Universitat de Lleida

Polyunsaturated fatty acids in amyotrophic lateral sclerosis: role of DHA, peroxidative modifications and sexual dimorphism

Daniel Cacabelos Barral

Dipòsit Legal: L.153-2015
http://hdl.handle.net/10803/285530

POLYUNSATURATED FATTY ACIDS IN AMYOTROPHIC LATERAL SCLEROSIS

ROLE OF DHA, PEROXIDATIVE MODIFICATIONS AND SEXUAL DIMORPHISM

by

Daniel Cacabelos Barral

October, 2014

Universitat de Lleida
Departament de Medicina Experimental

Manel Portero Otin, MD, PhD, Titular Professor of the University of Lleida at the Department of Experimental Medicine

Reinald Pamplona Gras, MD, PhD, Full Professor of the University of Lleida at the department of Experimental Medicine

CERTIFY:

That the work presented here, entitled **"Polyunsaturated Fatty Acids in Amyotrophic Lateral Sclerosis: Role of DHA, Peroxidative Modifications and Sexual Dimorphism"** was developed by Daniel Cacabelos Barral, B.Sc. Chem., under our supervision and was aimed to fulfil the requirements for the degree of Doctor at the University of Lleida following the PhD Program entitled "Estudis Avançats en Ciències Biomèdiques".

Hereby, to confirm this, we sign in Lleida, September 23rd, 2014

Dr. Manel Portero Otín Dr. Reinal Pamplona Gras

Financial support by the Spanish Ministry of Health (PI08-1843, PI11-01532 and PI13-00584) by the Catalonian Government (2009SGR-735) and by the Spanish Ministry of Science and Innovation (CENIT Program; BFU2009-11879/BFI, and AGL2006-1243). DC received a predoctoral fellowship from the Spanish Ministry of Health(PI08-00707).

ABSTRACT

In the present work we focus into the potential relevance of PUFAs in some models and human samples from patients suffering amyotrophic lateral sclerosis (ALS). Due to its pathological implication, oxidative stress was our first goal. We started from simple oxidative methodology screening to search for an antioxidant substance (among 21 different candidates) available in a Mediterranean diet. The results demonstrated high heterogeneity in carbonyl (measured by DNP) accumulation, regarding the oxidative source, substrate suffering it and the antioxidant structure. Further, thanks to GC/MS and LCQTOF, we detailed the protection over specific accrual of protein and lipid peroxidation markers as well as lipid profile modifications (as % of total fatty acids -FA) in oxLDL thanks to those dietary compounds. Moreover, we demonstrated its *in vitro* relevance, in terms of survival, when two cell lines (HMEC-1, HepG2) were treated with this oxidized (and protected) compounds, and finally address *in vivo* importance of those findings, demonstrating decreased carbonyl and oxidative accumulation in hamsters under an atherogenic diet supplemented with antioxidants. Once described the protective effect of antioxidants and specific signatures found regarding lipid oxidation markers, we extend the study focusing in different ALS samples. From previous work, we demonstrated an altered docosohexaenoic acid (DHA) composition in different location for patients suffering sporadic ALS. Hence, we though necessary to define whether the enzymatic machinery aimed to synthesize DHA from its precursors, are affected in sALS. Interestingly, we found a tissue specific variation (spinal cord *vs* cortex), compatible with our previous FA results. Further, thanks to inmunohistochemistry, differential involvement was unveiled for motor neurons (MN) and surrounding glia. Therefore, trying to depict cellular contribution, we switch to a neuronal model (N2A under oxidative stressors and/or aggregation-prone-TDP-43) and a tissular one (OT). There, we showed decreased desaturase (Δ6) and drebrin expression as well as increased DHA synthesis and an unreported inverse correlation of drebrin loss and aberrant p-TDP-43 expression under oxidative conditions in the cell culture. In the OT model, lipidomic analysis showed specific accretion of 8-iso-$PGF_{2\alpha}$ and NPD1 as well as increased DHA (and dramatically decreased precursors) and reduced AA concentrations (GC measured). Analysis of slice O_2 consumption showed decreased O_2 levels under excitotoxic treatment and alleviation by antioxidant (tocopherol) addition. Treatment of OT slices with Ω-3 precursors (better than final products) and DHA plus tocopherol ameliorated MNs number. Finally, we wanted to disclose PUFA's implication and phenothype of an animal model (SODG93A) under a dietary intervention with opposed FA unsaturation levels. Not surprisingly, FA profile was difficult to be altered in nervous system, although subtle specific variations were found. More importantly, differences in survival and clinical manifestations, UPR (Ubiquitin inclusions), mt-DNA (8-oxo-dG) and protein oxidative modifications revealed sex as a relevant factor in lipid handling for this model. Hence, whereas male under a low PUFA diet showed increased survival, females lack this beneficial outcome. Last but not least, we wanted to dig deeper regarding this sexual dimorphism. For this purpose, we focused in mitochondria and analyzed spinal cord oxygen consumption, oxidative damage to proteins and lipid profile along disease progression and also in a neuronal model (N2A overexpressing SODG93A, treated with 17β-estradiol).We could demonstrate a clear sexual implication, with females having late onset clinical symptoms concomitant to an upgraded mitochondrial function and lower protein and mitochondrial damaged proteins compared with males. Finally, to further confirmed steroid potential as a protective element in disease progression, *in vitro* estradiol pretreatmet of N2A showed increased oxygen consumption, with no relation with the mitochondrial complex expression.

RESUMEN

En el trabajo que aquí se presenta se ha profundizado en la relevancia que los ácidos grasos poliinsaturados (PUFA) puedan tener en el tratamiento de la esclerosis lateral amiotrófica (ALS). Dada su implicación en el desarrollo de la patología, el estudio del estrés oxidativo asociado fue uno de nuestros primeros objetivos. Para ello hemos empezado por un cribado metodológico simplista, tratando de encontrar una sustancia antioxidante (entre 21), biodisponible en una dieta Mediterránea equilibrada y que fuese capaz de reducir un daño oxidativo generado desde diversos frentes (medido como acumulación de carbonilos) y sobre diferentes substratos. Los resultados demostraron una alta heterogeneidad, dificultando así la elección de un único antioxidante. Aún así, gracias a la GCMS y la LCQTOF pudimos detallar la acumulación específica diferenciada de marcadores de daño oxidativo proteico y daño lipoxidativo producido cuando partículas de lipoproteínas de baja densidad (LDL) son oxidadas bajo la acción de diversos compuestos. Además se pudo objetivar el cambio en la composición lipídica de estas LDL (medida como % del total presente) y su relevancia biológica *in vitro*, medida en términos de supervivencia, cuando se exponen a dos líneas celulares (HMEC-1, HepG2). Por último se demostró la importancia in vivo, puesto que se pudo observar una menor acumulación de productos carbonílicos en hámsters alimentados con una dieta aterogénica, pero suplementada con antioxidantes. Una vez se demostró el papel jugado por estos antioxidantes en la acumulación diferenciada de productos de oxidación, extendimos el estudio a muestras y modelos de ALS. En trabajos previos habíamos evidenciado una composición tisular diferenciada en diversas localizaciones del sistema nervioso en pacientes diagnosticados de ALS. Por ello consideramos interesante el estudio de la expresión de la maquinaria enzimática necesaria para la síntesis lipídica. De un modo destacado, pudimos ver de nuevo una variación tisular, compatible con niveles reducidos de docosohexaenoico (DHA), y gracias a la inmunohistoquímica también se observaron diferencias entre las motoneuronas (MNs) y la glia circundante. Así pues, para poder revelar las aportaciones diferenciales de los distintos tipos celulares, utilizamos una línea celular (N2A) a la que sometíamos a diferentes estresores (daño oxidativo y sobreexpresión de una versión de TPD-43 que causa agregados) y a un cultivo tisular de médula espinal (OT), dónde se produce una muerta progresiva y selectiva de las MNs. Tras estos experimentos, observamos un descenso tanto en la expresión de la Δ6 desaturasa como de drebrin (marcador presináptico) tras la sobreexpresión de TDP-43, así como una mayor síntesis de DHA y una correlación inversa entre la pérdida de drebrina y la expresión de pTDP-43 bajo condiciones de estrés oxidativo. Por otro lado, en el modelo OT, el análisis lipidómico reveló la acumulación especifica de 8-iso-$PGF_{2\alpha}$ y NDPD1 (posiblemente en respuesta a un incremento del daño oxidativo) así como el aumento en la concentración de DHA (con un descenso muy marcado de sus precursores) y el descenso de araquidónico. Quisimos analizar también el consumo de oxígeno, tanto en tejido intacto como permeabilizado, pudiendo observar como la excitoxicidad reducía considerablemente su capacidad y como ésta era, en parte, rescatada con el uso de tocoferol. Además, el tratamiento del OT con precursores Ω-3 mejoró también el número de MNs. Por último, quisimos caracterizar la implicación que los PUFA dietarios podrían tener en un modelo animal bien conocido (SODG93A). No fue sorprendente encontrar que el perfil lipídico en el sistema nervioso fue muy difícil de alterar. Aún así, se observaron diferencias en supervivencia, en el devenir clínico, la respuesta UPR (con acumulaciones de Ub), el daño al DNA mitocondrial (8-oxo-dG) y modificaciones oxidativas en las proteínas y cómo éstas tenían un grado de afectación diferencial cuando considerábamos el sexo de los animales. Esto es, mientras que los machos sometidos a una dieta baja en PUFAs de cadena larga demostraron una mayor supervivencia, en las hembras no se

apreció mejoría. Por último, pero no menos importante, quisimos profundizar más respecto a este dimorfismo. Centrándonos en la mitocondria, pudimos hacer un seguimiento del consumo de oxígeno a lo largo de la enfermedad, el daño oxidativo a proteínas y el perfil lipídico. Por lo tanto pudimos demostrar una clara implicación sexual, siendo las hembras las que más tarde comienzan su manifestación clínica, con mejores funciones mitocondriales asociadas a un menor daño oxidativo. Finalmente, la relevancia del papel protector de los estrógenos se pudo comprobar *in vitro,* mediante el pretratamiento con 17β-estradiol en la línea N2A que sobreexpresa SOD1G93A, relacionado con la ALS familiar, proponiéndose el estradiol como un nuevo elemento que juega un papel relevante en el desarrollo de la enfermedad.

RESUM

En aquest treball s'ha intentat profunditzar en la possible rellevància dels àcids grassos poliinsaturats (PUFA) en el tractament de l'esclerosi lateral amiotròfica (ELA). Atesa la seva implicació en el desenvolupament de la patologia, l'estudi de l'estrès oxidatiu associat fou un dels primers objectius. Per això, es va començar amb un cribratge metodològic simplista, intentant trobar una substància antioxidant biodisponible en una dieta mediterrània equilibrada i que fos capaç de reduir el dany oxidatiu generat des de diferents fronts i sobre diferents substrats. Els resultats van demostrar una alta heterogeneïtat, dificultant així l'elecció d'un únic antioxidant. Malgrat això, mercès a tècniques de GC-MS i LC-QTOF, es va poder detallar l'acumulació específica diferenciada de marcadors de dany oxidatiu proteic i dany lipoxidatiu produït quan partícules de lipoproteïna de baixa densitat (LDL) s'oxiden per l'acció de diversos compostos. A més, es va poder objectivar el canvi en la composició lipídica d'aquestes LDL i la seva rellevància in vitro, mesurada en termes de supervivència, quan es cocultiven amb les línies cel·lulars. Per últim, es va demostrar la importància in vivo, atès que es va observar una menor acumulació de productes carbonílics en hàmsters alimentats amb una dieta aterogènica suplementada amb antioxidants. Un cop es va demostrar el paper d'aquests antioxidants en l'acumulació diferenciada de productes d'oxidació, es va estendre l'estudi a mostres i models d'ELA. En treballs previs s'havia evidenciat una composició tissular diferenciada en diverses localitzacions del sistema nerviós central en pacients d'ELA (respecte l'àcid docosahexaenoic (DHA), depleció en medul·la espinal i acumulació en còrtex). Per aquesta raó es va considerar interessant l'estudi de l'expressió de la maquinària enzimàtica necessària per la síntesi lipídica. D'una manera destacada, es va poder veure de nou una variació tissular, compatible amb nivells reduïts de DHA i, per tècniques d'immunohistoquímica, es van observar diferències entre les motoneurones i la glia circumdant. Per tant, per poder revelar les aportacions diferencials dels diferents tipus cel·lulars, es va utilitzar la línia cel·lular N2A, sotmesa a diferents estressos (dany oxidatiu i sobreexpressió d'una forma de TDP-43 que causa agregats) i un cultiu tissular de medul·la espinal, en el que es produeix una mort progressiva i selectiva de les motoneurones. Es va observar un descens en l'expressió de FADS2 i de drebrina, un marcador sinàptic, així com una major síntesi de DHA i una correlació inversa entre la pèrdua de drebrina i l'expressió de TDP-43 sota condicions d'estrès oxidatiu. Per altra banda, en el model organotípic, l'anàlisi lipidòmica va revelar l'acumulació específica de 8-isoPGF$_{2\alpha}$ i NDPD1, així com l'augment de la concentració de DHA i el descens motl marcat del seus precursors a mes del àcid araquidònic. Es va mesurar també el metabolisme oxidatiu, observant-se que l'excitotoxicitat reduïa considerablement la seva capacitat i, en part, es rescatava amb l'ús de tocoferol. A més, el tractament dels cultius organotípics amb precursors d'àcids grassos n-3 va millorar el nombre de motoneurones. Per últim, es va caracteritzar la implicació dels PUFA dietaris en un model animal d'ELA. Malgrat que el perfil lipídic del sistema nerviós central era difícil d'alterar, es van observar diferències en supervivència, fenotip clínic, resposta al malplegament de proteïnes (UPR) amb acumulació d'ubiquitina, dany en el DNA mitocondrial i modificacions oxidatives en les proteïnes, amb un grau d'afectació diferencial quan es considerava el sexe dels animals. En aquest sentit, mentre que els mascles sotmesos a una dieta baixa en PUFA de cadena llarga van mostra una major supervivència, en les femelles nomes va apreciar cap efecte millora. Per profunditzar en el dimorfisme sexual en ELA, i especialment la disfunció mitocondrial, es va analitzar mitjançant respirometria d'alta resolució la medul·la espinal durant tot el desenvolupament de la malaltia. Es va revelar una clara diferència de gènere, amb una manifestació clínica més tardana en femelles que correlaciona amb una millor conservació de la funció mitocondrial i un menor dany oxidatiu. El possible paper protector dels estròg ens es va

demostrar in vitro mitjançant el pretractament amb estradiol de cèl·lules N2A que sobreexpressen una forma de SOD1 humana mutada associada a l'ELA familiar (G93A-SOD1).

Os meus pais,
a miña irmá,
a minmesmo

Unas cuantas verdades que ya dijeron otros,…

La duda es la base de todo conocimiento.

Aristóteles

Mide lo que sea medible y haz medible lo que no lo sea.

Todas las verdades son fáciles de entender, una vez descubiertas.
El caso es descubrirlas.

Galileo Galilei

True ignorance is not the absence of knowledge, but the refusal to acquire it.

Science is always seeking, not a real discovery. It is continuous travelling for never arrival.

Karl Popper

Books permit us to voyage through time, to tap the wisdom of our ancestors.

There are many hypotheses in science that are wrong. That's perfectly alright; it's the aperture to finding out what's right. Science is a self-correcting process. To be accepted, new ideas must survive the most rigorous standards of evidence and scrutiny.

Carl Sagan

On the whole, human beings want to be good, but not too good and not quite all the time.

George Orwell

Cuando creíamos que teníamos todas las respuestas, de pronto,
cambiaron todas las preguntas.

Mario Benedetti

Existe al menos un rincón del universo que con toda seguridad puedes mejorar,
y eres tú mismo.

El secreto de la genialidad es el de conservar el espíritu del niño hasta la vejez, lo cual quiere decir nunca perder el entusiasmo.

Aldous Huxley

La religión explica los miedos del hombre, la ciencia descubre las verdades de la naturaleza, la ciencia es para valientes.

Anónimo

Agradecementos,

Well, bueno, ben, bè … Por fin me toca a mí!

Como es de rigor, pero además en este caso de verdad, me gustaría comenzar agradeciendo el calor y conocimientos que mis directores Manel y Reinald me han brindado, sin su apoyo esto hubiese sido imposible. Gracias Maestros!

Pero el grupo era una familia numerosa, muy extensa, por la que he ido viendo pasar mucha gente en estos años. Desde el principio, Mª Josep y Viki ya estabais por ahí, menudas charlas arreglamundos nos echamos en la mesada o en la cola del rancho por 2,12 leuros en el Santamaria, y los cafeses en el Kemdefer o en la Caseta… y cómo me olvidare de todo lo que aprendí para poder llevar la "colonia" de ratones, las inmmunos y el dichoso confocal, el OT y demás técnicas! También estaba ya por allí el "socio fundador" Joan Prat, recuerdo felizmente el día que encontramos una foto de los tres Manel, Reinald y él haciendo butifarras o que se yo… Muchos de mis compis ya son doctores, pero los comienzos conociendo la cuidad y sus recovecos nocturnos y descubriendo el mundo de la ciencia (y sus recovecos aún más oscuros ;) de la mano de Katia, Alba y Mariona son inolvidables. También andaban por allí el David y la Nuria -de aquellas todavía becaria de Calderó- y al poco llegaron la Saray, Mery y Jessica para darnos más apoyo, que buena falta hizo ;) . Pobre Jessica como la hacía sufrir con las bestias, pero bueno, le gustó … y se pudo quedar por el estabulario! Y al poco llego Jordi, siempre muy callado (pero por dentro se partía!), y tuve la suerte de compartir dos de sus pasiones (o tres si contamos el buen comer) a mitocondria y el monte, que tantas alegrías (!) me han dado. Y el Huget, Claro que si! Lo veía de prácticas con la Mª Angeles y lo fuimos llevando al lado (que no cuarto) oscuro fisiopato-ilógico… y cayó. Que pisazo teníamos… éramos la envidia del vecindario, jeje…días de vino y rosas!! Un abrazo para el Gus! Creo que fue mi segundo Octubre cuando apareció por aquí un cuate que nos conquistó con su saber hacer, buen humor y su "exo-estomago"; grande Chamaco! … Dulces recuerdos de congresos de médicos, a todo *Nu*-tren… historias preburbuja inmobiliaria y crisis…*snif*… DeLorenzo con la nutrigenomica por la casa y el Ribera con su radar! Y mientras iban viniendo gentes de estancias: el José y la Pilar de los madriles, el Jorge del grupo de Aurora Pujol (la Ione y el Stèphane después), la griega, el francés, los mexicanos (estos no pararon de llegar). Al ratín el Jose se pudo traer a la moza y vino una nueva pieza neuro- mitocondrial, la Ana y sus buenos embutidos de la querida extrema y dura… y como el Jose tenía debilidad, y era cosa de Annas (con una o dos enes), pues al poco también se vino p'acá la mejor Allmacellenca que conozco, *Uber Alles*! ;). Al tiempo, como ya habían pasado unos años sin "esclavos extranjeros", fichamos a un cubano, y cuando pensábamos encontrarnos a un macizo negrote, nos vino el Omarito "café con leche"! Pobre heredero de la "colonia" de gran corazón y mejor conversación! Unas buenas escapadas nos hemos hecho! Y con la Rosana y la Liliana se completó la cuadrilla, por ahora, quedando el grupo compuesto por médicos, biólogos /bioquímicos, químicos, farmacéuticos, nutricionistas tecnólogos alimentarios y hasta ingenieros, madremía que lío! Es pura suerte que nos entendamos! Y las nuevas generaciones pisan fuerte, la Rosana misma, anda que no le pega al cromatógrafo la tía!, y el Pascual y la Lara y quién sabe quién más pasaran por acá. Solo espero que puedan disfrutar y aprender a su costado, como yo he hecho.

Y las risas que nos echamos con los de BioQuímica!… No sé..., el hecho de estar tan cerca, ahí enfrente…pero lo suficientemente lejos también… Vero, Armando, Stef, MaAlbe y tod@s los becari@s de estancias que pasaron por allí, mimadriña! ... pero con todos congeniábamos… No fueron malos años, no! Myriam, no quedara muy bien, puesto que aun la tendrás que juzgar, pero que sepas que esoy encantado de los éxitos que te han brindado fuera, tú lo vales y lo mereces, animo! Lleida estará siempre aquí en nuestro corazón (y Álvaro también, jeje)...y que sepáis que el tipo del On the Rocks os hecha mucho de menos! Creo que Quim Ros me reservará un trocito de poyata, aunque también descansara al no verme tanto "revoloteando entre sus becarias". Yo ya lo echo de menos… Y las Rosas? Saludos a la Sta.

Decana, que me animo a calificar de amiga, Rosa Soler y las nuevas generaciones con AnaG y Nuria (y Rosa -la mujer del Sera y por supuesto de Roser y nuestros comienzos en el estabulario) y como no recordar als molt honorables presidents (Elia y David, herederos de una dinastía de justos representantes ;)) y los buenos momentos vividos (las grandes patxangas al futbol con Elia y los mano a mano de likor café con David… o era al revés) y mi compi de piso por unos meses, Saravanan, y Ambika… hay el idilio con la India … Indilio! El Bupesh abrió la brecha y anda que no ha habido éxito! Cuantas buenas gentes he podido conocer, que rica comida y cultura me brindaron, Hiren y Rinku, Charumathi, Arindan y Upasana, Depsheeka y Venki y Disha, un abrazo chavalada!

De la segunda planta recuerdo muy gustosamente a Sara, Xenia y Eli que la pelearon conmigo y pude disfrutar sus defensas de tesis, así como alguna que otra cenuki …, y al Dr Esquerda y Anna Casanovas que me ayudaron al poco de llegar con el microscopio y la perfusión de los pobres "voluntarios". Y del rock que se montan la Marta y Montse ni hablamos, no? Suerte que tienes Javi ;) . Del grupo de Loreta, con los que compartimos cuartucho algunos años, además del Bupesh no me quiero olvidar de Antonio y por supuesto de las mocicas: la rubia platino, compi hortolana y afines aficiones, Alba, y la morocha rizada albaceteña de profundos abrazos, que camina a Santiago más a menudo que yo, Bea, un beso a t'@s, maj@s!

De la tercera, como olvidar el grupo mindundi y las competis de disfraces de carnavales que nos hacíamos con los del Arnau!! Muchos ya se han ido (Martí y Carme, Rita, Marcelí, Galal o Seba), pero otros aun siguen por aquí (Paco-quillo!, Jordi, Eloy, Marta, Sonia o Neus C) han vuelto hace poco (Serafí and family, Neus P) y parece que no han pasado los años o refrescan con sangre nueva (Irene y Noel)… y el grupo de Carme Espinet, con Peter, Anton y Gerard, donde luego vino a parar el Egea con sus becarios… la Catherine (cuantas veces habre firmado en tu nombre, salerosa ^!^) la Mariajo (y sus melocotones-de comer-;)) y la Disha y, cómo no, la técnico mas viajera y con la que siempre puedes conversar, la Imma. Como olvidar la tarta que pasó tantos años en el arcón-congelador! (bueno si hubiese un historial de lo que allí ha habido, Dios!), poco más y se viene al IRB como uno más!

Este último año, ya recluido con la escritura de los papers y el ladrillo tesil, tuve la oportunidad de conocer a otra ilustre cubana, Idalmis, e intimar un poco más con Serafí, una suerte más que la vida me ha dado! Cuántas veces hemos desahogado las penas y arreglado el mundo con lixiviados germánicos fermentados por levaduras y destilados revolucionarios! Gracias majetes!

No puedo dejarme fuera a gente como Isu o Berta, y las buenas conversaciones que nos echamos en cultivos y a los currantes de secretaria de IRB. La gente de conserjería, sin duda a Emeterio, Fini, Trillo (el seleccionador) y María, Mónica y Montse los informáticos Josep y Toni y demás gentes de secretaría (David, Yolanda y Sandra), con sus buenos días y tardes siempre podías contar, y a veces hasta una sonrisa ;) Y por supuesto con el siempre excelentísimo e impecable servicio de limpieza, desde la Charo, Marcia o Daniela, pasando por Ani, Aisa y Loli, siempre estaban por ahí para amenizar cuando las tardes se hacían noches. Un gusto haberlas conocido! Algunos más que recuerdo,… el "gran jefe" de la biblioteca, Roland, Ánimo!, sin duda los maestros del estabulario Emilià y Manolo y los siempre eficientes JR y Joan ... Y otros que ya se han marchado, pero cerca Marina, Esteban, Maya o Alejandra...

Mención especial me gustaría hacer a una gran compañera y amiga que me temo lo será lo que me quede de vida, la Colman. No sé qué decir que no intuyas, mas que fue una suerte haberme encontrado contigo y lo bien que nos caímos. Siempre p'alante leona! No sé que más decirte, porque sabés boluda que siempre era sho el que te pedía consejo! Muchos éxitos y un abrazo al Ahmed, otro león también, y a mi familia postiza Argenta; la Carlota y el Chingol (que gloria de personas de verdad!!) Alois, Alexia y su bichito y a papa Colman y lo bien que disfrutó de la vida. Abrazos para todos! Malditos, me pegaron el gusto por el asado lento y el mate o un Malbec en buena compañía! Y siguiendo por el cono sur, como olvidarme al Gualano

y MªAlbe, dos corazones que no paran de bombear pensando en los demás, un placer chavales, que la vida les sonría! Y si hablamos de corazones y cono sur, me toca hablar de la Noe también, un recuerdo para ti, guapisísima, allá donde estés, estate bien y se buena!

And I would like also to thank the people in Norway. Lippid Gruppe kindly welcomed me a made my short stay in Bergen comfortable and very successful. Rolf, Bodil, Lena, Eline, Kari Wiliams, Pavol, Natalya, … thanks to everyone up there!

Pero por suerte mi paso por Lleida fue largo y tuve la oportunidad de mezclarme con otras buenas almas que me entretuvieron para que esta tesis fuera tan larga como ha sido, y yo feliz! Quiero recordar a Iñaki (Mc Mardigan), que está justo hoy de cumple, y emigrado de nuevo en London, gran aventurero rapitense y mejor ser humano, sabio. Así como el frente variado que he ido atrayendo a los Pirineos: Gonzalo (alpinista trovador) y Ana (por muchas cosas…y la portada, gracias!), Pintor (gracias por las correcciones, las escapadas montañeras y, en general, que decir, la amistad desde la guardería!), Dopo (que viajes macho! Solo se vive una vez, pero que bien que la pasemos oiga, gracias por los mejores veranos de mi vida), Patmos (gracias por conocer tu familia villafranquina, y que bien que te veré por Ou, a patear O Courel y tomar botillo!!), Oier (Sr concejal euskaldune, a sus pies), Dani y Carol (viticultores rompiendo la velocidad del sonido!), els veïns Oriol y Mariona (bon viatge xiquets) Nodar y Truji, (ya papas dior mio!! Os veo con la mochila-niño), Fariña, Pow, Jimmy Po (y respectivas señoras, jeje), Anita, Paula (y respectivos maridos, juju), Zintia, Pauliña … las gentes de Pontevedra, miña casiña, meu lar, … Juanin, Ansel, Javiere, Toñil, Berni y vicesberzas … bueno para el frente galaico podría hacer otra tesis así que agradecidos quedáis con este brindis: por todos los LK que no me pude tomar con vosotros, ya nos iremos actualizando!

También quisiera mencionar a una buena generación de estudiantes de medicina (Jesús, Samu, Cris, Paul, Jeong-Hu, Pili, Gonzalo, Clara, Ibai …) que, cuando todavía no era muy cantoso que me juntase con ellos (ahora ya me ven como bicho raro, o yo quizás ya no pego…), me acogieron, permitiéndome prolongar un poco más mis mejores años universitarios. Muchas gracias por la compañía, el equipo de fútbol la facultad, las fiestas, los asados, las eskiadas… y, bien pensado, no está mal tener unos cuantos amigos médicos, que todos nos avejentamos!

Por último quixera dedicarlle unhas verbas á miña familia. Os meus país, que me deron a vida e as ferramentas para afrontala e que incondicionalmente axudaronme a apoiaronme nas miñas decisións, ese compromiso e ese amor que me destes espero me fixeran bo rapaz, con iso xa sería dicir moito. Gracias porque moitas veces tivestes que elixir entre vos e os vosos fillos, e nunca o dudastedes. Ademáis, agora mesmo apetéceme moito pasar una tempadiña convosco y disfrutares de esta vida adulta que, como una parte mais do percorrer, teño o gusto de compartir con quen ma regalou. Así que xa sabes irmanciña, María, e certo que temén levamonos ben nas distancias, e que moitos días xuntos son como una bomba para nos, pero ben sabes o que che quero, e por un tempo xa é hora de tornares a casiña, pola miña parte estarei por ahí unha tempada, e sabes que alí sempre es (mais ben sodes, Miguel e mais ti ;)) benbidos. E os meus avos (Constantino e Carmen e mais Feliciano e Asunción), que tanto loitaron e que tiven a sorte de coñecer polo menos algún tempo. Aínda que agora so disfruto da compañía de Asunción, labrega emigrada tamén polo ben dos seus fillos, como tantos outros! pero esta e a miña! … Mans tinguidas de terra traballada, ollos espertos que coñeceron boas persoas e percorreron mundo e mais o lombo truncado de tratar de arranxalo. Celi, padriños Manolo e Fina, Angela, Tita, Diego e Carmen, (Manolo e Paquita, descansade), Dolores, Ricardo, Diego e Marta, Mundy, Celia, Laura e Alberto! Moitos bicos a todos, pronto poderedes contar conmigo a full!.

….Y mucha gente más me habrá ayudado, y quizás me los olvidé, pero vosotros sabéis quienes sois y lo que habéis hecho, a todos, GRACIAS y también a los que no me habéis ayudado, muchas gracias, puesto que con vuestra dejadez también me habré espabilado.

Hasta pronto Lleida-Pirineus

Index

Abbreviation List

Arachidonic Acid	AA	AminoAdipic SemiAldehyde	AASA
Acyl-CoAHydrolase	ACH	Acyl-CoASyntetase 2,4,6	ACS2, 4, 6
Alpha Linolenic Acid	ALA	Adenosin MonoPhosphate	AMP
Adenosin DiPhosphate	ADP	Adenosin TriPhosphate	ATP
AcetylCoA Acyltranferasa-1	ACAA1	Acyl-CoA Oxidase-1	ACOX1
Amyotrophic Lateral Sclerosis	ALS	Acyl-CoALysophospholipid acyltransferase	ACL
α-Amino-3-hydroxy-5-Methylisoxazole Proprionic Acid	AMPA		
Blood-Brain Barrier	BBB	B-cell lymphoma 2	Bcl-2
Cardiolipins	CL	Coenzime A	CoA
Cytochrome c	Cyt c	ClicloOXigenase 1 / 2	COX1, COX2
Cholesterol	CHO	CerebroSpinal Fluid	CSF
Central Nervous System	CNS	Calcium	Ca^{+2}
ClicloOXigenase 1 / 2	COX1, COX2	Cytochrome P450	CYP450
cytosolic Phospholipase A_2	$cPLA_2$	Carbohydrate-Response Element-Binding Protein	ChREBP
CarboxyMethyl Lysine	CML		
Cluster of Differentiation 36	CD36		
1,2-DiAcylGlycerol	DAG	DesoxiriboNucleid Acid	DNA
DocosoHexaenoic Acid	DHA	Double Bound Index	DBI
2,4 DiNitroPhenylhydrazine	DNP	8-oxo-desoxiGuanosine	8-oxo-dG
Double Strand Break	DSB	Dihomo-γ-Linolenic Acid	DGLA
DocosoPentaenoic Acid	DPA		
Elongases	ELOVL	Endoplasmic Reticulum	ER
EicosaPentaenoic Acids	EPA	Electron Transport Chain	ETC
Excitatory AminoAcid Transporter-2	EAAT2	Epoxy-EicosaTrienoic acids	ETT
Enzyme-Linked ImmunoSorbent Assay	ELISA	Extracellular-signal-Regulated Kinases	ERK1/2
Fatty Acid	FA	Fatty Acid Binding Protein	FABP
Δ5 Fatty Acid Desaturase	FADS1	Fatty Acid Transport Protein	FATP
Δ6 Fatty Acid Desaturase	FADS2	FrontoTemporal Dementia	FTP
FUsed in Sarcoma	FUS		
γ-AminoButiric Acid	GABA	Gas Chromatography	GC
GLutamete Transporter-1	GLT1	Glutamic SemiAldehyde	GSA
GlyceroPhosphoLipids	GPL	Glutamate	Glut
Glial Fibrilary Acidic Protein	GFAP		
Hepatic Nuclear Factor 4 α / γ	HNF-4α / γ	High Density Lipoprotein	HDL
4-HydroxyNonEnal	4-HNE	4-Hydroxy-2-HexEnal	HHE
Histone γ2AX	γ-2HAX	HydroxyEicosaTetraEnoic acid	HETE
Hepatocellular carcinoma G2	HepG2	Human dermal Microvascular Endothelial Cells-1	HMEC-1
IsoProstane	IsoP	Inositol 1,4-diPhosphate	IP_2
InterLeukin	IL	Inositol 1,4,5-triPhosphate	IP_3
independent Phospholipase A_2	$iPLA_2$	InmunoHistochemistry	IHQ
Linoleic Acid	LA	Low Density Lipoprotein	LDL
Long-Chain-PUFA	LCPUFA	LeucoTrienes	LT
LipoXines	LX	Lipoxigenase 1 / 2	LOX1,LOX2
Liver X Receptor α	LXR α	Liquid Chomatography Mass Spectrometry	LCMS

Neuro2A	**N2A**	**Neuroblastoma×SpinalCord 34**	**NSC-34**
N-Methyl-D-Aspartate	**NMDA**	**NeuroProtectin D-1**	**NPD1**
Nuclear Factor Kappa-light-chain-enhancer of activated B cells	**NFκB**	**NonSteroidal Anti-Inflammatory Drug**	**NSIAD**
Membrane Contact Sites	**MCS**	**MalonDiAldehyde**	**MDA**
MalonDiAldehyde Lysine	**MDAL**	**Mitogen Activated Protein Kinase**	**MAPK**
mamalial Target Of Rapamicyn	**mTOR**	**Motor Neuron**	**MN**
Mitochondria-Associated Membrane	**MAM**	**Macrophage mediators in Resolving Inflammation**	**Maresin**
mitochondrial DNA	**mtDNA**		
Organotypic	**OT**		
Patale Activated Factor	**PAF**	**Phosphatidil-Cholines**	**PC**
PhosphoLipid	**PL**	**Phosphatidil-Ethanolamines**	**PE**
ProstaGlandines	**PG**	**Prostacyclines**	**PGI**
Phosphatidil-Iinositols	**PI**	**PhosphoLipase B, C, D**	**PLB, PLC, PLD**
Protein Kinase B	**PKB, AKT**	**Phosphatidil-Serines**	**PS**
PeRoxidability Index	**PRI**	**Protein Kinase C**	**PKC**
Phosphatidyl-Inositol-4,5-bisphosphate 3-Kinase	**PI3K**	**PolyUnsaturated Fatty Acid**	**PUFA**
Reactive Lipid Species	**RLS**	**Reactive Nitrogen Species**	**RNS**
Reactive Oxygen Species	**ROS**	**RiboNucleid Acid**	**RNA**
Resolvin D, E	**RvD, RvE**	**Retinoid X Receptor α**	**RXR α**
Copper Zinc SuperOxide Dismutase	**SOD**	**Sterol Carrier Protein**	**SCP**
Sterol Regulatory Element-Binding Protein	**SREBP**	**Copper Zinc SuperOxide Dismutase Glynine93Alanine**	**SODG93A**
SphyngoMyelin	**SM**	**Syntaxin-3**	**STX3**
secreted Phospholipase A_2	**sPLA$_2$**	**Soluble N-ethylmaleimide-sensitive factor-Attachment protein REceptor**	**SNARE**
S-(2-succinyl)cysteine	**2SC**		
1,2,3-TriAcylGlycerol	**TAG**	**Tumor Necrosis Factor - α**	**TNF-α**
ThreoHydroxyAspartate	**THA**	**Tumor Growth Factor-β**	**TGF-β**
TransActive Response Binding Protein-43	**TDP-43**	**ThromboXane**	**TX**
Uncoupling Protein 3, 4	**UCP3, 4**	**Unfolding Protein Response**	**UPR**
VeryLongchainFA	**VLFA**	**UBiQuiliN-2**	**UBQN2**
Western Blot	**WB**	**Wild Type**	**WT**

List of Tables

List of Figures

1. Introduction

1.A PUFA Physiology

1.A1 PUFA structure and relevance in phospholipids

Polyunsaturated fatty acids (PUFA) belong to the lipid family. They are fatty acids (FA) having at least two or more carbon-carbon double bonds. But let's start from the beginning with a brief introduction of what a FA is, how many diverse forms there are and how this would have an influence in the different biological outcomes (for a review of the topic see (Tvrzicka et al. 2011).

Structurally, FA are composed of a carboxylic (organic acid) group at one end and a variable length aliphatic carbon chain on the other, seeded with none (saturated), one (monounsaturated) or multiple (polyunsaturated) carbon-carbon double bonds. The carbon atom on the acid group is chemically an electrophilic centre (electron deficient area) and therefore susceptible of being attacked by nucleophilic species (electron rich area). Hence, this carbon is termed alpha and the last one on the chain is named omega (see Figure 1). This nomenclature is often used in biomedicine and nutrition being referred as omega-x or n-x, meaning a double bond located on the x^{th} carbon–carbon bond, counting from the terminal methyl carbon (as said before, designated as n or ω) along the carbonyl chain towards the carboxylic region. For instance, linoleic acid (common nomenclature, LA) is a C18:2 fatty acid (meaning eighteen carbons and two carbon-carbon double bonds), belonging to the n-6 family (or omega-6, ω-6, specifying the position of the latest double bond). This point of view of the nomenclature is useful for nutritional purposes since the same family members, ω-6 or ω-3, share related biosynthetic pathways, and even similar outcomes.

Other classifications of FA are based on the length of the chain, being short (up to six carbons), medium (ranging between six and twelve), long (from thirteen to twenty-one) and very long (more than twenty two) FA, and/or the optical activity of those species (relying on the 3D structure). Stereospecifically, each particular double bond could be either *cis*, if the structural configuration on which adjacent hydrogen atoms are on the same side of the double bond (and therefore "bulky" sides are closeby) or *trans*, when the two hydrogen atoms are bound on opposite sides of the double bond.

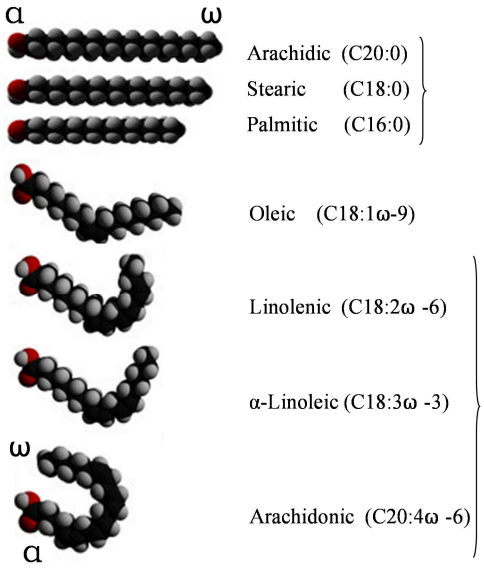


Figure 1. FA structure. 3D representation of different FA structures and its classification, according to the number of double bounds. It can be clearly seen that the number of unsaturations bend the aliphatic chain rendering each FA to specific steric impedances. Symbols α and Ω represented the first and the last carbon atom of the aliphatic chain respectively.

These variable situations (e.g. chain length, number of double bonds and/or stereospecifity of those) physically affect to the properties of different FA. This variability of the sterical incapacitances and packing difficulties at low scale are reflected, at macromolecular level, in terms of melting point temperature, fluidity and/or viscosity variations, as other hemorheological parameters. For instance, longer chains (increased number of carbon) are related to lower melting temperature. Double bonds also favour lower melting points and increased fluidity. Finally, *cis* configuration is another factor that caused melting point decrease, compared with the same FA in *trans* configuration. This data is summarized and presented in Table 1 and Figure 2.

Fatty Acids Melting Points			
Saturated	**T (ºC)**	**Unsaturated *(cis)***	**T (ºC)**
Butiric (C4:0)	-6	Myristoleic (C14:1 ω-5)	-4
Caproic (C6:0)	-3	Palmitoleic (C16:1n ω-7)	1
Caprilic (C8:0)	17	Oleic (C18:1 ω-9)	13
Capric (C10:0)	32	Gadoleic (C20:1 ω-11)	23
Lauric (C12:0)	44	**Unsaturated *(trans)***	
Miristic (C14:0)	54	Elaidic (C18:1 ω-9)	46
Palmitic (C16:0)	63	Vaccenic (C18:1 ω-7)	40
Estearic (C18:0)	69	Linoelaidic (C18:2 ω-6)	28
Araquidic (C20:0)	77	**Polyunsaturated**	
Behenic (C22:0)	80	Linoleic (C18:2 ω-6)	-5
Lignoceric (C24:0)	84	α-Linolenic (C18:3 ω-3)	-11
Cerotic (C26:0)	88	Araquidonic (C20:4 ω-6)	-50
		Docosohexaenoic (C22:6 ω-3)	-44

Table 1. Melting point of different FA. Celsius degrees.

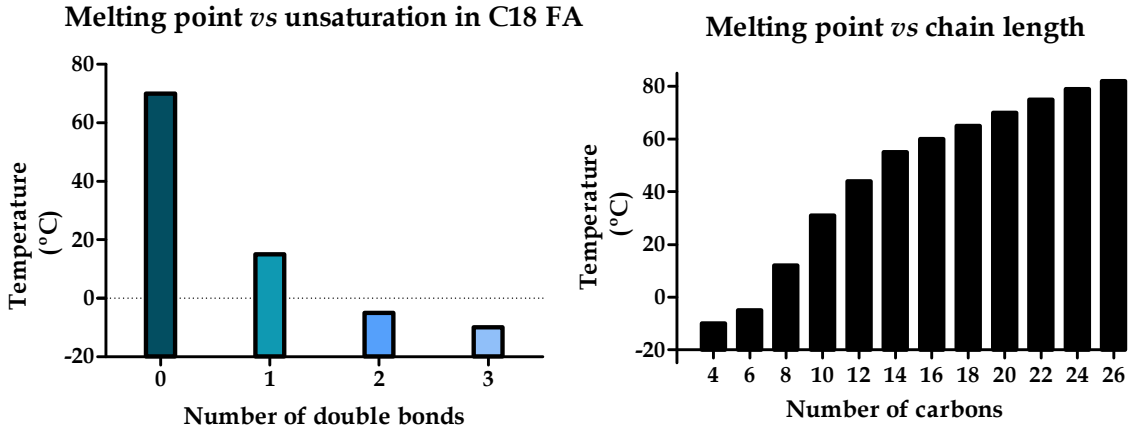


Figure 2. FA melting points. Graph representation of melting point temperatures of various FA in relation with the number of double bounds (left panel) or the number of carbon in the aliphatic chain (right panel). Data obtained from (Nelson DL, Cox 2005) and (Knothe and Dunn 2009).

As previously shown, structural differentiation confers a wide capacity for fine tuning modulation. This is biologically relevant since, within cells, free FA are often rapidly converted

into more complex forms, including phospholipids (PL), plasmalogens, triglycerides (TAG) and derivatives of those. We will later dig deeper into PL metabolism, biosynthesis and maintenance but just as an introduction for now, PL are major constituents of any membrane bilayer within a cell, also with sterols, sphingolipids and proteins. All those biomolecules are held together by coulombic, Van der Walls and hydrogen bonding forces. Regular composition of PL consists in diglyceride (two fatty acid chains covalently bonded to a glycerol molecule through ester linkages) and a phosphate group and a simple organic molecule (commonly choline) attached to it. This particular composition of glycerophospholipids (GPL), with one polar head (phosphate) and a non-polar (or low polar) chains allows an amphipathic behaviour in aqueous solution, since they possess one hydrophobic side (aliphatic chain tail) but also, a hydrophilic head on the molecule (phosphate group, represented as a red dot in Figure 3, next page). This organisation in aqueous solution is the key chemical PL property for being perfect candidates for, in general, cell organellae membrane scaffold.

Different GPL "families" are studied based on the structure of the polar group, finding phosphatidilcholines (PC), phosphatidilethanolamines (PE), phosphatidilinositols (PI), phosphatidilserines (PS) and cardiolipins (CL). Each family comprises numerous *molecular species* that keep the same head group but changed acyl chain; some relevant forms are showed in Figure 3. Synthesis location of those families differs, since CL and majority of PE are synthesized in mitochondria whereas other PL families are mostly generated in the endoplasmic reticulum (ER). This is also relevant for FA (discussed later in chapters 1.B2 and 1.C2) and plasmalogen synthesis, since many steps are started in peroxisomes to be finished at ER and/or mitochondria implying that a good communication between those organelles and a correct inner-cell trafficking is crucial for an optimal lipid balance.

Arousing from particular FA properties mention above, but also with increasing complexity by playing among combinations of different polar groups and/or residues, viscosity, curvature, permeability, elastic compressibility and fluidity of such membranes could therefore be successfully modified solely (but not only) by mixing specific amounts of particular FA incorporated to GPL.

1.A1.1.- Glycerophospholipids

Structurally, while the hydrocarbon chain at the *sn*-2 position is usually ester-linked to the glycerol moiety, the one in the *sn*-1 position can be linked via an ester, ether or a vinylether bond. Length of the alkyl chain typically varies from 14 to 24 carbons and, for the most relevant GPLs, the number of double bonds ranges from 0 to 6. Usually, alkyl chain in the *sn*-1 position is saturated or monounsaturated, while that in the *sn*-2 position is often polyunsaturated but again, the degree of unstauration would change wedge form of the acyl chains making it more or less bulky. Even more, since membrane bilayers (outer leaflet cell membrane, Golgi, ER, peroxisomes and/or mitochondria) are sprinkled with numerous proteins, interaction with those in specific regions (eg lipid rafts) would affect its association (itself but whiting other proteins too) and obviously its functional outcome (reviewed in (Stillwell and Wassall 2003) and (Nelson DL, Cox 2005)).

Due to the large number of different alkyl chain combinations, each GPL "family" consist of numerous structurally different molecular species, thus eukaryotic cells can contain hundreds of

different GPLs. It is important to note that approximately 5% of the genome on these cells is related to synthesis and maintenance of those molecules (Catalá 2012). Even though the meaning of such diversity is not fully understood, it is presumably related to the multiple functions of GPLs and their numerous ways for interacting within surrounding biomolecules, especially those with signalling purposes.

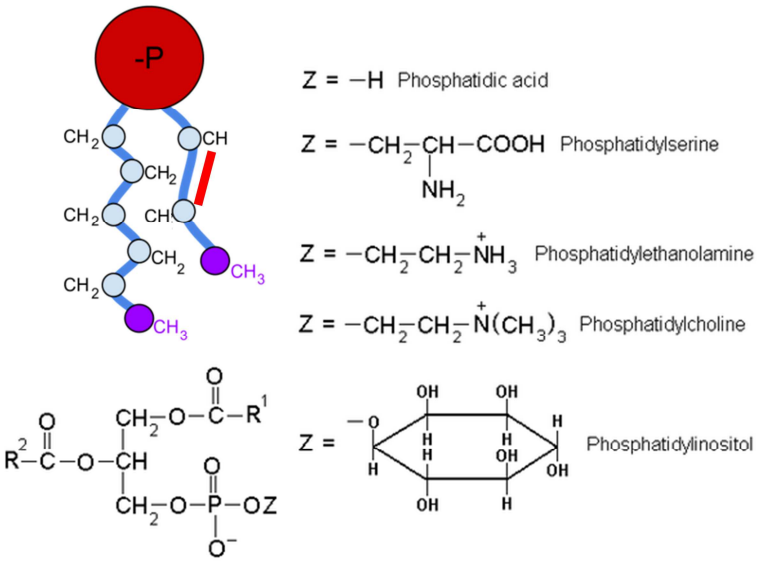


Figure 3. Glycerophospolipids structure. Representation of various GPLs. In the scheme, the red circle represents the polar head, which also corresponds to the Z substituents commonly found. Aliphatic chains could be saturated or unsaturated (red line) and are represented by the R^1 and R^2 substituents in the lower-letf panel.

GPL location within a specific membrane is asymmetric, differing from the inner leaflet to the outer bilayer in the majority of membranes. For instance, inner leaflet in eukaryotic cell membranes is rich in PE, PS, and PI whereas the outer is enriched with choline containing species, PC and SM. In fact, a widely used test for cell viability (early apoptosis) is based in the location change of PS and its interaction with AnexinV (Boas, Forman, and Beutler 1998). This membrane asymmetry is present in the ER, peroxisomes and mitochondria too and is actually a basic tenet for its correct performance. So, asymmetry maintenance, by translocases, flippases or phospholipases action (which will be discussed later in section 1.B1 and 1.B3) is tightly regulated.

Since Singer and Nicolson firstly described Mosaic Fluid model (Singer and Nicolson 1972) some revisions complete the picture to show a more complex and comprehensive scheme (Catalá 2012; Somerharju, Virtanen and Cheng 1999). In general, membrane surface can be subdivided into lipid-rafts containing motives and non-lipid-rafts ones. But, what is a lipid-ratf? Lipid rafts are micro domains enriched in sphingolipids and cholesterol (CHO) with an average diameter of about 50nm. Their formation occurs transiently (they are highly dynamic, but ordered) within inner and/or outer leaflet of membranes. They concentrate selected proteins serving therefore as platforms or scaffolds where selected cellular processes can occur more easily, ranging from cell signalling, pathogen entry, cell adhesion, motility and protein sorting and trafficking (reviewed in (Simons and Vaz 2004)). Lipid rafts are consequently so-called “liquid-ordered” phases, meanwhile, non-rafts membrane phases are “liquid-disordered” ones.

