UNIVERSIDAD DE GRANADA

DEPARTAMENTO DE FISIOLOGÍA

INSTITUTO DE NUTRICIÓN Y TECNOLOGÍA DE LOS ALIMENTOS
"JOSÉ MATAIX VERDÚ"

PROGRAMA DE DOCTORADO NUTRICIÓN HUMANA

Structural, functional and genomic changes associated to aging in the liver of rats fed life-long on virgin olive, sunflower or fish oils, as dietary fats

TESIS DOCTORAL

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2014

Editor: Editorial de la Universidad de Granada
Autor: Mª Patricia Pérez López
D.L.: GR 1883-2014
ISBN: 978-84-9083-067-3

La Memoria de esta Tesis Doctoral está incluida en el proyecto AGL2008-01057 (Ministerio de Ciencia e Innovación) al que fue asociado la ayuda Predoctoral de Formación de Personal Investigador BES-2009-013484 y la ayuda para estancias breves EEBB-I-12-04009. Así mismo la presente memoria está incluida en el proyecto de excelencia AGR802 (Junta de Andalucía).

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CERTIFICAN:

Que los trabajos de investigación que se exponen en la Memoria de Tesis Doctoral: **"Structural, functional and genomic changes associated to aging in the liver of rats fed life-long on virgin olive, sunflower or fish oils, as dietary fats",** han sido realizados bajo nuestra dirección por la licenciada en Ciencia y Tecnología de los Alimentos Dª. Mª Patricia Pérez López y la encontramos conforme para ser presentada y aspirar al Grado de Doctor por la Universidad de Granada con el Tribunal que en su día se designe.

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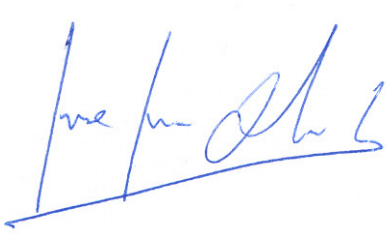

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Granada, 2014

Esta historia comenzó hace mucho tiempo. Cuando todas las niñas querían ser peluqueras o enfermeras, una soñadora le decía a su madre que quería descubrir una vacuna. Yo no sabía lo que implicaba eso, lo que único que quería era poder ayudar a mucha gente. Así empezó el viaje hasta aquí, un viaje en el que siempre he ido acompañada. Desde el principio, mi madre, Amparo, y mi hermana, Sandra, creyeron en mí y en que si trabajaba duro podría llegar donde quisiera. Y que razón tenían. Mi madre nos ha enseñado con su ejemplo a ser constantes, luchadoras y trabajadoras, a estudiar duro sin renunciar a disfrutar de la vida, y así conseguir grandes metas. Llegar hasta aquí era una de las mías, y con más o menos esfuerzo lo hemos conseguimos. Enhorabuena mamá!! no he inventado una vacuna, pero hoy estoy un poquito más cerca de poder ayudar a mucha gente, porque esa mi manera de entender la Ciencia. Y enhorabuena hermana!! Que habría sido de mí sin tu ayuda estas últimas semanas, dedicándome tu tiempo y tus cuidados. Sabes que para mí eres mucho más que una hermana, eres mi mejor amiga, la que me entiende y me acompaña siempre. Vuestro apoyo ha sido decisivo todo este tiempo, y más aún esta última etapa. Las dos sois un ejemplo para mí y por vosotras estoy aquí hoy.

Pero no han sido las únicas personas que han hecho conmigo este largo trayecto hasta aquí. Ya en el colegio conocí a los que han sido mis amigos de toda la vida. Nombrarlos a todos sería una locura, pero no puedo dejar de mencionar a mis niños, Juanje, Rami, Hugo… que me han tratado siempre como una hermana. Y especialmente Ñete y Fran, dos de los hombres más importantes que ha habido y habrá en mi vida y que tantos buenos momentos me han dado, además de una cuñada y un sobrino precioso! Nandi y Hugo, gracias por llenarme de buenos momentos. Y mis nenas Ana, Eli, Elisa, Eva, Fátima, Gloria, María José, Mari Cruz, Mari Loli, Tania, Cynthia y Merce, mis pilares dentro y fuera de este proyecto, siempre con una palabra de ánimo y una sonrisa que contagiarme. Las charlas con Cyn sobre los problemas del western ayudaban mucho, y nunca podré agradecerle lo suficiente que se prestara a enseñarme lo mejor que sabe hacer, su trabajo en el laboratorio. Que grande eres prima!!! Contagias tu optimismo y alegría a todo lo que te rodea. Una parte de esta tesis también es tuya. Y mi Erse, amiga, compañera de risas y compañera de sueños. Quedarnos dormidas estudiando historia era un clásico entre nosotras, y ahora míranos, cada una con su proyecto de vida pero siempre unidas. A todos, gracias por creer siempre en mí.

Si hay tres personas a las que les debo más que a nadie estar aquí, y ahora me vais a entender, son a mis tres florecillas: Isa, Encarni y Mary. A Isa la conozco de toda la vida, literalmente. Eso, bachillerato, selectividad, biología, tecnología de los alimentos… montones y montones de apuntes que no habrían sabido igual sin ella. Mi amiga y mi confidente, mi rival en el fútbol y mi compañera de comida china. Todo el mundo recuerda sus años de estudiante como los mejores de su vida, pero yo es que tengo motivos más que suficientes. Gracias petarda!!! Gracias por darme tantas anécdotas que recordar. Mi Mary Mary, te cruzaste conmigo con 13 o 14 años y mira por donde vamos ya! Junto con Isa fuimos las 3 locas que empezamos Biología y continuamos con CTA, qué de años juntas y qué de risas hemos compartido. A este trío se unió Encarni, y las 4 pasamos muuuchas tardes de estudio y de Tomeco, jeje, y compartimos meriendas, cenas, cumpleaños y lo más importante, el nacimiento de la pequeña Laura, qué bonita eres y que feliz nos haces! Pero si hay algo que no hay fortuna en el mundo que pueda pagar es lo que este súper equipo hizo por mí cuando más lo necesitaba. Para poder conseguir la beca que me ha traído hasta aquí tenía que aprobar una "pila" de asignaturas en "na y menos" de tiempo, y la recta se final se hizo más cuesta arriba de lo que yo esperaba. Pero allí estaban ellas, las que nunca me dejaron flaquear, las que me animaban a seguir estudiando, y las que no dudaron a ponerse a estudiar conmigo todos los exámenes, los tuvieran ellas o no! Las tardes estudiando DYN no se me van a olvidar nunca, porque sólo gracias a ellas conseguí

aprobar todo, me dieron mi beca y he podido llegar hasta aquí, escribiendo mi tesis doctoral!! Ay florecillas, que bonito todo lo que hicisteis por mí!! GRACIAS. Este trabajo es vuestro, y por vosotras lo he hecho lo mejor que he sabido.

En todos estos años de estudiante coincidí con muchos más compañeros, mis biololocos y mis aparejadores Fátima, Laura, Bea, Ángeles y Jesusma, grandes amigos y mejores personas que después de tantos años siguen tan importantes para mí como el primer día. Y la aventura de compartir piso me regaló a mis alcalainochiclaneras, Vero, Mónica, Rocío y Natalia. Pero que años más buenos me habéis hecho pasar!! Pero buenos buenos! Parte de lo que soy es gracias a vosotras, a todos esos buenos deseos que siempre habéis tenido hacía mí y que me han ayudado cuando lo he necesitado. No cambiéis nunca porque sois perfectas como sois.

Y cuando terminó la época de estudiante empezó la más emocionante de las etapas, mi etapa de predoctoral, y vaya manera de empezar! El destino quiso poner en mi camino a Pedro, un conductor loco de ambulancias de Cruz Roja que una tarde de guardia, después de una conversación sobre "qué queremos ser de mayor", me presentó a los que han dirigido mis pasos hasta aquí, Pepe y Mayca. Todavía me pregunto que verían en mí debajo de aquel uniforme gigante para ofrecerme la oportunidad que me cambió la vida: la posibilidad de formar parte de su equipo y más tarde la beca que me ha traído hasta aquí. Pedro, jamás voy a olvidar ese día y que tú formaste parte de él. Gracias.

Y así llegué al Insituto de Nutrición y Tecnología de los Alimentos y al departamento de Fisiología. Cualquier persona que empieza a trabajar en un sitio nuevo sueña con encontrar buenos compañeros y buen ambiente de trabajo. Pues ni en mis mejores sueños habría imaginado encontrarme a gente tan maravillosa. Sergio, mi mentor, con el que he pasado muchas horas trabajando en el laboratorio y muchos buenos ratos en nuestros descansos. Quisiste hacer de mí una buena aprendiz de científica y, sólo por ti, espero haberlo conseguido. Junto con Nuri, Nurification para los amigos, formábamos el mejor equipo, capaz de tener jornadas de 10 y 12 horas que terminaban con algún que otro video friki que siempre recordaré. Y Javi, un miembro más del 120. Es difícil conocer a alguien con más capacidad de sacrificio que él, y por supuesto es imposible encontrar a nadie que huela mejor. Los tres me han hecho sentir muy especial dentro y fuera del laboratorio, y los tres se merecen todo lo bueno que les pase. Poco a poco el 120 se fue completando con Adri, Alfonso, Mario y Naroa. Y con nuestros agregados!! Alicia, Encarni, Silvia, Jorge, Emilio, y tantos y tantos compañeros que han pasado por nuestro laboratorio ya sea para unas prácticas (no me puedo olvidar de mis niñas, Cristina y Marta) para un trabajo fin de máster o para alguna colaboración. Todos y cada uno de ellos me han dado lo mejor de sí mismos, siempre con una palabra de ánimo y una sonrisa para compartir. Son las mejores personas con las que se podría convivir tanto en el laboratorio como fuera de él, siempre atentos a mí y ayudándome cuando lo he necesitado y cuando no. No tengo palabras suficientes de agradecimiento a Adri por su optimismo, a Alfonso por su sencillez, a Mario por su fuerza, a Naroa por su cariño, a Silvia por su energía y a Alicia simplemente por ser Alicia, porque con eso se resume todo. No habría llegado hasta aquí sin su ayuda y espero poder estar siempre para ellos como ellos lo han estado para mí.

Si en el laboratorio conocí a grandes personas, en el departamento de Fisiología no ha sido menos. Desde la directora hasta la secretaria, todos me han tratado con mucho cariño y respeto. Para mí es una alegría entrar por esas puertas y encontrarme siempre con alguna palabra amable. Nombrarlos a todos sería complicado y a todos los estoy eternamente agradecida, pero no puedo dejar de mencionar a Elena Planells, María José, Inma, Tere, Cristina y Jesús Porres, que me han ayudado en todo lo que he necesitado. Elisa y Encarna, sin ellas el departamento no sería lo mismo. Alba, con la que he compartido muchas horas de clases y que siempre me lo ha puesto todo fácil. Ha sido un placer aprender de ti y tener siempre a mi disposición tu simpatía. Y

como no, Magdalena, siempre pendiente de mí, siempre dispuesta a ayudarme en lo profesional y en lo personal, y siempre con un buen consejo para ofrecerme. Toda vuestra energía y vuestros buenos deseos me van a acompañar siempre. Mil gracias. Y luego están los fisio-becarios, esa especie rara de jóvenes que deciden dedicarse a la investigación y que se apoyan y se ayudan sin esperar nada a cambio. Elena, Dani, Gary… Que sería de nosotros sin nosotros! El resto de personas que me han acompañado durante este viaje forman parte del Instituo de Nutrición y el CIBM. Esos pasillos que son un ir y venir de personas con los que da alegría cruzarse, como el grupo de Darío Acuña y Luis, a todos gracias!

Si es algo que tuve claro desde que escogí este camino es quien quería que guiara mis pasos hasta aquí: Pepe, Mayca y Julio. No hay nadie en este "mundillo" mejor de los que aprender. Julio me ha enseñado a que todo siempre tiene solución, y que normalmente es la solución más fácil, sólo hay que mantener los pies en la tierra y la cabeza fría. De Pepe he aprendido a que en la vida no todo es trabajar, es importante saber disfrutar del tiempo libre. Y Mayca, cuanto he aprendido de ella! La mejor lección que me ha dado es que a aquellos a los que la vida no nos regala nada, el esfuerzo y el sacrificio nos da lo que nos merecemos. Entre los tres han hecho posible que hoy me sienta más que orgullosa de estar donde estoy y de la tesis tan bonita que hemos conseguido y con ellos espero compartir todos los éxitos que seguro que llegarán.

A toda esta gente la escogí yo para que me acompañara, pero también hubo alguien que decidió aparecer hace 3 años ya sin que nadie le avisara. Su llegada no fue del todo bien recibida, era muy joven y no estaba preparada para que me cambiara la vida de esa manera, pero estas cosas no se eligen si no que se asumen y se aprende a convivir con ellas. Algunos la llaman artritis reumatoide, yo prefiero llamarla mi compañera de viaje, la que tantas cosas positivas me está enseñado y tanta fuerza me está dando, a pesar de todo, me ha dado mucho más de lo que me podría quitar. Sé que va a estar siempre conmigo y que pasaremos muy buenos ratos y quizás alguno menos bueno, pero también sé que en los buenos y en los menos buenos nunca estaré sola, y que estoy en manos de los mejores profesionales que podría haber conocido, el Dr. Utrilla y la Dra. Romaní. Sin su dedicación y su preocupación no estaría lo maravillosamente bien que estoy ahora (y mejor que voy a estar). Gracias Alfonso, gracias Lurdes, ojalá que cada vez tengamos que vernos menos.

Y pasito a pasito y rodeada de gente buena fui avanzando por este sendero, a veces un camino de rosas y otras veces más tortuoso, pero siempre gratificante. Al final del camino, como esa ráfaga de aire fresco que te da la energía para comerte el mundo, llegaste tú, y contigo más gente maravillosa a tu alrededor. Mi mejor amigo y mi compañero, mi mitad perfecta. La persona que hizo crecer la confianza en mí, que me alivió cuando mi salud más se resentía y que con su ejemplo de fuerza y vitalidad me ha hecho ver la vida como el regalo maravilloso que es. Manolo, puede que nos preguntemos por qué ahora, pero yo creo que lo tengo claro, porque no habría un momento mejor. Gracias por tu apoyo, tu energía, tu amor y tu paciencia infinita. Tú me has enseñado que puedo conseguir todo lo que me proponga, y por eso mis éxitos serán siempre tuyos también.

A todos los que habéis tenido una palabra de ánimo o un gesto de cariño hacia mí, GRACIAS. Ojalá la vida me da la oportunidad de devolveros todo lo bueno que me habéis dado.

EL ÉXITO EN LA VIDA NO SE MIDE
POR LO QUE LOGRAS,
SI NO POR LOS OBSTÁCULOS
QUE SUPERAS.

A MI ABUELO,
UNA DE LAS PERSONAS
QUE ESTARIA MÁS ORGULLOSO DE MI
GRACIAS POR TODO LO QUE NOS ENSEÑASTE

El interés sobre el envejecimiento ha crecido enormemente en las últimas décadas, debido a condicionantes de tipo social y científico. Una observación clave ha sido el aumento espectacular en las expectativas de vida en los seres humanos a lo largo de los últimos siglos. Además, el elevado número de personas ancianas en los países desarrollados junto al gran coste económico que conlleva los cuidados en salud para los mismos han estimulado a la comunidad científica y a la sociedad en general a conocer más sobre diversos aspectos asociados al envejecimiento. Una de las tareas más difíciles cuando se estudia el envejecimiento es definirlo. Harman define el envejecimiento como la progresiva acumulación de cambios adversos en las células y tejidos a medida que avanza la edad, lo cual aumenta el riesgo de enfermedad y muerte. Por su parte, Strehler define el envejecimiento mediante cuatro aspectos propios del fenómeno:

a) Universal. Con más o menos retraso ocurre en todos los individuos de una especie.

b) Intrínseco. Las causas que originan el envejecimiento son endógenas, no dependientes de factores extrínsecos. En otras palabras, aun en una urna de cristal, protegido de toda interacción con el medio, cualquier organismo envejecería.

c) Progresivo. Los cambios que originan el envejecimiento ocurren poco a poco a lo largo de la vida (también ocurren en individuos jóvenes, pero en menor proporción).

d) Deletéreo. Un fenómeno asociado al envejecimiento será considerado como parte del proceso de envejecimiento sólo si es pernicioso para el individuo.

Incluso con una buena definición y un grupo de características bien conocidas, el envejecimiento sigue siendo uno de los fenómenos biológicos menos conocidos, debido en parte a su complejidad inherente y su naturaleza multifactorial, así como a la dificultad de disociar los efectos de un envejecimiento normal de aquellos manifestados como consecuencia de una enfermedad asociada al envejecimiento. Como consecuencia de todo esto, diversas disciplinas científicas han desarrollado un amplio número de teorías que tratan de explicar por qué envejecemos, si bien, los mecanismos definitivos que explican el proceso permanecen confusos. Dos conceptos son muy importantes a la hora de estudiar el fenómeno del envejecimiento. El primer concepto hace referencia a la vida máxima o período vital, que representa la edad a la que el individuo más longevo de una especie determinada ha llegado. En el caso del ser humano ésta ha sido establecida en torno a los 110-120 años. El segundo de los conceptos es el de vida media (o esperanza de vida), edad a la cual el 50% de los individuos de una especie determinada sobreviven. Si bien la vida máxima para la especie humana es la misma desde hace miles de años, la vida media se ha incrementado drásticamente en el ser humano con el tiempo. Así, los humanos de hace 50.000 años raramente vivían por encima de los 40 años, encontrándose su vida media en torno a los 20 años, debido fundamentalmente a las enfermedades y a los accidentes. Sin embargo, los grandes avances en los campos de la medicina y la nutrición, entre otros, han dado lugar hoy en día a que en los países industrializados esta vida media sea al menos de 75 años. Se da la circunstancia de que la mayor parte de este incremento ha tenido lugar en los últimos 150 años.

Hasta hace no mucho, los científicos se hacían la siguiente pregunta: “si el envejecimiento es dañino para el organismo, ¿por qué afecta a todos los individuos de todas las especies?” En respuesta a esto, hoy se tiende a pensar que el envejecimiento tal vez sea el efecto colateral, dañino, de otros procesos biológicos inherentes a los organismos vivos. Si esto fuera cierto, cualquier gen capaz de reprimir los procesos biológicos responsables del fenómeno del envejecimiento, en teoría debería también ser capaz de retrasar el envejecimiento en sí. Al menos con un par de ejemplos, como son la reproducción y la alimentación, lo anterior parece cumplirse. En el caso de la reproducción hay dos hechos bien conocidos. En primer lugar, desde un punto de vista evolutivo, las especies más longevas presentan menores tasas reproductivas

que las que viven menos. Por otro lado, determinadas mutaciones puntuales que extienden la vida en ciertas especies son a la vez responsables de un descenso en la fertilidad de las mismas. En el caso de la alimentación, parece que este proceso representa otra importante fuente de daño siendo un destacado condicionante para la aparición de envejecimiento. Pues bien, muchos genes implicados en la reducción del envejecimiento participan a la vez en las respuestas fisiológicas al cambio en los niveles de determinados nutrientes. Además, es bien conocido que la reducción en la ingesta de alimentos (o restricción calórica) prolonga la vida en una amplia variedad de organismos, desde levaduras a mamíferos. En efecto, la nutrición parece tener una gran importancia desde el punto de vista del desarrollo del envejecimiento. Además, la población anciana puede presentar alteraciones nutricionales que pueden agravar el proceso de envejecimiento en sí y la aparición de enfermedades asociadas, e incluso acelerar su muerte.

Las teorías del envejecimiento se pueden agrupar de diversos modos. Así se puede hablar de teorías de un envejecimiento programado y teorías de error, de teorías genético-evolutivas y teorías estocásticas, etc. En general, se puede decir que las teorías del envejecimiento programado y las teorías genético-evolutivas asumen que el envejecimiento depende de "relojes biológicos" que regulan la longevidad controlando las etapas de crecimiento, desarrollo, maduración y senescencia. La regulación planteada por estas teorías depende de genes que envían o eliminan señales a los sistemas nervioso, endocrino e inmune responsables del mantenimiento de la homeostasis y la activación de las respuestas de defensa. Por su parte, las teorías estocásticas, de error o catastrofistas, identifican agresiones ambientales a los seres vivos que provocan el daño progresivo a diversos niveles de organización (ADN mitocondrial, proteínas en general, lípidos, etc.).

La teoría del estrés oxidativo se basa en la existencia de las especies reactivas del oxígeno (ERO) que son un producto normal de la vida aeróbica resultado de la oxidación de los macronutrientes sobre todo, con la finalidad de obtener energía, proceso que se lleva a cabo en la mitocondria. Es la acumulación del daño oxidativo debido a dichas especies reactivas lo que da lugar a cambios fundamentales que afectan al envejecimiento. Como se ha mencionado ya, esta teoría fue propuesta inicialmente por Harman en los años cincuenta del pasado siglo, y desde que fue enunciada se ha demostrado que el daño oxidativo tiene lugar en las células como resultado de la reacción entre las ERO y las diversas moléculas biológicas. Se ha descrito cómo las ERO y los radicales libres inducen peroxidación lipídica, modificaciones oxidativas en proteínas, inactivación de enzimas, modificación de bases y rotura de la cadena de ADN, dando lugar en general a lo que se conoce como estrés oxidativo.

El estrés oxidativo como agente etiopatogénico del envejecimiento podría estar pues en función del aumento en la producción de ERO y de la eliminación de dichas especies, lo cual estaría más o menos relacionado con los sistemas antioxidantes del organismo. Sin embargo, los antioxidantes, si bien podrían estar implicados en la protección frente a varias enfermedades asociadas al envejecimiento, parece ser que no son los responsables de controlar el grado de envejecimiento. A favor de esta hipótesis hay al menos cuatro líneas de evidencia:

a) Al contrario de lo que se pensó al principio, los antioxidantes no disminuyen con la edad; por el contrario, la literatura científica está llena de investigaciones que indican lo contrario.

b) Desde un punto de vista teórico, sería posible que las especies más longevas tuviesen maquinarias antioxidantes más potentes o eficaces que las que viven menos. Sin embargo, las evidencias científicas indican todo lo contrario.

c) Diferentes estudios en mamíferos en los cuales los niveles de antioxidantes son aumentados, bien mediante suplementación nutricional, a través de inducciones farmacológicas o mediante técnicas transgénicas no muestran cambios a nivel de la vida máxima de dichos animales en la mayoría de los casos.

d) Estudios en los cuales se produce el bloqueo a nivel genético de diversos antioxidantes mediante técnicas knock-out han demostrado una ausencia de resultados en relación al grado de envejecimiento.

El principal productor de ERO del organismo es la mitocondria. La mitocondria tiene dos membranas, una externa, que es porosa, y otra interna, muy rica en proteínas que se caracteriza por ser una barrera de permeabilidad para los iones. Ambas membranas, al igual que todas las membranas biológicas, se componen de lípidos y proteínas. Sin embargo, la membrana interna mitocondrial es muy diferente al resto de membranas biológicas por cuanto su contenido en proteínas supera el 80% (al contrario que la mayoría de las otras que no superan el 50%). Por su importancia y significado dentro del contexto del estrés oxidativo, merecen ser destacados los complejos proteicos que forman la cadena de transporte electrónico mitocondrial (CTEmt). Este sistema se encarga en los organismos aeróbicos de la producción metabólica de la energía necesaria para la vida. Básicamente, como se ha dicho, los macronutrientes (y también el alcohol) son oxidados mediante la pérdida de electrones, los cuales son aceptados por transportadores electrónicos que son oxidadas de nuevo por el oxígeno produciendo grandes cantidades de ATP.

Desde un punto de vista funcional, la CTEmt se compone principalmente de 5 complejos enzimáticos:

– Complejo I: NADH: ubiquinona oxidorreductasa.
– Complejo II: succinato: ubiquinona oxidorreductasa.
– Complejo III: ubiquinol: ferrocitocromo c oxidorreductasa.
– Complejo IV: ferrocitocromo c: oxidorreductasa o citocromo c oxidasa (COX).
– Complejo V: ATP sintasa.

Debido a esta reducción gradual del oxígeno, el complejo proteico debe asegurar que el oxígeno parcialmente reducido, altamente tóxico, no sea liberado al medio antes de ser convertido en agua. El responsable en la generación y aumento en la generación de ERO durante el envejecimiento parece ser el complejo I, más que el complejo III, tal y como se ha confirmado en diversos estudios. En cualquier caso, e independientemente del lugar preciso en el que se formen, muchas evidencias indican que lo que realmente dispara el proceso de envejecimiento es el aumento en la generación de ERO a nivel mitocondrial. De hecho, experimentalmente se ha observado un cambio progresivo a un entorno más prooxidante en muchas especies a medida que se envejece, pasándose de un escenario en el que hay un equilibrio redox a ese entorno más prooxidante en el que se va perdiendo progresivamente la capacidad para anular los ERO producidos durante la fisiología normal del individuo. Se produce, en suma, un desplazamiento en el estatus redox, lo cual podría ser uno de los primeros mecanismos moleculares que generan la aparición del envejecimiento, dado que esa situación puede alterar la actuación de muchas enzimas y vías metabólicas.

Además del aumento en la producción *in vivo* de ERO y de la alteración del equilibrio redox en los organismos viejos, una de las evidencias más comunes observadas por los investigadores es la fuerte correlación entre el envejecimiento y un aumento del daño oxidativo a diferentes tejidos del cuerpo en especies que van desde *Caenorhabditis elegans* a humanos. Así, estudios de daño asociado al estrés oxidativo durante el envejecimiento se han centrado en las modificaciones oxidativas de moléculas intracelulares, principalmente lípidos, proteínas y ADN. Muchos investigadores creen que este aumento de proteínas oxidadas es la consecuencia del aumento en la producción de ERO y de alteraciones en el recambio proteico.

La mitocondria cuenta con un genoma propio que es diferente en estructura y organización al nuclear. Se trata de un número variable de copias idénticas de ADN de cadenas doble y

circular, localizado en la matriz mitocondrial, próximo a determinadas zonas de la membrana mitocondrial interna (precisamente el lugar de máxima generación de ERO). Su tamaño es pequeño y codifica para 13 proteínas mitocondriales, 7 de las subunidades del complejo I, una proteína del complejo III, 3 del complejo IV, 2 del complejo V (la ATPasa), 22 ARN de transferencia y dos ARN ribosómicos. A diferencia del ADN nuclear, el mitocondrial no está protegido por histonas y tradicionalmente ha sido considerado de una elevada susceptibilidad a ser atacado oxidativamente. Durante mucho tiempo se pensó que la mitocondria carecía de un sistema de reparación de ADN. En la actualidad se reconoce la existencia de un sistema de reparación del ADN mitocondrial (ADNmt) que, aunque todavía es un gran desconocido, se sabe que es capaz de reparar el daño oxidativo (por ejemplo, daño a bases y roturas de una sola cadena). Además, se sabe que la vía de reparación de excisión de bases juega un papel predominante dentro del sistema de reparación de ADNmt.

El envejecimiento es uno de los procesos biológicos más complejos, determinado por factores genéticos y ambientales. Existen estudios en diversos organismos, desde los de tipo unicelular (levaduras) a vertebrados superiores, incluidos mamíferos como el ratón, en los que se observa que determinadas mutaciones genéticas (de un solo gen en muchos casos) alargan o acortan el período vital, y asimismo mejoran la calidad de su expectativa de vida. Hay estudios en los que se proponen posibles genes o grupos de éstos como candidatos a ser responsables de la longevidad en la especie humana, como son los genes TOR, RAS, LAG o SIR. En la actualidad, ya comienza a haber muchos datos disponibles procedentes de los modelos experimentales en relación a la genética del envejecimiento. Sin embargo, en el caso del ser humano, la situación es mucho más compleja, dado que el envejecimiento se debe a la interacción entre factores genéticos, epigenéticos, ambientales y culturales. Genes candidatos a ser estudiados son aquellos que participan en procesos inflamatorios y de respuesta inmune, como las interleucinas y el gen TNF-α, genes de respuesta al estrés, como la superóxido dismutasa y la paraxonasa, genes relacionados con la ruta de la insulina, genes relacionados con el ciclo celular y la apoptosis, como el p53, y genes del genoma mitocondrial.

El papel de la dieta en el envejecimiento, y en concreto en lo referente al tipo de grasa de la dieta, se centra en la posibilidad de construir membranas biológicas a la carta en función de un tipo específico de ácido graso, con objeto de modular la forma y velocidad a la que un órgano específico envejece representa un enfoque muy novedoso en el estudio del envejecimiento. El tipo de grasa de la dieta condiciona de manera importante numerosos parámetros bioquímicos en la membrana mitocondrial. La importancia del tipo de ácidos grasos de la dieta reside en el hecho de que la membrana mitocondrial (y en general todas las membranas biológicas) es capaz de adaptar la composición de sus fosfolípidos a la grasa ingerida de forma mayoritaria. De este modo, si un individuo ingiere mayoritariamente grasa de origen animal, sus membranas serán más ricas en ácidos grasos saturados que las de otro individuo cuya fuente grasa mayoritaria sea de origen vegetal.

Nuestra hipótesis es que las alteraciones asociadas a la estructura, la función y la susceptibilidad al estrés oxidativo en el hígado de ratas durante el proceso de envejecimiento pueden ser diferencialmente modulados por la alimentación de los animales durante toda la vida con diferentes grasas de la dieta (aceites de oliva virgen, girasol o pescado), en un patrón normolipídico (4% w / w). Las diferencias observadas con estas fuentes de grasa de la dieta se pueden explicar, al menos en parte, por los cambios producidos en el perfil de expresión génica en el hígado como respuesta a la interacción entre el envejecimiento y el tipo de grasa de la dieta.

De acuerdo con esta hipótesis, el principal objetivo de este trabajo ha sido estudiar el proceso de envejecimiento en el hígado de ratas alimentadas durante toda la vida con diferentes

fuentes grasas, en concreto aceite de oliva virgen, aceite de girasol y aceite de pescado. El estudio se ha llevado a cabo comparando los animales jóvenes (6 meses) con los viejos (24 meses).

Este objetivo principal ha sido desarrollado a través de los siguientes objetivos específicos:

1. determinar los efectos de la grasa de la dieta y el envejecimiento sobre aspectos relacionados con la histopatología y la ultraestructura hepáticas.

2. comparar cómo afectan diferentes fuentes grasas al estrés oxidativo mitocondrial a través de cambios en el sistema antioxidante hepático, tanto en antioxidantes de bajo peso (Coenzima Q y α-tocoferol) como en antioxidantes enzimáticos endógenos, además de través del análisis de productos de la oxidación.

3. estudiar la función de las mitocondrias hepáticas en respuesta a la adaptación del perfil lipídico de sus membranas, en animales jóvenes y viejos, en relación a la dieta.

4. identificar mecanismos que permitan explicar los cambios encontrados anteriormente descritos a través del análisis de la expresión génica.

Para ello, 72 ratas macho Wistar (*Ratus norvegicus*), con un peso inicial de 80-90 g, fueron aleatoriamente asignadas a tres grupos experimentales y alimentadas desde el destete hasta los 24 meses de edad con una dieta semi-sintética e isoenergética, de acuerdo con los criterios AIN93, pero modificada en relación a la fuente de grasa dietética. Una dieta AIN-93G modificada fue administrada a las ratas desde el destete hasta los dos meses de edad, y la AIN-93M durante el resto del experimento. Como se ha mencionado, las dietas AIN-93 fueron modificadas en cuanto a la fuente de grasa, sustituyendo el aceite de soja por los tres aceites de nuestro estudio: oliva virgen, girasol y pescado. La dieta fue administrada *ad libitum* durante los primeros dos meses, y después 25 gramos/rata/día el resto del experimento. Los animales tuvieron libre acceso al agua. La ingesta fuer indirectamente monitorizada mediante el control diario de la dieta sobrante y un control del peso semanal. En los dos puntos de edad del estudio 6 y 24 meses, se sacrificaron ratas por dislocación cervical seguida de decapitación. La sangre fue recogida, el plasma almacenado adecuadamente y el hígado extraído y preservado convenientemente para su estudio, tanto el tejido completo, como extracciones de mitocondrias hepáticas y cortes histológicos. Parte del tejido fue especialmente preservado para poder ser empleado en estudios de análisis de ARN.

Los siguientes análisis fueron llevados a cabo con el fin de obtener respuestas a los objetivos planteados:

1. Análisis del perfil de ácidos grasos de las membranas mitocondriales.
2. Análisis histopatológico del hígado.
3. Análisis ultraestructural de las mitocondrias.
4. Análisis de proteínas seleccionadas mediante Wester Blot.
5. Determinación de proteínas carbonilo.
6. Medida de la actividad de la Glutation peroxidasa y catalasa.
7. Determinación de coenzima Q_9, Q_{10} y de α-tocoferol.
8. Medida de la eficacia de la respiración de las mitocondrias hepáticas.
9. Medida de la actividad del complejo I de la cadena de transporte electrónica mitocondrial.
10. Análisis de expresión génica mediante microarray de ADN y análisis funcional mediante herramientas bioinformáticas.
11. Validación del microarray de ADN mediante técnicas de RT-PCR.

Después de los pertinentes tratamientos estadísticos de los resultados, se ha observado que, en relación al peso de los animales, no hay diferencias significativas entre las ratas alimentadas

con una u otra fuente grasa a 6 o 24 meses. Sin embargo, el peso del hígado y el ratio hígado-cerebro fue mayor en ratas viejas alimentadas con aceite de pescado con respecto a aquellas alimentadas con oliva virgen o girasol. El efecto del envejecimiento sobre el peso de los animales, el peso del hígado y el ratio hígado-cerebro mostraron aumentos significativos a los 24 meses para las tres grasas estudiadas.

Hubo una correcta adaptación de las membranas mitocondriales al tipo de grasa de la dieta puesto que las membranas mitocondriales de los animales alimentados con aceite de girasol mostraban una mayor cantidad de ácido linoleico, y las membranas de aquellos animales alimentados con aceite de pescado una mayor cantidad de EPA y DHA (ácidos grasos poliinsaturados n3). En el caso del análisis histopatológico del hígado, los resultados apoyan la idea de que la ingesta durante toda la vida de un tipo de grasa dietética en un patrón normolipídico afecta diferentemente a la morfología hepática, puesto que un mayor grado de esteatosis, NASH, inflamación y fibrosis fue observado en ratas viejas con respecto a las jóvenes. Además el tipo de grasa de la dieta también debe ser tomado en consideración, puesto que según dichos resultados, la ingesta de aceite de pescado provoca en el hígado una mayor tendencia a la esteatosis, NASH e inflamación. En el caso de la ingesta con aceite de girasol, mayores niveles de fibrosis fueron hallados en el hígado de ratas viejas.

Con respecto a la ultraestructura y biogénesis mitocondrial, mayores niveles de TFAM, uno de los marcadores específicos de biogénesis, fueron cuantificados en ratas alimentadas con aceite de oliva virgen y de girasol a los 24 meses con respecto a 6 meses. Mientras que en aquellas ratas viejas alimentadas con aceite de pescado, fueron cuantificados menores niveles de este factor de transcripción. Siguiendo con la investigación de los efectos de la grasa de la dieta sobre la ultraestructura, se observaron cambios con respecto a la edad en el área y perímetros mitocondriales, que fueron mayores en animales alimentados con aceite de girasol, mientras que la densidad mitocondrial fue mayor en aquellas ratas alimentadas con aceite de oliva virgen. El estudio del estado de estrés oxidativo se abordó desde distintas vías. Por un lado, se cuantificaron marcadores de estrés oxidativo como son las proteínas carbonilo. Y por otro se analizaron los sistemas antioxidantes tanto enzimáticos como no enzimáticos. En cuanto a las primeras, mayores cantidades de proteínas carboniladas fueron encontradas en ratas viejas con respecto a las jóvenes en los grupos de aceite de girasol y de pescado. Diferencias entre el tipo de grasa de la dieta también fueron detectadas en ratas viejas, donde el grupo de aceite de oliva virgen mostró los menos niveles de proteínas carbonilo. Con respecto a los sistemas antioxidantes enzimáticos, fueron analizadas las enzimas GPX, Se-GPX y catalasa. Para GPX, una mayor concentración fue determinada en ratas viejas que en jóvenes para los tres grupos de edad. Las diferencias entre el tipo de grasa de la dieta están observadas en las ratas alimentadas con aceite de pescado, que revelaron una mayor cantidad de enzima que las otras dos grasas tanto a 6 como a 24 meses. Con respecto a Se-PX, en relación al tipo de grasa de la dieta, tan sólo en ratas jóvenes fueron detectadas menores cantidades de enzima en ratas alimentadas con aceite de oliva virgen con respecto a las otras dos. Finalmente para catalasa, diferencias con respecto a la edad o al tipo de la dieta sólo fueron observadas en el grupo de aceite de pescado, cuya concentración de enzima aumentó con la edad, y aumentó con respecto al grupo de aceite de oliva virgen a los 24 meses de edad. Examinando los sistemas antioxidantes no enzimáticos, no fueron observadas diferencias para α-tocoferol. Sin embargo, un aumento significativo de CoQ fue detectado en los extractos mitocondriales procedentes de ratas viejas, con respecto a las jóvenes, en los tres grupos experimentales. A los 6 meses de edad, sólo los animales alimentados con aceite de pescado vieron incrementado su ratio entre CoQ_{10} y CoQ_9. Sin embargo este ratio fue menor a los 24 meses en el grupo alimentado con aceite de girasol con respecto al grupo alimentado con aceite de pescado. En general, menores cantidades de

diferentes formas de CoQ fueron halladas en las ratas alimentadas con aceite de pescado que en el grupo de girasol.

La función mitocondrial ha sido evaluada en este estudio a partir de la cuantificación del consumo de oxígeno de mitocondrias aisladas y de la actividad del complejo I de la CTEmt. De acuerdo con el envejecimiento, las ratas viejas alimentadas con aceite de oliva virgen y de girasol mostraron mayor actividad del complejo I que las ratas jóvenes. Según el tipo de grasa de la dieta, ratas viejas alimentadas con aceite de pescado presentaron menor actividad del complejo I que aquellas alimentadas con las otras dos grasas. La medida del consumo de oxígeno indicó diferencias entre los grupos experimentales a los 6 meses, donde los animales alimentados con aceite de oliva virgen registraron menos consumo que aquellos alimentados con girasol o pescado. A los 24 meses, fueron las ratas alimentadas con aceite de pescado las que presentaron menores valores de respiración que aquellas alimentadas con aceite de girasol. El efecto del envejecimiento sólo fue observado en ratas de los grupos de aceite de oliva virgen y de girasol, donde hubo mayores consumos de oxígeno con la edad.

Para intentar dar una explicación mecanística a todas estas observaciones se llevó a cabo un análisis de expresión génica mediante un microarray de ADN de Affymetrix®, usando el chip *RAE230_2.0* con 31099 sondas, en 3 muestras de cada grupo de 6 animales (18 muestras de hígado de rata en total). De esas 31099 sondas, fueron detectados cambios de expresión de las ratas viejas con respecto a las jóvenes (efecto del envejecimiento) en 9621 secuencias, que tras varios procesos de estandarización y normalización resultaron en 951 significativamente expresadas de forma distinta entre animales viejos con respecto a los jóvenes. De esas 951, 70 estaban presentes en las tres grasas mientras que 881 aparecían diferentemente expresadas en una o dos de las grasas, sufriendo por tanto el efecto tanto del envejecimiento como del tipo de grasa de la dieta. El análisis funcional y de rutas mediante la aplicación Ingenuity Pathways Analysis (IPA) (Ingenuity SystemsH, http://www.ingenuity.com/) ofreció una clasificación de esos genes dentro de rutas canónicas, que determinan los procesos metabólicos y de señalización celular significativamente más afectados por los genes de nuestra base de datos. De las 82 rutas seleccionadas por IPA, fueron seleccionadas y estudiadas en profundidad aquellas con mayor relevancia y más interesantes para el estudio, como son la ruta de la disfunción mitocondrial, la ruta de respuesta al estrés oxidativo mediada por Nrf2, rutas implicadas en el ciclo celular, rutas mediadoras de la apoptosis así como las relacionadas con la preservación de la longitud de los telómeros.

Como conclusión general del estudio se puede indicar que la alimentación con diferentes fuentes grasas insaturadas en forma normolipídica no induce cambios en el hígado durante la fase joven de la vida. Sin embargo, las dietas ensayadas a base de ácidos grasos insaturados llevaron a una acumulación de lípidos durante el envejecimiento en el hígado de las ratas. Las tres grasas estudiadas, pese al aumento similar en la cantidad de lípidos en el hígado, han dado lugar a numerosas diferencias en lo referente a la morfología del hígado, a la ultraestructura, y también en relación al nivel de estrés oxidativo y a la función mitocondrial. Algunos de esos cambios pueden ser asociados con variaciones en el perfil de expresión génica en el hígado de esos animales. Teniendo en cuenta todas las variables estudiadas, el aceite de oliva virgen puede ser considerado como la fuente grasa de la dieta que mejor preserva el hígado durante el proceso de envejecimiento. El aceite de pescado, a pesar de mostrar una supervivencia similar a la observada con el aceite de oliva virgen, da lugar a altos niveles de estrés oxidativo y una tendencia proinflamatoria del hígado. Esto, junto con un extraño comportamiento a nivel de la actividad de la cadena de transporte electrónico mitocondrial, podría suponer un problema para los animales alimentados con aceite de pescado en caso de que de forma paralela al envejecimiento se presentase un estrés adicional, en forma de patología, etc. Finalmente, el

aceite de girasol debe ser evitado como base de un patrón dietético prolongado, ya que para con esta grasa se encontraron los peores resultados durante el envejecimiento a nivel hepático, lo cual además fue seguido de la menor tasa de supervivencia en estos animales.

GENERAL INDEX Page

ABBREVATIONS

8-OH-dG: 8-hydroxydeoxyguanine
8-oxoG: 8-oxoguanine
AA: arachidonic acid
ADP: adenosine diphosphate
ALA: α-linoleic acid
AMPK: AMP-activated protein kinase
AP: apurinic/apyrimidinic
AP-1: activating protein-1
ARE: antioxidant response elements
BER: base excision repair
BSA: bovine serum albumin
CAT: catalase
CCO: cytochrome c oxidase
cDNA: complementary DNA
CETP: cholesteryl esters transfer protein
CGED: Cancer Gene Expression Database
CoQ: coenzyme Q
CoQ_{10}: coenzyme Q_{10}
$CoQ_{10}H_2$: reduced coenzyme Q_{10}
CoQ_9: coenzyme Q_9
CoQ_9H_2: reduced coenzyme Q_9
CR: caloric restriction
CREB: cAMP response element binding protein
CRM: caloric restriction mimetics
CRM: caloric restriction mimetics
cRNA: complementary RNA
CYP7A1: cholesterol 7α-hydroxylase
DDBJ: DNA Database of Japan
DGLA: dihomo-γ-linolenic
DHA: docosahexaenoic acid
DHODH: dihydroorate dehydrogenase
DMSO: dimethyl sulfoxide
DNPH: dinitrophenylhydrazine
EBI: Nucleotide Sequence Database at European Bioinformatics Institute
EDTA: ethylenediaminetetraacetic acid
EFA: essential fatty acids
Egr-1: early growth response protein-1
EMAP: The e-Mouse Atlas Project
EMBL: European Molecular Biology Laboratory
ENCODE: Encyclopedia of DNA Elements
EPA: eicosapentaenoic acid
ERKs: extracellular signal-regulated kinases
ETC: electronic transport chain
FAD: flavin adenine dinucleotide
FAD: flavin adenine dinucleotide
$FADH_2$: reduced flavin adenine dinucleotide
$FADH_2$: reduced flavine mononucleotide
FADS: fatty acid desaturase
FC: fold change
FCCP: carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FDR: false discovery rate
FFA: free fatty acids
FGDS: Functional Genomics Data Society
FMN: flavin mononucleotide
$FMNH_2$: reduced flavin mononucleotide
FO: fish oil
FRL: free radical leak
FWER: familywise error rate
GEO: Gene Expresión Omnibus
GPDH: glucose 6-phosphate dehydrogenase
GPx: glutathione peroxidase
GR: glutathione reductase
GRP78: glucose regulated protein 78
GSH: reduced glutathione
GSSG: oxidized glutathione
H&E: hematoxylin and eosin
H_2O_2: hydrogen peroxide
H3K27me3: trimethylated lysine 27 of histone H3
H3K4me3: trimethylated lysine 4 of histone H3
HDL: high-density lipoprotein
HPLC: High-performance liquid chromatography
IGF-1: insulin-like growth factor-1
IPA: Ingenuity Pathways Analysis
IU: international unit
KGDHC: alpha-ketoglutarate dehydrogenase complex
LAG: longevity-assurance genes
LC-PUFA: long chain PUFA
LDL: low-density lipoprotein
MAPKs: mitogen-activated protein kinases
MAS: mitochondrial assay solution
MES: morpholino ethanesulfonic acid

MetR: methionine restriction
Met-SO: methionine sulphoxide
mtDNA: mitochondrial DNA
mtETC: mitochondrial electron transport chain
mTOR: mammalian target of rapamycin
MTP: mitocrosomal transfer protein
mtROS: mitochondrial ROS
MUFA: monounsaturated fatty acids
MUGEN: Mutant Mouse Models Database
NAFLD: Non-alcoholic fatty liver disease
NASH: Non-alcoholic steatohepatitis
NCBI: National Center for Biotechnology information
NF-Y: nuclear factor-Y
NFκB: kappa nuclear factor B
NQO1: NADPH:quinone oxidoreductase 1
NTB: nitrotetrazolium blue
NTBH: reduced nitrotetrazolium blue
NTP: nucleoside triphosphate
O_2: oxygen
$O_2^{\cdot -}$: superoxide anion
OCR: oxygen consumption ratio
OGG1: 8-oxoguanine-DNA glycosylase
OXPHO: oxidative phosphorylation
PCR: polymerase chain reaction
PDB: Protein data Bank
PDBe: Protein Data Bank in Europe
PDBTM: Protein Data Bank of Transmembrane Proteins
PEPCK: phosphoenolpyruvate carboxy kinase
PGC1α: peroxisome proliferator-activated receptor-γ coactivator 1α
PGE2: prostaglandin E2
PIR: Protein Information Resource
pO_2: oxygen partial pressure
POLγ: mitochondrial DNA polymerase
PON1: paraoxonase
PPARα: peroxisome proliferator activator receptors alpha
PPARβ: peroxisome proliferator activator receptors beta
PPARγ: peroxisome proliferator activator receptors gamma
ProtCID: Protein Common Interface Database
PRXs: peroxiredoxins
PUFA: polyunsaturated fatty acids
PVDF: polyvinylidene fluoride membrane
qPCR: quantitative PCR
RARs: retinoic acid receptors
RIN: RNA integrity number
ROS: reactive oxygen species
RT-PCR: real time polymerase chain reaction
RXRs: retinoic X receptors
SASP: senescence associated secretory phenotype
SBMD: Systems Biology and Medicine Database
SCOP: Structural Classification of Proteins
SDH: succinate dehydrogenase
Se-GPX: selenium-dependent glutathione peroxidase
SEM: standard error of the mean
SFA: saturated fatty acids
SGD: Saccharomyces Genome Database
SLR: signal log ratio
SO: sunflower oil
SOD: superoxide dismutase
SREBP: sterol regulatory element binding proteins
STACs: sirtuins-activating compounds
TBARS: thiobarbituric acid reactive substances
TBP: tributylphosphine
TBS: tris buffered saline
TBS-T: tris-buffered saline and tween 20
TFAM: Mitochondrial transcription factor A
TMPD: N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine
TOPSAN: Open Protein Structure Annotation Network
TRX: thioredoxins
Tyr: tyrosine
U/mg: units per milligram
VLDL: very low-density lipoprotein
VOO: virgin olive oil
Ψ_{mt}: mitochondrial membrane potential

ABSTRACT

Aging, usually defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality, has aroused great interest in recent decades, due to social and scientific conditions. It is itself one of the most complex biological processes, determined by genetic and environmental factors. It has been estimated that 25% of the maximum lifespan is due to genetic factors and 75% to environmental and lifestyle factors. In the last years interest in this process has been growing as they have grown the theories that try to explain this process. One of the most widely accepted is the free radical theory which defends that free radicals produced at the mitochondria damage all biological tissues and lead to the aging phenotype. In fact, there is a direct relationship between accumulation of oxidative damage produced by reactive oxygen species and the mitochondrial alterations associated with aging.

Nutrition has been related to aging mainly from the point of view of caloric restriction and antioxidant supplementation. More recently dietary fat in relation to oxidative stress and anti-aging therapy has been studied. A new approach relates the dietary fat type to mitochondria and their sensitivity to experiencing oxidative stress because membranes adapt their lipid composition in response to dietary fat. The fatty acid composition of mitochondrial membranes affects in a direct way the properties of these membranes such as fluidity and permeability, which determines its susceptibility to oxidation, thus altering the susceptibility to oxidative stress-related phenomena. In addition, dietary fat type can modulate the age-related lipids accumulation at the liver, affecting the way and intensity in which this organ would age, through alterations in its histopathology or mitochondrial ultrastructure.

During aging, a progressive reduction in the response capacity to physiological needs occurs, reportedly due in part to changes in gene expression. Although evidences demonstrate that dietary fat may condition the aging process there is still little information about the specific mechanisms through which such role is exerted. Nevertheless, it begins to be clear that nutritional factors have a profound influence on gene expression. In this way, the application of massive-screening genomic tools, such as microarrays, and subsequent analysis with functional enrichment tools, offers new opportunities to investigate the role of dietary fat on the biology of aging from a deeper inside.

To investigate the role of dietary fat types on aging at the liver, male Wistar rats were fed lifelong on normolipidic diets which differed only in the fat source. Virgin olive, sunflower and fish oils were studied. Animals were sacrificed at 6 and at 24 months and different parameters related to liver histopathology, mitochondrial ultrastructure, oxidative stress status, mitochondrial electron transport chain activity as well as the associated gene expression profiles were studied.

As a general conclusion, normolipidic feeding on different unsaturated dietary fat sources does not induce changes at the liver during the youth life. However, any of the assayed diets based on unsaturated fatty acids, led to an age-related lipid accumulation in the liver of rats. Differences between three dietary fat sources, namely virgin olive, sunflower or fish oils, have been described concerning liver morphology, ultrastructure, as well as in relation to oxidative stress status and mitochondrial function. Some of these changes can be associated with observed variations in the gene expression profile at the liver of these animals. Taking into consideration all the studied variables, virgin olive oil, can be considered as the dietary fat source which better preserves liver during the aging process. Fish oil, despite to provide the same degree of survival than virgin olive oil, led to higher levels of oxidative stress and a preoccupant trend to inflammation at the liver. That, together with a strange behavior at the mtETC activity level might be deleterious for the animals under situations of potential additional stress situations,

apart from the aging itself. Finally, sunflower oil should be avoided as the basis for a lifelong dietary pattern, since the worse finding at the liver, as well as the lowest survival rate, were found for this dietary fat.

El envejecimiento, por lo general, puede ser definido como la pérdida progresiva de la función acompañada por la disminución de la fecundidad y el aumento de la mortalidad. Este fenómeno ha despertado un gran interés en las últimas décadas, debido a sus condicionantes sociales y científicas. Es en sí mismo uno de los procesos biológicos más complejos, determinado por factores genéticos y ambientales. Se ha estimado que el 25 % de la máxima vida se debe a factores genéticos y el 75% a los factores ambientales y de estilo de vida. En los últimos años el interés en el envejecimiento ha ido creciendo a medida que han crecido las teorías que tratan de explicarlo. Una de las teorías más ampliamente aceptada es la teoría de los radicales libres que defiende que los radicales libres producidos en la mitocondria dañan todos los tejidos biológicos y conducen al fenotipo de envejecimiento. De hecho, existe una relación directa entre la acumulación de daño oxidativo producido por las especies reactivas de oxígeno y las alteraciones fisiológicas asociadas con el envejecimiento.

La nutrición ha sido relacionada con el envejecimiento, principalmente desde el punto de vista de la restricción calórica y la suplementación antioxidante. Más recientemente se ha estudiado la relación de la grasa de la dieta con el estrés oxidativo y las terapias antienvejecimiento. En un nuevo enfoque la grasa es relacionada con la mitocondria y su sensibilidad a sufrir el estrés oxidativo debido a que las membranas mitocondriales son capaces de adaptar su composición lipídica en respuesta al tipo de grasa de la dieta. La composición de ácidos grasos de las membranas mitocondriales afecta de manera directa a las propiedades de estas membranas, tales como la fluidez y la permeabilidad, lo cual determina su susceptibilidad a la oxidación, alterando de este modo la susceptibilidad a los fenómenos relacionados con el estrés oxidativo. Además, el tipo de grasa de la dieta puede modular la acumulación de lípidos debidos al envejecimiento en el hígado. Esto produce alteraciones en su histopatología y su ultraestructura mitocondrial, lo que podría afectar la manera y la intensidad a la que éste órgano envejecería.

Durante el envejecimiento, una reducción progresiva de la capacidad de respuesta a las necesidades fisiológicas se produce, al parecer debido en parte a los cambios en la expresión génica. Aunque las evidencias demuestran que la dieta puede condicionar el proceso de envejecimiento aún hay poca información acerca de los mecanismos específicos mediante los cuales se ejerce tal función. Sin embargo, empieza a quedar claro que los factores nutricionales tienen una profunda influencia sobre la expresión génica. De esta manera, la aplicación de herramientas genómicas masivas de detección, tales como microarrays de ADN, ofrece nuevas oportunidades para investigar el papel de la dieta en la biología del envejecimiento desde un interior más profundo.

Para investigar el papel del tipo de grasa de la dieta en el envejecimiento en el hígado, ratas Wistar fueron alimentadas durante toda la vida con dietas normolipídicas que se diferenciaban entre ellas únicamente en la fuente de grasa. Aceite de oliva virgen, aceite de girasol y aceite de pescado fueron estudiados. Los animales fueron sacrificados a los 6 y 24 meses y fueron estudiados diferentes parámetros relacionados con la histopatología del hígado, la ultraestructura mitocondrial, el nivel de estrés oxidativo, la actividad de la cadena de transporte electrónico, así como los perfiles de expresión génica asociados.

Como conclusión general del estudio se puede indicar que la alimentación con diferentes fuentes grasas insaturadas en forma normolipídica no induce cambios en el hígado durante la fase joven de la vida. Sin embargo, las dietas ensayadas a base de ácidos grasos insaturados llevaron a una acumulación de lípidos durante el envejecimiento en el hígado de las ratas. Las tres grasas estudiadas, pese al aumento similar en la cantidad de lípidos en el hígado, han dado lugar a numerosas diferencias en lo referente a la morfología del hígado, a la ultraestructura, y también en relación al nivel de estrés oxidativo y a la función mitocondrial. Algunos de esos

cambios pueden ser asociados con variaciones en el perfil de expresión génica en el hígado de esos animales. Teniendo en cuenta todas las variables estudiadas, el aceite de oliva virgen puede ser considerado como la fuente grasa de la dieta que mejor preserva el hígado durante el proceso de envejecimiento. El aceite de pescado, a pesar de mostrar una supervivencia similar a la observada con el aceite de oliva virgen, da lugar a altos niveles de estrés oxidativo y una tendencia proinflamatoria del hígado. Esto, junto con un extraño comportamiento a nivel de la actividad de la cadena de transporte electrónico mitocondrial, podría suponer un problema para los animales alimentados con aceite de pescado en caso de que de forma paralela al envejecimiento se presentase un estrés adicional, en forma de patología, etc. Finalmente, el aceite de girasol debe ser evitado como base de un patrón dietético prolongado, ya que para con esta grasa se encontraron los peores resultados durante el envejecimiento a nivel hepático, lo cual además fue seguido de la menor tasa de supervivencia en estos animales.

HYPOTHESIS

Our **hypothesis** is that alterations associated to the structure, function and oxidative stress susceptibility at the liver of rats during the aging process can be differentially modulated by feeding animals lifelong on different dietary fats (virgin olive, sunflower or fish oils), in a normolipidic pattern (4% w/w). Differences observed with these dietary fat sources may be explained, at least in part, by the changes produced in the gene expression profile at the liver as a response to the interaction between aging and dietary fat type.

OBJECTIVES

According to the above mentioned hypothesis, the **main objective** of this work has been to study the aging process in the liver of rats fed lifelong on different dietary fats, namely, virgin olive, sunflower or fish oils. The study has been performed comparing young (6 months) *versus* old (24 months) animals.

This main objective has been carried out through the following **specific objectives**.

1. To determine the effects of dietary fat and aging on histopathological and ultrastructural features at the liver.

2. Compare how different fat sources affect mitochondrial oxidative stress status through changes in hepatic antioxidant system, both low weight antioxidants (Coenzyme Q and α-tocopherol) and enzymes endogenous antioxidants, as well as through the analysis of oxidation products.

3. To study the function of liver mitochondria by analyzing mitochondrial electron transport chain activity variations in response to dietary fat treatments in young and old animals.

4. To identify mechanisms that allow explaining observed changes related to the consecution of the previously described objectives by the analysis of gene expression profile.

INTRODUCTION

CHAPTER I: AGING

1. CONCEPT AND PRINCIPES OF AGING

Aging is considered an endogenous process characterized by the progressive loss of function with decreased fertility. This decline in the effectiveness of physiological processes after the reproductive phase of life due to accumulation of damage in molecules, cells and tissues over a lifetime, often decreases an organism's capacity to maintain homeostasis in stress conditions, and implies a greater risk for many diseases (cancer, cardiovascular and neurodegenerative disorders) and premature mortality (Camougrand and Rigoulet, 2001; Sohal *et al.*, 2002; Vijg and Suh, 2005; Fraga *et al.*, 2007; Quiles *et al.*, 2010a; Rodríguez-Rodero *et al.*, 2011; Barja, 2013; López-Otín, 2013).

All the different species of living organisms have the so-called maximum lifespan, which is defined as the maximum age reached by an individual of the specie. In humans, maximum lifespan has been established between 110 and 120 years. Currently a minimal part of humans are capable of achieving the maximum age. Meanwhile, life expectancy is the expected number of years of life remaining at a given age. Although maximum life span and life expectancy are different concept, they are fairly similar numerically in most developed countries (Fontana *et al.*, 2010; Mercken *et al.*, 2012; Capitaine *et al*., 2013; Steensma *et al*., 2013).

The interest in aging has grown enormously in recent decades, due to social and scientific conditions. It is one of the most complex biological processes determined by genetic and environmental factors. It is estimated that 25% of the maximum life span is due to genetic factors and 75% to other types of environmental and lifestyle habits. During aging, there is a progressive reduction in the response capacity to physiological needs, due in part to changes in gene expression (Rodríguez-Rodero *et al.*, 2011; Gems and Partridge, 2013).

Many hypotheses have been developed and tested to explain the aging process at the organismal, organ, cellular and molecular levels (Berman *et al*., 2012; Cosentino and Mostoslavsky, 2013; Vo *et al.*, 2013; Wang *et al.*, 2013a). As it is known, the genome determines life expectancy and even mutations in single genes can seriously affect the aging process, especially if they affect DNA repair genes or telomere length. However, not all events seen in aging can be explained by the information carried by the DNA sequence. Epigenetic marks, which control gene expression through changes beyond the DNA sequence, could provide a molecular explanation for the aging process. The progressive loss of cell functions could be due to a genetic program present in all the individuals or else to the stochastic accumulation of errors in the somatic cells. So, the discovery that the rate of aging is controlled, at least to some extent, by genetic pathways and biochemical processes conserved in evolution has involved an advance in aging research (Camougrand and Rose, 1991; Camougrand and Rigoulet, 2001; Fraga, 2009; Huidobro, 2013). In addition other possible triggers of the aging process have been defined, common in different organisms, especially in mammalian aging. López-Otín *et al.* have called "the hallmarks of aging": genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication (Kowald and Kirkwood, 2013, López-Otín *et al.*, 2013). In the study of aging it is important to considerate the added difficult to separate the process itself from diseases related to aging (Quiles *et al.*, 2004a; Ochoa *et al.*, 2011).

Different disciplines, from sociology to medicine, have studied the determinants of longevity (De Benedictis *et al.*, 2001; Capri, 2007). As stated above, life expectancy is affected by genetic as well as environmental factors, in addition to the stochastic component resulting from the interaction between individual changes for survival and unpredictable events that occur

over the course of a lifetime (De Benedictis *et al.*, 2001; Lai and Chan, 2011). In more advanced societies there are problems. Since the eighteenth century, life expectancy has grown at a rate of approximately three months per year; however, the cost of health has also grown and previously unknown diseases have appeared, such as Alzheimer's, cardiovascular diseases, etc. So, it can be said that the average lifespan has lengthened in developed countries with respect to undeveloped ones, although the maximum duration of life appears to be the same (Halliwell and Gutteridge, 1999; Partridge and Gems, 2002; McKeown, 2009).

If aging is deleterious for individuals and it exists all over the world, why does it happen? The answer to that question might be that aging is the side effect of something else. Thus, the genes that delay aging could do that through the repression of the causes that generates the damage associated to aging. Reproduction appears to be a source of this type of damage. Thus when aging decreases, fertility is frequently reduced. Food could be another source of damage associated to aging due to many genes that participate in the response to the change in nutrient levels. Furthermore, it is know that the reduction in the intake of food diminishes aging in a variety of organisms from yeast to mammals (Partridge and Gems, 2002; Quiles *et al.*, 2004a; Masoro, 2005, Barzilai *et al.*, 2012).

2. AGING THEORIES

The molecular basis of the age-related decline in organismal physiology is not well understood yet. A good evidence about the great difficulty to establish mechanisms operating in an organism to become aged is the large number of theories enunciated during the recent history of the research on biology trying to explain this process. More than 300 hypotheses have been proposed up to date (Medvedev, 1990, Pérez and Sierra, 2009, Cefalu, 2011). However any one of the "single cause" theories is able to explain the full complexity of this process in all living organisms (Sacher, 1980). Overall, any theory must explain, according to Sanz *et al.* (2006a) some characteristics of aging: its progressive, endogenous, irreversible and deleterious effect for the individual. It has defended that any theory should be able to (Sohal *et al*., 2002; Sohal and Orr, 2012):

- To explain how an organism loses the capacity of maintaining its homeostasis to the end of its life.
- To clarify the bases for the wide variations in the duration of life in the species.
- To identify the factors responsible for the extending of life by genetic mutations or through experimental variations such as caloric restriction in rodents or environmental temperature changes in poykilotherms.
- To demonstrate how variations in the factors suspected of causing senescence can alter the degree of aging.

We can group the existing theories on aging into three blocks: stochastic, evolutive and genetic theories.

2.1. Stochastic theories

These theories propose that a random damage accumulates over time in different biological molecules until reaching a level where the damage triggers the physiological decline known as aging.

In 1928 Pearl enunciated "The rate-of-living theory", based on the observation that species with high metabolic rates had shorter lives, stating that life expectancy is inversely proportional to the metabolic rate of the species. Harman (1956, 2006 and 2009) explained the biochemical

link between metabolism and longevity, which was unknown. He proposed "The theory of free radicals", which became one of the most widely accepted theories on aging.

This theory suggests that normal aging is the result of random damage to tissues mediated by endogenous oxidants. Although the concept of endogenous oxidative initially resulted in controversy, the discovery in 1969 of the enzyme superoxide dismutase (SOD) (McCord and Fridovich, 1969, 2013) provided the basis of this theory, showing the first evidence of *in vivo* generation of the superoxide radical and the subsequent need by developing cell antioxidant defense (Yu, 1994).

According to this theory, an elevated metabolism leads to high free radical generation, accelerating aging and shortening longevity, a link between the Pearl hypothesis and the Harman theory. Later, Harman began to focus his theory towards the mitochondria as the major source of free radicals in the organisms and the target of their damage (Harman, 1972). Miquel (Miquel *et al.*, 1980) was based in that to propose the mitochondrial theory (progressive damage to mitochondrial DNA by reactive oxygen species, ROS). As at present many ROS are known not to be free radicals, today we talk about "oxidative stress theory of aging" (Gomez-Cabrera *et al.*, 2012).

The use of SOD as a tool to locate subcellular compartments superoxide anion ($O_2^{\cdot -}$) generation led to the conclusion that the mitochondria is the major source of oxidants in endogenous production (Chance *et al.*, 1979; Cadenas and Davies, 2000, Gomez-Cabrera *et al.*, 2012). Based on the idea that the mitochondria produce most of the energy consumed in the cell and accordingly consumes a large volume of intracellular oxygen (O_2), the free radical theory remained linked to the rate of aging hypothesis, formulated beginning of the century (Kapahi *et al.*, 1999; Flynn *et al.*, 2013).

The study of the oxidants production, antioxidants systems and oxidative stress led to the confirmation of the theory proposed by Harman: "free radicals produced during aerobic respiration cause accumulation of oxidative damage accelerating the aging process and cell death."

There is another group of stochastic theories, called "Theories of damage accumulation", that could be explained by the "oxidative stress theory" due to certain similarities (Halliwell and Guteridge, 1999, 2007). These include the "crossing-over theory" (the random crossing-over of proteins and DNA alter cell functioning) (Padhukasahasram and Rannala, 2011); the "theory of the catastrophic error" (accumulation of random damage in protein synthesis) (Edelmann and Gallant, 1997); the "theory of glycosylation" (the formation of glycosylate proteins give rise to a serious disruption of cell functions) (Tsamis *et al*., 2011); the "theory of longevity determinants" (aging is caused by the products of metabolism and the capacity to protect oneself against such products determines the degree of aging) (Hayflick, 2007); the "membrane hypothesis" (damage to the cell membrane diminishes the capacity to eliminate waste products, a decreased protein synthesis, a loss of water for the cytoplasm and diminishes enzyme activity) (Yu, 2005); the "neuroendocrine theory" (no part of the body can act in isolation from the nervous and endocrine systems. So, if one of these is disturbed, the other systems are also affected. This theory is not universal because not all organisms have nervous or endocrine systems but and despite this, get older) (Maldonado, 2013).

There is a group of theories based on mathematics and physics (Mangel, 2001). Overall, there are based in the fact that the young normal state is characterized by the existence of a large number of regulatory factors interacting in a chaotic way; while aging is characterized by the loss of complexity and the tendency to shift towards non chaotic dynamical systems, simpler, which ultimately leads to a loss of adaptive capacity of the organism. More than an explanatory theory, the utterance corresponds to a model based on the chaos theory. The "entropy theory"

explains that mechanisms, such as caloric restriction, reduce the degree of entropy production, releasing energy more slowly and delaying the deterioration molecular (Gutierrez-Robledo, 1998; 2011).

2.2. Evolutive theories

In the evolutive theories, such as the "theory of immediate survival," aging occurs because of natural selection of genes which are important for immediate survival, even if they are deleterious long-term (Kirkwood and Austrad, 2000; Kirkwood 2005). It should be noted evolutive and stochastic theories are not mutually exclusive. In fact, it seems clear that the intrinsic and extrinsic processes that lead to aging have been established, through evolution, to give a maximum lifespan in all species.

Another example of this group of theories is the "immunological theory" (Walford, 1969; Effros, 2005), where aging appears because of the decreasing in the capacity of immune system to produce antibodies: as the immune response diminishes, the capacity of the system to discriminate between its constituents and foreign elements also declines, with an increase in autoimmune reactions. This theory only can be applied to the immune system, and does not rule out the possibility that these changes might be secondary to other appearing earlier, such as hormonal alterations.

The "reproductive-cell cycle theory" (Bowen and Atwood, 2004; Atwood *et al.*, 2011) proposes that the hormones that regulate reproduction act in an antagonistic pleiotropic manner to control aging via cell cycle signaling; promoting growth and development early in life in order to achieve reproduction. The problem appears when into adulthood the attempt to maintain reproduction is deregulated and leads to senescence. This theory also proposes a new definition of aging including not only the changes associated with the loss of function (i.e. senescence, the commonly accepted definition of aging), but also the changes associated with the gain of function (growth and development).

2.3. Genetic theories

Two main branches can be considered concerning genetic theories of aging. One branch proposes that aging is programmed under the control of genes that intelligently order a series of changes in the cell, changes that ultimately lead to the decline and destruction. Its mechanism of action is based on damaging gene activation and / or deactivation of other beneficial for survival. According to these theories, aging is genetically programed, either because the original program is altered (the "somatic mutation theory") or because the cellular changes associated with aging are included in the instructions contained in DNA from conception, so they are part of normal development. Another possibility is that genes coding for the production of a protein with useful functions become harmful at an advanced age (Catania and Fairweather, 1991; Kennedy *et al.*, 2012).

The second branch is based on modifications or damage in the DNA, more or less randomly. The cells have repair mechanisms but they are not 100% efficient. The damage will occur due to the effect of free radicals on the genetic material either by DNA demethylation or decrease of telomeres length.

The genetic theories of aging in general propose that aging is the continuation of the development and differentiation process encoded in the genome (Finch and Ruvkun, 2001; Robert *et al.*, 2010). According to that, aging would be the late consequence of the expression of genes selected by evolution for increasing reproductive success. As the young are in change

of reproduction and genetic transmission, they manifest principally the selection of beneficial genes, without take into account what occurs to these genes at later ages. Thus, a gene that promotes reproduction but that is harmful in the long term will not be selected for deletion.

One of the theories proposed in this group was the "hypothesis of the disposable soma" (Kirkwood, 1977, 2005), which defends that the utilization of energy over a lifetime should be used preferentially for reproduction, and suggests that among the main candidates determining the life expectancy of a species from the genetic standpoint are genes that regulate the repair and maintenance of somatic cells. According to this hypothesis, natural selection favors those genes that act in the early stages of life to permit the species reproduction, against those ones that are in charge of preserving non-germinal cells or available soma (Kirkwood and Rose, 1991; Lithgow and Kirkwood, 1996; Kirkwood and Austad, 2000; Drenos and Kirkwood, 2005; Hammers *et al.*, 2013). Therefore, somatic lines from all animals (contrary to germ cells) are declining and degenerate with age causing the phenotypic changes recognize as aging (Guarente and Kenyon, 2000; Barzilai *et al.*, 2012). At cellular and molecular level, the available soma theory predicts that the effort to cellular maintenance and repair processes vary in direct proportion to longevity, and it is associated to an exceptional longevity with decreased reproduction (Tabatabaie *et al.*, 2011). In this sense, Drenos and Kirkwood (2005) described a mathematical model that can make predictions about the optimal strategies for investment in somatic maintenance versus investments in growth and reproduction, and confirms the central prediction of the disposable soma theory that the optimum investment in somatic maintenance is less than what would be required for indefinite longevity. They also described how the optimal investment in maintenance is affected by varying the parameters that specify the times of reproduction and mortality.

The "telomere theory of aging" (Olovnikov, 1973, 2007) proposed that the shortening of the telomeres in each of the cycles of cell division is the factor responsible for the limitation in the proliferation of the cell cultures, the "Hayflick limit" (Hayflick and Moorehead, 1961). According this theory, aging could be fighted braking or blocking the process that shorten the telomeres (Mikhelson, 2001; Mikhelson and Gamaley 2012).

In 1998, Hayflick proposed a new hypothesis according to which telomere shortening and subsequent loss of cellular replicative capacity only determine the duration of the life of each species while aging itself is determined by the accumulation of cell damage. This hypothesis is based on the following observations:

- The length of telomeres in cells from patients with premature aging (such as Hutchinson-Gilford and Werner syndrome), is initially shortened in correlation with visibly decreased Hayflick limit. Patients showed remarkable features of accelerated aging at an early age compared with healthy individuals.
- Cell telomeres from extremely old individuals never reach the critical length, which determines that telomere shortening has no direct connection with aging.

To establish a correlation between aging and this theory, Hayflick proposed that senescence and death consequent need not reach the Hayflick limit in all tissues. The depletion of the proliferative potential in certain areas of some organs may be sufficient to promote the onset of certain diseases. The combination of these disorders gradually increases with age leading to aging.

The "telomere theory of aging" proposes that the biological clock that controls the life of all cells is precisely the telomere, a long DNA fragment consisting of the repetition of a series of 6 to 7 base high in guanine, located at the ends of all eukaryotic chromosomes. Telomeres function is to protect the chromosomal terminations avoiding fusions between them or that the stored genetic material can recombine improperly. Every time a cell divides, the telomere

shortens progressively until almost disappeared, reaching the cell senescence state in which the ability to divide and other metabolic functions are lost. To offset the loss of telomeres at each mitotic division, the enzyme telomerase adds DNA sequence repeats to the 3' end of DNA strands in the telomere regions (Olovnikov, 1996).

According to this theory, aging could be fought by retarding or blocking the process by which telomeres shorten. However, telomere dysfunction can also be a source of genomic instability and put cells at risk of developing into cancer (Artandi and DePinho, 2010; Martinez and Blasco, 2010; Shay and Wright, 2011; Davoli and de Lange, 2011). These because, in cells that escape senescence or apoptosis and divide, the DNA-repair activity at deprotected chromosome ends generates chromosome end-to-end fusions, whose initiate breakage-fusion-bridge cycles that generates complex unbalanced chromosome rearrangements (Murnane, 2012). In addition, telomere dysfunction can lead to tetraploidization and chromosome alterations (Davoli *et al.*, 2010; Davoli and de Lange, 2012).

Alternatively cells can couple the mechanism of "alternative lengthening of telomeres" to lengthen telomeres through recombination (Cesare and Reddel, 2010). Normal human somatic cells do not have enough telomerase or alternative lengthening activity to counteract the shortening with each cell division. Loss of telomere protection activates a DNA-damage-like signaling response that through induction of tumor suppressors p53 and p16 leads cells into senescence or apoptosis (Jacobs and de Lange, 2004, 2005; de Lange, 2010; O'Sullivan and Karlseder, 2010, Jacobs, 2013)

Some authors are considering the telomere shortening as the sole mechanism of aging. Mikhelson and Gamaley (2013) explain how telomere shortening might be the cause of aging and lifespan restriction, showing the inability of the oxidative theory to explain a number of indisputable facts, such as malignant growth of tumor cells and why children begin aging by themselves from zero and not from the level reached by the cells of their parents at the moment of conception, questions easily explained by telomere theory.

3. MITOCHONDRIA, OXIDATIVE STRESS AND AGING

Mitochondria are the energy source of the cell, obtaining ATP by oxidative phosphorylation. The importance of this organelle is such that even has its own DNA whose particular structure makes it vulnerable to the attack of ROS, and its damage is a fundamental process in the initiation of apoptosis. ROS are continuously generated in the body during normal metabolic functioning or as part of the body defense mechanism (Battino *et al*., 1999). Excessive production of ROS can cause oxidative damage to biological macromolecules such as DNA, proteins and lipids, which can be associated with, increased risk of chronic diseases, cancer, cardiovascular disease, immune-degenerative disorders and neurodegenerative pathologies (Halliwell and Gutteridge, 2007). Regardless of its pathophysiological effects, ROS fulfill some functions in the cell acting as a second messenger and redox regulators in the network of intracellular signal transduction in many physiological processes, such as hypothalamic regulation of energy metabolism, regulation of the proteasome via S-glutathionylation, T cell activation, induction of the mitochondrial permeability transition in cardiac myocytes, inter alia (Demasi *et al*., 2013; Diano, 2013; Fracchia *et al*., 2013; Wang *et al*., 2013a). In addition, overproduction of ROS is closely related to aging. The aging process is characterized with accumulation of mitochondria DNA mutations, impairment of oxidative phosphorylation as well as an imbalance in the expression of antioxidant enzymes, causing changes in its structure (which deregulates its internal homeostasis), in mitochondrial DNA

(causing the synthesis of defective molecules) or changes in the components of the electron transport chain can cause alterations in the function, which accelerate the aging process and lead to cell death by promoting the intrinsic apoptotic pathway through mitochondria (Sheckman, 2009; Wang *et al*., 2013a).

3.1. The role of mitochondria in aging

The role of mitochondria in normal aging has been extensively investigated from the free radical theory of aging, the oxidative stress theory of aging and the mitochondrial oxidative stress theory of aging, until the most recent researches to try to understand the regulation of mitochondrial energy, oxidative stress resistance mechanisms and signaling pathways in which mitochondria are involved (Pamplona and Barja, 2011; Schiff *et al.*, 2011; Hwang *et al.*, 2013).

Progressive loss of mitochondrial functionality is one of the common events associated with aging. As Harman proposed in 1956, aging is the result of the accumulation of oxidative damage throughout life. Some modifications (mainly those related to DNA) are not completely repaired and thus accumulate, leading to cell death, organism malfunction, and the "aging phenotype." In fact, oxidative damage is accepted as a primary event in aging (Pamplona, 2011). The mitochondrial free radical theory of aging (Harman, 1972) postulates that cellular aging is the product of mutations in the mitochondrial DNA (mtDNA) genome as a result of oxidative damage. As a result of accumulated damage to mtDNA, the mitochondrial blueprints are markedly altered, thus perpetuating the production of aberrant electronic transport chain (ETC) components (Gilmer *et al.*, 2010). According to this theory, the gradual and perpetual cycle of accumulation of damaged cellular components necessary for energy production could create an energy crisis situation in the cell, leading to its eventual dysfunction and demise (Hwang *et al.*, 2012).

Therefore, ROS generation by mitochondria play key roles in aging, because ROS damage indiscriminately macromolecules and lead to an age-dependent decline in biological function. For this reason, these organelles are considered the biological clock of aging (Pamplona and Barja, 2011; Hwang *et al.*, 2012; Cornelius *et al.*, 2013). In fact, mitochondria have been proposed as the link between the age-dependent accumulation of oxidative damage produced by ROS and the physiological alterations associated with aging, as cancer and neurodegenerative disorders (Leeuwenburgh *et al.*, 2011, Ochoa *et al.*, 2011).

Another question is the role of mitochondria as a key element in cell-signal transduction. Thus, mitochondria may also be considered an element of control for the nuclear gene expression. In this sense, a number of adaptation or regulation proteins have been found at the mitochondrial level or are translocated to the mitochondria to cope with this role, as with Nrf2, mitogen-activated protein kinases (MAPK), Akt, HIF-1, cytokines, AP-1, p53, JNK/SAPK, some caspases and several members of the Bcl2 family, such as Bid, Bax or Bim (Bolisetty and Jaimes, 2013).

Regarding mitochondria in the aging process, the control of apoptosis is vital. This control is frequently lost in aged cells, which in addition are more prone to suffer from oxidative stress. ROS decrease the mitochondrial membrane potential (Ψmt), allowing the opening of the transition pore and the subsequent escape to the outside of calcium and other substrates. Accumulation of ROS often associated with loss of activity of the antioxidant enzymes, as SOD, leads p53-mediated growth arrest and apoptosis (Ruffini *et al.*, 2013; Watanabe *et al.*, 2013).

3.2. Mitochondria as source of cellular energy and oxidative stress

The mitochondrial oxidative stress theory of aging (Harman, 1972, 2009; Miquel, 1998) is based on ROS, are a normal product of aerobic life and the accumulation of oxidative damage due to ROS results in changes that affect aging. ROS and free radical react with biological molecules inducing lipids peroxidation, oxidative changes in proteins, enzymatic inactivation and DNA alterations, lending to the socalled aging phenotype (Halliwell and Gutteridge, 1999; Sanz *et al.*, 2006a, 2006b, 2006c). This theory has been reviewed by Dr. Barja (Barja, 2013), emphasizing key aspects of the theory and including different issues, such as:

- The complex I as main ROS-generating site in the respiratory chain in relation to aging and longevity.
- The closeness or even contact between complex I and the mitochondrial DNA.
- The relationship between mitochondrial ROS production and oxygen consumption.
- The assumption that ROS are simple "by-products" of the mitochondrial respiratory chain.
- The unnecessary postulation of "vicious cycle" hypotheses of mitochondrial ROS generation which are not central to the free radical theory of aging.
- The role of DNA repair concerning endogenous versus exogenous damage.

In recent years, it has been ascertained that although ROS are produced through a large number of pathways of the aerobic metabolism, the main source of these species are mitochondria (Lenaz, 1998; Sastre *et al.*, 2000; Salvioli *et at.*, 2001, Van Remmen and Richardson, 2001, Cadenas, 2004; Vendelbo and Nair, 2011; Pamplona, 2011; Bolisetty and Jaimes, 2013; Sassi *et al.*, 2013).

3.2.1. Mitochondrial structure and function

Mitochondria are unique organelles as its structure provides compartmentalization of metabolism. They are complex organelles with two phospholipid bilayers that divide them into 4 different segments: the outer membrane, inter-membrane space, inner membrane and matrix (Green, 1983; Neupert, 1997; Watson *et al.*, 2000).

1. Outer membrane: it is a lipid bilayer with numerous pores (porins) and carrier proteins that allow the passage of substances through it. Its composition is very similar to the cytoplasmic membrane: proteins, carbohydrates, phospholipids and cholesterol in very similar proportions.

2. Inter-membrane space: it accumulates protons derived from passage of electrons through the electron transport chain that later will be used by the ATP synthase to produce ATP. In addition, certain enzymes, such as creatine kinase or carnitine are responsible for transporting fatty acids from the cytosol to the mitochondrial matrix to degrade through the β-oxidation.

3. Inner membrane: it is probably the most interesting part of the mitochondria because it allocated the mitochondrial electron transport chain (mtETC), the responsible machinery for the generation of energy in the form of ATP needed for life processes in cells. This membrane forms folds within the mitochondrial matrix called cristae, which serves to increase the membrane surface and so to house a larger number of enzymes. It is very selective to the passage of substances into the matrix, since it contains no pores as in the case of the outer membrane, but that the substances pass through specific transporters.

4. Matrix: with enzymes of the Kreb's cycle and the cycle of fatty acid oxidation as it is where reactions occur. It also has ribosomes and enzymes involved in protein synthesis, which are mtDNA products synthesize here.

Although both mitochondrial membranes are composed by lipids and proteins, they are different from the rest of biological membranes since its protein content goes beyond 80% while most of biological membranes are less than 50% (Quiles, 1995).

The protein complexes within the mtETC must be mentioned because of its importance and significance in the context of oxidative stress. In aerobic organisms, the mtETC produces the energy needed for life support. Basically, food is oxidized through the loss of electrons that are accepted by electronic carriers as nicotinamide adenine dinucleotide (NAD^+) and flavins (flavin mononucleotide, FMN, and flavin adenine dinucleotide, FAD). The reduced nicotinamide adenine dinucleotide (NADH) and the reduced flavins (reduced flavin mononucleotide, $FMNH_2$, and reduced flavin adenine dinucleotide, $FADH_2$) are oxidized again by oxygen, producing great amounts of ATP. Oxidation occurs with small jumps in which energy is gradually released (Lenaz, 1998). The mtETC is composed mainly by 5 lipoprotein complexes (Quiles, 1995; Lenaz, 1998; Cadenas, 2004; Ackerman and Tzagoloff, 2005, 2007; Mulkidjanian, 2005; Clason *et al.*, 2010, Hirst, 2013):

- Complex I or NADH dehydrogenase complex: NADH is oxidized to NAD^+, reducing FMN to $FMNH_2$. Electrons involved in the process will be a carrier of electron transport chain, ubiquinone, which diffuses through the membrane into the complex III to transport these two electrons. In the process they release 4 H^+ to the inter-membrane space.

- Complex II or succinate dehydrogenase: complex II transfers electrons from succinate to ubiquinone; it doesn't function as a proton pump.

- Complex III or bc1 complex: this complex receives two electrons from the ubiquinone and transferred them to 2 molecules of cytochrome c, which spread to the complex IV. In the process two H^+ pumped into space intermembrana.

- Complex IV or cytochrome c oxidase (CCO): the complex IV is the mtETC fraction that metabolizes oxygen. COO receives four electrons from cytochrome c and transferred to an O_2 molecule, generation 2 H_2O molecules. H^+ also pumped into space inter-membrane. This tetra-electronic reduction of oxygen is not tenable in a single step but rather must be done electron by electron. Because of this gradual reduction, the protein complex must be sure that partially oxidized oxygen, highly toxic, will not leak to the medium before being transformed into water.

- Complex V or ATPase: the ATP synthase is the final step in the electron transport chain. It uses the H^+ gradient generated to form ATP from ADP and obtain energy.

Mitochondria are the only know extra-nuclear cellular organelles in animal cells that contain their own DNA (mtDNA). MtDNA is different from nuclear DNA, is smaller and encodes different proteins; furthermore it has a different structure. For example, mtDNA is not associated with histones, which leaves him in a state of true vulnerability to ROS attack, to which must be added to their defense and repair systems are also lower than the nuclear DNA, so the mutation rate is 10 times greater than the DNA nuclear. In a mitochondrion there may be 2 to 10 mitochondrial genomes and replication is constant. The human mitochondrial genome is a small circular DNA molecule containing 16,569 bp in length containing 37 genes, 2 of which are ribosomal RNAs, transfer RNAs are 22 and 13 proteins of the electron transport chain (Desler *et al.*, 2011; Gredilla, 2011).

3.2.2. Mitochondria as an oxidative stress source

Mitochondria have been recognized as the organelle that produce the energy required leading the endergonic processes of cell life thought the respiratory chain, but for this reason it is also considered as the most important cellular source of free radicals (Cadenas and Davies, 2000; Cadenas *et al.*, 2004).

Mitochondria play a vital metabolic role by producing most of the cellular ATP via oxidative phosphorylation (OXPHOS) (Tzagoloff, 1982; Ackerman and Tzagoloff, 2005, 2007). OXPHOS is responsible for converting energy from macronutrients (carbohydrates, fatty acids, and amino acids) to ATP through reactions by which those macronutrients are oxidized, oxygen is reduced to water, and adenosine diphosphate (ADP) is phosphorylated to ATP (Treberg and Brand, 2011).

The products of the monovalent and bivalent reduction of oxygen are $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2), respectively, which are usually produced during aerobic metabolism, mainly at the mitochondrial level. The mitochondrial generation of these free radicals, as well as peroxynitrite ($ONOO^-$), represent the major intracellular source of ROS under physiological conditions (Halliwell and Cross 1994; Cadenas and Davies, 2000; Farrugia and Balzan 2012; Matés *et al.*, 2012). Mitochondria seem to be the most important cellular site of $O_2^{\cdot -}$ and H_2O_2 production in mammalian organs, and the steady state concentration of $O_2^{\cdot -}$ in the mitochondrial matrix is about five-to-ten-fold higher than that in the cytosol and nucleus (Cadenas and Davies, 2000; Cadenas *et al.*, 2004).

Over 95 % of all the oxygen we breathe undergoes a reduction to produce water in the mitochondrial electron transport chain; however it has been estimated that a 1-5% of the oxygen consumed by the mitochondria is not fully reduced to water and transformed to $O_2^{\cdot -}$. Superoxide anion is then transformed to H_2O_2 spontaneously or as the result of the action of SOD enzymes. Although CCO is the enzyme involved in the oxygen reduction, it hardly generates free radicals (Miriyala *et al.*, 2011).

There are different sources of ROS in cells. Exogenous sources include UV and visible light, ionizing radiation, drugs and environmental toxins. By the other hand, there are endogenous sites in the cell that produces ROS, such as xanthine oxidase, cytochrome P-450 enzymes in the endoplasmic reticulum, peroxisomal flavin oxidases and plasma membrane Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Lenaz, 2012). However, it is widely considered that the major source of ROS is the mitochondria. There are at least nine sites in mitochondria that possess the ability to produce $O_2^{\cdot -}$, summarizes in table 1 (Andreyev *et al.*, 2005; Jezek and Hlavata, 2005; Angermüller *et al.*, 2009; Brand, 2010; Venditti *et al.*, 2013). However, it is often assumed that the mitochondrial electron transport is the main biological process leading to ROS generation in most tissues in physiological conditions, with complexes I and III exhibiting the highest rate of $O_2^{\cdot -}$ production and the two main sites of free-radical production at the mtETC. (Cadenas *et al.*, 1977; Ksenzenko *et al.*, 1983; Shimomura *et al.*, 1985; Cross and Jones, 1991, Selivanov *et al.*, 2008; Lambert and Brand, 2009; Brand, 2010; Pamplona and Barja, 2011; Maranzana, 2013). The other seven sources to produce ROS are:

- Cytochrome b5 reductase: it is a protein located in the outer mitochondrial membrane that oxidizes cytoplasmic NADPH and reduces cytochrome b5 in the outer membrane. It may also reduce ascorbyl free radical and, therefore, be involved in regeneration of ascorbate in mammalian liver. Mitochondrial cytochrome b5 reductase may produce superoxide with a high rate of ~300 nmol/min per mg protein (Xu *et al.*, 2011; Mukherjee *et al.*, 2012).

- Monoamine oxidases A and B: they are mitochondrial bound isoenzymes located in the outer membrane which catalyze the oxidative deamination of dietary amines and monoamine neurotransmitters accompanied by release of H_2O_2. Its potential for H_2O_2 generation may far exceed that of other mitochondrial sources, even ~50 times higher than complex III. Monoamine oxidase may be an important source of H_2O_2 during tissues in ischemia, aging, and during oxidation of exogenous amines (Bortolato *et al.*, 2008; Ramsay, 2012; Wang *et al.*, 2013a).

- Dihydroorotate dehydrogenase (DHODH): it is located at the outer surface of the inner membrane and catalyzes conversion of dihydroorotate to orotate, a step in the synthesis of pyrimidine nucleotides. In the absence of its natural electron acceptor, coenzyme Q (CoQ) in the inner mitochondrial membrane, reduced DHODH can produce H_2O_2. Inhibition of complex II with antimycin A reduced DHODH activity and pyrimidine synthesis, suggesting that DHODH is functionally linked to complex III activity (Olgun and Akman, 2007; Fang *et al.*, 2012)

- Alpha-glycerophosphate dehydrogenase: this is a FAD-containing enzyme also located at the outer surface of the inner membrane. It catalyzes oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate, utilizing mitochondrial CoQ as electron acceptor. This reaction is involved in lipid metabolism and in the glycerol phosphate shuttle that regenerates cytosolic NAD^+ from the NADH formed in glycolysis, producing H_2O_2. The rates and sites of H_2O_2 production depend upon tissue source, concentrations of glycerol 3-phosphate and calcium, and the presence of different electron transport chain inhibitors (Orr *et al.*, 2012; Adam-Vizi and Tretter, 2013).

- Succinate dehydrogenase (SDH): this is a flavoprotein, located at the inner surface of the inner membrane, that oxidizes succinate to fumarate, with the concomitant production of $FADH_2$, using CoQ as electron acceptor. SDHA and SDHB subunits are bound to complex II in the inner mitochondrial membrane matrix, while the two other subunits, SDHC and SDHD, form the base of the enzyme as an intramembraneous complex within the inner mitochondrial membrane. SDHA and SDHB are probably hydrophilic with the former involved in substrate binding and oxidation and the latter in electron transfer (Ralph *et al.*, 2011; Ishii *et al.*, 2013).

- Mitochondrial aconitase: it is localized in the mitochondrial matrix and catalyzes conversion of citrate to isocitrate as part of the tricarboxylic acid cycle. A secondary role for mitochondrial aconitase is to act as a biosensor for ROS and iron. Mammalian aconitase contain a [4Fe-4S] prosthetic group in their catalytic centers which are susceptible to inactivation by ROS, particularly $O_2^{\cdot -}$.The enzyme is inactivated upon oxidation of its iron-sulfur cluster by superoxide. Upon inactivation, isolated aconitase induces production of hydroxyl radical, most likely mediated by released Fe^{2+}. (Fariss *et al.*, 2005; Cantu *et al.*, 2011).

- Alpha-ketoglutarate dehydrogenase complex (KGDHC): it is composed of multiple copies of three enzymes: alpha-ketoglutarate dehydrogenase, dihydrolipoamide succinyltransferase and lipoamide dehydrogenase, and is associated with the matrix side of the inner membrane. This complex catalyzes oxidation of alpha-ketoglutarate to succinyl-CoA using NAD^+ as electron acceptor. Several studies have demonstrated that KGDHC can generate superoxide and hydrogen peroxide. The source of ROS in KGDHC appears to be the dihydrolipoamide dehydrogenase component. The ROS production from KGDHC is stimulated by low availability of its natural electron acceptor, NAD^+ (Andreyev *et al.*, 2005; Shi *et al.*, 2011).

Table 1. *Mitochondrial sources of ROS.*

Enzyme / enzymatic complex	Location	Reactive
Complex I	Inner mitochondrial membrane	Rotenone
Complex III	Inner mitochondrial membrane	Ubisemiquinone anion radical
Cytochrome b5 reductase	Outer mitochondrial membrane	Cytochrome b5
Monoamine oxidase A and B	Outer mitochondrial membrane	Amines and monoamine neurotransmitters
DHODH	Inner mitochondrial membrane	Dihydroorotate
Alpha-glycerophosphate dehydrogenase	Inner mitochondrial membrane	Glycerol-3-phosphate
SDH	Inner mitochondrial membrane (complex II)	Succinate
Mitochondrial aconitase	Mitochondrial matrix	Citrate
Alpha-ketoglutarate dehydrogenase	Inner mitochondrial membrane	Alpha-ketoglutarate

The ROS production at the mtETC occurs when some electrons, during the pass from one complex to the other, escape and join the surrounding oxygen, generating $O_2^{\cdot -}$ (Lambert and Brand, 2009; Hirst, 2013). Complex III, furthermore, is the only site that can deposit $O_2^{\cdot -}$ into the inter-membrane space; the other sites deposit $O_2^{\cdot -}$ into the mitochondrial matrix. Inter-membrane space ROS seems to be easier access to the cytosol that matrix ROS, because they only need to cross the outer mitochondrial membrane and not both, as matrix ROS, and that could be an advantage to the cytosol signaling capacity (Sena and Chandel, 2012). Recently it has been reported production of ROS from nematode *Ascaris suum* (Paranagame *et al.*, 2010) and rat (Quinlan *et al.*, 2012) mitochondrial complex II. In addition, it has also been demonstrated that a substantial portion of H_2O_2 production is originated from electron flow into complex I and II and not from mitochondrial glycerol 3-phosphate dehydrogenase (mGPDH), as it was commonly attributed (Hagopian *et al.*, 2010; Orr *et al.*, 2012).

The physiological level of ROS production at the mtETC depends on the metabolic state of mitochondria. The state of mitochondrial rest (state 4), is characterized by a low respiration level and no ADP availability. This state is associated with a high rate of ROS production, probably as a consequence of the high degree of reduction of the chain components. On the contrary, at the state 3, the active mitochondrial state, there is a high oxygen expenditure and elevated ADP availability. This state shows relatively low ROS production. In the state of anoxia (state 5), with a limitation in the oxygen delivery and absence of respiration, no ROS production is observed (Cadenas and Davies, 2000, Cadenas, 2004).

More than 50 years ago, it was suggested for the first time that mitochondria might be involved in aging (Harman, 1956, 2006, 2009). At the time, aging was ascribed to the deleterious effects of ROS produced by mitochondria as a result of age-related OXPHOS dysfunction (Page *et al.*, 2010; Pamplona, 2011). From viewpoint of aging, the complex I seem to be the main site for ROS production (Sanz *et al.*, 2006a; Maranzana *et al.*, 2013). The importance of ROS study resides in its ability to oxidize other molecules, mainly protein though posttranscriptional modifications that alter its function in a signaling pathway. Several

phosphatases are regulated in this manner, as PTP1b, PTEN and MAPK phosphatases. Also, many studies have shown that ROS can act as cellular signaling molecules or second messengers that play a role in gene expression, cell survival and growth in various cell types (Tonks, 2005; Brown and Griendling, 2009; Bae *et al.*, 2011; Finkel, 2012).

There are some evidences about biological adaptations that determine the rate of mitochondrial free radical generation (Pamplona and Barja, 2006, 2007; Hulbert *et al.*, 2007):

- To adapt the amount of the respiratory complex/es responsible for ROS generation. Thus, a decrease in the amount of complex I protein will lead to a decreased rate of ROS generation.

- To adjust the amount of uncoupling proteins. The mitochondrial superoxide production is very sensitive to the proton motive force, so it can be strongly decreased by mild uncoupling, which diminishes mitochondrial superoxide production and protects against oxidative damage.

- To regulate the degree of electronic reduction of these generators: the higher their degree of reduction, the higher will be their rate of ROS production.

- To modify by enzymatic and nonenzymatic pathways such as S-nitrosation, acetylation, and glutathionylation specific peptides of the ROS generators.

Several studies have found an inverse correlation between the rate of respiratory ROS production and animal longevity. Species with short longevity could show high mitochondrial ROS production simply because their rates of mitochondrial oxygen consumption are higher, while it is generally lower in longer lived species (Page *et al.*, 2010, Pamplona and Barja 2011).

Three groups of homoeothermic vertebrates have an extraordinarily high longevity in relation to their body size and metabolic rate: birds, bats and mammals (Barja and Herrero, 2000; Holmes *et al.*, 2001). Birds can be a strong test to the free radical theory of aging because if a low rate of ROS production contributes to slow the aging rate, the mitochondria of birds should show a low rate of ROS generation in spite of the high rate of oxygen consumption of these animals (Holmes *et al.*, 2001). Negative correlation between ROS production in mammals and birds was described by Barja *et al*. (Barja, 2002), and later confirmed by Lambert *et al*. (Lambert *et al.*, 2007, 2010). Comparisons between rodents with different longevities but similar body size (Barja, 2004a), or between rodents and bats (Brunet-Rossinni, 2004) also showed that the longest lived species had lowest mitochondrial ROS (mtROS) production. Other studies have also found much lower rates of ROS generation in human, which also live much longer than expected for their body size and metabolic rate, than in rat brain mitochondria (Kudin *et al.*, 2004).

3.2.3. Mitochondria as a target for oxidative stress

Mitochondria are considered the main intracellular source of ROS and they often become the target of elevated ROS exposure with deadly consequences, such as oxidative damage of mtDNA, lipid and protein (Orrenius *et al.*, 2007; Circu *et al.*, 2009; Rachek *et al.*, 2009).

3.2.3.1. Lipid peroxidation by ROS

The polyunsaturated fatty acyl chains of phospholipids in the biological membranes are very sensitive to oxidation by ROS, which format carbon-centred radicals within the membranes, resulting in peroxidation of fatty acids (Halliwell and Gutteridge, 1999, 2007). The sensibility of biological membranes to oxidative stress is due to the presence of a double carbon-carbon bond in the lipid tails of its phospholipids (Milatovic *et al.*, 2011). Oxidative damage to membrane lipids may be directly generated by ROS as hydroxyl radicals or the superoxide anion, or indirectly by some products of lipid peroxidation such as some highly

reactive aldehydes (Esterbauer *et al.*, 1991). Oxidative damage of membrane lipids leads to its alteration and to changes in membrane fluidity and, in conclusion, alterations in membrane function (Halliwell and Gutteridge, 1999; 2007).

