JX, 2004, 2007).

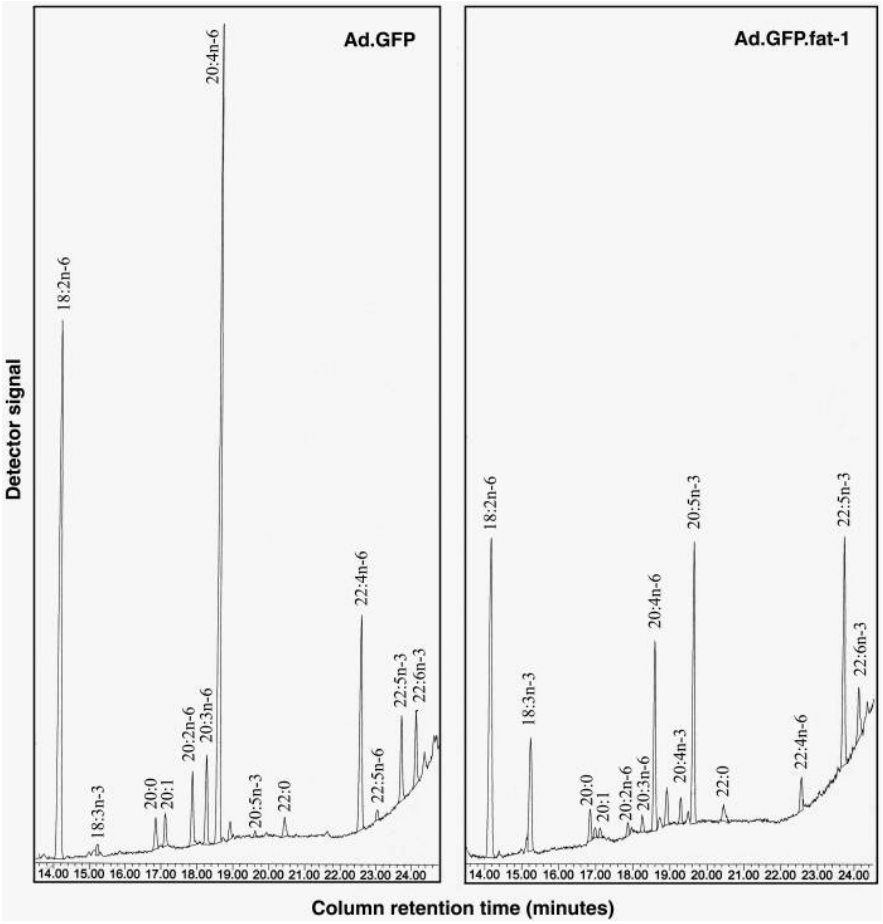

FIGURA 10. Perfil cromatográfico de ácidos grasos de la fracción total de lípidos en miocitos cardíacos de rata control infectadas con Ad.GFP y en células infectadas con Ad.GFP.fat-1. (Figura procedente de Kang ZB, 2001).

En comparación con el modelo de intervención dietética, la aproximación genética es más efectiva en alterar el balance tisular omega-6/omega-3 porque no sólo aumenta las cantidades absolutas de omega-3, sino que también disminuye los niveles de omega-6, promoviendo un balance omega-6/omega-3 equilibrado en todos los tejidos del organismo, sin cambiar la masa tisular de ácidos grasos (la cantidad total de omega-6 y omega-3 no varía pero la relación entre estos ácidos grasos es diferente entre los animales no transgénicos y los transgénicos) (Kang JX, 2004; 2007). Por ello, este modelo es ideal para dirigir alteraciones derivadas de un desequilibrio en la composición de ácidos grasos.

El uso del modelo de ratones *fat-1* permite la producción de dos perfiles de ácidos grasos diferentes (alto y bajo balance omega-6/omega-3) en una camada de ratones nacidos de la misma

madre utilizando una única dieta. Ambos ratones transgénicos y *salvajes* se mantienen bajo una dieta rica en ácidos grasos omega-6 con una pequeña cantidad de omega-3 (aproximadamente el 0.1% del suplemento lipídico total). Bajo este régimen dietético, los ratones *salvajes* contienen bajos niveles de omega-3 en sus tejidos. Además, en los ratones transgénicos, el cambio en la composición de ácidos grasos en los órganos (la conversión de omega-6 a omega-3) se produce en el estado embrionario y durará todo el tiempo de vida del animal (Kang JX, 2004; 2007).

III. OBESIDAD Y COMPLICACIONES ASOCIADAS: RESISTENCIA A LA INSULINA Y ENFERMEDAD DEL HÍGADO GRASO DE ORIGEN NO ALCOHÓLICO (EHGNA)

La obesidad está definida, según la OMS, como una acumulación excesiva de grasa que puede dañar la salud. La obesidad mundial ha doblado su prevalencia entre el 1980 y el 2014. De hecho, en el mismo año 2014 se registró que más de 1,9 billones (39%) de la población adulta mundial presentaban sobrepeso y que de entre ellos, unos 600 millones (13%) eran clínicamente obesos. La causa fundamental de la obesidad y el sobrepeso es el desequilibrio energético entre las calorías consumidas y las calorías gastadas. Globalmente, se ha producido un incremento de la ingesta energética a través de alimentos con elevado contenido graso, y una disminución de la actividad física debido al sedentarismo. Según la OMS, la obesidad y las enfermedades asociadas son prevenibles, y su prevención debe abordarse de modo individual, a través de cambios de hábitos más saludables (OMS, 2015).

Clasificación	IMC (kg/m^2)	Nivel de riesgo asociado
Delgadez	<18,5	Bajo (pero aumenta el riesgo de otros problemas clínicos)
Normal	18,5 – 24,9	Medio
Sobrepeso	**25,0 o superior**	
Pre-obesidad	25,0 – 29,9	Medio-Alto
Obesidad clase I	30,0 – 34,9	Alto
Obesidad clase II	35,0 – 39,9	Severamente alto
Obesidad clase III	40 o superior	Extremadamente alto

TABLA 3. Clasificación de sobrepeso y obesidad según el índice de masa corporal (IMC) reportado por la OMS.

La obesidad representa el mayor factor de riesgo de diversas enfermedades tales como las enfermedades cardiovasculares, la diabetes, los desórdenes musculoesqueléticos (osteoartritis), algunos tipos de cáncer (endometrial, mama y colon) y la enfermedad del hígado graso de origen no alcohólico (EHGNA) (Mirza MS, 2011).

La EHGNA representa el desorden hepático más común y la causa más frecuente de enfermedad hepática crónica. La EHGNA es un síndrome con etiología multifactorial y representa la manifestación hepática del síndrome metabólico. El síndrome metabólico es una constelación de factores de riesgo estrechamente relacionados entre si y caracterizados por la presencia de obesidad central, hiperglicemia, hipertrigliceridemia, bajos niveles de HDL e hipertensión arterial (Festi D, 2004; Youssef WI, 2002; Reaven G, 2002).

1. Prevalencia y epidemiología de EHGNA

La EHGNA es la condición definida por una excesiva acumulación de lípidos en los hepatocitos, principalmente TG, que excede el 5% del peso del hígado, en pacientes que no presentan infección por virus de la hepatitis B o C, ni una excesiva ingesta de alcohol (no superior a una ingesta de 20 g de alcohol al día) (Angulo P, 2002; 2007). La EHGNA incluye un amplio espectro morfológico de anormalidades hepáticas que van desde la esteatosis hasta la esteatohepatitis no alcohólica, y en algunos casos puede progresar a cirrosis (Angulo P, 2002).

La prevalencia de EGHNA está aumentando a nivel mundial paralelamente con el aumento de la obesidad. Concretamente, de entre la población obesa la prevalencia de hígado graso o esteatosis en pacientes obesos es de 70-100% mientras que la esteatohepatitis se encuentra entre el 20 y el 25% y la cirrosis entre el 2 y el 3% (Figura 11) (Haynes P, 2004; Dixon JB, 2001).

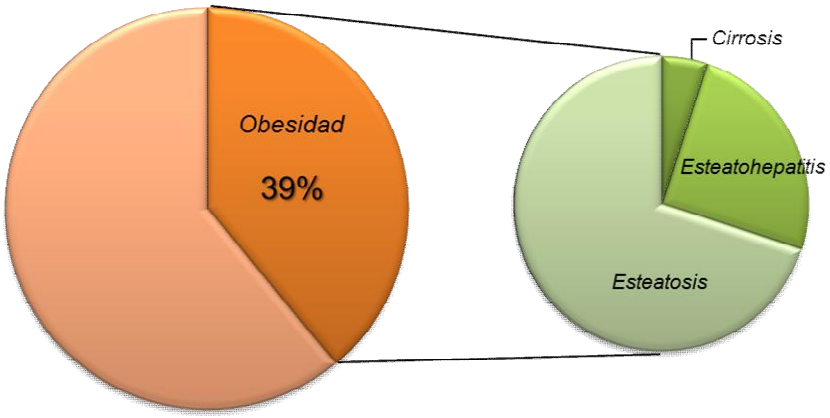

FIGURA 11. Prevalencia estimada de esteatosis, esteatohepatitis y cirrosis entre la población obesa mundial.

La EHGNA está considerada actualmente como una de las enfermedades hepáticas más comunes. A través de diversos estudios epidemiológicos (analíticos, histológicos y ecográficos) se ha calculado que entre el 20 y el 30% de la población de los países occidentales presenta EHGNA y mientras que un 15% la padece en los países orientales (Bellentani S, 2010). En los estudios *Dallas Heart Study* y el *Dionysios Nutrition and Liver Study* se publicó que el 30 y 25 % de la población adulta de EEUU y de Italia presentaban EHGNA, respectivamente (Browning JD, 2006; Bedogni G, 2005).

La prevalencia de EHGNA posiblemente está subestimada, debido a la gran proporción de pacientes asintomáticos, que presentan alteraciones biológicas discretas y no se someten a biopsia hepática. Sin embargo, esta prueba es difícil y costosa. Por ello, la ultrasonografía es la técnica comúnmente utilizada para el diagnóstico (Caballería L, 2010). En un estudio realizado en España empleando este método, se obtuvo una prevalencia del 25.8% en el país, valor coincidente con los porcentajes referidos a la prevalencia de los países occidentales en los últimos años (Caballería L, 2010).

2. Manifestaciones clínicas y progresión de EHGNA

La EHGNA es una forma crónica de enfermedad hepática histológicamente indistinguible de la hepatitis alcohólica (Angulo P, 2002). La principal característica histológica de EHGNA es la presencia de cambios lipídicos macrovesiculares en los hepatocitos que provocan un desplazamiento del núcleo hacia la membrana celular (Brunt EM, 1999).

En los años 70, se observó que la esteatohepatitis afectaba también a pacientes con obesidad mórbida sometidos a *bypass* yeyunoileal (Peters RL, 1975). A partir de entonces se reconoció como una nueva entidad patológica por Ludwig quien en 1980 acuñó el término de esteatohepatitis no alcohólica (EHNA) (Ludwig J, 1980). Mientras que la esteatosis es generalmente una condición benigna e irreversible, la EHNA se define como el punto de inflexión entre la esteatosis y la fibrosis avanzada (Haynes P, 2004; Reid AE, 2001). La EHNA se caracteriza por una infiltración lipídica intrahepática acompañada de necroinflamación, daño hepatocelular y fibrosis pericelular o perisinusoidal (Haynes P, 2004; Reid AE, 2001). La EHNA no resuelta puede culminar en formas más agresivas e irreversibles, como la cirrosis y el hepatocarcinoma celular (Figura 12) (Angulo P, 2002).

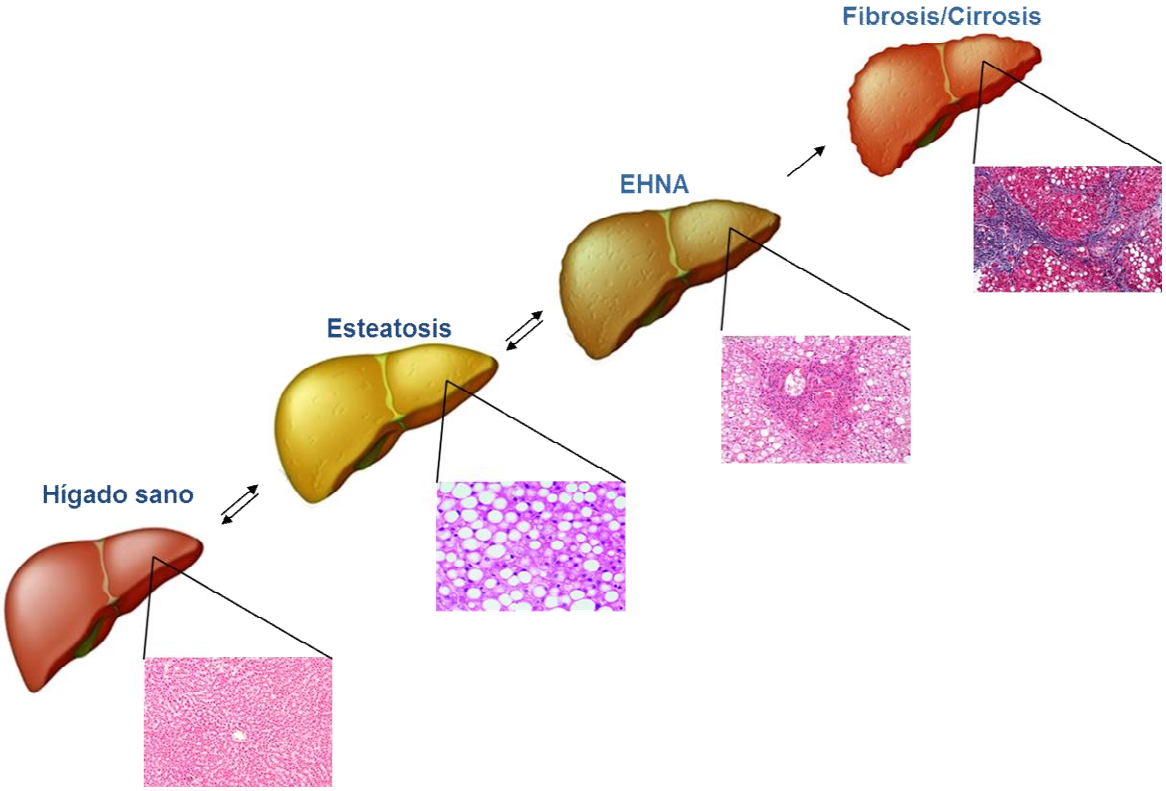

FIGURA 12. Progresión de la EHGNA en sus diferentes etapas (esteatosis, EHNA y fibrosis-cirrosis).

Asumiendo que la EGHNA es un espectro de patologías con incremento de grados de severidad, en 1998 Day y James presentaron la teoría de los dos "hits" para explicar la patogénesis de EHGNA (Day CP y James OF, 1998). De acuerdo con esta hipótesis, la acumulación de lípidos en el hígado origina el primer *"hit"* y es el prerrequisito para desarrollar daño hepatocelular, mientras que la disfunción mitocondrial, la liberación de citoquinas y el estrés del RE representan el segundo *"hit"* para la progresión de EHNA (Day CP y James OF, 1998; Levene AP, 2012).

Actualmente, esta hipótesis sigue liderando en el campo, sin embargo, se están proponiendo nuevos planteamientos debido a las reconocidas interacciones entre la resistencia a la insulina, las adipoquinas y la inflamación del tejido adiposo. En particular, se ha introducido el modelo de *"múltiples-hits",* en el cual la esteatosis hepática podría representar un epifenómeno de diferentes mecanismos patológicos (Figura 13) (Tilg H, 2010). En el modelo de *"múltiples-hits"*, el primer *"hit"* es

la resistencia a la insulina que provoca la aparición de esteatosis como consecuencia de alteraciones del metabolismo lipídico. La hiperinsulinemia, causada por la resistencia a la insulina resulta de un incremento de la lipogénesis *de novo* hepática. La disfunción del tejido adiposo también tiene consecuencias negativas en el hígado debido a que ambos tejidos tienen acceso inmediato a una vasta red de vasos sanguíneos que facilitan la conexión directa entre ellos. Por lo tanto, un defecto en la inhibición de la lipólisis del tejido adiposo promueve la liberación ácidos grasos libres (AGL) al torrente circulatorio. Esto produce un aumento en el flujo de AGL desde el tejido adiposo hasta el hígado. En el hígado, los AGL se oxidan para generar ATP o son esterificados para producir TG, los cuales pueden ser incorporados en lipoproteínas de muy baja densidad (VLDL) para ser exportados. Defectos en estos procesos producen la esteatosis hepática. La progresión a inflamación y fibrosis proviene del estrés oxidativo, el segundo *"hit"*, en el cual las sustancias oxidantes son poco controlables por los mecanismos antioxidantes del hígado. Las alteraciones en las adipoquinas como resistina, leptina, adiponectina y las citoquinas inflamatorias IL-6 y el factor de necrosis tumoral alfa (TNFα) también juegan un papel importante en este proceso (Sanyal AJ, 2005). Finalmente, el hígado se convierte extremadamente vulnerable a toda una serie de *"hits"* que pueden progresar al daño hepatocelular, fibrosis y cirrosis (Tilg H, 2010).

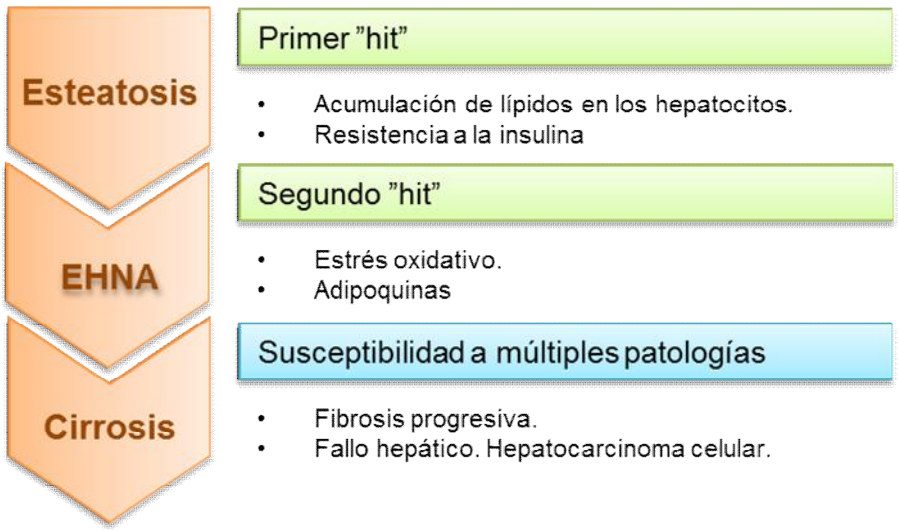

FIGURA 13. Hipótesis de múltiples "hits" de la patogénesis de EHGNA.

3. Alteraciones del tejido adiposo

La obesidad se caracteriza por una expansión de la masa del tejido adiposo como consecuencia de un aumento en el número y en el tamaño de los adipocitos (Kershaw EE, 2004). La obesidad es considerada en sí misma una condición heterogénea debido a que la distribución del tejido adiposo parecer ser más importante que la masa total. Hay una evidencia creciente que la distribución del tejido adiposo, especialmente en la zona abdominal, es importante para el desarrollo de complicaciones metabólicas asociadas (Kershaw EE, 2004).

El tejido adiposo es reconocido como un órgano endocrino con propiedades biológicas distintivas, siendo capaz de segregar una variedad de péptidos bioactivos o adipoquinas que ejercen múltiples efectos a nivel local y sistémico (Lyon JC, 2003; Kershaw EE, 2004). Las adipoquinas segregadas por el tejido adiposo influyen tanto en la homeostasis del peso corporal como en la inflamación, la coagulación, la fibrinólisis, la resistencia a la insulina, la aterosclerosis y el cáncer. Las principales adipoquinas son leptina, adiponectina, resistina, TNFα, TGF-β, PAI-1, angiotensina II, interleuquinas IL-6, IL-8, IL-10, entre otras (Hauner H, 2005).

En individuos obesos, el tejido graso se convierte insensible a la acción de la insulina produciendo la descomposición de los TG. La abundancia de los ácidos grasos circulantes derivados de una excesiva adiposidad contribuye al desarrollo de la resistencia a la insulina (Eckel RH, 2005). La liberación de las adipoquinas del tejido adiposo recibe la influencia de la insulina. Bajo condiciones de peso normal, las adipoquinas garantizan la homeostasis de la glucosa y del metabolismo lipídico. Sin embargo, una producción alterada en un estado obeso juega un papel importante en el desarrollo del síndrome metabólico. Por otro lado, las condiciones de obesidad con deficiencia o resistencia a la leptina promueven lipotoxicidad debido a la acumulación de TG en órganos no adiposos como hígado, músculo y páncreas (Eckel RH, 2005).

El estado de inflamación crónica de bajo grado en el tejido adiposo aumenta con la infiltración de macrófagos. Se ha demostrado que la reducción de la grasa produce un dramático descenso en la cantidad y distribución de los macrófagos acompañado de una disminución en la expresión de marcadores de la inflamación. Además de la infiltración de macrófagos, la hipertrofia de adipocitos y la hiperplasia son seguidas por una aumentada angiogénesis y producción de la matriz extracelular (Catalán V, 2012). Adicionalmente, la hipertrofia de los adipocitos genera un obstáculo para un

suficiente aporte de oxígeno a las células provocando un estado de hipoxia seguido de apoptosis celular (Trayhurn P, 2013).

En global, la obesidad está relacionada con una desregulación de la liberación de adipoquinas insulino-resistentes y proinflamatorias, acompañada por una reducción en la secreción de adipoquinas antiinflamatorias e insulino-sensibles en el tejido adiposo (Hotamisligil GS, 1995).

3.1 Resistencia a la insulina

El tejido adiposo requiere la presencia de la insulina para captar la glucosa, haciendo que la señalización de la insulina sea crítica para la regulación de la homeostasis de la glucosa. Una dieta no saludable y la obesidad inducen la resistencia a la insulina (Gordon S y Taylor PR, 2005; Ouchi N, 2011; Hotamisligil GS, 1995).

La mayor contribución al desarrollo de resistencia a la insulina es la abundancia de ácidos grasos circulantes. La albúmina plasmática se une a los AGL principalmente procedentes de los TG del tejido adiposo liberados a través de la acción de la enzima lipasa sensible a hormona (LSH). Los ácidos grasos provienen también de la lipólisis de las lipoproteínas ricas en TG por la acción de la lipoproteína lipasa (LPL). En un estado de resistencia a la insulina, la acumulación de ácidos grasos debido a un aumento de la lipólisis de moléculas de TG inhibe el efecto antilipolítico de la insulina, creando una lipólisis adicional. Los AGL son liberados del tejido adiposo en abundancia. En el hígado, los AGL aumentan la producción de glucosa y TG y la secreción de VLDL, acompañada de una reducción del colesterol HDL y un aumento del LDL. Paralelamente, también se produce una reducción de la síntesis de glicógeno y una acumulación de lípidos en los TG. El aumento de la glucosa circulante y de los AGL aumenta la secreción de insulina pancreática resultando en un estado de hiperinsulinemia (Gordon S y Taylor PR, 2005; Ouchi N, 2011; Hotamisligil GS, 1995).

Las vías de señalización de la insulina, quinasa c-jun-N-terminal (JNK) y quinasa k (IKK) se activan por sensores de estrés a través de mecanismos mediados por receptores (Shoelson SE, 2006). A su vez, la activación de JNK y IKK induce resistencia a la insulina por una alteración de la fosforilación de la serina en el receptor de la insulina IRS-1 (Shoelson SE, 2006).

Un factor primordial que se superpone y contribuye a la resistencia a la insulina producida por el exceso de AGL es el efecto paracrino y endocrino del estado proinflamatorio crónico establecido en la obesidad. Debido a una variedad de células en el tejido adiposo incluyendo adipocitos y

macrófagos derivados de monocitos, la secreción aumentada de IL-6 y TNFα es resultante del incremento de la lipólisis de los TG del tejido adiposo. Las citoquinas proinflamatorias y los AGL también aumentan la producción hepática de fibrinógeno y del inhibidor activador de plasminógeno-1 (PAI-1) que a su vez complementa una sobreproducción de PAI-1 en el tejido adiposo. Este estado viene acompañado por una disminución de la citoquina sensible a la insulina, la adiponectina (Shoelson SE, 2006).

3.2 Inflamación

El mecanismo patogénico clave responsable de una secreción alterada de adipoquinas que provocan la resistencia a la insulina y co-morbilidades asociadas a la obesidad, como EHGNA, es la existencia de un estado inflamatorio crónico denominado de "bajo grado" en el tejido adiposo (Shoelson SE, 2006; Gregor MF y Hotamisligil GS, 2011). La inflamación de "bajo grado" es por definición una respuesta inflamatoria crónica promovida por un excedente de energía (Shoelson SE, 2006; Gregor MF y Hotamisligil GS, 2011). La primera vez que se reconoció que el adipocito tiene la capacidad de segregar citoquinas fue cuando se describió la producción de TNFα estimulada por un estado de obesidad (Hotamisligil GS, 1993; 1995). De hecho, los adipocitos en obesidad tienen la capacidad de segregar una diversidad de adipoquinas que participan en la inflamación tales como TNFα, IL-1, IL-6, MCP-1, IL-8, IL-18, entre otras (Trayhurn P, 2004; Rajala MW, 2005).

Además, en estado de obesidad se produce la infiltración de células inmunológicas en el tejido adiposo que producen la síntesis y la secreción de factores proinflamatorios a la circulación sanguínea (Neels JG, 2006). La mayoría de citoquinas producidas en el tejido adiposo obeso son las secretadas por los macrófagos y actualmente se considera que las complicaciones asociadas son en gran parte debidas al reclutamiento y a la activación de los macrófagos (Neels JG, 2006). De hecho, se ha correlacionado positivamente la acumulación de macrófagos con el IMC y el tamaño del adipocito (Weisberg SP, 2003).

Los macrófagos presentan una función heterogénea y los factores ambientales condicionan sus propiedades y estados de activación (Gordon S y Taylor PR, 2005). La activación de los macrófagos se define entre dos estados de polarización diferentes, el M1 y el M2 (Figura 14) (Lumeng CN, 2007). Los macrófagos en estado M1 o "clásicamente activados" se inducen por mediadores proinflamatorios como LPS e IFNγ y son los que aumentan la producción de citoquinas

proinfllamatorias y especies reactivas de oxígeno como NO (óxido nítrico) mediante la activación de la óxido nítrico sintasa inducible (iNOS). Los macrófagos M2 o "alternativamente activados" se inducen *in vitro* ante la exposición a IL-4 e IL-13 y tienen una expresión reducida de citoquinas proinflamatorias y, a su vez, generan citoquinas antiinflamatorias como IL-10 (Gordon S y Taylor PR, 2005). Adicionalmente, aumentan la producción de arginasa y tienen la función de bloquear la actividad de iNOS (Lumeng CN, 2007).

No todos los macrófagos del tejido adiposo están programados para la promoción de la inflamación, sino que también se ha detectado en ratones normales la presencia de una cantidad moderada de macrófagos M2 (Lumeng CN, 2007). De hecho, en un estudio en modelo animal se ha demostrado que los macrófagos con fenotipo M2 se activan y se polarizan a un estado proinflamatorio M1 en estado de obesidad (Figura 14) (Weisberg SP, 2003)

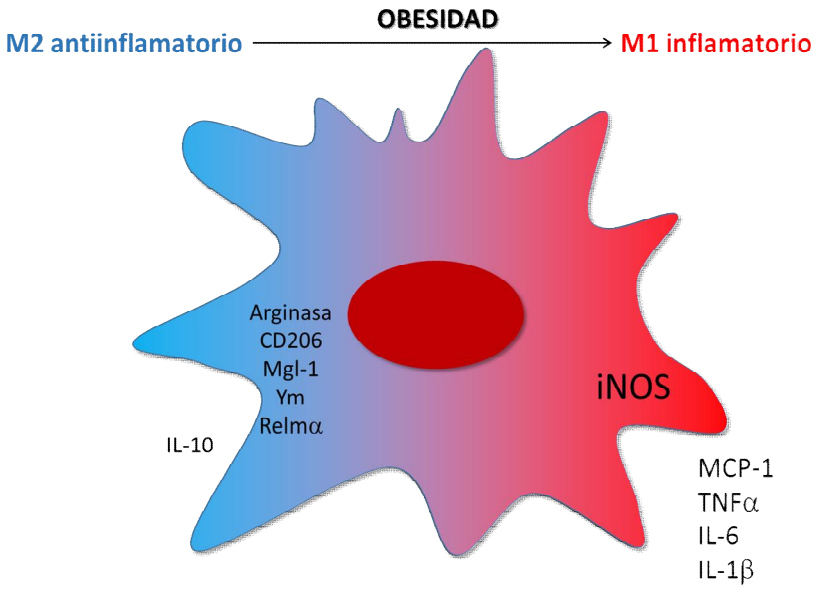

FIGURA 14. Estados de activación de los macrófagos en el tejido adiposo inflamado durante la obesidad. Polarización de marcadores antiinflamatorios (IL-10) a marcadores proinflamatorios (MCP-1, TNFα, IL-6 e IL-1β).

4. Estrés del retículo endoplasmático (ER) y autofagia

La obesidad también está caracterizada por el estrés del RE (Ozcan U, 2004). El estrés del RE induce el proceso de autofagia y por ello existen interacciones entre las vías canónicas de ambos procesos (Figura 15) (Kouroku Y, 2007; Ogata M, 2006).

El RE es un orgánulo membranoso que participa en el plegamiento y el tráfico proteico, en la síntesis lipídica y en el mantenimiento de la homeostasis del calcio, entre otras funciones celulares importantes (Smith MH, 2011). El RE mantiene la homeostasis celular actuando como sensor de cambios celulares, traducidos como estrés, a nivel intracelular y extracelular (Hotamisligil GS, 2010). Cuando las proteínas desplegadas o malformadas se acumulan en el lumen del RE, se activa la respuesta adaptativa conocida como UPR (del inglés *Unfolded Protein Response*) (Ron D, 2007). La UPR consiste en tres vías principales mediadas por tres proteínas asociadas a la membrana: PERK (PKR-like eukaryotic initiation factor 2a kinase), IRE1 (inositol requiring enzyme 1) y ATF6 (activating transcription factor-6) (Figura 15). Estos sensores pueden monitorizar cambios en el lumen del ER y activar vías de señalización. Bajo condiciones de no estrés, estos sensores se combinan y permanecen en su estado desactivado. Cuando las proteínas malformadas se acumulan en el lumen del RE, los sensores UPR se oligomerizan causando la activación de PERK y IRE1 que iniciará la cascada de señalización para aliviar el estrés del RE mediante la reducción de la síntesis de proteínas, promoviendo su degradación y produciendo chaperonas para asistir su plegamiento. Un estrés excesivo o prolongado puede provocar la muerte celular por apoptosis (Hampton RY, 2000). El estrés del RE participa en el curso de la activación de la inflamación asociada a la obesidad (Ozcan U, 2004; Hotamisligil GS, 2010). Durante la respuesta de proteínas desplegadas pueden producirse una respuesta inflamatoria y una transducción defectiva en la señalización de la insulina (Hotamisligil GS, 2008).

La autofagia participa en el proceso de degradación de las proteínas malformadas y facilita la homeostasis celular bajo condiciones de estrés como el estrés oxidativo o el estrés del RE (Mizushima N, 2008). Existen evidencias que muestran que la autofagia está asociada con el RE como parte importante de su función normal (Yin JJ, 2012; Yorimitsu T, 2007). La autofagia inducida por el estrés de RE juega un papel importante en el mantenimiento de la homeostasis celular a través de la mitigación del estrés y supone un proceso alternativo de degradación de proteínas desplegadas que se han acumulado en el lumen del RE (Ding WX y Yin XM, 2008).

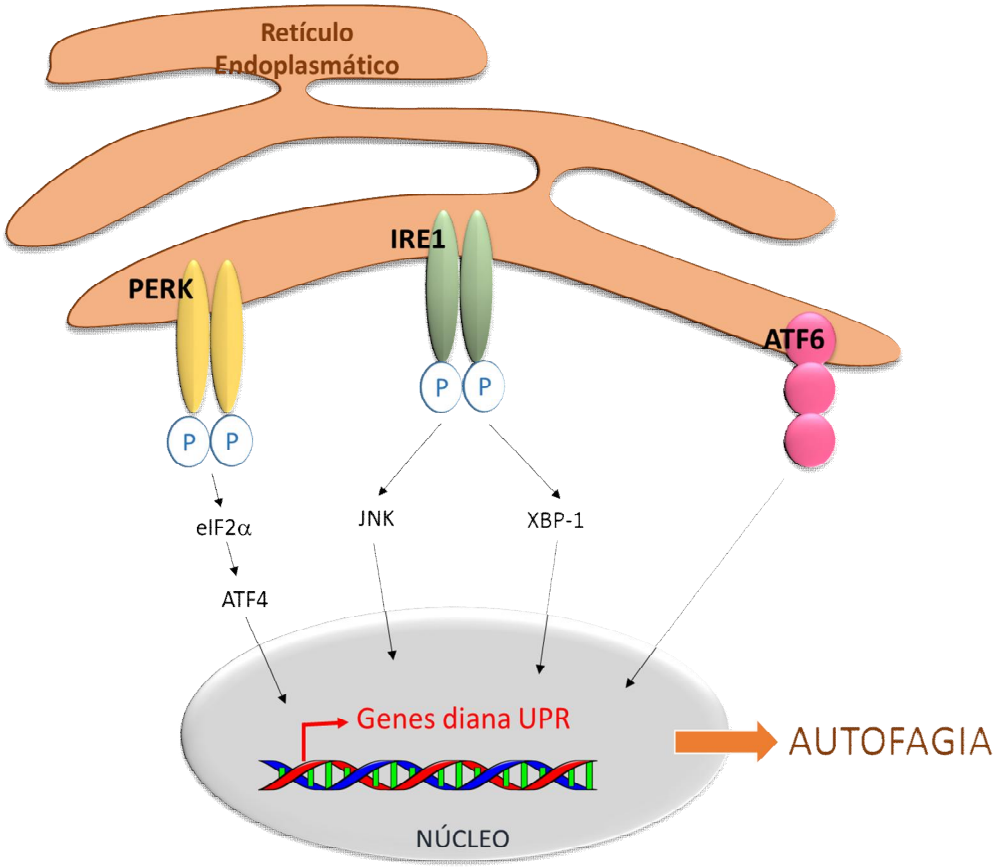

FIGURA 15. Cascada de señalización mediada por la UPR. Ante una situación de estrés del RE, la autofagia es inducida.

La autofagia es un proceso altamente regulado involucrado en el recambio de proteínas de larga vida, componentes citosólicos u orgánulos dañados (Yorimitsu T y Klionsky DJ, 2007). Existen tres tipos de autofagia: la autofagia mediada por chaperonas, la microautofagia y la macroautofagia (Yang Z y Klionsky DJ, 2010). La última es la más estudiada y referida aquí como autofagia. En ésta, se forman vesículas de doble membrana llamadas autofagosomas que secuestran sustratos para su degradación. La membrana externa del autofagosoma se fusiona con la del lisosoma, formando un autolisosoma, donde la membrana interna del autofagosoma y su contenido se digieren por acción de las hidrolasas lisosomales (Figura 16). Las macromoléculas resultantes son recicladas en el citoplasma celular para su reutilización (Abounit K, 2012; Yoshimori T, 2008). La elongación y formación de los autofagosomas son controladas por proteínas de autofagia denominadas ATG (ATG5, ATG7, ATG8, ATG12 y ATG16L1). El homólogo para los mamíferos del ATG8, LC3, se usa normalmente como marcador de autofagia debido a que se encuentra conjugado con el lípido

fosfatidiletanolamina formando LC3-II. (Yoshimori T, 2008). Las proteínas ATG son esenciales para el proceso de autofagia y participan en sus diferentes estadios conocidos como: inducción, reconocimiento, nucleación de la membrana, elongación de la membrana, formación del autofagosoma, fusión del autofagosoma con el lisosoma y degradación final (Abounit K, 2012).

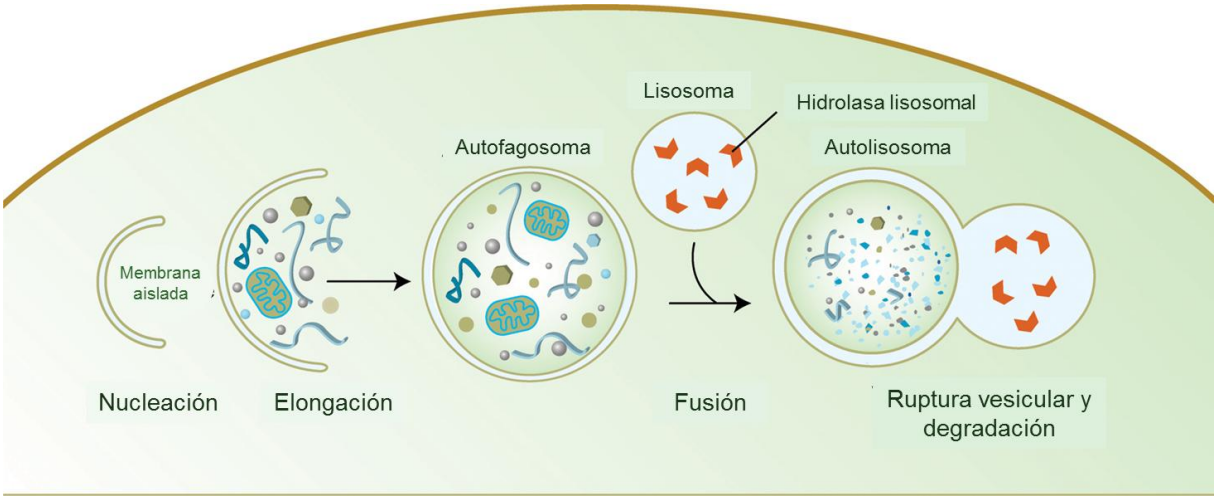

FIGURA 16. Diagrama esquemático del proceso de autofagia (Figura adaptada de Meléndez A y Levine B, Online Review of C. elegans Biology: Autophagy in C.elegans).

La autofagia está implicada en el desarrollo celular y la diferenciación, en varias enfermedades incluyendo cáncer y desórdenes neurodegenerativos, envejecimiento e inmunidad innata y adquirida (Wong ASL, 2011). Funciona principalmente como un proceso catabólico que genera energía para las células bajo condiciones de deficiencia de nutrientes y ayuda a mantener la homeostasis en un ambiente rico de nutrientes a través de su actividad constitutiva (Yorimitsu T y Klionsky DJ, 2007). De hecho, una desregulación del proceso puede participar en el desarrollo de enfermedades (Cecconi F y Levine B, 2008). En obesidad, el organismo no se puede adaptar y mantener la homeostasis bajo condiciones de continua exposición a energía y al aporte de nutrientes, y como consecuencia, el metabolismo y el estrés oxidativo promueven una respuesta inflamatoria y una disfunción de los orgánulos celulares (Hotamisligil GS, 2010). La exposición crónica a elevada energía y la ingesta de nutrientes aumenta la demanda de la maquinaria de degradación y síntesis celular en tejidos tales como hígado, tejido adiposo y páncreas. Un ineficiente recambio de

macromoléculas, como los lípidos o el glicógeno, puede comprometer al metabolismo hepático y a la acción de la insulina (Mizushima N, 2010; Reggiori F, 2005).

Hay evidencias que implican el proceso de autofagia en la regulación del almacenaje de lípidos entre los dos órganos principales involucrados en el mantenimiento de la homeóstasis lipídica, el hígado y el tejido adiposo. Una autofagia aberrante puede estar involucrada en la desregulación de la homeóstasis lipídica en desórdenes metabólicos (Christian P, 2013). Concretamente, en el tejido adiposo de pacientes obesos se encontró una actividad autofágica aumentada, indicando que la autofagia podría contribuir al desarrollo de la obesidad (Kovsan J, 2011; Ost A, 2010). Además, la autofagia también regula la acumulación lipídica en adipocitos. Un estudio en animales demostró que la deleción de los genes ATG5 y ATG7 generaba una limitada capacidad para almacenar TG en el tejido adiposo con una consecuente reducción de la grasa. Estos resultados, y en concordancia con otros estudios, sugirieron que la autofagia podría jugar un papel importante en el proceso de adipogénesis (Goldman S, 2010; Singh R, 2009; Zhang Y, 2009).

En hepatocitos, la función crítica de la autofagia es la ruptura de los “lipid droplets” citoplasmáticos, un proceso conocido como lipofagia. (Christian P, 2013). Se ha demostrado en modelo animal de ratón que la deleción de genes implicados en la autofagia produce un aumento de la acumulación lipídica hepática (Singh R, 2009). Además, la autofagia juega un papel importante en el hígado graso ya que es un proceso que también se encuentra directamente relacionado con la resistencia a la insulina. En pacientes obesos con esteatosis, la autofagia es insuficiente en hepatocitos y se ha demostrado que la sobreregulación de este proceso aumenta la sensibilidad a la insulina (Rautou PE, 2010).

Objetivos

La sociedad occidental ha sufrido cambios nutricionales en cuanto a la proporción en el consumo de ácidos grasos poliinsaturados ocasionando un balance omega-6/omega-3 elevado acompañado de una disminución en los niveles de omega-3. Esta relación en la dieta promueve en el organismo una mayor formación de mediadores derivados de los omega-6 con marcada función inflamatoria. La obesidad es la enfermedad inflamatoria que afecta a niveles epidémicos en los países occidentales. Desde que la obesidad es un problema emergente de salud pública, la identificación de nuevas estrategias terapéuticas es primordial para la prevención y el tratamiento de enfermedades metabólicas asociadas a la obesidad.

El objetivo esencial de este trabajo es investigar la efectividad de un balance equilibrado entre los ácidos grasos poliinsaturados esenciales omega-6 y omega-3 en la prevención de la enfermedad hepática no alcohólica asociada a la obesidad.

Con este propósito se ejecutaron los siguientes objetivos específicos:

1. *Investigar nuevas dianas moleculares para la prevención y el tratamiento de esteatohepatitis mediante la integración de datos en pacientes, experimentos en un modelo animal de la enfermedad e intervenciones farmacológicas con moléculas de nueva generación.*
    - *a.* Identificar vías génicas asociadas a la acumulación hepática de ácidos grasos y la inflamación y analizar el perfil hepático de ácidos grasos en modelo humano y animal de esteatohepatitis no alcohólica inducida por obesidad.
    - *b.* Investigar los efectos de la aportación endógena y exógena de omega-3 sobre esteatosis, inflamación y resistencia a la insulina hepáticas.
    - *c.* Investigar los efectos de la inhibición de las enzimas desaturasas en presencia de omega-3 en cultivo primario de hepatocitos.

2. *Modulación de la actividad de la epóxido hidrolasa soluble mediante su inhibición en ratones transgénicos fat-1 para investigar el papel de los metabolitos de la vía del CYP derivados de los omega-3, tanto en hígado como en tejido adiposo en estado obeso.*
    - *a.* Perfilar el patrón de expresión de las isoformas del CYP y los niveles de epóxidos derivados en hígado y tejido adiposo.
    - *b.* Investigar los efectos de la inhibición de la sEH sobre los parámetros histomorfométricos y bioquímicos en hígado y tejido adiposo.

c. Estudiar los efectos *in vivo* e *in vitro* de la inhibición de la sEH sobre los procesos de autofagia y de estrés del retículo endoplasmático.

Resultados

I. ARTÍCULO 1

Interacción molecular entre las Δ5/Δ6 desaturasas y los ácidos grasos en la patogénesis de la esteatohepatitis no alcohólica (EHNA).

1.1 Sobrerregulación de genes involucrados en la biosíntesis hepática de ácidos grasos insaturados en pacientes con EHNA.

Se obtuvieron biopsias hepáticas de ocho pacientes con esteatohepatitis asociada a obesidad mórbida y siete controles (donantes cadavéricos y resección de metástasis) y se analizaron mediante análisis transcriptómico de alto rendimiento. Se identificó un grupo de 157 genes diferencialmente expresados en pacientes con EHNA. El análisis jerárquico de los genes que se sobreexpresaron reveló 10 vías canónicas comprometidas de entre las cuales destacó la biosíntesis de ácidos grasos insaturados como vía implicada en el desarrollo de la enfermedad. En el análisis funcional se identificaron los genes candidatos FADS1 (Δ5-desaturasa), SCD-1 (Δ9-desaturasa), FADS2 (Δ6-desaturasa), ELOVL6 (fatty acid elongase 6), ELOVL5 (fatty acid elongase 5) y SC5DL (sterol-C5-desaturase-like). En concreto, se observó una sobreexpresión génica significativa de las desaturasas en hígado de pacientes con EHNA.

El análisis cromatográfico reveló el aumento de la relación omega-6/omega-3 y la reducción del índice omega-3 (EPA+DHA) en los pacientes con EHNA. En estos pacientes también se observó un aumento de la relación 18:1/18:0 como marcador indirecto de desaturación Δ9. Estos resultados se reprodujeron en un modelo de ratones *salvajes* C57BL/6 obesos tras recibir una dieta hipercalórica (60% cal de la grasa) durante 16 semanas.

1.2 Impacto del enriquecimiento endógeno y exógeno de omega-3 sobre los flujos de desaturación en el tejido hepático.

El análisis del perfil lipídico por cromatografía de gases reveló una disminución en los niveles hepáticos de DHA acompañada de un aumento significativo en los niveles de AA en el grupo de ratones obesos que recibieron una dieta hipercalórica (60% cal de la grasa) en comparación con el grupo de ratones que recibieron dieta normal (13% cal de la grasa) durante 16 semanas. Además, los ratones obesos presentaron una relación 20:4n6/20:3n6 más elevada y una relación 20:5n3/20:4n3

disminuida, como marcadores indirectos de la síntesis de AA y EPA, respectivamente. Este resultado muestra una alteración en la desaturación Δ5 a nivel de la ruta sintética de los omega-3 en la enfermedad de EHNA.

En el grupo de ratones *fat-1*, tras ser inducidos a obesidad con la dieta, se observó el reestablecimiento hepático de la relación omega-6/omega-3, y del índice omega-3. Estos resultados se compararon con un modelo de enriquecimiento exógeno de EPA/DHA a través de la dieta, el grupo dieta omega-3. Los flujos de saturación hacia la biosíntesis de los omega-3 aumentaron tanto en el grupo *fat-1* como en el grupo dieta omega-3. Sin embargo, el aumento del flujo de desaturación Δ5 hacia omega-3, estimado con la relación 20:5n3/20:4n3, resultó ser más evidente en el grupo dieta omega-3, probablemente como consecuencia del aporte de una dieta enriquecida con EPA, mientras que el aumento en el flujo de desaturación Δ6 hacia la vía de los omega-3, estimado con la relación 18:4n3/18:3n3, fue más evidente en los ratones *fat-1*, enriquecidos endógenamente. Además, la relación de desaturación mediante la Δ9 desaturasa entre los ácidos grasos moninsaturados y los saturados (16:1/16:0 y 18:1/18:0), disminuyó tanto por el enriquecimiento endógeno como exógeno de omega-3. Además, el enriquecimiento con omega-3 reprimió la expresión de las desaturasas tanto a nivel génico como proteico.

1.3 La expresión transgénica de la omega-3 desaturasa protege de la adiposidad y de la resistencia a la insulina inducida por una dieta hipercalórica.

La administración de una dieta hipercalórica durante 16 semanas produjo un aumento significativo del peso corporal y de los tejidos hepático y adiposo, así como también de los niveles séricos de colesterol y triglicéridos en los ratones control en comparación con los ratones que recibieron una dieta normal. Bajo estas condiciones, los ratones *fat-1* ingirieron una cantidad diaria de alimentos similar al resto de grupos pero presentaron una ganancia de peso menor en comparación con el grupo control. En general, los ratones con el transgen de la omega-3 desaturasa demostraron una mejora de los parámetros bioquímicos séricos y una resistencia a los efectos adipogénicos y esteatogénicos de una dieta hipercalórica.

El grupo de ratones de dieta omega-3 mantuvieron la ganancia de peso a pesar de controlar los niveles de colesterol y triglicéridos en suero. Además, los ratones *fat-1* corrigieron la hiperinsulinemia y la hiperglucemia. El análisis de la fosforilación de la JNK1/JNK2, como marcadores de la resistencia

a la insulina, reveló una mejora significativa en la sensibilidad hepática a la insulina en ratones *fat-1* obesos.

1.4 Prevención de la esteatosis hepática inducida por la obesidad tras recibir una dieta hipercalórica en el grupo de ratones *fat-1* y en el grupo dieta omega-3.

La dieta hipercalórica indujo en los ratones control un aumento de la tinción de los lípidos intrahepáticos mediante Oil-Red O en comparación a los ratones *fat-1* y los ratones suplementados con EPA/DHA a través de la dieta. Consistente con el efecto antiesteatótico observado en los ratones *fat-1*, estos mostraron una menor expresión de genes involucrados en la captación de ácidos grasos, CD36/FAT y L-FABP, y en la lipogénesis *de novo*, ACC, SREBP-1c y FASN. El efecto antiesteatótico observado en el grupo dieta omega-3 únicamente se reflejó con la supresión del gen FASN.

1.5 Efectos protectores de la expresión transgénica de la omega-3 desaturasa sobre la inflamación hepática y el estrés oxidativo.

El daño hepático necroinflamatorio fue atenuado en ambas aproximaciones de enriquecimiento con omega-3, evaluado mediante tinción hepática de hematoxilina-eosina. También se apreció una reducción significativa de la tinción positiva F4/80, como marcador de macrófagos infiltrados, en comparación con el grupo de ratones obesos control.

Cabe destacar que el grupo *fat-1* y el grupo dieta omega-3 presentaron una atenuación en los niveles séricos de ALT y AST, en la expresión hepática de MCP-1 e IL-1β y en la concentración de malondialdehído (MDA), marcador de estrés oxidativo. Sin embargo, el aporte exógeno de EPA/DHA a través de la dieta no moduló estos parámetros de forma significativa. Además, en el grupo de ratones *fat-1*, se observó una reducción de la expresión hepática de los marcadores inflamatorios TNFα y IL-6. Ambas aproximaciones de enriquecimiento de omega-3 no indujeron cambios en la expresión hepática de CYP2E1, como marcador de estrés oxidativo.

1.6 El enriquecimiento de omega-3 y la inhibición de las desaturasas ejercen efectos sinérgicos frente a la esteatosis y a la inflamación.

Se realizaron experimentos en cultivo primario de hepatocitos de ratón sometidos a modelos experimentales de esteatosis e inflamación. Los hepatocitos procedentes de ratones *salvajes* (WT),

fat-1 (Fat-1) o *salvajes* con suplemento de EPA (WT+EPA) se aislaron mediante perfusión del hígado *in situ* con colagenasa y fueron tratados durante 16 horas con el inhibidor de las delta-5/delta-6 desaturasas CP-24879 (0.1, 1 i 10 μM) o con el inhibidor de la delta-9 desaturasa CAY10566 (0.01, 0.1 i 1 μM). Después, se estimularon con ácido oleico (200 mM) y D-glucosa (25 Mm) durante 16 horas para inducir la acumulación intracelular de lípidos. Se observó que tanto los hepatocitos Fat-1 como los WT+EPA fueron sensibles a los efectos anti-esteatóticos de los inhibidores de las desaturasas. Este efecto fue asociado con una represión significativa en la expresión de DGAT2 que codifica para la enzima que participa en la biosíntesis de triglicéridos.

A su vez, los hepatocitos también fueron estimulados con LPS (10 ng/ml) durante 2 horas para inducir una respuesta inflamatoria. En este caso, se observó que los efectos de ambos inhibidores sobre la expresión de citoquinas inflamatorias IL-6, MCP-1, IL-1β y TNFα fue de mayor magnitud en los hepatocitos Fat-1 y WT+EPA.

Finalmente, se realizaron experimentos para comprobar los efectos protectores de la RvD1, un mediador lipídico derivado de los omega-3 con reconocida función antiinflamatoria. La RvD1 reprodujo las acciones sinérgicas de los omega-3 con los inhibidores de desaturasas sobre la expresión de los marcadores de inflamación.

ORIGINAL ARTICLE

Molecular interplay between Δ5/Δ6 desaturases and long-chain fatty acids in the pathogenesis of non-alcoholic steatohepatitis

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ABSTRACT

Objective The mechanisms underlying non-alcoholic steatohepatitis (NASH) are not completely elucidated. In the current study we integrated gene expression profiling of liver biopsies from NASH patients with translational studies in mouse models of steatohepatitis and pharmacological interventions in isolated hepatocytes to identify new molecular targets in NASH.

Design and results Using oligonucleotide microarray analysis we identified a significant enrichment of genes involved in the multi-step catalysis of long-chain polyunsaturated fatty acids, namely, Δ-5 desaturase (Δ5D) and Δ6D in NASH. Increased expression of Δ5D and Δ6D at both mRNA and protein level were confirmed in livers from mice with high-fat diet-induced obesity and NASH. Gas chromatography analysis revealed impaired desaturation fluxes toward the ω-6 and ω-3 pathways resulting in increased ω-6 to ω-3 ratio and reduced ω-3 index in human and mouse fatty livers. Restoration of hepatic ω-3 content in transgenic *fat-1* mice expressing an ω-3 desaturase, which allows the endogenous conversion of ω-6 into ω-3 fatty acids, produced a significant reduction in hepatic insulin resistance, steatosis, macrophage infiltration, necroinflammation and lipid peroxidation, accompanied by attenuated expression of genes involved in inflammation, fatty acid uptake and lipogenesis. These results were mostly reproduced by feeding obese mice with an exogenous ω-3-enriched diet. A combined Δ5D/Δ6D inhibitor, CP-24879, significantly reduced intracellular lipid accumulation and inflammatory injury in hepatocytes. Interestingly, CP-24879 exhibited superior antisteatotic and anti-inflammatory actions in *fat-1* and ω-3-treated hepatocytes.

Conclusions These findings indicate that impaired hepatic fatty acid desaturation and unbalanced ω-6 to ω-3 ratio play a role in the pathogenesis of NASH.

INTRODUCTION

Non-alcoholic fatty liver disease is a multifaceted condition that includes a wide spectrum of pathological manifestations ranging from steatosis, an asymptomatic triglyceride accumulation in the cytoplasm of hepatocytes, to non-alcoholic steatohepatitis (NASH), in which hepatic steatosis is accompanied by a marked inflammatory component.[1–3] NASH is the aggressive form of non-alcoholic fatty liver disease and increases the risk

Significance of this study

What is already known on this subject?

- Non-alcoholic fatty liver disease is considered the hepatic manifestation of the metabolic syndrome and its incidence is rapidly increasing worldwide in parallel with obesity.
- Abnormalities in the tissue content and metabolism of long-chain polyunsaturated fatty acids have been described in the metabolic syndrome, contributing to the progression of obesity-related disorders.
- There is limited information on the processing, biological roles and therapeutic manipulation of the long-chain fatty acid pathway in non-alcoholic steatohepatitis (NASH).

What are the new findings?