Studies from Atkinson and Harroum (Atkinson et al. 2010) hypothesized that, in addition to higher SM and CHO enrichment in lipid rafts, the liquid disordered ones had higher PUFAs concentrations (may be due to sterical impedances related to double bonds). Furthermore, when compared with lipid rafts, disordered areas had much higher levels of the main membrane resident antioxidant, the E vitamin (tocopherol). On the other side, CHO enriched phases (liquid

ordered) were more rigid and it is believed that oxygen diffusion events were reduced on those areas (Dumas et al. 1997; Subczynski, Hyde and Kusumi 1991). So, in general terms, at liquid ordered phases there was lower quantity of tocopherol and lower oxygen diffusion, hence, components were correctly localized, i.e. where more pexoxidation events can occur. Altogether this data suggest that specific and controlled positioning of all membrane constituents, GPL, SM, CHO, proteins and antioxidants, is crucial for a correct membrane function.

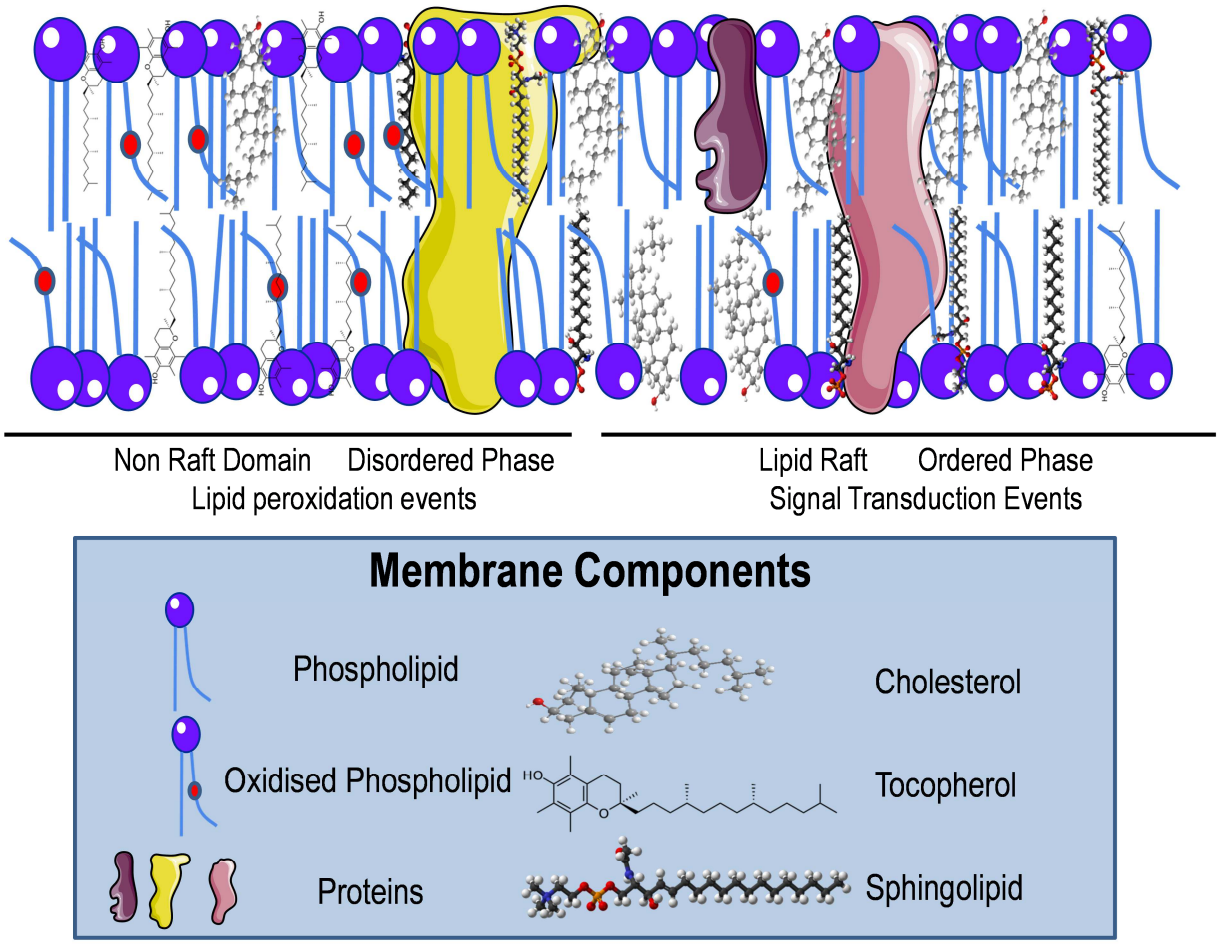


Figure 4. Membrane representation. Components of a membrane showing lipid raft domains and their characteristics (Higher CHO, SM and proteins enrichment), whereas non raft domains had higher levels of tochopherol (but also more oxidised PUFA). Not to scale.

In chapter 1.B3 we will dig deeper on membrane modification mechanisms by deacylation and reacylation processes and in the next section and 1.C2 we will say a few words about lipid signalling events. Just mention here that both are very active processes and thus FA biochemistry not only comprises the so cold “free” FA buy also GPL, TAG and much more complex derivatives (i.e. lipoxins, eicosanoids, docosanoids or plasmalogens; discussed in section 1.C1.2) whose powerful functions are still under debate.

1.A2 PUFA major functions

PUFAs have been related to interfere and/or regulate a huge variety of functions in very different organisms (i.e. birds, worms, humans). In general, functions could be clustered in a number of physiological roles, as: i) energy substrates; ii) structural and functional components

of cell membranes and, finally, iii) signalling purposes, themselves, and/or as precursors for lipid mediators, components affecting signal transduction pathways and gene transcription. Therefore, among others pathophysiological events, PUFAs have been linked to:

PUFA -related Processes	
Process	**References**
Learning and memory	(Hashimoto et al. 2006)
Neurotransmitter release	(Aïd et al. 2003)
Autocrine and paracrine communication	(Nicolas G Bazan 2008; Kitson et al. 2013)
Physiquiathric and mood disorders	(Freeman et al. 2006; McNamara et al. 2013)
Immunity and inflammation	(Calder n.d.; Serhan et al. 2004)
Membrane ion channels, enzymes and receptors	(Eckert et al. 2011; Gawrisch et al., 2008.; Im 2012)
Adhesion molecules	(Yates et al. 2011)
Apoptosis	(Akbar et al. 2005)
Neurogenesis and neurite growth	(Calderon and Kim 2004; Innis 2007)
Pro/antioxidative potentiation	(Nicolas G Bazan 2005; J.-M. Lee and Johnson 2004; Yavin, Brand, and Green 2002)

Table2. PUFA-related processes. List of processes and references in which PUFAs had relevant functions.

1.A2.1.- Energy substrates

Dietary FAs, in the form of TAG, are the primary source of the body's energy representing (in a balanced diet) 25-35% of the total energy intake in humans. Compared with proteins and carbohydrates, FAs have about twice as much energy value and their storage in adipose tissue needs less amount of water. The estimated energy value of long and very long chain FAs is about 38 kJ/g, and for those medium chain FAs approximately 29 kJ/g. While short chain FAs represent a local energy source for enterocytes and colonocytes, the others must be delivered (discussed in section 1.B1.1), generally in esterified forms or albumin-complexed. Also important, FAs take part in the process of transferring and depositing crucial lipid soluble molecules (such as vitamins A, D, E and K) and are precursors of lipid mediators.

Unfortunately, as fat intake has increased within recent years in western-type environments (reviewed in (Simopoulos 2011)), the percentage of FA in the energy intake could represent nowadays up to 40-45% of the total, depending on food sources and habitual consumption. Hence, the evolutionary positive role of FA as a energy storage for further necessities, altogether with the sedentary lifestyle of western population and their increased fat consumption turned nowadays fat into the focus of non-communicable diseases. Especially since epidemiological studies pointed that visceral adiposity (possibly emerged from fat intake unbalance) was a risk factor to developed metabolic syndrome (Björntorp 1993). Therefore, to conceal this major threat of western civilization, nutritional recommendations have been established about total fat and particular fat types, including essential FA and ω-3 PUFA (discussed in chapter 1.B4).

1.A2.2.- Structural membrane actions

As stated in the previous chapter, FAs, in the form of GPL, are the structural components of all cell membranes. Their profile have a profound influence in the biophysical properties of those membranes and, thus, the activity of membrane associated proteins (i.e. enzymes, ion channels, receptors and transporters) (Nelson DL, Cox MM. 2005). As indicated above, the tuning capacity of specific molecules (CHO and/or specific proteins) or the degree of unsaturation of FA present in GPL to promote higher or lower membrane fluidity should be considered (Chapkin et al. 2008; Stulnig et al. 2001). Finally, a very remarkable function of fats as insulators is found in many living organism, especially relevant, for instance, in marine mammals.

1.A2.3.- Signalling purposes

A) FA itself as second messengers

From preliminary studies by Berridge in 1984 (Berridge 1984), the hydrophilic inositol 1,4,5-triphosphate (IP_3) and the lipophilic 1,2-diacylglycerol (DAG) are considered second messengers. Both are generated from the membrane lipid phosphatidylinositol 4,5-bisphosphate (IP_2) by the action of a phospholipase C (PLC, for a review on its regulation see (Bunney and Katan 2011)). Mechanistically, IP_3 interacts with an intracellular IP_3 receptor and leads to the mobilization of Ca^{+2} from intracellular stores (ER and/or mitochondria), whereas DAG directly activates protein kinase C (PKC) and thereby initiates important down-stream signal transduction cascades (Nelson DL, Cox MM. 2005). Other phospholipases (discussed in section 1.B3.1) would also release some FA and derivatives, which had relevant signal transduction activities. For instance, phosphatidic acid (PA, excised by phospholipase D, PLD), is rapidly converter to DAG (with the aforementioned consequences). But, in parallel, this substance is necessary for appropriate mTOR (mamalial target of rapamicyn, a key protein representing a relevant survival cascade) signalling too (Fang et al. 2001). Through phospholipase A (PLA) action, docosahexaenoic acid (DHA, C22:6ω-3) or arachidonic acid (AA, C20:4ω-6) could be also released from the membranes and interact with multiple nuclear transcription factors (discussed below) and/or could be non-enzymatically oxidised too and those new lipid species (e.g. HETE, NPD1, 8-iso-$PGF_{2\alpha}$, among others) may interact with specific cellular receptors for achieving another different outcome.

From the complex lipid signalling overview seen before, which is in fact much more coiled, it seems clear that there is not a single mechanism for FA regulation of gene transcription. The classical point of view implied a specific FA to enter the cell in order achieve its regulation, but nowadays is well accepted that this could be overcome through membrane receptors (e.g. G-proteins coupled, reviewed in (Kostenis 2004)) which not only turn active by non-estified FA, but also very complex derivatives. It is therefore depending upon the cell-specific context, the target genes or the overall status of the cell, that FAs could take very different routes to alter transcription (Duplus and Forest 2002).

B) FAs as precursors of lipid mediators

Through the action of multiple enzymes, but through non-enzymatic oxidation too, FAs are converted into relevant signalling lipid species, such as eicosanoids (i.e. LT, PG, prostacyclines -PGI- and tromboxanes-TX), isoprostanes, endocannabinoids, docosanoids (maresins, docosatrienes, neuroprotectins, neuroprostanes), resolvins (D and E series) and sphingolipid derivatives (e.g. ceramide, sphingosine 1-phosphate, sphingosylphosphorylcholine). All those species compose an enormously wide family which exerts a myriad of different outcomes in multiple locations across the body, but which could be separated, in a very simplistic view, into pro or anti-inflammatory ones, and will be discussed in chapter 1.C.

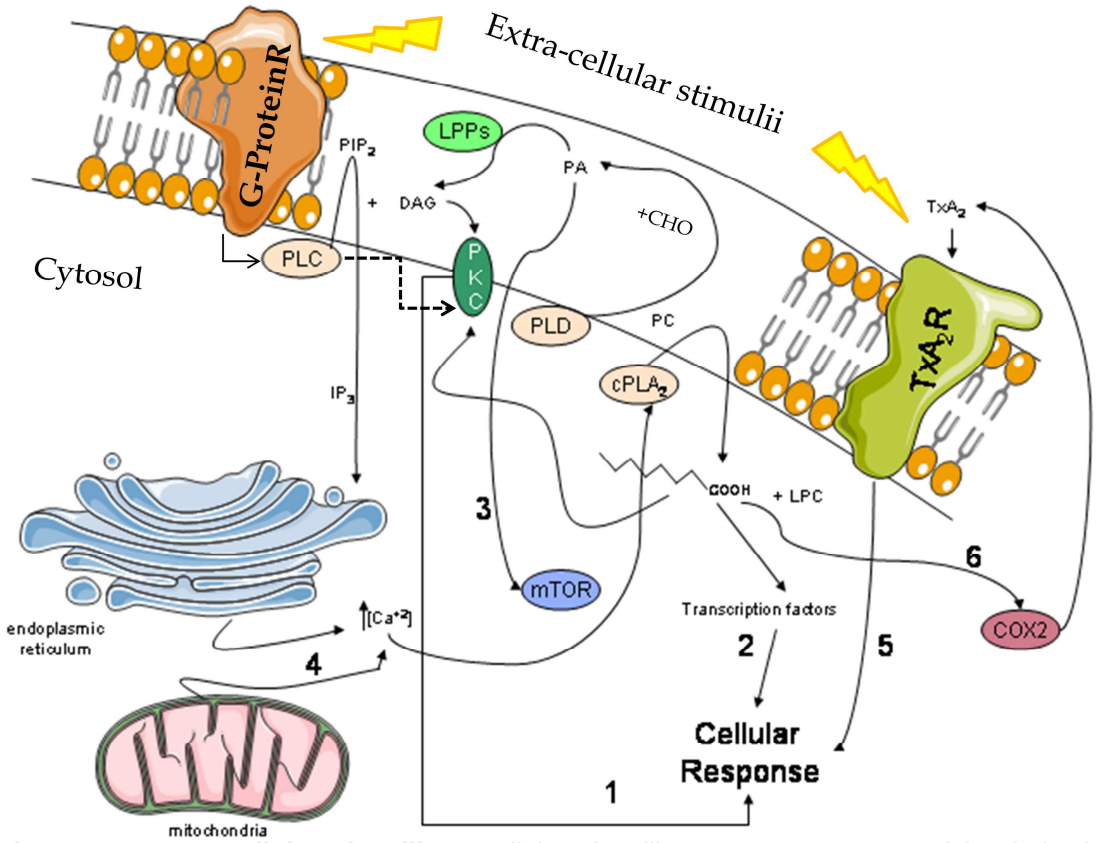


Figure 5. PUFAs cellular signalling. Cellular signalling response to FA and its derivatives could be achieved in multiple ways: (1) DAG direct action on protein kinase C (PKC) will trigger kinase pathways. (2) Free FA, cleaved form membrane by $cPLA_2$ (which is sensible to Ca^{+2} concentrations), would interact with different transcription factors (discussed below) and translocate to nucleus where they activate specific pathways. (3) PA (which cloud be released by phospholipase D- PLD, action under extracellular receptor stimulation) is necessary for the correct mTOR signalling. (4) Ca^{+2} release could be trigger by specific IP_3 (whose accretion could be PLC-dependent, through extracellular G-protein receptor stimulation) receptors present in ER and start signalling events. (5) Specific membrane receptors (e.g. TXA_2R and/or G-protein-coupled receptors, but also PG-dependent ones, among others) could themselves initiate an appropriate signalling cascade. Although, upon stimulation (e.g. PLC), they may influence PKC behaviour too, present in the surroundings, and ultimately affect cellular responses (e.g. nuclear factor kappa-light-chain-enhancer of activated B cells –NFκB-or mitogen-asctivated protein kinase -MAPK- cascades). (6) Finally, another possibility for free FA is to undergo further processing to achieve specific lipid products (through action of cycloxygenases-COX-, lipoxygenases-LOX- and/or cytochrome P450-CYP450) which potentially enhance paracrine and/or autocrine signalling. ($cPLA_2$, Ca^{+2}-dependent phospholipase A_2; LPC, lysophosphatidil-choline;PA, phosphatidic acid; PC, phosphatidil choline; TXA_2, tromboxane A_2; COX2, cycloxygenase 2; CHO, Cholesterol; LPPs, lysophosphatidic acid phosphatase).

C) Palmitoylation

Recently, a new form of post-translational modification involving FA attachment was found, the palmitoylation. This modification consists in the introduction of the palmitate lipid in the cysteine residues of a specific protein (a recent revision on the topic could be found (Blaskovic et al. 2014)). Another cellular lipidations such as myristoylation and prenylation, can also occur, opening a new field in signalling regulation, but due to its chemical properties (specially its reversivility), palmitoylation seems the most relevant. Lots of proteins have been shown to be palmitolated, therefore, multiple mechanisms of cellular metabolism are affected (e.g. signalling, ion transport across membranes, protein folding and degradation, synaptic plasticity, endocytosis, exit from the ER, transcription, bacterial and viral infection, among others; reviewed in (Nadolski and Linder 2007 and Planey and Zacharias 2009)). The obvious consequence of this modification is clear, to upgrade protein lipofilicity and help in any-membrane attachment, which ultimately results in a variation of its regular location. This was proposed to affect, for instance, synaptic plasticity (Conibear and Davis 2010) and Ras (proto-oncogene) regulation (Rocks et al. 2005). Among the identified 23 DHHC (core Asp-His-His-Cys motif which is essential for transferase activity, *in vitro* and *in vivo* (Huang et al. 2009)) mammalian palmitoyl-acyl-transferases, there was found several neuronal-specific, which exhibit a particularly marked substrate specificity, pointing that this post-translational modification is tightly regulated. However, due to the multiple mechanisms potentially affected, palmitoylation is still a wide open field for research.

D) FA direct interaction with transcription factors

Transcription factors are protein structures that bind to specific DNA sequences, usually close to the target genes, in order to control (commonly, by enhancement or repression at the promoter area) the flow of genetic information from DNA to messenger RNA. This specific DNA interaction is the defining feature of any transcription factor, that is, they must contain one or more DNA-binding domains. On the contrary, other molecular species, such as coactivators, chromatin remodelers, histone acetylases-deacetylases or methylases, which alter genetic flux too, lack those specific domains. FAs, specially long chain ones (C20-C22) but also medium and some lipid derivatives (e.g. epoxyeicosatrienoic acids, ETT (Liu et al. 2005)) could act as ligands of these cellular triggers. Hence, they could modulate gene transcription which, ultimately, influences cell signalling and fate (Chapkin et al. 2008). Two general mechanisms characterize FA control over transcription factors: one implies direct binding to the protein structure (see below) whereas the other refers to the greater stability and/or abundance of a specific transcription factor, but a direct binding is unlikely.

Therefore, among some others, non-esterified long chain unsaturated FA (C20-22) and some of their metabolites have been identified as effective ligands of nuclear receptors and/or transcription factors like:

PUFA Interactions Among Nuclear Receptors		
Nuclear Receptor / Transcription Factor		**References**
Direct Interaction	PPAR -α,-β/δ, -γ1 and -γ2	(Xu et al., 1999) reviewed in (Berger et al., 2002)
	LXR α	(Ou et al., 2001)
	RXR α	(Lengqvist et al. 2004; de Urquiza et al. 2000)
	HNF4α and HNF4γ	(Dhe-Paganon et al. 2002; Wisely et al. 2002)
Indirect	SREBP	(Xu et al. 1999)
	ChREBP	(Dentin et al. 2005)
	NFκB	(Camandola et al. 1996)

Table 3. PUFAs interactions among nuclear receptors. List of references and interactions founded between nuclear receptors/transcripcion factors and PUFAs and their derivatives.

We will discuss now some details of the aforementioned transcription factors:

1.-Peroxisome-proliferator activator receptor (PPARs) are members of the steroid hormone nuclear receptor superfamily and play important roles in lipid metabolism and homeostasis, generally through an induction of FA degradation (increasing peroxisomal and mitochondrial oxidation, reviewed elsewhere). Three subtypes of PPARs have been characterized in humans, PPAR-α, -γ, and -δ (the latter also known as PPAR-β; γ generates three isoforms) whose mechanism of action implies homo and/or heterodimerization (the latter, more frequently) with the 9-*cis*-retinoic acid receptor (RXR) and binding to PPAR-responsive elements in the promoter region of various target genes, ultimately regulating their transcription. Their DNA and protein sequences are highly conserved among them, especially upon the DNA binding domain, meanwhile the ligand binding pocket possess a wider variation to achieve their specific selectivity (Xu et al. 1999). There have been found homologous proteins in many different species, including all metazoan phyla, pointing out the relevance of their physiological functions across evolution (reviewed in (Escriva, Delaunay and Laudet 2000)).

In rodents, as well in humans, PPAR-α is highly expressed in the liver, skeletal muscle, heart, and kidney meanwhile PPAR-γ is predominantly found in white and brown adipose tissue, colon, endothelial and vascular smooth muscle cells and, to a lesser extent, in immune cells and liver. Finally, PPAR-δ is ubiquitously expressed, but its action is especially relevant in skeletal muscle. In the central nervous system (CNS), expression for all isoforms was found, but δ is the predominant (Basu-Modak et al. 1999). Thanks to knock out, inducible, deletion and silencing studies upon the family members performed in rodents over the past years, a complex time-dependent cell-specific distribution was found, in CNS too (reviewed in (Keller et al. 2000)). Whereas PPAR-α and γ are expressed at very low levels, predominantly in astrocytes and microglia respectively, PPARδ has been found in neurons of numerous brain areas as well as in immature oligodendrocytes, revealing a key feature helping a correct axon myelination (Peters et al. 2000). Interestingly, recent studies (Ciana et al. 2007) had demonstrated a differential male-to-female induction of PPAR, at least for the liver, which could be on the basis of the different lipid handling by each of the two sexes.

Regarding its activation, several FA and natural occurring derivatives (e.g. eicosanoids, PG, HETES) interact with PPARs (Krey et al. 1997), besides lots of synthetic agonist had been

proved to do so (reviewed in (Fruchart 2013; Derosa and Maffioli 2012; Bernardo and Minghetti 2006 and Bhurruth-Alcor et al. 2010) with particular preferences between the different isoforms. Synthetic thiazolidinediones (e.g. pioglitazone and rosiglitazone, already approved treatments for type-II diabetes) are preferred substrates for PPAR-γ induction, whereas fibrates do so to PPAR-α or thia-fatty acids (synthetic sulphur-containing FA with plasma lipid lowering effects) could have an effect on all of them (Bhurruth-Alcor et al. 2010).

2.- Liver-X-Receptor (LXRs) are crucial nuclear receptors for lipid homeostasis (for a review in this topic see (Jakobsson et al. 2012; Repa and Mangelsdorf 2002)). I*n vitro* LXR interaction with FA (serving as direct antagonists) has been proved for some of the PUFA family (Ou et al. 2001), but surprisingly not for the unsaturated stearic acid (C18:0). In response to oxysterols (derivatives of CHO), the two LXR isoformrs (α and β, with a 77% amino acids homology) formed heterodimers with retinoid X receptor to be fully activated and induces the expression of genes required for the reverse CHO transport. This fact explains the extensive research aimed to develop synthetic agonist to help in dyslipidemia treatments, in general, and more specifically in metabolic syndrome and related cardiovascular disarrangements, being statins of special relevance (for a review on the statins and their relevance in CNS see (A Farooqui et al. 2007). LXR activation induces genes for *de novo* lipogenesis in liver in two separate pathways: i) transcriptional induction of SREBP1c (Repa et al. 2000); ii) by up-regulation of ChREBP expression (Cha and Repa 2007). LXR-mediated increases in both ChREBP and SREBP-1c will raise expression of lipogenic enzymes to enhance fatty acid synthesis from glycolytic end-products, being therefore considered a master hepatic lipogenic factor.

3.-Sterol regulatory element-binding proteins (SREBPs) comprise another transcription factor family. Composed of three members (named 1a, 1c and 2 respectively), they are activated (by cleavage from ER-Golgi and nuclear membranes) when low levels of cellular CHO are present. In order to enhance CHO and FA biosynthesis, they bind specific DNA regions (10 bp length, named sterol regulatory element) located in promoter regions of various genes (for a review on the SREBP topic see (Xiao and Song 2013)). SREBP-2 is relatively selective for CHO biosynthesis enhancement, meanwhile SREBP-1c activates the FA pathway (e.g. promote expression of the fatty acid synthase) and SREBP-1a remains in between, with a mild activation of both. Two genes encode their protein sequences, asides the latter had different splicing to raise the two isoforms (Brown and Goldstein 1997). In relation with SREBP-1c, PUFA cause an activation, whereas, surprisingly, saturated or monounsaturated FA suppressed the induction of lipogenic genes by inhibiting its expression (reducing mRNA stability) and processing (Nakamura et al. 2004).

4.- Carbohydrate-response element-binding protein (ChREBP) heterodimerizes with Max-like protein X and bind to the carbohydrate response element regions of the DNA when a substantial decreaseof AMP/ATP ratio is present. Recently, it has been reported that ChREBP has two isoforms, ChREBP-α and ChREBP-β, and that they are located mainly in the cytosol and nucleus, respectively (Herman et al. 2012). Moreover, ChREBP is susceptible of being phosphorylated by various stimuli (e.g. protein kinase A, adenosine-monophosphate kinase), and therefore blocked in the cytosol, inhibiting the control over genes related to glycolysis, lipogenesis, and gluconeogenesis (e.g. liver type pyruvate kinase, fatty acid synthase and glucose-6-phosphatase catalytic subunit, respectively). Thus, the main function of ChREBP is to

regulate metabolic gene expression to convert excess carbohydrate into triglyceride, rather than glycogen. But this protein could repress PPAR-α, hypoxia inducible factor-1b and sirtuin-1 (among other transcription factors, for a review see (Iizuka 2013)) activities too, having a greater gene expression interference.

5.-Nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) pathway represents a key factor in cell survival. It had been implicated in a myriad of diverse situations, from the immune response to infection (the origin of its discovery) to cancer, as well as inflammatory and autoimmune diseases or synaptic plasticity and memory events (for a review see (Gilmore 2006)). Early studies about PUFA action on the pathway, showed a differential role for ω-3 and ω-6 fatty acids (Camandola et al. 1996) and it has been proved that some of PUFA derivatives affect it too (Ho et al. 2008; Sánchez-Galán et al. 2009). Remarkably, apart from the direct influence of PUFA and its derivatives in the signalling cascade, NFκB pathway is affected by PKC action. Hence, several members of the pathway are phosphorylated or helped to be stabilised (e.g. $IKK_{\alpha/\beta}$, RelA, among others) by most of the PKC isoforms studied (but with particular cell/tissue or pathology variations), providing another frame for PUFA's influence on cell fate.

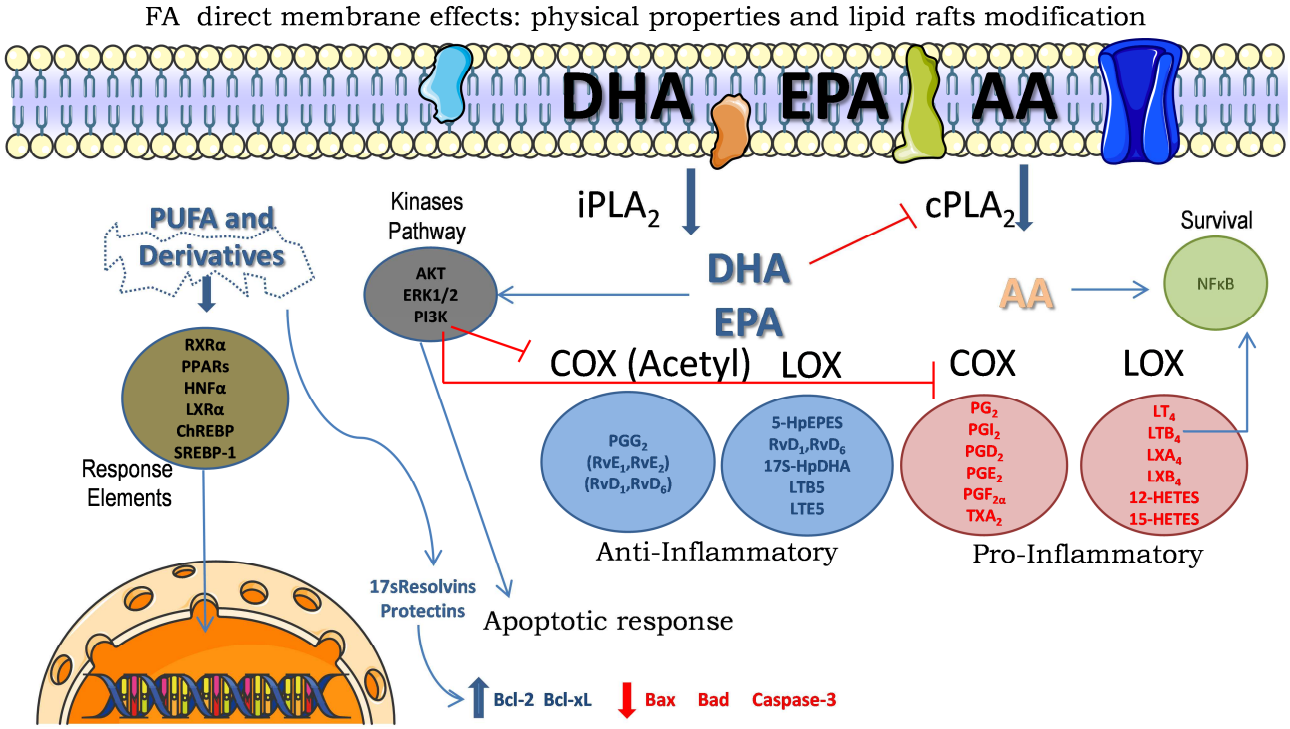


Figure 6. PUFAs actions. Apart from their specific membrane functions, under PLA_2 activity, PUFA are released from the membrane and could initiate various cascades and/or directly interact with response elements to enhance different cellular mechanisms. Importantly, PUFAs could also serve as substrates for the synthesis of multiple signalling molecules.

1A2.4.-Cell Survival

Akbar and co-workers (Akbar et al. 2005) linked DHA to crucial signalling cascades, PI3K-AKT/mTOR. As a consequence, it has been proved that cell survival is altered by various kinases pathways, susceptible to be PUFA and PUFA-derivative sensitive. For instance, the phosphoinositide 3-kinase (PI3K) and the MAPK pathways are activated in injured neurons

(Henshall et al. 2002) as well as in tumours (reviewed in (Bertucci and Mitchell 2013)). PUFA and their anti-inflammatory derivatives could alleviate PI3K mediated cell death, at least in yeast models (Couplan et al. 2009) or in lipopolysaccharide-induced microglial inflammatory response (Xu et al. 2013), but could also result in increased mortality in cancer cell-lines (Toit-Kohn, Louw and Engelbrecht 2009). Furthermore, in activated microglia, PI3K inhibition enhanced prostanoids production, and expression of COX-2 could be regulated by PI3K as a response to neuroinflammation (de Oliveira et al. 2012). Interestingly, a synthetic derivative of $PGF_{2\alpha}$, latanoprost, is able to promote retinal ganglion cell survival and neurite outgrowth through a PGF receptor-mediated modulation of the PI3K pathway (Zheng et al. 2011). Therefore, MAPK pathways are involved in neuroinflammation in many ways, including the enhancement of IL-6 synthesis in sensory neurons (St-Jacques and Ma 2011) and the regulation of IL-1-mediated COX-2 expression (Moolwaney and Igwe 2005) and those pathways could be affected by different lipid derivatives in a feedback loop.

Concluding: DHA and AA, two different motorways.

DHA and AA are chosen as paradigmatic examples from the two main eukaryotic fatty acid families, ω-3 and ω-6 respectively. They are "endproducts" themselves since their unique characteristics confer them special functions. Besides, they are crucial because they represent main precursors of a broad range of paracrine substances acting in a wide type of schemes, with different outcomes. In terms of inflammation, it is generally well accepted that both families symbolize two different actions in response to an insult (with the exception of AA-derived lipoxins). This is, AA and ω-6 derivatives initially predisposes to inflammation whereas DHA, EPA and ω-3 derivatives alleviate and, up to an extent, try to solve the urgency.

For one simple molecule, DHA, to have an effect in so many presumably unrelated processes, it should function in a fundamental level, common to most cells. On the other hand, ω-3 docosapentaenoic acid (DPA) is a DHA precursor and membranes enriched in this FA posse very subtle membrane fluidity alterations respect to those with DHA. Furthermore, ω -3 DPA is only two protons diferent, requires less energy (and reduced complexity) to be synthesized and it is more resistant to peroxidation than DHA. Why this molecule is tenaciously conserved through evolution (especially concerning its visual and cognitive virtues) is discussed in an interesting publication (Crawford et al. 2013).

It is been proved that ω-3, as well as ω-6 FA, are involved in: i) hormone production; ii) formation of potent lipid peroxidation products (which could be detrimental, but signalling useful too, reviewed in section 1.C2.3) ; iii) direct effectors on specific enzyme activity or as indirect effectors through transcription events and/or iv) membrane structure and function alterations (reviewed in (Stillwell and Wassall 2003)). All those general modes of action are not mutually exclusive, rendering ω-3 and ω-6 FA pleiotropic effects.

1.B PUFA Maintenance

1.B1 PUFA systemic and cellular delivery

1.B1.1.- Systemic delivery

Along digestion, phospholipase A_2 (discussed later in section 1B3.1), inespecific esterases and lipases, and bile acids are secreted, initially at the stomach and through small intestine. Most of those enzymes are originated in the pancreas and emulsified fats breakdown into free FA, mono and diglycerides and cholesterol-esters to form mixed micellaes. They are composed by bile acids, CHO (dietary, and bile-secreted too), the aforementioned break fats, PL and dietary liposoluble vitamins (A, D, E and K). This process takes place mostly in the upper part of the small intestine, and along it, increased amounts of lipolisis products enrich micellae composition. Those enzymes digregate lipids at neutral pH and the lipid enrichment of micellae renders then "soluble" and easily absorbed by enterocytes at microvelssels, in a passive manner. But this uptake could be also helped by specific proteins, such as CHO capturing proteins (i.e. Niemann Pick C1 like 1), ATP-binding cassette transporters or microvelssel membrane-FABP (fatty acid binding protein) (Berne and Levy 2000). ER of enterocytes resterifies FA and monoglicerids to obtain triglycerids (major chylomicron component, up to 77%), PL (up to 9%) and Cholesterol-esters (3%) to form, combined with apoliproteins (synthesized in the rough-ER and mainly composed by apoliprotein B, ≈100 KDa, 1%), the chylomicrons. These substances, conformed by a lipid core but protein coated, could range from 0.08 to 0.6 micrometers and are secreted to lymphatic system, bypasing portal vein transportation and liver, to arrive wherever they are needed along the lymph and finally re-entering into the blood stream at the thorax. This is the common transportation for long FA, but it's not the case for those short and medium chain lengths FA. Due to their higher aqueous solubility they avoid reacylation, chylomicron forming and secretion, being ready for use through blood stream from enterocyte, thus, entering directly to the liver by portal circulation.

Passing across adipose tissue and skeletal muscle capillaries, plasma chylomicorns are cleared. This could take around one to two hours after ingestion; meanwhile those substances could reach up to 2% of the plasma, conferring it turbidity and a yellow colour. Lipoprotein-lipase is responsible for this clearance, acting at capillar endothelium level. These enzymes hydrolysed triglycerids and PL at chylomicrons to form glycerol and free FA which immediately diffuses through membranes. Those FA are readily re-esterified to be stored for further requirements. Hence, the liver plays key roles: i) decomposing FA to obtain energy; ii) synthesising triglycerides from carbohydrates; iii) synthesising other lipid compounds from FA, specially CHO and PL. Meanwhile, adipose tissue: i) stores triglycerides forming fat; ii) is a major endocrine organ, synthezising or assisting in the synthesis of important hormones as adiponectin, TNFα, IL-6, leptin, resistin among many others; iii) regulates free FA levels; iv) the brown type one generates heat by uncoupling mitocondrial respiratory chain. Both tissues are therefore central organs controlling dietary fats and its further release when required.

Moreover, free FA transportation occurs, representing 5% of total lipids in plasma after chylomicron clearance. Nevertheless, it is not usually related with digestion processes, instead it is more commonly linked to energy expenditure recovery. There are at least two signals which cooperate to evoke FA delivery to form storage depots. One is the α-glicerophosphate decay in tissues with high energy demand and/or secondly, hormone-sensitive lipase activation.

However, free FA in plasma is scarce, regularly only 0.45 g in the whole circulatory system. Surprisingly, this low relative amount is enough, since its turnover is especially high. Half-life of a plasmatic FA is around 2-3 minutes, and when depleted, new FA is desorbed from storage depots. The flexible amounts of FA transported in plasma helped by albumin are also remarkable. Starvation or diseases such as type-I diabetes deplete carbohydrates and, under such conditions, FA/albumin ratio could peak to 30/1, when is normally around 3/1.

In fact, the majority of lipid delivery along the body is lipoprotein linked. Apart from chylomicrons, there are four types of lipoproteins, which could be classified attending its density after centrifugal distribution (expressing a ratio between triglycerides and proteins presented on them): i) very low density, containing high amounts of triglycerides, but moderate PL and CHO; ii) medium density, which are in fact very low density ones but half depleted of triglycerides (therefore raising its PL and CHO percentage); iii) low density, already triglycerides depleted, with very high amounts of CHO, but moderate PL quantity and iv) high density ones, where protein composition rises to 50%.

These are some important points related to lipid transport from dietary sources to the whole body through the circulatory system, but, what happens with lipids crossing cell membranes or the inner trafficking whiting the cell? These issues will be discussed below.

1.B1.2.- Regulation across cell membranes

The physical properties mentioned before claimed for very low FA solubility in aqueous solutions. For decades, there had been controversy on whether FA transportation across membranes occurs eventually by chance (by simple passive diffusion events) or facilitated by proteins. Nowadays the general idea is that both issues could happen, but for optimization, protein-mediated transport is more common.

Flip-flop and diffusion events take place naturally. Hamilton and co-workers (Kamp and Hamilton 2006; Kamp and Hamilton 1992; Hamilton 2007) demonstrate not only that proteins are not completely necessary for fatty acids to go across cytosolic membrane but also that they are not required for releasing those fatty acids into cytosol. Further, they demonstrated that extracellular membrane crossing could be much easier for fatty acids than for glucose, water or small non-electrolytes.

But driving forces for free diffusion are based on concentration and gradient generation, so it would be impossible to meet the requirements when high metabolic tissues demand it fast (e.g. muscle upon contraction). Thus, from a physiological point of view, it would be interesting to have a better control of FA trafficking to: i) ensure FA uptake when low levels are present inside cytosol; ii) avoid FA export when low levels are present outside the cell and uptake when inner quantities are sufficient (the latter may not take place because of gradient loss); iii) potentially select a particular FA type (specific enrichment) and finally iv) allow those rapid adjustments when metabolic fluctuations occurs. A scheme representing FA membrane trafficking is pictured next page in Figure 7.

So, in order to optimise transportation to and across biomembranes, FA and some of their derivatives, are helped by proteins. For instance, albumin levels in plasma and interstitial spaces

could range from 300 to 600µM and such an "unspecific" protein may help to "dissolve" 100 to 400µM (extreme cases up to 1,5mM) fatty acids (Richieri, Anel and Kleinfeld 1993). Finally, taking into account particularities found in the CNS (energetic demands, highly complex cell types, brain blood barrier intersection) its FA transportation and metabolism will be discussed later on section 1.B5.

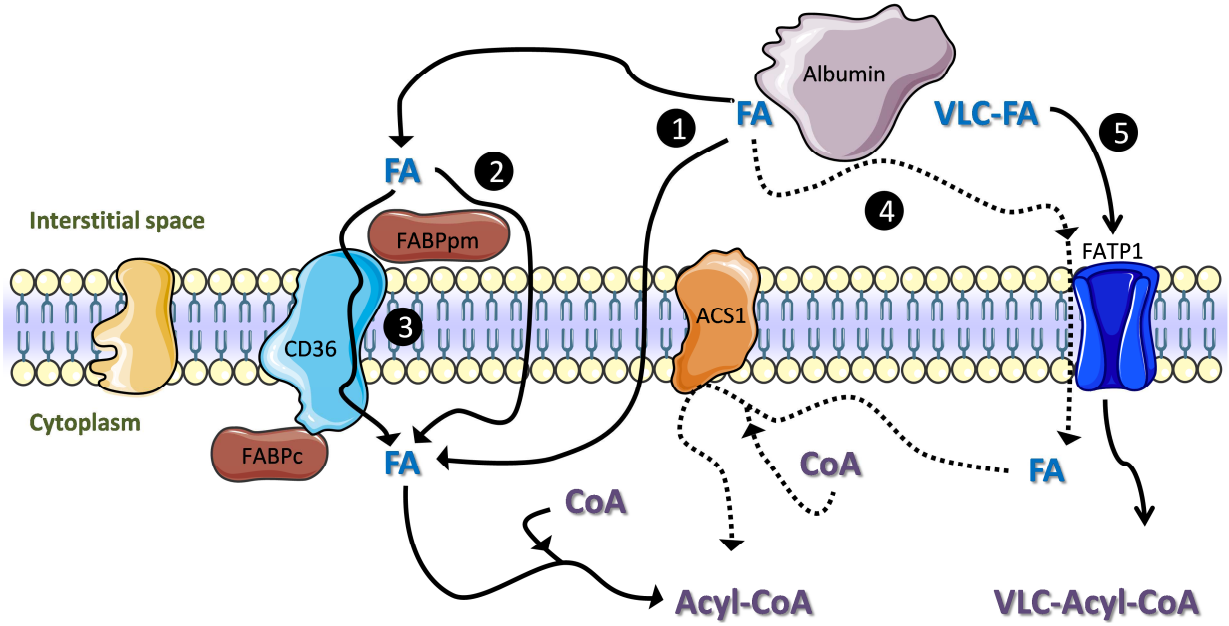


Figure 7. FA delivery across the membrane. Current scheme of FA transport mechanisms to intersect cell membranes. Since the exact mechanism of transmembrane translocation of FA is still unresolved, Figure 7 shows the most likely ones: (1) In view of their hydrophobic nature, Albumin-desorbed FA could cross the membrane by simple diffusion. (2) Alternatively, CD36 (88kDa; also referred as fatty acid translocase), alone or together with the peripheral membrane protein FABPpm (plasma membrane-associated fatty acid-binding protein; 43kDa) accepts FA at the cell surface to increase their local concentration and thus increase the number of FA diffusion events. (3) It is possible that FA membrane crossing is actively achieved through CD36 too. Once at the inner side of the membrane, FA are bound by cytoplasmic fatty acid binding protein (FABPc) before entering metabolic or signalling pathways and transformed into Acyl-CoAs. (4) Additionally, a minority of FA are thought to be transported by fatty acid transport proteins (FATP1) and rapidly activated by plasma membrane acyl-CoA synthetase (ACS1) to form acyl-CoA esters. (5) Very-long- chain fatty acids are preferentially transported by FATPs and, by action of their synthetase activity, directly converted into very-long-chain acyl-CoA esters.

1.B1.3.- FA inner trafficking

Different membranes in a single cell had clearly shown different lipid distribution. For instance, plasmatic membrane exhibits a transverse lipid composition asymmetry with high amounts of sterol and sphingolipids, whereas ER displays symmetrical distribution and contains low level of both species (Voelker 1991). Migration into and between organelles could be a difficult task because most lipid species lack any intrinsic motif that mediates their intracellular distribution like proteins do. However, some sort of connectivity between ER, Golgi, mitochondria and peroxisomes has to operate to interconnect FA fate. As commented previously, interplay between those organelles is necessary to achieve specific amounts of a required lipid for metabolic purposes or, if is not delivered, need to be synthesized. This internal connectivity could be, essentially, vesicular or non-vesicular mediated. It has been demonstrated that both systems occur but the latter is more likely to be restricted to membrane contact sites (MCS,

discussed in next section), where ER and virtually all the organelles "touch" each other (reviewed in (Holthuis and Levine 2005)).

Non vesicular spontaneous interconnection in between one of the mentioned areas and/or with the cytoplasmatic membrane could exist as i) total free desorption, stochastically diffusion across the cytosol and absorption to the target membrane or, on the contrary, ii) being helped by specific proteins (lipid transport proteins, LTP). Both processes have no net energy expenditure, but proteins can greatly facilitate (desorption and diffusion across aqueous cytoplasm are believed to be the more difficult steps) and more likely regulate final destination (reviewed in (Helmkamp 1986) and (Lev 2010; Lev 2012)). Finally, other possibility for FA exchange in between membranes includes collision, which could be "activated" when a substantial part of the lipid extends from the bilayer surface.

LTP are nowadays being extensively studied. From this research became clear that monomeric exchange (a single lipid specie intermembrane interchanged) proceeds even when vesicular pathways are blocked due to genetic manipulation or chemicals. For instance, a well-studied case is the delivery of ceramides (synthesized in the ER) to the *trans*-Golgi (Hanada et al. 2003) or the PI delivery form the ER to both the Golgi and the plasma membrane (Whatmore et al. 1999). Both routes are highly conserved throughout evolution and are both LTP mediated. Thanks to crystallographyseveral LTPs structures were elucidated (with and without their natural ligands). In general, their tunnel-shape determined lipid selectivity and more surprisingly, some of the elucidated structures had a "lid" to close the tunnel which, in turn, is opened through a conformation change when LTP is bound to a membrane. LTP targeting for a specific membrane is not clear yet, but studies in the past years pointed that Trp residues present at membrane binding sites helped in the correct positioning, but in a non-specific manner (Phillips et al. 2006).

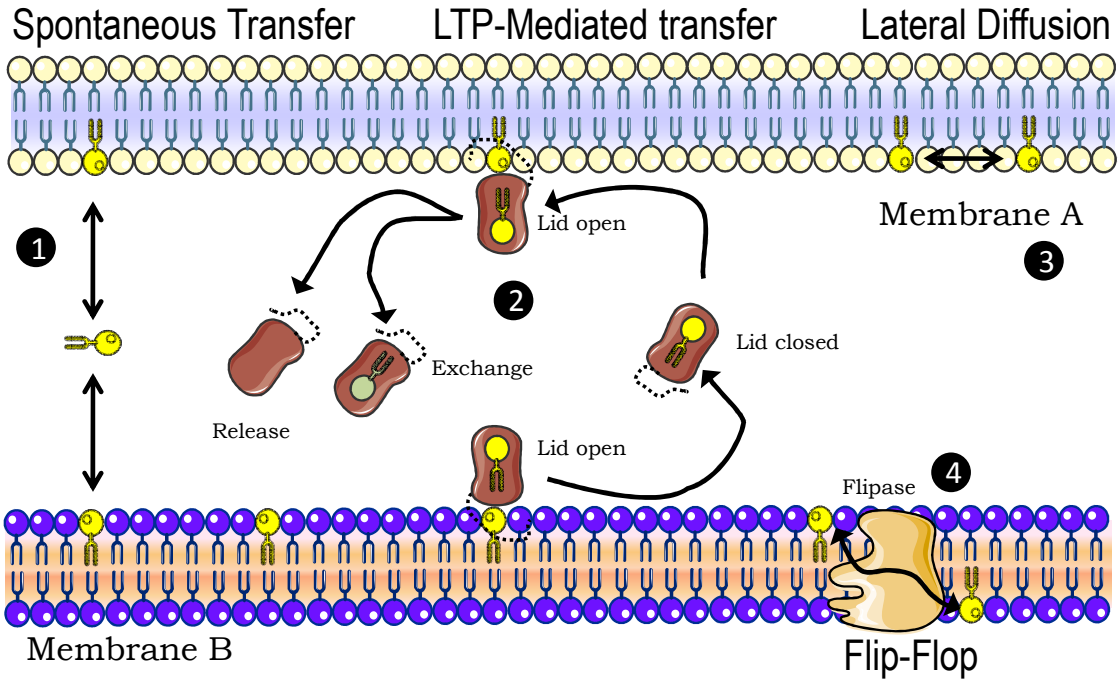


Figure 8. FA intermembrane transportation. There are four main mechanisms for non-vesicular lipid transport: (1) spontaneous transfer; (2) transbilayer LTP-mediated; (3) lateral diffusion and (4) flip-flop (which could be spontaneous or protein-helped, by flippases). The last two are not commonly involved on lipid layer exchange (only when membranes are connected through bridges), but also occurs naturally and remodelate lipid bilayers.

All in all LTP non vesicular transport represents a precisely, non-energy wasting and elegant way to translocate lipid species between two membranes probably much more efficiently than spontaneous processes, but further research is needed to clarify different aspects like membrane target specificity, its regulatory mechanism and cytoskeleton relationships, among others.

1.B1.4.- Lipid transport at membrane contact sites

Membrane contact sites (MCS) are defined as regions where two membranes are closely apposed but the membranes do not fuse and thus each organelle maintain its identity. MCS have been described for ER with mitochondria, Golgi, peroxisomes, endosomes, lysosomes and lipid droplets, as well as the plasma membrane (for a review see (Elbaz and Schuldiner 2011 and Rowland and Voeltz 2012)). The most studied ones are those between the ER and mitochondria. In fact, there is a fraction of the ER which can be isolated, that is attached to mitochondria (named the mitochondria-associated membrane, MAM) and this fraction is enriched in enzymes that are involved in lipid synthesis. Hence, for some lipid species (e.g. PC, PE or DHA, discussed later), synthesis started in the ER but mitochondria is required for several steps of the process.