Cumulative free radical generation with aging leads to significant damage to mitochondrial membranes constituents with subsequent mitochondrial dysfunction (Miquel and Fleming 1986; Shigenaga *et al.*, 1994; Navarro and Boveris 2007). An excess of ROS in mitochondria can damage several components affecting their metabolism (Lenaz, 1998; Maranzana *et al.*, 2013) and, thereafter, it can lead to decrease the activity of respiratory complex or even inactivate components of electron transport chain and loss of super-complex organization, as well as some enzymes of the Krebs cycle and other mitochondrial proteins, leading to mitochondrial dysfunction (Indo *et al.*, 2007, Lenaz, 2012; Venditti *et al.*, 2013). It has been suggested that cardiolipin neutralizes the charges of lysine residues in the interaction domain between cytochrome bc1 complex and COO, stabilizing the respiratory super-complex formation (Wiswedel *et al*., 2009).

Lipoproteins consist of lipids and apolipoproteins that have functional role in lipid metabolism. It has been proposed that oxidation of lipoproteins by ROS may be involved in several diseases, such as development of cardiovascular diseases. Amino acid residues of apolipoproteins can be oxidized by two pathways: by direct oxidation by ROS or through reactions with oxidized lipids. Low-density lipoprotein (LDL), high-density lipoprotein (HDL) and very low density lipoprotein (VLDL) contain a relatively high amount of n6 PUFAs, which are the major targets for ROS-mediated lipid peroxidation (Halliwell and Gutteridge, 2007). Lipid hydroperoxides formed can be decomposed in aldehydes. Both, LOOH and aldehydes can induce oxidative modifications in lipoproteins amino acid residues lead to protein dysfunction. LDL and VDLD oxidation seems are postulated as a central pathogenic mechanism in atherosclerosis. Oxidation of HDL, on its parts, impairs its ability to remove excess cellular cholesterol, decreasing its capacity of suppress the development of atherosclerosis (Arai, 2014).

3.2.3.2. Protein oxidation by ROS

There are several membrane proteins that are easily inactivated by oxidative stress (ATPase, ANT, COO, etc.). Ionizing radiation or the Fenton reaction can produce hydroxyl radicals able to attack the polypeptides to form carbon-centred radicals which may react with O_2 to form peroxyl radicals (Stadtman, 2004). All amino acids residues in proteins are susceptible to oxidation, but there are some of them most vulnerable to attack by ROS. They are arginine, glutamate, histidine, tyrosine (Tyr), valine, cysteine, proline, threonine and methionine. Mainly, cysteine and methionine residues are particularly susceptible to oxidation by ROS. ROS oxidizes methionine residues producing methionine sulphoxide (Met-SO). The oxidation of the amino acid residues generates carbonyl groups. The identification of protein carbonylation is the most widely used marker of protein oxidation (Stadtman 2004).

Damage to mitochondrial proteins, as result of direct oxidative stress or a consequence of covalent modification by products of lipid peroxidation, are associated to the formation of protein cross-linkages. This cross-linking may occur by addition of a lysine amino group to a carbonyl group of the oxidized protein, by interaction of two carbon-centred radicals of polypeptide backbones, by oxidation of cysteine residues to form S-S cross-links, or by oxidation of Tyr residues to form Tyr-Tyr cross-links, which lead the loss of protein function (Dalle-Donne *et al*., 2006a). Moreover, this damage leads to the opening of the permeability transition pore, a key step in the process of apoptosis. Therefore, mtETC protein alteration has two consequences: one direct consequence is the loss of mitochondrial functionality, but another

indirect consequence is a rise in the ROS production (Lippe *et al.*, 1991; Forsmark-Andree *et al.*, 1997; Battino *et al.*, 2002a; Lenaz, 2012). High levels of damaged mitochondrial proteins have been implicated in a growing number of diseases. A proteomic study has detected modified mitochondrial proteins in Alzheimer disease, founding a number of oxidatively modified brain proteins that are associated with the mitochondrial proteome, involving the mitochondrial oxidatively modified proteins in the Alzheimer disease progression (Sultana and Butterfield, 2009).

3.2.3.3. DNA damage by ROS

Mitochondrial DNA is not protected by histones, as opposed to nuclear DNA, and it has been traditionally considered of a high susceptibility to be oxidatively attacked by ROS (Richter *et al.*, 1988). For a long time, it was considered that mitochondria did not have a system to repair damaged DNA but actually it is recognized the existence of an mtDNA repair system, which can repair the oxidative damage (i.e. damage to bases and single-strand breaks) (Bohr *et al.*, 2007; Gredilla *et al.*, 2010; Maynard *et al.*, 2010; Glowacki *et al.*, 2013). However, mitochondria do not appear to contain the full range of DNA repair mechanisms that operate in the nucleus, although mtDNA contains types of damage that are targets of each nuclear DNA repair pathway. The reduced repair capacity could explain the high mutation frequency of the mitochondrial chromosome (Cline, 2012).

The main mtDNA lesions generated by ROS are base modifications, such as the ubiquitous 8-oxoguanine (8-oxoG) in 8-hydroxydeoxyguanine (8-OH-dG) due to the mispairing of 8-oxoG with adenine during replication, although base loss and stand breaks may also occur. 8-OH-dG is the most easily formed base alteration, is mutagenic and carcinogenic and is a good biomarker of oxidative DNA damage; this modification occurs in one in 10^5 guanine residues in normal cells, but increases in oxidative stress, ageing and a number of diseases. It has been considered that the 8-OH-dG is one of the most abundant oxidative lesions that accumulates in mtDNA over time. It has been reported that accumulation of 8-oxoG in mtDNA occurs with age possibly because 8-oxoguanine-DNA glycosylase (OGG1), a DNA glycosylase enzyme involved in base excision repair (BER) of 8-oxoG, accumulates in a unprocessed form in the mitochondrial intermembrane space and fails to be imported inside the matrix (Wei et al., 2009; Larsson, 2010; Lagouge and Larsson, 2013).

All of these lesions are repaired primarily by the BER pathway, which is catalyzed in mammalian mitochondria, similary that occur in nuclear BER, by DNA glycosylases, apurinic/apyrimidinic (AP) endonuclease, mitochondrial DNA polymerase (POLγ) and DNA ligase. POLγ is the only polymerase present in the mitochondria (Chevanne *et al.*, 2003; De Souza-Pinto *et al.*, 1999, 2008; Gredilla *et al.*, 2010; Maynard *et al.*, 2010)

As many reports suggest, oxidative damage to mtDNA is more important from the viewpoint of aging than that involving lipids and proteins, due to the ability of damaged mtDNA to be spread because of the division capacity of mitochondria and cells, which allows amplification of the physiological consequences of the damage. Furthermore, oxidative damage to mtDNA might be even more important than the damage to the nuclear DNA as the entire mitochondrial genome codifies for genes that are truly expressed, while the nuclear genome contains a huge amount of nontranscribed sequences (Van Remmen and Richardson, 2001). There are several ways through oxidative stress affects mtDNA, as alteration to bases, the rise in the number of deletions and the occurrence of punctual mutations. With aging, there is an accumulation of mtDNA damage and mutations, including point mutations and large deletions, increase with age (Caro *et al.*, 2010). The effects of mtDNA damage and mutations are

particularly important in tissues containing post-mitotic cells, because of these cells cannot be replaced by intact ones. It seems to be a relationship between age-associated accumulation of mtDNA mutations and bioenergy dysfunction, at least in postmitotic tissues, such as central neuron system and skeletal muscle, where is especially deleterious (Calabrese *et al.*, 2010; Pamplona, 2011). Therefore, it has been reported that oxidative damage to mtDNA in cardiac and neuronal tissue is inversely related to the maximum life span potential in mammals (Barja and Herrero, 2000), suggesting that such accumulation may play a role in the determination of lifespan (Gredilla *et al.*, 2010). In heart and muscle, there are evidences that suggest that mtDNA mutations increase as a function of age (Cornelius *et al.*, 2013).

Damage to mtDNA has been linked to aging and longevity in many studies performed in laboratory mammals, as well as in rhesus monkeys (Castro *et al.*, 2012). It has been reported that more than twenty different types of deletions accumulates in aging human tissues (Cornelius *et al.*, 2013). Some studies with mutant mice suggest that increasing the level of mtDNA mutations increases the aging rate (Trifunovic *et al.*, 2004; Kujoth *et al.*, 2005; Hiona *et al.*, 2010). It has been also described that the rate of accumulation of mtDNA mutations with age is much slower in humans than in mice (Wang *et al.*, 1997; Gredilla *et al.*, 2010).

ROS have mutagenic capacity, and the rate of accumulation of DNA mutations is known to be higher when the cellular oxygen partial pressure (pO_2) is increased, suggesting a role for ROS in their formation. Mitochondrial ROS generation can be among the causes of mtDNA accumulation *in vivo* and thus of aging (Al-Kafaji and Golbahar, 2013; Beltrami *et al.*, 2013; Motherwell and Zuo, 2013).

According to Halliwell and Gutteridge (1999; 2007), the steady state level of oxidative stress is the result of the balance between the levels of damage and the degree of repair or replacement of damaged molecules, so, in terms of balance, a net increase of oxidative stress during aging may be found in terms of higher levels of damage or as the result of defects in the repair system. Depending on the capacity of the affected tissue to repair the damage or to replace the altered cell, tissue function will be affected in a higher or lower extent (Quiles *et al.*, 2002b).There are certain factors that explain the steady-state level of mtDNA damage by mitochondrial ROS (Pamplona, 2011):

1. The long-lived animal species have low rates of mtROS generation; this should be reflected in the steady-state level of oxidative damage and the accumulation of somatic mutations in their mtDNA.

2. mtDNA is located very close to or even in contact with the site of mitochondrial ROS production.

3. The mtDNA molecular structure makes it more susceptibility to damage.

Actually, a good approach to studying oxidative alterations to bases is through the analysis of 8-OH-dG using liquid chromatography-tandem mass spectrometry (LC-MS/MS) in urine (Sauvain *et al.*, 2011). Most long-lived animal species studied have lower steady-state levels of 8-oxodG (a biomarker of DNA oxidation) in their mtDNA, in agreement with their low rates of mtROS production (Barja and Herrero, 2000; Sanz *et al.*, 2009, Caro *et al.*, 2010), and lower rates of urinary excretion of 8-OH-dG than short-lived ones (Dziaman *et al.*, 2013).

Concerning DNA deletions, it has been reported that rise with aging among a wide variety of post-mitotic tissues from several species. Moreover, the rise in the percentage of deletions has been directly correlated with oxidative damage (Greaves *et al.*, 2012). The "common deletion" is a 4,977-bp deletion that accumulates in many tissues during aging causing several sporadic diseases including Pearson's disease, Kearns-Sayre syndrome, mitochondrial myopathies and progressive external ophthalmoplegia. Nonetheless, since the percentage at

which the level of deletions increases does not exceed 2-3%, the physiological significance for aging is speculative (Schroeder *et al.*, 2008; Sadikovic *et al.*, 2010; Li *et al.*, 2012).

A wide spectrum of mtDNA mutations, including point mutations (Michikawa *et al.*, 1999; Khaidakov *et al.*, 2003), large scale deletions (Zang *et al.*, 1992; Lee *et al.*, 1994b; Yen *et al.*, 2001), and tandem duplications (Lee *et al.*, 1994a; Wei *et al.*, 1996) have been observed in the tissues of elderly subjects. The most prevalent aging-associated mutations of mtDNA are A3243G and A8344G transitions and large-scale deletions including the common 4,977bp deletion. These mtDNA mutations may occur alone or in various combinations in somatic tissues of humans during the aging process. However, the number of cells with predominantly mutated mtDNA is small in old animals, so a new theory has been proposed that defends that mtDNA fragments may insert into nuclear DNA contributing to aging and related diseases by alterations in the nucleus, suggesting that mitochondria are the source of the problem, but not the only target, if not that but the final target could also be the nucleus. Caro *et al*. quantified sequences of cytochrome oxidase III and 16S rRNA from mtDNA in nuclei from liver and brain in young and old rats, containing single nucleotide polymorphisms identical to those present in mtDNA of the same animal (Caro *et al*., 2010).

CHAPTER II: DIETARY FAT, HEALTH AND AGING

1. NUTRITION AND AGING

In recent years, a large body of evidence has shown that nutritional factors may play a role in the etiology of chronic diseases, cognitive decline, inflammation or cancer, suggesting a link between dietary patterns and increased longevity. Several nutrients, as dietary fat and antioxidants, have shown positive effects on age-related diseases and aging progression. In addition, intake control has been proposed as an effective tool to increase.

1.1. Caloric restriction

Caloric restriction (CR) is defined as a reduction in energy intake (typically 40% less than *ad libitum*) without malnutrition. Some observations prove that the CR positively affects longevity in several species. McCay and coworkers proposed first the limited food intake in 1935 (McCay *et al*., 1935), and they described the increase in longevity or life-span in several species and rodents, and also observed that age-related diseases appeared later. Since then, reduction of food intake by 20%-50% increases longevity in many species from yeast to nonhuman primates, and decreasing the development of age-related disease in rodents (McCay *et al.*, 1989; Masoro, 2005; Ingram *et al.*, 2006; Everitt *et al.*, 2010; Marchar *et al.*, 2012).

The more accepted hypothesis to explain CR effects is based on the possible oxidative stress reduction. CR appears to prolong life by reducing ROS-mediated oxidative damage (Cornelius *et al.*, 2013; Stankovic *et al.*, 2013). As reviewed in section I.1,aging is characterized by an exponential increase of oxidative damage to proteins, lipids and DNA. CR has been found to downregulate the expression of genes involved in oxidative stress and to ameliorate oxidative damage in different tissues (Lee *et al.*, 1999; Kayo *et al.*, 2001; Phillips and Leeuwenburgh, 2005; Hofer *et al.*, 2009; Marzetti *et al.*, 2009; Opalach *et al.*, 2010). CR is also associated with additional biological changes that may contribute to improve cellular quality control and increase the longevity through autophagy, improved function of the ubiquitin-proteosome system, and the maintenance of a healthy population of mitochondria though biogenesis, ultrastructural changes and markers of fission/fusion (Lee *et al.*, 1999; Kayo *et al.*, 2001; Wohlgemuth *et al.*, 2007, 2010; Rangaraju *et al.*, 2009; Dutta *et al.*, 2012; Aris *et al.*, 2013; Khraiwesh *et al.*, 2013).

CR prevents the majority of the age associated changes in expression of genes involved in inflammation, immune responses, and mitochondrial energy metabolism, the most affected in aging (Park S. K. and Prolla 2005; de Magalhaes *et al.*, 2009). It was observed that mice fed on a CR regimen generated less oxidative stress than those who were fed *ad limitum*, besides to having lower lipid, protein and DNA oxidation (Finckel and Holbrook, 2000, Le Couteur *et al.*, 2012). CR in rodents confers increased longevity, and it has been associated with reduced mitochondrial hydrogen peroxide production in isolated mitochondria from liver (Hagopian *et al.*, 2005), skeletal muscle (Bevilacqua *et al.*, 2004) and brain (Sanz *et al.*, 2005a) at the rat. In mice skeletal muscle, CR decreased apoptosis by reducing the activity of the plasma membrane neutral sphingomyelinase, which exerts a protective action against aging. This effect was higher when CR was combined with fish oil as dietary fat source (López-Domínguez *et al.*, 2013). In rat liver, moderate CR increased antioxidative capacity of hepatocytes due to increase in SOD activity and reduced glutathione (GSH) concentration, which might have a role in anti-aging mechanism of CR (Stankovic *et al.*, 2013). In addition, CR gives rodents higher capacity to withstand different physiological stress improves thermotolerance and diminishes heat-induced

damage in olds rats (Quiles *et al.*, 2004a). Most amino acids in proteins can be modified by one or more forms of ROS. Methionine is an essential amino acid that plays a relevant role in protein structure and function and metabolism. Residues commonly modified include tyrosine, cysteine, and methionine (Dean *et al.*, 1997). Oxidation of methionine residues generates Met-SO in proteins, which loss their biological function. Protein damage has direct physiological consequences due to their role as cellular structural components and biocatalysts. The lower protein methionine content increases the protein resistance to oxidative damage, in so much as methionine residues in proteins are particularly susceptible to oxidation by various reactive intermediates (Stadtman *et al.*, 2003). In accordance with this, it has been found t a negative correlation between methionine presence and mammalian longevity. Protein methionine content in the whole tissue proteome from heart, brain, skeletal muscle and liver are lower long-lived birds than in short-lived mammals of similar body size (Pamplona and Barja, 2011; Naudí *et al.*, 2013).

The effect of CR on mitochondrial ROS generation has been studied intensively in rats. It been demonstrated that long-term 40% CR decreases the rate of mtROS production in rat tissues, such as heart, brain, skeletal muscle, liver and kidney (Gredilla and Barja, 2005; Hagopian *et al.*, 2011). This decrease in mtROS production in CR rats mainly occurs in complex I, accompanied by a decrease in percentage of free radical leak (FRL), indicating that the mitochondria of CR animals avoid more efficiently the ROS production per unit electron flow (Sanz *et al.*, 2005a, 2005b; Gómez *et al.*, 2007; Hagopian *et al.*, 2011). ROS production by complex III in liver and skeletal muscle mitochondria is also decreased by CR, but it not markedly alters proton leak and ETC (electron transport chain) enzyme activities. Even complex II activity can be altered when CR is combined with a polyunsaturated fatty acids (PUFA)-rich diet compared with other fat sources such as soya bean oil or lamb fat, which indicates that ROS production, proton leak and ETC enzyme activities is affected by dietary lipid composition in CR animals (Chen *et al.*, 2012a; 2012b).

CR seems to slow down aging and to increase longevity in different animal species, such as *Caenorhabditis elegans*, *Drosophila melanogaster*, mice, rats, and probably even monkeys (Walker *et al.*, 2005; Gredilla and Barja, 2005; Colman *et al.*, 2009; Sanz *et al.*, 2010a, 2010b). Recent finding indicates that reduced glucose metabolism as a form of CR induce mitochondrial metabolism to extend life span in a number organisms including *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and possibly mice. These effects may be caused by an increase in ROS formation within the mitochondria, which lead to an adaptive response that increased stress resistance thought to a long-term reduction in oxidative stress (Calabrese *et al.*, 2010). This type of retrograde response is called mitochondrial hormesis or mitohormesis, and does not correspond to the free radical theory proposed by Harman. The concept of mitohormesis therefore provides a common mechanistic denominator for the physiological effects of physical exercise, CR and glucose restriction (Calabrese *et al.*, 2011, 2012).

CR can be considered an effective mechanism to act therapeutically against aging and related diseases (Roth *et al.*, 1999; Weindruch *et al.*, 2001; Colman *et al.*, 2009; Marchal *et al.*, 2012; Anton and Leeuwenburgh, 2013). Nevertheless, the possible use of CR as an anti-aging therapy in humans would involve practical and ethical difficulties that make it almost impossible to consider feasible or desirable (Finckel and Holbrook, 2000). Because of the difficulty in maintaining a CR diet for humans, there have been attempts to combine metabolic, hormonal and physiological effects that replicate the effects of CRs, the caloric restriction mimetics (CRM) (Ingram *et al.*, 2004, 2006; Minor *et al.*, 2010; Ribarič, 2012). CRM are

pharmacological agents that can replicate the beneficial effects of CR. Its characteristic activities have been defined (Kitada and Koya, 2013):

- CRM mimics the metabolic, hormonal, and physiological effects of CR.
- CRM activates the stress response pathways observed in CR and enhances stress protection.
- CRM produces CR-like effects on longevity, reduces age-related disease, and maintains more youthful function.
- CRM does not significantly reduce food intake in the short term (although a long-term reduction in food intake is expected).

The nutrient-sensing pathways that include sirtuins, especially SIRT1, the mammalian target of rapamycin (mTOR), and AMP-activated protein kinase (AMPK) appear critical for cellular homeostasis, cell growth and proliferation, mitochondrial function, autophagy, and survival. In addition, it been known that CR extends lifespan in mammals by inducing SIRT1 expression (Villalba and Alcaín, 2012). Candidate CRMs that modulate these pathways have been identified using animal models, which include sirtuin-activating compounds (STACs), AMPK activators, and mTOR inhibitors (Ingram and Roth, 2011). One of most representative STACs is resveratrol, a natural polyphenolic compound found in grapes and red wine which activates SIRT1. Resveratrol enhances the longevity of some short-lived organisms, including yeast, worms, flies, fish and rodent, shown also beneficial effects in the treatment or prevention of obesity, tumorigenesis and in the aging-related decline in heart function and neuronal loss (Lagouge *et al.*, 2006; Valenzano *et al.*, 2006; Villalba and Alcaín, 2012). Also in human administration oral resveratrol to obese patients had CR-like effects, improving obesity-related metabolic abnormalities (Timmers *et al.*, 2011). Rapamycin is known as not only an immunosuppressive or antitumor agent but also as an inhibitor of mTOR, so it has been proposed as CRMs for lifespan extension. It was reported that rapamycin extends the median and maximum lifespan of yeast and rodent accompanied with a decrease in TOR activity. However, it is important to note that there is evidence showing adverse side-effects of rapamycin such as an increase in the incidence of diabetes (Lee and Min, 2013). Metformin, a drug used for treatment of type-2 diabetes that increases insulin sensitivity and activates AMPK, is another CRM of interest. Metformin was shown to have a CR-related longevity benefit mediated by the activation of AMPK in *Caenorhabditis elegans* (Onken and Driscoll, 2010) and mice (Anisimov *et al.*, 2011), while having no significant effect in rats (Smith *et al.*, 2010). Moreover, metformin treatment has been shown to increase survival in individuals with type II diabetes and cardiovascular diseases (Eurich *et al.*, 2005). Alternatively, metformin has been reported to inhibit mTOR, thereby suggesting a potential overlap in the signaling pathways induced by both metformin and rapamycin (Mercken *et al.*, 2012).

Besides studying CR in general, there are also studies focused in the restriction of particular nutrients like carbohydrates, lipids, total protein and specific aminoacids. When CR only affects lipids (Sanz *et al.*, 2006a) or carbohydrates (Sanz *et al.*, 2006b) there were not changes in mitochondrial ROS production neither modify maximum longevity. However, when CR affects to protein methionine content, aging is affected by increased maximum longevity and lower mitochondrial oxidative stress (Naudí *et al.*, 2013). As the restriction of aminoacids, the most important studies are those from prof. Barja group, who has focused on methionine restriction (MetR). They have demonstrated that the lower mitochondrial ROS production in CR animals seems to be caused by the consequent decreased methionine ingestion, diminishing oxidative damage to mtDNA and mitochondrial protein. Studies in rats have been shown that 40 % of PR or 40 % of MetR decreased mtROS production and mtDNA oxidative damage in rats. So an

isocaloric MetR also increases rodent maximum longevity. 80% MetR also increase longevity in rats, and it decreases disease-associated markers and the incidence of age-related degenerative diseases through the reduction of serum glucose, insulin, insulin-like growth factor-1 (IGF1), cholesterol, triglycerides and leptin, among others (Sánchez-Román *et al*., 2012, Sánchez-Román and Barja, 2013).

1.2. Supplementation with antioxidants

Oxidative stress plays a very important role in the global process of aging. The free radical theory of aging (Harman, 1956, 2006, 2009) hypothesizes that oxygen-derived free radicals are responsible for the age-related damage at the cellular and tissues levels. In a normal situation, exits equilibrium between oxidants, antioxidants and biomolecules, however in aging the antioxidant capacity might decrease. Thus, the possible anti-aging therapy through the use of antioxidants is one of the alternatives to slow the process of senescence (Gomez-Cabrera *et al.*, 2012).

Antioxidant supplementation has been frequently indicated as a potential mean to improve health status and increase longevity (Harman, 2006). Antioxidants are substances capable of inhibit or delay oxidation of a substrate (Halliwell and Gutteridge, 1990). Endogenous antioxidant defenses are both non-enzymatic (uric acid, glutathione, nutritional factors including vitamins and phenols, etc) and enzymatic SOD, glutathione peroxidase, catalase, etc.). The most important source of natural antioxidants are foods. Numerous dietary components such as vitamin C, vitamin E, CoenzymeQ 10 (CoQ_{10}), and polyphenols are involved in the antioxidant defense system (Melov *et al.*, 2000; Quiles *et al.*, 2002a; Fusco *et al.*, 2007; Wojcik *et al.*, 2010). Nutritional antioxidants act through different mechanisms and in different compartments (Berger, 2005):

- Neutralizing free radicals.
- Reducing the peroxide concentrations and repairing oxidized membranes.
- Quenching iron to decrease reactive oxygen species production.
- Neutralizing ROS via lipid metabolism, short-chain free fatty acids (FFA) and cholesterol esters.

Among the earliest studies it should be noted Miquel and Economos (Miquel and Economos, 1979) in relation to the thiazolidine carboxylate capacity to increase vitality and prolong the lifespan in mice. Subsequently, Furukawa *et al*. (Furukawa *et al*., 1987) showed the protective role of oral administration of glutathione against the decline of immune function associated with aging. Many other antioxidants have been tested in relation to aging, with more or less positive results. Such antioxidants include vitamin E and vitamin C, coenzyme Q, herbal extracts rich in flavonoids and polyphenols, such as resveratrol, among others (Halliwel and Gutteridge, 1999; Huertas *et al.*, 1999; Fusco *et al.*, 2007; Bahadorani *et al.*, 2008; de Oliveira *et al.*, 2012; Gutiérrez-Mariscal *et al.*, 2012; Casani *et al.*, 2013). While the results obtained with these antioxidants have been successful in terms of attenuation of oxidative stress mediated by age or aged-related diseases, little or no success it has been related to increased longevity.

Vitamin C is one of the naturally occurring antioxidants; it acts as first defense against free radicals in whole blood and plasma, and combined to α-tocopherol (the major form of vitamin E in humans) is particularly effective in inhibiting oxidation (Bruno *et al.*, 2006; Niki 2014). However, it is rather unclear how ascorbic acid affects lifespan in humans and model organisms. Some studies suggest that vitamin C supplementation increase lifespan in different model organisms (such as worm, flies and rodents), while other studies not only do not find any beneficial effect of vitamin C on longevity, but even reported a decrease in lifespan following

vitamin C supplementation (Pallauf *et al*., 2013). In the case of tocopherol, supplementation with vitamin E in a correct doses and isoforms can restore the cellular homeostasis and protect against the deteriorating effects of oxidative stress, as those that affect to cell membranes, progression of degenerative diseases, and aging (Mocchegiani *et al*., 2014). Studies in *Caenorhabditis elegans*, *Drosophila melanogaster* and laboratory rodents support beneficial effect of vitamin E on lifespan (Ernst *et al*., 2013). Resveratrol is a polyphenol compound with antioxidant, anticarcinogenic, and anti-inflammatory properties. Scientific evidence has remarked its benefits in cardiovascular diseases and some cancers but also as an antiaging molecule, demonstrated the prolongation of life span by resveratrol supplementation in obese mammal models (Marchal *et al*., 2013). Green tea extract, curcumin, oxaloacetic acid and medium-chain triglyceride oil have reported have been evaluated without statistically significant effect on life span in mice (Strong *et al*., 2013), while acarbose, 17-α-estradiol and nordihydroguaiaretic acid showed a benefit increasing lifespan in male mice, but not in female (Harrison *et al*., 2013).

CoQ_{10} is an endogenous synthesized lipid, found in blood and in all organs, which transport electrons to complex III from complexes I and II and from the oxidation of fatty acids and branched-chain amino acids. CoQ_{10} has membrane-stabilizing and antioxidant properties and provides cell-protective effects, including inhibition of LDL oxidation and decreasing of proinflammatory cytokines production, as well as blood viscosity. Several studies have highlighted the beneficial effects of CoQ_{10} supplementation in a variety of clinical conditions, with emphasis on cardiovascular disease (Villalba *et al.*, 2010; Perez-Sanchez *et al.*, 2012; Potgieter *et al.*, 2013). Although CoQ_{10} is biosynthesized intracellularly, a small proportion is acquires through the diet. Results with these antioxidants were positives in terms of age-related oxidative stress attenuation, however, in relation to longevity increased only were observed positive effects in some cases. Long-term supplementation with a small dosage of CoQ_{10} might represent a good anti-aging therapy in rats fed on a PUFA-based diet, preserving beneficial aspects of PUFA on health and avoiding their deleterious aspects (Quiles *et al.*, 2004b). Furthermore, CoQ_{10} addition to dietary fat type modules age-related changes in mitochondrial DNA deletion and oxidative stress in heart of rats fed on a PUFA-based diet (Quiles *et al.*, 2010b) and in brain of rats fed on a monounsaturated fatty acid (MUFA)-based diet *versus* an n6 PUFA diet (Ochoa *et al.*, 2011) in rats fed on, as well as modified gene expression of antioxidant, proinflammatory and endoplasmic reticulum stress-related genes in peripheral blood mononuclear cells, which modules the inflammatory response and protects against the postprandial oxidative stress and decreases the free radical over-generation, improving the antioxidant system and the redox balance (Yubero-Serrano *et al.*, 2011, 2012, 2013).

A greater success with therapy based on antioxidants requires a deeper understanding of the pharmacological properties of the molecules studied, particularly in relation to absorption, tissue distribution, metabolism and dosage. Also, it should consider the role of ROS on cellular signaling, so must be adjust the antioxidants dose in order to avoid changes in redox state which could alter cell function. The above problems are being solved in part by using a new generation of synthetic antioxidants, superoxide dismutase and catalase mimetics. These substances are being tested with some success, and they have been effective in increasing longevity (Hosakote *et al*., 2012; Noritake *et al*., 2013; Lieb *et al*., 2014).

1.3. Fatty acids.

In addition to ROS production, there is another constitutive characteristic of long-lived animals that links aging with oxidative stress: the degree of fatty acid unsaturation of tissue

cellular membranes, which have had to adapt to protect themselves against reactive species (Quiles *et al.*, 2006c; Naudí *et al*., 2013). Biological membranes are dynamic structures that generally consist of bilayers of amphipathic molecules held together by non-covalent bonds (Vance and Vance, 1996). The predominant membrane lipids are phospholipids, whose acyl chains are either saturated, monounsaturated or polyunsaturated hydrocarbon chains with a chain length around 18 carbon atoms in eukaryotic cells from vertebrate species, and a relative distribution between saturated and unsaturated fatty acids follows a ratio of 40:60. The susceptibility of biological membranes to oxidative alterations is related to the chemical reactivity of their fatty acids composition (Pamplona, 2008).

Unsaturated fatty acids are the cellular macromolecules most sensitive to oxygen radical damage due to the presence of highly unstable electrons near their double bonds, and their sensitivity to lipid peroxidation is major in molecules with more number of double bonds (Holman, 1954; Bielski *et al.*, 1983). Thus, a low level of fatty acid unsaturation in cellular membranes (e.g., that of oleic acid) will decrease cellular oxidative stress. Some studies show that the degree of fatty acid unsaturation of mammalian tissues is negatively correlated with maximum longevity (Pamplona *et al.*, 2000a, 2008).

Moreover, lipid peroxidation products can produce protein covalent modifications. In this sense, lower levels of malondialdehyde-lysine and carboxymethyl-lysine protein adducts have been found both in mitochondria and tissues of the long-lived mammals and birds that display a low degree of fatty acid unsaturation (Pamplona *et al.*, 2002). Recent studies indicate that experimentally induced decreases in liver and brain fatty acid unsaturation also lower oxidative damage in mtDNA (Pamplona *et al.*, 2004).

The high concentration of unsaturated fatty acids in cellular membrane phospholipids not only makes them more sensible to oxidation reactions, also enables them to participate in long free radical chain reactions. So, a low degree of fatty acid unsaturation in biological membranes may decrease their sensitivity to lipid peroxidation, which can protect even other molecules against lipoxidation-derived damage (Mataix *et al*., 1998). Supporting this premise, it has been found that long-lived animals (birds and mammals, including humans) have a lower degree of total tissue and mitochondrial fatty acid unsaturation than short-lived ones (Pamplona *et al.*, 1996, 2002a, 2000b, 2000c; Pamplona and Barja, 2007; Hulbert *et al.*, 2007; Buttemer *et al.*, 2010). Also, it has been found evidence that in long-lived animal species a low degree of total tissue and mitochondrial fatty acid unsaturation is accompanied by a low sensitivity to lipid peroxidation and a low steady-state level of lipoxidation-derived adducts in both tissue and mitochondrial proteins from organs such as skeletal muscle, heart, liver and brain (Hulbert *et al.*, 2007; Pamplona, 2008).

Since fatty acids are directly related with the aim of the present study, a more detailed revision of their role in health, oxidative stress and aging will be accomplished in the forthcoming sections.

2. DIETARY FAT AND HEALTH

Nutrition has important long-term consequences for health that contributes to the development and progression of chronic diseases thus affecting life span. Fatty acids and other components of the diet may modulate, among others, mechanisms involved in homeostasis, aging, and age-related diseases, such as cancer, inflammation, diabetes, neurodegenerative diseases, cardiovascular pathologies, etc. The impact of the diet and dietary components on aging and age-associated degenerative diseases has been widely recognized in recent years.

Therefore, prevention through the introduction of lifestyle and proper nutrition habits (functional foods) is now considered a primary strategy for what we call healthy aging (Finkel and Holbrook, 2000; Meydani, 2001, 2005; Guo et al, 2009; Ribarič, 2012; Hammar and Ostgren. 2013, Santos-González *et al.*, 2013). Fatty acids are one of the dietary components most related to the maintenance of health as it is involved in the development of many disorders including cardiovascular diseases, diabetes, cancer, neurodegenerative disorders, gastrointestinal disturbances, allergies, etc (Quiles *et al*., 2006b).

Fatty acids can be classified into different series according to the position of the last double bond noted in each group member shorting form the terminal methylcarbon designated as n or w (Table 2). Linoleic acid, linolenic acid and oleic acid give rise to three major series, the n6, n3 and n9, respectively, after the last double bond in atoms is 6, 3 and 9 in fatty acid formed.

Table 2. *Different components of fatty acids n6; n3; and n9 series*

n6 series		**n3 series**		**n9 series**	
Linoleic	C18:2 n6	Linolenic	C18:3 n3	Oleic	C18:1 n9
Alpha-linoleic	C18:3 n6	Eicosatetraenoic	C20:4 n3	Eicosadienoic	C20:2 n9
Arachidonic	C20:4 n6	Eicosapentaenic	C20:5 n3	Eicosatrienoic	C20:3 n9
Docosatetraenoic	C22:4 n6	Docosapentaenoic	C22:5 n3	Docosatrienoic	C22:3 n9
Docosapentaenic	C22:5 n6	Docosahexaenoic	C22:6 n3	Docosatetraenoic	C22:4 n9

The main source of dietary fats is oil. Oil can be divided from the point of view of chemical composition into major and minor fractions. The major components, which include glycerols, represent more than 98% of total oil weight (table 3). Minor components, which are present in very low amounts (about 2% of oil weight) include more than 230 chemicals compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (carotenoids and phenolic compounds) (table 4) (Quiles *et al*., 2006b; Sánchez-Fidalgo *et al*., 2012;Cabello-Moruno *et al*., 2014).

Table 3. *Typical fatty acid profile (g/100g) of different oils and fats.*

Fatty acid	Palm oil	Butter	Margarine	Virgin olive oil	Canola oil	Sunflower oil	Corn oil	Soybean oil	Fish oil
C14:0	1,5	6,77	-	-	-	-	-	0,1	11,0
C16:0	45,1	11,94	7,83	8,7	5,4	6,35	12,6	10,6	26,7
C16:1n7	-	1,64	0	1,1	0,3	0,2	0,2	0,1	26,7
C18:0	4,8	10,44	5,81	1,9	1,6	4,5	1,9	3,8	4,1
C18:1n9	36,8	30,7*	44,59**	78,7	56,3	32,1	24,1	23,0	12,6
C18:2n6	10,2	19,67	30,5	8,3	25,0	55,92	60,1	52,4	1,7
C18:3n3	0,5	1,82	3,29	0,9	8,4	0,1	1,0	8,9	-
C20:5n3	-	-	-	0,03	-	0,03	-	-	13,8
C22:6n3	-	-	-	0,05	-	0,12	-	-	6,8
Total saturated	51,9	45,9	18,0	10,9	8,2	12,9	14,6	15,6	41,8
Total monounsaturated	37,0	29,0	29,2	79,8	58,4	32,3	24,3	23,1	27,2
Total polyunsaturated	10,7	25,5	33,8	9,3	33,4	54,8	61,1	61,3	31,0

* Of which 2,84 are trans. **Of which 15,64 are trans.

Table 4. *Minor components (mg/kg) profile of different oils.*

Component	VIRGIN OLIVE OIL	PALMA OIL	FISH OIL
Unsaponifiable fraction (%)	1,3		
Total sterols (ppm)	1696,0	423,0	1500,0
Cholesterol (%)	0,15	3,97	100,0
Campesterol (%)	3,80	7,66	--
Stigmasterol (%)	0,81	0,42	--
Brasicasterol (%)	--	0,14	--
α-Tocopherol (ppm)	34,0	310,0	300,0
γ-Tocopherol (ppm)	13,0	10,0	--
Squalene (ppm)	3000,0		7400,0
Aliphatic alcohols	60-200		
Triterpene alcohols	500-3000		
β-carotene	0,33-3,69		
Hydroxytyrosol	14,4	--	--
Tyrosol	27,45	--	--
Vit E (mg/kg)	5,10	12,00	
Vit K (mg/kg)	49,60	--	

2.1. Dietary fat type and the composition of biological membranes

Biological membranes are dynamic structures that generally consist of bilayers of amphipathic molecules held together by non-covalent bonds. The predominant membrane lipids are phospholipids, whose acyl chains are either saturated, monounsaturated or polyunsaturated hydrocarbons chains that normally vary from 14 to 22 carbons in length. The fatty acid desaturation pathway and the deacylation-reacylation cycle are the most important mechanisms responsible for the fatty acid composition of biological membranes (Vance and Vance, 1996; Wallis *et al.*, 2002). Biological membranes are composed of more than 50% of lipids, and the quantity and quality of dietary lipids are known to have an impact on the composition, characteristics and functions of the biological membrane (Rohrbach, 2009; Aoun *et al.*, 2012b).

Fatty acids as components of biological membranes strongly influence membrane fluidity, which, in turn, may influence many physiological processes involved in cell death and survival such as signal transduction, protein import, membrane receptor function and metabolite transport (Simkiss, 1998). In addition, fatty acids composition is the main responsible of the membrane phospholipids susceptibility to oxidative alterations (Pamplona, 2008). First, oxygen and free radicals are more soluble in the fluid lipid bilayer that in the aqueous solution, focusing in these organic regions and becoming membrane lipids primary target of oxidative damage (Gamliel *et al.*, 2008). Moreover, PUFA from membrane phospholipids are highly sensitive to oxidation, so membranes very rich in PUFA should be more prone to oxidation and ROS-

induced damage, and their sensitivity to oxidation increasing exponentially as a function of the number of double bonds per fatty acid molecule. Therefore, PUFAs side chains are much more easily attacked by radicals than saturated or monounsaturated side chains (Pamplona and Barja, 2011). The degree of fatty-acid unsaturation and the lifespan is not due to a low content of unsaturated fatty acids in long-lived mammals, but mainly to redistributions between components of the n3 fatty PUFAs series (Pamplona *et al.*, 1998, 1999). Dietary fat influences in relative fatty acid composition of the membrane, while the elongase and desaturase system also contributes to such an event through the inhibition of the various components of the different series of fatty acids (Quiles *et al.*, 1999a). As is well known, a fine control in the elongation and desaturation pathways is crucial for the maintaining of the membrane function through proper fluidity, signaling and other means (Sanz *et al.*, 2006).

Several studies showed that diet rich in olive oil stimulates the formation of membranes where the presence of n3 PUFAs when compared with diets rich in sunflower oil as fat source are increased (Navarro *et al.*, 1994; Escudero *et al.*, 1998). In diets rich in n3 PUFAs was observed a shift in the proportion of fatty acids of the n6 PUFAs too. There is a ratio between n9/n6 series suggesting that fatty acid from n9 series could be more easily incorporated in detriment to n6 series. Diets rich in unsaturated fatty acids increase levels of MUFAs and rich in n3 PUFAs increase the ratio n3/n6, which is also observed in long-lived animals (Ayre and Hulbert, 1997; Pamplona and Barja, 2003). The maintenance of membrane fluidity is achieved in mammals by a homeostatic mechanism and compensation control that maintains an optimal relationship between the proportion of saturated and unsaturated fatty acids (Gibson *et al.*, 1984; Girón *et al.*, 1992). No significant changes were observed in saturation of membrane lipids since an increase in the n6 series resulted in a decrease in the n3 and vice versa (Huertas *et al.*, 1991a; Quiles *et al.*, 1999a). Animals with a high longevity have a low degree of membrane fatty acid desaturation based in the redistribution between types of PUFAs without alteration in the total PUFAs content and phospholipids redistribution (Pamplona and Barja, 2011).

- There is a tendency that the proportion of SPAs remains stable, as C18:0, which is largely dependent on the concentration needs of each tissue. In the case of C16:0, adaptability is observed according to the composition of the diet, with a special ability to activate the Δ9 desaturase (Pamplona, 2008). In diets rich in fatty acids of the n9 series, it is shown a tendency to increase in MUFAs, mainly when oleic acid is administered (Ochoa *et al.*, 2002). In the case of the n6 series, their membrane levels only seem to increase when administered in the diet large number of their components (Quiles *et al.*, 2010b). The n3 series is present when good amounts is administrated, or when there is a deficiency of n6, after the two series have competed by the Δ6 desaturase, which determines the ratio n3/n6 (Martin, 2002).

2.2. Oil minor components and health

It is now well-established that not only the major fatty acid constituents, but also the minor phenolic components in oil have important health benefits for cancer, cardiovascular diseases, metabolic syndrome and inflammatory conditions due to their potent antioxidant and anti-inflammatory effects.

Much has been written about the health benefits of olive oil. However, traditionally, many of the beneficial properties associated with this oil have been ascribed to its high oleic acid content. Other diets are high in oleic acid, for example, oleic acid is present in pork, but these fail to promote the low cardiovascular disease profiles or other beneficial trends associated with

the Mediterranean diet. It is becoming increasingly clear that many of the benefits associated with the ingestion of virgin olive oil are due to its minor compounds (Granados-Principal *et al*., 2010). Because characteristics of minor components, these molecules are present almost exclusively in virgin olive oil since the process (mainly refining) involved in the production of these oils remove them. As mentioned above, minor components include more than 230 chemical compounds, such as non-glyceride esters, aliphatic alcohols, triterpene alcohols, sterols, hydrocarbons, pigments, lipophilic phenolics, hydrophilic phenolics and volatile compounds (Quiles *et al*., 2006b). Although the quantities of tocopherol and carotenoids present in a daily consumption of virgin olive oil are low, its chronic ingestion contributes to the overall pool of antioxidants in the body. Sterols consumption decreases levels of plasma LDL cholesterol. Triterpenes, such as erythrodiol and oleanolic acid have shown anti-inflammatory and antioxidant properties in addition to vasodilatatory activity (Pérez-Jiménez *et al*., 2005).

One of the most studied polyphenols is hydroxytyrosol, which comes from the hydrolysis of oleuropein. Hydroxytyrosol has been demonstrated in numerous studies to have antiatherogenic properties with strong antioxidant power. It acts as a powerful scavenger of free radicals with the superoxide anion, hydrogen peroxide, hypochlorous acid, etc. In addition, it has a strong chelating effect on metals such as iron, and thus diminishes the appearance of reactive oxygen species derived from reactions associated with this metal. This characteristic is the main factor responsible for the effect in atherosclerosis, characterized by the entrance of LDL particles oxidized in the interior of the arterial intima. Oxidation is probably due to the action of macrophages as well as endothelial and smooth-muscle cells. Hydroxytyrosol is capable of preventing oxidation of these lipoproteins by macrophages, since it increases the antioxidant capacity of these cells related to lowered glutathione levels (Granados-Principal *et al*., 2010). Hydroxytyrosol can be considered antithrombotic, since it significantly reduces platelet aggregation. It has been reported that hydroxytyrosol diminished the synthesis of thromboxane B2 in an *in vitro* model of platelet-rich plasma, probably as a result of reduced production of eicosanoids derived from arachidonic acid (AA) (such as 12-hydroxyeicosatetranoic acid). These antithrombotic effects would be helped by the decline in the production of vascular prostacyclin, effects similar to those presented by acetyl salicylic acid. Hydroxytyrosol also reduced the production of leukotriene B4 of leukocytes and the platelet aggregation (Granados-Principal *et al*., 2010).

More recently, hydroxytyrosol has demonstrated an anti-tumour effect *in vivo* in rats with experimental mammary tumours, inhibiting growth and cell proliferation in mammary tumours. Moreover, hydroxytyrosol alters several genes associated with cell proliferation (associated with a less nuclear Ki-67 immunostaining), apoptosis and the Wnt signalling pathway, promoting a high expression of Sfrp4 (Granados-Principal *et al*., 2011). Long-term studies on gingival vascular damage in atherosclerotic rabbits reported hydroxytyrosol and squalene (other natural product from the minor fraction of virgin olive oil) benefits reducing endothelial activation and decreasing fibrosis, which would be possible to modulate host response in gingivitis and periodontitis by using a dietetic approach (Bullón *et al*., 2009a).

2.3. Effects on the cardiovascular system

Research dating back to the 1950s reported an association between the fat of the diet and cardiovascular diseases, relating the consumption of saturated fatty acids (SFAs) with atherosclerosis and risk of coronary heart disease (Seiquer *et al.*, 1996; Ramírez-Tortosa *et al.*, 1999a; von Schacky, 2009; Torrejón and Uauy, 2011). By contrast, vegetable oils and especially

those derived from fish (rich in PUFAs) appear to have anti-atherogenic effects. Dietary recommendations have been made to decrease saturated and *trans*-fatty acids and increase unsaturated fatty acids, both MUFAs and PUFAs (Siegel and Ermilov, 2012).

Studies demonstrated the low incidence on cardiovascular diseases in countries with a Mediterranean diet, in which the fat source mainly is olive oil, rich in MUFAs (Menotti *et al.*, 1996, Altomare *et al.*, 2013). Thus, replacing a saturated diet by an unsaturated or polyunsaturated diet causes a decrease in plasma cholesterol as well as LDL cholesterol levels, increase HDL cholesterol levels, decrease atherosclerotic cardiovascular disease risk and lipid peroxidation, improve blood pressure control and favorable modifications of hemostasis (Baum *et al.*, 2012). In fact, a MUFAs rich diet prevents oxidative damage to lipoproteins more than a polyunsaturated. Fatty acids in the diet cause a more or less susceptibility to oxidation of lipoproteins exerting a large effect on the activation of adhesion molecules and other inflammatory factors. For example, oleic acid in the diet decreases the gene expression of protein adhesion to the endothelium, V-CAM1 and reduces LDL susceptibility to oxidation (Aguilera *et al.*, 2001; Salas-Salvadó *et al.*, 2008).

Several studies have reported a lower risk of cardiovascular disease when PUFA replaces SFA in the diet (Brouwer *et al.*, 2004; Wang *et al.*, 2006). At first it was thought that alpha-linolenic acid (ALA, C18:3n3) was the responsible for a lower risk of fatal coronary heart, but more recent analysis concluded that increased consumption of long-chain n3 PUFA (n3 LCPUFA) as eicosapentaenoic acid (EPA, C20:5n3) and docosahexaenoic acid (DHA, C22:6n3), but not of ALA, reduces the rates of sudden cardiac death (Vedtofte *et al.*, 2012).

Fish consumption enhances n3 PUFA, which constitutes a preventive element of thrombogenesis because it allows PGI3 prostaglandin formation, a vasodilator and platelet antiaggregant, and thromboxane TXA3, hardly vasoconstrictor and proaggregant. Furthermore, EPA and DHA inhibit the formation of prostaglandin PGI2, with effects similar to PGI3, but also thromboxane TXA2, vasoconstrictor and platelet aggregant. In this way they have an antithrombogenic character and justifies, in part, that fish consuming populations presented lower prevalence of cardiovascular disease (Sirtori *et al.*, 1998; Villalobos *et al.*, 2010).

Oleic acid from olive oil, the major dietary MUFA, also showed EPA effect from fish oil, but more attenuated, increasing PGI3 and TXA3 formation, while decreased PGI2 and TXA2 synthesis (Navarro *et al.*, 1994; Delgado-Lista *et al.*, 2012).

Meanwhile, n6 PUFA, mainly linoleic acid, has favorable effects on LDL-cholesterol levels and total to HDL-cholesterol ratio, and consequently decreases cardiovascular risk. However, linoleic acid alone and linoleic acid in combination with n3 PUFA may produce different findings, so mixed n3/n6 PUFA or specific n6 PUFA diets have significantly different effect on cardiovascular diseases: mixed n3/n6 PUFA for SFA found reduced risk of cardiovascular disease, whereas n6 specific PUFA interventions tended to increase it due to n6 PUFA decreases n3 PUFA by direct competence by the Δ6 desaturase (Vedtofte *et al.*, 2012).

2.4. Effects on the digestive system

Digestive functions are considered to be alterable by the ingestion of large amounts of fatty acids in the diet. Numerous studies have shown the ability of the dietary fats to affect gut functions, such as motility, secretion and propulsive function. It has been also described that chronic ingestion of fatty acids in the diet changes the sensitivity of satiation signals and luminal transit rates of gastrointestinal contents. Therefore, dietary fats are strongly considered to be potential factors that contribute to dysregulation of appetite through modulation of the motility of gastrointestinal tracts (Park *et al.*, 2013).

Studies in animals and in humans associated with digestive secretions (gastric, pancreatic and bladder) in the fasted and in response to food intake, showed that after adaptation or in response to diets high in olive oil there were a number of positive effects. Among these are reductions in intragastric acidity after ingestion of diets containing olive oil mixed against oils rich in PUFAs (Serrano *et al.*, 1997). Olive oil fat diet also ameliorated both protein concentration and amylase activity in exocrine pancreatic secretion (Díaz *et al.*, 2003). On the other hand, the type of dietary fat habitually consumed can influence bile composition, mainly cholesterol saturation index, which decreases significantly with the olive oil diet while in sunflower oil as majority fat of diet is supersaturated (Yago *et al.*, 2005).

Many studies have demonstrated that ghrelin and the ghrelin receptor agonist (growth hormone releasing peptide 6) accelerate gastric emptying and small intestinal transit (Levin *et al.*, 2006). Ghrelin expression and secretion seem to be influenced by the fat content of the diet. A diet with fish oil and olive oil estimulate fasting ghrelin expression and plasma levels, so consumption of these dietary oils can play an important role in ghrelin regulation, which might affect feeding behavior and energy intake (Saidpour *et al.*, 2012).

Changes in the fatty acid profile related to the biosynthesis of eicosanoids and inflammatory response have been described. These changes may have beneficial effects on inflammatory bowel disease by altering the lipid environment required for the production of these mediators in relation to the inflammatory response, being more resistant to lipid peroxidation processes (Bosco *et al*., 2013). Olive oil modifies basal and postprandial levels of several gastrointestinal peptides (cholecystokinin, peptide tyrosine tyrosine, gastrin and pancreatic polypeptide) associated with this disease (Yago *et al.*, 1997; Serrano *et al.*, 1997; Degen *et al.*, 2007; Zhou *et al.*, 2012).

Olive oil based diets also reduce the production of cytokines, the expression of adhesion molecules, and the levels of other endothelial markers in inflammatory bowel pathology (Sánchez-Fidalgo *et al*., 2012). MUFAs and phenolic compounds of olive oil increase the threshold of apoptosis in intestinal cells, decreasing necrosis and exerting a protective role (Mataix, 2001; Mataix *et al.*, 2008). The benefits of an olive oil diet in the management of inflammatory bowel disease are further enhanced by the addition of n3 PUFA, attenuating colonic inflammation (Camuesco *et al.*, 2005).

2.5. Effects on diabetes

Fatty acids and fatty acids-derived metabolites have long been implicated in the development of insulin resistance and type 2 diabetes. Randle (Radle, 1998) showed that the rise in lipolysis and resultant increase in circulating FAA in the fasted state contributes to increased dependence of tissues on fatty acid oxidation to provide energy. Glucose oxidation is reduced under these conditions in part via the generation of byproducts of fatty acid oxidation that suppress key steps of glucose metabolism. Instead, McGarry (McGarry, 2002) demonstrated that increased availability of glucose and insulin in the fed state leads to production of malonyl CoA, a potent allosteric inhibitor of fatty acid oxidation. Certain studies related insulin resistance of obese states with intrinsic mitochondrial deficiency, which lead the consequent accumulation of bioactive lipid species that interfere with insulin signaling (Savage *et al.*, 2007). Other researches have determinate that overloading of normally active mitochondria with lipid substrates lead to mitochondrial dysfunction and activation of alternative pathways for impairment of insulin action (Koves *et al.*, 2008).

Recently it has been shown the positive relationship between n3 PUFA from fish oil with both prevention and reversion of high-fat-diet–induced adipose tissue inflammation and insulin

resistance in rodents, suggesting that EPA and DHA might help delay the progression of metabolic syndrome to type 2 diabetes via peroxisome proliferator-activated receptors alpha (PPARα) and MAPKs (Kalupahana *et al.*, 2011). Fish oil diets improved glucose tolerance, decreasing insulin resistance, in addition to improve glucose tolerance by decreasing adipose tissue lipogenesis and inflammation (Jelinek *et al.*, 2013).

Several studies have manifested that the olive oil lowers blood glucose levels due to better utilization of glucose and likewise reduce insulin requirements. Garg in 1998 (Garg, 1998) performed a meta-analysis of ten studies in type 2 diabetes patients comparing the effect of a diet rich in MUFAs and other rich in carbohydrates, proving that the first decreased the glucose levels on fasting and postprandial, unchanged fasting insulin levels. The KANWU study (Vessby *et al*., 2001) confirmed a beneficial effect of MUFA compared with saturated fat on insulin sensitivity. A large European case-cohort study showed that the consumption of virgin olive oil, as part of the Mediterranean diet, was associated with a lower risk of developing type 2 diabetes (InterAct Consortium *et al*., 2011). Other researches demonstrated that a diet rich in olive oil reduced the glucose and insulin profile 24 hours and daily necessities hormone. Since then, many olive oil benefits on diabetes have been shown, such as the improvement of insulin sensitivity and the inverse association between serum phospholipid oleic acid and insulin resistance (Tierney and Roche, 2007; Sala-Vila *et al.*, 2011). So, the olive oil consumption has been postulated as a protector against the risk of type 2 diabetes (Salas-Salvadó *et al.*, 2011), improved the systemic lipid and DNA oxidative damage in metabolic syndrome patients (Mitjavila *et al.*, 2013). An examination of the association of dietary and membrane fatty acids with insulin secretion in the cross-sectional Pizarra study (Rojo-Martínez *et al*., 2006) showed that dietary MUFA from olive oil and PUFA contributed to the variability of β-cell function. A recent study has related the consumption of a virgin olive oil with the maintaining of pancreatic microstructure, which ameliorating the endocrine and exocrine function of this organ and tending to improve insulin sensitivity (Roche *et al*., 2013). This study of changes in pancreas associated to dietary fat will be discussed later.

2.6. Effects on cancer

Approximately 80% of human cancers (especially breast, ovary, prostate, colorectal and upper digestive and respiratory tract cancers) have been associated with unhealthy lifestyles (López-Miranda *et al*., 2010). In terms of fat intake and cancer, it must be taken into consideration both the amount of fat as the quality of it and the ratio of fatty acids present in the type of fat (Quiles *et al.*, 2004c). In this sense, there is experimental evidence that indicate the possible existence of a level of n6 PUFAs that can make a difference in the ability of different etiological types of fat in relation to the genesis of cancer. It seems that a 4% of weight of the diet in the form of linoleic acid (8% of total energy) sets a limit below which the fatty acids have a distinct carcinogenicity, while above this limit all have the same capacity (American Health Foundation, 1987).

Several studies have reported that olive oil consumption is associated with lower risk of cancer development (mainly breast, colorectal and prostate cancer), at variance from diets rich in total fat and in linoleic acid or SFA, which have been related to an increased cancer risk (López-Miranda *et al*., 2010). From the point of view of tumor initiation, the role of the fat is marked by oxidative type factors, such as the generation of free radicals conditioned by fat unsaturation. Moreover, lipids can alter the structure of the chromatin and act as co-carcinogens facilitating the accessibility and the genotoxic action of several agents (Escrich *et al*., 2006). In relation to the growth and progression of cancer, it is necessary to increase vascularization and

in parallel the immune system must be altered, in this process are key various derivatives of AA metabolites, including prostaglandin E2 (PGE2) generated from derivatives of linoleic acid. Olive oil attenuates growth by lower production of it. In the metastasis the gene expression of adhesion factors of cancer cells to tumor unit (E-cadherin) is decreased. At the same time another group of adhesion molecules, such as vascular endothelial I-CAM1, V-CAM1 and E-selectin are overexpressed (Aguilera *et al.*, 2001; Quiles *et al.*, 2001; Sugimura, 2001). In addition, compared with n6 fatty acids, n3 fatty acids seem to exert a protective effect on cell growth that may, at least partly, be explained by the formation of oxidation products, which leads to cell growth arrest and, as they are cytotoxic, apoptosis (Escrich *et al*., 2006). Taking on consideration the potent antioxidant activity of olive oil-phenols on different cellular systems, a significant part of the chemopreventive ability of olive oil could be attributable to its antioxidant properties (Di Benedetto *et al.*, 2007; Casaburi *et al.*, 2013). In fact, olive oil phenols could be successfully employed as therapeutic adjuvants against certain tumor phenotypes, such as breast cancer (Sirianni *et al.*, 2010; Granados-Principal *et al.*, 2010). As it has been mentioned in 2.2 section of this chapter, some virgin olive oil compounds, such as hydroxytyrosol, has anticancer effect inhibiting growth and cell proliferation in tumours (Granados-Principal *et al*., 2011)

The use of PUFAs from fish oil, mainly DHA and EPA fatty acids in a therapeutic context is also increasing in patients receiving treatment for a range of cancer types. EPA and DHA, can inhibit tumor growth through a variety of proposed mechanisms, including apoptosis, inhibition of angiogenesis, and alterations to cell signalling, all of which are implicated in the reduced risk of cancer development (Baracos *et al.*, 2004). The literature suggests that marine PUFAs have potential as an effective adjuvant to chemotherapy treatment, may have direct anticancer effects, and may help ameliorate some of the secondary complications associated with cancer (Merendino *et al.*, 2013; Vaughan *et al.*, 2013). EPIC study has evaluated how the Mediterranean diet decreases the risk of develop certain cancers, such as urothelial cell bladder cancer (Buckland *et al*., 2013), lymphoid and myeloid Leukemia (Saberi Hosnijeh *et al*., 2014), colorectal cancer (Murphy *et al*., 2013), breast cancer (Cottet *et al*., 2009), gastric, lung and prostate cancer, among other (Gonzalez and Riboli, 2010).

2.7. Effects on brain (cognitive functions)

The prevention of neurodegenerative diseases, such as Alzheimer's, Parkinson or Huntington diseases, is a growing public health concern, due to a rising global prevalence. Various potential risk or preventive factors have been suggested by epidemiological research, including modifiable lifestyle factors such as diet (Gillette-Guyonnet *et al.*, 2013). The lipids constitute majority of dry weight of mature human brain. 35% is comprised of PUFA with long chain (LC-PUFA), especially DHA of n3 family and AA of n6 family. N3 PUFA has been considered the main protective fatty acid in the prevention of cognitive decline because modulates immune and inflammatory processes in the brain, oxidative stress as well as its consequences (Zeman *et al.*, 2012). N3 PUFAs, including DHA and EPA are major components of neuronal membranes and have a wide range of functions, from modulating synaptic plasticity and neurochemistry, to neuroimmune-modulation and neuroprotection. Fatty acid composition of neuronal membranes can be directly altered by dietary modifications. Membrane fluidity also changes with age and neurodegenerative diseases, for various reasons, including increased content of cholesterol and ROS. Incorporation of PUFAs from diet to neuronal membranes is able to compensated neurodegenerative changes by maintaining membrane fluidity (Yehuda *et al.*, 2002; Frisardi *et al.*, 2011).

PUFAs are known to mediate widespread effects on gene expression in the neurodegenerative diseases, regulating gene expression of ligand-activated transcription factors as the retinoic acid receptors (RARs), and retinoid X receptors (RXRs). Retinoid signaling pathways have been implicated in regulating synaptic plasticity and learning and memory in rodent (Dyall *et al.*, 2010). Another important transcription factor involved with n3 PUFA is cAMP response element binding protein (CREB), which is important for synaptic integrity and synaptic transmission and largely belonging to the CREB1 transcription pathway, and it plays an important role in learning and memory in mammals (Sidhu *et al.*, 2011). PUFA is able to bind to peroxisome proliferator-activated receptors gamma (PPARγ) and directly downregulate inflammatory gene expression, but it also interferes with the activation of kappa nuclear factor B (NFκB), a major inflammatory transcription factor responsible for the induction of pro-inflammatory cytokines and enzymes such as COX-2 and iNOS (Calder, 2011), whereby the anti-inflammatory effects can be discussed. In fact, inappropriate amounts of dietary n6 and n3 fatty acids could promote neuroinflammation (Layé, 2010).

Effects of n3 PUFA deficiency and supplementation on cognitive development have been extensively researched in studies with rodents as well as humans. The conclusions have been similar; the n3 PUFA supplementation, especially DHA and EPA, in child- and adult-hood can modulate synaptic plasticity and neurochemistry, as well as cognitive performance, and hence improves neuronal function, while n6 PUFA contributed negatively to cognitive performance (Luchtman and Song, 2013). Recent studies have suggested that high MUFA intake and regular fish and n3 PUFA consumption, may play a role in the prevention of Alzheimer's disease but also cognitive decline associated with aging or predementia syndromes and their progression to overt dementia (Solfrizzi and Panza, 2013).

2.8. Effects on the immune system

Dietary fatty acids have effects on the immune system and inflammatory processes associated with it, therefore the primary and secondary immune responses to severe diseases might be changed by the nutritional condition, since the immune system has evolved a capacity to modify expenditure on inflammation to compensate for the effects of dietary fatty acids.

Fatty acids can affect the immune system via several mechanisms (Yaqoob, 2006; Jędrzejczak-Czechowicz and Kowalski, 2011):

- The first mechanism involves incorporation of lipids into the structure of cellular membranes. Alteration of the fatty acid composition of the diet can modulate de fatty acid composition of cells of the immune system and thus affecting its fluidity, permeability of ion channels and functions of membranous receptors.

- The second mechanism is associated with penetration of fatty acids to the cell where they can affect the production of eicosanoids, cytokines, pathways responsible for signal transduction into the cell, and expression of genes. Eicosanoids are important immunoregulatory molecules derivate from AA, which is released from membrane phospholipids.

- Finally, fatty acids may alter cell function by direct interaction with intracellular targets, including transcription factors that can modifies gene expression. Moreover, fatty acids can alter cell apoptosis and production of ROS.

Olive oil, due to its high content in MUFA n9 oleic acid, and PUFA n3, mainly DHA and EPA, have anti-inflammatory and antioxidant effects, which reduces levels of oxidative stress

and inflammation in chronic autoimmune diseases, as rheumatoid arthritis, by decreasing of PGE2 metabolites and thromboxane A2 (TXA2), reduction of plasma level in TNF-α, IL-1β, and IL-6, which reduce the production of C-reactive protein as well as ICAM-1 and VCAM-1 (Wardhana, 2011). As mentioned above, n3 fatty acids are important regulators and act as natural ligands of PPARγ, which activation by n3 fatty acids may play a role in innate or adaptive immunity by affecting dendritic cells migration that could contribute to the initiation and modulation of immune responses, and their subsequent accumulation in lymphoid organs (Szatmari and Nagy, 2008). Attending to lymphocyte proliferation and natural killer cell (NK cells) activity, consumption of a MUFA-rich diet, compared with diet based on hydrogenated coconut, sunflower or fish oils, decreases the proliferation of mesenteric lymph node lymphocytes in response to the T-cell migration and the NK cells activity, accompanied by an increase in the proportion of oleic acid in plasma phospholipids and peripheral blood mononuclear cells (Yaqoob, 2006).

Due to their anti-inflammatory properties, n3 PUFAs have been applied in the treatment of inflammatory disorders; although this may involve a reduction of immune protection against infectious microorganisms, except virgin olive oil that is able to modulate several immune functions without reduce host immune resistance to infectious microorganisms by increment of IL-4 and IL-12, cytokines involved in removing infectious agents with intracellular and extracellular growth, respectively (Puertollano *et al*., 2010).

2.9. Effects on the bone

There is a relationship between fat and bone. With aging, the composition of bone marrow shifts to favor the presence of adipocytes, osteoclast activity increases, and osteoblast function declines, resulting in osteoporosis. Other causes such as diabetes mellitus, glucocorticoids and immobility, are associated with bone-marrow adiposity (Rosen and Bouxsein, 2006). Dietary fat has been related with the bone remodeling process and prevention of osteoporosis. Animal studies have shown that dietary n3 LCPUFA may increase bone formation and reduce bone loss through alterations in the production of eicosanoids, expression of genes involved in osteoclast differentiation and calcium absorption. In other studies where young animals were fed with n3 LCPUFA combined with α-linolenic acid or n6 PUFA the positive effect on bone formation was not only smaller but could even disappear (Andersen *et al.*, 2013). Bone remodeling has been related with inflammatory states, being as the osteoclast is programmed to respond to inflammatory signal increasing osteoclastogenesis and bone resorption, so anti-inflammatory therapies can be used to reduce bone loss (Ferrari-Lacraz and Burger, 2010). The overconsumption n6 PUFA may contribute to the increased pathogenesis of osteoporosis by promoting low-grade chronic inflammation (LGCI), while dietary n3 PUFA, EPA and DHA, are potent anti-inflammatory which would inhibit the decline in bone mass (Kelly *et al.*, 2013). Recent findings have associated that the enhanced alveolar bone loss to age may be targeted by an appropriate dietary treatment. The mechanisms involved in this phenomenon are related with an ablation of the cell capacity to adapt to aging. Thus, MUFA or n3 PUFA might allow mitochondrial maintaining turnover through biogenesis or autophagy. They might also be able to induce the corresponding antioxidant systems to counteract age-related oxidative stress (Bullón *et al*., 2013). This study will be mentioned in detail in the section 3 of this chapter.

2.10. Effects on the skin and topic diseases

Changes in the appearance of the skin represent a visible sign of tissue alteration that occurs with age, which may result in the development of a large range of morbidities including non-melanoma skin cancers (Zouboulis and Makrantonaki, 2011). Skin is a major fat storage in humans, so numerous studies have focused on the possible role of diet in the capacity of the skin to resist damage induced by UV radiation and protect from photodamage. As skin damage is mainly driven by the production of ROS and related inflammation occurring in response to cumulated or intermittent intense sun exposure, high amount of MUFA and a low n6/n3 PUFA ratio in diets have been associated with major protective effect against free radical damage and skin dryness (Latreille *et al.*, 2012).

Relationship between the n3/n6 PUFAs in the diet and the development of atopic diseases such as asthma and eczema has been found. An important feature of atopic eczema is a decreased skin barrier function. The stratum corneum lipids, comprised of ceramides, FFA and cholesterol, fulfil a predominant role in the skin barrier function, and a change in the fatty acids chain length, with an increased presence of short-chain FFAs and a decreased presence of long-chain FFAs, alters lipid organization and increases the prevalence of skin lesions such as eczema and severe dermatitis (van Smeden *et al*., 2014). It has been reported an increased prevalence eczema, asthma and persistence of their symptoms in children of atopic mothers if the mother's milk contained low concentrations of ALA, EPA, DHA, total n3 fatty acids or a low n3/n6 ratio. The increased intake of these PUFAs in the diet significantly improved the asthma scores and airway responsiveness in children, and decreased allergic sensitization (Hageman *et al.*, 2012). Recently it has shown that dietary n3 LCPUFAs, EPA and DHA from fish oil, supplement daily during pregnancy and lactation reduce allergy and IgE-associated eczema due to sensitization for cow’s milk protein whey in children and prevent early childhood allergic disease in children at high hereditary risk (Makrides *et al*., 2013;van den Elsen *et al*., 2013).

3. EXPERIMENTAL STUDIES ON DIETARY FAT TYPE AND AGING

As stated above, the type of dietary fat affects mitochondrial structure and function as well as their susceptibility to oxidative stress. In this way, if we could build “customized” biological membranes according to a particular type of dietary fat, it would be possible to modify in a positive way the manner in which different organs age. This working hypothesis represents a new approach to the study of ageing from the point of view of nutrition, and it could have important implications for the study of the ageing phenomenon. We conducted experiments using virgin olive oil or sunflower oil as the source of dietary fat in relation to mitochondrial ageing in a rat model (Mataix *et al*., 2008). The research on dietary fat types and aging arose from our research group from the preliminary studies on fat and mitochondrial oxidative stress (Quiles *et al*., 2006a).

3.1. Dietary fat type and mitochondrial oxidative stress

The type of dietary fat influences several biochemical parameters at the mitochondrial membrane level (Mataix *et al*., 1998; Quiles *et al*., 1999a). The importance of fatty acids resides in the fact that the mitochondrial membrane (as do other biological membranes) adapts its lipid composition to some extent in response to dietary fat (Huertas *et al*., 1991a; Quiles *et al*., 1999c; Ochoa-Herrera *et al*., 2001). Thus, humans and animals fed on diets based on olive oil have membranes richer in oleic acid than those fed on diets based on sunflower oil, whose membranes are richer in linoleic acid. In addition, adaptations of the electron transport system in

response to the type of dietary fat have been widely reported (Huertas *et al*., 1991b; Quiles *et al*., 2001; Battino *et al*., 2002a). Moreover, oxidative stress is related to biological membrane composition. In that sense, a polyunsaturated fat source (e.g. sunflower oil) will lead to membranes becoming more prone to oxidation than a saturated (animal fat) or a monounsaturated (e.g. olive oil) source. This has been widely demonstrated under a wide range of physiological and pathological situations in both animal models and humans (Quiles *et al*., 1999b, 2002b; Ramírez-Tortosa *et al*., 1999a; Battino *et al*., 2002b; Ochoa *et al*., 2002). Table 5 shows the fatty acid profiles of the typical oils used in most of the experiments described in the following sections.

Table 5. *Typical fatty acid profile of edible oils in our experiments.*

Fatty acid composition	Virgin olive oil (g/100 g)	Sunflower oil (g/100g)
C16:0	8,9	7,2
C16:1n7	1,1	0,2
C18:0	1,9	4,5
C18:1n9	78,7	32,1
C18:2n6	8,4	54,3
C18:3n3	0,9	0,1
Total saturated	10,9	12,9
Total unsaturated	89,1	87,1
Total monounsaturated	79,8	32,3
Total polyunsaturated	9,3	54,8
MUFA:PUFA	8,6	0,6

3.1.1. Olive oil in the prevention of doxorubicin-mediated oxidative stress

Doxorubicin (Adriamycin, Pharmacia) is an anthracycline antibiotic obtained from *Streptomyces peucetius* that has been used for more than 30 years for the treatment of a wide variety of cancers. Doxorubicin is very important in the treatment of cancer patients, although its use may be complicated by the presence of acute and chronic side effects. Oxidative stress is involved in the action of doxorubicin, in terms of both antitumour effects and cardiotoxicity (Quiles *et al*., 2002a). Thus, it has been reported that doxorubicin leads to direct oxidative injury to DNA (Feinstein *et al*., 1993) and generates lipid peroxidation (Quiles *et al*., 1999a, 1999b; Huertas *et al*., 1991a, 19991b, 1992; Mataix *et al*., 1997). In relation to the cardiotoxicity of doxorubicin, the heart is very sensitive to free radical damage because it has a high rate of oxidative metabolism and a lower level of antioxidant defences than other organs, such as the liver. Additionally, it has been reported that doxorubicin has a very high affinity to cardiolipin, a phospholipid species mainly present in the mitochondrial membranes of the heart, which results in the accumulation of doxorubicin inside cardiac cells (Goormaghtigh and Ruysschaert, 1984). This may be enhanced by a very unsaturated diet, which results in cardiolipin becoming enriched with highly peroxidizable unsaturated fatty acids (Huertas *et al*., 19991b). How to prevent or attenuate the side effects of doxorubicin has been extensively studied. Several strategies have been adopted, including dosage optimization, synthesis and use of analogues or

combined therapy (Quiles *et al*., 2002a). In relation to combined therapy, although the antitumour action of doxorubicin may be mediated by a wide number of mechanisms, free radical production is among the main causes of cardiotoxicity mediated by this drug. Our laboratory has been working on the effects of antioxidant-rich virgin olive oil in relation to doxorubicin toxicity in rats over the last 15 years (Mataix, 2001). The use of virgin olive oil as dietary fat (8g/100g of diet) attenuates the toxic effects of doxorubicin in rats (20mg/kg intraperitoneally daily for 4 days before the rats were killed) at several points in the electron transport chain (Huertas *et al*., 19991b) and results in lower levels of peroxides in the mitochondria and microsomes of the liver (Huertas *et al*., 19991a; Mataix *et al*., 1997). To study the relative importance of the antioxidants present in virgin olive oil, we conducted an experiment comparing virgin olive oil with low-antioxidant "refined" olive oil. These experiments (Quiles *et al*., 1999b, 1999c) showed that supplementation of the refined olive oil with vitamin E to levels equivalent to those found in virgin olive oil improved the protective effect of this edible oil against doxorubicin toxicity in rats. From these experiments it is possible to conclude that the type of dietary fat affects the toxic effects of doxorubicin at the level of mitochondrial free radical production, which could be important in the chemotherapeutic treatment of patients with cancer.

3.1.2. The consequences of frying oils.

Thermal stressing of culinary oils rich in PUFA generates high levels of cytotoxic aldehydic products which arise from the fragmentation of conjugated hydroperoxy diene precursors. Such aldehydic products are harmful to human health. Moreover, several minor components (such as antioxidant vitamins) may be lost during the frying process. Because world-wide consumption of frying oils is high, the consequences of thermally modified oils are of growing interest. We found that the frying process decreased the content of vitamin E and phenolics in the oils while enhancing total polar materials. These effects were markedly higher in sunflower oil than in virgin olive oil. Furthermore, rats fed fried oils had higher levels of lipid peroxidation and lower concentrations of plasma antioxidants. Rats fed a fried sunflower oil-rich diet displayed a higher degree of lipid peroxidation in liver microsomes *in vivo* than rats fed fried virgin olive oil (Quiles *et al*., 2002c). Moreover, we found that the intake of fried oil greatly affected the hydroperoxide and thiobarbituric acid reactive substances (TBARS) contents of liver mitochondrial membranes, both being enhanced after the dietary treatments. Again, several mitochondrial respiratory chain components were affected (Battino *et al*., 2002b).

3.1.3. Oxidative stress related to physical exercise and olive oil.

Physical activity plays a critical role in growth and development. Epidemiological studies have shown that aerobic exercise reduces cardiovascular morbidity and mortality in the general population (Higashi and Yoshizyumi, 2004). However, involvement in sport is also a possible mechanism by which free radicals are generated, and it has been widely recognized as capable of producing peroxidative damage, mainly at the mitochondrial level, as a consequence of the net increase in oxygen consumption during sporting exercise.

We have been working for several years on the effects of dietary lipid sources with different degrees of unsaturation on oxidative stress and mitochondrial function during exercise (Mataix *et al*., 1998; Quiles *et al*., 1994, 1998, 1999a, 1999b) using the following approach. Two groups of rats were fed diets differing in the dietary fat source: virgin olive or sunflower oils. Each group was subdivided into four subgroups in relation to the physical exercise assigned.

Subgroup 1 consisted of sedentary rats. Subgroup 2 consisted of rats subjected to a programme of exercise based on training sessions on a treadmill without inclination over 8 weeks (the last 6 weeks the rats were running under conditions equivalent to 65–70% of their VO_2max). The animals in this subgroup were killed 24 h after the last training sessions. Subgroup 3 consisted of rats performing the same training programme as subgroup 2 except that on the last day, just before being killed, the rats were subjected to a special running session to exhaustion, and then were killed immediately. Subgroup 4 consisted of rats performing the same training programme as subgroup 3 except that after exhaustion they were given a 30-min rest before being killed. Thus, subgroup 2 represented a regular exercise model and subgroups 3 and 4 combined a regular model with an acute one. Regular exercise increased mitochondrial membrane hydroperoxide content in both the liver and skeletal muscle (Mataix *et al*., 1998). These effects were enhanced after the effort to exhaustion (subgroups with regular training model plus acute model). The hydroperoxide content of rats fed olive oil were lower than those of rats fed a diet rich in PUFA, being approximately twofold lower in the liver both after regular exercise and after regular exercise plus acute exercise. Plasma hydroperoxides showed a similar pattern (Quiles *et al*., 1998). In relation to antioxidant levels, CoQ was strongly affected by physical exercise and to a lesser degree by dietary lipid source. Sedentary rats fed on a sunflower oil diet showed the highest levels of this molecule in the plasma. However, after regular exercise these animals showed a sharp decrease in its content, which was not seen in the group fed olive oil. Exercise to exhaustion resulted in a similar decrease in plasma CoQ levels in both groups. However, mitochondrial membrane CoQ content, which increases after exercise, especially in skeletal muscle after exercise to exhaustion, was higher in animals fed sunflower oil as the dietary lipid source. The lower levels of CoQ in plasma, especially after exercise to exhaustion, appear to show the increased metabolic demand for this molecule by the tissues. This demand seems to be a response to, on the one hand, the extra need for protection against oxidative stress, which is in accord with the highest levels of hydroperoxides seen in rats fed sunflower oil. On the other hand, this demand of CoQ may be a response to a general increase in mitochondria and mitochondrial components induced by endurance exercise in response to the increased metabolic demands. This last aspect is supported by the fact that there is an increase in the content of cytochromes a+a3, b and c+c1 and in the activity of COO in exercised animals (Quiles *et al*., 2001). This increase in the content and/or activity of several components of the mitochondrial electron transport chain has been shown to be higher in animals fed sunflower oil, which could be correlated with the higher content of hydroperoxides in these animals (Quiles *et al*., 2001). Overall, these results show that the dietary lipid source should be considered in studies on exercise, since oxidative stress is directly affected by this factor.

3.2. Mitochondrial DNA deletions, oxidative stress, and ultrastructural alterations

3.2.1. Studies on the liver

The liver is the central metabolic organ of the body; therefore dietary changes can have a major impact on aging liver and on general health (Anantharaju *et al.*, 2002; Quiles *et al.*, 2002b). Moreover, the liver is critical in the protection from oxidative damage and plays a major role in the breakdown of potentially harmful lipophilic toxins (Thomas *et al.*, 2002). Although the aging liver appears to preserve its function relatively well (Anantharaju *et al.*, 2002), several changes have been associated with this organ during the process of aging. According to mitochondrial theory of aging, the aging process results from accumulation of mtDNA damage. Growing evidence indicates the role of mtDNA mutations in elevated production of reactive oxygen species, which are proved to increase the number of mtDNA

mutations (Ploszaj *et al.*, 2010). Quiles *et al*. investigated possible effects on the frequency of liver mitochondrial DNA deletions, oxidative stress and mitochondrial abnormalities in liver mitochondria during aging by following a model of feeding rats lifelong with two different dietary-fat sources (virgin olive or sunflower oils).

The study of the fatty-acid profile of mitochondrial phospholipids revealed no differences between both dietary treatments in relation to SPAs. It found a net increase in the amount of these types of fatty acids, individually as well as for total acids, with aging (except for C24:0 in olive-oil group). MUFA were higher in animals fed on olive oil both at 6 and 24 months. Aging increased these fatty acids in the virgin-olive-oil group. In animals fed sunflower oil, only the total amount of MUFAs was affected by aging, which increased. N6 PUFA was higher in animals fed on sunflower oil for both time periods. Aging led to a net increased in all studies PUFAn6 in both dietary groups, as well as PUFAn3, having virgin-olive-oil groups the higher content in these fatty acids, both individually and as a total. The total PUFA index showed no differences between dietary groups, but aging resulted in an enhancement. Finally, concerning the ratio between n3 PUFA and n6 PUFA, animals fed on sunflower oil led to an index seven-fold higher than animals fed on virgin olive oil; aging did not alter this index. In summary, the different lipid profile of the diets was properly reflected in liver mitochondrial phospholipids of young and old animal, suggesting a proper adaptation of the rats to dietary fats (Quiles *et al.*, 2006a).

There is substantial evidence from human and animal studies linking mtDNA deletions and aging. Deletion frequency is affected by age, tissue of origin, species and the presence of some age-related diseases (such as Alzheimer's) and these aspects also oscillate depending on the laboratory (Kang *et al.*, 1998). Quiles *et al*. found increased frequency of the so-called common deletion at the mtDNA levels in aged animals, with those fed on sunflower oil being twice as frequent as those fed on virgin olive oil (Quiles *et al.*, 2006a). This proved that the age-related rise in mtDNA deletions can be modulated by the dietary-fat profile. A similar effect was previously demonstrated for CR (Kang *et al.*, 1998). Also, mtDNA deletions corresponded to increased levels of oxidative stress with aging in both dietary groups, although this increases was greater in animals fed on sunflower oil. It found that sunflower oil led to deterioration in mitochondrial structure, as suggested by the lower percentage of cristae per μm of mitochondrial contour found in old animals fed on sunflower oil compared to the young animals fed on the same oil (Quiles *et al.*, 2006a). Additionally, animals fed on virgin olive oil had a higher number of mitochondrial cristae in both age periods. Mitochondrial circularly (the increase of which represents control loss) was higher in old animals fed on sunflower oil compared to those fed on virgin olive oil (Quiles *et al.*, 2006a; 2006b). These results demonstrate that the age-related increase in liver mtDNA-deletion frequency is differentially modulated by the intake of different dietary fats, with virgin olive oil leading to a lower frequency of deletions than the n6 polyunsaturated sunflower oil. On the other hand, mtDNA-deletion frequency could be correlated with mitochondrial oxidative-stress status and ultrastructural alterations.

3.2.2. Studies on the heart

Aging heart has unique histological and biochemical features, so that increased apoptosis and necrosis, greater myocyte volume, and connective-tissue accumulation are frequently found in the myocardium of old animals (Kajstura *et al.*, 1996). Heart is a postmitotic tissue that exhibits a highly aerobic metabolism due to the abundance of large mitochondria. Therefore, the physiology and bioenergetics of cardiomyocytes with age has been an important research for

many years (Judge and Leeuwenburhg, 2007). Quiles *et al*. investigated possible benefits on age-related mitochondrial DNA deletions and oxidative stress of two different dietary-fat sources (virgin olive oil or sunflower oils) and the supplementation of sunflower oil with CoQ_{10} in rat heart (Quiles *et al.*, 2010b).

After the study of the fatty-acid profile of rat heart mitochondrial phospholipids after 6 and 24 months of the experimental dietary treatment, in relation to the ration between oleic (C18:1n9) and linoleic (C18:2n6) acids, the higher ratio was found both at 6 and 24 months for the virgin-olive-oil group. At 24 months, aging increased the ratios in virgin olive and sunflower plus CoQ10 groups. No statistically significant difference was found between sunflower group for 6 and 24 months of age. CoQ addition to an n6 PUFA-rich diet improved the ratio between monounsaturated and polyunsaturated acids. The different lipid profile of the diets was properly reflected in heart mitochondrial phospholipids of young and old animals, that suggests a proper adaptation of the rats to dietary fats (Quiles *et al.*, 2010b).

It has been reported that mitochondria in aged rodent cardiomyocytes have a tendency to become larger and swollen in appearance and to exhibit lower amounts of cristae (Wessells and Bodmer, 2007). In this study, no changes were found in the mitochondrial area or perimeter during aging, which agrees with previous findings in liver (Quiles *et al.*, 2006a). The lower density of cristae for rats fed on sunflower oil, in comparison with those fed on virgin olive oil, has also been previously described in liver mitochondrial cristae (Quiles *et al.*, 2006a). The involvement of mitochondrial DNA (mtDNA) in the aging process stems mainly from its location, close to the mitochondrial respiratory chain, which is the main ROS source generation, thus explaining in part its susceptibility to oxidative damage. According to several authors, complex I is probably one of the complexes most affected by aging (Sanz *et al.*, 2006c). Quiles *et al*. measured the frequency of a particular deletion in complex I, found the lower values for animals fed on virgin olive oil at both 6 and 24 months. Concerning aging changes, sunflower and sunflower plus CoQ groups showed higher percentages at 24 months and no aging-associated changes were found for the virgin-olive-oil group, so aging led to higher values only for the animals fed on the n6 PUFA-rich diet, however, sunflower plus CoQ group shown lower values than sunflower group. Previously was reported that the dietary fat type can modulate the age-related increase in the frequency of this deletion in rat liver (Quiles *et al.*, 2006a), this study present similar results for the heart, but in addition, a positive effect of CoQ10 supplementation is also reported. These results demonstrate that the age-related rise in mtDNA deletions can be attenuated. Only CR has been previously demonstrated to have such a capacity in attenuating the age-related increase in mtDNA deletion frequency (Kang *et al.*, 1998).

One of goals was to determine whether low dosages of CoQ for long time periods raise CoQ levels. Three lipophylic antioxidants (α-tocopherol, CoQ9 and CoQ10) were measured in heart mitochondrial, and the results shown that aging led to higher concentrations at 24 months, compared with 6 months. Comparing the three antioxidants, it found no differences at 6 months between groups for α-tocopherol and CoQ9; however the sunflower plus CoQ10 group had the higher values of CoQ10. This evidences that the supplementation of sunflower-oil diet with CoQ10 increases the mitochondrial level of this antioxidant in heart. Another stress-oxidative marker measured was lipid peroxidation (lipidic hydroperoxides), where no differences were discovered at 6 months between groups. At 24 months, lower levels were found for the sunflower plus CoQ group. Aging led to higher concentrations at 24 months for the virgin-olive-oil and sunflower-oil group while no affects to sunflower plus CoQ group. Lipid-peroxidation values, as the level of mitochondrial hydroperoxides showed a common profile characterized by higher concentrations in all groups of aged rats. In opposition to other experiments performed previously by the same group (Ochoa *et al.*, 2003), animals fed on the MUFA-rich diet did not

reach lower values for lipid-peroxidation markers than those fed on the sunflower-rich diet. On this occasion, lipid-peroxidation results did not reflect the differences found in the above-mentioned mitochondrial-lipid profile. On the other hand, animals fed on the sunflower-rich diet and supplemented throughout life with CoQ10 showed lower lipid-peroxidation values during aging than in the rest of the groups. CoQ supplementation could be the responsible for the higher protection in this group against lipid peroxidation.

Protein oxidation is an important aspect of age-related oxidative stress. Several papers indicate that protein oxidation augments with aging (Yasuda *et al.*, 2006). Oxidative alterations to proteins include especially sensitive amino-acid residues in proteins, such as tyrosine, methionine, arginine, proline, and lysine, among others (Amici *et al.*, 1989). Quiles et al. investigated probes of oxidation of amino acids in protein, in particular carbonyl products of metal-catalyzed oxidation of proteins (Pamplona *et al.*, 2005). For the parameters used, aging led to higher values at 24 months compared with the same groups at 6 months. Overall, those data suggest that MUFA can partially protect mitochondria from age-related changes and that CoQ supplementation to n6 PUFA partially resembles some of the positive results found after the lifelong feeding of MUFA-based diet. Moreover, lipid-derived oxidative damage appears to be more important than the pure protein-derived oxidative damage during aging.

3.2.3. Studies on the brain

Mitochondria-related oxidative damage is a primary event in aging and age-related neurodegenerative disorders (Su *et al.*, 2010). Gilmer *et al*. recently reviewed that history of brain mitochondrial studies on aging (Gilmer *et al.*, 2010), examining many studies that have reported significant age-related decline in mitochondrial enzymatic activity and amount in various complexes isolated from whole brains or specific brain regions of mice, rats and primates. In these studies several groups report declines in enzymatic activities of certain ETC complexes with age, it is unclear whether these enzymatic changes translate into a functional decline in respiration or even a decline in ATP production (Van Remmen and Richardson, 2001). In the past few years it has been demonstrated that some antioxidants, as well as, interventions in relations to dietary fat, may be used as dietary antiaging therapies (Quiles *et al.*, 2006a). Already seen how n6 PUFA-rich diet lead to higher levels of lipid peroxidation and mtDNA damage in rat blood compared with MUFA-based diet (Quiles *et al.*, 2006a; 2010b); furthermore, it has been demonstrated that n6 PUFA diet supplemented with CoQ10 lengthens life span, decrease lipid-peroxidation levels and enhance antioxidant capacity (Ochoa *et al.*, 2007; Quiles *et al.*, 2010b). The next step taken by Quiles and his group was to evaluate the effect of dietary fat type and CoQ10 on age-related changes is brain mitochondrial DNA deletion and oxidative stress (Ochoa *et al.*, 2011). Studies of dietary fat (mainly n3 PUFA family) effects on neurobiology and neurodegeneration have a long history (Innis, 2000). The brain is one of the less adaptable organs concerning dietary fat other than n3 PUFAs. Only a few studies have analyzed the effects of dietary sunflower or virgin olive oil on the fatty acid profile in this organ (Barzanti, 1994; Yaqoob, 1995). On the other hand, studies using fish oil or any other n3 PUFA source, shown an important incorporation of these fatty acid into the brain fatty acid profile (Engström *et al.*, 2009), suggestion that the brain has a very strong preference for the incorporation of n3 PUFA fatty acids into its membranes. Ochoa *et al.* analyzed the fatty-acid profile of brain mitochondrial membranes. When oleic acid was observed, no difference between the two dietary fat types were to be seen, however animals fed sunflower oil shown higher percentages of this fatty acid, with the exception of animals fed virgin olive oil at 24 months. The ratio between oleic and linoleic acids is higher in all groups

fed virgin olive oil for both periods, which may suggest a good adaptation, although poorer than observed for these animals in other tissues (Quiles *et al.*, 2002c; 2010b). Respecting CoQ, animals supplemented with CoQ presented higher concentrations at 6 and 24 months than did the same groups without supplementation, so low dosages of CoQ for long time periods raise tissue CoQ levels. The fact that the animal membranes adapted well to dietary fat and CoQ10 supplementation is an interesting finding, because the benefits (if any) from being fed either of the two fat types and CoQ10 might persist throughout life, thus representing an opportunity for nutritional therapy during aging.

Although all groups presented higher levels of hydroperoxides at 24 months than at 6 months, animals fed diets rich in n6 PUFAs shown higher levels of lipid peroxidation than animals fed virgin olive oil. Even under aging, it has been shown that lipid peroxidation is lower in animals fed virgin olive oil (Ochoa *et al.*, 2003; Quiles *et al.*, 2010b). With these results, the protective action of virgin olive oil in brain of rats is demonstrated by means of lipid peroxidation markers. Furthermore, CoQ10 led to lower lipid-peroxidation values at all ages in the animals given the sunflower oil diet. There are various ways in which CoQ10 could induce these differences:

- Changes in the mitochondrial fatty-acid profile, thus affecting its susceptibility to lipid peroxidation.
- Increases in antioxidant defenses
- Effects on the production of free radicals.

Antioxidant defenses do not fall with physiological aging; indeed, increases with age have been described (Halliwell and Gutteridge, 1999). It has been postulated that a compensatory antioxidant defense system exists to counteract the oxidative stress associated with aging, and therefore some antioxidants would be expected to accumulate, such as α-tocopherol (Van der Loo *et al.*, 2002; Armeni *et al.*, 2003). In this study, Ochoa *et al*. found higher activities of antioxidant enzymes in older animals, irrespective of diet, which correlates with the increased oxidative stress found in older animals. Although CoQ10 increases α-tocopherol levels in the two diets, higher α-tocopherol amount were found in virgin olive oil group supplemented with CoQ10, both 6 and 24 months.

Aging has been associated with decay in the mitochondrial respiratory chain activity (Miró *et al.*, 2000). The involvement of mtDNA in the aging process is mainly from its location, close to the mitochondrial respiratory chain, which is the main source of ROS generation. The accumulation of somatic mtDNA mutations might play an important role in the aging process by producing cells with a decreased oxidative capacity (Harman, 1972). Ochoa *et al*. studied a particular deletion from a region coding for complex I proteins, which is probably among the complexes most affected by aging (Ochoa *et al.*, 2002). They found that the deletion frequency differed depending on the dietary fat, CoQ10 supplementation and aging, being lower in animals supplemented with CoQ10. Aging led to higher values with both dietary treatments, but they were higher for the animals fed the n6 PUFA-rich diet, what had previously been shown to liver and heart of rats (Quiles *et al.*, 2006a; 2010b). The MUFA diet prevents the age-related increase in levels of mitochondrial DNA deletions in the brain mitochondria from aged animals. In addition, a positive effect of CoQ10 supplementation is reported. These results demonstrate that the ager-related rise in mtDNA deletions can be attenuated. Only CR has previously been shown to have such a capacity in attenuating the age-related increase in mtDNA deletion frequency (Kang *et al.*, 1998).

3.2.4. Differences between mitotic and non-mitotic tissues

In 2003, Ochoa *et al*. studied differences between mitotic (liver) and non-mitotic (skeletal and cardiac muscles) tissues in relation to aging from the standpoint of mitochondrial oxidative stress and function. For this, they examined rats fed over 24 months on a diet differing in the lipid source (virgin olive oil or sunflower oil). To aging study animal was killed at 6, 12, 18 and 24 months and liver, heart and skeletal muscle mitochondria were isolated. Regarding the fatty-acid profiles of mitochondrial membranes, they found (Ochoa *et al.*, 2003) that the proportion of SFA in liver did not change with age in either group, although there was a higher proportion of these fatty acids in the sunflower group at 6, 12, 18 and 24 months. Only dietary fat affected liver SFA. In heart, the sunflower group increased SFA with age. They were found differences between diets at 12 and 18 months, with animals fed on sunflower oil showing the highest levels. However, in skeletal muscle only were detected differences between groups at 6 months with the olive group registering the highest SFA proportion. The effect on age differed in the rats fed virgin olive oil having lower SFA levels at 12, 18 and 24 months. Age also affected skeletal muscle SFA. Animals fed on olive oil hag higher MUFA levels in all tissues, except for 6 months in skeletal muscle. Aging did not affect the proportion of MUFA the same way, if not that depended on the tissue. In liver, age did not affect olive group, while in the sunflower group the rats 18 and 24 months old showed significantly lower values than in rats 12 months old. In heart, MUFA decreased in both dietary groups at 18 and 24 months with respect to 6 months. In skeletal muscle, age led to increased MUFA in both groups. The lipid source significantly affected the three tissues studied, while age was significant only for heart and muscle. For all tissues studied the proportion of PUFA proved higher in the sunflower group (except for heart at 18 months). No age effect was detected in liver. In heart, the olive group increased in PUFA at 18 and 24 months with respect to 6 and 12 months. The sunflower group showed a significant decrease at 12 months with respect to 6 and 24 months. In skeletal muscle, both groups registered a similar response, with the highest value at 18 months.

Oxidative stress in these animals was studied through the analysis of mitochondrial hydroperoxides (Ochoa *et al.*, 2003). In liver, differences between diets were found only at 12 months, with the sunflower group reaching the highest levels. Both groups showed a significant decrease at 24 months compared with 6 and 18 months. The non-mitotic tissues increased hydroperoxide levels with age. Animals fed on sunflower oil reached higher values for all time periods except for 6 months. So, the lipid source had a direct effect only on heart and muscle hydroperoxides.

Mitochondrial function in these animals was investigated through the study of some elements of the mitochondrial electron-transport chain (Quiles *et al.*, 2002b), namely, cytochrome b, CoQ and the amount and activity of the complex IV (the CCO system). In liver, CoQ levels for animals fed olive oil compared with sunflower oil were higher for 6 months of age and lower for 24 months. Age affected only the sunflower-oil group, with an increase in animals of 24 months of age. In heart, for both age periods, olive oil led to lower levels of CoQ than sunflower oil. In skeletal muscle, the olive-oil group registered lower levels of CoQ than did the sunflower-oil group at both ages. In terms of age, this molecule increased at 24 months for both dietary groups. Concerning cytochrome b in liver, the olive-oil group showed higher values for 6 months and lower levels for 24 months, compared with the sunflower-oil group. Age affected only the sunflower-oil group, with a sharp increase at 24 months. For heart, no differences were found between dietary treatments for 6 months of age, and higher levels were found in sunflower oil at 24 months. For skeletal muscle, both groups were similar at 6 months and the olive-oil group differed from the sunflower group at 24 months (with lower values).

Increased catalase activity is associated with a greater resistance to oxidative damage and, in this sense; catalase seems to be important in overall antioxidant enzymatic defense systems

with respect to lifespan (Beckman and Ames, 1998; Brown-Borg and Rakoczy, 2000). Ochoa *et al.* (2003) found that another factor must be considered when catalase activity during aging is considered, i.e., the dietary lipid source. Liver-catalase activity increased with virgin olive oil but remained unchanged with sunflower oil. In heart, both groups showed increased catalase activity with age, but this increase was higher in the olive-oil group. Finally, skeletal muscle decreased catalase activity in both dietary groups. With respect to heart, the increased activity found in olive group is important because it has been suggested that catalase can function as a major pathway for detoxification of H_2O_2 in cardiac tissue (Molina and García, 1997).

In addition, Quiles *et al*. (2002) have previously reported that aging depresses cytochrome oxidase activity, the final electron acceptor at the mitochondrial electron transport chain (METC), in heart and skeletal muscle but not in liver (Quiles *et al.*, 2002b). In parallel, the levels of other METC components such as cytochrome b increased a situation that could lead to the uncoupling of the METC and subsequently augment free-radical production. Cytochrome b enhancement is considered a marker of aging (Kipling, 2001) and occurs at the main site of free-radical production in the METC (Lenaz, 1998). Ochoa *et al*. study shown that with the PUFA-rich diet, cytochrome oxidase decreased and cytochrome b increased more than with the MUFA-rich diet (Ochoa *et al.*, 2003).