- Functional analysis of transcriptome data from human NASH identified a group of genes (ie, Δ-5/Δ-6/Δ-9 desaturases) associated with the processing of long-chain fatty acids as a pathway implicated in the pathogenesis of NASH.
- Perturbations in the hepatic balance of long-chain fatty acids in NASH were associated with impaired desaturation fluxes toward the ω-6 and ω-3 pathways.
- Transgenic *fat-1* mice engineered to express an ω-3 desaturase able to restore the endogenous content of ω-3 fatty acids showed reduced insulin resistance, hepatic steatosis and inflammation.
- Pharmacological interventions of Δ-5/Δ-6/Δ-9 desaturases together with balancing the ω-6 to ω-3 ratio produced antisteatotic and anti-inflammatory actions in hepatocytes.

How might it impact on clinical practice in the foreseeable future?

- In light of this interplay between Δ-5/Δ-6 desaturases and the tissue composition in long-chain polyunsaturated fatty acids, interventions targeting desaturation fluxes toward ω-6 and ω-3 pathways represent a promising strategy in the prevention and therapy of NASH.

for advanced liver disease culminating in hepatic fibrosis, cirrhosis and hepatocarcinoma.[1–3] Obesity, hyperglycaemia, insulin resistance and hypertriglyceridaemia are the best known risk factors for NASH.[4] Indeed, NASH is currently regarded as the hepatic manifestation of the metabolic syndrome, which closely correlates with the existence of a persistent inflammatory response, also known as 'metabolic-triggered inflammation' secondary to high intake of saturated fats and carbohydrates in obese individuals.[5] Since prevalence of obesity is an emerging public health issue in the Western society, identifying novel targets in NASH is of paramount importance in the prevention and therapy of metabolic liver disease.

NASH likely arises from the interaction of many different genes and life-style factors. In the current study, we used high-throughput microarray technology to identify gene pathways associated with hepatic fat accumulation and inflammation in patients with NASH. Using this approach we have identified a molecular signature of NASH that is involved in the multi-step catalysis of long-chain polyunsaturated fatty acids in the liver. In particular, we have identified a series of enzymes, namely, Δ desaturases (Δ5 and Δ6) that control the processing and availability of ω-6 and ω-3 fatty acids in mammalian tissues by inserting double bonds at specific carbon atoms in the fatty acid chain.[6] In addition to influencing the physical properties of membrane phospholipids and storing triglycerides, these polyunsaturated fatty acids serve as substrate precursors for the biosynthesis of short-lived lipid signalling molecules with either pro-inflammatory (ω-6 pathway) or anti-inflammatory (ω-3 pathway) properties.[7–9] Hence, we hypothesised that altered desaturation leading to an imbalance in the ω-6 to ω-3 ratio is likely to contribute to the pathogenesis of NASH. This hypothesis was tested in the current study by assessing (i) the hepatic fatty acid profile and desaturation indexes toward the ω-6 and ω-3 pathways in NASH patients and in a mouse model of high-fat diet (HFD)-induced obesity and NASH; (ii) the effects of the endogenous conversion of ω-6 into ω-3 fatty acids by transgenic expression of an ω-3 desaturase on HFD-induced insulin resistance, intrahepatic lipid accumulation, macrophage infiltration, hepatic necroinflammation and expression of genes involved in inflammation, fatty acid uptake and lipogenesis; (iii) the effects of exogenous supplementation of ω-3 fatty acids in HFD-induced obese mice; (iv) the effects of a mixed Δ5/Δ6 desaturase inhibitor on the response of hepatocytes to steatotic and inflammatory stimuli; and (v) the combined effects of the restoration of ω-6–ω-3 balance and inhibition of Δ5/Δ6 desaturases in hepatocytes. In summation, our findings indicate that impaired hepatic fatty acid desaturation and unbalanced ω-6 to ω-3 ratio play a role in the pathogenesis of NASH.

MATERIAL AND METHODS

RNA isolation, reverse transcription and real-time PCR

RNA isolation, cDNA synthesis and real-time PCR were performed as described in online supplementary material and methods. Validated and predesigned TaqMan primers and probes from Assays-on-Demand were used to quantify Δ9 desaturase (stearoyl-coenzyme A desaturase 1, SCD-1), Δ5 desaturase (FADS1), Δ6 desaturase (FADS2), interleukin (IL)-6, tumour necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), PPARγ, sterol response element-binding protein-1c (SREBP-1c), fatty acid synthase (FASN), liver fatty acid binding protein (L-FABP), fatty acid translocase (FAT/CD36), acyl-coenzyme A:diacylglycerol acyltransferase 2 (DGAT2) and cytochrome P450 2E1 (CYP2E1) expression, using β-actin as the endogenous control.

Microarray analysis and geneset enrichment analysis

RNA samples from eight patients with morbid obesity and associated NASH and seven control subjects (from optimal cadaveric liver donors (n=3) or resection of liver metastases (n=4) that had normal serum aminotransferase levels and liver histology) were obtained and preparation of cRNA probes, hybridisation and scanning of arrays were performed following the Affymetrix (Santa Clara, California, USA) protocol as described in online supplementary material and methods. Gene Set Enrichment Analysis (GSEA) was used to check statistically significant enrichment from Kyoto Encyclopedia of Genes and Genomes (KEGG) Canonical Pathways genesets deposited in the MSigDB c5 collection.[10] Weighted enrichment scores were calculated using gene expression lists ranked by signal to noise ratio. The maximum gene set size was set to 1000 genes, with the minimum gene set size set to 10 genes and the number of geneset permutations set to 1000.[11] The protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clinic. Only patients with signed informed consent were included.

Generation of *fat-1* mice colonies and DNA genotyping

Hemizygous *fat-1* mice were generated and backcrossed onto a C57BL/6 background as previously described.[12] Male *fat-1* mice were mated with wild-type female mice to obtain offspring, with a half being hemizygous *fat-1* mice. Genomic DNA from the ear was isolated using the Omni-Pure Genomic DNA System (Gene Link, Hawthorne, New York, USA) following the manufacturer's protocol and genotyped by PCR (see online supplementary material and methods).

Experimental studies

Male hemizygous *fat-1* (n=15) and wild-type (n=14) mice received either standard rodent chow diet (13% Kcal from fat) or HFD (custom diet D09031101, 60% Kcal from fat; Research Diets, New Brunswick, New Jersey, USA) for 16 weeks starting at 6 weeks of age. An additional group of wild-type mice (n=9, ω-3 diet group) received HFD supplemented with eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (custom diet D09031102, 60% Kcal from fat; Research Diets) (see online supplementary table 1). At the end of the study and after ~2 h of fasting, mice were euthanised via ketamine/xylazine injection (intraperitoneal, 4:1), blood was collected and serum obtained by centrifugation (3000 rpm, 10 min). The liver was excised, rinsed in Dulbecco's phosphate buffered saline, fixed in 10% formalin and paraffin embedded. Portions of liver tissue were placed in optimal cutting temperature compound (Sakura Finetek, Tokyo, Japan), immersed in 2-methylbutane on dry ice, and kept at −80°C. Portions of liver tissue were also snap-frozen in liquid nitrogen for RNA, protein and lipid analyses. Studies were conducted in accordance with the Investigation Committee of the Hospital Clinic and the EU laws governing animal experimentation.

Isolation and incubation of primary hepatocytes

Hepatocytes were isolated as previously described[13] and challenged for 16 h with oleic acid (200 μM), as an established in vitro model of steatosis,[14 15] or with lipopolysaccharide (LPS) (10 ng/ml) for 2 h, as an established in vitro model of inflammatory injury.[16] Hepatocytes were incubated with CP-24879, a specific Δ5/Δ6 desaturase inhibitor,[17] CAY10566, a selective Δ9 desaturase inhibitor,[18] EPA or resolvin D1 (RvD1) as detailed in online supplementary material and methods.

Histological analysis of hepatic steatosis and Oil Red-O staining of hepatocytes

Hepatic steatosis and the amount of Oil Red-O retained by hepatocytes were measured as described in online supplementary material and methods.

Fatty acid profiling by gas chromatography

Fatty acid methyl-esters from liver tissue were determined using a modification of the Folch method,[19] and were separated by gas chromatography with an Agilent 7890 system equipped with a flame-ionisation detector (Agilent Technologies) and a SupraWAX-280 capillary column (30 m×0.25 mm×0.25 µm) (Teknokroma, Barcelona, Spain). Each fatty acid was expressed as percentage of total fatty acids (see online supplementary material and methods).

Detection of F4/80 by immunohistochemistry

F4/80 detection was performed in liver tissue paraffin sections as described in online supplementary material and methods.

Biochemical analysis

Serum glucose, cholesterol, triglycerides and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined by standard laboratory procedures. Serum insulin was determined by enzyme immunoassay (Spi-Bio, Montigny Le Bretonneux, France).

Analysis by western blot of Δ5, Δ6 and Δ9 desaturases and JNK phosphorylation

Total liver protein was extracted with modified radio-immunoprecipitation assay and Δ5, Δ6 and Δ9 desaturases, GAPDH and c-Jun amino-terminal kinase (JNK)/phospho-JNK protein expression was analysed by western blot using specific primary rabbit antimouse antibodies (see online supplementary material and methods).

Lipid peroxidation analysis

Hepatic lipid peroxidation was determined using the TBARS Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA) for colorimetric measurement of malondialdehyde (MDA) (see online supplementary material and methods).

Statistical analysis of the results was performed by analysis of variance (ANOVA) (one-way or two-way ANOVA) or unpaired Student t test. Results are expressed as mean±SEM and differences considered significant at $p<0.05$.

RESULTS

We first analysed human livers using high-throughput transcriptome analysis to identify novel disease mechanisms. This analysis identified a group of 157 genes differentially expressed in livers from NASH patients (figure 1A). Unsupervised hierarchical analysis of genes upregulated in liver of NASH revealed 10 significant enriched canonical pathways (figure 1A). Functional analysis identified a group of genes associated with the biosynthesis of unsaturated fatty acids as a pathway implicated in the development of NASH. Among them, we identified FADS1 (Δ5 desaturase), SCD-1 (Δ9 desaturase), FADS2 (Δ6 desaturase), ELOVL6 (fatty acid elongase 6), ELOVL5 (fatty acid elongase 5) and SC5DL (sterol-C5-desaturase-like) as potential candidate genes (figure 1A). In particular, Δ5, Δ6 and Δ9 desaturases were overexpressed in liver samples from NASH patients (figure 1B). Gas chromatography analysis revealed increased hepatic ω-6 to ω-3 ratio and reduced ω-3 index (ie, EPA and DHA levels expressed as per cent of total fatty acids) in NASH patients (figure 1C). Of interest, a significant correlation ($r^2=0.96$, $p<0.0001$) between the percentage of hepatic fat deposition and the ω-6 to ω-3 ratio was seen in NASH patients (data not shown). In addition, the product to precursor ratio between the monounsaturated (18:1) to saturated (18:0) fatty acids, which is a surrogate marker of Δ9 desaturation, was increased in NASH patients (figure 1D). We also assessed the expression of desaturases in livers from mice fed a HFD, an experimental model of obesity-induced NASH. As shown in figure 1E, increased protein expression for Δ5, Δ6 and Δ9 desaturases was also seen by western blot analysis in murine NASH. In this model, upregulation of Δ5 and Δ6 desaturases was also detected at the mRNA level (see online supplementary figure 1). Consistent with patients, mice with HFD-induced NASH showed increased ω-6 to ω-3 ratio, reduced ω-3 index and enhanced 18:1 to 18:0 ratio (figure 1, F–G).

The Δ5/Δ6 desaturases are membrane-bound enzymes that catalyse the rate-limiting formation of long-chain polyunsaturated fatty acids, whereas Δ9 desaturase catalyses the endogenous biosynthesis of monosaturated fatty acids.[6] Since desaturase deregulation has been implicated in metabolic and inflammatory disorders,[6] we next analysed the profile of fatty acids in livers from mice with HFD-induced NASH. As compared with chow mice, hepatic levels of arachidonic acid (AA) were markedly increased while the levels of DHA were reduced in HFD mice (figure 2A). Consistent with this finding, HFD mice showed higher 20:4n-6 to 20:3n-6 and lower 20:5n-3 to 20:4n-3 ratios, indirect markers of AA and EPA biosynthesis, respectively (see online supplementary figure 2A), suggesting an impaired desaturation, predominantly through the Δ5 desaturase, toward the ω-3 pathway.

We next restored hepatic ω-3 levels by using transgenic *fat-1* mice engineered to express an ω-3 desaturase that catalyses conversion of ω-6 to ω-3 fatty acids without the need for dietary intervention.[12] The results were compared with those from wild-type mice supplemented with EPA/DHA (ω-3 diet group). Figure 2B shows representative chromatograms of liver samples from chow mice and wild-type mice, *fat-1* mice and mice of the ω-3 diet group after receiving a HFD. As expected, *fat-1* mice showed reduced hepatic ω-6 to ω-3 ratio and enhanced ω-3 index, with these effects being more pronounced in the ω-3 diet group (figure 2C). A detailed composition of hepatic ω-3 fatty acids is given in online supplementary table 2. The Δ5 (20:5n-3 to 20:4n-3) (figure 2D) and Δ6 (18:4n-3 to 18:3n-3) (figure 2E) desaturation indexes toward the ω-3 pathway were significantly reduced by the HFD. These indexes were restored in both *fat-1* and ω-3 diet group (figure 2, D–E). Of interest, the increase in Δ5 desaturase flux toward the ω-3 pathway, as estimated by 20:5n-3 to 20:4n-3, was more evident in the ω-3 diet group, probably as a consequence of feeding an EPA-enriched diet, whereas the increase in Δ6 desaturase flux toward the ω-3 pathway, as estimated by the 18:4n-3 to 18:3n-3 ratio, was more evident in *fat-1* mice (figure 2, D–E). On the other hand, the ratio between monounsaturated to saturated fatty acids (16:1 to 16:0 and 18:1 to 18:0), which reflexes flux through the Δ9 desaturase, was decreased by ω-3 tissue enrichment (figure 2F). Moreover, ω-3 enrichment repressed desaturase expression at both protein (figure 2G) and mRNA (see online supplementary 2B) levels.

We next analysed the impact of endogenous and exogenous ω-3 fatty acid enrichment on HFD-induced NASH. Under chow diet, transgenic *fat-1* mice showed similar body, liver and epididymal white adipose tissue (eWAT) weight, and serum leptin, triglycerides, AST and ALT levels, but lower serum cholesterol

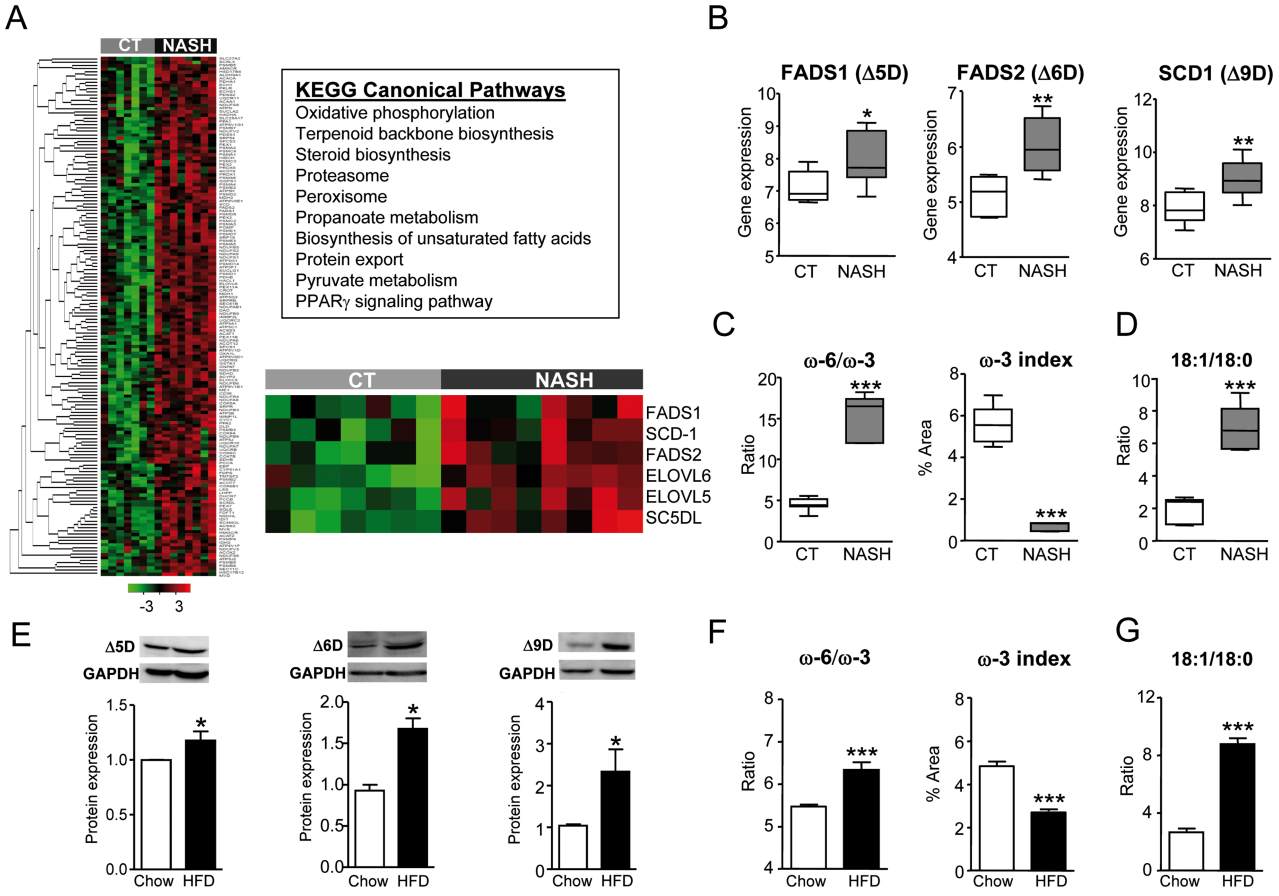

Figure 1 Upregulation of genes involved in the hepatic biosynthesis of unsaturated fatty acids in patients with non-alcoholic steatohepatitis (NASH). (A) Differential hepatic gene expression (Affymetrix GeneChip Human Genome U133 Plus 2.0) in patients with NASH. Results are expressed as a matrix view of gene expression data (heat map) where rows represent genes and columns represent hybridised samples. The intensity of each colour denotes the standardised ratio between each value and the average expression of each gene across all samples. Red pixels correspond to an increased abundance of mRNA in the indicated liver samples, whereas green pixels indicate decreased mRNA levels. Enriched KEGG canonical pathways and genes implicated in fatty acid biosynthesis, including FADS1 (Δ5 desaturase), stearoyl-coenzyme A desaturase 1 (SCD-1) (Δ9 desaturase), FADS2 (Δ6 desaturase), ELOVL6, ELOVL5 and SC5DL. (B) Expression of Δ5, Δ6 and Δ9 desaturases in livers from NASH patients (n=8) as compared with controls (CT, n=7). (C) Hepatic ω-6 to ω-3 ratio (given by C18:2n-6+C20:4n-6+C22:4n-6+C22:5n-6 to C18:3n-3+C18:4n-3 +C20:3n-3+C20:4n-3+C20:5n-3+C22:5n-3+C22:6n-3) and ω-3 index (eicosapentaenoic and docosahexaenoic levels expressed as per cent area of total fatty acids) in NASH patients as compared with CT. (D) Hepatic ratio between monounsaturated to saturated fatty acids (18:1 to 18:0) in CT and NASH patients. (E) Protein expression of Δ5, Δ6 and Δ9 desaturases as determined by western blot in livers from mice with high-fat diet (HFD)-induced NASH (n=7) and mice fed with chow diet (chow, n=7). The densitometric analyses of Δ5, Δ6 and Δ9 desaturase signals normalised to GAPDH are shown at the bottom. (F) Hepatic ω-6 to ω-3 ratio and ω-3 index in chow and HFD mice. (G) Hepatic 18:1 to 18:0 ratio in chow and HFD mice. Results are expressed as mean±SEM. *, p<0.05; **, p<0.01; and ***, p<0.001 versus CT or chow.

than wild-type mice (see online supplementary figure 3). HFD significantly increased body, liver and eWAT weight and serum cholesterol and triglyceride levels (figure 3, A–B). Under HFD conditions, *fat-1* mice showed similar food intake but lower weight gain than wild-type mice (see online supplementary figure 4). Consistently, *fat-1* mice were resistant to the adipogenic and steatogenic effects of the HFD and had lower body, liver, and eWAT weight and serum cholesterol and triglyceride levels than wild-type mice (figure 3, A–B). Decreased liver weight and serum cholesterol and triglyceride levels were also seen in the ω-3 diet group, although these mice were not resistant to the adipogenic effects of HFD (figure 3A). Moreover, transgenic expression of ω-3 desaturase in *fat-1* mice corrected HFD-induced hyperinsulinemia and hyperglycaemia (figure 3C). In addition, analysis of JNK1 and JNK2 phosphorylation, an established marker of insulin resistance,[20] revealed a significant improvement in hepatic insulin sensitivity despite downregulation of PPARγ (figure 3, D–E). Similar findings were observed in mice of the ω-3 diet group, except that changes in serum glucose and JNK2 phosphorylation did not reach statistical significance (figure 3). No changes in insulin receptor substrate 2 and GLUT-2 expression were observed (see online supplementary figure 5A).

As an established model of hepatic steatosis, HFD feeding induced extensive Oil Red-O staining (figure 4A). As compared with wild-type mice, however, hepatic steatosis was significantly less pronounced in *fat-1* mice (figure 4, A–B). Consistent with this antisteatotic effect, *fat-1* mice had reduced expression of genes involved in fatty acid uptake (CD36/FAT and L-FABP) and *de novo* hepatic lipogenesis (ACC, SREBP-1c and FASN) (figure 4, C–G). Although a remarkable antisteatotic effect was also seen in the ω-3 diet group (figure 4, A–B), the expression

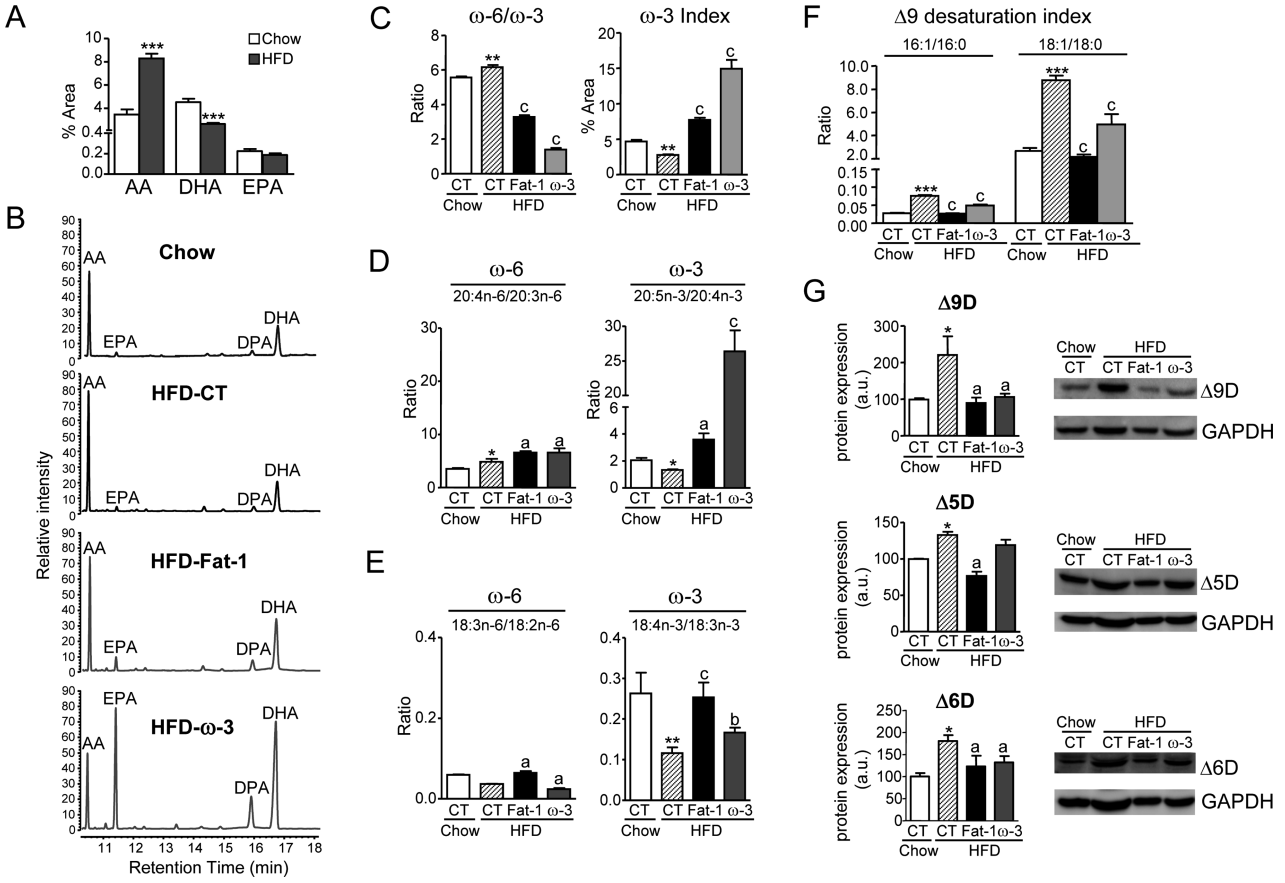

Figure 2 Impact of endogenous and exogenous ω-3 enrichment on liver desaturation fluxes. (A) Hepatic levels of arachidonic (AA), docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids in mice fed either chow or high-fat diet (HFD). (B) Representative gas chromatography chromatograms of the hepatic content of ω-6 (AA, C20:4n-6) and ω-3 (EPA, C20:5n-3; DPA: docosapentaenoic acid, C22:5n-3; and DHA, C22:6n-3) fatty acids in total lipid extracts from chow mice (n=7) and in wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an EPA/DHA rich diet (ω-3) (n=9) after receiving a HFD. (C) Hepatic ω-6 to ω-3 ratio and ω-3 index from the different study groups. (D and E) Hepatic product to precursor ratios as Δ5 and Δ6 desaturation indexes toward the ω-6 or the ω-3 pathways in the four groups of mice studied. (F) Hepatic Δ9 desaturation index (16:1 to 16:0 and 18:1 to 18:0) in the four groups of mice studied. (G) Hepatic protein expression of Δ9, Δ5 and Δ6 desaturases as determined by western blot in the study groups. The densitometric analysis of Δ5, Δ6 and Δ9 desaturases signals normalised to GAPDH are shown on the left panel. Results are expressed as mean±SEM. *, p<0.05; **, p<0.01; and ***, p<0.001 versus chow-CT and a, p<0.05; b, p<0.01; and c, p<0.001 versus HFD-CT.

of CD36/FAT, L-FABP, ACC and SREBP-1c remained unchanged (figure 4, C–F). Hepatic FASN expression was suppressed in both *fat-1* mice and ω-3 diet group (figure 4G). No changes were observed in the hepatic microsomal triglyceride transfer protein, the activity of which regulates triglyceride export from hepatocytes (see online supplementary figure 5B).

Hepatic inflammation and oxidative stress are, in addition to hepatic steatosis, hallmarks of NASH.[5] As shown in figure 5A, wild-type mice showed extensive necroinflammatory liver injury (upper panel) and extensive positive staining for F4/80, a murine macrophage marker (bottom panel) after receiving a HFD. The morphometric assessment of the area with positive staining for F4/80 is shown in figure 5B. In addition, HFD feeding increased serum ALT/AST and hepatic MCP-1, IL-1β and CYP2E1 expression and MDA levels (figure 5, C–H). Liver damage was attenuated in both *fat-1* mice and the ω-3 diet group (figure 5), although some differences were noticed between these two groups. Indeed, ALT/AST levels were decreased in *fat-1* mice and the ω-3 diet group, but in the latter changes did not reach statistical significance (figure 5, C–D). In contrast, the ω-3 diet group, but not *fat-1* mice, experienced a reduction in hepatic TNFα and IL-6 expression (see online supplementary figure 5C). Moreover, MDA levels were decreased in both groups of mice, but, again, changes did not reach statistical significance in the ω-3 diet group (figure 5H). No changes in CYP2E1 expression were observed (figure 5G).

To challenge the hypothesis that an alteration in the processing and availability of unsaturated fatty acids through Δ5, Δ6 and Δ9 desaturases plays a role in hepatic steatosis and inflammation, we performed studies in hepatocytes exposed to oleic acid. The addition of CP-24879, a combined Δ5/Δ6 desaturase inhibitor, to hepatocyte cultures abrogated oleic acid-induced accumulation of triglycerides (figure 6A). This antisteatotic effect was associated with a significant repression of the expression of DGAT2, the key enzyme in the biosynthesis of triglycerides (figure 6B).[21] To assess whether CP-24879 exerts synergistic actions with ω-3 fatty acids, we tested the effects of this inhibitor on hepatocytes from *fat-1* mice and hepatocytes treated with EPA. As shown in figure 6C, *fat-1* and EPA-treated hepatocytes were more sensitised to the antisteatotic effects of

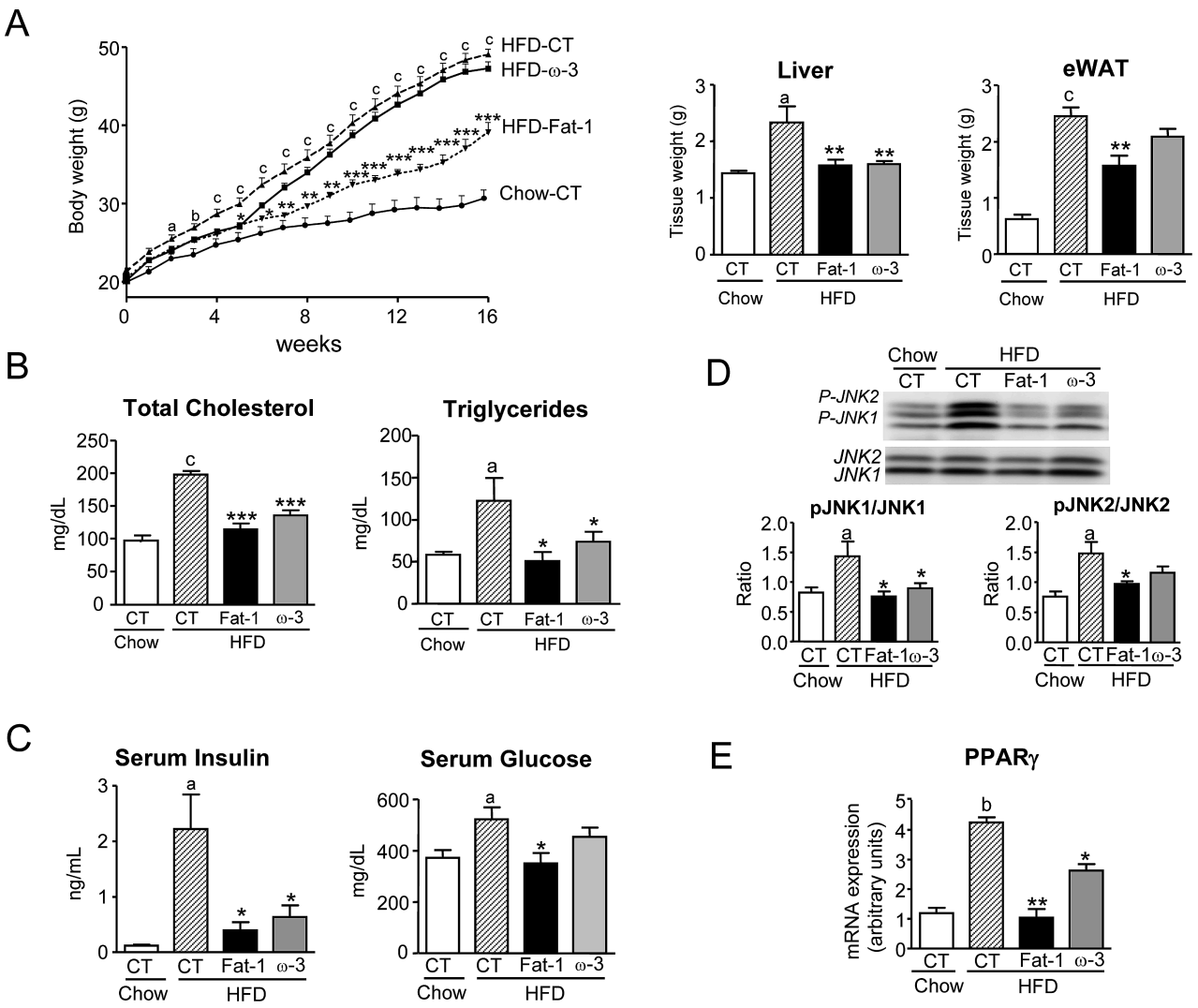

Figure 3 Transgenic expression of ω-3 desaturase protects mice from high-fat diet (HFD)-induced adiposity and insulin resistance. (A–C) Body, liver and epididymal white adipose tissue (eWAT) weight and serum cholesterol, triglycerides, insulin and glucose levels in chow mice (n=7) and in wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an eicosapentaenoic/docosahexaenoic rich diet (ω-3) (n=9) after receiving a HFD for 16 weeks. (D) Hepatic c-Jun amino-terminal kinase (JNK) phosphorylation (P-JNK1 and P-JNK2) and total JNK as determined by western blot. The densitometric analysis of P-JNK1 and P-JNK2 bands is shown at the bottom. (E) Hepatic PPARγ expression as determined by real-time PCR. Results are expressed as mean±SEM. a, p<0.05; b, p<0.01; and c, p<0.001 versus chow-CT; *, p<0.05; **, p<0.01; and ***, p<0.001 versus HFD-CT.

CP-24879. DGAT2 was also downregulated in these experiments (figure 6D). Representative photomicrographs for these experiments are shown in figure 6E. The Δ9 desaturase inhibitor CAY10566 also reduced oleic acid-induced accumulation of triglycerides and DGAT2 expression in hepatocytes (figure 6, F–G), and these antisteatotic effects were of higher magnitude in *fat-1* and EPA-treated hepatocytes (figure 6, H–I). Figure 6J shows representative images for experiments with CAY10566. Interestingly, the inhibitory responses of CP-24879 on oleic acid-induced triglyceride accumulation in hepatocytes were observed at starting concentrations as low as 0.1 μM (see online supplementary figure 6).

We next performed studies in hepatocytes stimulated with LPS. CP-24879 blocked LPS-induced expression of inflammatory cytokines in a concentration-dependent manner (figure 7A and online supplementary figure 7A). CAY10566 produced similar actions (figure 7A and online supplementary figure 7A). The inhibitory effects of CP-24879 and CAY10566 on cytokine expression were of higher magnitude in *fat-1* and EPA-treated hepatocytes (figure 7A). Interestingly, resting *fat-1* hepatocytes displayed lower IL-6, IL-1β, TNFα, MCP-1 and CXCL2 expression than wild-type hepatocytes (see online supplementary figure 7B). Moreover, *fat-1* and EPA-treated hepatocytes showed an attenuated response to LPS (see online supplementary figure 7B). We finally performed experiments with RvD1, an ω-3-derived lipid mediator with potent anti-inflammatory and pro-resolving properties.[9] RvD1 reproduced the inhibitory actions of ω-3 fatty acids and downregulated MCP-1 and TNFα expression in hepatocytes (figure 7B). Moreover, RvD1 exerted synergistic anti-inflammatory actions with the desaturase inhibitors CP-24879 and CAY10566 (figure 7B).

DISCUSSION

Mammals can desaturate fatty acids at positions Δ5, Δ6 and Δ9 (Δ indicates the number of the first carbon atom that forms the double bond) relative to the carboxyl group.[6] While Δ9

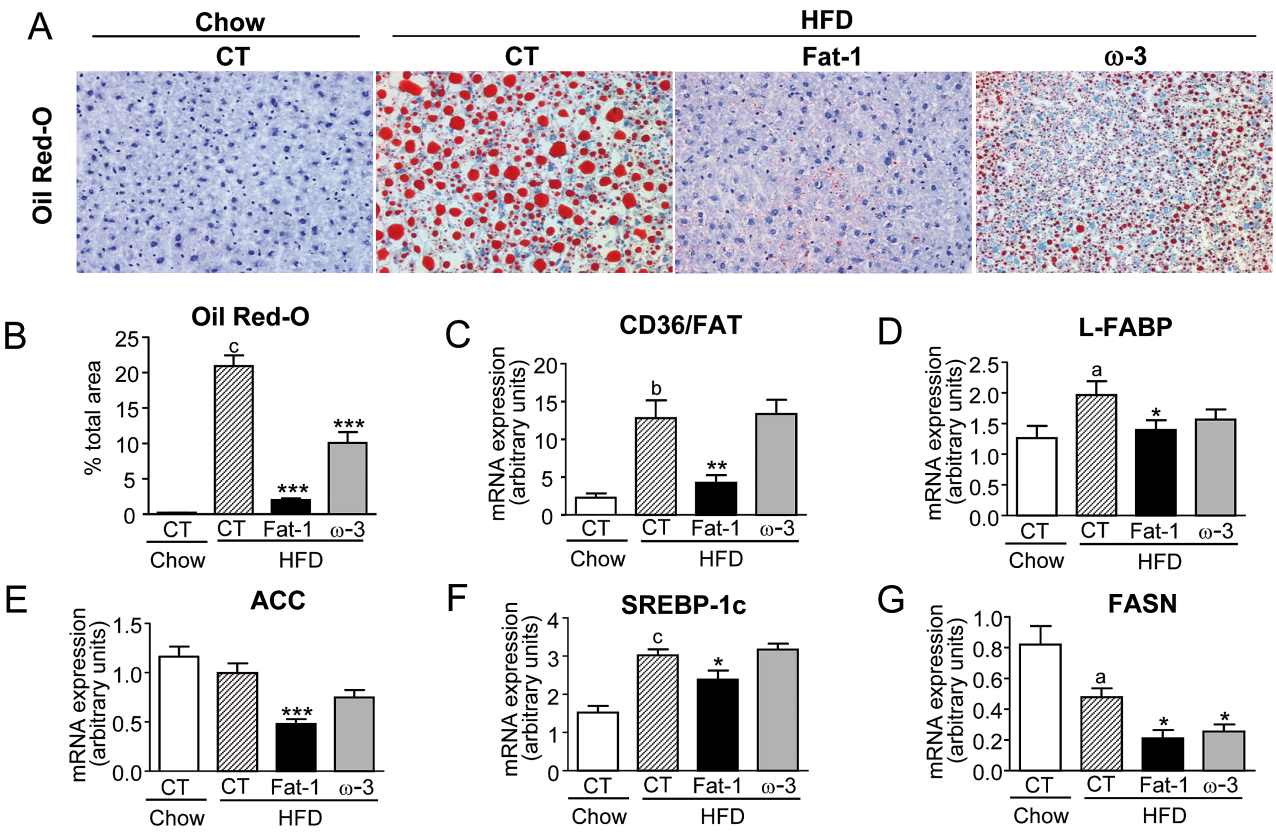

Figure 4 Prevention from HFD-induced hepatic steatosis in *fat-1* transgenic mice and in wild-type mice supplemented with eicosapentaenoic (EPA)/docosahexaenoic (DHA). (A) Representative photomicrographs (×200 magnification) and (B) histomorphometrical analysis of liver sections stained with Oil Red-O in chow mice (n=7) and in wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an EPA/DHA rich diet (ω-3) (n=9) after receiving a high-fat diet (HFD). Analyses of hepatic expression of genes involved in fatty acid uptake (CD36/FAT and L-FABP) (C and D) and *de novo* lipogenesis (ACC, SREBP-1c and fatty acid synthase) (E–G) were assessed by real-time PCR. Results are expressed as mean±SEM. a, p<0.05; b, p<0.01; and c, p<0.001 versus chow-CT; *, p<0.05; **, p<0.01; and ***, p<0.001 versus HFD-CT.

desaturase, also called stearoyl-CoA desaturase or SCD-1, converts the saturated fatty acid, stearic acid (18:0), into the monosaturated fatty acid, oleic acid (18:1), Δ5 and Δ6 desaturases are required for the processing of long-chain polyunsaturated fatty acids. The latter are classified as ω-6 or ω-3 on the basis of the location of the last double bond relative to the terminal methyl end of the molecule.[22] In humans, AA (20:4n-6) is the main ω-6 fatty acid, whereas EPA (20:5n-3) and DHA (22:6n-3) are the most representative members of the ω-3 family.[7] These fatty acids are essential because they cannot be synthesised in humans and must be obtained from the dietary linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3).[23]

Consistent with previous studies, the results of the current study demonstrate that patients with NASH have a hepatic imbalance between ω-6 and ω-3 levels.[24–27] This imbalance was reproduced in a murine model of HFD-induced NASH. An important strength of our study was the successful restoration of hepatic tissue ω-3 levels by using mice with transgenic expression of the *Caenorhabditis elegans fat-1* gene, which encodes an ω-3 desaturase capable of generating ω-3 fatty acids from the ω-6 type, leading to abundant tissue ω-3 distribution without the need for a dietary supply.[12 28] Another major finding of our study was that *fat-1* mice were protected from obesity (they showed reduced body weight gain) and obesity-induced hepatic insulin resistance, steatosis and inflammation. In particular, *fat-1* mice showed decreased intracellular hepatocyte triglyceryde accumulation, macrophage infiltration and inflammatory injury in response to a HFD, corroborating that a balanced hepatic ω-6 to ω-3 ratio is a preventive measure in NASH. The reduction in weight gain in *fat-1* mice observed in our study was consistent with the presence of lower body weight in other strains of *fat-1* mice.[29] This antiobesogenic effect was not previously recognised by White *et al*,[30] who fed *fat-1* mice with a HFD containing 55% Kcal from fat for 8 weeks whereas in our study we fed our mice with a HFD containing a higher caloric content (60% Kcal from fat) for 16 weeks.

Our study is very comprehensive in nature because we compared the effects in *fat-1* mice with those exerted by exogenous ω-3 supplementation. Although overall the responses were roughly the same in both groups, some differences were noticed. In this regard, the ω-3 diet group showed similar responses in metabolic parameters such as liver weight, serum cholesterol, triglycerides and insulin levels and hepatic insulin resistance (JNK1 phosphorylation) and in liver inflammation, assessed histologically by H&E and F4/80 (a specific macrophage marker) staining, as well as in MCP-1 and IL-1β expression. However, exogenous ω-3 supplementation failed to improve body and eWAT weight and serum glucose and ALT/AST levels. These mice showed a reduction in these parameters, but the differences did not reach statistical significance as it was the case for *fat-1* mice. In contrast, the ω-3 diet group experienced a statistically significant reduction in hepatic TNF-α and

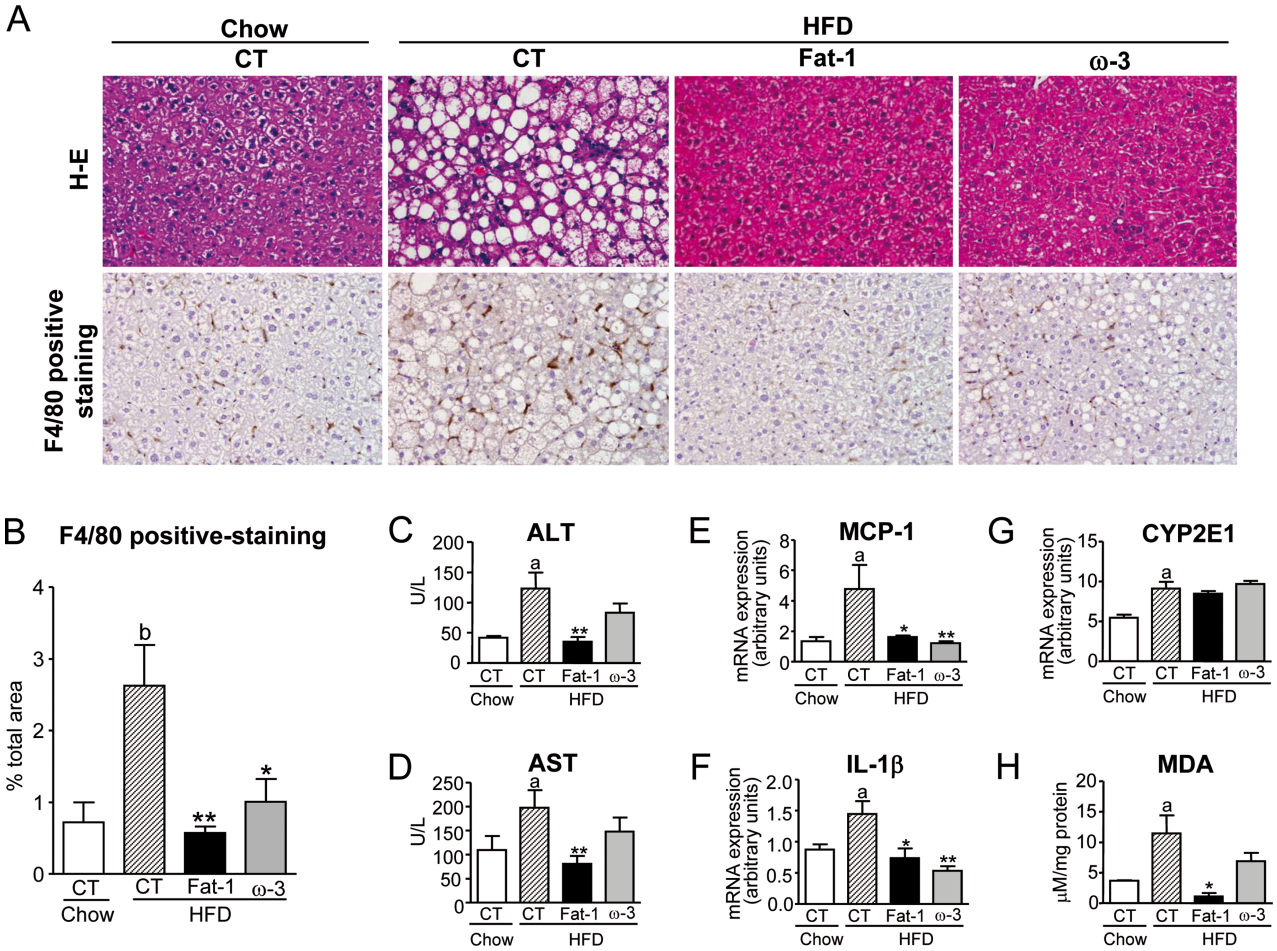

Figure 5 Prevention from high-fat diet (HFD)-induced hepatic inflammation in *fat-1* transgenic mice and in wild-type mice supplemented with eicosapentaenoic (EPA)/docosahexaenoic (DHA). (A) Representative photomicrographs of liver sections stained with H&E (upper panel) and the macrophage marker F4/80 (lower panel) in chow mice (n=7) and in wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an EPA/DHA rich diet (ω-3) (n=9) after receiving a HFD. (B) Histomorphometrical analysis of F4/80 staining. (C and D) Serum ALT and AST levels and (E and F) expression of monocyte chemoattractant protein-1 (MCP-1) and IL-1β in liver samples from the different study groups. (G) Hepatic CYP2E1 expression as determined by real-time PCR. (H) Hepatic malondialdehyde concentration expressed as micromoles per mg of protein. Results are expressed as mean±SEM. a, p<0.05 and b, p<0.01 versus chow-CT; *, p<0.05 and **, p<0.01 versus HFD-CT.

IL-6 expression, changes that were not significant in *fat-1* mice. Moreover, the antisteatotic actions of ω-3 supplementation were not associated with downregulation of CD36/FAT and L-FABP and the lipogenic genes ACC and SREBP-1c. Finally, we found that lipid peroxidation was reduced in both groups of mice, but, again, changes did not reach statistical significance in the ω-3 diet group. These findings are in agreement with the existence of a higher antioxidant potential in *fat-1* mice.[31] Since CYP2E1, a member of the oxido-reductase cytochrome family that oxidises a variety of substrates including fatty acids,[32] remained unchanged in *fat-1* mice, our findings support the concept that *fat-1* mice may at least partially oppose oxidative stress by the existence of an impaired mitochondrial electron transport chain.[33] In any event, the observed individual peculiarities of supplying ω-3 from either endogenous or exogenous sources were not the consequence of ineffective enrichment of hepatic tissues with EPA/DHA because the total ω-3 content and the ω-3 index were significantly higher in the ω-3 diet group than in *fat-1* mice. A potential explanation for this is that the existence in *fat-1* mice of a more balanced ω-6 to ω-3 ratio throughout their lives (since the embryonic stage) provides a superior protection than a sudden boost of exogenous ω-3.[28] This long-term fatty acid profile may allow *fat-1* mice to develop a gene profile that is significantly more suitable for protection. Further studies are needed to clarify this subject.

Another major finding of our study was that the desaturase gene-pathway is deregulated in NASH patients. Indeed, microarray analysis identified upregulated expression of Δ5, Δ6 and Δ9 desaturases in livers from patients with NASH. These findings are divergent from those previously reported by Araya *et al* showing decreased liver Δ5 and Δ6 desaturase activities, as estimated by product to precursor ratios, in obese patients with NAFLD.[34] However, in the study of Araya *et al*, subjects were predominantly female, while ours were predominantly male and a gender effect trending toward a decreased desaturase activity has been reported in women.[35] Moreover, we were able to confirm upregulated expression of Δ5 and Δ6 desaturases at both mRNA and protein levels as well as increased flux through the Δ9 desaturase in mice with HFD-induced NASH. Interestingly, our microarray analysis also identified deregulated expression in NASH of other genes implicated in the processing of unsaturated fatty acids, such as ELOVL6. ELOVL6 has been

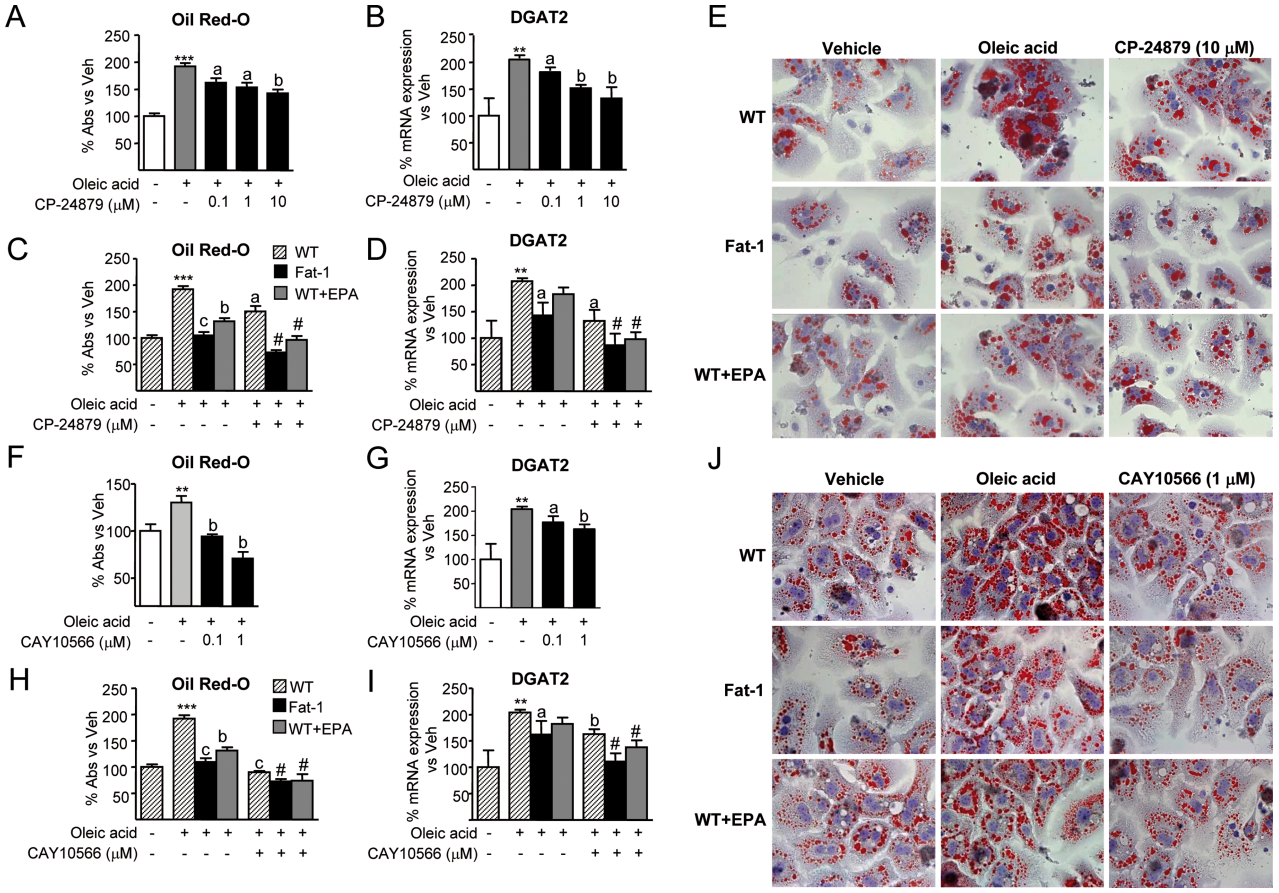

Figure 6 ω-3 Fatty acids amplify the antisteatotic actions of desaturase inhibitors. (A) Quantitation of Oil Red-O staining in wild-type hepatocytes incubated for 16 h with vehicle (0.01% ethanol) or oleic acid (200 μM) in the presence of increasing concentrations (0.1, 1 and 10 μM) of CP-24879, a combined Δ5/Δ6 desaturase inhibitor. (B) Real-time PCR analysis of acyl-coenzyme A:diacylglycerol acyltransferase 2 (DGAT2) expression in hepatocytes incubated under these conditions. (C and D) Quantitation of Oil Red-O staining and DGAT2 expression in hepatocytes isolated from wild-type (CT) mice (n=12), *fat-1* mice (n=12) and wild-type mice incubated with eicosapentaenoic (EPA) (10 μM) (n=16) and exposed to oleic acid and CP-24879 (10 μM), as described above. (E) Representative photomicrographs of Oil Red-O staining for these experiments. (F) Quantitation of Oil Red-O staining in wild-type hepatocytes incubated for 16 hours with vehicle (0.01% ethanol) or oleic acid (200 μM) in the presence of increasing concentrations (0.1 and 1 μM) of CAY10566, a Δ9 desaturase inhibitor. (G) Real-time PCR analysis of DGAT2 expression in hepatocytes incubated under these conditions. (H and I) Quantitation of Oil Red-O staining and DGAT2 expression in hepatocytes isolated from wild-type (CT) mice (n=12), *fat-1* mice (n=12) and wild-type mice incubated with EPA (10 μM) (n=16) and exposed to oleic acid and CAY10566 (1 μM), as described above. (J) Representative images of Oil Red-O staining for these experiments with CAY10566. Results are expressed as mean ±SEM. **, p<0.01 and ***, p<0.001 versus vehicle; a, p<0.05; b, p<0.01; and c, p<0.001 versus oleic acid; #, p<0.05 versus wild-type hepatocytes incubated with oleic acid and CP-24879 or CAY10566.

shown to promote NASH and its genetic deletion has been demonstrated to exert a protective role against insulin resistance.[36 37]

The availability of the required amount of desaturase inhibitors to perform in vivo experiments was a limiting factor in our study. However, we were able to carry out experiments in vitro with these compounds in hepatocytes exposed to either oleic acid or LPS. Oleic acid is a fatty acid that is accumulated in excess in the liver of patients with hepatic steatosis and this fatty acid is used at high concentrations to induce fat overloading in vitro.[14 15 26] On the other hand, LPS is a potent inducer of inflammatory injury.[16] The results of these experiments demonstrated antisteatotic and anti-inflammatory actions of a selective Δ5/Δ6 desaturase inhibitor in hepatocytes. Although manipulation of this pathway in mammals is challenging because both ω-6 and ω-3 fatty acids compete as substrates for the same desaturases,[22] our findings support the notion that manipulation of the hepatic desaturase pathway is of potential interest. Moreover, our findings demonstrate a synergy between desaturase inhibition and ω-3 fatty acids, suggesting that targeting desaturases combined with ω-3 supplementation is a feasible approach to restore the hepatic ω-6 to ω-3 ratio in NASH (figure 8).