ER-mitochondria MCS can have different structural features. Some contact sites are discrete, whereas others are more extensive, circumscribing almost completely around the mitochondrial membrane. Contact sites appear to be stable structures because the two organelles stay tethered to each other even as they move along the cytoskeleton, pointing out the relevance of this linkage. Spectacular live cell imaging videos show that the two organelles can traffic in a coordinated fashion without any noticeable disruption in their contact (Friedman et al. 2010). Lipid transport at the MCS could also occur as protein mediated or spontaneously. Again, when transport is LTP mediated, is more likely to be better regulated, hence more efficient. An example of this process is, for instance, the production of PE form PS in mammalian cells. PS is scarce in the mitochondria (2-3% of total membrane PL), but among other diverse functions, is required for the mitochondrial resident enzyme PS-decarboxylase. PS formation is ATP consuming and occurs through the exchange of a serine residue with either the choline moiety of PC or the ethnolamine moiety of PE. This process is catalysed by two enzymes, PS-synthase 1 (for both substrates) and 2 (specific for PE) which are mainly resident in the ER (specially at MAM, remarkable in hepatocytes) but could be found in the Golgi too (Daum and Vance 1997). Furthermore, most of the PE synthesized in mammalian cells comes from the decarboxylation of PS at the inner mitochondrial space (although two alternative mechanism of synthesis exists). Hence, the transport of PS from the ER to the mitochondria and PE back from the mitochondria to the ER is a remarkable example of bidirectional non-vesicular lipid transport mechanism at the MCS and how those regulate lipid biosynthesis and distribution.

1.B2 PUFA biosynthetic pathways: desaturase and elongase

De novo FA biosynthesis involved different enzymes (i.e. desaturases, elongases) distributed in various organelles across the cell (i.e. microsomes, ER, mitochondria, peroxisomes). For its obtention a coordinated cascade of processes (i.e. elongation, desaturation and β-oxidation) takes place in those precise locations. Because we lack the Δ12 and Δ15 desaturases present in

plants, mammals cannot synthesize all sort of PUFAs we need from the ω-3 and the ω-6 series. Hence, some FA must be dietary provided and consequently named "essential FA". We will focus in the two most relevant PUFAs as examples from both series, DHA (ω-3) and AA (ω-6), here we will discuss how these FA are formed arising from their dietary sources, that is, the C18:2ω-3 (alpha-linolenic acid, ALA) and the C18:3ω-6 (LA).

1.B2.1.-DHA synthesis

Stated long time ago by Dr Sprecher (Sprecher 1981), DHA synthesis has been recently revisited in non-mammalian species, for which certain Δ4 desaturase activity was proposed (Qiu 2003). Nevertheless, the canonical eukaryotic route for DHA synthesis, starting from its main precursor, ALA, involves a Δ6 desaturation step, performed by specific FADS2 (52KDa), followed by an elongation step, executed by a PUFA selective elongase, ELOVL5, to form C20:4ω-3. This reaction, in which malonyl-CoA is the two carbon donor and NADPH the reducing agent, takes place at the microsomes (ER) and involves four separate reactions: condensation (β-ketoacyl CoA synthase), reduction (β-ketoacyl CoA reductase), dehydratation (β-hydroxyacyl CoA dehydrase) and reduction once again (*trans*-2, 3-enoyl-CoA reductase).

The initial condensation step is the rate limiting factor and takes place at the cytosolic surface of the ER membrane. Chain length of the end-product is determined by a serine residue of the elongase at the luminal surface (Denic and Weissman 2007). It is therefore believed that end-product specificity is driven by a distinct chain length rather than by a number of repeated two-carbon additions.

After these two steps, another desaturation phase is required (Δ5, FADS1) followed by to consecutive elongation steps (ELOVL2 and 5) to form C22:5ω-3 and another ELOVL2 elongase cycle to achieve C24:5ω-3. Δ5 and Δ6 desaturases share up to a 75% sequence similarity in humans (for a review on those enzymes and their relation to FA abundance see (Lattka et al. 2010)). They introduce a double bound at position 5 (being substrates C20:3 and 20:4 for the in the ω-3 and ω-6 series respectively) or 6 (being substrates C18:3 and C18:2 for the in the ω-3 and ω-6 series respectively) of the acyl chain. Finally, FADS2 action desaturates once more to produce C24:6ω-3. The last step takes place in the peroxisomes and includes a β-oxidation to successfully synthesize DHA. A scheme of the described mechanism is pictured in Figure 9 in the next page.

FADS2 mRNA expression occurs principally in adrenal glands, but high amounts were found in the liver, brain and cerebellum too (Leonard et al. 2000). Interestingly, the level in foetal liver and brain was 6-fold greater than that found in the adult pointing to an early synthetic necessity. The same pattern of expression was observed for human FADS1, but with lower general levels, and again, the level in foetal tissues was several times higher than in adults. This may be related to their chromosomic location, since they share close positions in chromosome 11q12. Also remarkable, human Δ5 shares approximately 60% of their proteic sequence with mouse and rat Δ6. Nevertheless it is not clear whether these genes evolved through duplication, but since both desaturases were found to have been developed from a single protein in zebrafish, it seems clear that both had a common origin (Hastings et al. 2001). However, recently polymorphism findings showed an important role for these variations on the regulation of FA metabolism (reviewed in (Glaser, Heinrich and Koletzko 2010)). Similarities in the protein sequence are

reflected at structural levels, since both desaturases contain a cytochrome b_5-like domain, as well as three conserved histidine-rich domains (HXXHH histidine-box motif) (Cho et al. 1999a) as other membrane-bound desaturases usually do. A schematic representation of the structural and actual membrane attachment is shown in the next page, Figure 10. Finally, as soon as FADS1 and FADS2 were cloned (Cho et al. 1999b), researches could verify that dietary PUFAs downregulated their expression (at least in the liver). Besides, enzyme expression is influenced if essential FA is present on the diet. Therefore, increased expression is achieved upon consumption of FA rich on essential FA (e.g. corn oil). On the contrary, ingestion of food poor in those (e.g. butter), decreases their levels. Furthermore, hepatic activity of both desaturases was suppressed by fasting and induced by re-feeding carbohydrate and when agonist of PPAR (fenofibrate) is used, its expression also increases. However, this seems restricted to the liver. CNS played in "another league" and dietary changes have to be sustained across time to achieve a substantial change on their lipid profile or expression pattern of lipid-regulatory genes (Igarashi et al. 2007a; Hofacer et al. 2011 and Kitson et al. 2012).

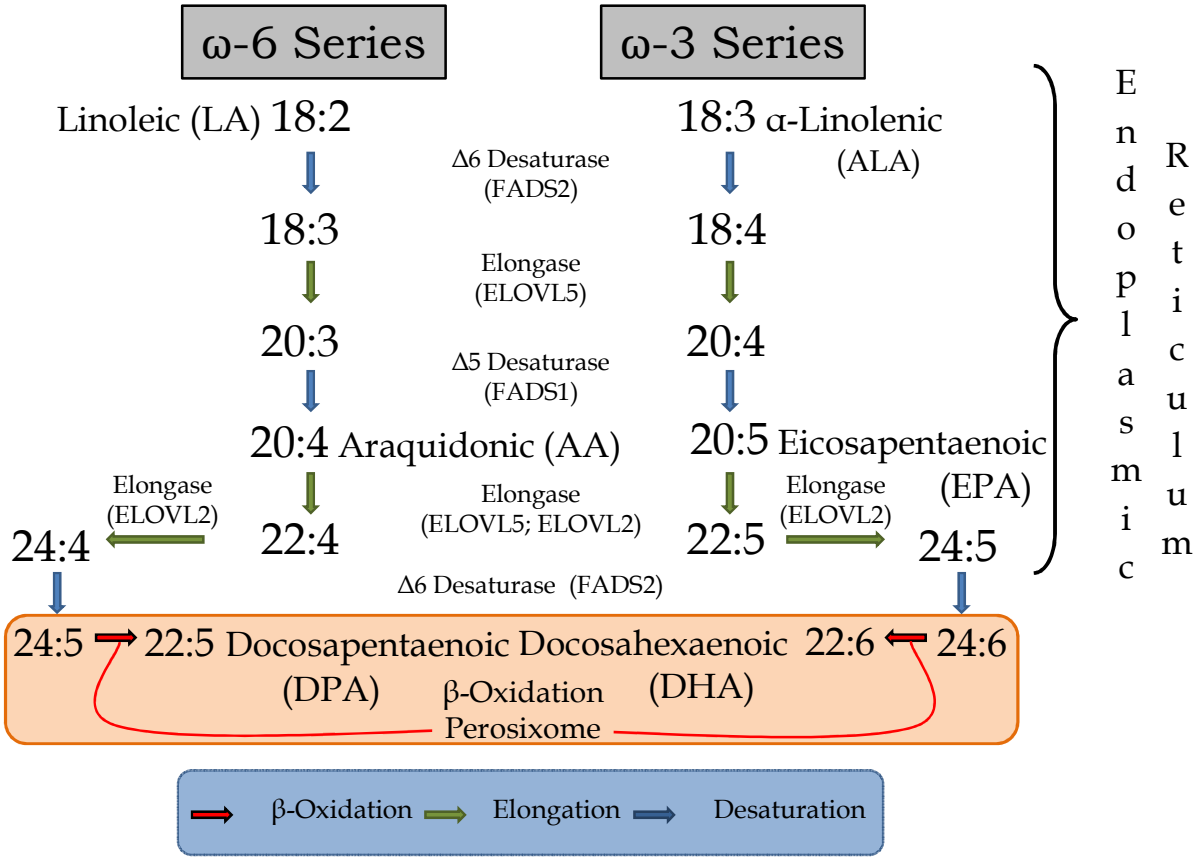


Figure 9. FA *de novo* synthesis. The scheme here represented the most common mechanism for representative PUFAs synthesis from their essential FA precursors, separated into the two *vias* (ω-3 and ω-6). Note that necessary enzymes are shared for both *vias*. Most of the synthesis is achieved in the ER, but for final steps mitochondrial translocation is required.

In mammals, seven ELOVL have been found, being 1, 3, 6 and 7 specific for saturated or monounsaturated FA and 2, 4 and 5 for PUFA ones. The expression pattern along the body differs, since 1, 5 and 6 are ubiquosly expressed (with high levels in liver, testis, adrenal glands and white adipose tissue) meanwhile 2, 3, 4 and 7 has a tissue specific distribution. Although the physiological consequences of such specificity are not clear yet, it is believed to respond to tissue-specific demand of long-chain-PUFA (LCPUFA). Therefore, whereas ELOVL 1, 4 and 7 may act as enzymes controlling the synthesis of LCPUFA as membrane components for optimal

membrane function, the ELOVL 3 and 6 are believed to take part in a more complex system controlling cellular and whole body lipid homeostasis (Guillou et al. 2010). For instance, ELOVL3 expression is under control of glucorticoids, norepinephine and the three isoforms of PPAR. In contrast, ELOVL1 (often named "housekeeping" elongase) liver expression is not regulated by PPARα, liver X receptor or SREBP-1c (sterol response element binding protein-1c) and not even subjected to nutritionally induced changes (Wang et al. 2006) meanwhile ELOVL7 is highly expressed in the kidney, pancreas, adrenal gland and prostate, where was found to be linked to cancer development in the latter (Tamura et al. 2009).

Finally, EPA and DHA have been proposed to have a complete different biosynthetic pathway for some marine eukaryotic and prokaryote microbes (Metz et al. 2001). Those species take advantage of a specialised poliketide synthase enzyme to form them and are very interesting as biotechnology tools for massive, cheap and clean FA production.

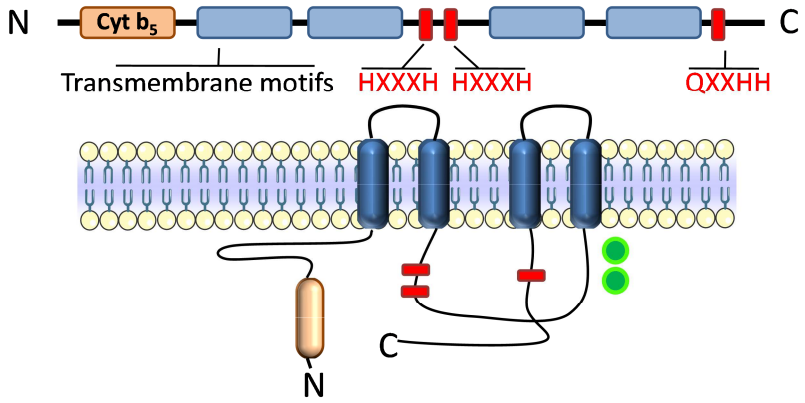


Figure 10. Desaturases structure. Schematic amino acid sequences of membrane-bound fatty acid desaturases from plants, animals and other organisms. All of them had three strongly conserved histidine-rich sequences (blue boxes) with the general motifs HXXXH, HXXHH and HXXHH. These boxes are required for enzyme activity and are separated by membrane-spanning domains that must provide the correct orientation for the active sequences. Many enzymes, including the mammalian FADS1 and FADS2, contain a cytochrome b_5-like N-terminal extension (brown box). Electrons acquired from NADH cytochrome b_5 reductase are transferred to cytochrome b_5 and then to the active site of the desaturase where the redox reaction proceeds through two iron atoms (green circles) that are stabilized by conserved histidine boxes interaction.

1.B2.2.- AA synthesis

Similarly, AA *de novo* synthesis was established long time ago (Sprecher 1981). It shares with DHA most of its enzymes, pathways and locations, being therefore "competitors". But, since AA only possess 2 more carbon atoms and 2 more double bond than its precursor, LA, elongation and desaturation steps comprises one and two steps, respectively. Thus, it starts with a Δ6 desaturation followed by elongation (ELOVL5) and finally a Δ5 desaturation again to form AA. All of these steps take place at the microsomes, in the ER. Nevertheless, AA synthesis is part of a longer process which leads to achieve 22:5ω-6 DPA, in a very similar way than it is done for DHA, hence including delivery to mitochondria to perform a β-oxidation step. This FA, DPA, is interesting since its accumulation in tissues (especially CNS) takes place when insufficient amounts of DHA and its ω-3 precursors are present in the diet, serving therefore as a marker for inadequate ω-3 intake.

Although it is now clear that FADS1, FADS2, ELOVL2 and ELOVL5 are involved in the metabolism of essential ω-3 and ω-6 FA, it is uncertain whether they play key roles (if any) in the synthesis of "non-essential" PUFAS (those of the ω-7 or ω-9), even when early works

(Mead 1958) pointed out the possibility of sharing the same pathways and enzymes. Further research is required to shed some light on this minoritary FA since, for instance, mead acid (C20:3ω-9) is known to be synthesized in animals fed with a PUFA deficient diet and is in fact used as a relevant indicator of such deficiency when accumulated.

Finally, it is important to remark the complex machinery involved in *de novo* synthesis of FA. As explained in this and previous chapter, not only specific enzymes and substrates are required for precise biosynthesis of the desired lipid specie, but also optimal interconnection with different organelles and their orchestrated timing.

1.B3 PUFA Reacylase and phospholipase systems

Delivery after ingestion, maintenance inside a specific PL, inner trafficking and *de novo* synthesis are part of a general mechanism for FA balance. Membrane structure modification by reacylase and phopholipase systems are both crucial to regulate and help to maintain correct levels of specific lipid species and to face both, physiological and pathophysiological conditions.

1.B3.1.- Phospholipase system

A phospholipase is an enzyme that hydrolyzes PLs into FAs and other lipidic substances. There are four major classes, termed A, B, C and D, distinguished by the cleaveage reaction location which they catalyze and therefore, the products obtained. Phospholipase A (PLA) comprises a superfamily of enzymes whose members cleave the *sn*-1 acyl chain (PLA_1) or the *sn*-2 acyl chain (PLA_2), releasing a FA. Phospholipase B cleaves both *sn*-1 and *sn*-2 acyl chains and is also known as a lysophospholipase. Phospholipase C cleaves before the phosphate, releasing DAG and a phosphate-containing head group (commonly, IP_3) which results in intracellular Ca^{+2} increase. Therefore, this enzyme plays a central role in signal transduction, releasing secondary messengers such as those aforementioned in chapter 1.A2 (Figure 5), which are related to multiple events directly, but to even many more indirectly (for an excellent review in PLC see (Kadamur and Ross 2013)). Finally, phospholipase D cleaves after the phosphate, releasing phosphatidic acid and an alcohol. Both types C and D are considered phosphodiesterases.

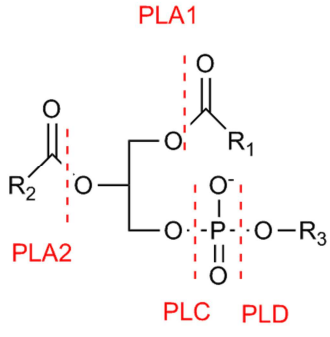


Figure 11. Phospholipases cleavage sites. Cleavage sites for each phopolipase family. Note that an enzyme that displays both PLA_1 and PLA_2 activities is called Phospholipase B.

PLA_2 is the more extensively studied family of phospholipases since both products, FA (specially AA, DHA and EPA) and lysophospholipid, represent first steps in generating important second messengers (whose formation is discussed in section 1.C1.2). It is especially relevant in inflammation and immune responses, besides sleep regulation, pain perception or

GPL-hydroperoxides detoxification and clearance associations. In fact, like other phospholipases (due to space limitation we will only focus on PLA_2), functions and implications related to them raised from the particular products that they generate on upon its activity and which serve as substrates for further synthesis of multiple bioactive lipidic compounds. PLA_2 superfamily is composed by several unrelated protein families with common enzymatic activity (for a review related to CNS, see (Sun et al. 2004)). In the classification based upon the Ca^{+2} requirement for their activity, they can be divided into three categories: i) secretory PLA_2 ($sPLA_2$) that require millimolar concentrations of Ca^{+2}; ii) the cytosolic PLA_2 ($cPLA_2$) that require micromolar concentrations of Ca^{+2}; and iii) the Ca^{+2} independent PLA_2 ($iPLA_2$). Regarding its location, $sPLA_2$ are stored in cytosolic granules or synthesized upon stimulation and then secreted extracellularly whereas $cPLA_2$ and $iPLA_2$ are intracellular enzymes. $cPLA_2$ are located in the cytosol and translocated into the membrane, while $iPLA_2$ are located both in the cytosolic and in the membrane fractions. Other members of the PLA_2 family are the lipoprotein-associated PLA_2 (lp-PLA_2), also known as platelet activating phospholipase and the recently discovered lysosomal phospholipase.

$sPLA_2$ are structurally characterised by their (relative) low molecular weight (14kDa average), histidine and Ca^{+2} requirements in the active site and six conserved disulfide bonds along the sequence. Their functions are diverse, since more than ten subfamilies have been found in different species (Burke and Dennis 2009) but some of the more remarkable are involved in the pathophysiology of inflammatory diseases, and have long been considered "inflammatory enzymes" (Yedgar, Lichtenberg and Schnitzer 2000). Nevertheless, some $sPLA_2$ isoforms have been reported to have a protective, anti-inflammatory potential too (Murakami et al. 2011) and an unambigual role in antimicrobial function and PLs digestion in the stomach (Eerola et al. 2006). For a more extensive study of $sPLA_2$ see (Lambeau and Gelb 2008 and Murakami and Lambeau 2013). Mammalian $sPLA_2$s do not show a distinct preference for particular fatty acids, whereas there is some specificity for certain head groups of the PL substrate: generally $sPLA_2$s show a high activity with anionic PL but some PLA_2 subfamilies also hydrolyze phosphatidylcholine (PC) vesicles (Singer et al. 2002). To date, the direct relevance of $sPLA_2$ in the eicosanoid production remains unclear, but it is true that those enzymes mediated AA release (which can be in an auto or paracrine manner) and accumulation would activate a variety of prostaglandin synthases, lipoxygenases and cytochrome P450 proteins which ultimately raise eicosanoids production.

Lysophopholipids (the other subproduct) can be converted into lyso-phosphatidic acid or acetylated to form platelet activated factor (PAF), a well-known specie that plays key roles in different pathophysiological events (Murakami and Lambeau 2013) and which mediates a feedback loop by the activation of another PLA family member, lp-PLA_2.

On the other hand, $cPLA_2$ (which are classified in six sub families) had longer protein sequences (refelected by 65 to105 kDa ranging weight; for a general classification on the PLA family see (Schaloske and Dennis 2006)) and Ca^{+2} loading is required for a complete functionality (not in the case of γ isoform). Surprisingly, this load is not required for catalysis, but rather for intracellular membrane translocation of the enzyme. Those enzymes have remarkable different specificity for the FA present in the PL *sn*-2 position. Therefore, $cPLA_2\alpha$ has specificity for the AA in this position, whereas $cPLA_2\delta$ is specific for LA-containing PL. In contrast, β and γ isoforms have very little FA discrimination and ε and ζ isoforms hydrolyze both, AA and LA (for a profound review on cPLA topic see (Ghosh et al. 2006 and Schaloske et al. 2006)).

The iPLA$_2$s are Ca^{+2}-independent enzymes and, like the cPLA$_2$s, utilize a serine residue for catalysis. The group consist of six enzymes and the most extensively studied one is the iPLA$_2$β which includes several splice variants which may exert an inhibitory response (Larsson, Claesson and Kennedy 1998). A structural feature of iPLA$_2$ is the presence of several ankyrin (structural anchors) repetitions which may be responsible for protein–protein interaction between monomers. Functionally, these enzymes are not specific with regard to the fatty acid being released but it seems that ATP helps to stabilize the protein (Lio and Dennis 1998). Main functions of this family are related with membrane homeostasis maintenance through its remodelling (reviewed in (Akiba and Sato 2004)). In addition, they play a role in processes connected with the metabolism of reactive oxygen species, at least in macrophages (Martínez and Moreno 2001) and astrocytes (Jianfeng Xu et al. 2003). They have been implicated in membrane transformation during apoptosis (Wissing et al. 1997) resulting in AA release and increasing caspase 3 activation, which could end up with the enzyme cleavage itself (Atsumi et al. 1998). Later on in we will discuss about the susceptibility of PUFAs to be oxidized if they are not effectively protected, so together with observations mentioned before, suggest that ROS could serve as a factor for regulating iPLA$_2$ activity, although the exact mechanism remains to be elucidated.

1.B3.2.- Reacylase system

The deacylation-reacylation cycle maintains a balance between free and esterified FA, resulting in normal membrane integrity and function, including optimal activity of membrane-bound enzymes, receptors and ion channels involved in normal signal-transduction processes. Using acyl-CoAs as donors, GPLs are formed by *de novo* pathway (Kennedy pathway (Kennedy and Weiss 1956)) and modified by a remodelling pathway (Lands' cycle (Lands 1958)) to generate membrane asymmetry and multiple lipid species (for an excellent review on GPL homeostasis see (Hermansson, Hokynar and Somerharju 2011)).

In neuronal tissues, four major enzyme families are responsible for membrane deacylation-reacylation cycle: acyl-CoAsyntetase (ACS), acyl-CoAhydrolase (ACH), acyl-CoAlysophospholipid acyltransferase (ACL) and phospholipaseA_2. The picture below shows a representation of the overall process.

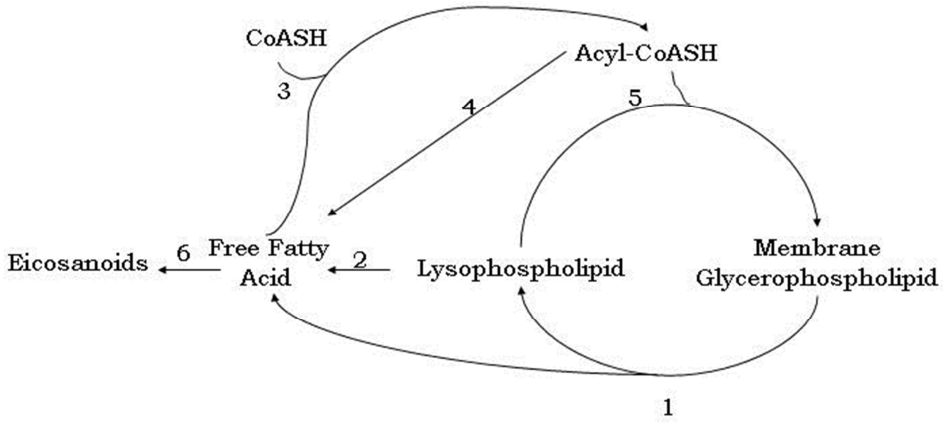


Figure 12. Deacylation-reacylation cycle. (1) PLA$_2$; (2) Lysophoslipase; (3) Acyl-CoA synthetase; (4) Acyl-CoA hydrolase; (5) Acyl-CoA lysophospholipid acyltransferase; (6) Cycloxigenases, lipoxigenases. CoASH is a cofactor required for the activation of a determined acyl group.

A) ACS

ACS converts free FA into acyl-CoA esters. Essentially, all cellular metabolic pathways in which FAs participate firstly require their activation into CoA derivatives (for a profound review see (Watkins 1997)), with a notable exception, pathways for conversion of PUFAs, AA and DHA to bioactive eicosanoids and docosanoids, respectively. Nowadays, 26 ACSs are encoded by the human and mouse genomes, and could be classified according to its FA specificity, being short (2 to 4 carbons), medium (4 to 12), long (12 to 20; the more studied ones, reviewed in (Watkins 2008)), bubblegum (14 to 24, whose name aroused form Drosophila mutant "bubblegum") and very long-chain, (18 to 26) synthetases. Another classification take into account its location within a cell, being therefore, cytosolic, microsomal (ER-resident), mitochondrial or peroxisomal (Watkins and Ellis 2012). The ACS reaction is ATP-dependent (Groot, Scholte and Hülsmann 1976), and in some cases Mg^{2+} is also required for a complete activation (Farooqui, Horrocks and Farooqui 2000). These enzymes are regularly membrane-bound and act on non-polar hydrophobic substrates. Their products (acyl-CoAs) are water-soluble and very powerful detergents. ACS function is especially relevant for the synthesis of unsaturated long chain acyl-CoA, since long term presence of free FA could result on its oxidation, with generation of potentially toxic (but also signalling) peroxides and hydroperoxides.

B) ACH

ACHs represent a group of enzymes that cleave acyl-CoAs into fatty acids and coenzyme A (CoA-SH). ACH relevance is therefore related to control levels of those species, because they participate in a large number of reactions, including lipid synthesis and oxidation, energy metabolism and potential regulation of a variety of signalling pathways (for a review see (Kirkby et al. 2010)). Although those enzymes have been less investigated than ACS, ACHs were localised in the cytosol, mitochondria, peroxisomes and ER. Within these compartments, they are distributed in a wide range of mammalian tissues, being detected in brain, liver, kidney, heart, lung, steroidogenic and brown adipose tissues, where they exert important functions, especially in the brain where its hydrolyzing activity is much higher than in any other organ in the body. Thirteen mammalian ACH genes are divided into two types, which differ according to the structural organization of the catalytic domains: type I enzymes contain an α/β-hydrolase domain and type II enzymes comprise one or two “hot dog-like” thioesterase domains (Han and Cohen 2012). Human orthologs are located within a very narrow region of chromosome 14 as a gene cluster (Hunt et al. 1999). Their activity is regulated by substrate for some of the family members, causing an inhibition, but they seem not to be affected by of non-esterified FA accumulation. Some of the family members have been shown to be under PPARs (Hunt et al. 1999), SREBP (especially long-chain, brain resident ACH (Takagi et al. 2005)) or HNF4α (Dongol et al. 2007) control. Finally, also noteworthy, a knock out animal model of ACOT7 (Ellis, Wong and Wolfgang 2013) was found to exhert hyperactive neural response when fasting and some kind of neurodegeneration, reflected by locomotor defects and increased GFAP inmunoreactivity in older animals, pointing its relevance in neuroprotection.

C) ACL

ACLs acylates (insertion of an acyl group) the *sn*-1 and/or the *sn*-2 positions of a given lysophopholipid. Because there are many species of GPL differing in the phosphoryl head groups, the fatty acids in chain lengths and degrees of saturation, many ACLs should exist and participate in both, *de novo* synthesis and remodelling. High selectivity for the activated FA (therefore, acyl-CoAs) desaturation degree and length is present in a particular enzyme. In general,there is higer prevalence for saturated (or monounsaturated) acyl-CoAs insertion in the *sn*-1 position of a given GPL, meanwhile, in the *sn*-2 position acyl-CoAs is more likely polyunsaturated. Combined actions of that enzyme family and the PLA_2 family would finally produce the great variety of GPL. ACL activities are widely distributed in various cells and tissues, and are tightly bound to microsomal and plasma membranes (for a review on ACL topic see (Shindou et al. 2009)).

All in all, membrane remodelling is a tightly regulated mechanism. In "resting" cells, one can assume that reacylation dominates, and hence, the bulk of cellular FA is found in the esterified form. But, upon stimulation (which could be caused by a myriad of factors), transient dominant reaction is the PLA_2-mediated deacylation, which results in dramatic releases of free FA (specially signalling relevant AA and DHA) that is now available for exerting its particular function (e.g. eicosanoid or docosanoid synthesis). However, activation conditions only last for a while (basics of any signalling event) and reacylation dominates again until replenishment of the intracellular pools of a particular FA is achieved.

1.B4 Essential FA dietary requirements

Essential FAs are required for a normal cell function and serve as a substrate/scaffold for further FA synthesis, if a specific need or depletion occurs. So, international organizations, nutritionists and medical doctors published some dietary recommendations for general healthy subjects (in different status; i.e. pregnancy, development, aging), but also for disease and to prevent them.

In a typical western society, adult intake ranges 1-2 g/day of ALA, meanwhile LCPUFAs is somewhere around 150-300mg/day (Lauritzen et al. 2001). In Spain, ω-3 FA consumption is approximately 380 mg/day (340 and 40 mg/day of DHA and EPA respectively (data obtained from Ministry of Agriculture, 2005)), the majority (67%) due to blue fish (tuna, sardine or salmon) consumption. Spanish dietary essential FA (ALA and LA) intake range between 0.4 and 3.4 g/day for ALA and 4 to 29 g/day for LA (with average values of 1,2 and 12 g/day for ALA and LA respectively (Sala-Vila et al. 2011)).

Omega 3 index (sum of DHA and EPA FA percentage in erythrocytes, extensively used in cardiovascular research) is remarkably high in Spanish population in comparison with other western countries (e.g. 7,1% in Spain *vs* 4,9% in USA-excluded Alaska Inuit population for whom reaches up to 20%). However, is lower than what is estimated for the highest marine product consumers worldwide (e.g. Iceland, Norway, Korea or Japan). Tables in the next page represent average values for FA daily intake recommended for healthy subjects (USA Institute of Medicine in 2002 (Trumbo et al. 2002)).

Omega 3 and 6 Essential FA Daily Dietary Requirements					
Vital Stage	Age(years)	Ω-6 (LA)		Ω-3 (ALA)	
		Male	Female	Male	Female
Babies	0-12 months	4,4*	4,4*	0,5*	0,5*
Childs	1-3	7	7	0,7	0,7
Childs	4-8	10	10	0,9	0,9
Childs	9-13	12	10	1,2	1
Teenagers	14-18	16	11	1,6	1,1
Adults	19-50	17	12	1,6	1,1
Elderly	≥ 51	14	11	1,6	1,1
Pregnancy	Any age	-	13	-	1,4
Breastfeeding	Any age	-	13	-	1,3

* Breast milk from a healthy woman (at least following above mentioned recommendations) provides enough ω-6 and ω-3 PUFAs. Data represented daily consumption in grams.

Table 4. Omega 3 and 6 essential FA dietary requirements.

More recently, WHO (World Health Organisation) has established a daily ingestion for healthy adults (included non-pregnant or non-breast feeding females) of around 0,250 g/ of EPA+DHA summation. This recommendation does not specify individual dosage for those FA, due to the lack of scientific relevant evidence. However, they suggest a maximum value (2 g/day), possibly due to lipid peroxidation and cytokine production affectance ("Fats and Fatty Acids in Human Nutrition. Report of an Expert Consultation." 2010). In this line, Mozaffarian and collaborators (Mozaffarian and Rimm 2006) showed, thanks to a risk-benefit study, that more than 500 mg/day of DHA+EPA intake has no net effect in diminishing cardiovascular risk. On the other hand, in 2009 EFSA (European Food Safety Authority, (European Food Safety Authority 2009)) proposed the same quantity for long ω-3 PUFAs as the labelling reference intake, but they suggest ALA (2g/day) and 10g/day for the ω-6 PUFA LA consumption too. Even so, differences in dietary recommendations reflect different nutritional purposes, since some advices are proposed to correct clinically evident deficiency in a given population, meanwhile other are based on cardiovascular and/or neurodevelopment health.

However, controversy arises, since some international organizations suggest avoiding intensive fish intake (especially bigger ones like tuna, emperor or big sharks) during pregnancy, breastfeeding and early childhood due to high risk of contaminants potentially present on them (toxic heavy metals and related species, particularly methyl mercury) (Mozaffarian and Rimm 2006). Anyway, this could be avoided by tiny blue fish ingestion (e.g. sardine or mackerel, since large and long-lived animals could accumulate those toxins) or by biotechnology-produced algae. The picture below is just to have an idea how nutritional status, regarding FA, vitamins E and C has evolved along humanity.

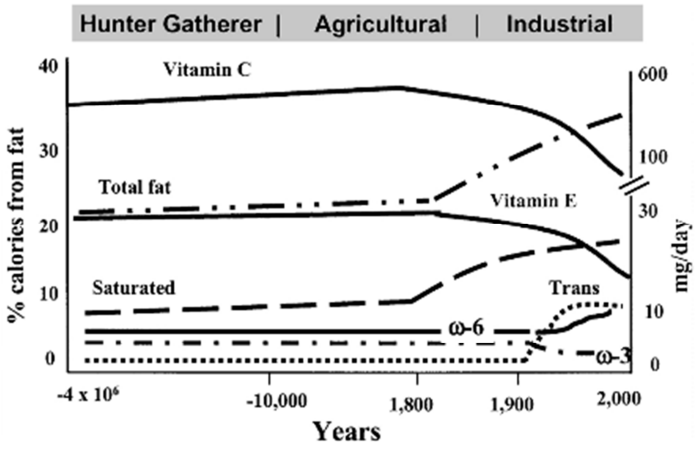


Figure 13. FA and antioxidants intake along human evolution. Hypothetical scheme of fat and fatty acid (ω-3, ω-6, *trans* and total) intakes (as % of calories from fat) and intake of vitamins E and C (mg/day) along human evolution Copied from (Simopoulos, Leaf and Salem 1999).

1.B4.1.-ω-3 defiency

Taking into account western society ω-3 FA consumption plus limited endogenous synthesis (currently under debate for CNS tissues), deficiency could arise both, on disease or by nutritional unbalance. However, this is hard to prove because i) there are no clear specific recognized symptoms for such deficiency; ii) ω-3 and ω-6 interdependency and interconnectivity could, up to an extent, mask such unbalance. However, impaired growth, reproductive disturbances and skin lesion are mostly related to ω-6 deficiency (Kremmyda et al. 2011). On the other hand, dysopsia and abnormal electroretinogram could point a specific ω-3 unbalance. Even so, the most noticeable marker for ω-3 deficiency is the accumulation of ω-6 long chain PUFAs, particularly ω-6 DPA (22:5ω-6) (Neuringer et al. 1986) and ω-6 adrenic acid (22:4 ω-6). The opposite (ω-6 deficiency), is not normally reflected by a PUFA ω-3 accretion. Finally, a concomitant deficiency (ω-3 and ω-6) would raise mead acid (C20:3ω-9). In general, even if ω-3 deficiency is hard to find, based on their potential positive outcome, some dietary recommendations regarding optimalω-3 status are offered in different conditions.

Finally but also remarkable, it is the fact that an unbalance in the ω-3/ω-6 dietary intake could enhance different signalling pathways, and therefore modulate responses to various stimuli, as recently demonstrated in terms of learning and memory processes (Hajjar et al. 2012) or inflammation (Liu et al. 2013).

1.B4.2.-Pregnancy and development

Since Lamptey and co-workers showed in 1976 that ω-3 deficient rats had decreased learning abilities (Lamptey and Walker 1976), lots of different test had also pointed out behavioural, olfactory or visual disarrangements in rats, mice or rhesus monkeys under such deficiency. Anyway, it is difficult to extrapolate those results to human infants and, in fact, some authors suggest that several of the differences encountered in early development stages are faded out along lifetime (Agostoni et al. 1997). In any case, most (80%) of the brain DHA accrues in the human foetus from week 26 until term (Sabel et al. 2009). The presence of LCPUFA-specific placental FABP (Fatty acid binding protein) as well as increased accretion of intrauterine DHA could determine why maternal DHA depots seem depleted after pregnancy (Lauritzen et al. 2001). Therefore, it is strongly recommended for preterm infants to be fed with DHA

supplements to improve their cognitive (Agostoni et al. 1997) and visual (Carlson et al. 1996) conditions, whether healthy termed ones should do, is still controversial.

1.B4.3.-Cardiovascular diseases

From long time ago (1965 by Sir Austin Bradford), outstanding evidences showed lower cardiovascular risk when high ω-3 index exist *in vivo*. Potential mechanisms for this reduced risk included anti-inflammatory, anti-thrombotic, antihypertensive, hypo-triglyceridemic and antiarrhythmic properties found in ω-3 FA rich diets, particularly in long chain ones. However, some recent meta-analyses studies of randomized trials pointed out low significance related to prevention (Rizos et al. 2012, Chowdhury et al. 2012 and Chowdhury et al. 2014), seeming that when damage is done, ω-3 PUFAs are beneficial to reduce its outcome but it fails preventing it when a balance diet is already present in a healthy individual. In any case, government health institutions still recommend two fish pieces per week in a balanced diet aimed to increase cardiovascular protection.

1.B4.4.- Neurodegenerative diseases

Studies on ω-3 function and levels in neurodegenerative diseases have been extensively performed, particularly in Alzheimer, Parkinson and some dementias (for reviews see, (Calon 2011; Sydenham, Dangour and Lim 2012; Saugstad 2006). In contrast to cardiovascular disarrangements, ω-3 supplementation seems to offer a preventive neuroprotection, especially in Alzheimer and mood disorders where adequate ω-3 correlates with a better cognitive function retention and better behaviour (Calon 2011).

Regarding amyotrophic lateral sclerosis (ALS), there is a recent paper that shows a deleterious effect in EPA supplementation diet in a wide used model of disease, copper zinc superoxide dismutase (with a glynine/alanine substitution in the 93 amino acid, SODG93A) overexpersion (Yip et al. 2013). Authors related this dimished survival to increased oxidative stress and accretion of 4-hydroxy-2-hexenal (HHE) in activated microglia. Other studies with valpronic acid (a FA acting as histone deacetylase inhibitor that showed antioxidative and antiapoptotic properties and reduced glutamate (Glut) toxicity in preclinical studies) failed to prolong lifespan in both, animal model (Crochemore et al. 2009) and patients (Piepers et al. 2009). Interestingly, the combination of a high intake of PUFAs and vitamin E has been related with a 50–60% decreased risk of developing ALS, and these nutrients appear to act synergistically (Veldink et al. 2007). Similarly, a very recent publication (Fitzgerald et al. 2014) epidemiologist focused also in the possible relation of dietary consumption of ω-3 FA and the risk of ALS development or a better outcome after diagnosis and, for both issues, higher levels of dietary ω-3 correlated with reduced ALS development probability, and also importantly, once diagnosed those patients had better progression. All in all, those researches suggest a possible prevention and better outcome after ω-3 PUFAs consumption for various neurodegenerative diseases, and may even be better if those levels are increased concomitant with substantial autoxidative protection.

1.B4.5.- Sex dimorphism

Data in Table 4 showed different FA necessities along a lifetime for male and female individuals. This is completely reasonable, and is believed to be linked to differential

peroxisomal β–oxidation rate and/or hormone synthesis and processing for both sexes. In fact, this could rely on the very different adipose tissue organization (reviewed in (White and Tchoukalova 2014 and Stevens et al. 2010)). Studies form professor Burdge group (Bakewell, Burdge and Calder 2006 and Childs et al. 2008) pointed that, in general, female have greater DHA synthesis from its precursors (possibly by stimulation at the transcriptional level) and circulatory (plasma) levels than male. From works studying women using oral contraceptives (Magnusardottir et al. 2009) or hormone-replacement therapy (Giltay et al. 2004b) and a study of sex-hormone treatment in trans-sexual subjects (Giltay et al. 2004a) was clear that hormone function had very relevant consequences in PUFA handling.

Also important, in response to dietary manipulation, ω-3 metabolism seems to be affected by gender, since desaturases Δ5 and Δ6 changed its activity and expression (Extier et al. 2010) (upregulated in female liver in response to ω-3 replenishment diet) as well as PUFA-related synthetic machinery, having increased Δ4 desaturation index (DHA/ω-3DPA ratio) for females too (Alessandri et al. 2012). Importantly, again CNS PUFA-synthetic enzymatic machinery was more difficult to be affected, and no differences were found regarding sexual dimorphism (Extier et al. 2010). Furthermore, the opposite occurs; FA synthesis is dependent on the hormone production, at least in neuroblastome cell line (Alessandri et al. 2008) or an elegant animal model of ovarictomized females treated with different dosage of 17-β estradiol (Alessandri et al. 2011) unveiling sex a as relevant factor regarding PUFA metabolism.

1.B5 PUFA and Central Nervous System

1.B5.1.- Delivery across brain blood barrier

As stated before, FA are important substrates for energy supply but also relevant as buildings block for membrane synthesis and signalling mediators (discussed in the next section). This is especially relevant in such a complex “orchestator” apparatus as the CNS, which is the second lipid accretion tissue (after adipose) whose PUFAs accounts up to 20% of dry weight (Owada 2008). In fact, there is an entire evolutionary theory which points out the relevance of shore based diets, particularly rich in DHA, trying to explain hominid encephalization burst (Cunnane et al. 2007). So, taking into account that particular insulation issues that apply for CNS and *in situ* synthesis which may not be sufficient (DeMar et al. 2006), we will discuss here how FA are transported to and passed through brain-blood barrier to overcome PUFA needs at CNS.

Blood-brain barrier (BBB) is the cellular structure that keeps CNS sectioned from the rest of the whole body and maintains cerebrospinal fluid (CSF) and blood separated. It occurs along all capillaries adjacent to CNS and could represent up to 12 m^2 surface in an adult human (Abbott et al. 2010). It consists of tight junctions (zipper-like) and high-density cells restricting passage around the capillaries and do not exist in regular circulatory system. Those endothelial cells, pericytes and astrocytes restrict the diffusion of microscopic particulae and large or hydrophilic molecules into the CSF, while allow diffusion of small hydrophobic molecules (O_2, CO_2, hormones, etc). Therefore, for crossing BBB, different epithelial walls and even cell types must be intersected. A schematic representation of BBB is shown below.

After a postprandial peak, plasma levels of triglycerides in healthy subjects are maintained stable. They are mainly distributed as sterificated lipids complexed with lipoproteins but there is a controversy on whether unesterificaded PUFAs are sufficient or not to maintain CNS lipid levels, in order to achieve their well-known neuroplasticity capacities (Purdon, Arai and Rapoport 1997). More than twenty years ago it was demonstrated that lipoprotein receptors are expressed at brain capillaries (Méresse et al. 1989) and that they show modulator capabilities related to PLs transfer, at least in those isolated brain capillaries. Nowadays it is believed that only unesterified forms are selectively incorporated (Rapoport, Chang and Spector 2001). Thus, it is demonstrated that dietary intake of PUFAS influence plasma derived levels, and that those indirectly influence brain PUFA metabolism, so BBB is unambiguously overcome by dietary fatty acids.

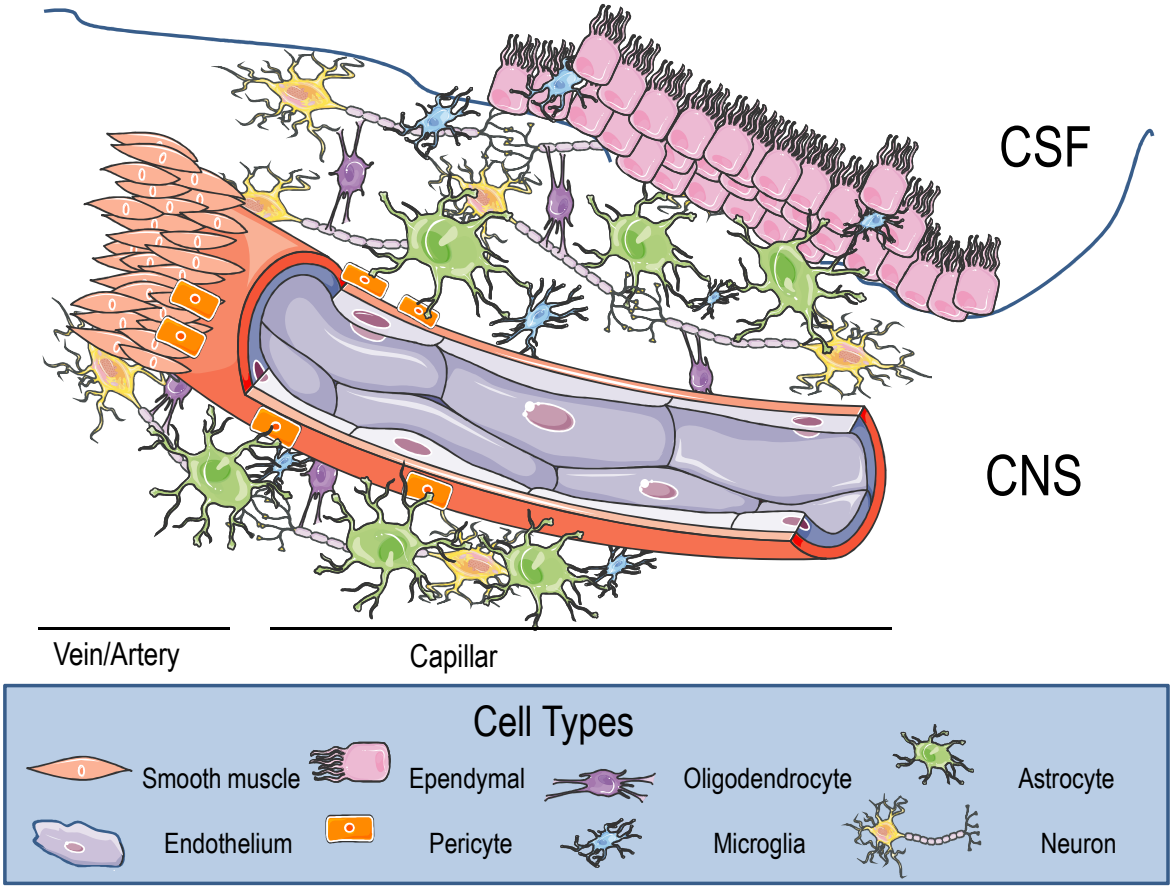


Figure 14. Blood-brain barrier scheme. Cell types encountered in the BBB and their relationships (CSF, cerebro-spinal fluid). Not to scale

It is believed that up to 3-5% of AA and 2-8% of DHA is daily replaced form rat brain GPL by the unesterified PUFAs presented in plasma (Rapoport, Chang and Spector 2001). The actual quantity in humans is estimated to be 0,3% for AA, but higher amounts are believed to be achieved by *de novo* synthesis through elongation and desaturation processes of their precursors or by deacylation and reacylation processes (see chapter 1.B2 and 1.B3, respectively). Dietary supplementation of human subjects with DHA (but also for AA) undoubtedly boost plasma levels of non esterificated DHA, remarkably with no net increase of serum lipoproteins, (Conquer and Holub 1998) but how this increase is affecting brain levels (whether its synthesis form ALA is significant) or which forms (stereoisomers specificity, preferential bioavailability, etc) are more prone to be uptake by the brain remains unclear.

Using DHA as a paradigmatic PUFA example, current models for delivering FA into the brain via plasma include passive diffusion of non-esterified DHA through the BBB (Spector 2001) and/or uptake by HDL or LDL containing DHA lipids via specific lipoprotein receptors on the luminal membrane of endothelial cells (Balazs et al. 2004). The first possibility, is supported by the of endothelial lipase that was found in brain capillary endothelial cells (Sovic et al. 2005) which was regulated (negatively, both protein and mRNA levels) when PPARα and γ agonists were applied. Surprisingly, those lipases possess a remarkable specificity for DHA positioning within the PL (Chen and Subbaiah 2007). Also remarkable is the preferential cleavage of ALA over LA by Δ6 desaturase present in endothelium (Cunnane et al. 1994) and how its evolution over a lifetime changed opposed to liver resident ones, Endothelium type FADS2 activity is enhanced during development and scarce in adults, contributing to brain-development-related DHA necessities.

Another possibility is that a sophisticated selective carrier mechanism exists for the transportation of ω-3 FA into the brain (Beisiegel and Spector 2001), including a BBB transport system that is highly selective for PUFAs esterified into PL such as DHA lysoPC (Bernoud et al. 1999). In fact, a recent study has demonstrated that lipoprotein particles can cross the BBB, at least in Drosophila (Brankatschk and Eaton 2010), suggesting that the lipoproteins could act as transporters of therapeutic lipid molecules across the BBB for the treatment of brain disorders.

Finally, in a recent published paper (Chen and Subbaiah 2013), Chen and collaborators proposed that DHA stereoespecificity in a given PL (being at *sn*-1 or *sn*-2 positions) may determine its destination. They claim for the DHA in *sn*-1 position at the PC to be more "protected" against the reacylase/deacylase cycle and therefore have more possibilities to be released at the BBB and, ultimately, uptake.

1.B5.2.- In situ synthesis

For a long time, neurons were believed to lack the ability to synthesize DHA from its ω-3 FA precursors (Igarashi DeMar et al. 2007b). In fact it wasn´t until recent studies (Kaduce et al. 2008) that PUFAs *in situ* synthesis was solely attributed to astrocyte action (Kaduce et al. 2008; Moore et al. 1991) rather than to a neuronal origin. In any case, this synthesis is believed to be scarce, so dietary DHA supplements in the forms of PL or/and TAG were (and still are) usually recommended in order to promote brain health across lifetime, but specially during early development (Guesnet and Alessandri 2011) and elderly.