Aging, considered as an endogenous and progressive phenomenon (Barja, 2002) leads throughout the lifespan to different disturbances in mitochondria and their components, such as mtDNA (Lee *et al.*, 1997; Michikawa *et al.*, 1999, Ploszaj *et al.*, 2010; Pamplona and Barja, 2011). These disturbances (which have a high oxidative component) have a negative impact on mitochondrial structure and function. Depending on the capacity of the affected tissue to repair the damage or to replace the altered cell, tissue function is affected to a greater or lesser extent (Quiles *et al.*, 2002b). In this way, mitotic tissues with the ability to regenerate their cells, such as liver, appear to be able to buffer the damage, at least in part, as suggested by the lack of alterations of mitochondrial function in terms of COO activity (Quiles *et al.*, 2002b). However, a loss in function is found in postmitotic tissues such as skeletal muscle, heart or brain, which lack de opportunity to replace damaged cells and are likely to have a less effective repair system (De Souza-Pinto *et al.*, 2008). This loss in function is reflected in the decrease in cytochrome c oxidase activity (Quiles *et al.*, 2002b; Ochoa *et al.*, 2003). Mitochondria from postmitotic tissues buffer the unfavorable situation by increasing in some elements of the mtETC, such as cytochrome b or PUFAs. The increase in polyunsaturation could enhance membrane fluidity and CCO activity by the presence of a more polyunsaturated cardiolipin, as has been previously suggested (Huertas *et al.*, 1991b; Quiles *et al.*, 2001). However, both actions raise ROS production. The role of dietary fatty acids in this mechanism could reside in the building of an environment more or less prone to the generation and propagation of ROS. Moreover, dietary fat could modulate the phenomenon through variations in the antioxidant system and overall upregulate or attenuate the process. Thus, as postmitotic tissues are the most affected by aging, the influence of diet should be particularly important in these tissues.

3.3. Dietary fat type and age-related alveolar bone loss

Periodontitis is a related to age disorder characterized by the breakdown of the tooth-supporting tissues, mainly alveolar bone loss. Increased evidences link periodontitis to systemic diseases like atherosclerosis. Metabolic syndrome and oxidative stress have been proposed as potential common links to explain this relationship (Bullon *et al.*, 2009b; 2011). Traditionally, much of the research on diet and periodontal diseases is focused on only a few nutrients with well-established roles in the formation and maintenance of structural

components of oral tissue such as collagen (vitamin C) and bone (calcium), integrity of epithelial tissue (vitamin A), or in promoting the formation of plaque that harbors periodontal pathogens (carbohydrates). More recently, research has expanded to include nutrients shown to attenuate the inflammatory process or to have anti-inflammatory properties, like some n3 and n6 PUFA. However, no research has been performed on the putative role of other well-documented healthy fatty acids such as MUFA (Kaye, 2010).

Bullón and coworkers, in their latest work in this line, have reproduced an age-dependent model of the periodontium, a fully physiological approach to periodontal conditions, to evaluate the impact of dietary fat type on gingival tissue of young (6 months old) and old (24 months old) rats (Bullón *et al*., 2013). Animals were fed life-long on diets based on MUFA as virgin olive oil, n6 PUFA, as sunflower oil, or n3 PUFA, as fish oil. They studied the alveolar bone level at two points at mesial and distal of second premolar and first molar, and the gingival histology in the molar gingival mucosa, finding differences regarding alveolar bone loss with aging depending on the dietary fat. A significantly highest bone loss, a high degree of fibrosis and a moderate degree of inflammation were observed in animals fed on sunflower oil, followed by those fed on fish oil. Several studies have investigated the potential effects of different fatty acids, mainly n3 PUFA, derived from diet or as supplements, on periodontal disease, both in humans and in experimental models (Bendyk *et al*., 2009; Naqvi *et al*., 2010).

Circulating levels of inflammatory cytokines were higher for older groups but not different depending on treatment and something similar happened for mRNA at the gum level. The lack of differences concerning cytokines could be ascribed to the fact that periodontal inflammation was not promoted. Also were measured mRNA and circulating levels of two bone resorption markers: RANKL and OPG. Relative quantity of RANKL was the highest for virgin olive oil fed animals at 6 months, while OPG relative quantity was the lowest value for fish oil fed young animals. Plasma level differences of RANKL were only associated to age, with animals at 6 months showing higher levels than those at 24 months. However, for plasma OPG levels differences between young and old animals fed on virgin olive oil were reported, showed the lowest concentration. It has been demonstrated that RANKL is up-regulated, whereas OPG is down-regulated in periodontitis, compared to periodontal health, resulting in an increased RANKL/OPG ratio (Jules *et al*., 2010).

It seems that age-related alveolar bone loss may be conditioned by dietary fat, so it was higher in n6 PUFA fed rats, probably as a consequence of the ablation of the cell capacity to adapt to aging. Gene expression analysis suggests that MUFA or n3 PUFA allowed mitochondria to maintain an adequate turnover through induction of biogenesis or autophagy. They might also be able to induce the corresponding antioxidant systems to counteract age-related oxidative stress, and do not inhibit mitochondrial electron transport chain. An appropriate dietary treatment could be reduced the excess of alveolar bone loss, characteristic of periodontal diseases (Bullón *et al.*, 2013).

3.4. Age-related changes in pancreas associated to dietary fat.

The pancreas is a vital organ for nutrient digestion and consumption. An adequate pancreatic structure is necessary for its optimal function (Ouyang *et al.*, 2011). Certain pathologies that disturb pancreatic cell mass and result in inflammation, ductal obstruction, fibrosis or even necrosis, accelerating the organ's aging process, have been related with habits in diet and lifestyle (Halter, 2012). The identification of factors that are capable of reverting or avoiding these pathological conditions is a useful instrumental to design prevention strategies against the different pancreatic disorders that occur during aging. The benefits of specific

unsaturated dietary fat sources against certain deleterious aspects of aging have been already described in other organs and tissues (Ochoa *et al*. 2003; Quiles *et al*. 2002b, 2004b, 2006c, 2010b; Bullón *et al*. 2013). In the same research line, Roche *et al.* (Roche *et al.* 2013) have compared three groups of rats fed during 2 years with an isocaloric diet but with different fat composition to study their involvement in the pancreas.

Rats were divided into three experimental groups fed on isoenergetic diet according to the AIN93 criteria but modified in relation to the dietary fat source, using virgin olive, sunflower or fish oil respectively. At the end of the experiment, no pathological symptom was detected, including obesity; however, the important finding of this study is that the type of fat can affect the exocrine and endocrine compartments of the pancreas differently. Taking islets of virgin olive oil fed rats as reference, islets from rats fed on sunflower olive oil contained a higher number of β cells, reflected by increased insulin contents, and hyperleptinemia. These signs, along with other parameters, have been described in obesity, glucose intolerance, insulin resistance, disruption of the adipoinsular axis or prediabetes (Sattar *et al*., 2008). In the immunohistochemical analysis (insulin and glucagon), the insulin immunopositive area per islet was significantly higher in the sunflower oil group compared with the virgin olive oil group. This increase seemed to correlate with an increase in the number of cells positive to the hormone per islet. On the other hand, the number of glucagon-positive cells was similar in all three groups, indicating that the islet changes seemed to be β-cell specific. It has been well documented that β-cell replication decreases with age in rodents and humans (Meier *et al*., 2008), but in compensation presents a minimal apoptosis (Teta *et al*., 2005). On the other hand, the presence of fish oil in animal diet seems to mainly affect the exocrine pancreas compartment, as observed by the intermediate alteration detected in the sunflower oil group. The histological pancreatic alterations observed in animals fed on fish oil resemble those observed in pancreatic fibrosis of the elderly (Klöppel *et al*., 2004). The insulin and glucagon content measured confirmed that the sunflower oil group contained twice the amount of insulin detected in the other groups, whereas glucagon levels remained unchanged.

It is well known that dietary fat can modulate the circulating fatty acids composition (Quiles *et al*., 2006b). As would be expected, the percentage of circulating MUFAs was significantly higher in rats fed on virgin olive oil, circulating n6 PUFAs were significantly higher in rats fed on sunflower oil, and n3 PUFAs were higher in rats fed on fish oil.

In order to elucidate the possible mechanisms involved in the previously mentioned pancreatic changes, several proliferation and apoptotic markers were analyzed. According to the number of $Ki67^{+}$ cells, it seems that proliferation events were very similar in the pancreases of all groups. The same occurred for the expression levels of the apoptotic markers Bcl2 and Bad but not for tumor necrosis factor-α, which presented lower expression levels in VOO group animals compared with SO group and FO group rats. Altogether, these results might suggest that the mitochondrial apoptotic pathway was not active in any of the animal groups and that the cytoplasmic pathway, where tumor necrosis factor-α is most likely participating (Chu, 2013).

The important finding of Roche and coworkers is the fact that the type of fat can affect the exocrine and endocrine compartments of the pancreas differently. The high β-cell number found in sunflower oil group could be interpreted as an increased replicative capacity of β cell or a longer life span of these cells due to reduced apoptosis. The exocrine pancreas compartment seems be affected mainly by fish oil-enrich diet (Roche *et al*., 2013). This study reinforces the already published favorable effects of olive oil in diet, in this case improving insulin sensitivity and showed a positive association between MUFA consumption and β-cell function (Rojo-Martínez *et al.*, 2006).

3.5. Liver apoptosis in relation to dietary fat type and age

The liver is the main organ regulating of mammalian metabolism, and due to its function and anatomical location is exposed to a multitude of toxins and xenobiotics, as well as to infection by viruses, and therefore, is highly susceptible to tissue injury. For this reason, the liver is one of the best models to study apoptosis *in vivo*. Apoptosis is the main mechanism of cell death in the liver, also being the physiologic route to eliminate damaged or infected cells and to maintain tissue homeostasis (Guicciardi *et al.*, 2013). Hepatocytes are particularly susceptible to death receptor-mediated apoptosis, which could be induced in two ways: intrinsic and extrinsic, initiated by different mechanisms (Ghavami *et al.*, 2005). The mitochondrial or intrinsic pathway is activated by a variety of extra and intracellular stresses, such as oxidative stress or treatment with cytotoxic drugs. Intrinsic factors include growth mediators, DNA damage and cytoplasm detachment could accumulate proapoptotic members of Bcl-2 family (Bax and Bak) in mitochondrial membrane, increasing its permeability and causing the output of the cytochrome c and certain proapoptotic proteins to cytoplasm, which activates caspases and finally results in formation of apoptotic bodies. In the extrinsic pathway could be involved death receptors in the cell surface whose activation induces recruitment of cytoplasmic adaptor proteins such as TRADD (TNF receptor-associated death domain) and FADD (Fas-associated death domain) that activates cascade of caspases. The final destination of apoptosis cascade is determined by interaction between proapoptotic and antiapoptotic proteins in Bcl-2 family in cell structure (Dabbagh and Rajaei, 2013).

Several evidences suggest that dysregulation of apoptosis is associated with the aging process (Hasty and Christy, 2013; Kincaid and Bossy-Wetzel, 2013; Qian and Chen, 2013). On the other hand, the effect of the diet in aging-related apoptosis have been studied, focused on calorie restriction that increases hepatic apoptosis, which allows for a decrease of spontaneous preneoplasic and neoplastic lesions in the liver and, therefore, is beneficial to the organism (Selman *et al.*, 2003a, 2003b; Miskin *et al.*, 2005). Bello *et al.* (Bello *et al.*, 2006) wanted to study how the predominant dietary oil affects the apoptotic response of rat liver during aging. Previously have been studied several factors involved in the regulation of apoptosis (caspases of the intrinsic and extrinsic pathways, Bcl-2 and Bax polypeptide levels, and neutral sphingomyelinase) in liver of rats fed lifelong with two experimental diets based of different lipid sources (Ochoa *et al.*, 2003; Quiles *et al.*, 2004b, 2006c). Bello *et al.* demonstrated that aging or diet do not affect the extrinsic pathway of apoptosis, but the intrinsic pathway is significantly depressed in liver from animals fed on a sunflower oil-based diet.

Two groups of rats were fed on two experimental diets, differing in the source of fat: virgin olive and sunflower oils. Animals were sacrificed at 6, 12 and 24 months and liver was removed for its analysis. They measured the proteolytic cytosolic activity of caspases by fluorimetry. Several caspases were determined, such as caspases-8/10 as representative of the extrinsic pathway, caspase-9 as representative of the intrinsic pathway, and caspase-3 as a well-established executioner caspase. Similar levels of caspases-8/10 activity were observed in liver from young and old rats for all the dietary groups. Activity in young and old rats fed on olive oil was not significantly different from that measured in animals fed on sunflower oil, but activity in middle age was lower in the olive oil group than in the sunflower oil group. However, the activity of caspase-9 and capase-3 was significantly decreased in animals fed on a sunflower oil-based diet at 24 months but not in animals fed on virgin olive oil. Detection of Bcl-2 and Bax polypeptides in liver membranes from aged animals was carried out by immunostaining of western blots. Bcl-2 levels were lower in the olive oil than in the sunflower oil group, while Bax levels were significantly higher in the olive oil group. As a result, Bcl-2/Bax ratio was

significantly lower in liver from rats fed on the olive oil-based diet. As oxidative stress is known to initiate the intrinsic pathway of apoptosis (Lawen, 2003), lipid hydroperoxides and CoQ levels were measured in membranes isolated from aged animals. Although the same basal levels of hydroperoxides were present in the membranes of the two dietary groups, they found that membranes displayed different sensitivity to induced oxidation depending on the predominant dietary oil. The virgin olive oil-based diet protected membranes from induced oxidation whereas that lipid hydroperoxides were significantly increased in membranes from the sunflower oil group. Levels of CoQ were particularly elevated in plasma membranes from old animals fed on olive oil.

These results are indicative that the extrinsic pathway of apoptosis (as represented by caspases-8/10 activity) does not experiment major changes with respect to either aging or dietary fat, whereas the intrinsic pathway (as represented by caspase-9 and then caspase-3 activities) is significantly depressed during aging in animals fed on the sunflower oil diet. Meanwhile, rats fed on a virgin olive oil-based diet resulted in significantly lower Bcl-2/Bax ratios. The data obtained by Bello *et al.* supporting that the type of fat source in the diet can alter significantly the susceptibility of hepatocytes to different apoptotic stimuli by altering both pro- and anti-apoptotic mediators.

3.6. Aspects related to longevity.

Many environmental, behavioral, socio-demographic and dietary factors influence the physiological pathways of aging and life-expectancy. Nutrition has an important impact on overall mortality and extending life expectancy. The membrane fatty acid composition and maximum lifespan have been related through the "membrane pacemaker" theory of aging (Hulbert, 2007), which highlights the importance of lipid peroxidation, whose reactive products could result in the oxidative damage to DNA and be determinant in longevity (Pamplona, 2008). One of the most important findings concerning fatty acids and longevity comes from the collaborative work between Dr. Pamplona and Dr. Barja, both in Spain. These scientists have proven through comparative studies that long-lived mammals and birds have low rates of mitochondrial ROS production and low levels of oxidative damage in their mitochondrial DNA (Pamplona *et al.*, 2002a; Sanz *et al.*, 2006c, 2009; Pamplona and Barja, 2011).

Oxidative damage to membrane lipid is extremely important in longevity. In spite of the fact that dietary lipids modulate the membrane lipid profile and peroxidation rate (Mataix *et al.*, 2006) their effects on aging have been only partially studied. Differences in peroxidizability between species lead to differences in oxidation at the level of lipids, DNA or protein. It has been found that long-lived birds and mammals, including humans, have a lower degree of total tissue and mitochondrial fatty acid unsaturation than short-lived ones (Pamplona *et al.*, 1996; 2002a, 2002b, 2002c; Sanz *et al.*, 2006c; Hulbert *et al.*, 2007; Pamplona and Barja 2007; Buttemer *et al.*, 2010). Furthermore, it has been demonstrated that in long-lived animal species a low degree of total tissue and mitochondrial fatty acid unsaturation is accompanied by a low sensitivity to lipid peroxidation and a low steady-state level of lipoxidation-derived adducts in both tissue and mitochondria from diverse organs (skeletal muscle, heart, liver and brain) (Hulbert *et al.*, 2007; Pamplona, 2008). In conclusion, animals with higher lifespan have membranes containing fatty acid less susceptible to lipid peroxidation, which helps to protect their tissues and mitochondria against lipid peroxidation and oxidative protein modification and can be a factor contributing to their slow rate of aging. This leads to lower levels of DNA damage from lipid peroxidation and of lipoxidation-derived protein modification in such species (Pamplona, 2011; Pamplona and Barja, 2011).

Quiles and coworkers have done several studies related dietary fat and longevity. On the one hand, studies with 8% of fat diets (containing 61% of total fatty acids as PUFA) differing n the supplementation or not with 0.7 mg/kg/day of CoQ_{10}, did not show differences on longevity. However, the addition of CoQ_{10} to the diet involved an improvement on longevity (Quiles *et al.*, 2004a). On the other hand, our group has also accomplished studies with diets containing 4% of different dietary fats (virgin olive, sunflower and fish), which data belong to the same set of experiments as part of this study. In these experiments it has been observed that there were differences among the dietary fat types when these were added to 4% of weight (data not published). Rats fed on sunflower oil presented lower longevity that those fed on virgin olive or fish oils, which show no differences between them.

More recent studies in senescence-accelerated mice have confirmed that long-term intake of n3 PUFA increases oxidative stress and decreases lifespan, because they are oxidized easily *in vivo* as *in vitro*, and therefore demonstrates the need for antioxidant supplementation (Tsuduki *et al.*, 2011).

CHAPTER III: AGING-RELATED GENE EXPRESSION STUDY

1. AGING AND GENES

Aging is one of the most complex biological processes determined by genetic and environmental factors. Studies in various organisms, from unicellular type (yeast) to higher vertebrates, including mammals, have reported that certain genetic mutations (single gene in most cases) enlarge or reduce maximum or mean life spam (Bartke, 2011). *Saccharomyces cerevisiae* is the organism less complicated in which longevity-related genes have been studied. RAS (1 and 2) are related to a signaling pathway associated with the response to the availability of nutrients and to different stresses. These genes are highly conserved in many organisms and are functionally interchangeable between yeast and human cells. The two Ras proteins are key regulatory molecules in an important cell signaling pathway (by regulation of adenylate cyclase), establishing a link between nutritional status and intracellular levels of cAMP with cell growth and division. Any of the two genes alone is only sufficient for the cell to be viable, however, deletion of the two at the same time it has lethal consequences. The Ras2 mutants are unable to grow on a nonfermentable carbon source (Tisi *et al*., 2014). They are involved in *S. cerevisiae* longevity in different ways. While RAS1 overexpression has not effect on the yeast life, its deletion supposes an increasing in life expectancy about 30%. RAS2 is involved in two different signaling pathways: one involves stimulation of adenylate cyclase, and the other is a classical type MAP kinase pathway. These pathways regulate the metabolic activity and cellular proliferation, in addition to the stress response (Quiles *et al*., 2010a). There is new evidence pointing to the importance of metabolic control on longevity. Thus, it has been described certain interaction between PHB1, homologue to human prohibitin gene, and the RAS2 gene in relation to the control of lifespan. This interaction has been observed petite only in *petite* yeast, which mitochondria are no functional. So, it appears that there is a connection between these two genes and mitochondria, and their effect on metabolic adjustments that determine longevity (Mishra *et al*., 2010). Longevity-assurance genes (LAG) 1 and 2 are expressed preferentially in young cells. LAG1 deletion increases cells replicative capacity while LAG2 deletion in haploid cells not affects to their growth but reduce the life expectancy. However, LAG2 overexpression increases significantly yeast longevity. In general, numerous experiments have demonstrated that the level and time of expression of LAG2 throughout life are important for the longevity of the yeast. It has been proposed that the protein derived from the gene, the lag2, acts at the mitochondrial level. It has also been proposed LAG2 interaction with Ras-cAMP pathway (Quiles *et al*., 2010a). Sir genes (Sir2, Sir 3 and Sir4) are related to the phenomenon of gene silencing in yeast acting on telomeres, and they are conserved in other species. The first link between gene silencing and aging was established following the identification of the mutation in longevity SIR4-42, which redirects the Sir2/3/4 complex away from telomeres and hidden MAT loci to the genome region that codes for ribosomal RNA. Later it was confirmed that the amount of Sir2 in rRNA predict longevity, so that Sir2 deletions reduce life expectancy whereas that its overexpression increase longevity. Sir2 main activity is as NAD-dependent ADP ribosyltransferase, function that activates a potent histone deacetylase activity, important in the process silenced. (Kaeberlein *et al.*, 2007; Quiles *et al*., 2010a, Longo *et al.*, 2012).

The nematode *Caenorhabditis elegans* has been probably the most widely used model in the study of longevity. Basically, three mechanisms that control the degree of aging in this nematode have been found (Back *et al.*, 2012):

- The first mechanism consists of genes that affect the route that stop development when the environmental conditions are not right, forming resistant larvae (dauer). The genes involved are the Age-1, Daf-2 and Spe-26. These genes control the situation by a signaling cascade similar to insulin which in turn regulates the activity of transcription factor daf-16.

- The second mechanism is formed by clock genes, specifically the Clk-1, Clk2, Clk3 and Gro-1. These genes affect metabolism and increases longevity through a reduction in the vital rate (among other effects influencing the synthesis of CoQ).

- The third mechanism is controlled by eat genes, very numerous. The mutant eat-2 creates defects in behavior and physiology of food, reducing food intake (something like calorie restriction). These genes are able to extend *Caenorhabditis elegans* life even 50% more (Quiles *et al*., 2010a).

In the fruit fly *Drosophila melanoganster* there are also genetic systems controlling the duration of life. Studies on mutations of specific genes have shown to be able to increase longevity in fruit flies. Thus, a mutation in the mth or methuselah gene increases the life of these flies at approximately 35%. Therefore, in the wild phenotype, this gene shorts life. However, individuals without the mth gene die before reaching to adulthood, suggesting that this gene plays an important role during development. Mth mutants are resistant to various types of stress, such as fasting, high temperatures and paraquat (a free radical generator). This gene encodes a membrane receptor coupled to a G protein, suggesting that it is a cellular signaling pathway which regulates longevity and resistance to stress in this animal. Another mutation of a single gene causes an increase almost twice the lifespan of the adult fly without alter their fertility and physical activity. This is the Indy gene ("I'm not dead yet"), that has homology to a mammalian membrane protein that transport Kreb's cycle intermediates. A mutation in the gene is able to generate a metabolic state similar to caloric restriction (Quiles *et al*., 2010a). Studies on sod and cat mutants, able to defend themselves better from oxidative stress, lived 30% longer than wild controls. It has been further demonstrated that this increase in longevity was due to the handling of both genes and not one only. Another example of transgenic mutants with high resilience to stress by fasting, high temperatures and free radicals are those associated with overexpression of the chaperone hsp70. Mutants with 12 copies for this gene were able to live between 8 and 12% at normal temperatures, when they have been previously charged with a slight heat stress. (Vermeulen and Loeschcke, 2007; Quiles *et al*., 2010a; Hoffmann *et al.*, 2013).

1.1. Aging-related genes in mammals and humans

There is a growing amount of information from experimental models regarding the genetics of aging. However, in the case of mammals in general and humans in particular, the situation is much more complex because aging is due to the interaction between genetic, epigenetic, environmental and cultural (including in the latter two the nutrition) factors.

The available studies allow describing the following groups of genes associated to aging and longevity in mammals and humans (Salvioli *et al.*, 2006; Cevenini *et al.*, 2008, 2010):

1. Genes of inflammation and immune response. "Inflamm-aging" is defined as the chronic low-grade inflammation typical of aging, and seems to be the common biological factor responsible for the decline and the onset of disease in the elderly. Aging is associated with a generalized increase in plasma levels of proinflammatory cytokines, coupled with a greater cellular capacity to produce them. This can lead to a chronic "proinflammatory" state, which can promote or enhance various pathologies associated with aging (cardiovascular diseases, atherosclerosis, Alzheimer's, arthritis, sarcopenia, type 2 diabetes, etc...). The five components

of the NF-κB family are prominent mediators of inflammation, acting as key transcriptional regulators of hundreds of genes that in turn control cell proliferation, cell survival, as well as innate and adaptive immune response. NF-κB signaling seems to be the culprit of inflamm-aging, as this signaling system integrates the intracellular regulation of immune responses in both aging and age-related diseases. The age-related "constitutive activation of NF-κB" has been verified in various tissues during aging. The senescent phenotype is accompanied by upregulation of the DNA damage-response system, and by the robust secretion of numerous growth factors, proinflammatory cytokines (such as IL-6, IL-8), proteases, and other proteins, globally named "Senescence Associated Secretory Phenotype" (SASP). Elevated levels of IL-6 have been correlated with increased disability, morbidity and mortality in older people. Moreover, elevated levels of IL-6, IL-1β and C-reactive protein are associated with muscle problems during aging. In turn, elevated levels of TNFα are considered a strong predictor of mortality in people aged 80 years and centenarians. These metabolic diseases have to be considered systemic diseases characterized by a pervasive and multisystemic state of inflammation. To this regard, it was proposed the concept of "metaflammation", defined as low-grade, chronic inflammation orchestrated by metabolic cells in response to excess nutrients and energy. Excessive levels of nutrients, in particular of glucose and free fatty acids, induce a stress in the pancreatic islets and in adipose tissue, liver and muscle, which are insulin-sensitive tissues, and induce the local release of cytokines, chemokines and adipokines (Donath and Shoelson, 2011; Gregor and Hotamisligil, 2011; Calabrese *et al.*, 2011, 2012; Cevenini *et al.*, 2013).

2. Oxidative stress response genes. Oxidative stress is important from a biomedical point of view because it is related to a wide variety of human diseases, such as neurodegenerative disease (e.g., Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis), inflammatory disease (e.g., rheumatoid arthritis), cardiovascular disease (e.g., muscular dystrophy), allergies, immune system dysfunctions, diabetes, aging and cancer. Nuclear factor E2-related factor 2 (Nrf2) is referred to as the "master regulator" of the antioxidant response, modulating the expression of hundreds of genes, including the familiar antioxidant enzymes such as SOD, catalase (CAT), glutathione peroxidases (GPXs), thioredoxins (TRX), peroxiredoxins (PRXs) and paraoxonase (PON1). The evidence of a direct link between aging and elevated ROS levels is not undisputed, and the discrepancies may be explained by the differential responses between species and cell types to a rise in ROS levels. Studies with mice showed that SOD2 overexpression, while resulting in decreased lipid peroxidation, did not alter either lifespan or age-related pathology. Meanwhile other studies performed with SOD polymorphisms revealed no age-related differences in genotype frequency. Sod2+/−Gpx1−/− double mutant mice, which in addition to low SOD2 levels lack one of the main mitochondrial GPXs, do not show a shortened lifespan. PON1 gene, an esterase associated with high HDL, hydrolyzed lipid peroxides and it has been suggested as a possible role in protecting LDL against oxidation and may therefore play an important role against the atherogenic process (Jaouad *et al.*, 2003; Landis and Tower, 2005; Jang *et al.*, 2009; Hybertson *et al.*, 2011; Vomhof-Dekrey and Picklo, 2012; Weyemi *et al.*, 2012; Sosa *et al.*, 2013).

3. Genes related to insulin/IGF-1 pathway. Different mutations in specific genes that share similarities with the route insulin/IGF-1 signaling can increase the life of the holders of such mutations. It has been observed that centenarians mantain their ability to respond to insulin as well as low levels of IGF-1 in serum. However, low levels of serum IGF-1 have also been correlated with increased disability and mortality in the elderly. Thus it has been observed that a high level of IGF-1 helps to maintain muscle mass and strength during aging, but at the expense of a higher incidence of cancer (Franceschi *et al.*, 2000; Salvioli *et al.*, 2009).

4. p53. This gene is important not only for its involvement in various molecular pathways (apoptosis, DNA repair, senescence or cell cycle arrest) and its antitumor activity, but also has considerable importance in the phenomenon of aging. Studies indicate that polymorphisms appear to exert no influence on the ability to achieve extremely advanced age. However, variations of the p53 gene at codon 72 affect elderly survival as well as cancer mortality. Although p53-mediated apoptosis is able to kill cancer cells, a role for cellular senescence in p53-dependent tumor suppression is becoming clear. Mouse studies shown that activation of p53-induced premature senescence promotes tumor regression *in vivo*. However, p53-mediated cellular senescence also leads to aging-related phenotypes, such as tissue atrophy, stem cell depletion, and impaired wound healing. In addition, several p53 isoforms and two p53 homologs, p63 and p73, have been shown to play a role in cellular senescence and/or aging (Matlashewski *et al.*, 1987; Zilfou and Lowe, 2009; Qian and Chen, 2013)

5. mTOR. The mTOR pathway plays a crucial role in the regulation of cell metabolism and cellular growth, although its role in senescence is still controversial. mTOR is essential for many developmental and physiological processes while deregulation in its signaling has been implicated in a wide variety of diseases. The mTOR pathway has been shown to have opposing roles on cellular senescence. On the one hand, by inhibiting autophagy, mTOR is considered as a negative regulator of senescence, which is in agreement with its widely accepted negative role in organismal aging. Moreover, as mentioned above, mTOR is inhibited by p53, a bona fi de pro-senescence positive regulator. On the other hand, the hypertrophic phenotype of senescent cells and increased production of secretory proteins, which requires high metabolic activity, are in apparent contradiction with cellular shrinkage that is usually associated with autophagy and mTOR inhibition. Indeed, in the case of oncogene or DNA damage-induced senescence, the onset of both senescence and autophagy correlates with inhibition of mTOR activity (Young *et al.*, 2009; Feng and Levine, 2010; Dazert and Hall, 2011; Kim *et al.*, 2011; Dublic, 2013).

6. Apolipoproteins. Along proteins associated to high density lipoprotein, such as PON1, a large number of studies have considered the involvement in the aging and longevity of allelic variants of genes encoding apolipoproteins, transfer proteins as mitocrosomal transfer protein (MTP) or the cholesteryl esters transfer protein (CETP), and transcription factors involved in lipid metabolism such as PPARγ. One of the most studied apolipoprotein-related genes has been the apoE gene, having found a negative correlation with longevity, and being its effects probably related to the occurrence of cardiovascular and neurodegenerative disease. Apolipoprotein E-deficient (apoE-/-) mouse has a propensity to spontaneously develop hypercholesterolemia and atherosclerotic lesions that are similar to those found in humans. ApoE normally plays a role in lipid metabolism and transport, however in Alzheimer disease it is believed to play a role in the clearance of amyloid β from brain (Meyrelles *et al.*, 2011; Tanzi, 2012).

7. Sirtuins. The mammalian sirtuin family is comprised by seven proteins (SIRT1-7) with a variety of subcellular locations: SIRT1 is predominantly nuclear; SIRT2 is located mainly in cytoplasm; SIRT3-5 are mitochondrial proteins, although SIRT3 has also been identified to move from the nucleus to mitochondria during cellular stress; finally SIRT6 and SIRT7 are nuclear sirtuins. These deacetylase enzymes are linked to gene repression, metabolic control, apoptosis and cell survival, DNA repair, development, inflammation, neuroprotection and healthy aging. The most studied sirtuin at the moment is SIRT1, which down-regulates p53 activity, increasing lifespan, cell survival, and neuroprotection; it also deacetylates PPARγ and its coactivator 1α, promoting fat mobilization, increasing mitochondrial size and number, and positively regulating insulin secretion (Alcaín and Villalba, 2009a) As mentioned in section 1.1, CR and resveratrol promote longevity by activation of SIRT-1 expression. On the other hand,

several specific inhibitors of sirtuins have been described. SIRT2 inhibitors might be useful for the treatment of cancer and neurodegenerative diseases by increasing p53 activity that stops the formation of tumours and induces apoptosis (Alcaín and Villalba, 2009b).

1.2. Aging-related mitochondrial genoma

The mitochondrial genome encodes 13 essential subunits of protein complexes belonging to the oxidative phosphorylation system, while most of the mitochondria-related genes are encoded by the nuclear genome. Coordination between the nucleus and mitochondria is crucial for the regulation of mitochondrial biogenesis and function (Liu *et al.*, 2013; Tseng *et al.*, 2013). An accumulation of mtDNA somatic mutations occurs with age, and many studies have reported an association between mtDNA mutations and aging, particularly in post-mitotic neuronal cells (Bohr *et al.*, 2007; Guo *et al.*, 2010). Progressive accumulation of defective mitochondria in cells is tightly linked to the decrease in physiologic function that occurs during aging and to the pathologies of aging-related diseases (Yen and Klionsky, 2008; Seo *et al.*, 2013). According to Salvioli *et al.* (2001) these mitochondrial evidence includes:

- Accumulations of deletions and certain mutations of the mtDNA in addition to decreased the mtDNA copy number in some tissues.
- Age-dependent decline in the activity of some enzymes in the mtETC.
- Greater production of free radicals, presumably as a consequence of the avove-described alterations.
- Changes in the morphology of mitochondria and collapse of the Ψmt.

The mtDNA shows a high mutation rate and the available literature suggests that a variety of deletions and mutations accumulate with age in mtDNA (Bohr *et al.*, 2007; Guo *et al.*, 2010; Pacheu-Grau *et al.*, 2011; Yang *et al.*, 2012). Studies with mutations in the POLγ have demonstrated that a deficient content of this protein accumulate mtDNA point mutations and display features of accelerated aging, such as mtDNA deletions in the substance nigra associated with neurodegeneration and parkinsonism. This data suggest that mtDNA deletions are associated to the aging phenotype (Luoma *et al.*, 2007; Eerola *et al.*, 2010). The majority of the current data agree that mtDNA somatic mutations are not only related to aging but also detrimental for longevity: mechanisms able to fight the accumulation of mtDNA somatic mutations, such as the expression in mice of mitochondria-targeted catalase, are associated with an increased lifespan (Schriner and Linford, 2006). Studies on age-related mitochondrial DNA deletions in liver, brain and heart of rats were performed by Quiles *et al*. and Ochoa *et al.*, over several years (Quiles *et al*., 2006a, 2006b, 2010b; Ochoa *et al.*, 2011), as it mentioned in chapter II, sections 3.2.1, 3.2.2 and 3.2.3, relating the aging from the viewpoint of the mitochondrial genome with the type of dietary fat in the diet.

Recently, a series of somatic mutations in mtDNA control region have been related to aging. It has been detected that A189G and T408A mutations accumulate in skeletal muscle during aging (Quiles *et al*., 2010a). Many researches have been done about heteroplasmy at position 150 in the mtDNA D-loop control region. The function for most of the control region is still unknown; however, the observation that the central domain of this region is highly evolutionarily conserved suggests that control region is selectively constrained. Studies on the mtDNA control region in leukocytes obtained from Italian centenarians we observed three intriguing findings:

- A homoplasmic C150T transition near an origin of heavy mtDNA-strand synthesis occurred more frequently in older than younger individuals.

- Somatic events seemed to contribute to the striking selective accumulation of the mutation in centenarians.

- An aging-related somatic expansion of the C150T mutation seems to occur according to human fibroblast longitudinal studies. In addition, a new replication origin at position 149, substituting for that at 151, occurs only in C150T mutation-carrying samples of fibroblasts or immortalized lymphocytes, suggesting that the striking accumulation of the C150T mutation in centenarians favors longevity due to the new replication origin may restore the efficiency of the mitochondrial machinery as the individual ages and stochastic damage accumulates in the mitochondrial genome (Zhang *et al.*, 2003; Iwata *et al.*, 2007; Quiles *et al.*, 2010a).

Variations in the functionality of mitochondria may determine adequate or inadequate aging, according to studies of specific population groups around the world. Most of data on mtDNA and longevity have been obtained on humans and particularly on centenarians. For example, an inherited variation in an mtDNA germ line (halogroup J) has been associated with a more adequate aging process and to an extended life in the Italian population (De Benedictis *et al.*, 1999, 2000), Northern Irish (Ross *et al.*, 2001), Belfast population (Rea *et al.*, 2013), Finnish population (Niemi *et al.*, 2003) and Spanish population (Domínguez-Garrido *et al.*, 2009; Pinós *et al.*, 2012); whereas the haplogroup U is more frequent in centenarians than in younger people in the European (Meinila *et al.*, 2001). The latest finding represents a novel association of Haplogroup X with successful aging (Courtenay *et al.*, 2012).

On other hand, in Japan, three mutations associated to an mtDNA germ line (3010A, 5178A and 8414T of mtDNA) have been found with a high frequency in centenarians in this part of the world (Tanaka *et al.*, 1998) while the mt9055A is reported to be more frequent in centenarians than in the younger population in French (Ivanova *et al.*, 1998) and Irish (Ross *et al.*, 2001). In China, mt146T/C and mt5178C/A polymorphisms have being related with Bama population longevity (Yang *et al.*, 2012).

Recent studies have indicated that certain mitochondrial haplogroups are correlated with the development of certain cancers, as oral squamous cell carcinoma, esophageal squamous cell carcinoma, lung cancer, colorectal cancer and pancreatic cancer inter alia (Wang *et al.*, 2007; Webb *et al.*, 2008; Singh *et al.*, 2009; Zhang *et al.*, 2010; Lai *et al.*, 2012; Zheng *et al.*, 2012). These data support the study of how certain mitochondrial DNA mutations may affect the survival of the organism.

Many mitochondria-centered theories have been proposes to explain human aging and longevity (Salvioli *et al.*, 2008). A number of non-pathogenic mutations have fixed in the mtDNA to form a series of population-specific lineages that can be identified by the presence of conserved groups of haplotypes (haplogroups). These haplogroups can be further subdivided into subhaplogroups and such classification is continuously updated and used for tracing back the origin of populations or for forensic purposes. These germline inherited mtDNA variants (haplogroups and subhaplogroups), considered to be biochemically neutral, are probably able to differently modulate the mitochondrial metabolism and, above all, the cross-talk supposed to exist between nuclear and mitochondrial genome (Sevini *et al.*, 2007; Lorente *et al.*, 2012). Haplogroups are coded with capital letters and subclusters with a running number. Three African (L1, L2, L3), seven Asian (C, D, G, E, A, B, F), and nine European (H, T, U, V, W, X, J, I, K) main mtDNA haplogroups have been identified. Haplogroups can be further subdivided in sub-haplogroups on the basis of specific mutations identified by sequence analysis of the D-loop region (Salvioli *et al.*, 2008). These genomic variants may not be as neutral as previously thought, exerting some influence on mitochondrial metabolism, so that could be selected based on factors such as climate, diet, or the availability of oxygen. Thus, mtDNA germ-line variants might be associated with human longevity or, on the contrary, with age-associated pathologies

and unsuccessful aging. On the other hand, mtDNA haplogroups have been associated with mitochondrial and complex diseases (Wallace, 2005). It has been observed that male centenarians in northern Italy, the halogroup J is over-represented, allowing suggest a protective effect of this halogroup from aging. However, when the study population expanded southern Italy, there was no correlation between halogroup J and longevity. These results might suggest that the effect of inherited variants mtDNA on longevity could be specific to each population, being this effect further subjected to a strong interaction with the environment. These observations have been confirmed in two other studies at European and Japanese centenarians, where the halogroup D is more common (Salvioli *et al.*, 2008).

2. GENE-NUTRIENT INTERACTIONS

Nutrition is probably the most important environmental factor that modulates the action of genes and the phenotypes being considered. The interaction between genotype and dietary exposure (nutritional genomics) has the potential to change dietary disease prevention and to have a major impact on public health. The concept of gene-diet interaction describes dietary modulation of the effect of genotype on a particular phenotype (for example obesity, insulin resistance and dyslipidemia) and/or modulation of the effect of a dietary factor on a particular phenotype by a genetic variant. There are two possibilities of nutrient-gene interaction (Finch and Ruvkun, 2001; Busuttil *et al.*, 2007; Phillips, 2013; Tucker *et al.*, 2013).

2.1. Transcription level interaction

The regulation is carried out mainly by the interaction between transcription factors and response elements, being on first where nutrients interact modifying the protein structure of the same, which increases the affinity of the gene regulatory regions. Nevertheless, in some cases certain nutrients or their metabolites may be directly interacting with response elements. Some response elements related with nutrients are sterol response element in genes regulating the same (Jeon and Osborne, 2012); calcitriol receptor response element related with genes regulating vitamin D (Lisse *et al.*, 2011); response elements for fatty acids and some of its metabolites in genes affecting the metabolism thereof (Benetti *et al.*, 2011) and metal response elements in regulatory genes zinc (Sharife *et al.*, 2012). An important example of nutrients acting on transcription level is the dietary fat, which causes diverse responses depending on the amount of fatty acids and structure of them (saturated, unsaturated, of greater or lesser chain length, etc.). Essential fatty acids (EFA) are nutrients that form an amazingly large array of bioactive mediators that act on a large family of selective receptors. The health consequences of specific gene-environment interactions with these nutrients are more extensive than often recognized. It also depends on derived from fatty acids such as eicosanoids, eicosanoids hydroxides, active forms of fatty acids (acyl CoA), etc. (Goto *et al.*, 2013).

PUFAs interact on PPAR α, β and γ, which exercises its action need to form a heterodimer with another nuclear receptor, the RXR binding finally PPRE response element located in the DNA molecule. Through this mechanism of regulation occurs expression of genes encoding proteins (enzymes) involved in the β-oxidation of fatty acids at both mitochondrial and peroxisomal level. Moreover, PUFAs can also interact with other transcription factors, the "sterol regulatory element binding proteins" (SREBP), and nuclear factor-Y (NF-Y). PUFA-dependent regulation of SREBP and NF-Y binding may represent a nutrient-sensitive motif through which PUFA selectively and coordinately targets subsets of hepatic genes involved in lipid metabolism (Benetti *et al.*, 2011; Ihunnah *et al.*, 2011; Jeon and Osborne, 2012).

The result of the interaction between fatty acids and byproducts with the different transcription factors constitute regulatory network of these nutrients on many aspects related to metabolic lipid metabolism, of eicosanoids and carbohydrates, with cell growth and differentiation, various cytokine production, adhesion molecules, etc. (Xu *et al.*, 2000; Clarke, 2004; Teran-García, 2007; Lands, 2012).

2.2. Post-transcriptional level interaction

Post-translational modifications of chromatin contribute to the epigenetic control of gene transcription. The response to food intake and individual nutrients also includes epigenetic events. The downstream effects of post-transcriptional modifications of histone proteins and other DNA-interacting proteins are emerging as crucial aspects contributing to the phenotypic response to food intake and to individual nutrients (Pham and Lee, 2012).

Fasting in rats decreases the activity and the amount of glycogen synthetase, and however the amount of its mRNA is the same as in well-fed rats. This difference may be explained by regulation at the translational level, through a regulatory protein that interacts with a region of the mRNA, preventing its translation. Other examples are glucose regulated protein 78 (GRP78), glucose 6-phosphate dehydrogenase (GPDH) and malic enzyme. Fasted mice also express higher levels of cholesterol 7α-hydroxylase (CYP7A1) and phosphoenolpyruvate carboxy kinase (PEPCK), the key genes in bile acid synthesis and gluconeogenesis respectively, in comparison to fed animals. In the same way, certain lipoproteins are expressed variously through changes in post-transcriptional processes modulated by nutrients. Glucose transporters and lipoprotein lipase are two proteins of key importance in metabolic response to nutrient intake (such as glucose), carrying out the regulation of the genes that express them through mRNA no translatable regions RNT5 'and RNT3'. These no translatable regions are also the key to post transcriptional regulation diverse proteins as of ferritin, transferrin protein, selenoprotein and metalloproteinase. When selenium intake is low, the RNT3 'of GPX is less effective in order to maintain the translation that of GPX, which may be the result of different ability to form complexes between tRNA seleno-cysteine and corresponding regulatory cytoplasmic proteins. Intake of significant amounts of zinc, cadmium and copper produces an induction of these enzymes in liver and kidney, result of regulation at transcription and post-transcriptional level (seems to depend also on the regulatory action of the RNT3') (Baldellou, 1998; De Fabiani *et al*., 2010).

Caloric restriction is established to influence lifespan, increasing longevity. Although the mechanisms are likely to be multiple and are not yet fully understood, one mechanism may involve upregulation of the NAD-dependent deacetylase sirtuin-1, that regulates the activities of a variety of transcription factors, coregulators, and enzymes that improve glucose uptake in skeletal muscle. A number of studies have reported that the levels of SIRT1 are increased in a range of tissues, including the brain, adipose tissue and kidney in rodents in response to CR. Transgenic mice overexpressing SIRT1 in tissues, including adipose tissue and brain, but not liver or muscle, have a phenotype that resembles that of CR, whereas SIRT1-null mice fail to respond to CR with an extended lifespan (Civitarese *et al.*, 2007; Holness *et al.*, 2010).

2.3. Nutritional modulation of aging: genomic aspects

During aging occurs a progressive reduction in the response capacity to physiological needs, which appears to be largely due to changes in gene expression. Thus, for example, the reduced stress tolerance ability in old people may be parallel to a decline in the ability of intracellular

protein synthesis in response to stress. Nutrition is believed to be an important modulator of aging, however, there is still little evidence about the role played by specific nutritional factors (except CR) related to longevity. Nevertheless begins to be clear that nutritional factors have a profound influence on gene expression (nutrigenomic) and the application of genomic tools of mass screening (microarrays, etc...). Nutritional research in the context of systems biology offers exciting new opportunities to investigate the role of diet on the biology of aging (Mathers, 2006; Busuttil *et al.*, 2007). The lifespan of some organisms can be extended by mutations that alter how DNA is packaged in their cells. Recent studies indicate that chromatin modifiers regulate lifespan in several organisms, raising the question as to whether changes in chromatin states in the parental generation could be incompletely reprogrammed in the next generation, and thereby affect the lifespan of descendants. The importance of appropriately maintaining or reprogramming histone methylation is illustrated by its links to disease and aging and possibly to transmission of traits across generations (Greer *et al.*, 2011, Greer and Shi, 2012).

Nutritional factor are part of a complex set of factors that come into play during epigenetic inheritance. Polymorphisms of the human Δ-5 (fatty acid desaturase, FADS1) and Δ-6 (FADS2) desaturase genes have been described as being associated with the level of several long-chain n3 and n6 PUFAs in serum phospholipids. An increased consumption of refined starches and sugar increases the generation of superoxide anion in the tissues and FFA in the blood. There is an increased amount and activity of NF-kB, a transcriptional factor regulating the activity of at least 125 genes, most of which are pro-inflammatory. The consumption of glucose may be associated with an increase in 2 other pro-inflammatory transcription factors such as activating protein-1 (AP-1), that regulates the transcription of matrix metallo-proteinases; and early growth response protein-1 (Egr-1), that modulates the transcription of tissue factor and plasminogen activator inhibitor-1 (Alam *et al.*, 2012). Recently it has been described the epigenetic regulation of lifespan in diverse model organisms, focusing on the role and mode of action of chromatin regulators that affect 2 epigenetic markers for longevity: trimethylated lysine 4 of histone H3 (H3K4me3), and trimethylated lysine 27 of histone H3 (H3K27me3), whose methylation may be regulate by epigenetic factors and affect lifespan (Han and Brunet 2011).

2.4. Nutritional modulation of aging: proteomic aspects

The term ‘proteomic’ was coined to make an analogy with genomics. Proteomic studies the expression of proteins, their structures and functions. The transcriptome from genome to proteome is not linearly proportional and many age-related diseases result from alterations in the proteome (Langley *et al.*, 2013). Several components of the diet, mainly fatty acids, can modulate the level of different plasma proteins involved metabolic process such as inflammation, homeostasis, oxidative stress, cardiovascular risk and cancer, which are of special concern in aging.

Santos-Gonzalez *et al.* have studied for years how dietary oil, supplemented or no with antioxidants, modifies the plasma proteome during aging in the rat. In order to determinate the effect of antioxidants supplementation on protein plasma levels during aging, they fed rats on a n6 PUFA-enriched diet with CoQ_{10}. Their results have shown that lifelong dietary supplementation with CoQ_{10} decreases significantly the plasma levels of proteins involved in inflammation, oxidative stress and cardiovascular risk during aging (Santos-Gonzalez *et al.*, 2007). Other of their studies showed how dietary oil affects the plasma proteins levels in young or old rats fed lifelong on two experimental diets enriched in either sunflower or virgin olive oil.

The results showed that the virgin olive oil diet, by providing oleic acid and minor constituents, induced significant decreases of plasma levels of acute phase protein, providing remarkable benefits on the antioxidant status, the anti-inflammatory state and the anti-atherogenic lipid profile during aging (Santos-Gonzalez *et al.*, 2011).

3. TOOLS FOR THE STUDY OF GENE EXPRESSION

Previous studies have investigated the gene expression changes that occur with age in some tissues and organs, such as brain, heart, liver, kidney and skeletal muscle (Ochoa *et al.*, 2007, 2011; Quiles *et al.*, 2010b; Khan *et al.*, 2013). The expression of some genes was found not only to change with age but also to reflect the biological function of the source organs, demonstrating that gene expression levels are not only markers of chronological age but also of tissue function. However, these studies cannot discriminate between genes showing expression changes in mid-life that may contribute to the aging process, from those showing expression changes later in life as a consequence of the aging process. A multitude of techniques have been set up for the analysis and validation of gene expression, among which we selected for our study those described in the following sections.

3.1. A high performance technique for the study of gene expression: Microarray technology

DNA microarrays are part of a technology that allows evaluate the expression of thousands of genes simultaneously in a single biological sample (Han and Hilsenbeck, 2001). A DNA microarray consists of an orderly arrangement of DNA fragments representing the genes of an organism. Each DNA fragment representing a gene is assigned a specific location on the array, usually a glass slide, and then microscopically spotted (less than 1 mm) to that location. Through the use of highly accurate robotic spotters, over 30,000 spots can be placed on one slide, allowing molecular biologists to analyze virtually every gene present in a genome (Stears *et al.*, 2003).

Gene expression array technology is extremely useful in studying a complex and multigenetic process as aging, where it needs to understand the interaction of a large number of genes. For gene expression array technology on aging studies three steps are necessary: the array hybridization; the biostatistical analysis; and the array result confirmation that differentially expressed genes are reflected by differential expression at the protein level.

There are several microarray platforms differing in their manufacture, labeling and assay protocol, however, in all cases the technique is based on specific binding or hybridization of DNA strands with exact complementary copies attached to a solid substrate (probes). First, the RNA sample is isolated, purified, retrotranscribed to complementary DNA (cDNA) and marked. Then the sample is hybridized to the microarray containing DNA fragments corresponding to the coding sequence of individual genes and arrayed on the solid surface of the microarray. Finally, the microarray is scanned and examined on a fluorescence microscope. The degree of fluorescence at the position of each probe can be measured, and represents the abundance of a specific transcript.

Currently, there are two microarray platforms used for gene expression analysis of performance: cDNA microarrays, or spotted microarrays, and *in situ* synthesized oligonucleotide microarrays. As the "gene chip" industry started to grow in the 1990's, with the establishment of companies, such as Affymetrix, Illumina, GenScriptand, or Roche NimbleGen among others, the technology of DNA microarrays has become the most sophisticated and the

most widely used. Affymetrix is the leader in this type of chips; it is generically called GeneChip® arrays. The main difference between the two platforms is in the length of probes arranged on the microarray; cDNA probes uses over 100 nucleotides and oligonucleotide probes are 25-70 nucleotides. Both strategies involve taking slightly different experimental protocols. In the case of cDNA microarray, RNA extracted from the sample and the RNA derived from a reference sample are reverse-transcribed in the presence of nucleotides labeled with two fluorophores of different colors and then hybridized to the microarray. In this bicolor system, the abundance of each transcript is expressed relative to the expression of the genes of the reference sample (Figure 1). In contrast, in the case of oligonucleotide microarrays, the RNA sample is initially retrotranscribed new cDNA and transcribed in vitro with biotin-labeled nucleotides complementary RNA (cRNA). The labeled cRNA is hybridized to the microarray and then stained with a solution containing streptavidin conjugated with a fluorophore. The result is a monochrome image in which the fluorescence intensity of each point represents the specific abundance of each transcript in absolute terms (Figure 2). Both methods generate reasonably consistent results, however, in recent years there has been widespread use of oligonucleotides microrrays because these platforms offer greater sensitivity than the cDNA, since its design minimizes the cross-hybridizations between homologous genes, and allows analysis of variants produced by splicing. Affymetrix GeneChip® arrays are the most commonly used microarray assays. It contains multiple oligonucleotide pairs for each gene, while requires special dedicated equipment to carry out the hybridization and quantitation processes. Likely advantages of the GeneChip® include specificity, and reproducibility due to automated control of labeling, hybridization, and quantitation (Brown and Botstein, 1999; Han and Hilsenbeck, 2001; Gresham *et al.*, 2006, 2008).

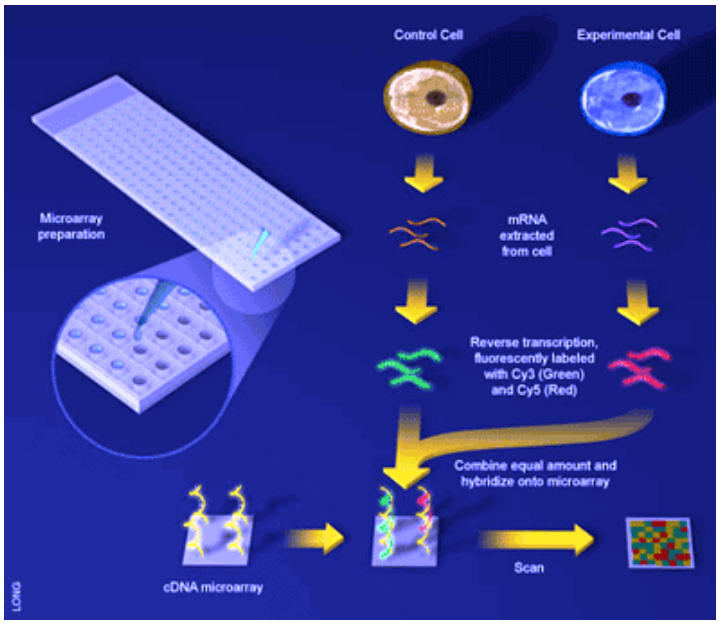


Figure 1. *Representation of cDNA microarray analysis. RNA from the experimental sample and the reference sample are extracted and labeled with two different fluorophores. After hybridization with the microarray, they are scanned and studied the resulting color pattern*.

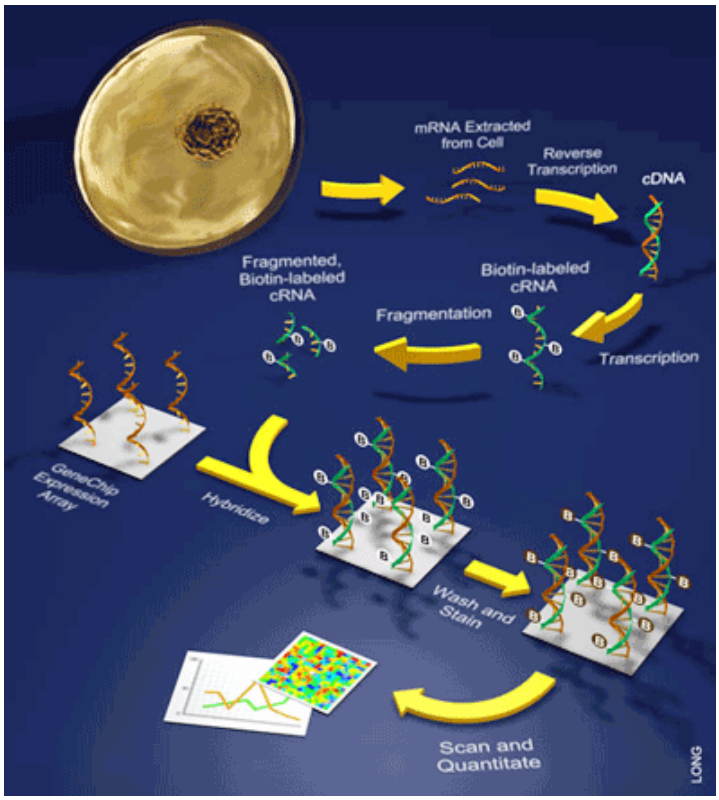


Figure 2. *Steps of oligonucleotide microarray analysis. The RNA of samples is isolated and purified, and then it is labeled with biotin and hybridized to the microarray. After the hybridization, washing and staining steps, the microarray is scanned and data are analyzed.*

3.2. A gene expression analysis technique to microarray validation: Real Time PCR (RT-PCR)

Another technique used in the analysis of gene expression in biological samples is the RT-PCR (real time polymerase chain reaction). This technique has developed considerably in recent years, allowing the analysis of several hundreds of genes simultaneously, so it has been included in the group of high performance analysis techniques under the name of multiplex RT-PCR. Moreover, many researchers have considered multiplex RT-PCR as a quantitative technique to confirm or validate the results obtained from the analysis of gene expression using microarrays (Deepak *et al.*, 2007).

RT-PCR is an adaptation of the technique of the polymerase chain reaction (PCR), originally developed by Mullis *et al.* in the 80s (Mullis, 1990). By PCR any nucleotide sequence present in the sample is amplified in a cyclic process for generating a large number of identical copies. This process essentially consists of three stages: the exponential phase, during which the product is doubled with each cycle; the linear stage, large variability phase, in which the reagents are consumed gradually and the product is not doubled with each cycle; and the plateau phase, in which the reaction is terminated and the product is not amplified.

PCR as a very sensitive analytical technique but is not quantitative, since after several cycles of amplification, the same amount of product is obtained, regardless of the initial concentration in the sample of DNA sequences of interest. Figure 3 shows the PCR of various dilutions of the same sample. Each dilution initially contains a different concentration of DNA; however, the end of the reaction gives the same amount of product of them all. This limitation was overcome in 1992 with the development of RT-PCR or qPCR (quantitative PCR) for Higuchi *et al.* (Higuchi *et al.*, 1992).

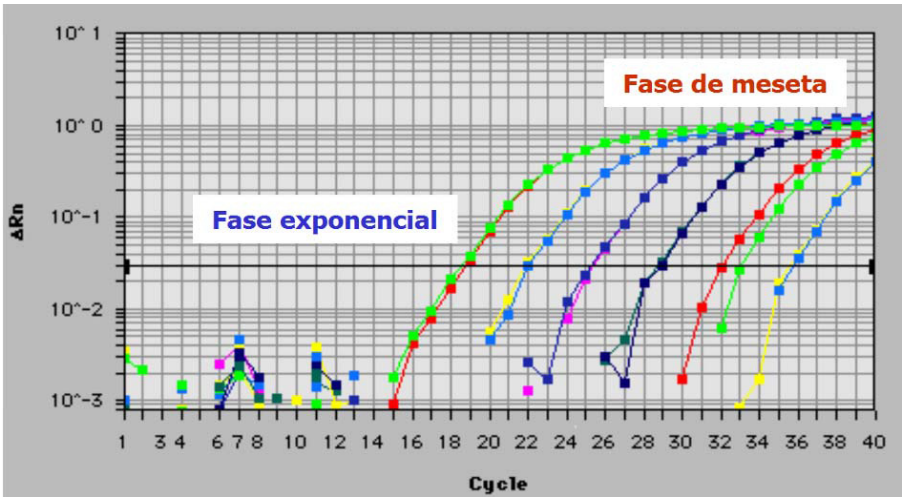


Figure 3. *Exponential and plateau phases during the course of reaction PCR, with serial dilutions of the same sample.*

RT-PCR allows us to monitor the amount of product formed during the course of the reaction and collect information while the reaction is occurring, and not only at the end of it. According figure 3 graph, the right time to collect data would be during the exponential phase, in which the DNA concentration differences between the samples are translated in different amplification speeds for each. Indeed, the RT-PCR reactions are characterized by the time in which the product of amplification begins to be detected; when the initial copy number in a specific DNA sequence is larger, it will first be detected in a RT-PCR (Kubista *et al.*, 2006).

The amount of DNA amplified in each cycle is measured by fluorescence detection methods. Amplification and detection are two simultaneous processes, such that the fluorescence emission produced by the reaction is proportional to the amount of product formed. Detection systems used are of two types: intercalating agents, such as Green SYRB or specific probes labeled with fluorophores, such as TaqMan probes. TaqMan probes are doubly labeled oligonucleotide: 5'-FAM as reporter or donor fluorophore which fluoresces when excited; and 3'-BHQ as fluorophore quencher or acceptor which absorbs the fluorescence released from the donor, while the two fluorophores are closely spaced. During the reaction the probe is aligned and hybrid with its target sequence in the DNA, remaining intact until the DNA polymerase of Thermus aquaticus (Taq polymerase), which travels along the complementary strand in 3'-5' sense during amplification, collides with the probe and its 5'-3' exonuclease activity hydrolyzes the 5' end of the probe releasing the emitter fluorophore that, spatially away from the acceptor fluorophore, emits a fluorescent signal detected by the reader (Figure 4) (Kubista, 2012).

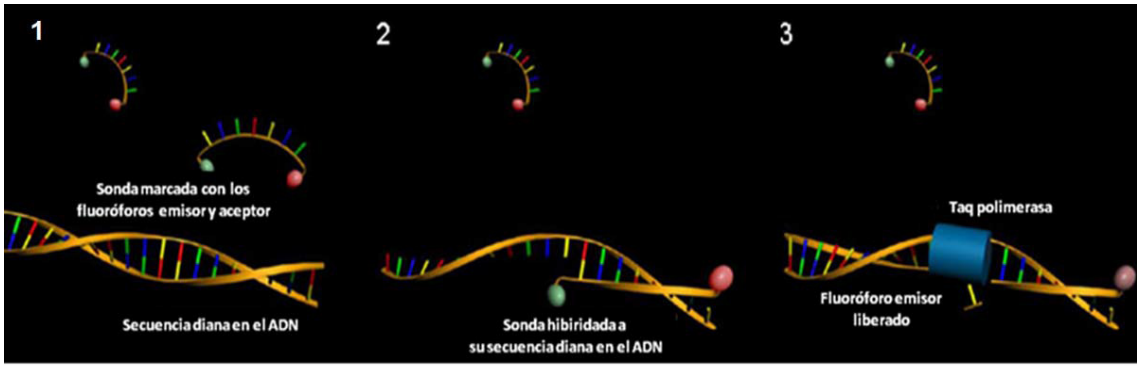


Figure 4. *TaqMan probe reaction in RT-PCR.*

3.3. Data analysis

The advances in sequencing techniques at the end of the last century allowed to perform sequencing studies aimed at the elucidation of the complete sequence of DNA from different organisms. This technological development has dramatically changed the approach of scientific hypotheses in the field of molecular biology, as we have gone from knowing a specific biological function, find the responsible gene and its encoded protein study, to have a large amount of information regarding the expression of thousands of genes and ask what is their function and how they interact with the proteins they encode (Kelmansky, 2013).

The study of genomics and proteomics involves the generation of a huge amount of information that needs to be analyzed and modeled to extract clinically relevant conclusions. In response to this need has emerged a new scientific discipline known as Bioinformatics or Computational Molecular Biology, which uses information technology to organize, analyze and distribute information of biomolecules in order to answer complex questions. Bioinformatics includes both the creation and maintenance of databases that store biological information, such as the processes of analysis and interpretation of data. The latter process is known as biocomputing and covers the development and application of mathematical tools (algorithms) and statistics able to relate parts of a data set (Martínez, 2006).

To date, a multitude of sophisticated bioinformatic methods have been developed with the aim of carrying out a comprehensive analysis of the data generated by gene expression studies with microarrays. Basically there are two types of approaches known as supervised and unsupervised analysis methods. Supervised analysis methods require prior biological or clinician knowledge to discriminate different genes distributed statistical probability between different sample groups. The unsupervised analysis methods are used for investigate different structures included in a data set, usually using pattern recognition algorithms to define groups of similar samples with molecular profiles (Gruvberger-saal *et al.*, 2006). Also, are currently available both commercially and academically, various bioinformatics tools and databases that facilitate functional analysis and biological interpretation of the data obtained in a study of global gene expression in aging. Among them are:

1. Nucleotide sequences databases:

- National Center for Biotechnology information (NCBI), Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore).
- Nucleotide Sequence Database at European Bioinformatics Institute (EBI), from European Molecular Biology Laboratory (EMBL) (http://www.ebi.ac.uk/embl).
- DNA Database of Japan (DDBJ), from the National Institute of Genetics (http://www.ddbj.nig.ac.jp).

These three primary nucleotide sequence databases form the International Nucleotide Sequence Database (http://www.insdc.org/). They include sequences submitted directly by scientists and genome sequencing group, and sequences taken from literature and patents. There are other nucleotide sequence databases containing subsets of the EMBL/GenBank databases. Some also contain more information or links than the primary ones, or have a different organization of the data to better suit some specific purpose. The databases below are secondary databases:

- UniGene (www.ncbi.nlm.nih.gov/UniGene/).
- Saccharomyces Genome Database (SGD) (www.stanford.edu/Saccharomyces/).
- EBI Genomes (www.ebi.ac.uk/genomes/).

- NCBI Genome Biology (www.ncbi.nlm.nih.gov/Genomes/).
- Ensembl (www.ensembl.org).

2. Protein structures and sequences databases:

- Protein data Bank (PDB) (http://www.rcsb.org/pdb).
- NCBI Protein Database (http://www.ncbi.nlm.nih.gov/sites/entrez).
- SWISS-PROT and TrEMBL (Translated EMBL) (http://www.expasy.org/sprot).
- Protein Information Resource (PIR) (http://pir.georgetown.edu).
- Database of Macromolecular Movements (http://www.molmovdb.org/).
- JenaLib (http://jenalib.fli-leibniz.de/IMAGE.html).
- ModBase (http://modbase.compbio.ucsf.edu/modbase-cgi/index.cgi).
- Protein Data Bank in Europe (PDBe) (http://www.ebi.ac.uk/pdbe/).
- OCA. The OCA integrates information from KEGG, OMIM, PDBselect, Pfam, PubMed, SCOP, SwissProt, and others (http://oca.weizmann.ac.il/oca-docs/oca-home.html).
- PDBsum. Pictorial database of 3D structures in the Protein Data Bank (http://www.ebi.ac.uk/pdbsum/).
- Protein Data Bank of Transmembrane Proteins (PDBTM) (http://pdbtm.enzim.hu/).
- ProteinLounge (http://www.proteinlounge.com/).
- Structural Classification of Proteins (SCOP) (http://scop.mrc-lmb.cam.ac.uk/scop/).
- Open Protein Structure Annotation Network (TOPSAN) (http://www.topsan.org/).
- Protein Common Interface Database (ProtCID) (http://dunbrack2.fccc.edu/protcid/).

3. Databases and software for the functional analysis:

- GeneOntology, which includes a link to access most bioinformatic tools available for analysis functional gene (DAVID, GoMiner, GFINDer, THEA, FIVA, Panther... (http://www.geneontology.org/GO.tools.microarray.shtml).
- Encyclopedia Of DNA Elements (ENCODE) (http://www.genome.gov/10005107).
- Biocarta (http://www.biocarta.com).
- KEGG (http://www.genome.jp/kegg/pathway.html).
- PharmGKB (http://www.pharmgkb.org/).
- GenMAPP (http://www.genmapp.org/).
- Ingenuity Systems (http://analysis.ingenuity.com).
- MetaCore (http://www.genego.com/metacore.php).
- Babelomics (http://babelomics.bioinfo.cipf.es/).
- Mutant Mouse Models Database (MUGEN) (http://www.mugen-noe.org/).

4. Public databases of gene expression:

- Gene Expresión Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/).
- Gene Expression Atlas (http://www.ebi.ac.uk/gxa/).
- Array Express (http://www.ebi.ac.uk/arrayexpress).
- CiBEX (http://cibex.nig.ac.jp/).
- BioGPS (http://biogps.org/#goto=welcome).
- The e-Mouse Atlas Project (EMAP) (http://www.emouseatlas.org/emap/home.html).
- Systems Biology and Medicine Database (SBMD) (http://www.lsbm.org/site_e/database/).
- Cancer Gene Expression Database (CGED) (http://lifesciencedb.jp/cged/).
- GUDMAP (http://www.gudmap.org/gudmap/pages/database_homepage.html).
- Functional Genomics Data Society (FGED) (http://www.fged.org/)

MATERIAL AND METHODS

CHAPTER IV: MATERIALS

1. CHEMICAL PRODUCTS

Reagents and solvents used, of the highest quality came from laboratories Fluka (St. Louis, Missouri, USA), Merck Millipore (Hohenbrunn, Germany), Sigma-Aldrich (St. Louis, Missouri, USA), Panreac (Barcelona, Spain), Boehringer (Manheim, Germany), Roche (Basel, Switzerland), Bio-Rad (Hercules, California, USA), Cayman Chemical Company (Michigan, USA), Macherey-Nagel (Durën, Germany) and Thermo Scientific (Rockfor, Illinois, USA). Standards to fatty acids determinations, CoQ_9 and CoQ_{10} were supplied by Sigma-Aldrich and Fluka. HPLC quality reagents and solvents were acquired in laboratories Merck and Panreac.

Virgin olive oil came from "Venta del Llano", from Research and Training Institute for Agricultural and Fisheries of Andalusia (IFAPA); sunflower oil was bought in a local supermarket, and fish oil was acquired from Roche (Basel, Switzerland). The other components used to elaborate diets were purchased to Musal Chemical (Granada, Spain). All the reagents and consumables to measure of the rate of oxygen consumption were acquired to Seahorse Bioscience (Billerica, Massachusetts, USA). Complex I Enzyme Activity Dipstick Assay Kit was acquired from Mitosciences (Eugene, Oregon, USA).

Western Blot buffers and reagents for protein electrophoresis were acquired from Bio-Rad (Hercules, California, USA). The following monoclonal antibodies were used: Mitochondrial transcription factor A (TFAM) (Santa Cruz Biotechnology®, California, USA); Anti-Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC1α) (Calbiochem®, Merck Millipore, Darmstadt, Denmark) and Anti-beta Actin antibody (β-Actin) (Abcam®, Cambridge, UK).

Specific horseradish peroxidase conjugated secondary antibodies anti-mouse were supplied by Abcam (Bio-Rad, Hercules, California, USA) or Santa Cruz Biotechnology (Santa Cruz, California, USA). The chemiluminescence reagents used were the Immun-Star™ HRP Chemiluminescence Kits, come from Bio-Rad (Hercules, California, USA).

DNA Microarray analyses were performed on the *RAE230_2.0* chip from Affymetrix®. RNA was extracted using RNeasy Mini kit from Qiagen (Düsseldorf, Germany). Chips and reagents to RNA integrity analysis were acquired to Agilent Technologies (Santa Clara, California, USA). cDNA was synthetized with One-Cycle cDNA Synthesis kit (Affymetrix®, Santa Clara, California, USA). cRNA was synthetized and biotinylated using the GeneChip® IVT Labelling Kit (Affymetrix®, Santa Clara, California, USA) and it was purified and fragmented with the GeneChip® Sample Cleanup Module Kit (Affymetrix®, Santa Clara, California, USA). Streptavidin-phycoerythrin was acquired to Invitrogen™ (Life Technologies Corporation, Carlsbad, California, USA). RNA was reverse-transcribed to cDNA with the High Capacity RNA to cDNA of Applied Biosystems (Life Technologies Corporation, Carlsbad, California, USA)

2. EQUIPMENT

- -20°C freezer Liebher (Liebher, Biberach, Denmark).
- -80°C freezer Revco VLT-1786-5-VUA (Revco, Asheville, North Carolina, USA).
- Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA).
- Automatic cutter model PT 10-35 POLYTRON (Kinematical AG, Lucerne, Switzerland).
- Automatic pipettes NICHIPET EX (Nichiryo, Tokyo, Japan).
- Automatic staining equipment Leica Autostainer XL (Leica, Wetzlar, Germany).
- Cold plate Leica EG1150C (Leica, Wetzlar, Germany).
- Computer Asus P6-P8H61E, Mini Tower, Core i3 2100 3.1 Ghz.
- Dako Automation Hematoxylin Histological Staining Reagent (Dako Denmark A/S, Denmark).

- Digital scale GRAM PRECISION (Gram Precisión, SL. Barcelona, Spain).
- Dipsticks reader Immunochromato reader MS1000 (Mitosciences Inc. Oregon, USA).
- Dry heat bath Dry block BD200-RE OVAN (Esper Group, Badalona, Spain).
- Electron microscope Carl Zeiss EM10C (Oberkochen, Germany).
- Electronic micropipettes EPPENDORF RESEARCH® PRO. (Eppendorf®, Hamburg, Denmark).
- Eppendorfs microcentrifuge MiniSpin® Rotor F45-12-11 (Eppendorf®, Hamburg, Denmark).
- Ethanol 100%, partially denatured (Panreac Química SA, Barcelona, Spain).
- Fully Automated Glass Coverslipper Leica CV5030 (Leica, Wetzlar, Germany).
- Gas-liquid chromatograph model HP-5890 Series II (Hewlett Packard, Palo Alto, California, USA).
- GeneChip® Scanner 3000 Targeted Genotyping System (Affymetrix®, Santa Clara, California, USA).
- Hamamatsu MS-1000 immunochromatographic dipsticks reader (Mitosciences, Eugene, Oregon, USA).
- Heater MEMMERT (Memmert, Schwabach, Denmark).
- High precision electronic scale SARTORIUS BP110S (Sartorius AG, Götingen, Denmark).
- Histological bath for inclusion in paraffin Termofin (JP Selecta, Barcelona, Spain).
- HPLC system:
    - 3mm ODS Supelcosil column (Beckman Instruments, Fullerton, California, USASigma-Aldrich, St. Louis, Missouri, USA).
    - Analytical cell model 5011 (Thermo Scientific, Rockfor, Illinois, USA).
    - Beckman System Gold 125 pump (Beckman Instruments, Fullerton, California, USA).
    - Electrochemical detector ESA Coulochem III (Thermo Scientific, Rockfor, Illinois, USA).
    - Guard cell model 5020 (Thermo Scientific, Rockfor, Illinois, USA).
    - Water 717 plus autosampler (Milford, Massachusetts, USA).
- Ice machine SCOTSMAN AF-10 (Scotsman Ice Systems, Vernon Hills, Illinois, USA).
- Incubator (JP Selecta, Barcelona, Spain).
- Isoparaffin H, Xylene substitute (Panreac Química SA, Barcelona, Spain).
- Luminescent Image Analyzer LAS-4000 mini (Fujifilm corp. Tokio, Japan).
- Luminescent image analyzer LAS-4000 Mini (Fujifilm. Tokyo, Japan).
- Magnetic shaker with heater, AGIMATIC-E. (JP Selecta, S.A, (Barcelona, Spain).
- Microplate's reader SYNERGY HT, MultidetectionMicroplate Reader (BioTek Instruments, Inc. Higland Park, Vermont, USA).
- Microplate's Shaker Heidolph Titramax 100 150-1350 1/min (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Microtome blades 250 µm thick (Feather Safety Razor Co, Osaka, Japan).
- Microtome Leica RM2235 (Leica, Wetzlar, Germany).
- Mounting Medium for Xylene Surrogates (Panreac Química SA, Barcelona, Spain).
- Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockfor, Illinois, USA).
- Optical microscope ECLIPSE E600 (Nikon Co., Tokio, Japan).
- pH-metre GLP21 (, Crison, (Barcelona, Spain).

- Platform-shaker Heidolph Polymax 1040 2-50 1/min (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Potter-Elvehjem PTFE glass tube (Sigma-Aldrich, St. Louis, Missouri, USA).
- PTM 2330 F.S column (Supelco Inc, Bellefonte, Palo Alto, California, USA).
- Refrigerated centrifuge BECKMAN model J-21 (Beckman Coulter, Inc. Fullerton, Palo Alto, California, USA).
- ScanSpeed MaxiVac Alpha (Neutec Group, New York, USA).
- Sterile distilled water (Fresenius Kabi AG, Sevres, France).
- Teflon pestle (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany).
- Thermal Cycler of Applied Biosystems model 2720 (Life Technologies Corporation, Carlsbad, California, USA).
- Ultracut S ultramicrotome EM UC6 (Leica, Illinois, USA).
- Vortex VWR (VWR International Eurolab S.L., Barcelona, Spain).
- Washing station Fluidics Station 400 (Affymetrix®, Santa Clara, California, USA).
- Western Blot equipment:
    - Critterion™ Blotter 560BR (Bio-RadBio-Rad, (Hercules, California, USA).
    - Critterion™ Cell 76S (Bio-Rad, Hercules, California, USA).
    - Power source PowerPac HCTM (250V, 30A, 300W) (Bio-Rad, Hercules, California, USA).
- XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica Massachusetts, USA).

3. SOFTWARE

- AnaliSYS Image Processing Software (Olympus, Hamburg, Germany).
- Autostainer equipment Software DakoLink, version 1.5.2.1 (System Dynamics, New Jersey, USA).
- Fluidigm technology (www.fluidigm.com).
- Gene Chip Operating Software (GCOS 1.4, Affymetrix®).
- GeNorm program (http://medgen.ugent.be/jvdsomp/genorm).
- Ingenuity Pathways Analysis (IPA) application (Ingenuity Systems, www.ingenuity.com).
- Partek Genomics Suite software.
- SPSS 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA).
- Windows 7 Home Premium.

CHAPTER V: METHODS

1. EXPERIMENTAL DESING: ANIMALS AND DIET

Seventy two male Wistar rats (*Ratus norvegicus*) initially weighing 80-90 g were used for this study. The rats were randomly assigned into three experimental groups and match-fed from weaning until 24 months of age on a semi-synthetic and isoenergetic diet according to the AIN93 criteria described by REEVES *et al*. (1993 and 1997), but modified in relation to the dietary fat source (see below). Modified AIN-93G diet was administered to rats from weaning to 2 months of age and AIN-93M was administered for the rest of the experiment. Components of AIN-93G (caloric content 401.4 Kcal/100g) and of AIN-93M (caloric content 362.4 Kcal/100g) are shown in tables 6 to 8. As indicated above, AIN93 diets were modified in relation to the fat source. Original AIN93 diets are composed by soya oil. We replaced this oil by virgin olive oil (virgin olive oil fed group), sunflower oil (sunflower oil fed group) or fish oil (fish oil fed group,) to formulate the experimental diets and groups. Table 9 shows the fatty acid profile of experimental oils. Diets were prepared weekly and stored in darkness at 4ºC to avoid lipid peroxidation. Animals were placed in a climate-controlled room (20ºC, 12 h dark/12h light cycle) for two years in collective cages, in groups of three animals per cage, with free access to water. Diet was delivered *ad libitum* for the first two months and then at 25 grams/rat/day for the rest of the experiment (in order to avoid overweight). Food intake was indirectly monitored through the weekly body weight control and daily spillage monitoring.

Table 6. *AIN93 diets composition.*

COMPONENTS	AIN-93G (%)	AIN-93M (%)
STARCH	38,7	46,6
CASEIN	20,0	14,0
DEXTROSE	13,2	15,5
SUCROSE	10,0	10,0
OIL	8,0	4,0
FIBER (CELULLOSE)	5,0	5,0
MINERAL MIXTURE	3,5	3,5
VITAMINS MIXTURE	1,0	1,0
L-CYSTEINE	0,3	0,18
METHIONINE	-	-
CHOLINE BIRTRATE	0,25	0,25

Table 7. *Vitamin mixture content.*

COMPONENTS	AIN-93G (%)	AIN-93M (%)
Nicotinic acid	0,3	0,3
Panthotenate Ca	0,16	0,16
Pirydoxine-HCl	0,07	0,07
Thiamine-HCl	0,06	0,06
Riboflavin	0,06	0,06
Folic Acid	0,02	0,02
D-Biotin	0,002	0,002
Vitamin K	0,0075	0,0075
Vitamin B-12	0,25	0,25
Vitamin A	0,08	0,08
Vitamin D	0,025	0,025
Vitamin E	1,5	1,5
Powder sucrose	97,5	97,5

Table 8. *Mineral mixture content.*

COMPONENTS	AIN-93G (%)	AIN-93M (%)
Essentials Elements		
Calcium biphasic phosphate	-	-
Calcium carbonate anhydride, 40,04% Ca	35,7	35,7
Potassium monophasic phosphate, 22,76% P; 28,73% K	19,6	25,0
Tripotassium monohydrate citrate	7,1	2,8
Sodium chloride	7,4	7,4
Potassium sulfate	4,7	4,7
Manganese oxide	2,4	2,4
Ferric Citrate	0,606	0,606
Zinc carbonate	0,165	0,165
Magnesium carbonate	0,063	0,063
Cupric carbonate	0,03	0,03
Iodate potassium	0,001	0,001
Selenate potassium	0,001025	0,001025
Ammonium paramolybdate	0,000795	0,000795
Potential Beneficial Elements		
Sodium metasilicate	0,145	0,145
Potassium chrome sulphate	0,0275	0,0275
Lithium chloride	0,00174	0,00815
Boric acid	0,00815	0,00635
Sodium fluorouridine	0,00635	0,00318
Nickel carbonate	0,00318	0,00174
Ammonium vanadate	0,00066	0,00066
Powder sucrosa	22,1	21,0

Table 9. *Fatty-acid composition of experimental oils (g/100g).*

Fatty Acid Composition	Virgin Olive Oil	Sunflower Oil	Fish Oil
Myristic (C14:0)	0,0	0,1	7,2
Palmitic (C16:0)	8,3	6,4	17,1
Stearic (C18:0)	3,2	4,7	2,7
Lignoceric (C24:0)	0,0	0,1	0,3
Total saturated	12,6	11,5	30,5
Hexadecenoic (C16:1n9)	1,1	0,1	9,6
Oleic (C18:1n9)	77,7	24,2	15,1
Nervonic (C24:1n9)	0,0	0,0	0,9
Total monounsaturated	83,7	24,4	30,1
Linoleic (C18:2n6)	3,2	62,8	2,8
DGLA* (C20:3n6)	0,1	0,9	0,1
AA (C29:4n6)	0,0	0,0	2,1
EPA** (C20:5n3)	0,2	0,1	18,6
DHA*** (C22:6n3)	0,0	0,0	10,5
Total polyunsaturated	3,7	64,1	39,4
Polyunsaturated (n3)	0,4	0,4	31,3
Polyunsaturated (n6)	3,3	63,7	8,2
Polyunsaturated (n6/n3)	5,5	171,0	0,3
Oleic/Linoleic	24,1	0,4	5,4

*Dihomo-γ-linolenic, **Eicosapentaenoic, ***Docosahexaenoic

The animals were treated in accordance with the guidelines of the Spanish Society for Laboratory Animals and the experiment was approved by the Ethical Committee of the University of Granada (permit number 20-CEA-2004). The rats were killed by cervical dislocation followed by decapitation, at the same time of the day to avoid any circadian fluctuation.

Blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated tubes and the plasma was centrifuged at 1750 x *g* for 10 min. Plasma samples were stored at -80ºC until analyzed. After exsanguination, livers were immediately removed and properly preserved to subsequent studies. Unspecified amounts of liver tissues were immediately frozen, first in liquid nitrogen and after at -80°C. About 1 g of liver tissue was used to mitochondria extraction (see below). Small cuts were fixed with glutaraldehyde buffer during 60 minutes, followed by a wash of 90 minutes in wash buffer, and preserved into wash buffer until electron microscopic analysis. About 50 mg of liver tissue was introduced in RNA later during 24 hours, and stored at -80°C for gene expression analyses. Finally, both longitudinal and transversal cuts of liver were placed into 3.9% formalin buffered at pH. After 24-48 hours fixing each of the fragments was cut and included in a cassette for processing in paraffin according to the conventional process after dehydration in growing up gradation alcohols until ethanol absolute, following histopathological analysis.

2. LIVER PROCESSING AND MITOCHONDRIA ISOLATION

After extraction, liver was washed with physiological saline, weighed and immediately processed. To obtain mitochondrial fraction according to Fleischer *et al*. (1979), hepatic tissue was homogenated with an automatic cutter model PT 10-35 (POLYTRON, Kinematical AG, Lucerne, Switzerland) and a mechanic homogenizer, formed by a Teflon pestle (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and glass potter-Elvehjem (Sigma-Aldrich, St. Louis, Missouri, USA).

Liver was cutted and suspended in sucrose-albumin buffer (sucrose 0.32M, Tris 10mM, $EDTANa_2$ 1mM and albumin 1%), passed through the automatic cutter to fractionate and then homogenized. Albumin was used in order to remove as much fat as possible.

Thereafter, homogenate was centrifuged at 756 x *g* for 10 minutes at 4°C, in a refrigerated centrifuge; model J-21 BECKMAN (Palo Alto, California, USA). The supernatant was filtered with gauze, and this filtered was centrifuged again at 7740 x *g* for 20 minutes at 4°C to precipitate mitochondria. A mitochondria-rich pellet was obtained at this stage. The pellet was stored at 4°C in a darkness ice bath. The supernatant, containing low weight mitochondria, was centrifuged at 12100 x *g* for 20 minutes to obtain mitochondria-rich pellet that was added to the previously preserved pellet. Pellets were centrifuged at 17400 x *g* for 10 minutes. This last pellet was suspended in sucrose buffer (the same as above, but without albumin), liquated in eppendorf vials and stored at -80°C until their analysis. The supernatant, that contained the cytosolic fraction, was also preserved in eppendorf vials and stored at -80°C for further antioxidants enzyme analysis. Figure 5 shows a diagram of the process.

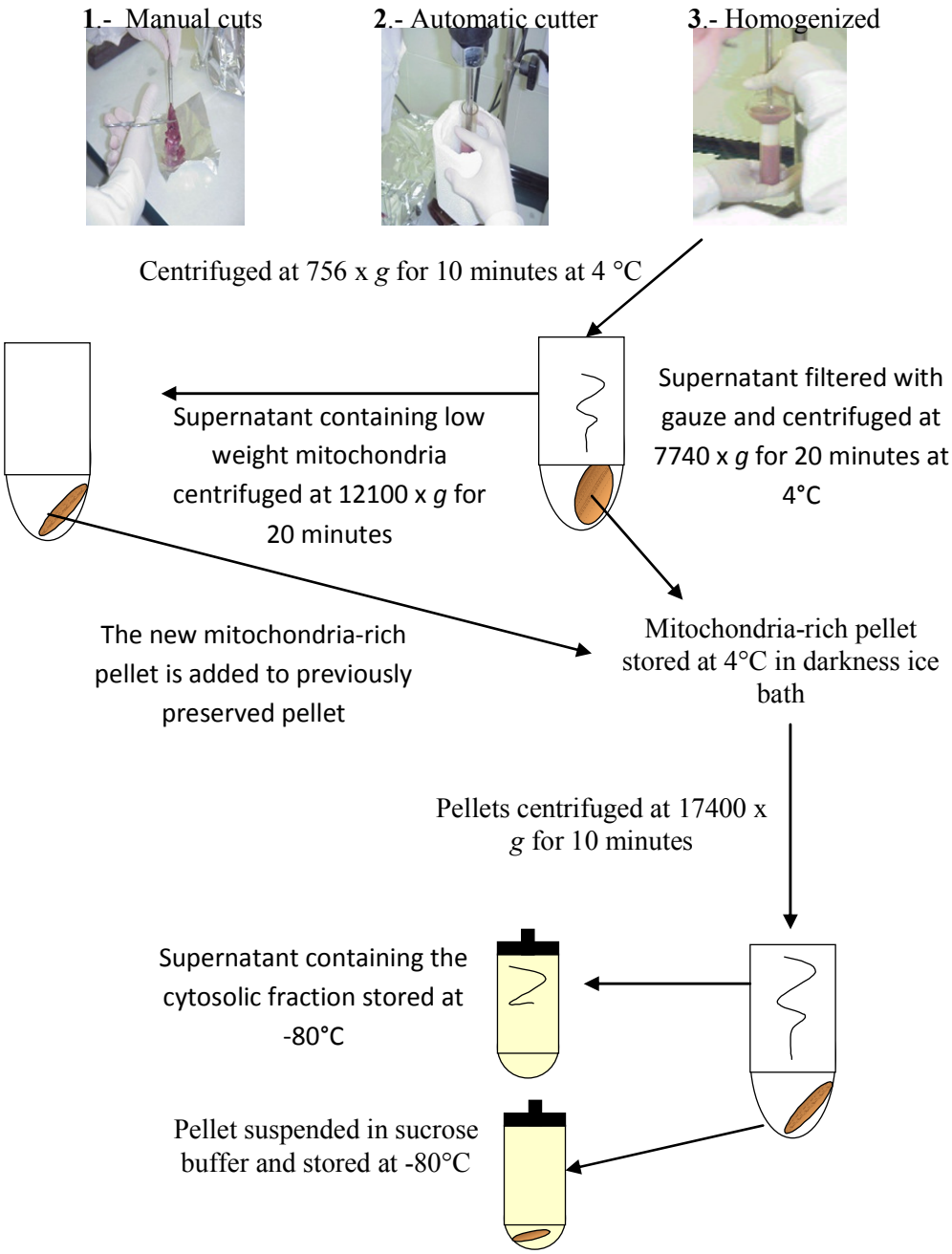


Figure 5. *Mitochondria isolation diagram.*

3. DETERMINATION OF MITOCHONDRIAL AND CYTOSOLIC PROTEIN CONCENTRATIONS IN LIVER TISSUE

Mitochondrial protein determination was performed by the Lowry assay (1951), based in two complementing reactions:

- Biuret reaction of NH_3 groups, that gives violet color.
- Folin reaction of phenolic groups with reducing OH, which gives blue color.

Original protocol was adapted to microplate reader. Both, sample and standards were prepared by triplicate. Optical density was measured by KC4 program in microplate's reader at 640nm.

Protein concentration was calculated according Lambert-Beer equation, modified for microplate reader, with albumin standard curve data.

The microplate design was as follows:

Table 10. *Microplate design for protein determination.*

	BLANK	**STD1**	**STD2**	**STD3**	**STD4**	**STD5**	**STD6**	**SAMPLE**
Water (µl)	30	30	28	25	20	15	10	20
Albumin*[1] (µl)	0	0	2	5	10	15	30	X*[2]
Albumin (µg)	0	0	2,72	6,8	13,6	20,4	40,8	?
Biuret (µl)*[3]	155	155	155	155	155	155	155	155
Cover microplate, wrap with aluminum foil and shake 1 minute at maximum speed. Incubate 15 minutes at room temperature, in darkness								
Folin (µl)*[4]	15	15	15	15	15	15	15	15
Shake 3 minutes in darkness, with the cover and aluminum foil. Incubate 20 minutes at room temperature, in darkness. Read in the microplate's reader SYNERGY HT BIO-TEK at 640nm.								

*[1] Bovine serum albumin 1,36mg/ml (Sigma) was used to prepare the standard curve. It was prepared 136mg of albumin standard with 100ml of distilled water, and kept for 1 week at 4°C.

*[2] A determinate volume of diluted sample were charged in each well. Two different samples were used, both obtained after mitochondrial isolation, so two different experiments, with a microplate in each case, was performed.

- Mitochondrial fraction: 10 µl of 1:20 diluted sample
- Cytosolic fraction: 10 µl of 1:10 diluted sample

*[3] Biuret reagent was prepared extemporaneously from the mixture of A, B1 and B2 solutions, in the ratio: 20ml A:200µlB:200µlB2.

- Solution A: sodium carbonate 2% in 0.1N NaOH 0.1N, stored in plastic container.
- Solution B1: copper sulfate pentahydrate 0,5%.
- Solution B2: sodium tartrate 1%.

*[4] Folin-Ciocalteau reagent (Sigma-Aldrich, St. Louis, Missouri, USA) was diluted at the time of use and kept in darkness.

4. TOTAL PROTEIN EXTRACTION AND DETERMINATION IN LIVER TISSUE

The total protein extraction was performed with the ReadyPrep™ protein extraction kit (Bio-Rad, Hercules, California, USA). Fifty milligrams of hepatic tissue were added to 1ml of extraction buffer, 10µl of tributylphosphine (TBP) reduction agent and 10µl of a protease inhibitor, and were homogenated with a mechanic homogenizer, formed by a Teflon pestle (Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and glass potter-Elvehjem (Sigma-Aldrich, St. Louis, Missouri, USA). The homogenate was centrifuged at 16.000 x *g* for 30 minutes at room temperature. The supernatant was transfered to a clean tube, and the pellet was discarded. Aliquots were made and stored at -80°C until its determination.

Total protein determination was performed using the RC DC protein assay kit (Bio-Rad, Hercules, California, USA).

The reagents were:

- DC Reagent S
- DC Reagent A
- DC Reagent B
- Reagent I
- Reagent II

Five microliters of DC Reagent S were added to 250µl of DC Reagent A, this solution is referred to as Reagent A'. Each standard or sample assayed required 127µl of Reagent A'. A standard curve was prepared with 3 dilutions of a protein standard from 0.2mg/ml to 1.5mg/ml. 25µl of standards and 1:10 diluted protein extraction were pipetted into microfuge tubes. One hundred and twenty five microliters of RD Reagent I were added into each tube, vortexed and incubated for 1 minute at room temperature. Then, 125µl of RC Reagent II was added into each tube, vortexed and centrifugated at 14.000 x *g* for 7 minutes. After centrifugation, supernatants were discarded and 127µl of Reagent A' were added into each tube, vortexed and incubated at room temperature for 5 minutes. The microfuge tubes were newly vortexed and 1ml of DC Reagent B were added into each tube and vortexed immediately. Microfuge tubes were incubated at room temperature for 15 minutes. After the 15 minutes incubation, 200µl of standards and samples were added into a microplate and absorbances were read at 750nm in the microplate's reader SYNERGY HT BIO-TEK.

5. FATTY-ACID PROFILE OF MITOCHONDRIAL MEMBRANES

Fatty acid analysis of biological specimens by gas-liquid chromatography requires solvent extraction, purification, and derivatization procedures that are both lengthy and heavy. Lepage and Roy proposed in 1986 a technique that avoids most of the preparative steps and consists in one step methylation and transesterification of samples.

One hundred microliters sample were added with 2 ml of a methanol:benzene (4:1) mixture and 200µl of acetyl chloride. Tubes were capped and vortexed, and they were incubated for 1 hour in a 100°C bath. After this time, the tubes were removed and allowed to cool at room temperature. Next, 2 ml of a solution of 5% potassium carbonate were added to stop the reaction and neutralize. After that, for 15 min at 2500 x *g* centrifugation in order to separate the phases was performed. The upper benzenic phase was removed with a Pasteur pipette and preserved with the lipid fraction in a separate tube.

Thereafter, tubes were evaporated in a speed vaccum system (ScanSpeed MaxiVac Alpha, Neutec Group, New York, USA) and the lipids remain in the bottom of the tube were resuspended in 50µl of hexane for analysis. A gas-liquid chromatograph model HP-5890 Series II (Hewlett Packard, Palo Alto, California, USA) equipped with a flame-ionization detector was used to analyze the fatty acids.

Chromatography was performed using a SPTM 2330 F.S column (Supelco Inc, Bellefonte, Palo Alto, California, USA), with 60-m-long, 32 mm id and 20 mm thickness. The method has a duration of 40 minutes and the following temperature ramp: initial temperature; 160°C for 5 min; 6°C/min to 195°C; 4°C/min to 220°C; 2°C/min to 230°C; hold 12 min at 230°C; and finally 14°C/min to 160°C.

Fatty acids analyzed were: C14:0, C16:0, C16:1n9, C18, C18:1n9, C18:2n6, C20:3n6, C20:4n6, C24:0, C24:1n9, C22:6n3. Post-analytical calculations were done to determinate SFA, MUFA, PUFA, n6 PUFA, n3 PUFA. The results have been expressed as a percentage of total fatty acids detected in the chromatogram. Figure 6 represents a typical chromatogram showing peaks and names of the fatty acids identified.

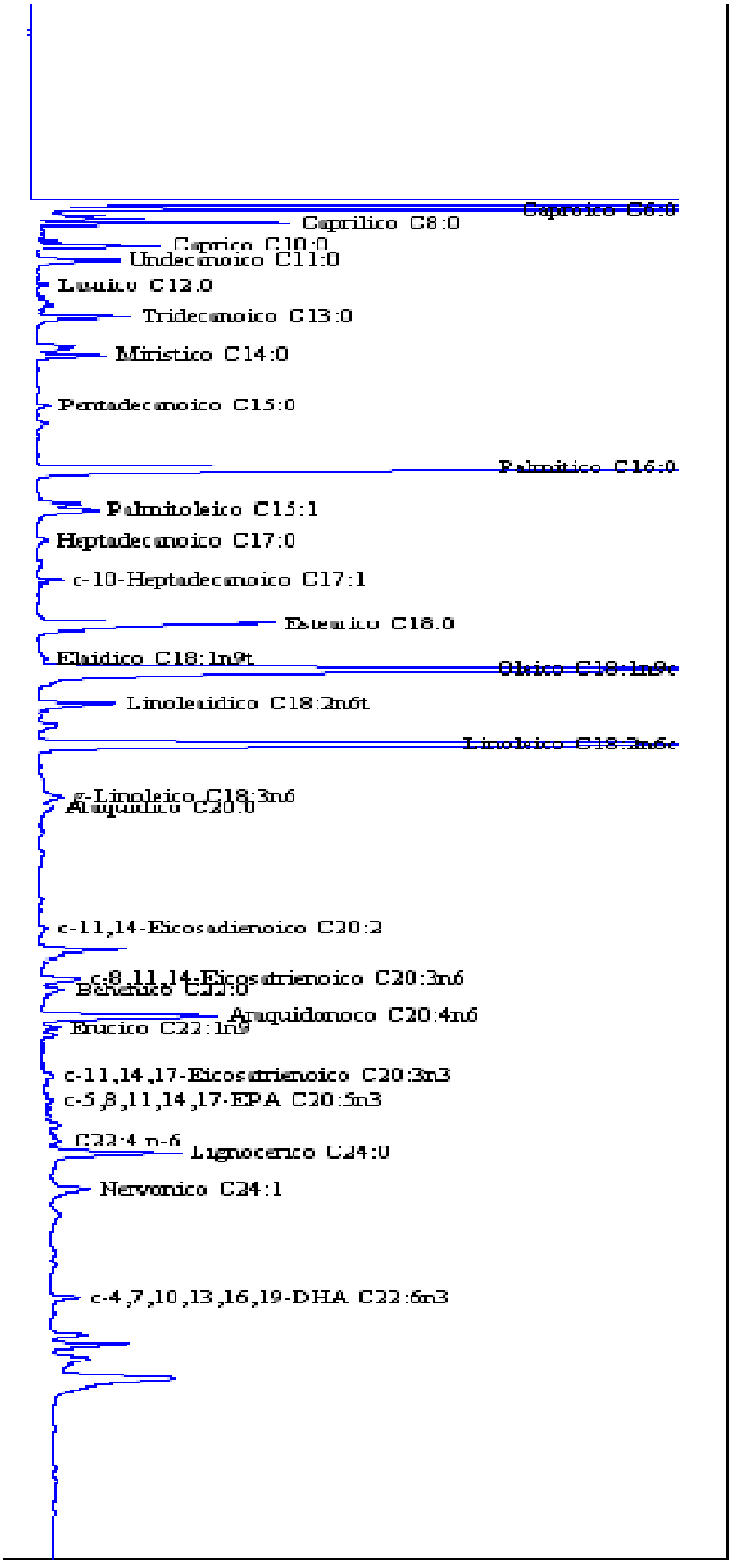


Figure 6. *Typical chromatogram showing peaks and names of the fatty acids identified.*

6. HISTOPATOLOGICAL ANALYSIS

At the time of animal sacrifice, a fragment of a liver lobe (always the same for all the animals) was included in formol 3,9 % at pH 7. After 24-48 hours fixation, each of the pieces was cuted and included in a cassette for processing in paraffin according to the conventional process, after dehydration in increasing graded alcohols to absolute ethyl alcohol.