Essential polyunsaturated fatty acids constitute an important component of cellular membranes and influence membrane fluidity and the response to injury of membrane-bound enzymes and receptors.[22] Since membranes contain mainly AA compared with EPA, AA is the predominant precursor for eicosanoid biosynthesis. In fact, after being released from cell membranes, AA is converted into potent inflammatory lipid mediators of the prostaglandin and leukotriene series.[7 8] In contrast, EPA and DHA are the biosynthetic precursors of potent anti-inflammatory lipid mediators, namely, resolvins and protectins.[9] Therefore, the protective actions observed in *fat-1* mice and in

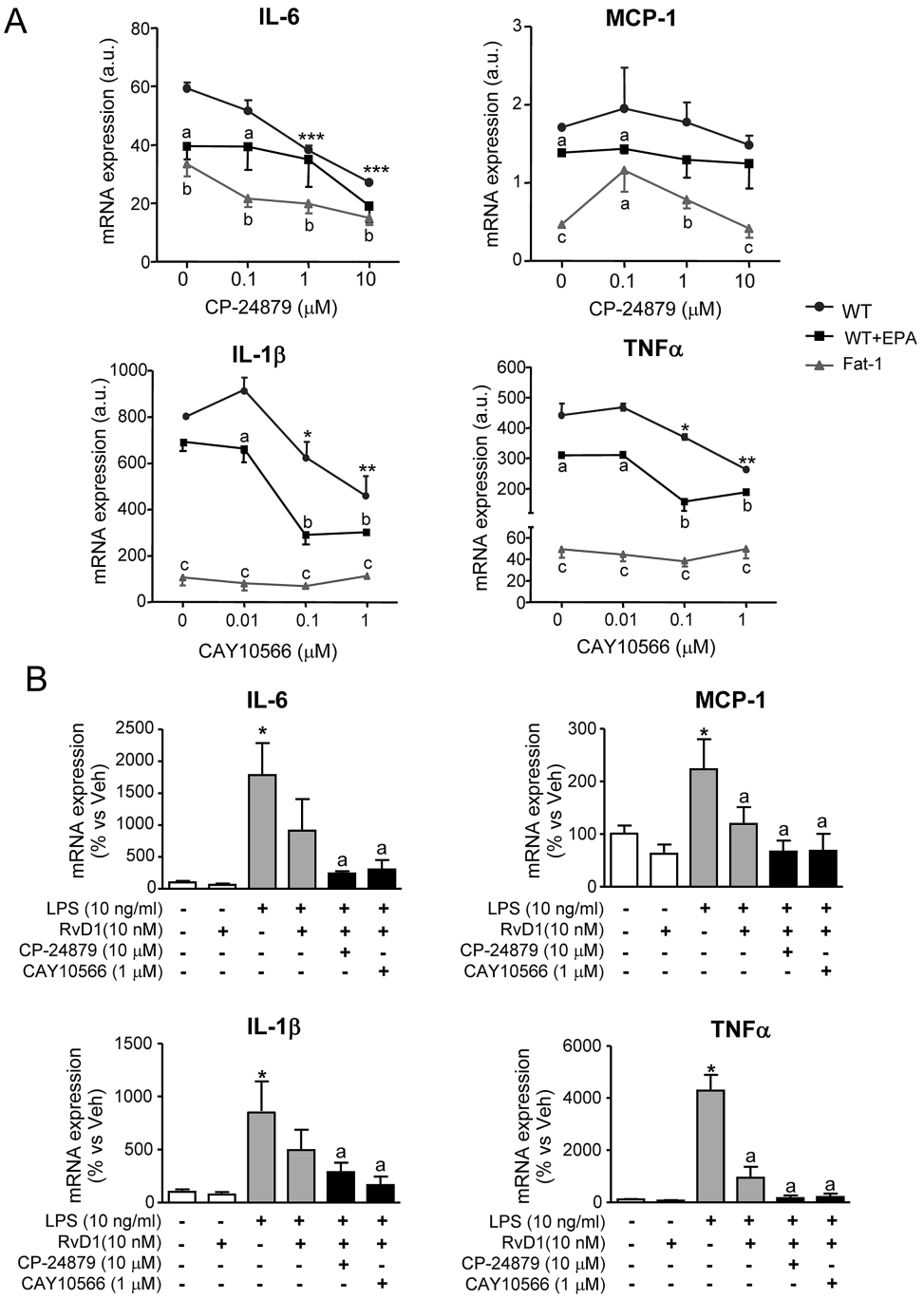

Figure 7 ω-3 Fatty acids amplify the anti-inflammatory actions of desaturase inhibitors. (A) IL-6, monocyte chemoattractant protein-1 (MCP-1), IL-1β and tumour necrosis factor α (TNFα) expression were assessed in hepatocytes isolated from wild-type (CT) mice (n=12), *fat-1* mice (n=12) and wild-type mice incubated with eicosapentaenoic (EPA) (10 μM) (n=16), pretreated with increasing concentrations of CP-24879 (0.1, 1 and 10 μM) or CAY10566 (0.01, 0.1 and 1 μM) for 16 h and stimulated with LPS (10 ng/ml) for 2 h. (B) Real-time PCR analysis of IL-6, MCP-1, IL-1β and TNFα expression in hepatocytes pretreated with CP-24879 or CAY10566 for 16 h and stimulated with LPS for 2 h in the presence of RvD1 (10 nM). Results are expressed as mean±SEM. *, p<0.05; **, p<0.01; and ***, p<0.001 versus vehicle; a, p<0.01 and b, p<0.001 versus wild-type hepatocytes stimulated with LPS.

mice of the ω-3 diet group could reflect either suppression of ω-6-derived inflammatory mediators or amplification of ω-3-derived anti-inflammatory compounds or both.[38 39] In fact, transgenic *fat-1* mice display increased formation of resolvins derived from ω-3 precursors.[40] Consistent with this, we reproduced the protective effects exerted by micromolar concentrations of ω-3 fatty acids against inflammatory injury by incubating hepatocytes with nanomolar concentrations of RvD1, a well-characterised member of this family of anti-inflammatory lipid mediators.

In summation, our findings strongly support the concept that re-establishment of the ω-6 to ω-3 balance contributes to the prevention of metabolic liver disease, similar to the improved outcomes reported in arthritis, cystic fibrosis, IgA nephropathy, diabetes, ulcerative colitis, Crohn's disease, asthma and sepsis.[7] Although the systematic review of the literature assessing the therapeutic role of

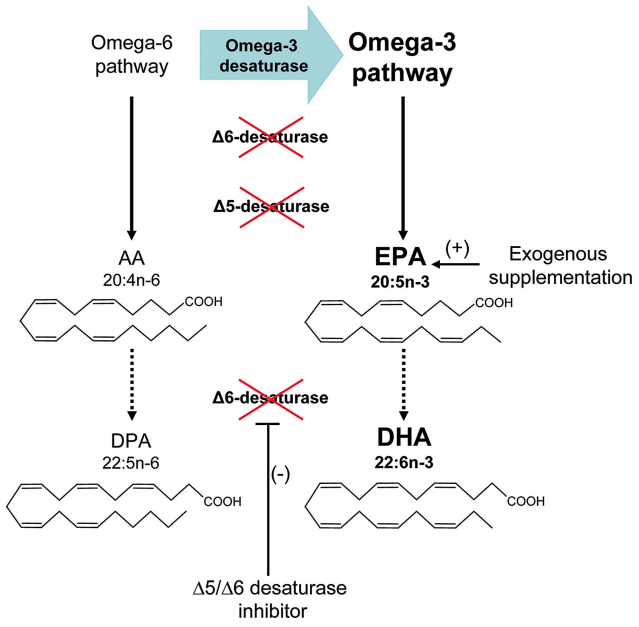

Figure 8 Schematic diagram summarising the existing interplay between Δ5/Δ6 desaturases and ω-3 fatty acids. Availability of arachidonic acid (AA), the representative polyunsaturated fatty acid of the ω-6 pathway, depends on the activity of Δ5 and Δ6 desaturases. These desaturases also control the availability of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are the most representative members of the ω-3 pathway. The existence of an imbalance between ω-6 and ω-3 fatty acids is a key factor in causing tissue inflammation. Unfortunately, humans lack an ω-3 desaturase capable of generating ω-3 fatty acids from the ω-6 type. An alternative approach to re-equilibrate the ω-6 to ω-3 ratio in humans is offered by inhibiting Δ5 and Δ6 desaturases accompanied by exogenous supplementation of ω-3 through the diet. DPA, docosapentaenoic acid.

ω-3 fatty acids in patients with NASH yields a significant heterogeneity in the outcomes between studies,[41–43] our findings suggest that restoration of the hepatic ω-6 to ω-3 balance accompanied by pharmacological modulation of fatty acid desaturation is a potential strategy for the prevention of metabolic liver disease.

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Contributors CL-V performed the experiments, collected and analysed data, interpreted the data and wrote the manuscript. JJL analysed data and interpreted the data of microarrays. AG-P, BR, EM-S and VG-A collected and analysed data. RB contributed to analyse human data and to the revision of the manuscript. MC analysed data and interpreted the chromatographic data. JXK and VA contributed to the revision of the manuscript. JC and ET designed the study, interpreted the data and wrote the manuscript.

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Competing interests None.

Patient consent Obtained.

Ethics approval Ethics Committee of the Hospital Clinic of Barcelona.

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SUPPLEMENTARY MATERIAL AND METHODS

RNA isolation, reverse transcription and real-time PCR

Isolation of total RNA from liver tissue and primary hepatocytes was performed using the TRIzol reagent. RNA concentration was assessed in a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and its integrity tested with a RNA 6000 Nano Assay in a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). cDNA synthesis from 1 μg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Validated and predesigned TaqMan primers and probes from Assays-on-Demand were used to quantify gene expression. Real-time PCR amplifications were carried out in an ABI Prism 7900HT Sequence Detection System and results were analyzed with the Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct method. The amount of target gene, normalized to β-actin and relative to a calibrator, was determined by the arithmetic formula $2^{-\Delta\Delta Ct}$ described in the comparative Ct Method (http://docs.appliedbiosystems. com/pebiodocs/04303859.pdf).

Microarray analysis and geneset enrichment analysis

RNA integrity was re-checked on the Bioanalyzer 2100 before performing microarray hybridization. Preparation of cRNA probes, hybridization, and scanning of arrays were performed according to the manufacturer's protocol (Affymetrix, Santa Clara, CA). Samples were hybridized onto Affymetrix GeneChip® Human Genome U133 Plus 2.0 containing 54,675 probes for 47,000 transcripts. Affymetrix gene expression data were normalized using the guanine-cytosine content–adjusted robust multiarray algorithm using a custom probe set definition that mapped probes directly to Entrez Gene Ids (HGU133Plus2_Hs_ENTREZG). We employed a filtering step excluding probes not reaching a coefficient of variation of 0.01

and the final number of probes reached a total of 12,373 probes. For the detection of differentially expressed genes, a linear model was fitted to the data and empirical Bayes moderated statistics were calculated using the Limma package from Bioconductor (Seattle, WA). Adjustment of p-values was done by the determination of false discovery rates (FDR) using the Benjami-Hochberg procedure. All computations were done using R statistical software.

Fatty acid profiling by gas chromatography (GC)

Total lipids of frozen liver tissue were extracted by the procedure similar to the Folch method.[1] Briefly, chloroform/methanol (2:1 vol/vol) containing 0.005% butylated hydroxytoluene (as antioxidant) was added and mixed vigorously for 30 seconds before adding 100 µl of 0.25% $MgCl_2$ and 1 ml of 0.01N HCl and mixed again. The chloroform phase containing lipids was collected. The remains were extracted with 3 ml of chloroform. The chloroform phases were pooled and dried under nitrogen and subjected to methylation. Fatty acid methyl esters were prepared by methods similar to those described previously,[2, 3] using methanol containing 14% boron trifluoride (BF_3/MeOH). Extracted lipid samples were mixed with BF3/MeOH reagent (1 ml) and the mixtures were heated at 100°C in a metal block for 1 hour, cooled to room temperature and methyl esters extracted twice in the upper (hexane) layer after addition of 1 ml H_2O. Samples were centrifuged at 3600 rpm for 10 min and then the upper hexane layer was removed and evaporated under nitrogen. Fatty acid methyl esters were analyzed by flame ionization GC. GC analysis was carried out with an Agilent 7890 Autosampler apparatus (Agilent Technologies, Santa Clara, CA) equipped with a capillary column (SupraWAX-280, Teknokroma, Barcelona, Spain), length 30 m, 0.25 mm i.d. and film thickness 0.25µm. Column conditions were: initial temperature, 120 ºC for 1.0 min; ramp 15 ºC/min to 210 ºC hold to 35 min; carrier gas, helium. A mixture of standard

fatty acids methyl esters (FAME) was included as an external standard with each run for peak identification by comparison of their retention times (Restek Corporation, Bellefonte, PA). Data acquisition and processing were performed with Agilent-Chemstation software for GC systems. Each fatty acid was expressed as percentage of total fatty acids.

DNA Genotyping

Genomic DNA from the ear was isolated using the Omni-Pure Tissue Genomic DNA System (Gene Link, Hawthorne, NY) following the manufacturer's protocol and genotyped by PCR. The primers used to probe the *fat-1* gene were: 5'-CTGCACCACGCCTTCACCAACC-3' (sense) and 5'-ACACAGCAGCAGATTCCAGAGATT-3' (antisense). The PCR reaction (20 μL) contained 0.2 μM of primers, 0.2 mM of dNTP Mix, 1.5 mM of $MgCl_2$, and 1 U of Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). PCR cycle conditions were 15 min at 95 ºC followed by 30 cycles of 20 s at 94 ºC, 40 s at 62 ºC and 1 min at 72 ºC, and a final step of 10 min at 72 ºC and then cooled to 4 ºC. PCR products were analyzed by electrophoresis in 2% agarose gels and visualized by GelRed staining (Biotium, Hayward, CA) using a 100-bp DNA ladder marker (Invitrogen).

Detection of F4/80 by immunohistochemistry

F4/80 detection was performed in liver tissue paraffin sections. Briefly, sections were deparaffinized, rehydrated, and pretreated with 0.05% trypsin-0.1% $CaCl_2$ for 20 min at 37°C to unmask the antigen, followed by incubation with 0.3% H_2O_2 for 25 min at room temperature and dark conditions to block endogenous peroxidase activity and with 2% BSA for 20 min at room temperature to avoid unspecific binding of the primary antibody. Sections were then incubated overnight at 4°C with the primary rat anti-mouse F4/80 antibody (1:400), followed by incubation for 30 min at room temperature with a biotinylated rabbit anti-rat IgG

secondary antibody (1:200) and incubation with ABC for 30 min at room temperature using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Color was developed using the diaminobenzidine substrate (Dako Diagnostics, Glostrup, Denmark), and sections were counterstained with hematoxylin. Sections were visualized at x200 magnification in a Nikon Eclipse E600 microscope.

Histological analysis of hepatic steatosis

Hepatic steatosis was assessed by Oil Red-O staining in OCT-embedded cryosections. Briefly, sections were fixed in 60% isopropanol for 10 min and stained with 0.3% Oil Red-O (Sigma, St. Louis, MO) in 60% isopropanol for 30 min and subsequently washed with 60% isopropanol. Sections were counterstained with Gill´s hematoxylin, washed with 4% acetic acid solution, and mounted with aqueous solution. Sections were visualized at x200 magnification in a Nikon Eclipse E600 microscope (Kawasaki, Japan). Relative areas of steatosis (expressed as percent Oil Red-O staining) were quantified by histomorphometry using a computerized image analysis system (AnalySIS, Munster, Germany). A minimum of 20 independent fields per sample were evaluated.

Isolation and incubation of primary hepatocytes

Hepatocytes were isolated from wild-type (n=16) and *fat-1* (n=12) mice by a three-step *in situ* perfusion procedure using 0.03% collagenase IV through the portal vein. Isolated hepatocytes were seeded on collagen I-coated plates and cultured in William's E medium supplemented with 10% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 μM insulin, 15 mM HEPES, and 50 μM β-mercaptoethanol. Primary hepatocytes were characterized by a combination of phase-contrast microscopy, immunocytochemical analysis and periodic acid-Schiff reaction, and their viability was determined by trypan blue exclusion. Hepatocytes

growing in 12-well plates ($4x10^5$ cells/well) in 1% FBS-William's E medium containing D-glucose (25 mM) and 1% FFA-free BSA were challenged with oleic acid (200 μM) and incubated with either vehicle (0.01 ethanol), the Δ5/Δ6 desaturase inhibitor CP-24879 (0.1, 1 and 10 μM) or the Δ9 desaturase inhibitor CAY10566 (0.01, 0.1 and 1 μM) (Cayman Chemical, Ann Harbor, MI) in the absence or presence of EPA (10 μM) (Solutex, Madrid, Spain) for 16 hours. At the end of the incubation, intracellular lipid content was measured by Oil Red-O staining as described below. In some experiments, hepatocytes were incubated with vehicle, resolvin D1 (RvD1, 10 nM) and EPA (10 μM) with or without desaturase inhibitors and stimulated with LPS (10 ng/ml) for 2 h. Thereafter, hepatocytes were resuspended in TRIzol and kept at -80°C for further analysis.

Oil Red-O staining of hepatocytes

Primary hepatocytes were washed twice with $DPBS^{-/-}$, exposed to 4% paraformaldehyde for 1 h, and then washed with 60% isopropanol before incubation with 0.2% Oil Red-O for 30 min at room temperature. To quantify the amount of Oil Red-O retained by the cells, hepatocytes were incubated with isopropanol for 30 min with shaking to elute the stain. The amount of stain was measured by optical density at 500 nm in a FluoStar Optima microplate reader (BMG Labtech, Offenburg, Germany). Hepatocytes were also grown ($2x10^5$ cells/well) in Permanox Lab-Teck Chamber Slides (Nalge Nunc, Rochester, NY), stained with Oil Red-O, counterstained with Gill's hematoxylin, washed with tap water, mounted with aqueous solution, and visualized under a Nikon Eclipse E600 microscope at x200 magnification.

Analysis by Western blot of Δ5, Δ6 and Δ9 desaturases and JNK phosphorylation

Total protein from liver was extracted using a modified RIPA buffer containing 50 mM TrisHCl, 150 mM NaCl, 1% Igepal, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM

PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and protease inhibitors. Homogenates were incubated on ice for 15 min with frequent vortexing and centrifuged at 16,100 *g* for 20 min at 4°C. Δ5, Δ6 and Δ9 desaturases, GAPDH and SAPK/JNK (56G8), phospho(Thr183/Tyr185)-SAPK/JNK protein expression was analyzed by Western blot. Total protein (40 mg) from the supernatants was placed in SDS-containing Laemmli sample buffer, heated for 5 min at 95°C, and separated by 12% SDS-PAGE. Proteins were electroblotted for 120 min at 400 mA at 4°C onto PVDF membranes, and the efficiency of the transfer was visualized by Ponceau S staining. Membranes were then soaked for 1 h at room temperature in 0.1% T-TBS and 5% (w/v) nonfat dry milk. Blots were washed 3 times for 5 min each with 0.1% T-TBS and subsequently treated overnight at 4°C with primary rabbit anti-mouse Δ5 desaturase (ab126706; dilution 1/1000; Abcam, Cambridge, UK), Δ6 desaturase (M-50; dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Δ9 desaturase (C12H5; dilution 1/1000; Cell Signaling, Danvers, MA) and phospho-SAPK/JNK antibody (dilution 1:250; Cell Signaling) in 0.1% T-TBS containing 5% BSA. Thereafter, the blots were washed 3 times for 5 min each with 0.1% T-TBS containing 5% nonfat dry milk and incubated for 1 h at room temperature with a horseradish-peroxidase-linked donkey anti-rabbit antibody (1:2000) in 0.1% T-TBS, and bands were visualized using the EZ-ECL chemiluminescence detection kit (Biological Industries, Haemek, Israel). To assess total JNK and GAPDH protein expression, membranes were stripped at 50°C for 20 min in 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.8) and reblotted overnight at 4°C with primary rabbit anti-mouse SAPK/JNK (56G8; dilution 1:250; Cell Signaling) or GAPDH (ab9485; dilution 1/2500; Abcam) antibodies. Membranes were visualized as described above.

Lipid peroxidation analysis

Measurement of Thiobarbituric Acid Reactive Substances (TBARS) was used as a well-established method for assessing tissue levels of lipid peroxidation.[4] Colorimetric measurement of malondialdehyde (MDA) concentrations were determined using the TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer's recommendations. In brief, liver tissue was homogenized using a modified RIPA buffer containing 50 mM TrisHCl, 150 mM NaCl, 1% Igepal, 1 mM EDTA, phosphatase and protease inhibitors. Samples were then centrifuged at 1600g for 10 min at 4°C, and the supernatant was preserved for analysis. Thereafter, 100 μl of provided SDS solution was added to 100 μl of each sample and standard curve, followed by 4 ml of color reagent. Samples and standard curve solutions were boiled for 1 hour, and the reaction was terminated by incubation on ice for 10 min followed by centrifugation at 1600g for 10 min at 4°C. Then, 150 μl of samples and standard curve solutions were loaded in duplicate onto a 96-well plate, and absorbance was read at 540 nm in a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany).

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Supplementary Table 1. Composition of the experimental diets[a]

Components	HFD	HFD+EPA/DHA
Casein	258.4	258.4
L-cystine	3.9	3.9
Maltodextrin	161.5	161.5
Sucrose	88.9	88.9
Cellulose	64.6	64.6
Soybean oil	32.3	32.3
Lard	316.5	272.6
EPA (70% EPA, 12.3% DHA)	-	32.3
DHA (74.5% DHA, 9.2% EPA)	-	11.6
Mineral mixture	12.9	12.9
Dicalcium phosphate	16.8	16.8
Calcium carbonate	7.1	7.1
Potassium citrate	21.3	21.3
Vitamin mixture	12.9	12.9
Choline Bitartrate	2.6	2.6
Vitamin E acetate	0.2	0.2
Detailed fatty acid composition		
C14:0, Myristic	2.8	2.4
C14:1, Myristoleic	1.5	1.4
C16:0, Palmitic	75.3	65.3
C16:1, Palmitoleic	11.9	10.3
C18:0, Stearic	43.0	37.2
C18:1, Oleic	137.0	119.0
C18:2n-6, Linoleic	44.1	40.4
C18:3n-3, Linolenic	5.6	5.3
C20:4n-6, Arachidonic	5.4	4.6
C20:5n-3, EPA	-	23.5
C22:6n-3, DHA	-	12.6
n-6	49.5	45.0
n-3	5.6	41.3
n-6/n-3 ratio	8.8	1.1

[a]*Both diets provide 60% Kcal from fat.*
HFD: high-fat diet; HFD+EPA/DHA: high-fat diet enriched with EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid).

Supplementary Table 2. Hepatic omega-3 fatty acid composition in HFD experimental groups.

ω-3 PUFA	CT	*Fat-1*	ω-3
C18:3n-3, Linoleic acid	6.20 ± 0.22	7.61 ± 0.65	16.17 ± 1.07 *,#
C18:4n-3, Stearidonic acid	0.73 ± 0.09	1.90 ± 0.20 *	2.65 ± 0.15 *
C20:3n-3, Eicosatrienoic acid	0.44 ± 0.02	4.89 ± 1.17 *	0.61 ± 0.07 #
C20:4n-3, Eicosatetraenoic acid	0.80 ± 0.02	3.96 ± 1.48 *	2.83 ± 0.36 *
C20:5n-3, EPA	2.06 ± 0.10	7.57 ± 0.60 *	68.8 ± 0.27 *,#
C22:5n-3, Docosapentaenoic acid	4.33 ± 0.17	7.53 ± 0.28 *	30.40 ± 3.77 *,#
C22:6n-3, DHA	25.67 ± 0.86	77.06 ± 3.91*	128.9 ± 1.31 *,#

All values are expressed as % area /gram liver tissue.
**P<0.05 vs. Control (CT) mice.*
#P<0.05 vs. Fat-1 mice.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Gene expression of delta-5 desaturase (Δ5D), Δ6D and Δ9D assessed by real-time PCR in livers from mice with high-fat diet (HFD)-induced NASH (n=7) and mice fed with chow diet (Chow, n=7). Results are expressed as mean ± SEM. *, P<0.05, ** and P<0.01 versus Chow.

Supplementary Figure 2. (A) Hepatic product to precursor ratios as Δ5 and Δ6 desaturation indexes toward the ω-6 or the ω-3 pathways in livers from mice with high-fat diet (HFD)-induced NASH (n=7) and mice fed with chow diet (Chow, n=7). **(B)** Gene expression of delta-5 desaturase (Δ5D), Δ6D and Δ9D assessed by real-time PCR in livers from wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an EPA/DHA rich diet (ω-3, n=9) after receiving a HFD. Results are expressed as mean ± SEM. *, P<0.05, **, P<0.01 and ***, P<0.001 versus Chow or CT.

Supplementary Figure 3. Body, liver and epididymal white adipose tissue (eWAT) weights, serum leptin, cholesterol, triglycerides, AST and ALT levels in wild-type (WT) (n=7) and *fat-1* (n=5) mice under chow diet (13% Kcal from fat). Results are expressed as mean ± SEM. *, P<0.05 versus WT.

Supplementary Figure 4. Weekly measurements of food intake and weight gain in wild-type (WT) and *fat-1* mice fed with HFD for 16 weeks. Results are expressed as mean ± SEM. *, P<0.05 and **, P<0.01 versus WT. a, P<0.001 versus week 4.

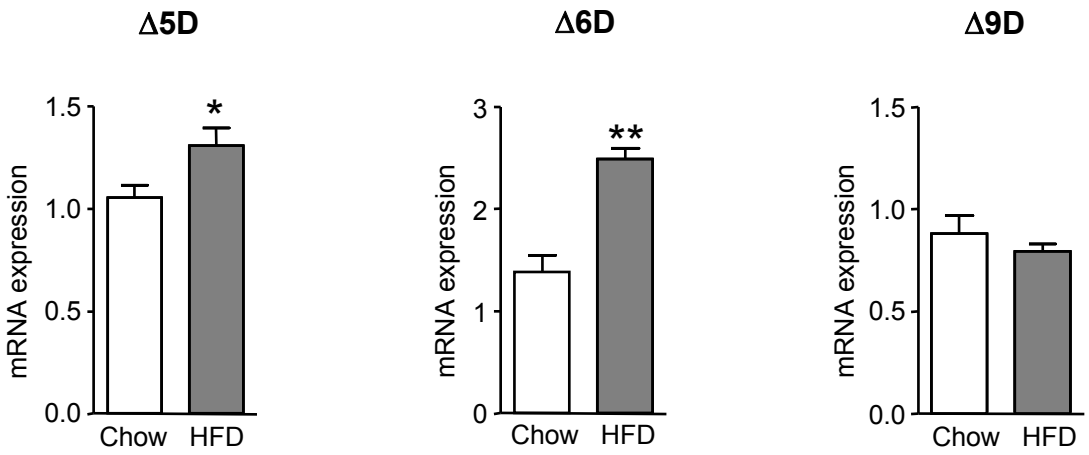
Supplementary Figure 5. Gene expression for IRS-2 and GLUT-2 **(A)**, microsomal triglyceride transfer protein (MTP) **(B)** and TNFα and IL-6 **(C)** assessed by real-time PCR in

liver samples from Chow mice (n=7) and in wild-type (CT) mice (n=7), *fat-1* mice (n=10) and wild-type mice fed an EPA/DHA rich diet (ω-3) (n=9) after receiving a HFD. Results are expressed as mean ± SEM. *, P<0.05 versus HFD-CT.

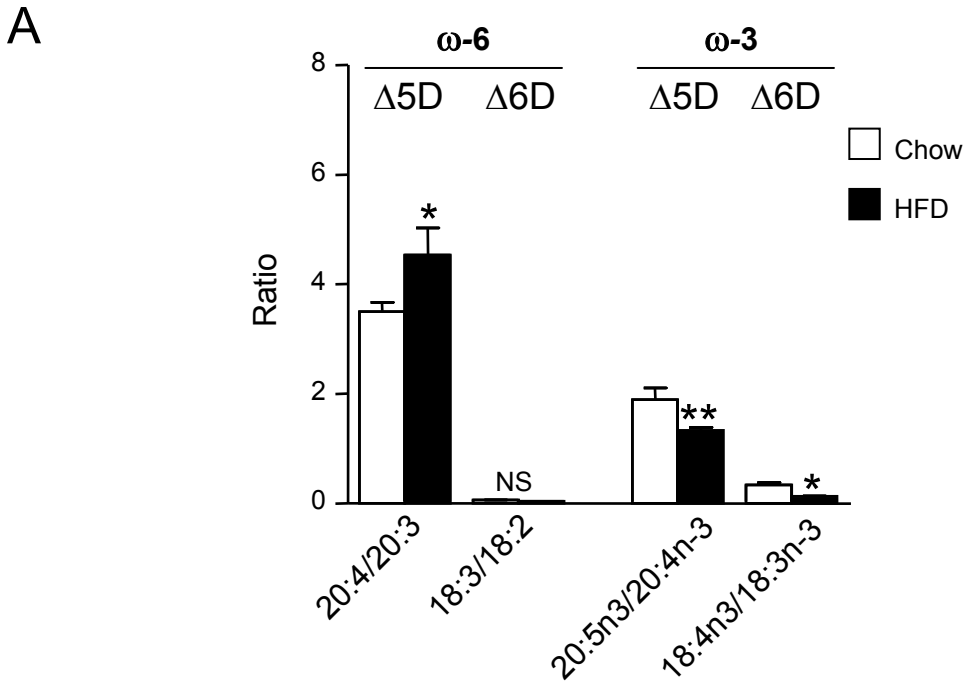
Supplementary Figure 6. Representative images and quantitation of Oil Red-O staining and DGAT2 expression in hepatocytes isolated from wild-type (WT) mice (n=12), *fat-1* mice (n=12) and wild-type mice incubated with EPA (10 μM) (n=16) and exposed to oleic acid (200 μM) and CP-24879 (0.1 μM and 1 μM) or CAY10566, (0.1 μM). Results are mean ± SEM and represent % inhibition versus oleic acid. a, P<0.05 and b, P<0.01 versus oleic acid and *, P<0.05, **, P<0.01 and ***, P<0.001 versus WT.

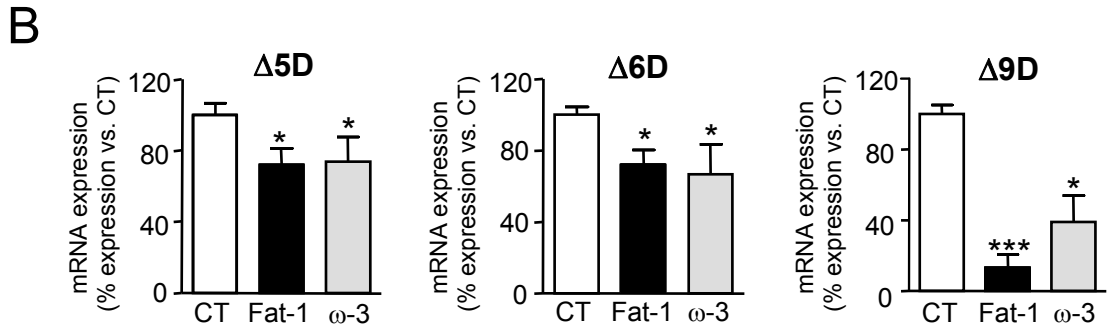
Supplementary Figure 7. (A) Gene expression for IL-1β, TNFα, IL-6 and MCP-1 was assessed by real time-PCR in hepatocytes pretreated with CP-24879 (0.1, 1 and 10 μM) or CAY10566 (0.01, 0.1 and 1 μM) for 16 hours and stimulated with LPS (10 ng/ml) for 2 hours. **(B)** IL-6, IL-1β, TNFα, MCP-1 and CXCL2 mRNA expression in hepatocytes from wild-type mice (WT) incubated with or without EPA (10 μM) (n=16) and *fat-1* mice (n=12) under resting conditions (vehicle, 0.01% ethanol) or following LPS stimulation (10 ng/ml). Results are expressed as mean ± SEM. a, P<0.01 and b, P<0.001 versus vehicle; *, P<0.05, **, P<0.01 and ***, P<0.001 versus WT or vehicle (0 concentration).

Supplementary Figure 1



Supplementary Figure 2





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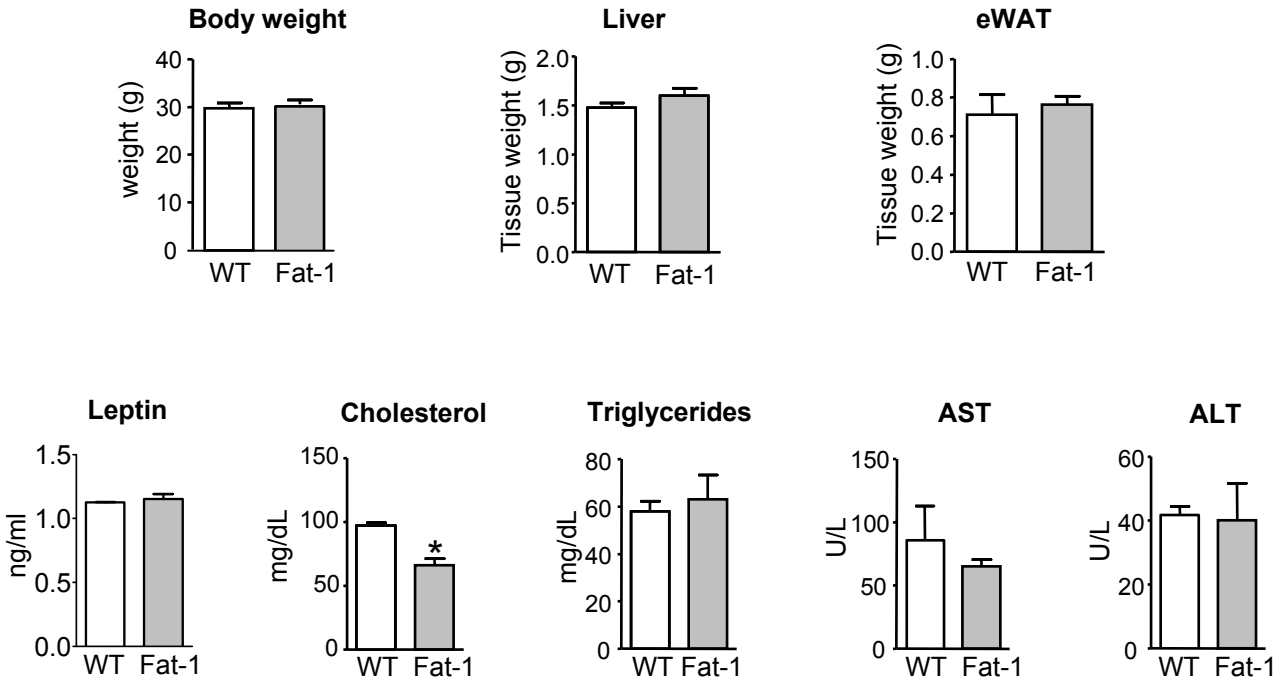

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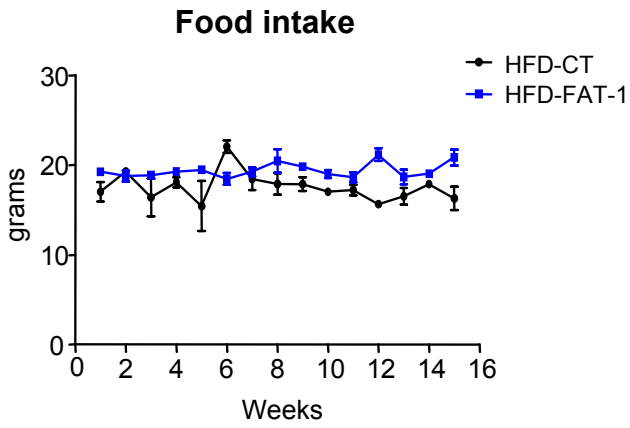
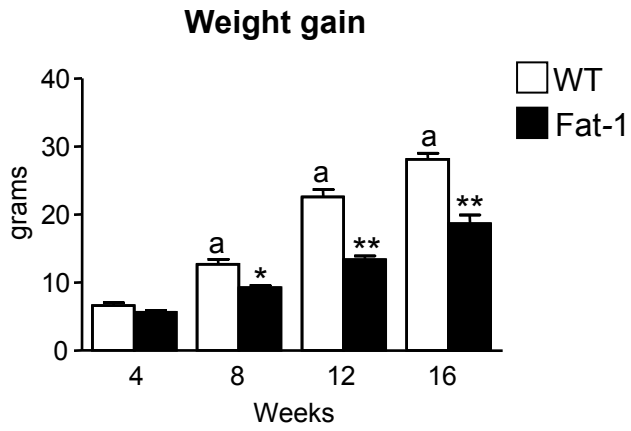
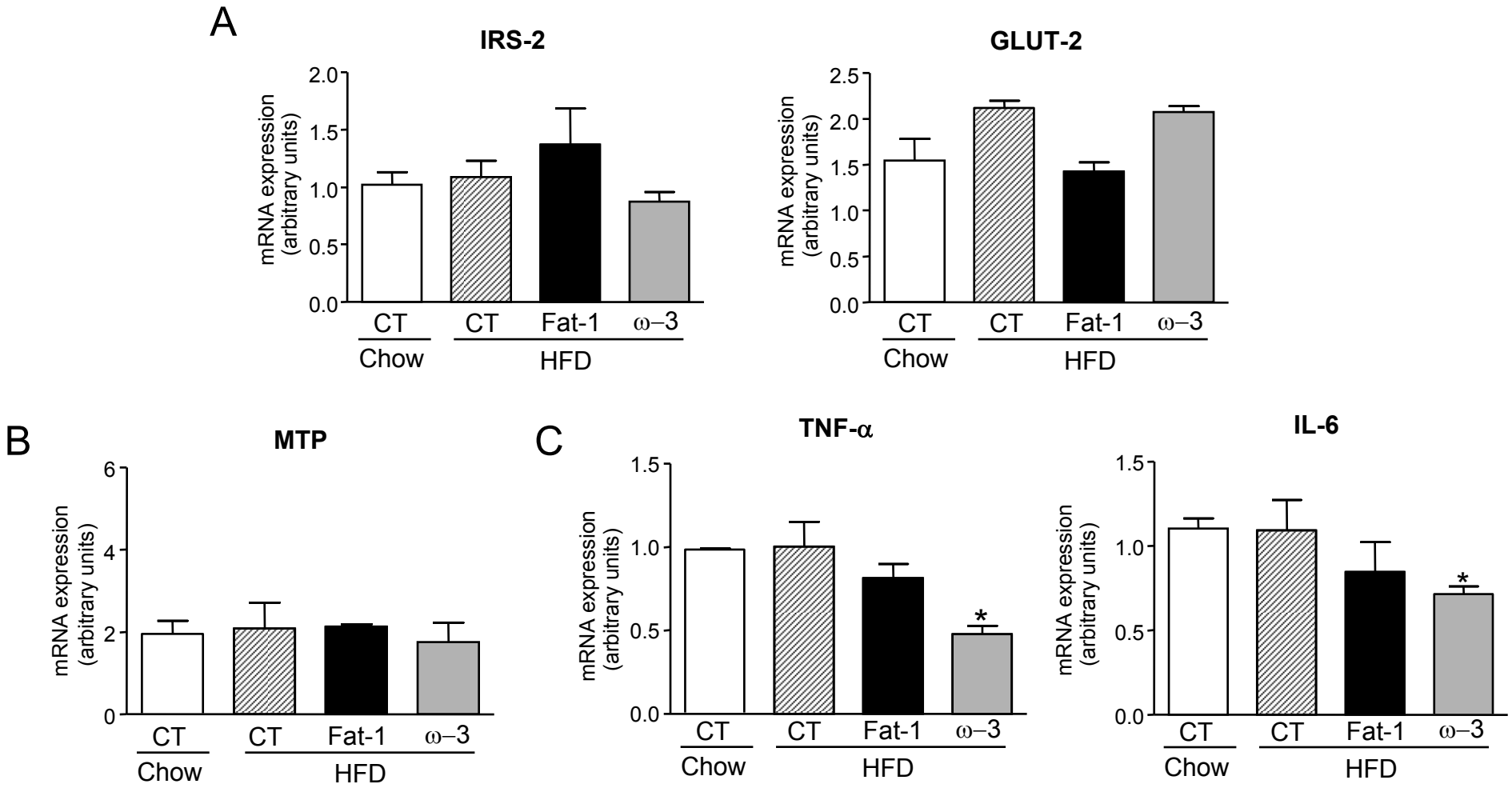
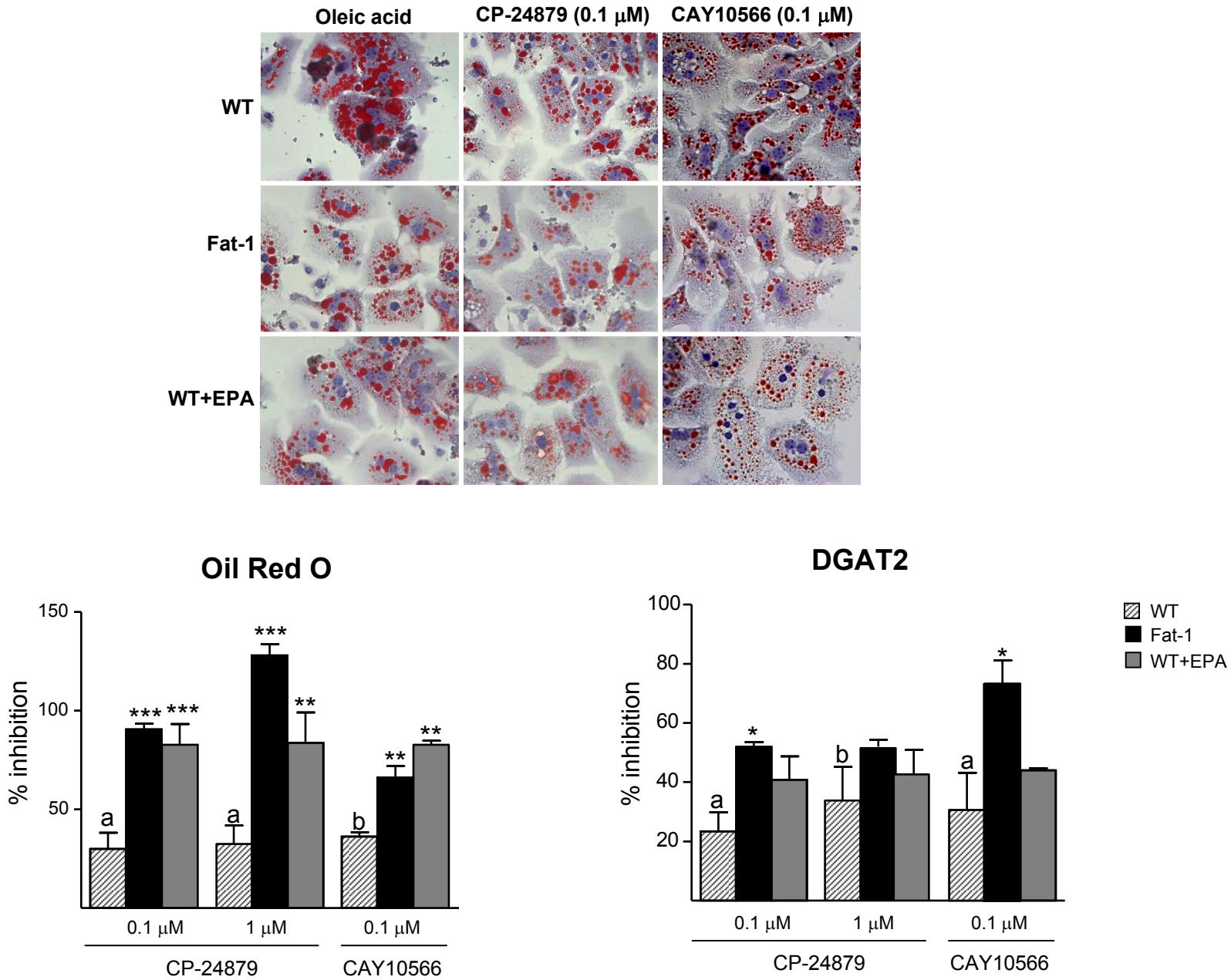




Supplementary Figure 5

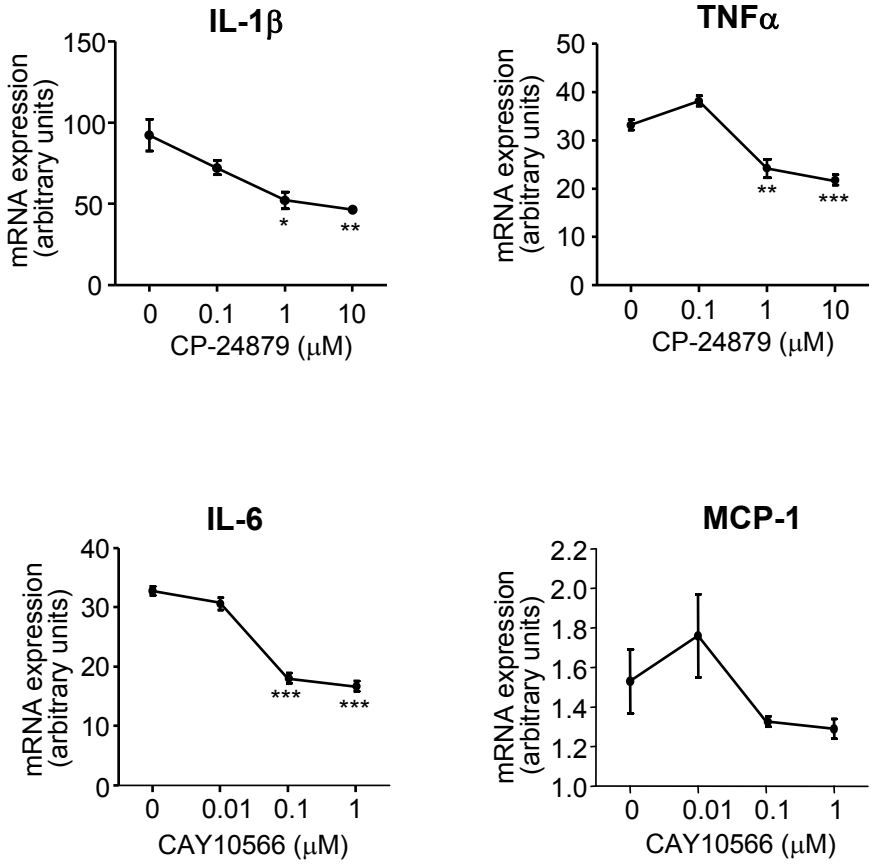


Supplementary Figure 6



Supplementary Figure 7

A



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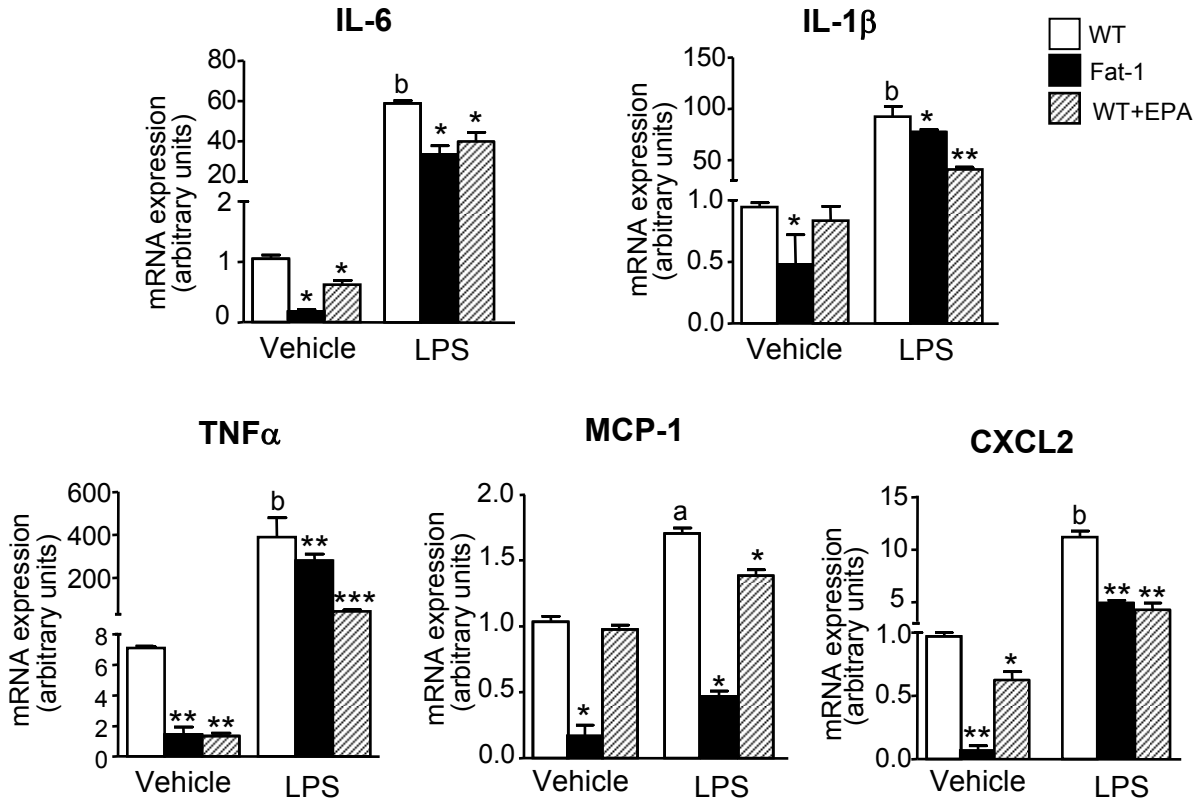

II. ARTÍCULO 2

La inhibición de la epóxido hidrolasa soluble (sEH) modula la inflamación y la autofagia en tejido adiposo obeso e hígado: El papel de los epóxidos de omega-3.

2.1 Patrón de expresión de las isoformas del CYP en el tejido adiposo de ratones obesos *fat-1*.

Tras recibir una dieta hipercalórica durante 16 semanas, los ratones *fat-1* obesos presentaron una reducción de la deposición de colágeno observado por el análisis histológico de secciones teñidas con Sirius Red. Además, los ratones *fat-1* presentaron en el tejido adiposo un incremento de la expresión de marcadores de macrófagos con fenotipo antiinflamatorio y pro-resolutivo M2 (CD206, IL-10 y MGL1). Por otro lado, los ratones *fat-1* presentaron una normalización de la expresión de las isoformas del CYP que epoxidan los PUFA omega-3, CYP1A1, CYP2E1 y CYP2U1.

2.2 Niveles tisulares de los metabolitos epóxidos derivados de los omega-3, 17,18-EEQ y 19,20-EDP, en ratones *fat-1*.

Mediante el análisis por LC-ESI-MS/MS se detectaron metabolitos epoxidados por la vía CYP en el hígado y en el tejido adiposo, siendo éstos más abundantes en hígado. En particular, los niveles de 17,18-EEQ y 19,20-EDP procedentes de EPA y del DHA, respectivamente, fueron más elevados en ratones obesos *fat-1* en comparación con el grupo de ratones control. El análisis de los regioisómeros derivados del AA (5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET) reflejó niveles elevados en ambos tejidos estudiados en el grupo control, un efecto que también fue observado en los ratones *salvajes* que recibieron una dieta omega-3.

2.3 Expresión de la sEH y modulación de los niveles tisulares de 17,18-EEQ y 19,20-EDP a través de un inhibidor selectivo de la sEH, el *t*-TUCB.

La proteína sEH se expresa preferentemente en el hígado y aumenta en la obesidad. Como la inhibición de la sEH evita la inactivación de los metabolitos epoxidados, se analizaron los niveles de los derivados de los omega-3 en un grupo de ratones *salvajes* y *fat-1* obesos tratados con un inhibidor selectivo de la sEH, el *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (*t*-TUCB, 10 μg/ml en el agua de bebida) durante 16 semanas.

La administración del *t*-TUCB en ratones obesos resultó, según el análisis por LC-ESI-MS/MS, en un aumento de los niveles hepáticos de 17,18-EEQ y 19,20-EDP, un efecto que fue más evidente en los ratones *fat-1*. Cabe destacar que la relación entre los metabolitos activos y sus respectivos dioles inactivos (17,18-EEQ/DiHETE y 19,20-EDP/DiHDPA) resultó incrementada con *t*-TUCB. Este inhibidor aumentó la expresión hepática de sEH, posiblemente por un efecto de retroalimentación en la síntesis de la proteína.

2.4 Efectos del *t*-TUCB sobre el tejido adiposo en ratones obesos *fat-1*.

El *t*-TUCB no produjo cambios sobre el peso corporal de los ratones después de ser tratados durante 16 semanas. Consecuentemente, la pérdida de peso fue atribuida al fenotipo de ratones *fat-1*. El análisis de las imágenes anatómicas por resonancia magnética nuclear 7.0T (RMN) mostraron una reducción en el volumen de la grasa corporal total en ratones *fat-1*. Cabe destacar un incremento en el volumen del tejido adiposo marrón del área interescapular en ratones *fat-1* obesos tratados con *t*-TUCB. En estos ratones también se observó una reducción en la hipertrofia de adipocitos, la infiltración de macrófagos y la fibrosis en el tejido adiposo. También se observó en este tejido una reducción significativa en la peroxidación lipídica y una polarización de los macrófagos hacia marcadores antiinflamatorios M2 (MGL1 y RELMα).

2.5 Efectos antiinflamatorios y anti-esteatóticos del *t*-TUCB en el tejido hepático de ratones obesos *fat-1*.

El *t*-TUCB bloqueó de forma efectiva la infiltración hepática de macrófagos en los ratones *salvajes, y este* efecto fue más intenso en ratones *fat-1*. Consistentemente, el *t*-TUCB disminuyó la expresión hepática de IL-1β y IL-6, mientras que aumentó la expresión de MGL1 y CD206. La polarización de macrófagos fue más evidente en ratones *fat-1* que recibieron el tratamiento con *t*-TUCB. Además, por espectroscopia de RMN se detectó que el tratamiento con el inhibidor redujo el contenido lipídico intrahepático e indujo un efecto anti-esteatótico sinérgico en los ratones *fat-1*.

2.6 El *t*-TUCB regula la autofagia en ratones obesos *fat-1* de forma diferenciada en hígado y tejido adiposo.

Los indicadores moleculares de autofagia, Atg12-Atg5 y LC3-II, incrementaron en tejido adiposo y disminuyeron en hígado en el modelo de obesidad. El análisis de la expresión proteica mediante Western blot demostró que el *t*-TUCB redujo en tejido adiposo los niveles conjugados Atg12-Atg5 y LC3-II, los cuales se requieren para la formación de los autofagosomas. Estos efectos fueron más evidentes en ratones *fat-1*, los cuales demostraron tener los niveles de estos marcadores disminuidos acompañados por una acumulación de p62, indicando un fallo funcional de la degradación autofagia. Por el contrario, en el hígado, *t*-TUCB restauró los niveles de Atg12-Atg5 y LC3-II y redujo la expresión proteica de p62, indicando una mejora en el flujo hepático de autofagia.