For optimal *in situ* synthesis, adequate amounts of precursors are needed, but also large batteries of different enzymes are required (commented in chapter 1.B2). Nevertheless, studies from Dr Rapoport's group (DeMar et al. 2005; DeMar et al. 2006) reveals that the contribution of *in situ* synthesis for both AA and DHA to brain PL is indeed very little. In those works, both with a non-deficient diet for the FA family studied, they estimated *de novo* synthesis represent less than 1% for both AA and DHA. The majority of unesterified precursors that cross the BBB undergo through β-oxidation (59% LA and 67% ALA, respectively) or acylation processes

(11% of LA, 12%ALA, respectively) rendering *de novo* synthesis residual and pointing out that delivery of final products (the required FA itself on its multiple forms; e.g. PC, PS, lysoPL, etc) but not its precursors must be the mechanism that avoids PUFAs depletion in the brain. In fact, a recently published review (Rapoport, Ramadan and Basselin 2011) using *in vivo* radiolabeled [1-^{14}C]DHA intravenously perfusion (for both rats and humans) they pointed out that the incorporation rate of DHA to brain equals its rate of metabolic consumption suggesting that if an increase need is required, *in situ* synthesis could be insufficient.

All in all, those data pointed out how complex regulatory machinery operate for FA delivery at the BBB and that FA destination after crossing differs enormously between them, further research is needed to clarify whether in physiological conditions, lipoproteins or phospholipases at the BBB are key factors to meet CNS FA delivery and requirements (it seems so) or, in the contrary, *in situ* synthesis (regardless neuronal or astrocytic origins) is sufficient. How this weak equilibrium is disrupted in disease and potential therapeutics regarding delivery and upgrading local synthesis are also major points to be elucidated.

1.B5.3.- Neurotransmitting

Vesicle formation, delivery to synapsis, exocytosis and substrate clearance represented essential compulsory processes for a correct neurotransmission. Synaptic proteins could be enhanced in order to optimize neurondialog, the basic tenet of the CNS. However, numerous events could interfere these chemical information interchange (e.g. oxidative stress, cytoesqueleton deregulation, membrane deareagments, among others) causing detrimental or missunderstanding connectivity and could even lead to denervation and ultimately, neuron death. In this sense endocannabinoid system demonstrated (Berghuis et al. 2007) to be very relevant for axon guidance and synaptogenesis. Further, astrocytic support fuction (commented in the next chapter) is also crucial in this delicate system. ω-3, but also ω-6 PUFA could enhance synaptic membrane expansion (Darios and Davletov 2006) with an increased expression of syntaxyn 3 (STX3, a protein involved in vesicle formation and exocitosis in synapsis) and seems clear the PUFA influence in the SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) protein complex formation and stability (García-Martínez et al. 2013 and Lang, Halemani and Rammner 2008; for an excellent revision on the FA-SNARE regulation see (Davletov, Connell and Darios 2007)). In fact, the SNARE proteins complex are believed to provide the required specificity for vesicle docking and sorting as well as they represent the minimal basic machinery required for membrane fusion (Weber et al. 1998). Finally, since membranes must adopt different curvatures during fusion, the commented membrane structural actions of PUFA are also crucial to complete exocytosis, as showed by cone-shaped lipids when they may favour the appropriate membrane geometry (reviewed in (Chernomordik and Kozlov 2003).

In this sense, deacylation-reacylation machinery was relevant, since, in cultured neurons (Marszalek et al. 2005), overexpression of ACS6 promoted an increased DHA-CoA accumulation (respect to oleic acid or AA) and, ultimately, neurite outgrowth (previously found for ACS2 too (Marszalek et al. 2004)). Interestingly, silencing its expression (by RNA interference) resulted in a decreased neurite outgrowth of various neuronal cell lines (Kim et al.

2009) and futher, *dAcsl* (ACS4 orthologue in drosophila) was found essential for correct axonal targeting and normal synaptic vesicle formation (Liu et al. 2011).

Also importantly, expression and function of Glut receptors (e.g. the glial excitatory amino-acid transporter 2, EAAT2, which is specially relevant since its excitoxicity involvement in the ALS pathogenesis and will be commented in section 1.D2.3), could be influenced by AA (Zerangue et al. 1995), causing a reduced Glut recovery. This mechanism seems to be triggered by AA itself, but whether PLAs play a role by influencing local membrane release is still controversial in this model. However, this opened the door for a more coiled synaptic-membrane-structural PUFA action. Therefore, all in all, PUFA action and regulation on the continuous synaptic remodeling represents important points for a correct neurotransmission and, possibly more important, promising therapeutic intervention in various diseases.

1.C PUFA Pathophysiology

1.C1 Inflammation induction and resolving phase

1.C1.1.- Inflammation partners

Since inflammation could play a key role in neurodegenaration, and particularly in ALS (reviewed in (Evans et al. 2013)), the importance of this phenomena should be discussed. Neuroinflammation refers to the rapid reaction on the CNS, through activation of the resident immune cells, in response to infections, trauma, toxins and ischemia, among others (Frank-Cannon et al. 2009). It is a complex combinatory mechanism of acute and chronic responses in which all CNS population (neurons, astrocytes and microglia) and infiltrating leukocytes take part. Innate immune response in CNS is responsible for microglia activation resulting in release of pro-inflammatory cytokines (Interleukins-IL-1β, -IL-6- and TNFα- among other), chemokynes (e.g. prostaglandin E_2, PGE_2) and nitric oxide (and other oxidative derived species such as superoxide and peroxinitrite), but also rescue anti-inflammatory signals (IL-4, IL-10, nerve growth factor-NGF-, BDNF, insulin-like growth factor-1, glia derived neurotrophic factor-GDNF-, leukemia inhibitory factor-LIF-, ciliary neurotrophic factor-CNF-, among others); for a review see (David and Kroner 2011). (Consilvio Vincent and Feldman, 2004)

Autoinflammation is a relative new concept (McDermott et al. 1999). It was proposed as an emerging family of clinical disorders which were characterized by seemingly unprovoked inflammation episodes without high number of neither autoantibodies nor T-lymphocytes activation. From this point, various diseases were related to developed the “autoinflammatory” imprint (recently reviewed in (Standing, Omoyinmi and Brogan, 2013)), like in the case of some granulomatous diseases, as chronic granulomatous synovitis with uveitis and cranial neuropathy (Blau syndrome) and Crohn’s disease, among others. Interestingly, both diseases have shown a disregulation in the NF-κB activation, but, as other autoinflammatory diseases, aetiology seemed more complex than initially (genetically) though. Also importantly, some multiple sclerosis patients showed complications compatible with autoinflammation episodes (Kümpfel et al. 2007) and, more recently, a significant host predisposition (Kastner, Aksentijevich and Goldbach-Mansky 2010) is proposed to open the “classical” definition to a broader one.

Focusing in neuroinflammation-related cell types, astrocytes play crucial roles supporting neuron activity, both under physiological conditions and disease. This cell type, with an ectodermal origin, could grow, in specific brain regions, up to ten times more numerous than neurons in the CNS, although in the whole brain, neuronal (counted as NeuN positive cells) and nonneuronal cells have almost equal number (Azevedo et al. 2009). Since they envelop synaptic terminals, astrocytes clear most of the synaptic neurotransmitters (Glut, γ-Aminobutyric acid -GABA- and glycine, among many others). They provide throphic factors (e.g. brain derived neurotrophic factor, BDNF, and tumor necrosis factor-α -TNFα), energy (lactate and FA among others) and precursors (e.g. adenosine, purines) for neuron optimal function. Astrocytes are not immune cells per se, but can, in specific conditions, contribute to the immune response (reviewed in (Philips and Robberecht 2011)). Evidences of astrocyte regulated intracellular Ca^{+2} concentration rise/fall accompanied firing synapses support the idea that astrocyte excitability is important not only for astrocyte-astrocyte crosstalk but also for astrocyte-neuron ones. Altogether, those findings gave rise to the “tripartite synapse theory” (Araque et al. 1999), in which not only pre and post-synaptic neurons are important for optimal synaptic function, but

also astrocyte population surrounding them. Ca^{+2} balance is also noteworthy related to ALS, since it is known to be deregulated and plays a key role on pathophysiology of the disease linking ER and mitochondria (recently reviewed in (Prell et al. 2013)). Finally, astrocytes are also responsible for glucose and FA uptake from the blood across BBB and therefore they regulate CNS energy flow (which is an important issue in ALS (Dupuis and Oudart 2004)).

Microglia are the resident macrophages of the CNS and have two main distinct roles: first, they help astrocyte population to support neurons in a concerted mechanism and the second is related to its immunological role, which could be cytotoxic-linked. They derive from primitive myeloid progenitors and, resembling to them, recent studies classify microglia according to its role as neuroprotective or detrimental for CNS (Henkel and Beers 2009) being M1 related to the latter and M2 to the former. Hence, their inflammatory outcome is believed to be based in both, by intrinsic factors and by microglia-neuron-astrocyte cross-talk. When activated, microglia undergoes morphological changes to an amoeboid shape and proliferates. They also migrate toward injured areas; release various powerful soluble factors and phagocyte foreign substances or unwanted self debris.

Like microglia, astrocytes also exhibit double edge sword behaviour. However, under neuroinflammation, shift from proinflammatory towards anti-inflammatory processes is poorly understood until date. When activated, they result in astrogliosis, usually identified by glial fibrilary acidic protein (GFAP) and aldehyde dehydrogenase-1 family-member-1 expression, concomitant with and cell body hypertrophy, and which could lead to pro-inflammatory cytokines release (mostly like microglia ones, but also producing neurotranmiters like Glut) and/or anti-inflammatory ones (transforming growth factor-β, IL-6, Interferon-γ, among others) (a complete review in astrocyte function could be found at (Sofroniew and Vinters 2010). Finally, other players in the inflammatory game are T cells, which could infiltrate CNS and modulate its outcome (regarding ALS those are a relevant papers (Chiu et al. 2008; Beers et al. 2008)) and oligodendrocyte and perycyte population, whose action regarding neuroinflammation is poorly understood yet.

It is important here to bear in mind that inflammation is a physiological response to an insult and, in general, acute neuroinflammation is a protective mechanism trying to “fix the problem” up to a extent, whereas chronic inflammatory status produces long-lasting and self-perpetuating mediators that remain after the insult, hence being more often related with the detrimental outcome (Glass et al. 2010). This is a plausible explanation why those cell populations could both, help to diminish or to exacerbate inflammatory damage, depending on how profound the aggression is.

Also noteworthy is the difference between anti-inflammatory and resolving actions of a specific molecule. Resolution is a relatively recent term (Serhan et al. 2007) and is defined, in contrast to anti-inflammatory species, by its action not only serving as agonist for inflammation responses (e.g. lowering lymphocyte infiltration and/or diminishing adhesion-molecule synthesis) but also by promoting active processes to achieve homeostasis/*catabasis*-return from disease (e.g. promoting uptake and clearance of apoptotic cells, stimulating expression of antimicrobial defense mechanism). Anyway, in general terms for all cases, first steps after an insult involved generation of proinflammatory mediators (classically, leukotrienes-LT- and prostaglandins-PG, mostly AA-derived) to be substituted afterwards by PGE_2 and PGD_2 which gradually induced anti-inflammatory and pro-resolution key enzymes (by LOX upregulation) which, in turn,

generate lipoxins, resolvins and protectins (Levy et al. 2001) for helping resolution. This new lipid profile is termed "lipid mediator switch" and is crucial for inflammation resolution. In fact recent studies (Schwab et al. 2007) have shown that COX-2 inhibition (classically pro-inflammatory, but signalling helpful) results in a delayed cleaning, and, therefore, slower recovery.

Broadly speaking in CNS, the mechanism could start by TNFα and Ilβ release by microglia and astrocyte followed by its binding to their specific receptors and upregulation of gene transcription via NFκB pathway (Blais and Rivest 2001). Among them, AA signalling cascade genes. Hence $cPLA_2$ and $sPLA_2$ in one side, and cyclooxygenases, lipoxygenases and thromboxane synthases (COX, LOX and TXS respectively, discussed below) release and modify AA to form powerful lipid species (generally termed eicosanoids for its 20 carbon content: LT, PG, prostacyclines -PGI- and tromboxanes -TX- in general). And, on the other hand, phospholipases (specially plasmalogen-selective phospholipase A_2 -PlsEtn-PLA_2) could also release DHA (and/or EPA) activating docosanoid synthesis which could, in turn, counteract those outcomes, since most of their derivatives (maresins, neuroprotectins, neuroprostanes) and closely related EPA-derived eicosanoids and dihomo-gamma-linolenic acid (DGLA) derived ones (less prominent) exhert anti-inflammatory properties. Below, there is a scheme of the aforementioned process and the cell types involved, and in the next chapter we will say some words about the synthesis of those derivatives, generally termed oxolipids.

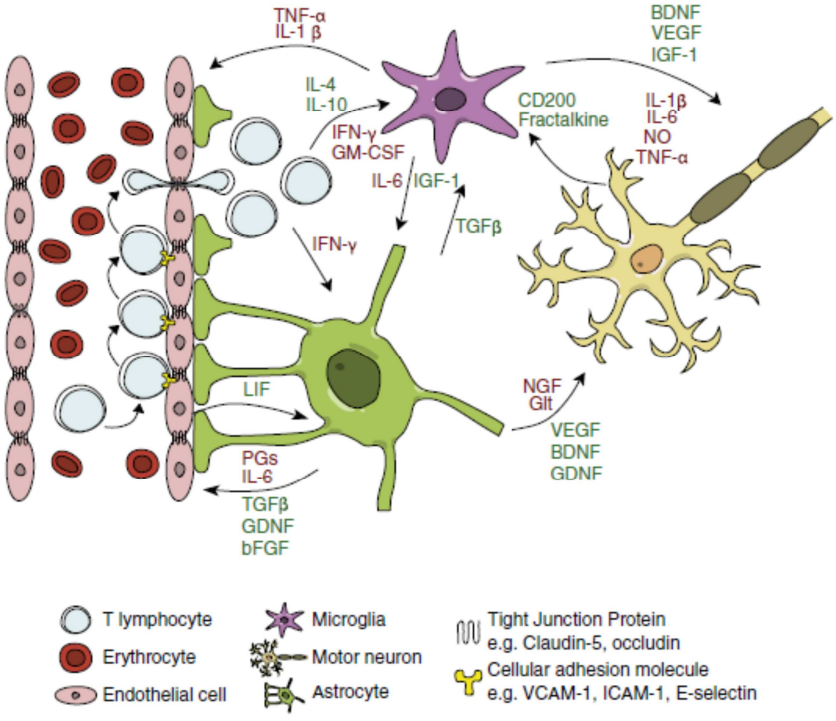


Figure 15. Inflammation partners in CNS. Inflammatory-derived and neurovascular changes in amyotrophic lateral sclerosis. VCAM-1 Vascular Cell Adhesion protein 1; ICAM-1 InterCellular Adhesion Molecule-1; GM-CSF Granulocyte-Macrophage Colony Stimulating Factor; BDNF Brain-Derived Neurotrophic Factor; CD200 Cluster of Differentiation 200; VEGF Vascular Endothelial Growth Factor; IGF-1 Insulin Growth Factor-1, IFN-γ Interferon-γ; bFGF basic Fibroblast Growth Factor. Copied from (Evans et al. 2013).

1.C1.2.- Oxidative-derived inflammatory-relevant lipid species

In common with other lipid mediators, all these lipid species are not stored in any cellular compartment, meaning they are quickly synthesized upon demand and therefore rely on the abundance of its precursors as well as the enzymes responsible for their synthesis (for an excellent review on oxolipids synthesis in CSN see (Phillis, Horrocks and Farooqui 2006).

Therefore, for some of them, the limiting factor is their relative abundance of the precursor, since they share, and therefore compete for the same enzymatic machinery with many other mediators.

A) Cyclooxygenases

COX-1 and COX-2, confined chromosomes nine and one respectively, catalysed the limitant step in the synthesis of PG, PGI and TX (collectively termed prostanoids). Those heme-containing bifunctional enzymes posses two catalytic centers. They are 65% homologous and, among FA, their preferred substrate is AA (other substrates include cannabinoids (Kozak, et al. 2001) and lipo-amino acids (Prusakiewicz et al. 2002)). However, their activities differ, since COX-1 is constitutively expressed in most tissues and produces derivatives that generally serve a housekeeping function, whereas COX-2 (constitutively expressed in the kidney, stomach, and CNS) was initially characterized as an inducible enzyme whose expression is enhanced in response to inflammatory stimuli, cytokines and transcription factors (e.g. IL-1β, TNFα, LPS, PAF, TGF-β, NF-κB) (O'Banion 1999). Under pathological conditions COX-2 expression was observed increased (respect to COX-1) in neurons and astrocyte, whereas in microglia, the latter is more often expressed (Consilvio, Vincent and Feldman 2004), although this cell type "selectivity" is not well understood. Nevertheless, COX-2 action was also reported to increase under excitotoxic (kainic acid) conditions in neurons (Adams et al. 1996) as well as in astrocytes (Sandhya and Farooqui 1998) and can be suppressed by Glut antagonists (e.g. MK-801, dexamethasone) implying that COX-2 regulation is dependent on synapse activity. Importantly, astrocyte and neuronal activation are sometimes non-simultaneous. Thus, neurons are more often rapidly induced and silenced, whereas astrocytes could sustained its induction for a longer period (Sandhya et al. 1998). These timing differences are crucial since it is know that the same PG could operate different responses (even opposed) depending upon the target cells and, of course, different PG could evoke opposed responses on the same cell.

Nomenclature of prostanoids aroused due to their structure, which is nowadays extremely wide, but originally resembled that of prostanoic acid. Subindex numbering of those molecules refers to the number of unsaturation along the aliphatic chains residues and the letter is related to the type, unsaturation degree and substituent present in the ring (Herrera E. 1993). During the conversion of substrates (we use AA as a representative) to oxolipids, there are two sequential steps. First, a pure cyclooxygenase phase operates where an O_2 molecule is added to form PGG_2; secondly, peroxidase action of the enzyme converts it to PGH_2, involving free radical release. This PG is subsequently metabolised by tissue-specific synthases to yield a variety of products (e.g. PGE_2, PGD_2, PGF_{2a} PGI_2, TXA_2, 15d-PGJ_2) which could be modified to generate a greater array of specific PG, with very different outcomes.

Finally, regarding COX substrate preferences, some differences have been reported. Although AA is the preferred substrate for COX-1 and COX-2, other fatty acids can be oxygenated by both isoforms with varying efficiencies (Wada et al. 2007), having COX-2 more extensive array of substrates compared with COX-1, presumably because of a ≈20% larger active site (Kurumbail et al. 1996). However, oxygenated products are scarce when DHA is the substrate (12%, versus 30% of EPA, respect to AA) and in fact, until recently DHA was not considered a valid substrate

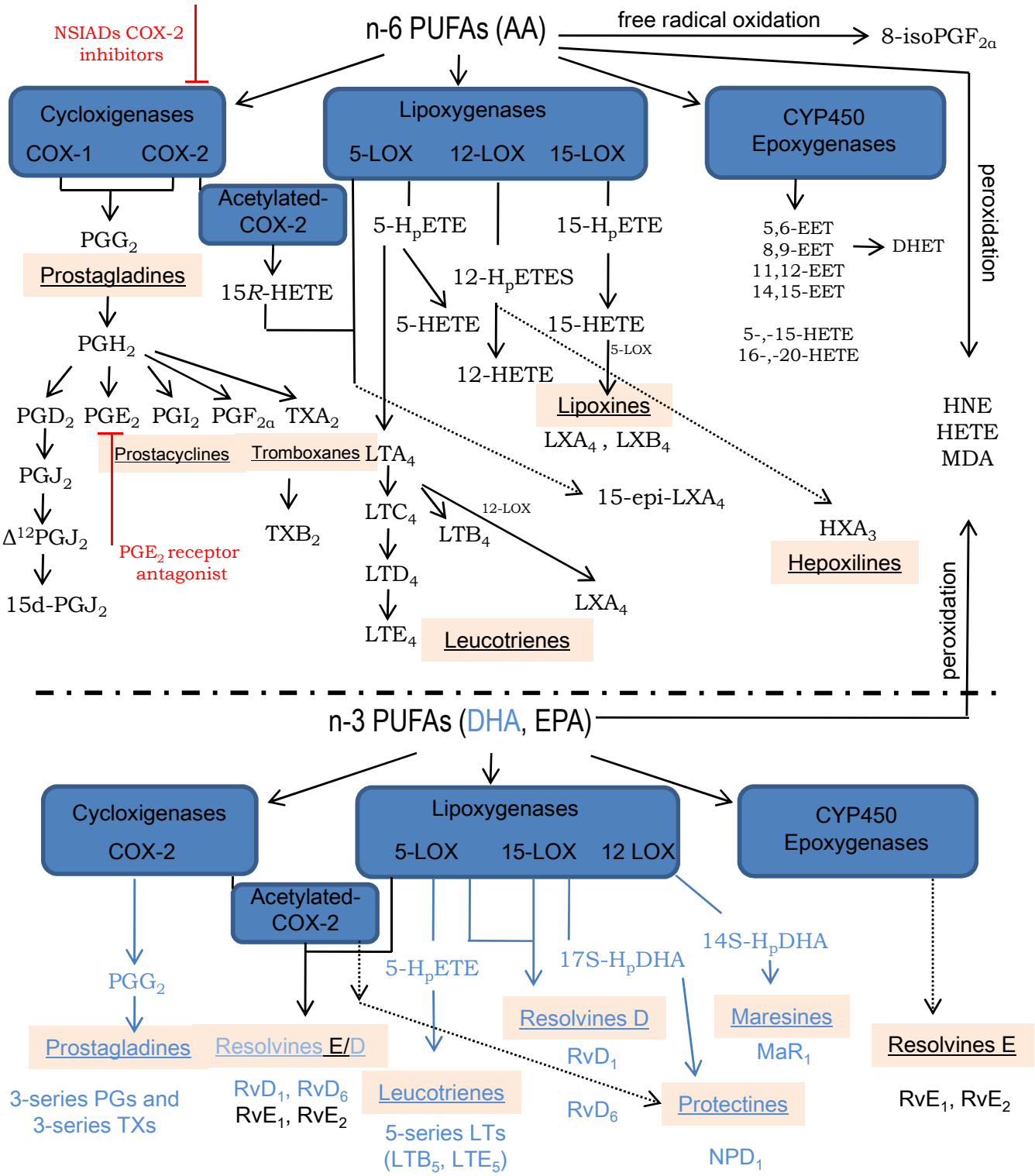


Figure 16. Inflammatory-stimulated PUFAs derivatives. Schematic representation of relevant lipid derivatives raised in inflammatory processes, depicted by FA family (ω-3 *vs*. ω-6) and responsible enzymes. In red, inhibitors, in blue, DHA derived and in black AA and EPA derived substances. NSIAD, Non-steroidal anti-inflammatory drug.

B) Lipoxygenases

Lipoxygenases (LOXs) constitute a heterogeneous family of lipid peroxidizing enzymes capable of oxygenating PUFAs containing *cis*, *cis* 1-4 pentadiene structures to their corresponding hydroperoxy derivatives. Lipoxygenase proteins have a single polypeptide chain (molecular mass ≈75–80 kDa) with an N-terminal β-barrel domain and a larger catalytic domain containing a single atom of non-heme iron critical for its optimal function (Brash 1999). Since 1974 (Hamberg and Samuelsson 1974), when the formation of 12-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) was described as being formed by exogenous AA addition to trombocytes, it was proved that those enzymesflamilies were found in both, superior animals and plants. In mammals, LOXs could be classified according to their positional specificity of AA oxygenation into 5-, 8-, 12-, and 15-LOXs or attending its positional specificity of arachidonate oxygenation into *S* and *R* isoforms (Meruvu et al. 2005). In recent years, LOX biochemistry and its regulation had gained increaseing attention due to its intimate relation with inflammation-resolution processes and, especially in allergic responses. Hence, intensive research has been done, mainly in 5, 12 and 15-LOX

The 5-LOX is a Ca^{+2}-dependent membrane-binding which is translocated to the nuclear membrane and whose fully activation also requires a helper protein, known as 5-LOX-activating protein (FLAP) (Peters-Golden and Brock 2003). This location close to the nucleus, and the high probability of ROS leak on their catalysis could influence redox tone and thereby modulate, for example, the activity of oxidant-sensitive transcription factors such as NF-κB (Bonizzi et al., 1999). the Ca^{+2} dependence of this enzyme is also noteworthy, since this ion is largely known to be deregulated in various neurodegenative processes (reviewed in (Calì, Ottolini and Brini 2013 and Prell et al. 2013)). 5-LOX is the key enzyme for the LT (the name comes from its discovery being generated from “leuko”cytes and conserved 3 carbon-carbon double-bond, “trienes”) synthesis (Funk et al. 2002) and catalyzes the oxygenation of AA to form 5-HEPETE, and finally the epoxide intermediate LTA_4. This LT can be later hydrolyzed by LTA_4 hydrolase to leukotriene B4 (LTB_4). Another possibility renders LTA_4 to be conjugated with glutathione by leukotriene C4 synthase to yield LTC_4 (other possibility of LTA_4 is to serve as substrate of 12-LOX to form LXA_4). Then, LTC_4 can be converted by γ-glutamyl transpeptidase to LTD_4. In turn, LTD_4 could be also converted to LTE_4 by dipeptidases. Those derivatives (LTC_4, LTD_4, LTE_4), are termed cysteinyl LT and are mainly produced by eosinophils, basophils, and macrophages cells. LTD_4 and LTE_4 are secreted (not having an intracellular function to date) and the three of them are relevant in allergic responses as slow-reacting substances of anaphylaxis (Austen et al. 2009).

Finally, using a DHA derivative, 17-hydroperoxy-docosahexaenoic (17-(R,S)-HPDA) acid, 5-LOX also catalyzes its conversion into resolvines one and two (RvD1, RvD2, discussed below). Interestingly, this synthesis could be also aspirin triggered, and when is under such conditions, predominant lipid specie posses *R* configuration (Hong et al. 2003). These potent species have been proved to reduce neutrophil recruitment and infiltration as well as functioning as pain reduction agents in mice (Hong et al. 2003; Spite et al. 2009) possibly serving as new “antinflammatory therapy”.

Two types of 15-LOX have been characterized in humans: reticulocyte-type (15-LOX-1) and epidermal-type (15-LOX-2). This enzymes displayed differential substrate specifity, LA is the preferred substrate for 15-LOX-1 whereas AA is the preferential election for 15-LOX-2. Thus,

LA is metabolized to 13S-hydroperoxyoctadeca- (9Z, 11E)-dienoic acid, which is later reduced to 13S-hydroxyoctadeca-(9Z,11E)-dienoic acid (13-HODE). On the other hand, 15-LOX-2oxygenates AA to form 15(S)-HPETE, which is reduced to 15(S)-HETE (Mahipal et al., 2007). One important action of 15-LOX-1 is related with the synthesis of Neuroprotectin D1 (NPD1) (Calandria et al. 2009) since its action upon DHA generates 17-(S)-HPDHA as an intermediate, which after epoxidation and hydrolysis reaction generated NPD1. It was demonstrated that this lipid specie protects against oxidative stress-induced cell death and to exert pro-resolution signals, in retinal pigment epithelial cells. In the brain, NPD1 was related to attenuated protein misfolding responses (Calandria et al. 2012) which are specially relevant in Alzheimer disease (Lim et al. 2005; Zhao et al. 2011) but also in ALS (Ilieva et al. 2007; Walker and Atkin 2011) since it is largely known an alteration in unfolding protein response (UPR) of the ER involved in its pathogenia (recently reviewed in (Matus et al. 2013; Prell et al. 2013).

Three types of 12-LOX have been described and they catalyze the stereo-specific oxygenation of AA to yield 12(S)- hydroperoxyeicosatetraenoic acid (HPETE, a well known thromboxane suppressor by inhibition of platelet cyclooxygenase (Brüne and Ullrich 1991)) and 12(S)-hydroxyeicosatetraenoic acid (HETE). Both 12 and 15-HETE have been linked with hypertension (González-Núñez et al. 2001), endothelial pathology (Anning et al. 2005) and even renal function (reviewed in (Hao and Breyer 2007)). 12-HPETE is also a substrate for the synthesis of HXA_3 mediated by hepoxilin synthases (even when this could also be obtained by the CYP450-mediated catalysis, reviewed in (Nigam and Zafiriou 2005)). Lots of functions have been related to be hepoxilin modulated, most of the research has been done in neutrophils (Nigam and Zafirio 2005) but they were also related to hyperpolarization in rat hippocampal slices (Carlen et al. 1994).

C) Acetyl-salicylic acid-stimulated ω-6 anti-inflammatory mediators: Lipoxins

Lipoxins are generated in humans from AA via LOX enzymes and comprise 2 distinct regioisomers, lipoxin $(LX)A_4$ and LXB_4. They were among the first lipid mediators recognized to have both antiinflammatory and proresolving actions (Serhan et al. 2008) by stimulating monocytes and macrophages to perform phagocytosis without releasing cytokines or chemokines. Classically, their biosynthesis proceeds via 15-LOX and 5-LOX mediated conversion of AA during cell-cell interactions (e.g. epithelial-leukocyte, leukocyte-leukocyte) or, in the contrary, 12-LOX mediated in the vasculature during platelet-leukocyte interactions and their actions proceed via activation of specific receptors (found in murine and human CNS brain atlas; http://human.brain-map.org; http://mousespinal.brain-map.org/ imageseries/ detail/100028215.html). However, an intriguing novel lipoxin route involving COX-2 and 5-LOX under the presence of aspirin was discovered, the 15-epi-LXA_4 (Clària and Serhan 1995). Its biosynthesis was recently proposed to be alternatively initiated by CYP450 enzymes and this pathway may underlie its generation in the absence of aspirin. For a review on lipoxin synthesis and resolving functions see (Ryan and Godson 2010). Since in one hand, aspirin was proved to acetylate both COXs (Loll, Picot and Garavito 1995) (more recently CYP450 too) causing its inhibition by active site alteration and, on the other, DHA and EPA are known to be substrates for acetylated COX, this opened a new route for more aspirin triggered lipoxins, which, in fact have been found (EPA derived resolvines, RvE1 and DHA-derived 17*R*-

hydroxydocosahexaenoic acid, among others, discussed below) with novel powerful resolution properties.

D) ω-3 Pro-resolution mediators: Resolvins, Protectins and Maresins

Resolvins represent a novel potent lipid mediator family *in vivo* arising from ω-3 FA at final stages of acute inflammation. Those local, short-lived lipids species exerted their function, together with protectins and the previously described lipoxins, on recently discovered membrane receptors (Krishnamoorthy et al., 2010) but, interestingly, do not activate PPAR (all isoforms) or RXR factors (at least in HEK-293 cells). As pointed before, resolution is an active process and resolvins are termed so since, i) they are produced during cell-cell interactions occurring in the resolution phase of acute inflammatory response; ii) they "stop" further neutrophil entry to sites of inflammation, and iii) reduce proinflammatory exudates (Bannenberg et al. 2005). All those lipid mediators, generally termed docosatrienes (twenty-two carbons, 3xC-C double bounds), were found thanks to a complex bioinformatics approach and its *in vivo* relevance was confirmed by LC-MS/MS. Depending on the ω-3 source (EPA or DHA), Resolvins are classified as E and D series. On the other hand, Protectins and Maresins were described as DHA hydroxyl derivates (for a review (Ariel and Serhan 2007;Spite and Serhan 2010)); the latter discovered in macrophages (Macrophage mediators in resolving inflammation (Serhan et al. 2009) and the former found in inmmune cells and/or neural tissues (Mukherjee et al. 2004). In the past years (for a recent rewiew see (Serhan, Dalli and Colas 2014)) research was focused in structural and biosinthesis elucidation of those potent mediators, trying to clarify whether this substances could be responsible for the "inflammatiory switch".

E) Isoprostanes

Isoprostanes are lipid species emerging from nonenzymatic free-radical oxidation of PUFAs, sometimes (but not always) when they are esterified in biomembranes. Those compounds are named prostanes since, when firstly described (70's decade (Porter and Funk 1975)), its structure had a cyclopentane (prostane) ring. However, nowadays compound family has grown a lot and many different structures could be found (for profound review on isoprostanes generation and function see (Milne et al. 2011)). In fact, there is controversy just on its nomenclature (Mueller 2010), in simple, IUPAC proposed C20 derivatives (mainly AA source, but also EPA ones) are termed isoprostanes (IsoP), C22 derived ones (often DHA derived) neuroprostanes (NeuroP, firstly described by Roberts in 1989 (Roberts et al. 1998)) and C18 (could araise from α and/or γ linolenic acids) Phytoprostanes -founded in plants too. A sub classification of those species was needed and F_2, E_2, D_2, A_2, J_2-IsoP (AA derived with a closed cyclopentane ring but also open structures like E_2- and D_2-IsoK; EPA derived, F_3-IsoP; DHA derived, F_4-IsoP) and NeuroP were subdivided according to where oxygenation takes place, arising 5, 8, 11, 12 and 15 series (this is the case for the simplest AA-derived IsoP; the more double bonds the more possibilities). The myriad of different structures arises since initial stochastically-radical-oxidation takes place close to a high number of double bounds (along those FA) and those reactions could terminate in a great variety of options. However, there are some C-C double bounds more likely prone (based on their hydrogen pK) and/or exposed (depending on structural configuration or their degree of concealment) to oxidation, and, also importantly, some of those "unspecific" products were in fact substrates for further synthetic processes. For instance, in the AA-derived IsoP generation, series 5 and 15 are predominant, but

the 12 and 8 series (minority) could serve for dioxolanes (another IsoP type, with two 5-vertex-ring) synthesis and therefore, reduced its accretion (Yin, Morrow and Porter 2004). For a more detailed study of EPA derived IsoP see (Yin, Brooks, Gao and Porter 2007).

IsoP, and some of the related family members, are considered a new class of biomarkers for oxidative stress measurements (Roberts and Morrow 2000) and are especially relevant among lipid peroxidation ones. Besides the major lipid oxidation products (HNE and MDA (Spickett 2013), IsoP fullfills the requirements to be a good marker: They are (i) frequently accumulated up to detectable quantities in biological fluids and tissues, allowing the definition of a normal range; (ii) stable compounds; (iii) their formation increases dramatically *in vivo*, in a number of models of oxidant injury; (v) their formation is modulated by antioxidant status.

Nevertheless, IsoP family have been proved to affect very different aspects of cell fate, from actions on vascular system (where F_2-IsoP are specially relevant (Hou et al. 2004)), muscle contraction, nociception or cell proliferation. Those actions, are believed to take place through "specific" membrane receptors, since some have been shown to bind to classical prostanoid (for PG) membrane receptors (for instance TX receptor is known to bind F_2-IsoP), although most IsoP are "orphan" receptors to date.

1.C2 Lipid peroxidation: Friend or foe?

1.C2.1.-Chemistry of oxidative stress

Oxidative stress is a term used to explain a situation when, an unbalance in between physiological oxidative production and antioxidant defence counteract (enzymatic and non enzymatic too) and/or reparatory systems is present. In principle, this situation could be therefore achieved when antioxidant defence and/or repairing systems fail to detoxify physiological noxious by-products or, on the other hand, when regular occurring oxidation is no longer under strict control, hence, promoting damage to cell molecules constituents (proteins, sugars, lipids and nucleic acids). This, among others, is the basic tenet of several pathophysiological states, such as neurodegeneration (reviewed in (Barber and Shaw 2010)), cancer or aging (Harman 1956). Although any biomolucule is susceptible to receive this modofication, proteins, due to its high overall abundance, are more probably affected by oxidative processes and are therefore believed to account (as final acceptors) for the majority reactive species generated (50%–75%) (Davies, Fu and Wang 1999). However, damage to DNA, often measured by the accretion of 8-oxo-deoxiguanosine (8-oxo-dG) as well as proteic systems designed to detect (double strand break -DSB-, e.g. phosphorylation of histone γH2AX (Rogakou et al. 1998)) and respond to DNA-oxidative damage are relevant mechanisms, especially in the "germ" cells, since any DNA mutation could be therefore inherited.

From an evolutionary point of view, oxidative biochemistry (phosphorylation), which in superior eukaryotes is mainly taken part in mitochondria or chloroplasts, turned out to be a very satisfactory mechanism. Ancient archeas, bacteria and cyanobacteria started oxygen production, as an undesirable metabolic by-product, around 3400 million year ago. Approximately 1000 million years later, Earth atmosphere reflected the "work" done. This event was known as "the

oxygen burst", which ultimately caused one of the first massive extinctions (almost complete) of obligated anaerobiotic organisms, but on the other side helped to promote a massive biodiversity. In any case, from this oxygenated atmosphere, and helped by aforementioned organellas (whose eukaryotic evolution was elegantly explained by the Russian biologist Konstantin Mereschkowsky in the endosymbiotic theory (Mereschkowsky 1910), organisms evolved to live in an oxygenated ambient, taking enormous benefits, specially, in terms of energy production.

However, there are hundreds (possibly thousands, since extremophiles research is a an enormously growing field) of organisms which obtain energy without oxygen being the final electron acceptor, through multiple ways, from methane or acetate to sulphur or even halogens or uranium (*Methanothrixthermophila*, *Sulfolobus* genus, *Geobacteraceae*, *Geobacter-metallireducens* respectively, reviewed in (Schäfer, Engelhard and Müller 1999)). In any case, those organisms also grapple with side effects of oxidative reactions and represent an interesting research field, because those different options to optimize energy sources give rise to various approaches in terms of antioxidant capacity design. Either way, eukaryotic anaerobic cell respiration, consisting in glycolysis, produces 2 ATP per unit of glucose consumed. On the contrary, aerobic respiration (composed by glycolysis, plus Krebs cycle and oxidative phosphorylation) could produce up to 36 ATP per glucose. All in all, aerobic respiration in eukaryotes is roughly 18 times more efficient than anaerobic. Furthermore, efficiency of the process for energy extraction from nutrients is believed to be about 40%. The remaining 60% is converted in heat, which is useful for poikylothermic organisms, but could become an issue to deal with for homoeothermic ones.

Nevertheless those energetic benefits pay a bill, which is the production of undesired oxidative species. But let's start from the beginning, what is and oxidant? What is a free radical? Or where do they come from? Chemically, redox reactions represent a group of natural occurring reactions which involve electron transference between two species. So oxidant processes have to be understood as a pair of coupled reactions, reduction of one counterpart with the oxidation of the other. Under this situation, oxidant is the specie which "receives" those electrons, being itself therefore reduced on its oxidation state. On the contrary, a reducing agent will decrease its oxidation state (because it is electron donor) causing the oxidation of the pair. Hence, is important to bear in mind that any specie is oxidant or reducing depending on the counterpart which is confronted. Nonetheless, in biological systems, those conditions are in an homeostatic range, so the oxidant behaviour of a given substance is normally "permanent".

Among oxidants, free radicals are one of the most powerful agents. In a simple definition, a free radical is any atom (e.g oxygen, nitrogen, sulphur) or group of atoms (e.g. $\bullet O^-_2$ superoxide anion, HO• hydroxyl radical) or molecular species (e.g. ascorbate radical, lipid radical, LO• or $LO_2\bullet$) capable of independent existence (commonly extremely short due to its high instability) that contains at least one or more unpaired valence electrons (the ones in the more exterior atom shell) or a open outer shell configuration (Halliwell B, Gutteridge JMC 2000). In biology, due to its relevance, free radicals are also known as reactive oxygen species (ROS), reactive nitrogen species (RNS) (Halliwell B, Gutteridge JMC 2000). and more recently, reactive lipid species (RLS), although, in nature, all sort of radicals can be found.

In eukaryotic biota, those radical species are mainly generated along the electron transport chain (ETC) in mitochondria and chloroplasts. Mitochondrial ETC comprises very sophisticated and

complicated proteic systems which could be “divided” in five complexes (each composed by multiple subunits, but interestingly not all encoded in the mitochondrial DNA, mtDNA) and electron donors-transporters (e.g. Ubiquinone, Coenzyme Q). All of them are cristae resident, thus, embedded in the inner mitochondrial membrane. ETC bioenergetics, composition and physiology will be discussed below, just say here that they orchestrate a controlled transfer of energy from the substrate (e.g. pyruvate, malate, succinate) to molecular oxygen (the final acceptor) forming, along this process, the corresponding reduced equivalents (e.g. $NADPH^{+}$, $FADH_2$) and generating an intermembrane electrochemical gradient ($\Delta\Psi$) which ultimately results in ATP production (trough ATP synthase) and/or heat (uncoupling proteins, UCP).

Hence, mitochondrial ETC complex I and III have been blamed for main ROS production long time ago (Turrens and Boveris 1980). However, there is still controversy on which of them are “the more responsible”. Nowadays, it is believed that CI ROS production could be counteracted more efficiently by mitocondrial antioxidant defence, but due to its location and functionality, affects mtDNA more significantly (reviewed in (Stefanatos and Sanz 2011)). On the other hand CIII ROS are more prone to affect extra-mitochondrial molecules (Chen et al. 2003), and possibly related with signalling events to control this excess.

However, during millions of years, those processes where refined to avoid undesired leaks in the system, which could generate the mentioned radicals and/or oxidant species with those harmful consequences. Pointed long time ago (Richter 1997), taking into account this evolutionary process and its statistically-stochastically-linked physiological leak, organisms evolved to understand and react when situations turned dangerous. There are sensitive mechanisms which detect the unbalance and operate towards solving it. This fact changed the classical harmful focus into, up to an extent, benign signalling. So nowadays, ROS, RNS and RLS are known to have double edge-sword behaviour. Nobody is saying that general indiscriminate damage to neither nucleotides nor proteins is beneficial, but, along million of years, is reasonable to think that organisms developed a wide array of mechanisms in order to ascertain their status and therefore orchestrate a reasonable response, among them oxidative signalling (for a review see (Martindale and Holbrook 2002)). In fact antioxidant supplementation, aimed to minimize radical production, is nowadays controversial (Bjelakovic et al. 2013 and Dolara et al. 2012). Some studies have shown that antioxidant supplementation could have no effect in health subjects with a balanced diet and a reasonable active lifestyle (Taghiyar et al. 2013) or even, in turn, exacerbate oxidative damage (Schnorr et al. 2011 and Bjelakovic and Gluud 2007). This may be explained, since if dietary intake is exceeded, local synthesis and detoxifying mechanisms would render “unnecessary” and the body would reach another homeostatic situation, potentially harmful if this dietary supplements failed to be at the right time and location.

This relates to a relative new concept, mitohormonesis (Tapia 2006), which, in brief, refers to the already mentioned minimal oxidative damage needed to achieve a balanced status of the organism's natural response to the oxidant compounds. This was partially validated by finding an increased life expectancy in *C elegans* (Schulz et al. 2007). However, it seems pretty clear that those antioxidant-dietary supplements are beneficial when an altered diet or pathology is present (Kim, Kim and Choue 2013 and Miyamae et al. 2013) and there is no discussion on the positive role of radical production for pathogen control, like physiologically produced oxidant derivatives by myeloperoxidase action in neutrophiles, Nitric Oxide signalling or NRF2 translocation, among other beneficial properties.

1.C2.2.- Mitochondrial physiology

We will present here a brief description of mitochondrial bioenergetics and components trying to shed some light on its physiology. As stated before, ETC could be subdivided into five complexes (complex I, CI; II, CII; III, CIII; IV, CIV or cytochrome-c oxidase and V, CV, or ATP synthase) each one composed of multiple subunits and mobile electron shuttles - ubiquinone (Coenzyme Q, CoQ), a lipidic quinone and cytochrome c (Cyt c), a heme-containing peptide -. A representation is shown in page 74, Figure 17.

mtDNA encodes several, but not all, subunits of the ETC. Apart from 2 ribosomal and 22 transfer RNAs, mammalian mtDNA encodes only 13 proteins (7 from CI, 1 from CIII, 3 from CIV, and 2 from CV) (Mitochondrial Oxidative Phosphorylation 2012). Also remarkable, for a correct mitochondrial functionality, this organelle needs from, approximately, 1000 (Elstner et al. 2009) nuclear-coded cytosol-synthesed proteins, delivered through two classical "pathways", but with increasing novel partners (both issues recently reviewed in (Schmidt, Pfanner and Meisinger 2010)).The vast majority of mitochondrial proteins (taking into account mtDNA encoded and nuclear encoded ones) shows a prokaryotic origin, as reflected by the findings of orthologous phylogenetically related ones. However, a large number of proteins were incorporated during the evolution of eukaryotic cells, with increased complexity, probably for optimising regulation since those cells had also higher networking and their functioning proceed more specifically. Thus, actual eukaryotic mitochondrial proteome represents a mosaic of ancestral components and components emerged though evolution to adapt their new functionality, for which no orthologous are found in prokaryotes.

In mammalian mitochondria, CI catalyzes the oxidation of reduced NADH by CoQ. This complex is the largest in the ETC system, consisting of ≈45 subunits in mammals. Ultrastructural studies of purified CI revealed an "L" shaped object, consisting of two arms, a hydrophilic "peripheral arm" protruding into the matrix, and a hydrophobic "membrane" arm, embedded in the mitochondrial inner membrane (Efremov, Baradaran and Sazanov 2010). This two arms harbour the three functional modules: i) the dehydrogenase site, responsible for the oxidation of NADH to NAD+; ii) the Q module, in the hinge region between the two arms, contains the CoQ reduction site; ii) the P module, which is responsible for proton translocation. Various human diseases have been linked to mutated or malfunction of CI (reviewed in (Fassone and Rahman 2012)), most of them with an early clinical manifestation. Interestingly in a recent paper (Ghiasi et al. 2012) researchers find a reduced complex I activity concomitant to an increase in ADP content, in ALS patients' circulating lymphocytes. Finally another SOD1 mutation linked to ALS was found to alter C I activity and ATP production (in animal model (Coussee et al. 2011) suggesting a CI involvement in the ALS pathogenic mechanism.

CII is a succinate dehydrogenase which represented the only membrane-bound member of the tricarboxylic acid (TCA) cycle. This enzyme catalyzes the oxidation and dehydration of succinate to fumarate coupling this reaction to the reduction of ubiquinone to ubiquinol. CII is the smallest among the ETC complexes, consisting of four subunits and three Fe–S centers and having a FAD (Flavin-adenin-dinucleotide)-binding domain. The crystal structure consists of a hydrophilic head protruding into the matrix, a hydrophobic tail embedded in the inner

membrane and a short segment projecting into the intermembrane space (Sun et al. 2005). Interestingly, mRNA expression levels of both, FAD and succinate dehydrogenase complex subunits were found decreased in ALS patients (Lin et al. 2009).

Finally, CIV (which is believed to represent a rate limiting step of the ETC (Pacelli et al. 2011)) is the unique responsible of final electron acceptor of oxygen, but unlikely responsible of ROS production (Yu et al. 2011), and represents the only mitochondrial complex that has been found to have different isoforms on 5 of the 10 nuclear-encoded proteins. The existence of tissue and developmental specific forms is believed to occur to provide a platform for tissue-specific cell signalling and for a better adaptation to tissue/age demands too (Hüttemann et al 2008). In line with this, this complex was found to undergone accelerated evolution in primates (Uddin et al. 2008 and Grossman et al. 2004) respect to the other complexes. CIV is the only complex to be ATP/ADP-binding-regulated, also probably due to an increased adaptation of its activity to the particular energy demand. This complex also developed a special Ca^{+2} sensitivity. Apart from a direct effect (mamalial Cyt c have a Ca^{+2} binding site (Kirichenko et al. 2005), high extra-mitochondrial Ca^{+2} levels promote CIV dephosphorylation (Hopper et al. 2006) and this could lead to a mitochondrial hyperpolarization and increased ROS production.

Also remarkable, very relevant due to its function in transduction events leading to apoptosis, is the role of Cyt c (recently reviewed in (Wu and Bratton 2013)). This intrinsic pathway for self-controlled cellular death is conserved from worms to humans and represents one of the most likely forms of suicide in a specific cell when deleterious effect of various stimuli can no longer be confronted. Interestingly a significant Cyt c deletion was found in CNS of SOD1G93A mouse at endpoint (120 days but not in early stages (Kirkinezos et al. 2005)), linked to an increased MDA accretion suggesting an involvement of this ETC element in ALS development too.

For evaluation of relative contributions of mitochondrial complexes to oxygen consumption, several mitochondrial specific inhibitors and substrates could be added sequentially in an experiment, as routinely performed in our laboratory (López-Erauskin et al. 2013). Therefore, one can depict each-complex oxygen consumption in permeabilized samples, as well as the overall contribution in intact tissue or isolated mitochondria. This is a valuable and helpful tool for specific detection of the question, when the overall process (respiration) is believed to be altered.

1.C2.3.- Lipid peroxidation events

Let's focus now on lipids, particularly PUFAs, and their potentially harmful oxidative amplification. Particular composition of mitochondrial membrane lipids was extensively studied along the 90's at the laboratory of Dr Shalme and Dr Kligenberg (Schlame et al. 1991) and more recently revisited thanks to lipidomic analyses (Kiebish, Han and Seyfried 2009). Both studies showed big local differences from mitochondria of different sources. There are adaptive variations depending on the tissue, which is up to an extent reasonable, but even for the same tissue differences were found (e.g. fast or slow stretch muscle (Stefanyk et al. 2010)), or the synaptic location (e.g. pre or postsynaptic (Kiebish, Han and Seyfried 2009)) and of course, pointed long time ago, related to temperature where the specie is adapted to (Dey et al. 1993).