Subsequently, cuts at 4µm were performed following by hematoxylin and eosin (H&E) staining. Gomori trichromic, periodic acid–Schiff (PAS, figure 7), Gomori's reticulin silver impregnation stains and Sirius red.

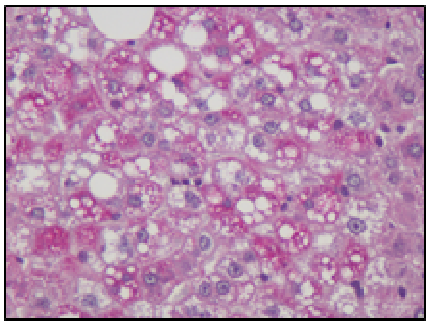


Figure 7. *Intracytolasmic PAS-negative deposits in hepatocytes. PAS, 40X.*

Histological lesions were evaluated by the grading system proposed by Yeh and Brunt (2007) focused on Non-Alcoholic Steatohepatitis (NASH) analysis and also hepatic fibrosis was studied. NASH grade was analyzed as follows:

1. Steatosis, predominantly as Macrovesicular (also microvesicular).

Figure 8 shows steatosis gradation in liver.

- Grade 0: absence of steatosis or minimum (<5%).
- Grade 1: >5-33% of observed area with steatosis.
- Grade 2: >33-66% of observed area with steatosis.
- Grade 3: >66% of observed area with steatosis.

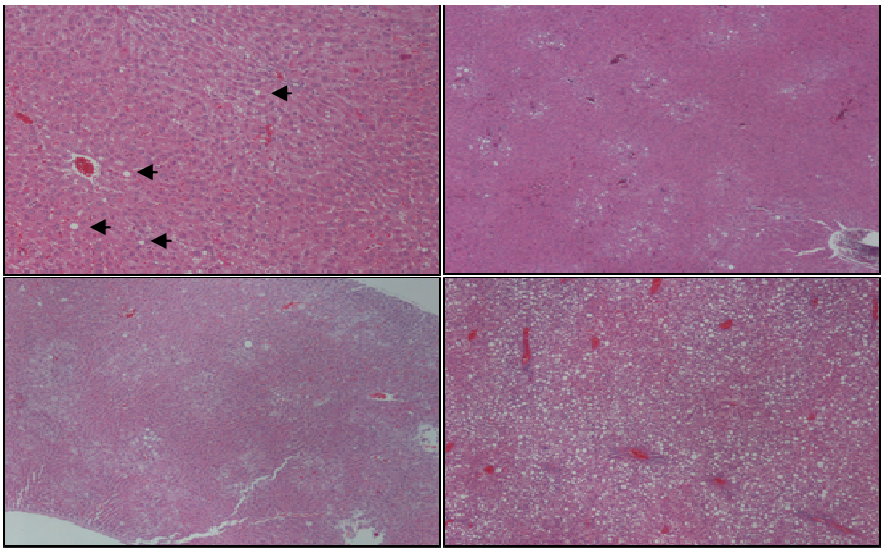


Figure 8. *Steatosis gradation. Top-Left: grade 0 = minimum macrovacuoles (arrows) H&E 10X. Top-Right: grade 1 (H&E 4X); Lower-Left: grade 2 (H&E 4X); Lower-Right: grade 3 (H&E 10X).*

2. Inflammation, evaluating the centrolobulilar inflammation.

It counts the number of inflammatory foci present in a 20X field, it samples until twenty 20X fields and it calculates the mean of these fields with the following grading system:

- Grade 0 = 0/20 C20X
- Grade 1 = 1-2/20 C20X
- Grade 2 = 2-4/20 C20X
- Grade 3 = >4/20 C20X

3. Ballooning Degeneration.

It indicates the accumulation of fluid and other toxic substances in the cytoplasm of hepatocytes causing their swelling and rarefaction of the same.

- Grade 0: absence of ballooning.
- Grade 1: minimum ballooning.
- Grade 2: prominent ballooning.

With these data, the NASH grade proposed by Brunt in 1999 was applied. Brunt *et al.* (Brunt *et al*., 1999) established a semiquantitative grading scheme for NASH based on the constellation of lesions considered important in steatohepatitis: steatosis, ballooning, and lobular and portal inflammatory infiltrates, such table 11 summarizes.

Table 11. *NASH grading.*

Grade	Steatosis	Ballooning	Inflammation
Mild (No NASH)	1-2	Minimal	L=1-2, P=0-1
Moderate (Boderline)	2-3	Present	L=1-2, P=1-2
Severe (NASH)	2-3	Marked	L=3, P=0-1

Steatosis: grade 1 ≤ 33%, grade 2 =33–66%, grade 3 > 66%; Ballooning: zonal location noted; L, lobular inflammation (0–3), 0: none, 1: < 2 foci/20X field, 2: 2–4/20X field, 3: > 4/20X field; P, portal inflammation (0–3), 0: none, 1: mild, 2: moderate, 3: marked.

Concerning liver fibrosis, Sirius red stained slides were used (Huang *et al*., 2013). The area and the percentage of tissue positive for Sirius red (collagen) was calculated for each sample in comparison with hepatic parenchyma. Ten fields for sample were analysed at a magnification of 10X. Fort the analysis, Olympus (Hamburg, Germany) *AnaliSYS Image Processing* software was used. This software has an option that allows getting the collagen percentage and area by the quantification of colour pixels in which collagen is stained. The parameters applied for all the samples were as presented in the table 12.

Table 12. *AnaliSYS Image Processing set up parameters.*

Colour	Phase 1	Phase 2
Red	0-255	130-255
Green	0-255	0-65
Blue	0-255	0-255

To eliminate nuclear stain, colour intensity caption at the picture was reduced, both for collagen as for hepatic parenchyma, by the capture of the picture with a 680x510 parameter (figure 9).

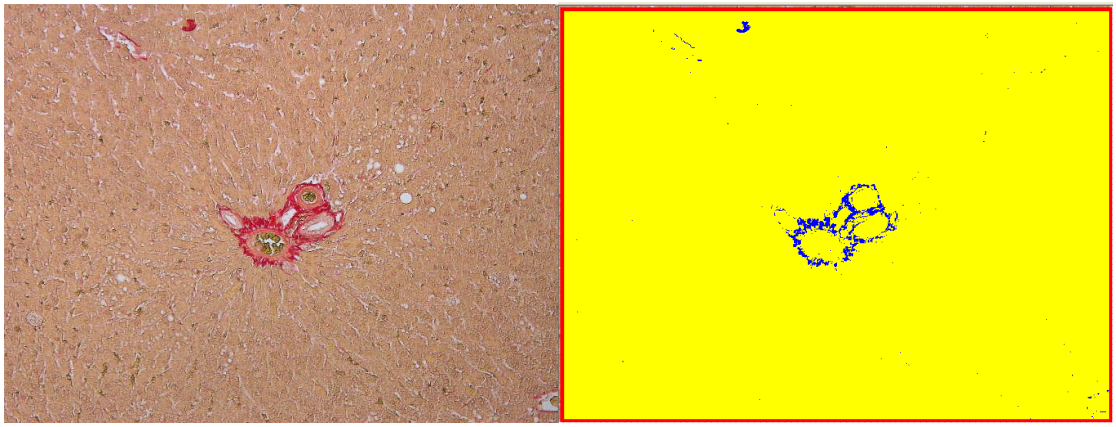


Figure 9. *Liver fibrosis. Left: Sirius Red Stain. Right: Filtered image used for AnaliSYS Image Processing software quantification*.

Collected data were as follows:

- ✓ Collagen and hepatic parenchyma area for each field.
- ✓ Collagen and hepatic parenchyma percentage for each field.

7. MITOCHONDRIAL ULTRA-STRUCTURAL ANALYSES

Immediately after liver isolation, a small piece of tissue was prefixed in 1.5% formaldehyde in 1% cacodylate buffer, pH 7.4 for 2h at 4ºC. After 3 washes in cacodylate buffer, extracts were fixed in 1% osmium tetroxide for 60min at 0-4ºC. The samples were dehydrated in graded ethanol and embedded in Epon resin. After overnight incubation at 65ºC, ultrathin sections (70nm) were cut with a diamond knife using an Ultracut S ultramicrotome and placed on 200-mesh copper grids. All sections were stained with uranyl acetate, counterstained with lead citrate, and viewed using a Carl Zeiss (Oberkochen, Germany) EM10C electron microscope at 4000X, 7500X and 40000X magnifications in the Biomedical Research Center at the University of Granada's Health Technology Park.

The ImageJ 1.46r, a public domain program from U.S. National Institutes of Health (Rasband, 2008) was used to determinate the following calculations:

- Mitochondrial area
- Mitochondrial perimeter
- Mitochondrial density
- Autophagosome density

Figure 10 shows a mitochondrial ultra-structural analysis example.

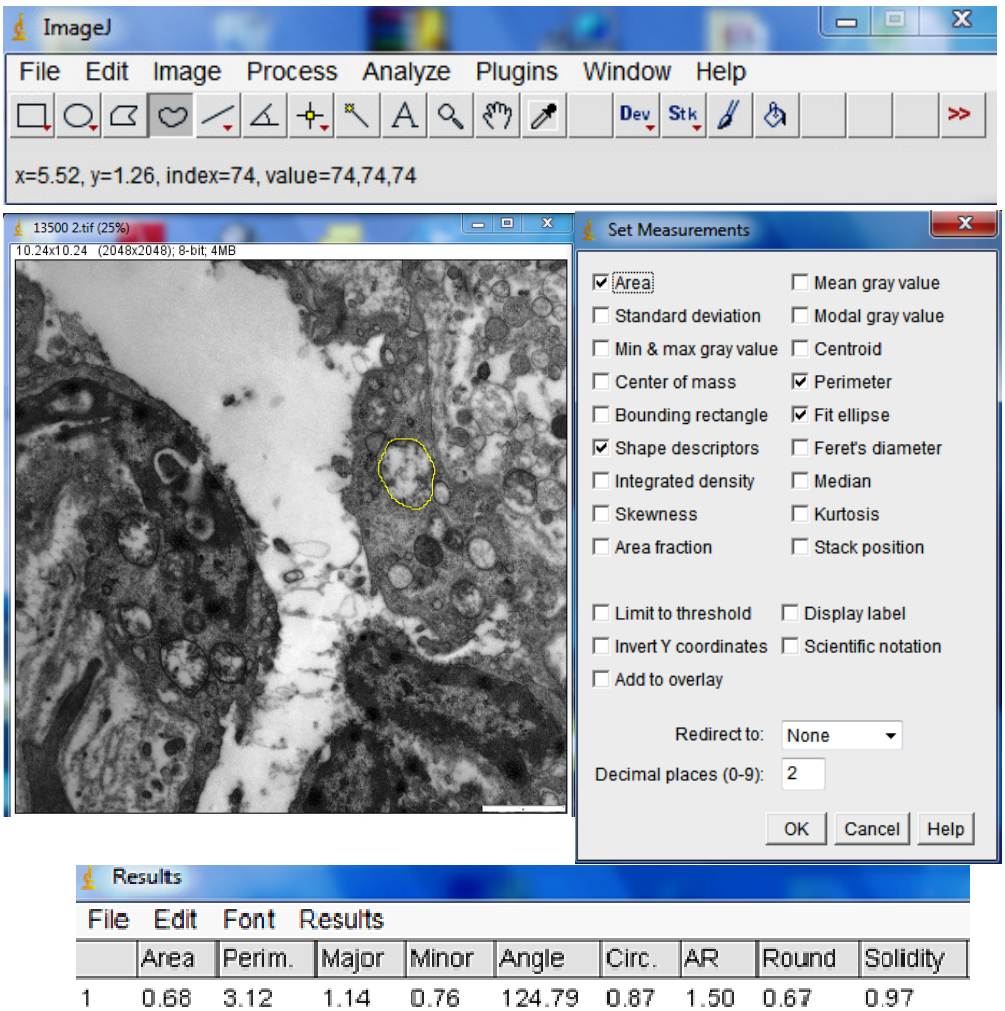


Figure 10. *Mitochondrial ultra-structural analysis example.*

8. WESTERN BLOT ANALYSIS OF SELECTED PROTEINS

Western blot assay is a widely accepted analytical technique used to detect specific proteins in the given sample of tissue homogenate or extract. It uses acrylamide gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a polyvinylidene fluoride membrane (PVDF), where they are stained with antibodies specific to the target protein (primary antibodies). Secondary antibody horseradish peroxidase-linked binds primary antibody and reacts with chemiluminescence reagent to give a detectable light reaction by a luminescent image analyzer.

Samples containing 20µg of liver tissue protein, extracted and determinated as described in section 4, were subjected to electrophoretic fractionation on a 4 to 12% Bis–Tris Criterion XT Precast gel (Bio-Rad, Hercules, California, USA). Separated fractions were transferred onto a polyvinylidene difluoride 0.2 µm membrane (Bio-Rad Laboratories, Hercules, California, USA) by using the trans-blot whet electrophoretic transfer cell (Bio-Rad, Hercules, California, USA) (Figure 11).

Figure 11. *Bio-Rad Laboratories Western Blot equipment.*

Membranes were blocked with TBS-T containing 5% nonfat milk and incubated with primary monoclonal antibodies against PGC1α and TFAM (Santa Cruz and Abcam) (Table 13), under appropriate dilutions and incubation times. For all membranes and antibodies, incubation with β-Actin (from Abcam, Cambridge, UK) was performed, as protein loading control.

Table 13. *Primary monoclonal antibodies dilutions and incubation times.*

Antibody	Manufacturer	Type	Dilution	Incubation time
Mitochondrial transcription factor A (TFAM)	Santa Cruz Biotechnology®	Mouse monoclonal	1:200	overnight
Anti-Peroxisome Proliferator-Activated Receptor-γ Coactivator 1α (PGC1α)	Calbiochem®	Mouse monoclonal	1:1000	overnight
Anti-beta Actin antibody (β-Actin)	Abcam®	Mouse monoclonal	1:500	overnight

Following incubation with the antibodies, the membranes were washed three times for 5 min in TBS-T and incubated with the specific horseradish peroxidase conjugated secondary antibodies Bio-Rad (Hercules, California, USA) or Santa Cruz Biotechnology (Santa Cruz, California, USA). After washing two times for 5 min with Tris-buffered saline and tween 20 (TBS-T) and one time with Tris buffered saline (TBS), specific proteins were detected using a chemiluminescence reagent Immun-Star™ HRP Chemiluminescence Kits Bio-Rad (Hercules, California, USA). Signal quantification and recording was performed with a Luminescent Image Analyzer LAS-4000 mini (FUJIFILM corp. Tokio, Japan) (Figure 12). Coomasie brilliant blue and Ponceau S (Sigma-Aldrich, St. Louis, Missouri, USA) staining were used for markers of the protein loading in the gel and protein transfer control in the PVDF membrane, respectively.

Figure 12. *Luminescent Image Analyzer LAS-4000 mini.*

9. PROTEIN CARBONYLS ASSAY

Oxidative stress and ROS lead protein oxidation producing carbonyl groups (aldehydes and ketones) on protein side chains (especially of proline, arginine, lysine and threonine) when they are oxidized. The usage of protein carbonyl groups as biomarkers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins.

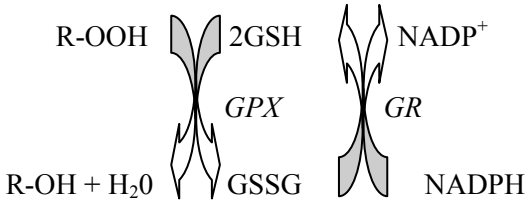
The levels of protein carbonyl groups in liver were assessed using Protein Carbonyl Kit (Cayman Chemical Company, Michigan, USA), which is a convenient colorimetric assay for the measurement of oxidized proteins. Protein samples are derivatized by making use of the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. Formation of a Schiff base produces the corresponding hydrazone which can be analyzed spectrophotometrically at 360-385 nm

Briefly, 100µl of liver protein extract were transferred to two tubes. One tube was the sample tube and the other was the control tube. After adding 400µl of DNPH to the sample tube and 400µl of 2.5 M HCl to the control tube, both of them were incubated in the dark at room temperature for 1 h, stirring them shortly every 15 minutes. Afterwards, 0.5 ml of 20% trichloroacetic acid was added to each tube and incubated in ice for 5 min. This mixture was centrifuged at 10.000 x *g* for 10 min at +4ºC, obtaining a pellet that was resuspended in 0.5 ml of 10% trichloroacetic acid and incubated in ice for 5 min and again centrifuged at 10.000 x *g* for 10 min at +4ºC. The pellet obtained was resuspended in 0.5 ml of (1:1) ethanol/ethyl acetate mixture and centrifuged at 10.000 x *g* for 10 min at +4ºC twice. Finally, the pellet obtained was resuspended in 250µl of guanidine hydrochloride and centrifuged at 10.000 x *g* for 10 min at +4ºC, obtaining a supernatant of which 220µl were transferred to a 96-well plate and absorbance read in a microplate's reader SYNERGY HT BIO-TEK at 370 nm. Total protein concentration in liver samples extracts was measured using Pierce® BCA Protein Assay (Thermo scientific, Rockford, Illinois, USA) following the manufactures recommendations.

The results were expressed as nmol of carbonyl proteins per mg of total proteins in the extract.

10. GLUTATHIONE PEROXIDASE ACTIVITY MEASUREMENT (TOTAL GPX)

GPX is a selenoprotein that catalyzes the reduction of a large number of hydroperoxides from H_2O_2 to organic hydroperoxides. It was determined according to Flohé and Günzler (1984), adapting original method to microplate reader performance. That method is based on the instantaneous formation of oxidized glutathione during the reaction catalyzed by GPX. Oxidized glutathione (GSSG) is continually reduced by an excess of glutathione reductase (GR) and NADPH present in the microplate's well. The subsequent oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm, considering an indicator of GPX activity.



Here it was monitorized the NADPH-dependent enzymatic decrease. Oxidation associated to NADPH was monitorized by spectrophotometric measures at short intervals, obtaining an enzymatic spectrum for each of the samples and using a Tert-butil hydroperoxide solution as source of peroxides.

Two blanks are needed:

- Blank 1: sample without Tert-butil hydroperoxide, which removes independent NADPH absorbance decrease.
- Blank 2: without sample neither Tert-butil, which removes possible no enzymatic decrease.

The reagents were:

- Working buffer: 50mM Tris-ClH pH 7,6 + 5mM EDTA.
- GSH 3,2mM diluted in buffer or distilled water.
- NADPH 0,64mM diluted in 0,1% $NaHCO_3$.
- GR.
- Tert-butil hydroperoxide 0,78mM, diluted in distilled water.

To prepare the reaction mixture, GSH and NADPH were added in 20ml of working buffer. Then, 5 ml of this mixture were added with 13,3 µl of glutathione reductase to obtain 1,6 U/ml.

The reactive mixture was prepared in the same day, and stored covered at 4°C in darkness. Measures were done at a constant temperature of 25°C, incubating solutions in a digital dry bath.

In a microplate, 75µl of working buffer, 75µl of reactive mixture and 15µl of sample (diluted in working buffer to obtain a concentration of 30µg of protein per sample) were mixed. Then 75µl of Tert-butil were added, quickly shaked and the measure at the microplate's reader SYNERGY HT BIO-TEK started. Reading were done for 3-5 minutes at 340nm, discarding the first 15 seconds.

Results are expressed in units per milligram (U/mg) of protein. A unit of GPX is defined as the activity that oxide 1 µmol of NADPH, and it is calculated according the equation:

$$U/mg = (\Delta \log [GSH]/t \times (\Delta[NADPH]/ [GSH]_0 \times t)) \times (V_i/ V_f)$$

11. CYTOSOLIC CATALASE K ACTIVITY

Cytosolic catalase activity in liver was determined following the method described by Aebi (1984), based on monitoring at 240nm the H_2O_2 decomposition, as a consequence of the catalytic activity of catalase.

As described by Aebi (1984) catalase has double function:

1. Decomposition from H_2O_2 to O_2 (catalytic activity). Catalase forms an active complex with H_2O_2, working as a proton donor.

2. Oxidation of hydrogen donors (for example methanol, ethanol, formic acid and phenols), consuming 1 mol of peroxide (peroxide activity).

The predominant activity will depend on the concentration of protons donors and H_2O_2 formation rate, and the first order reaction rate will depend on peroxide concentration; therefore peroxidation reactions are slower than catalytic reactions.

In the ultraviolet range, H_2O_2 exhibits continuous absorption increase when wavelength is decreasing. H_2O_2 decomposition can be directly monitored by absorbance decreasing at 240nm. Catalase activity is provided by absorbance difference per time unit. This activity depends on system temperature; accordingly, all tests must be carried out between 0 and 37°C, at pH 7.0.

Used reagents were:

- Working reagent: phosphate buffer 50mM, pH 7.0, stored at 4°C for 5 days max.
- Working reagent with Triton X-100, stored at 4°C for 5 days max. Used for sample dilution to 0.5mg/ml protein concentration. A higher concentration of protein may interfere with catalase and decrease its function.
- H_2O_2 solution, 30mM, prepared in working reagent 15-30 minutes before make the measurement, and stored in ice.

In tissues with a high catalase activity, as liver, this activity can be determined by spectrophotometry following the method described by Cohen *et al*. (1970) to tissue extracts, adapted to measure optical density in a microplate's reader SYNERGY HT BIO-TEK.

Final volume per well was 225µl, containing 125µl of phosphate buffer, 25µl of sample (diluted previously in phosphate buffer with Triton until the protein concentration indicated above) and 75µl of H_2O_2 solution. Absorbance at 240nm was measured during one minute in a speed reading.

It is not possible to define the catalase international unit (IU) due to its abnormal kinetic. Instead, it is recommendable to use the rate constant (*k*) of a first order constant reaction, because with a very short reaction times and relatively high enzyme concentrations the decrease of the H_2O_2 concentration with time due to the action of catalase corresponds to a first order reaction.

$$K\ (sec^{-1}) = (1/\Delta t)\ (\ln S_1/S_2) = (2.3/\Delta t)\ (\log A_1/A_2)$$

In which $\Delta t = t_2 - t_1$ is the time interval, and S_1 y S_2 is the H_2O_2 concentration at t_1 and t_2 time.

The rate constant (*k*) related to the protein content (*k*/mg protein) can serve as a measure of the specific activity of catalase.

12. COENZYME Q_9, Q_{10} AND α-TOCOPHEROL DETERMINATION

Coenzyme Q_9 (CoQ_9), reduced Coenzyme Q_9 (CoQ_9H_2), CoQ_{10} and reduced Coenzyme Q_{10} ($CoQ_{10}H_2$) and α-tocopherol were assayed by high-performance liquid chromatography (HPLC) combined with electrochemical detection, following Battino *et al*. (2004). This method was modified to include a novel system requiring no preanalytical extraction of the sample, a very limited handling of the samples and a direct injection into the HPLC apparatus. Altogether, these modifications, allow for the first time, a systematic and simultaneous determination of these very sensitive and unstable compounds, especially in their reduced forms, that displays the highest antioxidant potentiality, in rat liver mitochondria.

The HPLC system consisted of a Beckman System Gold 125 pump (Beckman Instruments, Fullerton, California, USA), a Water 717 plus autosampler (Milford, Massachusetts, USA), and a stainless-steel column (15cm long and 4.6mm I.D.) packed with 3mm ODS Supelcosil. An ESA Coulochem III electrochemical detector, a model 5020 guard cell and a model 5011 analytical cell were also used. Chromatograms were integrated using the System Gold Beckman system.

The mobile phase consisted of 20mM lithium perchlorate, 10mM perchloric acid, 20% ethanol, 80% methanol at 1 ml/min; electrode 1 was set at 20.5V, electrode 2 was set at 0.35V. Briefly, 20µl of the sample were precipitated with 200 µl of 1-propanol (1:10 ratio), and vortexed for 60 s. After centrifugation at 11,200 x *g* for 5 min at 4°C in a bench-top centrifuge for Eppendorf vials, 3 µl of supernatant were injected into the HPLC.

Oxidized CoQ_9, CoQ_9H_2, oxidized CoQ_{10}, $CoQ_{10}H_2$ and α-Tocopherol were identified by predetermining the retention times of individual standards. Retention times were around 3.3min for α-Tocopherol, 7.5min for CoQ_9H_2, and 9.7min for $CoQ_{10}H_2$, 12min for CoQ_9 and 17.9min for CoQ_{10}. Figure 13 shows a HPLC chromatogram example.

Post-analytical calculations were done to determinate Total CoQ_9, Total oxidized CoQ_9, Total CoQ_9H_2 , Total CoQ_{10}, Total oxidized CoQ_{10}, Total $CoQ_{10}H_2$, Total summatory of CoQ_9+Q_{10}, oxidized CoQ_9:CoQ_9H_2 ratio, oxidized CoQ_{10}:$CoQ_{10}H_2$ ratio, Total oxidized CoQ: Total $CoQH_2$ ratio, CoQ_9H_2:$CoQ_{10}H_2$ ratio, oxidized CoQ_{10}:oxidized CoQ_9 ratio and Total CoQ_9:Total CoQ_{10} ratio.

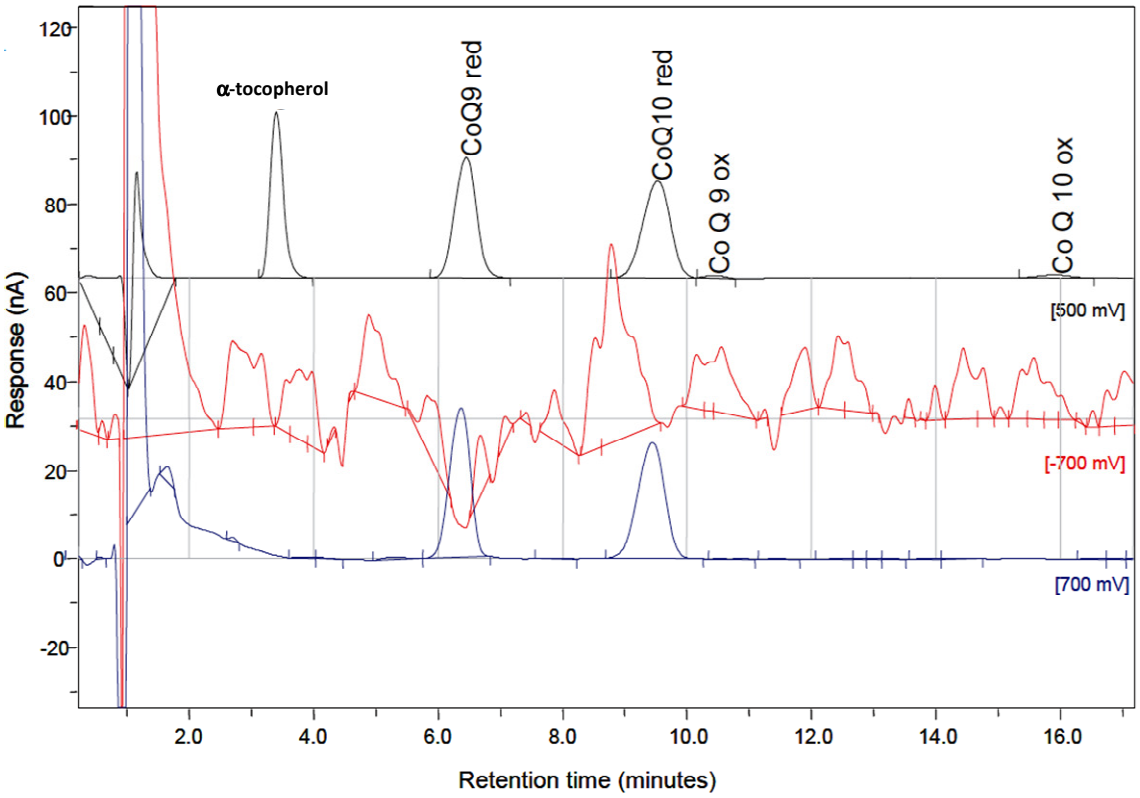


Figure 13. *HPLC chromatogram example showing retention times of individual standards and Coenzymes Q analyzed.*

13. LIVER MITOCHONDRIAL RESPIRATORY EFFICIENCY

Measurement of the oxygen consumption rate (OCR) of mitochondria is valuable, since electron transport and oxidative phosphorylation consume oxygen, and reflect the mitochondrial and nuclear genomes' expression of functional components of the electron transport chain. OCR in liver mitochondria was measured with a XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, Massachusetts, USA) (Figure 14). XF24 Analyzer has developed a high throughput assay in which both energy demand and substrate availability can be tightly controlled for mechanistic studies with isolated mitochondria. The optimized conditions enable sensitive measurement of the rates of OCR and proton extrusion or extracellular acidification, by isolated mitochondria attached to the bottom of an XF Cell Culture Microplate. This approach is uniquely capable of analyzing small quantities of sample (1-10 µg of mitochondrial protein per well). Sequential measurement of Basal, State 3, State 4, and uncoupler-stimulated respiration or any other combination can be made through additions of reagents from the drug injection ports of the XF assay cartridge.

Figure 14. *XF24 Extracellular Flux.*

Here, we were interested in Electron Flow Assay, which examines sequential electron flow through different complexes of the electron transport chain which can identify the mechanism of mitochondrial dysfunction or modulation. To minimize variability between wells, mitochondrial suspension were first diluted 10x in cold MAS buffer added with 10mM pyruvate, 2mM malate and 4µM FCCP to achieve the final concentration of 10µg of mitochondria in 50µl of MAS solution. Then, 50µl of the mitochondria diluted solution were delivered to each well (except for background correction wells) in a XF24 cell culture microplate (Seahorse Bioscience) while the microplate was on ice. The microplate was then transferred to a centrifuge equipped with a swinging bucket microplate adaptor, and spun at 2000 x *g* for 20 minutes at 4°C. After centrifugation, 450µl of MAS + substrate was added to each well. The mitochondria were then placed at 37°C for 10 minutes in the XF Prep Station incubator (Seahorse Bioscience, Billerica, Massachusetts, USA) (Figure 15).

Figure 15. *XF Prep Station incubator.*

Working solutions:

1. Mitochondrial assay solution (MAS, 1X): 70 mM sucrose (12 g/0.5L), 220 mM mannitol (20 g /0.5L), 10 mM KH_2PO_4 (0.68 g/0.5L), 5 mM $MgCl_2$ (0.23 g/0.5L), 2 mM HEPES (0.23 g/L), 1 mM EGTA (0.18 g/0.5 L and 0.2% (w/v) fatty acid-free BSA, pH 7.2 at 37°C. 2X stock MAS is needed for dilution of substrates, ADP and respiration reagents.

2. ADP & Substrate Stocks: 0.5 M succinate, 0.5 M malate, 0.5 M glutamate, 0.5 M pyruvate, and 1 M ADP in H20 adjusted to pH 7.2 with potassium hydroxide. Pyruvate should be made fresh the day of the assay and pH adjusted to 7.0 - 7.2 with KOH.

3. Respiration Reagent Stocks: 10 mM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), 2 mM rotenone, 5 mg/ml oligomycin and 40 mM antimycin A in 95% ethanol. Do not use 100% ethanol as it contains traces of benzene which is detrimental to mitochondrial function. 1.0 M ascorbate in H_2O, pH 7.2. 10 mM N1,N1,N1,N1-tetramethyl-1,4-phenylene diamine (TMPD) in H_20, pH 7.2, mix with an equimolar concentration (10 mM) ascorbate to ensure TMPD remains reduced.

All reagents were stored at -20°C, except pyruvate which was prepared fresh on the day of each experiment.

The plate was transferred to the XF24 instrument and the experiment initiated. Changes to the concentrations of dissolved oxygen in the media were measured for each 2 second intervals by solid state sensor probes. After determining the basal mitochondrial respiration, rotenone (2 µM), succinate (10mM), antimycin A (4 µM) and ascorbate plus 1mM TMPD (10 mM and 100 µM) were sequentially added to each well (figure 16).

- Port A, 50 µl of 20 µM rotenone (2 µM final)
- Port B, 55 µl of 100 mM succinate (10 mM final)
- Port C, 60 µl of 40 µM antimycin A (4 µM final)
- Port D, 65 µl of 100 mM ascorbate plus 1 mM TMPD (10 mM and 100 µM final, respectively)

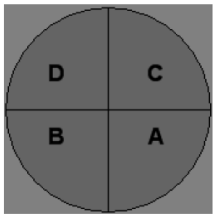


Figure 16. *Well ports.*

XF software analysed data, presented as the average of 3 replicates per wells ± SE. Results are expressed as OCR in picomoles of consumed oxygen per minute and were adjusted for protein concentration (figure 17).

The general work flow for this experiment is illustrated in Figure 18.

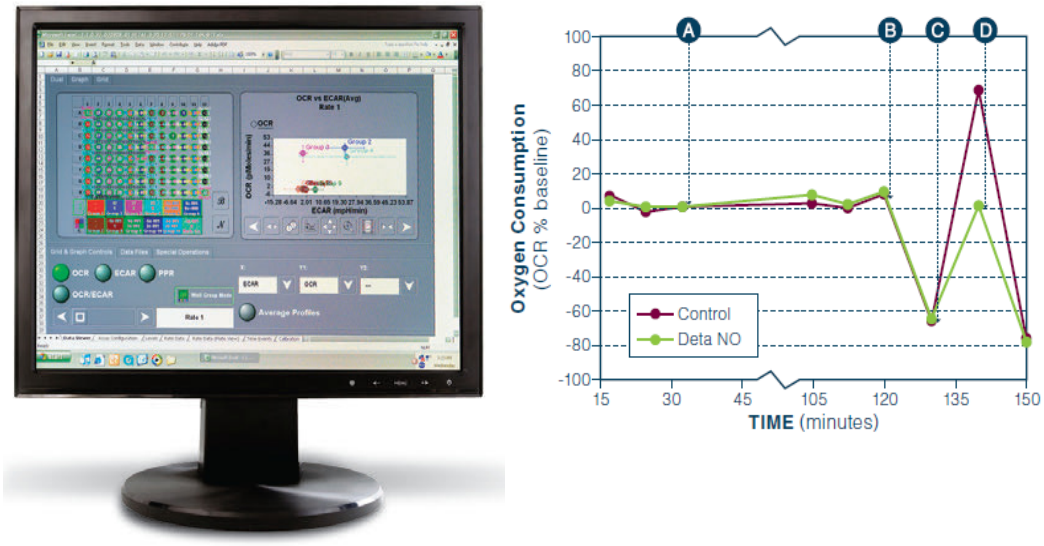


Figure 17. *XF24 Software.*

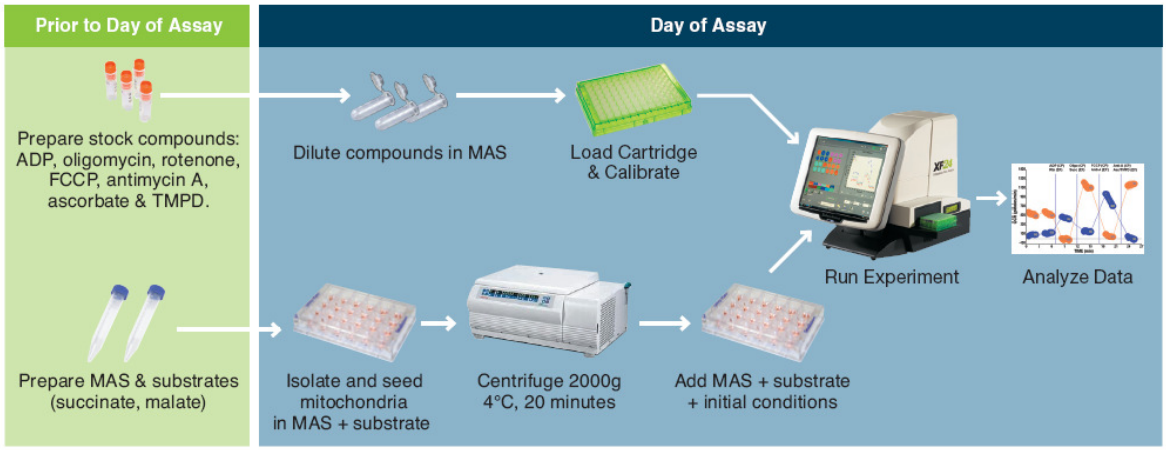


Figure 18. *General work flow for XF24 Extracellular Flux.*

14. MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEX I ACTIVITY

Mitochondrial electron transport chain enzyme activity was studied by enzyme activity immunocapture assay. This enzyme activity assay applies a novel approach, whereby target enzymes are first immunocaptured from tissue samples before subsequent functional analysis. Dipstick ELISA Kit extend this concept by utilizing the well-established lateral flow concept, wherein capture antibodies are striped onto nitrocellulose membrane and a wicking pad draws the sample through the antibody bands.

Complex I Enzyme Activity Dipstick Assay Kit was acquired from Mitosciences (Eugene, Oregon, USA). Figure 19 shows a complex I dipstick.

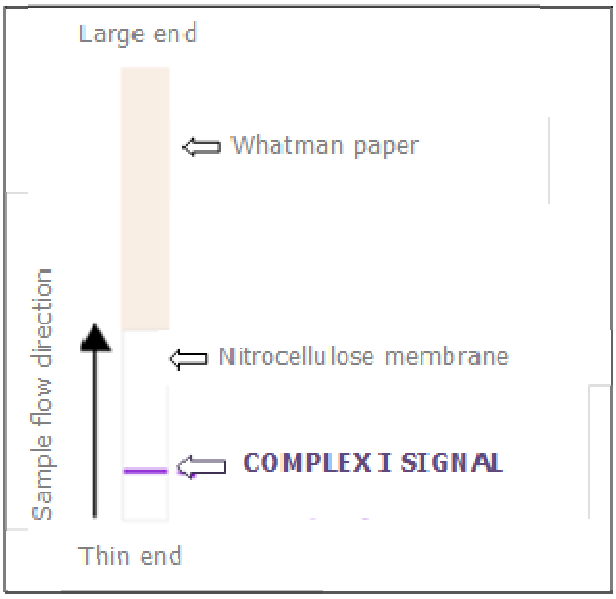


Figure 19. *Complex I dipstick.*

Complex I activity dipsticks contain a zone of anticomplex I mAb 18G12BC2 striped at 1mg/ml. Dipstick assays were performed by inserting individual dipsticks into 50µl samples. The entire sample was then allowed to pick up laterally through the membrane, passing through the zone where the target enzymes are immunocaptured and concentrated (approximately 15 min). The dipsticks were then cleared by allowing 30µl of wash buffer (50mM Tris–Cl, 150mM NaCl, pH 7.4) to pick up through the dipstick (approximately 10 min). The dipsticks were then transferred into the appropriate enzyme substrate buffer (described below) and enzyme activity calculated by measuring the optical density of precipitating, colorimetric enzyme reaction products. Figure 20 shows the dipstick assay.

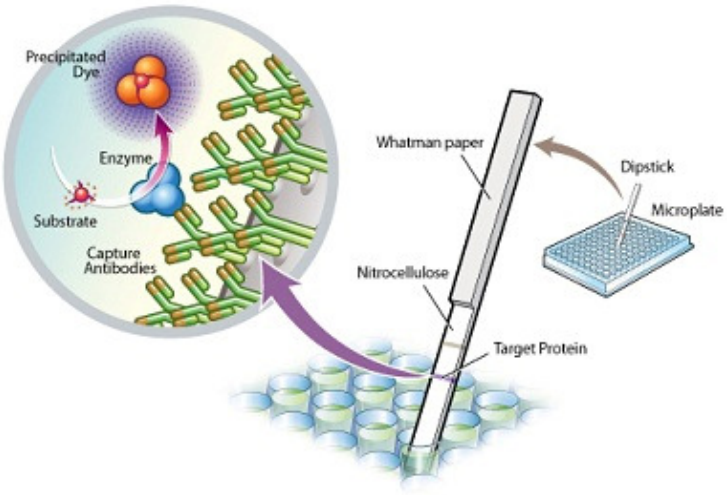


Figure 20. *Dipstick assay.*

A Hamamatsu MS-1000 immunochromatographic dipsticks reader was used for densitometry (Figure 21).

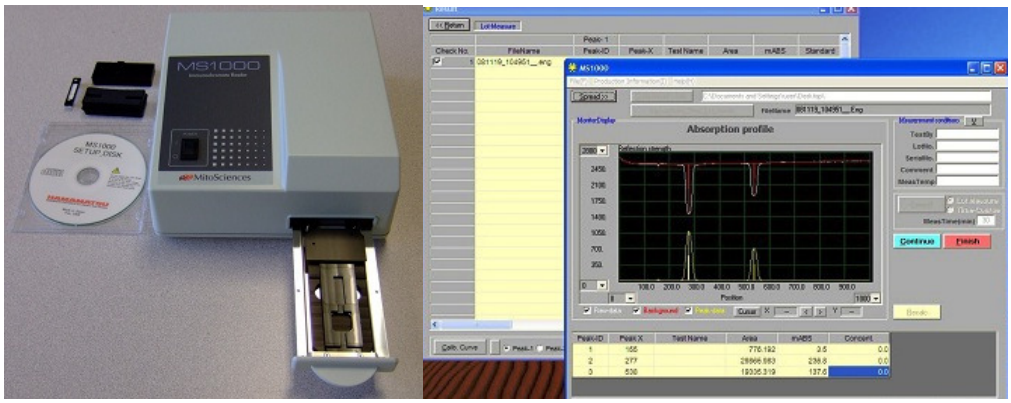


Figure 21. *Dipsticks reader and software.*

Complex I activity was revealed and measured by immersing each complex I activity dipstick in 500µl of complex I reaction buffer (20 mM Tris–Cl, pH 7.4 containing 0.1mg/ml NADH (Sigma) and 0.3mg/ml Nitrotetrazolium blue (NBT, BioRad) for 40 min, stopping the reaction by immersing the dipsticks in 300 µl of distilled water for 10 minutes and then measuring the amount of intensely colored, reduced NBT (NTBH), which precipitates at the site of immunocapture of functional complex I (figure 22).

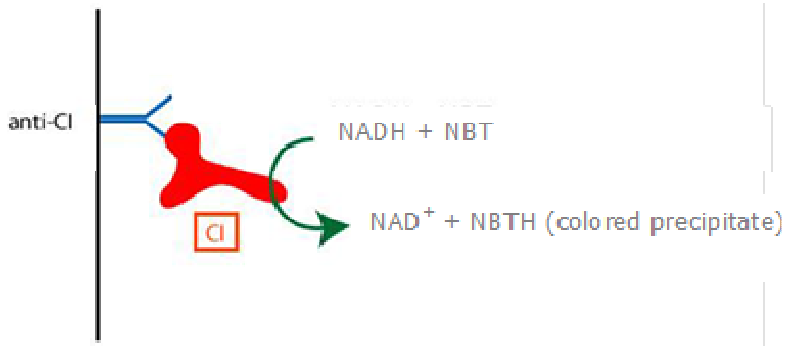


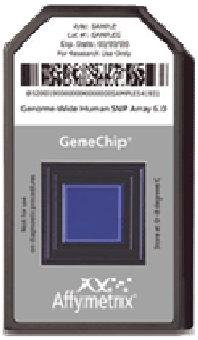
Figure 22. *Complex I reaction.*

15. DNA MICROARRAY ANALYSIS

15.1. INTRODUCTION

The gene expression analysis was performed using GeneChip® by Affymetrix® technology at Progenika Biopharma S.A. (Derio, Vizcaya, Spain). Both, the sample processing such as hybridization, revealed chip scanning and analysis of the results has been done following the protocols and equipment recommended officially by Affymetrix®. (Santa Clara, California, USA). The used chip was *RAE230_2.0* from Affymetrix®, with 31099 probes. Figure 23 shows an example of Affymetrix® hybridization chip.

For scanning of the chips it was used the GeneChip® Scanner 3000 Targeted Genotyping System (Affymetrix®, Santa Clara, California, USA), shown in Figure 24. The software employed to processing of chips and results has been Gene Chip Operating Software (GCOS 1.4, Affymetrix®). For bioinformatics analysis, several programs have been used: Partek Genomics Suite (Partek Inc., Saint Louis, Missouri, USA) and dChip (Harvard School of Public Health).



Three samples from each group of six animals were chosen to perform the microarray assay (a total of 18 samples of rat liver). Table 14 describes how each sample was named.

Figure 23. *Example of Affymetrix® hybridization chip.*

Figure 24. *GeneChip® Scanner 3000 Targeted Genotyping System.*

Table 14. *Description of the conditions of each group of samples.*

GROUP	AGE	OIL	SAMPLES
1	Young	Virgin olive	07SE930, 07SE936, 07SE942
2	Young	Sunflower	07SE931, 07SE937, 07SE943
3	Young	Fish	07SE932, 07SE938, 07SE944
7	Old	Virgin olive	07SE948, 07SE954, 07SE960
8	Old	Sunflower	07SE949, 07SE955, 07SE961
9	Old	Fish	07SE950, 07SE956, 07SE962

15.2. RNA EXTRACTION

15.2.1. Samples processing

The hepatic tissue was pulverized in a homogenizer in the presence of liquid nitrogen. Total RNA was extracted with RNeasy Mini kit (Qiagen, Düsseldorf, Germany) following manufacturer recommendations.

15.2.2. Measuring the concentration and purity of RNA

The concentration of RNA extracted was determined by spectrophotometrical assay that measures sample absorbance at 260nm (A260). In this case, it was used the Thermo Scientific Nanodrop Spectrophotometer ND-1000. (Thermo Fisher Scientific, Waltham, Massachusetts, USA) (Figure 25). A beam of light passes through 1µl of sample and measures absorbance at 260nm and recalculates RNA concentration (ng/ml).

Figure 25. *Nanodrop Spectrophotometer ND-1000.*

To determinate purity of RNA extracted, absorbance at 280nm (A280) it was also measured to calculate the ratio A260/A280. Values between 1.9 and 2.1 indicate a good purity, but a smaller value can indicate protein contamination.

15.2.3. RNA integrity

Once quantified, we proceeded to the analysis of RNA quality. Traditionally it is carried out a horizontal electrophoresis on 0.8% agarose gel to verify that the intensity ratio between the bands corresponding to the 28S and 18S ribosomal subunits was 2:1. This process is now optimized using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) (Figure 26), which provides sizing, quantitation and quality control of DNA, RNA,

proteins and cells on a single platform, providing high quality digital data with minimum expenditure sample (4µl).

Figure 26. *Agilent 2100 Bioanalyzer.*

Chips and reagents to RNA integrity analysis (Figure 27) were acquired to Agilent and were used according to manufacturer recommendations:

- RNA Nano Chip Ref. 5067.1511.

- Agilent RNA 6000 Nano Reagents Part I Ref. 5067.1511.

Figure 27. *Agilent RNA Nano Chips and Reagents.*

The procedure is as follows:

1. Gel preparation: pipette 550µl of RNA 6000 Nano gel matrix within a spin filter; centrifuge at 1500 x *g* ± 20% for 10 minutes at room temperature and aliquot 65µl of gel filtration in 0.5 tubes RNase-free. Use gels within 4 weeks.

2. Mix gel-dye preparation: equilibrate the RNA 6000 Nano dye concentrate at room temperature for 30 minutes; vortex for 10 seconds; add 1µl dye in an aliquot of filtered gel; vortex again, and shake by 13000 x *g* for 10 minutes at room temperature. Use the mixture on the same day.

3. Gel-loading dye mix: place a new chip in the station; pipette 9µl gel-dye mix in the apropiate well; ensure that the plunger is set to 1 ml and then close the station; push the plunger until it is subject to the clip; wait exactly 30 seconds and raise the clip; wait exactly five

seconds. Slowly back the plunger to 1ml position. Open the station and pipette 9µl of gel-dye mix in the **G** wells. Discard any leftover mix.

4. Loading the Agilent RNA 6000 Nano Marker: pipette Marker in all 12 wells and the well indicated.

5. Loading the Ladder and samples: pipette 1µl of ladder prepared on indicated well; pipette 1µl of sample in each of the 12 wells and 1µl of Marker in each well without using. Vortex the chip horizontally for 1 minute at 2400 rpm. Finally, run the chip in the Agilent 2100 Bioanalyzer in 5 minutes.

The computer software results in an estimate of the extract RNA degradation represented by the parameter value RNA Integrity Number (RIN). RIN values above 7 were considered as optimums to our study.

15.3. BIOTINYLATED cRNA SYNTHESIS

The target molecule from gene expression study is mRNA. Total RNA extracted from hepatic tissue has a mix with different RNA types, so it's necessary to purify mRNA by reverse transcription from RNA to cDNA, and then to cRNA, which will be biotin-labeled.

15.3.1. cDNA synthesis

cDNA was synthetized from 1µg of RNA with One-Cycle cDNA Synthesis kit (Affymetrix®, Santa Clara, California, USA), adding, for each sample, 2µl poly-A RNA, 2µl of primers T7 oligo-dT (deoxy-thymine nucleotides) and RNase-free water until final volume of 11µl. The mix was incubated for 10 minutes at a 70ºC, and thereafter, 2 minutes at 4ºC. Then were added 7µl of First-Strand Master Mix and the mix was incubated at 42ºC for 2 minutes. When incubation finished, it added 1µl of SuperScript II enzyme to each sample and were incubated again at 42ºC for 1 hour, and the at 4ºC for 2 minutes. In the next step, it added to each sample 130µl of Second-Strand master Mix and was incubated for 2 hours at 16ºC. Finally, 2µl of DNA-Polymerase T4 were added and incubated at 16ºC for 5 minutes, after which it added 10µl of ethylenediaminetetraacetic acid (EDTA) to finish the reaction.

15.3.2. cRNA synthesis and biotin labeling

This process was carried out using the GeneChip® IVT Labelling Kit (Affymetrix®, Santa Clara, California, USA), adding to each cDNA sample 4µl of 10X binding buffer, 12µl of nucleoside triphosphate (NTP) mix, 4µl of enzymatic mix and RNase-free water until 40µl final volume. Samples were incubated at 37ºC for 16 hours.

15.3.3. cRNA purification

Synthetized cRNA was purified with the GeneChip® Sample Cleanup Module Kit (Affymetrix®, Santa Clara, California, USA).

Six hundred microliters of binding buffer were added to each sample. Five hundred microliters of this mix were transferred to a column, which was centrifuged at 8000 x *g* for 1 minute. This step was repeated loading the remaining sample. The column was introduced in a

new tube and filled with 750µl of wash buffer. After centrifugation for 1 minute at 8000 x *g*, the eluate was discarded and the centrifugation repeated at maximum speed for 5 minutes.

Finally, the columns were introduced in 1,5ml tubes; it was added 22µl of RNase-free water and centrifuged at maximum speed for 5 minutes to recollect the purified cRNA.

After synthesis of biotin-labeled cRNA, quantification and assessment of the integrity of the same was performed. The amount was calculated by spectrophotometric measurement, and the quality was evaluated by checking an aliquot on an agarose gel electrophoresis. Table 15 shows the results of this process. Figure 28 shows the testing of the quality of purified cRNA.

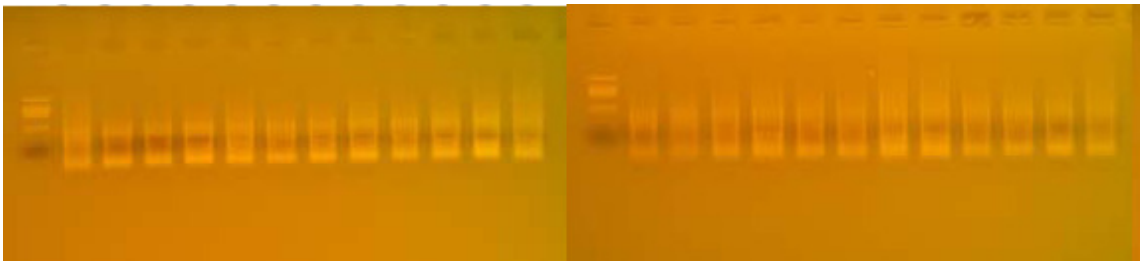


Figure 28. *Verification by agarose gel electrophoresis of the purified cRNA.*

Table 15. *Concentration and purity of extracted cRNA measure.*

Muestra	[ ] µg/µl	A260/A280	µgtotales	Muestra	[ ] µg/µl	A260/A280	µgtotales
07SE930	1,949	2,02	35,04	07SE948	2,193	2,00	37,48
07SE931	1,951	2,00	3,13	07SE949	2,551	1,96	43,92
07SE932	1,880	1,96	31,83	07SE950	2,290	2,01	39,21
07SE936	1,218	1,97	18,70	07SE954	2,161	1,99	34,73
07SE937	1,391	2,00	23,04	07SE955	2,098	1,95	33,67
07SE938	1,534	1,97	25,61	07SE956	1,511	1,98	25,19
07SE942	1,743	2,09	31,12	07SE960	3,939	2,03	64,97
07SE943	3,421	2,06	59,59	07SE961	3,172	2,06	55,09
07SE944	3,148	2,05	51,52	07SE962	2,946	2,03	53,97

15.4. BIOTINYLATED cRNA FRAGMENTATION

cRNA fragmentation is a previous step to ensure correct chips hybridization. The protocol based in a hydrolysis reaction induced by metals that obtains 35-200 nucleotides length fragments.

Eighteen microliters of fragmentation buffer from GeneChip® Sample Cleanup Module was added to cRNA biotinylated, and mix was incubated for 35 minutes at 94ºC. After this, samples were stored at –20ºC until chips hybridization. The efficiency of this process was evaluated by agarose gel electrophoresis, showing the images represented in figure 29.

Figure 29. *Verification by agarose gel electrophoresis of the fragmented cRNA.*

15.5. GENECHIP® RAT GENOME 230 2.0 ARRAY HYBRIDIZATION

After cRNA fragmentation, it was prepared the hybridization mixtures: 0,05µg/µl of cRNA fragmented; 50pM of oligo controls B2; 1,5, 5, 25 y 100 pM of bioB, bioC, bioD y Cre controls respectively; 0,1mg/ml of Herring sperm DNA; 0,5mg/ml of bovine serum albumin (BSA), hybridization buffer 1X; 10% of dimethyl sulfoxide (DMSO).

Mixtures were heated at 99ºC for 5 minutes. Then they were transferred to a dry bath programed at 45ºC for 5 minutes. Chips were wet with hybridization buffer 1X and incubated at 45ºC for 10 minutes. Last, mixtures were put on the chip and were incubated in hybridization oven at 45ºC for 16 hours.

15.6. WASHING, STAINING AND SCANNING

15.6.1. Washing

Chip washing is essential to remove cRNA fragments which have not hybridized with the probes on the chip. Chips were extracted from the hybridization oven and transferred to a washing station Fluidics Station 400 (Affymetrix®, Santa Clara, California, USA), where it did two washed:

- A first washed at 25ºC with wash buffer A: NaCl 0,9M, NaH_2PO_4 0,06M, EDTA 6mM, Tween-20 0,01%.

- A second washed at 50ºC with wash buffer morpholino ethanesulfonic acid (MES) 100mM, [Na^+] 0,1M, Tween-20 0,01%.

15.6.2. Staining

Solution SAPE (MES 100mM, [Na^+] 1M, Tween-20 0,05%, BSA 2mg/ml, streptavidin - phycoerythrin 10 µg/ml) was applied on hybridized chips at 25ºC for 10 minutes, time during which the streptavidin-phycoerythrin joined the biotinylated cRNA. After this, the chips were washed with wash buffer A at 25ºC and was applied on the chips the antibody solution (MES 100mM, [Na^+] 1M, Tween-20 0,05%, BSA 2mg/ml, IgG Goat Antibody 0,1mg/ml, biotinylated antibody 3µg/ml) and they were incubated for 10 minutes at 25ºC.

The last staining was done with SAPE solution, with a final washing with wash buffer A at 30ºC.

15.6.3. Scanning

After removing excess of buffer A, chips were introduced into the GeneChip® Scanner 3000 Targeted Genotyping System (Affymetrix®, Santa Clara, California, USA).

The result is a dark background image and bright points each corresponding to a particular probe set, and whose intensity is quantified to provide a measure of expression of each transcript in samples (figure 30).

Visual inspection of microarrays gives us a general idea of experiment quality. A dark background image with bright hybridization signals is validate; however sample problems, poor hybridization or incorrect acquisition by the scanner, can result in high background signals and noise to conceal the actual signal.

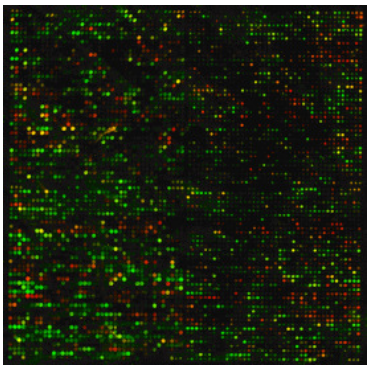


Figure 30. *Example of a good image of the scan of a microarray.*

After observing the images of each chip, the evaluation of the quality of the hybridization mixtures was carried out by analyzing spike control BioB and the relationship 3'/5' GAPDH gene. The quality test results are shown in table 16.

Table 16. *Values of the quality parameters for each of the chips analyzed.*

Sample	Spike Control BioB	GAPDH (3'/5')	Scaling Factor (TGT=100)	Sample	Spike control BioB	GAPDH (3'/5')	Scaling Factor (TGT=100)
07SE930	Present	1,38	0,975	07SE948	Present	1,38	0,963
07SE931	Present	1,51	0,998	07SE949	Present	1,31	0,979
07SE932	Present	1,64	0,854	07SE950	Present	1,34	0,862
07SE936	Present	1,33	1,222	07SE954	Present	1,31	1,063
07SE937	Present	1,59	1,249	07SE955	Present	1,37	0,944
07SE938	Present	1,39	0,969	07SE956	Present	1,50	0,977
07SE942	Present	1,81	1,019	07SE960	Present	1,31	0,898
07SE943	Present	1,27	1,173	07SE961	Present	1,64	1,244
07SE944	Present	1,37	1,181	07SE962	Present	1,45	1,181

Observing images of each of the arrays hybridized and considering the quality criteria (presence of spike controls and relationships 3'/5' housekeeping genes), the eighteen arrays hybridized surpassed the quality criteria.

15.7. BIOINFORMATICS ANALYSIS

It was analyzed the gene expression result obtained from 18 RNA samples from two age groups rats ("young and old") and under three treatment conditions (virgin olive, sunflower and

fish oil), given a total of 6 groups. The used chip was the RAE230_2.0 of Affymetrix®, with 31099 probes.

The objective of this analysis was to obtain sequences differentially expressed between the treatment conditions studied within each age group and the comparison between the two age groups as a treatment.

15.7.1. Quality controls

After observing the images of each chip, there are two essential parameters for assessing the quality of the hybridization mixtures: presence of spike controls and 3'/5' ratio of housekeeping. P call % and Array Outlier provides an estimate of the reliability of results.

- Array Outlier %: indicates the percentage of transcripts whose levels in an array are inconsistent with the remaining arrays of the experiment. The analysis program used, dChip (www.dchip.org), recommends taking caution those samples that exceed the value of 5%, and eliminate those that exceed 15%. A higher value would indicate an image potentially contaminated by traces of bubbles mainly, scratches or a problem with the sample preparation or hybridization.

15.7.1.1. Spike controls

Spike controls are control probes to sequences that are included in the hybridization mixture. The presence of these controls indicates that the process of hybridization, washing, and scanning has resulted correct. The controls used were BioB spike, BioC, BioD and Cre. The control BioB was less represented in the mixture, so it was mainly used to assess the sensitivity of the experiment

15.7.1.2. Housekeeping controls

Housekeeping genes are probes to expressed genes constitutively in all tissues. In the microarray were included probes corresponding to 3' and 5'-ends. The 3'/5' ratio of hybridization signal indicates the integrity of synthetized cRNA, been an original RNA quality indicator. A 3'/5' ratio close to 1 indicates integrity of total RNA and synthetized cRNA. Depending on the organ and the applied treatment it is difficult to obtain this ratio, so following Affymetrix® recommendations it will be taken the experiments as satisfactory if 3'/5' ratio does not exceeds the value of 3.

Moreover, the constitutive character of genes represented as housekeeping is not always true for all types of tissues or experimental conditions, as is widely reported in the literature. Among the genes represented on the microarray, the most often behaves as true housekeeping is that which encodes enzyme GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

15.7.1.3. P call %

P call % indicates the percentage of transcripts detected in a microarray. Under appropriate processing conditions, this value is used to assess the quality of the sample. All samples analyzed in this study have an acceptable P call %.

15.7.1.4. Array Outlier

Array outlier indicates the percentage of transcripts whose levels in a microarray are inconsistent with respect to the levels of the rest microarrays of the experiment. The analytical program used, dChip (www.dchip.org), advised to take caution those samples that exceed the value of 5%, and eliminate those that exceed 15%. A higher value will indicate a potentially contaminated image, mainly with bubbles, scratches or a problem in preparation or hybridization sample. All values of analyzed arrays indicated good quality of cRNA and the data obtained, and data consistency.

Table 17 shows the values of the quality indicators of the data in the arrays.

Table 17. *Values of the quality indicators of the data in the arrays.*

SAMPLE	% P CALL	%ARRAY OUTLIER	SAMPLE	% P CALL	%ARRAY OUTLIER
07SE930	47,4	0,09	07SE948	46,4	0,36
07SE931	46,8	0,09	07SE949	49,4	0,03
07SE932	46,9	0,41	07SE950	48,9	0,97
07SE936	45,8	0,37	07SE954	48,9	0,17
07SE937	46,0	0,03	07SE955	47,9	0,31
07SE938	47,8	0,13	07SE956	46,8	0,06
07SE942	45,7	0,25	07SE960	47,2	0,44
07SE943	44,9	0,01	07SE961	46,1	0,04
07SE944	45,0	0,04	07SE962	44,9	0,39

15.7.2. Data standardization by RMA method

To measure gene expression with accurately and reliably, it is important to take into account the experimental variability (due to the samples) and systematic (due to processing) that can be associated to an experiment of this nature. The normalization is the process carried out to minimize the effects of such variations on the analysis.

The intensity values of each probe in the microarray are processed and standardized by the method RMA (Robust Multichip Average) to obtain a single intensity value for each probe set, using the analysis program v7.3.1 Partek Genomics Suite (Partek Incorporated, Missouri, USA).

15.7.2.1. First filtering of data

GeneChip® data from 31099 probes were filtered to remove the control sequences and those sequences with hybridization signal near the bottom (background).

15.7.2.2. Standardization

It was performed using the quartile normalization method (as part of the standardization process by RMA) applied to microarray data stored in the *.cel files (Affymetrix® output files).

15.7.2.3. Second filtering of data

Finally, this sequence list with 9621 sequences was filtered to eliminate those probes that do not submit any change of expression between the different experimental conditions, based on the standard deviation of normalized intensity data. This filtering results in a final list of sequences forming the worklist with 7248 sequences.

15.7.3. Principal Components Analysis and hierarchical clustering

PCA (Principal Components Analysis) is a statistical technique of synthesis of information, or reducing the size of the data (in this case, the expression values of each probe). The primary objective of the PCA is the representation of numerical measures of the probes on a low-dimensional space where our senses can perceive relationships that would otherwise remain hidden in higher dimensions. This representation should be such that the discard higher dimensions (usually the third or fourth below) the loss of information is minimal. That is, with a database with many variables (each of the expression values of each gene), the aim will be reduce to fewer losing as little information as possible.

Choosing factors or components is performed such that the first factor picks up the largest proportion of the original variability present in the experiment; the second factor must collect maximum variability not reflected by the first, and so on. The number of factors or main components that taken by default is 3 and it explains a percentage of existing variance specified in the graphic.

The resulting image is compared with the known experimental factors in order to associate the observed variability (or what is the same, differences) to one of them. The results are shown in figure 31.

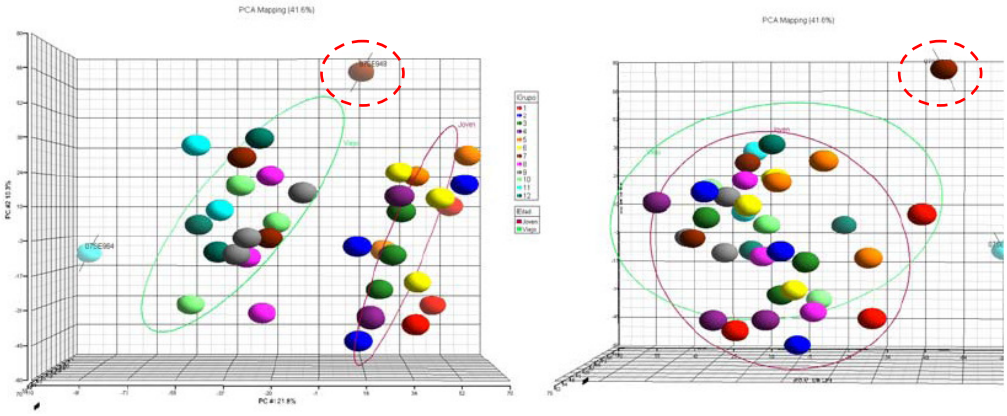


Figure 31. *Two views of PCA: A) First view of PCA. B) View rotated 90º to the left of the vertical axis (z axis, component 3) taken from the worklist. The ellipses represent age groups, red for young and green for old animals. Samples are colored according to the group they belong to. Italic rounded sample corresponds to the possible outlier. Percentage of variability: 21.8% (axis 1), 10.3% (axis 2), 9.5% (axis 3).*

Figure 31 may indicate that the greater variability or differences observed are due to the age of the samples, which coincides with the maximum separation between groups of samples. It can also appreciate how the replicas generally belonging to the same experimental group are close to each other, except the samples 07SE948 and 07SE964. This could indicate the

existence of two possible sample "outlier", we decided to keep within the experiment the sample 07SE948, but 07SE964 was removed.

A cluster or hierarchical tree is a statistical method that allows the classification of objects, in this case gene expression profiles or samples, in groups or clusters of similarity between them. For this it is calculated the distance of each gene with all other and grouped the two genes that present a higher correlation. Then it is calculated the average expression profile of the two and with this gene o synthetic sample repeats calculating the distance for all other objects.

This process is repeated until all the genes have been grouped by pairs. Then, it is calculated the separation ratios and the similarity measure that will organize the tree forming discrete groups and ramification, respectively.

The hierarchical clusters of the present study were performed using the Partek Genomics Suite software, using as distance measure the Euclidean distance and as clustering method the "Average Linkage" method. Figure 32 shows the cluster of samples using normalized intensity values of the selected and filtered sequences (the list of work).

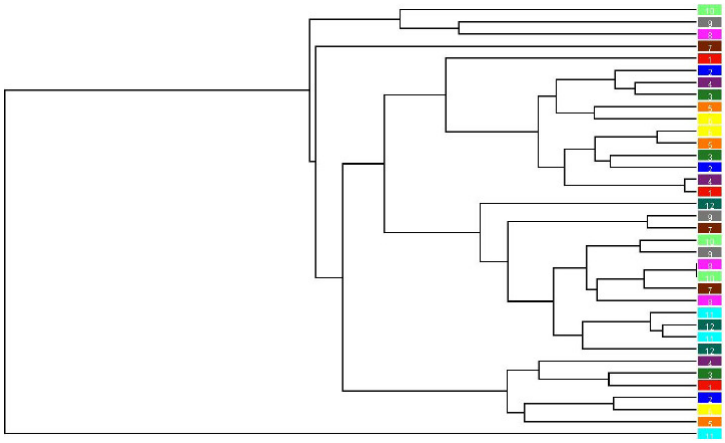


Figure 32. *Hierarchical cluster of samples made from the worklist using the Euclidean distance as distance measure. Each sample is marked by a color that represents the experimental group.*

The elaboration of this unsupervised cluster (genes are not the result of applying a statistical test) gives an approximate idea of the differences between each of the conditions, as well as the variability within each condition. Overall, it appears that the experimental replicas are quite similar, although there is no clear trend grouping by experimental condition.

Then, a supervised hierarchical cluster was performed to the worklist “todos *vs.* todos” using the Pearson correlation for clustering genes and the Euclidean distance for samples) Figure 33 shows the two-dimensional hierarchical cluster obtained. A cluster of these characteristics is not useful for finding relationships between samples.

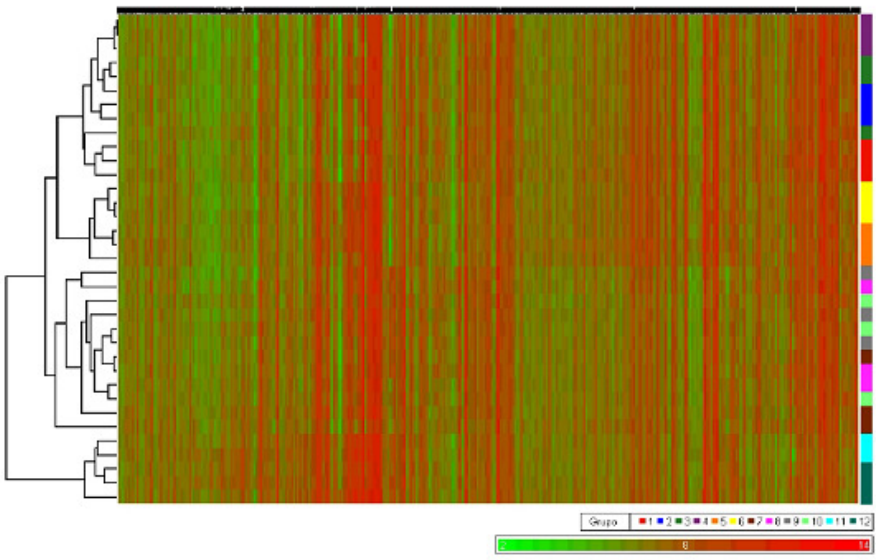


Figure 33. *Two-dimensional hierarchical cluster of genes and samples made from the worklist "todos vs. todos", using the Euclidean distance as a measure of clustering of samples and the Pearson correlation for clustering sequences. Each sequence is represented by a color between red and green. Green indicates repressed gene relative to the median for all samples, and red over-expressed gene. Each sample is marked by a color that represents the experimental group.*

15.8. FUNCTIONAL ENRICHMENT

Once the filtering and statistical analysis of expression data was done, we obtained several datasets of genes up-regulated or down-regulated statistically significant under our comparisons. To process all this information and translate it into useful biological information, it is necessary to carry out a functional analysis of the data obtained. There are numerous public databases and bibliographical sources to the study of genic function; however, the large amount of information as a result of a microarray experiment makes this approach not viable for a functional study. For this reason, in recent years some bioinformatics tools have been development to allow the classification of genes according to the biological processes in which they are involved, the molecular functions of proteins encoded and the signaling pathways implicated. This type of analysis is known as "functional enrichment", and it consists in the processing of a list of genes defined by the researcher and further classified by the program in different functional categories according to thresholds *P* values, FC or both.

In our case, we had only one selected dataset genes to the functional analysis. The dataset was composed of those genes differentially expressed after applying the Benjamini-Hochberg correction test, a statistical test that controls the false positives less restrictively than Bonferroni test.

15.8.1. Functional networks and pathways analyses with Ingenuity Pathways Analysis

Ingenuity Pathways Analysis (IPA) application (Ingenuity Systems, www.ingenuity.com) is a web-based software application that enables comprehensive pathway and network analysis to interpret the biological meaning of complex genomic data.

IPA performs up to five different types of analysis focused either on biological processes, pathways and molecular networks, metabolomics, toxicity assessment or biomarker analysis for drug discovery or disease research. In addition, it has a large set of features that allow us to understand and visualize datasets of interest. Then, IPA offers different analysis modalities and tools to be selected by the researchers, in accordance to their work hypothesis and the specific questions they intend to answer through deep biological insight.

To proceed with IPA analysis, the first step is the uploading of a gene dataset. An .xls archive, containing the probeset ID and the FC value for each analysis was imported into IPA. The FC expression value cutoff selected in the present study was 1.5, so expression changes lower than 1.5 were not considered for the analysis.

Two different type dataset were uploaded:

- Genes with changes in their expression but that were common in the three experimental groups: virgin olive, sunflower and fish oil. So these gene expression changes were due only to age. This dataset was called "Non Common aging genes"
- Genes which changes in their expression appeared only in one or two experimental groups, so these gene expression changes were influenced by the dietary fat type, as well as by age. These datasets were called "Non Common virgin" "Common sunflower" and "Non Common fish".

Once this, the select identifier (ID) and the array platform was chosen. Then the program mapped the probeset ID and related with a gene symbol, building a table as the shown in figure 34. With the mapped ID several analyses can be selected: core analysis, tox analysis, metabolomics analysis, etc.

After running a Core Analysis, IPA generated the following results (Figure 35):

- ✓ Summary (highlighting the most significant results)
- ✓ Functions, including Downstream Effects Analysis
- ✓ Canonical Pathways
- ✓ Transcription Factors
- ✓ Networks (Optional)
- ✓ Network Explorer (Optional)
- ✓ Overlapping Networks (Optional)
- ✓ Molecules
- ✓ Lists (relevant if we have created our own molecule lists)
- ✓ My Pathways (relevant if we have created our own custom pathways)

For the present study, focus was placed on Canonical Pathways Analysis, which provides an approach to identifying crucial pathways in the control of cellular function and processes that are affected by changes in the expression of our focus genes. Canonical Pathways analysis determines the most significantly affected metabolic and cell signaling pathways across the entire dataset of focus genes. Canonical pathways were identified from IPA Knowledge Base on the basis of the significance of the association between the dataset and the canonical pathway, which is determined based on the ratio of the number of focus genes that map to the canonical pathway divided by the total number of genes that map to the canonical pathway; and a *P* value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone. This ratio provides an idea about the percentage of genes in a pathway that were also found in the uploaded list. The *P* value refers the probability that a canonical pathway may be affected by the experimental

treatment. If a *P* value is very small, the pathway is more probably associated with the uploaded dataset.

When a pathway has a high ratio and a very low *P* value, it means that probably this pathway is associated with the data and a large proportion of the pathway may be involved or affected, and it may be a good candidate for an explanation of the observed phenotype.

How the Fisher's Exact Test is calculated?

First, it raises a null hypothesis: is the proportion of genes mapping to a function or pathway in a considered sample (those that are significant) similar to the proportion that map in the entire population (reference set)?

If the proportions are similar, there is no biological effect, so Fisher's Exact Test calculates exactly the significance of the difference. For it, a 2x2 contingency table is created based on the total population, the sample, and how many genes map to the function/pathway, as table 18.

Table 18. *Example of 2x2 contingency table to Fisher's Exact Test calculate*

	Inflammation	Non-inflammation	Raw total
In sample	k	n-k	N
Not in sample	m-k	N+K-n-m	N-m
Column total	m	N-m	N

M = total that map to function or pathway; N = total; K = number that map to function or pathway in sample; n = total sample

$P = [(a+b)!\ (c+d)!\ (a+c)!\ (b+d)!] / [a!\ b!\ c!\ d!\ n!]$

If $P < 0.05$, differences are considered significant.

In a typical Core Analysis, IPA only considers whether a gene is significantly changed, and does not consider the directional change of expression. IPA answers the question of what networks, biological functions, diseases, and canonical pathways do the significant molecules in a particular experiment.

Canonical pathways analysis is graphically represented in a bar chart which displays the most significant Canonical Pathways across the entire dataset (x-axis) with respect -log of *P*-value which is calculated by Fisher's exact test right-tailed (y-axis). Y-axis also represents the ratio to each canonical pathway (Figure 36). Further customization of bar chart provides a more intuitive representation named "Stacked bar chart", which displays the number of up-regulated (red), down-regulated (green), and unchanged molecules (gray) in each canonical pathway (Figure 37).

Following the identification of relevant pathways in which our focus genes are involved, we proceeded to select the most significant canonical pathway in each gene dataset for further analysis. A pathway representation, which integrates the expression profile of the experimental data set, can be obtained from IPA (Figure 38).

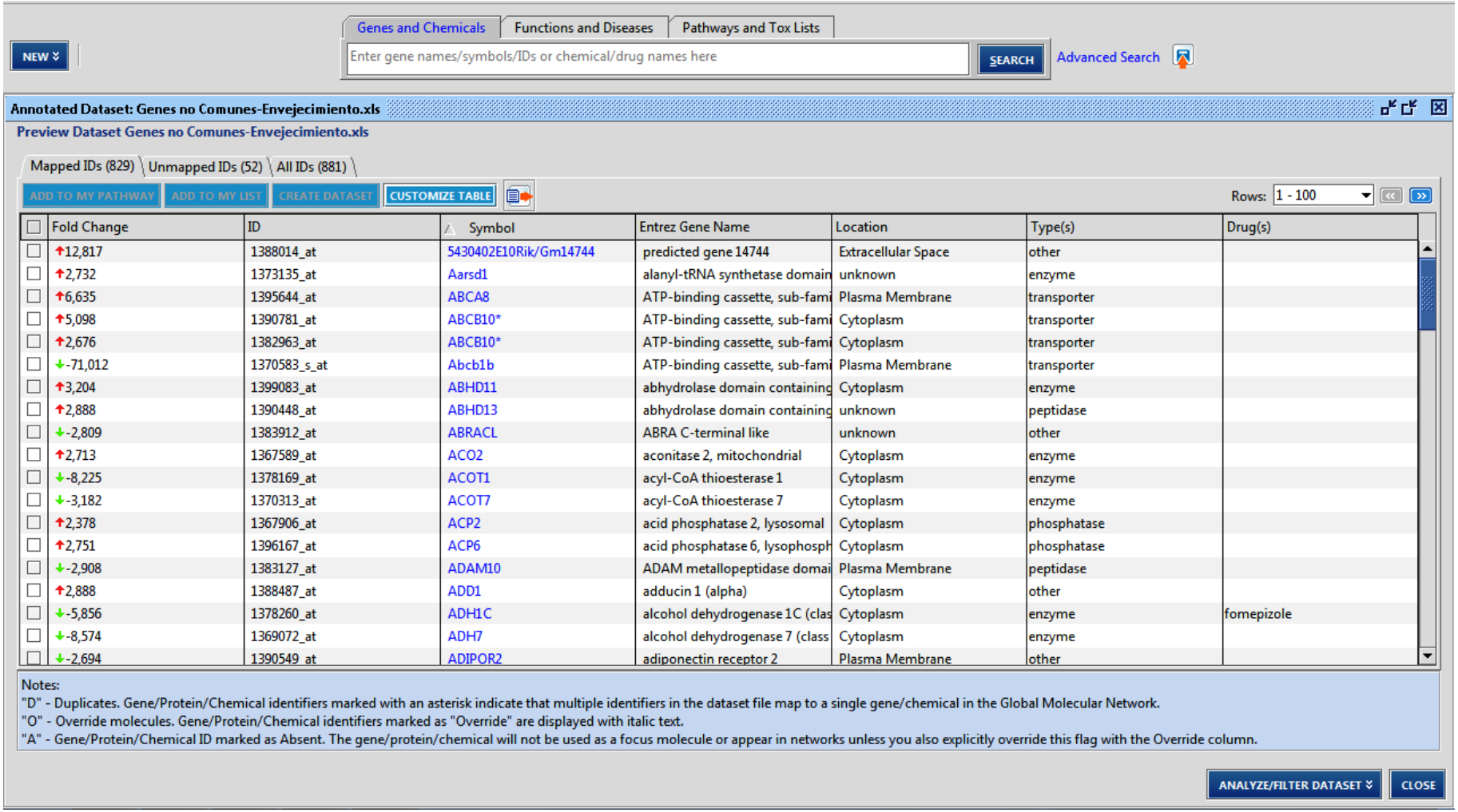


Figure 34.*Select genes table example.*

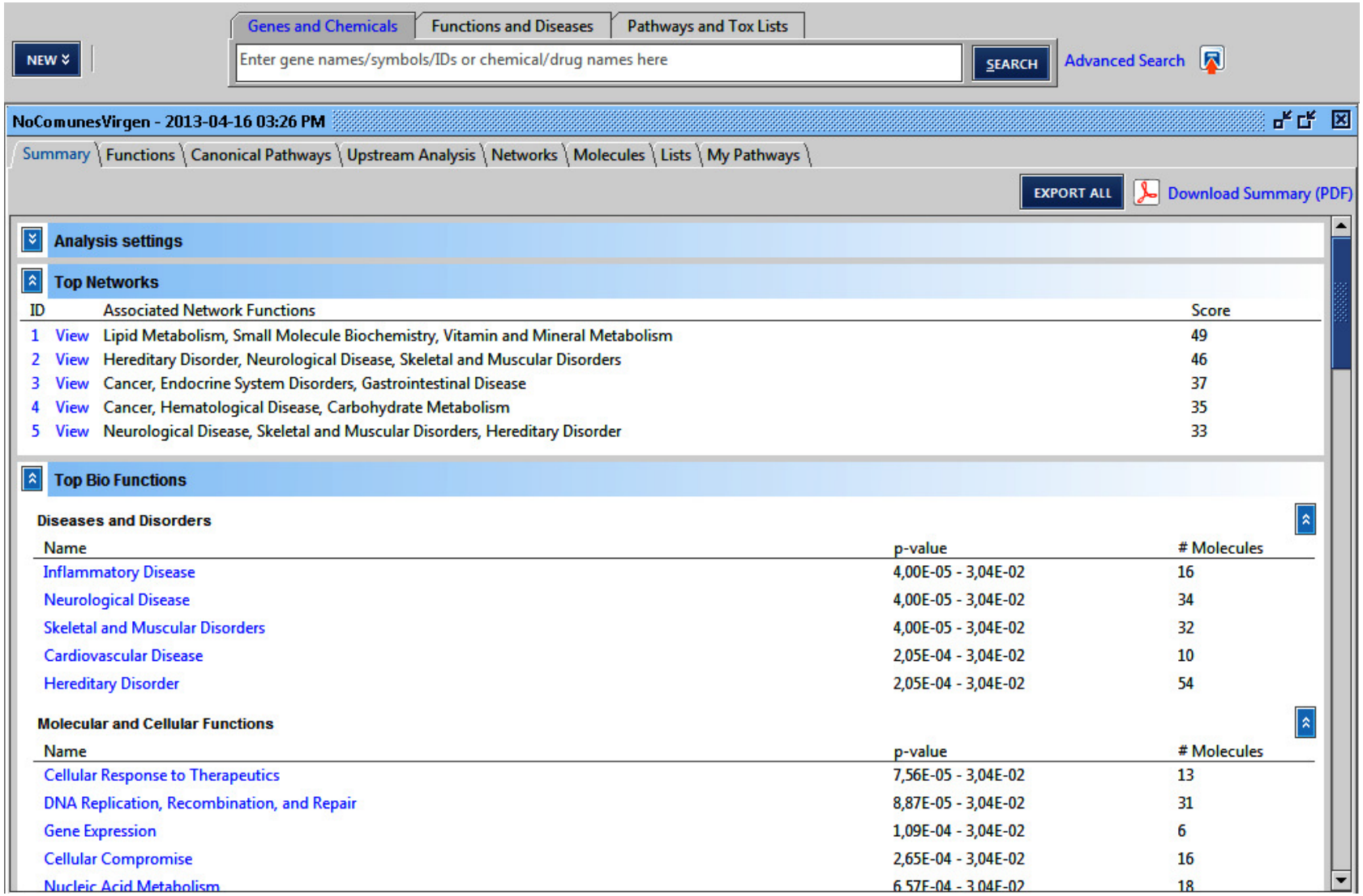


Figure 35.*Core analysis example.*

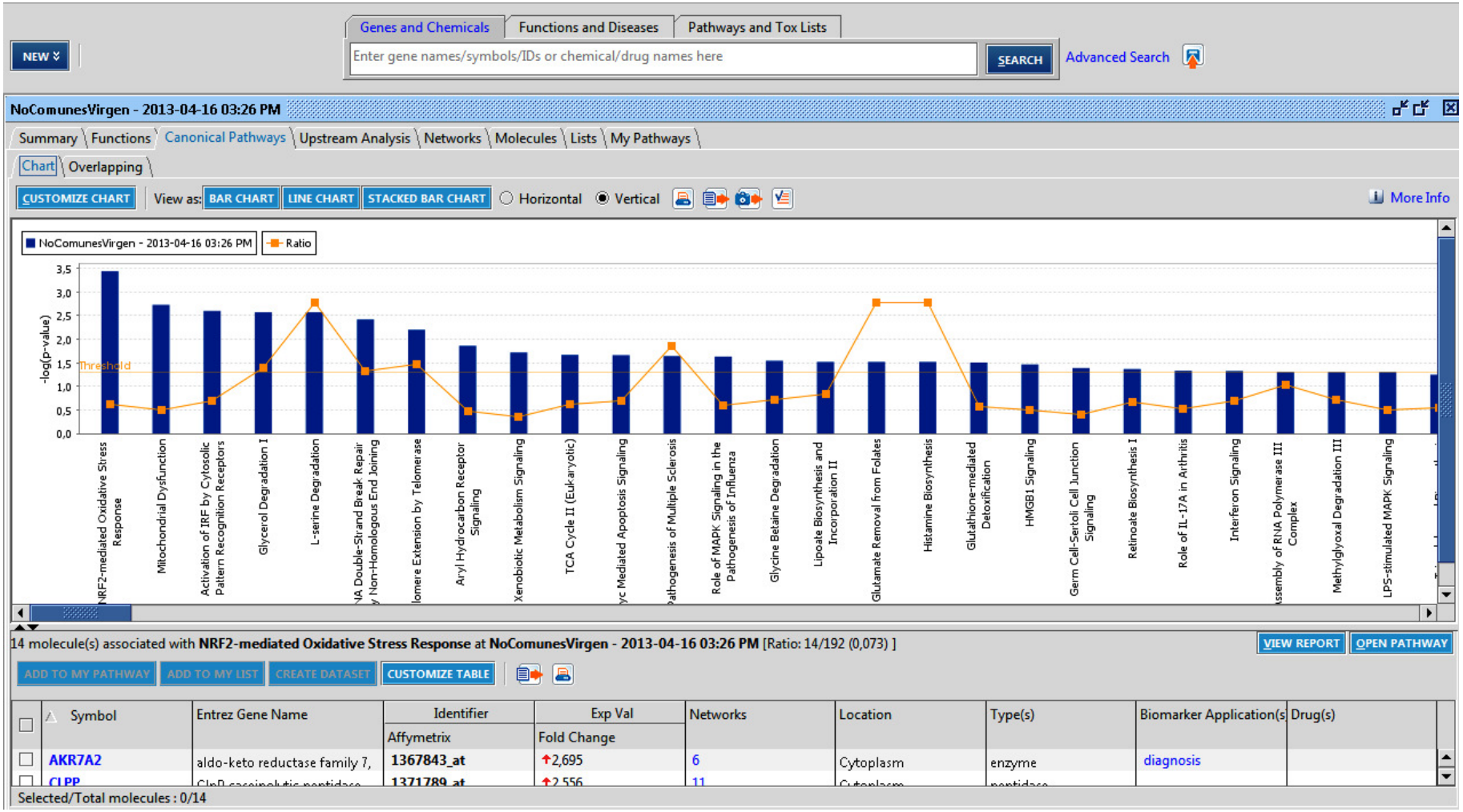


Figure 36. *Canonical pathways bar chart example.*

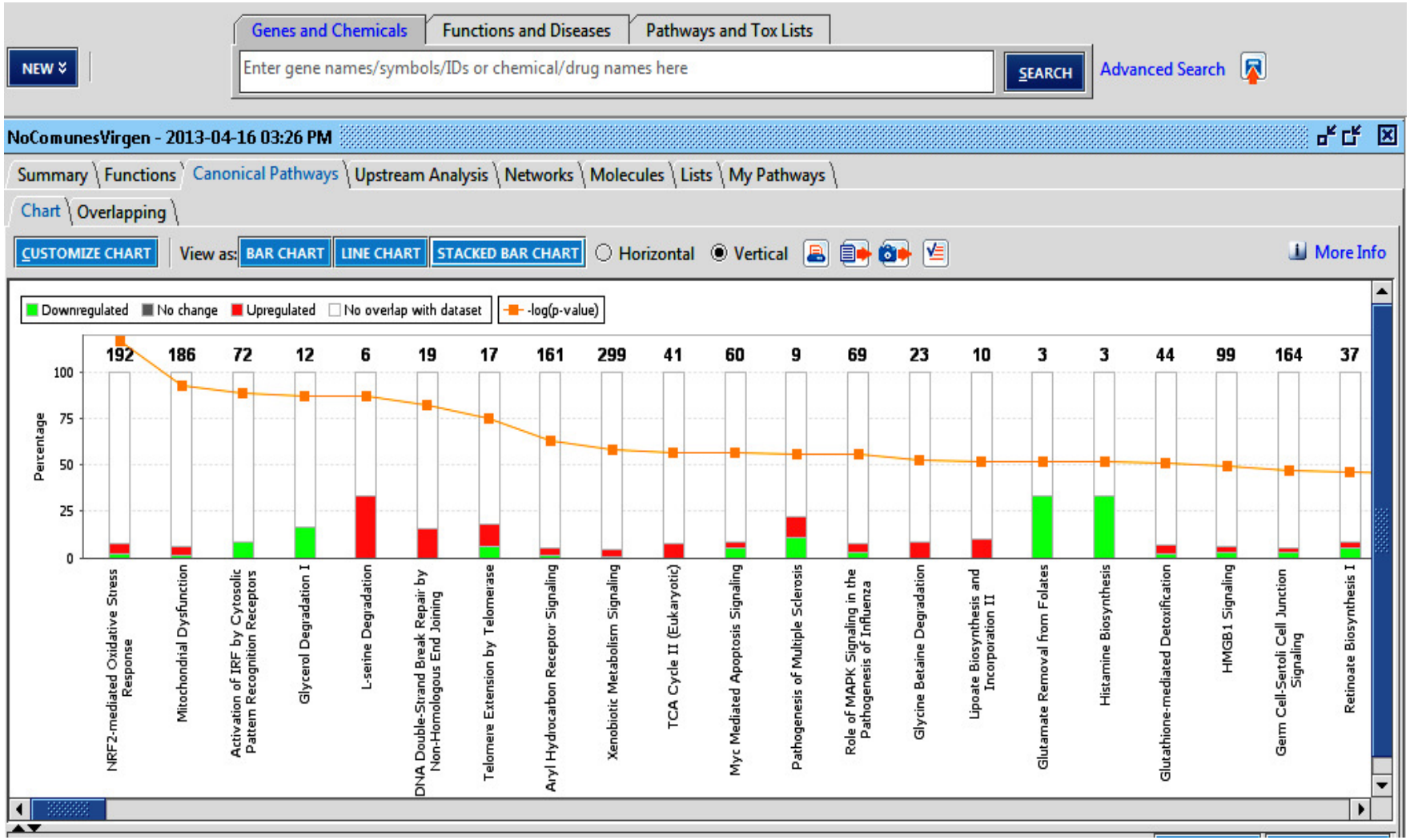


Figure 37. *Canonical pathways stacked bar chart customizer example.*

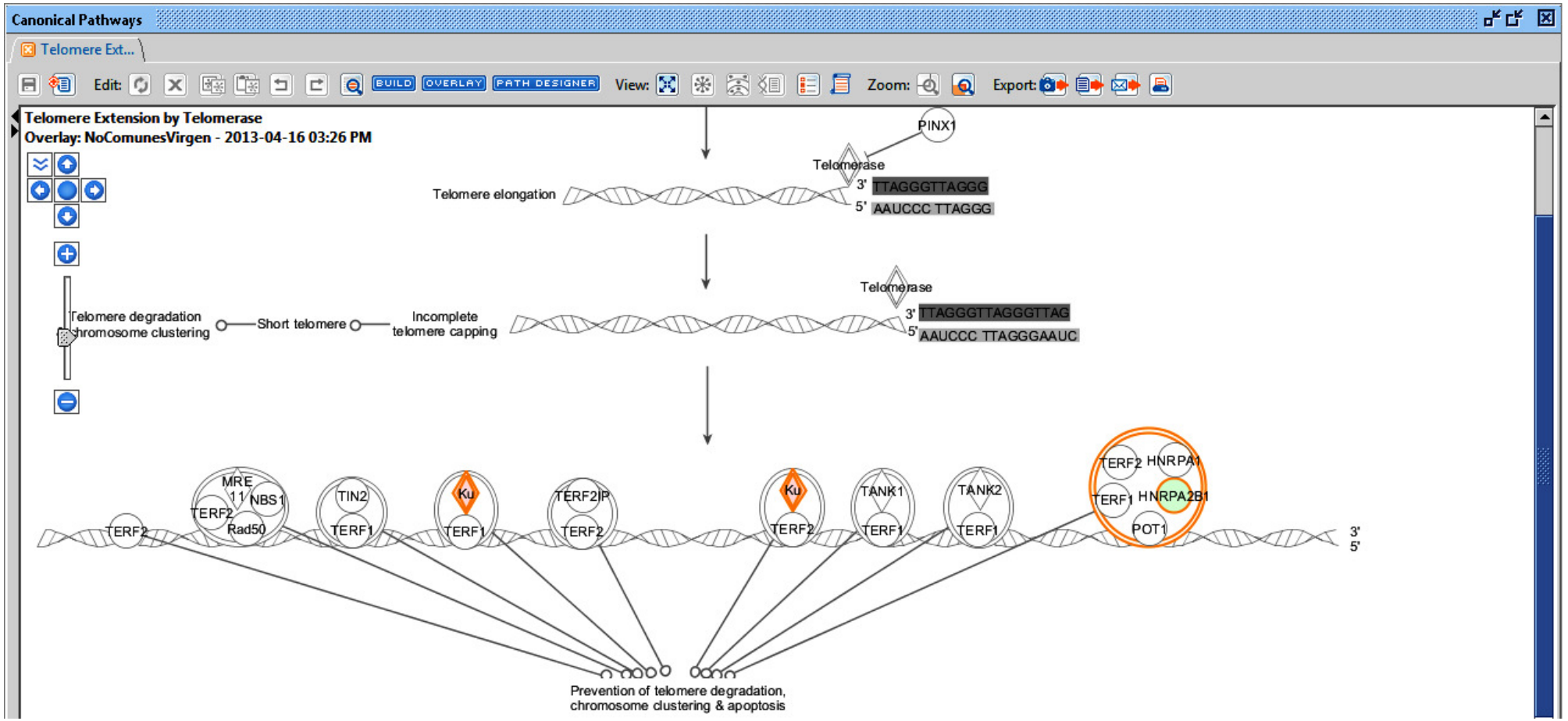


Figure 38.*Canonical pathway representation.*

16. REAL TIME QUANTITATIVE PCR (RT-PCR) FOR MICROARRAY VALIDATION.

From each group of six animals, the three samples that were not considered for microarray screening were used for RT-PCR validation (Port *et al*., 2012). One hundred and fifty nanograms of RNA from each of the 18 samples, previously extracted from liver tissue as is indicated in section 15.2, were reverse-transcribed into cDNA using Affinity Script Multiple Temperature cDNA Synthesis kit (Agilent Technologies, Santa Clara, California, USA). cDNA was obtained in a final volume of 20µl. cDNA was aliquoted and stored at -20°C, in accordance with the manufacturer's protocol. The quantity and purity of the cDNA were determined form the absorbance at 260/230 nm in the Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockfor, Illinois, USA). Previous to RT-PCR pre-amplification was performed with the kit QIAGEN® Multiplex PCR Kit (Düsseldorf, Germany) and the pool of all 20x TaqMan® Gene Expression Assays.

Analysis of gene expression by RT-PCR was performed using the Fluidigm technology (www.fluidigm.com). The BioMarck Fluidigm system utilizes TaqMan technology to detect the PCR amplified product. TaqMan probes detection is based on the use of a DNA fragment (probe) complementary to an intermediate portion of the DNA to be amplified. This probe is affixed to a fluorescent molecule and another molecule inhibiting the fluorescence ("quencher"), so that only when the probe is displaced from its position by action of the DNA polymerase liberates the fluorescent molecule with "quencher" action and fluoresces when illuminated with a laser. Quantification of the fluorescence emitted during each PCR cycle will be proportional to the amount of DNA being amplified and also turn proportional to the amount of RNA that exists in the sample.

All PCR reactions were run in real time in microfluidic format, 1 array of Fluidigm 48.48. This experiment consists of 18 samples and 20 genes problem + 3 genes housekeeping, which probes were acquired to Applied Biosystems (table 19). To get the most out of all positions of the array samples were placed in triplicate (the first 8 samples) or duplicate, and the assays were placed in duplicate in the array (Figure 39). For the PCR reactions, the TaqMan Universal PCR Master Mix from Applied Biosystems was used, according to the specifications provided by the supplier. A negative control (NTC) was included for quadruplicate. A total of 2024 score points (46-well assays x 44-well samples) were obtained. For each sample and assay there are 4 or 6 technical replicates and at both 4 or 6 Ct values.

Table 19. *Genes to RT-PCR assay.*

Gen Assay	Probe ID
Atp5d	Rn00756371_g1
Btg1	Rn00820872_g1
Casp3	Rn00563902_m1
Ccnd1	Rn00432360_m1
Fis1	Rn01480911_m1
Keap1	Rn00589292_m1
Map2k6	Rn00586764_m1
Mapk9	Rn00569058_m1
Ndufa8	Rn01438607_m1
Ndufa9	Rn01462923_m1

Pik3c2g	Rn00588317_m1
Ppp2r1b	Rn01422402_g1
Prdx5	Rn00586040_m1
Rb1	Rn01753308_m1
Rras	Rn01454699_g1
Stat1	Rn00583505_m1
Tap1	Rn00709612_m1
Tp53	Rn00755717_m1
Xrcc1	Rn01457689_m1
Xrcc6	Rn00594589_m1
Gapdh	Rn01775763_g1
B2m	Rn00560865_m1
18S	Hs99999901_s1

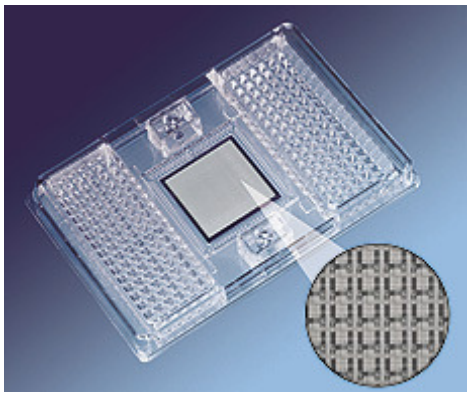


Figure 39. *48.48 Fluidigm Array*.

16.1. DATA ANALYSIS

Data analysis was performed by the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001). Briefly, the average C_T of 4 to 6 technical available replicates was calculated. As reference genes (housekeeping genes) Gapdh, 18S and B2m were used. By the use of GeNorm program (http://medgen.ugent.be/~jvdesomp/genorm/) the most appropriate housekeeping gene was selected (Schlotter *et al*., 2009).