Como el proceso de autofagia está integrado en la homeóstasis del retículo endoplasmático (RE), la administración con *t*-TUCB resultó en la reducción del estrés del RE, tal y como se observó por la atenuación de la fosforilación de IRE-1α y el eIF2α en el hígado y en el tejido adiposo.

2.7 El metabolito 19,20-EDP ejerce efectos *in vitro* sobre la autofagia inducida por el palmitato sódico, la resistencia a la insulina y la acumulación intracelular de lípidos en células 3T3-L1 diferenciadas a adipocito.

La incubación de adipocitos diferenciados con el 19,20-EDP en presencia de *t*-TUCB produjo en los marcadores de autofagia cambios similares a aquellos observados *in vivo* en el tejido adiposo de ratones obesos *fat-1* que recibieron el inhibidor. También se produjo una reducción de la fosforilación de IRE-1α y eIF2α en respuesta al 19,20-EDP y *t*-TUCB en cultivo de adipocito. Adicionalmente, el 19,20-EDP mejoró la captación de glucosa bajo el estímulo con palmitato, un efecto que alcanzó la significación en presencia de *t*-TUCB. Este metabolito epoxidado derivado del DHA, también indujo la sobreexpresión de los genes involucrados en la señalización de la insulina, IRS-1 y GLUT-4, de forma concentración-dependiente. Por otro lado, el 19,20-EDP significativamente redujo la acumulación intracelular de lípidos en adipocitos incubados con palmitato.

2.8 El 19,20-EDP ejerce efectos *in vitro* sobre la autofagia inducida por el palmitato sódico y la acumulación intracelular lipídica en cultivo de hepatocito primario de ratón. Comparación con el 17(S)-HDHA.

La incubación de hepatocitos primarios con *t*-TUCB resultó en un aumento de la formación de autofagosomas revelado por la desaparición de la expresión de LC3-I y su conversión a LC3-II mediante la conjugación con la fostatidiletanolamina. Esta mejora de la autofagia se reflejó con la atenuación de los marcadores de estrés del RE, phospho-IRE-1α y phospho-eIF2α. Estos efectos se compararon con los del metabolito 17(S)-HDHA, un precursor de la biosíntesis de las resolvinas y protectinas derivadas del DHA a través de la vía de la LOX. Este mediador también demostró mejorar la autofagia y reducir la acumulación intracelular de lípidos estimulada por el palmitato.

Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver: Role for omega-3 epoxides

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Soluble epoxide hydrolase (sEH) is an emerging therapeutic target in a number of diseases that have inflammation as a common underlying cause. sEH limits tissue levels of cytochrome P450 (CYP) epoxides derived from omega-6 and omega-3 polyunsaturated fatty acids (PUFA) by converting these antiinflammatory mediators into their less active diols. Here, we explored the metabolic effects of a sEH inhibitor (*t*-TUCB) in *fat-1* mice with transgenic expression of an omega-3 desaturase capable of enriching tissues with endogenous omega-3 PUFA. These mice exhibited increased CYP1A1, CYP2E1, and CYP2U1 expression and abundant levels of the omega-3–derived epoxides 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) and 19,20-epoxydocosapentaenoic (19,20-EDP) in insulin-sensitive tissues, especially liver, as determined by LC-ESI-MS/MS. In obese *fat-1* mice, *t*-TUCB raised hepatic 17,18-EEQ and 19,20-EDP levels and reinforced the omega-3–dependent reduction observed in tissue inflammation and lipid peroxidation. *t*-TUCB also produced a more intense antisteatotic action in obese *fat-1* mice, as revealed by magnetic resonance spectroscopy. Notably, *t*-TUCB skewed macrophage polarization toward an antiinflammatory M2 phenotype and expanded the interscapular brown adipose tissue volume. Moreover, *t*-TUCB restored hepatic levels of Atg12-Atg5 and LC3-II conjugates and reduced p62 expression, indicating up-regulation of hepatic autophagy. *t*-TUCB consistently reduced endoplasmic reticulum stress demonstrated by the attenuation of IRE-1α and eIF2α phosphorylation. These actions were recapitulated in vitro in palmitate-primed hepatocytes and adipocytes incubated with 19,20-EDP or 17,18-EEQ. Relatively similar but less pronounced actions were observed with the omega-6 epoxide, 14,15-EET, and nonoxidized DHA. Together, these findings identify omega-3 epoxides as important regulators of inflammation and autophagy in insulin-sensitive tissues and postulate sEH as a druggable target in metabolic diseases.

obesity | inflammation | autophagy | omega-3–derived epoxides | soluble epoxide hydrolase

Cytochrome P450 (CYP) epoxygenases represent the third branch of polyunsaturated fatty acid (PUFA) metabolism (1). CYP epoxygenases add oxygen across one of the four double bonds of PUFA to generate three-membered ethers known as epoxides (1). In the case of arachidonic acid, CYP epoxygenases convert this omega-6 PUFA into epoxyeicosatrienoic acids (EETs), which act as autocrine or paracrine factors in the regulation of vascular tone, inflammation, hyperalgesia, and organ and tissue regeneration (2, 3). In addition to omega-6s, CYP epoxygenases also convert the omega-3 PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) into novel epoxyeicosatetraenoic (EEQs) and epoxydocosapentaenoic (EDPs) acids, respectively (4, 5). These omega-3–derived epoxides also exert salutary actions and are even more effective and potent than omega-6–derived EETs (4–8).

Because the predicted in vivo half-lives of fatty acid epoxides (EpFA) are in the order of seconds (9), drugs that stabilize their levels by targeting the enzyme soluble epoxide hydrolase (sEH) are currently under investigation. sEH is a cytosolic enzyme with epoxide hydrolase and lipid phosphatase activities that catalyzes the rapid hydrolysis of EETs, EEQs and EDPs by adding water to these EpFA and converting them into inactive or less active 1,2-diols (10). Accordingly, inhibition of sEH exerts beneficial actions in controlling vascular tone, inflammation, and pain, and this strategy has shown its therapeutic potential for long-term use in hypertension, diabetes, renal disease, organ damage, and vascular remodeling (6, 9–12).

The aim of the present study was to investigate the potential metabolic benefits of sEH inhibition in obesity. Specifically, this study addresses the question as to whether sEH inhibition increases the effectiveness of omega-3–derived epoxides in obese adipose tissue and liver in the context of enriched omega-3 tissue content. *fat-1* mice with transgenic expression of the *Caenorhabditis elegans* omega-3 fatty acid desaturase gene represent a useful model to address this question because these mice have abundant tissue omega-3 distribution from their embryonic stage

Significance

Our study demonstrates that stabilization of cytochrome P-450 epoxides derived from omega-3 polyunsaturated fatty acids through inhibition of the inactivating enzyme soluble epoxide hydrolase (sEH) exerts beneficial actions in counteracting metabolic disorders associated with obesity. In addition, our study sheds more light on the role of sEH in cellular homeostasis by providing evidence that omega-3 epoxides and sEH inhibition regulate autophagy and endoplasmic reticulum stress in insulin-sensitive tissues, especially the liver. Therefore, administration of a sEH inhibitor is a promising strategy to prevent obesity-related comorbidities.

Author contributions: C.L.-V., V.A., and J.C. designed research; C.L.-V., J.A.-Q., V.G.-A., B.R., E.T., A.L., and J.C. performed research; C.L.-V., S.H.H., and B.D.H. contributed new reagents/analytic tools; C.L.-V. and J.C. analyzed data; and C.L.-V., B.D.H., and J.C. wrote the paper.

Reviewers: K.G., University of California, Berkeley; and S.W., Lipomics Technologies Inc.

Conflict of interest statement: B.D.H. and S.H.H. are authors on a patent held by the University of California on the synthesis of soluble epoxide hydrolase (sEH) inhibitors. B.D.H. founded a company, EicOsis, to move these inhibitors to the clinic to treat neuropathic and inflammatory pain. The published and freely available sEH inhibitor was provided by University of California, Davis to the Claria group in Spain along with additional reagents and data. The inhibitor was a key tool to test the hypothesis that the omega-6 and 3 fatty acid epoxides were responsible for biological effects. It is conceivable that use of an sEH inhibitor could be of some benefit to EicOsis or GSK, both of which are working to develop these materials clinically. However, numerous papers have been published already implicating these inhibitors in diabetes treatment, and other inhibitors of similar structure and potency are commercially available from Cayman Chemical and CalBiochem. B.D.H. is an author on University of California patents in the area, has stock in EicOsis, which has licensed these patents, but has no salary from EicOsis.

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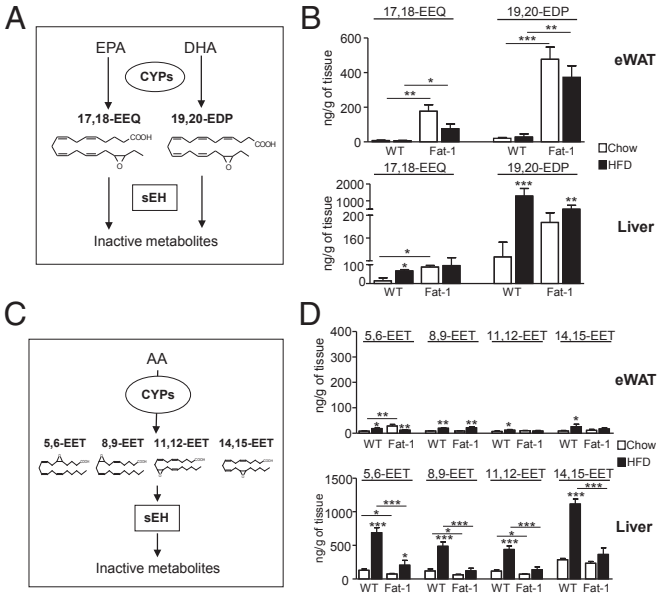

Fig. 1. Tissue levels of omega-3–derived epoxy metabolites, 17,18-EEQ and 19,20-EDP, in *fat-1* mice. (*A*) Schematic diagram of the CYP epoxygenation pathway generating the omega-3 EpFA, 17,18-EEQ, and 19,20-EDP and conversion into their inactive diols by sEH. (*B*) LC-ESI-MS/MS analysis of 17,18-EEQ and 19,20-EDP levels in eWAT and liver from WT and *fat-1* mice. (*C*) Schematic diagram of the CYP epoxygenation pathway generating omega-6 EpFA (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) from arachidonic acid (AA) and their inactivation by sEH. (*D*) LC-ESI-MS/MS analysis of omega-6 EpFA levels in eWAT and liver. Results are mean ± SEM from WT ($n = 28$) and *fat-1* ($n = 20$) mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

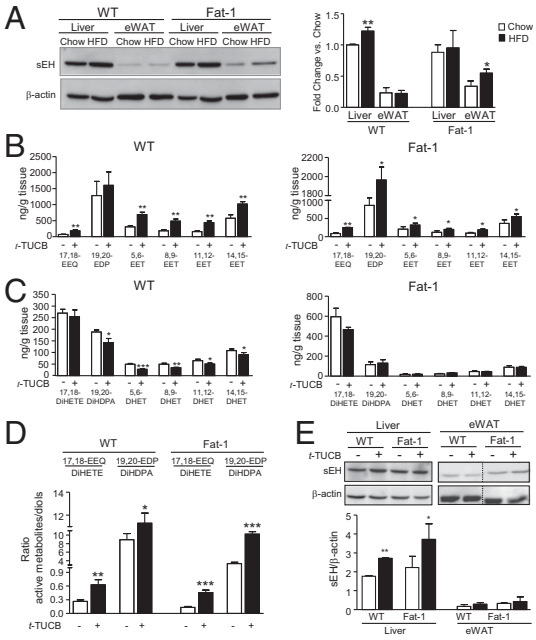

Fig. 2. sEH overexpression and modulation of 17,18-EEQ and 19,20-EDP tissue levels by the selective sEH inhibitor, *t*-TUCB. (*A*) Protein expression of sEH in liver and eWAT from WT and *fat-1* mice. Densitometry of sEH signals normalized to β-actin is shown on the right. (*B*) LC-ESI-MS/MS analysis of omega-3 and omega-6 epoxides in liver from HFD-induced obese WT and *fat-1* mice receiving *t*-TUCB (10 μg/mL) for 16 wk. (*C*) LC-ESI-MS/MS analysis of inactive diols in livers from obese WT and *fat-1* mice receiving *t*-TUCB. (*D*) Hepatic ratios of active metabolites to inactive diols. (*E*) Protein expression of sEH in liver and eWAT from obese WT and *fat-1* mice receiving *t*-TUCB. Densitometry of sEH signals normalized to β-actin is shown below. Results are mean ± SEM from WT ($n = 28$) and *fat-1* ($n = 20$) mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

and throughout their lives (13, 14). This study builds on previous work by our laboratory demonstrating that *fat-1* mice replicate the protection against insulin resistance and hepatic inflammation and steatosis observed in obese mice nutritionally enriched with exogenous omega-3 PUFA (13, 15). The results of the present investigation indicate that inhibition of sEH when there is an increased content of omega-3 PUFA exerts a more favorable role in counteracting the metabolic disorders associated with obesity. In addition, our findings expand focus to include EpFA to the protective actions described for those lipid mediators derived from omega-3s through lipoxygenase- and cycloxygenase-initiated pathways (i.e., resolvins, protectins, and maresins) (16, 17).

Results

WT and *fat-1* mice had similar body and epididymal white adipose tissue (eWAT) weights under Chow conditions (Fig. S1*A*). However, *fat-1* mice exhibited smaller adipocyte size (Fig. S1*B*). The administration of a high-fat diet (HFD) resulted in increased adipocyte hypertrophy and extensive positive F4/80 staining (Fig. S1*B*). HFD-fed mice also displayed enhanced fibrosis (Fig. S1*B*). Compared with WT, *fat-1* mice were more resistant to HFD-induced obesity (body weight: 45.6 ± 0.8 vs. 49.7 ± 1.0 g, $P < 0.01$; eWAT weight: 1.5 ± 0.1 vs. 1.8 ± 0.1 g, $P < 0.01$) and showed reduced adipocyte size, macrophage infiltrate, and fibrosis (Fig. S1*B*). Changes in adiposity, inflammation and fibrosis were confirmed by morphometric analysis and by assessing F4/80 expression by real-time PCR (Fig. S1*B*). *fat-1* mice also showed reduced monocyte chemoattractant protein 1 (MCP-1) and increased CD206, IL-10, and macrophage galactose-type C-type lectin 1 (MGL1) (Fig. S1*C*). These results were confirmed in HFD-induced obese mice receiving omega-3 PUFA through the diet (Fig. S1*D*). No changes in IL-6, IL-1β, arginase-1 (Arg1), resistin-like molecule-α (RELMα), and Ym1 were observed (Fig. S1*D*). eWAT from both lean WT and *fat-1* mice had constitutive expression of CYP epoxygenases with preference for omega-3 PUFA (Fig. S1*E*). In response to the HFD, CYP expression was repressed in WT mice, whereas CYP1A1

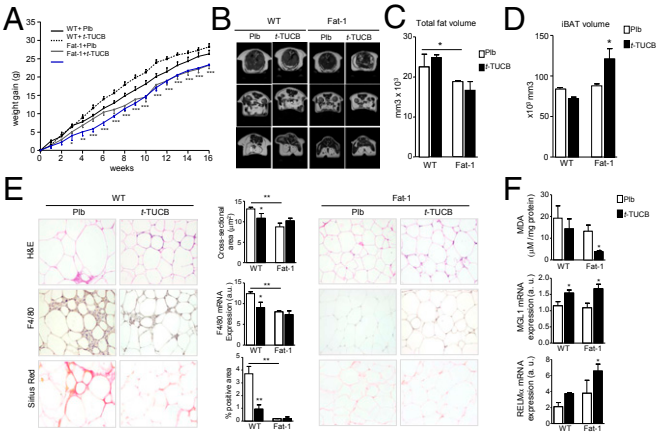

Fig. 3. Effects of *t*-TUCB on adipose tissue in obese *fat-1* mice. (*A*) Body weight curves for WT ($n = 28$) and *fat-1* ($n = 20$) mice receiving a HFD and treated with either placebo (Plb) or *t*-TUCB for 16 wk. (*B*) Representative MR images of coronal sections. Total fat volume (*C*) and iBAT volume (*D*) assessed by MRI analysis. (*E*) Representative photomicrographs of adipose tissue sections stained with H&E (*Top*), F4/80 (*Middle*), and Sirius red (*Bottom*) and adipocyte cross-sectional area, Sirius red staining, and F4/80 mRNA expression. (Magnification: 200×.) (*F*) Malondialdehyde (MDA) concentration and MGL1 and RELMα mRNA expression in adipose tissue. Results are mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

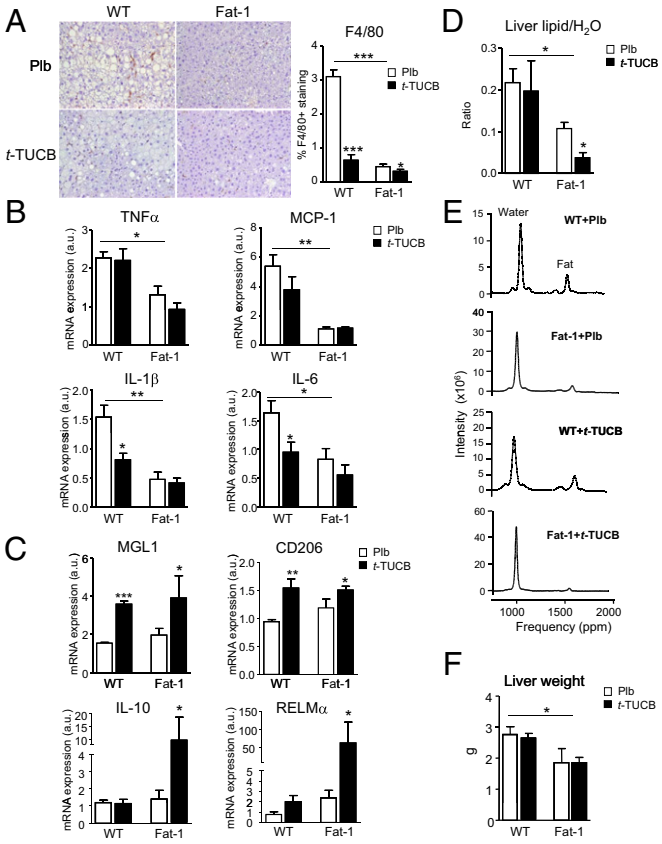

Fig. 4. Antiinflammatory and antisteatotic actions of *t*-TUCB in livers from obese *fat-1* mice. (*A*) Representative photomicrographs of liver sections stained with F4/80 and histomorphometrical analysis in HFD-induced obese WT and *fat-1* mice receiving placebo (Plb) or *t*-TUCB (10 μg/mL) for 16 wk. (Magnification: 200×.) (*B*) Hepatic mRNA expression of M1 proinflammatory markers. (*C*) Hepatic mRNA expression of M2 antiinflammatory markers. (*D*) Hepatic steatosis assessed by MR spectroscopy. (*E*) Representative spectra. (*F*) Liver weight. Results are mean ± SEM from WT ($n = 28$) and *fat-1* ($n = 20$) mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

was induced in *fat-1* mice with no changes in CYP2E1 and CYP2U1 (Fig. S1*E*).

Because—in addition to adipose tissue—the liver plays a pivotal role in metabolic homeostasis, we also examined the hepatic phenotype of *fat-1* mice. Consistent with our recent finding that *fat-1* mice are protected against HFD-induced hepatic inflammation and steatosis (13), HFD-fed *fat-1* mice presented lower serum ALT/AST and reduced F4/80 and Oil Red-O staining (Fig. S2*A*). Moreover, HFD-fed WT mice exhibited a unique hepatic pattern of CYP expression characterized by up-regulation of CYP1A1, CYP2E1, and CYP2U1 (Fig. S2*B*). In contrast, only CYP2U1 was up-regulated by HFD feeding in *fat-1* mice (Fig. S2*B*). A distinct pattern of CYP expression in response to HFD was also detected in skeletal muscle (Fig. S2*C*). Direct comparison of CYP expression among insulin-sensitive tissues revealed that CYP2E1 and CYP2U1 are preferentially expressed in the liver, whereas CYP1A1 appears as the major isoform in muscle (Fig. S2*D*). The observation that changes in CYP expression in HFD-fed mice in response to omega-3 enrichment are tissue-dependent was confirmed in obese WT mice receiving omega-3 PUFA through the diet (Fig. S3).

Because many CYP epoxygenases can form epoxides converting omega-3 PUFA into biologically active 17,18-EEQ and 19,20-EDP metabolites (Fig. 1*A*), we next analyzed these epoxides by liquid chromatography-electrospray ionization (LC-ESI)-MS/MS. Both EPA-derived 17,18-EEQ and DHA-derived 19,20-EDP were detected in eWAT and liver (Fig. 1*B*). Importantly, 17,18-EEQ levels were significantly increased in eWAT and liver from *fat-1* mice (Fig. 1*B*). In these tissues, 19,20-EDP was also increased in *fat-1* mice and changes in this epoxide reached statistical significance in eWAT (Fig. 1*B*). Unexpectedly, HFD triggered omega-3–derived epoxides in the liver, especially in WT mice (Fig. 1*B*). Increased tissue levels of omega-3–derived epoxides were also seen in eWAT and liver from obese WT mice after receiving an omega-3–enriched diet (Fig. S4*A*). On the other hand, EETs regioisomers (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET), formed from arachidonic acid by CYP epoxidation (Fig. 1*C*), were also detected in eWAT and especially in liver (Fig. 1*D*). Hepatic EET levels were also increased by HFD (Fig. 1*D*), an effect also seen in WT mice receiving an omega-3–enriched diet (Fig. S4*B*). The fact that omega-3– and omega-6–derived epoxides were more abundant in liver (Fig. 1 *B* and *D*) was consistent with the presence of a higher content of PUFA in this organ (Fig. S5*A*). Moreover, although the rate-limiting enzymes involved in long-chain fatty acid desaturation (i.e., Δ5 and Δ6 desaturases) were constitutively expressed in liver, the expression of Δ6 desaturase in eWAT was undetectable and residual at protein (Fig. S5*B*) and mRNA (Fig. S5*C*) levels, respectively.

We next sought to establish the role of sEH, the key enzyme in the inactivation of EpFA. Consistent with previous findings, the sEH protein was preferentially expressed in liver (18) and slightly

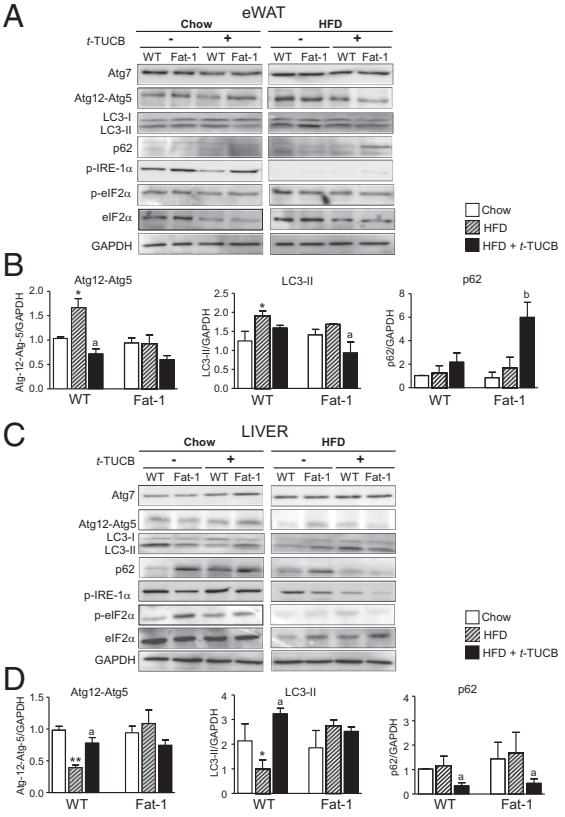

Fig. 5. *t*-TUCB regulates in a tissue-specific manner autophagy and ER stress in obese *fat-1* mice. (*A*) Protein expression of Atg7, Atg12-Atg5, LC3-I/II, p62, phosphoIRE-1α (p-IRE-1α), phosphoeIF2α (p-eIF2α), total eIF2α (eIF2α), and GAPDH as determined by 10% SDS/PAGE Western blot in eWAT from WT and *fat-1* mice receiving Chow or HFD and treated with *t*-TUCB (10 μg/mL) for 16 wk. (*B*) Densitometric analysis of Atg12-Atg5, LC3-I/II, and p62 signals normalized to GAPDH in eWAT. (*C*) Hepatic protein expression of Atg-7, Atg12-Atg5, LC3-I/II, p62, p-IRE-1α, p-eIF2α, eIF2α, and GAPDH. (*D*) Densitometric analysis of Atg12-Atg5, LC3-I/II, and p62 signals normalized to GAPDH in liver. Results are mean ± SEM from WT ($n = 28$) and *fat-1* ($n = 20$) mice. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Chow. $^{a}P < 0.05$ and $^{b}P < 0.001$ vs. HFD.

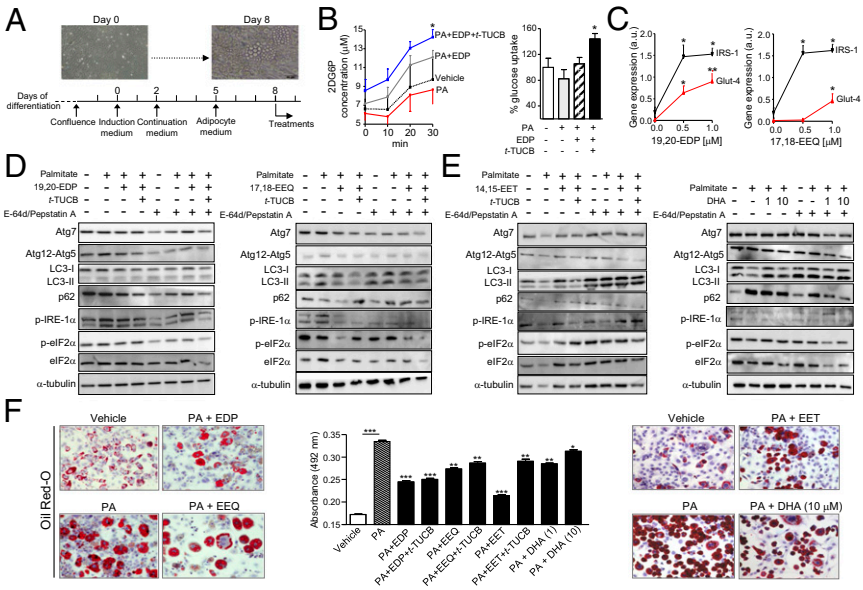

Fig. 6. In vitro effects of EpFA on glucose uptake, autophagy, ER stress, and lipid accumulation in differentiated 3T3-L1 adipocytes. (*A*) Scheme of the in vitro experiments. Visible microscope images at days 0 and 8 of differentiation are shown. (*B*, *Left*) Glucose uptake assessed by the 2-deoxyglucose assay in cells incubated with vehicle or sodium palmitate (PA) alone or in combination with 19,20-EDP (EDP) and *t*-TUCB for 30 min. (*Right*) Quantitation of endpoint glucose uptake. (*C*) GLUT-4 and IRS-1 expression in cells exposed to 19,20-EDP (*Left*) or 17,18-EEQ (*Right*) in the presence of PA. (*D* and *E*) Western blots (15% SDS/PAGE) of Atg7, Atg12-Atg5, LC3-I/II, p62, phosphoIRE-1α (p-IRE-1α), phosphoeIF2α (p-eIF2α), total eIF2α (eIF2α), and α-tubulin in cells incubated with vehicle or PA (0.5 mM) alone or in combination with *t*-TUCB (1 μM), 19,20-EDP (1 μM), 17,18-EEQ (1 μM), 14,15-EET (1 μM), and DHA (1 and 10 μM) in the absence or presence of the protease inhibitors E-64d/pepstatin A for 24 h. (*F*) Representative photomicrographs of Oil Red-O–stained cells incubated with the same conditions as *D* and *E*. (Magnification: 200×.) The amount of Oil Red-O retained is shown on the middle. Results are mean ± SEM from three independent experiments assayed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$.

increased by HFD feeding (Fig. 2*A*). In *fat-1* mice, HFD-feeding also increased sEH expression in eWAT (Fig. 2*A*). Because inhibition of the sEH prevents the inactivation of EpFA by epoxide hydration, we next analyzed the omega-3–derived epoxides in mice treated with the selective sEH inhibitor, *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid (*t*-TUCB). Administration of *t*-TUCB to HFD-induced obese mice resulted in increased 17,18-EEQ levels in liver (Fig. 2*B*) and eWAT (Fig. S5*D*) from both WT and *fat-1* mice. Tissue levels of 19,20-EDP were increased in livers from *fat-1* mice and eWAT from WT animals (Fig. 2*B* and Fig. S5*D*). *t*-TUCB also increased levels of EETs in liver and eWAT from both WT and *fat-1* mice, although the extent of stimulation was less pronounced than that of 19,20-EDP (Fig. 2*B* and Fig. S5*D*). Hepatic levels of 19,20-DiHDPA, the inactive or less active 1,2-diol from 19,20-EDP, as well as the respective EETs diols were significantly reduced by *t*-TUCB in WT mice (Fig. 2*C*). No changes in diol levels were observed in *fat-1* mice (Fig. 2*C*). Of note, the hepatic ratios of each active epoxide to the corresponding inactive diol were significantly increased by *t*-TUCB (Fig. 2*D*). Compared with placebo, *t*-TUCB did not induce any significant effect on the hepatic and adipose tissue levels of arachidonic acid, DHA and EPA (Table S1). Coincident with previous findings (19), *t*-TUCB up-regulated hepatic sEH protein expression (Fig. 2*E*).

Because sEH inhibition is associated with salutary effects, we next assessed the metabolic actions of *t*-TUCB in mice with HFD-induced obesity. As shown in Fig. 3*A*, *t*-TUCB did not modify weight gain in WT mice or alter the resistance of *fat-1* mice to become obese. Consequently, endpoint body weight was only influenced by the *fat-1* phenotype (Fig. S6*A*). Anatomical 7.0T magnetic resonance (MR) imaging analysis confirmed reduced total fat volume in *fat-1* mice and absence of changes in this parameter following *t*-TUCB treatment (Fig. 3 *B* and *C* and Fig. S6*B*). Interestingly, interscapular brown adipose tissue (iBAT) volume was increased in *fat-1* mice treated with *t*-TUCB (Fig. 3*D* and Fig. S6*C*). Despite the absence of changes in total fat volume, *t*-TUCB significantly reduced adipocyte hypertrophy, macrophage infiltration, and adipose tissue fibrosis in obese WT mice (Fig. 3*E*). No further reduction in these adiposity parameters was observed in obese *fat-1* mice receiving *t*-TUCB (Fig. 3*E*). These findings were confirmed by morphometric analysis and the assessment of F4/80 mRNA expression (Fig. 3*E*). Changes in adipose tissue fibrosis were also confirmed by Masson's trichrome staining (Fig. S6*D*). Of interest, *t*-TUCB significantly reduced lipid peroxidation in *fat-1* mice and up-regulated the expression of MGL1 and RELMα in both WT and *fat-1* mice (Fig. 3*F*).

The effects of *t*-TUCB on the liver are shown in Fig. 4. *t*-TUCB effectively blocked HFD-induced hepatic macrophage infiltration in WT mice, an effect that was more intense in *fat-1* mice (Fig. 4*A*). Consistently, *t*-TUCB decreased hepatic IL-1β and IL-6 expression (Fig. 4*B*), while up-regulating MGL1 and CD206 (Fig. 4*C*). M2 polarization of hepatic macrophages was more evident in *fat-1* mice receiving *t*-TUCB (Fig. 4*C*). In addition to reducing inflammation, *t*-TUCB decreased the hepatic lipid content and induced a synergistic antisteatotic action in *fat-1* mice, as detected by MR spectroscopy (Fig. 4*D*). Representative spectra are depicted in Fig. 4*E*. The reduction of liver weight in *fat-1* mice was not further decreased by *t*-TUCB (Fig. 4*F*).

Because dysregulation of autophagy is a critical component of liver and eWAT dysfunction in obesity (20), we next investigated the effects of sEH inhibition on autophagy and the emergence of endoplasmic reticulum (ER) stress, insulin resistance, and lipid deposition in obesity. Consistent with previous studies (21, 22), HFD-induced obesity increased the activity of the molecular indicators of autophagy, Atg12-Atg5, and LC3-II, in eWAT from WT mice, effects that were reversed by *t*-TUCB treatment (Fig. 5 *A* and *B*). No changes in Atg12-Atg5 and LC3-II conjugates were observed in *fat-1* mice after HFD feeding, but LC3-II was reduced and p62 was increased by *t*-TUCB (Fig. 5 *A* and *B*). On the other hand, in livers from WT mice, HFD-induced obesity was associated with reduced Atg12-Atg5 and LC3-II levels, and these markers of autophagy were restored by *t*-TUCB (Fig. 5 *C* and *D*). Reduced autophagy was not observed in livers from *fat-1* mice, suggesting that these mice were already protected from HFD-induced autophagy dysfunction (Fig. 5 *C* and *D*). Taken together, and considering that Atg12-Atg5 and LC3-II conjugates are required for autophagosome formation and that the induction of p62 levels indicates a lack of functional autophagic degradation (23), our data suggest that sEH inhibition improves the autophagy flux in obese insulin-sensitive tissues. Finally, consistent with the view that autophagy is integrated to ER homeostasis (20), *t*-TUCB administration resulted in reduced ER stress, as shown by the attenuation of inositol-requiring enzyme 1α (IRE-1α) and eukaryotic initiation factor 2 (eIF2α) phosphorylation in both eWAT and liver (Fig. 5 *A* and *C*). The extent of suppression of ER stress by *t*-TUCB was roughly similar in WT and *fat-1* mice (Fig. 5 *A* and *C*).

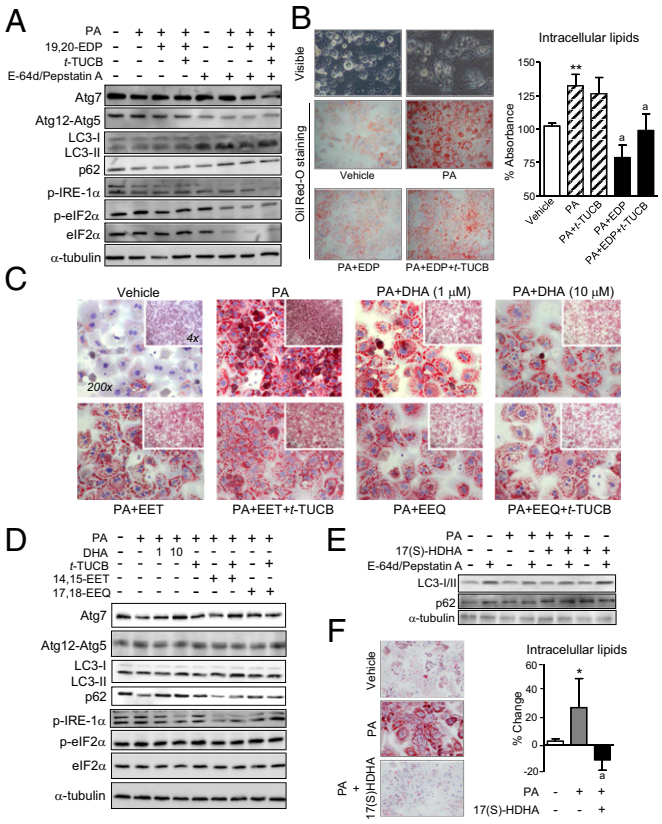

Fig. 7. In vitro effects of EpFA on autophagy, ER stress, and lipid accumulation in primary hepatocytes. (*A*) Western blots (15% SDS/PAGE) of Atg7, Atg12-Atg5, LC3-I/II, p62, phosphoIRE-1α (p-IRE-1α), phosphoeIF2α (p-eIF2α), total eIF2α (eIF2α), and α-tubulin in hepatocytes incubated with vehicle or palmitate (PA) (0.5 mM) in combination with 19,20-EDP (1 μM) and *t*-TUCB (1 μM) in the absence or presence of E-64d/pepstatin A for 24 h. (*B*) Representative photomicrographs of visible microscope (*Top*) and Oil Red-O–stained (*Middle* and *Bottom*) hepatocytes exposed to vehicle or PA alone or in combination with 19,20-EDP (EDP) and *t*-TUCB for 24 h. The amount of Oil Red-O retained is shown on the right. (*C*) Representative photomicrographs of Oil Red-O–stained hepatocytes exposed to either vehicle or PA alone or in combination with 17,18-EEQ (EEQ, 1 μM), 14,15-EET (EET, 1 μM), DHA (1 and 10 μM), and *t*-TUCB (1 μM) for 24 h. (*D*) Western blot analysis of Atg7, Atg12-Atg5, LC3-I/II, p62, p-IRE-1α, p-eIF2α, eIF2α, and α-tubulin in hepatocytes incubated with the same conditions as in *C*. (*E*) Protein expression of LC3-I/II, p62 and α-tubulin in hepatocytes incubated with vehicle or PA in combination with 17(S)-HDHA (1 μM). (*F*) Amount of Oil Red-O retained by hepatocytes in response to PA and 17(S)-HDHA. Results are mean ± SEM from four independent experiments assayed in triplicate. $^{*}P < 0.01$ and $^{**}P < 0.001$ vs. vehicle. a, $P < 0.05$ vs. PA. (Magnification: 200×.)

To provide direct evidence linking omega-3 EpFA to autophagy and ER stress, we next performed in vitro experiments in adipocytes incubated with the saturated fatty acid palmitate, a major contributor to lipotoxicity and insulin resistance (24). A schematic diagram of the experimental in vitro procedure is shown in Fig. 6*A*. Incubation of adipocytes with 19,20-EDP, the most abundant omega-3 epoxide in eWAT, in the presence of *t*-TUCB, stimulated glucose uptake in adipocytes (Fig. 6*B*). Moreover, in these cells, 19,20-EDP and 17,18-EEQ induced a concentration-dependent up-regulation of insulin receptor substrate-1 (IRS-1) and glucose transporter-type 4 (GLUT-4) (Fig. 6*C*). Interestingly, in the presence of *t*-TUCB, both 19,20-EDP and 17,18-EEQ produced similar changes in autophagy to those reported in vivo in eWAT from obese *fat-1* mice receiving *t*-TUCB (Fig. 6*D*). With *t*-TUCB on board, reduction of IRE-1α and eIF2α phosphorylation in response to these omega-3 EpFA also paralleled that seen in vivo (Fig. 6*D*). In these experiments, the omega-6 EpFA, 14,15-EET, and the nonoxidized form of DHA were either ineffective or less active than omega-3 EpFA in regulating authophagy and ER stress in adipocytes (Fig. 6*E*). All compounds tested significantly reduced palmitate-induced accumulation of lipids in adipocytes, with the EpFA being more potent than the nonoxidized form of DHA (Fig. 6*F*). *t*-TUCB alone did not modify ER stress, autophagy, and intracellular lipid levels (Fig. S6*E*).

Finally, we incubated hepatocytes with palmitate in the presence of EpFA and *t*-TUCB. As shown in Fig. 7*A*, incubation of hepatocytes with 19,20-EDP and *t*-TUCB resulted in increased autophagosome formation, as revealed by the disappearance of LC3-I and its conversion to LC3-II by conjugation to phosphatidylethanolamine. This improvement in autophagy was mirrored by a reduction in the phosphorylated forms of IRE-1α and eIF2α, indicative of attenuated ER stress in hepatocytes (Fig. 7*A*). In these cells, 19,20-EDP also overrode palmitate-induced accumulation of intracellular lipids (Fig. 7*B*). Similar actions on intracellular lipid levels were observed with 17,18-EEQ as well as with DHA and 14,15-EET, although this omega-6 EpFA was less potent than the omega-3 epoxides (Fig. 7*C*). However, compared with 19,20-EDP, 17,18-EEQ, DHA, and 14,15-EET were less active in regulating autophagy and unable to modulate ER stress in hepatocytes (Fig. 7*D*). Finally, we explored the effects of 17 (S)-HDHA, a lipid mediator acting as a precursor and marker of the biosynthesis of the D-series resolvins and protectins derived from DHA through the lipoxygenase pathway (16). This hydroxylated DHA-derived product also enhanced autophagy in palmitate-treated hepatocytes (Fig. 7*E*), and reduced the accumulation of intracellular lipids in palmitate-primed hepatocytes (Fig. 7*F*). *t*-TUCB alone did not induce any change in the response of hepatocytes to palmitate (Fig. 7 *B* and *D*).

Discussion

Special attention has been given to the effects of omega-3 PUFA on the chronic "low-grade" inflammatory state driven by the expansion of adipose tissue mass in obesity (15, 25). This persistent inflammation in adipose tissue of obese individuals is deleterious and increases the incidence of comorbidities, including insulin resistance and nonalcoholic fatty liver disease, a condition in which a recent systematic meta-analysis reported the benefits of omega-3 PUFA therapy (26). In addition, our understanding the recognized therapeutic values of omega-3s has been challenged by the discovery that these fatty acids can be converted to a novel class of proresolving lipid mediators (i.e., resolvins, protectins, and maresins) (reviewed in ref. 16). Indeed, these proresolving mediators are able to counteract inflammation and to prime the resolution process in obesity-induced nonalcoholic fatty liver disease (17). A common feature of these proresolving lipid mediators is that their biosynthesis from omega-3 PUFA is initiated through the interaction of lipoxygenase and cyclooxygenase pathways, the two classic branches of PUFA metabolism (16). Recent findings indicate that CYP epoxygenases, the so-called third branch of PUFA metabolism, can also convert omega-3 PUFA into bioactive lipid mediators (1, 4, 5). Indeed, CYP1A1, CYP2E1, and CYP2U1 have been described to generate a number of CYP-derived epoxides (namely EEQs and EDPs) from omega-3 PUFA (4). In our study, we used *fat-1* mice as an optimal model of omega-3 enrichment, in which the stabilization of CYP-derived epoxides by a sEH inhibitor reinforced the omega-3–dependent reduction in hepatic inflammation and intrahepatic lipid deposition. Taken together, our findings expand to the metabolic field the initial observation that an omega-3–rich diet in combination with a sEH inhibitor exerts antihypertensive actions (6).

A salient feature of our study was that the sEH inhibitor restored autophagy in livers from obese *fat-1* mice. Defective autophagy contributes to a variety of diseases, because efficient sequestration and clearance of damaged cellular components in stress conditions is crucial for cell homeostasis (27). Moreover, consistent with the view that autophagy is a primordial cellular

adaptive mechanism that mitigates ER-associated unfavorable conditions in insulin-sensitive tissues (20), sEH inhibition in *fat-1* mice was accompanied by an attenuated hepatic ER stress. Down-regulation of ER stress was also seen in conjunction with decreased autophagy in adipose tissue from *fat-1* mice receiving the sEH inhibitor, suggesting dissociation between these two cellular processes in this tissue. Because inhibition of autophagic function in adipose tissue is related to reduced fat mass and improved insulin sensitivity (28), our findings in adipose tissue can be regarded as beneficial in terms of lipid homeostasis and metabolic control. A strong asset of our study was that we were able to recapitulate the effects on autophagy and ER stress seen in vivo following sEH inhibition, by exposing hepatocytes and adipocytes in vitro to the omega-3 epoxides 19,20-EDP and 17,18-EEQ. Our findings are consistent with those reported by Bettaieb et al., who showed attenuation of ER stress in adipose and liver tissues in mice either receiving a sEH inhibitor or deficient for sEH (29). However, our data cannot exclude other EpFA, such as the case of arachidonic acid-derived EETs as well as the potential implication of other oxidized lipid mediators derived from omega-3 PUFA through the interaction of lipoxygenase and cyclooxygenase pathway. Additionally, our data cannot exclude the potential implication of nonoxidized DHA in the observed favorable metabolic phenotype of *fat-1* mice. In this regard, although less potent than EpFA, DHA was active in our cell bioassays. This finding is consistent with the reported biological properties of DHA (30) and with findings reported by Caviglia et al. showing that DHA was able to rescue rat hepatoma cells from palmitate-induced ER stress (31). However, these studies did not address whether the protective effects of DHA were mediated by the parent nonoxidized molecule or by any other DHA oxidized metabolite (30, 31).

In summary, the results of the present study demonstrate that stabilization of omega-3 epoxides through inhibition of sEH exerts beneficial actions in counteracting the metabolic disorders associated with obesity. Of particular interest are the findings demonstrating the ability of sEH inhibition to restore autophagy in the liver with the consequent reduction of obesity-induced liver ER stress. Our observations highlight the potentiality of small bioactive lipid mediators to modulate autophagy, serving as templates for the exploitation of this housekeeping cellular process for therapeutic interventions against obesity and obesity-related comorbidities, such as fatty liver disease.

Materials and Methods

Studies in *fat-1* mice, hepatocytes and 3T3-L1 adipocytes, measurement of 2-deoxyglucose uptake, mRNA and protein expression, and histology and immunohistochemistry analysis, MR imaging and spectroscopy, and LC-ESI-MS/MS and gas chromatography analysis are described in detail in *SI Materials and Methods*. The LC-MS/MS conditions used to profile the omega-3 and omega-6 epoxides are described in Table S2 and a schematic diagram of the experimental design of the study can be found in Fig. S7.

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Supporting Information

López-Vicario et al. 10.1073/pnas.1422590112

SI Materials and Methods

Generation of *fat-1* Mice Colonies and DNA Genotyping. Hemizygous *fat-1* mice were generated and backcrossed onto a C57BL/6 background as previously described (1). Male *fat-1* mice were mated with WT female mice to obtain offspring, with half being hemizygous *fat-1* mice. Genomic DNA from the ear was isolated using the Omni-Pure Tissue Genomic DNA System (Gene Link) following the manufacturer's protocol and genotyped by PCR. The PCR (20 μL) contained 0.2 μM primers (FAM fluorescent-labeled 5′-CTGCACCACGCCTTCACCAACC-3′ for forward direction and unlabeled 5′-ACACAGCAGCAGATTCCAGA-GATT-3′ for reverse), 0.2 mM dNTPs mix, 1.5 mM $MgCl_2$, and 1 U Platinum Taq DNA Polymerase (Invitrogen). PCR cycle conditions were 15 min at 95 °C followed by 30 cycles of 20 s at 94 °C, 40 s at 62 °C and 1 min at 72 °C, and a final step of 10 min at 72 °C and then cooled to 4 °C. PCR products were analyzed by capillary electrophoresis in a 3130 Genetic Analyzer (Applied Biosystems) (Fig. S7*A*). Genotyping was also performed by conventional PCR in the conditions described above using unlabeled primers, and PCR bands were separated by electrophoresis in 2.5% LM Sieve agarose gels and visualized by GelRed Nucleic Acid Gel Stain (Biotium), using a 100-bp DNA ladder marker (Invitrogen).

Experimental Studies. Male hemizygous *fat-1* (n = 31) and WT (n = 46) mice were housed in wood-chip bedding cages with 50–60% humidity and 12-h light/dark cycles. At 6 wk of age, mice were placed on either a standard rodent chow diet (Chow) (13% kcal from fat) or a HFD (60% kcal from fat; Research Diets) and randomly assigned into two treatment groups receiving either the sEH inhibitor *t*-TUCB or placebo (1% polyethylene glycol 400) for 16 wk. *t*-TUCB was given in the drinking water (10 μg/mL) to yield ~1.67 mg·kg·d. Drinking water was changed every 2 d and water consumption was monitored throughout the experiment. A schematic diagram of the experimental design is shown in Fig. S7*B*. At the end of the intervention period, mice were euthanized via ketamine/xylazine injection (intraperitoneally, 4:1), and the liver and adipose tissue were excised, rinsed in DPBS, fixed in 10% (vol/vol) formalin, and embedded in paraffin. In addition, portions of liver and adipose tissue were snap-frozen in liquid nitrogen for RNA, protein and fatty acid analyses. An additional group of WT mice (n = 9) received HFD supplemented with EPA/DHA as described in ref. 2. All animal studies were conducted in accordance with the Investigation and Ethics Committee criteria of the Hospital Clínic and European Union legislation.

MRI and Spectroscopy. MR imaging was conducted on a 7.0T BioSpec 70/30 horizontal animal scanner (Bruker BioSpin), equipped with a 12-cm inner diameter actively shielded gradient system (400 mT/m) and a receiver/transceiver coil covering the whole mouse volume. Animals were placed in supine position in a Plexiglas holder with a nose cone for administering anesthetic gases (isofluorane in a mixture of 30% O_2 and 70% CO_2), fixed using a tooth bar, ear bars, and adhesive tape. Tripilot scans were used for accurate positioning of the animal in the isocenter of the magnet. High-resolution T2-weighted images were acquired by RARE (rapid acquisition with rapid enhancement) sequence applying repetition time = 4,534 ms, echo time = 19 ms, RARE factor = 8, eight averages, number of slices = 19 for horizontal view, field-of-view = 100 × 40 mm, matrix size = 640 × 256 pixels, resulting in a spatial resolution of 0.156 × 0.156 mm in 1.5-mm slice thickness. Coronal images included 40 slices, field-of-view = 40 × 40 mm, matrix size = 256 × 256 pixels, with the same slice thickness and same spatial resolution. These two sets of images (coronal and horizontal) were acquired with and without fat suppression to subtract them and quantify the total fat volume. Images were processed in ImageJ software, normalized for signal intensity, subtracted, and binarized. A threshold mask was used over the binary images to select all pixels belonging to fat tissue. Localized ^{1}H MR spectroscopy of the liver was acquired with the same system. The liver slice with the largest gross dimensions was chosen, and MR spectroscopy for water and fat quantification was obtained using a point-resolved spectroscopy sequence. After line broadening and phase and baseline correction, the peak area of the water at 4.77 ppm and fat resonance (FR) at 1.40 ppm were measured. Quantification of the fat content was done by comparing the area of the FR with that of the unsuppressed water. Spectroscopic data were processed using the Paravision 5.1 software (Bruker BioSpin). The hepatic fat percentage was calculated by dividing (FR) by the sum of FR and peak area of water.

Histology and Immunohistochemistry Analysis. Adipose tissue samples fixed in 10% formalin were embedded in paraffin, cut into 5-μm sections, and stained with H&E. Detection of F4/80 by immunohistochemistry was performed in deparaffinized adipose tissue sections which were rehydrated, and pretreated with 0.05% trypsin-0.1% $CaCl_2$ for 20 min at 37 °C to unmask the antigen. Thereafter, sections were incubated in 0.3% H_2O_2 for 25 min at room temperature and dark conditions to block endogenous peroxidase activity and in 2% (wt/vol) BSA for 20 min at room temperature to avoid unspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with the primary rat anti-mouse F4/80 antibody (dilution 1:400; AbD Serotec), followed by incubation for 30 min at room temperature with a biotinylated rabbit anti-rat IgG secondary antibody (1:200) and incubation with ABC for 30 min at room temperature using the Vectastain ABC Kit (Vector Laboratories). Color was developed using the diaminobenzidine substrate (Dako Diagnostics), and sections were counterstained with Gill's hematoxylin. Fibrosis in adipose tissue was assessed in paraffin sections by Sirius red staining. Briefly, sections were incubated for 10 min in 0.5% thiosemicarbazide and stained in 0.1% Sirius red F3B in saturated picric acid for 1 h, and subsequently washed with an acetic acid solution (0.5%). Fibrosis was also assessed by Masson's trichrome staining at the Pathology Department of the Hospital Clínic. Hepatic lipid content was assessed by Oil Red-O staining in optimal cutting temperature compound-embedded samples. Briefly, cryosections were fixed in 60% (vol/vol) isopropanol for 10 min and stained with 0.3% Oil Red-O in 60% isopropanol for 30 min and subsequently washed with 60% isopropanol. Sections were counterstained with Gill's hematoxylin, washed with acetic acid solution (4%, vol/vol) and mounted with aqueous solution. Sections were visualized at 200× magnification in a Nikon Eclipse E600 microscope and the relative areas of macrophage infiltration, steatosis, and fibrosis were quantitated by histomorphometry using Olympus Cell (Olympus). Adipocyte cross-sectional area was assessed using ImageJ.

Isolation and Incubation of Primary Hepatocytes. Hepatocytes were isolated from WT and *fat-1* mice by a three-step in situ perfusion procedure using 0.03% collagenase IV through the portal vein, as described previously (2). Isolated hepatocytes were seeded on

collagen I-coated plates and cultured in William's E medium supplemented with 10% (vol/vol) FBS, 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 μM insulin, 15 mM Hepes, and 50 μM β-mercaptoethanol. Primary hepatocytes were characterized by a combination of phase-contrast microscopy and immunocytochemical analysis, and their viability was determined by Trypan blue exclusion. Hepatocytes were maintained in a humidified 5% CO_2 incubator at 37 °C and cultured in 12-well plates (4×10^5 cells per well) in 1% FBS-William's E medium with 1% fatty acid-free (FAF) BSA and incubated with either vehicle (18.4% FAF-BSA) or 0.5 mM sodium palmitate freshly prepared in DMEM containing 18.4% FAF-BSA for 24 h to induce autophagy. In some experiments, the protease inhibitors E64d (10 μg/mL) and pepstatin A (10 μg/mL) were added. In selected experiments, the cells were also treated with *t*-TUCB (1 μM) alone or in combination with 19,20-EDP (1 μM), 17,18-EEQ (1 μM), 14,15-EET (1 μM), 17(S)-HDHA (1 μM), and DHA (1 and 10 μM) (Cayman Chemical) for 24 h. At the end of the incubation period, hepatocytes were scraped into ice-cold $DPBS^{-/-}$ and resuspended in lysis buffer for protein extraction or stained with Oil Red-O for assessment of intracellular lipid content, as described below.

Differentiation and Incubation of 3T3-L1 Adipocytes. Mouse 3T3-L1 cells were seeded onto six-well plates (250,000 cells per well) in DMEM supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin/streptomycin and 4 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C and allowed to grow to confluence for 2 d, as described previously (3). Confluent 3T3-L1 cells were cultured in adipocyte induction medium containing insulin (5 μg/mL), isobutylmethylxanthine (0.5 mM), dexamethasone (0.25 μM), penicillin/streptomycin (100 U/mL), and L-glutamine (4 mM) in DMEM supplemented with 10% FBS. After 2 d, the cells were cultured in continuation medium (5 μg/mL insulin) for 72 h and then maintained in DMEM supplemented with 10% FBS until exhibiting an adipocyte phenotype, as characterized by a combination of phase-contrast microscopy and Oil Red-O staining (see below). At day 7–8 of differentiation, 0.5 mM sodium palmitate was added for 24 h to induce autophagy in the absence or presence of protease inhibitors E-64d (10 μg/mL) and pepstatin A (10 μg/mL). In some experiments, the cells received *t*-TUCB (1 μM) alone or in combination with 19,20-EDP (1 μM), 17,18-EEQ (1 μM), 14,15-EET (1 μM), and DHA (1 and 10 μM) for 24 h. At the end of the incubation period, adipocytes were either stained with Oil Red-O or scraped into ice-cold $DPBS^{-/-}$ and resuspended in lysis buffer or TRIzol for protein extraction or RNA isolation, respectively.