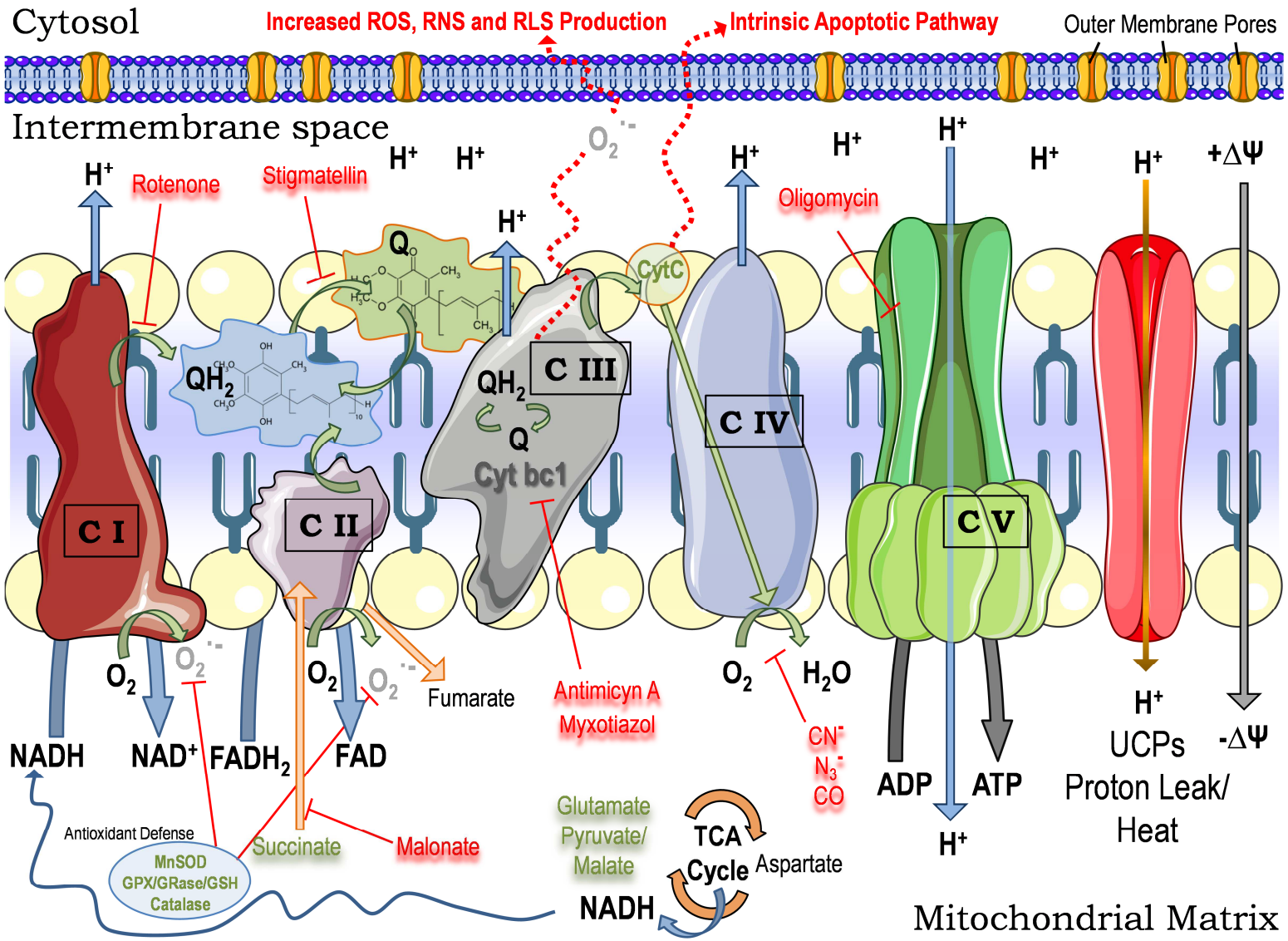


Figure 17. ETC depiction and physiology. From left to right CI (NADH dehydrogenase) generates reduced equivalents of Ubiquinone (QH2) and pumps protons from the NADH generated in the TCA cycle (tri carboxylic acid) into the intermembrane space. The CII (succinate dehydrogenase activity) also generates Ubiquinone, while consuming $FADH_2$. CIII complete the Ubiquinol (Q) cycle (helped by the cytochrome bc1) as well as transmit electrons to CytC (cytochrome c) and releases protons to the intermembrane. All those complexes generate free radicals as by products which, for CI and CII, are in part counteracted by the mitochondrial antioxidant defence machinery (catalase, manganese superoxide dismutase, glutathione, glutathione peroxidase, and glutathione reductase; MnSOD, GSH, GPX, and GRase respectively). Complex IV (cytochrome c oxidase) reduced oxygen to water and sends protons to the intermembrane space. The electrochemical gradient ($\Delta\Psi$) generated by the pumped protons is used by CV to couple its entrance (protons) back to the mitochondrial matrix to ATP generation. Other possibility, helped by the uncoupling proteins (UCPs), is to "waste" the electrochemical gradient formed along the ETC to generate heat. Radical generated in CIII affects more easily extramitocondrial species and, under specific harmful conditions, Cyt c could be launched out from the mitochondria triggering the intrinsic apoptotic pathway. Inhibitors are shown in red, substrates in green and products in black. Electron flux is represented by green arrows and proton flux by blue arrows. Not to scale.

Surprisingly, temperature could in fact regulate the rate of incorporation of FA into those mitochondrial PLs, at least in *in vitro* astrocyte cultures (Tocher and Sargent 1990). However, broadly speaking, one can assume that mitochondrial membranes are principally enriched in PE, PC, CHO and CL, representing, approximately 26%, 27%, 21% and 7% of lipids, respectively.

Among them, mitochondrial CHO levels are funded the lowest founded in any membrane (probably to minimize sterical impedances and reduce cholesteryl oxidation) and CL is the only mitochondrial-specific one. Remarkable, CL dysplayed an extremely precise acyl composition for a given particular specie and/or tissue and its accretion could rise to represent up to 25% of total GPL in the inner mitochondrial membrane. CL contains normally only one or two types of sterified FA (i.e. LA and 18:1 are the most frequently found in humans, raising up to 80% of the CL FA (Schlame et al. 2005)). Although CL composition has a strong difficulty to be dietary modified, could be changed up to an extent specially in highly metabolic tissues (e.g. liver, heart (Stavrovskaya et al. 2013)). This strong uniformity in the FA pattern, also reflected by the identical tretaenoyls found in majority of CL, had stereochemical implications (e.g. symmetry) that very likely affect the physico-chemical molecular properties: i) symmetry will increase molecular entropy since increases the freedom of rotational and vibrational motion and thus may affect interactions between CL and other components of the mitochondrial membrane as well as the physical state of the membrane itself; ii) symmetric CL may freely adopt the optimal binding conformation with respect to the central carbon atom (in contrast to the asymmetric one which could be more sterically constrained); iii) chiral properties of the central carbon atom may affect the capacity of CL to act as a local proton buffer (Kates, Syz, Gosser, & Haines, 1993), which is believed to represent a key factor on its biological function.

Also noteworthy is the incorporation of different FA into other GPL. As a component of them, DHA mainly takes the *sn-2* position, especially in PS, PE and PC; while AA is also incorporated into the *sn-2* position, but the majority in PI and PC. Different from DHA and AA ALA, LA and EPA have lower incorporation rate into the membrane GPL and lower resterification rates when consumed (Zhang et al. 2011).

From the previously commented location of the mitochondrial ETC complexes and carriers, embedded in the membrane, and their composition, it seems clear that those lipid species are potentially targets of the mitochondrial ROS and RNS, thus converting themselves in RLS. Particularly, unsaturated FAs, due to their double bounds (bis-allyllic hydrogen are much more prone to be abstracted by radicals), are more susceptible to be oxidised. Importantly, if this oxidation occurs *via* radical chemistry, consequences are potentially more harmful, since breakdown of those double bounds would generate higher amounts on new radicals, and therefore amplify damage. However, two interesting issues emerged here since, as stated before, i) a minimal damage is believed to be necessary to establish a balance point where a drastic increase could be sensed and up to an extent, orchestrate an ordered response, and ii) multiple signalling events raised related to coordinated (e.g. LOX and COX mediated) oxidation of PUFAs. Also noteworthy, in the already discussed mechanism, in case of increased peroxidation, esterified FA exchange from the GPL membranes could be performed by PLA and acyltrensferaese and possibly account for sufficient turnover.

Furthermore, it is plausible to speculate with a potential antioxidant role of PUFAs, understanding it as previously commented prevalence to be a preferred oxidation substrate, in order to “protect” other biological species. From this point of view, the high vulnerability to

peroxidation of free PUFAs in CNS (where there is a lack of intense antioxidant defences) and its close-to-damage mitochondrial location, coupled with their relative abundance in those CNS tissues could point that PUFAs might play a passive protective role of nucleotides, hence limiting their oxidation, fact already demonstrated, at least in vitro (Kim et al. 2010).

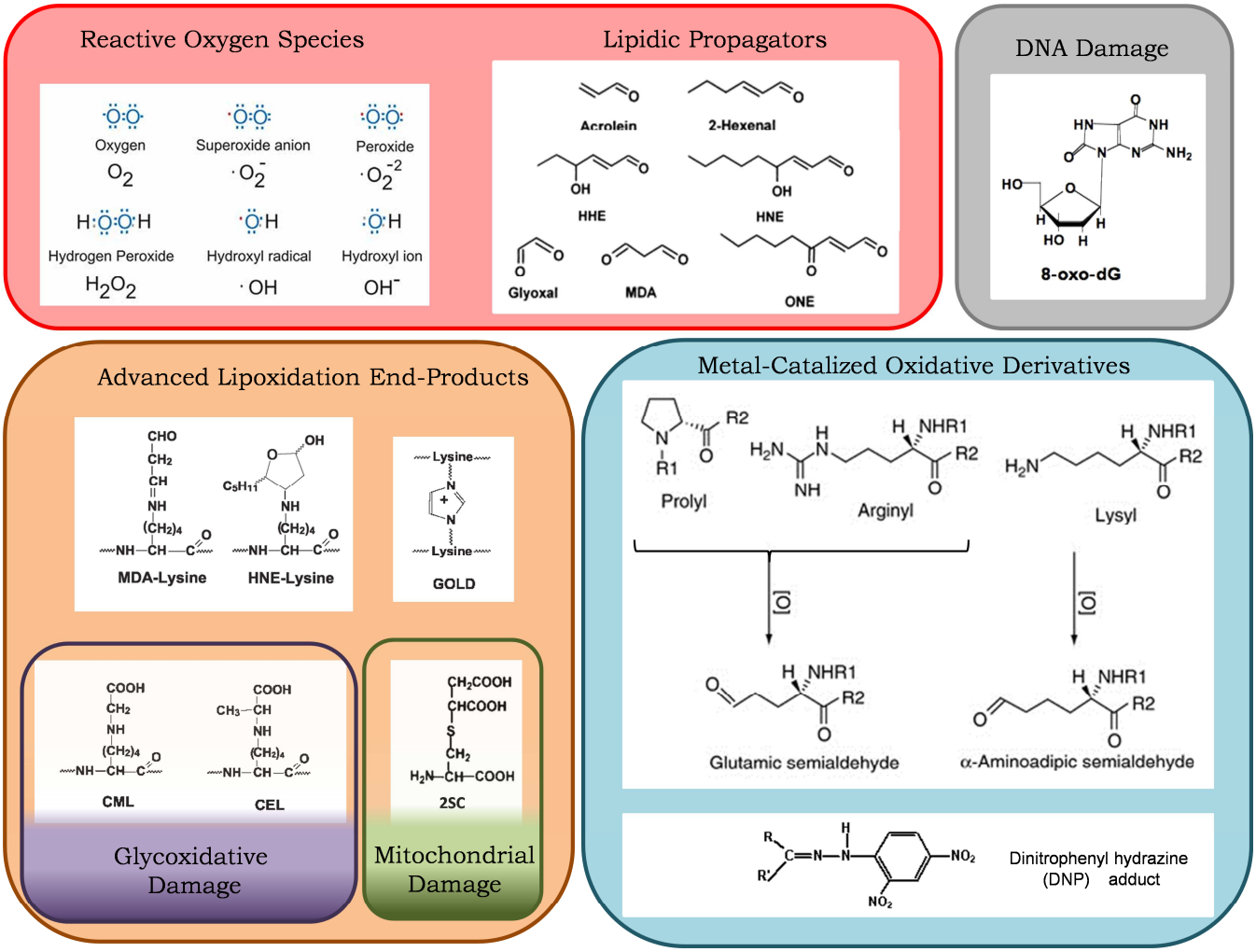


Figure 18. Representative oxidative products structure scheme. ROS formed in the mitochondria could interact with different lipid structures to form multiple lipidic propagators. These species are characterized by the presence of highly reactive carbonyl groups (remarkable for those α/β unsaturated), hence potentially increasing and spreading the oxidative damage. Those propagators could form stable adducts with different biological substances, especially with protein residues and those substances could be used to ascertain the oxidative status of a given sample as they fulfil the previously commented requirements to be a successful oxidative damage marker. Further, according to the source of the oxidative damage, we could account for lipoxidation (MDA-Lys, HNE-Lys), glicoxidation (CML, CEL), DNA (8-oxo-dG) or mitochondrial (2SC) damage or metal catalysed carbonylation (GSA, AASA or DNP adducts) as oxidative-affected pathways.

Finally, for a better support of this concept, the formation of some lipoxidation (and also nitrosilated FA) products were demonstrated to be reversible (Batthyany et al. 2006), specially through GSH function. Also relevant, some enzimatically-synthesized lipid species could be also generated through a non-enzymatically pathway (Morrow et al. 1990). Hence, those facts open the possibility for higher regulation of enzymatic functioning and/or signalling in relation to oxidised PUFAs and their derivatives.

In any case, lipid oxidation (GPL, but also free FA and PUFAs) occurs physiologically and specific products are usually found. Furthermore, from lipidic intermediates, highly reactive aldehydes could be generated, and also relevant, some PG could undergo dehydratation and oxidative modifications which would also interact with other cellular components. All those species could be sometimes crosslinked (the most frequently are covalently bounded) with virtually all sort of protein residues (but especially to cysteine, histidine and lysine groups *via* Michael addition (Requena et al. 1997; Uchida and Stadtman 1992)). Since they form stable adducts with them, this substances could be very useful as biomarkers of oxidative status/stress (for a profound review on the lipoxidation adducts formation see (Domingues et al. 2013) and regarding PL oxidation see (Reis and Spickett 2012). 4-hydroxy-alkenals (especially nonenal, 4-HNE, and hexenal, HHE), malondialdeyde (MDA) and 2-propenal (acrolein) protein adducts are the most commonly studied oxidative-produced lipidic derivatives (and also more often accumulated in biological samples) and meet the commented features to be considered valuable oxidative stress markers. Furthermore, unespecific carbonylated products (insercion of C=O duble bound) are often accumulated and commonly detected through 2,4 dinitrophenylhydrazine (DNP) incubation and WB. Anyway, multiple methodological approaches (e.g. WB, ELISA, HPLC, GC/MS, LC/MS/MS) compose a more or less accurate and reliable satate-of-art in a specific situation. However, origin of the damage (e.g. glucidic, purely lipidic or metal catalysed protein-protein), even when is sometimes difficult to interpret (e.g. MDA is generated as by-product of TX and PG synthesis too) gives relevant information helping to better represent the whole situation and possibly pointing to a metabolic pathway which could be more affected or susceptible. In addition, as commented before, when a specific accretion of those molecules occurs, could be either due to i) failure of the detoxification system; ii) for an increased indiscriminate damage and/or iii) an specific signalling purpose.

All in all, those data reinforce the complex machinery operating in the physiology of lipids related to the oxidative status in a specific situation. Moreover, when pathology overcomes, this tightly cross linked net had multiple ways to try to resolve the situation. Finally, in order to reduce damage to the minimum, this homeostatic mechanism could lead to a lipid profile adjustment. How dietary lipid could influence this outcome is an interesting question and is one the focus of this work.

1.D Amyotrophic Lateral Sclerosis

1.D1 ALS Introduction

The amyotrophic lateral sclerosis (OMIM #105400) is an adult onset neurodegerative disease, characterised by selective loss of both upper (comprising the cortex and the corticospinal tract) and lower motor neurons (MN) arising from the brainstem nuclei and ventral roots of the spinal cord. This devastating disease was initially described by Charcot in 1869 and represented the most common MN disorder with a prevalence of 2-3 per 100,000 people. ALS is, in reality, a member of heterogeneous disorders covering different forms of heritance, age and location for initial onset and different markers and proteins are involved to be deregulated. Initially, this illness was classified to be sporadic (when a familial linked heritage is missing or etiopathogenesis remains unclear) or familial (genetically linked, representing up to 15%), but whereas is generally acknowledged that the clinical presentations of sporadic and familial ALS are indistinguishable, there are subtle differences in pathology (Ajroud-Driss and Siddique 2014).

The first relevant research finding was established more than 20 years ago when Rosen and colleagues demonstrated (Rosen 1993) that mutations in copper/zinc superoxide dismutase (SOD) were a primary cause for ALS, which led to development of mouse models (Gurney et al. 1994) of the familial form. Since then, more than 100 different heterozygous mutations in SOD have been found (reviewed in (Gaudette, Hirano and Siddique 2000)) in patients with ALS (representing approximately 15-20% of the familial forms), and, for many of them, an animal model was developed. Also noteworthy, along the past years, multiple enzymes were demonstrated to be mutated and/or deregulated up to an extent, showing a more broad spectra than was initially thought (an excellent review on the genetics behind the pathophysiology of ELA could be found in (Al-Chalabi et al. 2012 and Ajroud-Driss and Siddique 2014)). In figure 20 there is a picture of the yearly progression of genetic/protein involvement in the pathology.

Another breakthrough point was recently found thanks to geneticists, when an intronic expansion of a hexanucleotide (GGGGCC) repeats in the chromosome 9 open reading frame 72 (C9ORF72) gene was demonstrated (DeJesus-Hernandez et al. 2011 and Renton et al. 2011) to represent the major cause (35%) of familial form of ALS, and very importantly, frontotemporal dementia (FTD) too. Although, surprisingly, the ALS penetrance in people carrying this repetition varies enormously along lifetime (Majounie et al. 2012), raising from almost zero (for young individuals, below 34 years) to 50% at 58 years and finally almost complete for those individuals who reached 80 years old. This suggests that this characteristic predisposition to suffer the disease required extra unknown-yet events to be finally developed. In between these two discoveries, more than 19 different proteins were related to participate in ALS development, with different particular features. Hence, mutated and/or misslocalized or phosphorylated forms of TDP-43 (TransActive Response DNA Binding Protein-43 kDa, TARDBP), FUS (FUsed in Sacroma protein) and UBQNL2 (Ubiquilin-2, Ubiquitin-like protein) were described for various phenotypes, from purely “typical” ALS to FTD. Further, disease-associated gene variants (e.g. encoding *elongator protein-3* (Simpson et al. 2009), *inositol 1,4,5-trisphosphate receptor 2* (van Es et al. 2007), *dipeptidyl-peptidase 6* (van Es et al. 2009)) were also described suggesting that ALS is not a simple disease implying only one or two groups of related proteins, monogenic mutations or so. Instead, it comprises several distinct hallmarks, which make more difficult to establish a precise coiling the data interpretation of those treatments when they fail.

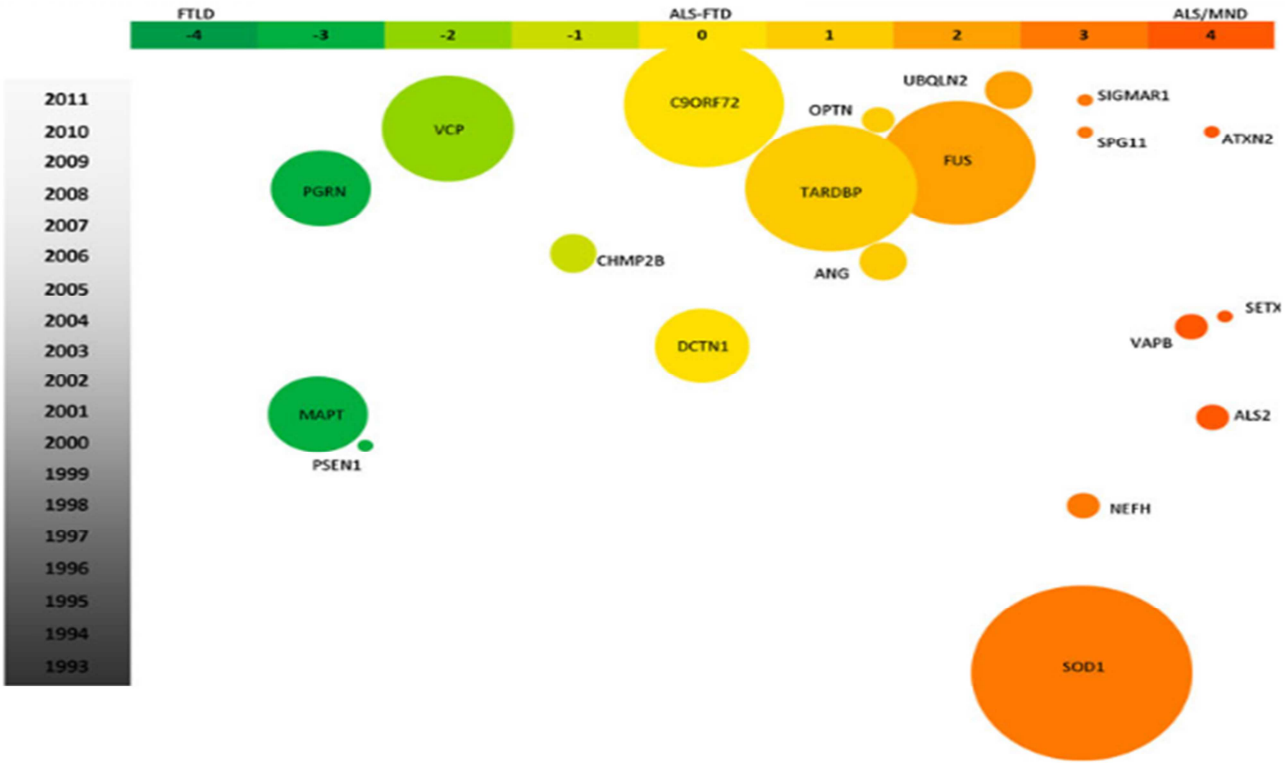


Figure 19. Genes founded implied in ALS/FTD development. Copied from (Al-Chalabi et al. 2012). NEFH, NEuroFilament Heavy polypeptide; ATX2, ATaXin-2; SIGMAR1 SIGMAR1 gene variant; DCTN1, DynaCTiN heavy subunit-1; PSEN1, PreSENilin-1; PGRN ProGRaNulin; VCP, Valosin Containing Protein.

Epidemiological studies showed a little increased disease prevalence in European ancestry (Cronin, Hardiman and Traynor 2007; Zaldivar et al. 2009), in contrast, for instance, to Latin American, native American or ethnically mixed populations. Dealing with gender dimorphism, a higher incidence in men, respect to women (even this difference is faded out in the elderly, reviewed in (McCombe and Henderson 2010)) is known. This sexual dimorphism was relevant since some specific differences in disease progression are also found. Some of them were reproduced in murine models of the disease (Suzuki et al. 2007) and were modified by estrogens therapy (Choi et al. 2008). From those epidemiologic studies, many different situations had been shown to affect illness development. Environmental risk factors (recently reviewed in (Trojsi, Monsurrò and Tedeschi 2013)), veterans of the Army (Gulf war (Cox et al. 2009)), grass-field sportsman players (Beghi 2013), western-Asian populations (Kaji et al. 2012; Lee 2011), exposure to pesticides (Furby et al. 2010) and some occupancies (McGuire et al. 1997; Park et al. 2005) were among many other cited that play a role in the incidence or development of this disease.

1.D2 Mechanism involved

As pointed previously, many different tissular and cellular events are related to ALS development. These can be clustered as: i) mitochondrial-Ca^{+2}-ER deregulation; ii) energetic unbalance; iii) non-cell-autonomous inflammation/excitotoxicity; iv) proteinopathy; v) heavy metals and toxic/ environmental agents exposure; etc. Here, we will discuss briefly about them.

1.D2.1.-Mitochondrial-Calcium-ER dysfunction

Regarding ALS and mitochondria, relevant amount of research has been done (nice reviews were recently published (Cozzolino et al. 2013; Tan, Pasinelli and Trotti 2014)). Very closely-related to mitochondrial function, decreased Ca^{+2} buffer capacity was extensively described both in patients and animal models (Guatteo et al. 2007; Siklós et al. 1996). Miss-localization of mutated SOD (usually found in the cytosol, but also in nucleus, ER and intermembrane space in disease models) have been also described in animal models, linked to anti-apoctotic Bcl-2 in the mitochondria (Pasinelli et al. 2004) as well as aberrant mitochondrial swollen and vacuolization, both in animals and human patients (Bendotti et al. 2001; Mattiazzi et al. 2002; Siklós et al. 1996). Using the SODG93A murine model, decreased mitochondrial respiration was also found (Mattiazzi et al. 2002) in animals under clinical stages, but whether preclinical ones are affected is still under debate. Further, regarding oxidative damage, an impairment of Cyt c was found and reduced respiration complexes linked to an increased MDA accretion (also in preclinical animals) in isolated forebrain mitochondria of this mouse model (Kirkinezos et al. 2005). Of note, it should be reminded that mitochondrial respiration is very sensitive to lipid peroxidation (Echtay et al. 2003) and links with the "energetic unbalance theory", which will be discussed later in this section.

Anyway, in these early works, increased carbonylation and lipid hydroperoxdes were also found for brain and spinal cords of older animals, however, the methodology used could be criticised This increased oxidative damage, even whether to be cause or consequence is controversial yet, was initially found as augmented carbonylation in spinal cord (Shaw et al. 1995) and motor cortex (Ferrante et al. 1997) of patients. Higher glyco- and lipoxidative products (Shibata et al. 2001) were found in all CNS population in the spinal cord, as well as damage to DNA was verified, as pointed the accretion of 8-oxodG (as previously commented, extensively-studied as oxidative biomarker of DNA damage) (Ferrante et al. 1997). The latter was also found increased in CSF of patients (Ihara et al. 2005), concomitant with an increased 4-HNE too (Smith et al. 1998). Regarding oxidative-derived IsoP, increased 15-F_2t-IsoP levels were found in CSF from patients suffering Alzheimer, but not in those ALS patients, respect to the control ones (Montine et al. 1999). However, in a later study (Mitsumoto et al. 2008), significant accretion of urinary 8-oxodG and 15-F_2t-IsoP, (both ELISA measured) was demonstrated, but interestingly none of those levels were correlated with an increased plasma carbonyl content (DNP measured) nor a better clinical outcome. However, one should take into account that increased excreted levels of IsoP or 8-oxodG could have two sides. One can assume that there is a raised production of those species, but is also reasonable to think in a better clearance of those substances in these individuals. So, whenever is possible, a more reliable measure of such species, aimed to express an increased oxidative damage, should be done in situ. In any case, collectively, all those outstanding findings pointed towards oxidative stress as a relevant factor in the ALS pathogeny.

Some of those human hallmarks were recapitulated in the wide variety of transgenic animal models (e.g. TDP-43, SODG93A, FUS, in mouse, rat, drosophila and zebrafish species, among others), tissular (lumbar slice organotipic culture) and cellular models developed. Taking into account their limitations, organotypic (OT) culture model stands out, since it conserves cell to cell interactions. This is relevant since not only one cell type but a concert of them (e.g. MNs, astrocytes, microglia, interneurons and muscular junctions) are involved in the degeneration. Also interesting, but just for the opposite, MN primary cultures may clarify what specific MN

signature would help to better confront different stimuli, aimed to reproduce the different ALS features.

As pointed before, in ALS there is an unbalance in the Ca^{+2} homeostasis (reviewed in (Grosskreutz, Van Den Bosch and Keller 2010)). MNs are known to be vulnerable to membrane depolarization and increased intracellular calcium concentration (Arakawa et al., 2002). Hence, higher levels of such ion increased their susceptibility to undergo cell death (Gou-Fabregas et al. 2009; Kaiser et al. 2006), but, even when some specific responsible enzymes (e.g. protein phosphatases, endonucleases, or proteases) are known, the exact mechanism is not fully understood yet. We (Ilieva et al. 2007) and many others (recently reviewed in (Prell et al. 2013 and Roussel et al. 2013) had pointed to deregulated ER, UPR stress and Ca^{+2} unbalance as key partners on pathophysiology of ALS, linking ER and mitochondria. These two cellular locations are the main ones responsible for Ca^{+2} regulation and a combined reduction of calcium buffering capacity and a high number of Ca^{+2}-permeable α-amino-3-hydroxy-5-methylisoxazole proprionic acid (AMPA) receptors in several animals models and in patients was demonstrated (reviwed in (Kwak et al. 2010)).

The relative importance of vesicular transport in cellular and sub-cellular lipid homeostasis and sorting remains unclear. As previously commented, it is a key mechanism required for a correct neurotransmission. But it is also believed to be an important connexion route from trans-Golgi and plasma membrane, helping to maintain especially high levels of SM and PS on that membrane. However no direct evidence of such transportation has been proved for mitochondrial or peroxisomes-ER connection even when for mitochondrial and peroxisomes exists (Schumann and Subramani 2008). Furthermore, fragmented Golgi apparatus was already described for preclinical classical mouse model (Gonatas, Stieber and Gonatas 2006) and patients (Stieber et al. 1998). Vesicular transport requires intact cytoesqueleton organization and functionality. It is also energy dependent (opposed to LTP mechanism) and comprises a very complex mechanism involved in different processes, ranging from lipid droplet formation (Jacquier et al. 2011) to autophagy (reviewed in (Knævelsrud and Simonsen 2012), which has been linked to various neurodenerative diseases (Salminen et al. 2013; Shen et al. 2013), including ALS (Zhang et al. 2013; Zhang et al. 2014). In fact, the finding of a relatively new familial form (named ALS8) of this disease related with mutations (P56S and T46I) in the gene encoding an ER-resident vesicle-associated membrane protein (Nishimura et al. 2004 and Kanekura et al. 2006) opened a new possible terapeutic route. This protein is related to regulation of vesicle trafficking (Rocha et al. 2009) but also plays a critical role in the UPR and, its malfunction, results in increased MN vulnerability to ER stress (Prosser et al. 2008). Interestingly, defects in this protein does not lead to morphological defects at the neuromuscular junction nor to muscle denervation in mice (and zebrafish, Kabashi et al. 2013), and only leads to a mild, late-onset motor impairments. However, in flies, this mutation caused a marked ER stress, with an accumulation of ubiquitinated proteins in brain neurons (Moustaqim-Barrette et al. 2014) and, furthermore, a misslocalization of oxyterol and ceramide binding proteins, suggesting an integral mechanism of sterol and SM metabolism malfunction.

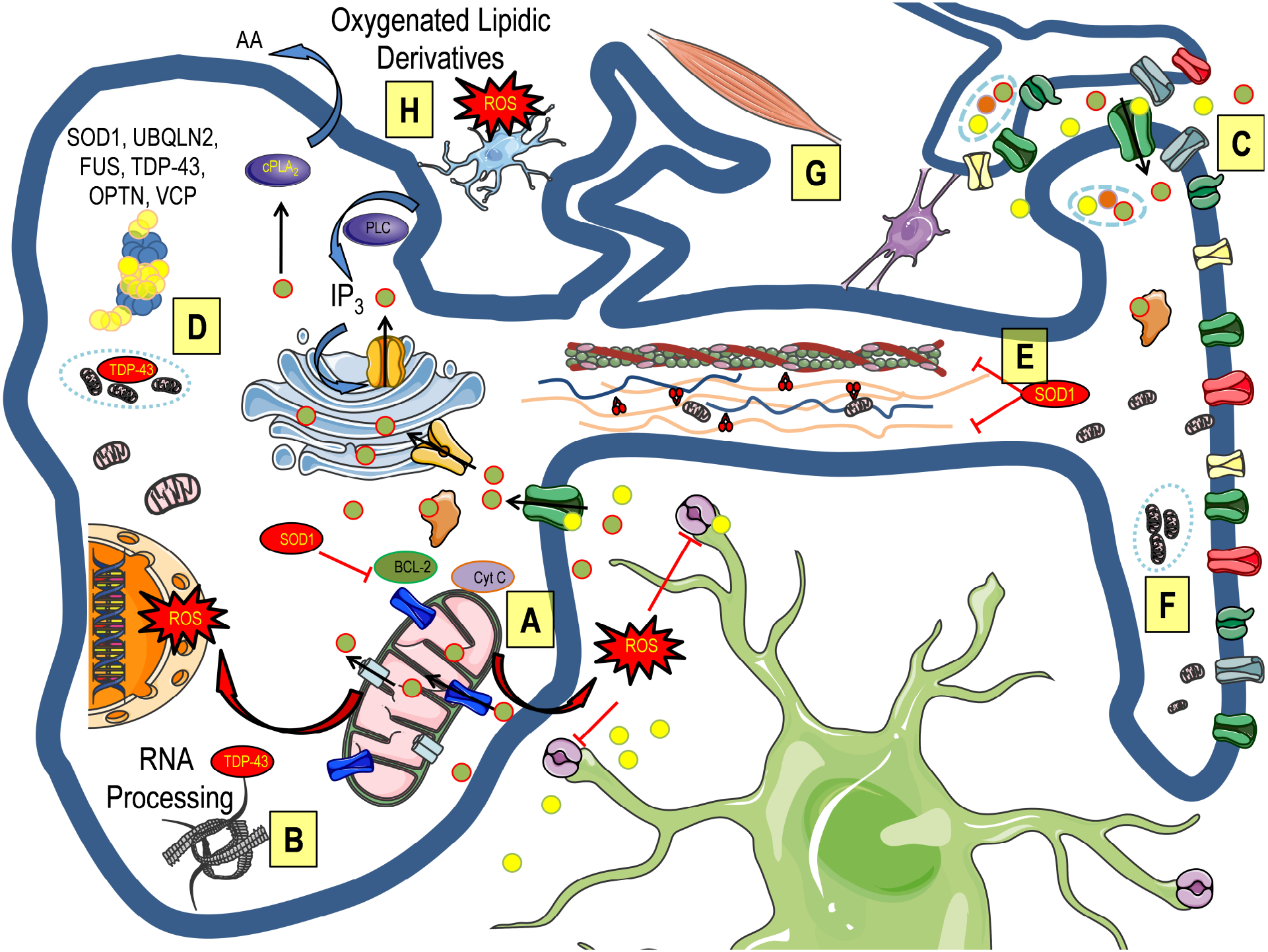
Finally, we discuss some issues regarding pro-survival signalling. Some years ago it was demonstrated that NFκB inhibition leads to neuron protection from the Glut-mediated injury (at least in cultured cells (Pizzi et al. 2005)), a mechanism already involved in phatophysiology of ALS. In the SODG93A mouse model, it was demonstrated (Prell et al. 2014) that microglial activation of the NF-κB pathway accounts for the MN loss, both *in vitro* and *in vivo*.

Furthermore, Julien and collaborators (Swarup et al. 2011) shown that synthetic inhibition of NF-κB in another mouse model of ALS (TDP-43) reduced denervation in the neuromuscular junction and TDP-43 silencing (by siRNA transfection in microglial cells) increased neuron survival whereas, the contrary, TDP-43 up-regulation, resulted in increased microglia-mediated neurotoxicity in primary cultures. Also importantly, a relation between inflammatory signalling, TNF-α mediated, and NFκB was shown in other model of ALS, the threohydroxyaspartate (THA) excitotoxicity (developed by Rothstein (Rothstein et al. 1993) and commented in the next section) on OT spinal cord slice cultures (Tolosa et al. 2011). In this work, researches showed increasing production of COX-2 concomitant with MN loss along the culture as well as drastic decay of them when co cultures were performed with TNF-α. Furthermore they demonstrate down-regulation of the main astrocitic Glut transporter (GLT-1, ortologous to EAAT2) in the combined excitotoxic and TNF-α treatment. Altogether, this data reinforces the link of various prominent pathogenic mechanisms of ALS, excitotoxicity, neuroinflammation and prosurvival siganalling, at least for some models.

Also noteworthy, results from our group refer to an altered phospho-ERK1/2 signalling upon TDP-43-mediated toxicity in cell lines under different stress stimuli, spinal cord OT culture as well as human patients (Ayala et al. 2011). This is relevant since implication of PI3K pathway was demonstrated some years ago (Wagey et al. 1998) and more recently confirmed for humans and animal models (Dewil et al. 2007). Patients were reported to have increased spinal cords protein levels and activity of PI3K respect to controls, but no changes in activity nor expression in ERK1/2 was found in this early work. Interestingly, those two pro-survival pathways (PI3K-AKT and ERK1/2) were recently proved to be enhanced by palmitic acid (in muscle cell line, but surprisingly, both pathways at the same time (Pu et al. 2011)) and DHA (in neuron cell line, activates AKT but not upstream PI3K (Akbar et al. 2005)). In another recent paper (Peviani et al. 2014) researchers showed that activation of AKT3 (one of the three isomers of protein kinase B/AKT pathway) increased survival of MNs both in *in vitro* co-cultured (astrocyte and MNs) and *in vivo* "fast" SODG93A model although in previous paper, the same group found no substantial changes in AKT pathway in this murine model of ALS (Peviani et al. 2007). Unfortunately, this MN preservation does not ameliorate disease progression of the animals (although only females were studied in both papers). On the other hand, DHA pretreatment was observed to significantly increase neuronal survival by promoting ERK-related survival pathways, at least in Alzheimer disease models (Florent-Béchard and Koziel 2007). However, further research is needed and, to our knowledge, no deep examination have been developed regarding PUFA's action alleviating excitotoxicity, reducing oxidative spread and/or counteracting inflammatory ALS components even when compiling evidences pointed an specific altered lipid status in patients tissues (Ilieva et al. 2007).

1.D2.2.- Energetic unbalance: ALS hypermetabolism

Once a patient is diagnosed, their body mass index, better than dyslipidemia (Paganoni et al. 2011), is predictor of its outcome, with worst prognosis for lower body mass individuals (Greenwood 2013). Hence, dietary interventions focusing in a correct energetic status have been shown to be beneficial in animal models (e.g. high fat diet (Dupuis et al. 2004), L-carnitine (Kira et al. 2006) and caprylic-TAG (Zhao et al. 2012), among others) as well in humans (Dorst, Cypionka and Ludolph 2013; Wills et al. 2014 and Beghi et al. 2013) (for a review on this topic see (Dupuis et al. 2011)). Not surprisingly, on the contrary, caloric restriction in SODG93A


AA
Oxygenated Lipidic Derivatives
H
ROS
SOD1, UBQLN2, FUS, TDP-43, OPTN, VCP
cPLA2
PLC
IP3
G
C
D
TDP-43
E
SOD1
SOD1
BCL-2
Cyt C
A
ROS
ROS
F
RNA Processing
TDP-43
B

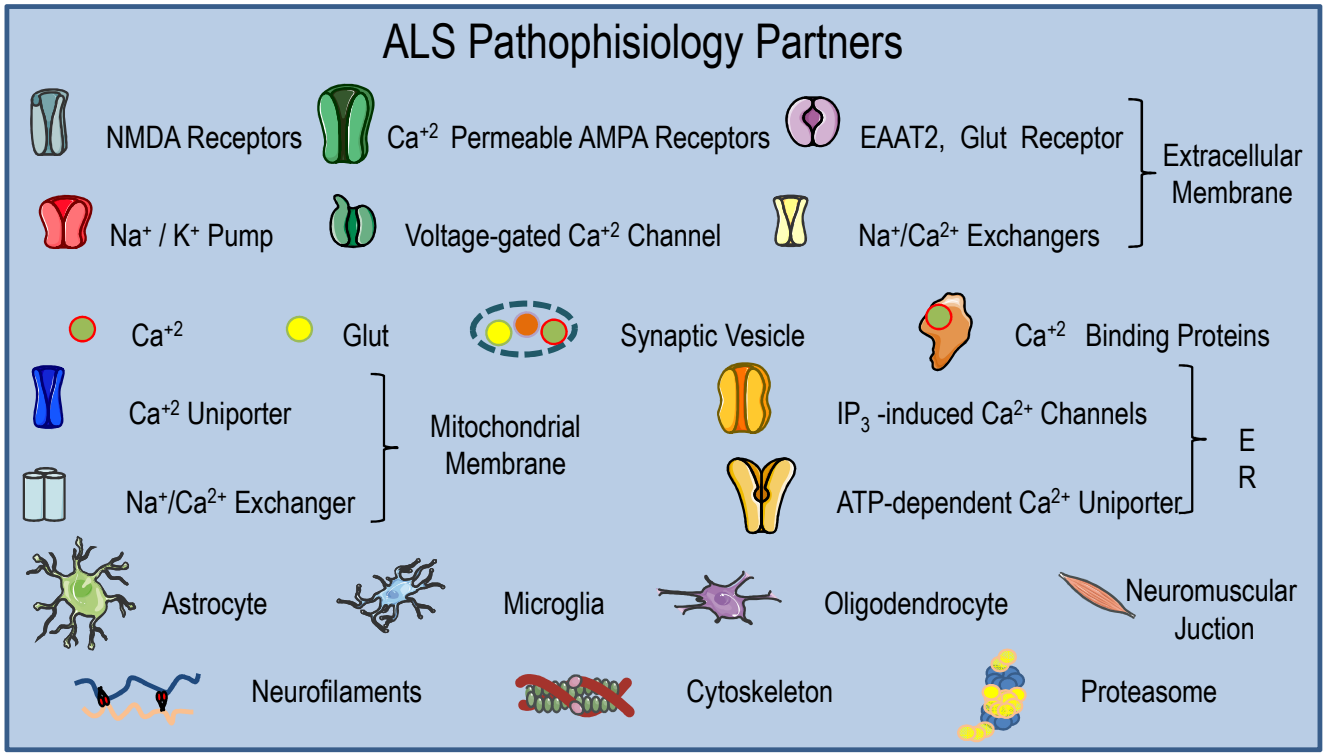


Figure 20.-ALS phatophisiology partners scheme.(A) Mitochondrial dysfunction increased ROS production, which could reduce astrocyte EAAT2 Glut buffering capacity, leading to excitotoxycity, as well as damage to DNA.Further, CytC could be launched from mitochondria, altogheter with mutated SOD1 blocking Bcl-2 prosurvival signalling would seriously comprise cell viability. (B) Altered RNA processing was demostrated in patients. TDP-43 aggregates and mislocalized could be found (with degenerated mitochondria too) vacuolized and/or inside proteasome (D) when the UPR is exhausted. Those facts altered normal ER fuction. Other proteins were found accumulated inside proteasome (i.e.valosin-containing protein (VCP), optineurin (OPTN)). (C) Ca^{+2} metabolismin MN is different than in regular neurons. They possess high number of membrane Ca^{+2} permeable AMPA receptors concomitant with lower cytoplasmic Ca^{+2} binding proteins, rendering them to be very sensitive to Ca^{+2} overload. Furthermore, Glut binding to AMPA receptors, promote increased Ca^{+2} entrances. Those situations, together with IP_3 signaling (which could be triggered by PLC activation), could result in Ca^{+2} desorption from ER depots and, ultimately, its uptake by mitochondria, comprising this organelle too. Mitochondrial (i.e. entrance through the Ca^{2+} uniporter; release through the Na^{+}/Ca^{2+} exchanger) and ER (Ca^{+2} intake is mediated by the sarco-endoplasmic reticulum calcium ATPases -i.e. through ER Ca^{+2} uniporter- Ca^{+2} depletion could occur through IP_3 dependent channels) Ca^{+2} trafficking is represented. Ca^{+2} is required for $cPLA_2$ activity. High plasmatic Ca^{+2} content could result in higher plasma membrane FA desorption, potentially causing enhanced lipoxidative products formation. Altogether, this claimed for a key role for this ion along ALS pathophysiology. (E) Mutated SOD1 altered cytoskeleton structure (some neurofilaments) and blunted axonal transport too. This would alter neurotransmission (less number of synaptic vesicles or altered configuration). (F) Fewer, smaller and sometimes vacuolated mitochondria were found in patients synapses. (G) Muscle denervation (whether a cause or a consequence) leads to neuron degeneration and apoptosis. (H) ROS generated by mitochondrial malfunction could also activate microglia and initiate a pro-inflammatory lipid derivatives cascade, possibly resulting in a feedback loop by astroglial activation (with lees pro-surviving factor release, e.g. BDNF, IGF, VEGF and enhancing inflammatory intermediates).A similar mechanisms may operate in non MN cells. Not to scale

animal model have been shown detrimental (Patel et al. 2010) but some interesting issues also aroused. Oxidative carbonylation of proteins in skeletal muscle (measured by WB) was reduced in caloric restricted animals compared with *ad libitun* ones (both sexes), but lipoxydation (MDA, spectrophotometrically measured) was increased, showing complex crosslinked oxidative responses to diet. Furthermore, both types of superoxide dismutase (mitochondrial and cytoplasmic) and TNF-α were drastically raised as a result of caloric restriction, again in both sexes, but only restricted females raised their uncoupling protein 3 levels, possibly trying to reduce de oxidative production. However, the same group had previously reported (Hamadeh and Tarnopolsky 2006) that only females were protected against detrimental effects of short-term mild caloric restriction (60%, from postnatal days 40 to 55) in the same mice strain.

The previously commented preclinical outcomes in animal's models (e.g. increased oxidative damage, lower mitochondrial capacity, reduced Ca^{+2} buffering) as well as premature weight loss (seven days after birth, unpublished observations), altogether with some clues for hypermetabolic status (probably due to skeletal muscle origin), both in animal models (Capitanio et al. 2012) and patients (Vaisman et al. 2009 and Funalot et al. 2009) suggest that early events are very relevant in the pathogenesis, which, ultimately, reflect the MN loss, and finally leading to motion and behavioural consequences. Those features could also point to a mitochondrial deregulation possibly linking those two pathogenic mechanisms. In any case, the pharmacological studies focused in hypermetabolism "reduction" through thyroid hormone regulation failed to improve survival, with no gender differences found (Li et al. 2012). Research focussed in dietary optimization for energetic balance could be beneficial in terms of a better handling of disease, once diagnosed. In this sense, transplantation with adipose-tissue derived human stem cell to a SODG93A mouse model showed alleviation (Kim et al. 2013) of clinical onset and prolonged life span concomitant with increased levels of neurotrophic factors.

Since the anti-inflammatory implication of PPARγ agonists, and the well documented participation of inflammatory processes underlying the pathogenesis of ALS, promising research has shown an alleviated response, not only in survival, but also disease progression (better MN and muscular preservation) in the SODG93A murine model under pioglitazone supplementation (Schütz et al. 2005). Unfortunately, translation to humans revealed no effect on survival (Dupuis et al. 2012). Anyway, despite this disappointing result, research on this field, aimed to a better lipid status control and signalling is still promising, and more efforts should be done since PPAR expression and regulation have been recently studied regarding its influence upon natural occurring neuronal stem cells (reviewed in (Cimini and Cerù 2008)). Thus, even when physiological localization of those neuronal stems cell seems is far from mainly affected disease location, they represented another potential therapeutic field of action. In fact, another recent publication (Benedusi et al. 2012), demonstrates, in females of the SODG93A mouse model, that there is an activation of PPARγ (nuclear localization) in MNs concomitant with clinical symptoms and this activation is produced (at least *in vitro*) by lipid peroxidative subproduct 4-HNE, as mentioned, present in CSF of ALS patients too.

Of note, statin treatment, aimed to control CHO status by reducing LXR action, had shown contradictory results, increasing confusion to clinicians. General statin function is the mevalonate synthesis blockade, therefore resulting in reduced CHO and their derivatives. (24(S)-hydroxycholesterol) concentration. But interestingly, the later is produced in large amounts in CNS and is the principal endogenous LXR agonist therein. Furthermore, side effects of statins have been proved in some individuals, including muscle toxicity, such as myopathy

(Sathasivam and Lecky 2008). Initially, no harmful effects had been published (diminished survival) linking ALS and statins usage (Edwards, Star and Kiuru 2007; Drory et al. 2008; Colman et al. 2008). However later studies suggest a gender specific variation (Nefussy et al. 2011) and a recent review (Beltowski 2010) also revealed that statins may increase the risk of ALS by inducing LXR-dependent perturbations of cholesterol/phytosterol metabolism in the CNS.

Regarding FA delivery, in a very recent paper developed with a TDP-43 knock-in mouse model (Stribl et al. 2014) researchers showed impaired lipid metabolism as reflected by donwregulation of the CD36 protein expression in old animal brains. Also remarkable, the transcriptome analisis of those mice (brain and muscle tissues) showed a disregulation of many mithocondrial-related genes and electron microscopy images confirmed impaired morphology in motor cortex and hippocampus, although this cristae morphology disruption was not observed in spinal cords. In this line, lipid-raft domains in ALS, were recently described in the SODG93A mouse model (Zhai et al. 2009) comparing the specific lipid-raft-resident protein changes. This study identified 154 proteins. Among them, 41 had quantitative statistical differences. Also important, they found proteins uniquely expressed in SODG93A (17; 29% related to cytoesqueletal regulation, 24% microglia/inflammation, 17% vesicular transport, respectivelly), and, on the contrary, only few expressed in the WT animals (9 proteins). Importantly, a majority of the identified proteins were found donwregulated in the SODG93A animals. Hence, among them vesicular transport, neurotransmitter synthesis and release represented approximately 20%, but also metabolism (19%), cytoesqueletal regulation (15%) and microglial activation/inflammation (9%) where represented, all of them major threats in pathophysiolgy of this devasting disease.

Finally, a link between fat storage location and functional and survival alleviation in patients was recently observed (Lindauer et al. 2013) suggesting that not only having enough energy depots is relevant but also where those are located. All in all, is therefore believed that optimal energetic equilibrium along lifetime could help to maintain a better status, with upgraded responses, hopefully ultimately alleviating pathology and extending survival.