CHAPTER VI. STATISTICAL TREATMENT OF RESULTS

1. STATISTICAL ANALYSIS FOR ALL VARIABLES EXCEPT FOR DNA ARRAY EXPERIMENT

All results are expressed as mean ± standard error of the mean (SEM) for six animals. Before making comparisons, we evaluated normality of variables by the method of Kolmogorov-Smirnov test and homogeneity of variance by the method of Levene. All those variables related to histopathological analyses, activities of complexes I and those that did not follow normality, were analyzed by the use of nonparametric Kruskal-Wallis and U-Mann-Whitney test.

Variables following normal distribution were analyzed for differences between dietary treatments at 6 months and at 24 months, significant differences between groups ($P<0.05$) were established by analysis of variance (ANOVA), using a "post-hoc" test of comparisons between groups, Bonferroni and the minimum difference test to homogenous variances. When the variance was not homogeneous, Tamhane's T2 test was followed. For differences between 6 and 24 months of age, for each dietary treatment, Student-T test for related sample was applied ($P<0.05$).

Statistical analysis was performed with SPSS 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

2. STATISTICAL ANALYSIS FOR DNA ARRAY EXPERIMENT

To identify statistically significant changes in expression between groups of samples, a linear regression model was performed, taking into account the factors age (two levels) and experimental group (six levels).

Thus, the mathematical model is of the form:

$$Y_i = age + grup + \square\square$$

where "Y_i" is the intensity Y for each sequence i, "age" refers to the effect of age on the observed intensity, "group" refers to the effect of the group on the observed intensity, and "□" is the error is not measurable and it meets the non-predicted part of the response variable (intensity). Thus, it can obtain the differentially expressed sequences due to aging and to the effect of experimental group.

Figure 40 shows a graph with the major sources of variation that lead to differences between samples. According to figure 51, the effect of aging is that which produces the greatest change; while on the other hand, there are no large differences between the experimental groups.

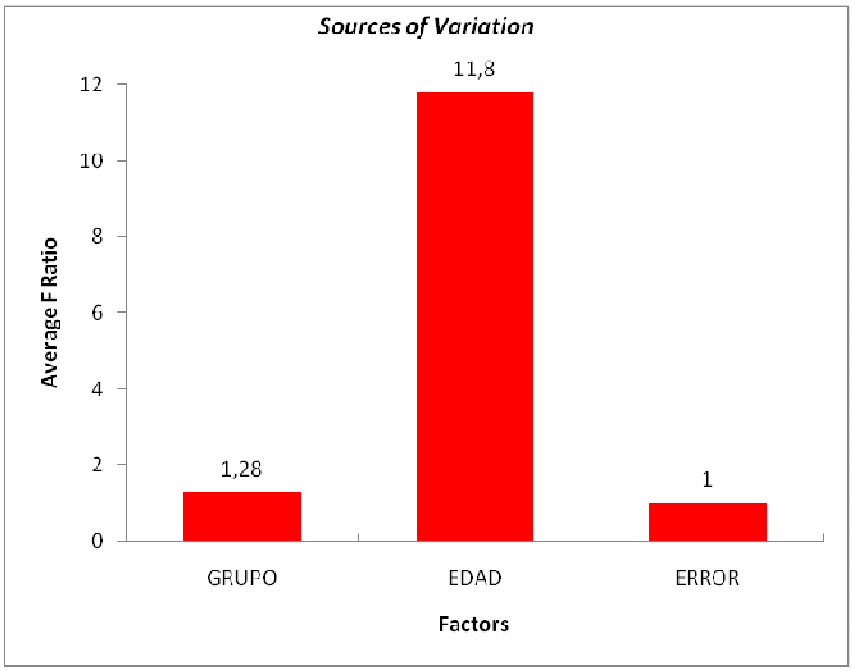


Figure 40. *Sources of variation causing the differences between samples, based on the average of the F statistic of all probes analyzed.*

After statistical analysis, lists of significant genes were obtained to all possible combinations between the 6 groups, named "Lista de significativos".

When looking at significant differences between various conditions are searched, some sequences may appear as significant by chance, i.e. be false positives (Type I error). The purpose of the application of a multiple correction test is to try to decrease the likelihood of false positives more rigorously than which is specified by the significance level. The correction applied was the Benjamini and Hochberg correction test, which controls the FDR (False Discovery Rate), one of the most relevant statistical genomics studies used in multiple hypotheses testing to correct for multiple comparisons. FDR controls the expected proportion of incorrectly rejected null hypotheses (false positives). It refers to the error rate of the selected sequences as discriminants. FDR is the percentage of false positives in each of the factors studied. In our case, by applying 0,05 significance, it is estimated that 5% of the significant sequences could be a false positive. Finally, without applying any correction is estimated that 5% of the statistical tests performed could have been selected by chance.

FDR controlling procedures exert a less stringent control over false discovery compared to familywise error rate (FWER) procedures (such as the Bonferroni correction).

Benjamini and Hochberg correction test calculates a significance threshold *P* value (included in table 20), which is contrasted with the significance value *P* original, from which the probe is not considered significant. As an example, if we take the list "*P*<0.05 all *vs.* all" from de Excel file with the results, the Benjamini and Hochberg threshold was calculated as 0.0002185. With this value we have to go to the column *P*-value and find the value of *P* that is closest to this threshold. Those probes that have the value of *P* less than 0.0002185 remain significant after Benjamini and Hochberg correction. It is assumed that a 5% of the number of

sequences result in the comparison may have been selected by chance after this correction. After this correction, the significant sequences list age-related in each treatment was reduced to 1412 sequences. Table 18 shows the statistical analyses results.

Table 20. *Statistical analysis results.*

Comparison	Significant sequences	Significant sequences after Benjamini y Hochberg	Benjamini y Hochberg threshold
P<0.05 old *vs*. young	3571	3107	0,022626
1 vs. 2	362	0	7,28E-06
1 vs. 3	457	0	7,28E-06
2 vs. 3	185	0	7,28E-06
7 vs. 8	504	0	7,28E-06
7 vs. 9	473	0	7,28E-06
8 vs. 9	237	0	7,28E-06
1 vs. 7	1821	574	0,00418
2 vs. 8	1050	76	0,000553
3 vs. 9	1285	240	0,001748

The normalized data from the lists of differentially expressed genes are shown as value of change between conditions -change of order of magnitude or Fold Change (FC)-. For this, data from the two experimental conditions being compared are normalized versus the geometric mean of the array of samples and that found in the second comparison (eg. A *vs.* B, normalizes A and B on B). The value used in the lists will be the Signal Log Ratio (SLR) which corresponds to the $\log_2$ of FC. A greater absolute value of SLR (negative for positive sequences for repressed and overexpressed) the exchange rate will be higher.

The mathematical relationship between SLR and FC is the following:

$$FC = 2^{(-1*SLR)}$$

RESULTS

I. WEIGHT OF RATS AND LIVERS

Differences in the body weight of rats, liver weight and liver to brain ratio are shown in figures 41A, 41B and 41C, respectively. For any of the studied parameters at 6 months, no differences were found between the three experimental groups: virgin olive, sunflower and fish oil. At 24 months, no differences were found between virgin olive, sunflower and fish oil groups in the body weight of animals (Figure 41A). However, liver weight (figure 41B) and liver to brain ratios (figure 41C) were higher in fish oil group than in the rats fed on virgin olive and sunflower oil.

Regarding the aging effect, differences were found between virgin olive oil, sunflower and fish groups in the three parameters examined: body weight (figure 41A), liver weight (figure 41B) and liver to brain ratio (figure 41C). In the three variables, old rats presented higher values than young rats.

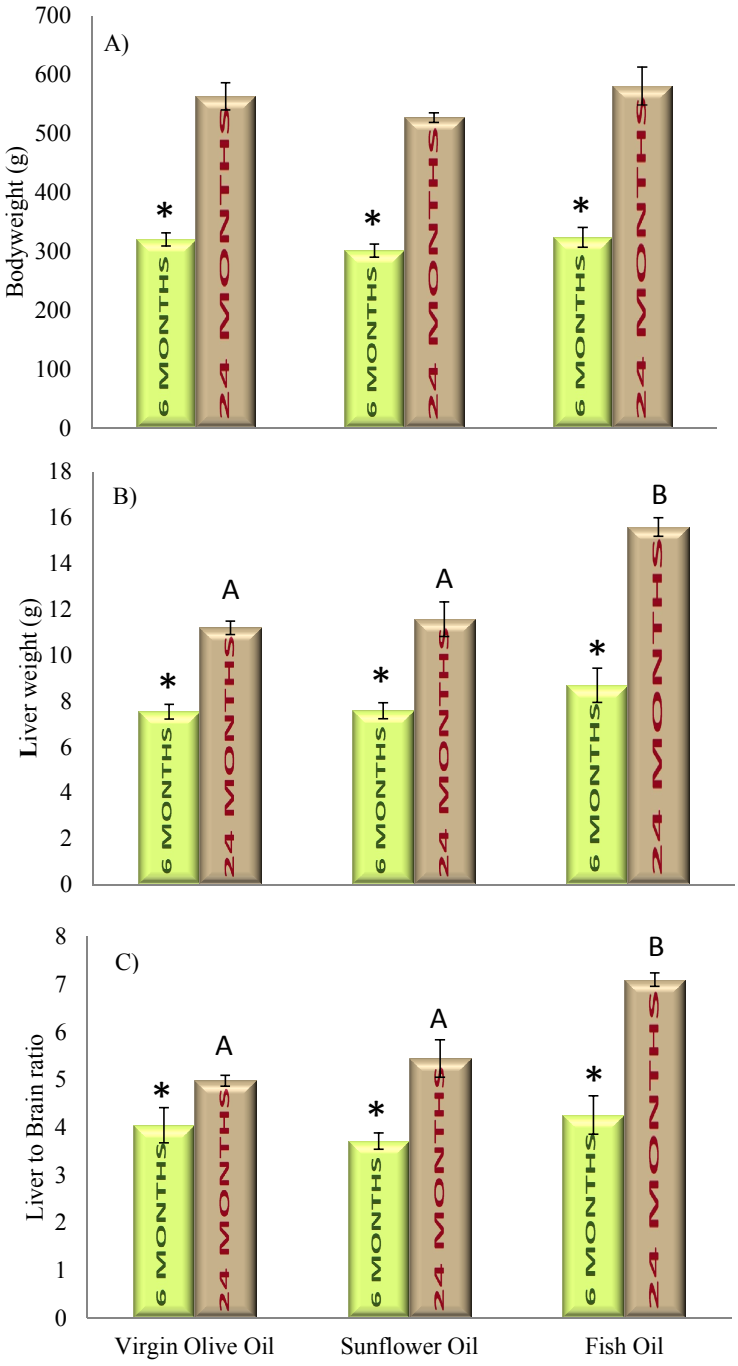


Figure 41 . Body weight, liver weight and live to brain ratio of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

II. FATTY ACID PROFILE OF MITOCHONDRIAL MEMBRANE

Differences in the percentage of fatty acids of mitochondrial membranes are presented in table 21.

At 6 months, the percentage of C14 fatty acid was lower in fish oil group than virgin olive oil. Percentage of C16, C22:6n3, EPA and n3 PUFA was higher in rats fed on fish oil as fat source than in virgin olive and sunflower oil fed animals.

C18:2n6, C20:4n6 and C24:1n9 showed higher percentages in rats fed on sunflower oil than in the rats fed on virgin olive and fish oils. Higher percentages for C20:3n6 were found in rats fed on virgin olive oil than in those fed on sunflower or fish oil.

Rats fed on fish oil showed lower percentage of n6 PUFA than virgin olive and sunflower oil groups.

Table 21. *Effects of dietary fat type (virgin olive oil, sunflower oil or fish oil) on the fatty acid profile of liver mitochondrial membrane from young (6 months old) rats.*

Fatty acid or index (g/100g)	VIRGIN OLIVE OIL	SUNFLOWER OIL	FISH OIL
C14:0	24,6±6,6 [b]	11,7±3,9 [ab]	6,2±2,5 [a]
C16:0	17,8±1,0 [a]	20,7±0,8 [a]	32,6±9,6 [b]
C16:1n9	3,9±0,8	2,5±0,5	4,6±0,9
C18:0	14,2±1,7	18,3±3,0	15,4±2,1
C18:1n9	14,4±4,0	8,8±1,2	10,2±1,7
C18:2n6	4,8±0,5 [a]	9,0±2,2 [b]	2,3±0,3 [a]
C20:3n6	0,5±0,1 [b]	0,2±0,1 [a]	0,1±0,1 [a]
C20:4n6	6,3±0,6 [a]	13,5±2,8 [b]	4,4±0,6 [a]
EPA	0,2±0,1 [a]	0,5±0,5 [a]	6,2±1,0 [b]
C24:0	0,7±0,2	0,9±0,2	0,5±0,2
C24:1n9	0,2±0,1 [a]	1,6±0,3 [b]	0,4±0,2 [a]
C22:6n3	3,7±0,9 [a]	1,4±0,9 [a]	16,0±2,5 [b]
%SFA	57,5±4,7	51,9±1,6	54,8±6,7
%MUFA	22,9±3,6	23,5±4,2	16,2±2,6
%n-6 PUFA	15,7±1,8 [b]	22,8±4,9 [b]	6,8±1,0 [a]
%n-3 PUFA	3,9±0,9 [a]	1,9±1,4 [a]	22,2±3,5 [b]
%PUFA	19,6±2,6	24,6±3,9	29,0±4,4

Notes: results show means ± standard error of the mean (n = 6).
Abbreviations: EPA = Eicosapentaenoic acid; SAF = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
Statistical significances: lower-case letters, when different, represent statistically significant differences (P < 0, 05) between the three dietary treatments at 6 months of age.

III. HISTOLOPATOLOGICAL STUDY OF THE LIVER

III.1. STEATOSIS

Figure 42 represents the results found concerning liver steatosis. No differences were found at 6 and 24 months between the three experimental groups. According with aging, at 24 months values were higher than at 6 months in all groups.

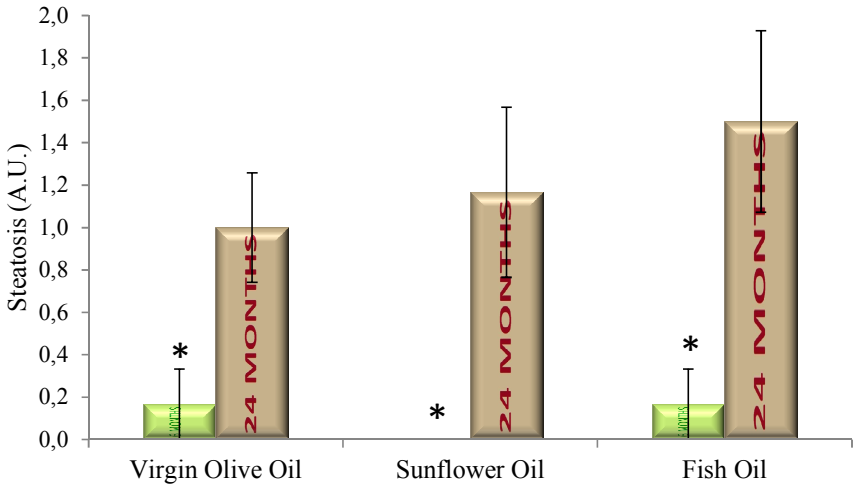


Figure 42. Steatosis analysis of the liver of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment between 6 and 24 months of age. A.U. means arbitrary units.*

III.2. CENTROLOBULILAR INFLAMMATION GRADE

Figure 43 presents the differences in centrolobulilar inflammation grade. No differences were found between groups at 6 or 24 months.

Regarding aging effect on centrolobulilar inflammation, no differences were found in virgin olive oil, but differences between 6 and 24 months were reported in sunflower and fish groups, showing higher values in older animals than in younger.

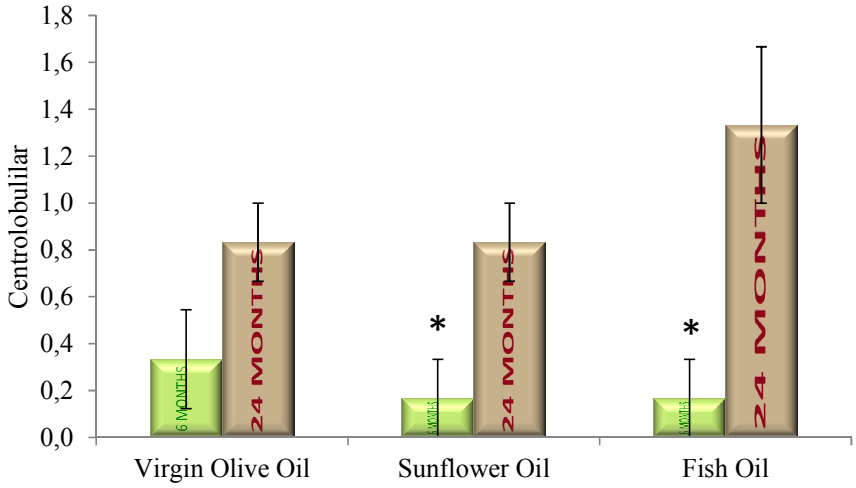


Figure 43. Centrolobulilar inflammation grade of liver of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age.*

III.3. NASH GRADE

Figure 44 shows NASH grade in the liver of rats. No differences were found between the three groups at 6 or 24 months. Concerning the effect of aging on this parameter, all groups reported higher values for old animals compared to 6 months groups.

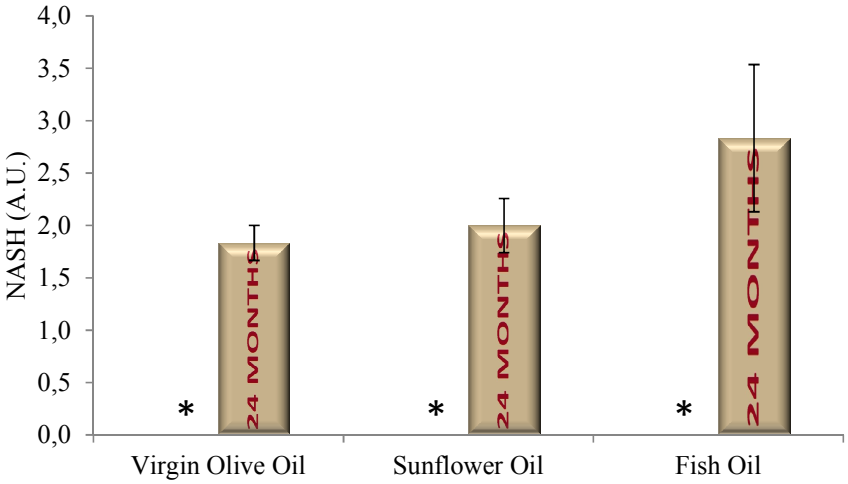


Figure 44. NASH grade of liver of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. A.U. means arbitrary unit.*

III.4. LIVER FIBROSIS

Figure 45 represents the results found concerning liver fibrosis. No differences were found between the three experimental groups: virgin olive, sunflower and fish oil. However, at 24 months, higher value of liver fibrosis, calculated as percentage of collagen, was found in sunflower oil group, compared with virgin olive and fish groups.

Regarding the effect of aging on liver fibrosis, all groups reported higher values for old animals compared to 6 months groups.

Figure 46 shows liver fibrosis in liver from rats fed on the three dietary treatments.

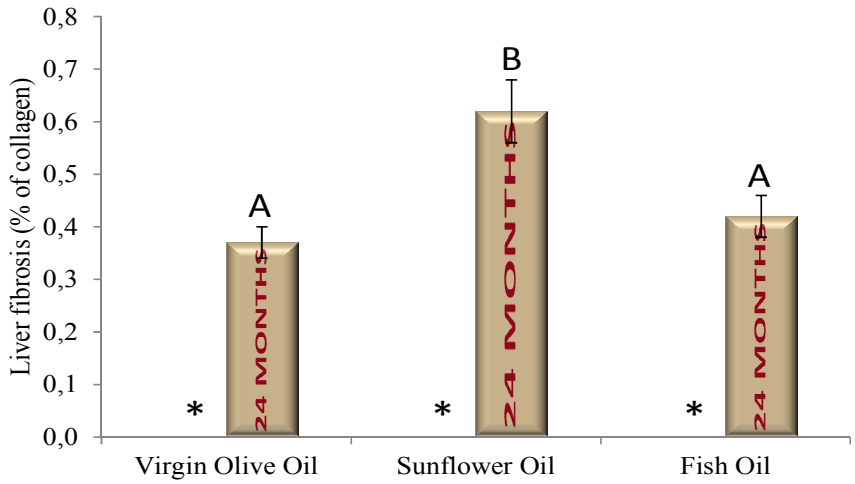


Figure 45. Liver fibrosis of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

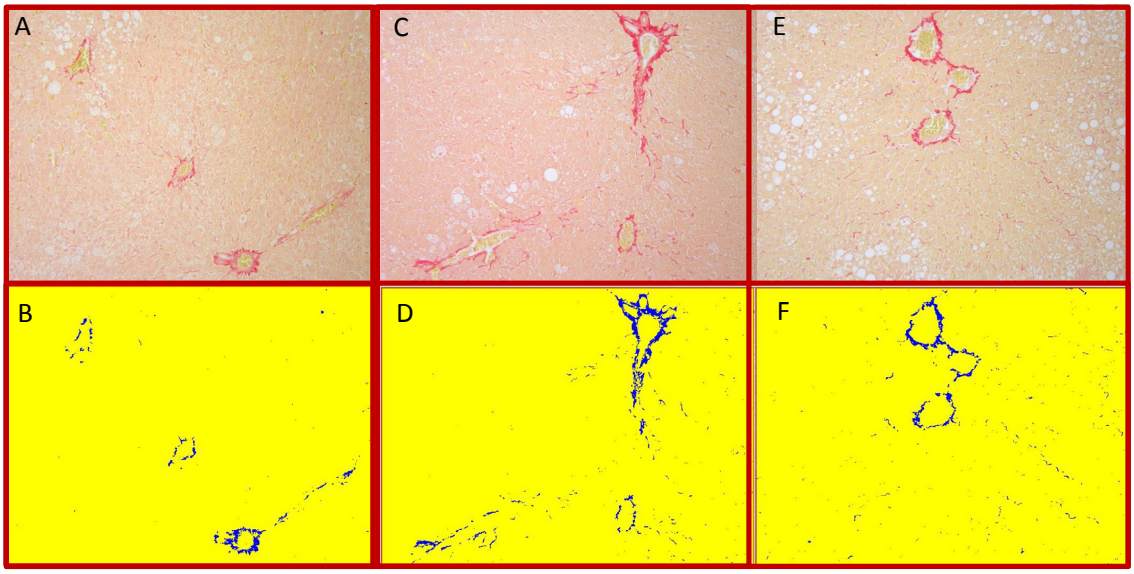


Figure 46. *Liver fibrosis.* ***A****: Sirius red stained (10X). Liver of rat at 24 months fed on virgin olive oil. Note the low macro-microvesicular steatosis with the collagenous reinforcement in red. Quantification (****B****) by image analysis resulted in a collagenous deposit percentage of 0,28%.* ***C****: Sirius red stained (10X). Liver of rat at 24 months fed on sunflower oil. Note the macro-microvesicular steatosis with the collagenous reinforcement in red. Quantification (****D****) by image analysis resulted in a collagenous deposit percentage of 1,52%.* ***E****: Sirius red stained (10X) Liver of rat at 24 months fed on fish oil. Note the low macro-microvesicular steatosis with the collagenous reinforcement in red. Quantification (****F****) by image analysis resulted in a collagenous deposit percentage of 0,76%.*

IV. MITOCHONDRIAL ULTRASTRUCTURAL STUDY.

The ultra-structural analysis of liver mitochondria is shown in table 22. No significant differences between the three groups at 6 months were found. At 24 months, higher values of mitochondrial area and perimeter were found in sunflower oil group, compared with virgin olive and fish groups. Rats fed on virgin olive oil showed higher values of mitochondrial denstity than those fed on sunflower oil. Figure 47, 48 and 49 shows ultrastructural images of hepatic mitochondria of young and old rats fed with virgin olive (VOO), sunflower (SO) or fish oil (FO) at 8.000X, 20.000X and 40.000X respectively. Attending aging, no differences were detected between 6 and 24 months to each fat group.

Table 22. *Effects of dietary fat type (virgin olive oil, sunflower oil or fish oil) on the mitochondrial ultrastructure in liver of young (6 months old) and old (24 months) rats.*

	6 months			**24 months**		
	VOO	SO	FO	VOO	SO	FO
Area (μm^2)	0,34±0,05	0,42±0,05	0,32± 0,07	$0,32\pm0,04^{A}$	$0,49\pm0,05^{B}$	$0,32\pm 0,03^{A}$
Perimeter (μm)	2,08±0,18	2,36±0,13	2,05± 0,18	$2,06\pm0,10^{A}$	$2,56\pm0,08^{B}$	$2,06\pm 0,09^{A}$
Mitochondrial density (unit/ μ^2)	0,73±0,29	0,42±0,04	1,00± 0,14	1,10±0,29 B	0,55±0,05 A	0,60± 0,09 AB
Autophagosome density (unit/ μ^2)	0,04±0,02	0,04±0,01	0,11± 0,04	0,18±0,1	0,26±0,18	0,07± 0,03

Notes: results show means ± standard error of the mean (n = 6).
Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.
Abbreviations: VOO = virgin olive oil; SO = sunflower oil; FO = fish oil.

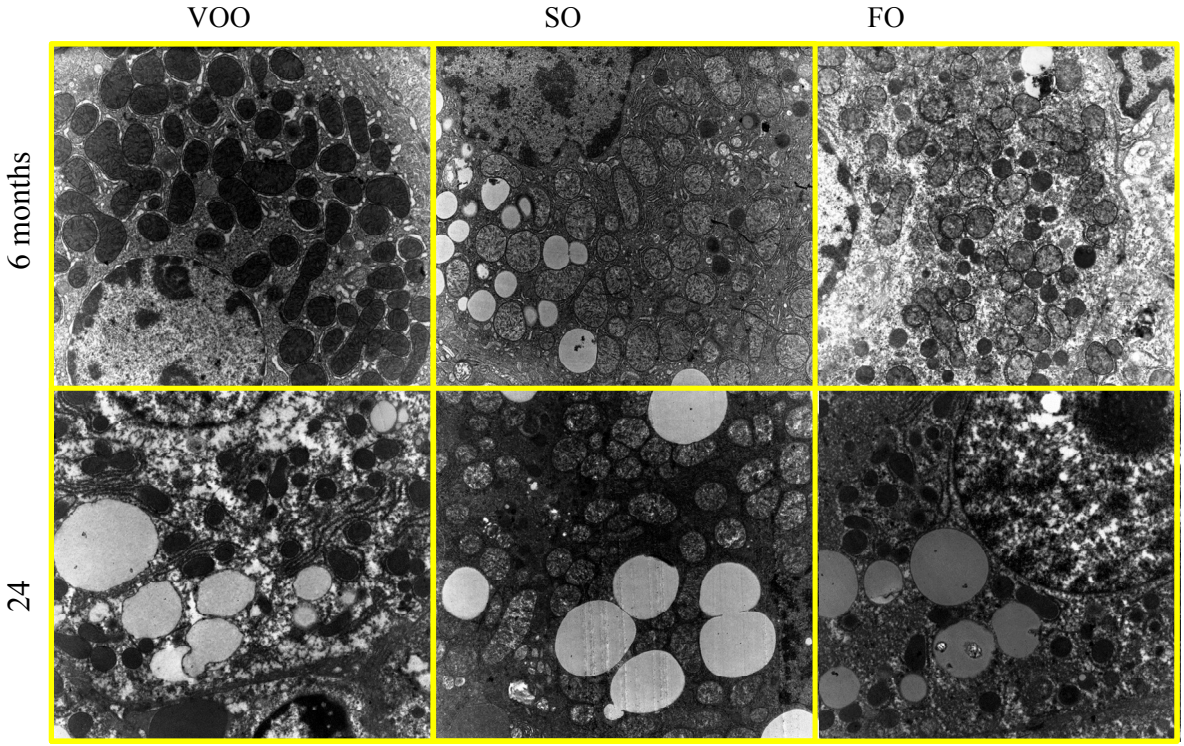


Figure 47. *Ultrastructural images of hepatic mitochondria of young and old rats fed on virgin olive, sunflower or fish oil at 8.000X. Abbreviations: VOO = virgin olive oil; SO = sunflower oil; FO = fish oil.*

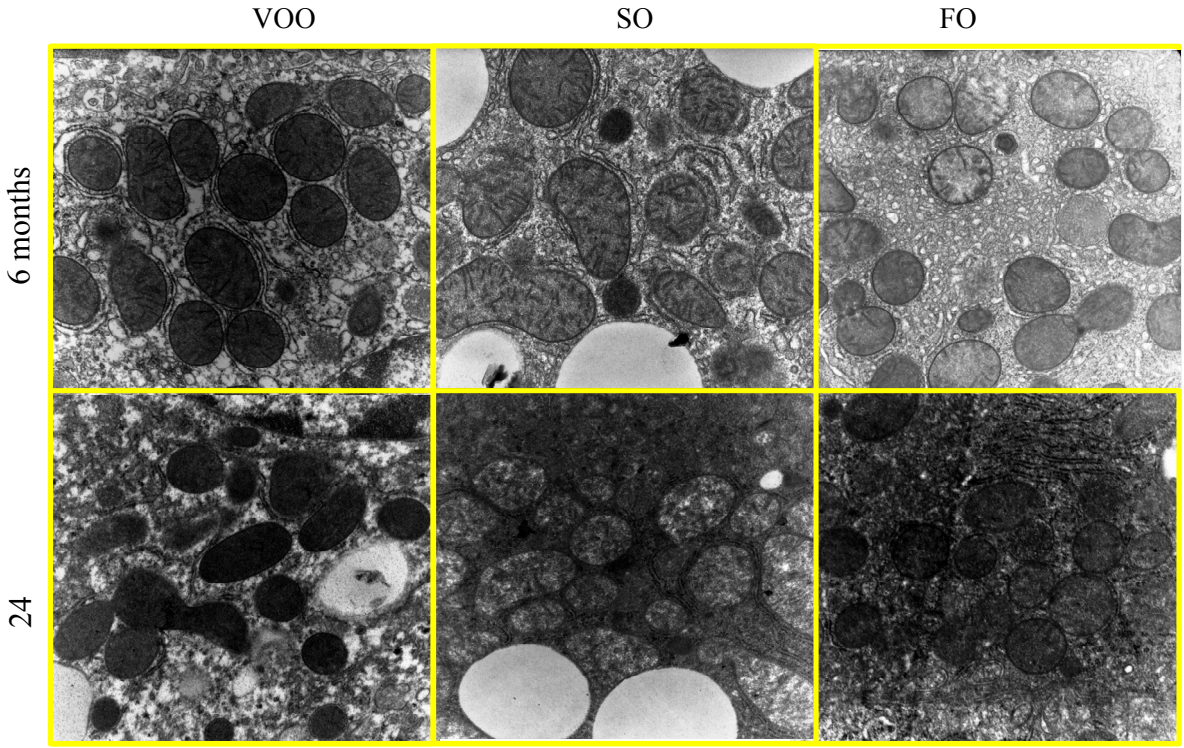


Figure 48. *Ultrastructural images of hepatic mitochondria of young and old rats fed on virgin olive, sunflower or fish oil at 20.000X. Abbreviations: VOO = virgin olive oil; SO = sunflower oil; FO = fish oil.*

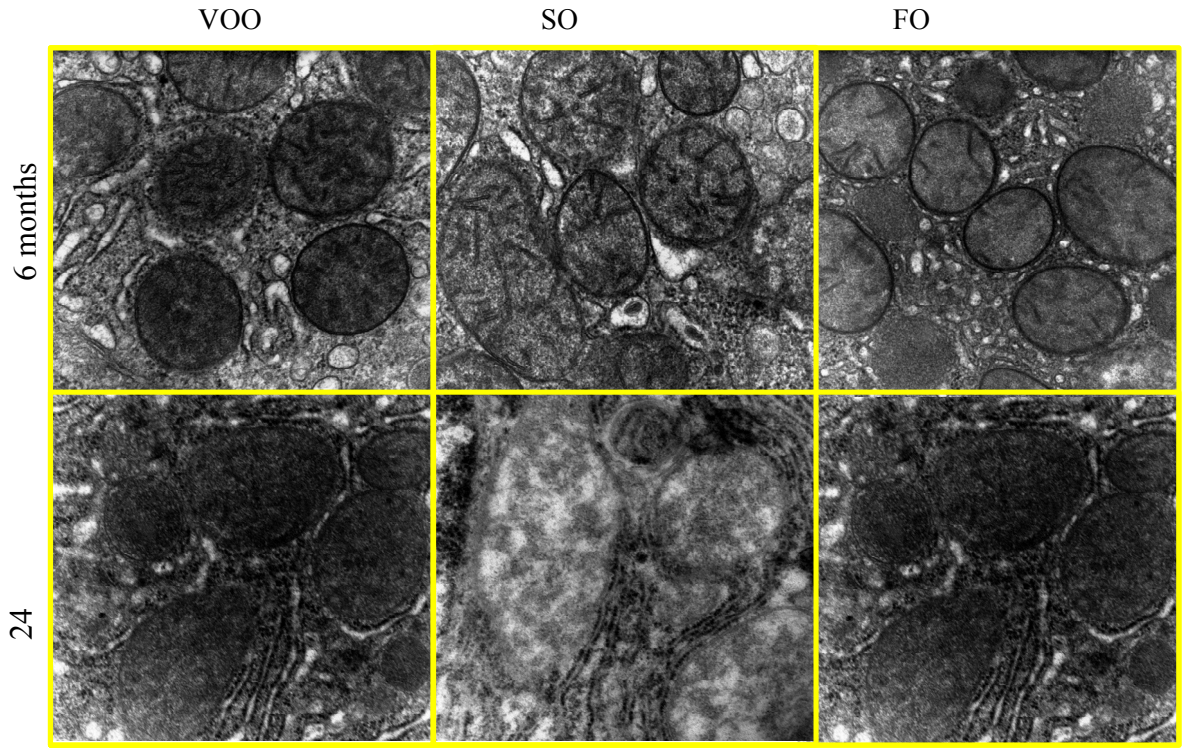


Figure 49. *Ultrastructural images of hepatic mitochondria of young and old rats fed on virgin olive, sunflower or fish oil at 40.000X. Abbreviations: VOO = virgin olive oil; SO = sunflower oil; FO = fish oil.*

V. PGC1α AND TFAM PROTEIN LEVELS.

PGC1α and TFAM protein levels are shown in Figure 50A and 50B, respectively. PG1α did not exhibit differences at 6 or 24 months between any groups. For TFAM, no differences were found at 6 months. However, at 24 months fish oil group reported lower relative amounts than virgin olive and sunflower oil fed rats.

According to aging, relative amount of PG1α did not show significantly differences with aging. Older animals from sunflower oil group showed higher relative amounts of TFAM than young animals.

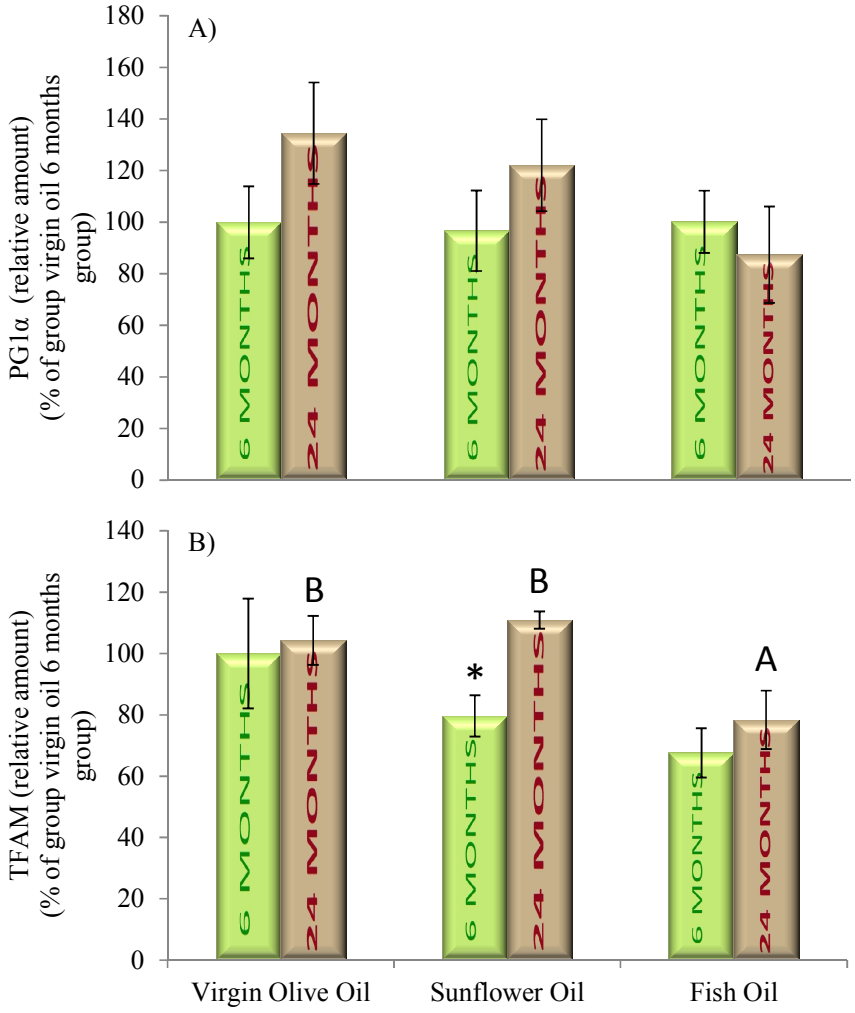


Figure 50. PGC1α and TFAM protein levels liver mitochondria of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

VI. PROTEIN CARBONYLS

Differences in values of carbonyls protein are shown in figure 51. At 6 months, no differences were found between the experimental groups. At 24 months rats from virgin olive oil group showed lower values than those fed on sunflower and fish oils.

Differences due to aging between young and old rats were found in two experimental groups: sunflower oil and fish oil, where old rats showed higher protein carbonyls concentration than young animals.

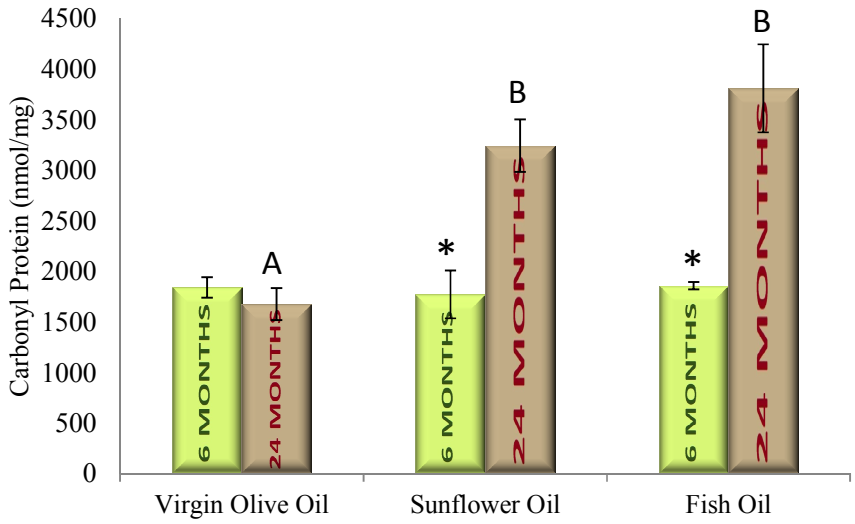


Figure 51. Protein carbonyls plasma concentration of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

VII. ANTIOXIDANT ENZYMES

GPX, selenium-dependent glutathione peroxidase (Se-GPX) and catalase are shown in figure 52A, 52B and 52C, respectively. At 6 months, higher values in rats fed on fish oil than in those fed on virgin olive and sunflower oils were found for total GPX (figure 52A). The amount of Se-GPX was lower, also at 6 months, in virgin olive oil group than in sunflower and fish oil groups (figure 52B). No differences were found in catalase amount (figure 52C).

At 24 months, more differences were identified. Old rats showed again higher values of total GPX in fish oil group than in virgin olive and sunflower oil groups (figure 52A). The activity of catalase at 24 months was lower in rats fed on virgin olive oil than in those fed on fish oil (figure 52C). No differences were found in Se-GPX amount (figure 52B).

Differences due to aging between young and old rats were found in the three experimental groups (virgin olive, sunflower and fish oil) in GPX and Se-GPX, where the highest values were found at 24 months (figures 52A and B). Catalase values at 24 about 6 months were higher only in rats fed on fish oil (figure 52C).

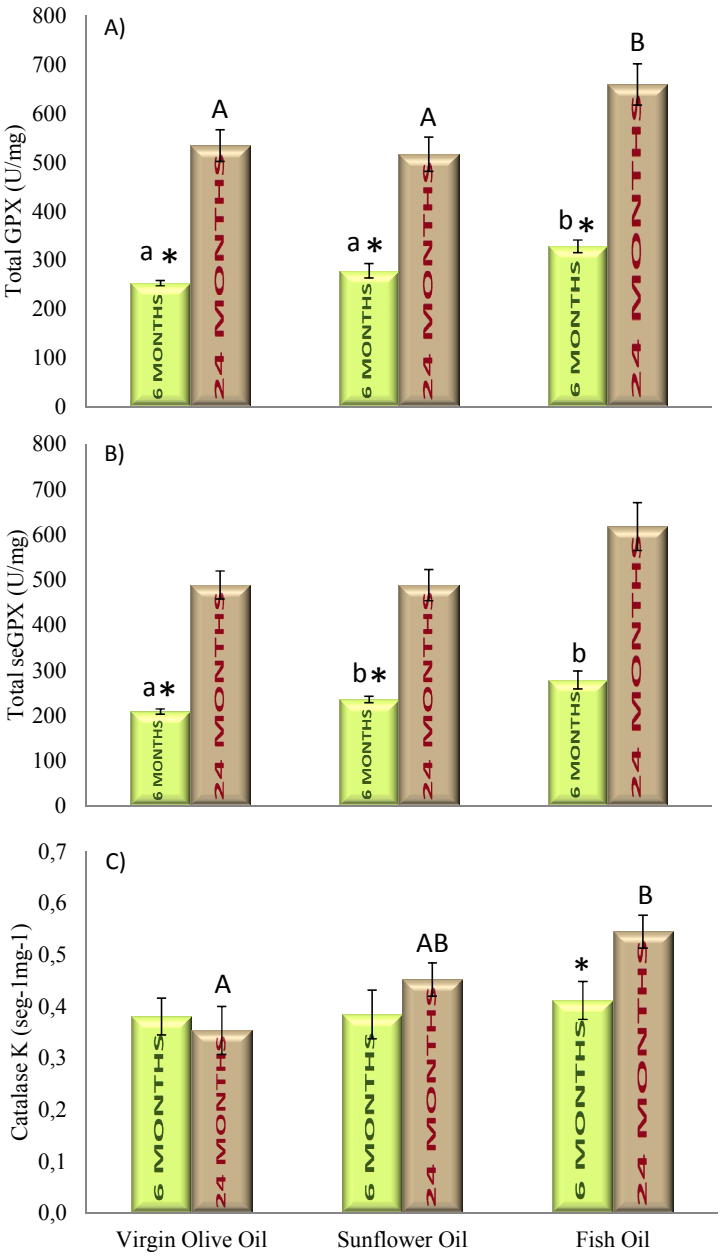


Figure 52. Antioxidant enzymes plasma concentration of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Lower-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 6months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

VIII. VITAMIN E AND CoQ

Vitamin E and CoQ are shown in table 23. At 6 months, only differences between dietary groups were found for the ratio between CoQ_{10} and CoQ_9; with higher values for animals fed on fish oil. At 24 months, lower values were found in fish oil group than sunflower oil group for CoQ_9H_2, total CoQ_9 (CoQ_9H_2+CoQ_9), summatory of the oxidized plus reduced forms of CoQ_9 and CoQ_{10}, $CoQ_{10}H_2$ to CoQ_{10} ratio and $CoQH_2$ to CoQ ratio. The value of CoQ_9H_2 to CoQ_9 ratio was higher in sunflower oil than in virgin olive oil and fish oil groups. The value of CoQ_{10} to CoQ_9 ratio was lower in sunflower oil than in fish oil group.

Differences due to aging between young and old rats for $CoQ_{10}H_2$, CoQ_9, CoQ_{10}, $CoQ_{10}H_2$+CoQ_{10}, CoQ_9H_2+CoQ_9, and summatory of the oxidized plus reduced forms of CoQ_9 and CoQ_{10}were found in the three experimental groups (virgin olive, sunflower and fish oils), where the highest values were found at 24 months. $CoQH_2$ to CoQ ratio values at 24 about 6 months were higher only in rats fed on fish oil. Differences between 6 and 24 months in CoQ_9H_2 were found in sunflower and fish oil groups, that shown lower values in young animals. CoQ_{10} to CoQ_9 ratio values from sunflower oil group was lower at 6 months than at 24 months.

Table 23. *Effects of dietary fat type (virgin olive oil, sunflower oil or fish oil) on the mitochondrial concentration of α-tocopherol and coenzyme Q of liver and cytosol of young (6 months old) and old (24 months) rats.*

	6 months			24 months		
	VIRGIN OLIVE OIL	SUNFLOWER OIL	FISH OIL	VIRGIN OLIVE OIL	SUNFLOWER OIL	FISH OIL
α-tocoferol (µg/mg)	6,2±0,8	6,1±0,7	5,1±0,9	3,8±0,5	4,6±0,7	3,8±0,7
CoQ_9H_2 (µg/mg)	19,5±1,3	13,7±3,5*	13,4±2,9*	48,2±12,7^{AB}	73,8±14,7^{B}	26,2±4,0^{A}
$CoQ_{10}H_2$ (µg/mg)	0,8±0,2*	1,0±0,2*	0,8±0,1*	3,5±0,9	3,9±1,0	1,8±0,1
CoQ_9 (µg/mg)	0,4±0,1*	0,5±0,1*	0,3±0,1*	1,3±0,1	1,1±0,2	0,9±0,1
CoQ_{10} (µg/mg)	0,0±0,0*	0,0±0,0*	0,2±0,1*	0,5±0,1	0,3±0,1	0,4±0,1
CoQ_9H_2 + CoQ_9 (µg/mg)	16,7±3,3*	14,2±3,4*	13,7±2,9*	49,5±12,7^{AB}	74,9±14,7^{B}	27,1±4,0^{A}
$CoQ_{10}H_2$ + CoQ_{10} (µg/mg)	1,0±0,3*	1,1±0,2*	0,9±0,1*	3,9±0,9	4,3±1,1	2,2±0,2
Total CoQ $^{(1)}$ (µg/mg)	17,7±3,5*	15,3±3,5*	14,6±3,0*	52,5±12,9^{AB}	79,1±15,7^{B}	29,3±4,1^{A}
CoQ_9H_2:CoQ_9	73,7±24,0	38,2±10,9	55,1±11,2	38,9±9,7^{A}	84,0±21,2^{B}	29,9±4,9^{A}
$CoQ_{10}H_2$:CoQ_{10}	30,8±13,9	24,4±4,9	7,1±1,7	10,9±4,1^{AB}	14,1±2,8^{B}	4,2±0,4^{A}
[CoQ_9H_2+$CoQ_{10}H_2$]:[CoQ_9+CoQ_{10}]	81,4±25,2	42,5±9,7	48,5±5,1*	36,0±10,4^{AB}	67,2±16,7^{B}	21,8±3,7^{A}
$CoQ_{10}H_2$:CoQ_9H_2	0,0±0,0	0,1±0,0	0,1±0,0	0,1±0,0	0,1±0,0	0,1±0,0
CoQ_{10}:CoQ_9	0,1±0,0^{a}	0,1±0,0^{*a}	0,5±0,1^{b}	0,4±0,1^{AB}	0,3±0,1^{A}	0,5±0,0^{B}
[CoQ_9H_2+ CoQ_9]:[$CoQ_{10}H_2$+CoQ_{10}]	20,0±5,6	14,9±3,4	15,5±2,5	16,2±3,3	18,5±1,9	12,6±1,6

Statistical significances: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Lower-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 6months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.
(1) Summatory of the oxidized plus reduced forms of CoQ_9 and CoQ_{10}.

IX. LIVER MITOCHONDRIAL RESPIRATORY EFFICIENCY

The different ratios of oxygen consumption in isolated mitochondria are shown in figure 53. Differences between groups were found at 6 months; virgin olive oil group exhibited lower OCR than sunflower and fish oil groups. At 24 months, rats fed on fish oil showed lower values of OCR than those fed on sunflower oil.

Concerning the aging effect, higher OCR values were found for aged animals fed on virgin olive or sunflower oil compared with their respective young counterparts, but not for fish oil fed animals.

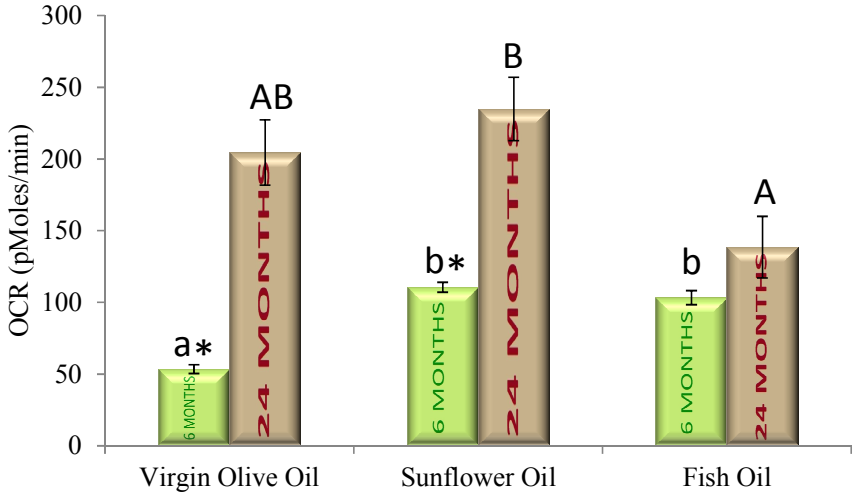


Figure 53. Oxygen consumption ratio in isolate mitochondria of liver of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Lower-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 6months of age. Upper-case letters, when different, represent statistically significant differences (P < 0.05) between the three dietary treatments at 24 months of age.*

X. MITOCHONDRIAL ELECTRON TRANSPORT CHAIN COMPLEX I ACTIVITY

The differences in complex I activity are exposed in figure 54. At 6 months no differences were manifested, however at 24 months fish oil group showed lower activity than virgin olive and sunflower oil groups.

According to aging, older animals fed on virgin olive oil and sunflower oil reported higher activity than animals at 6 months of age.

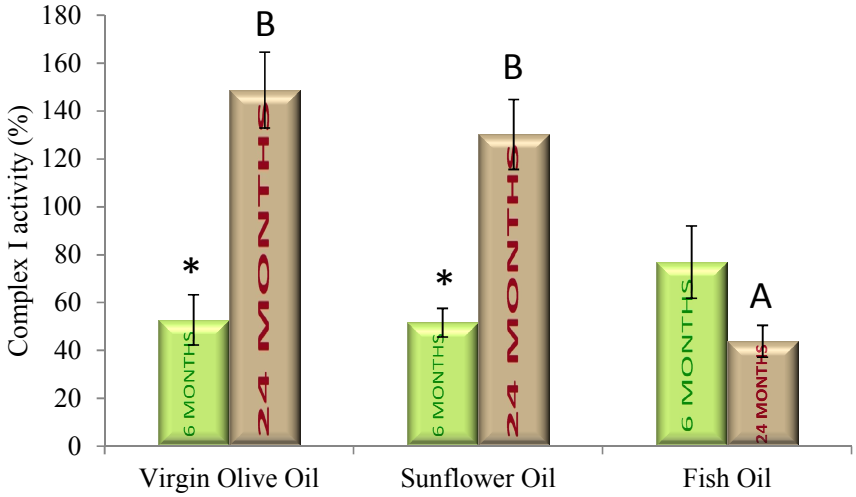


Figure 54. Complex I activity of liver mitochondria of rats fed on different fat sources (virgin olive, sunflower or fish oils) at 6 and 24 months. *Results are mean ± SEM of 6 animals. Statistical significance: asterisk (*) means statistically significant difference between the same dietary treatment for 6 and 24 months of age. Upper-case letters, when different, represent statistically significant differences ($P < 0.05$) between the three dietary treatments at 24 months of age.*

XI. GENE EXPRESSION ANALYSIS

XI.1 Worklist gene selection

To obtain a final worklist, sequences expressed differently with age effect but common among three experimental groups were removed (a total of 70 sequences), so a final list of uncommon sequences among the three experimental groups was obtained, with a total of 882 sequences that are over-expressed or suppressed.

A new supervised hierarchical cluster was made with this new definitive worklist (Figure 55). With this new image some green or red groups are identifiable, indicating gene overexpression or repression of the samples depending on the experimental group.

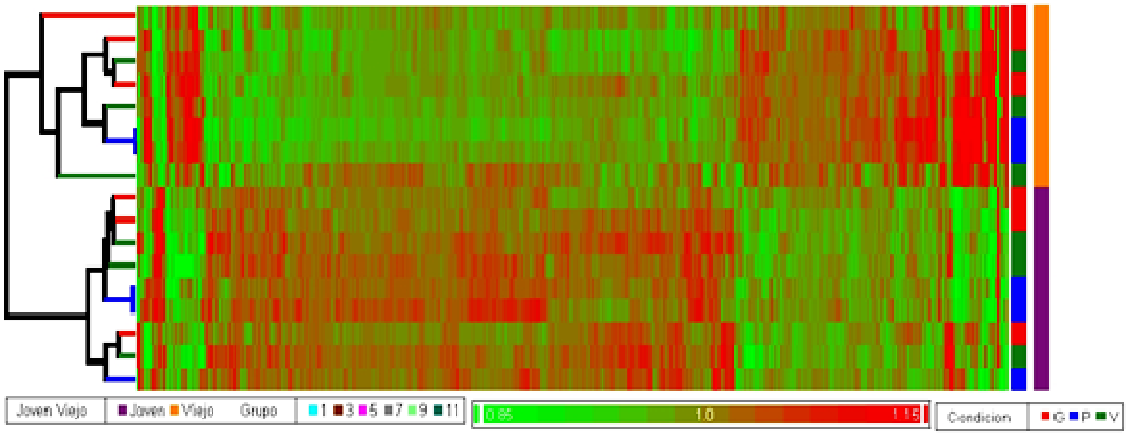


Figure 55. *Two-dimensional hierarchical cluster of genes and samples made from the worklist "uncommon genes", using the Euclidean distance as a measure of clustering of samples and the Pearson correlation for clustering sequences. Each sequence is represented by a color between red and green. Green indicates repressed gene relative to the median for all samples, and red over-expressed gene. Each sample is marked by a color that represents the experimental group: red-sunflower, blue-fish, green-virgin olive.*

XI.2 Functional networks and pathways analyses with Ingenuity Pathways Analysis.

Ingenuity Pathways Analysis (IPA) application (Ingenuity SystemsH, http://www.ingenuity.com/) was used to made two different analyses. On the one hand, we studied the common genes that showed changes in gene expression in the three experimental fats (virgin oil, sunflower and fish), therefore we can think that these changes are due only to aging, and are not affected by the effect of a type of fat. So, we elaborated a dataset containing common genes between the three fats.

By the other hand, we wanted to study the effect of the fat on aging. Accordingly, we built a dataset with uncommon genes, genes whose expression changes were due to the type of fat consumed in the diet.

The results generated by IPA alter running a Core Analysis and that are interesting to our study were the canonical pathways. We used this program to classify genes according the intracellular signaling pathways in which their proteins encoded are involved. The results are shown in a bar chart. To each analysis (common and uncommon), we only selected those

canonical pathways whose bar exceeds the established threshold, and therefore we take as significantly affected.

XI.2.1. Functional networks and pathways analyses to common genes.

The dataset containing 70 genes identifiers in the three fats and the corresponding expression values was uploaded into the IPA application. These genes, called "Common Genes", were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base.

Networks of the focus genes were algorithmically generated based on their connectivity and expression values. Significance of each network and function was assessed using the Fisher's exact tests ($P \leq 0.05$). Functional and transcriptional regulation was predicted by the Activation z-score. The *Z*-score is based on relationships in the molecular pathways (networks), which represent experimentally observed gene expression or transcription events and function annotation data, as derived from the information compiled in the Ingenuity Knowledge Base. These relationships are associated with a direction of change that is either activating (*Z*-score ≥ 2) or inhibiting (*Z*-score ≤ -2).

Our main interest in the IPA analysis is based on the recognition of the canonical pathways which are involved in our selected genes, both common and uncommon. For pathways analysis, the Search feature in IPA was used to create gene lists associated with molecular and cellular processes of interest in aging. IPA identified 61 genes of the 70 sequences introduced, showed in table 24. The bar chart with the canonical pathways in which these genes are involved are shown in figure 56.

Table 24. *Sequences from the worklist "Common Genes" identified by IPA.*

Symbol	Affymetrix	FC
ABCB6	1368159_at	2,48
ABCF3	1371456_at	2,49
ABTB1	1389493_at	2,51
AKR1C4	1393902_at	2,68
ANAPC2	1389087_at	0,06
APOA1BP	1367520_at	2,60
ATP1B3	1398300_at	0,35
ATXN10	1398874_at	2,87
B2M	1371440_at	0,39
BCKDHA	1370897_at	3,17
BTD	1386773_at	3,07
C19orf60	1372746_at	3,21
CARKD	1379759_at	2,62
CASP12	1387605_at	0,21
CELA1	1387819_at	3,77
COQ6	1372753_at	2,64
CPSF3L	1390967_a_at	2,51
CYB561D2	1399111_at	2,72
CYP4A22	1368607_at	0,00
DAPK3	1367621_at	2,45
DHRSX	1388832_at	2,51
DNPEP	1398954_at	2,44

ECSIT	1373420_at	2,63
FAM82A1	1379636_at	0,19
FARS2	1390267_at	2,89
FASTK	1372551_at	2,65
HOGA1	1390421_at	3,83
INMT	1373975_at	1688487,42
INPP5K	1389570_at	2,48
IRF1	1368073_at	0,19
KARS	1371662_at	2,49
MAL2	1372755_at	0,22
MAN2C1	1387920_at	2,79
MED25	1374973_at	2,47
MRPL23	1368004_at	2,59
MRPS5	1388877_at	2,62
MTMR14	1378853_at	2,65
NAGLU	1389746_at	2,69
NDUFS8	1388326_at	2,50
NFE2L2	1367826_at	0,24
OSGEP	1389117_at	2,91
P2RX4	1369743_a_at	3,16
PLK2	1368106_at	0,34
PMPCA	1371008_at	2,41
PSMD2	1398858_at	2,61
PYCRL	1381832_at	2,78
RDH10	1393351_at	0,35
RFT1	1381421_at	2,50
RNASE3	1370777_at	0,00
SLC17A4	1397268_at	15,68
SLC25A11	1370060_at	2,42
SLC34A2	1368168_at	0,00
TMEM180	1388633_at	2,86
TPD52L1	1372626_at	0,07
TSPAN33	1372980_at	3,92
TXNRD2	1368309_at	2,80
UBE2V2	1372143_at	0,36
VAMP5	1398840_at	2,98
WDR18	1375439_at	2,55
YIF1A	1370305_at	2,51

Notes: symbol refers to NCBI gene symbol; Affymetrix refers to Affymetrix gene identification.

Abbreviations: FC = fold change.

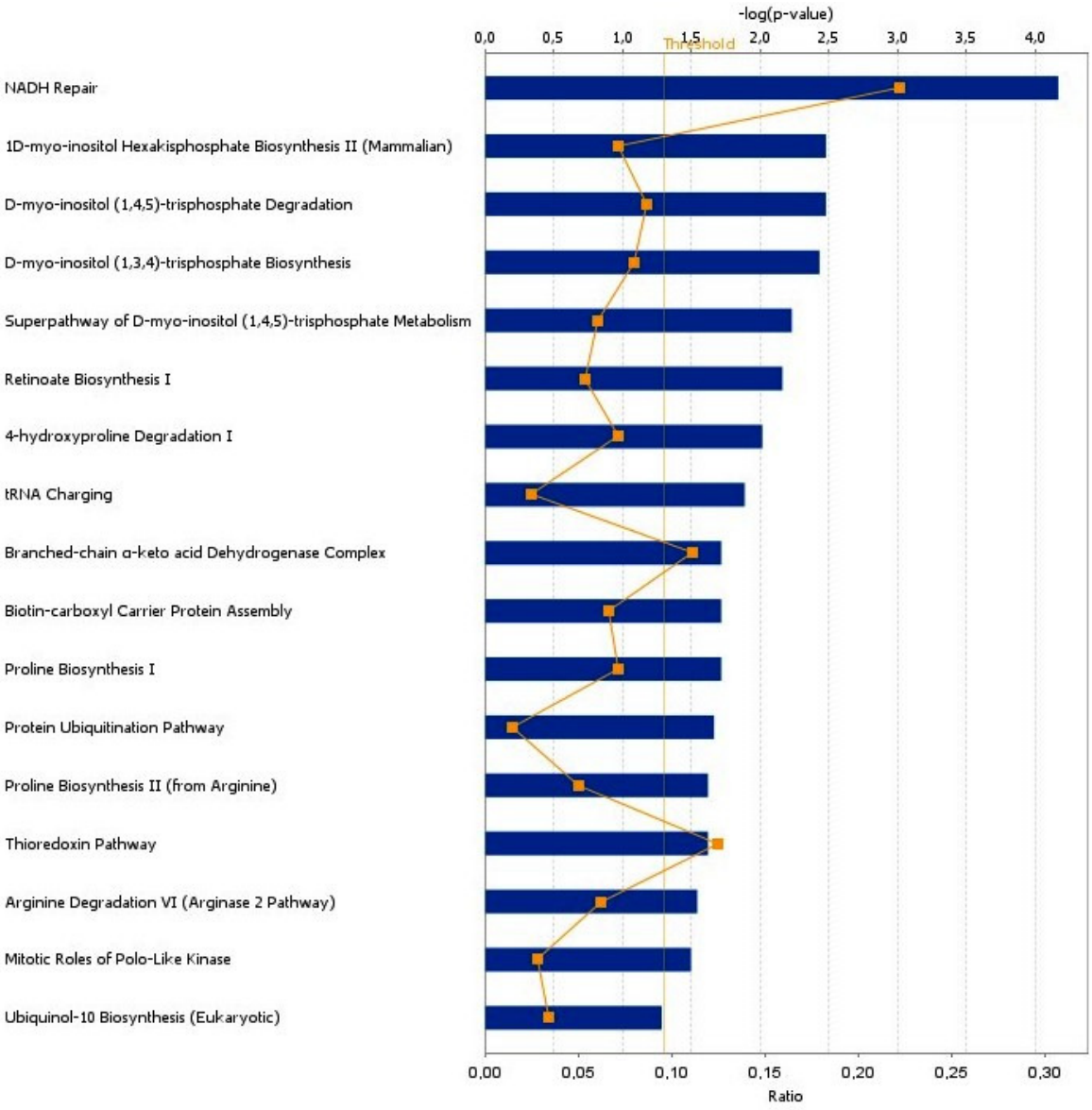


Figure 56. *Canonical pathways, whose –log(P-value) is greater than the threshold, identified from the "Common Genes" list.*

The bar chart analysis did not reveal any canonical pathway of interest in our studied physiological processes, so that we could discard the aging effect as solely responsible for the molecular and structural changes detected in the previous analyzes of this study.

XI.2.2. Functional networks and pathways analyses to uncommon genes.

The dataset containing 881 genes identifiers in one or two of fats and the corresponding expression values was uploaded into the IPA application (annex III). These genes, called "Uncommon Genes", were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. IPA identified 697 genes of the total of sequences introduced, shown in annex IV.

The canonical pathways study of uncommon genes was considerably more complex and the bar chart cannot be represented in a picture, so the list of 506 canonical pathways selected by IPA from de worklist "Uncommon Genes" are shown in annex V. This table also shows in which of the three dietary treatments is present each pathway. As next step, this list was reduced by removing those canonical pathways whose –log (*P*-value) was lower than the threshold. The resulted list is shown in annex VI, with 82 pathways that include all genes from each dietary fat (virgin olive, sunflower or fish oils).

The next step consisted in the selection of the six canonical pathways with the highest signification (higher –log of the *P*-value) together with another six pathways that were considered of interest to our study. With these 12 canonical pathways (table 25) a more detailed study was performed, locating genes, analyzing to what extent their expression was altered and indicating the relative position (in terms of IPA interest) for each canonical pathway and dietary fat. In table 26 are shown the 90 uncommon genes differently expressed identified in the 12 canonical pathways.

Table 25. *Non common genes of selected canonical pathways.*

	VIRGIN OLIVE OIL				SUNFLOWER OIL				FISH OIL			
Ingenuity canonical pathways	-log (*P*-value)[1]	Ratio[2]	Relative CP position[3]	Genes	-log (*P*-value)[1]	Ratio[2]	Relative CP position[3]	Genes	-log (*P*-value)[1]	Ratio[2]	Relative CP position[3]	Genes
Mitochondrial dysfunction	2,73	5,91 E-02	2	APH1B,ATP5D,ATP5G2,GPD2,MAPK9,NDUFA9,NDUFS2,NDUFS3,NDUFS7,SURF1,UQCRC1			-		7,88	1,02 E-01	1	SDHB,NDUFB3,NDUFA9,ATP5D,UQCRH,UQCR11,NDUFS7,PRDX5,SURF1,ATP5G2,NDUFS3,FIS1,NDUFA6,TXN2,NDUFB7,NDUFS2,CYC1,UQCRC1,NDUFA8
Cell cycle regulation by BTG family proteins			-		3,73	1,11 E-01	1	PPP2CA,BTG1,PPP2R5E,PPP2R1B	1,89	1,11 E-01	9	RB1,PPP2R5E,PPP2R1B,CCND1

Nrf2-mediated oxidative stress response	3,44	7,29 E-02	1	AKR7A2,MAP2K6,RRAS,NQO1,PIK3C2G,MAPK9,DNAJA1,CLPP,TXNRD1,GSTO1,MGST2,KEAP1,VCP,UBE2E3			-				-	
ERK/MAPK signaling			-		3,09	3,4 E-02	2	PRKCI,ATF1,PPP2CA,DUSP1,PPP2R5E,PPP2R1B,EIF4E			-	
DNA double-strand break repair by non-homologous end joining	2,42	1,58 E-01	6	XRCC6,XRCC5,XRCC1			-		1,37	1,05 E-01	27	XRCC6,XRCC1
mTOR signaling			-		2,35	2,84 E-02	8	PRKCI,RHOQ,PPP2CA,PPP2R5E,PPP2R1B,EIF4E			-	

Telomere extension by telomerase	2,2	1,76 E-01	7	XRCC6,HNRNPA2B1,XRCC5			-				-	
Xenobiotic metabolism signaling	1,72	4,35 E-02	9	MAP2K6,ALDH4A1,Ces1c,RRAS,NQO1,PIK3C2G,MAPK9,GSTO1,PNPLA7,MGST2,KEAP1,RXRA,ALDH16A1	2,23	2,34 E-02	10	Ces1c,PRKCI,MGST2,PPP2CA,PPP2R5E,ALDH16A1,PPP2R1B			-	
Protein ubiquitination pathway			-				-		1,72	4,85 E-02	14	PSMA7,DNAJC15,USP2,TAP1,PSMB6,FZR1,PSMB7,HSCB,DNAJC4,STUB1,PSMB1,UBE2J1,USP25
TCA cycle II (eukaryotic)	1,67	7,32 E-02	10	ACO2,DLST,FH			-		1,65	7,32 E-02	17	SDHB,IDH3G,DLST
Myc mediated apoptosis	1,66	8,33 E-02	11	RRAS,YWHAZ,PIK3C2G,M			-				-	

signaling		APK9,BAX					
Cyclins and cell cycle regulation	-		1,54	3,37 E-02	29	PPP2CA,PPP2R5E,PPP2R1B	-

[1] *P* value: probability that a canonical pathway be affected by our treatment and it's calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.
[2] Ratio: percentage of genes in a pathway that were also found in our uploaded list. It's calculated as the number of focus genes that map to the canonical pathway divided by the total number of genes that map to the canonical pathway.
3 Relative position of the canonical pathway within all canonical pathways identified for this fat.
Abreviations: CP = canonical pathway

Table 26. *85 uncommon genes that were differentially expressed between one or two of fats in the 12 selected canonical pathways.*

Symbol	Affymetrix	FC	Oil
ACO2	1367589_at	2,71	V
AKR7A2	1367843_at	2,69; 2,69	V;F
ALDH16A1	1374661_at	2,57; 2,53	V;S
ALDH3A2	1368365_at	0,38	F
ALDH4A1	1372277_at	2,99	V
APH1B	1388440_at	4,20	V
ARAF	1368390_at	2,51; 2,46	V;F
ATF1	1389623_at	0,41; 0,38	V;S
ATG13	1388336_at	2,75	V
ATP5D	1370278_at	2,39; 2,79	V;F
ATP5G2	1370207_at	2,41; 2,48	V;F
BAX	1369122_at	0,31	V
BTG1	1367657_at	0,36; 0,37	V;S
CCND1	1383075_at	0,00	F
Ces1c	1371100_at	4,47; 5,10	V;S
CLPP	1371789_at	2,55; 2,79	V;F
CYC1	1371342_at	2,91	F
DLST	1370879_at	2,58; 2,89	V;F
DNAJA1	1368852_at	0,36	V
DNAJC15	1373064_at	2,89	F
DNAJC4	1372428_at	2,85	F
DUSP1	1368147_at	0,09	S
EIF4E	1398799_at	0,39; 0,39	V;S
EIF4G2	1367469_at	0,41	F
FIS1	1371594_at	2,53	F
GPD2	1387670_at	0,10	V
GSTO1	1389832_at	2,69; 2,62	V;F
HERPUD1	1367741_at	3,71	F
HNRNPA2B1	1398883_at	0,35; 0,37	V;F
IDH3G	1370865_at	2,71	F
JUND	1393138_at	0,38	F
KEAP1	1370066_at	2,51; 2,55	V;F
MAP2K6	1387809_at	6,96	V
MAPK9	1368646_at	0,26	V
MGST2	1372599_at	3,25; 3,20	V;S
MKNK2	1372038_at	6,54; 5,13	V;F
MYC	1368308_at	0,10	F
NDUFA6	1379243_at	2,64	F
NDUFA8	1371355_at	2,69	F
NDUFA9	1388323_at	2,57; 2,69	V;F
NDUFB3	1373041_at	2,77	F
NDUFB7	1388343_at	2,75	F
NDUFS2	1371482_at	2,50	V
NDUFS3	1388364_at	2,62; 2,91	V;F
NDUFS7	1371912_at	2,57; 2,73	V;F

NQO1	1387599_a_at	15,35	**V**
PIK3C2G	1369050_at	5,06	**V**
PPP2CA	1388805_at	0,40	**S**
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**
PRDX5	1367677_at	3,14	**F**
PRKCI	1373047_at	0,32	**S**
PSMA7	1371869_at	2,57	**F**
PSMB1	1398812_at	2,46	**F**
PSMB6	1398777_at	2,62	**F**
PSMB7	1367656_at	2,77	**F**
PSMB9	1370186_at	0,32	**V**
RAC2	1372404_at	0,33	**F**
RB1	1388185_at	0,17	**F**
RHOA	1399027_at	0,38	**V**
RHOB	1369958_at	0,22; 0,16	**V**;**F**
RHOQ	1386967_at	0,29; 0,27	**S**;**F**
RND2	1372521_at	3,12	**F**
RRAS	1388729_at	2,66	**V**
RXRA	1371668_at	2,83	**V**
SDHB	1372123_at	2,73	**F**
STAT1	1372757_at	0,33	**F**
STUB1	1398910_at	2,87	**F**
SURF1	1398857_at	2,53; 2,69	**V**;**F**
TAP1	1388149_at	0,21	**F**
TLN1	1389002_at	2,69	**V**
TXN2	1398844_at	2,79	**F**
TXNRD1	1386958_at	0,29	**V**
UBE2E3	1389534_at	0,38	**V**
UQCR11	1375197_at	2,69	**F**
UQCRC1	1388301_at	2,60; 2,68	**V**;**F**
UQCRH	1371415_at	2,71	**F**
USP2	1387703_a_at	27,67	**F**
USP25	1378679_at	0,38	**F**
VCP	1367455_at	2,50	**V**
XIAP	1369248_a_at	0,03	**V**
XRCC1	1387129_at	2,69; 2,81	**V**;**F**
XRCC5	1370931_at	2,83	**V**
XRCC6	1370537_at	2,75; 2,95	**V**;**F**
YWHAZ	1387774_at	0,36	**V**

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

Figures 57 to 68 show images provides by IPA to the selected canonical pathways, reflecting the relations between the genes involved. For each canonical pathway, genes differentially expressed in each dietary fat were highlighted.

XI.2.2.1. Mitochondrial dysfunction canonical pathway analysis.

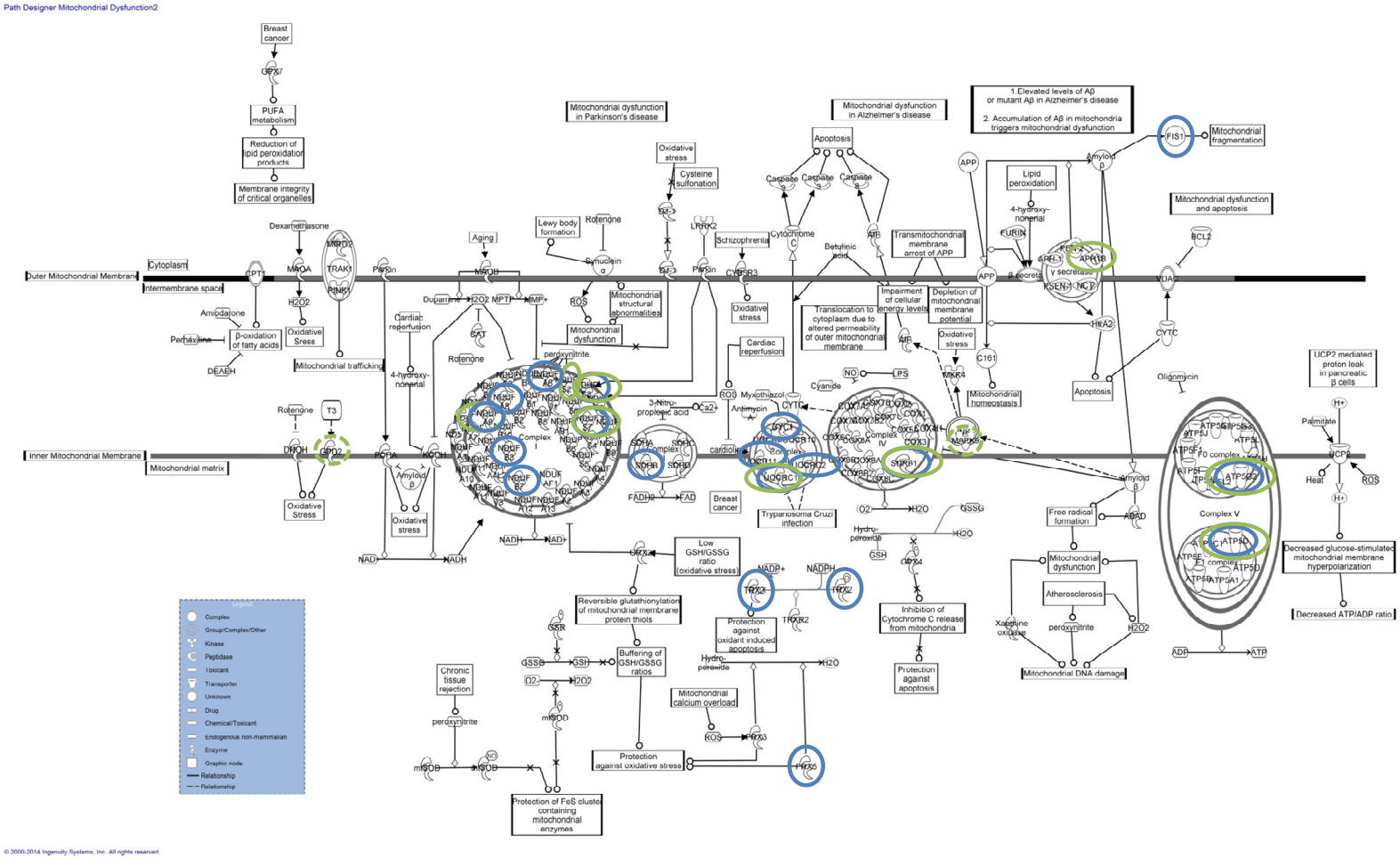


Figure 57. *Mitochondrial dysfunction canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Mitochondrial dysfunction canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the mitochondrial dysfunction canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 57.

Differences in gene expression due to virgin olive or fish oil treatments were found in 22 genes. Most of these differentially expressed genes were located in complexes of the mtETC at the inner membrane, but also some of them were found in the other mitochondrial compartments. Rats fed on sunflower oil did not present genes differentially expressed in mitochondrial dysfunction canonical pathway (table 27).

Table 27. *Twenty two microarray-differentially expressed genes present at the Mitochondrial dysfunction canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
APH1B	1388440_at	4,20	**V**	Outer mitochondrial membrane
ATP5D	1370278_at	2,39; 2,79	**V**;**F**	Complex V
ATP5G2	1370207_at	2,41; 2,38	**V**;**F**	Complex V
CYC1	1371342_at	2,91	**F**	Complex III
FIS1	1371594_at	2,53	**F**	Cytoplasm
GPD2	1387670_at	0,10	**V**	Inner mitochondrial membrane
MAPK9	1368646_at	0,26	**V**	Intermembrane space
NDUFA6	1379243_at	2,64	**F**	Complex I
NDUFA8	1371355_at	2,69	**F**	Complex I
NDUFA9	1388323_at	2,57	**F**	Complex I
NDUFB3	1373041_at	2,77	**F**	Complex I
NDUFB7	1388343_at	2,75	**F**	Complex I
NDUFS2	1371482_at	2,50	**V**	Complex I
NDUFS3	1388364_at	2,62; 2,91	**V**;**F**	Complex I
NDUFS7	1371912_at	2,57; 2,73	**V**;**F**	Complex I
PRDX5	1367677_at	3,14	**F**	Mitochondrial matrix
SDHB	1372123_at	2,73	**F**	Complex IV
SURF1	1398857_at	2,53; 2,69	**V**;**F**	Complex II
TXN2	1398844_at	2,79	**F**	Mitochondrial matrix
UQCR11	1375197_at	2,69	**F**	Complex III
UQCRC1	1388301_at	2,60; 2,68	**V**;**F**	Complex III
UQCRH	1371415_at	2,71	**F**	Complex III

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.2. Nrf2-mediated oxidative stress response canonical pathway analysis

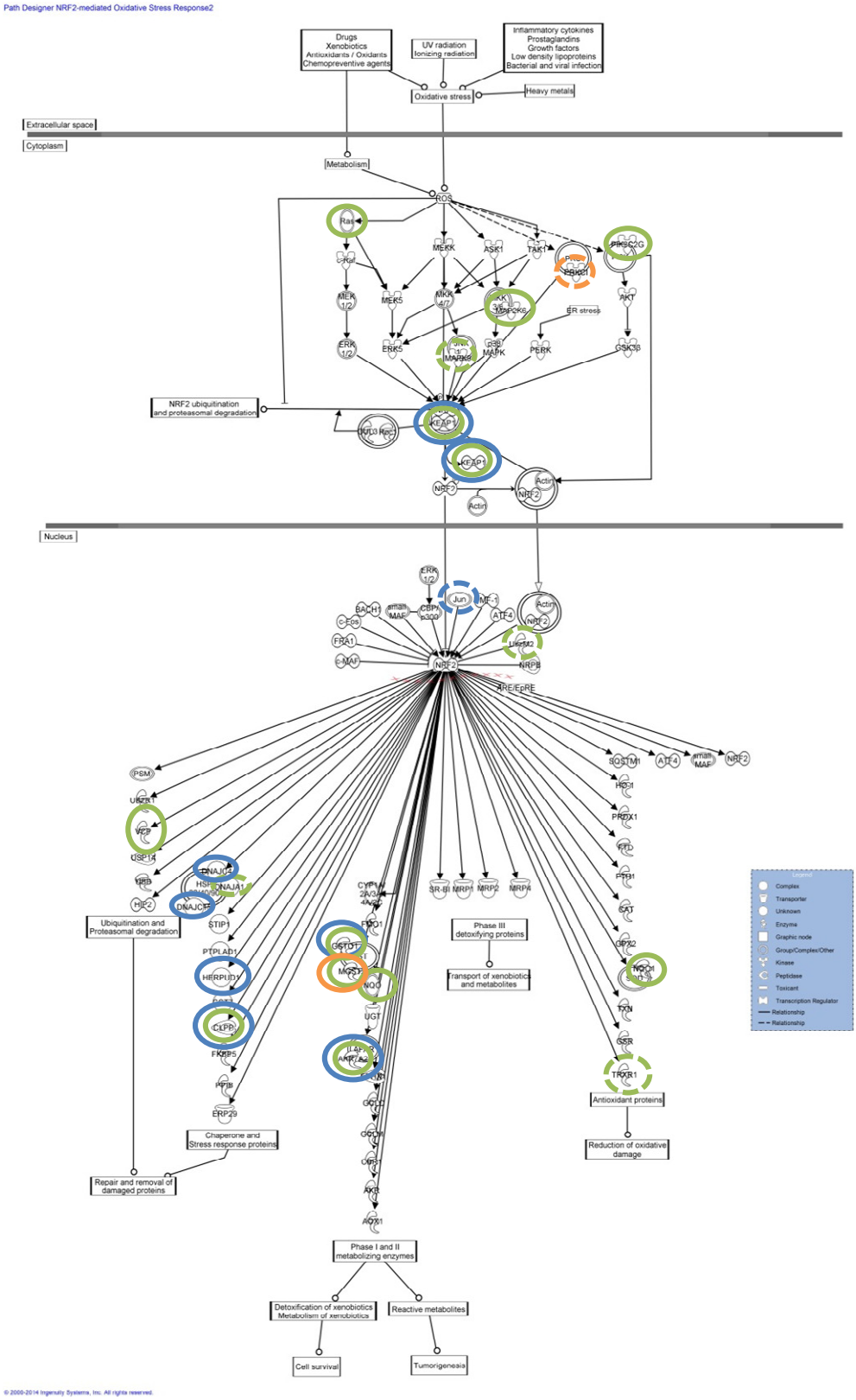


Figure 58. *Nrf2-related oxidative stress response canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish.*

To the Nrf2-mediated oxidative stress response canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Nrf2-mediated oxidative stress response canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish, as is shown in figure 58.

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 19 genes. From the 14 differentially expressed genes in virgin olive oil treatment, 10 were overexpressed and 4 of them were repressed. Most of them were located in the cellular nucleus. Only 2 genes were differentially expressed in sunflower oil treatment, one of them, located in the cellular nucleus, was overexpressed. The other one, located in the cytoplasm, was repressed. Eight differentially expressed genes were found in rats fed on fish oil in the cytoplasm and the cellular nucleus. All of them were overexpressed except one located in the cellular nucleus (table 28).

Table 28 *Nineteen microarray-differentially expressed genes present at the Nrf2-mediated oxidative stress response canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
AKR7A2	1367843_at	2,69; 2,69	V;F	Nucleus
CLPP	1371789_at	2,55; 2,79	V;F	Nucleus
DNAJA1	1368852_at	0,36	V	Nucleus
DNAJC15	1373064_at	2,89	F	Nucleus
DNAJC4	1372428_at	2,85	F	Nucleus
GSTO1	1389832_at	2,69; 2,62	V;F	Nucleus
HERPUD1	1367741_at	3,71	F	Nucleus
JUND	1393138_at	0,38	F	Nucleus
KEAP1	1370066_at	2,51; 2,55	V;F	Cytoplasm
MAP2K6	1387809_at	6,96	V	Cytoplasm
MAPK9	1368646_at	0,26	V	Cytoplasm
MGST2	1372599_at	3,25; 3,20	V;S	Nucleus
NQO1	1387599_a_at	15,35	V	Nucleus
PIK3C2G	1369050_at	5,06	V	Cytoplasm
PRKCI	1373047_at	0,32	S	Cytoplasm
RRAS	1388729_at	2,66	V	Cytoplasm
TXNRD1	1386958_at	0,29	V	Nucleus
UBE2E3	1389534_at	0,38	V	Nucleus
VCP	1367455_at	2,50	V	Nucleus

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

XI.2.2.3. Cell cycle regulation by BTB family proteins canonical pathway analysis

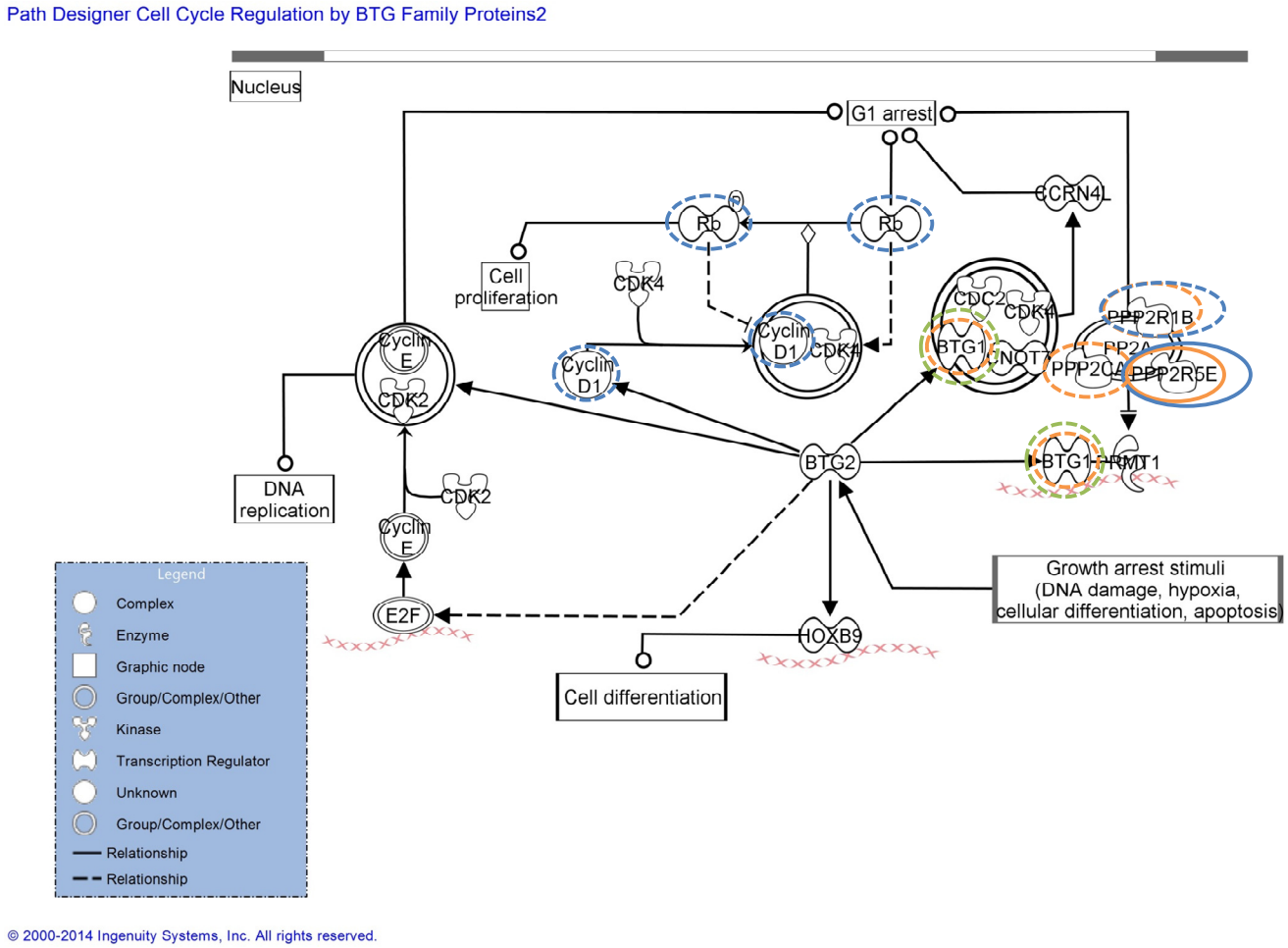


Figure 59. *Cell cycle regulation by BTG family proteins canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish.*

To the Cell cycle regulation by BTB family proteins canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Cell cycle regulation by BTB family proteins canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish, as is shown in figure 59.

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 6 genes, all located at the nucleus and involved in the cell cycle G1 phase. Only one gene was differentially expressed in virgin olive oil treatment. From the 4 differentially expressed in sunflower oil treatment, 3 of them were repressed and only one of them was overexpressed. Rats fed on fish oil presented 4 differentially expressed genes, again 3 of which were repressed and only one of them was overexpressed (table 29).

Table 29. *Six microarray-differentially expressed genes present at the Cell cycle regulation by BTG family proteins canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
BTG1	1367657_at	0,36; 0,37	**V**;**S**	Nucleus
CCND1	1383075_at	0,00	**F**	Nucleus
PPP2CA	1388805_at	0,40	**S**	Nucleus
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**	Nucleus
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**	Nucleus
RB1	1388185_at	0,17	**F**	Nucleus

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

XI.2.2.4. Cyclins and cell cycle regulation canonical pathway analysis

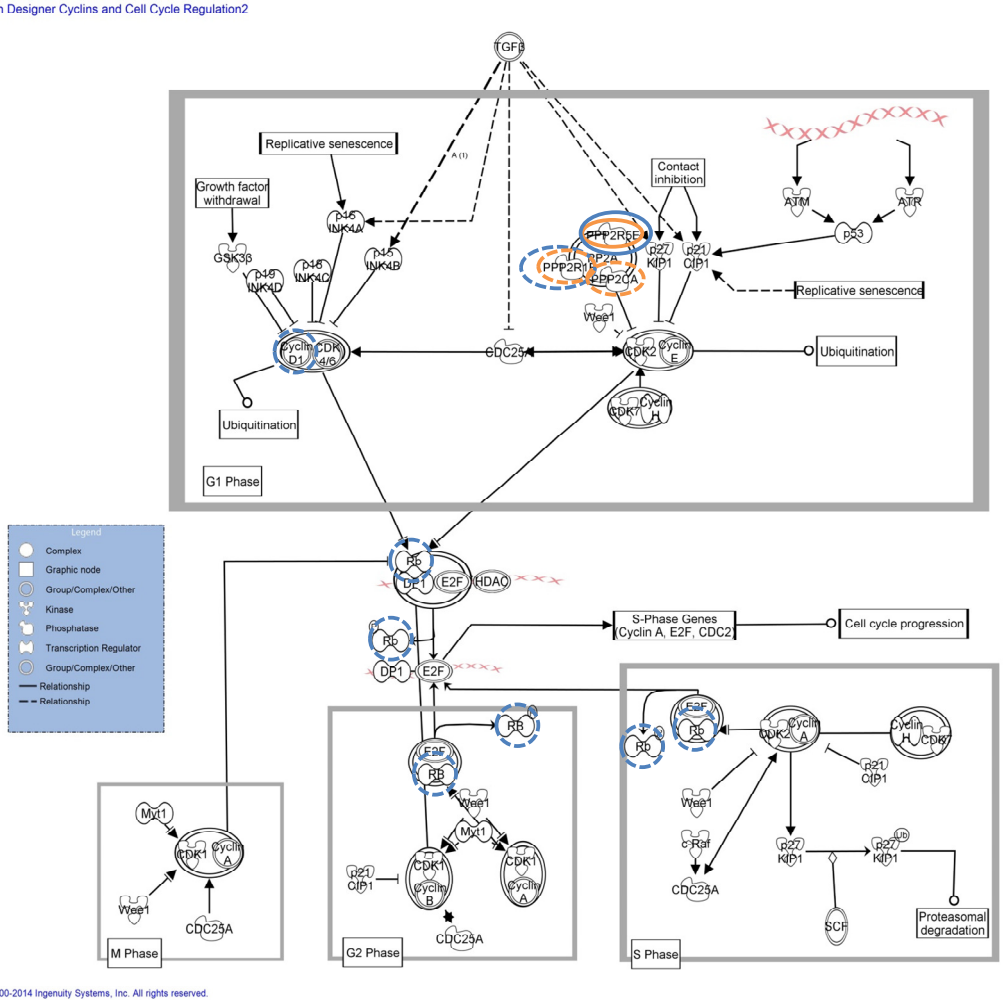


Figure 60. *Cyclins and cell cycle regulation canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: orange to sunflower and blue to fish.*

To the Cyclins and cell regulation canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Cyclins and cell regulation canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: orange to sunflower and blue to fish, as is shown in figure 60.

Expression differences due to dietary treatment with sunflower or fish oil were found in 5 genes, all located at the nucleus and participating in the G1, S and G2 phases of the cell cycle. The 3 differentially expressed genes in sunflower oil treatment participate in G1 phase of the cell cycle. Two of them were repressed and the other was overexpressed. From the 4 differentially expressed genes in fish oil treatment, 3 of them participate in G1 phase of the cell cycle, 2 being repressed and the other one being overexpressed. The other differentially expressed gene to this fat participates in G2 and S phases of the cell cycle and it was repressed. Rats fed on virgin olive oil did not show differences in the expression of genes involved in this canonical pathway (table 30).

Table 30. *Five microarray-differentially expressed genes present at the Cyclins and cell regulation canonical pathway.*

Symbol	Affymetrix	FC	Oil	Cell cycle phase
CCND1	1383075_at	0,00	**F**	G1 Phase
PPP2CA	1388805_at	0,40	**S**	G1 Phase
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**	G1 Phase
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**	G1 Phase
RB1	1388185_at	0,17	**F**	G2 and S Phase

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; S = sunflower; F = fish oil.

XI.2.2.5. DNA double-strand break repair by non-homologous end joining canonical pathway analysis

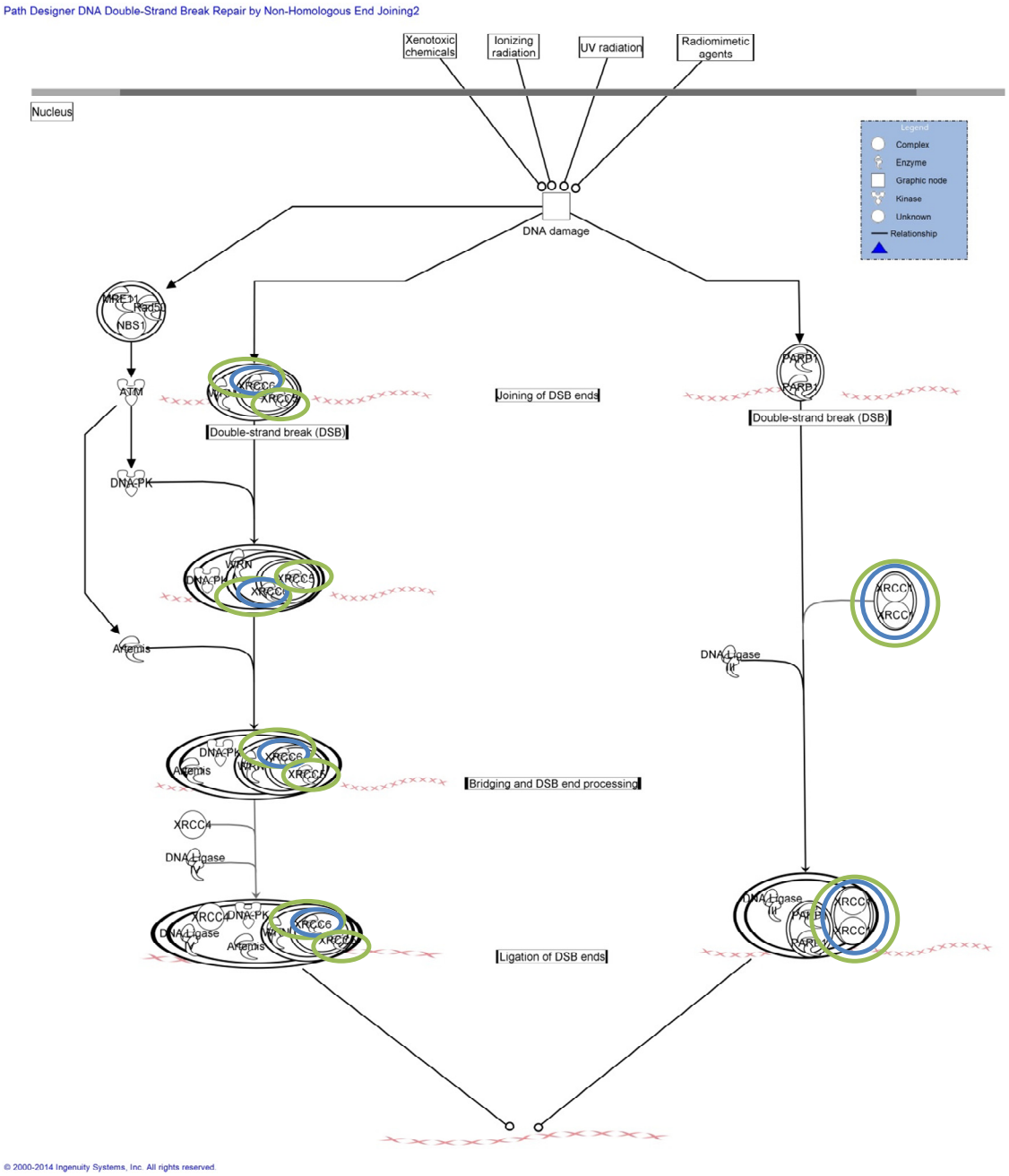


Figure 61. *DNA double-strand break repair by non-homologous end joining canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the DNA double-strand break repair by non-homologous end joining canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the DNA double-strand break repair by non-homologous end joining canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 61.

Expression differences due to dietary treatment with virgin olive or fish oil were found in 3 genes, all located at the nucleus and participating throughout all the repair process. The 3 were overexpressed in virgin olive oil treatment, and only 2 of them were overexpressed in fish oil treatment. Rats fed on sunflower oil did not show differences in the expression of genes involved in this canonical pathway (table 31).