Oil Red-O Staining of Hepatocytes and Adipocytes. Cells were washed twice with $DPBS^{-/-}$, exposed to 4% (vol/vol) paraformaldehyde for 1 h, and then washed with 60% isopropanol before incubating with 0.2% Oil Red-O for 30 min at room temperature. To quantify the amount of Oil Red-O retained by the cells, hepatocytes and adipocytes were incubated with isopropanol for 30 min with shaking to elute the stain. The amount of staining was measured by optical density at 500 nm in a FluoStar Optima microplate reader (BMG Labtech). Cells were also grown (1×10^5 cells per well) in Permanox Lab-Teck Chamber Slides (Nalge Nunc), stained with Oil Red-O, counterstained with Gill's hematoxylin, washed with tap water, mounted with aqueous solution, and visualized under a Nikon Eclipse E600 microscope at 200× magnification.

Measurement of 2-Deoxyglucose Uptake. Adipocytes were incubated with either vehicle (18.4% FAF-BSA) or 0.5 mM sodium palmitate in the absence or presence of 19,20-EDP (1 μM) or *t*-TUCB (1 μM) for 24 h. Thereafter, the cells were washed twice with $DPBS^{-/-}$ and incubated with 2-deoxyglucose (2DG) (1 mM) with or without insulin (1 μM) in Krebs Ringer Phosphate Hepes buffer containing 2% BSA for 20 min at 37 °C. Cells were washed three times with cooled PBS containing the glucose uptake inhibitor phloretin (200 μM), scraped and resuspended in Tris·HCl buffer (10 mM, pH8). Cell lysates were heat-treated (80 °C, 15 min) and centrifuged (15,000 × *g*, 20 min, 4 °C), and 2DG uptake was determined in supernatants (diluted 1:4) using a 2DG Uptake Measurement Kit from Cosmo Bio, based on an enzymatic method for the direct measurement of 2DG-6-phosphate.

RNA Isolation, Reverse Transcription, and Real-Time PCR. Isolation of total RNA from liver, adipose tissue, hepatocytes, and adipocytes was performed using the TRIzol reagent. RNA concentration was assessed in a NanoDrop-1000 spectrophotometer (NanoDrop Technologies), and its integrity tested with a RNA 6000 Nano Assay in a Bioanalyzer 2100 (Agilent Technologies). cDNA synthesis from 1 μg of total RNA was performed using the High-Capacity cDNA Archive Kit (Applied Biosystems). Validated and predesigned TaqMan primers and probes from Assays-on-Demand were used to quantify CYP1A1 (Mm00487218), CYP2E1 (Mm00491127), CYP2U1 (Mm01310397), F4/80 (Emr1; Mm00802529), MCP-1 (*Ccl2*; Mm00441242), Δ5 and Δ6 desaturases (*Fads1*; Mm00507605 and *Fads2*; Mm00517221), TNFα (Mm00443258), IL-1β (Mm01336189), IL-6 (Mm00446190), MGL1 (Mm00546124), CD206 (Mannose Receptor type 1; Mm00485148), IL-10 (Mm00439614), RELMα (Mm00445109), Arg1 (Mm00475988), Ym1 (Chitinase-3 like protein 3; Mm00657889), GLUT-4 (Mm00436615), and IRS-1 (Mm01278327), using β-actin (ID Mm00607939) as the endogenous control. Real-time PCR amplifications were carried out in an ABI Prism 7900HT Sequence Detection System and the results were analyzed with the Sequence Detector Software version 2.1 (Applied Biosystems). Relative quantification of gene expression was performed using the comparative Ct method. The amount of target gene, normalized to β-actin and relative to a calibrator, was determined by the arithmetic equation $2^{-\Delta\Delta Ct}$ described in the comparative Ct Method (docs.appliedbiosystems.com/pebiodocs/04303859.pdf).

Analysis of Protein Expression by Western Blot. Total protein from liver and adipose tissue was extracted using a lysis buffer containing 50 mM Hepes, 20 mM β-glycerophosphate, 2 mM EDTA, 1% Igepal, 10% (vol/vol) glycerol, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 150 mM NaCl, supplemented with protease inhibitor (Complete Mini; Roche Diagnostics) and phosphatase inhibitor (PhosSTOP; Roche Diagnostics) mixtures. For protein isolation from hepatocytes and adipocytes, cells were scraped into ice-cold DPBS and resuspended in 150 μL of lysis buffer. Homogenates were incubated on ice for 10–15 min and centrifuged at either 1,000 × *g* for 2 min (cells) or 9,300 × *g* for 20 min (tissue) at 4 °C. Total protein (50 μg) from supernatants was placed in SDS-containing Laemmli sample buffer, heated for 5 min at 95 °C, and separated by 10% (tissue) or 15% (cells) (vol/vol) SDS/PAGE for 90 min at 120 V. Transfer was performed by the iBlot Dry Blotting System (Invitrogen) onto PVDF membranes at 20 V over 5–7 min, and the efficiency of the transfer was visualized by Ponceau S staining. The membranes were then soaked for 1 h at room temperature in 0.1% T-TBS and 5% (wt/vol) nonfat dry milk. Blots were washed three times for 5 min each with 0.1% T-TBS and subsequently incubated overnight at 4 °C with primary rabbit anti-mouse Δ5 desaturase (ab126706; dilution 1/1000; Abcam), Δ6 desaturase (M-50; dilution 1:200; Santa Cruz Biotechnology), sEH (Ab155280; Abcam), Atg7 (8558; dilution 1/1000; Cell Signaling), Atg12/Atg5 (4180; dilution 1/1000; Cell Signaling), rabbit polyclonal phospho-IRE-1α (NB100-2323; dilution 1/1000; Novus Biologicals), rabbit polyclonal phospho-eIF2α (ab4837; dilution 1/1000; Abcam), mouse monoclonal eIF2α (sc-133132; dilution 1/200; Santa

Cruz Biotechnology), p62 (Ab109012; dil 1/10,000, Abcam), and LC3I/II (12741S; dil 1/1000; Cell Signaling) in 0.1% T-TBS containing 5% BSA. Thereafter, the blots were washed three times for 5 min each with 0.1% T-TBS and incubated for 1 h at room temperature with a horseradish-peroxidase-linked donkey anti-rabbit antibody (1:2,000) in 0.1% T-TBS containing 5% nonfat dry milk, and the bands were visualized using the EZ-ECL chemiluminescence detection kit (Biological Industries). To assess housekeeping protein expression, the membranes were stripped at 50 °C for 20 min in 100 mM β-mercaptoethanol, 2% (vol/vol) SDS, and 62.5 mM Tris·HCl (pH 6.8) and reblotted overnight at 4 °C with primary rabbit anti-mouse GAPDH (ab9485; dilution 1/2500; Abcam) antibody, β-actin HRP conjugate (5125; dilution 1/1000; Cell Signaling), and primary mouse anti-mouse α-tubulin (T6074; dilution 1/1000; Sigma-Aldrich) and the membranes visualized, as described above.

Analysis of CYP Metabolites by LC-ESI-MS/MS. Thirty milligrams of frozen adipose tissue were homogenized in liquid nitrogen and 14,15-EET-d8 (Cayman Chemical) (10 ng) were added as internal standard in 300 μL of 10 M sodium hydroxide solution and heated for 30 min at 60 °C for alkaline hydrolysis. The samples were brought to pH6 with 500 μl of 1 M sodium acetate buffer and acetic acid for hydrolyzed samples. After centrifugation, the supernatant was added to Bond Elute Certify II columns (Agilent Technologies) for solid-phase extraction (SPE) in a SPE Vacuum Manifold. The columns were preconditioned with 3 mL methanol, followed by 3 mL of 0.1 M sodium acetate buffer containing 5% (vol/vol) methanol (pH6). The SPE-columns were then washed with 3 mL methanol/H_2O (50/50, vol/vol) and eluted with 2 mL of n-hexane:ethyl acetate (25:75, vol/vol) with 1% acetic acid. The eluate was evaporated on a heating block at 40 °C under a stream of nitrogen to obtain a solid residue. The residue was dissolved in 70 μL acetonitrile and analyzed using an Agilent 1200 HPLC system with binary pump, degasser, autosampler and column thermostat with a Kinetex C-18, 2.1 × 150 mm, 2.6 μm column (Phenomenex), using a solvent system of aqueous formic acid (0.1%) and acetonitrile. The elution gradient was started with 5% (vol/vol) acetonitrile, which was increased within 0.5 min to 55, 14.5 min to 69%, 14.6 min to 95% and held there for 5.4 min. The flow rate was set at 0.3 mL/min; the injection volume was 7.5 μL. The HPLC was coupled with an Agilent 6460 Triplequad mass spectrometer (Agilent Technologies) with ESI source. Analysis was performed with Multiple Reaction Monitoring in negative mode with the following source parameters (drying gas: 250 °C/10 l/min, sheath gas: 400 °C/10 l/min, capillary voltage: 4500 V, nozzel voltage: 1500 V and nebulizer pressure: 30 psi). The LC-MS/MS conditions used to profile the omega-3 and omega-6 epoxides are described in Table S2. Quantification was performed using standard calibration curves for each corresponding synthetic epoxide.

Fatty Acid Profiling by Gas Chromatography. Total lipids were extracted from frozen liver and adipose tissues by the Folch method, with modifications (4). Briefly, chloroform/methanol (2:1 vol/vol) containing 0.005% butylated hydroxytoluene (as antioxidant) was added and mixed vigorously for 30 s before adding 100 μL of 0.25% $MgCl_2$ and 1 mL of 0.01N HCl and mixed again. The chloroform phase containing lipids was collected. The remains were extracted with 3 mL of chloroform. The chloroform phases were pooled and dried under nitrogen and subjected to methylation. Fatty acid methyl esters were prepared by methods similar to those described previously (2, 3) using methanol containing 14% boron trifluoride (BF_3/MeOH). The extracted lipid samples were mixed with BF_3/MeOH reagent (1 mL), and the mixtures were heated at 100 °C in a metal block for 1 h, cooled to room temperature, and methyl esters were extracted twice in the upper (hexane) layer after addition of 1 mL H_2O. The samples were centrifuged at 1,200 × *g* for 10 min and then the upper hexane layer was removed and evaporated under nitrogen. Recovery yields were similar for tissue samples from both WT and *fat-1* mice (85.9 ± 0.9 for WT and 93.3 ± 0.8 for *fat-1*). Fatty acid methyl esters were analyzed by flame ionization gas chromatography (GC). GC analysis was carried out with an Agilent 7890 Autosampler apparatus (Agilent Technologies) equipped with a capillary column (SupraWAX-280, Teknokroma), length 30 m, 0.25 mm i.d., and film thickness 0.25 μm. Column conditions were: initial temperature, 120 °C for 1.0 min; ramp 15 °C/min to 210 °C hold to 35 min; carrier gas, helium. A mixture of standard fatty acids methyl esters (Restek Corporation) was included as an external standard with each run for peak identification by comparison of their retention times. Data acquisition and processing were performed with Agilent-Chemstation software for GC systems. Each fatty acid was expressed as percentage of total fatty acids.

Statistical analysis of the results was performed by analysis of variance (one-way or two-way ANOVA) or unpaired Student's *t* test. Results are expressed as mean ± SEM and differences considered significant at $P < 0.05$.

1. Kang JX, Wang J, Wu L, Kang ZB (2004) Transgenic mice: *fat-1* mice convert n-6 to n-3 fatty acids. *Nature* 427(6974):504.
2. López-Vicario C, et al. (2014) Molecular interplay between Δ5/Δ6 desaturases and long-chain fatty acids in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 63(2):344–355.
3. García-Alonso V, et al. (2013) Coordinate functional regulation between microsomal prostaglandin E synthase-1 (mPGES-1) and peroxisome proliferator-activated receptor γ (PPARγ) in the conversion of white-to-brown adipocytes. *J Biol Chem* 288(39): 28230–28242.
4. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 226(1):497–509.

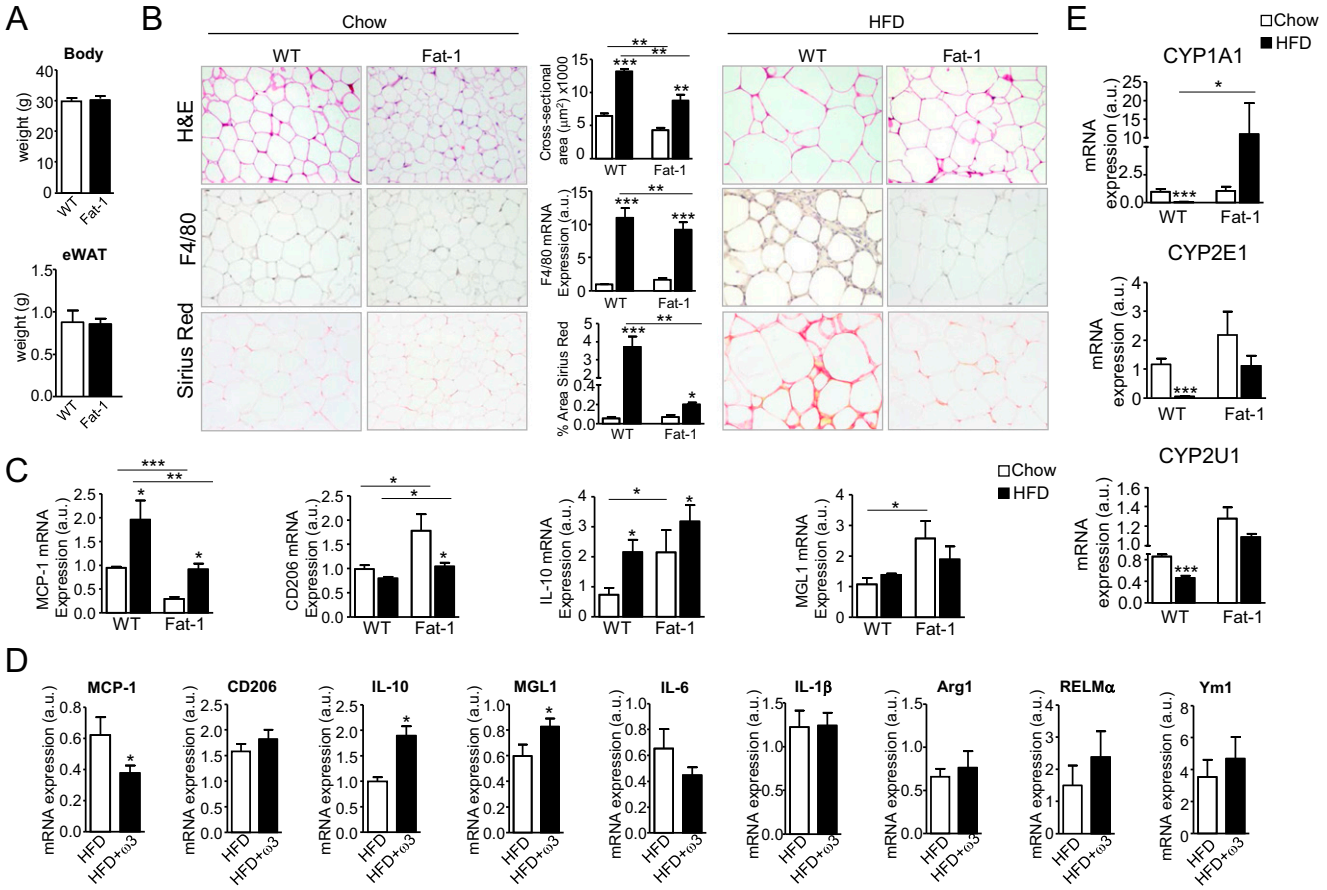

Fig. S1. CYP epoxygenase expression in adipose tissue from *fat-1* mice. (*A*) Body and eWAT weights in WT ($n = 5$) and *fat-1* ($n = 8$) mice under Chow diet for 16 wk. (*B*) Representative photomicrographs of H&E-stained adipose tissue sections (*Top*), F4/80 (*Middle*), and Sirius red (*Bottom*) in WT ($n = 25$) and *fat-1* ($n = 12$) mice after receiving either Chow or HFD for 16 wk. (Magnification: 200×.) Quantification of adipocyte cross-sectional area, Sirius red staining and F4/80 mRNA expression is also shown. (*C*) MCP-1, CD206, IL-10, and MGL1 expression in adipose tissue. (*D*) Gene expression analysis of MCP-1, CD206, IL-10, MGL1, IL-6, IL-1β, Arg1, RELMα, and Ym1 in eWAT from WT mice (HFD) ($n = 10$) and WT mice fed an omega-3–rich diet (HFD+ω3) ($n = 9$) receiving a HFD for 16 wk. (*E*) CYP1A1, CYP2E1 and CYP2U1 expression in adipose tissue. Results are mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

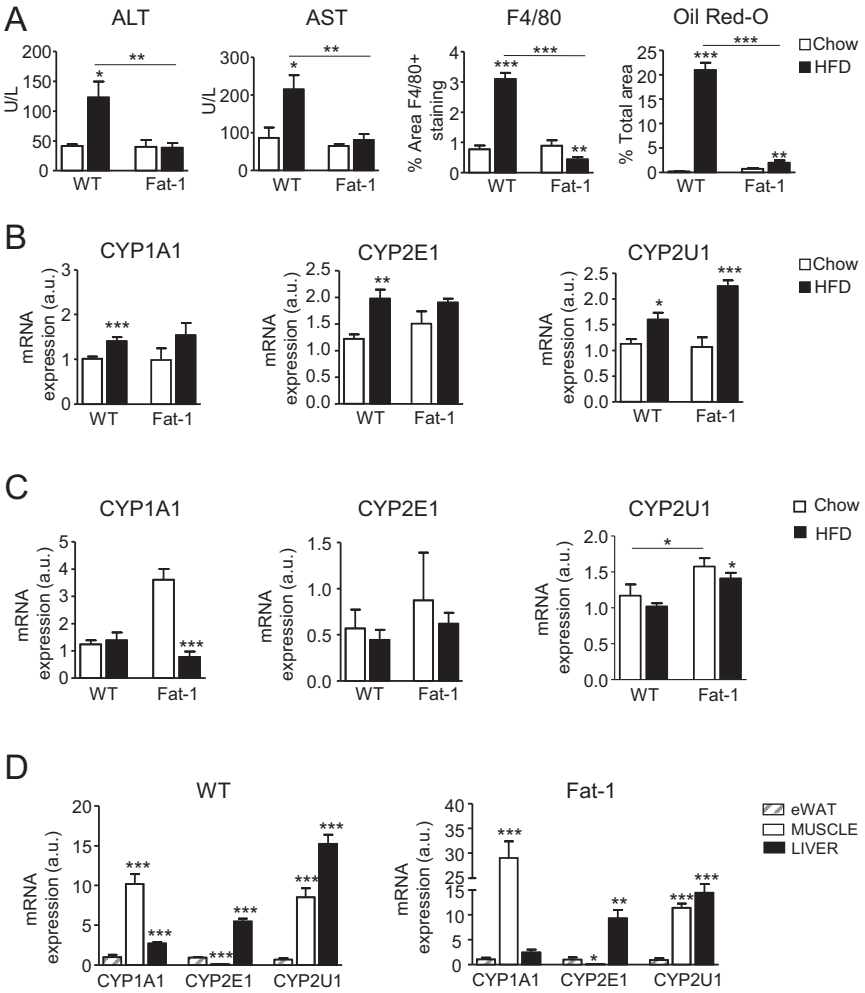

Fig. S2. CYP epoxygenase expression in liver from *fat-1* mice. (*A*) Serum ALT and AST and analysis of hepatic F4/80 and Oil Red-O staining in WT ($n = 25$) and *fat-1* ($n = 12$) mice after receiving either Chow or HFD for 16 wk. (*B*) Hepatic CYP1A1, CYP2E1, and CYP2U1 expression. (*C*) Gene expression of CYP1A1, CYP2E1, and CYP2U1 in skeletal muscle. (*D*) Gene expression of CYP1A1, CYP2E1 and CYP2U1 in eWAT, skeletal muscle and liver from WT and *fat-1* mice under Chow diet. Results are mean ± SEM $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

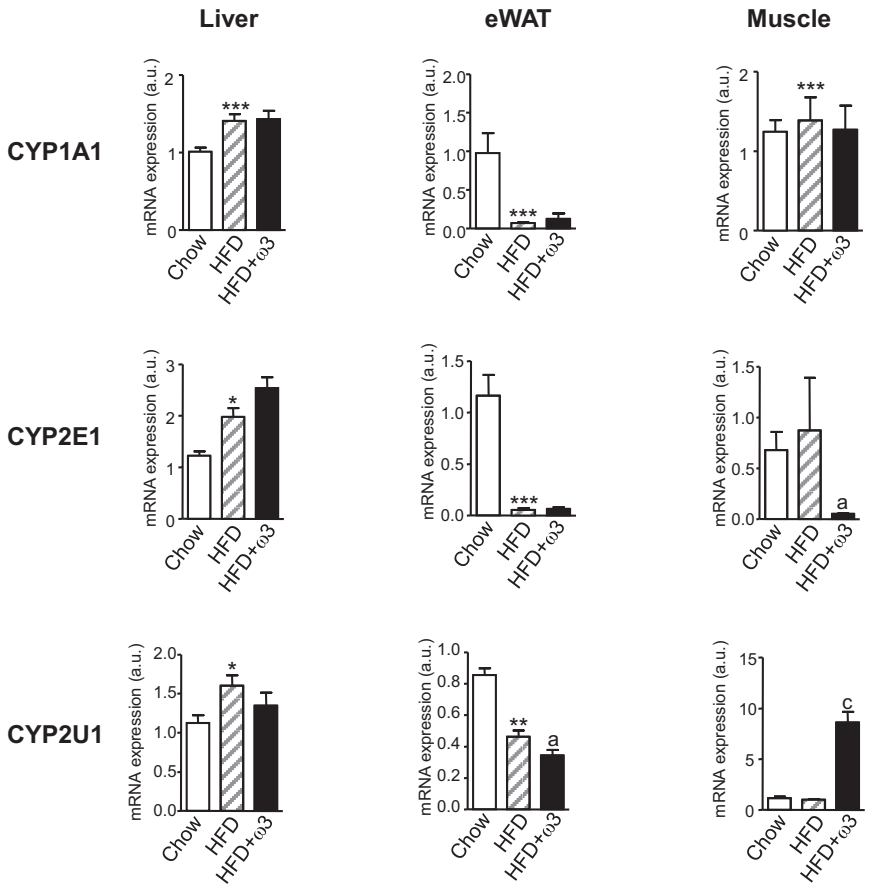

Fig. S3. Gene expression of CYP1A1, CYP2E1, and CYP2U1 in liver, eWAT and skeletal muscle from WT mice receiving either Chow diet ($n = 7$), HFD ($n = 10$), or HFD enriched with omega-3 fatty acids (HFD+ω3) ($n = 9$) for 16 wk. Results are expressed as mean ± SEM *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs. Chow and $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ vs. HFD.

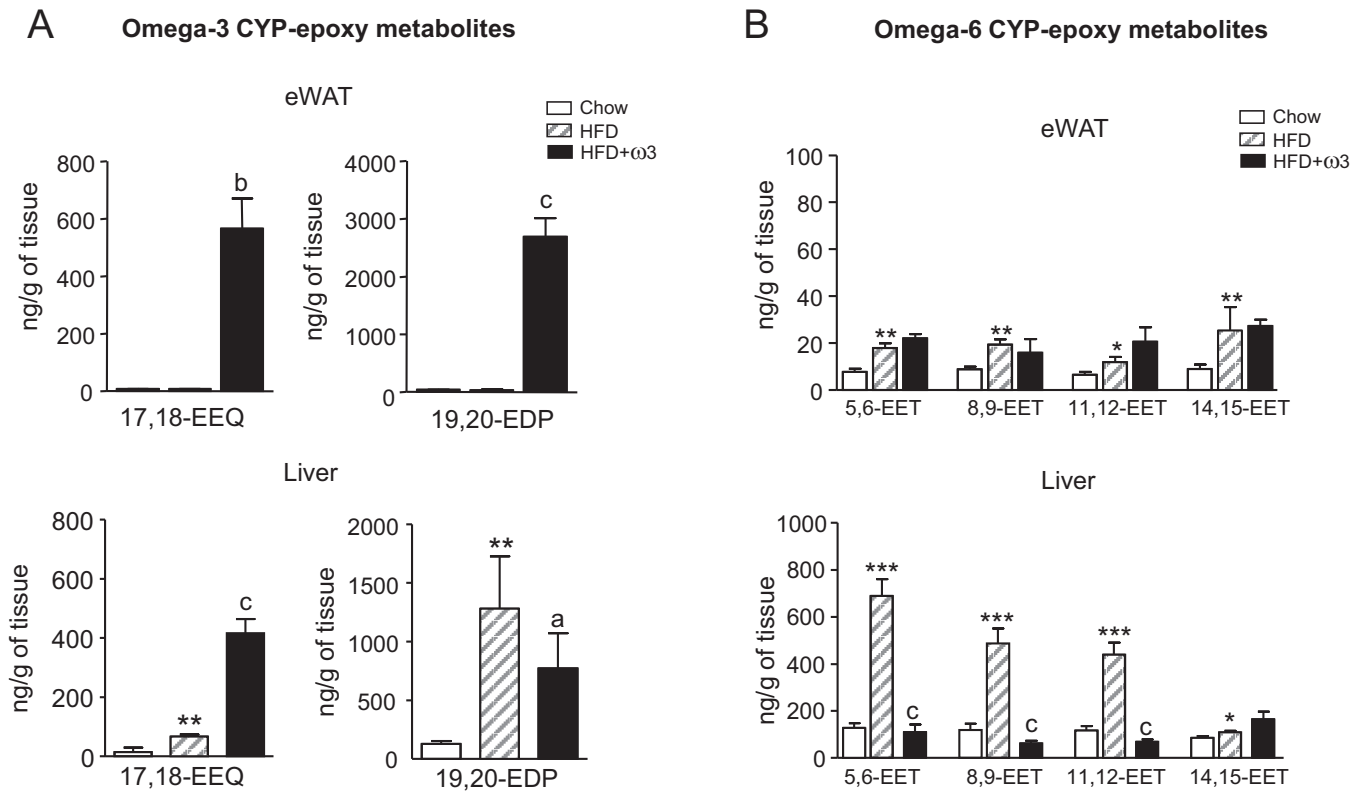

Fig. S4. (*A*) Analysis of the levels of omega-3 CYP-epoxy metabolites (17,18-EEQ and 19,20-EDP) by LC-ESI-MS/MS in eWAT and liver from WT mice receiving either Chow diet ($n = 7$), HFD ($n = 10$), or HFD enriched with omega-3 fatty acids (HFD+ω3) ($n = 9$) for 16 wk. (*B*) Analysis of the levels of omega-6 CYP-epoxy metabolites (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) in these animals by LC-ESI-MS/MS. Results are expressed as mean ± SEM $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. Chow and $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$ vs. HFD.

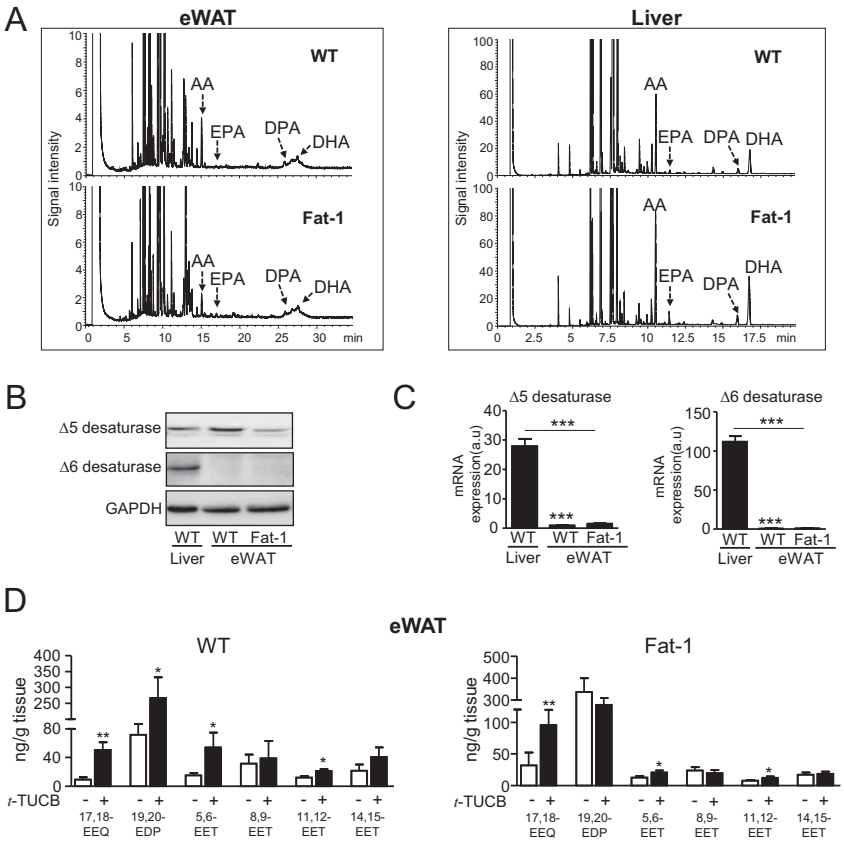

Fig. S5. (*A*) Representative GC chromatograms of eWAT and liver content of omega-6- and omega-3 PUFA in WT and *fat-1* mice. Protein (*B*) and mRNA (*C*) expression of Δ5 and Δ6 desaturases in eWAT and liver from WT and *fat-1* mice. (*D*) LC-ESI-MS/MS analysis of omega-3 and omega-6 epoxides in eWAT from HFD-induced obese WT and *fat-1* mice receiving *t*-TUCB (10 μg/mL) for 16 wk. Results are mean ± SEM $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

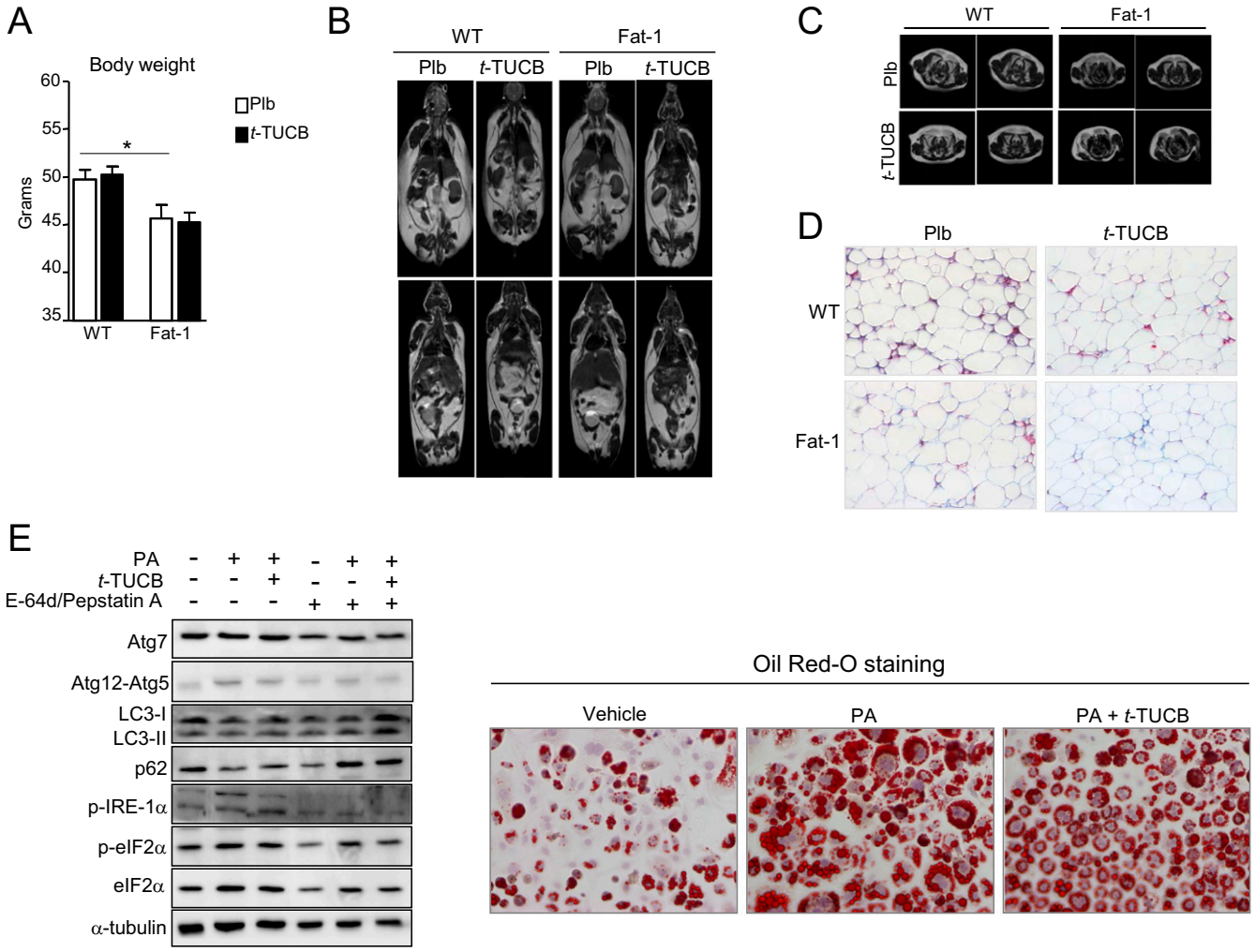

Fig. S6. (*A*) Endpoint body weight for WT (n = 28) and *fat-1* (n = 20) mice receiving a HFD and treated with either placebo (Plb) or *t*-TUCB for 16 wk. (*B*) Representative MR images of axial sections. (*C*) Representative MR images of coronal sections from the interscapular area. (*D*) Representative photomicrographs of Masson's trichrome-stained adipose tissue sections. (*E*) Protein expression of Atg7, Atg12-Atg5, LC3-I/II, p62, phosphoIRE-1α (p-IRE-1α), phosphoeIF2α (p-eIF2α), total eIF2α (eIF2α), and α-tubulin in differentiated 3T3-L1 adipocytes incubated with either vehicle or sodium palmitate (PA, 0.5 mM) alone or in combination with *t*-TUCB (1 μM) in the absence or presence of the protease inhibitors E-64d/pepstatin A for 24 h. Western blots were performed in 15% SDS/PAGE. Representative photomicrographs of Oil Red-O–stained cells incubated with either vehicle or PA alone or in combination with *t*-TUCB are shown on the right. Results are mean ± SEM $*P < 0.05$. (Magnification: 200×.)

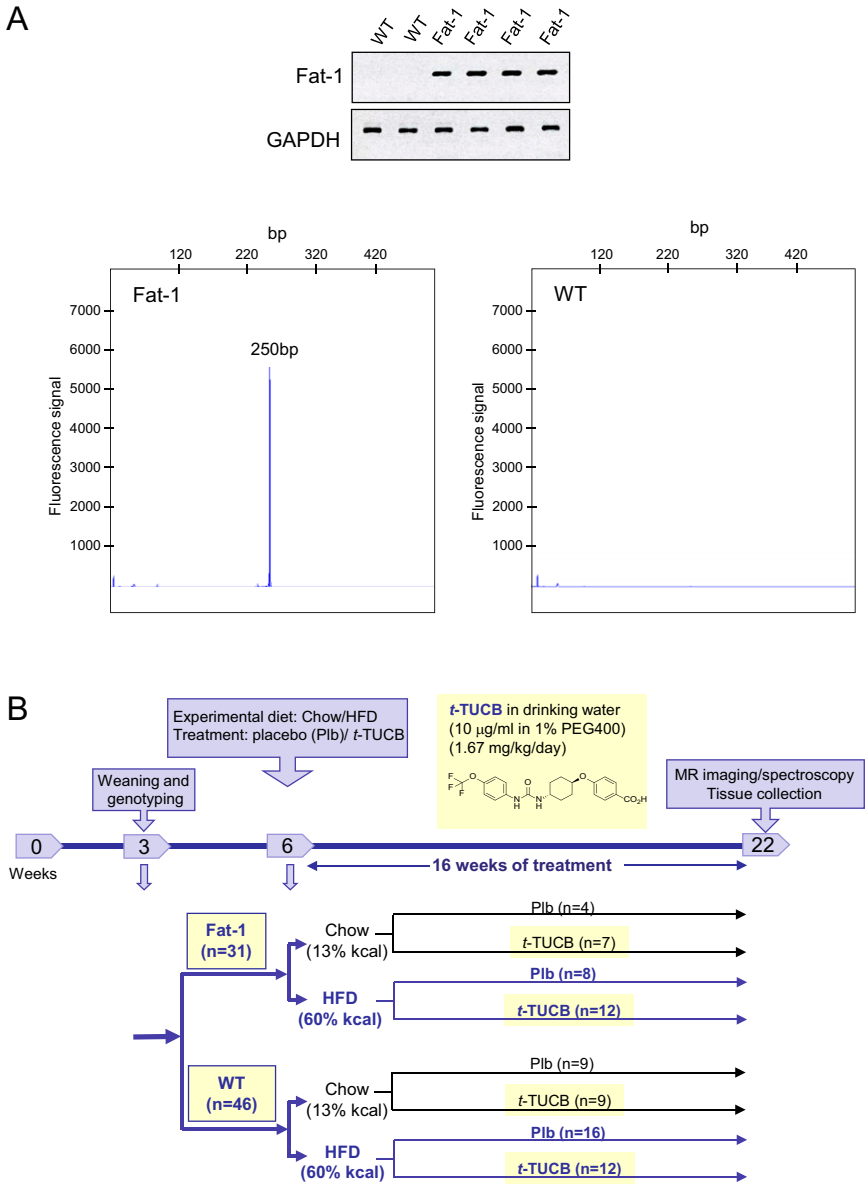

Fig. S7. (*A*) DNA genotyping of transgenic *fat-1* mice by conventional PCR (*Upper*) and by fluorescent-labeled fragment analysis in a ABI 3130 capillary electrophoresis analyzer (*Lower*). (*B*) Schematic diagram of the experimental design of the study. At 3 wk of age, after weaning, male hemizygous *fat-1* and WT mice were genotyped. At 6 wk of age, these animals were placed on either a standard rodent chow diet (Chow) (13% kcal from fat) or HFD (60% kcal from fat) and randomly assigned into two treatment groups receiving either the sEH inhibitor *t*-TUCB or placebo (Plb; 1% polyethylene glycol 400) for 16 wk. *t*-TUCB was given in the drinking water (10 μg/mL) to yield ~1.67 mg·kg·d. Tissue collection and MR imaging and spectroscopy analysis were carried out at the end of the experimental period.

Table S1. Polyunsaturated fatty acid composition in HFD experimental mice groups

	WT				*fat-1*			
	Liver		eWAT		Liver		eWAT	
PUFA	Placebo	t-TUCB	Placebo	t-TUCB	Placebo	t-TUCB	Placebo	t-TUCB
AA (C20:4n6)	3.47 ± 0.03	3.78 ± 0.53	0.45 ± 0.12	0.43 ± 0.077	3.57 ± 0.08	3.14 ± 0.20	0.42 ± 0.03	0.32 ± 0.05
EPA (C20:5n3)	0.25 ± 0.03	0.28 ± 0.042	1.50 ± 0.54	1.89 ± 0.44	0.33 ± 0.03*	0.39 ± 0.08	1.87 ± 0.01	1.54 ± 0.03
DPA (C22:5n3)	0.51 ± 0.07	0.58 ± 0.08	0.22 ± 0.016	0.26 ± 0.03	0.74 ± 0.05*	0.73 ± 0.04	0.11 ± 0.06	0.18 ± 0.05
DHA (C22:6n3)	1.97 ± 0.02	2.36 ± 0.25	0.58 ± 0.72	0.42 ± 0.11	3.49 ± 0.64*	2.9 ± 0.07	0.33 ± 0.21	0.37 ± 0.11

All values are expressed as percent area/gram tissue.*$P < 0.05$ vs.WT-placebo. AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table S2. LC-MS/MS conditions used to determine the epoxides of AA, EPA, and DHA

Compound name	Precursor ion	Product ion	Fragmentor (V)	Collision energy	Cell accelerator (V)	Retention time* (min)
17,18-EEQ	317.2	259.1	120	3	1	10.63
19,20-EDP	343.3	241.0	160	8	2	12.44
14,15-EET	319.2	219.1	120	3	1	12.89
11,12-EET	319.2	167.1	120	2	1	13.67
8,9-EET	319.2	155.1	120	3	1	13.87
5,6-EET	319.2	191.1	120	4	1	14.05
14,15-EET-D8[†]	327.2	226.1	120	3	8	12.65

*Retention times refer to the Kinetex C-18, 2.1 x 150 mm, 2.6-μm column using a solvent system of aqueous formic acid (0.1%) and acetonitrile. Gradient elution was started with 5% acetonitrile, which was increased within 0.5 min to 55, 14.5 min to 69%, 14.6 min to 95% and held there for 5.4 min. The flow rate was set at 0.3 mL/min.

[†]Used as internal standard for all epoxides.

Discusión

En este proyecto de investigación se demuestra que en la esteatohepatitis no alcohólica (EHNA) existe un desequilibrio de los niveles hepáticos de ácidos grasos omega-6 y omega-3. El desequilibrio producido en pacientes obesos con EHNA es consistente con el observado en estudios previos en los que se analizó el contenido y la distribución de ácidos grasos en hígado, en plasma y en suero de pacientes que padecían esta enfermedad (Puri P, 2007 y 2009; Araya J, 2004; Barr J, 2010). El desequilibro de ácidos grasos a nivel hepático ha sido reproducido por primera vez en nuestro modelo murino de EHNA inducida por una dieta hipercalórica.

Un aspecto importante del proyecto fue demostrar con éxito la capacidad de reestablecer los niveles hepáticos de omega-3 utilizando ratones transgénicos que expresan el gen *fat-1* procedente del nematodo *Caenorhabditis elegans*. Este gen codifica para la enzima omega-3 desaturasa que presenta la función de catalizar la síntesis de ácidos grasos omega-3 ultilizando como sustrato los ácidos grasos omega-6 (Kang JX, 2004). Esta capacidad confiere a estos ratones una abundante distribución tisular de omega-3 sin necesidad de un suplemento dietético (Kang JX, 2007). Uno de los hallazgos más importantes fue demostrar que los ratones *fat-1* presentan protección frente a los efectos de la obesidad tras ingerir una dieta hipercalórica. Esta observación se manifestó con una menor ganancia de peso, una reducción de la resistencia a la insulina, a la acumulación lipídica intrahepática y a la inflamación tras ingerir la dieta hipercalórica. La reducción de la ganancia de peso en el grupo de ratones *fat-1* es consistente con la presencia de un peso corporal más bajo en otras cepas de ratones *fat-1* (Ji S, 2009). Sin embargo, este efecto antiobesogénico no fue observado en un estudio realizado por White y colaboradores (White PJ, 2010) en el que los animales *fat-1* recibieron una dieta con un contenido de grasa distinto durante 8 semanas.

Para investigar los efectos en un modelo similar a un aporte nutricional de omega-3, se compararon los efectos observados en los ratones *fat-1* con los ejercidos por un grupo de ratones *salvajes* que recibieron un suplemento exógeno de omega-3 (EPA y DHA). En global, las respuestas obtenidas en ambas aproximaciones de enriquecimiento endógeno y exógeno de omega-3 fueron equiparables. La administración de EPA/DHA a través de la dieta demostró una respuesta similar a la observada en el modelo transgénico *fat-1* en cuanto a parámetros metabólicos como el peso del hígado, los niveles de colesterol, TG e insulina en suero y la resistencia a la insulina hepática (fosforilación JNK). También se observó una mejora de la inflamación hepática evaluada

histológicamente mediante tinción H&E y F4/80, y una reducción en la expresión génica de los marcadores de la inflamación, MCP-1 y IL-1β.

Aparte de las similitudes encontradas entre ambos grupos, las diferencias merecen ser pronunciadas. En primer lugar, la administración de omega-3 a través de la dieta no resultó ser antiobesogénica (no hubo reducción en la ganancia de peso ni en el peso corporal del tejido adiposo) ni tampoco fue antidiabética (hubo una tendencia no significativa en los niveles de glucosa en suero) ni se normalizaron los niveles de ALT/AST, pero se redujo, aunque de forma menos destacable, la acumulación lipídica intrahepática observada histológicamente por tinción Oil-Red O. Por el contrario, este grupo experimentó una reducción estadísticamente significativa de la expresión de TNFα e IL-6. Además, los efectos antiesteatóticos de los ratones suplementados con omega-3 no se asociaron a una reducción de la expresión de los genes CD36/FAT, L-FABP, ACC y SREBP-1c. Finalmente, los ratones *fat-1* fueron protegidos frente a la peroxidación lipídica hepática de forma significativa.

Estas observaciones, en conjunto, indican que el enriquecimiento endógeno con omega-3 en los ratones *fat-1* presenta una protección superior frente a EHNA, a pesar de que el enriquecimiento hepático de omega-3 a través de la dieta resultó ser efectivo, debido a la presencia de un incremento en el contenido total de omega-3. Una posible explicación para esta observación es que los ratones transgénicos *fat-1* mantienen un balance omega-6/omega-3 equilibrado durante toda la vida del animal, desde su etapa embrionaria (Kang JX, 2007) y este aspecto podría manifestarse con un efecto protector mayor al conseguido por un aporte exógeno de omega-3 abundante durante un periodo corto de tiempo. Por lo tanto, el perfil de ácidos grasos que presentan los ratones *fat-1* en sus tejidos de forma crónica permite un perfil genético que es significativamente más efectivo para la prevención de enfermedades metabólicas, tales como la EHNA.

Otra novedad que se ha publicado durante la realización de este proyecto es la desregulación de las vías metabólicas de los ácidos grasos insaturados en pacientes con EHNA. De hecho, en el análisis transcriptómico se identificó una expresión diferencial de las desaturasas Δ5, Δ6 y Δ9 en el hígado de pacientes con EHNA inducida por obesidad. Estos resultados divergen de los obtenidos previamente por Araya y sus colaboradores (Araya J, 2010) en el que presentaron una disminución de la actividad de las desaturasas Δ5 y Δ6 en pacientes con EHGNA. Sin embargo, este estudio fue

realizado mayormente en mujeres, indicando que un efecto de género puede estar influyendo sobre la actividad de las desaturasas (Warensjö E, 2006).

La sobreexpresión génica y proteica de las desaturasas delta-5/delta-6/delta-9 se confirmó en un modelo animal de EHNA inducida por la dieta hipercalórica. Además, también se identificó en el análisis transcriptómico una desregulación en la expresión de otros genes implicados en la biosíntesis de los ácidos grasos poliinsaturados, como es el caso de la elongasa ELOVL6. Según estudios previos, esta enzima metabólica promueve la progresión de EHNA y su deleción genética ejerce efectos protectores frente a la resistencia a la insulina (Matsuzaka T, 2007; Matsuzaka T, 2012).

La inhibición de las desaturasas *in vivo* fue un factor limitante para este proyecto, por ello, se diseñaron estudios *in vitro* para demostrar la efectividad de los inhibidores de estas enzimas en hepatocitos primarios expuestos a ácido oleico y a LPS. El ácido oleico es un ácido graso que se acumula en exceso en el hígado de pacientes con esteatosis hepática y se utilizó a grandes concentraciones para inducir *in vitro* una acumulación del lípido en el citoplasma celular (Araya J, 2004; Morán-Salvador E, 2011; Gómez-Lechón MJ, 2007). Por otro lado, el LPS se utilizó como un potente inductor de daño inflamatorio (Olinga P, 2001). Ante la dificultad de manipular las vías de biosíntesis de los omega-6 y los omega-3 porque ambas rutas son altamente competitivas, se planteó la inhibición de la vía de las desaturasas en combinación con un aporte de omega-3, para contrarrestar la imposibilidad de producir omega-3 a partir de los omega-6 en humanos. Los resultados de estos experimentos demostraron que la inhibición selectiva de las desaturasas ejerce una acción anti-esteatótica y antiinflamatoria y existe un efecto sinérgico en presencia tanto de un aporte de omega-3 endógeno (hepatocitos procedentes de ratones *fat-1*) como exógeno (hepatocitos incubados con EPA). Los efectos beneficiosos observados en los ratones *fat-1* y en los ratones que recibieron la dieta enriquecida con omega-3 podrían reflejar una supresión de los mediadores derivados de los omega-6 o una amplificación de los derivados de los omega-3 (Hellmann J, 2011; González-Périz A, 2009). Además, los efectos antiinflamatorios ejercidos en los hepatocitos procedentes de ratones *fat-1* fueron reproducidos en hepatocitos incubados con RvD1, un mediador pro-resolutivo derivado del DHA, debido a que los *fat-1* presentan en sus tejidos una elevada formación de resolvinas derivadas de los precursores de omega-3 (Hudert C, 2006). En global, los resultados obtenidos corroboran que un balance omega-6/omega-3 equilibrado en los tejidos es una medida preventiva en la aparición de la enfermedad de EHNA.

Las propiedades biológicas de los ácidos grasos omega-3 sobre la inflamación crónica de bajo grado que se da en la obesidad están recibiendo especial atención en el campo (González-Périz A, 2009; Buckley JD y Howe PR, 2009). La inflamación persistente en el tejido adiposo de individuos obesos aumenta la incidencia de co-morbilidades, incluyendo la resistencia a la insulina y la EHGNA, y por ello, se está tomando en consideración el tratamiento de estos pacientes con omega-3 (Parker HM, 2012). La mayor parte de los efectos beneficiosos de los omega-3 han sido ampliamente atribuidos a los mediadores lipídicos sintetizados a través de la vías de la COX y de la LOX, las dos vías clásicas del metabolismo de los PUFA (Serhan CN, 2014).

En este proyecto también se ha investigado la vía del citocromo P450, descrita recientemente como la tercera vía del metabolismo de los ácidos grasos, la cual también tiene la capacidad de convertir los precursores de los omega-6 y de los omega-3 en mediadores bioactivos (Spector AA, 2015; Arnold C, 2010; Zhang G, 2013). De hecho, las isoformas CYP1A1, CYP2E1 y CYP2U1 presentan actividad epoxigenasa y generan epóxidos de los ácidos grasos omega-3 convirtiendo el DHA y el EPA en EEQ y EDP, respectivamente (Arnold C, 2010). Con la utilización de los ratones *fat-1* enriquecidos con omega-3 se demostró que la estabilización de los epóxidos con el inhibidor de las sEH amplificó los efectos antiesteatóticos y antiinflamatorios de los omega-3. Estos resultados amplían el campo de los efectos de los mediadores derivados del CYP con actividad epoxigenasa. En este sentido, una publicación reciente demuestra como una dieta rica en omega-3 en combinación con la inhibición de la sEH ejerce acción antihipertensiva (Ulu A, 2014).

Una desregulación de la autofagia contribuye a una variedad de enfermedades metabólicas, debido a que el secuestro y la degradación de los componentes celulares dañados en condiciones de estrés son procesos cruciales para la homeostasis celular (Schneider JL, 2014). En este trabajo se ha observado que la inhibición de la sEH en ratones *fat-1* restauró los procesos de autofagia en hígado. En consecuencia, en este modelo también se mitigó la respuesta del estrés del retículo endoplasmático, reflejando una recuperación de la homeostasis lipídica y el control metabólico en ambos tejidos. Estos resultados están en consonancia con el hecho que la autofagia es un mecanismo primordial que mitiga las condiciones desfavorables que derivan del estrés de RE (Yang L, 2010). Paralelamente, la disminución del estrés del RE fue acompañada de un flujo autofágico reducido en el tejido adiposo de ratones *fat-1* que recibieron el inhibidor de la sEH, sugiriendo una disociación entre estos dos procesos en este tejido. Como la reducción de la función autofágica en

tejido adiposo está relacionada con una reducción de la grasa y una mejora de la sensibilidad a la insulina (Singh R, 2009), estos resultados pueden considerarse en beneficio de la homeóstasis lipídica del organismo.

Este trabajo se complementó con experimentos *in vitro*, exponiendo hepatocitos y adipocitos a los epóxidos de omega-3, 19,20-EDP y 17,18-EEQ. Los resultados obtenidos fueron consistentes con los obtenidos por Bettaieb y sus colaboradores en los que se demostró una atenuación del estrés del RE en el hígado y el tejido adiposo en ratones que recibieron un inhibidor de la sEH o presentaban una deleción de la proteína (Bettaieb A, 2013). La comparación con otros lípidos bioactivos como el 14,15-EET, el 17S-HDHA y el DHA, resultó evidenciar la implicación potencial de los mediadores epoxidados de los omega-3 y el papel preventivo en la progresión de las diferentes afecciones del metabolismo derivadas de la obesidad.

Conclusiones

Los resultados del primer estudio indican que el enriquecimiento tisular con omega-3 mediante la expresión transgénica del gen *fat-1* representa efectos protectores frente a la esteatohepatitis no alcohólica. El reestablecimiento del balance omega-6/omega-3 acompañado de intervenciones farmacológicas dirigidas a enzimas específicas delta-5/delta-6/delta-9 desaturasas producen un efecto protector sobre la esteatosis hepática y la inflamación.

Los resultados del segundo estudio demuestran que la estabilización de los epóxidos derivados de los omega-3 generados a partir del CYP mediante la inhibición de la sEH ejerce efectos beneficiosos en contrarrestar los desórdenes metabólicos asociados con la obesidad. Además, en este estudio se lanza más luz sobre el papel de la sEH en la homeostasis celular mostrando evidencias que los epóxidos de los omega-3 y la inhibición de la sEH regula la autofagia en los tejidos sensibles a la insulina, especialmente en el hígado.

En conclusión, este proyecto de investigación pone de manifiesto que la modulación de la composición tisular en mediadores lipídicos derivados de los omega-3 para reestablecer el balance omega-6/omega-3 representa una estrategia prometedora en la prevención y la terapia de las co-morbilidades relacionadas con la obesidad, como es el caso de la esteatohepatitis no alcohólica.

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ANEXO I:
Otras publicaciones

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
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New insights into the role of macrophages in adipose tissue inflammation and fatty liver disease: modulation by endogenous omega-3 fatty acid-derived lipid mediators

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Obesity is causally linked to a chronic state of "low-grade" inflammation in adipose tissue. Prolonged, unremitting inflammation in this tissue has a direct impact on insulin-sensitive tissues (i.e., liver) and its timely resolution is a critical step toward reducing the prevalence of related co-morbidities such as insulin resistance and non-alcoholic fatty liver disease. This article describes the current state-of-the-art knowledge and novel insights into the role of macrophages in adipose tissue inflammation, with special emphasis on the progressive changes in macrophage polarization observed over the course of obesity. In addition, this article extends the discussion to the contribution of Kupffer cells, the liver resident macrophages, to metabolic liver disease. Special attention is given to the modulation of macrophage responses by omega-3-PUFAs, and more importantly by resolvins, which are potent anti-inflammatory and pro-resolving autacoids generated from docosahexaenoic and eicosapentaenoic acids. In fact, resolvins have been shown to work as endogenous "stop signals" in inflamed adipose tissue and to return this tissue to homeostasis by inducing a phenotypic switch in macrophage polarization toward a pro-resolving phenotype. Collectively, this article offers new views on the role of macrophages in metabolic disease and their modulation by endogenously generated omega-3-PUFA-derived lipid mediators.