1.D2.3.-Non cell autonomous inflammation/excitotoxicity

Non-cell-autonomous toxicity, meaning, MN damage derived from other cell types (e.g. astrocytes, microglia, muscle cells, among others) than MN solely was elegantly demonstrated by Clement and colleagues (Clement et al. 2003) by creating a chimeric mice. It had been anyway speculated before, since protein mutations, already recognized to evoque a fALS-like disease, were ubiquitously expressed along the body and among different cell types. Experiments developed by Lino and collaborators (Lino, Schneider and Caroni 2002) and Pramatrova (Pramatarova et al. 2001), where mutant SOD expression was restricted to motorneuron or astrocytes (Gong et al. 2000) failed to cause specific neuron degeneration nor death. Therefore, from those early works, deleterious mutations were shown to act in a non-cell automomous specificity. Subsequent molecular biology developments, such as Cre/Lox cassettes to selectively turn on and off specific genes in interesed locations and for a limited time, confirmed that MN neighbourhood really matters. Hence, all CNS population, microglia (Corcia et al. 2012), astrocyte (Rothstein et al. 1995), oligodendroglia (Kang et al. 2013) and

even perycyte (Winkler et al. 2013) as well as neuromuscular junctions (Shindo et al. 1995) of patients were shown to be affected, up to an extent, along disease progression.

Therefore, shortly after the discovery of SOD1 mutations, a defective Glut transporter protein EAAT2 (Rothstein et al. 1995) was found in spinal cord and cortex glia from sALS patients. Thus, this provided one of the first lines of evidence that indicate a possible role of Glut-mediated excitotoxicity in this disease (recently reviewed in (Philips and Rothstein 2014)). Further, the same group rapidly established the previously commented excitoxity tissular model (Rothstein et al. 1993), useful thanks to its preservation of cell-to-cell interactions. In this model of explanted lumbar region spinal cords, the addition of THA blockades Glut reuptake, leading to MNs loss in 30 days. In this widely used model, as previously commented, we demonstrated a relation between TDP-43 phophorylation and ERK1/2 signalling and, up to our knowledge, we were among the first to show a decreased O_2 consumption under THA treatment, which, up to an extent, could be rescued by antioxidant treatment (the second paper of this thesis).

Anyway, Glut neurotransmission also involves other receptors. Furthermore, Riluzole©, the unique approved drug for ALS treatment, directly interacts with GABA and glycinere ceptors (Mantz et al. 1994 and Umemiya and Berger 1995, respectivelly) in addition to its antiglutamatergic action. Of note, both substrates (GABA and glycine) represented the major ionotropic inhibitory neurotransmistters in MNs. In any case, and leaving apart all metabotropic Glut receptors (for further immersion see (Caraci et al. 2012)), MNs express all three types of ionotropic Glut receptors too, AMPA, N-methyl-D-aspartate (NMDA) and kainate receptors, with different expression patterns and interactions among them (for a profound review see (Rekling et al. 2000)). Other excitatory molecules may play a role and, from the tripartite synapse theory, not only MNs but astrocyte and surrounding cells support a correct neurotransmission (Araque et al. 1999). Alltogether with differential expression of some of synaptic enzymes found in patients, and sometimes recapitulated in models, there are compiling evidences conforming a wider picture of neutotranmission malfunction and excitotoxic involvement in ALS.

We previously commented the powerful machinery acting in membrane remodelling (e.g. PLAs, ACS and SNARE actions, among others), thus it is worthy describing how these weak equilibriums could be modified by PUFAs' actions in the synaptic cleft. Extensive research has been done in Alzheimer disease and PLAs (for a review see (Schaeffer et al. 2010)) and in Parkinson too, where a mutation in PLA_2 (group VI) was recently found to produce an early-onset rare form (Shi et al. 2011). However, ALS research in this field is still poor, even when higher expression and activation (phosphorylated) of $cPLA_2$ (110kDa) was found in motor neurons, astrocytes and activated microglia of patients (Noriyuki Shibata et al. 2010) as well as lumbar sections of the SODG93A and the SODG85R mouse models (Kiaei et al. 2005). In a model of spinal cord injury, (Liu et al. 2006) downregulation of $cPLA_2$, already described to be present in all along spinal cord motor neurons (Ong, Horrocks and Farooqui 1999) was demonstrated to be beneficial in terms of reduced inflammatory cytokine production, lower lipoxidative damage and neuronal loss. Moreover, PLA_2 injection after injury induced a massive demyelination, a fact that is counteracted by administration of an inhibitor. Altogether, this could open a potentially interesting terapeutic point of action, but precaution should be taken, since chronically inactivation of both PLAs ($cPLA_2$ and $iPLA_2$) was demonstrated to be detrimental, at least in cultured hippocampal neurons (Forlenza et al. 2007). Definite proof for this membrane remodelling therapeutic potential comes from a recent work (Staats et al. 2013).

They firstly found a microsatellite polymorphism marker for association with sALS, which was related to phospholipase C delta 1 (PLCδ1). Hence they focus the study in the SODG93A mouse model, finding increased gene expression of *plcd1* in spinal cords and further confirmed by WB in end-stage animal. Crossing PLCδ1$^{-/-}$ animal and mutated SOD significantly increased survival, with alleviated nuclear shrinkage, without changes in glial activation, nor aggregation or MNs number at end stage, compared to ALS mice expressing PLCδ1. Possible mechanism behind those findings imply the commented function in second messenger action of PLC, this is, the hydrolysation of IP_2 to form DAG and IP_3.which may create a feedback loop by the increased Ca^{+2} release through IP_3 specific receptors in ER, resulting in increased excitoxicity.

Finally, as pointed before, inflammation's implication is clear, since early involvement and release of various cytokines and chemokynes was found in patients (for a list in CSF see (Tateishi et al. 2010)). Hence, the modulation of its duration and intensity represent a potential beneficial treatment. In this sense, the influence of potent lipid mediators have been found to be relevant in Alzheimer's disease (recently reviewed (Bazan 2013)) and, interestingly, also in ALS (Liu et al. 2012) although to date this is the unique paper we found regarding those mentioned potent lipid intermediates and ALS. In this work, ResolvinD1 minimizes macrophage phagocytosis in ALS patients *in vitro* as well as promotes a reduced transcription of inflammatory cytokines and chemokines, revealing an interesting therapeutic approach to be applied in early detection.

Furthermore, recent studies (Abraki et al. 2013) demonstrate an important cross-talk in response to different stimuli for achieving an orchestrate pro/anti-inflammatory response, since both PPAR-γ stimulation via 15d-PGJ_2, product of COX-2 action (found increased in spinal cord of ALS patients (Kondo et al. 2002)), and the inhibition of the latter enzyme could lead to similar standards of neuroprotecion though the activation of nuclear factor E2-related factor 2 signalling pathway, decreasing NF-κB and reducing pro-apoptotic Bax/Bcl-2 ratio. And, for instance, PGE_2 suppression was demonstrated to increase motor function and lifespan in SOD1 mutant mice in mechanism implying a ROS reduction (Shin et al. 2012). Other example of inter-cellular dialogue in response to inflammation using stem cells to generate motor neurons and cocultered them with mutated (SODG93A) glia, demonstrate its sensitization towards PGD_2, since its inhibition partially rescues motor neuron loss (Di Giorgio et al. 2008). Finally, other examples to stress the relevance of this interconnectivity emerged from the finding of NF-κB immunoreactivity depletion from neuronal nucleus in spinal cord of patients, concomitant to an increased accretion in microglia (Sako et al. 2012). This might render neurons more vulnerable as well as prone microglia to inflammation-related processes.

Globally, all this multiple factors interfering between each other render ALS to have many faces. This may be among the most difficult point to be confronted. Hence, like most biological systems, unravel the complexity and interconnectivity of processes involved to finally rise ALS-like phenotype, challenge us and gives encourage force to keep researching. In any case, due to intrinsic ramification, a definite cure would probably emerge from cutting off several of different aspects rather than focusing in a single frame of the scene. In this sense, based on the multifactorial functions in FA metabolism, there are plenty of possibilities to be explored trying to alleviate disease sintomatology.

2. Hypothesis

2. Hypothesis

As commented in the introductory section, PUFA physiology is very important for global homeostasis. Numerous biologically relevant processes have been shown to be dependent on PUFA or its derivatives, and/or their actions are fundamental for a healthy condition.

On the other hand, CNS is particularly exquisite in terms of FA handling, further, special requirements of this tissue (e.g. high energetic demands, low antioxidant capacity, particular vascular and immune systems, extremely high cellular specificity, among others) points to a critical regulation of lipid metabolism for optimal functioning.

Finally, both ALS patients and models have shown mitochondrial, ER and Ca^{2+} malfunction in numerous cell types in the CNS linked to a hypermetabolism status, also in the muscular tissue. Furthermore, relevant inflammatory parameters as well as oxidative status (closely impinged by PUFA status) are raised along the disease progression in multiple locations.

Knowing the potential role of PUFA in all those processes and the sexual differences found in both, ALS and PUFA metabolism, altogether, we therefore hypothesized that *PUFA and/or their derivatives could alter oxidative status and responses to it in biological samples. Therefore they might play a role in the modulation and spread of oxidative damage and inflammation in various models of ALS disease and, further, those outcomes could be dietary modified in animal models*.

3. Objectives

3. Objectives

In order to validate the above mentioned hypothesis, we propose the following objectives:

GOAL 1.- To determine the potential of phenolic antioxidants as modulators of oxidative stress in a wide variety of biomolecules and their biological significance.

- **AIM 1.1-**To establish, by using immunological methods, a suitable methodology to ascertain the degree of protection which different phenolic antioxidants could offer to representative biomolecules (LDL, BSA, Human plasma) against various oxidative stress conditions.
- **AIM 1.2.-**To describe the effects of different phenolic antioxidants in the oxidative modification of proteins and lipids in human LDL under the Cu^{+2} oxidative system.
- **AIM 1.3.-**To establish the biological relevance (measured by cell viability test in two different cell lines-HMEC-1 and HepG2) of the antioxidant potential of various phenolic antioxidants against the noxious effect of *in vitro* oxidized LDL or oxidative stress inducers in human plasma.
- **AIM 1.4.-**To evaluate the biological relevance of an *ex vivo* oxidative system operated in both, a diet-induced hypercolesterolemic hamsters feed with a polyphenol extract and human plasma oxidized with Fe-Asc and protected with luteolin.

GOAL 2.- To describe the relevance of DHA and TDP-43 related processes in sALS and different ALS models.

- **AIM 2.1-**To describe DHA related enzymatic machinery (FADS1/2, ACAA1/2, ACOX1, SCP2) in sALS samples and different ALS models.
- **AIM 2.2-**To characterize DHA-related proteins (drebrin and syntaxin-3), its synthesis and the responsible enzymatic machinery in sALS samples and its *in vivo* and *in vitro* relation under stress conditions (TDP-43 and oxidative stress).
- **AIM 2.3-**To establish the relevance of FA precursors in OT model of ALS in terms of cell survival, fatty acid and lipidomic signatures and mitochondrial functionality (O_2 consumption) and lipid-phase antioxidants influence.

GOAL 3.- To define and describe the possible beneficial outcome of a nutritional intervention, regarding lipid unsatutration degree, in a mouse model of ALS.

- **AIM 3.1.-**To determine the effects of a dietary intervention (consisting in different lipid unsaturation degrees) on behavior, clinical phenotype and survival in a mouse model of ALS.

- **AIM 3.2.-**To test the biochemical effectiveness of the above-mentioned dietary intervention by measuring lipid profiles in different tissues, as well as surrogates of PUFA abundance (sintaxyn) and unfolding protein response elements (Ubiquitin).
- **AIM 3.3.-**To measure dietary interference in terms of protein oxidative modifications and its possible relationship with tissular lipid profile changes in above mentioned model.
- **AIM 3.4.-**To establish the potential relationship of nuclear damage response and protein damage crosstalk in such dietary intervention.

GOAL 4.- To clarify, in the same mouse model, the relationship between oxidative damage and mitochondrial dysfunction along the disease, with particular relevance on sexual dimorphism found.

- **AIM 4.1.-**To define a novel method for evaluation of mitochondrial function, based on O_2 consumption, in fresh slices of mice's spinal cord.
- **AIM 4.2.-**To evaluate, through the aforementioned methodology, the effect on the O_2 consumption of the SODG93A overexpression mouse model, dissected into the different mitochondrial complexes.
- **AIM 4.3.-**To determine the relationship between general *vs*. mitochondrial-specific protein oxidative damage measurements and the mitochondrial dysfunction in both genders of commented mouse model, in various points along disease progression.
- **AIM 4.4.-**To investigate whether estrogens treatment may recapitulate the modulated mitochondrial functionality in an *in vitro* cell line (N2A) model of ALS.

In order to answer all those questions, the main objectives were depicted into different research works, and fortunately were successfully written (but not all of them already published) to compose the Thesis core:

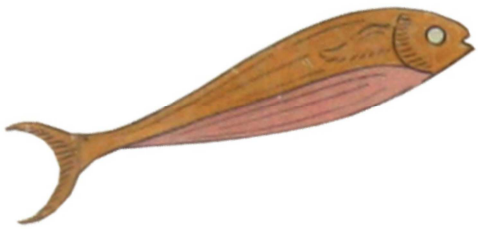
Paper one. Title: "Plant-derived Phenolics Inhibit the Accrual of Structurally Characterised Protein and Lipid Oxidative Modifications".

Paper two. Title: "Interplay between TDP-43 and docosohexaenoic acid-related processes in amyotrophic lateral sclerosis".

Paper three. Title: "Dietary Lipid Unsaturation Influences Survival and Oxidative Modifications of an Amyotrophic Lateral Sclerosis Model in a Gender-Specific Manner".

Paper four. Title: "Gender specific differences in spinal cord mitochondrial function and oxidative damage markers in a mouse model of ALS in early stages of disease".

4. Articles



4.1 Article 1

Title: “Plant-derived Phenolics Inhibit the Accrual of Structurally Characterised Protein and Lipid Oxidative Modifications”.

Authors: Soler-Cantero A, Jové M, Cacabelos D, Boada J, Naudí A, Romero MP, Cassanyé A, Serrano JC, Arola L, Valls J, Bellmunt MJ, Prat J, Pamplona R, Portero-Otin M, Motilva MJ.

Journal: Public Library of Science, PLoS ONE 7(8): e43308
Received: April 5th 2012 / Accepted: July 23rd 2012. Published: August 29th 2012.
DOI:10.1371/journal.pone.0043308

OPEN ACCESS Freely available online

PLOS ONE

Plant-Derived Phenolics Inhibit the Accrual of Structurally Characterised Protein and Lipid Oxidative Modifications

Arantza Soler-Cantero[1☯], Mariona Jové[2☯], Daniel Cacabelos[2☯], Jordi Boada[2☯], Alba Naudí[2], Maria-Paz Romero[1], Anna Cassanyé[2], José C. E. Serrano[2], Lluis Arola[3], Josep Valls[4], Maria Josep Bellmunt[2], Joan Prat[2], Reinald Pamplona[2], Manuel Portero-Otin[2]*, Maria-José Motilva[1]*

1 Departament of Food Technology, CeRTA-TPV, Escola Tècnica Superior d' Enginyeria Agrària, Universitat de Lleida, Lleida, Spain, **2** NUTREN-Nutrigenomics Center, Department of Experimental Medicine, Parc Científic i Tecnològic Agroalimentari de Lleida-Universitat de Lleida-IRBLLEIDA, Lleida, Spain, **3** Department of Biochemistry and Biotechnology, Nutrigenomic Research Group, Universitat Rovira i Virgili, Tarragona, Spain, **4** Shirota Functional Foods, S.L., Reus, Spain

Abstract

Epidemiological data suggest that plant-derived phenolics beneficial effects include an inhibition of LDL oxidation. After applying a screening method based on 2,4-dinitrophenyl hydrazine- protein carbonyl reaction to 21 different plant-derived phenolic acids, we selected the most antioxidant ones. Their effect was assessed in 5 different oxidation systems, as well as in other model proteins. Mass-spectrometry was then used, evidencing a heterogeneous effect on the accumulation of the structurally characterized protein carbonyl glutamic and aminoadipic semialdehydes as well as for malondialdehyde-lysine in LDL apoprotein. After TOF based lipidomics, we identified the most abundant differential lipids in Cu^{++}-incubated LDL as 1-palmitoyllysophosphatidylcholine and 1-stearoyl-sn-glycero-3-phosphocholine. Most of selected phenolic compounds prevented the accumulation of those phospholipids and the cellular impairment induced by oxidized LDL. Finally, to validate these effects in vivo, we evaluated the effect of the intake of a phenolic-enriched extract in plasma protein and lipid modifications in a well-established model of atherosclerosis (diet-induced hypercholesterolemia in hamsters). This showed that a dietary supplement with a phenolic-enriched extract diminished plasma protein oxidative and lipid damage. Globally, these data show structural basis of antioxidant properties of plant-derived phenolic acids in protein oxidation that may be relevant for the health-promoting effects of its dietary intake.

Citation: Soler-Cantero A, Jové M, Cacabelos D, Boada J, Naudí A, et al. (2012) Plant-Derived Phenolics Inhibit the Accrual of Structurally Characterised Protein and Lipid Oxidative Modifications. PLoS ONE 7(8): e43308. doi:10.1371/journal.pone.0043308

Editor: Jose Vina, University of Valencia, Spain

Received April 5, 2012; **Accepted** July 23, 2012; **Published** August 29, 2012

Funding: This study was supported in part by Research and Development grants from the Spanish Ministry of Education and Science (BFU2006-14495/BFI) and the Generalitat of Catalunya (2005SGR00101) to RP; the Spanish Ministry of Health (FIS 04-0355, 05-2214 and 05-2241) to MPO, Spanish Ministry of Education and Science (AGL2005-07881-C02-01 and AGL2006-12433) to MJM and MPO. Supported partially by the COST B-35 Action. The present work was supported by the CENIT program from the Spanish Minister of Industry and by a consortium of companies led by La Morella Nuts S. A. (Reus, Catalonia, Spain) with the following companies: Shirota Functional Foods, S. L.; KRAFT; BTSA, Biotecnologías Aplicadas, S. L.; Selección Batallé, S. A.; Industrial Técnica Pecuaria, S. A.; Neuron BioPharma, S. A.; Grupo Leche Pascual, S. A. U.; Innaves, S. A. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: This study was partly funded by a consortium of companies led by La Morella Nuts S. A. (Reus, Catalonia, Spain) with the following companies: Shirota Functional Foods, S. L.; KRAFT; BTSA, Biotecnologías Aplicadas, S. L.; Selección Batallé, S. A.; Industrial Técnica Pecuaria, S. A.; Neuron BioPharma, S. A.; Grupo Leche Pascual, S. A. U.; Innaves, S. A. JV is employed by Shirota Functional Foods. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: manuel.portero@mex.udl.cat (MPO); motilva@tecal.udl.cat (MJM)

☯ These authors contributed equally to this work.

Introduction

The oxidative stress hypothesis of atherosclerosis is based on the fact that free radical-derived damage participates in atherogenesis pathophysiology by means of LDL modification, among other mechanisms [1–3]. Supporting this hypothesis, LDL modified by oxidation (oxLDL) has been detected in atherosclerotic lesions [4–6] and oxLDL exhibits various proatherogenic activities *in vitro* [3,7].

It is important to emphasize that oxLDL represents a heterogeneous population of modified forms of LDL that differ greatly in their chemical composition and biological properties. The conversion of native LDL into highly modified LDL via oxidative processes can occur by two major mechanisms: In the first case, the events start with the complete loss of LDL's endogenous antioxidants (i.e., α-tocopherol, ubiquinol-10), followed by the conversion of a majority of the polyunsaturated fatty acids (PUFA) into their corresponding hydroperoxides. Then, these primary lipid oxidation products are fragmented into secondary lipid oxidation products, such as malonyldialdehyde or 4-hydroxynonenal, which can react with the N$\sum$-amino group of lysine residues from LDL apoprotein (Apo B-100). Consequently, the particle's electrophoretic mobility increases and the lipoprotein becomes "high uptake" [8]. Additionally, in the subendothelial space, oxLDL may exert a "Trojan horse effect", e.g. allowing the diffusion of those lipid mediators modifying

endothelial and vascular smooth muscle cells. The second case is characterized by the immediate and preferential oxidation of amino acid residues from Apo B-100 in the absence of substantial consumption of lipid soluble antioxidants and/or occurrence of lipid peroxidation [9]. Nevertheless, apoprotein oxidative modifications have been not studied as extensively as lipid-phase ones.

The beneficial effects of Mediterranean diet concerning cardiovascular diseases are well known. Most of them are associated with olive oil and wine consumption [10,11], nutrients with a high content of phenolic acids. With regard to the influence of diet on atherosclerosis, the ingestion of fruits and vegetables (phenolic-rich vegetal sources) is related to a lesser development of atheroma plaque [12,13]. This effect is mainly attributed with their protective effects against LDL oxidation [14–17]. Despite a great deal of research having been devoted to the prevention of lipid peroxidation in LDL by antioxidants, including phenolic acids [18–21], few studies have reported the prevention of protein oxidation in LDL by exogenous antioxidants. Apo B-100 modifications, e.g., the binding of lipid peroxidation products or direct oxidation of amino acid side-chain residues, are thought to finally result in the formation of new epitopes that are specifically recognized by scavenger receptors [22–25], but the potential preventive effect of nutritional compounds on apoprotein oxidative modifications have not been studied so extensively.

To fill those gaps, the antioxidant effect of 21 different vegetal-derived phenolic compounds (mainly founded in olive oil and grape-derived products) in the oxidation of apolipoprotein of human LDL was assessed in this study. For that purpose, the quantification of carbonyl groups (detected by Western-Blot) was carried out in LDL-model systems. The antioxidant behaviour of the more active phenols was then further characterized by measuring the protection of the lipidome changes induced by Cu^{++}, the accumulation of specific oxidation and lipid peroxidation markers in LDL apoproteins. The preventive role on the loss of cell viability induced by Cu^{++}-treated LDL was tested using these phenolic compounds. Finally, in order to test whether those or related phenolic compounds would have similar effects *in vivo* in an atherosclerosis model, carbonyl content of plasma proteins and lipid oxidation markers were analysed in hypercholesterolemic hamsters fed with a phenolic- enriched diet.

Methods

Reference Compounds

α-Tocopherol was purchased from Sigma-Aldrich Chemical Co (St. Louis, MA, USA). Phenolic standards from the following sources were used without further purification: 2-(3,4-dihydroxyphenyl)-4,5-dihydroxy-3-[3,4,5-trihydroxy-6-[(3,4,5-trihydroxy-6-methyl-oxan-2-yl)oxymethyl]oxan-2-yl]oxy-chromen-7-one (rutin), 4′,5,7-tihydroxyflavone (apigenin), apigenin 7-*O*-glucoside, 3′,4′,5,7-tetrahydroxyflavone (luteolin), luteolin 7-*O*-glucoside, *trans*-4-hydroxycinnamic acid (*p*-coumaric acid), 2-(3,4-dihydroxyphenyl) ethyl alcohol (OH-tyrosol) (3,4-DHPEA), 2-(4-hydroxyphenyl) ethyl alcohol (tyrosol) (*p*-HPEA), oleuropein, verbascoside and vanillin from Extrasynthese (Genay, France); 3,4-dihydroxycinnamic acid (caffeic acid), ferulic acid, and 3,4,5-trihydroxybenzoic acid (gallic acid) from Fluka Co. (Buchs, Switzerland); Pinoresinol from Arbonova Sales (Turku, Finland). Phenolic enriched grape seed extract was obtained as previously described [26].

Isolation of Phenolic Compounds by Semipreparative HPLC

Secoiridoid derivatives 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), 4-hexenoic acid, 4-formyl-3-(2-oxoethyl)-2-(3,4 dihydroxyphenyl) ethyl ester (3,4-DHPEA-EDA), methylated form of the oleuropein aglycone (ME 3,4-DHPEA-EA) and 4-hexenoic acid, 4-formyl-3-(2-oxoethyl) 2-(4-hydroxyphenyl) ethyl ester (*p*-HPEA-EDA) were isolated from virgin olive oil phenolic extract by semi-preparative HPLC method according to the method of Artajo et al. [27]. Stock solutions of commercial standards and phenolic compounds isolated from virgin olive oil were dissolved in methanol/H_2O (80:20 v/v) and stored at $-40^{\circ}C$ before the evaluation of their antioxidant activity. The chemical structures of the phenols included in the study are shown in Figure S1.

Hypercholesterolemic hamsters and human plasma

Twelve male Gold Syrian hamster weighing 127.55 ± 6.75 g were randomly assigned to two groups ($n = 6$ for each group) with approximately equal mean group body weights. They were caged and maintained in a 12:12 (light:dark) cycle at $22 \pm 2^{\circ}C$ and $50 \pm 10\%$ relative humidity with free access to both food and water. Food intake and body weight were controlled every week.

The control group ate an atherogenic diet [28] in which the cholesterol content had been set at 5% and which was supplemented with 15% of lard, and the experimental group ate this atherogenic diet supplemented with a 0.2% of phenolic-enriched vegetal extract (Table 1). The diets were maintained during 12 weeks and the animals were deprived of food for 15 h. Hamsters were anesthetized and then sacrificed by heart puncture and plasma and serum were collected and stored at $-20^{\circ}C$ until analysis. For metabolomic analyses, the method of Wikoff et al was used [29]. The plasma triglycerides, cholesterol, HDL, LDL, and alkaline phosphatase content were quantified by enzymatic

Table 1. Control and supplemented diet composition.

Component	Control (g)	Supplemented (g)
Casein	200	200
L-Cysteine	3	3
Corn starch	362	362
Sugar	140	140
Corn oil	0	0
Cellulose	50	48
Minerals	35	35
Lard	150	150
Vitamins	10	10
Cholesterol	50	50
Polyphenol extract	0	2
Total weight	**1000**	**1000**
Energy (Kcal/g diet)	4.62	4.62
Carbohydrates (g/Kg)	**502**	**502**
% Energy	43.5	43.5
Proteins (g/Kg)	**203**	**203**
% Energy	17.6	17.6
Lipids	**200**	**200**
% Energy	39	39

The control diet is an atherogenic diet.
doi:10.1371/journal.pone.0043308.t001

colorimetric reactions using commercial kits (Spinreact, Girona, Spain).

Human plasma was obtained from 6 different, healthy male donors after an overnight fast (mean age: 25±3) by standard venepuncture and centrifugation using EDTA coated Vacutainer tubes. Both plasma obtention and animal experiments were supervised and approved by the Experimental and Ethics Committee of the University of Lleida.

Protein oxidation screening method: Protein oxidation and Western blot analysis

Aliquots of phenolic acid compounds dissolved in methanol were transferred to Eppendorf tubes and desiccated under a nitrogen current at room temperature. The dried phenols were redissolved to a final concentration of 5 μM with PBS (except when indicated) containing dissolved protein. Then, protein (700 μg/ml) was oxidized by exposure to different prooxidants. We used i) $CuSO_4$ (5 μmol/l free Cu^{++}); ii) Hemin:H_2O_2 (30 μmol/l: 5 μmol/l); iii) H_2O_2 (5 μmol/l); iv) Ascorbate:Fe^{3+} (1.5 mmol/l: 8 μmol/l); v) ultraviolet radiation (λ 254 nm) and v) myeloperoxidase (MPO) (0.77 U/mL and H_2O_2 100 μmol/l) in phosphate buffered media at 37°C for 3 hours, according published procedures [30]. All samples were used immediately or stored at −80°C for further analysis.

To assess the extent of protein oxidation, 2,4-dinitrophenylhydrazine (DNP)-reactive carbonyls were measured by Western Blot as previously described [31] (See Methods S1 to further information).

Measurement of glutamic (GSA) and aminoadipic (AASA) semialdehydes and malondialdehyde lysine (MDAL)

The concentration of chemically characterized markers of protein oxidative modification GSA, AASA, and MDAL, in LDL apoproteins was evaluated by gas chromatography/mass spectrometry (GC/MS) as previously described [31] (See Methods S1 to further information).

Lipidome analyses

Lipid composition was assessed by both fatty acid analysis (see Methods S1 to further information) and time of flight mass spectrometry (TOF)-based lipid molecular species analyses. In both cases the total lipids from LDL were extracted with chloroform:methanol (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene as previously described [31].

For TOF-based lipid molecular species analyses, lipid extracts (from LDL) or methanolic extracts (from hamster's plasma) were submitted to mass-spectrometry using a LC ESI-QTOF MS/MS 6520 (Agilent Technologies, Barcelona, Spain), coupled to a capillary LC module using an untargeted approach as described [29] (see Methods S1 to further information). In order to offer a relative quantification of 1-palmitoyl-2-(5-oxovaleryl)-*sn*-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-*sn*-glycero-3-phosphocholine (PGPC), bioactive lipids present in oxLDL mass profiles [32] were integrated for an m/z of 594.3 for POVPC and 610.2 for PGPC with a Δ of 0.01 Da.

FRAP assay

The ferric reducing antioxidant power of the samples was estimated according to the procedure previously described [33,34]. Briefly, FRAP reagent, was mixed with distilled water and either of sample or appropriate reagent blank. The readings at 30 min were selected for calculation of FRAP values. Reduction power activities were as μmol of Trolox equivalents, per gram of dry matter.

Cell viability

Both HMEC (kindly providen by Anne Negre-Salvayre, INSERM, Toulouse [35] and HepG2 viability were measured with the MTT-based Cell Toxicity Colorimetric Assay Kit (Sigma-Aldrich, St.Louis, MA, USA) according to the manufacturer's instructions after oxLDL tert-butylhydroperoxide (t-BOOH) as described [36,37]. The results were expressed as the percentage of viability versus cells exposed to non-oxidized LDL or untreated with t-BOOH. Further details are described in the Methods S1 section.

Statistical analyses

All statistics were analysed using the SPSS software (SPSS Inc., Chicago, IL, USA). Differences between the groups were analysed by the Student's T tests or ANOVA (with post-hoc analyses for detecting differences between specific pairs), after assessment of normal distribution of variables by the Kolmogorov-Smirnoff test. Correlations between variables were evaluated by the Pearson statistic and plotted with the Metaboanalyst software [38] The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

Effect of individual phenolic compounds on LDL oxidation. LDL oxidation by different methods induces accumulation of carbonyl in LDL apoproteins: differential inhibitory potential of phenolic compounds

The antioxidant capacity was quantified after Western Blot of DNP reactive carbonyls in LDL apoproteins (Figure 1A) being the value corresponding to oxLDL considered 0% of antioxidant capacity. The anti-DNP immunoreactivities which are lower than that found in oxLDL were interpreted as anti-oxidant activity and that which are higher as prooxidant activities. The different phenols were tested at three concentrations (5, 50 and 100 μM) (Figure 1B), using α-tocopherol as a reference. Apoprotein oxidation was significantly inhibited by the majority of the phenols tested. The OH-tyrosol showed the maximal efficiency even at 5 μM, higher than the efficiency of α-tocopherol. Luteolin (flavonoid), pinoresinol (lignan), gallic and caffeic acids showed a good efficiency which was concentration-dependent. These phenols reduced the Cu^{++} inducted oxidation by between 60 and 80%. The secoiridoid derivatives (3,4-DHPEA-EDA and *p*-HPEA-EDA) showed a slight activity, similarly to α-tocopherol. Other phenols, such as verbascoside, vanillin, 3,4-DHPEA-AC and the methylated form of the oleuropein aglycone (ME 3,4-DHPEA-EA) showed lower antioxidant activity with oxidation inhibition values below 20%. The prooxidant effect shown by some phenols, such as oleuropein, tyrosol and apigenin in its aglycone and glucosidic forms should also be noted.

As Figure 1B shows, different phenolic compounds exhibit differential effects in a dose dependent fashion, in the apoprotein moiety of human LDL. According to this first screening, the more active phenols selected were: OH-tyrosol (OH-tyrosol), 3,4-DHPEA-EDA (3,4-DHPEA) as secoiridoid derivative; pinoresinol (Pin) as a lignane; luteolin (Lut) as a flavonoid; and gallic (Gallic) acids as a phenolic acid.

In order to extend those findings from Cu^{++} to other oxidation systems, we measured the effects of phenolics in different systems ranging from ultraviolet radiation to enzymatic paradigm (Figure 1C, D, E, F and G). Of those, only UVA, Fe^{+3}-Asc and H_2O_2 increased carbonyl content more than 50% in LDL (Figure 1D, E and F). In those systems, all phenols analysed had

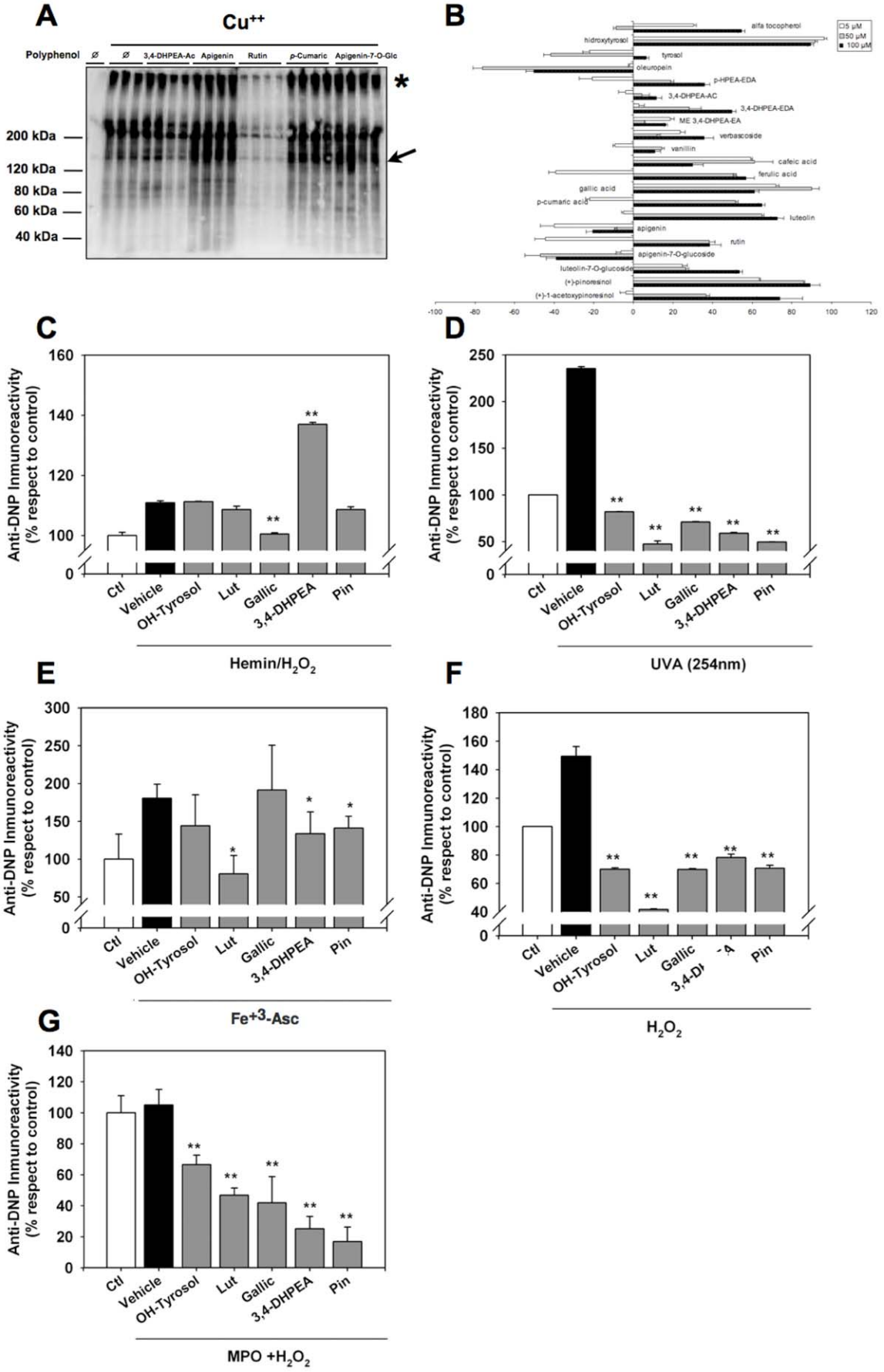

A
Cu++
Polyphenol
Ø
Ø
3,4-DHPEA-Ac
Apigenin
Rutin
p-Cumaric
Apigenin-7-O-Glc
200 kDa
120 kDa
80 kDa
60 kDa
40 kDa
B
alfa tocopherol
hidroxytyrosol
tyrosol
oleuropein
p-HPEA-EDA
3,4-DHPEA-AC
3,4-DHPEA-EDA
ME 3,4-DHPEA-EA
verbascoside
vanillin
cafeic acid
ferulic acid
gallic acid
p-cumaric acid
luteolin
apigenin
rutin
apigenin-7-O-glucoside
luteolin-7-O-glucoside
(+)-pinoresinol
(+)-1-acetoxypinoresinol
C
Anti-DNP Inmunoreactivity (% respect to control)
Ctl
Vehicle
OH-Tyrosol
Lut
Gallic
3,4-DHPEA
Pin
Hemin/H2O2
D
UVA (254nm)
E
Fe+3-Asc
F
H2O2
G
MPO +H2O2

Figure 1. Plant-derived phenolic acids inhibit protein oxidation in isolated human LDL to varying degrees. A. Representative Western Blot of the screening phase for LDL apoprotein protection in oxLDL. Data are presented for 3,4-DHPEA-AC, apigenin, rutin, *p*-cumaric acid and apigenin-7-O-glucoside at 100 μM. Veh:Vehicle, * indicates Apo B-100 molecular weight and arrow indicates protein fragmentation induced by oxidation. **B:** Effect of plant-derived phenolic acids in protein carbonylation in Cu^{++}-incubated LDL. All phenols were tested at three concentrations (5 μM, 50 μM and 100 μM). LDL and Cu^{++} incubated LDL were used as control of native and oxidized LDL. The antioxidant capacity of each phenol at different concentrations is expressed as percentual values, considering that the immunoreactivity of Cu^{++}-incubated LDL is considered 0% of antioxidant capacity - positive values reveal decreased carbonyl formation and negative values reveal increased protein carbonylation-. LDL Apoprotein oxidation was also prevented to various extents in oxidation induced with Hemin:H_2O_2 (**C**), Ultraviolet radiation (254 nm) (**D**), Fe^{3+}:Ascorbate (**E**), H_2O_2 (**F**) and MPO: H_2O_2 (**G**) as indicated in the *Methods and Materials* section. In the case of C-G, phenols were tested at 5 μM. In these, carbonyl contents are expressed as percentual values, considering that the immunoreactivity of LDL incubated without oxidant (Ctl) as 100% of carbonyl content. *p<0,05 and ** p<0,01 by ANOVA respect to oxidant-incubated LDL. Data shown are mean±S.D., (n = 4 for each data point).
doi:10.1371/journal.pone.0043308.g001

a strong antioxidant action, with the exception of Fe^{+3}-Asc, where only luteolin (and 3,4-DHPEA and pinorresinol to a minor extent) had a significant effect. Of note, pathogenically relevant systems such as MPO+H_2O_2 do not offer a significant increase in carbonyl staining (Figure 1G). Most importantly, all selected phenolic compounds are able to significantly inhibit the accrual of carbonyl modification after MPO+H_2O_2 incubation, being pinoresinol the most active.

In order to extent those results to other proteic systems, BSA and human plasma were oxidized using Hemin-H_2O_2, UVA and Fe^{3+}-Asc. These results (Figure S2) do not allow to distinguish any individual phenol as a general antioxidant, i.e. its effect being independent of the protein and oxidation-system used. Generally, BSA is less oxidizable than LDL in same conditions, and only pinoresinol and luteolin diminish significantly protein oxidation in specific systems (Figure S2B and S2C). In contrast, human plasma is more oxidizable than BSA (under UVA and Fe^{+3}-Asc). Under Fe^{3+}-Asc, again pinoresinol and luteolin stand out as significant inhibitors of protein oxidation in plasma (Figure S2E and S2F). Of note, several polyphenols (notably gallic acid) exhibited prooxidant actions under these conditions (Figure S2B and S2D).

Cu^{++} incubation induces accumulation of metal-catalyzed oxidation (MCO), lipoxidation markers in LDL apoproteins and changes in LDL lipidome: differential inhibitory potential of phenolic acid compounds

Taking into account that DNP reactive carbonyls could arise from either lipid peroxidative damage or the direct modification of aminoacid residues by MCO [39] specific probes for each of those oxidative modifications were measured by using GC/MS. The results show that Cu^{++} incubation led to significant increases in the MCO markers GSA, AASA and an even greater increase in the lipoxidation marker MDAL (Figure 2A, B and C). OH-tyrosol and the lignane were the most effective compounds for inhibiting GSA accumulation. Luteolin also prevented its accumulation in Cu^{++}-treated LDL. Neither gallic acid, nor 3,4-DHPEA-EDA were effective (Figure 2A). A similar pattern was observed for AASA accumulation, but in this case, gallic acid was significant inhibitors of its formation (Figure 2B). Finally, OH-tyrosol and pinoresinol were potent antioxidants in considering MDAL accumulation, while 3,4-DHPEA-EDA, and specially luteolin (with no significant effect), were among the lowest in this sense (Figure 2C). To reinforce the importance of the lipid composition in relation with the lipoxidative modifications of proteins, a significant correlation was observed ($r^2 = 0.91$; $p<0.0001$) between PI and MDAL formation (Figure 2D).

Carbonyl modification of apoproteins can arise from lipid peroxidation. In such a case, the LDL fatty acid composition exhibits PUFA consumption. To test this, the fatty acid composition of LDL was analysed after Cu^{++}-induced oxidation, demonstrating significant losses in PI and PUFA content (Table 2). For this reason we examined the potential for prevention of this phenomenon. OH-tyrosol, and the lignane were among the most potent compounds, almost preventing the effect of Cu^{++}. Gallic acid was less potent, and luteolin and 3,4-DHPEA-EDA had almost no effect on the oxidative modification of LDL

After TOF-based analyses of oxLDL, palmitoyllysophosphatidylcholine (PLPC), 1-stearoyl-sn-glycero-3-phosphocholine (SGPC) and 1-oleoylglycerophosphocholine (OGPC) comprised more than 90% of the differentially present lipids in Cu^{++}-treated LDL (Figure 2E). Similarly to the fatty acid analyses, luteolin and gallic acid exhibit the lowest capacity for preventing the build-up of PLPC, while the other compounds prevented the accumulation of this compound almost completely. Luteolin was the only compound unable to inhibit the formation of SGPC (Figure 2F and 2G).

Taking into account that 1-palmitoyl-2-(5-oxovaleryl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC) have been identified as biological effectors of oxLDL [32], the presence of those compounds was ensured. As expected, their level increased in Cu^{++}-treated samples (12 and 4-fold over untreated samples, respectively). Luteolin and gallic acid prevented the accumulation PLPC to a lower extent when compared with the other compounds (Figure 2F).

Biological relevance of plant-derived phenolics antioxidant effects: Loss of cell viability induced by Cu^{++}-treated LDL and in vivo evidences of protein and lipid antioxidant activity

To further reinforce the biological relevance of the antioxidant potential of those compounds and the methodology described here for its identification, we examined the cytotoxic potential of Cu^{++} incubated LDL in an endothelial cell culture model. For this purpose, the endothelial cell line HMEC-1 was treated with Cu^{++}-treated LDL and 18 h later, the viability of the cultures was assessed with the MTT assay. The results demonstrate that oxLDL led to a loss of 60% of viability and OH-tyrosol prevented partially those effects (Figure 3A), inducing only a 10% of viability loss. Unexpectedly, luteolin, a compound with a low antioxidant potential, based on lipidome changes, showed a significant preventive effect on the oxLDL induced loss of viability.

To ascertain whether this was due to a specific cellular effect (e.g. by modulation of antioxidant cellular responses in endothelial cells), we examined the potential influence of those compounds in *tert*-BOOH-mediated cell death in a HepG2 cell line, an unrelated cell line. Those analyses demonstrate that luteolin, OH-tyrosol and 3,4-DHPEA-EDA were the only compounds able to prevent significantly the loss of viability secondary to treatment with *tert*-BOOH (Figure 3B).

The correlation of cell viability with antioxidant capacity reinforced the pathogenic importance of LDL apoprotein oxidative modification, as that GSA amount showed the most significant correlation with the loss of viability induced by oxLDL (Figure 3C).

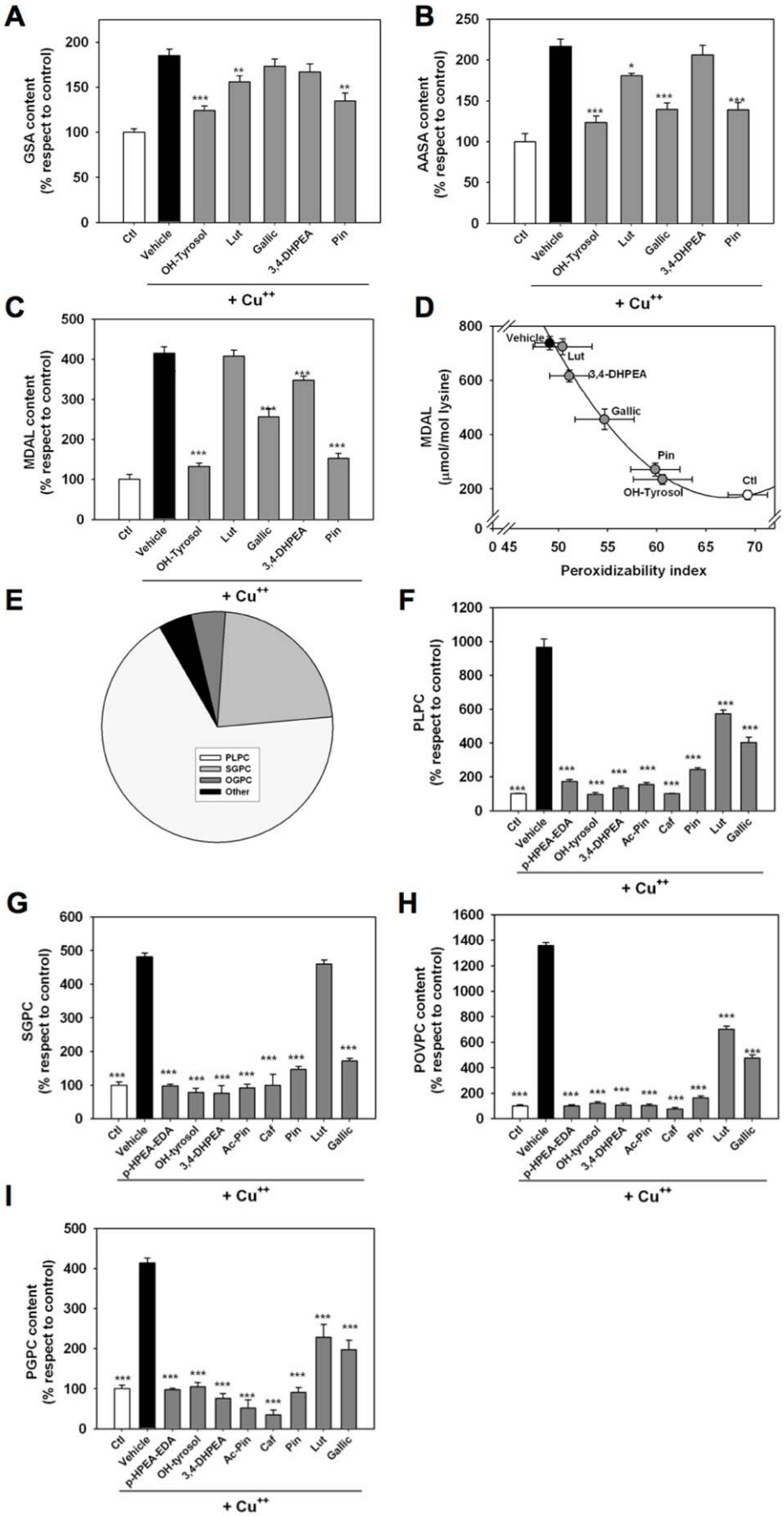

A
GSA content (% respect to control)
Ctl
Vehicle
OH-Tyrosol
Lut
Gallic
3,4-DHPEA
Pin
+ Cu++
B
AASA content (% respect to control)
C
MDAL content (% respect to control)
D
MDAL (μmol/mol lysine)
Peroxidizability index
E
PLPC
SGPC
OGPC
Other
F
PLPC (% respect to control)
p-HPEA-EDA
Ac-Pin
Caf
G
SGPC (% respect to control)
H
POVPC content (% respect to control)
I
PGPC content (% respect to control)

Figure 2. Cu^{++}-incubated LDL show significant increases in the amounts of mass-spectrometry characterized protein and lipid oxidation markers, whose formation is inhibited by selected plant-derived phenolic acids. A–C show GC/MS analyses of GSA and AASA (markers of metal-catalyzed oxidation), and MDAL, originated from lipoxidation. Values shown are % changes of mean ± S.E.M. over values from non-oxidized LDL (GSA: 5945±61 μmol/mol lysine; AASA: 1128±20 μmol/mol lysine; and MDAL: 177±23 μmol/mol lysine); *p<0,05; ** p<0,01 and *** p<0,001 respect to Cu^{++} incubated LDL. D: protein lipoxidative damage shows a quadratic relationship with peroxidizability index (PI) (r^2 = 0,911;p<0,0001; model:[MDAL] = 1,03*[PI]^2-151,26*[PI]+5698) **E:** Pie plot showing distribution of differential (Student's T test p<0.001) lipid species between control and Cu^{++}-incubated LDL, after TOF-based lipidome analyses. **F:** Effect of plant-derived phenolic acids in the accumulation of PLPC induced by Cu^{++}-incubation. **G:** Effect of plant-derived phenolic acids in the accumulation of SGPC induced by Cu^{++}-incubation. **H:** Effect of plant-derived phenolic acids in the accumulation of POVPC induced by Cu^{++}-incubation. **I:** Effect of plant-derived phenolic acids in the accumulation of PGPC induced by Cu^{++}-incubation. Values shown are mean ± S.E.M. over values from Cu^{++}-incubated LDL, *** p<0,001 (n = 4 for each data point).
doi:10.1371/journal.pone.0043308.g002

Protein antioxidant activity of phenolic acids should be bioavailable. For this reason we analysed the plasma proteins from a hypercholesterolemic model (Golden Syrian Hamsters under hypercholesterolemic diet, showing an increase around 125% in cholesterol levels respect animals under standard chow) under a diet enriched in vegetable-derived phenols. The polyphenol supplementation did not induce any signs of toxicity nor changes in alkaline phosphatase, indicating that it was well tolerated. Despite an effect in LDL cholesterolemia was noted, no significant differences were evidenced in other values analysed (Table S1). Nevertheless, the results (Figure 3D) demonstrate that phenolic-enriched diet significantly diminishes steady state levels of circulating protein carbonyls. Furthermore, their intake reduces oxidability of proteins *ex vivo* under Fe^{3+}-Asc system, both in hypercholesterolemic hamsters (Figure 3E) and in human plasma with exogenously added luteolin at 5 μM (Figure 3F). Noteworthy, compounds with masses compatible with oxidized phospholipids as PLPC, OGPC and PGPC were detected in the methanolic extract of hamster plasma (Figure S3, S4, S5). The levels of those metabolites were diminished in animals under phenolic-enriched diet (Figure 3G, H and I). Reinforcing the importance of specificity of oxidative damage measurements, FRAP assay of plasma demonstrated that polyphenol-enriched diet did not change antioxidant capacity (Figure 3J), although it changed significantly profiles of metabolic parameters by hierarchical clustering (Figure 3K).