Table 31. *Three microarray-differentially expressed genes present at the DNA double-strand break repair by non-homologous end joining canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
XRCC1	1387129_at	2,69; 2,81	**V**;**F**	Nucleus
XRCC5	1370931_at	2,83	**V**	Nucleus
XRCC6	1370537_at	2,75; 2,95	**V**;**F**	Nucleus

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.6. mTOR signaling canonical pathway analysis

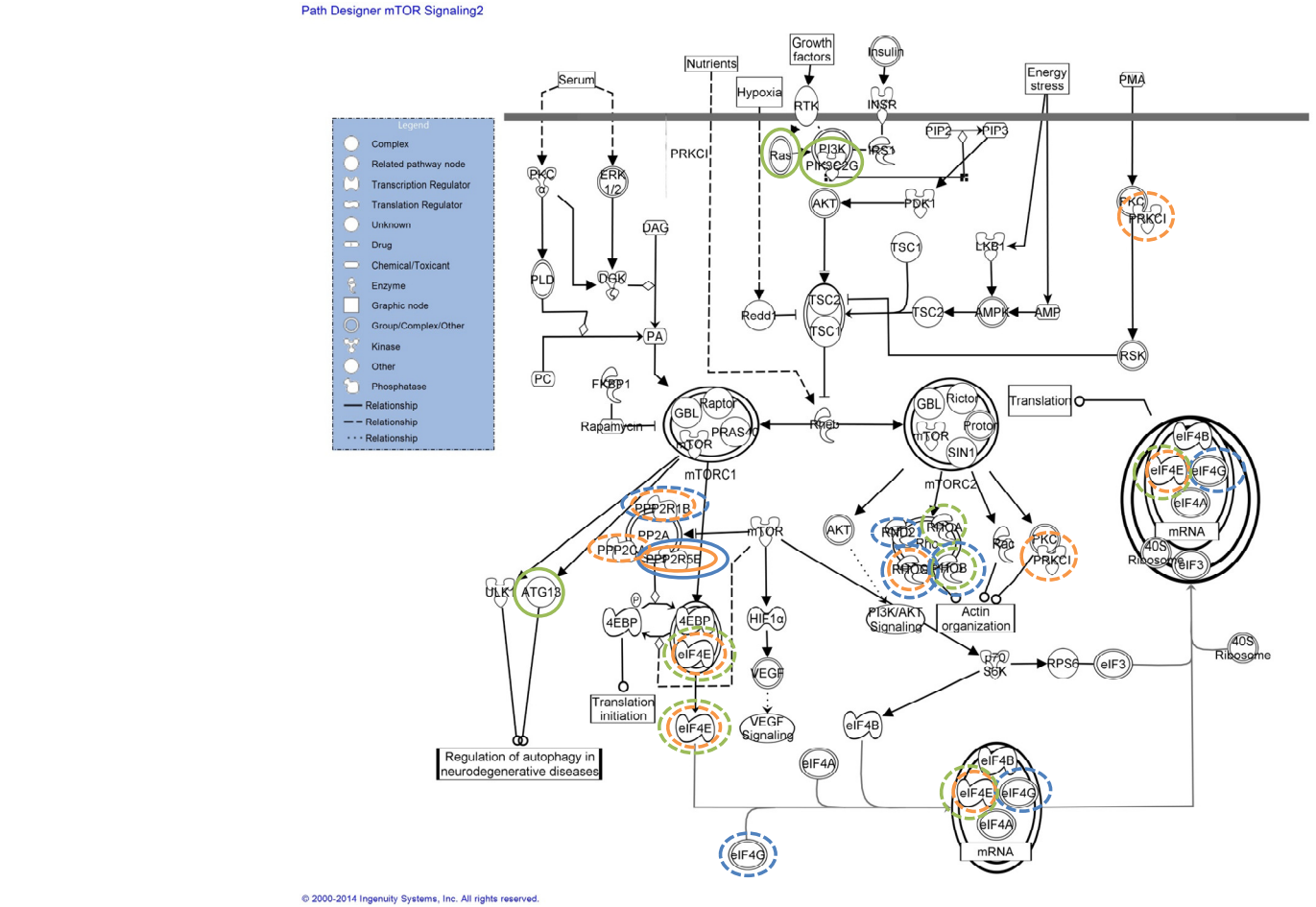


Figure 62. *mTOR signaling canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish.*

To the mTOR signaling canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the mTOR signaling canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish, as is shown in figure 62.

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 13 genes, all located at the cytoplasm and related with the ribosome. Six genes were differentially expressed in virgin olive oil treatment, 3 of them overexpressed and the other 3 repressed. From the 6 differentially expressed genes in sunflower oil treatment, only one of which was repressed while the other 5 genes were overexpressed. Fish oil treatment presented 6 differentially expressed genes, two of them overexpressed and the other 4 repressed (table 32).

Table 32. *Thirteen microarray-differentially expressed genes present at the mTOR signaling canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
ATG13	1388336_at	2,75	**V**	Cytoplasm
EIF4E	1398799_at	0,39; 0,39	**V**;**S**	Cytoplasm
EIF4G2	1367469_at	0,41	**F**	Cytoplasm
PIK3C2G	1369050_at	5,06	**V**	Cytoplasm
PPP2CA	1388805_at	0,40	**S**	Cytoplasm
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**	Cytoplasm
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**	Cytoplasm
PRKCI	1373047_at	0,32	**S**	Cytoplasm
RHOA	1399027_at	0,38	**V**	Cytoplasm
RHOB	1369958_at	0,22; 0,16	**V**;**F**	Cytoplasm
RHOQ	1386967_at	0,29; 0,27	**S**;**F**	Cytoplasm
RND2	1372521_at	3,12	**F**	Cytoplasm
RRAS	1388729_at	2,66	**V**	Cytoplasm

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

XI.2.2.7. Myc mediated apoptosis signaling canonical pathway analysis

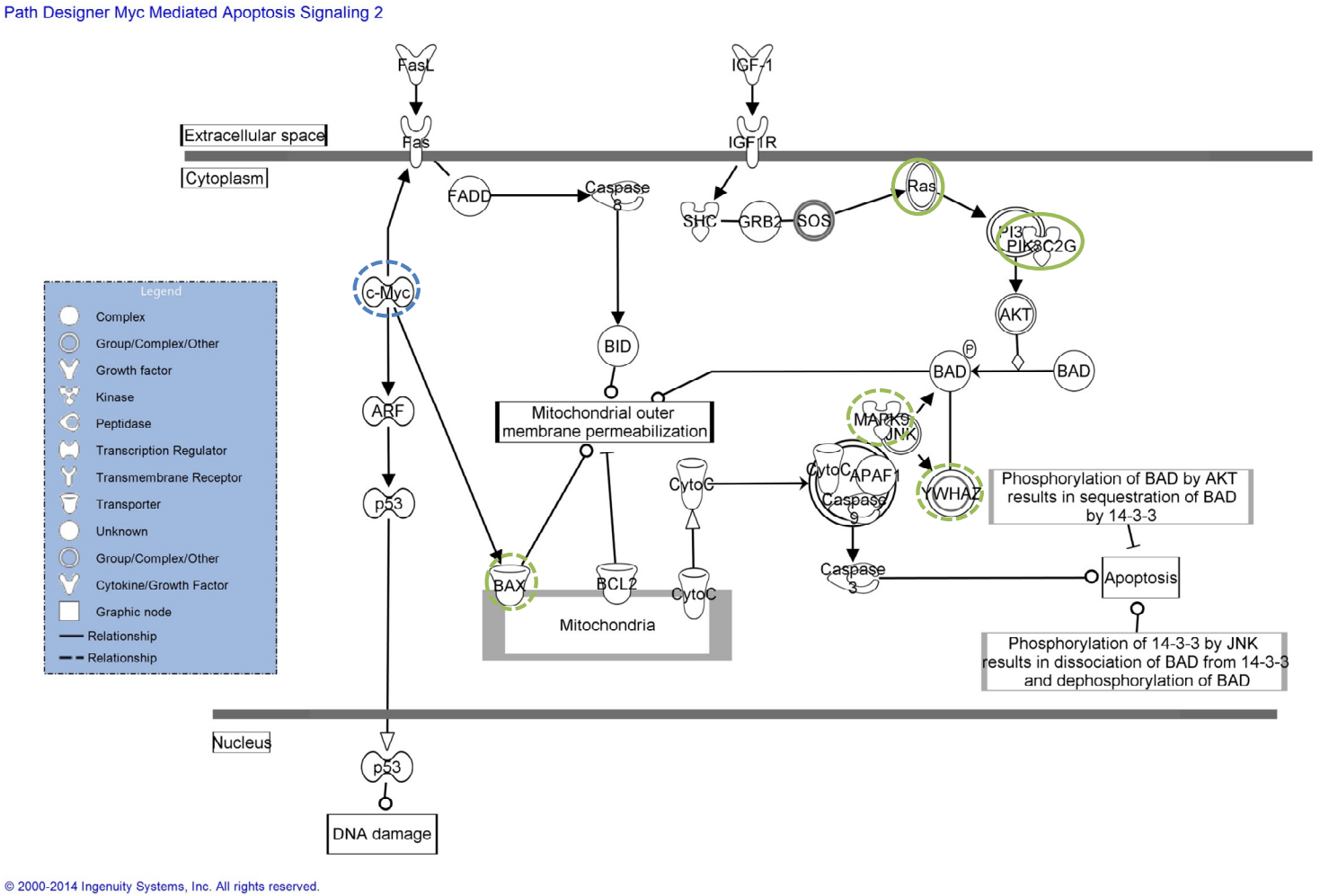


Figure 63. *Myc mediated apoptosis canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Myc mediated apoptosis canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Myc mediated apoptosis canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 63.

Expression differences due to dietary treatment with virgin olive or fish oil were found in 6 genes, all located at the cellular cytoplasm and related with mitochondrion. Five of them were differentially expressed in virgin olive oil treatment, 3 repressed and the other 2 overexpressed. Only one gene was repressed in the fish oil treatment. Rats fed on sunflower oil did not show differences in the expression of genes involved in this canonical pathway (table 33).

Table 33. *Six microarray-differentially expressed genes present at the Myc mediated apoptosis canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
BAX	1369122_at	0,31	**V**	Cytoplasm
MAPK9	1368646_at	0,26	**V**	Cytoplasm
MYC	1368308_at	0,10	**F**	Cytoplasm
PIK3C2G	1369050_at	5,06	**V**	Cytoplasm
RRAS	1388729_at	2,66	**V**	Cytoplasm
YWHAZ	1387774_at	0,36	**V**	Cytoplasm

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.8. Telomere extension by telomerase canonical pathway analysis

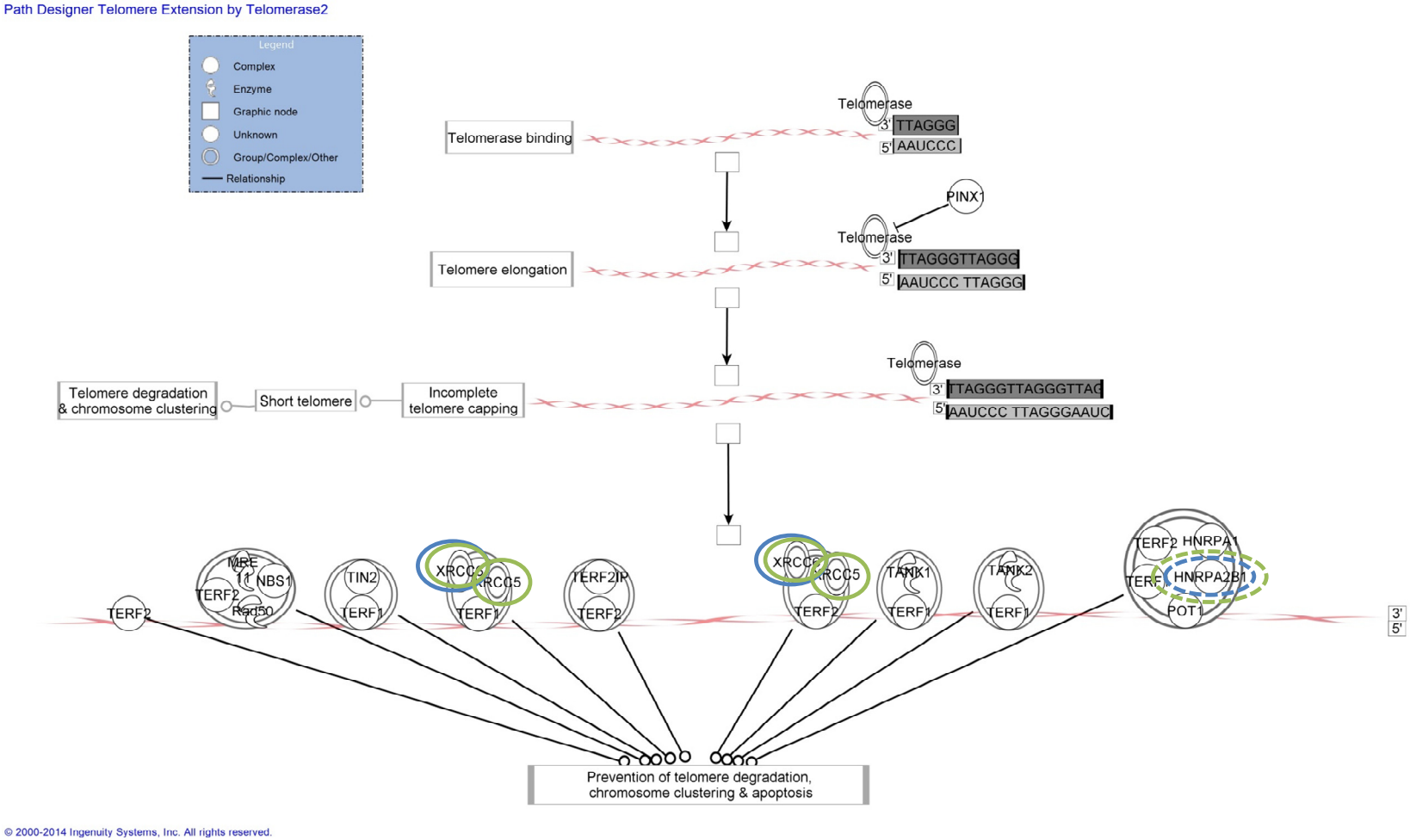


Figure 64. *Telomere extension by telomerase canonical pathway picture differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Telomere extension by telomerase canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway, without indicating that those genes are expressed differently. Using this picture, the microarray-differentially expressed genes present at the mitochondrial dysfunction signaling canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 64.

Expression differences due to dietary treatment with virgin olive or fish oil were found in 3 genes, all located at the cellular nucleus and acting directly on the DNA double-strand. The 3 genes were differentially expressed in virgin olive oil treatment, two of them overexpressed and one repressed. Treatment with fish oil presented 2 differentially expressed genes, one repressed and the other one overexpressed. Rats fed on sunflower oil did not show differences in the expression of genes involved in this canonical pathway (table 34).

Table 34. *Three microarray-differentially expressed genes present at the Telomere extension by telomerase canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
HNRNPA2B1	1398883_at	0,35; 0,37	**V**;**F**	Nucleus
XRCC5	1370931_at	2,83	**V**	Nucleus
XRCC6	1370537_at	2,75; 2,95	**V**;**F**	Nucleus

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.9. Xenobiotic metabolism signaling canonical pathway analysis

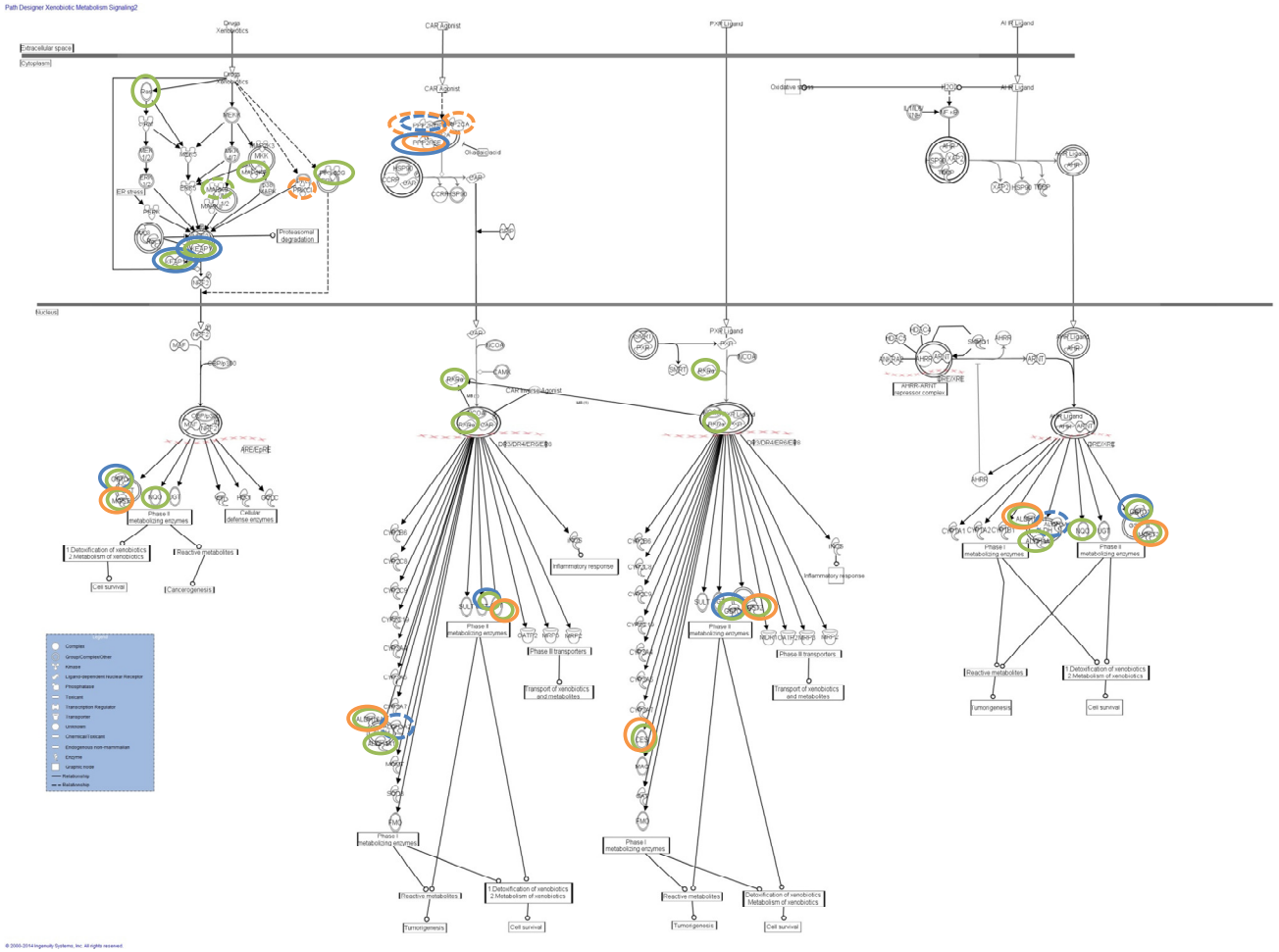


Figure 65. *Xenobiotic metabolism signaling canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish.*

To the Xenobiotic metabolism signaling canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Xenobiotic metabolism signaling canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish, as is shown in figure 65.

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 17 genes, located both at the cellular nucleus and cytoplasm, including the phase I and II at the metabolizing enzymes of xenobiotic detoxification as well as the Nrf2-mediated stress response. Twelve genes were differentially expressed in virgin olive oil treatment, most located in the cellular nucleus. All of them were overexpressed except one which was repressed. The 7 genes differentially expressed in sunflower oil treatment are located mainly in the cytoplasm. Four of them were overexpressed and 3 were repressed. Rats fed on fish oil presented 5 genes differentially expressed in this canonical pathway, most of them located in the cytoplasm. Three of these genes were overexpressed and two were repressed (table 35).

Table 35. *Seventeen microarray-differentially expressed genes present at the Xenobiotic metabolism signaling canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
ALDH16A1	1374661_at	2,57; 2,53	**V**;**S**	Nucleus
ALDH3A2	1368365_at	0,38	**F**	Nucleus
ALDH4A1	1372277_at	2,99	**V**	Nucleus
Ces1c	1371100_at	4,47; 5,10	**V**;**S**	Nucleus
GSTO1	1389832_at	2,69; 2,62	**V**;**F**	Nucleus
KEAP1	1370066_at	2,51; 2,55	**V**;**F**	Cytoplasm
MAP2K6	1387809_at	6,96	**V**	Cytoplasm
MAPK9	1368646_at	0,26	**V**	Cytoplasm
MGST2	1372599_at	3,25; 3,20	**V**;**S**	Nucleus
NQO1	1387599_a_at	15,35	**V**	Nucleus
PIK3C2G	1369050_at	5,06	**V**	Cytoplasm
PPP2CA	1388805_at	0,40	**S**	Cytoplasm
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**	Cytoplasm
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**	Cytoplasm
PRKCI	1373047_at	0,32	**S**	Cytoplasm
RRAS	1388729_at	2,66	**V**	Cytoplasm
RXRA	1371668_at	2,83	**V**	Nucleus

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

XI.2.2.10. Protein ubiquitination canonical pathway analysis

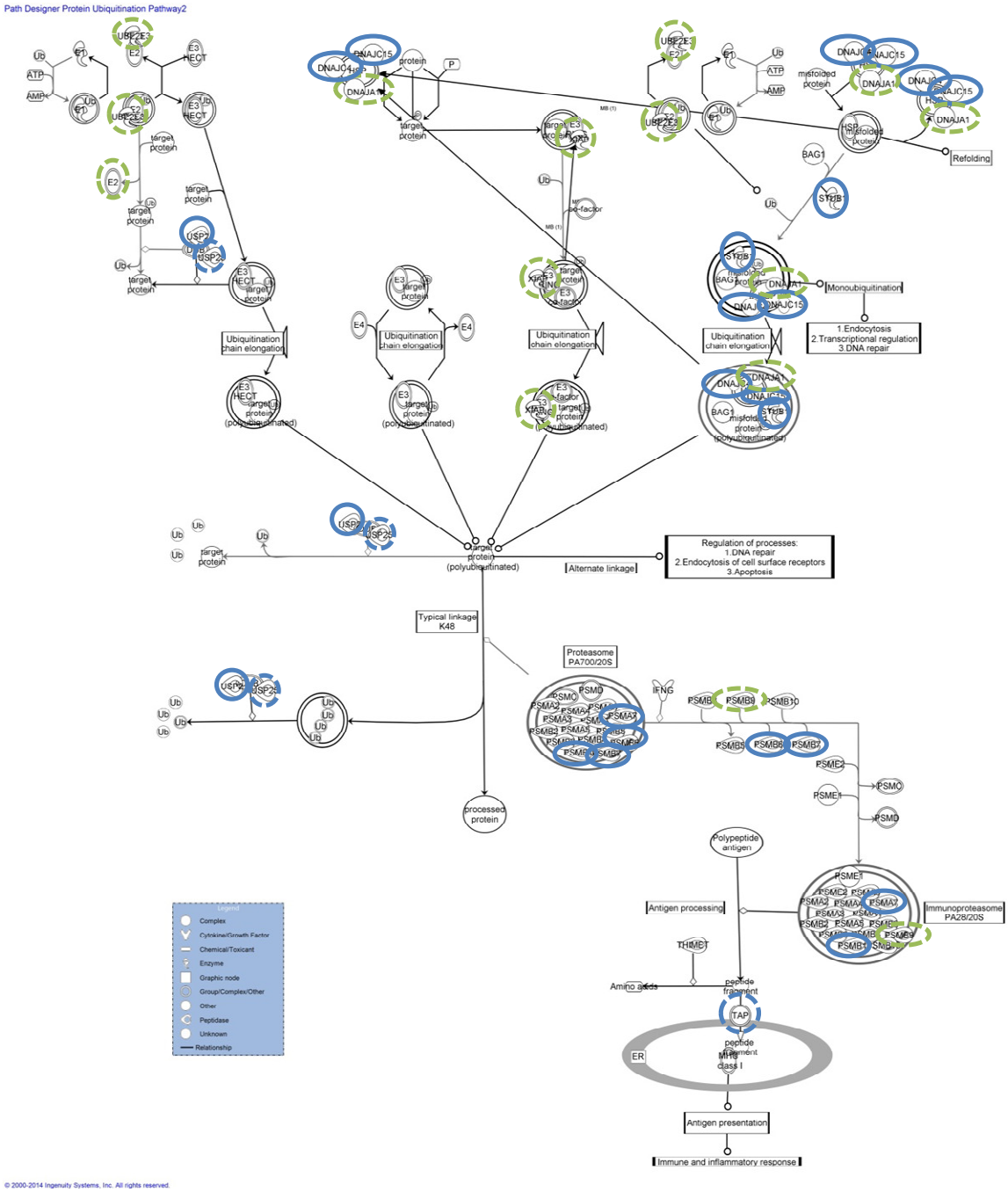


Figure 66. *Protein ubiquitination canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Protein ubiquitination canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the Protein ubiquitination canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 66.

Expression differences due to dietary treatment with virgin olive or fish oil were found in 14 genes, located mainly at the cellular cytoplasm and closely related to proteasome and endoplasmic reticulum. Four genes were repressed in the virgin olive oil treatment, most of them located in the cytoplasm. The 10 genes differentially expressed in the fish oil treatment are located in the cytoplasm. Eight of them were overexpressed and 2 were repressed. Rats fed on sunflower oil did not show differences in the expression of genes involved in this canonical pathway (table 36).

Table 36. *Fourteen microarray-differentially expressed genes present at the Protein ubiquitination canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
DNAJA1	1368852_at	0,36	**V**	Nucleus
DNAJC15	1373064_at	2,89	**F**	Cytoplasm
DNAJC4	1372428_at	2,85	**F**	Cytoplasm
PSMA7	1371869_at	2,57	**F**	Cytoplasm
PSMB1	1398812_at	2,46	**F**	Cytoplasm
PSMB6	1398777_at	2,62	**F**	Cytoplasm
PSMB7	1367656_at	2,77	**F**	Cytoplasm
PSMB9	1370186_at	0,32	**V**	Cytoplasm
STUB1	1398910_at	2,87	**F**	Cytoplasm
TAP1	1388149_at	0,21	**F**	Cytoplasm
UBE2E3	1389534_at	0,38	**V**	Cytoplasm
USP2	1387703_a_at	27,67	**F**	Cytoplasm
USP25	1378679_at	0,38	**F**	Cytoplasm
XIAP	1369248_a_at	0,03	**V**	Cytoplasm

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.11. Tricarboxylic acid cycle (TCA) canonical pathway analysis

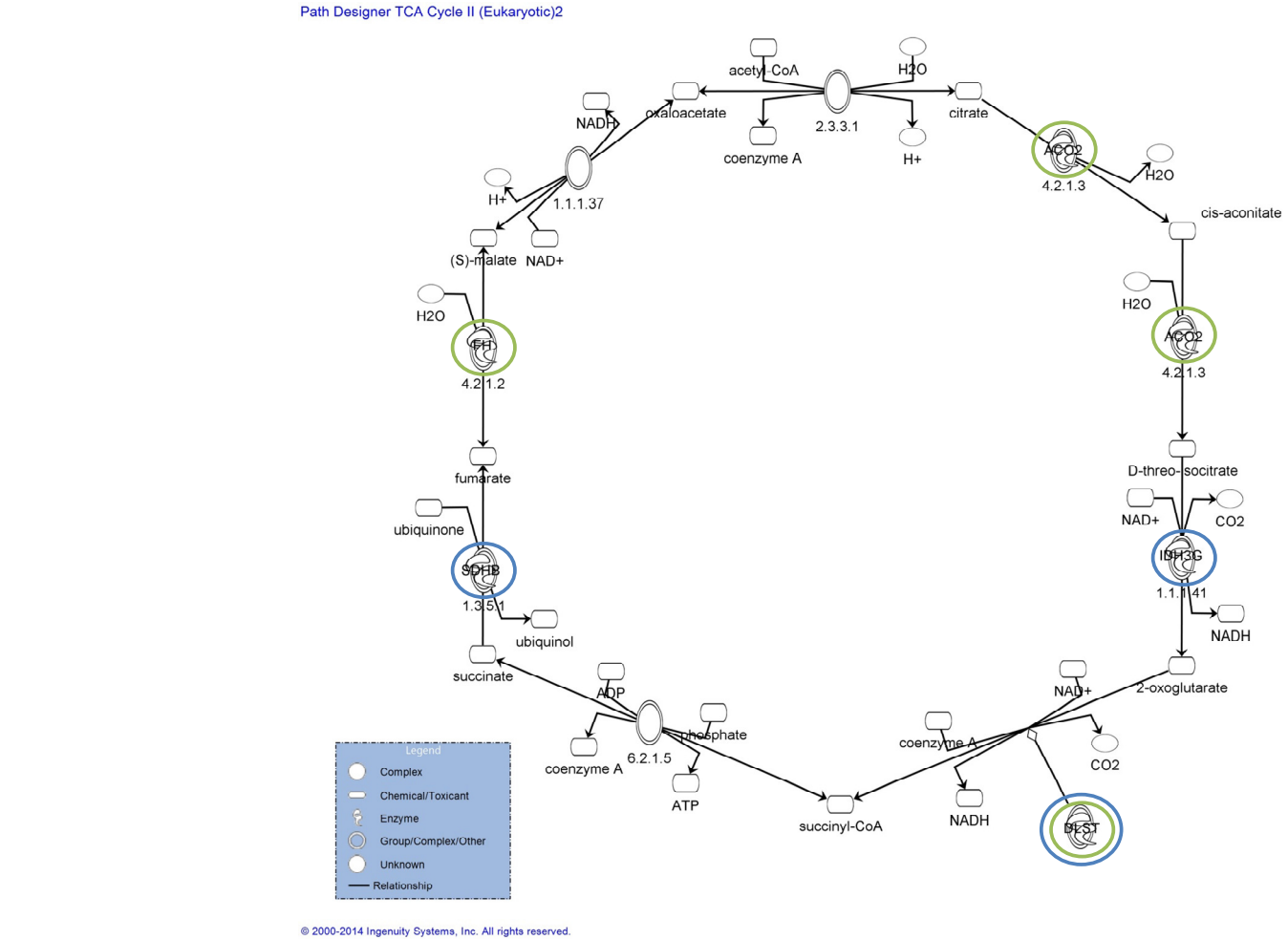


Figure 67. *TCA cycle canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the TCA cycle canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the TCA cycle canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish, as is shown in figure 67.

Expression differences due to dietary treatment with virgin olive or fish oil were found in 4 genes that encode four enzymes of the TCA cycle, also called Krebs cycle, in the matrix of the mitochondrion. Two genes were overexpressed in the virgin olive oil treatment while 3 genes were overexpressed in rat fed on fish oil. Rats fed on sunflower oil did not show differences in the expression of genes involved in this canonical pathway (table 37).

Table 37. *Four microarray-differentially expressed genes present at the TCA cycle canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
ACO2	1367589_at	2,71	**V**	Mitochondrion
DLST	1370879_at	2,58; 2,89	**V**;**F**	Mitochondrion
IDH3G	1370865_at	2,71	**F**	Mitochondrion
SDHB	1372123_at	2,73	**F**	Mitochondrion

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; F = fish oil.

XI.2.2.12. ERK/MAPK signaling canonical pathway analysis

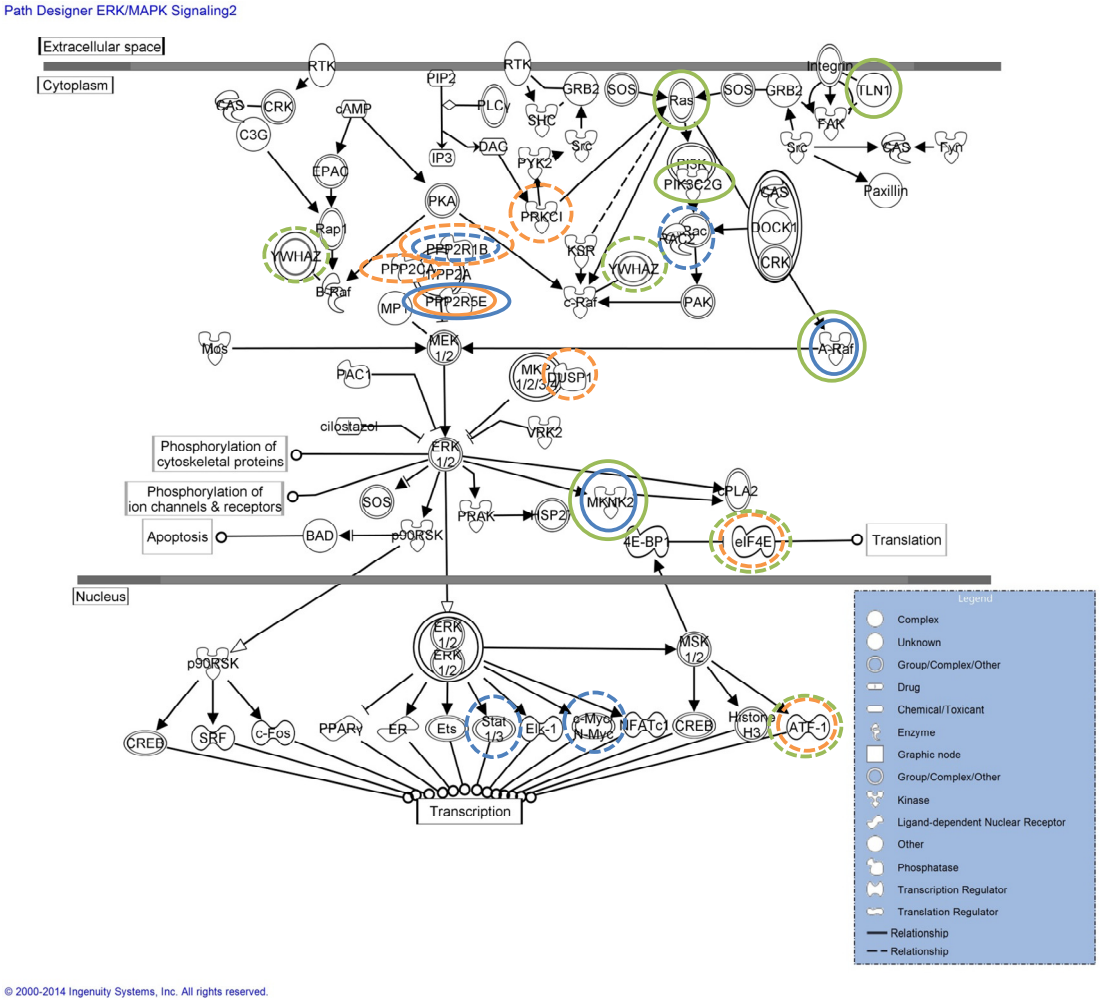


Figure 68. *ERK/MAPK signaling canonical pathway picture with differentially expressed genes. A continuous circle indicates overexpressed genes, and one discontinuous those repressed. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish.*

To the ERK/MAPK signaling canonical pathway analysis, IPA provided a picture that represented all the genes involved in this pathway. Using this picture, the microarray-differentially expressed genes present at the ERK/MAPK signaling canonical pathway according to IPA were hand-picked with a continuous circle surrounding the overexpressed genes. The color of the circle indicates the type of dietary fat: green to virgin olive, orange to sunflower and blue to fish, as is shown in figure 68.

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 16 genes, located both in cellular cytoplasm and nucleus involved in translation and transcription inter alia. Eight genes were differentially expressed in the virgin olive oil treatment, most located in the cytoplasm. Five of them were overexpressed while 3 were repressed. Rats fed on sunflower oil presented 7 genes differentially expressed, most located in the cytoplasm. Except one gene that was overexpressed, all of them were repressed. Six genes were differentially expressed in the fish oil treatment, most located in the cytoplasm. Three genes were overexpressed and the other three were repressed (table 38).

Table 38. *Sixteen microarray-differentially expressed genes present at the ERK/MAPK signaling canonical pathway.*

Symbol	Affymetrix	FC	Oil	Location
ARAF	1368390_at	2,51; 2,46	**V**;**F**	Cytoplasm
ATF1	1389623_at	0,41; 0,38	**V**;**S**	Nucleus
DUSP1	1368147_at	0,09	**S**	Cytoplasm
EIF4E	1398799_at	0,39; 0,39	**V**;**S**	Cytoplasm
MKNK2	1372038_at	6,54; 5,13	**V**;**F**	Cytoplasm
MYC	1368308_at	0,10	**F**	Nucleus
PIK3C2G	1369050_at	5,06	**V**	Cytoplasm
PPP2CA	1388805_at	0,40	**S**	Cytoplasm
PPP2R1B	1373959_at	0,19; 0,10	**S**;**F**	Cytoplasm
PPP2R5E	1388965_at	2,81; 2,99	**S**;**F**	Cytoplasm
PRKCI	1373047_at	0,32	**S**	Cytoplasm
RAC2	1372404_at	0,33	**F**	Cytoplasm
RRAS	1388729_at	2,66	**V**	Cytoplasm
STAT1	1372757_at	0,33	**F**	Nucleus
TLN1	1389002_at	2,69	**V**	Cytoplasm
YWHAZ	1387774_at	0,36	**V**	Cytoplasm

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. When there are two fold change values to the same gene (belonging to two different fats), they are separated by semicolon.

Abbreviations: FC = fold change; V = virgin olive oil; S = sunflower; F = fish oil.

XI.3 Validations by RT-PCR assay.

After a detailed study of the genes involved in every canonical pathway and the way which the treatment affected their expression, 18 genes were selected for validation by RP-PCR, shown in table 39, specifying the fat which are they from and the expression change registered. The selection criteria were mainly choose genes present at a key point on the pathway, such as in the case of complex in mitochondrial dysfunction and genes involved in more than one pathway, considering its FC. Two more genes were added to the list (Tp53 and Casp3), they are present in the selected canonical pathways but IPA did not identify differences in their expression among the three fats, however we considered them interesting to our study to be closely related with apoptosis. Three housekeeping (Gapdh, 18S and B2m) were included to normalize results. The total of the genes was not validated by mainly economic reasons, and in turn to avoid redundancies.

Once performed genes validations by RT-PCR, the results were summarized in table X. Table 40 shows results to the three selected housekeeping. After analysis, 18S was eliminated because changes in its expression due to aging were detected; in fact, it was validated by B2m as a one more gene. Between Gapdh and B2m, the latter showed greater stability, so B2m was chosen as housekeeping in our RT-PCR assay, validating 16 of the 20 genes at least in one of the fat, constituting a validation of 80 percent. Of those, 12 (Atp5d, Ccnd1, Keap1, Map2k6, Ndufa8, Pik3c2g, Rras, Xrcc1, Fis1, Prdx5, Stat1 and Tap1) were validated in all comparisons in which they appeared, while 4 (Ndufa9, Ppp2r1b, Xrcc6 and Casp3) only were validated in in one of the two fats that showed expression change. Btg1, Mapk9, Rb1 and Tp53 were not validated by RT-PCR. This validate genes are highlighted in bold in table 39. In total, 18 of the 20 comparisons in which the array detected expression changes have been validated by RT-PCR assay. Figure 69 to 80 shows a representation of each canonical pathway with genes validated by RT-PCR identified.

Table 39. *Genes selected to validate by RP-PCR and housekeeping.*

Symbol	Affymetrix	Probe ID	FC	*P*-value	SLR	OIL
Atp5d	1370278_at	Rn00756371_g1	2,40	0,00375476	1,26	**V**
Atp5d	1370278_at	Rn00756371_g1	2,79	6,8159E-05	1,48	**F**
Btg1	1367657_at	Rn00820872_g1	0,36	0,00039725	-1,46	**V**
Btg1	1367657_at	Rn00820872_g1	0,37	0,00053258	-1,45	**S**
Casp3		Rn00563902_m1				
Ccnd1	1371150_at	Rn00432360_m1	0,01	0,00238062	-6,66	**F**
Fis1	1371594_at	Rn01480911_m1	2,53	0,00175883	1,34	**F**
Keap1	1370066_at	Rn00589292_m1	2,52	0,00115385	1,33	**V**
Keap1	1370066_at	Rn00589292_m1	2,54	0,00219433	1,35	**F**
Map2k6	1387809_at	Rn00586764_m1	6,95	0,00198514	2,80	**V**
Mapk9	1368646_at	Rn00569058_m1	0,26	0,00149475	-1,97	**V**
Ndufa8	1371355_at	Rn01438607_m1	2,70	0,00222909	1,43	**F**
Ndufa9	1388323_at	Rn01462923_m1	2,57	0,00155416	1,36	**V**
Ndufa9	1388323_at	Rn01462923_m1	2,69	0,00125995	1,43	**F**
Pik3c2g	1369050_at	Rn00588317_m1	5,05	0,00023968	2,34	**V**
Ppp2r1b	1373959_at	Rn01422402_g1	0,19	0,00012574	-2,40	**S**
Ppp2r1b	1373959_at	Rn01422402_g1	0,10	7,4916E-06	-3,39	**F**

Prdx5	1367677_at	Rn00586040_m1	3,14	0,00035419	1,65	**F**
Rb1	1388185_at	Rn01753308_m1	0,17	0,00074395	-2,53	**F**
Rras	1388729_at	Rn01454699_g1	2,66	2,2491E-05	1,41	**V**
Stat1	1372757_at	Rn00583505_m1	0,33	0,00098244	-1,62	**F**
Tap1	1388149_at	Rn00709612_m1	0,21	0,00070689	-2,24	**F**
Tp53		Rn00755717_m1				
Xrcc1	1387129_at	Rn01457689_m1	2,70	0,00299616	1,43	**V**
Xrcc1	1387129_at	Rn01457689_m1	2,81	0,00304118	1,49	**F**
Xrcc6	1370537_at	Rn00594589_m1	2,75	0,00192154	1,46	**V**
Xrcc6	1370537_at	Rn00594589_m1	2,95	0,00120469	1,56	**F**
Gapdh		Rn01775763_g1				
18S		Rn00560865_m1				
B2m		Hs99999901_s1				

Notes: symbol refers to NCBI gene symbol. Affymetrix refers to Affymetrix gene identification. Probe ID refers to probe identification.

Abbreviations: FC = fold change; SLR = signal log ratio; V = virgin olive oil; S = sunflower; F = fish oil.

Table 40. *RT-PCR validation result of the three housekeeping.*

	Virgin olive oil (Old *vs.* Young)								Sunflower oil (Old *vs.* Young)								Fish oil (Old *vs.* Young)							
	ARRAY		RT-PCR 18S		RT-PCR GAPDH		RT-PCR B2M		ARRAY		RT-PCR 18S		RT-PCR GAPDH		RTPCR B2M		ARRAY		RT-PCR 18S		RT-PCR GAPDH		RT-PCR B2M	
Symbol	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***
Atp5d	0,42	0,004	10,60		0,98	0,75	0,44	0,00									0,36	0,000	11,19		0,73	0,24	0,53	0,034
Btg1	2,75	0,000	29,23		2,44	0,04	0,93	0,62	2,72	0,001	259,89		0,76	0,20	0,52	0,11								
Casp3			13,14		1,21	0,92	0,53	0,00			196,05		0,57	0,04	32,41	0,235			8,88		0,89	0,65	0,59	0,06
Ccnd1																	101,34	0,002	172,38		6,96	0,05	5,82	0,032
Fis1																	0,40	0,002	9,30		0,68	0,24	0,49	0,002
Keap1	0,40	0,001	10,38		0,92	0,79	0,48	0,05									0,39	0,002	5,05		0,63	0,22	0,36	0,031
Map2k6	0,14	0,002	0,79		0,21	0,01	0,12	0,02																
Mapk9	3,91	0,001	32,00		1,90	0,25	0,87	0,37																
Ndufa8																	0,37	0,002	11,27		0,71	0,25	0,52	0,026
Ndufa9	0,39	0,002	9,97		1,00	0,77	0,46	0,01									0,37	0,001	16,08		0,82	0,36	0,65	0,143
Pik3c2g	0,20	0,000	7,34		0,64	0,18	0,30	0,01																
Ppp2r1b									5,28	0,000	353,73		1,50	0,48	1,03	0,38	10,51	0,000	49,02		3,88	0,00	2,73	0,005
Prdx5																	0,32	0,000	7,65		0,69	0,29	0,44	0,003
Rb1																	5,78	0,001	21,59		1,07	0,99	0,86	0,492
Rras	0,3	0,00	13,3		0,9	0,7	0,4	0,0																

	8	0	4	6	0	4	0											
Stat1												3,07	0,001	34,74	2,42	0,01	1,88	0,002
Tap1												4,73	0,001	34,96	3,54	0,05	2,48	0,035
Tp53			26,19	1,80	0,22	0,80	0,23	707,95	1,42	0,15	0,95	0,762		34,92	1,65	0,42	1,28	0,957
Xrcc1	0,37	0,003	9,92	0,89	0,44	0,53	0,05						0,36	0,003	6,82	0,26	0,03	0,05	0,045
Xrcc6	0,36	0,002	11,58	0,82	0,40	0,37	0,00						0,34	0,001	13,31	0,84	0,13	0,60	0,43
18S			1,00	0,61	0,48	0,38	0,27	1,00	0,06	0,03	0,03	0,017			1,00	0,19	0,04	0,13	0,025

Notes: symbol refers to NCBI gene symbol.
Abbreviations: FC = fold change. P = P-value.

Table 39. *RT-PCR validation results with the selected housekeeping (B2m). Results in bold shows validate genes.*

		Virgin olive oil (Old *vs.* Young)				Sunflower oil (Old *vs.* Young)				Fish (Old *vs.* Young)			
		ARRAY		RT-PCR B2M		ARRAY		RT-PCR B2M		ARRAY		RT-PCR B2M	
Symbol	**Probe ID**	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***
Atp5d	Rn00756371_g1	0,42	0,004	**0,44**	**0,004**					0,36	0,000	**0,53**	**0,034**
Btg1	Rn00820872_g1	2,75	0,000	0,93	0,622	2,72	0,001	0,52	0,107				
Casp3	Rn00563902_m1			**0,53**	**0,001**			32,41	0,235			**0,59**	**0,06**
Ccnd1	Rn00432360_m1									101,34	0,002	**5,82**	**0,032**
Fis1	Rn01480911_m1									0,40	0,002	**0,49**	**0,002**
Keap1	Rn00589292_m1	0,40	0,001	**0,48**	**0,046**					0,39	0,002	**0,36**	**0,031**
Map2k6	Rn00586764_m1	0,14	0,002	**0,12**	**0,020**								
Mapk9	Rn00569058_m1	3,91	0,001	0,87	0,372								
Ndufa8	Rn01438607_m1									0,37	0,002	**0,52**	**0,026**
Ndufa9	Rn01462923_m1	0,39	0,002	**0,46**	**0,007**					0,37	0,001	0,65	0,143
Pik3c2g	Rn00588317_m1	0,20	0,000	**0,30**	**0,013**								
Ppp2r1b	Rn01422402_g1					5,28	0,000	1,03	0,380	10,51	0,000	**2,73**	**0,005**
Prdx5	Rn00586040_m1									0,32	0,000	**0,44**	**0,003**
Rb1	Rn01753308_m1									5,78	0,001	0,86	0,492
Rras	Rn01454699_g1	0,38	0,000	**0,44**	**0,004**								
Stat1	Rn00583505_m1									3,07	0,001	**1,88**	**0,002**
Tap1	Rn00709612_m1									4,73	0,001	**2,48**	**0,035**
Tp53	Rn00755717_m1			0,80	0,228			0,95	0,762			1,28	0,957
Xrcc1	Rn01457689_m1	0,37	0,003	**0,53**	**0,049**					0,36	0,003	**0,05**	**0,045**
Xrcc6	Rn00594589_m1	0,36	0,002	**0,37**	**0,001**					0,34	0,001	0,60	0,43
18S	Hs99999901_s1			0,38	0,272			**0,03**	**0,017**			**0,13**	**0,025**

Notes: symbol refers to NCBI gene symbol. Probe ID refers to probe identification. Data highlights in bold belong to validated genes by RT-PCR.
Abbreviations: FC = fold change. P = P-value.

XI.3.1. RTPCR-validated genes at the Mitochondrial dysfunction canonical pathway

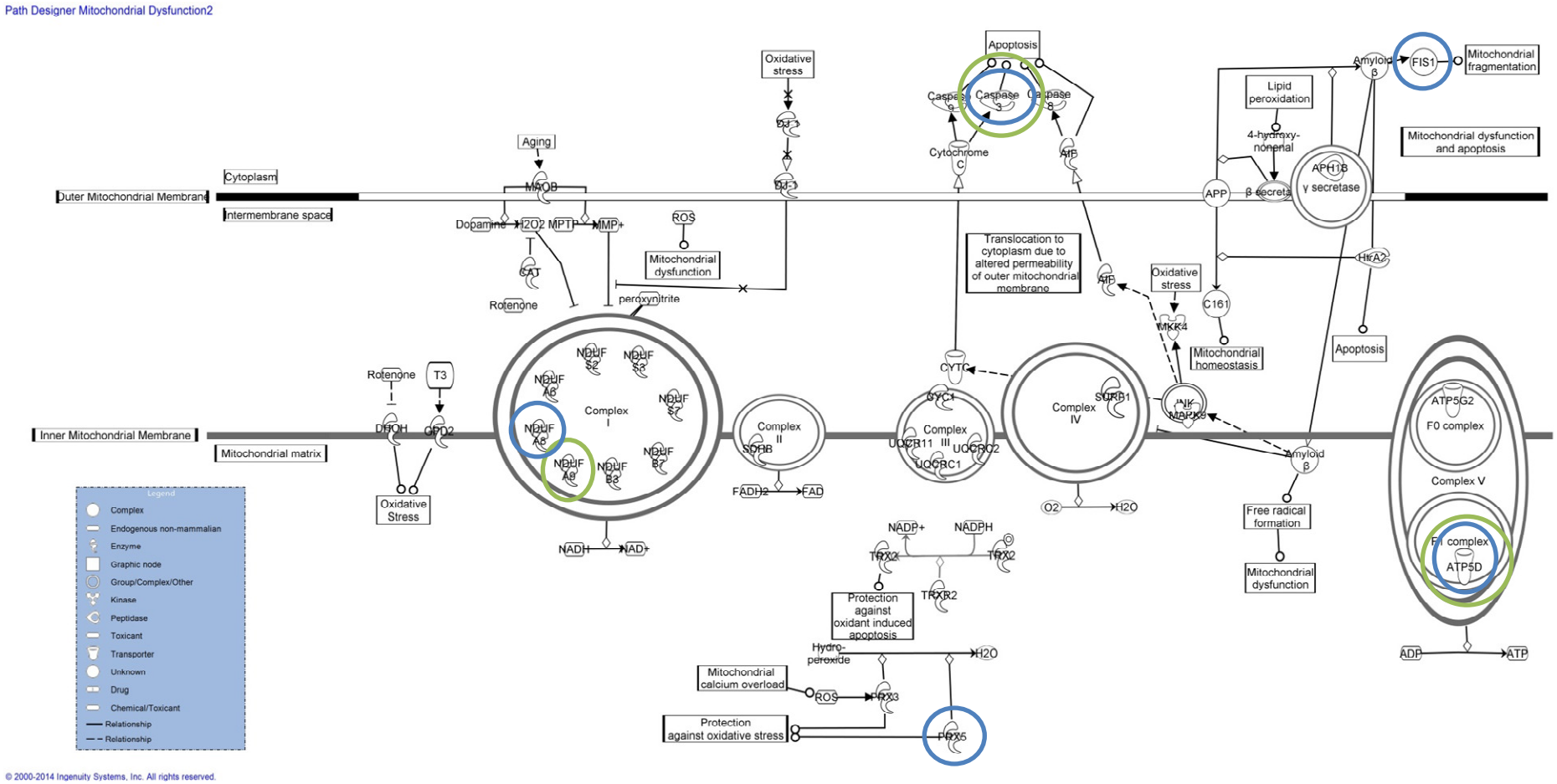


Figure 69. *Mitochondrial dysfunction canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive, and blue to fish.*

To the Mitochondrial dysfunction canonical pathway analysis, from the 22 genes expressed differently between virgin olive and fish oil, 6 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Atp5d, Fis1, Mapk9, Ndufa8, Ndufa9 and Prdx5. One more gene, Casp3, was added to the validation list due to its interest in apoptosis process, so finally RT-PCR assay were made to 7 genes. Of them have been validated 6 genes: Atp5d, Casp3, Fis1, Ndufa8, Ndufa9 and Prdx5, representing more than 85 percentage of successful validation. Genes were validated in all fats where IPA identified the expression change except Ndufa9, whose validation only occurred in virgin olive oil, while in fish oil its expression change was not confirmed by RT-PCR.

Figure 69 is a canonical pathway picture, simpler than original picture (figure 57), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.2. RTPCR-validated genes at the Nrf2-mediated oxidative stress response canonical pathway

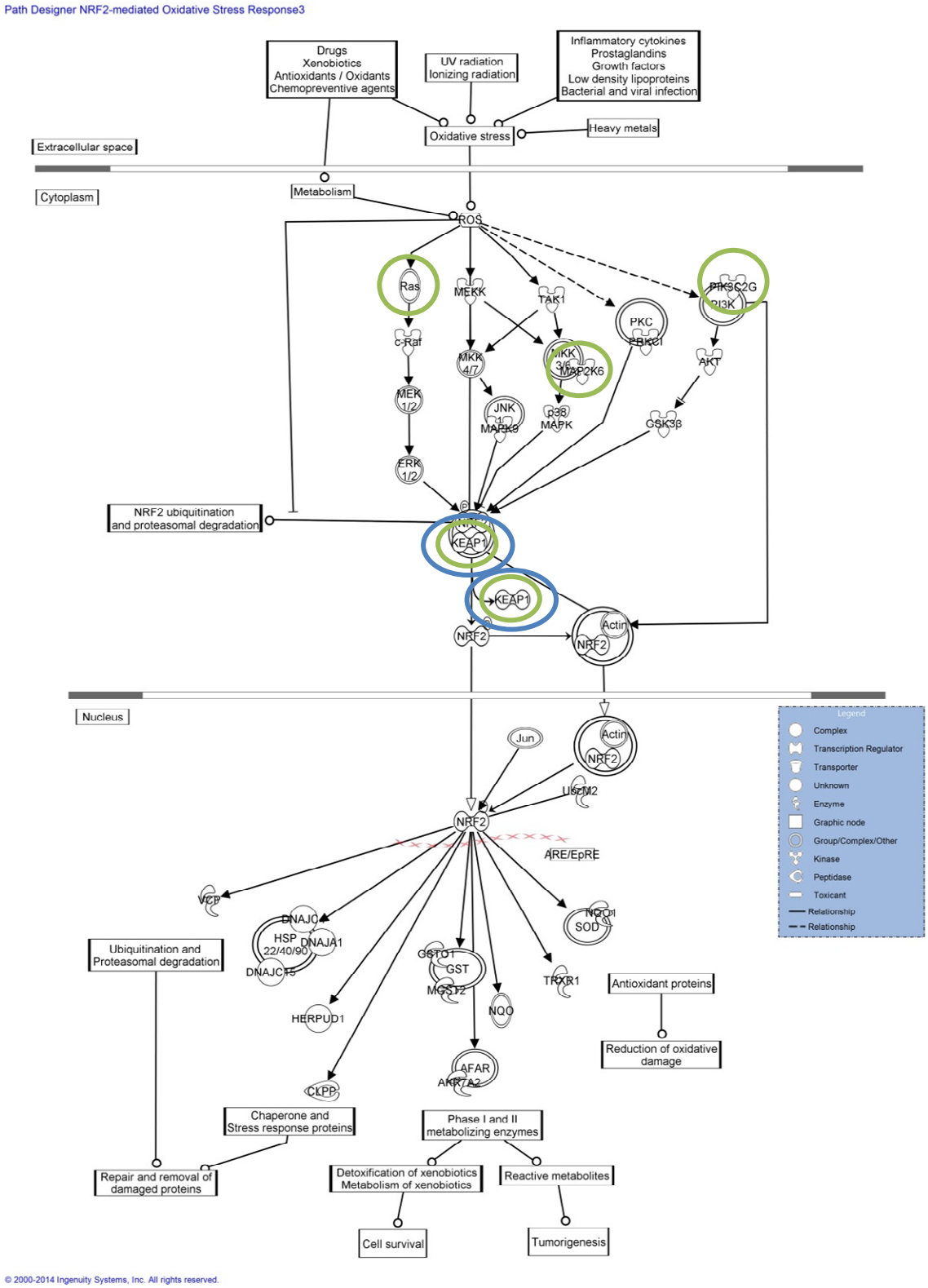


Figure 70. *Nrf2-mediated oxidative response canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive, and blue to fish.*

To the Nrf2-mediated oxidative stress response canonical pathway analysis, from the 19 genes expressed differently among virgin olive, sunflower and fish oil, 5 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Keap1, Map2k6, Mapk9, Pik3c2g and Rras. Of them have been validated 4 genes: Keap1, Map2k6, Pik3c2g and Rras, representing 80 percentage of successful validation. Expression changes identified by IPA were confirmed by RT-PCR in all cases.

Figure 70 is a canonical pathway picture, simpler than original picture (figure 58), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.3. RTPCR-validated genes at the Cell cycle regulation by BTG family proteins canonical pathway

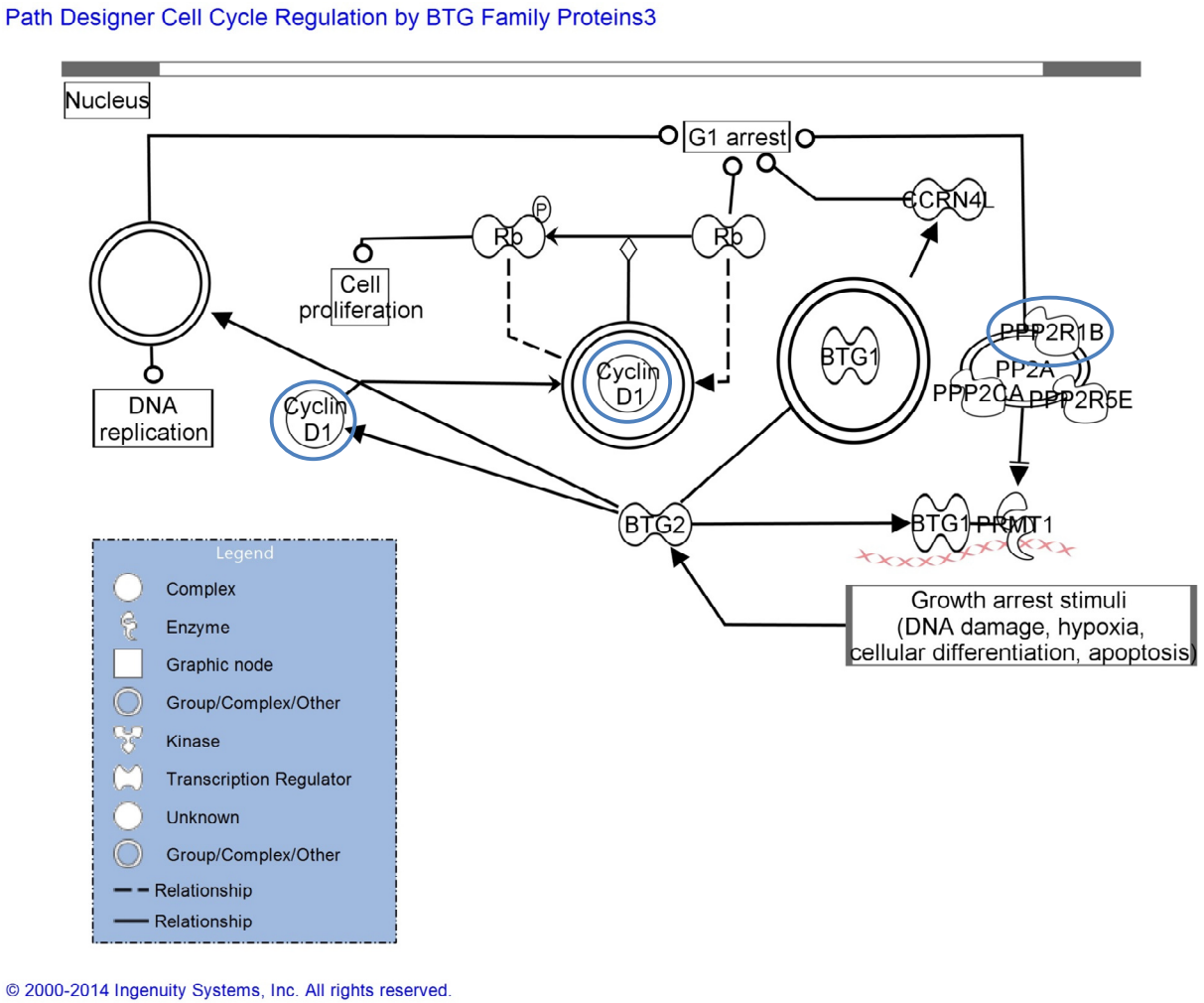


Figure 71. *Cell cycle regulation by BTG family proteins canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: blue to fish.*

To the Cell cycle regulation by BTG family proteins canonical pathway analysis, from the 6 genes expressed differently among virgin olive, sunflower and fish oil, 4 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Btg1, Ccnd1, Ppp2r1b and Rb1. Of them have been validated 2 genes: Ccnd1 and Ppp2r1b, representing 50 percentage of successful validation. Expression changes identified by IPA were confirmed by RT-PCR only in fish oil, expression change in Ppp2r1b gene observed in rats fed on sunflower oil did not validate.

Figure 71 is a canonical pathway picture, simpler than original picture (figure59), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: blue to fish.

XI.3.4. RTPCR-validated genes at the Cyclins and cell cycle regulation canonical pathway

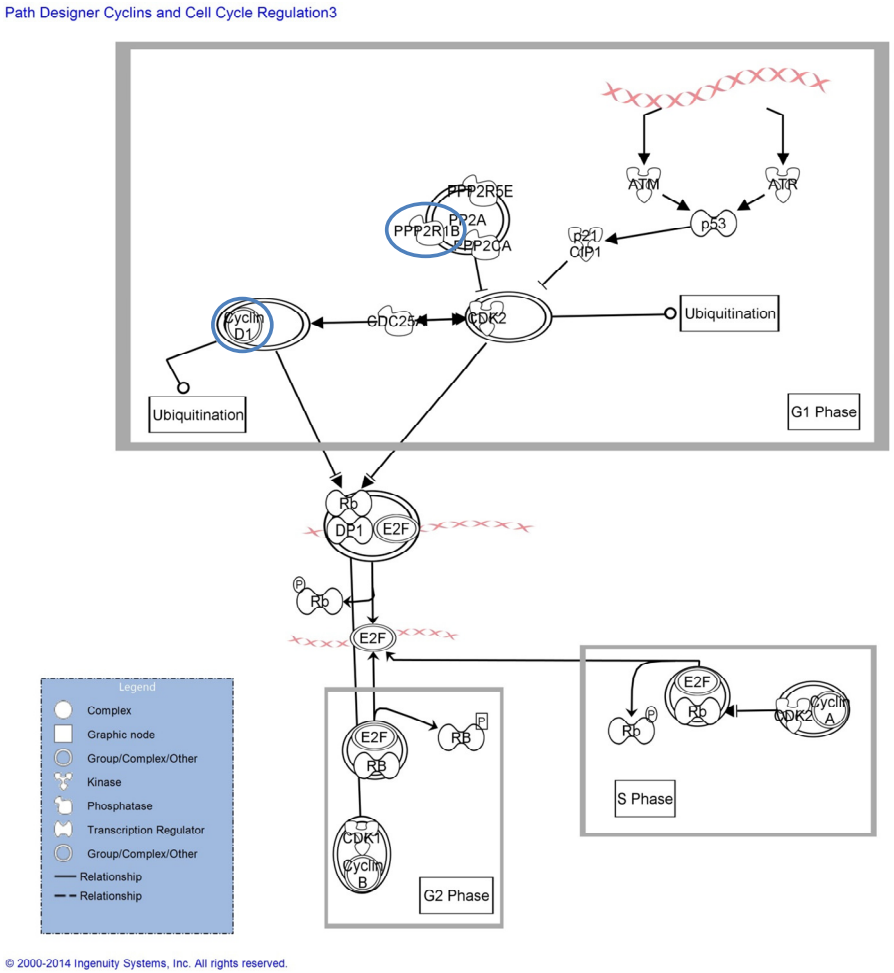


Figure 72. *Cyclins and cell cycle regulation canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: blue to fish.*

To the Cyclins and cell cycle regulation canonical pathway analysis, from the 5 genes expressed differently among virgin olive, sunflower and fish oil, 3 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Ccnd1, Ppp2r1b and Rb1. Of them have been validated 2 genes: Ccnd1 and Ppp2r1b, representing more than 65 percentage of successful validation. Expression changes identified by IPA were confirmed by RT-PCR only in fish oil, expression change in Ppp2r1b gene observed in rats fed on sunflower oil did not validate.

Figure 72 is a canonical pathway picture, simpler than original picture (figure 60), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: blue to fish.

XI.3.5. RTPCR-validated genes at the DNA double-strand break repair by non-homologous end joining canonical pathway

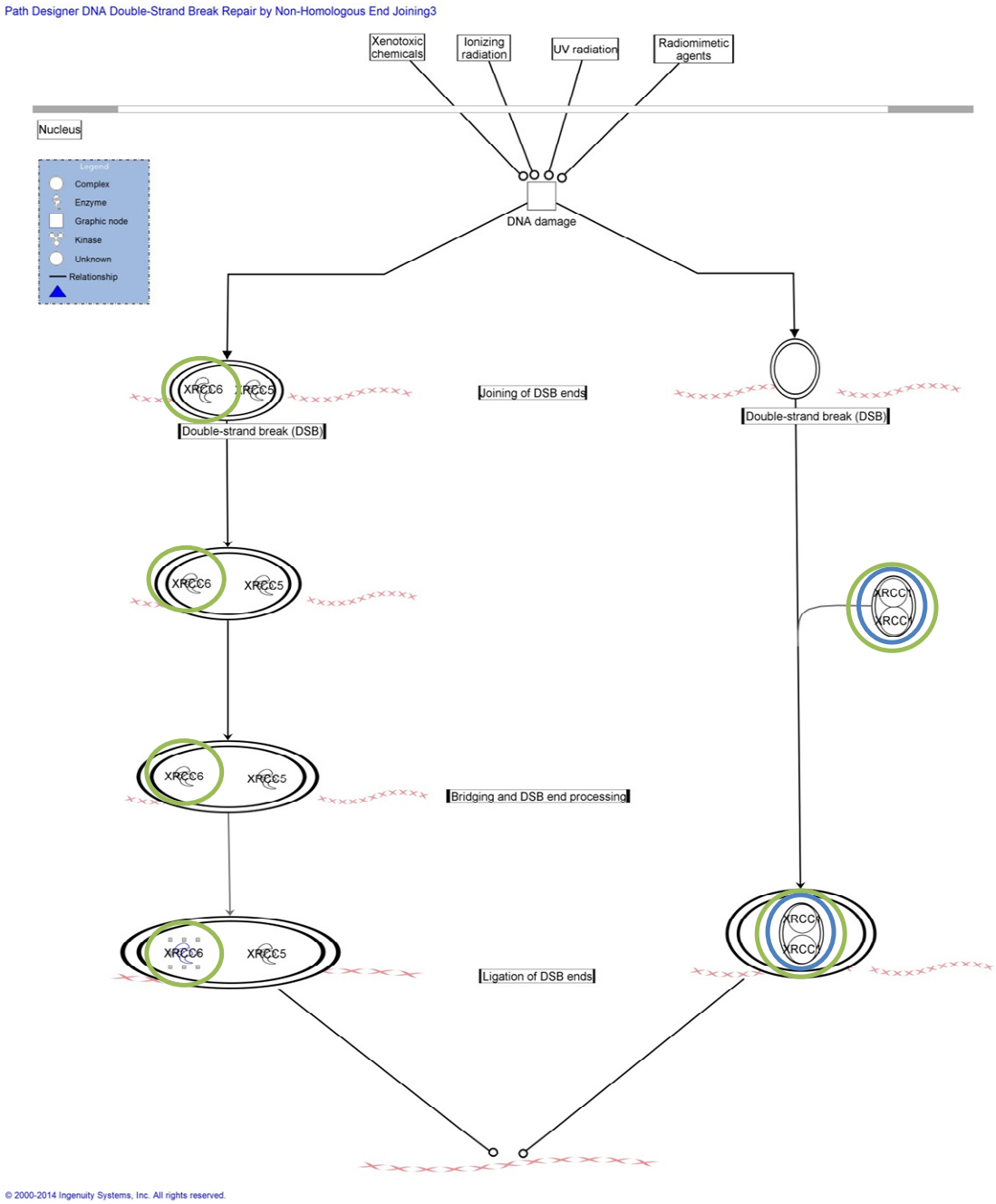


Figure 73. *DNA double-strand break repair by non-homologous end joining canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the DNA double-strand break repair by non-homologous end joining canonical pathway analysis, from the e genes expressed differently among virgin olive, sunflower and fish oil, 2 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Xrcc1 and Xrcc6. Both have been validated, representing 100 percentage of successful validation. Xrcc1 was validated in all fats where IPA identified the expression change while Xrcc6 validation only occurred in virgin olive oil, while in fish oil its expression change was not confirmed by RT-PCR.

Figure 73 is a canonical pathway picture, simpler than original picture (figure 61), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.6. RTPCR-validated genes at the mTOR signaling canonical pathway

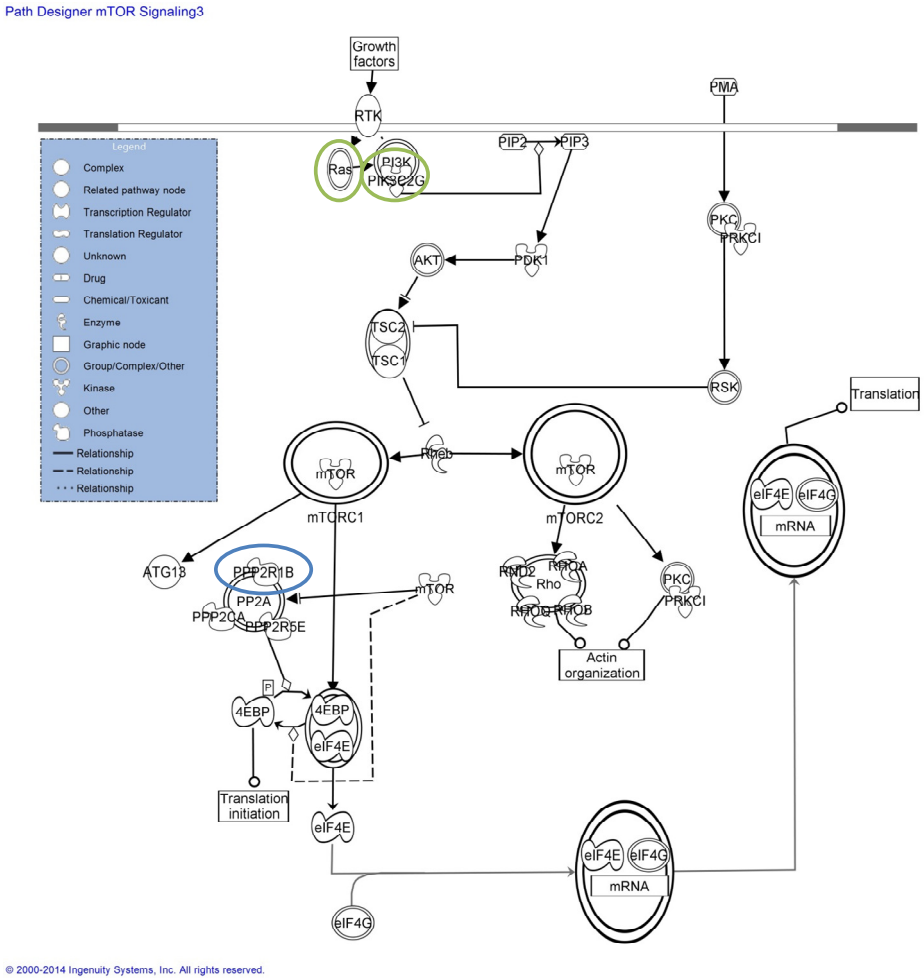


Figure 74. *mTOR signaling canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the mTOR signaling canonical pathway analysis, from the 13 genes expressed differently among virgin olive, sunflower and fish oil, 3 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Pik3c2g, Ppp2r1b and Rras. All have been validated, representing 100 percentage of successful validation. Ppp2r1b validation only occurred in fish oil, while in sunflower oil its expression change was not confirmed by RT-PCR.

Figure 74 is a canonical pathway picture, simpler than original picture (figure 62), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.7. RTPCR-validated genes at the Myc mediated apoptosis signaling canonical pathway

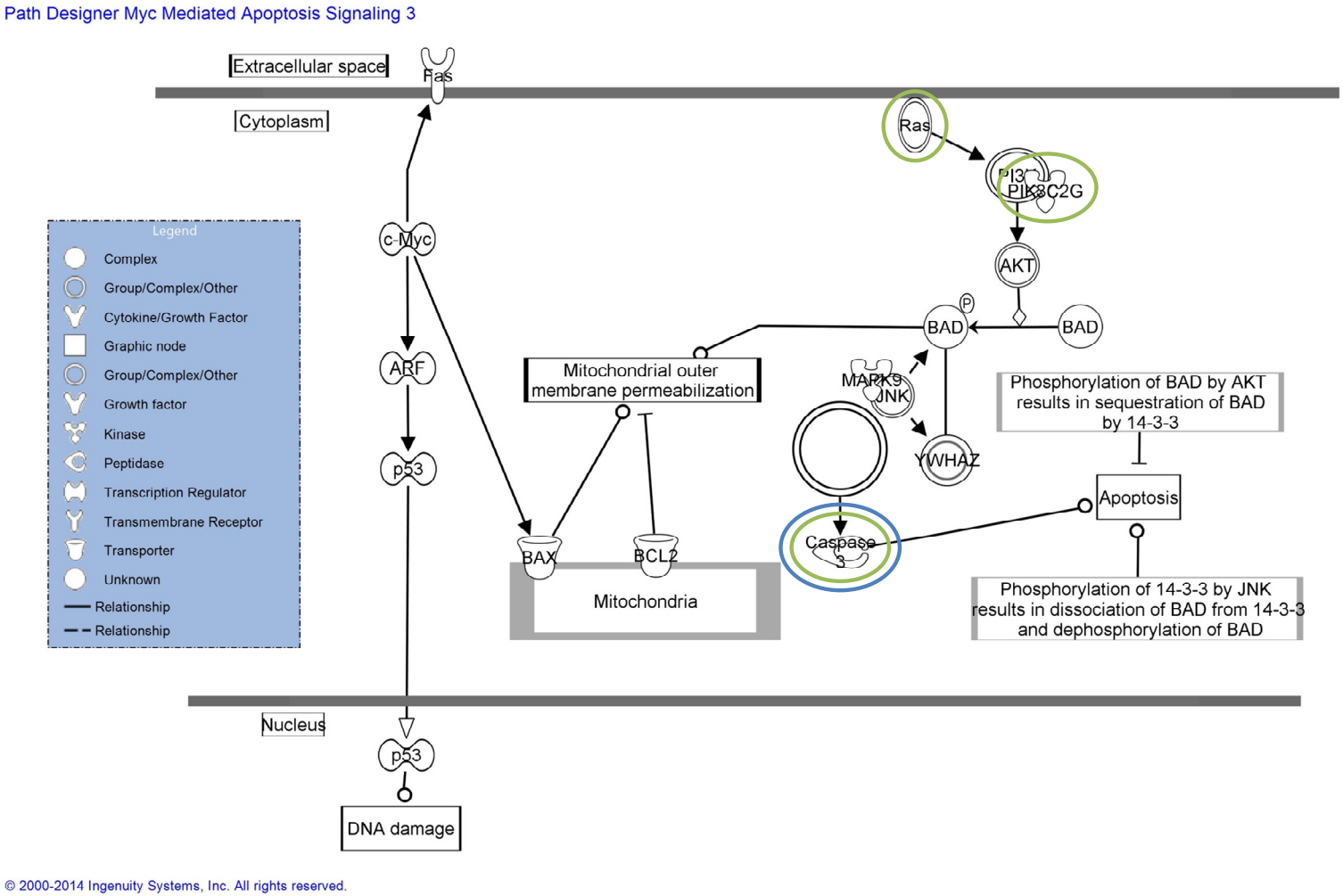


Figure 75. *Myc mediated apoptosis signaling canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Myc mediated apoptosis signaling canonical pathway analysis, from the 6 genes expressed differently among virgin olive, sunflower and fish oil, 3 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Mapk9, Pik3c2g and Rras. Two more genes, p53 and Casp3 were added to the validation list, so in total RT-PCR assay was performed to 5 genes, of which were validated 3: Casp3, Pik3c2g and Rras, representing 60 percentage of successful validation. Expression change of all of them was confirmed in all fats where IPA identified them.

Figure 75 is a canonical pathway picture, simpler than original picture (figure 63), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.8. RTPCR-validated genes at the Telomere extension by telomerase canonical pathway

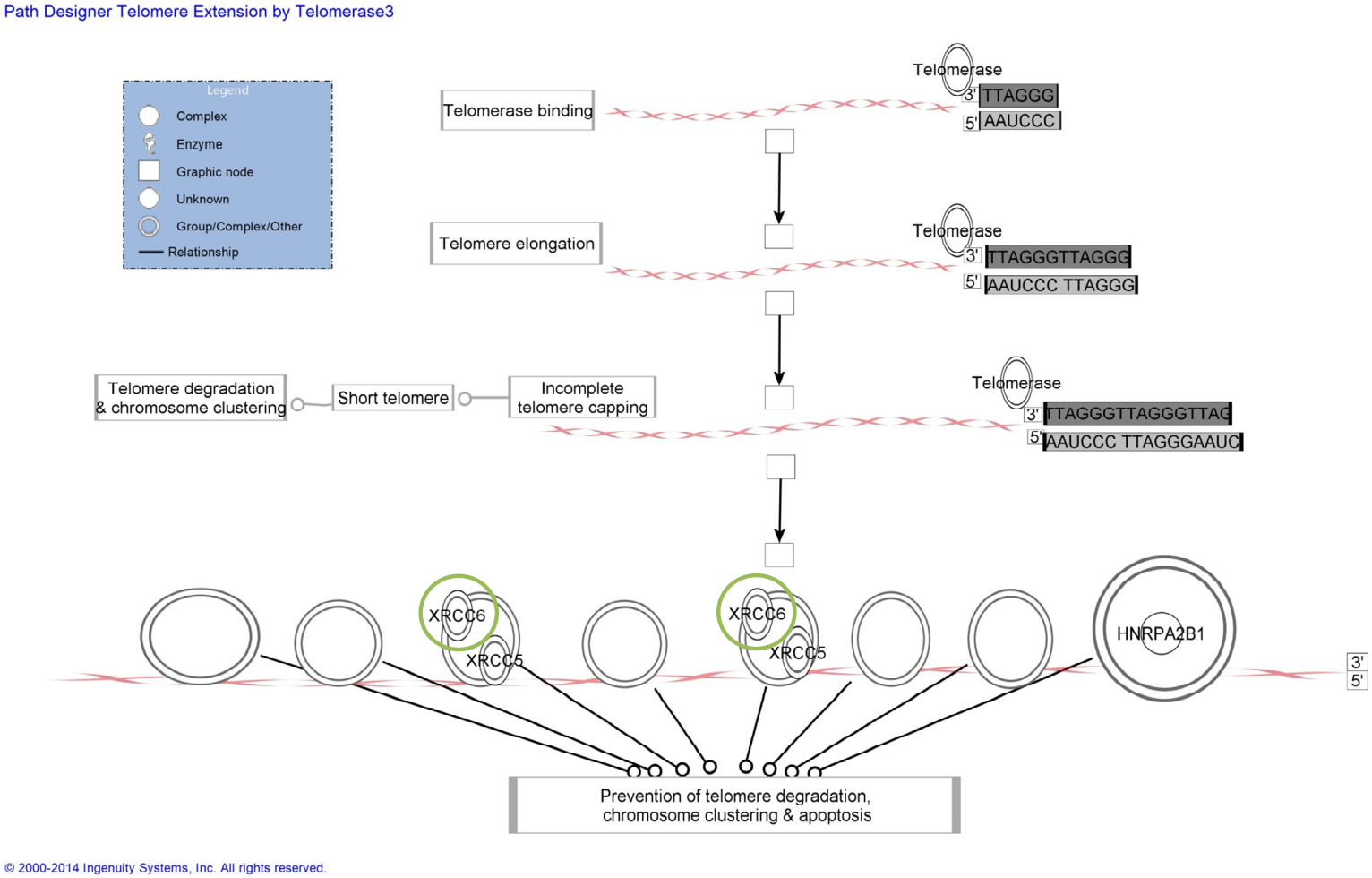


Figure 76. *Telomere extension by telomerase canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive.*

To the Telomere extension by telomerase canonical pathway analysis, from the 3 genes expressed differently among virgin olive, sunflower and fish oil, only Xrcc6 was selected to validate by RT-PCR due to its location and importance in this and other pathways, and it was validated, representing 100 percentage of successful validation; however expression changes only were confirmed in rats fed on virgin olive oil, while expression change present in rats fed on fish oil was not validate.

Figure 76 is a canonical pathway picture, simpler than original picture (figure 64), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive.

XI.3.9. RTPCR-validated genes at the Xenobiotic metabolism signaling canonical pathway

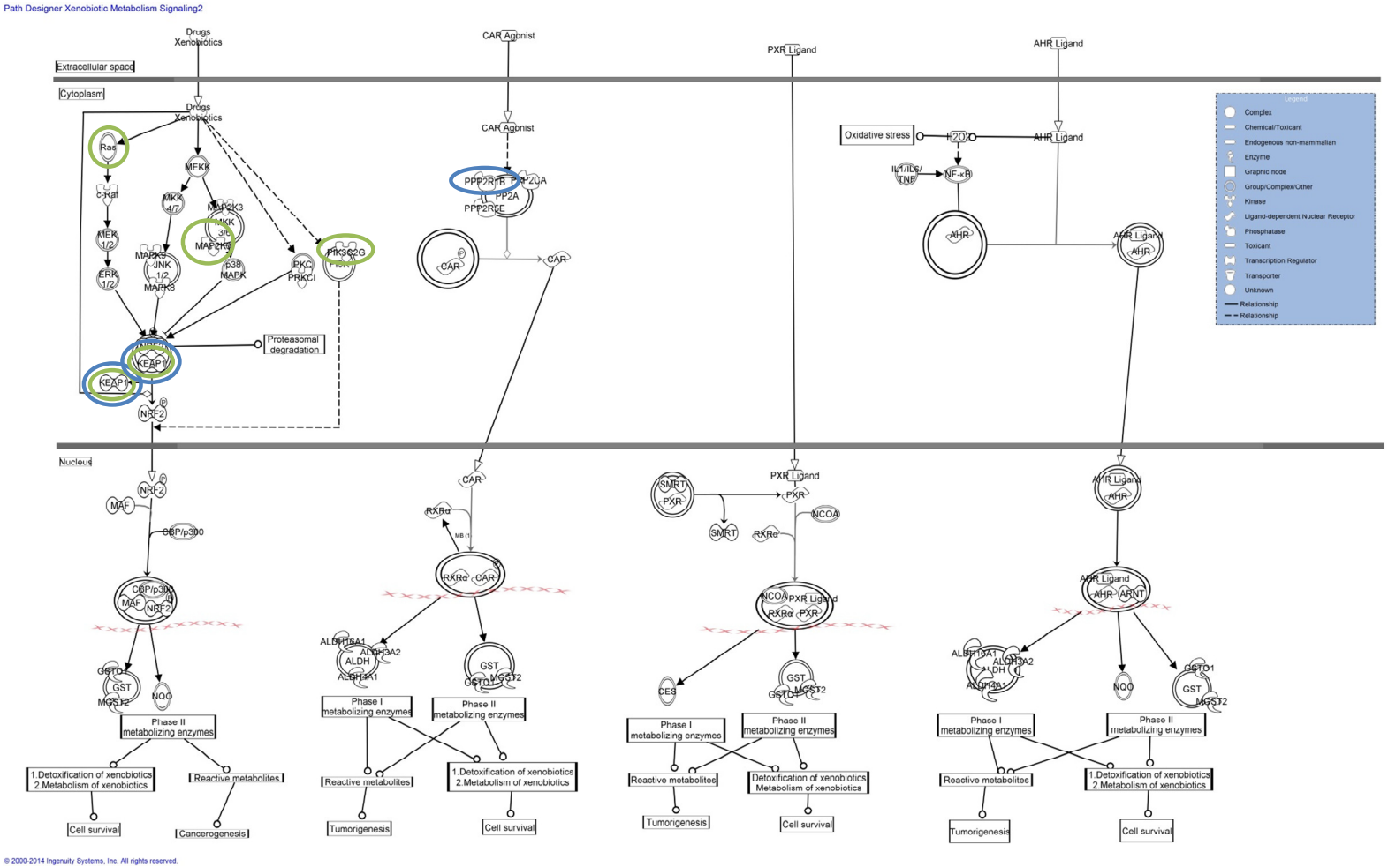


Figure 77. *Xenobiotic metabolism signaling canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the Xenobiotic metabolism signaling canonical pathway analysis, from the 17 genes expressed differently among virgin olive, sunflower and fish oil, 5 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Map2k6, Mapk9, Pik3c2g, Ppp2r1b and Rras. Of them have been validated 4 genes: Map2k6, Pik3c2g, Ppp2r1b and Rras, representing 80 percentage of successful validation. Genes were validated in all fats where IPA identified the expression change except Ppp2r1b, whose validation only occurred in fish oil, while expression change in rats fed on sunflower oil was not confirmed by RT-PCR.

Figure 77 is a canonical pathway picture, simpler than original picture (figure 65), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.10. RTPCR-validated genes at the Protein ubiquitination canonical pathway

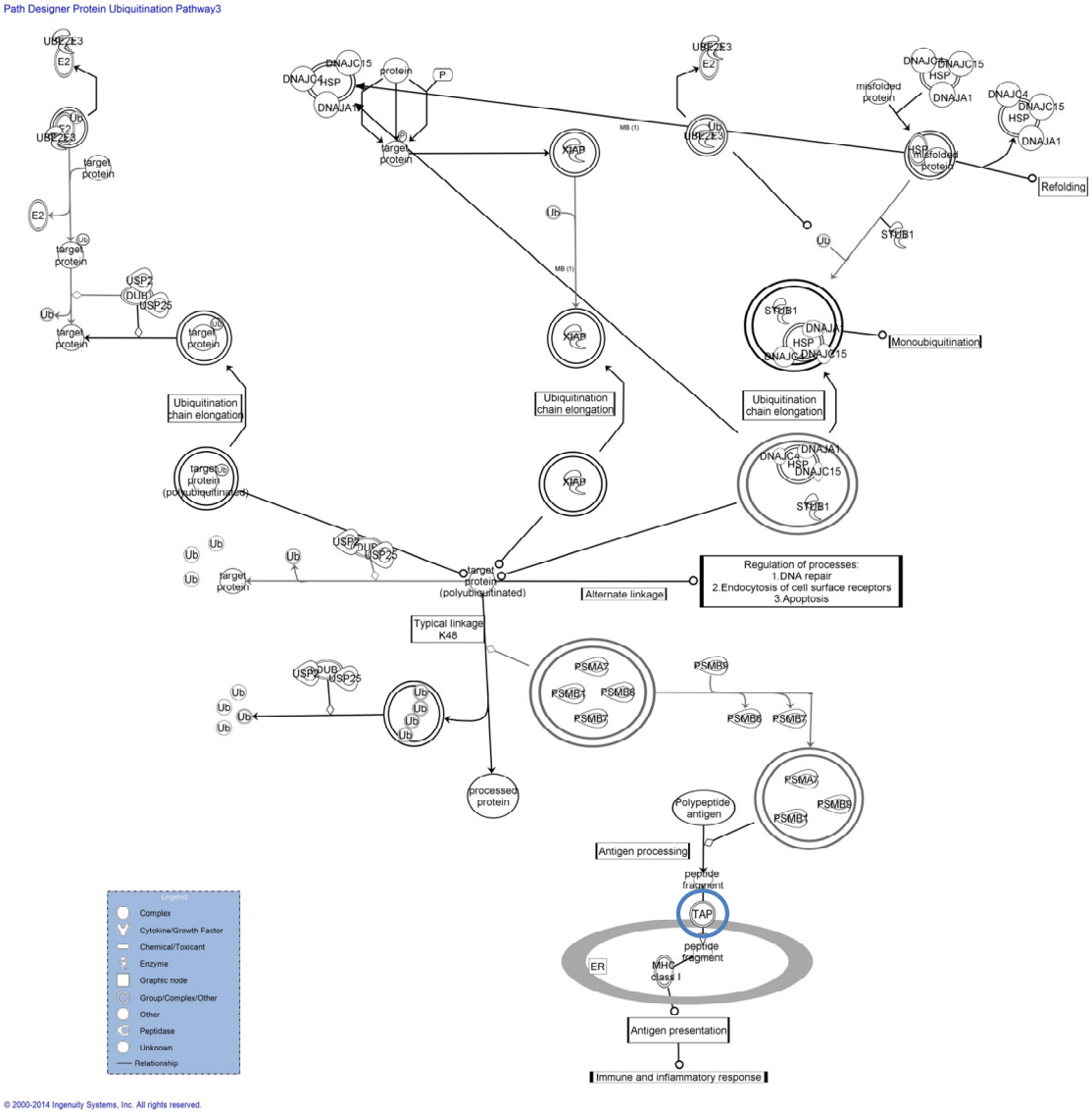


Figure 78. *Protein ubiquitination canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: blue to fish.*

To the Protein ubiquitination canonical pathway analysis, from the 14 genes expressed differently among virgin olive, sunflower and fish oil, only one was selected to validate by RT-PCR due to its location and importance in this and other pathways: Tap1, and it was validate in the fat where IPA identified the expression change. This represents 100 percentage of successful validation.

Figure 78 is a canonical pathway picture, simpler than original picture (figure 66), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

XI.3.11. RTPCR-validated genes at the TCA cycle II canonical pathway

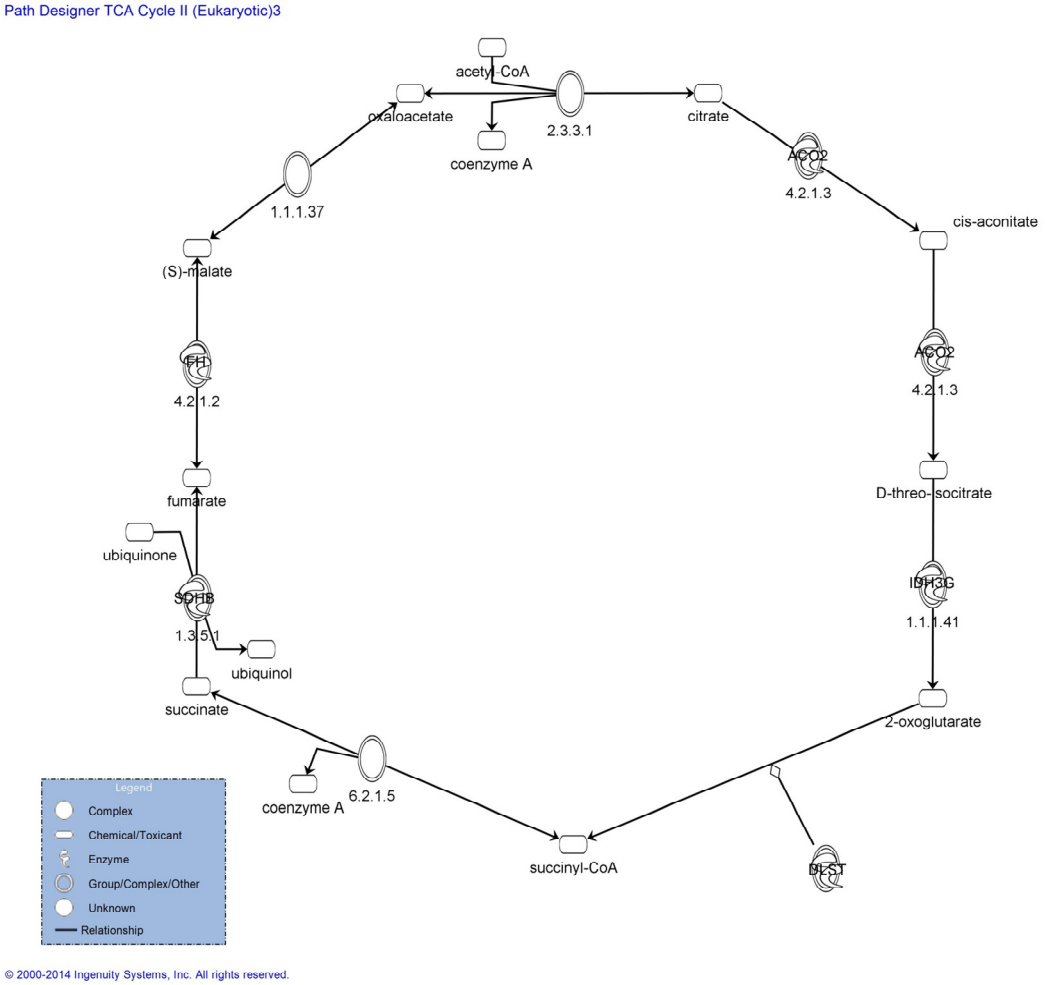


Figure 79. *TCA cycle II canonical pathway picture with genes validated by RT-PCR.*

Any gene from the TCA cycle II canonical pathway analysis was selected to validate by RT-PCR due to its location on the pathway was not decisive, nor appeared on any other pathway.

Figure 79 is a canonical pathway picture, simpler than original picture (figure 67), in which any gene has been surrounding since none has been selected to validate.

XI.3.12. RTPCR-validated genes at the ERK/MAPK signaling canonical pathway

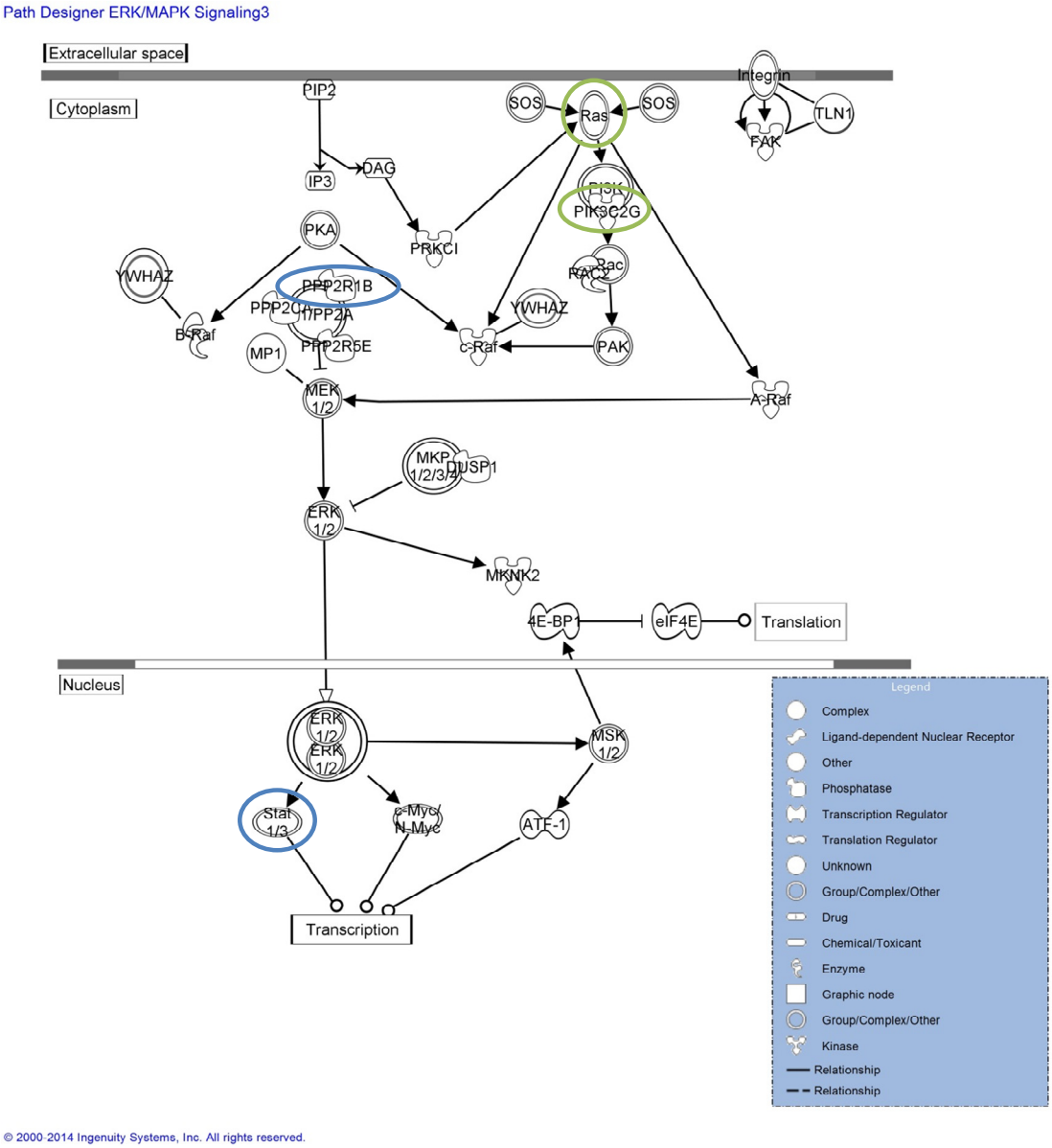


Figure 80. *ERK/MAPK signaling canonical pathway picture with genes validated by RT-PCR. The color of the circle indicates the type of dietary fat: green to virgin olive and blue to fish.*

To the ERK/MAPK signaling canonical pathway analysis, from the 16 genes expressed differently among virgin olive, sunflower and fish oil, 4 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Pik3c2g, Ppp2r1b, Rras and Stat1. All of them have been validated, representing 100 percentage of successful validation. Genes were validated in all fats where IPA identified the expression change except Ppp2r1b, whose validation only occurred in fish oil, while expression change in rats fed on sunflower oil was not confirmed by RT-PCR.

Figure 80 is a canonical pathway picture, simpler than original picture (figure 68), in which validated genes have been surrounding with a continuous circle whose color indicates the type of dietary fat: green to virgin olive and blue to fish.

DISCUSSION

Aging is a normal event in the lives of all organisms with the exception of the germ cells and malignant cell lines. Senescence is characterized by progressive decline of the function which concludes with cell death by loss of cell capacity response against oxidative damage (Sohal and Orr, 2012; Barja, 2013).