Keywords: obesity, adipocytes, M2 macrophages, resolvins, docosahexaenoic acid, Kupffer cells

OBESITY AND ADIPOSE TISSUE INFLAMMATION

White adipose tissue, once considered a mere storage depot of energy in the form of fat, is today recognized as an important endocrine organ. In fact, the adipocyte or fat cell is actively involved in the balance of our body homeostasis by releasing a number of factors, collectively known as adipokines (Ouchi et al., 2011). However, the expansion of adipose tissue mass seen in obesity inadvertently interrupt the interplay among these factors and other intracellular components yielding a chronic "low-grade" inflammatory scenario in this tissue (Ferrante Jr., 2007; Ouchi et al., 2011). This "low-grade" inflammation, also known as "metabolic-triggered inflammation" or "metainflammation," can be described as a long-term inflammatory response triggered by nutrients and metabolic surplus (Hotamisligil, 2006). It involves a similar set of molecules/signaling pathways to those involved in classical inflammation, but in this case these molecules/signaling pathways have a dual role as inflammatory mediators as well as regulators of energy metabolism. In fact, a rise in pro-inflammatory adipokines such as tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-1β, monocyte chemoattractant protein (MCP)-1, leptin, and resistin, accompanied by a reduction in the anti-inflammatory and insulin-sensitizing adipokine, adiponectin has been reported to signal the onset of metabolic dysfunction (Ouchi et al., 2011).

One of the most important sequela of adipose tissue inflammation is insulin resistance (**Figure 1**). In fact, stress sensors activate both the c-jun-N-terminal kinase (JNK) and inhibitor of κ kinase (IKK) pathways through classical receptor-mediated mechanisms (Shoelson et al., 2006). JNK and IKK activation induce insulin resistance by disrupting serine phosphorylation of IRS-1, a protein that connects the insulin receptor to the PI(3)K signaling cascade. In parallel to the activation of these kinases and their downstream signaling cascades, there is an increased production of pro-inflammatory adipokines (i.e., TNFα, IL-6, and MCP-1) in obese subjects, whose levels directly correlate with the degree of insulin resistance (Hotamisligil et al., 1996). Adipose tissue inflammation leading to insulin resistance also has negative consequences on the liver. In fact, adipose tissue and liver have immediate access to a vast network of blood vessels that facilitate a direct connection between these two organs. The exact mechanisms linking adipose tissue dysfunction and insulin resistance with metabolic liver disease are not completely understood, but several processes have been implicated. First, increased lipolysis from visceral fat resulting in increased free fatty acid efflux to the liver (Sanyal, 2005). Second, increased secretion of pro-inflammatory and insulin-resistant adipokines (TNFα and IL-6) by adipose tissue in parallel with a reduced release of adiponectin (Sanyal, 2005; **Figure 1**). Finally, a combined hepatic

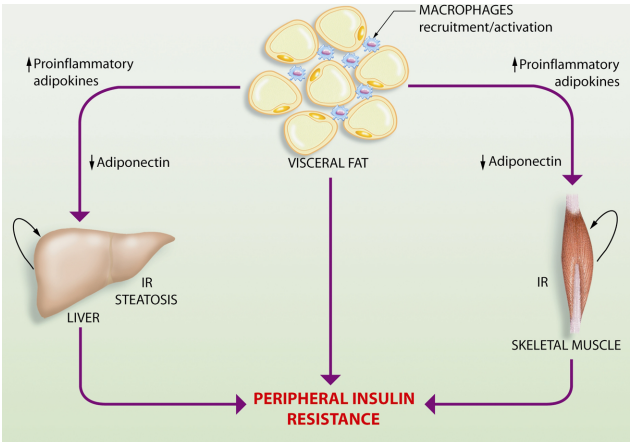

FIGURE 1 | Schematic representation of the interplay between adipose tissue, skeletal muscle, and the liver in the obesity-related perturbation of systemic metabolic control. Obesity results in expansion of adipose tissue mass that eventually leads to a characteristic inflammatory response driven by macrophage infiltration and aberrant production and release of pro-inflammatory adipokines, accompanied by a reduction in the anti-inflammatory and insulin-sensitizing adipokine, adiponectin. This altered profile of adipokine secretion leads to insulin resistance (IR) in the liver and skeletal muscle, which are the major organs contributing to the development of peripheral insulin resistance. Hepatic insulin resistance also triggers the progression of hepatic steatosis or fatty liver.

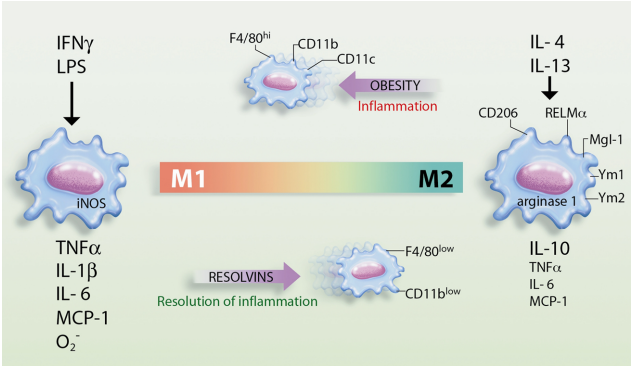

FIGURE 2 | Schematic representation of macrophage polarization in the adipose tissue and the actions of resolvins. Obesity promotes the polarization of macrophages into the M1 phenotype, which are highly inflammatory in nature and release pro-inflammatory cytokines/chemokines [e.g., tumor necrosis factor (TNF) α, interleukin (IL)-1β, IL-6, and monocyte chemotactic peptide (MCP)-1] and superoxide anion (O_2^-). These macrophages express inducible nitric oxide synthase (iNOs) and cell surface markers such as F4/80, CD11b, and CD11c and act as classically activated macrophages expressing interferon (IFN) γ and lipopolysaccharide (LPS)-responsive genes. Conversely, resolvins promote the resolution of inflammation by skewing macrophages toward the M2 phenotype, which release high levels of IL-10 in parallel with reduced levels of TNFα, IL-6, and MCP-1. M2 macrophages are alternatively activated macrophages, originally identified after IL-4 and IL-13 stimulation, that up-regulate scavenger, mannose (CD206), and galactose (Mgl-1) receptors, resistin-like molecule (RELM)-α, and chitinases Ym1 and Ym2 expression and arginase 1 activity.

dysregulation in free fatty acid oxidation and *de novo* lipogenesis secondary to altered hepatic insulin sensitivity (Tilg and Moschen, 2008).

MACROPHAGES AND ADIPOSE TISSUE INFLAMMATION

Obesity-induced adipose tissue inflammation is a unique process characterized by an inflammatory response driven by tissue macrophages (Lumeng and Saltiel, 2011). In fact, a pathological hallmark of obesity is the presence of an increased number of adipose tissue-infiltrating macrophages, which form the characteristic "crown-like structures" that surround necrotic adipocytes and perpetuate a vicious cycle of macrophage recruitment and exacerbated production of pro-inflammatory mediators (Weisberg et al., 2003; Wellen and Hotamisligil, 2003; Cancello et al., 2005; Lesniewski et al., 2007).

Tissue macrophages display an extensive receptor repertoire and a versatile biosynthetic capacity that confer them the plasticity to adapt to different tissue microenvironments (Gordon and Taylor, 2005). Accordingly, tissue macrophages are phenotypically heterogeneous and can exhibit either pro- or anti-inflammatory properties depending on the disease stage and the signals they are exposed. Although the classification based on the Th1/Th2 nomenclature needs to be revised, macrophages are broadly characterized by their activation (polarization) state according to the M1/M2 classification system (Mantovani et al., 2007; Martínez et al., 2009). According to this classification, the M1 designation is reserved for classically activated macrophages following stimulation with interferon (IFN) γ and LPS, whereas the M2 designation is applied to the alternatively activated macrophages after *in vitro* stimulation with IL-4 and IL-13 (**Figure 2**). M1 macrophages display enhanced microbicidal capacity and secrete high levels of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) and increased concentrations of superoxide anion (O_2^-) and oxygen and nitrogen radicals to increase their killing activity (Gordon and Taylor, 2005). Conversely, M2 macrophages dampen pro-inflammatory cytokine levels, secrete components of the extracellular matrix, and may be essential for the immune response to parasites, tissue repair, and resolution of inflammation (Gordon, 2003). In this classification system, M1 and M2 macrophages are merely regarded as two extremes of a continuum of functional stages (Mosser and Edwards, 2008). For instance, M2a designation defines those macrophages stimulated by IL-4/IL-13; M2b refers to macrophages activated by stimuli such as apoptotic cells in concert with LPS; and M2c relates to polarization in response to IL-10, transforming growth factor (TGF)-β, or glucocorticoids (Martínez et al., 2008). In mice, M1/M2 macrophage polarization can be monitored by assessing the expression of selected markers. M1-associated genes include inducible nitric oxide synthase (iNOs), the interferon responsive CXC chemokines, and classical pro-inflammatory mediators such as TNFα, IL-1β, IL-6, and MCP-1 as well as increased production of O_2^- (Gordon, 2003; Martínez et al., 2008; **Figure 2**). M2 macrophages display up-regulation of scavenger, mannose (CD206), and galactose (Mgl-1) receptors, arginase 1, which antagonizes iNOS activity, and IL-10, in parallel with down-regulation of IL-1β and other pro-inflammatory cytokines (Gordon, 2003; Scotton et al., 2005; Martínez et al., 2008). In addition, the panel of M2 markers comprises up-regulation of other genes with unknown function such

as chitinases Ym1 and Ym2, and resistin-like molecule (RELM)-α, also known as FIZZ (**Figure 2**).

In addition to the augmented infiltration of macrophages into the adipose tissue, obesity also induces a phenotypic switch in these cells toward the classically activated M1 phenotype (Olefsky and Glass, 2010). In fact, the majority of macrophages that accumulate in obese adipose tissue are M1-like and selectively express the cell surface markers F4/80, CD11b, and CD11c (Lumeng et al., 2007; Nguyen et al., 2007). In our laboratory, we have recently gathered data indicating the presence of a specific subset of macrophages with high expression of the surface glycoprotein F4/80 (F4/80*hi*) in adipose tissue from obese mice (Titos et al., 2011). This finding is consistent with that reported by Bassaganya-Riera et al. (2009) who identified two functionally distinct subsets of macrophages in adipose tissue based on their surface expression of F4/80 (F4/80*lo* macrophages predominate in adipose tissue of lean mice, obesity causes accumulation of both F4/80*lo* and F4/80*hi*). Importantly, lean adipose tissue macrophages are M2-like, display F4/80 and CD11b but are negative for CD11c and do not exhibit activation of the inflammatory pathways. In a series of elegant studies, Lumeng et al. (2007) and Nguyen et al. (2007) have demonstrated that adipose tissue macrophages undergo a phenotypic switch from the M2 polarization state to a more M1-like, $CD11c^+$ polarization state upon high-fat feeding. Moreover, Patsouris et al. (2008) have reported that selective depletion of $CD11c^+$ macrophages in adipose tissue reverses insulin resistance in high-fat diet-induced obese mice. Recently, Li et al. (2010) have reported that the M1-like, $CD11c^+$ macrophage subset can exhibit phenotypic plasticity between inflammatory and non-inflammatory states, depending on the presence or absence of insulin resistance.

MACROPHAGES AND LIVER DISEASE

Kupffer cells are specialized macrophages located in the liver lining the walls of the sinusoids (Ramadori et al., 2008). Kupffer cells are uniquely positioned within the liver and their location enables intimate contact with circulating blood and the clearance of pathogens and parasites by receptor-mediated phagocytosis or release of TNFα, reactive oxygen species, or proteinases. Kupffer cells are also professional antigen-presenting cells that trigger the adaptive immune system. Therefore, Kupffer cells act as true sentinels of the adaptive and immune system in the liver and protect our body from the extracorporeal environment. In cases of pathogenic infection or tissue damage, Kupffer cells act as the predominant inflammatory effector cell type to initiate the inflammatory cascade leading to liver injury (Ramadori et al., 2008). In fact, activation of Kupffer cells and the subsequent release of cytokines, reactive oxygen species, and inflammatory lipid mediators (i.e., eicosanoids) are considered an early step in the pathogenesis of liver damage and tissue remodeling, as they stimulate inflammatory and fibrogenic events in the liver (Titos et al., 2003, 2005; Ramadori et al., 2008; **Table 1**). Depletion of Kupffer cells by treatment with either gadolinium chloride, liposomal clodronate, or conditional ablation of the diphtheria toxin receptor appears to confer a protective role in the liver by reducing the production of inflammatory mediators and collagen content (Ramadori et al., 2008).

Recent studies have revealed a novel role for Kupffer cells in metabolic liver disease. In fatty livers, similar to that occurring

Table 1 | Kupffer cell-derived mediators and associated liver pathologies.

Mediators	Biological effects	Liver pathology	References
CYTOKINES/CHEMOKINES			
IL-1β, TNFα, IL-6	Hepatotoxicity, endothelial activation, steatogenic, hepatocyte proliferation	Alcoholic liver disease, acute liver injury, NAFLD, NASH, crucial for liver regeneration	Miura et al. (2010), Ramadori and Armbrust (2001), Taub (2004)
TGF-β, PDGF	Myofibroblast transformation and activation	Hepatic fibrosis and cirrhosis	Bataller and Brenner (2005), Pinzani (2002)
MCP-1, IL-8	Neutrophil, monocyte recruitment, angiogenesis, steatogenic	Acute liver injury, alcoholic liver disease, hepatic fibrosis	Devalaraja et al. (1999), Domínguez et al. (2009)
IL-12	Lymphocyte, natural killer activation	Alcoholic liver disease, viral hepatitis	Leifeld et al. (2002)
IL-10, IL-18, IFNα/β	Immunoregulatory, anti-inflammatory, anti-proliferative	Ischemia-reperfusion injury, viral hepatitis	Ellett et al. (2010), Takeuchi et al. (2004), Neuman et al. (2008)
EICOSANOIDS			
PGE_2, PGD_2	Cytoprotection/cytotoxicity	Ischemia-reperfusion injury	Quiroga and Prieto (1993), Planagumà et al. (2005)
LTB_4, cysteinyl-LTs	Vasoactive, hepatic stellate cell activation, chemotactic, steatogenic	Hepatic fibrosis and cirrhosis, NAFLD	Titos et al. (2000), Titos et al. (2003), Horrillo et al. (2010)
REACTIVE OXYGEN SPECIES			
O_2^-, H_2O_2, $ONOO^-$	Hepatotoxicity and necrosis, pro-inflammatory	Alcoholic liver disease, hepatic cirrhosis, ischemia-reperfusion injury, steatohepatitis	Lieber (1997), Muriel (2009)
OTHER			
Gelatinases	Extracellular matrix remodeling, collagen synthesis	Liver fibrosis	Wynn and Barron (2010)
Complement proteins	Pathogen destruction	Chronic liver disease	Bilzer et al. (2006)

in obese adipose tissue, macrophages are in close proximity to fat-laden parenchymal cells (the hepatocytes) and may establish a cross-talk by secreting insulin-resistant cytokines such as TNFα and IL-6, thus regulating hepatic fat and glucose homeostasis and the progression of fatty liver (Baffy, 2009). In fact, excessive exposure of Kupffer cells to fatty acids may induce the activation of these cells via Toll-like receptors thus connecting an important mechanism by which lipids regulate inflammation and immune response in the liver (Kim, 2006). In a mouse model of steatohepatitis, Miura et al. (2010) convincingly showed that TLR9 signaling induces production of IL-1β by Kupffer cells, leading to steatosis, inflammation, and fibrosis. These authors have also shown that JNK activation in Kupffer cells contribute to the development of chronic inflammation and fibrosis in an experimental model of diet-induced steatohepatitis (Kodama et al., 2009). Lanthier et al. (2010) have elegantly demonstrated that early hepatic insulin resistance and steatosis are concurrent with Kupffer cell activation, and that selective Kupffer cell depletion through intravenous clodronate injection is sufficient to improve hepatic insulin signaling. Interestingly, as earlier described for adipose tissue macrophages, alternative M2 activation of Kupffer cells appears to ameliorate insulin resistance and to retard the progression to steatohepatitis in mice (Odegaard et al., 2008).

CLINICAL IMPACT OF OMEGA-3-PUFAs IN DIABETES AND METABOLIC LIVER DISEASE

The first evidences of beneficial actions of omega-3-PUFAs in humans were provided by Endres et al. (1989). Since then, several *in vivo* and *in vitro* studies both in human and rodents have demonstrated the therapeutic potential of omega-3-PUFAs in pathologies with an important inflammatory component (Dinarello, 2010). A number of pre-clinical and clinical studies have demonstrated that regular consumption of modest amounts of omega-3-PUFAs ($\leq$3 g/day) improves serum lipid profiles, exerts cardiovascular protective actions, and may reduce the risk of conversion from impaired glucose tolerance to type-2 diabetes (Nettleton and Katz, 2005). The use of enriched omega-3-PUFA diets in patients with non-alcoholic fatty liver disease could also represent an important nutritional strategy for their clinical management (Shapiro et al., 2011). However, there is a concern that most of studies addressing the effects of omega-3-PUFAs on glucose metabolism and insulin sensitivity did not have a control group and that dosages of fatty acids were sometimes higher than those sufficient to obtain beneficial end-points in these patients (De Caterina et al., 2007). This point out that new, more specific approaches are needed (i.e., compare potency and specificity of resolvins to their substrate precursors, see below).

EFFECTIVE RESOLUTION OF INFLAMMATION: ROLE OF MACROPHAGES

Since prolonged inflammation is detrimental to the host, higher organisms have evolved protective mechanisms to ensure resolution of the inflammatory response in a limited and specific time- and space-manner (Serhan et al., 2007). Once thought as a mere passive process of dilution of inflammation, resolution is today envisioned as a highly orchestrated process coordinated by a complex regulatory network of cells and mediators. Among the molecules that facilitate resolution, lipoxins generated from the omega-6-PUFA arachidonic acid, and resolvins and protectins generated from omega-3-PUFAs, are the lipid mediators that have attracted most attention. These endogenous anti-inflammatory and pro-resolving mediators counteract the effects of pro-inflammatory signaling systems and act as "braking signals" of the persistent vicious cycle leading to unremitting inflammation (Serhan et al., 2008). In fact, the same pro-inflammatory factors that initially trigger the inflammatory response also signal the termination of inflammation by stimulating the biosynthesis of pro-resolving lipid mediators (Serhan et al., 2008). For instance, both PGE_2 and PGD_2 transcriptionally activate the expression of 15-LO in human PMN, switching the mediator profile of these cells from the pro-inflammatory LTB_4 to the anti-inflammatory lipoxin A_4, which was the first identified omega-6-PUFA-derived anti-inflammatory lipid mediator (Serhan et al., 2007, 2008). Another example of this class switch is the displacement of pro-inflammatory lipid mediators derived from omega-6-PUFAs by anti-inflammatory mediators (i.e., resolvins and protectins) derived from omega-3-PUFAs (Serhan, 2011). These anti-inflammatory and pro-resolving mediators exert a strict control of the resolution process and pave the way for monocyte migration and their differentiation to phagocytosing macrophages, which remove dead cells and then terminate the inflammatory response (Tabas, 2010; Serhan, 2011).

RESOLVINS

Resolvins are a novel family of anti-inflammatory and pro-resolving mediators generated from the omega-3-PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). By using a lipidomics-based approach that combines liquid chromatography and tandem-mass spectrometry, Serhan et al. (2000, 2002) identified a library of omega-3-PUFA-derived lipid mediators present within exudates obtained from mice dorsal skin pouches during the "spontaneous resolution" phase of acute inflammation. These novel bioactive lipid autacoids were termed resolvins and were classified as either resolvin E-series, if the biosynthesis is initiated from EPA, or resolvin D-series, if they are generated from DHA. Schematically, the biosynthesis of resolvin E1 is initiated when EPA is converted to 18R-hydroperoxy-EPE by endothelial cells expressing COX-2 treated with aspirin (Serhan et al., 2000). Alternatively, 18R-hydroperoxy-EPE can be produced through cytochrome P450 activity (Haas-Stapleton et al., 2007). By transcellular biosynthesis, 18R-hydroperoxy-EPE generated by endothelial cells is transformed by 5-LO of neighboring leukocytes into resolvin E1 (5S,12R,18R-trihydroxy-EPA) via a 5(6)epoxide intermediate (Serhan et al., 2000, 2002). Resolvin D1 biosynthesis is also initiated in endothelial cells expressing COX-2 treated with aspirin, which transform DHA into 17R-hydroxy-DHA which is further transformed by leukocyte 5-LO into resolvin D1 (Serhan et al., 2000, 2002). More importantly from a physiological point of view, resolvin D1 can also be formed from endogenous sources of DHA without the requirement of aspirin. In this case, endogenous DHA is converted via 15-LO/5-LO interactions that give rise to a 17S alcohol-containing series of resolvins, including resolvin D1 and resolvin D2 (Hong et al., 2003). Finally, DHA is also transformed into a dihydroxy-containing DHA derivative,

17S-hydroxy-DHA via an intermediate epoxide that opens via hydrolysis and subsequent rearrangements to form protectin D1 (10R,17S-dihydroxy-docosa-DHA) (Serhan et al., 2000, 2002; Hong et al., 2003).

Unlike their precursors DHA and EPA, resolvins exert biological actions at the nanomolar range. Resolvin E1, decreases PMN infiltration and T cell migration, reduces TNFα and IFNγ secretion, inhibits chemokine formation and blocks IL-1-induced NF-κB activation (Gronert et al., 2005; Schwab et al., 2007; Bannenberg and Serhan, 2010). Resolvin E1 also stimulates macrophage phagocytosis of apoptotic PMN and is a potent modulator of pro-inflammatory leukocyte expression adhesion molecules (i.e., L-selectin) (Schwab et al., 2007; Dona et al., 2008). *In vivo* resolvin E1 exerts potent anti-inflammatory actions in experimental models of periodontitis, colitis, and peritonitis and protects mice against brain ischemia-reperfusion (Arita et al., 2005; Bannenberg and Serhan, 2010). Furthermore, Haworth et al. (2008) have identified a resolvin E1-initiated resolution program for allergic airway responses. Finally, a recent study has identified resolvin D2 as a potent endogenous regulator of excessive inflammatory responses in mice with microbial sepsis (Spite et al., 2009).

Our laboratory has recently provided evidence that adipose tissue expresses all the enzymes necessary for the formation of bioactive lipid mediators derived from both omega-6 and omega-3-PUFAs (i.e., 12/15-LO, 5-LO, FLAP, LTA4 hydrolase, and LTC4 synthase; Horrillo et al., 2010). Importantly, by means of liquid chromatography–tandem mass spectrometry (LC/MS/MS) analysis we have detected the presence of the omega-6 products PGE_2, $PGF_{2\alpha}$, TXB_2, 5-HETE, 12-HETE, and 15-HETE as well as the formation of the omega-3-derived mediators resolvin D1, protectin D1, and 17-hydroxy-DHA (González-Périz et al., 2009). Interestingly, the administration of a DHA-enriched diet to *ob/ob* mice, an experimental model of obesity-induced insulin resistance and fatty liver disease, resulted in the amplification of the formation of resolvin D1, protectin D1, and 17-hydroxy-DHA, accompanied by an inhibition of the formation of omega-6-derived inflammatory mediators (González-Périz et al., 2009). In these animals, DHA significantly increased adipose tissue levels of adiponectin which alleviated hepatic steatosis and insulin resistance (González-Périz et al., 2009). Of interest, intraperitoneal injection of resolvin E1 at the nanomolar levels elicited significant insulin-sensitizing effects by inducing adiponectin, GLUT-4, and IRS-1 expression in adipose tissue and conferred significant protection against hepatic steatosis (González-Périz et al., 2009). There is also evidence that omega-3-PUFAs may also signal independently of the 12/15-LO pathway by exerting potent anti-inflammatory and insulin-sensitizing actions through a G-protein-coupled 120 receptor (GPR120) (Oh et al., 2010).

Recent findings from our laboratory also indicate that DHA (at micromolar concentrations) and resolvin D1 (at nanomolar concentrations) consistently induce hallmarks of alternative macrophage activation in adipose tissue including stimulation of arginase 1 expression and non-phlogystic macrophage phagocytosis and attenuation of IFNγ/LPS-induced Th1 cytokine secretion (Titos et al., 2011). These results are in agreement with those reported by Schif-Zuck et al. (2011) who recently identified a novel phenotype of macrophages with pro-resolving properties emerging during the resolution of murine peritonitis. These macrophages had a low marker expression of CD11b ($CD11b^{low}$), engulfed significantly higher numbers of apoptotic PMN than $CD11b^{high}$ macrophages, responded poorly to activation by different TLR ligands in terms of cytokine and chemokine secretion, lost their phagocytic potential and were prone to migrate to lymphoid organs (Schif-Zuck et al., 2011). In addition, these $CD11b^{low}$ macrophages expressed low or moderate levels of COX-2, metalloproteinase-9, and 12/15-LO, but not detectable levels of iNOS and arginase 1. Importantly, *in vivo* administration of resolvin E1, resolvin D1, and glucocorticoids to peritonitis-affected mice clearly enhanced the appearance of $CD11b^{low}$ macrophages by reducing the number of engulfment-related events required for macrophage deactivation and by reducing the ability of peritoneal macrophages to produce pro-inflammatory cytokines upon LPS stimulation (Schif-Zuck et al., 2011). The ability of resolvins to modify tissue macrophage plasticity has also been demonstrated by Hellmann et al. (2011). These authors were able to improve insulin resistance by administering resolvin D1 to obese-diabetic mice, which reduced macrophage $F4/80^{+}CD11c^{+}$ cell accumulation and increased the percentage of positive F4/80 cells expressing Mgl-1, a marker of alternatively activated macrophages, in adipose tissue (Hellmann et al., 2011).

Studies on experimental models of liver injury have elucidated a protective role of DHA and DHA-derived lipid mediators against hepatic inflammation. In fact, feeding of a DHA-enriched diet ameliorated hepatotoxic-induced necroinflammatory liver injury in mice (González-Périz et al., 2006). The hepatoprotective actions of DHA were associated with a decrease in the hepatic formation of PGE_2 and a concomitant increase in the generation of protective DHA-derived lipid mediators (i.e., PD1 and 17*S*-HDHA). The beneficial role of these DHA-derived lipid signals was further supported by experiments *in vitro* demonstrating attenuated DNA damage and oxidative stress in hepatocytes. More important, DHA and DHA-derived autacoids reduced TNFα release in macrophages, recognized as the predominant effector cells involved in the inflammatory cascade leading to hepatocyte damage (Decker, 1990). A significant down-regulation of 5-LO protein expression was also noticed in macrophages treated with 17*S*-HDHA and in liver tissue from mice receiving DHA in the diet (González-Périz et al., 2006). This is relevant because the presence of an active 5-LO pathway in the liver is restricted to Kupffer cells and inhibition of the 5-LO pathway in these resident macrophages has been shown to attenuate necroinflammatory liver injury and fibrosis (Titos et al., 2000, 2003, 2005).

CONCLUSION

Obesity is not only a matter of appearance and beauty, but a serious health issue because the global obesity epidemic will result in increased incidence and risk of cardiovascular disease, type-2 diabetes, dyslipidemia, and fatty liver disease. The prevalence of obesity-related metabolic disorders is tightly associated with the appearance of a chronic "low-grade" inflammatory state in the adipose tissue, which severely disrupts the endocrine function of this organ. Indeed, a number of studies have appreciated

that expansion of adipose tissue during weight gain is associated with an inflammatory phenotype characterized by the recruitment of inflammatory cells, mainly macrophages, in this tissue. A very provocative strategy to manipulate this exacerbated inflammatory response is to replace the use of drugs that inhibit the formation of pro-inflammatory mediators by the use of endogenous-generated autacoids that boost the resolution of inflammation. Therefore, adipose tissue inflammation in obesity appears to be the perfect scenario for testing the novel anti-inflammatory and pro-resolving lipid mediators, designated resolvins. Notably, these inflammation-resolving factors can induce a proper skew of macrophages toward a unique pro-resolving phenotype, thus ameliorating the incidence of obesity-related metabolic disorders.

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Pro-resolving mediators produced from EPA and DHA: Overview of the pathways involved and their mechanisms in metabolic syndrome and related liver diseases

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ABSTRACT

A novel genus of pro-resolving lipid mediators endogenously generated from omega-3 polyunsaturated fatty acids has been identified in exudates obtained during the resolution phase of acute inflammation. The term specialized pro-resolving mediators (SPM) has been coined for these lipid mediators, comprising four novel chemical mediator families designated resolvins of the E series (if derived from eicosapentaenoic acid) and resolvins of the D series, protectins and maresins (if generated from docosahexaenoic acid). These SPM act not only as "stop-signals" of inflammatory response, but also as facilitators of the ability of macrophages to clear apoptotic cells (efferocytosis) and migrate to peripheral lymph nodes (efflux), thus, expediting their removal from sites of inflammation. In this review, we provide an overview of the current efforts to elucidate the structure-function, biosynthesis and actions of these omega-3-derived SPM in the context of inflammatory diseases. We specifically highlight the role of these SPM as endogenous counter-regulators of the persistent inflammatory status present in adipose tissue of obese individuals and describe the potential therapeutic impact of these bioactive lipid autacoids on the prevention of hepatic co-morbidities associated with obesity and the metabolic syndrome.

1. Introduction

Inflammation plays a vital role in host defense against invasive pathogens and tissue and wound repair. Inflammation is part of the innate immune response and is initiated by a cascade of signals in response to an infection or injury that leads to the recruitment of specialized inflammatory cells, particularly neutrophils (PMN), into injured tissue to neutralize and eliminate the injurious stimuli (Barton, 2008; Chen and Nuñez, 2010). The innate immune response not only acts as the first line of defense against an insult, but it also provides the necessary signals to instruct the adaptive immune system as to an effective response to deal with the noxious agent. Although inflammation is important in the eradication of pathogens, unresolved, chronic inflammation that occurs when the offending agent is not removed or contained, is detrimental to the host, resulting in tissue damage, fibrosis and loss of function (Barton, 2008; Chen and Nuñez, 2010).

Recent evidence indicates that unresolved, chronic inflammation in abdominal adipose tissue is the predominant underlying risk factor for the development of co-morbidities (i.e. insulin resistance, type 2 diabetes, dyslipidemia and non-alcoholic fatty liver disease (NAFLD)) in obese patients with underlying metabolic syndrome (Elks and Francis, 2010; Ferrante, 2007). In this condition, unresolved, chronic inflammation is characterized by a persistent "low-grade" state of inflammation, which is aggravated by the recruitment of macrophages into the adipose tissue (Elks and Francis, 2010; Ferrante, 2007). As a consequence of this unresolved inflammatory response, the production of pro-inflammatory adipokines (i.e. IL-6, TNFα and MCP-1) is increased while the secretion of adiponectin, an anti-inflammatory and insulin-sensitizing adipokine, is reduced (Elks and Francis, 2010; Ferrante, 2007). Based on these findings, the regulation of chronic adipose tissue inflammation has been established as a target priority in the management of adipose tissue dysfunction and related co-morbidities.

A rapidly evolving field in managing inflammatory response is to modulate its dynamic resolution. Since unresolved

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inflammation is detrimental to the host, higher organisms have evolved protective mechanisms to ensure resolution of the inflammatory response in a specific time-limited manner (Serhan et al., 2007). Once considered a mere passive process of dilution, resolution is today envisioned as a highly-orchestrated process coordinated by a complex regulatory network of cells and mediators. Among the mediators that control the resolution process, lipid mediators derived from the metabolism of essential omega-3-polyunsaturated fatty acids have attracted the most attention. They were initially identified using a lipidomics-based approach that combined liquid chromatography and tandem mass spectrometry (LC–MS/MS) within self-limited inflammatory exudates captured during the "spontaneous resolution" phase of acute inflammation (Serhan et al., 2000). These mediators, generically known as "specialized pro-resolving mediators" or SPM, comprise molecules designated as resolvins, protectins and maresins, and contrary to their metabolic substrates, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), exert their biological actions at the nanomolar range. Indeed, the potency of these SPM is notable, with concentrations as low as 10 nM producing a 50 percent reduction in PMN transmigration in model systems (Bannenberg and Serhan, 2010). Moreover, these SPM expedite the resolution process by paving the way for monocyte migration and their differentiation to phagocytosing macrophages, which remove dead cells (efferocytosis) and then terminate the inflammatory response by promoting macrophage efflux into the lymph nodes (Schif-Zuck et al., 2011).

Recent studies indicate that the formation of SPM is severely deregulated in inflamed obese adipose tissue (Clària et al., 2013; Spite et al., 2014). Since SPM act as "braking signals" of the persistent vicious cycle leading to unremitting inflammation, in recent years these endogenous lipid mediators have attracted attention as a novel strategy to expedite the resolution process of inflamed adipose tissue in obesity. In this review, we present a summary of the most recent evidence that demonstrate that enhancement of local SPM production in adipose tissue or the administration of these resolution agonists reduces the inflammatory tone of obese adipose tissue.

2. Omega-3 fatty acids attenuate inflammation

Fatty acids are increasingly being recognized as a central feature of many biological processes (Wallis et al., 2002). Fatty acids are categorized as either saturated or unsaturated depending on the absence or the presence of a carbon-to-carbon double bond, respectively (Wallis et al., 2002). Polyunsaturated fatty acids (PUFAs) harbor two or more double bonds and are classified into two distinct families, namely omega-6 and omega-3 PUFAs (Wallis et al., 2002). Contrary to other fatty acids, they cannot be synthesized de novo by mammalian cells; therefore they are termed "essential" and must be obtained in adequate amounts from the diet (Wallis et al., 2002; El-Badry et al., 2007).

Numerous studies have demonstrated that PUFAs, whether released from membrane phospholipids by cellular phospholipases or made available to the cell from the diet or other extracellular sources, are important cell-signaling molecules (Shaikh and Edidin, 2006). They can act as second messengers or as modulator molecules mediating responses of the cell to extracellular signals (Shaikh and Edidin, 2006; Simopoulos, 1999). In particular, the most characteristic omega-6-PUFA, arachidonic acid (AA), exerts inflammatory, atherogenic and prothrombotic effects by conversion to potent lipid mediators, generically known as eicosanoids. Eicosanoids are generated from AA through two major enzymatic routes: the cyclooxygenase (COX) pathway, which metabolizes AA to form prostaglandins and thromboxane A_2 (TXA_2); and the lipoxygenase (LOX) pathway, which produces leukotrienes and hydroxyeicosatetraenoic acids (HETEs) (Bergstrom and Samuelsson, 1962; Hamberg and Samuelsson, 1967; Samuelsson et al., 1987). By sequential LOX–LOX and COX–LOX interactions AA can also give rise to the biosynthesis of lipoxins, which were the first class of anti-inflammatory and pro-resolving lipid mediators described in the field of inflammation resolution (Samuelsson et al., 1987). In addition to the COX and LOX pathways, CYP epoxygenases (the so-called third branch of AA metabolism) can also convert AA into epoxyeicosatrienoic acids (EETs), which have consolidated properties as regulators of vascular tone, inflammation, hyperalgesia and tissue regeneration (Zeldin, 2001; Panigrahy et al., 2013). A detailed description of the biosynthetic pathways and biological actions of eicosanoids is beyond the scope of this article, and comprehensive reviews can be found elsewhere (Romano and Clària, 2003; Capra et al., 2015; Zeldin, 2001).

The typical omega-3-PUFAs, EPA and DHA also regulate both innate and acquired immune responses (reviewed in De Caterina (2011) and Calder (2013)). However, in contrast to omega-6-PUFAs, omega-3-PUFAs are chemically linked to the formation of anti-inflammatory lipid mediators (Schmitz and Ecker, 2008). In fact, these omega-3-PUFA act as substrates for the same COX and LOX pathways described previously for omega-6-PUFA, but in this case, EPA and DHA are converted into potent anti-inflammatory and pro-resolving mediators designated as resolvins, protectins and maresins (see Section 3, below, for more details) (reviewed in Serhan (2014)). These omega-3-derived mediators evoke organ protection and tissue remodeling, enhance microbial clearance and function as immunoresolvents in host defense and pain (see Section 3, below). It is still a subject of debate whether the parent non-oxidized omega-3-PUFAs are also biologically active or the omega-3-PUFA-derived pro-resolving lipid mediators account for all protective effects seen with these fatty acids. However, identification of the chemical structures and stereochemical assignments, mapping of their biosynthetic pathways and elucidation of their biological actions, collectively indicate that formation of omega-3-PUFA-derived pro-resolving lipid mediators underlies most of the beneficial effects attributed to their precursors, EPA and DHA (see Section 3, below) (Serhan and Chiang, 2013).

A number of studies in both humans and rodents have demonstrated the therapeutic potential of omega-3-PUFAs in pathologies with an important inflammatory component, such is the case for coronary artery disease, arthritis, cystic fibrosis, IgA nephropathy, diabetes, ulcerative colitis, Crohn's disease, asthma and sepsis (Calder, 2015). A potential role for omega-3-PUFAs in cancer has also been suggested (Wang et al., 2014). It is also well documented that omega-3 PUFAs are essential for normal growth and the development of cognitive function in both rodents (Abdel-Wahab et al., 2015) and humans (Widenhorn-Müller et al., 2014) as well as in neurotransmission, cell survival and neuroinflammation (Bazinet and Layé, 2014). Omega-3-PUFAs have also attracted much attention in the regulation of body weight and body composition and in the prevention of obesity-associated metabolic syndrome features (Lorente-Cebrián et al., 2013). Indeed, clinical interventions have provided supporting data for the beneficial effects of omega-3 fatty acids in the prevention and management of hyperinsulinemia, type 2 diabetes and NAFLD (Lou et al., 2014; El-Badry et al., 2007; Parker et al., 2012; Masterton et al., 2010; Scorletti et al., 2014). Of particular interest is a recent study which described that serum phospholipid omega-3-PUFA levels are significantly decreased in patients with type 2 diabetes and NAFLD, whose levels are negatively related to insulin resistance (Lou et al., 2014). Although systematic review of the literature assessing the therapeutic role of omega-3-PUFAs in patients with NAFLD yields a significant heterogeneity in the outcomes among studies (El-Badry et al., 2007; Parker et al., 2012;

Masterton et al., 2010), a recent publication established substantial anti-steatotic actions of purified EPA and DHA in patients with NAFLD (Scorletti et al., 2014).

3. Omega-3-derived lipid mediators and resolution of inflammation

The specific roles of endogenous omega-3-PUFA-derived lipid mediators acting as counter-regulators of inflammation and activators of resolution have recently been established (reviewed in Serhan (2014)). The term SPM has been coined for these omega-3-derived pro-resolving lipid mediators, a term that includes several families of chemically and functionally distinct mediators (i.e. resolvins, protectins and maresins). These lipid mediators were originally identified by Serhan et al. (2000) using a lipidomics-based approach that combined liquid chromatography and tandem mass spectrometry in exudates obtained from mice dorsal skin pouches during the "spontaneous resolution" phase of acute inflammation. These novel bioactive lipid autacoids were termed resolvins (derived from resolution phase interaction products) and were classified as either resolvins of the E-series if the biosynthesis is initiated from EPA or resolvins of the D-series if they are generated from DHA (Serhan et al., 2000). Protectins and maresins, macrophage mediators in resolving inflammation, are also biosynthesized from DHA (Serhan et al., 2009).

The biosynthesis of resolvins of the E-series is schematically represented in Fig. 1. Their biosynthesis is initiated when EPA is converted into 18R-hydroperoxy-EPE (18R-HEPE) by endothelial cells expressing aspirin-acetylated COX-2 (Serhan et al., 2000). 18R-HEPE is transformed by transcellular biosynthesis in neighboring 5-LOX-containing leukocytes into RvE1 (5S,12R,18R-trihydroxy-EPA) via a 5S,6-epoxide intermediate (Serhan et al., 2000) (Fig. 1). This finding is consistent with previous studies showing that when aspirin acetylates the inducible COX isoform, this enzyme is able to convert AA into 15*R*-HETE, which is further transformed into potent pro-resolving molecules, namely 15-epi-lipoxins (Clària and Serhan,1995). Alternatively, 18R-HEPE can be produced through CYP450 activity (Haas-Stapleton et al., 2007). In addition, reduction of 5-LOX derived hydroperoxide intermediate via a peroxidase leads to the formation of RvE2 (5S,18R-dihydroxy-EPA). Recently, a novel member of the E-series resolvin family, RvE3 (17S,18R-dihydroxy-EPA) generated via the 12/15-LOX pathway from 18R HEPE, has been identified (Isobe et al., 2012). Similarly, a novel series of 18S-RvE1 (5S,12R,18S-trihydroxy-EPA) and 18S-RvE2 (5S,18S-dihydroxy-EPA) derived from EPA have also been identified using chiral LC–MS/MS-based lipidomics (Oh et al. 2011). Importantly, EPA is also an alternative substrate for CYP epoxygenases, which efficiently add oxygen across one of the five double bonds of EPA to generate epoxy fatty acids, in particular into a predominant epoxide form, 17,18-epoxyeicosatetraenoic acid (17,18-EEQ) (Spector and Norris, 2007; Arnold et al., 2010). 17,18-EEQ is chemically stable but metabolically unstable (its predicted in vivo half-live is in the order of seconds) due to its rapid metabolism by the enzyme soluble epoxide hydrolase (sEH), which converts this epoxide into the inactive diol 17,18-

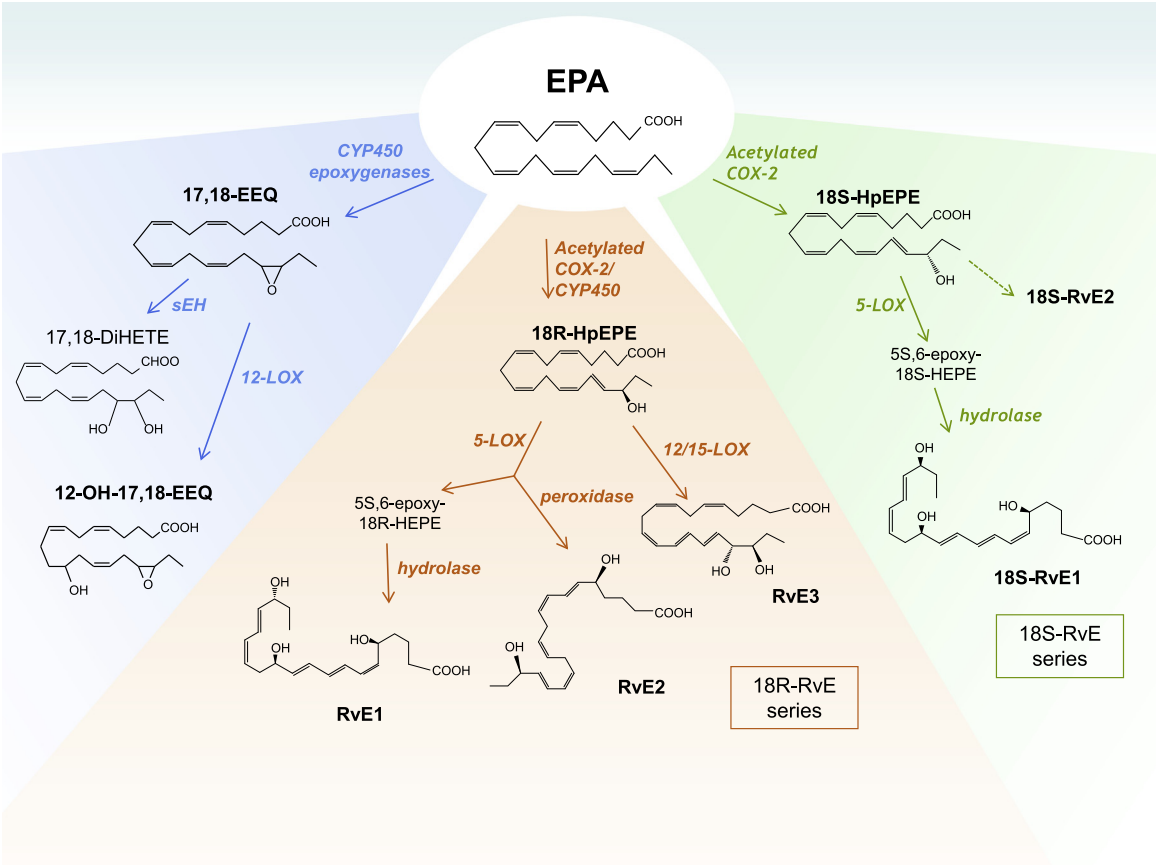

Fig. 1. Biosynthesis of specialized pro-resolving mediators (SPM) from omega-3 eicosapentaenoic acid (EPA). During the resolution process, EPA is converted by aspirin-acetylated cyclooxygenase-2 (COX-2) or cytochrome P450 (CYP450) into 18R-hydro(peroxy)-EPE (18R-HEPE) and subsequently transformed by 5-lipoxygenase (5-LOX) into RvE1 and RvE2. Conversion of 18R- HEPE via the 12/15-LOX pathway gives rise to a novel member of the E-series resolvin family, RvE3. Members of the 18S-RvE series, including 18S-RvE1 and 18S-RvE2 derived from EPA, have also been identified. On the other hand, CYP epoxygenases convert EPA into a predominant epoxide form, 17,18-epoxyeicosatetraenoic acid (17,18-EEQ), which is rapidly metabolized by enzyme soluble epoxide hydrolase (sEH) into the inactive diol 17,18-dihydroxyeicosatetraenoic acid (17,18-DiHETE). Finally, a novel 12-LOX-derived metabolite of 17,18-EEQ, 12-hydroxy-17,18-epoxyeicosatetraenoic acid (12-OH-17,18-EEQ), has been identified.

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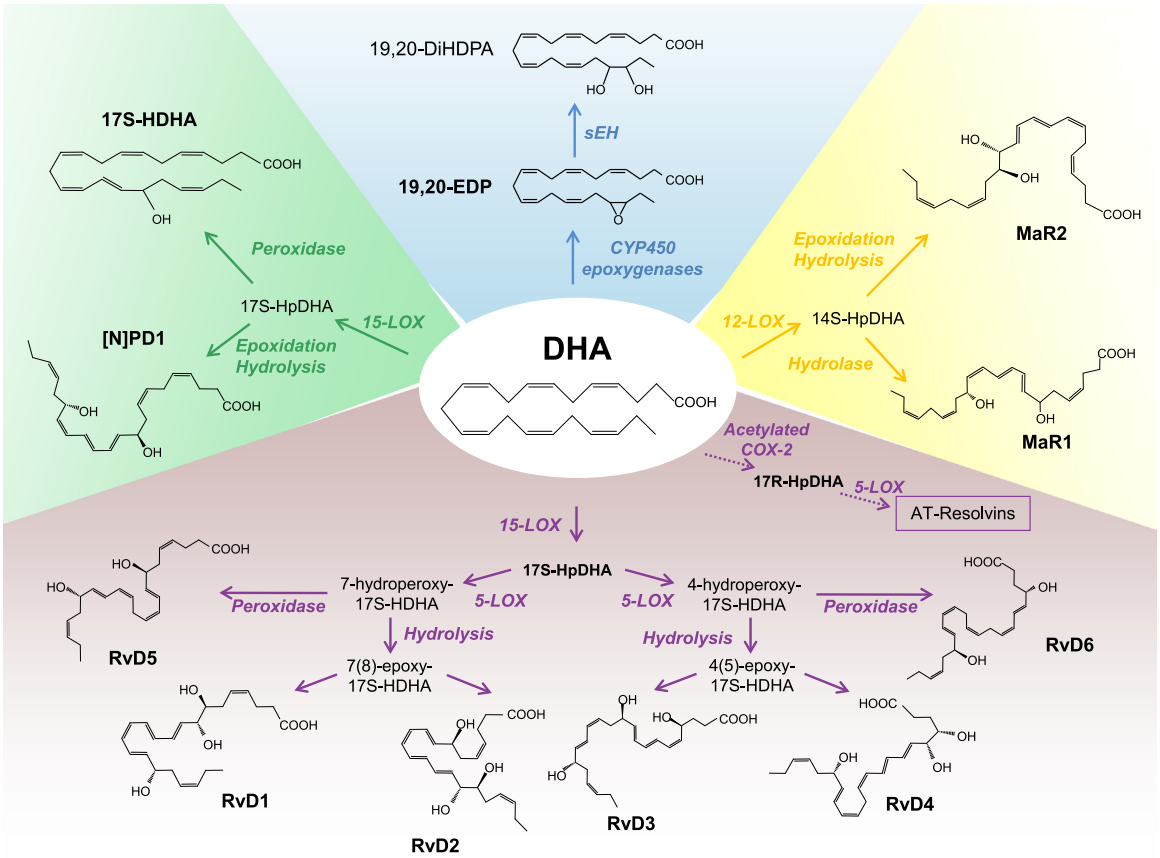

Fig. 2. Biosynthesis of specialized pro-resolving mediators (SPM) from omega-3 docosahexaenoic acid (DHA). During the resolution process, DHA is converted into 17-hydro(peroxy)-DHA (17-HpDHA) by 15-lipoxygenase (15-LOX), which is subsequently transformed by 5-LOX into a hydroperoxide intermediate (7-hydroperoxy-17S HDHA) that is then hydrolyzed into a 7S,8S-epoxide and subsequently converted into either RvD1 or RvD2. Reduction of the 5-LOX derived hydroperoxide intermediate via a peroxidase leads to the formation of RvD5. Alternatively, 5-LOX forms 4-hydroperoxy-17S HDHA from 17-HpDHA, which is subsequently converted to RvD3, RvD4 and RvD6. DHA can also be transformed by aspirin-acetylated COX-2 into 17R-HpDHA, which is further converted by 5-LOX into the corresponding aspirin-triggered (AT)-resolvins. On the other hand, DHA can be transformed by 15-LOX into a dihydroxy-containing derivative termed [neuro]protectin D1 ([N]PD1). In macrophages, lipoxygenation of DHA by 12-LOX gives rise to a 14S-hydro(peroxy)-DHA (14S-HpDHA) intermediate, that can be converted by epoxidation and/or hydrolysis into maresins MaR1 and MaR2. Finally, CYP epoxygenases convert DHA into a predominant epoxide derivative, 19,20-epoxydocosapentaenoic acid (19,20-EDP), which is rapidly metabolized by sEH into the inactive or less active metabolite, 19,20-dihydroxydocosapentaenoic acid (19,20-DiHDPA).

dihydroxyeicosatetraenoic acid (17,18-DiHETE) (Shen, 2010; Morisseau and Hammock, 2013). In addition, a novel 12-LOX-derived metabolite of 17,18-EEQ, 12-hydroxy-17,18-epoxyeicosatetraenoic acid (12-OH-17,18-EEQ), has been identified as one of the major CYP epoxygenase-derived EPA metabolites, which retains anti-inflammatory properties (Kubota et al., 2014).

The biosynthesis of resolvins of the D-series is graphically summarized in Fig. 2. Schematically, the biosynthesis of D-series resolvins is initiated by 15-LOX which transforms DHA into 17*S*-hydro(peroxy)-DHA (17S-H(p)DHA) intermediate, which is further transformed by leukocyte 5-LOX into 7-hydroperoxy-17S-HDHA that is converted into 7S,8S-epoxide and hydrolyzed to either RvD1 (7S,8R,17S-trihydroxy-DHA) or RvD2 (7S,16R,17S-trihydroxy-DHA) (Fig. 2) (reviewed in Serhan (2014)). Reduction of the 5-LOX derived hydroperoxide intermediate via a peroxidase also leads to the formation of RvD5 (7S,17S-dihydroxy-DHA) (Fig. 2). Alternatively, lipoxygenation at the C-4 position by the enzyme 5-LOX forms 4-hydroperoxy-17S HDHA that is subsequently converted to RvD3 (4S,11R,17S-trihydroxy-DHA), RvD4 (4S,5S,17S-trihydroxy-DHA) and RvD6 (4S, 17S-dihydroxy-DHA) (reviewed in Serhan and Petasis (2011)) (Fig. 2). Similar to that described for AA and the biosynthesis of aspirin-triggered lipoxins (Clària and Serhan, 1995), Endothelial cells expressing COX-2 acetylated by aspirin also transform DHA into 17*R*-HDHA which is further converted by 5-LOX into the corresponding 17*R*-RvD1, 17R-RvD2 and other 17R-D resolvins, which are collectively known as aspirin-triggered (AT)-resolvins (Serhan and Petasis, 2011; Serhan, 2014). On the other hand, DHA can be transformed by 15-LOX into a dihydroxy-containing DHA derivative termed protectin D1 (PD1) (10R,17S-dihydroxy-DHA) via an intermediate epoxide that opens via hydrolysis and subsequent rearrangements (Fig. 2) (Serhan et al., 2000). This lipid mediator is also known as neuroprotectin ([N]PD1) when formed in neural tissues (Bazan, 2009). Importantly, in macrophages, lipoxygenation of DHA by 12-LOX gives rise to a 14S-hydroperoxy-DHA intermediate that can be converted into maresins MaR1 (7R,14S-dihydroxy-DHA) and MaR2 (13R,14S-dihydroxy-DHA) by epoxidation and/or hydrolysis (Fig. 2) (Serhan et al., 2009; Deng et al. 2014). Finally, similar to that reported for EPA, the omega-3-fatty acid DHA also serves as an efficient substrate for CYP epoxygenases, which convert this omega-3 fatty acid into a predominant DHA epoxide derivative, 19,20-epoxydocosapentaenoic acid (19,20-EDP) (Arnold et al., 2010). 19,20-EDP is rapidly metabolized by sEH resulting in the inactive or less active metabolite, 19,20-dihydroxydocosapentaenoic acid (19,20-DiHDPA) (Morisseau and Hammock, 2013) (Fig. 2).

The discovery and the structural identification of this novel family of SPM derived from endogenous sources of omega-3 fatty acids has confirmed the existence of previously unappreciated pro-resolving mechanisms, supporting the concept that the resolution phase of acute inflammation is an active signaling process

(Serhan and Savill, 2005). In general terms, initiation of acute inflammation is controlled by a number of autacoids including lipid mediators derived from omega-6-PUFAs, such as prostaglandins and leukotrienes that play key roles in regulating blood flow, endothelial permeability and PMN diapedesis (Samuelsson et al., 1987). These pro-inflammatory mediators, especially LTB_4, also induce transendothelial migration and chemotaxis of PMN towards injured tissue and/or pathogens (Samuelsson et al., 1987). During the whole process of inflammation, there is a temporal shift in lipid mediators from the initiation phase to resolution, indicating that different lipid mediators are generated at different times during the evolution of the inflammatory response and these mediators coincide with distinct cellular traffic and events. For example, while maximal levels of LTB_4 occur as PMN infiltrate tissues, other eicosanoids such as *prostaglandin* E_2 and *prostaglandin* D_2 initiate a switch in the class of lipid mediators, by which endogenous circuits modulate leukocyte responses in such a way that they promote the activation of enzymes required to produce pro-resolving lipid mediators (Levy et al., 2001). Thus, signals that mediate resolution of a contained acute inflammatory response are tightly linked to mediators of the initiation phase, i.e. the beginning programs the end of inflammation (Serhan and Savill, 2005). The majority of pro-resolving SPM are generated via transcellular biosynthesis and their levels peak when macrophages are actively clearing apoptotic PMN (Dalli and Serhan, 2012). Interestingly, macrophage phagocytosis of apoptotic cells also leads to the biosynthesis of pro-resolving lipid mediators, which act in an autocrine manner to facilitate phagocytosis (Dalli and Serhan, 2012). Of interest, there are also temporal relationships between different SPM families and individual mediators produced in response to different pathogens (e.g. viral versus bacterial products), which trigger the production of different SPM in the host (Chiang et al., 2012; Koltsida et al., 2013). This finding suggests that, even within the SPM genus, there are distinct roles for individual SPM and that the complexity of the biosynthesis of these bioactive autacoids is just now beginning to be studied and fully appreciated.