Discussion

The present work demonstrates the antioxidant properties of selected plant-derived phenols (mainly found in olive oil and grape-derived products) in protein oxidation both *in vivo* and *in vitro*, and the existence of some heterogeneity in the mechanisms. We offer a novel approach for analysing and screening the antioxidant properties of natural products over LDL modification, especially in its protein components. After a screening phase, five different compounds were selected for further analysis. We have demonstrated for the first time, the effect of those nutritional compounds on the accrual of structurally characterized markers of both protein (GSA, AASA and MDAL) and lipid peroxidation (POVPC, PGPC) in oxLDL. The pathogenic importance of LDL protein oxidation is shown by the fact that it was the only factor that correlated with oxLDL-induced cell death. To extend the validity to an *in vivo* setting, we have shown that phenolic intake in a hypercholesterolemic context diminishes protein carbonyl content in plasma as well as the concentration of lipid peroxidation markers.

Table 2. Effect of Cu^{++} and phenolic acids in LDL fatty acid composition.

	Ctl	Cu^{++}	OH-Tyrosol	Lut	Gall	3,4-DHPEA	Pin
16:0	21,11±0,59*	24,78±0,78	23,39±0,84	26,55±0,91	24,25±0,65	24,77±0,8	23,32±0,10
16:1n-7	3,06±0,28*	3,27±0,55	3,00±0,66*	3,33±0,26	3,25±0,87	3,10±0,7	3,26±0,15
18:0	11,55±0,16	11,08±0,93	8,90±0,30*	11,63±0,35	10,08±0,41	11,41±0,0	9,36±0,46*
18:1n-9	22,77±0,97**	26,38±0,26	24,91±0,27	25,37±0,06	25,24±0,93	25,74±0,0	24,61±0,69
18:2n-6	28,84±0,50**	24,97±0,21	30,39±0,33**	23,11±0,81	28,02±0,8**	26,09±0,9*	29,78±0,13**
20:3n-6	1,59±0,47*	0,98±0,07	1,39±0,85*	0,88±0,02	1,18±0,14*	1,01±0,1	1,24±0,22*
20:4n-6	6,05±0,96**	3,08±0,70	4,62±0,19*	2,67±0,25	3,77±0,14*	3,32±0,6	4,29±0,65*
ACL	17,74±0,9	17,5±0,7	17,6±0,2	17,5±0,1	17,5±0,8	17,5±0,7	17,6±0,1
SFA	34,41±1,2*	38,1±0,3	33,8±0,0*	40,2±0,6*	36,1±0,4	37,9±0,8	34,6±0,3*
UFA	65,59±2,2*	61,8±1,7	66,2±1,0*	59,7±0,4	63,8±0,6	62,0±2,2	65,4±1,2*
MUFA	26,07±0,7*	30,3±0,5	28,0±0,2*	28,8±0,8	28,7±0,1	29,0±0,2	28,0±0,2*
PUFA	39,52±0,8**	31,5±0,2	38,2±0,1**	30,8±0,6	35,1±0,6*	33,0±0,0	37,4±0,2*
PUFAn-3	1,67±0,02*	0,9±0,08	1,2±0,1	0,8±0,1	1,0±0,5	1,0±0,7	1,3±0,1
PUFAn-6	37,85±1,2*	30,5±0,4	36,9±0,9*	30,05±0,1	34,1±0,1*	31,9±0,3	36,1±0,1*
DBI	124,58±3,2**	104,6±0,6	119,0±0,6**	103,2±0,0	111,6±0,8*	106,6±0,5	117,3±0,7**
PI	69,26±2,3**	49,1±0,1	60,6±0,1**	50,4±0,2	54,6±0,9*	51,1±0,1	59,8±0,6*

Values: mean±S.E. ACL, average chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; PUFA n-6/n-3, polyunsaturated fatty acids n-6 or n-3 series; MUFA, monounsaturated fatty acids; DBI, double bond index; PI, peroxidizability index,
*p<0,05 and ** p<0,01 respect to values in Cu^{++} incubated LDL by ANOVA post-hoc analyses (n = 4 for each data point).
doi:10.1371/journal.pone.0043308.t002

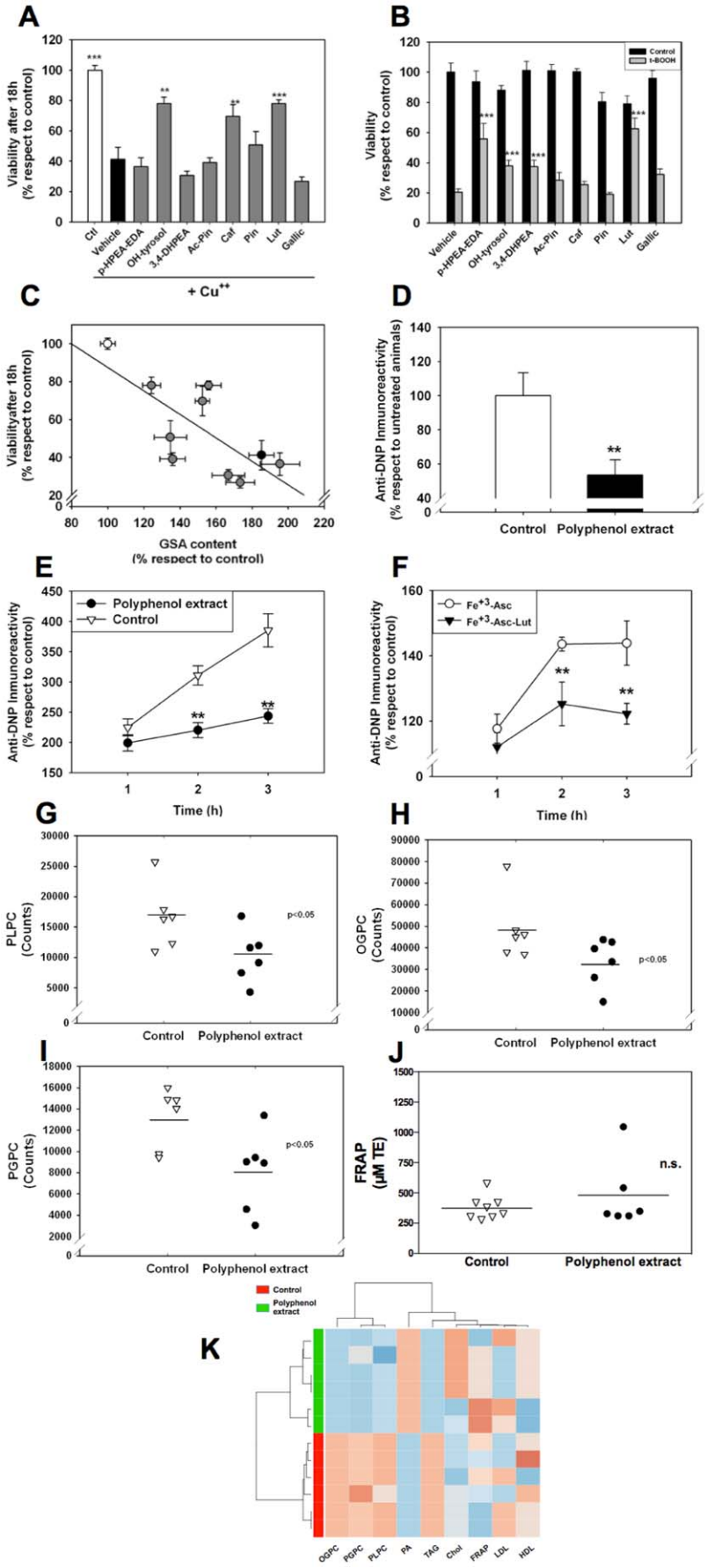

A
Viability after 18h (% respect to control)
Ctl
Vehicle
p-HPEA-EDA
OH-tyrosol
3,4-DHPEA
Ac-Pin
Caf
Pin
Lut
Gallic
+ Cu++
B
Viability (% respect to control)
Control
t-BOOH
C
Viabilityafter 18h (% respect to control)
GSA content (% respect to control)
D
Anti-DNP Inmunoreactivity (% respect to untreated animals)
Control
Polyphenol extract
E
Anti-DNP Inmunoreactivity (% respect to control)
Polyphenol extract
Control
Time (h)
F
Fe+3-Asc
Fe+3-Asc-Lut
Time (h)
G
PLPC (Counts)
p<0.05
Control
Polyphenol extract
H
OGPC (Counts)
p<0.05
Control
Polyphenol extract
I
PGPC (Counts)
p<0.05
Control
Polyphenol extract
J
FRAP (μM TE)
n.s.
Control
Polyphenol extract
K
Control
Polyphenol extract
OGPC
PGPC
PLPC
PA
TAG
Chol
FRAP
LDL
HDL

Figure 3. Plant-derived phenolic acids inhibit both the effects of oxidative stress in endothelial and HepG2 cell culture and protein oxidation in diet-induced hypercholesterolemia in vivo. A: Effect of plant-derived phenolic acids in cell death in HMEC (endothelial cell line) induced by Cu^{++}-incubated LDL; ** $p<0,01$ and *** $p<0,001$ respect to cell death induced by Cu^{++} incubated LDL ($n=6$ for each data point). Values shown are mean ± S.E.M. over values from samples treated with control LDL **B:** Effect of plant-derived phenolic acids in HepG2 capacity to withstand t-BOOH induced cell death; *** $p<0,001$ respect to cell death induced by t-BOOH ($n=6$ for each data point). Values shown are % changes of mean ± S.E. over values from control samples. **C:** Endothelial cell death induced by Cu^{++}-incubated LDL is strongly correlated with GSA content ($r=-0.734$; $p<0,016$ by the Pearson correlation test). To validate this in vivo, proteins from hypercholesterolemic hamsters were analyzed for carbonyl content (**D**). Both plasma from hypercholesterolemic hamsters with a dietary supplement of phenolic acid-enriched vegetal extract (**E**) and pooled human plasma (**F**) were oxidized with Fe^{3+}-Asc as indicated in the *Methods and Material* section. Aliquots were taken at indicated time points and processed for determination of carbonyl content. **G:** Effect of phenolic acids-enriched diet intake in the steady state levels of a compound compatible with PLPC in plasma from hypercholesterolemic hamsters. **H:** Effect of phenolic acids-enriched diet intake in the steady state levels of a compound compatible with OGPC in plasma from hypercholesterolemic hamsters. **I:** Effect of phenolic acids-enriched diet intake in the steady state levels of a compound compatible with PGPC in plasma from hypercholesterolemic hamsters. J: Effect of phenolic acids-enriched diet intake in FRAP activity of plasma. K: Heat-map showing that phenolic acids-enriched diet induces a significant hierarchical clustering of measured parameters, including both oxidative damage and metabolic (cholesterol, HDL and LDL-cholesterol, triacilglyceride and alkaline phosphatase activities), revealing an important biological effect. The carbonyl content is expressed as a percentual value, considering that the immunoreactivity of plasma from hypercholesterolemic hamsters individuals (Controls in **D**) or protein incubated without oxidant (Ctl) (in **E**) as 100% of carbonyl content. ** $p<0,01$ by Student's T test respect to untreated animals (***E,G,H,I,J***) or oxidant-incubated human plasma (**F**). Data shown are mean±S.E.M., ($n=4$ for each data point). For clustering analyses, values of samples were log transformed and hierarchical clustering was performed using Pearson correlation coefficient as distance measure and the Ward's clustering algorithm. Colors in K express intensity/abundance with red colors showing high levels and blue colors lower levels.
doi:10.1371/journal.pone.0043308.g003

In order to establish whether structural features determine the effect on the resistance of LDL to Cu^{++}-induced oxidation, the chemical structures of the phenolic compounds should be considered (Figure S1). Important differences in the protection of the apoprotein oxidation were observed in this study, similar to those observed by other authors using the lag time of LDL as marker oxidation [40,41]. The differences between those phenols are attributable to their chemical structure, e.g. OH-tyrosol possesses a 3,4-dihydroxy structure linked to an aromatic ring that confers the moiety with a higher proton dislocation, facilitating a higher scavenging activity than observed with tyrosol, which only has a hydroxyl group linked to an aromatic ring (Figure S1). The protective effect of the OH-tyrosol structure has biological relevance as OH-tyrosol and tyrosol are the more simple phenols detected in plasma and LDL after the ingestion of virgin olive oil and red wine [42,43]. It should be noted that OH-tyrosol, with the highest potential for inhibition of MDAL, has been previously described as a potent inducer of cellular antioxidant responses, both in oxLDL-induced and in tert-BOOH-induced cell stress [36,44].

The antioxidant capacity shown by oleuropein and its secoiridoid derivatives was quite different. The aglicon derivative of oleuropein (3,4-DHPEA-EDA) and the ligstroside derivative (*p*-HPEA-EDA) showed a positive result as antioxidants, mainly at the maximum concentration tested. These compounds exhibit a high selectivity for lipid-related changes since they mainly prevented Cu^{++} induced changes in lipidome and MDAL in LDL. Although no previous results were available on lipid or protein antioxidant properties, 3,4-DHPEA-EDA is a compound of special interest because of its presence as one of the major phenolic antioxidant compounds in virgin olive oil. Moreover, it is an important source of OH-tyrosol in plasma. In contrast with these data, a similar phenolic structure, the 3,4-DHPEA-AC showed no significant antioxidant activity in this system. This may be attributed to the potential proton dislocation due to double bonds in the elenolic structure in 3,4-DHPEA-EDA. Reinforcing the importance of conjugation, oleuropein, or glucose-conjugated elenolic acid, showed the more remarkable prooxidant activity of all phenols included in the study in agreement with its previous reported pro-oxidant activities [45,46]. Most interestingly, as oleuropein is considered a proapoptotic agent with potential use as an anti-tumoral agent [47,48], it may be suggested that those protein prooxidant properties could be involved in the beneficial effects of oleuropein.

Flavonoids such as rutin, flavones (apigenin and luteolin), and their glucosides (apigenin-7-*O*-glucoside and luteolin-7-*O*-glucoside) can scavenge reactive oxygen radicals, by donating a hydrogen atom or electron [49,50]. Their radical scavenging activity seems to be substantially dependent on three structural groups: (i) the *orto*-dihydroxyl structure (catechol structure) in the B ring, which is the obvious radical target site, (ii) the 2,3 double bond in conjunction with 4-oxo function, which is responsible for electron delocalization, and (iii) the additional presence of both the 3- and the 5-OH groups for maximal radical scavenging potential and the strongest radical absorption. Our data suggest the importance of the *ortho*-dihydroxyl structure in the prevention of protein modification, as luteolin and rutin -both containing this structure- act as more effective antioxidants than apigenin, a very similar molecule. Comparing the antioxidant activity in the copper-induced LDL oxidation of the luteolin and luteolin-7-*O*-glucoside, the antioxidant capacity of both structures was similar. Despite the described preventive effects of those flavonoids in the diminution of the lag-phase of LDL induced by Cu^{++} and other oxidants [51,52] no data were available on their role as inhibitors of protein oxidation markers. In fact, luteolin can be classified as an agent with more potential for preventing direct oxidation (i.e. GSA and AASA accumulation) than for preventing lipid peroxidation, as it shows a lower power for protecting against Cu^{++}-induced lipidome changes in LDL, in agreement with previous data showing a modest inhibition in the formation of thiobarbituric acid-reactive substances driven by Cu^{++} [53]. In contrast with this relative low *in vitro* potential, it shows a high efficiency in both cell systems as it is able to completely block tert-BOOH-induced changes in cell viability and it shows a high potential against oxLDL-induced cell death, in agreement with the reported inhibitory effect of luteolin in oxidized LDL-induced endothelial monocyte adhesion and/or oxidised LDL uptake [53].

Other major phenols quantified in virgin olive oils and grape-derived products are lignans, with a 2,3-dibenzylbutane skeleton, whose concentration is related to the olive cultivar of origin [54]. Although they are important as sources of lignans enterodiol and enterolactone by colonic flora metabolism, our data reveal that acetoxypinoresinol and pinoresinol possess antioxidant activity, in agreement with previous reports, where it was shown that some vegetable extracts, rich in pinoresinol, were able to inhibit LDL oxidation [55,56]. Their antioxidant activity could be more closely related to their chelating properties than scavenging activity, as they only exhibit a hydroxyl group linked to an aromatic ring.

Globally, acetoxypinoresinol and pinoresinol exhibit similar potencies in the inhibition of direct protein oxidation and lipid peroxidative damage, as well as in the prevention of lipidome changes induced by Cu^{++}.

With regard to the phenolic acid group (ferulic, cumaric, caffeic and gallic acids) (Figure S1), all the phenols showed antioxidant capacity at 50 and 100 μM. Ferulic and *p*-cumaric acids showed a slight prooxidant activity at 5 μM, that may be attributed to H_2O_2 *in vitro* production from phenolic compounds [57,58]. According to their mechanism of action, the phenolic acids may be classified as free radical terminators interfering with lipid oxidation by rapid donation of a hydrogen atom to peroxy radicals. Their antioxidant activity is related to the molecule containing at least two neighbouring phenolic hydroxyl groups; three such groups are even more desirable facilitating the interference. Results of the present study showed that the caffeic (with two neighbouring phenolic hydroxyl groups) and gallic acids (three hydroxyl groups) shown to have the highest antioxidant capacity. Previous results have demonstrated the antioxidant capacity of gallic and caffeic acids on lipid peroxidation [59–61], but only one reported the effect of gallic acids on protein modification, and that was related to nitrosative stress [62]. Concerning their cellular effects, gallic and caffeic acid differed in their effects: while gallic acid was unable to prevent oxLDL-induced or tBOOH-induced loss of viability, caffeic acid inhibited the toxic effects of oxLDL (data not shown). This agrees with the known effect of gallic acid as a proapoptotic agent [63–65] and the reported protective effect of caffeic acid in endothelial cell survival after oxLDL treatment [66].

All the phenols studied showed antioxidant capacity in the LDL model, the OH-tyrosol being the more effective. In general, all the phenols showed higher antioxidant activity than α-tocopherol, which could be attributed to the hydrophilic nature of the phenolic structures. In the biphasic microenvironment constituting core lipids and water phase, such as biomembranes and plasma lipoproteins, the location of phenols should be taken into account for understanding their antioxidant activity. Vitamin E (α-tocopherol) seems to be located within the membrane lipids or lipoprotein particles because of its high lipophilicity. However, it is demonstrated that flavonoid aglycones interact in the polar surface region of the phospholipids bilayers in membranes [67], offering a higher protection. This is specially relevant assuming that the antioxidant profile is completely dependent on the system. As shown by experiments with BSA and human plasma, phenolic interaction in protein oxidation is a very complex phenomenon, which depends on not only of the system used for oxidation, but also on the protein, the presence of lipids and the several mechanisms between protein-phenol and free radical source.

In summary, these data show novel antioxidant properties of plant-derived phenolic acid compounds in LDL oxidation and demonstrates phenolic activity in protein oxidation *in vivo*. It is also demonstrated that *in vitro* antioxidant measurements could only partially predict biologic responses to oxidized LDL, thus reinforcing the importance of a multidisciplinary approach for the description of oxidative phenomena in atherosclerosis pathogenesis and its dietary modulation.

Supporting Information

Figure S1 Structures of plant-derived phenolics used in the present study.
(DOCX)

Figure S2 Representative phenolic acids inhibit protein carbonylation in different oxidative systems up to various extents. All phenols were tested at 5 μM. Oxidation of BSA (**A,B,C**) or a human plasma pool (**D,E,F**) was induced with Hemin:H_2O_2 (**A, D**), Ultraviolet radiation (λ 254 nm) (**B,E**), and Fe^{3+}:Ascorbate (**C,F**) as indicated in the *Methods and Materials* section. Carbonyl contents are expressed as percentual values, considering that the immunoreactivity of protein incubated without oxidant (Ctl) as 100% of carbonyl content. ** $p<0,01$ by ANOVA respect to oxidant-incubated protein. Data shown are mean±S.D., (n = 4 for each data point).
(DOCX)

Figure S3 Chromatographic and mass-spectra evidence for the presence of PLPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 496.3398 (magnified in **B**) showing the peak quantified as PLPC (arrow in **A**). **C:** Mass spectra of the peak quantified as PLPC (arrowhead showing m/z peak with $\Delta<0.05$ Da in comparison to a theoretical mass of $C_{24}H_{50}NO_7P$)
(DOCX)

Figure S4 Chromatographic and mass-spectra evidence for the presence of OGPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 522.3554 (magnified in **B**) showing the peak quantified as OGPC (arrow in **A**). **C:** Mass spectra of the peak quantified as OGPC (arrowhead showing m/z peak with $\Delta<0.02$ Da in comparison to a theoretical mass of $C_{26}H_{52}NO_7P$)
(DOCX)

Figure S5 Chromatographic and mass-spectra evidence for the presence of PGPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 610.3715 (magnified in **B**) showing the peak quantified as PGPC (arrow in **A**). **C:** Mass spectra of the peak quantified as PGPC (arrowhead showing m/z peak with $\Delta<0.01$ Da in comparison to theoretical mass of $C_{29}H_{56}NO_{10}P$)
(DOCX)

Methods S1 Supplementary methods.
(DOCX)

Table S1
(DOCX)

Author Contributions

Conceived and designed the experiments: RP MPR MJM MPO. Performed the experiments: ASC MJ DC AN JB JCES AC. Analyzed the data: MPO ASC JP MJB. Contributed reagents/materials/analysis tools: MJM MPR LLA JV. Wrote the paper: MJ MPO.

References

1. Goldstein JL, Brown MS (1977) The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 46: 897–930.
2. Goldstein JL, Ho YK, Basu SK, Brown MS (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. Proc Natl Acad Sci U S A 76(1): 333–337.
3. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL (1989) Beyond cholesterol. modifications of low-density lipoprotein that increase its atherogenicity. N Engl J Med 320(14): 915–924.
4. Hazell LJ, Arnold L, Flowers D, Waeg G, Malle E, et al. (1996) Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J Clin Invest 97(6): 1535–1544.
5. Hazen SL, Heinecke JW (1997) 3-chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density

lipoprotein isolated from human atherosclerotic intima. J Clin Invest 99(9): 2075–2081.
6. Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, et al. (1997) Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. J Biol Chem 272(3): 1433–1436.
7. Berliner JA, Heinecke JW (1996) The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 20(5): 707–727.
8. Esterbauer H, Gebicki J, Puhl H, Jurgens G (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med 13(4): 341–390.
9. Hazell LJ, Stocker R (1993) Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages. Biochem J 290 (Pt 1)(Pt 1): 165–172.
10. Stupans I, Kirlich A, Tuck KL, Hayball PJ (2002) Comparison of radical scavenging effect, inhibition of microsomal oxygen free radical generation, and serum lipoprotein oxidation of several natural antioxidants. J Agric Food Chem 50(8): 2464–2469.
11. Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, et al. (2003) Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: Antiatherogenic properties of mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol 23(4): 622–629.
12. Grassi D, Aggio A, Onori L, Croce G, Tiberti S, et al. (2008) Tea, flavonoids, and nitric oxide-mediated vascular reactivity. J Nutr 138(8): 1554S–1560S.
13. Natella F, Nardini M, Belelli F, Scaccini C (2007) Coffee drinking induces incorporation of phenolic acids into LDL and increases the resistance of LDL to ex vivo oxidation in humans. Am J Clin Nutr 86(3): 604–609.
14. Nakagawa K, Ninomiya M, Okubo T, Aoi N, Juneja LR, et al. (1999) Tea catechin supplementation increases antioxidant capacity and prevents phospholipid hydroperoxidation in plasma of humans. J Agric Food Chem 47(10): 3967–3973.
15. Kamiyama M, Kishimoto Y, Tani M, Andoh K, Utsunomiya K, et al. (2009) Inhibition of low-density lipoprotein oxidation by nagano purple grape (vitis viniferaxVitis labrusca). J Nutr Sci Vitaminol (Tokyo) 55(6): 471–478.
16. Hayek T, Fuhrman B, Vaya J, Rosenblat M, Belinky P, et al. (1997) Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. Arterioscler Thromb Vasc Biol 17(11): 2744–2752.
17. Ness AR, Powles JW (1997) Fruit and vegetables, and cardiovascular disease: A review. Int J Epidemiol 26(1): 1–13.
18. Abbey M, Nestel PJ, Baghurst PA (1993) Antioxidant vitamins and low-density-lipoprotein oxidation. Am J Clin Nutr 58(4): 525–532.
19. Jialal I, Grundy SM (1993) Effect of combined supplementation with alpha-tocopherol, ascorbate, and beta carotene on low-density lipoprotein oxidation. Circulation 88(6): 2780–2786.
20. Suarna C, Hood RL, Dean RT, Stocker R (1993) Comparative antioxidant activity of tocotrienols and other natural lipid-soluble antioxidants in a homogeneous system, and in rat and human lipoproteins. Biochim Biophys Acta 1166(2–3): 163–170.
21. Hou L, Zhou B, Yang L, Liu ZL (2004) Inhibition of human low density lipoprotein oxidation by flavonols and their glycosides. Chem Phys Lipids 129(2): 209–219.
22. Chen CY, Milbury PE, Chung SK, Blumberg J (2007) Effect of almond skin polyphenolics and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation. J Nutr Biochem 18(12): 785–794.
23. Oorni K, Pentikainen MO, Annila A, Kovanen PT (1997) Oxidation of low density lipoprotein particles decreases their ability to bind to human aortic proteoglycans. dependence on oxidative modification of the lysine residues. J Biol Chem 272(34): 21303–21311.
24. Wagner P, Heinecke JW (1997) Copper ions promote peroxidation of low density lipoprotein lipid by binding to histidine residues of apolipoprotein B100, but they are reduced at other sites on LDL. Arterioscler Thromb Vasc Biol 17(11): 3338–3346.
25. Yan LJ, Lodge JK, Traber MG, Packer L (1997) Apolipoprotein B carbonyl formation is enhanced by lipid peroxidation during copper-mediated oxidation of human low-density lipoproteins. Arch Biochem Biophys 339(1): 165–171.
26. Terra X, Pallares V, Ardevol A, Blade C, Fernandez-Larrea J, et al. (2011) Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J Nutr Biochem 22(4): 380–387.
27. Artajo LS, Romero MP, Morello JR, Motilva MJ (2006) Enrichment of refined olive oil with phenolic compounds: Evaluation of their antioxidant activity and their effect on the bitter index. J Agric Food Chem 54(16): 6079–6088.
28. Auger C, Gerain P, Laurent-Bichon F, Portet K, Bornet A, et al. (2004) Phenolics from commercialized grape extracts prevent early atherosclerotic lesions in hamsters by mechanisms other than antioxidant effect. J Agric Food Chem 52(16): 5297–5302.
29. Wikoff WR, Gangoiti JA, Barshop BA, Siuzdak G (2007) Metabolomics identifies perturbations in human disorders of propionate metabolism. Clin Chem 53(12): 2169–2176.
30. Balla G, Jacob HS, Eaton JW, Belcher JD, Vercellotti GM (1991) Hemin: A possible physiological mediator of low density lipoprotein oxidation and endothelial injury. Arterioscler Thromb 11(6): 1700–1711.
31. Pamplona R, Dalfo E, Ayala V, Bellmunt MJ, Prat J, et al. (2005) Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. effects of alzheimer disease and identification of lipoxidation targets. J Biol Chem 280(22): 21522–21530.
32. Watson AD, Leitinger N, Navab M, Faull KF, Horkko S, et al. (1997) Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/endothelial interactions and evidence for their presence in vivo. J Biol Chem 272(21): 13597–13607.
33. Benzie IF, Strain JJ (1996) The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal Biochem 239(1): 70–76.
34. Pulido R, Bravo L, Saura-Calixto F (2000) Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J Agric Food Chem 48(8): 3396–3402.
35. Bouguerne B, Belkheiri N, Bedos-Belval F, Vindis C, Uchida K, et al. (2011) Antiatherogenic effect of bisvanillyl-hydralazone, a new hydralazine derivative with antioxidant, carbonyl scavenger, and antiapoptotic properties. Antioxid Redox Signal 14(11): 2093–2106.
36. Goya L, Delgado-Andrade C, Rufian-Henares JA, Bravo L, Morales FJ (2007) Effect of coffee melanoidin on human hepatoma HepG2 cells. protection against oxidative stress induced by tert-butylhydroperoxide. Mol Nutr Food Res 51(5): 536–545.
37. Goya L, Mateos R, Bravo L (2007) Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. protection against oxidative stress induced by tert-butylhydroperoxide. Eur J Nutr 46(2): 70–78.
38. Xia J, Psychogios N, Young N, Wishart DS (2009) MetaboAnalyst: A web server for metabolomic data analysis and interpretation. Nucleic Acids Res 37(Web Server issue): W652–60.
39. Berlett BS, Stadtman ER (1997) Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 272(33): 20313–20316.
40. Vissers MN, Zock PL, Katan MB (2004) Bioavailability and antioxidant effects of olive oil phenols in humans: A review. Eur J Clin Nutr 58(6): 955–965.
41. Leenen R, Roodenburg AJ, Vissers MN, Schuurbiers JA, van Putte KP, et al. (2002) Supplementation of plasma with olive oil phenols and extracts: Influence on LDL oxidation. J Agric Food Chem 50(5): 1290–1297.
42. Covas MI, Miro-Casas E, Fito M, Farre-Albadalejo M, Gimeno E, et al. (2003) Bioavailability of tyrosol, an antioxidant phenolic compound present in wine and olive oil, in humans. Drugs Exp Clin Res 29(5–6): 203–206.
43. Covas MI (2007) Olive oil and the cardiovascular system. Pharmacol Res 55(3): 175–186.
44. Di Benedetto R, Vari R, Scazzocchio B, Filesi C, Santangelo C, et al. (2007) Tyrosol, the major extra virgin olive oil compound, restored intracellular antioxidant defences in spite of its weak antioxidative effectiveness. Nutr Metab Cardiovasc Dis 17(7): 535–545.
45. Briante R, Febbraio F, Nucci R (2004) Antioxidant/prooxidant effects of dietary non-flavonoid phenols on the Cu2+-induced oxidation of human low-density lipoprotein (LDL). Chem Biodivers 1(11): 1716–1729.
46. Mazziotti A, Mazzotti F, Pantusa M, Sportelli L, Sindona G (2006) Pro-oxidant activity of oleuropein determined in vitro by electron spin resonance spin-trapping methodology. J Agric Food Chem 54(20): 7444–7449.
47. Menendez JA, Vazquez-Martin A, Colomer R, Brunet J, Carrasco-Pancorbo A, et al. (2007) Olive oil's bitter principle reverses acquired autoresistance to trastuzumab (herceptin) in HER2-overexpressing breast cancer cells. BMC Cancer 7: 80.
48. Hamdi HK, Castellon R (2005) Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. Biochem Biophys Res Commun 334(3): 769–778.
49. Rice-Evans CA, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20(7): 933–956.
50. Bors W, Heller W, Michel C, Saran M (1990) Flavonoids as antioxidants: Determination of radical-scavenging efficiencies. Methods Enzymol 186: 343–355.
51. Kostyuk VA, Kraemer T, Sies H, Schewe T (2003) Myeloperoxidase/nitrite-mediated lipid peroxidation of low-density lipoprotein as modulated by flavonoids. FEBS Lett 537(1–3): 146–150.
52. Brown JE, Khodr H, Hider RC, Rice-Evans CA (1998) Structural dependence of flavonoid interactions with Cu2+ ions: Implications for their antioxidant properties. Biochem J 330(Pt 3)(Pt 3): 1173–1178.
53. Jeong YJ, Choi YJ, Choi JS, Kwon HM, Kang SW, et al. (2007) Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL. Br J Nutr 97(3): 447–457.
54. Brenes M, Garcia A, Garcia P, Rios JJ, Garrido A (1999) Phenolic compounds in spanish olive oils. J Agric Food Chem 47(9): 3535–3540.
55. Chen CC, Chen HY, Shiao MS, Lin YL, Kuo YH, et al. (1999) Inhibition of low density lipoprotein oxidation by tetrahydrofurofuran lignans from forsythia suspensa and magnolia coco. Planta Med 65(8): 709–711.
56. Vuorela S, Kreander K, Karonen M, Nieminen R, Hamalainen M, et al. (2005) Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. J Agric Food Chem 53(15): 5922–5931.
57. Hou Z, Sang S, You H, Lee MJ, Hong J, et al. (2005) Mechanism of action of (−)-epigallocatechin-3-gallate: Auto-oxidation-dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE 150 cells. Cancer Res 65(17): 8049–8056.
58. Long LH, Clement MV, Halliwell B (2000) Artifacts in cell culture: Rapid generation of hydrogen peroxide on addition of (−)-epigallocatechin, (−)-

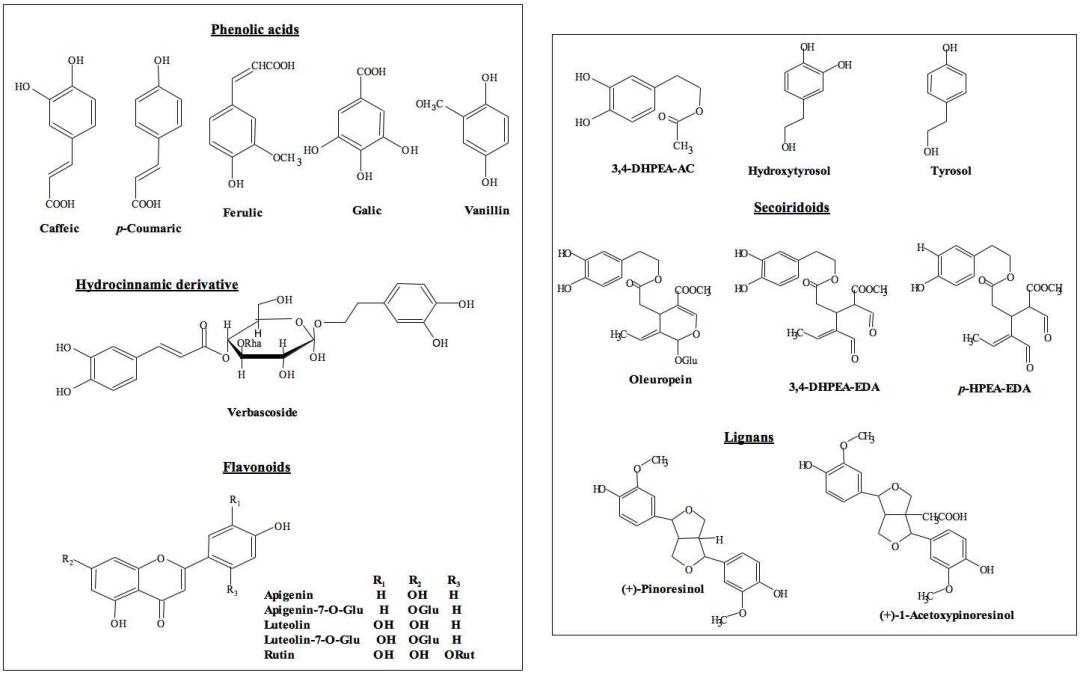
epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. Biochem Biophys Res Commun 273(1): 50–53.
59. Hsieh CL, Yen GC, Chen HY (2005) Antioxidant activities of phenolic acids on ultraviolet radiation-induced erythrocyte and low density lipoprotein oxidation. J Agric Food Chem 53(15): 6151–6155.
60. Hseu YC, Chang WH, Chen CS, Liao JW, Huang CJ, et al. (2008) Antioxidant activities of toona sinensis leaves extracts using different antioxidant models. Food Chem Toxicol 46(1): 105–114.
61. Laranjinha JA, Almeida LM, Madeira VM (1994) Reactivity of dietary phenolic acids with peroxyl radicals: Antioxidant activity upon low density lipoprotein peroxidation. Biochem Pharmacol 48(3): 487–494.
62. Pannala AS, Rice-Evans CA, Halliwell B, Singh S (1997) Inhibition of peroxynitrite-mediated tyrosine nitration by catechin polyphenols. Biochem Biophys Res Commun 232(1): 164–168.
63. Locatelli C, Rosso R, Santos-Silva MC, de Souza CA, Licinio MA, et al. (2008) Ester derivatives of gallic acid with potential toxicity toward L1210 leukemia cells. Bioorg Med Chem 16(7): 3791–3799.
64. Hsu CL, Lo WH, Yen GC (2007) Gallic acid induces apoptosis in 3T3-L1 pre-adipocytes via a fas- and mitochondrial-mediated pathway. J Agric Food Chem 55(18): 7359–7365.
65. Agarwal C, Tyagi A, Agarwal R (2006) Gallic acid causes inactivating phosphorylation of cdc25A/cdc25C-cdc2 via ATM-Chk2 activation, leading to cell cycle arrest, and induces apoptosis in human prostate carcinoma DU145 cells. Mol Cancer Ther 5(12): 3294–3302.
66. Vieira O, Escargueil-Blanc I, Meilhac O, Basile JP, Laranjinha J, et al. (1998) Effect of dietary phenolic compounds on apoptosis of human cultured endothelial cells induced by oxidized LDL. Br J Pharmacol 123(3): 565–573.
67. Fukuzawa K, Matsuura K, Tokumura A, Suzuki A, Terao J (1997) Kinetics and dynamics of singlet oxygen scavenging by alpha-tocopherol in phospholipid model membranes. Free Radic Biol Med 22(5): 923–930.

Supplemental Table 1

	Control	Polyphenol extract	P (By Student's T test)
Final weight (g)	118.12±7.6	114.18±8.6	0.062
Total cholesterol (mg/dL)	455,0 ± 36,39	421,9 ± 12,07	0.108
LDL-Cholesterol (mg/dL)	403,8 ± 50,57	345,9 ± 44,09	0.4
HDL-Cholesterol (mg/dL)	327,7 ± 17,63	305,4 ± 10,03	0.27
Triacilglyceride (mg/dL)	225,0 ± 35,95	142,6 ± 9,492	0.051
Alkaline phosphatase (U/L)	54.07±18.3	47.63±6.6	0.56

Shown is mean±S.E.M (n=6)

Figure S1

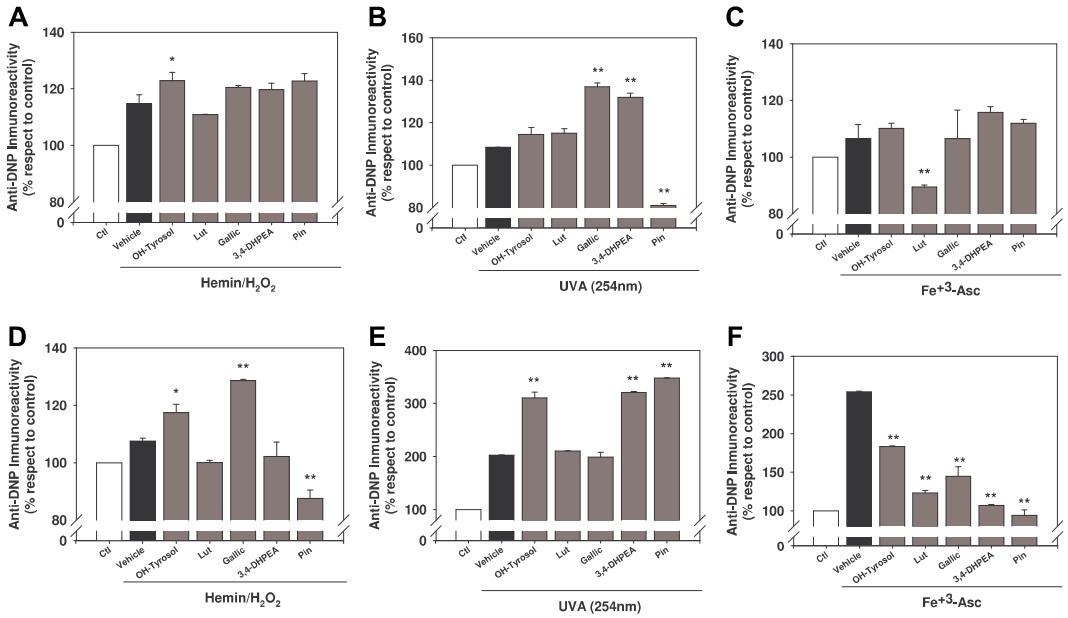


Structures of plant-derived phenolics used in the present study.

doi:10.1371/journal.pone.0043308.s001

(DOCX)

Figure S2

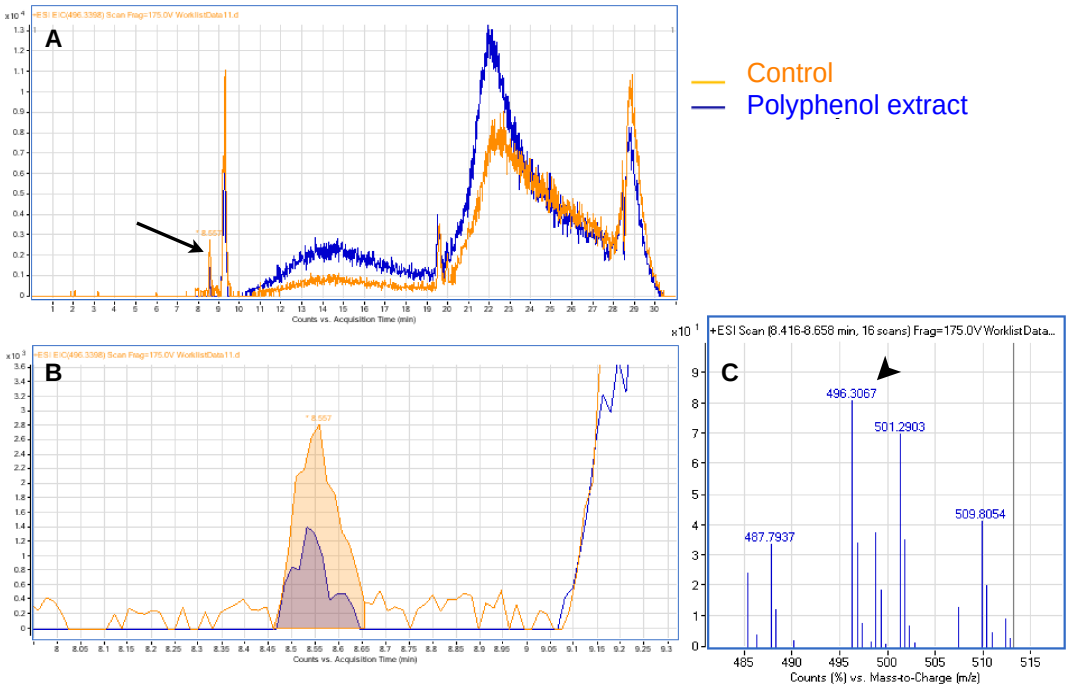


Representative phenolic acids inhibit protein carbonylation in different oxidative systems up to various extents. All phenols were tested at 5 µM. Oxidation of BSA (**A,B,C**) or a human plasma pool (**D,E,F**) was induced with Hemin:H_2O_2 (**A, D**), Ultraviolet radiation (λ 254 nm) (**B,E**), and Fe^{3+}:Ascorbate (**C,F**) as indicated in the *Methods and Materials* section. Carbonyl contents are expressed as percentual values, considering that the immunoreactivity of protein incubated without oxidant (Ctl) as 100% of carbonyl content. ** p<0,01 by ANOVA respect to oxidant-incubated protein. Data shown are mean±S.D., (n = 4 for each data point).

doi:10.1371/journal.pone.0043308.s002

(DOCX)

Figure S3

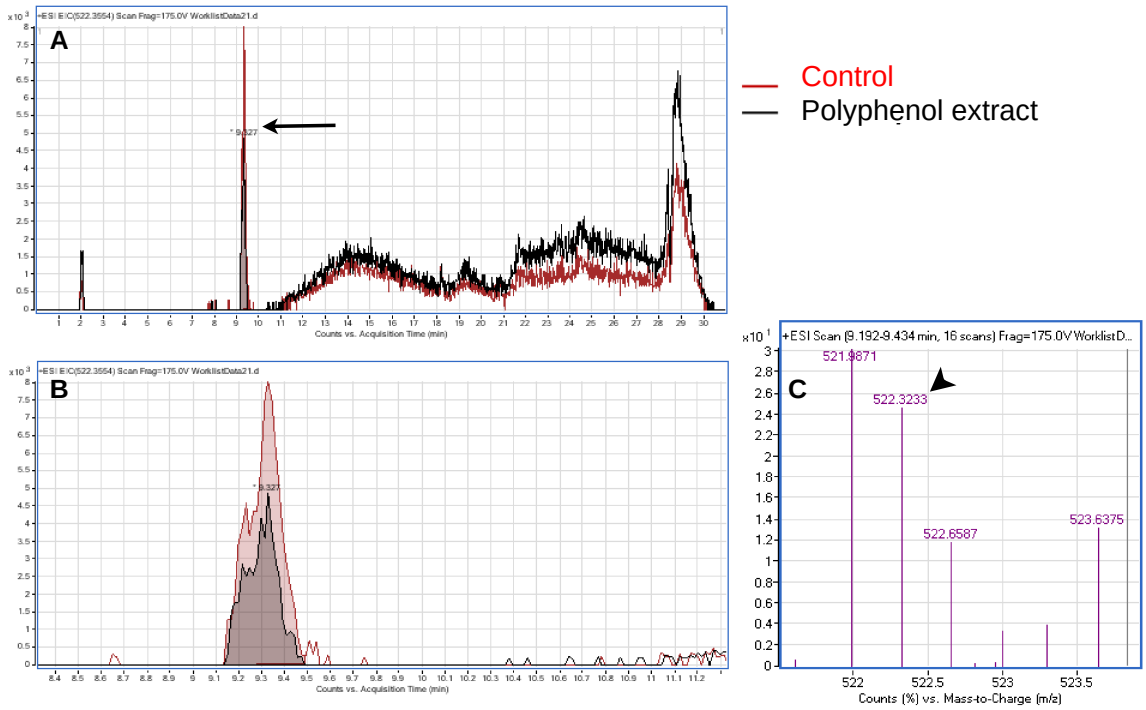


Chromatographic and mass-spectra evidence for the presence of PLPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 496.3398 (magnified in **B**) showing the peak quantified as PLPC (arrow in **A**). **C:** Mass spectra of the peak quantified as PLPC (arrowhead showing m/z peak with Δ<0.05 Da in comparison to a theoretical mass of $C_{24}H_{50}NO_7P$)

doi:10.1371/journal.pone.0043308.s003

(DOCX)

Figure S4

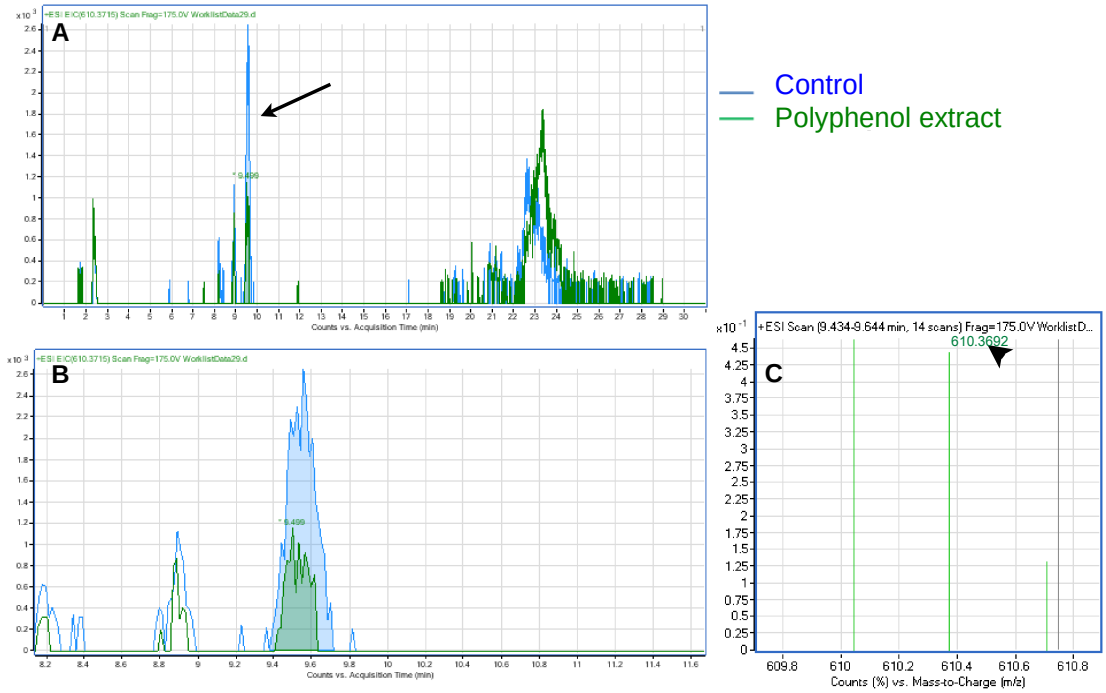


Chromatographic and mass-spectra evidence for the presence of OGPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 522.3554 (magnified in **B**) showing the peak quantified as OGPC (arrow in **A**). **C:** Mass spectra of the peak quantified as OGPC (arrowhead showing m/z peak with Δ<0.02 Da in comparison to a theoretical mass of $C_{26}H_{52}NO_7P$)

doi:10.1371/journal.pone.0043308.s004

(DOCX)

Figure S5



Chromatographic and mass-spectra evidence for the presence of PGPC in methanolic extracts of plasma from hypercholesterolemic hamsters. A: Extracted ion chromatogram of m/z 610.3715 (magnified in **B**) showing the peak quantified as PGPC (arrow in **A**). **C:** Mass spectra of the peak quantified as PGPC (arrowhead showing m/z peak with Δ<0.01 Da in comparison to theoretical mass of $C_{29}H_{56}NO_{10}P$)

doi:10.1371/journal.pone.0043308.s005

(DOCX)

SUPPLEMENTAL ONLINE MATERIAL

Supplemental Methods

Protein oxidation screening method: Protein oxidation and Western blot analysis.