Many organ systems exhibit significant age-related deficits, but, based on studies in old rodents and elderly humans, the liver appears to be relatively protected from such changes, being the tissues without the ability to regenerate (postmitotic tissues) the most affected by aging (Bellanti *et al*., 2013). However, the liver is one of the most metabolically active organs, so their morphological, structural and functional alterations confer it a special importance from a nutritional point of view. On the one hand, the liver does not exhibit marked changes in either structure or function during the aging process. In aging, there is an increase in rat hepatocytes volume and decrease the hepatocyte number. Other changes in hepatocellular structure include a loss of smooth surfaced endoplasmic reticulum, an increase in the volume of the dense body compartment, such as secondary lysosomes, residual bodies, or lipofuscin; and an increase in hepatocyte polyploidy (Irrisarri and Hollander, 1992). On the other hand, there are data that demonstrate specific age-related changes, including a loss of hepatic volume between 25% and 35%, and a decline in hepatic perfusion; both of which may affect certain liver functions, such as first pass pharmacokinetics (Schmucker and Sanchez, 2011; Lopez-Cruzan and Herman, 2013).

Aging has been associated with multiple changes in liver function while the clinically most relevant biochemical parameters of this organ function remain normal in senescent individuals. Therefore, these biochemical parameters should be used to study liver diseases rather than to evaluate the aging (Anantharaju and Van Thiel, 2003). It has been shown that hepatic triglyceride and cholesterol content increases with age, altering cholesterol homeostasis, while phospholipids remaining quantitatively unchanged (Bose *et al*., 2005). It has been demonstrated a reduction in both proliferative capacity and resistance to oxidative stress in aged rat hepatocytes (Ikeyama *et al*., 2003), although enzymatic activity and respiratory capacity almost remain the same during aging in the liver (Quiles *et al*., 2002b). The widely demonstrated "free radical theory of aging" postulated that ROS are part of the mechanisms involved in the aging process and in many mitochondrial disorders, as well as in alterations in proteins, lipids and mtDNA and mitochondrial apoptosis (Barja, 2013). Hepatocyte has a wild of antioxidant defense system, including small molecule antioxidants and antioxidant enzymes, protecting mitochondria from oxidative damage and leading in some extent to lifespan extension. Some of these antioxidant compounds are in one way or another influenced by diet (Chinnadurai *et al*., 2013; Gruber *et al*., 2013; Stankovic *et al*., 2013). Several studies have reported that the levels of different markers of oxidative stress increase with ageing, although other studies did not find such an increase (Halliwell and Gutteridge, 2007). Such a disparity in the results may be due to the nature of the biological sample or in the studied biomarker. Concerning the nature of the biological sample, many studies have been carried out using tissue homogenates or whole cells. In relation to this, it should be noted that most of the oxidative stress in the cell occurs in mitochondria. Therefore, a poor choice of biological sample could give rise to misleading results, since mtDNA represents approximately 5% of nuclear DNA. Furthermore, the net oxidative stress seems to be dependent on the sex of the animal, the species, the studied tissue, the lipid profile of mitochondrial membranes, etc. Enhanced levels of exhaled pentane and ethane related to age in old rats have been reported. In a similar way, higher levels of carbonyl radicals and 8-OH-dG have been found in the brain and other tissues from rats, mice and humans (Kadiiska *et al*., 2005; Sohal and Orr, 2012).

According to Halliwell and Gutteridge (2007), the steady state level of oxidative stress is the result of the balance between the levels of damage and the degree of repair or replacement of damaged molecules. According to that, in terms of balance, a net increase in oxidative stress during ageing may be found in terms of higher levels of damage or as the result of defects in the repair system. In that sense, a positive correlation between the efficiency of DNA repair systems and species longevity has been observed (de Souza-Pinto *et al*., 2008). On other hand, it has been reported that the capacity of several cell lines to degrade abnormal proteins and to repair DNA seems to diminish with ageing. Thus, conceptually, the term "oxidative stress", as well as considering the level of ROS production and the fall in the antioxidant capacity, should be extended to the complex, and not yet well understood, framework of the damage repairing systems (Gredilla *et al*., 2010).

In relation to the response of antioxidant defenses, it seems that overall protection does not decline with aging (Quiles et al., 2004b, 2010b; Ochoa et al., 2007, 2011), although several exceptions have been described.

Since the liver is the central organ of metabolism, changes in diet have a great impact on this organ and overall on health with aging (Anantharaju, *et al*., 2002). It is well known that dietary fat source strongly influences many parameters of the hepatic mitochondria. These changes includes modification of lipid composition of mitochondrial membrane, affecting the mtETC functions, oxidative stress and mtDNA alterations (Quiles *et al*., 1999a, 2001, 2002b, 2004c, 2006b; Ochoa *et al*., 2001, 2003, 2011; Bullón *et al*., 2013; Roche *et al*., 2013).

According to all the above mentioned features of aging at the liver, the main objective of this work has been to extensively study the aging process in the liver of rats fed lifelong on different dietary fats, namely, virgin olive, sunflower or fish oils. The study has been performed comparing young (6 months) *versus* old (24 months) animals.

1. CONSTATATION OF THE AGING PROCESS

According scientific literature, Wistar rat lifespan has been stabilized around 26 months depending on diet and sex of the animal (Hubert *et al*., 2000). Although mean lifespan for rodents is considered around 50% at 2 years old, it has been found that this age has been decreasing for several species of laboratory rats, including Wistar rats, for the last decades, which may be due to consanguinity, increased caloric intake leading to a greater tendency to obesity, and maybe others (Keenan *et al*., 1998). Considering many studies that deal senescence in rats, it can be established a starting point for study beginning with young animals into adulthood, taking the first age period at 6 months and the old age at 24 months, although some studies extend their research at 28 and 30 months. However, at this age rats survival decreases drastically, which could involve methodological difficulties to finish the study (Vollmar *et al*., 2002). Furthermore, many of the considerations related to aging should be treated differently from certain ages, as occurs in human centenarians, where the empirical models predicting survival are not valid (Kowald, 2002). Sequencing of the experimental period at two points, taking as initial and final values at 6 months (young rats) and 24 months (old rats) respectively, is therefore justified.

The evolution of animal and liver weights in the two selected ages has been studied. Also the relationship between the liver and brain weights has been analyzed, since this parameter is considered to be more stable than liver weight and it is considered a good indicator of aging (Marton *et al*., 2013). Figure 41 A, B and C show that both in general and independently each diet there is an increase in body weight, liver weight and liver to brain ratio in old animals respectively, depending accordingly on age effect. These results are in agreement with

preliminary studies on aging at the liver (Quiles *et al*., 2004a, 2004b, 2006c; Ochoa *et al*., 2011; Bullón *et al*., 2013; Roche *et al*., 2013). Besides these effects due to the age, one of the studied dietary fats (fish oil) seems to induce additional changes at the liver weight which would be analyzed later.

In addition to weight parameters, some others analyzed markers that will be discussed in further sections, also helps us to corroborate the effect of aging on animal's liver. Among these aging markers, histopathological and ultrastructural parameters should be included. Even, the main effect found in the genomic analysis by microarrays came from animal aging.

Overall these considerations allow concluding that the selected age points (6 and 24 months) are good reference points for young and old animals, as it was the target of the study.

2. LIVER ADAPTATION TO DIETARY FAT TREATMENT

The objective of this study was to investigate the effect of virgin olive, sunflower and fish oils on aging at the liver. To achieve this goal male Wistar rats were fed lifelong on these dietary treatments. Consequently, it is necessary to evaluate if animals have adapted their hepatic mitochondrial membranes to dietary lipid profile. Lipid profile adaptation means that dietary fatty acid profile should be reflected at least in part at the liver biomembranes level. To corroborate this issue, lipid profile was analyzed at the liver mitochondrial membranes, since that we have been previously demonstrate that this is a good marker of dietary fat intervention at different tissues levels, including liver, brain, heart and skeletal muscle (Ochoa *et al*., 2003, 2007; Quiles *et al*., 2004b; 2006c, 2010b). In fact, in previous works based on virgin olive or sunflower oils treatments in rat liver (Quiles *et al*., 2002b; Ochoa *et al*., 2003), mitochondrial membranes lipid profile of animals aged 6, 12, 18 and 24 months clearly reflected differences based on dietary treatments, including higher levels of MUFA in animals fed on virgin olive oil and higher PUFA levels in those fed on sunflower oil. These differences were more pronounced as the duration of treatment increased and therefore the age of the animal. Table 22 shows mitochondrial membranes fatty acid profile in young animals from the present study. In this table it can be shown differences between animals fed on a specific dietary fat in most of fatty acid content as well as in n6 and n3 PUFA indices. In particular, animals fed on sunflower oil led to the main representative fatty acids in this oil, namely linoleic acid. Something similar happened with animals fed on fish oil concerning EPA and DHA fatty acids. These data suggest a proper adaptation of experimental animals to dietary treatments as expected from previous reports (Quiles *et al*., 2002b, 2006c; Ochoa *et al*., 2005, 2007, 2011; Priore *et al*., 2012).

In summary, it can be stated that liver of the animals from the present study show a good adaptation to the diet. These finding are noteworthy since any benefit or damage observed at any the two studied experimental periods might come from the differences between lipid sources properties.

3. HISTOPATHOLOGY

Despite the fact that the liver is one of the organs less affected by aging, it has been found that lifelong changes in diet, either in quality or quantity of fat, alters fatty acid composition in liver and turn the liver more or less susceptible to external aggressions and to develop non-alcoholic fatty liver disease (NAFLD). Several liver pathologies are included in NAFLD, from benign hepatic lipid accumulation (steatosis) to NASH (inflammation), fibrosis, cirrhosis, and ultimately hepatocellular cancer (De Minicis *et al*., 2013). The increased prevalence of NAFLD during the last decades was associated with deep modifications of dietary habits, especially

increased fat intakes (Ramírez-Tortosa *et al*., 2009; Molendi-Coste *et al*., 2010). NASH is increasingly recognized as a precursor to a more severe liver disease and that in 15 to 25% of patients evolves into cirrhosis or even to hepatocellular carcinoma (Angulo, 2002). In NASH, fibrosis may or may not be present (McCullough, 2006). In the general population, NASH has increased from previous estimates and is now in the range of 7,7 to 17%, which makes this condition one of the most common liver diseases in the United States (Lieber *et al*., 2004). In Europe, data refers to NAFLD and are in the range of 20 to 30,14 % and until 69,5% in patient with type 2 diabetes (Blachier *et a*l., 2013). In addition, NASH is responsible for asymptomatic elevation of serum aminotransferases in 40 to 90% of cases; also, it represents a frequent cause of abnormal liver tests in blood donors (Portincasa *et al*., 2004), and recent research suggests that obesity-related fatty-liver disease is intimately related to metabolic syndrome (Marchesini and Babini, 2006), which in addition is markedly increased by aging (Sheedfar *et al*., 2013).

Although much is known about how fat accumulates in the liver, much remains unknown about how this causes hepatocellular injury. The consequences of injury are recognized as NASH and progressive fibrosis. The pathogenesis of NASH is not fully understood, and the "two hit" hypothesis proposed by Day and James (Day and James, 1998) remains the prevailing theory. The "first hit" is the development of steatosis because of prolonged overnutrition that leads to accumulation of liver free fatty acids and triglycerides. In the "second hit," steatosis progresses to NASH and this progression is associated with factors such as oxidative stress, mitochondrial dysfunction, lipid peroxidation and inflammatory cytokines. However, it has not been established so far a relationship or a pathogenic link between NASH and oxidative stress (Ramírez-Tortosa *et al*., 2009). The presence of oxidizable fat in the liver led to hepatic lipid peroxidation, which products react with mtDNA and mitochondrial respiratory chain to partially block the transfer of electrons within the respiratory chain and increase mitochondrial ROS formation. So, animals with NASH have a decreased ability to re-synthesize ATP after a fructose challenge, and their hepatic mitochondria exhibit liver ultrastructural lesions, depletion of mtDNA, and decreased activity of respiratory chain complexes (Pessayre *et al*., 2004). Lipid peroxidation products and high leptin levels, as well as ROS, activate hepatic stellate cells that acquire the characteristics of contractile cells (myofibroblasts). These cells secrete large amounts of extracellular matrix proteins that accumulate and led to fibrogenesis (Kisseleva and Brenner, 2006; Tanaka *et al*., 2008).

Hepatic steatosis is the most frequent and initially observed morphological feature of NASH. Along with ballooning and lobular and portal inflammatory infiltrates degeneration, they are the lesions considered important in steatohepatitis. In general, steatosis, inflammation and fibrosis are the major alterations of liver during NASH (Basaranoglu *et al*., 2013), being thus the appropriate biomarkers when studying this pathological state.

Dietary fat has been shown to play an important role in the pathogenesis of liver disease. In that sense, high fat diets have been directly related to the development of NASH, both in humans (Skamarauskas *et al*., 2013) and in animals models (Ramírez-Tortosa *et al*., 2009). Concerning dietary fat type, research has been mainly focused on the treatment of rodents with particular fats but delivered on a high amount intake. On the opposite, the study of different dietary fat types on a normolipidic base (as in the present research) is scarce.

Overall, experimental studies suggest that high fat diets mainly derived from PUFA and SFA exacerbate hepatic steatosis, leading to the development of NASH (usually associated to lipotoxicity, increased ROS and endoplasmic reticulum stress). However, when high fat diet is composed mainly on MUFA (especially when derived from virgin olive oil) certain degree of protection is observed. This positive effect might probably come from avoiding decrease in fatty acid beta oxidation, lower oxidative stress production, lower VLDL cholesterol accumulation

and higher insulin sensitivity, altogether leading to a less hepatic steatosis. In addition, MUFA diet decreases activation of hepatic stellate cells, resulting in a liver less prone to lipid peroxidation and with a lower fibrosis development, when compared to PUFAs (Assy *et al*., 2009; Wu *et al*., 2011; Hanke *et al*., 2013). Nevertheless, above mentioned detrimental PUFA effects on NASH should not be considered universal for all PUFA types. In animal models, an association between n3 PUFA deficiency and the development of hepatic steatosis has been described (Molendi-Coste *et al*., 2010). In this sense, Popescu *et al*. (2013) reported that a normolipidic diet supplemented with n3 PUFA reversed medium hepatopathy to normal liver architecture in mice with high fat diet-induced NAFLD. Also in humans, n3 PUFA intake has been reported to prevent high fat-diet induced NAFLD (Espinosa *et al*., 2013). Differences between n3 and n6 PUFA have been described concerning liver disease. So, an imbalanced dietary intake of n3 and n6 PUFAs plays a crucial role in the appearance of liver steatosis, while dietary fish oil or EPA/DHA supplementation regulates hepatic lipid metabolism, adipose tissue function, and inflammation, reducing liver steatosis in NASH patients (Bouzianas *et al*., 2013; Scorletti and Byrne, 2013). Even not all n3 PUFA has the same beneficial effect. It has been described that DHA has more capacity to reduce inflammation, oxidative stress and fibrosis in liver than EPA in diet-induced NASH, so dietary DHA might be useful in combating NASH in obese human (Depner *et al*., 2013).

Results from the present study offer differences concerning several aspects. The first and more evident effect comes from the age of the animals. In fact, irrespective of dietary treatment, there is an overall increase in steatosis, inflammation (NASH) and fibrosis for all groups of aged animals when compared with the young counterparts (with the exception of inflammation for virgin olive oil group, where no differences were found between young and old animals). It is well know that aging provides a physiological increase in lipid accumulation in non-adipose tissues, including the liver (Bose *et al*., 2005) compromising their normal functionality by promoting organ-specific toxic reactions, also known as lipotoxicity (Slawik and Vidal-Puig, 2006). Concerning NAFLD and aging, there is accumulating evidence that show an increased prevalence of NAFLD with older age in humans (Floreani, 2007). Aging has also been reported to significantly increase the progression of NAFLD to NASH and fibrosis, thus predisposing to increased mortality in elderly subjects with NAFLD (Frith *et al*., 2009). Our results also support the claim that the lifelong intake of a type of dietary fat in a normolipidic schedule differently affects hepatic morphology. In support of that, a higher degree of NASH, steatosis, centrolobulilar inflammation grade and fibrosis have been shown in aged rats.

Concerning dietary treatments, despite significant differences among dietary treatments composition, no changes were found concerning NASH. There is a trend to increase steatosis, inflammation and NASH in old rats fed on fish oil. The suspect that the type of dietary fat affects hepatic histopathology is confirmed with the study of fibrosis, where rats fed on sunflower oil shown higher value of liver fibrosis. This data are in agreement with previously reported studies showing that thermally oxidized sunflower oil induces fibrosis at the liver (Penumathsa *et al*., 2006; Clichici *et al*., 2011).

In conclusion it might be said that virgin olive oil could be the best dietary fat from the point of view of liver morphology. This assumption is based in the fact that although there is an overall increase in the accumulation of fat in the liver with aging, the three fats behave differently with respect to liver susceptibility to develop a disease associated with NAFLD, such as NASH, fibrosis, etc. In particular it can be concluded the following: one hand, the trend (formerly difference) to increased inflammation during aging observed in animals fed on fish oil does not appear even as a trend in those fed on virgin olive oil. So, virgin olive oil avoids age-related inflammation increase. On the other hand, from the point of view of fibrosis, sunflower

oil shown the worst response during aging, reporting significantly higher levels of fibrosis, despite having a response more similar to virgin olive oil than to fish olive, regarding moderate levels of inflammation.

4. ULTRASTRUCTURE

Although the liver appears to preserve its function through life relatively well, several changes have been associated with this organ during the process of aging (Anantharaju, 2002). Elucidating the nature and mechanisms of these changes in the aged liver no doubt should lead us to a better understanding and treatment of hepatic dysfunctions. Hepatocytes contain a large number of mitochondria which play an important role in the energy homeostasis (Le *et al*., 2013), which shape, size, distribution, transport and number is controlled by a balance among several events, including fusion and fission processes (Zhao *et al*., 2013). It has been demonstrated that liver mitochondria decreases in number but increases in size per unit with aging (Nagata *et al*., 2010).

Nutritional treatments have been historically associated with aging basically from the standpoint of CR and antioxidant therapy. Magwere *et al*. (2006) noticed that dietary restriction alters mitochondrial morphology (size, shape, and number), and also it has been reported that protein restriction in early life causes long-lasting changes in mitochondria, and this change is more evident in the liver and skeletal muscle (Wei *et al*., 2006). Diet has been also analyzed in relation to ultrastructure from the point of view of the fat (amount and type). Studies based on high-fat diets in rodent models have shown that, at the liver, mitochondria expanded and their number increased (Altunkaynak and Ozbek, 2009). In recent years, it has been demonstrated that dietary fat, through changes in membrane-lipid profiles, may help attenuate some deleterious aspects of aging, such as those related to exacerbated oxidative stress or mitochondrial dysfunction (Quiles *et al*., 2010b). Khraiwesh *et al*., (2013) combined the study of CR and dietary fat but in young animals. They separated animals into three CR groups with soybean oil (also in controls), fish oil, and lard. CR increases both density and number of cristae per mitochondrion, and concerning dietary fat treatments, lard increased also the mitochondrial size. In relation to dietary fat diets and aging, studies on fatty hepatocytes *in vitro*, treated with high-fat diets, have been reported that n3 PUFAs may change mitochondrial morphology having beneficial effects on recovery of mitochondrial function (Zhang *et al*., 2011). Quiles *et al*. (2006c) compared age-related alterations in isolated liver mitochondria from rat fed on MUFA (from olive oil) and n6 PUFA (from sunflower oil) delivered at an 8% w/w in the diet. Both, size and mitochondrial contour were similar irrespective of diet but lower in the old groups. The circularity parameter was similar for both dietary groups at 6 months of age, while it was higher in 24 months old animals fed sunflower oil. The number of cristae per micrometer of mitochondrial contour was lower in animals fed sunflower oil compared to those fed virgin olive oil, both at 6 and 24 months of age. Aging diminished the number of cristae in the sunflower oil-fed group. These authors also compared the age-related alterations in isolated heart mitochondria under similar experimental conditions (Quiles *et al*., 2010b). Less mitochondrial area and perimeter were reported for 24 months old n6 PUFA-fed animals while MUFA led to a higher density of mitochondrial cristae. Results from these studies suggest that MUFA can protect liver and heart mitochondria from age-related changes when compared with n6 PUFA related diets.

Following with the research of dietary fat effects on mitochondrial ultrastructure, as a part of the present study we investigated liver mitochondrial ultrastructure changes after lifelong feeding of rats with three different dietary fat sources (as a novelty in relation to the preliminary

studies we included fish oil). In the present research, dietary fat was delivered at 4% w/w (previous studies were at 8%) and the ultrastructural analysis was performed on liver tissue instead of isolated mitochondria. Presents results shown that lifelong intake on different dietary fat led to ultrastructural variations in aged animals. Briefly, animals fed on sunflower oil showed higher mitochondrial area and perimeter than those fed on virgin and fish oils, and less mitochondrial density than rats fed on virgin olive oil. Compared with above-mentioned studies on dietary fat type at 8% (Quiles *et al*., 2006c, 2010b), results from the present study are in agreement, as far as MUFA and n6 PUFA are concerning, i.e., better preserved ultrastructure after feeding MUFA diet. Regarding fish oil, response found at the liver is closer to that reported on MUFA than to the observed in n6 PUFA. Summarizing, dietary fat type conditions mitochondrial structure and number through life, not only when fat is present in the diet at high percentage (Quiles *et al*., 2006c, 2010b) or associated to CR (Khraiwesh *et al*., 2013), but also under a normolipidic schedule as in the present work. Specifically, sunflower oil might be deleterious in two senses. On one hand, it leads to an increase in mitochondrial size, typical aging marked related to a loss of function through swelling condition and Ψ_{mt} loss (Sastre *et al*., 2003). On the other hand, it promotes a decreasing in mitochondrial density, which might be trying to be compensated through the stimulation of the biogenesis machinery.

The complexity of mitochondrial biogenesis regulation cannot be understated; it involves changes in the expression of more than 1000 genes, the cooperation of two genomes, and alters the level of approximately 20% of cellular proteins (López-Lluch *et al*., 2008). At the molecular level, several transcription factors and cofactors are involved in the activation and regulation of mitochondrial biogenesis. Despite the complexity of the various signaling pathways that converge to regulate mitochondrial biogenesis, they all seem to share the common key component of the PGC1 family of co-transcription factors. Several studies suggest that PGC1 family of coactivators is of particular importance in the control of liver metabolism. PGC1α stimulates mitochondrial biogenesis and respiration in multiple cell types and modulates biological programs normally associated with increased oxidative metabolism, such as hepatic gluconeogeogenesis. It does it also by coactivating transcription factors in a tissue-specific manner, such as FOXO1 in the liver, and the MEF2 family members in skeletal muscle (Lin *et al*., 2005). In liver, several reports have been published concerning lower levels for PGC1α and PGC1α in patients affected by NAFLD and in several models of obesity (Lin *et al*., 2005). Another reason that reinforces the importance of PGC1 in liver metabolism comes from a study in which a mutation in PGC1β causes mitochondrial dysfunction and liver insulin resistance (Vianna *et al*., 2006). In the present study, no significant differences associated to aging or to dietary fat type were observed. The transcriptional coactivator PGC1α plays important roles in the expression of genes not only involved in mitochondrial biogenesis, but also in some related to fatty acid metabolism and lipid accumulation. Since in this work liver fat accumulation is also observed, maybe biogenesis aspect of PGC1α might be masked.

PGC1α appears to act as a master regulator of energy metabolism and mitochondrial biogenesis by integrating and coordinating the activity of multiple transcription factors, such as NRF-1, -2, PPARα and mtTFA (also referred to as TFAM) (Puigserver *et al., 1998*). We also analyzed the expression of TFAM, crucial in mitochondrial transcription and biogenesis (Håkansson *et al*., 2011). TFAM is a stimulatory factor that unwinds DNA allowing mtDNA transcription. TFAM knockout mouse displays embryonic lethality and a depletion of mtDNA confirming an essential role for the protein in mtDNA maintenance in mammals (Scarpulla, 2008). In other words, TFAM is considered one of the most specific marked of mitochondrial biogenesis (Viña *et al*., 2009). Our results show that aging has effect on TFAM only in animals fed on sunflower oil, reporting higher TFAM levels in old animals when compared with their

younger counterparts. On other hand, at 24 months fish oil group reported lower relative amounts than virgin olive and sunflower oil fed rats.

Among the plethora of biological phenomena affected by aging, the malfunction and decrease of biogenesis of mitochondria seem to exert some of the most potent effects on the organism. If biogenesis is affected, it is reasonable to expect mitochondrial turnover must be slower and the accumulation of modified lipids, proteins and DNA must also increase, further aggravating the situation resulting from the deficient activity of aged mitochondria. The precise reason for the decrease in the rate of mitochondrial biogenesis during aging is currently unknown. However, it seems that both, extra- and intra-cellular regulatory factors of mitochondrial biogenesis are implicated (Navarro and Boveris, 2007). In our conditions, lipid accumulation at the liver indicated as steatosis is similarly present with the three dietary treatments. Despite that, mitochondrial ultrastructure and biogenesis markers are differentially affected. This question leads us wonder if biogenesis and ultrastructural disruptions are the cause or the consequence of the lipid accumulation at the liver. Nevertheless, dietary fat type seems to be important because, despite for the three treatments lipid accumulation is the same, ultrastructural disruptions followed by biogenesis activation is only present for n6 PUFA diet. In this sense, a variety of strategies are currently been considered to alleviate deficits in mitochondrial activity and biogenesis during aging. For example, it has been observed that several conditions that promote survival such as vitamin E dietary supplementation, CR, polyphenols and moderate physical exercise ameliorate mitochondrial dysfunction in aging (López-Lluch *et al*., 2008). Altogether, results from the present study are fully in agreement, since those dietary fat leading to the highest lifespan, namely virgin olive and fish oils (data not shown) fully correlates with those reporting the better response from the point of view mitochondrial morphology and density.

5. OXIDATIVE STRESS

Oxidative stress as etiopathogenic agent of aging could be based on the increase in ROS production and the removal of these species, which would be more or less, related to the cellular antioxidant systems. However, although antioxidants are involved in the protection against several diseases associated with aging, it seems that they are not responsible to control the degree of aging (Cutler and Rodríguez, 2003). In agreement with this hypothesis there are at least four lines of evidence (Quiles *et al*., 2010a):

a) Antioxidants not decrease with aging, quite the contrary.

b) From a theoretical point of view, it would be expected that the long-lived species had more potent or effective antioxidant machinery than those living less. However, the scientific evidence indicates the opposite.

c) Different studies in mammals with increased antioxidants levels, through nutritional supplementation or by pharmacological induction or transgenic techniques, show no change at the level of maximum lifespan in most the cases.

d) Studies with knock-out animals in which several antioxidants have been blocked at the genetic level have shown no changes concerning aging.

ROS production and ROS-mediated damage are counteracted in cells by different mechanisms. These include DNA (Hazra *et al*., 2007), mtDNA (de Souza-Pinto *et al*., 2008), and oxidized protein-repairing mechanisms (Ugarte *et al*., 2010). Also, systems that degrade oxidized molecules (Ghazi *et al*., 2007), autophagy machinery recognizing damaged organelles (Chen and Gibson, 2008), defense mechanisms to reduce ROS production (Barja, 2007), and

biosynthesis of macromolecules resistant to oxidative stress (Pamplona and Barja, 2007). The present study is mainly based on the last two mechanisms, i.e., to build biological systems more resistant to oxidative stress production. Finally, cells and body fluids contain molecules that neutralize or scavenge ROS, collectively termed antioxidant systems. Antioxidants may be divided into antioxidant enzymes that neutralize ROS by forming less toxic species, and antioxidant small molecules: the latter may either be endogenous metabolites and biosynthesized cofactors, or exogenous molecules taken up from the diet (Zimniak, 2008).

Briefly, concerning enzymatic antioxidants, SOD catalyzes the dismutation of superoxide to H_2O_2 and O_2 (Landis and Tower, 2005). SOD must work in concomitance with enzymes removing H_2O_2, such as catalases and peroxidases. Catalase is localized in peroxisomes and is very efficient having one of the highest turnover numbers known for an enzyme. Although mitochondria in most cells appear to lack catalase, the enzyme has been detected in mitochondria from rat liver (Salvi *et al*., 2007). Mitochondrial GSH emerges as the main line of defense for the maintenance of the appropriate mitochondrial redox environment to avoid or repair oxidative modifications leading to mitochondrial dysfunction and cell death (Marí *et al*., 2009). A vital component of the enzymatic antioxidant barrier is GPX and its selenium-dependent form, Se-GPX. In general, the highest activity of GPX is found in the liver, which is associated with a very high rate of oxygen metabolism in this organ and the detoxification and biotransformation of many compounds of endogenous origin and xenobiotics. In the presence of GSH, GPX catalyzes the reaction of H_2O_2 to water or the reaction of lipid peroxides to alcohols. Se-GPX has a fourfold higher enzymatic activity than selenium-independent peroxidases (Pilarczyk *et al*., 2011). PRXs and TRX belongs to this antioxidant enzymatic system (Rhee *et al*., 2005; Conrad, 2009).

Among non-enzymatic antioxidants, some of these are water-soluble and act in the cell's soluble fraction or in plasma, others being lipid-soluble act within membranes, while amphipathic molecules can act in both environments. Vitamin C (ascorbic acid) is water-soluble and cooperates with lipid-soluble Vitamin E to regenerate α-tocopherol from tocopheroxyl radical produced during the Vitamin E radical scavenging activity; the product of the reaction is a very stable ascorbate radical (Valko *et al*., 2006). α-tocopherol, one of the vitamin E active molecules, is the most important lipid-soluble antioxidant. It is distributed in all cellular membranes, including mitochondria, preventing lipid peroxidation (Saito *et al*., 2009). CoQ is an essential electron carrier in the mtETC, but is also present in serum lipoproteins, endomembranes and the plasma membrane (Turunen *et al*., 2004). CoQ is the only lipidic antioxidant that is synthesized in mammals, and its biosynthesis is a complex process which involves the participation of at least eight gene products. Besides the mtETC, several enzymes catalyze CoQ reduction to achieve its antioxidant reduced state in eukaryotic cells, such as NADH-cytochrome b5 and NADPH:quinone oxidoreductase 1 (NQO1) (Arroyo *et al*., 1998). In turns, CoQ also participates in the recycling of vitamin E. Finally there are a great number of dietary antioxidants contained in fruit and vegetables that are not vitamins or part of coenzymes, such as carotenoids and flavonoids. They may act as quenchers of singlet oxygen and peroxyl radicals forming more stable radicals that do not propagate and may act as chain terminators by reacting with other radicals (Valko *et al*., 2006).

Despite antioxidant systems, oxidative stress can be also quantified by assessing production degree but especially through analysis of fingers prints. Among the late, one of the most used tools is protein carbonylation. Free radicals enhance the level of carbonylation in some proteins, causing alterations in protein structure and function (Dalle-Donne *et al*., 2006b), and this can be monitored (Ramos *et al*., 2013). Age-related changes in protein carbonyl levels have been found in the liver of old mice, which showed significantly higher protein carbonyl amount

than young counterparts (Amano *et al*., 2013). Something similar has also been found in liver of old rats compared to young control groups (Cakatay *et al*., 2013). In this context of aging analysis, and regarding to oxidative stress, this physiological process has been associated to an increase in the activity of GPX in the liver of mice and rats (Bakala *et al*., 2012; García-Valles *et al*., 2013). Also CAT is considered an aging indicator, since its activity increased with age, as it has been observed in liver mitochondrial extract from old rats (Ochoa *et al*., 2011; Bakala *et al*., 2012).

Results from our laboratory conclude that besides the effect of age itself, dietary fat source is of utmost importance when assessing oxidative stress during aging. However, not all dietary fats have the same effect (Quiles *et al*., 2002c, 2004b, 2006c; Ochoa *et al*., 2003, 2007, 2011). Regarding the present work, protein carbonylation offered differences due to aging between young and old rats into two experimental groups, namely sunflower and fish oils, where old rats showed higher protein carbonyls concentration than young animals. Dietary fats effect on oxidative alterations to protein, assessed as carbonyl products, were not found in young animals, but in old rats the virgin olive oil group reported lower values than the sunflower and fish oils groups. Concerning oxidative stress and aging, opposing results have been reported in past years (Halliwell and Gutteridge, 2007). However, after contradictory artifacts are avoided and appropriate biomarkers are used, overall, it seems that free radical damage increases during aging (Chevanne *et al*., 2003). In general, our study agrees with the abovementioned assumption, which is additionally in accord with the free radical theory of aging of Harman (Harman, 2006), except for virgin olive oil group. Thus, we found increased levels of damage with aging in n6 and n3 PUFA-fed groups. Previous studies comparing lifelong feeding on MUFA *vs.* n6 PUFA-diets at 8% w/w during aging offered partially similar results that those found in the present study. In that sense, lipid peroxidation at the liver of animals fed on virgin or sunflower oils were higher than those found in young animals (Quiles *et al*., 2006c). Similarly, the same animals presented higher degree of protein oxidation at the brain, irrespective of the assayed dietary fat (Quiles *et al*., 2010b).

In relation to dietary fat type and oxidative stress, it has been well established that unsaturation level is responsible for differences concerning this issue. Particularly, high unsaturation degrees are associated with higher oxidative stress status (Quiles *et al*., 2002c, 2004b, 2006c; Ochoa *et al*., 2003, 2007, 2011). Here, dietary fat showed differences only in old animals. These differences followed the above mentioned concept, with MUFA-fed animals reporting the lowest oxidative stress status. However, despite unsaturation levels were different between sunflower and fish oils no differences were found in protein carbonylation. At this point, several considerations have to be taken. First, why we did not find differences among dietary fat for young animals? Second, why virgin olive oil-fed animals did not increase oxidative stress with age? Third, why old animals fed on sunflower or fish oils did not reporter differences concerning protein carbonyl status? Perhaps the third question is the easiest to answer, since it has been previously demonstrated that the increasing *in vitro* susceptibility to oxidation offered by fatty acids with increasing unsaturation has not direct correlation *in vivo* (Sevanian and Hochstein, 1985). This is particularly true when comparing n6 *vs*. n3 PUFA, and has its origin in the production of pro- or anti-inflammatory prostaglandin-related products of both dietary fat types (cita nuestra). As far as the second question is considered, preliminary studies including those above mentioned (Quiles *et al*., 2002c, 2004b, 2006c; Ochoa *et al*., 2003, 2007, 2011) offered differences among dietary fat types concerning oxidative stress status under several physiological or pathological conditions. However, in most of these studies dietary treatment was based on higher dietary fat proportions. In the present study, dietary fat was delivered at 4% of w/w and the animals were not undergoing additional stress situations.

According to that, low dietary fat level would not lead to differences on oxidative stress status for short periods. The same consideration might be done in relation to second question above established concerning lack of increasing protein carbonylation for animal fed on virgin olive oil.

The second element of analysis as the oxidative stress status is concerned is that related to the antioxidant system. So, results from the present research in liver are in agreement with previous finding of this group (Ochoa *et al*., 2003, 2007, 2011; Quiles *et al*., 2006c), even though the experimental conditions were varied slightly, as we have noted on several occasions, as to the amount of fat (from 8% to 4% w/w) and the dietary treatments (from two to three fat sources). At 24 months rats from virgin olive oil group showed lower values than those fed on sunflower and fish oils. Meanwhile, in 6 months old rats, GPX showed higher values in rats fed on fish oil than in those fed on virgin olive and sunflower oils, Se-GPX was lower in virgin olive oil group than in sunflower and fish oil groups, and no differences were found in catalase amount. At 24 months, more differences were identified. Old rats showed again higher values of total GPX in fish oil group than in virgin olive and sunflower oil groups. The activity of CAT was lower in rats fed on virgin olive oil than in those fed on fish oil, and no differences were found in Se-GPX amount. Differences due to aging between young and old rats were found in the three experimental groups in GPX and Se-GPX, where the highest values were found at 24 months. Catalase values at 24 about 6 months were higher only in rats fed on fish oil.

Regarding non-enzymatic antioxidant analyses in the present research, no differences were found for α-tocopherol, the most important vitamin E element. Previous studies showed higher levels of this molecule associated to aging but in studies based on higher dietary fat content. Since, it has been established that vitamin E content at the membrane level has a direct correlation with lipid content (Saito *et al*., 2009), this would be in the basis for the lack of differences found here. In relation to CoQ, it has been described that mitochondrial levels of this molecule at the liver increases in animals with aging (Quiles *et al*., 2004a, 2006c; Ochoa *et al*., 2005, 2007). It has been widely described the dietary treatment effect on CoQ mitochondrial and plasmatic levels in rodents. In that sense, CoQ content was increased in plasma of young rats fed on a high fat diet (Safwat *et al*., 2009; Bravo *et al*., 2012), as well as old mice (Kohli *et al*., 2010). Effect of the dietary fat source on CoQ mitochondrial and plasmatic levels in rats has been researched by our group in several times. There is an influence of dietary fat on the contents of CoQ_9 and CoQ_{10} which must be taken into account when considering the levels of CoQ in mitochondrial membrane. It has been reported that the amount of CoQ in different tissues varies considerably according dietary fat type. In heart from rats fed on 8% w/w of fat, both at 6 and 24 months, In liver, CoQ levels for animals fed olive oil compared with sunflower oil were higher for 6 months of age and lower for 24 months. Age affected only the sunflower-oil group, with an increase in animals of 24 months of age. In heart, for both age periods, olive oil led to lower levels of CoQ than sunflower oil. A time-course increase was found for olive-oil-fed animals. In skeletal muscle, the olive-oil group registered lower levels of CoQ than did the sunflower-oil group at both ages. In terms of age, this molecule increased at 24 months for both dietary groups (Quiles *et al*., 2002b). Studies on brain also showed diet-related differences but only in old rats, where higher CoQ levels were reported in animals fed on sunflower oil (Ochoa *et al*., 2011).

In the present work, a significant increase in the levels of CoQ it has been observed in mitochondrial extracts from 24 months old rats, respecting their young counterparts, in the three experimental groups (virgin olive, sunflower and fish oils). These increased CoQ levels are in agreement with those previously described (Quiles *et al*., 2004a, 2006c; Ochoa *et al*., 2005, 2007). At 6 months, only differences between dietary groups were found for the ratio between

CoQ_{10} and CoQ_9, with higher values for animals fed on fish oil. However this ratio was lower in sunflower oil than in fish oil group at 24 months. In general, in aged rats lower values for different CoQ forms were found in fish oil group than sunflower oil group. Sunflower oil led to lower CoQ_{10} and CoQ_9 ratio due to a higher CoQ_9H_2 to CoQ_9 ratio in old rats.

Taking into consideration that oxidative stress results from an imbalance between the production of free radicals and the scavenger antioxidant system (Malik and Storey, 2009), the explanations for these accumulation of antioxidants and CoQ in liver mitochondria might be due to the increased need for antioxidant molecules that protect against oxidative stress increased (Lenaz, 2012), or as secondary effect due to the accumulation of lipids with age (Quiles *et al*., 2006b). Finally, CoQ enhance could also be due to an increase in needs of the molecule as mitochondrial electron carrier, as a result of an age-related effect to bioenergetic function (Hargreaves, 2014).

Finally, the message obtained from the analysis of oxidation markers and antioxidants systems is that dietary fat is important especially as the animals age. In this sense, the aging process *per se* implies a condition of stress for the organism, and is under this situation when dietary fat is of particular relevance. So there is one particular fat that seems to prevent, at least in part, some of the effects of this stress. This is evident when analyzing both protein carbonyl levels as well as most of the antioxidant defense system, which generally increase as does the oxidative stress itself.

6. MITOCHONDRIAL FUNCTIONALITY

Aging, considered as an endogenous and progressive phenomenon, leads throughout the lifespan to different disturbances in mitochondria and their components, such as mtETC. These disturbances (which have a high oxidative component) have a negative impact on mitochondrial structure and function (Barja, 2013). Mitochondria are essential for several biological processes including energy production by generating ATP through the mtETC located on the inner mitochondrial membrane. The “oxidative stress theory of aging” suggests that mitochondria play a key role in aging as they are the main cellular source of ROS as well as the main ROS-target, which indiscriminately damage macromolecules leading to an age-dependent decline in biological function (Romano *et al*., 2013). Mitochondria are especially sensitive to oxidative damage in the pathogenesis of disease and aging, exhibiting different degrees of mitochondrial dysfunction probably due to changes in the activity of key components of the respiratory chain (Ralph *et al*., 2011). It has been noted the mitochondrial dysfunction in aged rats, characterized by depletion of membrane potential with increased proton leak rates and intramitochondrial free radical production, and a significant reduction of ATPase and complex activities (Puche *et al*., 2008). Age-related degradation of mitochondrial respiratory chain reactions deregulates the balance between generation and elimination of ROS by the antioxidant defense system (Sanz *et al*., 2006a), which might increase the emission of reactive molecules and their detrimental consequences on nucleic acids, proteins and lipid, leading to age-related detrimental effects, such as reduction of hepatic mitochondrial membrane fluidity (Moghadam *et al*., 2013). Rat hepatocytes have shown a reduction in resistance to oxidative stress as a function of aging as well as alterations in the mtETC (Ikeyama *et al*., 2003). Studies with hepatocytes from young and old mice have shown different response to mtETC dysfunction being reported that aging is associated with chronic dysfunction of mitochondrial respiratory chain, either at site I or III (Lopez-Cruzan and Herman, 2013). Moreover, it has been demonstrated that complex I and III activities are significantly increased in the aged liver mitochondria (Zhao *et al*., 2014). Several reports suggest that complex I has a central role in the regulation of longevity through two

mechanisms: an ROS-dependent mechanism that leads to mitochondrial DNA damage (Barros *et al*., 2010) and an ROS-independent mechanism controlling the NAD^+ to NADH ratio, which allow the regulation of glycol- and lipoxidative-damage (Trifunovic, 2006) and the activation of sirtuins (Pamplona and Barja, 2007). All of this suggests that aging in the liver is accompanied by a dysfunctional mitochondrial network that can be due to structural or functional respiratory defects, or both.

It is well know that dietary fat type determines several biochemical parameters at the mitochondrial membrane level (Quiles *et al*., 2006b). The importance of fatty acids resides in the fact that mitochondrial membrane adapts its lipid composition to dietary fat. Biological membranes are composed of more than 50 % of lipids, and composition, characteristics and functions of the biological membrane depends on the quantity and quality of dietary lipids, (Ochoa *et al*., 2003, 2007; Quiles *et al*., 2004b; 2006c, 2010b). The mitochondrial respiratory chain enzymes are membrane protein complexes whose function depends on the composition and fluidity of the mitochondrial membrane lipid (Lemieux *et al*., 2008; Aoun *et al*., 2012a; Bullón *et al*., 2013). So, the dietary fat type is able to condition the mtETC efficiency through, among other, mitochondrial membrane lipid composition. In that sense, Aoun *et al*. (2012a) have reported that fish oil-rich diets at 30% w/w decreased complex I activity in liver of rats in comparison to others diets (basal diet with 5 % or 30 % fat, lard-rich diet with 5 % or 30 % fat and fish oil-rich diet with 5 % fat). Our group has previously investigated the role of dietary fat and aging on several aspects of mitochondrial function. Thus, in the previously described study we analyzed the role of 8% of fat during aging on mitochondrial function through cytochrome b and the amount and activity (CCO system) and concentration (cytochrome a+a3) of the complex IV (Quiles *et al*,. 2002b). Concerning cytochrome b in liver, the olive-oil group showed higher values for 6 months and lower levels for 24 months, compared with the sunflower-oil group. Age affected only the sunflower-oil group, with a sharp increase at 24 months. For heart, no differences were found between dietary treatments for 6 months of age, and higher levels were found in sunflower oil at 24 months. For skeletal muscle, both groups were similar at 6 months and the olive-oil group differed from the sunflower group at 24 months (with lower values). Aging boosted cytochrome b values in both groups at 24 months. Regarding the cytochrome a+a3 concentration for liver, dietary treatment had an effect only at 24 months, with higher levels found in rats fed sunflower oil. Age affected only the sunflower-oil group, with higher values at 24 months. In heart, no differences between diets were found for both age periods. Age affected both dietary groups, which decreased cytochrome a+a3 levels at 24 months. In skeletal muscle, no differences were found between dietary treatments. The age effect was reflected as higher values at 24 months for both groups. In terms of CCO activity in liver, differences were found between the diets at 24 months (higher activity for sunflower oil). No age effect was found for olive-oil-fed animals but CCO activity increased at 24 months in sunflower-oil group. In heart, no differences between diets were found. Aging led to a decrease in both groups. For skeletal muscle, diet had an effect only at 6 months, with higher activity for the sunflower-oil group. Aging led to a reduction in CCO activity in the sunflower-oil group. Complex IV turnover for liver registered no differences with regard to diet or age. In heart, a higher turnover was found at both age periods for sunflower-oil rats. Aging led to a sharp decrease in turnover for both dietary groups. In skeletal muscle, diet resulted in differences only at 24 months, with higher levels for the sunflower-oil group. Age decreased the turnover in both dietary groups, showing lower values for 24 months (Quiles *et al*,. 2002b, Quiles and Ramírez-Tortosa, 2007). In the present study, to expand the knowledge about the mtETC and its role in mitochondrial function in relation to dietary fat, we have analyzed the complex I activity. As

above mentioned, complex I seem to have a central role in the regulation of aging as major producer of ROS (Barros *et al*., 2010).

In our experimental model, according to aging, older animals fed on virgin olive oil and sunflower oil reported higher complex I activity than animals at 6 months of age. No differences were manifested among the three dietary treatments at 6 months, however at 24 months fish oil group showed lower activity than virgin olive and sunflower oil groups.

In addition, through the development of new and more specific methodologies, we were able to measure the oxygen consumption in isolated mitochondria. Determination of the OCR in isolated mitochondria (measured in picomoles/minute) is a good measure of the rate of mitochondrial oxidative phosphorylation (Horan *et al*., 2012). The definitions of the mitochondrial respiratory measured states first proposed by Chance and Williams have evolved, and new conventions regarding the active measurement of mitochondrial function can be derived by the sequential injection of different substrates to isolated mitochondria (Schildgen *et al*., 2011). The addition of different substrates is important because it stimulates the flow of electrons from different complexes of the electron transport system, thus allowing the identification of a specific malfunctioning complex. Mitochondrial parameters are measured over five principle respiratory states, which in combination with different substrates are used to determine the functional activity of the electron transport system. Malfunctioning mitochondria isolated from disease tissue can therefore be identified in comparison to the normal respiratory activity of isolated control mitochondria (Brand and Nicholls, 2011). The study of the functional properties of mitochondria is likely to provide a greater understanding of the underlying metabolic malfunctions associated with the progression of aging. Decreasing in the mitochondrial respiratory function and mtETC activity have been reported in the liver of old rats, which might means that the mitochondria are not well-coupled and the mitochondrial membrane loses its integrity during aging (Meng *et al*., 2007). However, measurements of mitochondrial function in liver of senescence accelerate mice shown increased respiration rate and uncoupling, suggesting that mitochondrial function is altered in the aged mice. In this sense, increased mitochondrial OCR might be a compensatory mechanism or just part of the pathological aging process (Cogger *et al*., 2013).

Fat intake alters mitochondrial membrane lipid composition which can affect mtETC function, since its function depends on the composition and fluidity of the mitochondrial membrane (Yu *et al*., 2013). Moreover, a slight enhancement in lipid flux through the liver promotes changes in hepatic mitochondrial metabolism (Pereira *et al*., 2012). This effect should be added to the higher thermogenic response induced by high fat-diet, leading together to increased energy requirements (So *et al*., 2011). In fact, higher OCR in rats fed on high fat-diet has been reported (Ciapaite *et al*., 2007; So *et al*., 2011; McAllan *et al*., 2014). Nevertheless, other studies in mitochondria isolated from the liver of high-fat fed rodents exhibited decreased oxygen consumption (Martínez-Morúa *et al*., 2013; Teodoro *et al*., 2013; Yu *et al*., 2013). This somehow contradictory reports could suggest that mitochondrial function picture is quite complex. Concerning dietary fat types, OCR variations have been reported among mice fed on different fat sources. Thus, mice fed for 8 weeks on diets enriched with fish oil led to lower rates of oxygen consumption that those fed on lard (Fiamoncini *et al*., 2013). Other results concerning differences in mitochondrial function depending on fatty acids variations comes from experiments feeding mice on normolipidic diet supplemented with 0,1 ml of conjugated linoleic acid. Under these conditions, liver mitochondrial oxygen consumption was higher than in control animals supplemented with linoleic acid (Pereira *et al*., 2012). On the contrary, in other studies, no differences were found when investigated dietary fat quality in liver of young rats fed on 5% and 30% fat during 12 weeks (Aoun *et al*., 2012a). The measurement of OCR in

isolated mitochondria from the present work reported differences between groups at 6 months, with virgin olive oil fed animals exhibiting lower OCR than those fed on sunflower and fish oils. At 24 months, rats fed on fish oil showed lower values of OCR than those fed on sunflower oil. Concerning the aging effect, higher OCR values were found for aged animals fed on virgin olive or sunflower oils (compared with their respective young counterparts), but not for fish oil fed animals.

The role of dietary fat in this mechanism could reside in the building of an environment more or less prone to the generation and propagation of ROS, especially when as the result of events such as aging, failures in the mtETC start to appear. Moreover, dietary fat could modulate the phenomenon through variations in the antioxidant system and overall upregulate or attenuate the process. In that sense, it seems like virgin olive and sunflower oils are able to meet the growing energetic needs of aging, while the fish oil seems not be able to respond efficiently. As it has been reported in previous sections, fatty acids from fish oil led to higher antioxidant system activity as well as higher oxidative biomarkers, while mitochondrial function was reduced as indicated the lower complex I activity and oxygen consumption. Alterations in energy production by generating ATP through the mtETC affect to other mitochondrial process such as beta-oxidation of lipids, increasing the accumulation of lipids, in this case in the liver, which has been associated as one of the key step during NASH establishment (Ramírez-Tortosa *et al*., 2009).

7. GENES EXPRESSION ANALYSIS

An analysis of the results evaluated to date indicates that fat feed lifelong to an amount considered as normolipidic (4% w/w) but differing in fatty acid profile, lead to different structural features, oxidative stress and mitochondrial functionality. These observations indicate that one of the three studied fat sources presents some qualitative advantages over the other two. This, despite for the three fats, a similar lipid accumulation was observed during aging. Specifically, the monounsaturated fat shows no structural alterations of inflammatory or fibrotic origin. Likewise, this fat did not lead to ultrastructural changes associated with aging, also generating lower levels of oxidative stress. On the contrary, the two polyunsaturated fat sources reported different deleterious features associated to age. In this respect, n6 PUFA show a significantly higher degree of fibrosis than the other two dietary fats, as well as higher levels of oxidation associated with aging. Meanwhile, the n3 PUFA tend to generate a liver with more proinflammatory characteristics, with significant alterations at the mitochondrial function and also with the highest levels of oxidative stress.

In order to learn about mechanisms or causes associated with the above mentioned observations, the next step of our study was a detailed gene expression profile analysis in the liver of the studied animals. To perform this analysis, first, a massive screening was performed using DNA microarray techniques to detect age-related differently expressed genes among the dietary fats. Next, an analysis with functional enrichment tools (Ingenuity Pathways Analysis (IPA) application, http://www.ingenuity.com/) was performed. Finally, subsequent validation of differently expressed genes by RT-PCR was followed. In the forthcoming paragraphs we will proceed to the discussion of the findings from the gene expression study, and table 42 shows the main changes observed for the three dietary fat groups after the validation study.

Table 42. *Changes with age in the expression of validated genes and related pathways.*

Gene	Pathway	Virgin olive oil	Sunflower oil	Fish oil
Atp5d	Mitochondrial dysfunction	↑	--	↑
Fis1	Mitochondrial dysfunction	--	--	↑
Nudfa8	Mitochondrial dysfunction	--	--	↑
Ndufa9	Mitochondrial dysfunction	↑	--	--
Keap1	Oxidative stress	↑	--	↑
Map2k6	Oxidative stress	↑	--	--
Pik3c2g	Oxidative stress	↑	--	--
Rras	Oxidative stress	↑	--	--
Prdx5	Oxidative stress	--	--	↑
Ccnd1	Cell cycle	--	--	↑
Ppp2r1b	Cell cycle	--	--	↑
Casp3	Apoptosis	↑	--	↑
Xrcc6	Telomere length	↑	--	--
Xrcc1	Telomere length	↑	--	↑

The DNA microarray analysis was performed on liver from both young and old rats fed on the three dietary fats (virgin olive, sunflower and fish oils). For any gene from any fat, the expression levels of the young animals were used as baseline, establishing comparisons "young *vs*. old". After the comparative analysis of gene expression data provided by DNA microarray analysis, we joined a list of genes whose differential expression was common for the three dietary treatments. Consequently, these so-called "common genes" should be a direct effect of aging. A total of 70 sequences with common genes were introduced in IPA to perform a functional network and pathways analysis. On the other hand, microarray analysis also provided a list of genes that were differently expressed in one or two of the experimental treatments. These so-called "uncommon genes" (881 sequences) should be the consequence of differential effect provided for a particular dietary fat although modulate by aging. In relation to the analysis of our experiment, we mainly focused on the deep analysis of these uncommon genes. The 881 uncommon sequences were introduced in IPA to perform a canonical pathways analysis.

Canonical pathways analysis provided an approach to identifying crucial pathways in the control of metabolism and cellular function and processes that are affected by changes in the expression of our focus genes. Canonical pathways were identified from IPA Knowledge Base on the basis of the significance of the association between the dataset and the canonical pathway, which is determined based on the ratio of the number of focus genes that map to the canonical pathway divided by the total number of genes that map to the canonical pathway; and a *P* value calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone. This ratio provides an idea about the percentage of genes in a pathway that were also found in the uploaded list. The *P* value refers the probability that a canonical pathway may be affected by the experimental treatment. If a *P* value is very small, the pathway is more probably associated with the uploaded dataset. When a pathway has a high ratio and a very low *P* value, it means that probably this pathway is associated with the data and a large proportion of the pathway may be involved or affected, and it might be a good candidate for an explanation of the observed phenotype.

From the 82 canonical pathways identified by IPA, we selected 8 (ordered by decreasing level of significance) that were relevant to our study, because they had the highest –log of the *P*-

value, or because were considered of interest to our study based on the investigated issue (aging, mitochondria, nutrition, etc.):

- Mitochondrial dysfunction
- NRF2-mediated oxidative stress response
- DNA double-strand break repair by non-homologous end joining
- Cell cycle regulation by BTG family proteins
- Cyclins and cell cycle regulation
- Telomere extension by telomerase
- mTOR signaling
- Myc mediated apoptosis signaling

A more detailed study was performed on these genes, including the location of the genes, analyzing to what extent their expression was altered and indicating the relative position (in terms of IPA interest) for each canonical pathway and dietary fat. After this analysis, 17 genes were selected for validation by RT-PCR. Results from this study shown that a total of 14 genes were validated, at least in one of the dietary fat.

The functional enrichment study with IPA showed that the most relevant canonical pathway, in which the higher number of our differently expressed genes was involved, was "mitochondrial dysfunction" canonical pathway. Mitochondria are the primary consumers of oxygen in a cell and contain a multitude of redox carriers that are capable of transferring single electrons to oxygen through the mtETC. This results in the formation of the ROS (Gredilla *et al*., 2010). Mitochondria also contain an extensive antioxidant defense system to detoxify ROS, which would otherwise cause oxidative damage to cellular components (Barja, 2013). Thus, in structurally and functionally intact mitochondria, there is little net ROS production. Mitochondrial dysfunction occurs when the ROS-mediated oxidative stress, as cause or effect of mtETC alterations, overpowers the antioxidant defense system. The factors for triggering oxidative stress can be genetic defects, environmental factors like radiation and toxins and metabolic fluctuations. The predominant physiological function of mitochondria is the generation of ATP by oxidative phosphorylation. Additional functions include the generation and detoxification of ROS, regulation of cytoplasmic and mitochondrial matrix calcium, synthesis and catabolism of metabolites. Also, these organelles are involved in their own transport to their correct locations within the cell, in apoptosis, mitochondrial DNA damage and lipid peroxidation, as well as in the regulation of their homeostasis and structure (Brand and Nicholls, 2011).

In view of the studied parameters and the obtained results, changes in this pathway could explain most of the observations related to the accumulation of lipids in the liver, functional variations in mtETC, oxidative stress status and ultrastructural alterations in the own mitochondria. As it has been described in the previous sections, age and dietary treatments led to a number of changes in several aspects associated to mitochondrial homeostasis. Thus, it is not surprising that the canonical pathway presented as the most relevant after the IPA study is that related to mitochondrial dysfunction. So, probably a deep analysis of the changes found at this pathway might us allow to decipher mechanisms involved in experimental observations.

DNA microarray analysis offered a total of 22 genes differentially expressed in relation to the mitochondrial dysfunction pathway. As it can be shown in figure 57, several processes are involved in this pathway. In the present study, the highest number of differently expressed genes belonged to one of these processes, namely that related with mtETC. From these 22 genes, 6 were selected to validate by RT-PCR due to its location and importance in this and other pathways: Atp5d, Fis1, Mapk9, Ndufa8, Ndufa9 and Prdx5. One more gene that did not appear in the DNA microarray analysis, Casp3, was added to the validation list due to its

interest in apoptosis process, so finally RT-PCR assay was made on 7 genes. Six of these genes were validated: Atp5d, Casp3, Fis1, Ndufa8, Ndufa9 and Prdx5.

Atp5d is a gene that encodes a subunit of mitochondrial ATP synthase or complex V of mtETC. Complex V catalyzes ATP synthesis, utilizing an electrochemical gradient of protons across the inner membrane during oxidative phosphorylation. The most important role of mitochondria is the production and cell's supply of ATP, which is used as a source of energy. Its dysfunction may play an important role in the aging progress (Johannsen and Ravussin, 2009). The quantity and quality of dietary lipids may modulate cellular metabolism and energy store and expenditure, particularly by modulating lipid metabolism and mitochondrial functions (Quiles *et al*., 2006b). The function of proteins that regulate membrane flux across the mitochondrial membrane depends on their position and conformation, which are influenced by membrane phospholipid composition. Moreover, the respiratory chain enzymes are membrane protein complexes whose function depends on mitochondrial membrane fluidity, in particular the complex V (Aoun *et al*., 2012a). A decreased Atp5d expression is related to aging (Bakala *et al*., 2013). It has been reported that patients with steatohepatitis present ultrastructural mitochondrial alterations and impairment of hepatic ATP synthesis (Serviddio *et al*., 2008). In our work, both virgin olive and fish oils overexpressed Atp5d. Overexpression in Atp8a and Atp6a, subunits of complex V, has been reported in cell incubated with hydroxytyrosol, one of the most important phenolic compounds of virgin olive oil (Hao *et al*., 2010).

Fis1 is a component of a mitochondrial complex that promotes mitochondrial fission. The balance between fission and fusion regulates the morphology of mitochondria. It can induce cytochrome c release from the mitochondrion to the cytosol, ultimately leading to apoptosis. Age-related increased expression levels of Fis1 have been described (O'Leary *et al*., 2013). High-fat diet lowered Fis1 mRNA levels in brown and white adipose tissue in mice (Wu *et al*., 2014) while a significant increase of Fis1 expression in hepatocyte in CR-fed animals has been described (Khraiwesh *et al*., 2013). In this same study, authors did not find differences in Fis1 expression among the different experimental groups (soybean oil, fish oil and lard as fat source of a CR-diet). However, our results shown that samples from rats fed on fish oil overexpressed Fis1 expression.

Ndufa8 and Ndufa9 encode proteins belong to the complex I of mtETC. The protein encoded by Ndufa8 has NADH dehydrogenase activity and oxidoreductase activity, and it plays an important role in transferring electrons from NADH to the respiratory chain. The protein encoded by Ndufa9 is a subunit of the hydrophobic protein fraction of the NADH:ubiquinone oxidoreductase (complex I), the first enzyme complex in the electron transport chain located in the inner mitochondrial membrane. As above mentioned, complex I seem to have a central role in the regulation of aging as major producer of ROS (Barros *et al*., 2010). It has been demonstrated that complex I and III activities are significantly increased in the aged liver mitochondria (Zhao *et al*., 2014). Fish oil diets have shown lower complex I activity in liver of rats (Aoun *et al*. 2012a). In our work, virgin olive oil led to overexpression on Ndufa9 and fish oil led to overexpression on Ndufa8. This increase in complex I expression has been also observed in rats fed on virgin olive oil through the overexpression on other complex I subunits, such as Ndufs1 (Ronis *et al*., 2012).

Prdx5 encodes a member of the PRX family of antioxidant enzymes, which reduce hydrogen peroxide and alkyl hydroperoxides. The encoded protein may play an antioxidant protective role in mammalian cells under normal conditions and during inflammatory processes (Conrad 2009). The amount of PRX proteins is significantly reduced with aging in liver of rats (Picca *et al*., 2013). It has been reported that overexpressed mitochondrial PRDX5 prevents mitochondrial apoptosis (De Simoni *et al*., 2013). In our study, rats fed on fish oil showed an

overexpression on Prdx5 expression. Overexpression in others members of PRX family, namely Prdx6, has been described in liver of rats fed on virgin olive oil (Ronis *et al*., 2012).

As mentioned above, alterations on mtETC are related to ROS-mediated oxidative stress. Most of genes differently expressed in the DNA microarray analysis and related to mitochondrial dysfunction canonical pathway are located at the mtETC. Specifically, virgin olive oil overexpressed 4 genes of complex I, one gene of complex III, one gene of complex IV and two genes of complex V. Meanwhile, fish oil overexpressed 7 genes of complex I, one of complex II, 4 genes of complex III, one of complex IV and 2 genes of complex V. In view of these results, fish oil is the dietary fat that overexpressed more percentage of genes at the mtETC, despite these rats fed on fish oil presented the lowest complex I activity and OCR among all rats.

One or more feedback mechanisms could explain variations at the amount or activity of mtETC complexes leading to the gene expression induction of mtETC components, in order to try to compensate changes in energy production and/or requirements at the mitochondria, as it has been described in other mechanism of response to oxidative stress (Jousse *et al*., 2007). In the present study the activation of feedback loops may help us to explain observed changes at the mtETC gene expression. Overall, these feedback mechanisms might be directly or indirectly related to aging itself, or to the interactions between the aging process and the age-related lipid accumulation at the liver (figure 81). A striking question is the lack of response by sunflower oil fed animals concerning variations at the gene expression level regarding mitochondrial function. It would be hypothesized that under tolerable stress levels (oxidation, inflammation, and maybe others), mitochondria could activate the necessary machinery to compensate changes in bioenergetics requirements. But under situations in which stress level exceeds the response capacity of mitochondria, as might be occurring for sunflower oil fed animals, other response or fate of the cell might happen.

As indicated repeatedly, mitochondrial dysfunction is closely related to ROS (Barja, 2013). So, it was expected that genes involved in antioxidant systems and in the protection against oxidative stress shown changes in their expression. IPA analysis identified a key canonical pathway in the response to oxidative stress, the Nrf2-mediated oxidative stress response canonical pathway. Reactive intermediates such as peroxides and free radicals can produce damage to many parts of cells such as proteins, lipids and DNA. Even, severe oxidative stress can trigger apoptosis and necrosis. The cellular defense response to oxidative stress includes induction of detoxifying enzymes and antioxidant enzymes. Nrf2 binds to the antioxidant response elements (ARE) within the promoter of these enzymes and activates their transcription. Inactive Nrf2 is retained in the cytoplasm by association with Keap1. Upon exposure of cells to oxidative stress, Nrf2 is phosphorylated in response to the protein kinase C, phosphatidylinositol 3-kinase and MAP kinase pathways. After phosphorylation, Nrf2 translocate to the nucleus, binds AREs and transactivates detoxifying enzymes and antioxidant enzymes, such as glutathione S-transferase, cytochrome P450, NAD(P)H quinone oxidoreductase, heme oxygenase and SOD (Digaleh *et al*., 2013; Gan and Johnson, 2013).

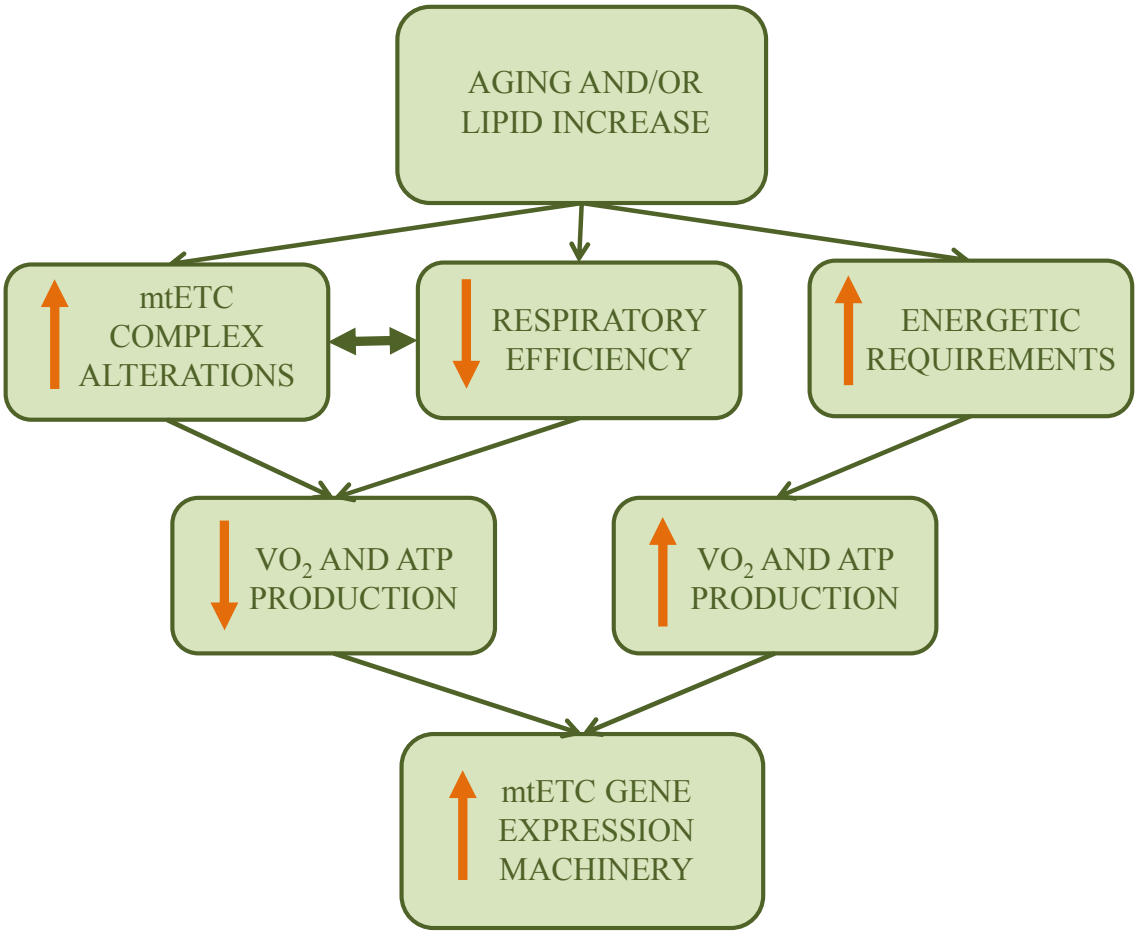


Figure 81. *Aging and/or lipid increase alterations.*

Expression differences due to dietary treatment with virgin olive, sunflower or fish oil were found in 19 genes, as it can see in figure 58. Five genes were selected to validate by RT-PCR: Keap1, Map2k6, Mapk9, Pik3c2g and Rras, from which were validated 4 genes: Keap1, Map2k6, Pik3c2g and Rras.

Keap1 is a negative regulator of Nrf2 expression. Nrf2 production appears to decline with age, while free radical production increases (Suh *et al*., 2004). Thus the regulation of Nrf2 may be very important to the management of the so-called 'diseases of aging' (Mercado *et al*., 2014). The intake of a high-SFA diet induced a higher postprandial increase in Keap1 mRNA levels in the adipose tissue in humans more than after the consumption of the high-MUFA or high-complex carbohydrate diet supplemented with n3 polyunsaturated fatty acids diets (Peña-Orihuela *et al*., 2013). It has been reported that cells treated with hydroxytyrosol activates Keap1-Nrf2 pathway, enhancing the antioxidant defense (Liu *et al*., 2007). In our results, both virgin olive and fish oils overexpressed Keap1 at the liver.

Map2k6 encodes a member of the dual specificity protein kinase family, which functions as MAPKs. MAPKs, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals pathways regulating cellular functions including cell proliferation, differentiation, migration, and apoptosis. This protein phosphorylates and activates p38 MAP kinase in response to inflammatory cytokines or environmental stress. As an essential component of p38 MAP kinase mediated signal transduction pathway, this gene is involved in many cellular processes such as stress induced cell cycle arrest, transcription activation and apoptosis. Map2k6 activity is elevated in aged

mouse liver (Hsieh *et al*., 2003). It has been reported that DHA inhibits the activation of ERK and p38 MAP kinase in UVB-irradiated mouse skin (Rahman *et al*., 2011). Our results shown an increase in expression of Map2k6 in rats fed on virgin olive oil. Overexpression in other MAPKs, such as Mapk14 and Mapk1 has been described in liver of rats fed on virgin olive oil (Ronis *et al*., 2012).

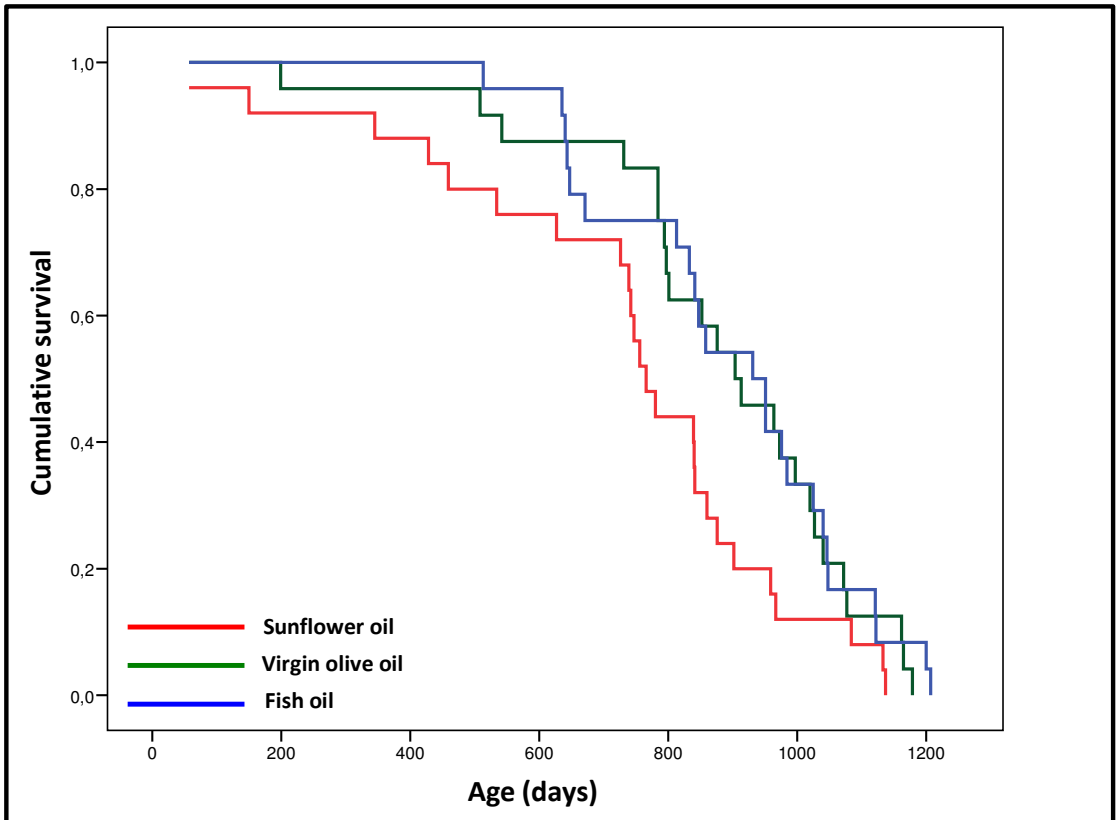
Pik3c2g belongs to the phosphoinositide 3-kinase (PI3K) family. PI3K play roles in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking through generation of second messengers. It has been described that PI3Kc protein kinase activity leading to MAPK activation seems to be restricted to a cytosolic localization (Dolle *et al*., 2011). In addition, the PIK3/AKT pathway plays an important role in both the inhibition of the apoptotic cascade and the promotion of cell growth and proliferation. Its expression is decreased in the liver of old rats (Wang *et al*., 2009; Straszewski-Chavez *et al*., 2010). Concerning dietary fat, high-fat diet appears to inhibit the PI3K/Akt signaling pathway, which may lead to hepatocellular injury through activation of the mitochondrial membrane pathway of apoptosis (Han *et al*., 2010). In our study, rats fed on virgin olive oil reported overexpression of Pik3c2g. Expression of other genes from this family has been analyzed in liver of rats fed on this fat source, with similar results. For example, Pik3c3 was also overexpressed (Ronis *et al*., 2012).

Rras is a member of the RAS superfamily of small GTP-binding proteins whose physiologic function has not been fully elucidated. Among the Ras-family proteins, HRas, KRas, and NRas are well known as regulators of cell growth and differentiation through the kinase-mediated pathways of ERK and PI3K. Rras–subfamily proteins have different functions owing to distinct effector coupling. Rras activates PI3K but not ERK, regulating integrin activation, cell migration, and angiogenesis (Iwasawa *et al*., 2012). It belongs to the Ras proto-oncogenes family, which are central regulators of intracellular signal transduction pathways involved in malignant transformation. Abnormal activation of Ras signaling has been found in mammary tumourigenesis and metastasis, i.e. in human hepatocellular carcinoma (Nguyen *et al*., 2011). Despite it has described that virgin olive oil diet decreases Ras activation and downregulated the Ras/PI3K/Akt pathway (Solanas *et al*., 2010), our results show an overexpression in Rras. In addition, as stated upstream, fish oil led to overexpression of some genes involved in the protection against oxidative stress, such as Prdx5.

Concerning oxidative stress biomarkers, data obtained in the present study (carbonyl proteins and antioxidants) should correlate with expression of oxidative stress-related genes. In that sense, virgin olive oil group led to the lowest level of carbonyls and antioxidants (CoQ, GPX and CAT). These results at the gene expression level might be related to the higher degree of expression of Keap1 and upstream regulators. In that sense, we could speculate that increased level of expression of these genes might be in the way to a higher blockade of Nrf2 out de the cell nucleus. In other words, it might be understood that virgin olive oil which is producing lower oxidative stress, is leading to a lower level of expression of the Nrf2 pathway. According to that, the two dietary fats with the highest levels of oxidative stress should in turn lead to the overexpression of antioxidant-related genes. What we found when antioxidant were analyzed was that only fish oil led to higher values for enzymatic antioxidants activity, namely GPX and CAT. As for fish oil is concerned, gene expression results could be correlated at the observational level mainly because Prdx5 is overexpressed in this group and no changes in expression is shown for Nrf2 pathway. However, at this point, no explanation for the lack of changes at the gene expression of antioxidant-related genes observed for sunflower oil group. Might be, different mechanisms would be involved in the higher level of oxidation reported by these animals. One explanation could be that once again, sunflower oil ablated the adaptation

capacity of the cell to answer to the oxidative stress challenge via gene expression modulation. In support of this hypothesis is the fact that antioxidant mechanisms independent to gene expression regulation, as it can be considered non-enzymatic antioxidants (in particular, CoQ) are increased in sunflower oil fed animals.

Again, the gene expression analysis of results from oxidative stress shows that sunflower oil incapacitates the cell to respond to the age-related changes. We have previously observed that for mitochondrial dysfunction pathway, and now for oxidative stress response pathways. In such a situation as the proposed here, the cell might be doomed to take decisions or to follow pathways probably incompatible with cell or even with organism survival. To examine this possibility, survival analysis for these animals is available. Although these data have not been presented in the "material and methods" and "results" sections because we focused on the liver study, survival curves might help us to better understand gene expression results. Figure 82 shows that rats fed on sunflower oil presented a lower survival rate than those fed on virgin olive or fish oils (which showed no significant differences between them). These results can be inferred from the Log-Rank Test analysis, which provides a very conservative analysis of the survival curve as a whole.



Mean Lifespan (days)	**Maximum lifespan** (days)	**Log-Rank test** (*P*)
Virgin olive oil: 882	Virgin olive oil: 1179	Virgin olive oil *vs.* Sunflower oil: 0.028
Sunflower oil: 732	Sunflower oil: 1137	Virgin olive oil *vs.* Fish oil: 0.685
Fish oil: 898	Fish oil: 1207	Sunflower oil *vs.* Fish oil: 0.048

Figure 82. *Survival analisys*.

In relation to the liver, the effects on cell survival can be analyzed from the point of view of the canonical pathways offered by IPA with statistical significance, specifically those related to cell cycle, apoptosis and telomere length. The IPA analysis of differently expressed sequences indicated that a large number of genes were involved in canonical pathways related to the maintaining of cell survival and proliferation, such as cell cycle regulation by BTG family proteins, cyclins and cell cycle regulation, mTOR signaling and Myc mediated apoptosis signaling.

The mammalian cell cycle consists of four discrete phases: S-phase, in which DNA is replicated; M-phase, in which the chromosomes are separated over two new nuclei in the process of mitosis; and G1 and G2 phases, in which the so-called "daughter cells" continue to grow. BTG proteins are novel regulators of transcription. Although little is known about the biological functions of BTG proteins, it is likely that they constitute a family of functionally related genes that are involved in the control of the cell cycle. Btg1 acts as a growth arrest gene responsible for the maintenance of the quiescent state. Cyclins are positive regulators of progression through the eukaryotic cell cycle (Salama *et al*., 2014). mTOR pathways is also involved in cell survival and proliferation through the PI3K/AKT signal transduction pathway. It has a central role as modulator of proliferative signal transduction (Betz and Hall, 2013). c-Myc plays a critical role in multiple cellular processes including cell growth, proliferation, differentiation and apoptosis. c-Myc activity is sufficient to drive cells into the cell cycle in the absence of growth factors but also induces apoptosis when survival factors are missing (Prochownik, 2008).

Several genes with expression changes in our study are located in these canonical pathways, namely Ccnd1, Pik3c2g, Ppp2r1b, Rras, Xrcc1 and Xrcc6. Btg1 and Ppp2r1b were down-regulated by sunflower oil (although they were not validated).

Cyclin Ccnd1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Ccnd1 gene is a marker of proliferation, and aging is related to an increase in Ccnd1 expression (Tao *et al*., 2012). It has been shown that high fat-diet decreased the gene expression of Ccdn1 (Al-Dwairi *et al*., 2012). Changing n3:n6 fatty acids ratio in the diet through feeding n3 rich-diets oil, decreased Ccnd1 and reduced cell proliferation in a rat model of chemically induced mammary carcinoma has been described (Jiang *et al*., 2012). In our experiment, Ccnd1expression was down-regulated in rats fed on fish oil.

Ppp2r1b encodes a constant regulatory subunit of protein phosphatase 2. Protein phosphatase 2 is one of the four major Ser/Thr phosphatases, and it is implicated in the negative control of cell growth and division. Mutations in Ppp2r1b lead to carcinogenesis and likely other human diseases (Liu *et al*., 2012). Downstream regulation of its expression has been related to mitochondrial apoptosis and the development of liver injury (Chou *et al*., 2007; Lin *et al*., 2007). Fish oils repressed Ppp2r1b expression in our work, probably leading to an induction of cell cycle. We could wonder if some age-related gene expression variations like that (or those found at the mtETC) might be responsible for high survival rates of fish oil-fed animals, despite their high oxidative stress levels and lower mtETC function.

In addition to the pathways associated to cell cycle, other pathways related to survival were also significantly identified by IPA analysis, namely those related to apoptosis, telomere length and DNA repair (the last one also involved in some types of oxidative stress defense).

Xrcc1 play a key role in the repair pathway. It encodes 633 amino acids protein that acts as a scaffold to stabilize the BER proteins in both single-strand break repair and BER. XRCC1 not only interacts with other proteins during the repair process but also coordinates with several repair proteins to enhance the efficiency of DNA repair. Cells lacking functional XRCC1

protein display genetic instability, and frequent spontaneous chromosomal aberration and deletion (Bardia *et al*., 2012). Both,virgin olive and fish oils, overexpressed this gene in our work. Xrcc6 encodes a single stranded DNA-dependent ATP-dependent helicase. It is involved in DNA non-homologous end joining required for double-strand break repair, induced by both exogenous and endogenous DNA-damaging agents. A lower XRCC6 mRNA and protein expressions has been found in HCC samples (Hsu *et al*., 2013). However, overexpression of XRCC6 protects against HCC initiation and progression by restoring the cellular senescent response and activation of immune networks, which induces an effective autophagic degradation, removes the accumulated ROS, decreases DNA damage, attenuates proliferation, and promotes programmed cell death in livers (Wang *et al*., 2013b). Both virgin olive and fish oils, overexpressed this gene in our work, but only samples from liver of rats fed on virgin olive oil validated this change in the expression.

These gene expressions might indicate that both virgin olive and fish oils tend to protect the age-related telomere shortening, which is not observed in animals fed on sunflower oil. According to this gene expression and canonical pathways analysis, results seem to indicate that sunflower oil, as it has been previously mentioned, might be leading to an overall process in which cell death could be increased, and even promoting a shortened in animals lifespan.

Despite not having demonstrated expression changes related to age, Caps3 was selected for its involvement in the activation cascade of caspases responsible for cell apoptosis execution, as well as in the signal transduction pathways of necrosis and inflammation. They have been implicated in the pathogenesis of many disorders including mitochondrial apoptosis. Aging is associated with increases in the Casp3 expression (Fannin *et al*., 2013). It has been also described that rat fed on high-fat diet exhibits increases in the expression levels of Casp3 in the liver (Wang *et al*., 2010). This gene is involved in mitochondrial dysfunction and Myc mediated apoptosis signaling canonical pathways. Both virgin olive and fish oils overexpressed Casp3 expression in liver of rats at the present study. An increasing in Casp3 expression has been described in cells maintain in medium with DHA (Sun *et al*., 2013) and in colon adenocarcinoma cells exposed to hydroxytyrosol (Mateos *et al*., 2013), as well as in TNF-α-induced oxidative stress cardiomyocytes from adult rat fed on virgin olive oil (Al-Shudiefat *et al*., 2013). In the present study, the response of virgin and sunflower oil fed animals with an increased Casp3 expression, might indicate just that these animals are able to keep under control the controlled cycle of programmed cell death, meanwhile, animals fed on sunflower oil had a lack of response in this aspect. Nevertheless, since the experiment was not designed to investigate apoptosis in deep, no precise conclusions can be obtained from this aspect.

In summary, unsaturated normolipidic diets lead to an age-related lipid accumulation in the liver of rats. However, differences between three dietary fat sources, namely virgin olive, sunflower or fish oils, have been described concerning liver morphology, ultrastructure, as well as in relation to oxidative stress status and mitochondrial function. Some of these changes can be associated with variations in the gene expression profile at the liver of these animals. Taking into consideration all the studied variables, virgin olive oil can be considered as the dietary fat source which better preserves liver during the aging process.

CONCLUSIONS

CONCLUSIONS

Conclusion 1: on the histological finding at the liver

There is an increase in the accumulation of lipids at the liver associated to aging. This fact is true for animals fed on the three experimental fat sources. However, from the histopathological point of view, virgin olive oil provided the best protection to the liver. This conclusion is based on the observation that virgin olive oil avoided the trend to increase inflammation related to age shown in fish oil fed animals. Moreover, animals fed on virgin olive oil reported lower fibrosis score than rats fed on sunflower oil.

Conclusión 1: hallazgos histológicos en el hígado

Hay un aumento de la acumulación de lípidos en el hígado asociados a la edad. Este hecho se cumple en los animales alimentados con las tres fuentes grasas experimentadas. Sin embargo, desde el punto de vista histopatológico, el aceite de oliva virgen proporciona la mejor protección al hígado. Esta conclusión está basada en la observación de que el aceite de oliva evita la tendecia a aumentar la inflamación con la edad observada en los animales alimentados con aceite de pescado. Además, los animales alimentados con aceite de oliva virgen registraron un menor grado de fibrosis que las ratas alimentadas con aceite de girasol.

Conclusion 2: on the ultrastructural finding at the mitochondrial level

Sunflower oil fed lifelong leads at the liver mitochondrial level to a set of deleterious features. On one hand, it leads to an increase in mitochondrial size (typical aging marker related to a loss of function through swelling condition and Ψ_{mt} loss). On the other hand, it promotes a decreasing in mitochondrial density, which might be trying to be compensated through the stimulation of the biogenesis machinery. Dietary fats leading to the highest lifespan, namely virgin olive and fish oils, fully correlates with those reporting the better response from the point of view mitochondrial morphology and density.

Conclusión 2: hallazgos ultraestructurales a nivel mitocondrial

La alimentación durante toda la vida con aceite de girasol da lugar a una serie de características deletéreas a nivel de las mitocondrias del hígado. Por un lado, da lugar a un aumento en el tamaño mitocondrial (un típico marcador de envejecimiento relacionado con la pérdida de la función debido al hinchamiento y a la pérdida de potencial de membrana). Por otro lado, promueve una disminución en la densidad mitocondrial, lo cual podría estar tratando de ser compensado a través de la estimulación de la biogénesis. El aceite de oliva virgen y el aceite de pescado fueron las grasas que permitieron la mayor vida media, lo cual se corresponde con la mejor respuesta desde el punto de vista de la morfología y densidad mitocondrial.

Conclusion 3: about the oxidative stress status

As it has been described previously, dietary fat differences may lead to changes in the oxidative stress status through the life. In the present study, no differences were found for young animals, although old animals fed on the polyunsaturated dietary fats led to higher oxidative stress status. The administration of a monounsaturated fat source in the form of virgin olive oil prevents, at least in part, the age-related oxidative stress increase. This is evident when analyzing both protein carbonyl levels as well as most of the antioxidant defense system, which generally increase as does the oxidative stress itself.

Conclusión 3: acerca del nivel de estrés oxidativo

Como se ha descrito anteriormente, las diferencias en la grasa de la dieta pueden dar lugar a cambios en el nivel de estrés oxidativo durante la vida. En el presente estudio, no se encontraron diferencias para los animales jóvenes, aunque los animales viejos alimentados con dietas ricas en grasas poliinsaturadas dieron lugar a mayores niveles de estrés oxidativo. La administración de una fuente grasa monoinsaturada en forma de aceite de oliva virgen previene, al menos en parte, el aumento del estrés oxidativo asociado al envejecimiento. Esto se hace evidente cuando analizamos tanto los niveles de proteínas carbonilo como la mayoría de los sistemas de defensa antioxidantes, los cuales generalmente aumentan como lo hace el estrés oxidativo por sí mismo.

Conclusion 4: on the functional aspects of the liver mtETC

Higher levels of mitochondrial activity were found in old animals fed on virgin olive or sunflower oils compared with their younger counterparts and with old animals fed on fish oil. According to these results, fish oil might attenuate mtETC activity to adapt to the increased oxidative stress found for this dietary fat during aging. That in opposition to what happened for sunflower oil group which, despite higher levels of oxidative stress led to an increased mtETC activity, probably showing a lack of capacity for the regulation of mitochondrial function.

Conclusión 4: respecto a los aspectos funcionales de la CTEmt del hígado

Altos niveles de actividad mitocondrial fueron encontrados en animales viejos alimentados con aceite de oliva virgen o aceite de girasol comparados con los animales jóvenes de su mismo grupo experimental y también comparados con los animales viejos alimentados con aceite de pescado. De acuerdo con estos resultados, el aceite de pescado podría atenuar la actividad de la CTEmt para adaptarse al aumento de estrés oxidativo encontrado en animales alimentados con esta grasa durante el envejecimiento. Lo opuesto ocurrió en el grupo de aceite de girasol, el cual a pesar de mostrar altos niveles de estrés oxidativo dio lugar a un aumento en la actividad de la CTEmt, lo cual probablemente demuestra la falta de capacidad para regular la función mitocondrial por parte del aceite de pescado.

Conclusion 5: concerning the gene expression analysis

The conclusion at the gene expression analysis level was that sunflower avoided animals fed on this dietary fat to adapt their liver to aging. This compared with virgin olive and fish oil fed animals, in which an important overexpression of genes associated to mitochondrial dysfunction, oxidative stress control, cell cycle regulation, apoptosis and telomere length maintaining was found. The adaptation to age allowed by virgin olive and fish oils at the liver gene expression might be responsible, at least in part, for the higher survival found in these animals compared with those fed on sunflower oil.

Conclusión 5: en lo relative al estudio de expresión génica

La conclusion a nivel del análisis de expresión génica fue que el aceite de girasol impide a los animales alimentados con este grasa adaptar su hígado al envejecimiento, comparadazo con los animales alimentados con aceite de oliva virgen y aceite de pescado, en los que se encontró una importante sobreexpresión de genes asociados a la disfunción mitocondrial, al control del estrés oxidativo, a la regulación del ciclo celular, a la apoptosis y al mantenimiento de la longitud de los telómeros. La adaptación al envejecimiento permitida por el aceite de oliva virgen y el aceite de pescado en la expresión génica en el hígado podría ser responsable, al menos en parte, de la mayor superviciencia hallada en esos animales comparados con aquellos alimentados con aceite de girasol.

General conclusion

As a general conclusion, normolipidic feeding on different unsaturated dietary fat sources does not induce changes at the liver during the youth life. However, any of the assayed diets based on unsaturated fatty acids, led to an age-related lipid accumulation in the liver of rats. Differences between three dietary fat sources, namely virgin olive, sunflower or fish oils, have been described concerning liver morphology, ultrastructure, as well as in relation to oxidative stress status and mitochondrial function. Some of these changes can be associated with observed variations in the gene expression profile at the liver of these animals. Taking into consideration all the studied variables, virgin olive oil, can be considered as the dietary fat source which better preserves liver during the aging process. Fish oil, despite to provide the same degree of survival than virgin olive oil, led to higher levels of oxidative stress and a preoccupant trend to inflammation at the liver. That, together with a strange behavior at the mtETC activity level might be deleterious for the animals under situations of potential additional stress situations, apart from the aging itself. Finally, sunflower oil should be avoided as the basis for a lifelong dietary pattern, since the worse finding at the liver, as well as the lowest survival rate, were found for this dietary fat.

Conclusión general

Como conclusión general del estudio se puede indicar que la alimentación con diferentes fuentes grasas insaturadas en forma normolipídica no induce cambios en el hígado durante la fase joven de la vida. Sin embargo, las dietas ensayadas a base de ácidos grasos insaturados llevaron a una acumulación de lípidos durante el envejecimiento en el hígado de las ratas. Las tres grasas estudiadas, pese al aumento similar en la cantidad de lípidos en el hígado, han dado lugar a numerosas diferencias en lo referente a la morfología del hígado, a la ultraestructura, y también en relación al nivel de estrés oxidativo y a la función mitocondrial. Algunos de esos cambios pueden ser asociados con variaciones en el perfil de expresión génica en el hígado de esos animales. Teniendo en cuenta todas las variables estudiadas, el aceite de oliva virgen puede ser considerado como la fuente grasa de la dieta que mejor preserva el hígado durante el proceso de envejecimiento. El aceite de pescado, a pesar de mostrar una supervivencia similar a la observada con el aceite de oliva virgen, da lugar a altos niveles de estrés oxidativo y una tendencia proinflamatoria del hígado. Esto, junto con un extraño comportamiento a nivel de la actividad de la cadena de transporte electrónico mitocondrial, podría suponer un problema para los animales alimentados con aceite de pescado en caso de que de forma paralela al envejecimiento se presentase un estrés adicional, en forma de patología, etc. Finalmente, el aceite de girasol debe ser evitado como base de un patrón dietético prolongado, ya que para con esta grasa se encontraron los peores resultados durante el envejecimiento a nivel hepático, lo cual además fue seguido de la menor tasa de supervivencia en estos animales.

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ANNEX

ANNEX I. Table index

ANNEX II. Figure index

ANNEX III. 881 genes from the worklist "Uncommon Genes" uploaded into the IPA application to be identified.