Like other small molecules, SPM evoke stereospecific bioactions mediated in the nanomolar range by their binding to specific G-protein coupled receptors (GPCRs). Systematic receptor screening approaches along with radioligand specific binding resulting from competition studies identified GPCRs activated by SPM. Two receptors (ALX/FPR2 and GPR32) have been shown to transmit RvD1 signals (Krishnamoorthy et al., 2010), whereas a GPCR (ChemR23) signals for RvE1 (Arita et al., 2005). Of interest, GPR32 is also activated by RvD3 and RvD5 (Dalli et al., 2013), while other pro-resolving mediators, including Annexin A1, activate ALX/FPR2 (Norling and Perretti, 2013). NPD1/PD1 displays specific binding to PMN and human epithelial cells in which no other SPM competes for its specific binding, indicating that PD1 actions are likely mediated by separate receptors. Binding studies using specific receptor expression constructs corroborate their potent actions on isolated cell-types, with K_d values in the picomolar–nanomolar range (Serhan and Chiang, 2013). RvE1 is also an endogenous receptor antagonist for the *leukotriene* B_4 receptor, BLT_1, which likely explains its ability to potently regulate PMN trafficking to sites of inflammation (Arita et al., 2007). The endogenous role of these specific GPCRs in transmitting SPM signals has now been elucidated in vivo in mice with transgenic overexpression of ChemR23 and ALX/FPR2, as well as in mice deficient for Fpr2 (the murine isoform of ALX/FPR2), and this has provided insight into the biological role of pro-resolving receptors (Serhan and Chiang, 2013). It should be noted that the substrate precursors of SPM, namely EPA and DHA, do not activate these receptors (Arita et al., 2005; Chiang et al., 2012; Dalli et al., 2013), but rather other receptors (i.e. GPR120), although these sensors are activated by essential omega-3 fatty acids at high micromolar concentrations. Other fatty acids, including monounsaturated fatty acids e.g., palmitoleic acid and oleic acid as well as branched fatty acid esters of hydroxy fatty acids also appear to activate GPR120 (Oh et al., 2010; Yore et al., 2014), and thus, receptor-mediated actions of fatty acids remain to be fully elucidated.

Unlike their precursors EPA and DHA, which act at the micromolar to millimolar range, SPM (i.e. resolvins, protectins and maresins) exert potent actions in the picomolar to nanomolar range (Bannenberg and Serhan, 2010). Indeed, the potency of these SPM is notable, with concentrations as low as 10 nM producing a 50 percent reduction in PMN transmigration (Bannenberg and Serhan, 2010). As the first-ever described omega-3-derived pro-resolving lipid mediators, these SPM were shown to stimulate the active resolution of inflammation and the return of inflamed tissue to homeostasis (Bannenberg and Serhan, 2010). In parallel with full structural elucidation and stereochemical assignment, the biological actions of these SPM were first completed for RvE1. RvE1 was readily shown to decrease PMN infiltration and T cell migration, to reduce TNFα and IFNγ secretion, to inhibit chemokine formation and to block IL-1-induced NF-κB activation (Bannenberg and Serhan, 2010; Schwab et al., 2007). RvE1 was also shown to stimulate macrophage phagocytosis of apoptotic PMN and to be a potent counter-regulator of L-selectin expression (Schwab et al., 2007; Dona et al., 2008). RvE1 displayed potent anti-inflammatory actions *in vivo*, protecting mice against experimental periodontitis, colitis, peritonitis and brain ischemia–reperfusion (Arita et al., 2005; Bannenberg and Serhan, 2010). A RvE1-initiated resolution program for allergic airway response was also identified (Haworth and Levy, 2008). Similarly, RvD1 and RvD2 were reported to reduce inflammatory pain, to block IL-1β transcripts induced by TNFα in microglial cells and to function as potent regulators limiting PMN infiltration into inflamed brain, skin and peritoneum (Sun et al., 2007; Hong et al., 2003). RvD2, in particular, has been shown to be a potent endogenous regulator of excessive inflammatory responses in mice with microbial sepsis (Spite et al., 2009). On the other hand, PD1 has been reported to exert protective actions in acute models of inflammation by blocking PMN migration and infiltration into the inflammatory site (Serhan et al., 2006). Finally, MaR1 displays potent anti-inflammatory and pro-resolving actions inhibiting PMN infiltration *in vivo*, and stereoselectively stimulating macrophage phagocytosis and efferocytosis (Serhan et al., 2009, 2012). MaR1 also exerts potent tissue regenerative and anti-noniceptive actions (Serhan et al., 2012).

A novel aspect in the resolution process is that SPM are able to take advantage of macrophage plasticity by inducing changes in the phenotype of macrophages toward a pro-resolution state. For example, Schif-Zuck et al. (2011) reported that administration of RvD1 or RvE1 to peritonitis-affected mice enhances the appearance of $CD11b^{low}$ macrophages by reducing the number of engulfment-related events required for macrophage deactivation and by reducing the ability of peritoneal macrophages to produce pro-inflammatory cytokines upon LPS stimulation. Another interesting aspect of the pro-resolving actions of SPM is that they pave the way for monocyte differentiation into phagocytosing macrophages, facilitating the removal of dead or dying cells (efferocytosis) as well as bacterial clearance (Schif-Zuck et al., 2011; Serhan and Savill, 2005; Chiang et al., 2012). SPM also regulate PMN apoptosis and stimulate chemokine scavenging during resolution via up-regulation of CCR5 expression on apoptotic PMN and T cells (Ariel et al. 2006). Notably, SPM enhance phagocyte efflux from inflamed tissues to draining lymph nodes to aid in host defense (Schwab et al., 2007). When leukocytes exit the inflamed site or exudate, they traverse perinodal adipose tissue en route to local lymph nodes (Schwab et al., 2007). Excessive and persistent

inflammation during this lipo-passage or failure of leukocytes to reach the lymphatics (Schwab et al., 2007) and hence, getting stuck while activated within adipose, can lead to adipose inflammation that may contribute to the metabolic syndrome (see Section 4, below).

4. Obesity, insulin resistance and NAFLD

Obesity results in expansion of adipose tissue mass that is causally linked to a dysregulated release of pro-inflammatory and insulin-resistant adipokines, accompanied by a reduction in the secretion of the anti-inflammatory and insulin-sensitizing adipokine, adiponectin (Reviewed in Clària et al. (2011)). This altered profile of adipokine secretion leads to insulin resistance in liver and skeletal muscle, which are the major organs contributing to peripheral insulin resistance (Clària et al., 2011). In fact, in obese subjects, increased levels of pro-inflammatory adipokines (i.e., TNFα, IL-6, and MCP-1) are directly correlated with the degree of insulin resistance (Gordon and Taylor, 2005; Ouchi et al., 2011; Hotamisligil et al., 1996). In parallel to the heightened secretion of inflammatory adipokines, there is an activation of the c-jun-N-terminal kinase (JNK) and inhibitor of κ kinase (IKK) pathways and their downstream signaling cascades by stress sensors through classical receptor-mediated mechanisms (Shoelson et al., 2006). In turn, JNK and IKK activation induce insulin resistance by disrupting serine phosphorylation of insulin receptor substrate-1 (IRS-1), a protein that connects the insulin receptor to the phosphoinositide 3-kinase (PI3K) signaling cascade (Shoelson et al., 2006).

Adipose tissue dysfunction leading to insulin resistance also has negative consequences on the liver, since hepatic insulin resistance is a major factor triggering the progression of hepatic steatosis or fatty liver (i.e. NAFLD) (reviewed in Clària et al. (2011)). Although the exact mechanisms linking adipose tissue dysfunction, insulin resistance and NAFLD are not completely delineated, several factors and processes are implicated. First, adipose tissue and liver have immediate access to a common vast network of blood vessels that facilitate a direct connection between these two organs (Sanyal, 2005). Second, increased lipolysis from visceral fat results in increased free fatty acid efflux to the liver (Sanyal, 2005). Third, increased secretion of pro-inflammatory and insulin resistance inducing cytokines (TNFα and IL-6) accompanied by reduced release of adiponectin by adipose tissue has a direct impact on liver cells (Sanyal, 2005). Finally, altered hepatic insulin sensitivity is a driving factor for dysregulated free fatty acid oxidation and de novo lipogenesis, which contribute to the development of NAFLD (Tilg and Moschen, 2008).

The key pathogenic mechanism responsible for dysregulated adipokine secretion leading to insulin resistance and obesity-associated co-morbidities (i.e. NAFLD) is the existence of a chronic "low-grade" inflammatory state in the adipose tissue (Shoelson et al., 2006; Gregor and Hotamisligil, 2011). This "low-grade" inflammation, also known as "metabolic-triggered inflammation" or "metainflammation", can be defined as a long-term inflammatory response triggered by nutrients and metabolic surplus (Shoelson et al., 2006; Gregor and Hotamisligil, 2011). It involves a similar set of molecules/signaling pathways to those involved in classical inflammation, but in obesity-induced inflammation these molecules/signaling pathways have a dual role as inflammatory mediators as well as regulators of energy storage and metabolism (Shoelson et al., 2006; Gregor and Hotamisligil, 2011). A particularity of this obesity-induced chronic state of adipose tissue inflammation is that the inflammatory response is driven by tissue macrophages (Lumeng and Saltiel, 2011). In fact, an increased number of adipose tissue-infiltrating macrophages, which form the characteristic "crown-like structures" surrounding necrotic adipocytes is one of the pathological hallmarks of obesity (Cancello et al., 2005). The presence of these infiltrated macrophages in the inflamed fat perpetuates a vicious cycle of exacerbated production of pro-inflammatory mediators and extensive macrophage recruitment (Weisberg et al., 2003; Wellen and Hotamisligil, 2003; Xu et al., 2003; Lumeng et al., 2007; Chawla et al., 2011; Wentworth et al., 2010).

5. Impaired omega-6/omega-3 balance in obesity and NAFLD

Essential PUFAs have been part of our diet since the beginning of human life. Before the agricultural revolution, human beings evolved consuming a diet that contained small and roughly equal amounts of omega-6 and omega-3 PUFAs (ratio omega-6/omega-3 PUFAs: 1-4:1) (Simopoulos, 1999). However, the current Western diet is very high in omega-6 and the ratio of omega-6/omega-3 PUFAs has risen to up to 20–30:1 (Simopoulos, 1999). Changes in nutrition such as lower consumption of omega-3-PUFA and modern agriculture (animals fed on diets rich in omega-6s) are believed to be the origin of the changes in the human metabolic pattern in Western societies (Simopoulos, 1999).

Because of the increased intake of omega-6-PUFAs in the Western diet, pro-inflammatory eicosanoids generated from AA, specifically prostaglandins, TXA_2 and leukotrienes, are formed in larger quantities than those derived from omega-3-PUFA (Schmitz and Ecker, 2008). In this regard, eicosanoids generated from AA contribute to the formation of thrombi and atheromas, the development of allergic and inflammatory disorders and uncontrolled cell proliferation (Schmitz and Ecker, 2008). Thus, a diet rich in omega-6-PUFAs shifts the physiologic state to one that is prothrombotic and proaggregatory, hyperlipidemic and proinflammatory (Schrör, 1990; Bagga et al., 2003; Levy et al., 2001; Calder, 2006). Indeed, the prevalence of metabolic diseases including type 2 diabetes, hyperlipidemia, hypertension and cardiovascular disease is closely related to the increased omega-6 to omega-3-PUFA ratio found in the human population (reviewed in Simopoulos, 1999). The close association between a high omega-6/omega-3 ratio and the onset of metabolic diseases has contributed to our understanding of the pathogenesis of many diseases and has encouraged the routine profiling of the content of PUFAs in different body tissues. In this regard, in a pioneering lipidomic analysis, Puri et al. (2007) described an altered hepatic lipid composition in patients with NAFLD. This finding was recently refined by our group in a study demonstrating increased omega-6 to omega-3 PUFA ratio in livers from patients with NAFLD (López-Vicario et al., 2014). Similar findings were observed in a mouse model of high-fat-diet (HFD)-induced obesity and NAFLD (López-Vicario et al., 2014). Of interest, when the ingestion of omega-3-PUFA surpasses the intake of omega-6, the content of AA in cell membranes is partially reduced as it is replaced by EPA and DHA (Shaikh and Edidin, 2006). That is why feeding an exogenous omega-3-enriched diet (EPA and DHA, 65/35) is beneficial in HFD-induced obese mice (López-Vicario et al., 2014). The beneficial actions of exogenous omega-3 supplementation were mirrored by transgenic *fat-1* mice expressing an omega-3 fatty acid desaturase from the worm *Caenorhabditis elegans*, which endogenously enriches tissues with omega-3 PUFAs (López-Vicario et al., 2014). In particular, transgenic *fat-1* mice have shown an increased hepatic content of omega-3-PUFAs, and consequently a lower omega-6/omega-3 PUFA ratio, which conferred protection against HFD-induced obesity, hepatic insulin resistance, steatosis, macrophage infiltration, necroinflammation and lipid peroxidation, accompanied by attenuated expression of genes involved in inflammation, fatty acid uptake and lipogenesis (López-Vicario et al., 2014). Similar findings were previously reported by our group in *ob/ob*

mice, a genetic model of obesity (González-Périz et al., 2009). In this study, we provided evidence that dietary intake of omega-3-PUFAs by obese *ob/ob* mice produces anti-steatotic effects associated with improved glucose tolerance and insulin sensitivity and changes in the expression of adipokines, in particular adiponectin (González-Périz et al., 2009). In addition, the hepatoprotective properties exerted by omega-3-PUFA enrichment were also seen in mice receiving a diet enriched in omega-3-PUFAs, which were protected against carbon tetrachloride-induced liver injury and oxidative damage (González-Périz et al., 2006). Overall, experimental studies by our laboratory and others indicate that an inadequate dietary intake of omega-3-PUFAs, and/or an unbalanced omega-6/omega-3 ratio are common findings in obesity and NAFLD (Krebs et al., 2006; Todoric et al., 2006; Nobili et al. 2011; Teague et al., 2013). In summary, these studies support the idea that a balanced omega-6 and omega-3 tissue content is essential for health and highlight the potential benefits of supplementing omega-3-PUFAs in patients with obesity and related hepatic comorbidities.

6. Altered profile of omega-3-derived lipid mediators in unresolved inflamed tissues

Prolonged, unremitting inflammation in adipose tissue has a negative impact on insulin-sensitive tissues; hence, its timely resolution is a critical step toward regaining metabolic balance. Given that balancing the omega-6 to omega-3 PUFAs ratio is a good strategy for the maintenance of human health, it can be hypothesized that unbalanced formation of omega-3-derived lipid mediators is a contributing factor for inflammation-triggered metabolic complications. In other words, the presence of unresolved inflammation could be the consequence of a deregulated balance between the exacerbated levels of pro-inflammatory mediators and the reduced levels of mediators with anti-inflammatory and pro-resolution properties (i.e. SPM). This hypothesis has received support from experimental studies showing a compromised capacity to produce local SPM in obese tissues with a heightened pro-inflammatory phenotype. In this regard, a deficit in tissue SPM levels (RvD1, PD1 and 17-HDHA) has been characterized in inflamed visceral and subcutaneous fat compartments from obese *ob/ob* and obese/diabetic *db/db* mice (Clària et al., 2012; González-Périz et al., 2009; Neuhofer et al., 2013). In humans, a deficit in PD1 and its precursor 17S-HDHA has been reported in subcutaneous fat from patients with peripheral vascular disease in whom the inflammatory status in adipose tissue is remarkably exacerbated compared with healthy subcutaneous fat (Clària et al., 2013). Moreover, LC–MS/MS-based metabolo–lipidomic analyses of fat from selected human anatomic locations have identified unique signature profiles in the content of bioactive lipids (Clària et al., 2013). Importantly, these analyses demonstrated a heterogeneous capacity for SPM biosynthesis among different adipose tissue depots, with higher activation of resolution circuits in perivascular fat compared with subcutaneous fat (Clària et al., 2013). This is relevant for vascular pathologies with perivascular adipose tissue playing an emerging role in vascular biology homeostasis because of its tissue mass and anatomic proximity surrounding systemic vessels.

In the setting of obesity, a deficit in pro-resolving mediators in adipose tissue could be the consequence of a structural deficiency in the tissue content of omega-3-PUFAs as established substrates for SPM biosynthesis. Indeed, there are evidence that SPM are generated in humans taking omega-3 dietary supplements and that SPM levels are increased above those produced normally in transgenic *fat-1* mice (Hudert et al., 2006; Mas et al., 2012). This is consistent with the observation that transgenic restoration of omega-3 fatty acids in *fat-1* mice reversed the inefficient resolution capacity of their obese adipose tissue and produced anti-inflammatory and pro-resolution actions in adipose and liver tissues (White et al., 2010; López-Vicario et al., 2015). Alternatively, the loss of SPM in obesity may reflect accelerated tissue SPM conversion and clearance to inactive further metabolites because 15-PG-dehydrogenase/eicosanoid oxidoreductase, the key enzyme in SPM inactivation, is markedly up-regulated in obese adipose tissue (Clària et al., 2012). In this tissue, omega-3-derived SPM are readily metabolically converted to oxo-resolvin products, some of which are biologically inactive (Clària et al., 2012). In addition, sEH, the enzyme that catalyzes the rapid hydrolysis of omega-6 and omega-3 epoxides by converting them into inactive or less active diols, is invariably overexpressed in HFD-induced obese mice (López-Vicario et al. 2015). It is noteworthy that SPM deficiencies in obesity appear to be a generalized defect in all metabolic tissues, because in addition to adipose, deficiencies are noted in the liver, cutaneous wounds and skeletal muscle (White et al., 2010; Tang et al., 2013). Collectively, these findings are consistent with the notion that unresolved chronic "low grade" inflammation in obese adipose tissue is the result of an inappropriate SPM resolution-capacity allowing the inflammatory response to proceed without controlled checkpoints.

The role of SPM in adipose tissue homeostasis has been investigated by the administration of synthetic SPM in several *in vivo*, *ex vivo*, and *in vitro* studies. In this regard, the administration of nanogram doses of RvD1 to obese/diabetic *db/db* mice improves glucose tolerance, decreases fasting blood glucose, and increases insulin-stimulated Akt phosphorylation in adipose tissue (Hellmann et al., 2011). This SPM also reduces the formation of macrophage-containing crown-like structures in adipose tissue (Hellmann et al., 2011). Similarly, intraperitoneal injection (nanogram amounts) of RvE1 to obese *ob/ob* mice confers significant insulin-sensitizing effects by mechanisms related to the AMPK-adiponectin axis and the induction of GLUT-4 and IRS-1 expression (González-Périz et al., 2009). In addition, 17S-HDHA treatment (intraperitoneal injection of nanogram doses) reduces adipose tissue expression of inflammatory cytokines (MCP-1, TNFα, IL-6 and osteopontin), increases adiponectin expression and improves glucose tolerance in parallel to insulin sensitivity in obese/diabetic *db/db* mice (Neuhofer et al., 2013). *Ex vivo*, in fat explants, both RvD1 and RvD2 rescue the impaired phenotype of obese adipose tissue by enhancing the expression and secretion of adiponectin in parallel with decreasing the secretion of pro-inflammatory adipokines/cytokines including leptin, TNFα, IL-6 and IL-1β (Clària J et al., 2012). *In vitro*, at nanomolar concentrations, RvD1 stimulates macrophage nonphlogistic phagocytosis, a critical step in the resolution of the inflammatory process, and enhances the phagocytic activity of macrophages isolated from the adipose tissue stromal vascular cell fraction (Titos et al., 2011). In human monocyte-adipocyte co-incubations, both RvD1 and RvD2 reduce MCP-1 and *leukotriene* B_4-stimulated monocyte adhesion to adipocytes as well as monocyte transadipose migration, which are two critical processes in the recruitment of monocytes/macrophages into the inflamed adipose tissue (Clària et al., 2012). In addition to reducing macrophage recruitment, RvD1 skews polarization of adipose tissue macrophages from a classical activation inflammatory profile (M1 phenotype) toward an alternatively anti-inflammatory M2-like state (Titos et al., 2011). This phenotypic switch is characterized by a reduction of secreted pro-inflammatory adipokines, such as TNFα and IL-6 accompanied by an up-regulation of a complete panel of M2 markers including IL-10, CD206, RELM-α and Ym1 (Titos et al., 2011). In addition, RvD1 remarkably increased arginase-1 expression, a well established M2 marker, while attenuating IFNγ/LPS-induced Th1 cytokine secretion (Titos et al., 2011). Of particular interest, changes in the

expression of M1/M2 markers appeared to be confined to the adipose tissue stromal vascular fraction, which is highly populated by macrophages (Titos et al., 2011). These results are in agreement with those reported by Hellmann et al. (2011), who showed the ability of RvD1 to improve insulin resistance in obese-diabetic mice by reducing macrophage F4/80$^+$CD11c$^+$ cell accumulation and increasing the percentage of *F4/80 positive* cells expressing the M2 marker Mgl-1 in adipose tissue. The switch of recruited macrophages toward a M2 phenotype is interpreted as anti-inflammatory and pro-resolving (Olefsky and Glass, 2010).

In addition to adipose tissue, we have recently demonstrated that RvD1 also acts as a facilitator of resolution of inflammation in the liver (Rius et al., 2014). In this study, RvD1 was shown to stimulate macrophage polarization toward the pro-resolving M2 phenotype, which facilitated the return of the hepatic tissue to homeostasis during calorie restriction in a murine model of obesity-induced NAFLD. On the other hand, RvE1 was shown to reduce hepatic triglyceride accumulation and macrophage infiltration in obese *ob/ob* mice (González-Périz et al., 2009). Moreover, using precision-cut liver slices, which override the influence of circulating factors, RvD1 was shown to attenuate hypoxia-induced mRNA and protein expression of inflammatory genes, namely COX-2, IL-1β, IL-6 and CCR7 (Rius et al., 2014). Of interest, the anti-inflammatory actions of RvD1 were completely absent in tissue slices in which macrophages were depleted with chlodronate liposomes (Rius et al., 2014), a finding that highlights the critical role of macrophages in the resolution phase of inflammatory response. A salient feature of this study was the identification of a specific microRNA signature (i.e., miR-219-5p and miR-199a-5p, which have putative targets in genes of the cytokine network and the innate immune response) in the liver in response to RvD1 (Rius et al., 2014). These findings are in accordance with a previous study demonstrating a specific microRNA signature (i.e. miR-21, miR-146b, miR-208a, miR-203, miR-142, miR-302d, and miR-219) for RvD1 in self-limited, self-resolving, inflammatory exudates from a murine model of peritonitis (Recchiuti et al., 2011). Finally, in a very elegant study in humans and rodents with NAFLD, the anti-inflammatory and insulin-sensitizing adipokine, adiponectin, was shown to rescue the reduced expression of the receptor for RvE1, ChemR23, in hepatocytes (Wanninger et al., 2012). A summary of the most relevant actions of RvD1 and RvE1 on adipose tissue and liver macrophages is illustrated in Fig. 3.

A common feature of the SPM described so far is that their biosynthesis from omega-3-PUFAs is initiated through the interaction of the LOX and COX pathways, the two classical branches of PUFA metabolism (Serhan, 2014). However, CYP epoxygenases, the so-called third branch of PUFA metabolism, can also convert EPA and DHA into novel epoxides, which exert salutary actions and are even more effective and potent than omega-6-derived EETs (Arnold et al., 2010; Zhang et al., 2013; Ulu et al., 2014; Morisseau et al., 2010; Falck et al., 2011). In a recent study, we used *fat-1* mice as an optimal model of omega-3 tissue enrichment, in which the stabilization of CYP-derived epoxides by a sEH inhibitor reinforced the omega-3-dependent reduction in hepatic inflammation and intrahepatic lipid deposition (López-Vicario et al., 2015). Data from this study also uncovered the ability of a sEH inhibitor to stabilize the levels of 19,20-EDP epoxide and to restore autophagy in the liver with the subsequent reduction of obesity-induced liver ER stress (see Fig. 3). Autophagy is a cellular degradation process by which toxic aggregate-prone proteins and damaged organelles are cleared from the cytoplasm (Scheider and Cuervo, 2014). Defective autophagy contributes to a variety of diseases since efficient sequestration and clearance of damaged cellular components in stress conditions is crucial for cell homeostasis (Scheider and Cuervo, 2014). Therefore, our observations highlight the potential of small bioactive lipid mediators to modulate autophagy, serving as templates for the exploitation of this cellular housekeeping process in therapeutic interventions against obesity and obesity-related co-morbidities.

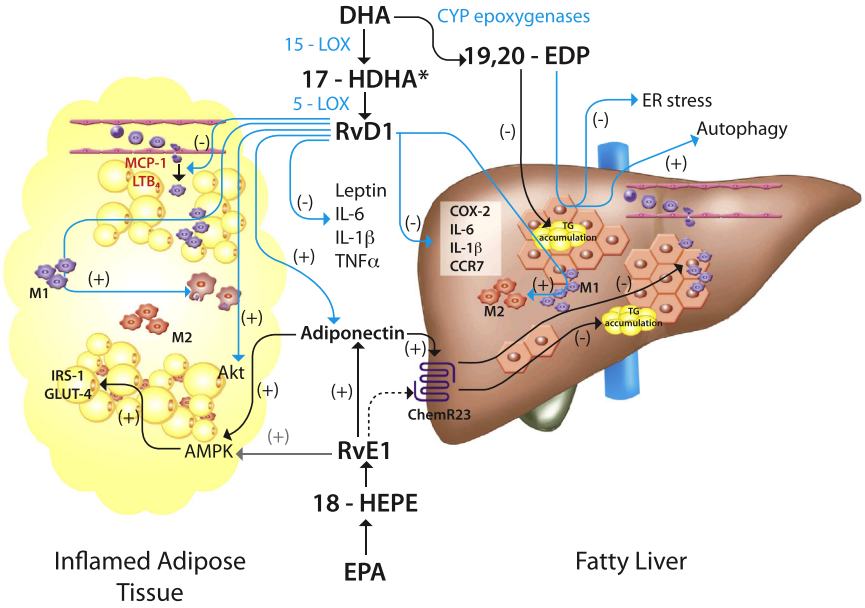

Fig. 3. Schematic diagram summarizing the actions of omega-3-derived SPM on inflamed adipose tissue and fatty liver. DHA is converted into 17-hydroxy-DHA (17-HDHA) by 15-lipoxygenase (15-LOX) and subsequently transformed into RvD1 by 5-LOX. Left panel. In inflamed adipose tissue RvD1 inhibits monocyte chemoattractant protein-1 (MCP-1) and leukotriene B_4 (LTB_4)-induced transadipose monocyte adhesion and migration, promotes polarization of macrophages into the anti-inflammatory M2 phenotype and stimulates efferocytosis. RvD1 activates protein kinase B, also known as Akt, and inhibits adipose tissue secretion of leptin, interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF) α, while stimulating the release of adiponectin. RvE1, generated from EPA-derived 18-hydro-EPE (18-HEPE), up-regulates adiponectin, AMP-activated protein kinase (AMPK), insulin receptor signaling 1 (IRS-1) and glucose transporter-4 (GLUT-4). Left panel In fatty liver, RvD1 down-regulates cyclooxygenase-2 (COX-2), IL-6, IL-1β and C-C chemokine receptor type 7 (CCR7) and induces anti-inflammatory M2 macrophage polarization. After binding to its receptor (i.e. ChemR23; which is up-regulated by adiponectin), RvE1 inhibits both triglyceride (TG) accumulation in hepatocytes and macrophage infiltration. Finally, the cytochrome P450 (CYP) epoxygenase-derived DHA metabolite, 19,20-EDP, reduces TG accumulation while restoring autophagy in hepatocytes, with the subsequent reduction of endoplasmic reticulum (ER) stress in these liver cells. *Most of the above reported actions are also reproduced by 17-HDHA.

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7. Clinical implications and future directions

This review article describes state-of-the-art knowledge on the biosynthetic and signaling pathways and biological actions of the recently described SPM (i.e. resolvins, protectins and maresins) derived from omega-3-PUFAs. This review also provides novel insight into the role of these SPM as potent immunoresolvents that may work as endogenous "stop signals" associated with the resolution of the chronic "low-grade" inflammatory state present in obesity and related metabolic syndrome. In particular, this review summarizes the current evidence indicating that the persistent inflammation in adipose tissue in obese individuals is likely the consequence of a failure of resolution capacity secondary to impaired SPM levels that prevent the return of the fat tissue to homeostasis. The loss of SPM contributing to obesity-linked inflammation is likely the result of the lack of the intrinsic capacity of adipose tissue to generate appropriate endogenous "stop signals" and pro-resolving mediators for catabasis and the return to complete resolution. This deficit in SPM in obese adipose tissue appears to be a likely consequence of the structural deficiency in the tissue content of omega-3 fatty acids, namely DHA and EPA, as substrates of SPM biosynthesis. Therefore the use of dietary supplements enriched in omega-3-PUFAs, which favor the formation of endogenous anti-inflammatory and pro-resolving SPM in clinical practice, is more than justified (Naqvi et al.,2014; Scorletti et al., 2014). In this context, the supplementation of highly pure EPA and DHA to patients with NAFLD within a balanced nutritional guide is rapidly becoming an effective dietary intervention to prevent the hepatic complications associated with obesity and the metabolic syndrome (Scorletti et al., 2014).

Another optimal strategy to reduce inflammation and to promote the timely resolution of inflamed adipose tissue, thereby, preventing obesity-associated co-morbidities, would be the exogenous administration of a functional set of SPM. The first human clinical trials evaluating oral resolvin therapeutic analogs and mimetics are currently underway for ocular disorders and these resolution agonists are gaining momentum for improving several clinically-relevant outcomes in inflammatory diseases. This review posits that resolution agonists may represent a novel pharmacologic genus that is distinct from traditional anti-inflammatory therapies which impair host-defense and have potential side effects. In this regard, a major advantage of replacing conventional anti-inflammatory drugs by endogenously-generated SPM is that these mediators expedite the resolution process and terminate inflammatory response by promoting macrophage efflux into the lymph nodes. In summary, current evidence suggest that enhancing local SPM production in adipose tissue or exogenously administering these resolution agonists could reduce the inflammatory tone of obese adipose tissue, thus, facilitating the prevention of the adverse effects of the metabolic syndrome. Obesity-induced adipose tissue inflammation and associated metabolic disorders appear to be the perfect scenario for testing these novel omega-3-derived pro-resolving lipid mediators.

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Review

Role of bioactive lipid mediators in obese adipose tissue inflammation and endocrine dysfunction

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ABSTRACT

White adipose tissue is recognized as an active endocrine organ implicated in the maintenance of metabolic homeostasis. However, adipose tissue function, which has a crucial role in the development of obesity-related comorbidities including insulin resistance and non-alcoholic fatty liver disease, is dysregulated in obese individuals. This review explores the physiological functions and molecular actions of bioactive lipids biosynthesized in adipose tissue including sphingolipids and phospholipids, and in particular fatty acids derived from phospholipids of the cell membrane. Special emphasis is given to polyunsaturated fatty acids of the omega-6 and omega-3 families and their conversion to bioactive lipid mediators through the cyclooxygenase and lipoxygenase pathways. The participation of omega-3-derived lipid autacoids in the resolution of adipose tissue inflammation and in the prevention of obesity-associated hepatic complications is also thoroughly discussed.

Contents

Abbreviations: BAT, brown adipose tissue; COX, cyclooxygenase; CYP, cytochrome P450; DHA, docosahexaenoic acid; EDPs, epoxydocosapentaenoic acids; EEQs, epoxyeicosatetraenoic acids; EETs, epoxyeicosatrienoic acids; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; FFA, free fatty acid; HFD, high-fat diet; HSL, hormone sensitive lipase; IL, interleukin; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LD, lipid droplet; LOX, lipoxygenase; LT, leukotriene; LX, lipoxin; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PG, prostaglandin; PUFA, polyunsaturated fatty acids; sEH, soluble epoxide hydrolase; TAG, triacylglycerides; TNFα, tumor necrosis factor α; WAT, white adipose tissue.

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1. Introduction

White adipose tissue is now well recognized as a highly active metabolic tissue and an important endocrine organ that plays a major role in balancing the homeostasis of our body. Unfortunately, this balance is lost in obese individuals in whom the excessive expansion of adipose tissue gives rise to a chronic state of "low-grade" inflammation. This unresolved inflammation of adipose tissue in obesity is deleterious and leads to many pathological sequelae including insulin resistance and type 2 diabetes, hypertension, dyslipidemia and non-alcoholic fatty liver disease (NAFLD).

Bioactive lipids play a major role in the inflammatory process. Among the different lipid mediators, polyunsaturated fatty acids and especially the essential omega-6 arachidonic acid are the prime precursors for the biosynthesis of inflammatory mediators, generically known as eicosanoids (from the Greek *eicosa* = twenty; for 20-carbon fatty acid derivatives). Arachidonic acid is primarily found esterified in the 2-acyl position of phospholipids in all mammalian cell membranes. The intracellular levels of unesterified arachidonic acid are remarkably low and in its free form this fatty acid is readily available as a substrate for the intracellular biosynthesis of eicosanoids. With the exception of lipoxins, the majority of eicosanoids have pro-inflammatory properties. In contrast, another family of essential polyunsaturated fatty acids, the omega-3 family, is linked to the biosynthesis of lipid mediators with anti-inflammatory properties. Among the different lipid mediators generated from the omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), resolvins, protectins and maresins have attracted much attention in recent years because they act as 'braking signals' of the persistent vicious cycle leading to unremitting inflammation. An important aspect of these endogenous omega-3-derived lipid autacoids is their critical role in the dynamic resolution of tissue inflammation.

The aim of this review is to highlight the role of bioactive lipids as key protagonists of the intracellular and intercellular communication networks in white adipose tissue (WAT). Special emphasis is placed on the novel opportunities offered by omega-3-derived lipid mediators to prevent the "low-grade" state of mild inflammation present in adipose tissue of obese individuals. This review also covers different aspects of adipose tissue physiology and pathophysiology, including the metabolic consequences of adipose tissue expansion in obese subjects, the structural and storage lipid composition of adipose tissue and a detailed enumeration of the principal and most common bioactive lipids present in adipose tissue and their biosynthesis and actions on adipocytes and other insulin-sensitive cells.

2. Expansion of adipose tissue in obesity: metabolic consequences

WAT is an anatomical term for loose connective tissue composed of adipocytes or fat cells. Adipocytes are nucleated cells comprising a characteristic unilocular lipid droplet mainly composed of triglycerides (TAG) and cholesterol esters, which occupy most of the cell, and a thin rim of cytoplasm displaced to the periphery (Redinger, 2009). The physical adaptability and the storage capacity of adipocytes are key components of their function. Indeed, during times in which energy intake is higher than the metabolic demand, adipocytes can expand nearly 1000-fold in volume and 10-fold in diameter in order to store the excess of fuel as TAG (Redinger, 2009). In contrast, in periods of food restriction or in periods demanding more energy expenditure, adipose tissue serves, via lipolysis, as the major source of energy. Under starving conditions, lipolysis is an essential mechanism whereby rate-limiting enzymes such as hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) catalyze the hydrolysis of TAG to release free fatty acids (FFA) into the circulation (Carmen and Victor, 2006). Circulating FFA are subsequently taken up via the fatty acid binding protein (FABP) and fatty acid translocase (FAT/CD36) by metabolically active and insulin-sensitive tissues (primarily skeletal muscle and liver). These tissues use FFA as substrates for the generation of the high-energy nucleotide adenosine triphosphate (ATP) (Redinger, 2009).

The expansion of WAT occurring in obese individuals leads to prevailing high levels of hypoxia and chronic inflammation in this tissue. This inflammation is described as "metainflammation" and is characterized by a "low-grade", "long-term" inflammatory res ponse triggered by nutrients and metabolic surplus (Hotamis ligil, 2006). It involves the rise in pro-inflammatory cytokines (i.e. tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1)) and adipokines (i.e. leptin and resistin) (Ouchi et al., 2011). In parallel, a reduction in anti-inflammatory and insulin-sensitizing adipokine adiponectin signals the onset of metabolic dysfunction in obese individuals (Ouchi et al., 2011). Among the metabolic consequences of this persistent state of inflammation insulin resistance leading to type-2 diabetes and hepatic steatosis leading to NAFLD are the most clinically relevant (Hotamisligil, 2006; Ouchi et al., 2011).

2.1. Insulin resistance and type 2 diabetes

Insulin resistance is one of the most important sequelae of obesity. Insulin resistance is defined as a reduced response of target tissues, such as the skeletal muscle, liver, and adipose tissue, to insulin, compared with subjects with normal glucose tolerance without a family history of diabetes (DeFronzo and Tripathy, 2009). Although skeletal muscle is the predominant site of insulin-mediated glucose uptake in the postprandial state, adipose tissue plays a major role in the development of peripheral insulin resistance. In fact, in obese subjects, the degree of insulin resistance is directly correlated with the serum levels of pro-inflammatory adipokines (i.e. TNFα, IL-6, and MCP-1) (Ouchi et al., 2011; de Luca and Olefsky, 2008). In parallel to the heightened secretion of inflammatory adipokines, there is an activation of the c-jun-N-terminal kinase (JNK) and inhibitor of κ kinase (IKK) pathways and their downstream signaling cascades by stress sensors through classical receptor-mediated mechanisms (Shoelson et al., 2006).

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JNK and IKK activation in turn induces insulin resistance by disrupting tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1), a protein that connects the insulin receptor to the phosphoinositide 3-kinase (PI3K) signaling cascade (DeFronzo and Tripathy, 2009). In addition, the increased release of FFA by obese adipose tissue is a major determinant of impaired insulin-stimulated glucose uptake into muscle (Ebbert and Jensen, 2013).

2.2. Hepatic steatosis and NAFLD

Dysregulated adipose tissue function also has negative consequences in the liver. In fact, adipose tissue and the liver have immediate access to a vast network of blood vessels that implicate direct connection between these two organs. This connection is exemplified by the observation that NAFLD is one of the major metabolic consequences of obesity (Angulo, 2002; Sanyal, 2005). NAFLD is a condition ranging from simple accumulation of TAG in the cytoplasm of hepatocytes (steatosis or fatty liver) to steatosis combined with inflammation (steatohepatitis or NASH) (Angulo, 2002; Sanyal, 2005). Although generally asymptomatic, hepatic steatosis is no longer regarded as a neutral bystander, but rather as a pre-morbid condition that increases the vulnerability of this organ to progress to steatohepatitis and to more severe forms of liver damage (Angulo, 2002; Sanyal, 2005). Indeed, steatotic livers are more susceptible to the tissue-damaging effects of oxidative stress and inflammatory mediators, and transition to steatohepatitis represents a critical step in the progression to hepatic fibrosis and cirrhosis (Angulo, 2002; Sanyal, 2005). Although the exact mechanisms linking adipose tissue dysfunction and NAFLD have not been completely delineated, the exacerbated secretion of FFA and pro-inflammatory and insulin resistant adipokines (TNFα and IL-6) accompanied by reduced release of adiponectin by adipose tissue has a direct impact on liver cells (Angulo, 2002; Sanyal, 2005). Moreover, altered hepatic insulin sensitivity is a driving force for impaired hepatic FFA oxidation and *de novo* lipogenesis, which also contribute to the development of NAFLD (Tilg and Moschen, 2008).

3. Composition of adipose tissue in structural and storage lipids

The lipid composition of adipose tissue is strongly dependent on the diet. Differences in adipose tissue composition reported between racial groups, infant vs. adult and gender rapidly disappear when a diet of similar fatty acid composition is consumed (Damsgaard et al., 2013; Field et al., 1985). This section describes the lipid composition of fat cells categorized as structural lipids (i.e. lipids located within the cell membranes) or storage lipids (located within the unilocular lipid droplet).

3.1. Cell membranes

As with any other type of cell in the body, adipocytes are bound by a plasma membrane composed of carbohydrates, proteins and especially lipids. The carbohydrates in the cell membranes appear on the outside bound to lipids (glycolipids, such as cerebrosides and gangliosides) or to proteins (glycoproteins) forming the glycocalix that provides adhesion properties to the cell and participates in lymphocyte homing (Dejana et al., 1994; Ekyalongo et al., 2015). Apart from their structural and biophysical membrane functions, membrane lipids bound to carbohydrates have no bioactive effects on the cell, and therefore are not discussed in this review. Proteins are the second most abundant component of the cell after lipids. They can be transmembrane proteins with an extracellular part that interacts with the extracellular environment and an intracellular domain interacting with signal transducers or with cytosolic proteins in the cytoplasmic layer of the membrane (Harvey Lodish et al., 2000). There are also proteins that temporally interact with the membrane, including phospholipases (PL) A1 and A2 (PLA1 and PLA2) that release fatty acids in the SN1 and SN2 positions from the phospholipids; PLC, which releases diacylglycerol (DAG) from phospholipids; and sphingomyelinase (SMase), which hydrolyzes sphingomyelin to ceramide (see Section 4) (Balsinde and Dennis, 1997).

Phospholipids are the most abundant lipid components of the cell membrane. Phospholipids are amphipathic molecules containing both hydrophilic and hydrophobic moieties. For example, phosphoglycerides or glycerophospholipids are composed of a glycerol backbone with two fatty acids esterified to the SN1 and SN2 positions, and a phosphate group bound to the third hydroxyl group. This phosphate group is esterified to another hydroxyl group on another hydrophilic compound, such as choline, ethanolamine, serine or inositol, forming different phospholipids with unique properties (Balsinde et al., 1997). The fatty acids in glycerophospholipids can be saturated or unsaturated, and within these either mono or polyunsaturated, or a combination of both. Phospholipids are the most important precursors of fatty acids released intracellularly upon the action of PLA1 and PLA2. FFAs within the cytoplasm are toxic and are rapidly converted into biologically active lipid mediators by lipoxygenases and cyclooxygenases. The different derivatives and biological actions of these lipid mediators are extensively discussed in Section 4 of this review. On the other hand, sphingolipids such as sphingomyelin are formed by ceramide and phosphocoline or phosphoethanolamine that lacks the glycerol backbone. In this case, lipids are bound to a single sugar residue or to an oligosaccharide forming cerebrosides or gangliosides, respectively (Kolter et al., 1999). SMase activity hydrolyzes sphingomyelin into ceramide, sphingosine and phosphorylcoline (See Section 4.6 for more details). Finally, sterols are also lipid components of the cell membranes. Sterols are composed of a four-ring hydrocarbon structure with different groups at the two ends of the structure (Cantafora and Blotta, 1996). The most common sterol is cholesterol, which has a hydroxyl substituent on one end of the ring and a six-carbon tail with two methyl groups at positions 1 and 5 in the other. Membrane cholesterol may exert some biological activity since this lipid can post-translationally modify hedgehog signaling (Alcedo and Noll, 1997; Beckers et al., 2007).

3.2. Lipid droplets

Lipid droplets (LD) (also called lipid bodies, oil bodies or adiposomes) are considered a *bona fide* organelle present in almost all vertebrate cells, some plant cells and several yeasts and prokaryotes (Waltermann and Steinbuchel, 2005; Murphy, 2001). LD are composed of a hydrophobic core, mainly consisting of neutral lipids such as TAG and cholesterol esters surrounded by a phospholipid monolayer (as opposed to the normal bilayer of the other organelles) (Fujimoto and Parton, 2011). The hydrophilic phosphate group of phospholipids faces the aqueous cytoplasmic space, whereas the hydrophobic acylic tails are towards the inner part of the LD in contact with lipids accumulated in its core (Thiam et al., 2013). Several proteins are associated with LD, giving structural stability to its phospholipid monolayer. These proteins are implicated with lipid metabolism and signaling (Brasaemle, 2007). Interestingly, some proteins that are normally associated with the cytoplasm have been identified to be located in the LD core; but how their structure or function remains active in such a hydrophobic environment is unclear (Robenek et al., 2005). The major role of LD is the storage of lipids and cholesterol for energy purposes and for the formation and renewal of membranes (Thiam et al., 2013). LD can also participate in the inflammatory response

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and are potentially involved in the pathogenesis of metabolic disorders such as obesity and atherosclerosis (Greenberg et al., 2011; Bozza and Viola, 2010; Krahmer et al., 2013).

The most widely accepted model of LD formation establishes the cellular origin in the endoplasmic reticulum (ER), where the enzymes catalyzing the end-steps of neutral lipid biosynthesis are located (Murphy, 2001; Buhman et al., 2001; Martin and Parton, 2006; Robenek et al., 2004). In the ER, nascent neutral lipids accumulate in the leaflets of the ER membrane forming a protuberance at the outer leaflet of the membrane that closes forming the LD surrounded by a phospholipid monolayer serving as an emulsifying agent (Thiam et al., 2013). Although the exact mechanisms are not yet clear, it is likely that several proteins contribute to the stabilization and release of the nascent LD. Among them, it has been proposed that members of the perilipin family (or PAT family) including perilipin, adipophilin and TIP47 control LD lipolysis and stabilization. Perilipin 1 (Plin1) was the first member of the PAT family described and is the best characterized. Upon formation, Plin1 is recruited to LD where it has a half-life exceeding 70 h under basal non-lipolytic conditions (Kovsan et al., 2007). Plin1 activates fat-specific protein 27 (Fsp27, or CIDEC) which promotes droplet fusion, emptying small LD into large droplets (Gong et al., 2011). Another protein of the PAT family that is present in the nascent LD after budding is TIP47 (or perilipin 3) (Wolins et al., 2005, 2003), which is recruited to the nascent LD on the ER surface when cells are incubated with fatty acids (Skinner et al., 2009). As the droplet starts growing, it migrates from the ER to the center of the adipocyte gradually losing TIP47 content while gaining adipophilin (Wolins et al., 2005). Adipophilin (or perilipin 2) is only stable while in contact with the LD, and it is rapidly degraded by the proteasome in the cytoplasm (Gross et al., 2006; Masuda et al., 2006; Xu et al., 2005). As the LD grows further, perilipin 1 replaces the other proteins in such a way that the mature adipocyte unilocular LD is coated almost exclusively by perilipin 1 (Wolins et al., 2005).

In the cell, LD store TAG and cholesteryl esters, which are hydrolyzed when cholesterol or fatty acid levels are depleted. Cholesterol is released for the production of steroid hormones and for renewal of the cellular membrane (Hu et al., 2010). On the other hand, fatty acids serve either as scaffolds of the membrane phospholipids, for energy production through β-oxidation or for the biosynthesis of bioactive lipid mediators. HSL is the first and best characterized enzyme involved in TAG hydrolysis (lipolysis) (Holm et al., 1988). HSL is highly expressed in adipocytes and exerts hydrolytic activity over TAG, diacylglycerol (DAG) and cholesteryl and retinol esters (Fredrikson et al., 1981; Pittman et al., 1975; Wei et al., 1997). For decades HSL was thought to be the major adipocyte lipase until some studies unexpectedly revealed that HSL null mice retain TAG lipase activity with accumulation of DAG, suggesting that other lipases are implicated in TAG lipolysis (Haemmerle et al., 2002; Osuga et al., 2000; Wang et al., 2001). This controversy was solved when adipose TAG lipase (ATGL) was later identified (Jenkins et al., 2004; Zimmermann et al., 2004). ATGL hydrolyzes TAG to DAG releasing a FFA, whereas HSL hydrolyzes DAG to monoacylglycerol (MAG) again releasing another fatty acid. Finally, another enzyme acting downstream and designated MAG lipase converts MAG into a fatty acid and glycerol (Yang et al., 2010). Plin1 and PKA appear at the center of this process regulating the activity of both HSL and ATGL either directly or indirectly. PKA-mediated phosphorylation of HSL induces the docking of HSL to the LD monolayer in close association with Plin1, thus favoring lipolysis (Sztalryd et al., 2003; Granneman et al., 2007). Under basal conditions, Plin1 is associated with CGI-58 on the LD surface, and when Plin1 is phosphorylated by PKA, CGI-58 is released and can interact with ATGL, thereby increasing its activity (Lass et al., 2006). Fsp27 also plays a double role in the regulation of ATGL activity: in the LD it inhibits lipolysis by binding to ATGL (Grahn et al., 2014), whereas in the nucleus it potentiates Erg1-negative regulation of the ATGL promoter (Singh et al., 2014).

The increased release of FFA from LD by TAG lipolysis in obese adipose tissue contributes to a great extent to the development of insulin resistance. For example, a reduction in Plin1 expression is associated with an increased rate of lipolysis that promotes systemic insulin resistance (Greenberg et al., 2011). In this regard, adipocytes from Plin1-null mice have an increased rate of lipolysis, and despite these mice being leaner than wild-type mice, they develop insulin resistance with aging (Tansey et al., 2001; Martinez-Botas et al., 2000). In humans, three individuals carrying a missense heterozygous mutation in the Plin1 gene (*PLIN1*) showed partial lipodystrophy, with loss of adipose tissue, along with insulin-resistant diabetes, hypertriglyceridemia and hepatic steatosis (Gandotra et al., 2011).

In addition to Plin1, knockdown of FSP27 expression increases the rate of basal lipolysis, an effect that is reversed by overexpressing FSP27 (Puri et al., 2007; Keller et al., 2008; Liu et al., 2009; Nordstrom et al., 2005; Ranjit et al., 2011; Kim et al., 2008a). Moreover, FSP27-null mice have a similar phenotype to that of Plin1-null mice, having reduced fat mass and increased lipolysis (Nishino et al., 2008). In humans, a homozygous nonsense mutation in FSP27 has been reported in a patient with lipodystrophy, insulin-resistant diabetes, hypertriglyceridemia, and hepatic steatosis. Another two proteins (seipin and caveolin-1) related to LD biology are involved in the development of lipodystrophy. Mutations in the seipin gene reduce TAG storage capacity in adipocytes, with a nearly complete absence of adipose tissue and with insulin resistance, diabetes, hypertriglyceridemia and hepatic steatosis (Magre et al., 2001). Similar features have been reported in a patient with a homozygous nonsense mutation in the gene coding for caveolin-1, which is part of the LD proteome (Kim et al., 2008b).

It has been proposed that LD have a role in the production of inflammatory and anti-inflammatory lipid mediators (Bozza and Viola, 2010; Dichlberger et al., 2013). In this regard, the number of LD has been shown to increase during the interaction of macrophages with pathogens, such as parasites (Melo et al., 2003), bacteria (Peyron et al., 2008; Daniel et al., 2011; Cardona et al., 2000; Tanigawa et al., 2008; Cao et al., 2007) and viruses (Barba et al., 1997; Samsa et al., 2009). Moreover, during infection, LD are mobilized into phagosomes, so the pathogen can use them as energy sources and for increased production of inflammatory mediators.

4. Bioactive lipids: biosynthesis and actions on WAT

Bioactive lipid mediators have been increasingly recognized as important endogenous regulators of key cellular processes. Fig. 1 illustrates a schematic representation of the most commonly described lipid mediators generated by adipocytes. The majority of these bioactive mediators originate from the cleavage of lipid constituents of cellular membranes under the activity of PLs, in particular PLA1 and PLA2, which release fatty acids from phospholipids, and PLC, which generates DAG from membrane phospholipids. In the free form, fatty acids are toxic in the cytosol, and are therefore readily converted into a number of lipid mediators, including omega-6 and omega-3 derivatives, fatty acid hydroxyl fatty acids (FAFH), nitroalkenes and endocannabinoids (Fig. 1). On the other hand, sphingolipids found in animal cell membranes are converted by the enzyme SMase into ceramide, which is subsequently hydrolyzed by ceramidase into a sphingosine molecule plus a FFA. Both ceramide and sphingosine are phosphorylated into ceramide 1-phosphate or sphingosine 1-phosphate, respectively (Fig. 1). In addition to cell membrane-derived lipids, TAG stored in

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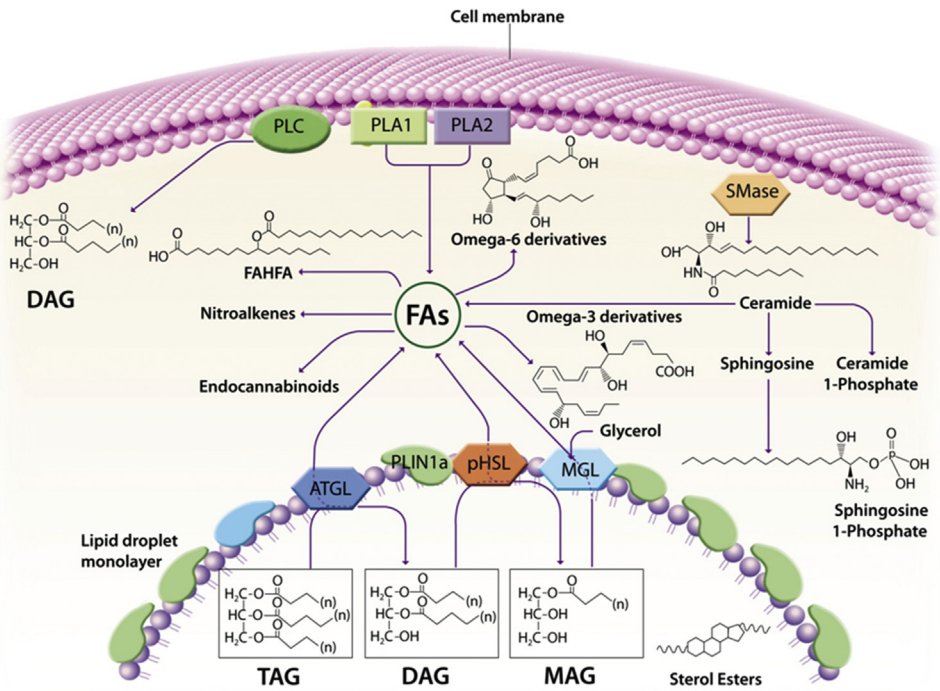

Fig. 1. Representative bioactive lipid mediators in adipocytes. Most of the bioactive lipids endogenously generated by adipocytes are primarily derived from cell membrane phospholipids and sphingolipids and secondarily from neutral lipids stored inside monolocular lipid droplets. Fatty acids (FAs), especially omega-6 and omega-3 polyunsaturated FAs, released from membrane phospholipids by phospholipase (PL) A1 and 2 (PLA1 and PLA2) are readily transformed by cyclooxygenases, lipoxygenases and cytochrome P450 epoxygenases into a broad spectrum of omega-6 and omega-3 derivatives. FA modifications also occur in the cytoplasm including nitrations (nitrated FAs or nitroalkenes), esterifications (fatty acid-hydroxy fatty acids, FAHFA), or ethanolamide additions (endocannabinoids). In the cell membrane, sphingomyelin is a substrate for sphingomyelinase (SMase), releasing ceramide to the cytoplasm. Ceramide can be phosphorylated into ceramide 1-phosphate or enzymatically hydrolyzed releasing a fatty acid and sphingosine (which can be phosphorylated to sphingosine 1-phosphate). In the cell membrane, PLC also hydrolyzes phospholipids to release diacylglycerol (DAG) that participates in different signaling pathways. FAs are also released from triacylglycerides (TAG) in perilipin 1a (PLIN1a)-coated lipid droplets. In the lipid droplet monolayer, adipose triclyceride lipase (ATGL) converts TAG into DAG by losing a FA. DAG is subsequently hydrolyzed by phosphorylated hormone-sensitive lipase (pHSL) to monoacylglycerol (MAG) releasing another FA. MAG is finally hydrolyzed into a third FA and glycerol by the actions of monoacylglycerol lipase (MGL).

the unilocular LD of adipocytes can release FFA into the cytosol by the concerted activities of ATGL, HSL and MGL present in the phospholipid monolayer coating the LD (Fig. 1).