Symbol	Affymetrix	FC
Aarsd1	1373135_at	2,73
ABCA8	1395644_at	6,63
ABCB10	1382963_at	2,68
ABCB10	1390781_at	5,10
Abcb1b	1370583_s_at	0,01
ABHD11	1399083_at	3,20
ABHD13	1390448_at	2,89
ABRACL	1383912_at	0,36
ACO2	1367589_at	2,71
ACOT1	1377037_at	0,20
ACOT1	1378169_at	0,12
ACOT7	1370313_at	0,31
ACP2	1367906_at	2,38
ACP6	1396167_at	2,75
Adam10	1383127_at	0,34
Adcy9_predicted	1374419_at	4,20
ADD1	1388487_at	2,89
ADH1C	1378260_at	0,17
ADH4	1371470_at	2,62
ADH7	1369072_at	0,12
ADIPOR2	1390549_at	0,37
ADSS	1399050_at	0,41
ADTRP	1379326_at	0,37
AEN	1383491_at	0,29
AGMAT	1383395_at	2,66
AGPAT6	1372919_at	3,63
AGPAT6	1384383_at	2,81
AK3	1368095_at	2,83
AKIP1	1383175_a_at	3,25
AKIP1	1392938_s_at	2,75
Akr1b7	1368569_at	0,00
AKR7A2	1367843_at	2,69
ALDH16A1	1374661_at	2,57
ALDH3A2	1368365_at	0,38
ALDH4A1	1372277_at	2,99
ALG12	1372902_at	3,61
ALKBH3	1371772_at	3,01
ALKBH7	1388692_at	3,71
AMY1A	1370359_at	0,33
ANAPC13	1372718_at	2,71
ANGPTL6	1383018_at	3,48
ANO10	1371738_at	2,91
ANXA6	1387673_a_at	2,51

ANXA7	1368143_at	5,58
Ap3s1	1373024_at	0,33
APBA3	1368389_at	2,69
APH1B	1388440_at	4,20
Apip	1367536_at	2,79
Apoa1	1368335_at	3,03
Apol9a	1376496_at	0,19
APOO	1375297_at	2,91
AQP9	1368621_at	2,60
ARAF	1368390_at	2,51
ARF5	1367748_at	2,79
ARG1	1368266_at	2,60
ARGLU1	1372522_at	0,37
ARHGAP18	1390406_at	3,41
ARHGDIB	1373881_at	0,35
Arpp19	1393008_at	0,29
Asmtl	1372228_at	2,48
ASPDH	1378819_at	2,68
ASPG	1387959_at	0,24
ASS1	1370964_at	2,79
ATF1	1389623_at	0,41
Atf7ip	1382070_at	0,38
ATG13	1388336_at	2,75
ATG2A	1377665_at	3,10
Atg4d	1385512_at	2,58
ATP11C	1391211_at	4,08
ATP1A1	1367585_a_at	2,73
ATP5D	1370278_at	2,39
ATP5G2	1370207_at	2,41
ATP5SL	1375461_at	2,85
ATP5SL	1389192_at	2,87
ATPIF1	1369588_a_at	3,25
ATRAID	1371807_at	3,12
AURKAIP1	1398916_at	2,75
AVPR1A	1369664_at	0,19
BABAM1	1371992_at	2,53
BAG6	1387175_a_at	2,79
BANF1	1367820_at	2,58
BAX	1369122_at	0,31
BC026585	1377899_at	3,43
BCL2A1	1368482_at	0,11
Bcl9l_predicted	1384335_at	0,30
BCS1L	1374886_at	2,95
BET1L	1372950_at	3,03
BOLA1	1372157_at	2,83
BST2	1390738_at	0,26
BTBD2	1372617_at	2,60

BTG1	1367657_at	0,36
C11orf65	1393464_at	3,03
C11orf71	1375346_at	2,81
C14orf1	1377730_at	3,32
C15orf38	1375435_at	2,53
C16orf58	1389330_at	2,75
C18orf8	1374047_at	2,64
C19orf66	1383662_at	0,26
C1GALT1	1387446_at	0,29
C1orf50	1392485_at	4,56
C1orf50	1394327_at	3,92
C21orf33	1371609_at	2,77
C3orf38	1373961_at	0,38
C3orf58	1389199_at	0,09
C6orf62	1372711_at	0,37
C7orf60	1389558_at	0,33
CAPRIN1	1371938_at	0,35
CAPRIN1	1371939_at	0,38
CARS	1376754_at	0,31
CCDC125	1393346_at	0,29
CCDC84	1376992_a_at	0,38
CCL5	1369983_at	3,89
CCND1	1371150_at	0,01
CCND1	1371643_at	0,01
CCND1	1383075_at	0,00
CCNG1	1367764_at	0,23
Ccnl2	1393213_at	0,30
CCNY	1374220_at	0,40
CCS	1387038_at	3,20
CCSER2	1382265_at	0,27
CD36	1386901_at	0,08
CD5L	1385635_at	0,23
CD63	1367709_at	12,91
CD68	1375010_at	0,30
CD74	1367679_at	0,12
CD83	1390529_at	0,16
CDC42EP1	1389157_at	3,01
CDC42SE1	1388468_at	0,36
CDC42SE1	1388530_at	0,35
CDK2AP1	1388443_at	0,40
Cebpa	1384126_a_at	3,29
Cebpa	1384127_at	3,48
CENPL	1398608_at	2,64
Ces1c	1371100_at	4,47
CFH	1387029_at	0,33
CHCHD4	1373001_at	2,66
CHCHD5	1373890_at	2,99

CHCHD6	1372134_at	5,98
CHD1L	1389469_at	3,46
CHUK	1373517_at	0,38
CKS1B	1376346_at	3,36
CLDN3	1368115_at	9,92
CLN3	1378479_at	2,95
CLPP	1371789_at	2,55
CMIP	1382064_at	0,36
CNOT1	1398951_at	2,73
CNPY3	1382110_at	2,73
COA4	1374416_at	2,58
COG4	1372819_at	3,23
COG7	1379876_at	2,60
COG8	1374045_at	2,75
COL18A1	1388459_at	3,25
COL3A1	1370959_at	4,29
COMMD8	1373294_at	0,36
COPS7A	1374460_at	2,57
COPZ2	1372305_at	3,23
COQ5	1388790_at	2,91
COQ9	1388570_at	2,91
CORO1A	1369964_at	0,19
CORO1B	1398769_at	2,58
COX10	1373649_at	3,18
COX14	1371460_at	2,89
CPOX	1377998_at	0,36
CPPED1	1373924_at	2,53
CPSF1	1373531_at	2,48
CPT2	1386927_at	0,30
CRELD1	1388594_at	3,01
CRLS1	1379525_at	0,35
CRLS1	1390717_at	0,36
CROT	1368426_at	0,01
CROT	1387183_at	0,00
CRTC2	1373828_at	2,60
CRY1	1392640_at	0,06
CTC1	1373665_at	2,85
Ctdspl	1374349_at	5,21
CTF1	1387083_at	0,35
CTSD	1367651_at	2,45
CTSS	1387005_at	0,34
Ctsz	1370885_at	3,10
Cxcl12	1369633_at	0,18
Cxcl12	1387655_at	0,10
CXCL9	1373544_at	0,08
CXCL9	1382454_at	0,18
CXorf40A/CXorf40B	1372946_at	2,68

Cyc1	1371342_at	2,91
CYP27A1	1387914_at	2,75
Cyp3a13	1370387_at	39,95
CYP4F3	1387916_at	3,16
CYP7A1	1368458_at	5634,22
CYTH2	1367727_at	2,50
DAG1	1371430_at	0,31
DBNDD2	1372250_at	2,95
DBT	1373201_at	3,34
DCTPP1	1370308_at	3,43
DDB1	1371648_at	2,53
DDB1	1399162_a_at	2,57
DDOST	1372247_at	3,07
DDX21	1375901_at	0,35
Ddx47	1375554_at	2,66
DDX49	1374332_at	2,81
DGAT2	1371615_at	0,37
DGCR6	1373424_at	2,85
DGUOK	1372799_at	2,68
DHPS	1388803_at	2,85
DHX36	1379101_at	0,32
DLST	1370879_at	2,58
DNAJA1	1368852_at	0,36
DNAJC15	1373064_at	2,89
DNAJC4	1372428_at	2,85
DNASE2B	1368491_at	0,02
DNTTIP1	1370914_at	2,58
DNTTIP1	1370915_s_at	2,81
DPAGT1	1372270_at	2,55
Dpcd	1374358_at	2,51
DPP3	1367966_at	2,50
Dpysl2	1371668_at	2,83
DPYSL2	1371694_at	0,33
DTD1	1388428_at	2,97
DUSP1	1368146_at	0,12
DUSP1	1368147_at	0,09
DUSP22	1375967_a_at	0,36
DUSP3	1389050_at	0,37
DUSP6	1377064_at	0,09
ECE1	1367801_at	2,85
ECI2	1388908_at	0,38
EDEM1	1372533_at	7,16
EDEM2	1376315_at	3,20
EEF2	1370003_at	2,43
EGLN1	1389207_at	0,35
EHD1	1372317_at	2,68
EIF1B	1373411_at	0,39

EIF2AK2	1387242_at	0,30
EIF2D	1372769_at	2,77
EIF4E	1398799_at	0,39
Eif4ebp3	1373692_at	6,50
EIF4G2	1367469_at	0,41
EIF5	1398846_at	0,33
ELAVL1	1375892_at	0,39
ELOVL1	1398360_at	0,38
ERCC1	1399032_at	3,16
ERGIC1	1389319_at	0,31
ERI3	1388475_at	2,62
EXOSC5	1372942_at	3,07
EXOSC7	1372688_at	2,53
FABP7	1370024_at	167, 73
FAF1	1369995_at	2,50
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FAM126B	1382818_at	5,10
FAM134B	1373011_at	60,97
FAM173A	1372786_at	2,43
FAM92A1	1377616_at	3,14
FARP2	1374239_at	2,95
FBXO21	1369972_at	4,14
FBXO31	1372600_at	4,23
FBXO8	1373835_at	0,39
FBXW9	1389106_at	3,18
FCER1G	1373575_at	0,31
FDFT1	1367839_at	5,28
FDFT1	1389906_at	4,63
FDX1L	1372496_at	2,69
FDXR	1368011_at	2,58
FEM1A	1398417_at	3,20
FGFR2	1373829_at	3,39
FH	1367670_at	2,55
FIBP	1399072_at	2,79
FIG4	1390289_at	2,60
FIS1	1371594_at	2,53
FKBP4	1389844_at	0,33
FKBP8	1371528_at	2,43
FKBPL	1390995_at	3,01
FLOT2	1367746_a_at	2,81
FOLR2	1390348_at	0,31
FOXN3	1384728_at	0,29
Foxred1	1376921_at	2,85
FSTL1	1368822_at	2,93
FUOM	1377830_a_at	5,66
Fzr1	1375546_at	2,36
GAA	1371799_at	2,85

GABARAPL2	1380170_at	2,75
GATAD1	1389390_at	0,34
GBF1	1389044_at	3,12
GCAT	1382325_at	4,50
GEMIN2	1368229_at	2,77
GET4	1372596_at	2,45
GET4	1376016_at	2,51
GFRA1	1387007_at	17,63
GGH	1368470_at	0,25
GGNBP2	1372201_at	2,93
GHR	1373803_a_at	0,38
GK	1387491_at	0,28
GLIPR1	1373504_at	0,19
GLT8D1	1388893_at	2,95
GLTPD1	1391437_at	3,63
Glyctk	1377009_at	0,37
Gm20267	1398341_at	2,73
GMPPA	1372859_at	2,93
GNG10	1388455_at	0,31
GOLGA2	1386942_at	2,81
GPAM	1382986_at	0,23
GPD2	1387670_at	0,10
GPM6A	1373773_at	0,00
GPNMB	1368187_at	0,02
GPR107	1384602_at	3,27
GPR146	1382266_at	4,99
GSDMD	1385001_at	2,55
GSTO1	1389832_at	2,69
GTF2H5	1367509_at	2,75
GTF3C1	1370231_at	2,91
GTF3C2	1389574_at	2,73
Gulo	1369837_at	3,20
H1FX	1371376_at	6,59
HAAO	1368794_at	2,48
HBS1L	1372344_at	2,71
HDC	1370491_a_at	0,07
Hddc3	1372762_at	2,89
HEATR4	1391070_at	3,03
HERPUD1	1367741_at	3,71
HEXB	1372706_at	4,11
HEXIM1	1374846_at	0,36
HGFAC	1381006_at	2,60
HIP1	1390082_at	2,85
HIST1H2BN	1390021_at	0,33
HLA-B	1371209_at	0,01
HLA-B	1388255_x_at	0,01
HLA-DQA1	1370822_at	0,15

HLA-DQA1	1377334_at	0,19
HLA-DRA	1370883_at	0,12
HLA-DRB1	1370383_s_at	0,20
HMBS	1386983_at	2,73
HMGCS1	1367932_at	0,10
HMGN2	1371352_at	2,93
HNRNPA2B1	1398883_at	0,35
HNRNPA3	1370871_at	0,38
HNRNPU	1370171_at	0,40
HPN	1368431_at	2,39
Hs3st3b1_predicted	1383661_at	3,01
HSCB	1382051_at	2,75
HSD11B2	1368102_at	0,26
HYI	1384103_at	2,83
IAH1	1372087_at	2,51
ICK	1387948_at	2,97
ICT1	1374443_at	2,89
ID2	1368870_at	0,24
IDH2	1388403_at	3,41
IDH3G	1370865_at	2,71
Ifi27l2b	1376845_at	0,24
IFIT1B	1369836_at	0,02
IFIT2	1379568_at	0,08
IFIT2	1384180_at	0,12
IFITM1	1372013_at	0,26
Ift20	1375425_at	2,62
Igf2bp3	1377824_a_at	0,15
IGFBP2	1367648_at	134,36
Igtp	1375796_at	0,08
Igtp	1396163_at	0,07
Igtp	1397304_at	0,13
IL18BP	1369031_at	0,33
IL7	1369208_at	0,08
IMP4	1379407_at	2,75
INPP4A	1387078_at	3,12
INSIG2	1393690_at	0,19
IP6K1	1387904_at	2,73
IPO5	1373955_at	2,48
IRF3	1371560_at	0,38
IRF7	1383564_at	0,12
IRF9	1383448_at	0,31
IRGM	1391489_at	0,11
ISG15	1382314_at	0,09
ITGA2B	1380600_at	2,71
ITGB2	1383131_at	0,19
JUND	1393138_at	0,38
Kat3	1374006_at	2,83

KAT5	1372366_at	2,62
KCNN2	1370111_at	0,09
Kctd12_predicted	1373818_at	0,25
KDM2B	1393101_at	0,40
KEAP1	1370066_at	2,51
KIAA0922	1391414_at	3,51
KIFAP3	1389556_at	2,75
KISS1	1398516_at	3,63
KLF15	1368249_at	3,63
KLF9	1370209_at	3,78
Kptn	1381992_at	2,89
KRT8	1371530_at	3,81
LAMTOR4	1398915_at	2,83
LAPTM5	1383658_at	0,36
Large	1372884_at	0,28
LCP1	1389210_at	0,22
LENEP	1387067_at	2,66
LEPRE1	1367967_at	2,85
LEPROTL1	1374876_at	2,71
LGALS3BP	1387946_at	0,14
LIAS	1389154_at	2,77
LITAF	1370928_at	0,23
LOC100362008	1381063_at	4,47
LOC100910885	1372604_at	0,25
LOC100910973	1372585_at	0,18
LOC290071	1371016_at	0,23
LOC291411	1370925_at	3,03
LOC494529	1371420_at	3,14
LOC679583	1383643_at	3,01
LOC681282	1372644_at	3,20
LOC683897	1389667_at	2,91
LOC687295	1374877_at	2,62
LOC689176	1376691_at	0,29
LOC689176	1393414_at	0,24
LOC690000	1377893_at	0,35
LOC691143	1389222_at	2,66
LONP1	1398247_at	2,38
LRP3	1368239_at	3,07
LRPPRC	1373500_at	2,77
Lrrc45	1382248_at	2,57
LSG1	1373293_at	2,66
Lsm2	1371712_at	2,57
LY6E	1388347_at	0,31
Maf	1385243_at	0,27
Maf	1392566_at	0,29
MAGED1	1386895_at	3,58
MAGI3	1398866_at	0,31

MAL2	1384361_at	0,06
MAN1A1	1383574_at	0,37
MAN2B1	1388699_at	2,93
MANBAL	1398924_at	2,64
MAP1LC3B	1367669_a_at	2,95
MAP2K6	1387809_at	6,96
Map7	1392916_at	2,57
MAPK9	1368646_at	0,26
MARC1	1376847_at	2,58
MARCKS	1373432_at	0,32
MARCKS	1375523_at	0,33
MARK3	1368831_at	2,64
MAT1A	1371031_at	2,68
Mawbp	1390993_at	3,34
MCCC1	1376852_at	3,03
MDFIC	1378482_at	0,20
MED29	1374851_at	3,51
MEN1	1398785_at	2,30
METAP1D	1375457_at	3,63
MGC112830	1388867_at	0,39
MGST2	1372599_at	3,25
MKNK2	1372038_at	6,54
MMAA	1377302_a_at	3,05
MMACHC	1376214_at	2,77
MMP14	1378225_at	0,35
MOCS2	1372177_at	0,33
MPG	1368410_at	3,53
Mpnd	1371990_at	3,01
MPV17	1393110_at	2,89
MRPL10	1395526_at	2,75
MRPL12	1372431_at	2,81
Mrpl15	1398891_at	2,85
MRPL16	1371967_at	2,66
MRPL19	1373477_at	2,64
MRPL2	1398911_at	2,62
MRPL21	1367502_at	2,64
MRPL24	1371888_at	2,73
MRPL27	1399047_at	2,77
MRPL28	1373705_at	2,64
MRPL3	1371709_at	2,60
MRPL30	1398983_at	2,55
MRPL35	1371955_at	2,77
MRPL38	1383618_at	3,05
Mrpl39	1372730_at	2,60
MRPL40	1388517_at	2,77
MRPL41	1371423_at	2,69
MRPL42	1371853_at	2,58

MRPL48	1371586_at	2,99
Mrpl52	1374411_at	2,53
MRPL53	1367477_at	2,83
MRPL9	1398912_at	2,71
MRPS15	1372697_at	2,48
MRPS16	1398946_at	3,18
MRPS18A	1371591_at	2,87
MRPS18B	1371809_at	2,79
MRPS24	1371649_at	2,62
Mrps27	1372916_at	3,12
MRPS31	1372456_at	3,03
MRPS35	1372839_at	3,23
MRPS9	1373002_at	2,87
MSN	1371575_at	0,33
MTFP1	1388661_at	3,14
MTHFD1	1368181_at	3,27
MTX1	1399077_at	2,71
MX1	1369202_at	0,00
MX1	1387283_at	0,04
MYC	1368308_at	0,10
MYCL	1395781_at	0,16
N6AMT1	1392330_at	2,91
NABP1	1392579_at	0,18
NABP2	1374631_at	2,39
NADSYN1	1379472_at	2,89
NAGA	1382285_at	2,87
Naglt1	1383518_at	0,25
NDUFA6	1379243_at	2,64
NDUFA8	1371355_at	2,69
NDUFA9	1388323_at	2,57
NDUFAF3	1368230_a_at	3,12
NDUFB3	1373041_at	2,77
NDUFB7	1388343_at	2,75
NDUFS2	1371482_at	2,50
NDUFS3	1388364_at	2,62
NDUFS7	1371912_at	2,57
NEK6	1374565_at	0,24
NELFE	1372290_at	2,83
Nfib	1389601_at	3,81
NFIX	1370946_at	0,30
NFU1	1389162_at	2,85
NIF3L1	1383289_at	0,40
NIPSNAP1	1388537_at	2,77
NOB1	1388878_at	2,77
NOLC1	1368032_at	0,33
NOSIP	1371397_at	2,48
Notch2	1398362_at	0,15

NOX4	1368543_at	111,43
NQO1	1387599_a_at	15,35
NR1H4	1369073_at	0,33
NSMCE1	1374210_at	2,83
NSMCE2	1389011_at	2,79
NTHL1	1393963_at	2,79
NTMT1	1372185_at	3,18
NTPCR	1390108_at	2,95
NUCB2	1370000_at	5,66
NUDT16	1375847_at	2,97
NUDT19	1371919_at	3,14
NUDT22	1367507_at	2,77
NUDT6	1370435_a_at	3,46
Nudt7	1389251_at	0,22
NUDT9	1373840_at	2,75
NUP37	1376703_at	2,85
NUPR1	1367847_at	6,41
NUTF2	1371246_at	0,33
NXF1	1398861_at	0,34
OASL	1377497_at	0,15
OBFC1	1373295_at	2,81
OR51E2	1387981_at	0,09
OSBP	1373974_at	2,81
OSBPL9	1371978_at	2,50
OTUB2	1376118_at	0,26
OXA1L	1372291_at	2,91
OXSM	1391530_a_at	2,75
PAFAH1B2	1369642_at	0,38
PAIP1	1379272_at	0,23
PAOX	1372212_at	3,46
PAOX	1399093_at	2,60
PAQR9	1382569_at	0,19
Parp12	1380071_at	0,38
PBLD	1370320_at	3,48
PBXIP1	1379868_at	2,83
Pcbd2	1379329_at	3,01
Pcdhga1	1388551_at	0,31
PCMTD1	1377742_at	0,36
PCNA	1367671_at	0,41
Pctk3	1382318_at	2,64
Pctk3	1386817_at	2,60
Pde4dip	1379811_at	3,46
Pde4dip	1382254_at	4,03
PDIA5	1374828_at	3,58
PDK2	1367892_at	3,34
PDK2	1386975_at	3,51
PDK4	1369150_at	0,22

PEX1	1376595_at	0,34
PEX11G	1393668_at	3,01
PEX16	1383960_at	2,71
PEX6	1368264_at	2,89
PFDN1	1398926_at	2,85
PFKFB1	1369467_a_at	6,06
Phb	1367926_at	2,60
PHB2	1367463_at	2,81
PHF11	1382546_at	0,24
PHLDA3	1375224_at	0,03
PHYHD1	1371974_at	2,79
PI4KA	1370318_at	2,95
PIK3C2G	1369050_at	5,06
Pink1_predicted	1372199_at	3,94
PLA1A	1370445_at	7,46
PLEC	1386941_at	3,03
PLEKHA1	1376762_at	0,25
PLIN2	1390383_at	0,22
PMPCB	1387781_at	2,77
PMVK	1373243_at	4,50
PNKP	1393367_at	2,51
PNPLA7	1377049_at	3,92
POGLUT1	1389197_at	0,41
POLB	1368341_at	0,31
POLD4	1388883_at	3,46
POLE4	1388367_at	0,37
POLG2	1375504_at	5,13
POLL	1389268_at	2,89
POLR1C	1374289_at	2,87
Pom121	1386957_at	2,73
POMT1	1368267_at	2,60
POP7	1373597_at	2,69
PPIL3	1375686_at	0,34
PPP2CA	1388805_at	0,40
PPP2R1B	1373959_at	0,10
PPP2R3C	1378741_at	2,60
PPP2R5E	1388965_at	2,81
PPP3CA	1373479_at	0,36
PRDX5	1367677_at	3,14
PREB	1370300_at	2,71
PREPL	1374004_at	2,69
PRKCI	1373047_at	0,32
PRLR	1369493_at	0,03
PRLR	1370384_a_at	0,00
PRLR	1370789_a_at	0,01
Prlr	1376944_at	0,01
PRODH	1372920_at	5,66

PROSC	1389549_at	2,66
PRPF3	1372432_at	2,68
PRR13	1388408_at	0,38
Prss32	1393241_at	0,00
PSMA7	1371869_at	2,57
PSMB1	1398812_at	2,46
PSMB6	1398777_at	2,62
PSMB7	1367656_at	2,77
PSMB9	1370186_at	0,32
PSPH	1375964_at	0,17
PTDSS2	1372663_at	3,12
PTOV1	1388430_at	2,64
PTPN11	1388379_at	0,40
PTPN3	1376537_at	3,34
PTPRC	1390798_at	0,19
PUF60	1367464_at	2,46
PYCRL	1392535_at	3,14
R3HCC1	1372567_at	2,83
RAB21	1383089_at	0,37
RAB3IP	1387821_at	2,95
RAB43	1394486_at	2,83
RABGGTA	1387869_s_at	2,99
RAC2	1372404_at	0,33
RANGRF	1371752_at	3,16
RAPH1	1376658_at	0,17
RB1	1388185_at	0,17
RBCK1	1386966_a_at	2,64
RBFA	1388946_at	2,83
RBL2	1368403_at	3,23
RBM42	1371502_at	2,51
RCBTB1	1390433_at	0,32
RDH10	1384391_at	0,35
RDH5	1379587_at	7,67
RFFL	1385504_at	0,35
RGD1305094_predicted	1372698_at	0,27
RGD1308513	1398991_at	2,62
RGD1559600	1392825_at	0,26
RGD1562136_predicted	1373049_at	0,33
RGD1564316_predicted	1382601_at	0,30
RHOA	1399027_at	0,38
RHOB	1369958_at	0,22
RHOQ	1386967_at	0,29
RIN3	1375020_at	0,32
RINT1	1382797_at	4,11
RIOK3	1394363_at	2,51
RND2	1372521_at	3,12
RNF11	1373015_at	0,23

Rnf113a2	1377837_at	2,62
RNF181	1371373_at	2,79
RNF181	1398359_at	2,62
RNF217	1393047_at	0,30
RPIA	1389468_at	0,32
RPL7L1	1389013_at	2,51
Rpn1	1398766_at	2,71
RPRM	1390672_at	0,01
RPUSD3	1380562_at	3,10
RRAS	1388729_at	2,66
RT1-Aw2	1375850_at	0,38
RT1-EC2	1369110_x_at	0,21
RT1-EC2	1371078_at	0,34
Rusc2	1388384_at	3,66
RUVBL1	1370386_at	2,57
S1PR1	1371840_at	0,23
SALL1	1391194_at	0,22
SAMM50	1388380_at	2,68
SCAMP4	1367688_at	2,89
SCAP	1389567_at	2,77
SCN1B	1387010_s_at	4,03
SDHB	1372123_at	2,73
Sdhb_predicted	1380813_at	3,48
SDPR	1375638_at	0,37
SDPR	1382452_at	0,30
SDS	1369864_a_at	279018,26
SDSL	1385670_at	4,86
SDSL	1393422_at	3,63
Sectm1b	1376976_at	2,89
SEMA4G	1380248_at	3,51
SERPINB9	1373592_at	0,33
SERPINF2	1377033_at	2,58
SERPING1	1372254_at	0,37
SH3BGRL	1383085_at	0,28
SH3BP5	1387294_at	4,14
SHISA5	1388776_at	0,38
SHMT2	1388695_at	3,18
Sik2	1376649_at	0,26
SIL1	1376431_at	2,71
SIVA1	1383962_at	3,12
SKIV2L	1399112_at	2,64
SLC12A2	1367853_at	0,27
SLC13A3	1368047_at	7,52
SLC16A1	1386981_at	0,20
SLC22A1	1368191_a_at	2,81
SLC22A9	1369840_at	0,08
SLC25A29	1390483_at	2,79

SLC25A33	1373282_at	4,92
SLC27A1	1367789_at	2,62
SLC37A4	1386960_at	3,34
SLC38A10	1399102_at	2,69
SLC38A3	1370824_at	3,41
SLC39A3	1377933_at	2,79
SLC39A7	1389089_at	3,66
SLC46A3	1383471_at	3,43
SLC4A4	1369756_a_at	0,19
SLC6A12	1387295_at	3,05
Slfn2	1377916_at	0,35
SMARCD2	1370159_at	3,32
SMIM13	1383325_at	4,17
SNRK	1386282_x_at	2,55
Snrpd3	1371848_at	2,83
Snx11	1383653_at	2,95
Snx11	1396278_at	3,27
SPA17	1370088_at	3,29
SPG7	1374839_at	3,03
Spred2	1389008_at	3,14
SRGN	1368655_at	0,33
SS18	1391628_at	0,31
SSR2	1373185_at	3,34
SSU72	1371768_at	2,77
ST5	1373288_at	2,77
STAP2	1389420_at	3,58
STARD4	1396521_at	3,89
STARD5	1381461_at	0,37
STARD5	1393183_at	0,34
STARD5	1394275_at	0,33
STAT1	1372757_at	0,33
STIM1	1376024_at	2,66
STK17B	1373164_at	0,30
STK24	1374584_at	0,34
STOML2	1398908_at	2,51
STT3A	1398599_at	2,97
STUB1	1398910_at	2,87
STX5	1367878_at	2,81
STX5	1386853_s_at	3,18
SUB1	1398880_at	0,40
SULF2	1388753_at	3,76
Sult2a2	1387936_at	0,00
SUMF2	1381979_at	2,69
SUPT7L	1373871_at	2,93
SURF1	1398857_at	2,53
SWSAP1	1372396_at	3,29
SYS1	1367543_at	2,73

TAP1	1388149_at	0,21
TARS2	1373760_at	2,77
TBC1D17	1379901_at	2,64
TBC1D9B	1372129_at	2,48
TBL1XR1	1394079_at	0,20
TBRG1	1371509_at	2,58
TCEA3	1388611_at	4,76
TEF	1390819_at	4,82
TEN1	1383230_at	0,13
Tenc1	1371356_at	3,73
TENM2	1395836_at	7,62
TGM2	1369943_at	0,30
THNSL2	1389114_at	3,39
THOC7	1398385_at	0,40
TIMM13	1398868_at	2,68
TIMM22	1370353_at	2,71
TLN1	1389002_at	2,69
TM2D2	1398984_at	2,64
TM6SF2	1381196_at	3,20
TMCO6	1374637_at	2,68
TMEM140	1390042_at	0,35
TMEM203	1379323_at	2,60
TMEM214	1382596_a_at	2,81
TMEM218	1389561_at	4,82
TMEM223	1398931_at	2,73
Tmem234	1375064_at	2,95
TMEM238	1383056_a_at	2,75
TMEM30B	1379513_at	0,04
TMEM33	1369076_at	0,40
TMEM41B	1382689_at	2,85
TMEM43	1373595_at	0,35
TMEM66	1398879_at	2,77
TMEM98	1373161_at	3,76
TMSB10/TMSB4X	1387883_a_at	0,38
TMTC4	1379541_at	0,30
TMUB1	1372961_at	2,53
TNFSF13	1379677_at	0,27
TNS1	1371954_at	3,25
TOMM40	1389969_at	2,77
TOMM70A	1379186_at	0,37
TOP1MT	1380060_at	2,93
TOR1A	1375357_at	2,53
Tpm3	1387918_at	0,37
Tpm4	1368838_at	0,21
Tprkb	1376007_at	2,93
TRAP1	1374467_at	2,97
TRAPPC1	1372252_at	2,58

TRAPPC11	1399129_at	2,58
TRAPPC9	1376416_at	2,75
TRIM2	1375278_at	3,36
TRMT1	1389056_at	2,87
TRUB2	1378326_at	3,05
Tsfm	1398603_at	2,83
TSPAN12	1372455_at	2,62
TSPAN7	1371702_at	0,36
TSTA3	1398953_at	2,51
TTC36	1393061_at	2,93
TUBB2A	1373718_at	0,03
TXN2	1398844_at	2,79
TXNRD1	1386958_at	0,29
U2AF1L4	1373205_at	2,64
UBE2E3	1389534_at	0,38
Ube2j1	1373495_at	2,68
UBL5	1371590_s_at	2,69
UBN1	1381878_at	0,27
UBR2	1381349_a_at	2,60
UBR2	1382970_at	2,55
UBXN6	1389260_at	2,85
UFC1	1371758_at	2,53
UFSP2	1376086_at	2,93
UGP2	1388410_at	3,41
UNC5B	1393799_at	0,21
UQCR11	1375197_at	2,69
UQCRC1	1388301_at	2,60
UQCRH	1371415_at	2,71
USP2	1387703_a_at	27,67
USP25	1378679_at	0,38
UXS1	1376262_at	0,33
VANGL1	1394435_at	2,87
VCP	1367455_at	2,50
VIM	1367574_at	0,31
VIMP	1387915_at	2,85
Vipas39	1367490_at	2,68
VPS11	1373498_at	2,50
Vps13c	1379941_at	3,43
VPS16	1388934_at	2,57
VPS33A	1385081_s_at	2,66
VPS33A	1387557_s_at	2,89
VPS51	1383664_a_at	2,87
WBP1L	1375369_at	2,77
WBP1L	1397681_at	2,43
WDR24	1373938_at	2,55
WDR74	1373263_at	2,68
WFDC2	1390765_at	0,37

WFS1	1368839_at	3,29
WHAMM	1377000_at	3,36
WRAP53	1373694_at	2,81
XIAP	1369248_a_at	0,03
XKR8	1376642_at	2,68
XRCC1	1387129_at	2,69
XRCC5	1370931_at	2,83
XRCC6	1370537_at	2,75
YWHAZ	1387774_at	0,36
ZCCHC11	1390820_at	3,23
ZER1	1383389_at	2,55
Zfp238	1389252_at	0,33
Zfp446_predicted	1391702_at	5,31
ZMAT5	1371788_at	3,10
ZNF318	1389340_at	2,99
ZNF467	1391507_at	4,63
ZNF496	1373950_at	2,60
ZNF496	1384517_at	3,63
ZNHIT1	1371660_at	3,01
5430402E10Rik/Gm14744	1388014_at	12,82
---	1372054_at	3,68
---	1372347_at	0,24
---	1373626_at	0,24
---	1374003_at	2,71
---	1375887_at	0,38
---	1376848_at	0,32
---	1378828_at	6,41
---	1379252_at	0,28
---	1381811_at	16,34
---	1383486_at	0,02
---	1385029_at	0,14
---	1385739_at	0,16
---	1388880_at	0,19
---	1389613_at	0,30
---	1390117_at	0,31
---	1392842_at	0,22
---	1393342_at	3,63
---	1395362_at	0,35
---	1398713_at	0,35

Notes: symbol refers to NCBI gene symbol; Affymetrix refers to Affymetrix gene identification.
Abbreviations: FC = fold change.

ANNEX IV. Sequences from the worklist "Uncommon Genes" identified by IPA.

Symbol	Affymetrix	FC
ABCA8	1395644_at	6,63
ABCB10	1390781_at	5,10
Abcb1b	1370583_s_at	0,01
ABHD11	1399083_at	3,20
ABRACL	1383912_at	0,36
ACO2	1367589_at	2,71
ACOT1	1378169_at	0,12
ACOT7	1370313_at	0,31
ACP2	1367906_at	2,38
ACP6	1396167_at	2,75
ADAM10	1383127_at	0,34
ADD1	1388487_at	2,89
ADH1C	1378260_at	0,17
ADH7	1369072_at	0,12
ADIPOR2	1390549_at	0,37
ADSS	1399050_at	0,41
ADTRP	1379326_at	0,37
AEN	1383491_at	0,29
AGMAT	1383395_at	2,66
AGPAT6	1372919_at	3,63
AK3	1368095_at	2,83
AKIP1	1383175_a_at	3,25
Akr1b7	1368569_at	0,00
AKR7A2	1367843_at	2,69
ALDH16A1	1374661_at	2,57
ALDH3A2	1368365_at	0,38
ALDH4A1	1372277_at	2,99
ALG12	1372902_at	3,61
ALKBH3	1371772_at	3,01
ALKBH7	1388692_at	3,71
AMY1A	1370359_at	0,33
ANAPC13	1372718_at	2,71
ANGPTL6	1383018_at	3,48
ANO10	1371738_at	2,91
ANXA6	1387673_a_at	2,51
ANXA7	1368143_at	5,58
Ap3s1	1373024_at	0,33
APBA3	1368389_at	2,69
APH1B	1388440_at	4,20
APIP	1367536_at	2,79
APOA1	1368335_at	3,03
AQP9	1368621_at	2,60
ARAF	1368390_at	2,51
ARF5	1367748_at	2,79
ARG1	1368266_at	2,60

ARGLU1	1372522_at	0,37
ARHGAP18	1390406_at	3,41
ARHGDIB	1373881_at	0,35
Arpp19	1393008_at	0,29
ASPG	1387959_at	0,24
ASS1	1370964_at	2,79
ATF1	1389623_at	0,41
ATF7IP	1382070_at	0,38
ATG13	1388336_at	2,75
ATG2A	1377665_at	3,10
ATP11C	1391211_at	4,08
ATP1A1	1367585_a_at	2,73
ATP5D	1370278_at	2,39
ATP5G2	1370207_at	2,41
ATP9B	1370925_at	3,03
ATPIF1	1369588_a_at	3,25
ATRAID	1371807_at	3,12
AURKAIP1	1398916_at	2,75
AVPR1A	1369664_at	0,19
BABAM1	1371992_at	2,53
BAG6	1387175_a_at	2,79
BANF1	1367820_at	2,58
BAX	1369122_at	0,31
BCL2A1	1368482_at	0,11
BCS1L	1374886_at	2,95
BET1L	1372950_at	3,03
BOLA1	1372157_at	2,83
BST2	1390738_at	0,26
BTBD2	1372617_at	2,60
BTG1	1367657_at	0,36
C11orf71	1375346_at	2,81
C14orf1	1377730_at	3,32
C15orf38/C15orf38-AP3S2	1375435_at	2,53
C16orf58	1389330_at	2,75
C18orf8	1374047_at	2,64
C19orf12	1377893_at	0,35
C19orf66	1383662_at	0,26
C1GALT1	1387446_at	0,29
C1orf50	1392485_at	4,56
C21orf33	1371609_at	2,77
C3orf38	1373961_at	0,38
C3orf58	1389199_at	0,09
C6orf62	1372711_at	0,37
CAPRIN1	1371938_at	0,35
CARS	1376754_at	0,31
CCL5	1369983_at	3,89
CCND1	1383075_at	0,00

CCNG1	1367764_at	0,23
CCNL2	1393213_at	0,30
CCNY	1374220_at	0,40
CCS	1387038_at	3,20
CCSER2	1382265_at	0,27
CD36	1386901_at	0,08
CD5L	1385635_at	0,23
CD63	1367709_at	12,91
CD68	1375010_at	0,30
CD74	1367679_at	0,12
CD83	1390529_at	0,16
CDC42EP1	1389157_at	3,01
CDC42SE1	1388530_at	0,35
CDK2AP1	1388443_at	0,40
CENPL	1398608_at	2,64
Ces1c	1371100_at	4,47
CFH	1387029_at	0,33
CHCHD5	1373890_at	2,99
CHCHD6	1372134_at	5,98
CHD1L	1389469_at	3,46
CHUK	1373517_at	0,38
CKS1B	1376346_at	3,36
CLDN3	1368115_at	9,92
CLN3	1378479_at	2,95
CLPP	1371789_at	2,55
CMIP	1382064_at	0,36
CNOT1	1398951_at	2,73
COA4	1374416_at	2,58
COG4	1372819_at	3,23
COG7	1379876_at	2,60
COG8	1374045_at	2,75
COL18A1	1388459_at	3,25
COL3A1	1370959_at	4,29
COPS7A	1374460_at	2,57
COQ5	1388790_at	2,91
CORO1A	1369964_at	0,19
CORO1B	1398769_at	2,58
COX14	1371460_at	2,89
CPOX	1377998_at	0,36
CPPED1	1373924_at	2,53
CPSF1	1373531_at	2,48
CPT2	1386927_at	0,30
CRELD1	1388594_at	3,01
CRLS1	1379525_at	0,35
CROT	1387183_at	0,00
CRTC2	1373828_at	2,60
CRY1	1392640_at	0,06

CTC1	1373665_at	2,85
CTDSPL	1374349_at	5,21
CTF1	1387083_at	0,35
CTSD	1367651_at	2,45
CTSS	1387005_at	0,34
Cxcl12	1387655_at	0,10
CXCL9	1373544_at	0,08
CYC1	1371342_at	2,91
CYP27A1	1387914_at	2,75
Cyp3a13	1370387_at	39,95
CYP4F3	1387916_at	3,16
CYP7A1	1368458_at	5634,22
CYTH2	1367727_at	2,50
DAG1	1371430_at	0,31
DBT	1373201_at	3,34
DCTPP1	1370308_at	3,43
DDB1	1399162_a_at	2,57
DDOST	1372247_at	3,07
DDX21	1375901_at	0,35
DDX47	1375554_at	2,66
DDX49	1374332_at	2,81
DGAT2	1371615_at	0,37
DGCR6	1373424_at	2,85
DGUOK	1372799_at	2,68
DHPS	1388803_at	2,85
DHX36	1379101_at	0,32
DLST	1370879_at	2,58
DNAJA1	1368852_at	0,36
DNAJC15	1373064_at	2,89
DNAJC4	1372428_at	2,85
DNTTIP1	1370915_s_at	2,81
DPAGT1	1372270_at	2,55
DPCD	1374358_at	2,51
DPP3	1367966_at	2,50
DPYSL2	1371694_at	0,33
DTD1	1388428_at	2,97
DUSP1	1368147_at	0,09
DUSP22	1375967_a_at	0,36
DUSP3	1389050_at	0,37
DUSP6	1377064_at	0,09
ECE1	1367801_at	2,85
ECI2	1388908_at	0,38
EDEM1	1372533_at	7,16
EDEM2	1376315_at	3,20
EEF2	1370003_at	2,43
EGLN1	1389207_at	0,35
EHD1	1372317_at	2,68

EIF1B	1373411_at	0,39
EIF2AK2	1387242_at	0,30
EIF4E	1398799_at	0,39
EIF4G2	1367469_at	0,41
EIF5	1398846_at	0,33
ELAVL1	1375892_at	1,00
ELOVL1	1398360_at	0,38
ERCC1	1399032_at	3,16
ERGIC1	1389319_at	0,31
ERI3	1388475_at	2,62
EXOSC5	1372942_at	3,07
EXOSC7	1372688_at	2,53
FABP7	1370024_at	167,73
FAF1	1369995_at	2,50
FAM117A	1376177_at	0,36
FAM126B	1382818_at	5,10
FAM134B	1373011_at	60,97
FAM173A	1372786_at	2,43
FAM92A1	1377616_at	3,14
FARP2	1374239_at	2,95
FBXO21	1369972_at	4,14
FBXO31	1372600_at	4,23
FBXO8	1373835_at	0,39
FBXW9	1389106_at	3,18
FCER1G	1373575_at	0,31
FDFT1	1367839_at	5,28
FDX1L	1372496_at	2,69
FDXR	1368011_at	2,58
FGFR2	1373829_at	3,39
FH	1367670_at	2,55
FIBP	1399072_at	2,79
FIG4	1390289_at	2,60
FIS1	1371594_at	2,53
FKBP4	1389844_at	0,33
FKBP8	1371528_at	2,43
FKBPL	1390995_at	3,01
FLOT2	1367746_a_at	2,81
FOLR2	1390348_at	0,31
FOXN3	1384728_at	0,29
FOXRED1	1376921_at	2,85
FSTL1	1368822_at	2,93
FZR1	1375546_at	2,36
GAA	1371799_at	2,85
GABARAPL2	1380170_at	2,75
GBF1	1389044_at	3,12
GCAT	1382325_at	4,50
GEMIN2	1368229_at	2,77

GET4	1376016_at	2,51
GFRA1	1387007_at	17,63
GGH	1368470_at	0,25
GGNBP2	1372201_at	2,93
GHR	1373803_a_at	0,38
GK	1387491_at	0,28
GLIPR1	1373504_at	0,19
GLT8D1	1388893_at	2,95
GMPPA	1372859_at	2,93
GNG10	1388455_at	0,31
GOLGA2	1386942_at	2,81
GPAM	1382986_at	0,23
GPD2	1387670_at	0,10
GPM6A	1373773_at	0,00
GPNMB	1368187_at	0,02
GPR107	1384602_at	3,27
GPR146	1382266_at	4,99
GSDMD	1385001_at	2,55
GSTO1	1389832_at	2,69
GTF2H5	1367509_at	2,75
GTF3C1	1370231_at	2,91
GTF3C2	1389574_at	2,73
Gulo	1369837_at	3,20
H1FX	1371376_at	6,59
HAAO	1368794_at	2,48
HBS1L	1372344_at	2,71
HDC	1370491_a_at	0,07
HDDC3	1372762_at	2,89
HERPUD1	1367741_at	3,71
HEXB	1372706_at	4,11
HEXIM1	1374846_at	0,36
HGFAC	1381006_at	2,60
HIP1	1390082_at	2,85
HLA-B	1371209_at	0,01
HLA-DQA1	1370822_at	0,15
HLA-DRA	1370883_at	0,12
HLA-DRB1	1370383_s_at	0,20
HMBS	1386983_at	2,73
HMGCS1	1367932_at	0,10
HMGN2	1371352_at	2,93
HNRNPA2B1	1398883_at	0,35
HNRNPA3	1370871_at	0,38
HNRNPU	1370171_at	0,40
HPN	1368431_at	2,39
HSCB	1382051_at	2,75
HSD11B2	1368102_at	0,26
HYI	1384103_at	2,83

IAH1	1372087_at	2,51
ICK	1387948_at	2,97
ICT1	1374443_at	2,89
ID2	1368870_at	0,24
IDH2	1388403_at	3,41
IDH3G	1370865_at	2,71
IFIT1B	1369836_at	0,02
IFIT2	1379568_at	0,08
IFITM1	1372013_at	0,26
IFT20	1375425_at	2,62
IGF2BP3	1377824_a_at	0,15
IGFBP2	1367648_at	134,36
Igtp	1396163_at	0,07
IL18BP	1369031_at	0,33
IL7	1369208_at	0,08
IMP4	1379407_at	2,75
INPP4A	1387078_at	3,12
INSIG2	1393690_at	0,19
IP6K1	1387904_at	2,73
IPO5	1373955_at	2,48
IRF3	1371560_at	0,38
IRF7	1383564_at	0,12
IRF9	1383448_at	0,31
IRGM	1391489_at	0,11
ISG15	1382314_at	0,09
ITGA2B	1380600_at	2,71
ITGB2	1383131_at	0,19
JUND	1393138_at	0,38
KAT5	1372366_at	2,62
KCNN2	1370111_at	0,09
KDM2B	1393101_at	0,40
KEAP1	1370066_at	2,51
KIAA0922	1391414_at	3,51
KIFAP3	1389556_at	2,75
KISS1	1398516_at	3,63
KLF15	1368249_at	3,63
KLF9	1370209_at	3,78
KPTN	1381992_at	2,89
KRT8	1371530_at	3,81
LAPTM5	1383658_at	0,36
LARGE	1372884_at	0,28
LCP1	1389210_at	0,22
LENEP	1387067_at	2,66
LEPRE1	1367967_at	2,85
LEPROTL1	1374876_at	2,71
LGALS3BP	1387946_at	0,14
LIAS	1389154_at	2,77

LITAF	1370928_at	0,23
LONP1	1398247_at	2,38
LRP3	1368239_at	3,07
LRPPRC	1373500_at	2,77
LSG1	1373293_at	2,66
LSM2	1371712_at	2,57
LY6E	1388347_at	0,31
MAGED1	1386895_at	3,58
MAGI3	1398866_at	0,31
MAL2	1384361_at	0,06
MAN1A1	1383574_at	0,37
MAN2B1	1388699_at	2,93
MANBAL	1398924_at	2,64
MAP1LC3B	1367669_a_at	2,95
MAP2K6	1387809_at	6,96
MAP7	1392916_at	2,57
MAPK9	1368646_at	0,26
MARC1	1376847_at	2,58
MARCKS	1373432_at	0,32
MARK3	1368831_at	2,64
MAT1A	1371031_at	2,68
MCCC1	1376852_at	3,03
MDFIC	1378482_at	0,20
MED29	1374851_at	3,51
MEN1	1398785_at	2,30
METAP1D	1375457_at	3,63
MGST2	1372599_at	3,25
MKNK2	1372038_at	6,54
MMACHC	1376214_at	2,77
MMP14	1378225_at	0,35
MOCS2	1372177_at	0,33
MPG	1368410_at	3,53
MPV17	1393110_at	2,89
MRPL10	1395526_at	2,75
MRPL12	1372431_at	2,81
MRPL15	1398891_at	2,85
MRPL16	1371967_at	2,66
MRPL19	1373477_at	2,64
MRPL2	1398911_at	2,62
MRPL21	1367502_at	2,64
MRPL24	1371888_at	2,73
MRPL27	1399047_at	2,77
MRPL3	1371709_at	2,60
MRPL30	1398983_at	2,55
MRPL35	1371955_at	2,77
MRPL38	1383618_at	3,05
MRPL40	1388517_at	2,77

MRPL41	1371423_at	2,69
MRPL42	1371853_at	2,58
MRPL52	1374411_at	2,53
MRPL53	1367477_at	2,83
MRPL9	1398912_at	2,71
MRPS15	1372697_at	2,48
MRPS16	1398946_at	3,18
MRPS18A	1371591_at	2,87
MRPS18B	1371809_at	2,79
MRPS24	1371649_at	2,62
MRPS27	1372916_at	3,12
MRPS35	1372839_at	3,23
MRPS9	1373002_at	2,87
MSN	1371575_at	0,33
MTFP1	1388661_at	3,14
MTHFD1	1368181_at	3,27
MTX1	1399077_at	2,71
MX1	1369202_at	0,00
MYC	1368308_at	0,10
MYCL1	1395781_at	0,16
N6AMT1	1392330_at	2,91
NABP1	1392579_at	0,18
NABP2	1374631_at	2,39
NADSYN1	1379472_at	2,89
NAGA	1382285_at	2,87
NDUFA6	1379243_at	2,64
NDUFA8	1371355_at	2,69
NDUFA9	1388323_at	2,57
NDUFAF3	1368230_a_at	3,12
NDUFB3	1373041_at	2,77
NDUFB7	1388343_at	2,75
NDUFS2	1371482_at	2,50
NDUFS3	1388364_at	2,62
NDUFS7	1371912_at	2,57
NEK6	1374565_at	0,24
NFIX	1370946_at	0,30
NFU1	1389162_at	2,85
NIF3L1	1383289_at	0,40
NIPSNAP1	1388537_at	2,77
NOLC1	1368032_at	0,33
NOSIP	1371397_at	2,48
NOTCH2	1398362_at	0,15
NOX4	1368543_at	111,43
NQO1	1387599_a_at	15,35
NR1H4	1369073_at	0,33
NSMCE1	1374210_at	2,83
NSMCE2	1389011_at	2,79

NTHL1	1393963_at	2,79
NTMT1	1372185_at	3,18
NTPCR	1390108_at	2,95
NUCB2	1370000_at	5,66
NUDT19	1371919_at	3,14
NUDT22	1367507_at	2,77
NUDT6	1370435_a_at	3,46
NUDT7	1389251_at	0,22
NUDT9	1373840_at	2,75
NUP37	1376703_at	2,85
NUPR1	1367847_at	6,41
NUTF2	1371246_at	0,33
NXF1	1398861_at	0,34
OASL	1377497_at	0,15
OBFC1	1373295_at	2,81
OR51E2	1387981_at	0,09
OSBP	1373974_at	2,81
OSBPL9	1371978_at	2,50
OTUB2	1376118_at	0,26
OXSM	1391530_a_at	2,75
PAFAH1B2	1369642_at	0,38
PAIP1	1379272_at	0,23
PAOX	1372212_at	3,46
PAQR9	1382569_at	0,19
PARP12	1380071_at	0,38
PBLD	1370320_at	3,48
PBXIP1	1379868_at	2,83
Pcbd2	1379329_at	3,01
PCMTD1	1377742_at	0,36
PCNA	1367671_at	0,41
PDIA5	1374828_at	3,58
PDK2	1386975_at	3,51
PDK4	1369150_at	0,22
PEX1	1376595_at	0,34
PEX11G	1393668_at	3,01
PEX16	1383960_at	2,71
PEX6	1368264_at	2,89
PFDN1	1398926_at	2,85
PFKFB1	1369467_a_at	6,06
Phb	1367926_at	2,60
PHB2	1367463_at	2,81
PHF11	1382546_at	0,24
PHLDA3	1375224_at	0,03
PHYHD1	1371974_at	2,79
PI4KA	1370318_at	2,95
PIK3C2G	1369050_at	5,06
PLA1A	1370445_at	7,46

PLEC	1386941_at	3,03
PLEKHA1	1376762_at	0,25
PLIN2	1390383_at	0,22
PMPCB	1387781_at	2,77
PMVK	1373243_at	4,50
PNKP	1393367_at	2,51
PNPLA7	1377049_at	3,92
POLB	1368341_at	0,31
POLD4	1388883_at	3,46
POLE4	1388367_at	0,37
POLG2	1375504_at	5,13
POLL	1389268_at	2,89
POLR1C	1374289_at	2,87
POMT1	1368267_at	2,60
POP7	1373597_at	2,69
PPIL3	1375686_at	0,34
PPP2CA	1388805_at	0,40
PPP2R1B	1373959_at	0,10
PPP2R3C	1378741_at	2,60
PPP2R5E	1388965_at	2,81
PPP3CA	1373479_at	0,36
PRDX5	1367677_at	3,14
PREB	1370300_at	2,71
PREPL	1374004_at	2,69
PRKCI	1373047_at	0,32
PRLR	1370384_a_at	0,00
PRODH	1372920_at	5,66
PROSC	1389549_at	2,66
PRPF3	1372432_at	2,68
PRR13	1388408_at	0,38
PSMA7	1371869_at	2,57
PSMB1	1398812_at	2,46
PSMB6	1398777_at	2,62
PSMB7	1367656_at	2,77
PSMB9	1370186_at	0,32
PSPH	1375964_at	0,17
PTDSS2	1372663_at	3,12
PTOV1	1388430_at	2,64
PTPN11	1388379_at	0,40
PTPN3	1376537_at	3,34
PTPRC	1390798_at	0,19
PUF60	1367464_at	2,46
PYCRL	1392535_at	3,14
RAB21	1383089_at	0,37
RAB3IP	1387821_at	2,95
RABGGTA	1387869_s_at	2,99
RAC2	1372404_at	0,33

RAPH1	1376658_at	0,17
RB1	1388185_at	0,17
RBCK1	1386966_a_at	2,64
RBFA	1388946_at	2,83
RBL2	1368403_at	3,23
RBM42	1371502_at	2,51
RCBTB1	1390433_at	0,32
RDBP	1372290_at	2,83
RDH10	1384391_at	0,35
RDH5	1379587_at	7,67
RFFL	1385504_at	0,35
RHOA	1399027_at	0,38
RHOB	1369958_at	0,22
RHOQ	1386967_at	0,29
RINT1	1382797_at	4,11
RIOK3	1394363_at	2,51
RND2	1372521_at	3,12
Rnf113a2	1377837_at	2,62
RNF181	1371373_at	2,79
RNF217	1393047_at	0,30
RPIA	1389468_at	0,32
RPL7L1	1389013_at	2,51
RPN1	1398766_at	2,71
RPRM	1390672_at	0,01
RPUSD3	1380562_at	3,10
RRAS	1388729_at	2,66
RT1-EC2	1369110_x_at	0,21
RUVBL1	1370386_at	2,57
RXRA	1371668_at	2,83
S1PR1	1371840_at	0,23
SALL1	1391194_at	0,22
SAMM50	1388380_at	2,68
SCAMP4	1367688_at	2,89
SCAP	1389567_at	2,77
SCN1B	1387010_s_at	4,03
SDHB	1372123_at	2,73
SDPR	1382452_at	0,30
SDS	1369864_a_at	1,00
SDSL	1385670_at	4,86
Sectm1b	1376976_at	2,89
SEMA4G	1380248_at	3,51
SERPINB9	1373592_at	0,33
SERPINF2	1377033_at	2,58
SERPING1	1372254_at	0,37
SH3BGRL	1383085_at	0,28
SH3BP5	1387294_at	4,14
SHISA5	1388776_at	0,38

SHMT2	1388695_at	3,18
SIK2	1376649_at	0,26
SIL1	1376431_at	2,71
SIVA1	1383962_at	3,12
SKIV2L	1399112_at	2,64
SLC12A2	1367853_at	0,27
SLC13A3	1368047_at	7,52
SLC16A1	1386981_at	0,20
SLC22A1	1368191_a_at	2,81
SLC22A9	1369840_at	0,08
SLC25A33	1373282_at	4,92
SLC27A1	1367789_at	2,62
SLC37A4	1386960_at	3,34
SLC38A10	1399102_at	2,69
SLC38A3	1370824_at	3,41
SLC39A3	1377933_at	2,79
SLC39A7	1389089_at	3,66
SLC46A3	1383471_at	3,43
SLC4A4	1369756_a_at	0,19
SLC6A12	1387295_at	3,05
Slfn2	1377916_at	0,35
SMARCD2	1370159_at	3,32
SNRK	1386282_x_at	2,55
SPG7	1374839_at	3,03
SRGN	1368655_at	0,33
SS18	1391628_at	0,31
SSR2	1373185_at	3,34
SSU72	1371768_at	2,77
ST5	1373288_at	2,77
STAP2	1389420_at	3,58
STARD4	1396521_at	3,89
STARD5	1394275_at	0,33
STAT1	1372757_at	0,33
STIM1	1376024_at	2,66
STK17B	1373164_at	0,30
STK24	1374584_at	0,34
STOML2	1398908_at	2,51
STT3A	1398599_at	2,97
STUB1	1398910_at	2,87
STX5	1386853_s_at	3,18
SUB1	1398880_at	0,40
SULF2	1388753_at	3,76
Sult2a2	1387936_at	0,00
SUPT7L	1373871_at	2,93
SURF1	1398857_at	2,53
SWSAP1	1372396_at	3,29
SYS1	1367543_at	2,73

TAP1	1388149_at	0,21
TARS2	1373760_at	2,77
TBC1D17	1379901_at	2,64
TBC1D9B	1372129_at	2,48
TBL1XR1	1394079_at	0,20
TBRG1	1371509_at	2,58
TCEA3	1388611_at	4,76
TEF	1390819_at	4,82
TEN1	1383230_at	0,13
TGM2	1369943_at	0,30
THNSL2	1389114_at	3,39
THOC7	1398385_at	0,40
TIMM13	1398868_at	2,68
TIMM22	1370353_at	2,71
TLN1	1389002_at	2,69
TM2D2	1398984_at	2,64
TMEM140	1390042_at	0,35
TMEM203	1379323_at	2,60
TMEM214	1382596_a_at	2,81
TMEM218	1389561_at	4,82
TMEM223	1398931_at	2,73
TMEM30B	1379513_at	0,04
TMEM33	1369076_at	0,40
TMEM41B	1382689_at	2,85
TMEM43	1373595_at	0,35
TMEM66	1398879_at	2,77
TMEM98	1373161_at	3,76
TMSB10/TMSB4X	1387883_a_at	0,38
TMTC4	1379541_at	0,30
TMUB1	1372961_at	2,53
TNFSF13	1379677_at	0,27
TNS1	1371954_at	3,25
TOMM40	1389969_at	2,77
TOMM70A	1379186_at	0,37
TOR1A	1375357_at	2,53
Tpm3	1387918_at	0,37
Tpm4	1368838_at	0,21
TRAP1	1374467_at	2,97
TRAPPC1	1372252_at	2,58
TRAPPC11	1399129_at	2,58
TRAPPC9	1376416_at	2,75
TRUB2	1378326_at	3,05
TSPAN7	1371702_at	0,36
TSTA3	1398953_at	2,51
TTC36	1393061_at	2,93
TUBB2A	1373718_at	0,03
TXN2	1398844_at	2,79

TXNRD1	1386958_at	0,29
U2AF1L4	1373205_at	2,64
UBE2E3	1389534_at	0,38
UBE2J1	1373495_at	2,68
UBL5	1371590_s_at	2,69
UBN1	1381878_at	0,27
UBR2	1381349_a_at	2,60
UBXN6	1389260_at	2,85
UFSP2	1376086_at	2,93
UGP2	1388410_at	3,41
UNC5B	1393799_at	0,21
UQCR11	1375197_at	2,69
UQCRC1	1388301_at	2,60
UQCRH	1371415_at	2,71
USP2	1387703_a_at	27,67
USP25	1378679_at	0,38
UXS1	1376262_at	0,33
VANGL1	1394435_at	2,87
VCP	1367455_at	2,50
VIM	1367574_at	0,31
VIMP	1387915_at	2,85
VPS11	1373498_at	2,50
VPS13C	1379941_at	3,43
VPS16	1388934_at	2,57
VPS33A	1387557_s_at	2,89
VPS51	1383664_a_at	2,87
WBP1L	1375369_at	2,77
WDR74	1373263_at	2,68
WFDC2	1390765_at	0,37
WFS1	1368839_at	3,29
XIAP	1369248_a_at	0,03
XRCC1	1387129_at	2,69
XRCC5	1370931_at	2,83
XRCC6	1370537_at	2,75
YWHAZ	1387774_at	0,36
ZER1	1383389_at	2,55
ZMAT5	1371788_at	3,10
ZNF318	1389340_at	2,99
ZNF467	1391507_at	4,63
ZNF496	1384517_at	3,63
ZNHIT1	1371660_at	3,01

Notes: symbol refers to NCBI gene symbol; Affymetrix refers to Affymetrix gene identification.
Abbreviations: FC = fold change.

ANNEX V. Canonical pathways selected by Ingenuity from the worklist "Uncommon Genes".

Metabolic pathways	V	S	F
• Activation/Inactivation/Interconversion			
○ Activation			
▪ Fatty Acid Activation	X		
• Biosynthesis			
○ dTMP De Novo Biosynthesis			X
○ Amines and Polyamines Biosynthesis			
▪ Histamine Biosynthesis	X		
○ Amino Acids Biosynthesis			
▪ Diphthamide Biosynthesis	X		
▪ Hypusine Biosynthesis			X
▪ Superpathway of Serine and Glycine Biosynthesis I	X		X
▪ Individual Amino Acids Biosynthesis			
▪ Arginine Biosynthesis			
▪ Arginine Biosynthesis IV	X		
▪ Citrulline-Nitric Oxide Cycle	X		
▪ Cysteine Biosynthesis			
▪ Cysteine Biosynthesis III (mammalia)			X
▪ Glutamate Biosynthesis			
▪ Arginine Degradation I (Arginase Pathway)	X		X
▪ Glycine Biosynthesis			
▪ Glycine Biosynthesis I			X
▪ Methionine Biosynthesis			
▪ S-methyl-5-thio-α-D-ribose 1-phosphate Degradation	X		X
▪ Other Amino Acid Biosynthesis			
▪ Citrulline Biosynthesis			
▪ Citrulline Biosynthesis			X
▪ Superpathway of Citrulline Metabolism	X		X
▪ Proline Biosynthesis			
▪ Arginine Degradation VI (Arginase 2 Pathway)	X		X
▪ Proline Biosynthesis I	X		X
▪ Proline Biosynthesis II (from Arginine)	X		X
▪ Serine Biosynthesis			
▪ Serine Biosynthesis	X		
▪ Tyrosine Biosynthesis			
▪ Tyrosine Biosynthesis IV			X
▪ B-Alanine Biosynthesis			
▪ Uracil Degradation II (Reductive)			X
○ Carbohydrates Biosynthesis			
▪ Polysaccharides Biosynthesis			

▪ Dolichyl-diphosphooligosaccharide Biosynthesis		X	
▪ Glucogen and Starch Biosynthesis			
▪ Glycogen Biosynthesis II (from UDP-D-Glucose)	X		
▪ Sugars Biosynthesis			
▪ Sugar Nucleotides Biosynthesis			
▪ GDP-L-fucose Biosynthesis I (from GDP-D-mannose)			
▪ GDP Mannose Biosynthesis			
▪ GDP-mannose Biosynthesis		X	
▪ UDP-D-xylose and UDP-D-glucuronate Biosynthesis	X		X
o Cell Structures Biosynthesis			
▪ Colanic Acid Building Blocks Biosynthesis	X	X	X
o Cofactors, Prosthetic Groups and Electron Carriers Biosynthesis			
▪ Mevalonate Pathway I	X		X
▪ Lipoate Biosynthesis			
▪ Lipoate Biosynthesis and Incorporation II	X		X
▪ Molybdenum Cofactor Biosynthesis	X		
▪ Molybdenum Cofactor Biosynthesis	X		
▪ NAD Metabolism			
▪ NAD Phosphorylation and Dephosphorylation	X		X
▪ NAD Biosynthesis			
▪ NAD Biosynthesis from 2-amino-3-carboxymuconate Semialdehyde		X	X
▪ NAD Salvaαe Pathway II	X		X
▪ NAD biosynthesis II (from tryptophan)	X	X	X
▪ Polyprenyl Biosynthesis			
▪ Geranylgeranyldiphosphate Biosynthesis			
▪ Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	X		X
▪ Porphyrin Compounds Biosynthesis			
▪ Heme Biosynthesis			
▪ Heme Biosynthesis II	X		X
▪ Heme Biosynthesis from Uroporphyrinogen-III I	X		
▪ Reductants Biosynthesis			
▪ Glutathione Redox Reactions I	X	X	
▪ Thioredoxin Pathway	X		
▪ Tetrapyrrole Biosynthesis			
▪ Tetrapyrrole Biosynthesis II			X
▪ Vitamins Biosynthesis			
▪ Folate Biosynthesis			
▪ Folate Polyglutamylation	X		X
▪ Folate Transformations I	X		X
▪ Glutamate Removal from Folates	X		

▪ Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	X		
▪ Vitamin A Biosynthesis			
▪ Retinoate Biosynthesis I	X	X	X
▪ Retinol Biosynthesis	X	X	
▪ The Visual Cycle	X	X	
▪ Vitamin B6 Biosynthesis			
▪ Pyridoxal 5'-phosphate Salvage Pathway	X		X
o Fatty Acids and Lipids Biosynthesis			
▪ Acyl-CoA Hydrolisis		X	X
▪ Fatty Acid Activation	X		
▪ Mevalonate Pathway I	X		X
▪ Triacylglycerol Biosynthesis	X	X	X
▪ Fatty Acid Biosynthesis			
▪ Fatty Acid Biosynthesis Initation II			X
▪ Oleate Biosynthesis			
▪ Oleate Biosynthesis II (Animals)			X
▪ Palmitate Biosynthesis			
▪ Palmitate Biosynthesis I (Animals)			X
▪ Stearate Biosynthesis			
▪ Stearate Biosynthesis I (Animals)	X	X	X
▪ γ-Linolenate Biosynthesis			
▪ γ-linolenate Biosynthesis II (Animals)	X		
▪ Phospholipid Biosynthesis			
▪ 3-phosphoinositide Biosynthesis	X	X	X
▪ D-myo-inositol-5-phosphate Metabolism	X	X	X
▪ Superpathway of Inositol Phosphate Compounds	X		X
▪ CDP-diacylglycerol Biosynthesis			
▪ CDP-diacylglycerol Biosynthesis I	X		X
▪ Phosphatidylethanolamine Biosynthesis			
▪ Phosphatidylethanolamine Biosynthesis III			X
▪ Phosphatidylglycerol Biosynthesis			
▪ Phosphatidylglycerol Biosynthesis II (Non-plastidic)	X		X
▪ Sterol Biosynthesis			
▪ Bile Acid Biosynthesis, Neutral Pathway	X		X
▪ Cholesterol Biosynthesis I	X		
▪ Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol)	X		
▪ Cholesterol Biosynthesis III (via Desmosterol)	X		
▪ Superpathway of Cholesterol Biosynthesis	X		X
o Hormones Biosynthesis			
▪ Leukotriene Biosynthesis	X	X	
▪ Thyroid Hormone Biosynthesis	X		
o Metabolic Regulators Biosynthesis			
▪ Nitric Oxide Biosynthesis			

▪ Citrulline-Nitric Oxide Cycle	X		
o Nucleosides and Nucleotides Biosynthesis			
▪ Purine Nucleotide Biosynthesis			
▪ Purine Nucleotide De Novo Biosynthesis			
▪ Purine Nucleotides De Novo Biosynthesis II	X		
▪ Pyrimidine Nucleotide Biosynthesis			
▪ Pyrimidine Nucleotides Salvage			
▪ Salvage Pathways of Pyrimidine Ribonucleotides	X		X
o Secondary Metabolites Biosynthesis			
▪ Sugar Derivatives Biosynthesis			
▪ Cyclitols Biosynthesis			
▪ D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	X	X	X
▪ D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	X	X	X
▪ D-myo-inositol-5-phosphate Metabolism	X	X	X
▪ Inositol Pyrophosphates Biosynthesis	X		X
▪ Superpathway of Inositol Phosphate Compounds	X	X	X
▪ Terpenoids Biosynthesis			
▪ Diterpenoids Biosynthesis			
▪ Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	X		X
▪ Hemiterpenes Biosynthesis			
▪ Mevalonate Pathway I	X		X
▪ Triterpenoids Biosynthesis			
▪ Epoxysqualene Biosynthesis	X		
• Degradation/Utilization/Assimilation			
o Alcohols Degradation			
▪ Ethanol Degradation			
▪ Ethanol Degradation II	X	X	X
▪ Ethanol Degradation IV	X		X
▪ Oxidative Ethanol Degradation III	X		X
▪ Glycerol Degradation			
▪ Glycerol Degradation I	X		
o Aldehyde Degradation			
▪ Methylglyoxal Degradation III	X		X
o Amines and Polyamines Degradation			
▪ Dopamine Degradation	X		X
▪ Glycine Betaine Degradation	X		X
▪ Histamine Degradation	X		X
▪ Phenylethylamine Degradation I			X
▪ Putrescine Degradation			
▪ Putrescine Degradation III	X		X
▪ Spermine and Spermidine Degradation			

▪ Spermine and Spermidine Degradation I	X	X
o Amino Acids Degradation		
▪ 2-ketoglutarate Dehydrogenase Complex	X	X
▪ Branched-chain α-keto acid Dehydrogenase Complex	X	
▪ 4-Hydroxyproline Degradation		
▪ 4-hydroxyproline Degradation I	X	
▪ Arginine Degradation		
▪ Arginine Degradation I (Arginase Pathway)	X	X
▪ Arginine Degradation VI (Arginase 2 Pathway)	X	X
▪ Citrulline-Nitric Oxide Cycle	X	
▪ Asparagine Degradation		
▪ Asparagine Degradation I	X	
▪ Histidine Degradation		
▪ Histidine Degradation III	X	
▪ Isoleucine Degradation		
▪ Isoleucine Degradation I	X	X
▪ Leucine Degradation		
▪ Leucine Degradation I	X	X
▪ Methionine Degradation		
▪ Methionine Degradation I (to Homocysteine)		X
▪ Phenylalanine Degradation		
▪ Phenylalanine Degradation I (Aerobic)		X
▪ Phenylalanine Degradation IV (Mammalian, via Side Chain)		X
▪ Proline Degradation		
▪ Proline Degradation	X	X
▪ Serine Degradation		
▪ L-serine Degradation	X	X
▪ Tryptophan Degradation		
▪ Tryptophan Degradation III (Eukaryotic)	X	X
▪ Tryptophan Degradation X (Mammalian, via Tryptamine)	X	X
▪ Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	X	X
▪ Valine Degradation		
▪ Valine Degradation I	X	X
o Aromatic Compounds Degradation		
▪ Phenolic Compounds Degradation		
▪ Phenylethylamine Degradation		
▪ Phenylethylamine Degradation I		X
o Carboxylates Degradation		
▪ Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	X	
o Cofactors, Prosthetic Groups and Electron Carriers Degradation		
▪ Ascorbate Recycling (Cytosolic)	X	X

○ Degradation/Utilization/Assimilation - Other			
▪ Glutathione-mediated Detoxification	X	X	
▪ α-tocopherol Degradation		X	X
○ Fatty Acid and Lipids Degradation			
▪ 3-phosphoinositide Degradation	X	X	X
▪ Glycerol Degradation I	X		
▪ Mitochondrial L-carnitine Shuttle Pathway	X	X	
▪ Fatty Acids Degradation			
▪ Fatty Acid α-oxidation	X		X
▪ Fatty Acid β-oxidation I	X	X	X
▪ Fatty Acid β-oxidation III (Unsaturated, Odd Number)		X	
○ Hormones Degradation			
▪ Noradrenaline and Adrenaline Degradation	X	X	X
▪ Serotonin Degradation	X	X	X
○ Inorganic Nutrients Metabolism			
▪ Nitrogen Compounds Metabolism			
▪ Urea Cycle	X		X
○ Nucleosides and Nucleotides Degradation			
▪ S-methyl-5-thio-α-D-ribose 1-phosphate Degradation	X		X
▪ Pyrimidine Degradation			
▪ Thymine Degradation			X
▪ Uracil Degradation			
▪ Uracil Degradation II (Reductive)			X
○ Polymeric Compounds Degradation			
▪ Polysaccharides Degradation			
▪ Glycogen Degradation			
▪ Glycogen Degradation III	X	X	
▪ Glycosaminoglycan Degradation			
▪ Chondroitin Sulfate Degradation (Metazoa)	X		
▪ Dermatan Sulfate Degradation (Metazoa)	X		
• Detoxification			
○ Glutathione-mediated Detoxification	X	X	X
○ Superoxide Radicals Degradation	X		
○ Arsenate Detoxification			
▪ Arsenate Detoxification I (Glutaredoxin)	X		X
○ Methylglyoxal Detoxification			
▪ Methylglyoxal Degradation III	X		X
• Generation of Precursor Metabolites and Energy			
○ Glycerol-3-phosphate Shuttle	X		
○ Acetyl-CoA Biosynthesis			
▪ Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	X		
○ Ketogenesis			X
○ Pentose Phosphate Pathways			
▪ Pentose Phosphate Pathway		X	
▪ Pentose Phosphate Pathway (Non-oxidative Branch)		X	

○ Respiration	X		
▪ 2-ketoglutarate Dehydrogenase Complex	X		X
▪ Branched-chain α-keto acid Dehydrogenase Complex	X		
○ TCA cycle			
▪ TCA Cycle II (Eukaryotic)	X		X
Signaling Pathways			
• Apoptosis			
○ 14-3-3-mediated Signaling	X	X	X
○ Apoptosis Signaling	X	X	X
○ April Mediated Signaling	X	X	
○ Aryl Hydrocarbon Receptor Signaling	X	X	X
○ CD27 Signaling in Lymphocytes	X	X	
○ Calcium-induced T Lymphocyte Apoptosis		X	X
○ Ceramide Signaling	X	X	X
○ Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	X		X
○ IL-15 Production	X	X	X
○ IL-9 Signaling	X		X
○ Induction of Apoptosis by HIV1	X	X	
○ JAK/Stat Signaling	X	X	X
○ LPS-stimulated MAPK Signaling	X	X	
○ Myc Mediated Apoptosis Signaling	X		X
○ Nur77 Signaling in T Lymphocytes	X	X	X
○ PTEN Signaling	X	X	X
○ SAPK/JNK Signaling	X		X
○ TNFR2 Signaling	X	X	
○ TWEAK Signaling	X	X	
○ Telomerase Signaling	X	X	X
○ Telomere Extension by Telomerase	X		X
○ Toll-like Receptor Signaling	X	X	X
○ Tumoricidal Function of Hepatic Natural Killer Cells	X		X
○ Type I Diabetes Mellitus Signaling	X	X	X
• Cancer			
○ Acute Myeloid Leukemia Signaling	X		X
○ Bladder Cancer Signaling	X		X
○ Chronic Myeloid Leukemia Signaling	X	X	X
○ Colorectal Cancer Metastasis Signaling	X	X	X
○ ERK/MAPK Signaling	X	X	X
○ Endometrial Cancer Signaling	X		X
○ Glioblastoma Multiforme Signaling	X	X	X
○ Glioma Invasiveness Signaling	X	X	X
○ Glioma Signaling	X	X	X
○ HER-2 Signaling in Breast Cancer	X	X	X
○ Melanoma Signaling	X		X
○ Non-Small Cell Lung Cancer Signaling	X		X
○ PI3K/AKT Signaling	X	X	X
○ PTEN Signaling	X	X	X
○ Prostate Cancer Signaling	X	X	X

○ Renal Cell Carcinoma Signaling	X	X	X
○ Role of BRCA1 in DNA Damage Response	X	X	X
○ Role of p14/p19ARF in Tumor Suppression	X		X
○ Small Cell Lung Cancer Signaling	X	X	X
○ Telomerase Signaling	X	X	X
○ Telomere Extension by Telomerase	X		X
○ Thyroid Cancer Signaling	X		X
• Cardiovascular Signaling			
○ Aldosterone Signaling in Epithelial Cells	X	X	X
○ Angiopoietin Signaling	X	X	X
○ Inhibition of Angiogenesis by TSP1	X	X	X
○ Intrinsic Prothrombin Activation Pathway	X	X	
○ Renin-Angiotensin Signaling	X	X	X
○ nNOS Signaling in Skeletal Muscle Cells			X
• Cell Cycle Regulation			
○ 14-3-3-mediated Signaling	X	X	X
○ Antiproliferative Role of Somatostatin Receptor 2	X	X	X
○ Aryl Hydrocarbon Receptor Signaling	X	X	X
○ Cell Cycle Regulation by BTG Family Proteins	X	X	X
○ Cell Cycle: G1/S Checkpoint Regulation	X		X
○ Cell Cycle: G2/M DNA Damage Checkpoint Regulation	X		X
○ Ceramide Signaling	X	X	X
○ Cyclins and Cell Cycle Regulation		X	X
○ Estrogen-mediated S-phase Entry			X
○ GADD45 Signaling		X	X
○ Integrin Signaling	X	X	X
○ Mitotic Roles of Polo-Like Kinase		X	X
○ Role of CHK Proteins in Cell Cycle Checkpoint Control		X	X
• Cellular Growth, Proliferation and Development			
○ AMPK Signaling	X	X	X
○ Angiopoietin Signaling	X	X	X
○ B Cell Activating Factor Signaling	X	X	
○ B Cell Development		X	X
○ CNTF Signaling	X	X	X
○ Cdc42 Signaling	X	X	X
○ Cleavage and Polyadenylation of Pre-mRNA	X		
○ GM-CSF Signaling	X	X	X
○ Germ Cell-Sertoli Cell Junction Signaling	X	X	X
○ Growth Hormone Signaling	X	X	X
○ HGF Signaling	X	X	X
○ Hematopoiesis from Multipotent Stem Cells		X	
○ Hematopoiesis from Pluripotent Stem Cells		X	X
○ IGF-1 Signaling	X	X	X
○ ILK Signaling	X	X	X
○ Integrin Signaling	X	X	X
○ JAK/Stat Signaling	X	X	X
○ Mouse Embryonic Stem Cell Pluripotency	X	X	X

○ Oncostatin M Signaling	X		X
○ PDGF Signaling	X		X
○ PEDF Signaling	X	X	
○ PI3K/AKT Signaling	X	X	X
○ RAN Signaling	X		
○ RANK Signaling in Osteoclasts	X	X	
○ Regulation of Cellular Mechanics by Calpain Protease	X		X
○ Regulation of eIF4 and p70S6K Signaling	X	X	X
○ Role of p14/p19ARF in Tumor Suppression	X		X
○ Semaphorin Signaling in Neurons	X	X	X
○ Thrombopoietin Signaling	X	X	X
○ Vitamin-C Transport	X		X
○ mTOR Signaling	X	X	X
○ p70S6K Signaling	X	X	X
• Cellular Immune Response			
○ 4-1BB Signaling in T Lymphocytes	X	X	
○ Activation of IRF by Cytosolic Pattern Recognition Receptors	X	X	X
○ Allograft Rejection Signaling	X		X
○ Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	X	X	X
○ Antigen Presentation Pathway	X		X
○ Autoimmune Thyroid Disease Signaling	X		X
○ CCR5 Signaling in Macrophages	X	X	X
○ CD27 Signaling in Lymphocytes	X	X	
○ CD28 Signaling in T Helper Cells	X	X	X
○ CD40 Signaling	X	X	
○ CTLA4 Signaling in Cytotoxic T Lymphocytes	X	X	X
○ CXCR4 Signaling	X	X	X
○ Calcium-induced T Lymphocyte Apoptosis		X	X
○ Caveolar-mediated Endocytosis Signaling	X	X	X
○ Communication between Innate and Adaptive Immune Cells	X		X
○ Crosstalk between Dendritic Cells and Natural Killer Cells	X		X
○ Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	X		X
○ Dendritic Cell Maturation	X	X	X
○ GM-CSF Signaling	X	X	X
○ Graft-versus-Host Disease Signaling	X		X
○ Granzyme A Signaling	X		X
○ HMGB1 Signaling	X	X	X
○ IL-12 Signaling and Production in Macrophages	X	X	X
○ IL-15 Production	X	X	X
○ IL-17 Signaling	X		
○ IL-2 Signaling	X	X	X
○ IL-22 Signaling	X		X
○ IL-3 Signaling	X	X	X
○ IL-4 Signaling	X		X
○ IL-6 Signaling	X	X	X
○ IL-8 Signaling	X	X	X
○ IL-9 Signaling	X		X

○ Interferon Signaling	X		X
○ Leukocyte Extravasation Signaling	X	X	X
○ Lipid Antigen Presentation by CD1			X
○ MIF Regulation of Innate Immunity	X		X
○ MSP-RON Signaling Pathway	X		X
○ Macropinocytosis Signaling	X	X	X
○ NF-κB Activation by Viruses	X	X	X
○ NF-κB Signaling	X	X	X
○ Natural Killer Cell Signaling	X	X	X
○ Nur77 Signaling in T Lymphocytes	X	X	X
○ OX40 Signaling Pathway	X		X
○ PI3K Signaling in B Lymphocytes	X	X	X
○ PKCθ Signaling in T Lymphocytes	X	X	X
○ Primary Immunodeficiency Signaling			X
○ Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	X	X	X
○ Role of NFAT in Regulation of the Immune Response	X	X	X
○ Role of PKR in Interferon Induction and Antiviral Response	X	X	X
○ Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	X	X	X
○ Role of RIG1-like Receptors in Antiviral Innate Immunity	X	X	X
○ T Cell Receptor Signaling	X	X	X
○ T Helper Cell Differentiation			X
○ Toll-like Receptor Signaling	X	X	X
○ Tumoricidal Function of Hepatic Natural Killer Cells	X		X
○ fMLP Signaling in Neutrophils	X	X	X
○ iCOS-iCOSL Signaling in T Helper Cells	X	X	X
○ p38 MAPK Signaling	X	X	
• Cellular Stress and Injury			
○ DNA Double-Strand Break Repair by Non-Homologous End Joining	X		X
○ HMGB1 Signaling	X	X	X
○ Intrinsic Prothrombin Activation Pathway	X	X	
○ Mismatch Repair in Eukaryotes		X	
○ Nrf2-mediated Oxidative Stress Response	X	X	X
○ Nucleotide Excision Repair Pathway			X
○ Regulation of eIF4 and p70S6K Signaling	X	X	X
○ Role of BRCA1 in DNA Damage Response	X	X	X
○ Role of CHK Proteins in Cell Cycle Cehckpoint Control		X	X
○ Type II Diabetes Mellitus Signaling	X	X	X
○ UVA-Induced MAPK Signaling	X		X
○ UVB-Induced MAPK Signaling	X	X	
○ UVC-Induced MAPK Signaling	X	X	X
○ p38 MAPK Signaling	X	X	X
○ p70S6K Signaling	X	X	X
• Cytokine Signaling			
○ Acute Phase Response Signaling	X	X	X
○ CCR5 Signaling in Macrophages	X	X	X

○ CNTF Signaling	X	X	X
○ CXCR4 Signaling	X	X	X
○ Chemokine Signaling	X		
○ Dendritic Cell Maturation	X	X	X
○ Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	X		
○ Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	X		
○ FLT3 Signaling in Hematopoietic Progenitor Cells	X	X	X
○ GM-CSF Signaling	X	X	X
○ HMGB1 Signaling	X	X	X
○ IL-12 Signaling and Production in Macrophages	X	X	X
○ IL-15 Production	X	X	X
○ IL-17 Signaling	X		
○ IL-17A Signaling in Gastric Cells	X		
○ IL-2 Signaling	X	X	X
○ IL-22 Signaling	X		X
○ IL-3 Signaling	X	X	X
○ IL-4 Signaling	X		X
○ IL-6 Signaling	X	X	X
○ IL-8 Signaling	X	X	X
○ IL-9 Signaling	X		X
○ Interferon Signaling	X		X
○ NF-κB Signaling	X	X	X
○ Oncostatin M Signaling	X		X
○ Prolactin Signaling	X	X	X
○ Role of IL-17A in Arthritis	X		
○ Role of JAK family kinases in IL-6-type Cytokine Signaling	X	X	X
○ Role of JAK1 and JAK3 in γc Cytokine Signaling	X	X	X
○ Role of JAK2 in Hormone-like Cytokine Signaling	X	X	X
○ T Helper Cell Differentiation			X
○ TNFR2 Signaling	X	X	
○ TWEAK Signaling	X	X	
○ fMLP Signaling in Neutrophils	X	X	X
○ p38 MAPK Signaling	X	X	X
• Disease-Specific Pathways			
○ Acute Myeloid Leukemia Signaling	X		X
○ Allograft Rejection Signaling	X		X
○ Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	X	X	X
○ Autoimmune Thyroid Disease Signaling	X		X
○ Bladder Cancer Signaling	X		X
○ Chronic Myeloid Leukemia Signaling	X	X	X
○ Colorectal Cancer Metastasis Signaling	X	X	X
○ Endometrial Cancer Signaling	X		X
○ Glioblastoma Multiforme Signaling	X	X	X
○ Glioma Invasiveness Signaling	X	X	X
○ Glioma Signaling	X	X	X

○ Graft-versus-Host Disease Signaling	X		X
○ HER-2 Signaling in Breast Cancer	X	X	X
○ Hepatic Cholestasis	X	X	X
○ Hepatic Fibrosis / Hepatic Stellate Cell Activation	X	X	X
○ Induction of Apoptosis by HIV1	X	X	
○ Mitochondrial Dysfunction	X		X
○ Neuroprotective Role of THOP1 in Alzheimer's Disease	X		
○ Non-Small Cell Lung Cancer Signaling	X		X
○ Pathogenesis of Multiple Sclerosis	X		X
○ Primary Immunodeficiency Signaling			X
○ Prostate Cancer Signaling	X	X	X
○ RANK Signaling in Osteoclasts	X	X	
○ Renal Cell Carcinoma Signaling	X	X	X
○ Role of IL-17A in Arthritis	X		
○ Role of MAPK Signaling in the Pathogenesis of Influenza	X		
○ Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	X		
○ Small Cell Lung Cancer Signaling	X	X	X
○ Systemic Lupus Erythematosus Signaling	X		X
○ Thyroid Cancer Signaling	X		X
○ Type I Diabetes Mellitus Signaling	X	X	X
○ Type II Diabetes Mellitus Signaling	X	X	X
• Growth Factor Signaling			
○ Angiopoietin Signaling	X	X	X
○ ErbB Signaling	X	X	
○ ErbB2-ErbB3 Signaling	X		X
○ ErbB4 Signaling	X	X	X
○ GDNF Family Ligand-Receptor Interactions	X	X	
○ GM-CSF Signaling	X	X	X
○ Growth Hormone Signaling	X	X	X
○ HGF Signaling	X	X	X
○ IGF-1 Signaling	X	X	X
○ NGF Signaling	X	X	X
○ Neurotrophin/TRK Signaling	X	X	X
○ PDGF Signaling	X		X
○ Renin-Angiotensin Signaling	X	X	X
• Humoral Immune Response			
○ Antigen Presentation Pathway	X		X
○ Autoimmune Thyroid Disease Signaling	X		X
○ B Cell Activating Factor Signaling	X	X	
○ B Cell Development			X
○ B Cell Receptor Signaling	X	X	X
○ CD40 Signaling	X	X	
○ Complement System			X
○ Fc Epsilon RI Signaling	X	X	X
○ FcγRIIB Signaling in B Lymphocytes	X		
○ HMGB1 Signaling	X	X	X
○ IL-4 Signaling	X		X

○ NF-κB Signaling	X	X	X
○ Primary Immunodeficiency Signaling			X
○ Role of NFAT in Regulation of the Immune Response	X	X	X
○ Toll-like Receptor Signaling	X	X	X
○ p38 MAPK Signaling	X	X	X
• Ingenuity Toxicity List Pathways			
○ Acute Phase Response Signaling	X	X	X
○ Apoptosis Signaling	X	X	X
○ Aryl Hydrocarbon Receptor Signaling	X	X	X
○ Cell Cycle: G1/S Checkpoint Regulation	X		X
○ Cell Cycle: G2/M DNA Damage Checkpoint Regulation	X		X
○ FXR/RXR Activation	X		X
○ Hepatic Cholestasis	X	X	X
○ Hepatic Fibrosis / Hepatic Stellate Cell Activation	X	X	X
○ LPS/IL-1 Mediated Inhibition of RXR Function	X	X	X
○ LXR/RXR Activation	X	X	X
○ Mitochondrial Dysfunction	X		X
○ NF-κB Signaling	X	X	X
○ Nrf2-mediated Oxidative Stress Response	X	X	X
○ PPARα/RXRα Activation	X	X	X
○ RAR Activation	X	X	X
○ TR/RXR Activation	X		X
○ Xenobiotic Metabolism Signaling	X	X	X
○ p53 Signaling	X	X	X
• Intracellular and Second Messenger Signaling			
○ AMPK Signaling	X	X	X
○ Actin Nucleation by ARP-WASP Complex	X	X	X
○ Docosahexaenoic Acid (DHA) Signaling	X		X
○ ERK/MAPK Signaling	X	X	X
○ ERK5 Signaling	X	X	X
○ Gαq Signaling	X	X	X
○ Integrin Signaling	X	X	X
○ JAK/Stat Signaling	X	X	X
○ PI3K/AKT Signaling	X	X	X
○ Phospholipase C Signaling	X	X	X
○ Protein Ubiquitination Pathway	X		X
○ Rac Signaling	X	X	
○ Regulation of eIF4 and p70S6K Signaling	X	X	X
○ Role of NFAT in Regulation of the Immune Response	X	X	X
○ Signaling by Rho Family GTPases	X	X	X
○ Sphingosine-1-phosphate Signaling	X	X	X
○ Tec Kinase Signaling	X	X	X
○ p38 MAPK Signaling	X	X	X
• Neurotransmitters and Other Nervous System Signaling			
○ Agrin Interactions at Neuromuscular Junction	X		X
○ CNTF Signaling	X	X	X
○ Cholecystokinin/Gastrin-mediated Signaling	X	X	X

○ Docosahexaenoic Acid (DHA) Signaling	X		X
○ Ephrin A Signaling	X	X	X
○ ErbB Signaling	X	X	
○ ErbB2-ErbB3 Signaling	X		X
○ ErbB4 Signaling	X	X	
○ GDNF Family Ligand-Receptor Interactions	X	X	
○ NGF Signaling	X	X	X
○ Netrin Signaling		X	X
○ Neurotrophin/TRK Signaling	X	X	X
○ Reelin Signaling in Neurons	X		X
○ Regulation of Actin-based Motility by Rho	X	X	X
○ Semaphorin Signaling in Neurons	X	X	X
• Nuclear Receptor Signaling			
○ Aldosterone Signaling in Epithelial Cells	X	X	X
○ Aryl Hydrocarbon Receptor Signaling	X	X	X
○ Estrogen-mediated S-phase Entry			X
○ FXR/RXR Activation	X		X
○ LPS/IL-1 Mediated Inhibition of RXR Function	X	X	X
○ LXR/RXR Activation	X	X	X
○ Nur77 Signaling in T Lymphocytes	X	X	X
○ PPARα/RXRα Activation	X	X	X
○ RAR Activation	X	X	X
○ TR/RXR Activation	X		X
• Organismal Growth and Development			
○ BMP signaling pathway	X	X	
○ Caveolar-mediated Endocytosis Signaling	X	X	X
○ Chemokine Signaling	X		
○ Ephrin A Signaling	X	X	X
○ HGF Signaling	X	X	X
○ Hematopoiesis from Multipotent Stem Cells		X	
○ Hematopoiesis from Pluripotent Stem Cells		X	X
○ Inhibition of Matrix Metalloproteases	X		X
○ Macropinocytosis Signaling	X	X	X
○ Mouse Embryonic Stem Cell Pluripotency	X	X	X
○ NF-κB Signaling	X	X	X
○ Netrin Signaling		X	X
○ Paxillin Signaling	X	X	X
○ Prolactin Signaling	X	X	X
○ Reelin Signaling in Neurons	X		X
○ Regulation of the Epithelial-Mesenchymal Transition Pathway	X	X	X
• Pathogen-Influenced Signaling			
○ Caveolar-mediated Endocytosis Signaling	X	X	X
○ Dendritic Cell Maturation	X	X	X
○ IL-17 Signaling	X		
○ LPS-stimulated MAPK Signaling	X	X	
○ LPS/IL-1 Mediated Inhibition of RXR Function	X	X	X
○ NF-κB Activation by Viruses	X	X	X

○ Role of MAPK Signaling in the Pathogenesis of Influenza	X		
○ Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	X		
○ Role of PKR in Interferon Induction and Antiviral Response	X	X	X
○ Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	X	X	X
○ Role of RIG1-like Receptors in Antiviral Innate Immunity	X	X	X
○ Toll-like Receptor Signaling	X	X	X
○ Virus Entry via Endocytic Pathways	X	X	X
• Transcriptional Regulation			
○ Assembly of RNA Polymerase I Complex			X
○ Assembly of RNA Polymerase III Complex	X		
• Xenobiotic Metabolism			
○ Aryl Hydrocarbon Receptor Signaling	X	X	X
○ Xenobiotic Metabolism Signaling	X	X	X

Abbreviations: V = virgin olive oil; S = sunflower oil; F = fish oil.

ANNEX VI. Canonical pathways from the "Uncommon Genes" worklist whose –log (*P*-value) is higher than the threshold, identifying the genes involved to each fat (virgin olive, sunflower or fish oil).

	VIRGIN OLIVE OIL			SUNFLOWER OIL			FISH OIL		
Ingenuity canonical pathways	-log(*P*-value)[1]	Ratio[2]	Genes	-log(*P*-value)[1]	Ratio[2]	Genes	-log(*P*-value)[1]	Ratio[2]	Genes
Mitochondrial dysfunction	2,73	5,91 E-02	NDUFA9,APH1B,ATP5D,NDUFS7,GPD2,SURF1,NDUFS2,MAPK9,ATP5G2,UQCRC1,NDUFS3				7,88	1,02 E-01	SDHB,NDUFB3,NDUFA9,ATP5D,UQCRH,UQCR11,NDUFS7,PRDX5,SURF1,ATP5G2,NDUFS3,FIS1,NDUFA6,TXN2,NDUFB7,NDUFS2,CYC1,UQCRC1,NDUFA8
Antigen presentation pathway							4,28	1,5 E-01	HLA-DRA,HLA-DQA1,HLA-DRB1,CD74,TAP1,PSMB6
Cell cycle regulation by BTG family proteins				3,73	1,11 E-01	PPP2CA,BTG1,PPP2R5E,PPP2R1B	1,89	1,11 E-01	RB1,PPP2R5E,PPP2R1B,CCND1
Nrf2-mediated oxidative stress response	3,44	7,29 E-02	AKR7A2,MAP2K6,RRAS,NQO1,PIK3C2G,MAPK9,DNAJA1,CLPP,TXNRD1,GSTO1,MGST2,KEAP1,VCP,UBE2E3						

B Cell development							3,16	1,21 E-01	PTPRC,HLA-DRA,HLA-DQA1,HLA-DRB1
ERK/MAPK signaling				3,09	3,4 E-02	PRKCI,ATF1,PPP2CA,DUSP1,PPP2R5E,PPP2R1B,EIF4E			
Autoimmune thyroid disease signaling							2	6,56 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1
Role of CHK proteins in cell cycle checkpoint control				2,98	7,02 E-02	PCNA,PPP2CA,PPP2R5E,PPP2R1B			
Retinoate biosynthesis I	1,37	8,11 E-02	ADH7,RDH10,RDH5	2,72	8,11 E-02	ADH7,RDH10,RDH5			
Activation of IRF by cytosolic pattern recognition receptors	2,6	8,33 E-02	IRF7,MAPK9,IRF9,IRF3,IFIT2,ISG15	1,52	2,4 E-02	PPP2CA,CPT2,PPP2R5E,PPP2R1B			
Glycine betaine degradation	1,54	8,7 E-02	SDS,SDSL				2,68	1,3 E-01	SDS,SHMT2,SDSL
Dopamine-DARPP32 reedback in cAMP signaling				2,5	3,28 E-02	PRKCI,ATF1,PPP2CA,PPP2R5E,PPP2R1B,PPP3CA			
Glycerol degradation I	2,57	1,67 E-01	GPD2,GK						

L-serine degradation	2,57	3,33 E-01	SDS,SDSL				2,56	3,33 E-01	SDS,SDSL
Production of nitric oxide and reactive oxygen species in macrophages				2,46	2,84 E-02	PRKCI,RHOQ,PPP2CA,CHUK,PPP2R5E,PPP2R1B			
PI3K/AKT signaling				2,43	3,47 E-02	PPP2CA,CHUK,PPP2R5E,PPP2R1B,EIF4E			
DNA double-strand break repair by non-homologous end joining	2,42	1,58 E-01	XRCC6,XRCC5,XRCC1				1,37	1,05 E-01	XRCC6,XRCC1
mTOR signaling				2,35	2,84 E-02	PRKCI,RHOQ,PPP2CA,PPP2R5E,PPP2R1B,EIF4E			
Telomere extension by telomerase	2,2	1,76 E-01	XRCC6,HNRNPA2B1,XRCC5						
CTLA4 signaling in cytotoxic T lymphocytes				2,27	4,08 E-02	PTPN11,PPP2CA,PPP2R5E,PPP2R1B	2,16	7,14 E-02	PTPN11,HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1,PPP2R5E,PPP2R1B
Xenobiotic metabolism signaling	1,72	4,35 E-02	MAP2K6,ALDH4A1,Ces1c,RRAS,NQO1,PIK3C2G,MAPK9,GSTO1,PNPLA7,MGST2,KEAP1,RXRA,ALDH16A1	2,23	2,34 E-02	Ces1c,PRKCI,MGST2,PPP2CA,PPP2R5E,ALDH16A1,PPP2R1B			

The visual cycle				2,17	7,14 E-02	RDH10,RDH5			
Tight junction signaling				2,05	3,11 E-02	PRKCI,PPP2CA, PPP2R5E,PPP2R 1B,CLDN3			
Graft-versus-host disease signaling							1,95	8 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1
D-myo-inositol (1,4,5,6)-tetrakisphosphate biosynthesis				1,8	2,86 E-02	PTPN11,DUSP1, PPP2R5E,PPP3C A			
D-myo-inositol (3,4,5,6)-tetrakisphosphate biosynthesis				1,8	2,86 E-02	PTPN11,DUSP1, PPP2R5E,PPP3C A			
Type II diabetes mellitus signaling				1,8	2,48 E-02	PRKCI,CD36,CH UK,ADIPOR2			
Arginine degradation VI (arginase 2 pathway)							1,89	1,25 E-01	PYCRL,ARG1
Role of JAK2 in hormone-like cytokine signaling				1,47	5,56 E-02	PTPN11,PRLR	1,89	1,11 E-01	GHR,PTPN11,PRLR,STA T1
Aryl hydrocarbon receptor signaling	1,86	5,59 E-02	ALDH4A1,CTSD, NFIX,MGST2,NQ O1,BAX,ALDH1 6A1,RXRA,GST O1						

Allograft rejection signaling				1,84	4,35 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1
RAR activation	1,77	2,65 E-02	ADH7,PRKCI,RDH10,DUSP1,RDH5			
Mitotic roles of polo-like kinase	1,74	4,29 E-02	PPP2CA,PPP2R5E,PPP2R1B			
ILK signaling				1,73	5,21 E-02	RND2,MYC,ITGB2,RHOQ,TMSB10/TMSB4X,RHOB,VIM,PPP2R5E,PPP2R1B,CCND1
Asparagine degradation I	1,72	2 E-01	ASPG			
Protein ubiquitination pathway				1,72	4,85 E-02	PSMA7,DNAJC15,USP2,TAP1,PSMB6,FZR1,PSMB7,HSCB,DNAJC4,STUB1,PSMB1,UBE2J1,USP25
T Helper cell differentiation				1,71	6,94 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1,STAT1
D-myo-inositol-5-phosphate metabolism	1,6	2,55 E-02	PTPN11,DUSP1,PPP2R5E,PPP3CA			
Estrogen-mediated S-phase entry				1,6	1,07 E-01	MYC,RB1,CCND1
p70S6K signaling	1,68	3,1 E-02	PRKCI,PPP2CA,PPP2R5E,PPP2R1B			

TCA cycle II (eukaryotic)	1,67	7,32 E-02	ACO2,DLST,FH				1,65	7,32 E-02	SDHB,IDH3G,DLST
Cytotoxic T lymphocyte-mediated apoptosis of target cells							1,66	4,88 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1
Myc mediated apoptosis signaling	1,66	8,33 E-02	RRAS,YWHAZ,PIK3C2G,MAPK9,BAX						
Pathogenesis of multiple sclerosis	1,64	2,22 E-01	CXCL9,CCL5						
PI3K signaling in B lymphocytes				1,64	2,86 E-02	PRKCI,ATF1,CHUK,PPP3CA			
Citrulline biosynthesis							1,63	7,69 E-02	PRODH,ARG1
NAD phosphorylation and dephosphorylation							1,63	1,18 E-01	ACP2,ACP6
Role of MAPK signaling in the pathogenesis of influenza	1,63	7,25 E-02	MAP2K6,RRAS,MAPK9,BAX,CCL5						
Glutathione-mediated detoxification	1,5	6,82 E-02	MGST2,GGH,GSTO1						
3-phosphoinositide degradation				1,58	2,53 E-02	PTPN11,DUSP1,PPP2R5E,PPP3C			

						A
Insulin receptor signaling				1,57	2,82 E-02	PRKCI,RHOQ,PTPN11,EIF4E
Dopamine receptor signaling				1,56	3,19 E-02	PPP2CA,PPP2R5E,PPP2R1B
Prolactin signaling				1,56	3,75 E-02	PRKCI,PTPN11,PRLR
Cyclins and cell cycle regulation				1,54	3,37 E-02	PPP2CA,PPP2R5E,PPP2R1B
Fatty Acid β-oxidation III (unsaturated, odd number)				1,54	2 E-01	ECI2
α-tocopherol degradation				1,54	1 E-01	CYP4F3
3-phosphoinositide biosynthesis				1,52	2,27 E-02	PTPN11,DUSP1,PPP2R5E,PPP3CA
Ceramide signaling				1,52	3,37 E-02	PPP2CA,PPP2R5E,PPP2R1B
Glutamate removal from folates	1,52	3,33 E-01	GGH			
Histamine biosynthesis	1,52	3,33 E-01	HDC			

Lipoate biosynthesis and incorporation II	1,52	1 E-01	LIAS				1,51	1 E-01	LIAS
Synaptic long term depression				1,52	2,52 E-02	PRKCI,PPP2CA, PPP2R5E,PPP2R 1B			
Phosphatidylethano lamine biosynthesis III							1,51	2 E-01	PTDSS2
Regulation of eIF4 and p70S6K signaling				1,51	2,29 E-02	PPP2CA,PPP2R5 E,PPP2R1B,EIF4 E			
OX40 signaling pathway							1,47	4,4 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1
HMGB1 signaling	1,46	6,06 E-02	MAP2K6,RHOB, RRAS,RHOA,PIK 3C2G,MAPK9						
Retinol biosynthesis				1,45	3,45 E-02	Ces1c,RDH10			
Gαq signaling				1,44	2,38 E-02	PRKCI,RHOQ,C HUK,PPP3CA			
NAD biosynthesis II (from tryptophan)							1,44	5,88 E-02	NADSYN1,HAAO
Nur77 signaling in T lymphocytes							1,44	6,35 E-02	HLA-DRA,HLA-DQA1,FCER1G,HLA-DRB1

Pentose phosphate pathway (non-oxidative branch)				1,42	7,69 E-02	RPIA			
Protein kinase A signaling				1,42	1,76 E-02	PRKCI,DUSP3,ATF1,PTPN11,DUSP1,CHUK,PPP3CA			
Semaphorin signaling in neurons							1,41	7,69 E-02	RND2,DPYSL2,RHOQ,RHOB
Assembly of RNA polymerase III complex	1,3	1,25 E-01	GTF3C1,GTF3C2						
LPS-stimulated MAPK signaling	1,3	6,1 E-02	MAP2K6,ATF1,RRAS,PIK3C2G,MAPK9	1,59	3,66 E-02	PRKCI,ATF1,CHUK			
Methylglyoxal degradation III	1,3	8,7 E-02	AKR7A2,Akr1b7						
CDK5 signaling				1,39	3,19 E-02	PPP2CA,PPP2R5E,PPP2R1B			
Germ cell-Sertoli cell junction signaling	1,39	4,88 E-02	MAP2K6,RHOB,RRAS,KEAP1,RHOA,TUBB2A,PIK3C2G,MAPK9						
Netrin signaling				1,35	3,51 E-02	UNC5B,PPP3CA			

NAD biosynthesis from 2-amino-3-carboxymuconate semialdehyde				1,33	5,88 E-02	NADSYN1			
Role of IL-17A in arthritis	1,33	6,35 E-02	MAP2K6,PIK3C2G,MAPK9,CCL5						
Interferon signaling	1,32	8,33 E-02	MX1,IRF9,BAX				1,31	8,33 E-02	IFITM1,STAT1,TAP1

[1] *P* value refers the probability that a canonical pathway be affected by our treatment, and it's calculated using Fischer's exact test determining the probability that the association between the genes in the data set and the canonical pathway is due to chance alone.
[2] Ratio gives an idea of the percentage of genes in a pathway that were also found in our uploaded list. It's calculated as the number of focus genes that map to the canonical pathway divided by the total number of genes that map to the canonical pathway.