4.1. Polyunsaturated fatty acid (PUFA)-derived lipid mediators

PUFAs cannot be synthesized *de novo* by mammalian cells and are therefore termed as "essential" because they must be obtained in adequate amounts from the diet (Lopez-Vicario et al., 2015a; Simopoulos, 1999). PUFAs are of utmost importance for mammalian cellular processes as they are the precursors of most cell-signaling molecules bearing a lipid structure, that is, lipid mediators. Indeed, PUFAs are unique fatty acids in the sense that they have multiple double bonds with a low dissociation energy rendering them very reactive to become easily oxidized to hydroperoxy and hydroxy derivatives (Lopez-Vicario et al., 2015a). There are two families of PUFAs depending on the carbon position of the first double bond from the methyl end (omega end): omega-6 and omega-3. In general terms, lipid mediators derived from omega-6-PUFA exert inflammatory, atherogenic and prothrombotic effects whereas those derived from omega-3-PUFA are anti-inflammatory and pro-resolving. Given that the current Western diet is very high in omega-6 and the ratio of omega-6/omega-3 PUFAs has risen to up to 20–30:1 (Simopoulos, 1999), pro-inflammatory and pro-thrombotic eicosanoids generated from omega-6 fatty acids are produced in larger quantities than those derived from omega-3 fatty acids (Lopez-Vicario et al., 2015a; Schmitz and Ecker, 2008). This explains why dietary changes characterized by a lower consumption of omega-3-PUFA and modern agriculture (animals fed on diets rich in omega-6s) are believed to be the origin of the higher incidence of thrombotic, inflammatory and carbohydrate and lipid disorders in our society (Schror, 1990; Bagga et al., 2003; Calder, 2006). The biosynthetic and signaling pathways and the cellular actions of lipid mediators derived from essential omega-6 and omega-3 fatty acids are described in detail in the following paragraphs.

4.1.1. Omega-6-derived lipid mediators

Arachidonic acid, an essential omega-6 PUFA, is the precursor of the biosynthesis of eicosanoids (Astudillo et al., 2012). There are two classical routes of eicosanoid biosynthesis in mammals: the cyclooxygenase (COX) pathway that results in the formation of prostaglandins (PGs) and thromboxane (TX); and the lipoxygenase (LOX) pathway that catalyzes the formation of leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) (Fig. 2) (Samuelsson et al., 1987; Romano and Claria, 2003). Apart from the COX and LOX pathways, arachidonic acid can also be converted into biological active mediators by cytochrome P-450 (CYP) epoxygenases (Fig. 2) (Spector and Norris, 2007). CYP epoxygenases are the so-called third branch of arachidonic acid metabolism and add oxygen across one of the four double bonds of this PUFA to generate three-membered ethers known as epoxyeicosatrienoic acids (EETs) (Spector and Norris, 2007). These epoxides act in an autocrine and paracrine fashion regulating vascular tone, inflammation, hyperalgesia and organ and tissue regeneration (Zeldin, 2001; Panigrahy et al., 2013). Finally, the eicosanoid family includes the lipoxins (LXs), a unique class of potent bioactive lipid mediators resulting

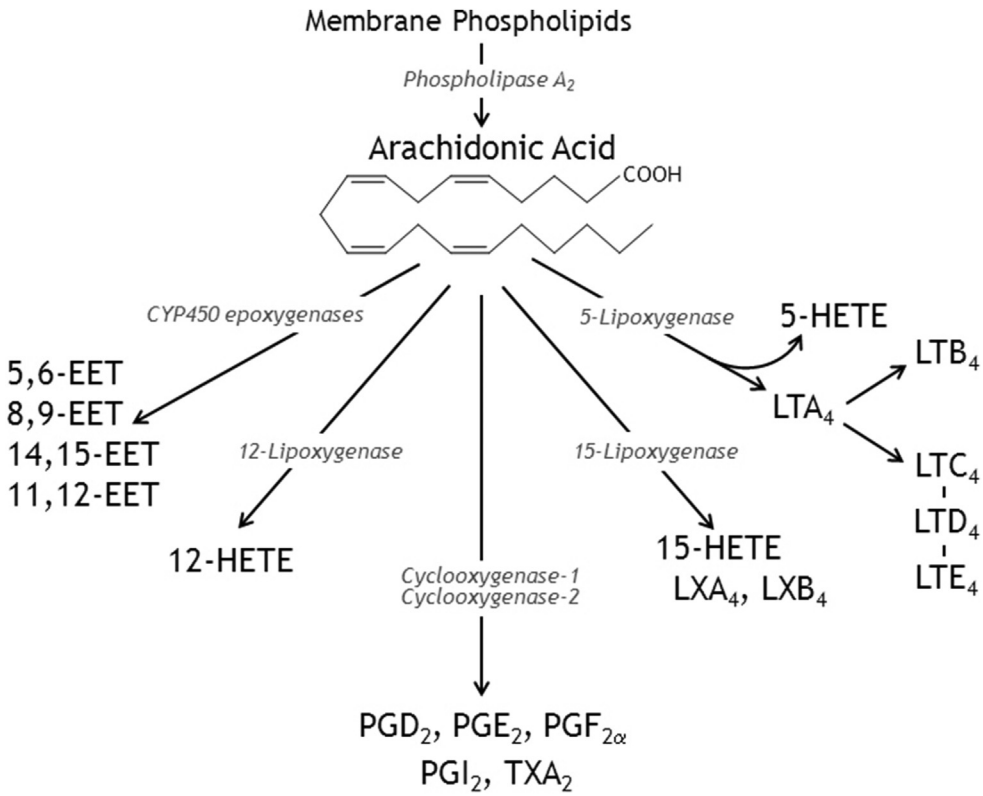

Fig. 2. Schematic diagram summarizing the biosynthesis of eicosanoids derived from the omega-6 polyunsaturated fatty acid arachidonic acid. Upon activation of phospholipase A_2, arachidonic acid is released from membrane phospholipids and converted into biologically active eicosanoids through the cyclooxygenase and lipoxygenase pathways. The cyclooxygenase pathway comprises two isoforms (cyclooxygenase-1 and -2) that convert arachidonic acid into prostaglandin (PG) D_2, PGE_2, PGF_2, PGI_2 and thromboxane (TX) A_2. On the other hand, the lipoxygenase pathway comprises three different dioxygenases (5-, 12- and 15-lipoxygenases) that catalyze the oxygenation of the 5-, 12- or 15-carbon atoms of arachidonic acid resulting in the formation of the respective hydroxyeicosatetraenoic acids (i.e. 5-, 12- and 15-HETEs). 5-lipoxygenase also gives rise to the unstable allylic epoxide leukotriene (LT) A_4, which is either hydrolyzed to LTB_4 or converted into LTC_4/LTD_4/LTE_4. During cell–cell interactions, mechanisms involving transcellular routes can transform 15-HETE into lipoxin (LX) A_4 and LXB_4, which, contrary to PGs and LTs, are potent endogenous anti-inflammatory eicosanoids. A so-called third branch of arachidonic acid metabolism is represented by the CYP450 epoxygenases, which convert this fatty acid into epoxyeicosatrienoic acids (EETs).

from the interaction between individual LOXs and, in the presence of aspirin, between COX-LOX interactions (Fig. 2) (Samuelsson et al., 1987).

4.1.1.1. The COX pathway. COX, the key enzyme in the biosynthesis of PGs, has two different isoforms: COX-1, which is constitutively expressed, and COX-2, which is inducible (Kujubu et al., 1991; Xie et al., 1991). Both COX isoforms convert arachidonic acid into PGG_2 and subsequently into PGH_2, which is finally converted by specific terminal synthases into PGs of the D_2, E_2, F_2 and I_2 series as well as into TXA_2 (Chandrasekharan and Simmons, 2004). Both PGI_2 and TXA_2 have a very short half-life and are rapidly hydrolyzed to the inactive compounds 6-keto-$PGF_{1\alpha}$ and TXB_2, respectively. PGD_2 is also readily dehydrated to the cyclopentenone PGs of the J_2 series (PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2)) (Chandrasekharan and Simmons, 2004). COX products act as autocrine or paracrine hormones, maintaining homeostasis within their cells of origin or in neighboring cells in the tissue. These eicosanoids bind to at least ten types and subtypes of prostanoid receptors belonging to the family of the seven transmembrane G protein-coupled receptors. Four of the receptor subtypes bind PGE_2 (EP1, EP2, EP3 and EP4), two bind PGD_2 (DP1 and DP2), two bind TXA_2 (TPα and TPβ) and the rest are single receptors for $PGF_{2\alpha}$ and PGI_2 (FP and IP, respectively) (Breyer et al., 2001).

4.1.1.2. The LOX pathway. There are three major LOXs in humans: 5-, 12- and 15-LOX. 5-LOX converts arachidonic acid into 5(*S*)-hydroxyeicosatetraenoic acid (5(*S*)-HETE) and LTs whereas 12- and 15-LOXs generate the corresponding 12- and 15-HETEs, respectively (Fig. 2). The best characterized and most relevant LOX is arachidonate 5-LOX. Arachidonate 5-LOX is a 674-amino acid protein, which upon cellular activation translocates to the nuclear envelope where it interacts with five lipoxygenase-activating protein (FLAP), an 18 kDa resident integral protein which functions as a transfer protein facilitating the binding of arachidonic acid to 5-LOX (Dixon et al., 1990). In the nuclear envelope, 5-LO transforms arachidonic acid into 5(*S*)-HpETE, which is subsequently reduced to either 5(*S*)-HETE or converted to the highly unstable allylic epoxide LTA_4 (Rouzer et al., 1986). Once formed, LTA_4 is rapidly transformed to either LTB_4 by LTA_4 hydrolase or to LTC_4 by LTC_4 synthase (Radmark et al., 1984). LTC_4 is subsequently deaminated into LTD_4 and LTE_4 (Fig. 2). These 5-LOX products bind G-protein coupled receptors (GPCRs), in particular B-LT_1 and B-LT_2 receptors for LTB_4 and Cys-LT_1 and Cys-LT_2 for LTC_4 and LTD_4 (Back et al., 2014).

On the other hand, 15-LOX exists in two isoforms: 15-LOX-1 and 15-LOX-2, which transform arachidonic acid into 15(*S*)-HETE (Kuhn et al., 2015). 12/15-LOX is the murine ortholog of human 15-LOX-1 (Kuhn et al., 2015). The physiologic role of 15-LOX is dependent on the context in which it is expressed, but this pathway is essential for the formation of products able to antagonize and properly resolve inflammatory responses (see Section 4.1.3). Regarding 12-LOX, this enzyme exists in three isoforms designated after the cells in which they were first identified: platelet-type, which metabolizes arachidonic acid into 12(*S*)-HETE; leukocyte-type, which converts

arachidonic acid or linoleic acid into 12(*S*)-HETE and also small quantities of 15(*S*)-HETE; and epithelial- or epidermis-type, that catalyzes the synthesis of both 12(*S*)- and 15(*S*)-HETE from arachidonic acid (Kuhn et al., 2015; Cole et al., 2013).

The interaction between individual LOX gives rise to the formation of lipoxins (LXs) by transcellular biosynthesis, eicosanoids that exert potent anti-inflammatory and immunoresolving actions in mammalian cells (Chiang et al., 2005). Three routes of transcellular LX biosynthesis have been described. The first route is initiated by the release of the epoxide intermediate LTA_4 formed by 5-LOX in activated leukocytes, which is then converted by platelet 12-LOX to LXA_4 and LXB_4 (Serhan and Sheppard, 1990). The second route takes place mainly in tissues in which endothelial and epithelial cells expressing 15-LOX can interact with 5-LOX-containing leukocytes (Chiang et al., 2005). Finally, a third major route of LX biosynthesis initiated by aspirin has been described (Claria and Serhan, 1995). Aspirin acetylates COX-2 and switches its catalytic activity from a PG synthase to a 15-LOX in such a way that PG biosynthesis is inhibited and arachidonic acid is instead transformed to 15(*R*)-HETE (Claria and Serhan, 1995). 15(*R*)-HETE is subsequently transformed by activated leukocytes possessing 5-LOX to a new series of carbon-15 epimers of LXs that carry their 15 alcohol in the *R* configuration (15-epi-LXs) (Claria and Serhan, 1995). The formation of these lipid mediators is specific for aspirin treatment, and the term aspirin-triggered LXs, abbreviated as AT-LXs, has been coined for these compounds (Claria and Serhan, 1995).

4.1.2. Biosynthesis and actions of omega-6-derived lipid mediators in WAT

The ability of WAT to generate bioactive lipid mediators was first described in the late 1960s when Shaw and Ramwell identified a group of hydroxyl C20 carboxylic acids, later identified as PGs derived from the oxygenation of arachidonic acid by COX in rat epipidymal fat pads (Shaw and Ramwell, 1968). Among the different COX-derived products, PGE_2 was recognized as one of the most abundant PGs in WAT. This finding was consistent with studies pointing to PGE_2 as a negative regulator of hormone-stimulated lipolysis (Steinberg et al., 1963). Along these lines, pre-incubation of adipocytes with COX inhibitors has been reported to enhance lipolysis (Chatzipanteli et al., 1992). Given that the lipolytic actions of catecholamines are mediated by cAMP, which in turn activates HSL, the anti-lipolytic actions of PGE_2 are likely associated with the modulation of adipocyte cAMP levels (Kather et al., 1985). PGE_2 has also been shown to suppress 3T3-L1 adipocyte differentiation by binding to EP4 and eliciting an increase in intracellular cAMP levels in preadipocytes (Tsuboi et al., 2004). Recently, we gathered data supporting a coordinated negative regulation between PGE_2 and PPARγ (Garcia-Alonso et al., 2013). Indeed, mice deficient in PPARγ showed increased expression of COX-2 and mPGES-1 and augmented PGE_2 levels, whereas the addition of exogenous PGE_2 suppressed PPARγ expression (Garcia-Alonso et al., 2013). Opposite effects were seen after the inhibition of endogenous PGE_2 biosynthesis with a selective mPGES-1 inhibitor (Garcia-Alonso et al., 2013). Of note, PGE_2 diverted preadipocyte differentiation to beige/brite mature adipocytes accompanied by up-regulation of UCP1, whereas a selective pharmacological mPGES-1 inhibitor and a siRNA directed against mPGES-1 resulted in the reduction of browning markers (i.e. UCP1, CIDEA and PGC-1α) and browning determination factors (i.e. PRDM16) in preadipocytes (Garcia-Alonso et al., 2013).

In addition to PGE_2, it has been postulated that another COX-derived product, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), plays a relevant role in adipose tissue by regulation of the adipogenic process. 15d-PGJ_2 is a cyclopentenone metabolite produced by dehydration of PGD_2 (Bell-Parikh et al., 2003). The predominant enzymatic source of 15d-PGJ_2 formation *in vivo* is COX-2, and unlike other PGs, no specific membrane receptor has been identified for this lipid mediator (Forman et al., 1995). Instead, 15d-PGJ_2 exerts its anti-inflammatory and adipogenic actions through binding and activation of the nuclear receptor PPARγ (Forman et al., 1995). Exposure of human adipocytes to 15d-PGJ_2, inhibits the secretion of pro-inflammatory adipokines and stimulates the production of macrophage inhibitory cytokine-1, a protective adipokine, by adipose tissue (Ding et al., 2009). In addition, a significant down-regulation in the expression and secretion of the pro-inflammatory adipokine leptin has been reported in adipocytes exposed to exogenous 15d-PGJ_2 (Sinha et al., 1999). Importantly, 15d-PGJ_2 stimulates adipogenesis (Sinha et al., 1999) and also exerts proadipogenic actions in fibroblasts, although in this case lymphocytes are the source of this cyclopentenone PG (Feldon et al., 2006). Surprisingly, an impaired adipogenic program has been identified in 3T3-L1 cells with stable transfection of PGD synthase and appreciably higher levels of endogenous PGD_2-derived metabolites, suggesting a complex regulatory interaction between PPARγ and pro-adipogenic lipid mediators (Hossain et al., 2012).

Apart from COX products, WAT also has the ability to produce and release LOX products, especially LTB_4, which apparently is the predominant LOX metabolite in this tissue (Horrillo et al., 2010). In this regard, WAT expresses all the enzymes necessary for the formation of 5-LOX products (5-LOX, FLAP, LTA_4 hydrolase, and LTC_4 synthase), as well as all the receptors involved in LT signaling (BLT-1, BLT-2, CysLT1, and CysLT2) (Horrillo et al., 2010). LTB_4 has been identified as an inflammatory factor in WAT, and FLAP overexpression and excessive generation of 5-LOX products is a common finding in WAT of obese patients and animals with insulin resistance (Horrillo et al., 2010; Li et al., 2015; Pardo et al., 2015). Moreover, a direct relationship has been reported between LTB_4 and enhanced release of inflammatory adipokines (i.e. MCP-1 and IL-6) in obese WAT (Horrillo et al., 2010). Consistent with this finding, mice deficient in the LTB_4 receptor BLT-1 show reduced monocyte recruitment to hypertrophied adipose tissue, whereas inhibition of the 5-LOX pathway with a selective FLAP inhibitor or genetic deletion of the BLT-1 receptor alleviates adipose tissue inflammation and insulin resistance in obesity (Horrillo et al., 2010; Spite et al., 2011).

4.1.3. Omega-3-derived lipid mediators

EPA and DHA are the archetypal omega-3-PUFAs that are substrates of the same COX and LOX pathways described previously for omega-6-PUFA. However, in the case of EPA and DHA, they are converted into potent anti-inflammatory and pro-resolving mediators, generically known as specialized pro-resolving mediators (SPM). This family includes a number of functionally distinct mediators such as resolvins, protectins and maresins (Serhan, 2014; Serhan and Chiang, 2013). These novel bioactive lipid mediators are further classified as either resolvins of the E-series if the biosynthesis is initiated from EPA or resolvins of the D-series if they are generated from DHA (Serhan et al., 2000). Protectins and maresins are also biosynthesized from DHA (Serhan et al., 2006, 2009). A schematic diagram of DHA and EPA-derived lipid mediators is shown in Fig. 3. The biosynthesis of omega-3-derived lipid mediators and their role in the metabolic syndrome and related liver disease have been described in detail in a recent review by our group (Lopez-Vicario et al., 2015a).

4.1.4. Biological actions of omega-3-derived lipid mediators in WAT

Human and mouse WAT express all the enzymes necessary for the biosynthesis of resolvins, protectins and maresins derived from omega-3 PUFAs, as well as all the receptors necessary for their

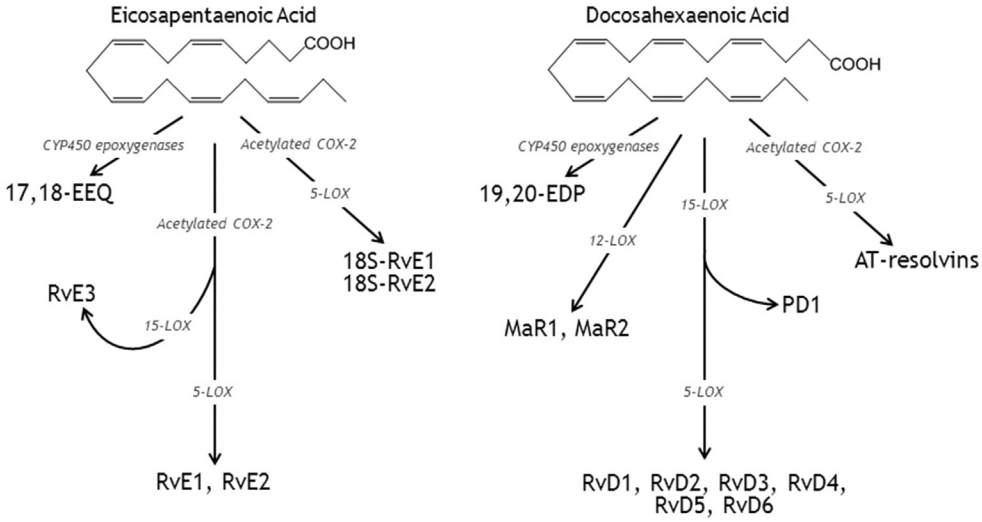

Fig. 3. Schematic diagram summarizing the biosynthesis of lipid mediators from the omega-3 polyunsaturated fatty acids eicosapentaenoic acid and docosahexaenoic acid. Eicosapentaenoic acid is converted by aspirin-acetylated cyclooxygenase-2 (COX-2) into 18R-HEPE (not shown), which is subsequently transformed by 5-lipoxygenase (5-LOX) into resolvin E1 (RvE1) and RvE2. Conversion of 18R-HEPE via the 15-LOX pathway gives rise to a novel member of the E-series resolvin family, RvE3. Members of the 18S-RvE series, including 18S-RvE1 and 18S-RvE2 derived from eicosapentaenoic acid, have also been identified. In addition, CYP epoxygenases convert eicosapentaenoic acid into a predominant epoxide form, 17,18-epoxyeicosatetraenoic acid (17,18-EEQ). On the other hand, docosahexaenoic acid is converted into 17-HDHA (not shown) by 15-LOX, which is subsequently transformed by 5-LOX into the D-series resolvins (i.e. RvD1, RvD2, RvD3, RvD4, RvD5 and RvD6). 15-LOX-derived 17-HDHA can also be converted into a dihydroxy-containing derivative termed protectin D1 (PD1). In macrophages, lipoxygenation of docosahexaenoic acid by 12-LOX gives rise to maresins (MaR1 and MaR2). Alternatively, CYP450 epoxygenases can convert docosahexaenoic acid into a predominant epoxide derivative, 19,20-epoxydocosapentaenoic acid (19,20-EDP). Finally, docosahexaenoic acid can be metabolized by aspirin-acetylated COX-2 and 5-LOX into the so-called aspirin-triggered (AT)-resolvins.

signaling (reviewed in (Spite et al., 2014)). In the setting of obesity, prevailing data support an unbalanced formation of these omega-3-derived lipid mediators in obese WAT. Indeed, a deficit in tissue SPM levels (RvD1, PD1 and 17-HDHA) has been characterized in inflamed visceral and subcutaneous fat compartments from obese *ob/ob* and obese/diabetic *db/db* mice (Gonzalez-Periz et al., 2009; Claria et al., 2012; Neuhofer et al., 2013). In humans, a deficit in PD1 and its precursor 17S-HDHA has been reported in subcutaneous fat from patients with peripheral vascular disease in whom the inflammatory status in adipose tissue is remarkably exacerbated compared with healthy subcutaneous fat (Claria et al., 2013). Moreover, LC-MS/MS-based metabolo-lipidomic analyses of fat from selected human anatomic locations have identified unique signature profiles in the content of bioactive lipids (Claria et al., 2013). Importantly, these analyses demonstrated a heterogeneous capacity for SPM biosynthesis among different adipose tissue depots, with higher activation of resolution circuits in perivascular fat compared with subcutaneous fat (Claria et al., 2013). This is relevant for vascular pathologies, with perivascular adipose tissue playing an emerging role in vascular biology homeostasis because of its tissue mass and anatomic proximity surrounding systemic vessels.

The compromised capacity to produce local SPM in obese tissues with a heightened pro-inflammatory phenotype could be the consequence of a structural deficiency in the tissue content of omega-3-PUFAs as established substrates for SPM biosynthesis. Indeed, there is evidence that SPM are generated in humans taking omega-3 dietary supplements and that SPM levels are increased above those produced normally in transgenic *fat-1* mice (Hudert et al., 2006). This is consistent with the observation that transgenic restoration of omega-3 fatty acids in *fat-1* mice with high-fat diet (HFD)-induced obesity reversed their inefficient resolution capacity and produced anti-inflammatory and pro-resolution actions in adipose tissue (Lopez-Vicario et al., 2015b; White et al., 2010). Alternatively, the loss of SPM in obesity may reflect accelerated tissue SPM conversion and clearance to inactive further metabolites because 15-PG-dehydrogenase/eicosanoid oxidoreductase, the key enzyme in SPM inactivation, is markedly upregulated in obese adipose tissue (Claria et al., 2012). In this tissue, omega-3-derived SPM are readily converted metabolically into oxo-resolvin products, some of which are biologically inactive (Claria et al., 2012). In addition, sEH, the enzyme that catalyzes the rapid hydrolysis of omega-6 and omega-3 epoxides by converting them into inactive or less active diols is invariably over-expressed in HFD-induced obese mice (Lopez-Vicario et al., 2015b). Collectively, these findings are consistent with the notion that unresolved chronic inflammation in obese adipose tissue is the result of an inappropriate SPM resolution-capacity allowing the inflammatory response to proceed without controlled checkpoints.

The use of synthetic SPM in different *in vivo*, *ex vivo*, and *in vitro* studies has provided clues of the role of SPM in adipose tissue homeostasis. In this regard, the administration of nanogram amounts of RvD1 to obese/diabetic *db/db* mice improves glucose tolerance, decreases fasting blood glucose, and increases insulin-stimulated Akt phosphorylation in adipose tissue (Hellmann et al., 2011). This SPM also reduces the formation of macrophage-containing crown-like structures in adipose tissue (Hellmann et al., 2011). Similarly, intraperitoneal injection (nanogram amounts) of RvE1 to obese *ob/ob* mice confers significant insulin-sensitizing effects by mechanisms related to the AMPK-adiponectin axis and the induction of GLUT-4 and IRS-1 expression (Gonzalez-Periz et al., 2009). In addition, 17S-HDHA treatment (intraperitoneal injection of nanogram doses) reduces adipose tissue expression of inflammatory cytokines (MCP-1, TNFα, IL-6 and osteopontin), increases adiponectin expression and improves glucose tolerance in parallel with insulin sensitivity in obese/diabetic *db/db* mice (Neuhofer et al., 2013). Similar beneficial actions in adipose tissue physiology have been described for LXA_4 in an experimental model of adipose tissue inflammation associated with aging (Borgeson et al., 2012). *Ex vivo*, in fat explants, both RvD1 and RvD2 rescue the impaired phenotype of obese adipose tissue by enhancing the expression and secretion of adiponectin in

parallel with decreasing the secretion of pro-inflammatory adipokines/cytokines including leptin, TNFα, IL-6 and IL-1β (Claria et al., 2012). *In vitro,* nanomolar concentrations of RvD1 stimulate macrophage nonphlogistic phagocytosis, a critical process in the resolution of inflammation, and enhance the phagocytic activity of macrophages isolated from the adipose tissue stromal vascular cell fraction (Titos et al., 2011). In human monocyte-adipocyte co-incubations, both RvD1 and RvD2 reduce MCP-1 and LTB_4-stimulated monocyte adhesion to adipocytes as well as monocyte transadipose migration, which are critical for the recruitment of monocytes/macrophages into the inflamed adipose tissue (Claria et al., 2012).

In line with SPM-protective actions against excessive macrophage recruitment, RvD1 skews polarization of adipose tissue macrophages from a classical activation inflammatory profile (M1 phenotype) toward an alternative anti-inflammatory M2-like state (Titos et al., 2011). This phenotypic switch is characterized by a reduction of secreted pro-inflammatory adipokines, such as TNFα and IL-6 accompanied by an up-regulation of a complete panel of M2 markers including IL-10, CD206, RELM-α and Ym1 (Titos et al., 2011). In addition, RvD1 remarkably increases arginase-1 expression, a well-established M2 marker, while attenuating IFNγ/LPS-induced Th1 cytokine secretion (Titos et al., 2011). Of particular interest, changes in the expression of M1/M2 markers appear to be confined to the adipose tissue stromal vascular fraction, which is highly populated by macrophages (Titos et al., 2011). These results are in agreement with those reported by Hellmann et al. (2011), who showed the ability of RvD1 to improve insulin resistance in obese-diabetic mice by reducing macrophage $F4/80^+CD11c^+$ cell accumulation and increasing the percentage of $F4/80^+$ cells expressing the M2 marker Mgl-1 in adipose tissue. The switch of recruited macrophages toward a M2 phenotype is interpreted as anti-inflammatory and pro-resolving (Olefsky and Glass, 2010). Of particular interest is the fact that when leukocytes exit the inflamed site or exudate, they traverse perinodal adipose tissue *en route* to local lymph nodes (Schwab et al., 2007). Excessive and persistent inflammation during this lipo-passage or failure of leukocytes to reach the lymphatics and hence, getting stuck while activated within adipose, can lead to adipose inflammation that may contribute to the metabolic syndrome. Finally, in a recent study, we used *fat-1* mice as an optimal model of omega-3 tissue enrichment, in which the stabilization of the CYP-derived 19,20-EDP epoxide by a sEH inhibitor down-regulated obesity-induced ER stress and autophagy in adipose tissue (Lopez-Vicario et al., 2015b). Since inhibition of autophagic function in adipose tissue is related to reduced fat mass and improved insulin sensitivity (Singh et al., 2009), these findings can be regarded as beneficial in terms of lipid homeostasis and metabolic control.

Apart from adipose tissue, SPM also exert beneficial roles in other insulin-sensitive tissues such as the liver. Indeed, hepatocytes from transgenic *fat-1* mice, which have increased levels of resolvins, and wild-type hepatocytes incubated with nanomolar concentrations of RvD1, show a reduction in neutral lipid accumulation (steatosis) as well as inflammation (Lopez-Vicario et al., 2015b, 2014). In addition, PD1 and 17*S*-HDHA are able to attenuate DNA damage and oxidative stress in hepatocytes and reduce TNFα release in macrophages (Gonzalez-Periz et al., 2006), whereas LXA_4 and AT-LXA_4 efficiently block IL-8 secretion by hepatocytes (Planaguma et al., 2002). Moreover, in precision-cut liver slices, an *ex vivo* model that overrides the influence of extrahepatic circulating factors, Rius and collaborators have demonstrated that RvD1 reduces hypoxia-induced mRNA and protein expression for inflammatory genes including COX-2, IL-1β, IL-6 and CCR7 (Rius et al., 2014). Of interest, the anti-inflammatory actions of RvD1 were completely absent in tissue slices in which macrophages were depleted with chlodronate liposomes (Rius et al., 2014), a finding that highlights the critical role of macrophages in the resolution phase of inflammatory response. Finally, the DHA derived 19,20-EDP epoxide was shown to restore autophagy in hepatocytes with the subsequent reduction of ER stress in these liver cells (Lopez-Vicario et al., 2015b). In summary, these findings highlight the potential of small bioactive lipid mediators to not only modulate lipid homeostasis and inflammation in insulin sensitive tissues, but also to serve as templates for the exploitation of cellular housekeeping processes (i.e. autophagy) in therapeutic interventions against obesity-related hepatic co-morbidities.

4.2. DAG

Diacylglycerol (DAG) biosynthesis is initiated from glycerol-3-phosphate generated by glycolysis in the cytoplasm of liver or adipose tissue cells (Li et al., 2010). Glycerol-3-phosphate is acylated with acyl-CoA to form lysophosphatidic acid (LPA), which is again acylated to give phosphatidic acid (PA). Subsequently, PA dephosphorylation forms DAG (Li et al., 2010). DAG is the precursor of TAG which is generated by another acylation catalyzed by diglyceride acyltransferase. This synthetic pathway can be reversed, and by the action of lipases (ATGL or HSL) TAG is hydrolyzed to DAG. Moreover, phospholipase C (PLC) can directly release DAG from phospholipids, by a hydrolytical reaction between the phosphate and the glycerol backbone (Gresset et al., 2012). All these reactions give rise to the 1,2 or the 2,3 DAG isoform; however, in some edible oils DAG can be found as a minor constituent in its 1,3 isoform, which is produced during high temperature, manufacturing processes (Flickinger and Matsuo, 2003). PLC can generate DAG from phosphatidylinositol 4,5-bisphosphate (PIP2) hydrolysis. This reaction releases inositol triphosphate (IP3) to the cytosol (Gresset et al., 2012), which stimulates calcium release. On the other hand, DAG is hydrophobic and remains in the membrane, acting as a signal messenger for activation of protein kinase C (PKC), and facilitates its translocation to the cellular membrane. DAG-mediated PKC activation in adipocytes through β-adrenergic receptors, in turn, increases glycogenolysis and gluconeogenesis (Lafontan et al., 1997).

Compared with TAG oils, dietary DAG oils suppress postprandial hypertriglyceridemia and decrease body fat mass (Nagao et al., 2000; Yamamoto et al., 2006). In animal studies this anti-obesity effect was attributed to an increase in β-oxidation of fatty acids, major energy expenditure and suppression of TAG synthesis (Meng et al., 2004; Kimura et al., 2006; Murase et al., 2001). DAG enriched structural lipids containing conjugated linoleic acid and capric acid (a medium chain fatty acid, MCFA) given in the diet to Sprague–Dawley rats, lowered the concentration of plasma TAG and decreased fat pads, simultaneously enhancing lipoprotein lipase activity (Kim et al., 2006). On the other hand, dietary supplementation with α-linoleic acid-rich DAG induced the up-regulation of β-oxidation of fatty acids, resulting in reduced body weight and fatty liver (Murase et al., 2002, 2005). For further information on this class of lipids see reference (Finck and Hall, 2015).

4.3. Endocannabinoids

The endocannabinoid system is involved in the regulation of WAT metabolism, energy homeostasis and appetite. The two classical endocannabinoids are anandamide (AEA) and 2-arachidonoglycerol (2-AG), both derived from the omega-6-PUFA arachidonic acid (sn-1 and sn-2 positions for AEA and 2-AG, respectively) (Bab et al., 2009). Three routes for AEA synthesis have been described. The major route is by arachidonic acid cleavage

from phosphatidylethanolamine and involves an acyltransferase and a specific phospholipase D (Fezza et al., 2005). The second route includes the action of phospholipase C and the formation of a phosphorylated intermediate that is then hydrolyzed by a phosphatase to form AEA. A third route involving arachidonic acid and ethanolamine condensation pathway has been described. The enzyme fatty acid amide hydrolase is a reversible enzyme that normally cleaves AEA to AA and ethanolamine, but it can also catalyze the AA and ethanolamide condensation into AEA. Three pathways have been described for 2-AG synthesis. It can be synthesized from phosphatidylinositol hydrolysis by phospholipase A that yields lysophosphatidylinositol, that is further hydrolyzed by phospholipase C to produce 2-AG. A second pathway involves phospholipase C action generating DAG with AA in its composition, which is further hydrolyzed by DAG lipase releasing 2-AG. The third pathway for 2-AG formation is catalyzed by a monoacylglycerol kinase acting over 2-arachidonoyl lysophosphatidic acid forming 2-AG (Sugiura et al., 1995).

Endocannabinoids exert their functions by binding to two cannabinoid receptors: CB-1 and CB-2 (Nogueiras et al., 2009). Apart from regulating appetite, and thus food intake, endocannabinoids participate in the control of lipid and glucose metabolism, and its dysregulation in obesity contributes to fat accumulation and associated metabolic disorders (Bluher et al., 2006; Di Marzo, 2008). Circulating levels of 2-AG are increased in obese compared to lean individuals (Bluher et al., 2006; Cote et al., 2007), and their levels are correlated with BMI and intra-abdominal adiposity (Cote et al., 2007; Cable et al., 2011). The expression of the CB-1 receptor is also correlated with BMI and the metabolic syndrome (Sarzani et al., 2009). Overall the endocannabinoid system is dysregultaed in peripheral tissues in obesity, altering lipid and glucose metabolism (Pagano et al., 2007; You et al., 2011; Izzo et al., 2009). In visceral adipose tissue there is greater mRNA expression of CB-1 than in subcutaneous adipose tissue (Bluher et al., 2006). Moreover, in obese individuals, 2-AG, CB-1 and MGL are up-regulated in abdominal adipose tissue, whereas their expression is down-regulated in gluteal adipose tissue (Pagano et al., 2007; You et al., 2011). Collectively, current evidence indicates that the endocannabinoid system is dysregulated in obesity and greatly influences the storage of energy in different adipose tissue depots. For further information on endocannabinoids see reference (Silvestri and Di Marzo, 2013).

4.4. Nitroalkenes of fatty acids

Nitro fatty acids are formed by nitration of unsaturated fatty acids. For this process, the formation of $\bullet NO_2$ during gastric acidification or in the presence of nitric oxide ($\bullet NO$) and nitrite (NO_2^-) is necessary (Bonacci et al., 2012). The nitration of PUFAs results in the formation of electrophilic fatty acid nitroalkene derivatives (NO2-FA) with biological properties including nitro-oleic acid (NO2-OA), nitro-linoleic acid (NO2-LA) and nitro-conjugated linoleic acid (NO2-CLA). NO2−FAs have been detected in plasma and urine from healthy subjects at nano-to micromolar concentrations (Baker et al., 2005; Khoo and Freeman, 2010; Schopfer et al., 2011). Transcriptional responses to NO2−FAs account for a broader array of signaling events than fatty acid derivatives generated by enzymatic oxygenation (Rudolph and Freeman, 2009; Kansanen et al., 2009). This is due to the pleiotropic effects of the Michael addition reaction by which the electrophilic NO2−FAs can react with the nucleophilic center of a protein (for example a cysteine or histidine residue) that regulates their structure, function and subcellular distribution (Schopfer et al., 2010).

NO2−OAs display anti-inflammatory properties *in vitro* and *in vivo*. Indeed, NO2−OAs limit LPS-induced inflammation and multi-organ dysfunction and have a reduced expression of MCP-1, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, xanthine oxidase, inducible NO synthase and COX-2 (Wang et al., 2010a; Kelley et al., 2008). NO2−FAs have been reported to inhibit NF-κB-regulated gene expression, as a consequence of adduction of a cysteine in the p65 subunit (Cui et al., 2006). NO2−FAs also mediate the adduction of a cysteine residue in Keap1, an Nrf2 regulatory protein, activating Nrf2-dependent phase 2 gene expression, thereby protecting the cells against oxidative damage and inflammation (Kansanen et al., 2009). NO2−FAs can regulate glucose and lipid metabolism by binding to PPARγ (Li et al., 2008; Schopfer et al., 2005). Recently, NO_2−FAs were reported to inactivate the 5-LOX enzyme, resulting in a lower production of inflammatory eicosanoids (Awwad et al., 2014). More recently, Kelley et al. have reported that NO_2−FAs improve glucose tolerance and reduce the expression of inflammatory cytokines and circulating leptin levels while increasing adiponectin in a HFD model of obesity (Kelley et al., 2014). In a previous study, Wang and collaborators administered NO_2-OA to obese Zucker rats to reduce body weight, TAG and FFA levels, and TBARS and proteinuria (Wang et al., 2010b). For further information on this class of lipids see reference (Rubbo, 2013; Narala et al., 2014).

4.5. Fatty acid-hidroxy fatty acids (FAHFA)

A new class of fatty acid derivatives designated as FAHFA has recently been identified by Yore et al. (2014). In WAT, these lipid mediators are synthesized in response to carbohydrate-responsive element-binding protein (ChREBP) and released to the blood, enhancing insulin secretion and glucose uptake (Yore et al., 2014). These authors identified FAHFA in adipose tissue of AG4OX mice, a transgenic model of Glut-4 over-expression, using an untargeted mass spectrometry lipidomic approach. Among the 16 species of FAHFA identified, palmitic acid-hydroxy stearic acid (PAHSA) was the most up-regulated. Eight isomers of this branched fatty acid (depending on the carbon position of the ester) were detected, among which the most relevant were 5- and 9-PAHSA (Yore et al., 2014). In this study, PAHSA levels were reduced in mice and humans with obesity and insulin resistance, and oral administration of 5- and 9-PAHSA was able to enhance glucose tolerance (Yore et al., 2014). Additionally, PAHSA increased Glp-1 secretion in intestinal enteroendocrine cells and enhanced pancreatic insulin secretion. Apart from these metabolic effects, PAHSA exhibited anti-inflammatory properties (Yore et al., 2014). Apparently, PAHSA exert all the above-described biological activities by binding to the long-chain fatty acid receptor GPR120 (Yore et al., 2014). Nonetheless, it remains unclear whether FAHFA biosynthesis is limited to adipocytes or it is produced in other tissues as well.

4.6. Sphingolipids

Sphingolipids contain a backbone of sphingosine and other aliphatic amino alcohols. Sphingolipids play different roles in the cell as structural lipids of the cell membrane by forming a stable and chemically resistant outer protective layer as well as in cell recognition and intracellular signaling. Ceramide is released from the hydrolysis of sphingomyelin (located in the cell membrane), which in turn can be converted to sphingosine and sphingosine-1-phosphate (S1P) (Fig. 1). Both ceramide and S1P are involved in different signaling cascades that participate in proliferation, differentiation, senescence, stress response, necrosis, apoptosis, autophagy, and inflammation.

Apart from the mentioned hydrolytic formation of ceramide from sphingomyelin by the action of acid or neutral SMases

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(Hannun and Obeid, 2008), ceramide can be biosynthesised *de novo*. In this case, ceramide biosynthesis is initiated by the condensation of serine and palmityl CoA by serine palmitoyltransferase, and subsequent actions of 3-ketosphinganine reductase, ceramide synthase and dihydroceramide desaturase. Alkaline or acid ceramidase deacetylates ceramide to generate sphingosine, and sphingosine kinase phosphorylates sphingosine to S1P. Similarly, ceramide kinase phosphorylates ceramide to ceramide 1 phosphate (C1P) (Sugiura et al., 2002). The availability of free fatty acids and inflammatory cytokines increases ceramide synthesis (Samad et al., 2006; Schilling et al., 2013), suggesting that ceramide metabolism may be altered in obesity. In obese rodents and humans ceramide is elevated in the liver, the hypothalamus and skeletal muscle (Adams et al., 2004; Holland et al., 2007). In leptin-deficient ob/ob mice, total sphingomyelin and ceramide levels in adipose tissue are reduced, whereas circulating levels of sphingomyelin, ceramide, sphingosine and S1P are increased in plasma, since ceramide secretion by adipose tissue is increased (Samad et al., 2006). In addition, the inflammatory status associated with obesity overlaps with ceramide production. TNF-α administration to mice results in an up-regulation of ceramide synthetic enzymes in adipose tissue, thus increasing ceramide production (Samad et al., 2006).

Adiponectin, the levels of which are generally reduced in obesity and diabetes (Liu et al., 2007), is linked to sphingolipid metabolism. The receptors for adiponectin contain ceramidase activity, thereby reducing ceramide levels (Holland et al., 2011), an activity that depends on adiponectin levels and binding to the receptors. This ceramidase activity leads to the release of a fatty acid and sphingosine that can be phosphorylated to S1P which has anti-apoptotic and anti-diabetic effects. S1P is released and through S1P receptors elevates intracellular calcium and activates AMPK, which participates in FA oxidation (Fang and Sweeney, 2006; Matsuzawa, 2010), thus alleviating lipotoxicity and reducing metabolic dysregulation. Moreover, adiponectin transgenic mice have better insulin tolerance under a HFD (Luo et al., 2010).

Obesity is associated with insulin resistance and type 2 diabetes. Ceramide appears to be elevated in these conditions, correlating positively with the degree of insulin resistance (Haus et al., 2009). *In vitro,* ceramide and sphingosine can inhibit insulin signaling by inhibiting Akt and AMPK (Hajduch et al., 2001; Liu et al., 2004; Summers, 2006). *In vivo*, the administration of myoricin, a serine palmitoyltransferase inhibitor, results in an improvement in insulin resistance in different disease models due to the inhibition of *de novo* ceramide biosynthesis (Holland et al., 2007). Heterozygous deficiency of dihydroceramide desaturase, another protein of the ceramide synthetic pathway, results in improved insulin sensitivity, and protection against dexamethasone-induced insulin-resistance (Holland et al., 2007). In 3T3-L1 adipocytes, ceramide blocks the phosphorylation of Akt and IRS-1 (Summers et al., 1998; Chavez et al., 2003); activates protein phosphatase 2A and PKCξ; and inhibits Akt translocation to the membrane (Powell et al., 2003, 2004). In NAFLD patients, ceramide biosynthesis and levels are also significantly increased (Kolak et al., 2007). Moreover, in ob/ob mice hepatic ceramide levels correlated with the degree of steatosis (Kolak et al., 2007). For further information on this class of lipids see reference (Hla and Dannenberg, 2012; Larsen and Tennagels, 2014).

4.7. Short- and medium-chain fatty acids

Short-chain fatty acids (SCFA) and MCFA are not generated within adipose tissue and are generally derived from fiber fermentation in the colon. These fatty acids enter the blood directly through the portal vein during lipid digestion, as opposed to long chain fatty acids which require proper absorption into the blood circulation (Papamandjaris et al., 1998). An important feature of SCFA and MCFA is that they remarkably differ in the number of carbons. In this regard, MCFA contain 8-10 carbon atoms, as in caprylic (C8:0) and capric (C10:0), whereas SCFA contain less than 8 carbons in their structure. Relevant members of the SCFA are formic acid, acetic acid, propionic acid, butyric acid and valeric acid, among others. Among these, butyric acid is the best known for its biological functions. Butyric acid is a substrate for growth and regeneration of cells in the large intestine; it has anti-cancer properties in colon cancer, probably enhancing apoptosis of tumoral cells, and more importantly for the purpose of this review, it increases thermogenesis, energy expenditure and contributes to the reduction of body weight and other factors present in the metabolic syndrome (Goncalves and Martel, 2013; Liu et al., 2011; Xue et al., 2009).

5. Concluding remarks

Much progress has been made in identifying some of the triggers of adipose tissue inflammation in obesity. Among the factors that are involved in this uncontrolled “low-grade” inflammatory response, lipid mediators play a pivotal role. Indeed, a number of lipid mediators derived from membrane phospholipids, including PUFA derivatives, are emerging as key regulators of adipose tissue homeostasis. Any strategy targeting mediators of inflammation such as omega-6 derivatives (i.e. PGs and LTs) and sphingolipids (i.e. LPA, S1P and ceramide) would be beneficial in reducing obesity-associated comorbidities. Another strategy to combat inflammation in adipose tissue is based on fostering the endogenous production of anti-inflammatory and pro-resolving mediators derived from omega-3 fatty acids, such as resolvins, protectins and maresins. Alternatively, the exogenous administration of stable analogs of this novel functional set of lipid autacoids would promote the timely resolution of inflamed tissues.

Interestingly, other fatty acid-derived molecules have come into focus in the last few years, giving us more tools to regulate obesity-related metabolic diseases. For example, nitroalkene derivatives such as nitro-oleic acid have been reported to exert pleiotropic effects, which overall reduce the inflammatory state and improve glucose tolerance. Another example is the recently identified fatty acid hydroxy fatty acids described by Yore et al. in adipose tissue. These authors have reported some metabolic effects of these novel lipids, such as enhanced glucose tolerance, and anti-inflammatory actions. Nonetheless, more studies are needed to demonstrate their potential. However, not only have these novel lipids opened and expanded new avenues in the field of lipid mediators, but they have also encouraged the search for new therapeutic molecules, thereby providing more options for the pharmacological regulation of obesity related diseases.

In summary, the current review provides up-to-date insight on the wide variety of lipid mediators present in mammalian cells, with special emphasis on the role of these bioactive lipids in the regulation of adipose tissue homeostasis.

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ANEXO II:
Presentaciones en congresos

Los resultados de la tesis doctoral han sido presentados en los siguientes congresos y conferencias:

- European PhD School of Bioactive Lipids. Inhibition of soluble epoxide hydrolase modulates inflammation and autophagy in obese adipose tissue and liver. Role for omega-3 epoxides. 23-27 noviembre, 2014. Pescara. Oral.

- 5th European Workshop on Lipid Mediators. The role of omega-3 fatty acid epoxides in obesity-induced adipose tissue inflammation and liver autophagy. 23-24 octubre, 2014. Istambul. Póster.

- DSM Science and Technology Award in the Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2014. Omega-3 fatty acids exert protective actions in obesity-related metabolic complications. 28 junio - 2 julio. Stockholm. Oral.

- 11th Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) 2014. The role of omega-3 fatty acid epoxides in obesity-induced adipose tissue inflammation and liver autophagy. 28 junio - 2 julio. Stockholm. Póster.

- XXXIV Congreso de la Asociación Española para el Estudio del Hígado (AEEH) 2014. La inhibición de la epóxido hidrolasa soluble modula la Inflamación y autofagia en la esteatohepatitis asociada a obesidad. Papel de los metabolitos epóxido derivados de los ácidos grasos Omega-3. 18-21 febrero, 2014. Madrid. Oral.

- 13th International conference on Bioactive Lipids in Cancer Inflammation, and Related Diseases. Molecular interplay between omega-3 fatty acids and delta-5/delta-6 desaturases in the pathogenesis of non-alcoholic steatohepatitis. 3-6 noviembre, 2013. Puerto Rico. Póster.

- 4th European Workshop on Lipid Mediators, Pasteur Institute. A regulatory loop between desaturases and omega-3 fatty acids plays a role in non-alcoholic steatohepatitis. 27-28 septiembre, 2012. París. Oral.

- 47th Annual Meeting of the European Association for the Study of the Liver. Fat-1 transgenic mice expressing an omega-3 fatty acid desaturase are protected from steatohepatitis.18-22 abril, 2012. Barcelona. Póster.

- XXXVII Congreso de la Asociación Española para el Estudio del Hígado. Los ratones transgénicos para la ácido graso omega-3 desaturasa codificada por el gen fat-1 estan protegidos frente e la esteatohepatitis. 15-17 febrero, 2012. Madrid. Oral.

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Todos estos años en los que he crecido profesionalmente y personalmente han sido posibles gracias a todas las personas que me rodean durante esta etapa de mi vida. Realizar la tesis no sólo ha sido importante para mí a nivel curricular, también a nivel personal.

No soy la misma joven de 24 años que vio el anuncio en la pared de la facultad de Farmacia. Aquella estudiante en la que Joan Clària depositó su confianza y adoptó en su grupo de investigación. Joan te mereces un gran reconocimiento, gracias por tu paciencia y por tu esfuerzo diario. Gracias por tus consejos y por los ánimos, sin ellos no habría desarrollado los talentos propios de una científica y mejorar el inglés, hablar en público sin miedo, conocer personas del campo gracias a los congresos y realizar una estancia en Suecia poniendo a prueba mi capacidad de adaptación día a día. Eres un ejemplo de dedicación a la ciencia.

Debo reconocer también el talento de todos y cada uno de mis compañeros, de todos he aprendido muchísimo trabajando codo con codo en el laboratorio. Me guardo grandes momentos y también ¡cómo no mencionarlo! Esos piques que siempre acaban de buen rollo y que nos ayudan a conocernos mejor. ¡Sois geniales! Desde los que ya no estáis… Ana González, Raquel Horrillo, Marta López, Marcos Martínez, Eva Morán, Mireia Casulleras… Hasta los que seguís aguantándome día a día… Esther Titos, Bibiana Rius, Verónica García, José Alcaraz, Aritz Lopategi y Anabel Martínez. Muchas gracias por ayudarme siempre que lo he necesitado experimentalmente hablando o advertirme de cuando he estado a punto de cometer errores y hacerme reflexionar cuando ya es demasiado tarde para rectificar. Pero también muchas gracias por ser mi hombro en el que llorar cada vez que me he sentido demasiado preocupada por alguna exageración de las mías. Tenéis esa capacidad de empequeñecer los problemas hasta hacerlos desaparecer ¡Porque sólo existen en mi cabeza! Nunca voy a olvidar los momentos que comparto con vosotros en el comedor y después ese ratito en el café para desconectar… me hacéis reír muchísimo ¡He tenido mucha suerte en teneros como compañeros! ¡Y la voy a seguir teniendo!

Los momentazos compartidos con el resto de compañeros de planta han sido muy muy grandes! En estos espacios abiertos en que los resfriados y los períodos femeninos celebran festivales del contagio. Y como buenos vecinos… la escasez de reactivos es menos escasa… y esto lo entenderán Dani, Delia, Cristina Millán, Pau, Isa Graupera, Mar, Denisse, Sílvia, Jordi, Guillermo, los que ya no están Uri, Sílvia Affó, Montse Pauta, Jon… y como persona importantísima para mi… ¡¡¡Pepa Ros!!!

La mejor Lab manager del mundo. Por favor no dejes tu iniciativa de pastel por artículo… es de lo más motivador!!

Nunca olvidaré una etapa muy importante durante la realización de la tesis, aprendí mucho a todos los niveles y esa etapa fue cuando trabajé con Montse Cofán ¡Muchas gracias por tu ayuda! Contigo he aprendido una nueva acepción del significado de trabajo, y es pasión.

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Y gracias también a toda la familia y amigos que habéis llevado con orgullo mi dedicación. Por darme ánimos en los momentos en los que os he necesitado… En especial a mis sobrinas Ariadna y Astrid por proporcionarme descansos obligatorios en casa. Para jugar… para reñiros… ¡sí, sí! Cuando leáis esto que sepáis que me encanta estar con vosotras… ¡¡¡sois mis favoritas!!!! A mis padres les debo todo, creo que está de más mencionarlo pero sobre todo gracias por enseñarme a ser fuerte y valiente. Porque "el mundo es de los vivos". Y especialmente quisiera agradecer la existencia de esta tesis a mi hermano, sin él no habría decidido realizar los estudios de doctorado. ¡Eres mi motor y mi ejemplo a seguir!

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¿La oda al egocentrismo?

¡El Arte que crea uno mismo!

Arte repelente.

Parece inocuo,

Pero estremece.

Único hijo inmortal…

¡Edipo enloquece!

Cristina López Vicario, Malgrat de Mar 2015

Abres la puerta y aquí me encuentras,

Naranjean los ojos, los últimos rayos de luz,

La espera no duele si duermo mientras

Las flores regaladas resisten la senectud.

“Debí alejarme en el ocaso,

Erosionar las afiladas voces con el viento,

Debí aguardar bajo otro amparo,

Pero anidas mi cielo y he vuelto”.

Enmudeces y hablan tus pensamientos,

Ahora negrean los ojos, el crepúsculo,

Las flores grises suspiran al tiempo,

Que absorben un fotón de luz minúsculo.

“Háblame de las leyendas de tu ausencia,

Embellece la noche, en recital de fados,

Mis labios raídos por la senescencia,

Al alba besarán versos olvidados”.

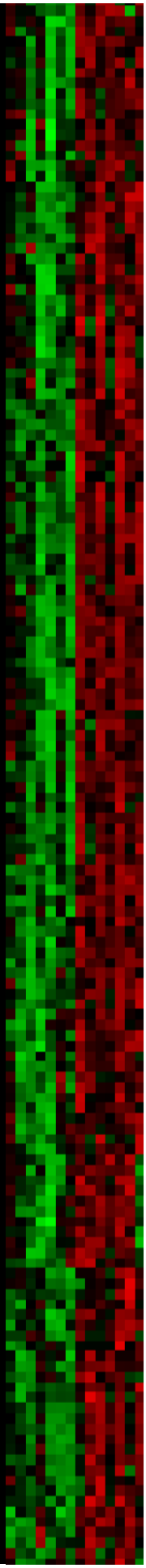
Tus pasos indecisos al fin te acercan,

Azulean los ojos, este rayo de luna,

Las flores se abren para que merezcan,

Ser regaladas a la infancia más pura.

Cristina López Vicario, Barcelona 2014



Cristina López Vicario
Barcelona
2015