Briefly, to 15 µl of sample SDS was added to a final concentration of 6%, and, after boiling for 3 min, 20 µl of 10 mM DNP in 10% trifluoroacetic acid were added. After 7 min at room temperature, 20 µl of a solution containing 2M Tris base, 30% glycerol and 15% β-mercaptoethanol was added for neutralization and sample preparation for loading onto 4-20% gradient pre-made SDS-PAGE gels (Invitrogen, Barcelona, Spain). For immunodetection after SDS-PAGE, proteins were transferred using a Mini Trans-Blot Transfer Cell (BioRad Laboratories, Barcelona, Spain) to PVDF membranes (Immobilon-P Millipore, Bedford, MA, USA). Immunodetection was performed using a rabbit anti-DNP antiserum as the primary antibody (1:4000, Dako, Carpenteria, CA, USA). Peroxidase-coupled secondary antibodies were used from the Tropixchemiluminiscence kit (TropixInc, Bedford, MA, USA). Luminiscence was recorded and quantified in Chemidoc equipment (BioRad, Barcelona, Spain), using the Quantity-one analysis software (Biorad). The average mode of background subtraction was chosen to quantify. Control experiments showed that omission of derivatization step, primary or secondary antibody addition produced blots with no detectable signal (data not shown). After developing, the blots were silver-stained as described to check the protein load (1).

Measurement of glutamic (GSA) and aminoadipic (AASA) semialdehydes and malondialdehyde lysine (MDAL).

Samples containing 0.4 mg of protein were extensively delipidated using chloroform:methanol extraction (2:1 v/v, 3x) in the presence of 0.01% butylatedhydroxytoluene, and the proteins were precipitated by adding 10% of trichloroacetic acid (final concentration) and subsequent centrifugation. The protein samples were reduced overnight with 500 mM NaBH4 (final concentration) in 0.2M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. The proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and then centrifuging this. The following isotopicallylabelled internal standards were then added: [2H8]Lysine (d8-Lys; CDN Isotopes); and [2H8]MDAL (d8-MDAL), [2H5] 5-hydroxy-2-aminovaleric acid (for GSA quantization) and [2H4]6-hydroxy-2-aminocaproic acid (for AASA quantization) as described(1). The samples were hydrolysed at 155º C for 30 min in 1ml of 6N HCl, and then vacuum dried. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described(1). The GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30m HP-5MS capillary column (30m x 0.25mm x 0.25 µm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent Technologies, Barcelona, Spain). The injection port was maintained at 275º C; the temperature program was 5 min at 110º C, then rising by 2º C/min to 150º C, then by 5º C/min to 240º C, then 25º C/min to 300º C, and finally hold at 300º C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. The analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and d8-lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d5-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and d4-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; and MDAL and d8-MDAL, m/z 474 and 482, respectively. The amounts of products were expressed as the ratio µmol GSA, AASA, or MDAL/mol lysine.

Lipidome analyses.

Fatty acid profiles

For fatty acid analyses, the chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2.5 ml of 5% methanolicHCl for 90 min at 75º C. The resulting fatty acid methyl esters were extracted by adding 2.5 ml of n-pentane and 1 ml of saturated NaCl solution. The n-pentane phase was separated, evaporated under nitrogen, redissolved in 75 µl of hexane and 1 µl was used for the GC/MS analysis. Separation was performed in a SP2330 capillary column (30m x 0.25mm x 0.20µm) in a Hewlett Packard 6890 Series II gas chromatograph (Agilent Technologies, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer (Agilent Technologies, Barcelona, Spain) was used as the detector in the electron-impact mode. The injection port was maintained at 220º C, and the detector at 250º C; the temperature program was 2 min at 100º C, then rising by 10º C/min to 200º C, then 5º C/min to 240º C, and finally held at 240º C for 10 min. Identification of fatty acid methyl esters was done

by comparison with authentic standards and based on mass spectra. Results are expressed as mol %. The following indexes were calculated from the fatty acid composition: Saturated Fatty Acids (SFA)= Σ % of saturated fatty acids; Unsaturated Fatty Acids (UFA)= Σ % unsaturated fatty acids; Monounsaturated Fatty Acids (MUFA)= Σ % of monoenoic fatty acids; Polyunsaturated n-3 Fatty Acids (PUFAn-3)= Σ % of polyunsaturated fatty acids n-3 serie; Polyunsaturated n-6 Fatty Acids (PUFAn-6)= Σ % of polyunsaturated fatty acids n-6 serie; Average Chain Length (ACL)= [(Σ %Total14 x 14) + …+ (Σ %Total n x n)]/100 (n= carbon atom number); Peroxidizability index (PI) = [(Σ mol% Monoenoic x 0.025) + (Σ mol% Dienoic x 1) + (Σ mol% Trienoic x 2) + (Σ mol% Tetraenoic x 4) + (Σ mol% Pentaenoic x 6) + (Σ mol% Hexaenoic x 8)].

LC-TOF based lipidome analyses

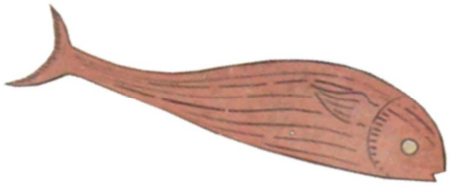
Briefly, lipid samples, extracted as above, after drying and reconstitution with mobile phase (see below) were injected into a reversed-phase chromatography system, using a Zorbax C18 column (150 x 0.5 mm x 5 μm) at a flow rate of 8 μl/min in a LC system consisting of a capillary pump (Agilent 1200). Buffer A was water with 0.1% formic acid, and buffer B was acetonitrile with 0.1% formic acid. The column was equilibrated in 5% B and the gradient was 5%--95%B over 50 min. The eluant was directed to an ESI source, with a nebulizer gas flow of 15 l/min at 300º C with a capillary voltage of 3500 V operating in positive mode, with a dual spray for reference mass (m/z 121.05087 and m/z 922.0098). The instrument was calibrated immediately prior to the test. For the data analysis with the Agilent MassHunter Profiling software, five repeated injections of each extract sample were measured by the same LC/MS method. The acquired MS data were extracted by the Molecular Feature Extractor in the Agilent MassHunter Qualitative software. In this process, the identified ions were clustered to "Molecular features" comprising different isotopic distribution and adducts from the same molecule. The obtained files were grouped according to the samples into two respective groups (control samples *vs* Cu^{++} treated samples or control hypercholesterolemic hamsters *vs*hypercholesterolemic phenolic-enriched vegetal extract supplemented hamsters) and loaded into the Agilent MassHunter Profiling software. For the differential analysis of both groups the features of each group were compared using Student's T Test. The resulting masses were searched for in the Lipid Maps Databases (http://www.lipidmaps.org/tools/index.html), and among 73 differential molecular features found, 17 were significantly increased ($p<0.01$) over values found in control incubations of LDL (being the remaining 56 diminished). Among those, the three most abundant had masses compatible with 1-palmitoyllysophosphatidylcholine –PLPC- (measured mass: 495.3328, theoretical mass: 495.3325), 1-stearoyl-sn-glycero-3-phosphocholine –SGPC- (measured mass: 523.364, theoretical mass: 523.3638) and 1-oleoylglycerophosphocholine –OGPC- (measured mass: 521.3476, theoretical mass: 521.3481).

Cell viability

HMEC cell line (endothelial cell), kindly donated by Dr. A. Negre-Salvayre (INSERM, Toulouse, France) were cultured in 100mm plates with high glucose DMEM containing 10% FBS until they were 70-80% confluent. Afterwards, the cells were harvested, counted and seeded in 96-well microplates (25,000 cells/well) with the same culture medium, leaving a strip of wells free of cells to be used as a blank. The cells were immediately centrifuged at 1000 rpm for 4 min, without stop brake, in order to obtain a fast and homogeneous attachment to the bottom of the wells. After 6 h, the cell culture medium was removed and serum-free DMEM was added to the cells. One hour later, the culture medium was removed again and 100 μl of serum-free DMEM containing non-oxidized LDL, oxLDL, oxLDL in the presence of phenolic acids or the vehicles alone, was added to each well. The final concentration of LDL in all conditions was 200 μg/ml. Eighteen hours later, the culture medium was removed and 100 μl of PBS was added to each well in order to avoid possible interferences, immediately before determining viability. The effect of phenolic compounds on the cellular capacity to withstand tert-butylhydroperoxide (t-BOOH) challenge was measured. The HepG2 cells were serum starved (0.5% FCS) in the presence of selected phenolics (5 μM) for 8 h. The cells were then challenged with 200 μM t-BOOH as peroxide donor, and 2 h later, cell viability was estimated with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, according manufacturer instructions.

Supplemental Literature Cited

1. Pamplona R, Dalfo E, Ayala V, Bellmunt MJ, Prat J, Ferrer I, Portero-Otin M. Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. effects of alzheimer disease and identification of lipoxidation targets. J Biol Chem. 2005;280:21522-30.



4.2 Article 2

Title: “Interplay Between TDP-43 and Docosohexaenoic Acid-Related Processes in Amyotrophic Lateral Sclerosis”.

Authors: Cacabelos D, Ayala V, Granado-Serrano AB, Jove M, Boada J, Cabre R, Ramírez-Nuñez O, Gonzalo H, Torres P, Soler Cantero A, Serrano JC, Bellmunt MJ, Romero MP, Motilva MJ, Nonaka T, Hasegawa M, Ferrer I, Pamplona R, Portero-Otín M.

Journal: Proceedings of the National Academy of Sciences, PNAS
Submitted 28th August.

Interplay between TDP-43 and docosahexaenoic acid-related processes in amyotrophic lateral sclerosis

Daniel Cacabelos[a1], Victòria Ayala[a1], Ana Belén Granado-Serrano[a1] Mariona Jové[a1], Jordi Boada[a], Rosanna Cabré[a], Omar Ramírez-Núñez[a], Hugo Gonzalo[a], Pascual Torres[a], Aranzazu Soler-Cantero[b], José Carlos Enrique Serrano[a],Maria Josep Bellmunt[a], María Paz Romero[b], María José Motilva[b], Takashi Nonaka[c], Masato Hasegawa[b], Isidre Ferrer[d,e], Reinald Pamplona[a,2] and Manuel Portero-Otín[a,2]

[a]Departament de Medicina Experimental, Facultat de Medicina,IRBLLEIDA-UDL, Avda Rovira Roure, 44, 25008, Lleida, Spain [b]Departament de Tecnologia d'Aliments, XaRTA-TPV, Escola Tècnica Superior d' Enginyeria Agrària, UdL, Avda Rovira Roure, 85, 25008, Lleida, Spain [c]Department of Neuropathology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Setagaya-ku, Tokyo 156-8506, Japan [d] Institut de Neuropatologia, Hospital Universitari de Bellvitge - IDIBELL, Universitat de Barcelona, [e] CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas), Instituto Carlos III, Spanish Ministry of Health, Spain. L'Hospitalet de Llobregat, c/La Feixa Llarga, S/N 08908 Hospitalet de Llobregat, Barcelona,Spain [1]These authors contributed equally to the work. [2]To whom correspondence should be addressed: Reinald Pamplona or Manuel Portero-Otin, IRBLLEIDA-UdL, Facultat de Medicina-Edifici Biomedicina I, Avda Rovira Roure, 80. 25008 Lleida, SPAIN Tel +(34) 973702442-2048; Fax: +(34) 973702426; E-mail: reinald.pamplona@mex.udl.cat or manuel.portero@mex.udl.cat

Submitted to Proceedings of the National Academy of Sciences of the United States of America

Docosahexaenoic acid (DHA), a key fatty acid in nervous system homeostasis, is depleted in spinal cord of sporadic amyotrophic lateral sclerosis (sALS) patients. In this work we show that sALS leads to changes in spinal cord distribution of DHA synthesis enzymatic machinery. This is closely related to pathological deposits of Tar-DNA binding protein 43 (TDP-43), since transfection of neurons with aggregation prone C-terminal fragments of TDP-43 recapitulates diminished levels of these enzymes in motor neurons with TDP-43 aggregates in vivo. Further, drebrin, a DHA dependent synaptic protein, is depleted in sALS samples and it is associated to TDP-43 aggregation in vitro.,However, chronic excitotoxicity, preceeding motor neuron disease, increases DHA amount, with both enhanced concentrations of neuroprotective DHA-derived resolvin D, and higher lipid peroxidation-derived moelcules such as 8-iso-prostaglandin-F_2-α (8-iso-PGF2α) levels. Since α-tocopherol improved mitochondrial respiratory function and motor neuron survival in these conditions, it is suggested that oxidative stress could boost motor neuron loss. Cell culture experiments, showing enhanced expression of desaturases (FADS2) and β-oxidation enzymes after H_2O_2 challenge shows that DHA production can be an initial response to oxidative stress, driven by TDP-43 aggregation and drebrin loss. The finding of altered levels of desaturases and peroxisomal and mitochondrial β-oxidation enzymes in human sALS suggest that in early stages of the disease increased DHA could act as a response over chronic excitotoxicity in spinal cord, involving several DHA synthethic enzymes, but later on this could contribute, by lipid peroxidation, to motor neuron loss.

lipid peroxidation | lipidomics | drebrin | desaturases

Introduction

Polyunsaturated fatty acids (PUFA) are essential for neuronal function. Neuronal membranes host the highest amount of highly unsaturated PUFA, whose function is multiphaceted: modulation of biomembrane physical characteristics, precursors of bioactive paracrine regulators, control of neurotransmitter and synapses physiology, among other (1). As an example, docosahexaenoic acid (DHA), a member of the PUFA familiy, is involved in synaptic transmission, as its depletion by nutritional deficiencies leads to downregulation of the synaptic components drebrin and syntaxin-3(2, 3). DHA can be produced from n-3 precursors by a series of different elongation and desaturation steps, finally comprising a peroxisomal beta-oxidation step(4). On the other hand, PUFAs, and particularly DHA –due to the high number of unsaturations in its aliphatic chain- are also preferential targets of oxidative modifications (5). Thus, a trade-off between PUFA function and its sensitivity to lipid peroxidation has been achieved through hominid evolution(6–8). In this equilibrium, an adequate tissular status of lipophilic antioxidants plays a key role in the protection of those PUFA. In this line it should be reminded that DHA supplementation, described as neuroprotective, may be more beneficial with α-tocopherol addition(9, 10). Besides α-tocopherol, several reports have hypothesized the neuroprotective role of other antioxidant micronutrients, such as those present in virgin olive oil in conditions such as ageing, hypoxia-reperfusion, Alzheimer's disease, and other neurodegenerative conditions(11, 12)

Recent data demonstrates that the intake of foods high in n-3 PUFAs could help to prevent or delay the onset of ALS (13). In line with this, prior data showed decreased levels of DHA in spinal cord from sporadic ALS (sALS) patients (14), a fatal neurodegenerative disease. However, the potential changes in DHA synthesis machinery behind those alterations were unknown. Further, no information was available about the potential protective role of PUFA and antioxidants in chronic excitotoxicity, a pathogenic mechanism in sALS and other neurodegenerative diseases. Acute excitotoxocity shows that there are relevant interactions between PUFA and lipophilic or hydrophilic antioxidants(15, 16) .

Significance

Dietary polyunsaturated fatty acids of the n-3 family, such as docosahexaenoic acid or linolenic acid, could play a role in prevention of amyotrophic lateral sclerosis, a motor neuron degenerative disease. This may be due to its essential role in maintenace of synaptic machinery. We demonstrate that in ALS patients enzymes for the synthesis of this fatty acid are altered, with closelly resembling TDP-43 aggregates, a pathologic hallmark of the disease. Further, we show that TDP-43 aggregates diminish the levels of these enzymes, uncovering a novel pathway potencially therapeutic target for the study of ALS pathogenesis. of ALS pathogenesis.

Reserved for Publication Footnotes

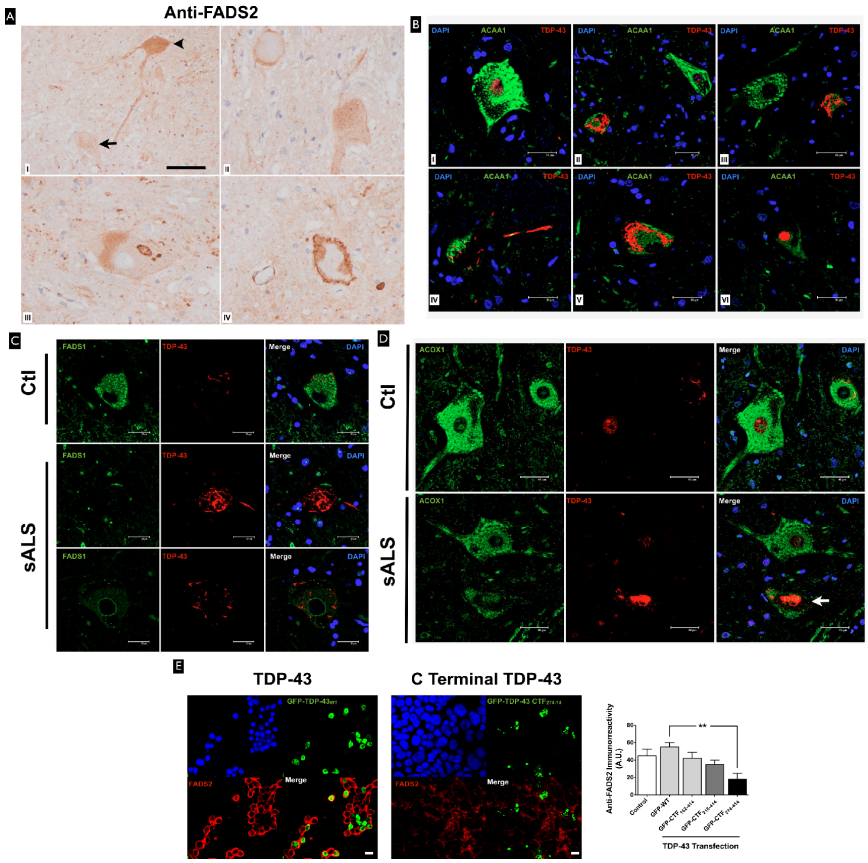


Fig. 1. Enzymes for DHA synthetic pathways are influenced by sALS in a close relationship with TDP-43 aggregation. FADS2 immunoreactivity is distorted in motor neurons from sALS samples, as shown by (A), withreduced FADS2 immunoreactivity in certain cells (arrowhead) while preserved in others (arrow) (I, magnification x200). This is accompanied by abnormal aggregation of FADS2 in a few motorneurons with reduced FADS2 immunoreactivity (II-IV, magnification x400).Confocal imaging in B shows that, in comparison with motor neurons from healthy individuals (I) motor neurons in sALS with TDP-43 aggregates show reduced ACAA1 content (II-VI). The same phenomena is present for FADS1 (C) and ACOX1 (D), showing reduced ACOX expression in sALS motor neurons with TDP-43 inclusions (arrow in D). As indicated by transfection experiments, CTF fragments of TDP-43 are able to reproduce the loss of FADS2 (E). Right panel in E shows the immunoreactivity quantification, with bars representing the means±S.E.M. of immunofluorescence values for different transfections (n=3-4 different experiments). ** Indicates p<0,001 with reference to values of GFP-WT-TDP-43 after one way ANOVA test. Paraffin sections slightly counterstained with haematoxylin. Bar in A = 25 µm.

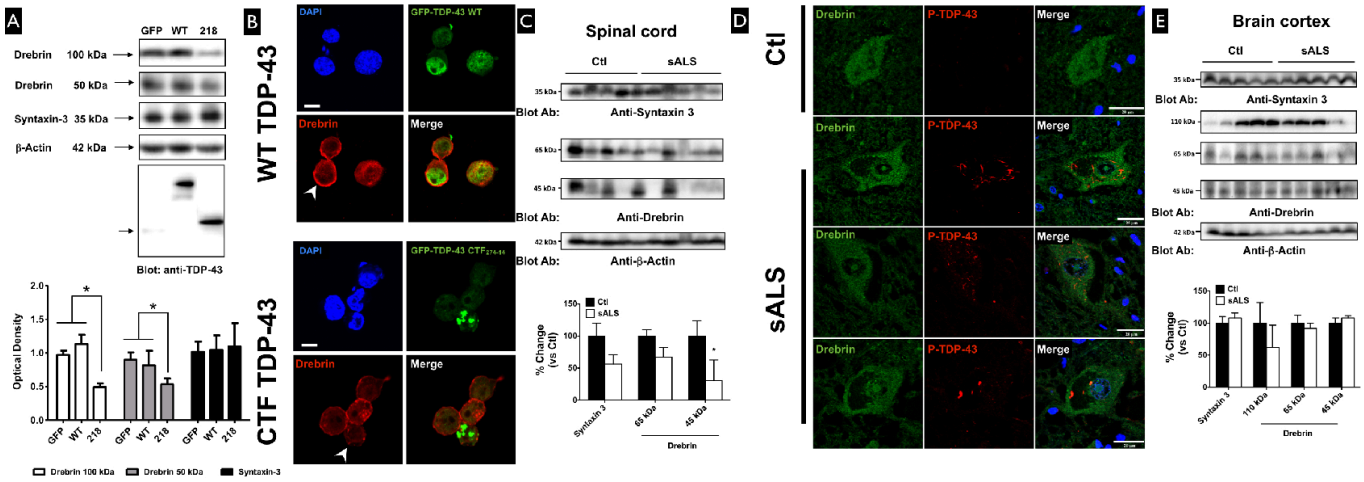


Fig. 2. TDP-43 aggregation and sALS are related to decreased content of the DHA-dependent protein drebrin in neurons. (A) shows that transfection with aggregation prone TDP-43 CTF leads to decreased amounts of the DHA dependent protein drebrin, but not syntaxin. This is cell-specific, as shown by coexistence of TDP-43 aggregates and drebrin depletion in confocal microscopy (B). Note the different abundance of drebrin in cells transfected with WT-TDP-43 (arrowhead in upper panel) and that in a cell showing CTF-TDP-43 aggregates (arrowhead in lower panel). This could explain changes present in samples from human spinal cord and brain cortex, revealing that sALS is associated to specific changes in the content the DHA dependent synaptic proteins drebrin in spinal cord (C), though immunofluorescence analyses show that motor neurons in sALS samples conserve drebrin levels (D), even with P-TDP-43 aggregates. In line with maintained DHA levels, brain cortex lysates do not show changes in drebrin nor in syntaxin levels. Lower panels in A,C and E show the densitometric analyses of the above western-blots. Numbers in the left of blots show the approximate molecular weight. Bars in A,C and E represent the means±S.E.M. being *p<0,05 with reference to values of GFP-WT-TDP-43 (A) spinal cord (C) or brain cortex (E) from control individuals after ANOVA (A) or Student's t test (C,E).

Besides excitotoxicity, it shall be reminded that most sALS patients exhibit Tar-DNA binding protein (TDP-43) aggregates. Gene deletion of *tardbp*, encoding TDP-43, leads to a phenotype consisting with altered lipid homeostasis (e.g. distorted fat storage and membrane trafficking mechanisms)(17). This suggest that, perhaps, DHA synthetic machinery can be influenced by TDP-43 pathogenic events.

To fill these gaps, we have studied the enzymes responsible for DHA-synthesis as well as the potential loss of drebrin and syntaxin 3 associated to the ALS-linked DHA loss. We correlated

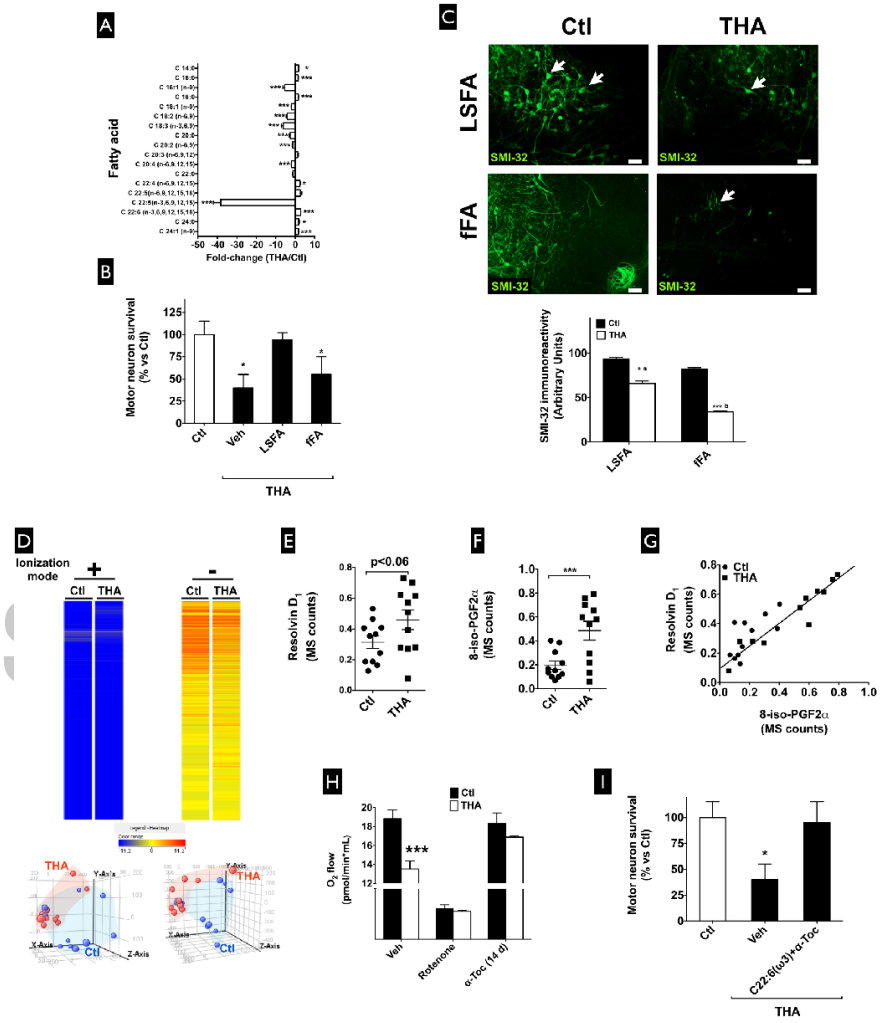


Fig. 3. Chronic excitotoxicity increases the concentration of DHA and biologically related compounds impinging selective changes in the lipidome with depletion of ω3 precursors and mitochondria-derived oxidative stress. A)Variations in fatty acid composition (shown by fold-change) induced by chronic excitotoxicity in spinal cord reveal that the concentrations of the DHA precursor docosapentaenoic acid (C22:5(n-3,6,9,12,15)) and LNA (C18:3(n-3,6,9)) are severely decreased under these conditions. ω3 depletion is pathogenically relevant, as shown by the fact that loss in motor neuron number induced by chronic excitotoxicity is prevented by linenseed fatty acids (LSFA) incubation(B). C shows representative micrographs with the effect of LSFA and fish fatty acids (fFA) in the number of neurofilament positive (SMI-32 immunoreactive) cells in ventral horns of spinal cord organotypic cultures, identified as motor neurons (arrows). D) Lipidome analyses after chronic excitotoxicity reveal relatively minor changes as shown by heatmap of lipids in positive (left) and negative (right) ionization modes. Lower panels show tridimensional Principal Component Analyses of these ionized lipids (blue spheres are control specimens, while red spheres are THA-treated cultures). E) Consisting with increased DHA availability, one of its enzymatic products, resolvin D1, shows a tendency for increased concentrations in cultures under excitotoxicity. F) Levels of the lipid peroxidation marker isoprostane 8-iso-PGF2α are increased by chronic excitotoxicity, and directly correlated (G) to the resolvin D1 amount (y=-0.2+0.75x, p<0.0001, Rsq=0.79). H) In line with this, high-resolution respirometry suggests the potential implication of lipid peroxidation afecting mitochondrial function in the motor neuron loss induced by chronic excitotoxicity, as α-tocopherol treatment during 14 days (α-toc, 100 μM) is able to prevent this function loss, which can be attributed to complex I activity. Further, I) indicates that oxidative stress can be pathophysiologically relevant, as loss of motor neuron induced by excitotoxicity is prevented by addition of DHA (combined with α-tocopherol (α-toc+DHA, 100 μM both) –but not DHA alone (data not shown)- is able to prevent motor neuron loss associated with chronic excitotoxicity. Bars in A,B,C, H and I represent the means±S.E.M. (n=11-15 slices/condition) being *p<0,05; **p<0,001 and *** p<0,0001 with reference to Ctl values by Student's t test (A,E,F,I), one-way ANOVA (B,I) and two-way ANOVA (C). In C [a] and [b] superindexes indicate significant differences between the fatty acids used (p<0,0001). Bars in C are 50μm long.

these findings with TDP-43 aggregates in spinal cord samples from sALS patients (i.e. where motor neuron death is almost complete). We have also used the chronic excitoxicity model, at a stage preceeding motor neuron death. In this system we studied the changes of lipidomic profile and the fatty acid composition to evaluate changes in DHA availability. In this line, we established that oxidative stress is able to modulate DHA synthesis and DHA-linked proteins. Finally, to shed light upon a potential novel therapeutic pathway, we have studied if the addition of α-linolenic acid (ALA), and/or DHA combined with several antioxidants is able to interact with diverse fatty acids rescuing motor neuron-like cells from oxidative stress induced cell death and motor neurons from cell death in chronic excitotoxicity.

Results

sALS is associated with spinal cord specific changes in enzymes for DHA synthesis, associated in a cell-specific fashion to TDP-43 aggregates.

In a previous work, we have shown that sALS is associated with changes in fatty acid composition (14): we detected decreased DHA content in spinal cord and increased levels in brain cortex. DHA synthesis is dependent on the so called Sprecher's

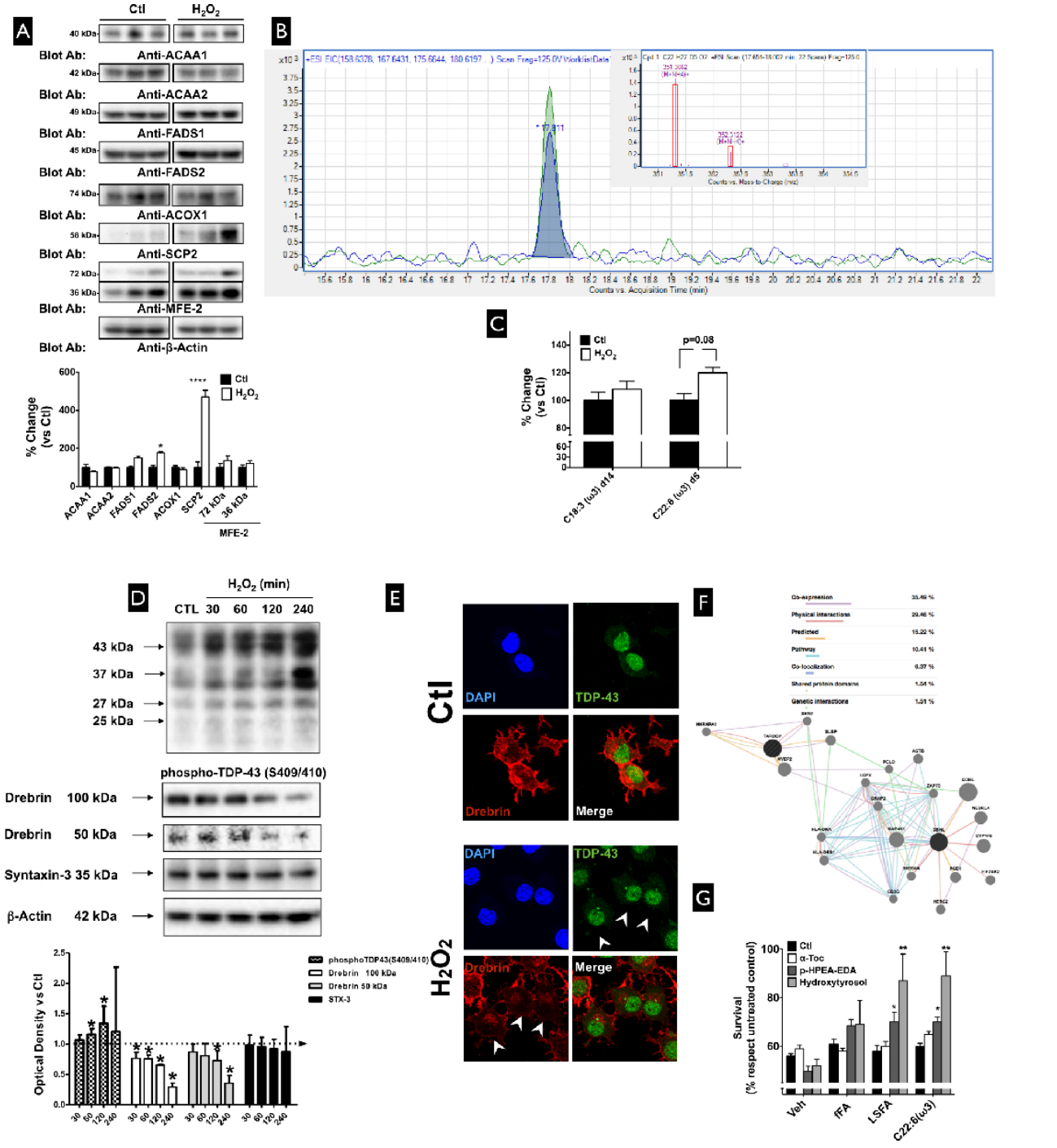


Fig. 4. Oxidative stress enhances DHA synthesis, associated to TDP-43 pathological events and drebrin loss, and its neuroprotective role is enhanced by exogenous antioxidants. A) Representative western-blot showing that H_2O_2 incubation of N2A cells, also changes the expresion of FADS2 and SCP2. B) Representative chromatogram for d_5 labelled DHA (as evidenced by compatible mass spectra of the NH_4^+ adduct in the inset), showing that levels are higher in H_2O_2 treated cells (green trace) in comparison with control cells (blue trace). Omission of d_{14} labelled ALA resulted in indetectable signal of d_5 labelled DHA C) In line with these changes, capacity for conversion of d_{14} labelled ALA to d_5DHA is slightly increased. This increased DHA synthesis can be a response to an H_2O_2-driven TDP-43 phosphorylation and aggregation, which preceedes drebrin loss as shown by western blot (D) and immunocytochemical analyses (E), demonstrating loss of drebrin in those cells with TDP-43 aggregates (arrowheads in E). Interactomic analyses demonstrate that drebrin is mainly linked to TDP-43 by co-expression, with HLA-DRA, HLA-DRB1 and XRN2 as linking nodes. E) In contrast to organotypic spinal cord cultures H_2O_2-induced loss of NSC-34 viability is not prevented by α-tocopherol (α-toc, 200 μM), but by the phenolic compounds derived from virgin olive oil p-HPEA-EDA (5 μM) and hydroxytyrosol (25 μM), in a fatty-acid specific fashion. Lower panels in A and D show the densitometric analyses of the above western-blots. Numbers in the left of blots show the approximate molecular weight. Bars in A, C, D and F are the means±S.E.M (3-4 experiments) being *p<0,05; **p<0,01; *** p<0,001 and **** p<0.0001 by Student's t test (A,C) or one-way ANOVA (D and F) with reference to Ctl.

shunt which coordinates a series of elongation and desaturation steps including a coordinated action of endoplasmic reticulum and peroxisomal functions (4). Interestingly, FADS2 expression in tissue was affected by sALS, with some cells showing preserved

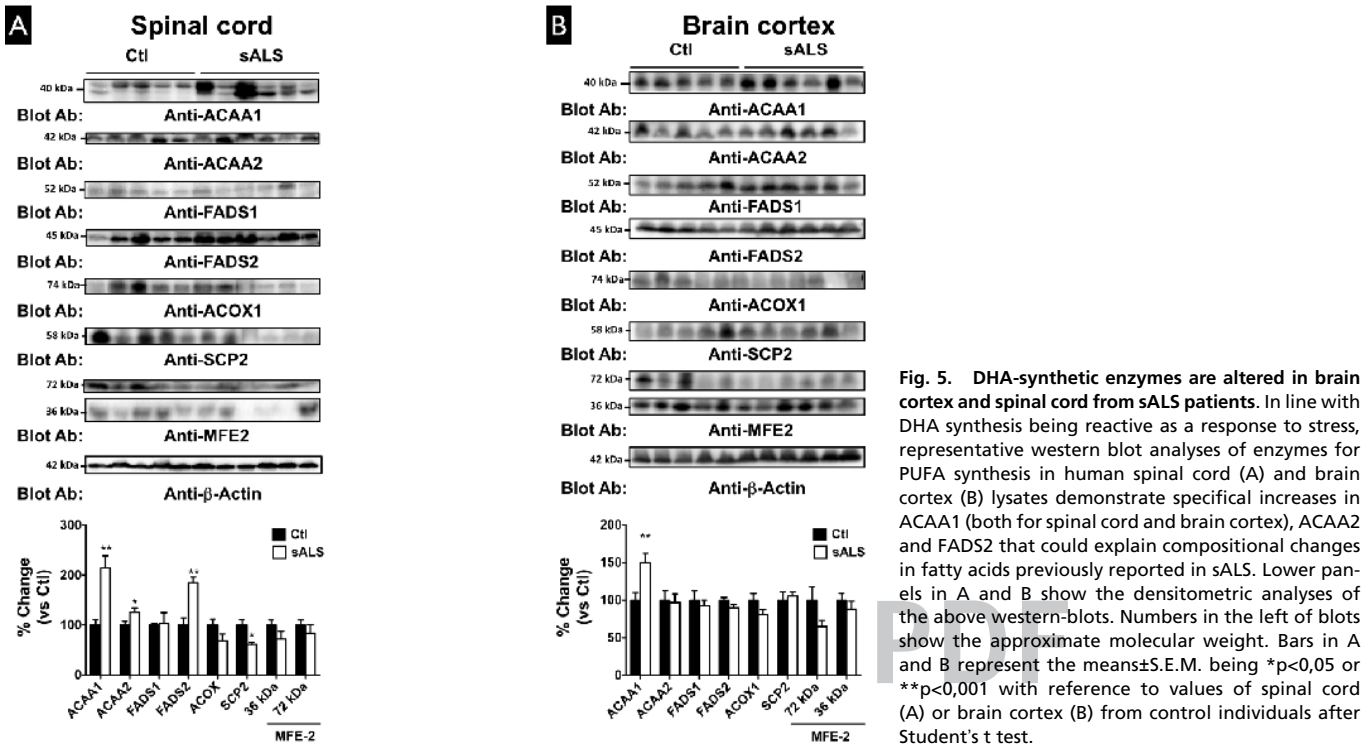


Fig. 5. DHA-synthetic enzymes are altered in brain cortex and spinal cord from sALS patients. In line with DHA synthesis being reactive as a response to stress, representative western blot analyses of enzymes for PUFA synthesis in human spinal cord (A) and brain cortex (B) lysates demonstrate specifical increases in ACAA1 (both for spinal cord and brain cortex), ACAA2 and FADS2 that could explain compositional changes in fatty acids previously reported in sALS. Lower panels in A and B show the densitometric analyses of the above western-blots. Numbers in the left of blots show the approximate molecular weight. Bars in A and B represent the means±S.E.M. being *p<0,05 or **p<0,001 with reference to values of spinal cord (A) or brain cortex (B) from control individuals after Student's t test.

immunoreactivity and others with marked depletion of this enzyme (Figure 1A). Phospho-TDP-43 aggregation is a hallmark for motor neuron demise in sALS. We found that cytosolic TDP-43 aggregates are associated with changes (generally decreased expression) of DHA synthetic enzymes such as ACAA1 (Figure 1B), FADS1 (Figure 1C) and ACOX1 (Figure 1D). Furthermore, *in vitro* experiments showed that the expression of the aggregation prone TDP-43 carboxy-terminal fragments(CTF)(18) leads to a significantly diminished expression of FADS2 ($p<0{,}008$ ANOVA between groups, Figure 1E), specially evident in those fragments with higher tendency to aggregate, such as CTF 274-414, thought it was also present in the other CTF (data not shown).

TDP-43 aggregation decreases the expression of the DHA-dependent synaptic protein drebrin, whose levels are low in spinal cord from sALS patients.

DHA is functionally linked to synaptic proteins, such as drebrin and syntaxin-3, as the expression of those proteins is directly related to DHA concentration (2, 3). In line with TDP-43 aggregation potentially interferring with DHA availability, overexpression of aggregation prone TDP-43 CTF (shown for CTF 218-414) induces a significant decrease in drebrin expression ($p<0.05$), but not in syntaxin-3 (Figure 2A and 2B). Reinforcing the validity of these *in vitro* approaches, western-blot analyses confirmed decreased contents of drebrin in spinal cord from sALS patients, while brain cortex did not show these changes (Figure 2C and 2E), in line with higher DHA content in this later location. Interestingly, this is not linked to direct loss of drebrin in motor neurons, since those showing TDP-43 aggregates do not appear to have decreased drebrin expression (Figure 2D). This would agree with other cells (non motor neurons) having decreased drebrin levels. Thus, while single cell type cell culture demonstrated that TDP-43 aggregation is linked to drebrin depletion, in vivo measurements suggest that other cells could help motor neurons to maintain homeostatic drebrin amounts, stressing the importance of intercellular relationships in spinal cord.

Chronic excitotoxicity leads to changes in whole lipidome and fatty acid profiles in lumbar spinal cord, compatible with increased DHA synthesis, associated to mitochondrial dysfunction and lipid peroxidation.

Chronic excitotoxicity in lumbar spinal cord slices is considered a model of slow neurotoxicity with similar features as sALS pathogenesis (19). Since we wanted to evaluate in a tissue paradigm (i.e. with capacity to reproduce intercellular relationships) the potential involvement of DHA changes in motor neuron demise, we evaluated some of the above mentioned parameters. Drebrin distribution, but not global content –in contrast with sALS-, was significantly altered in chronic excitotoxicity (Supplemental Figure 1). Furthermore, increased syntaxin-3 levels suggested increased DHA levels, a fact that was confirmed by fatty acid compositional analyses (Figure 3A), with a selective decrease of the DHA precursor DPA from ω3 family (without variations in the equivalent of the ω6 family). Estimation of desaturase activities showed that DHA synthesis is the most sensitive pathway of fatty acid synthesis under this chronic excitotoxicity model (Supplemental Figure 1). Therefore, chronic excitotoxicity could lead to a depletion of DHA ω3 precursors. To evaluate whether chronic excitotoxicity could lead to a depletion of DHA ω3 precursors we examined whether exogenously ω3 precursors (linenseed fatty acids, LSFA) could enhance motor neuron survival in this model in comparison with final products (added as fish fatty acids, fFA). The results show that the presence of LSFA enhanced motor neuron survival and expression of motor neuron neurofilament markers in ventral horn of the spinal cord organotypic cultures, in comparison with fFA (Figure 3B and 3C).

To ascertain if these changes affected a particular lipid specie, the effects of chronic excitotoxicity on the lipidomic profile of lumbar spinal cord organotypic culture were studied. The detected changes (Figure 3D) mainly included decreased contents of specific lipids belonging to phosphatidyl-ethanolamine and phosphatidyl-serine families (Supplemental Table 1). Levels of specific ceramides and other quantitatively minor lipids were

also down-regulated. Nevertheless, the global profile did not show a high number of differentiating molecules (144 differential molecules between control and THA-treated samples, in a total of 17832 molecular features, e.g. less than 1% of total lipids).

Since DHA is a precursor of the resolvin family of neuroprotective molecules we evaluated whether increased DHA would sustain increased resolvin concentration. The results show that levels of a lipid specie with mass compatible with the neuroprotective DHA-derived resolvin D1 were increased in chronic excitotoxicity treatment with THA(Figure 3E). This could suggest that increased DHA synthesis in chronic excitotoxicity could be part of a neuroprotector adaptive mechanism. However, in this model PUFA content increased could also increase lipid peroxidizability because double bonds in aliphatic chain confers high susceptibility to peroxidation. Confirming this, the concentration of the lipid peroxidation intermediate isoprostane 8-iso-PGF2α was increased in chronic excitotoxic conditions (Figure 3F), and it correlated significantly with resolvin D1 levels (Figure 3G). Other lipid peroxidation products -indicated in the Experimental Methods section- did not increase or were detected (data not shown), suggesting specificity of those oxidative changes.

Given this increased lipid peroxidation, we searched for potential causes of it. The results (Figure 3H) demonstrate that chronic excitotoxicity leads to a significant loss of oxygen consumption (*ca* 30%). This is caused by a selective loss in mitochondrial complex I activity (Figure 3H), as they were completelly abrogated by rotenone. Thus, potential protective effects of DHA could be limited by potentially enhanced free radical production, compromising the availability of lipophilic antioxidants. Therefore, we tested if a lipophilic antioxidant-DHA combination could prevent motor neuron loss induced by chronic excitotoxicity. The results demonstrated that a DHA-α-tocopherol combination (but not DHA alone –data not shown-) prevents chronic excitotoxicity-induced loss of motor neurons (Figure 3I).

All in all, these results suggest that spinal cord responses to chronic excitotoxicity comprise increased DHA content, sustained by an enhanced desaturase expression leading to increases in DHA derived neuroprotective factors, linked to loss of drebrin amount and distribution as well as to specific increases in lipoxidation likelly fuelled by a complex I related mitochondrial dysfunction.

DHA synthesis at a cellular level is upregulated by oxidative stress and its neuroprotective role is enhanced by exogenous antioxidants.

The above mentioned results are compatible with DHA synthesis being upregulated as a response to oxidative stress induced by a chronic excitotoxicity-derived mitochondrial decay. To test this, we evaluated whether DHA synthesis and drebrin expression were influenced by H_2O_2, a signal derived from complex I dysfunction (20). H_2O_2 incubation increased significantly levels of FADS2 (in accordance to the above described changes in spinal cord ALS samples) and SCP2 (Figure 4A). Synthesis of DHA was evaluated by metabolic flux experiments by measuring the conversion from exogenously added d_{14}ALA to d_5DHA (Figure 4B). The results suggest that there is an almost significant tendency for increased DHA synthesis (Figure 4C). This enhanced DHA production could be a response to compromised DHA availability.

To demonstrate this, we evidenced an early TDP-43 phosphorylation after H_2O_2 challenge, preceeding drebrin loss in N2A cells (Figure 4D and 4E). Globally, after interactomic analyses, few nodes separate TDP-43 from drebrin, confirming the feasibility of the proposed hypothesis (Figure 4F).

To extend these findings to other cellular systems, we studied the effects of exogenously added DHA or other fatty acids in the loss of viability in the motor neuron like line NSC-34. Cell death induced by H_2O_2 was prevented significantly by the use of p-HPEA-EDA and hydroxytyrosol (natural hydrophilic antioxidants of hydrophilic nature present in virgin olive oil) in the presence of DHA and LSFA, in contrast with α-tocopherol (Figure 4G).

ALS leads to significative changes in the levels of enzymes for DHA synthesis in spinal cord and brain cortex.

These results pointed out that increased expression of several enzymes for DHA synthesis can be a response to oxidative stress. Based on previous findings of oxidative damage in ALS samples, we analyzed the tissular concentration of enzymes responsible for DHA synthesis. The results (Figure 5A) showed that in spinal cord the enzymes related to β-oxidation acetyl CoA acyltransferase 1 and 2 (ACAA1 and ACAA2) as well as those related to early steps of desaturation -FADS2- were all increased in spinal cords from sALS samples, perhaps as a compensation for motor-neuron dysfunction of FADS2(Figure 1A). In contrast, SCP2 levels were decreased. In brain cortex, significant increases were only seen for ACAA1 (Figure 5B). This increase could provide the biochemical basis for increased amounts of DHA present in brain cortex(14).

Globally, these results suggest that in sALS, neuronal responses to oxidative challenge comprise increased expression of DHA synthesis enzymes, preceeded by TDP-43 dysfunction and drebrin loss. Furthermore, they also show that motor neuron survival is dependent on the interaction of both motor neuron and surrounding cells with fatty acid sources, with preference of DHA and its precursors, and that phenolic antioxidants could enhance these beneficial properties.

Discussion

The results presented here evidence an important role of the interaction between PUFA synthesis, TDP-43, antioxidants and motor neuron disease. Thus, despite increased machinery for DHA synthesis in spinal cord, apparently it is not able to fully supply increasing needs of this important PUFA (based on data in drebrin) in sALS samples. The resulting increased PUFA amount (non-DHA) could serve as a substrate to lipid peroxidation (under stress conditions). In this sense, data from our own group and others show increased lipid peroxidation in several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Creutzfeld-Jacobs disease, Pick's disease and ALS (21). In this work it is demonstrated that the organotypic spinal cord culture, when affected by chronic excitotoxicity induced by the glutamate uptake inhibitor THA, shows increased DHA concentrations and reduced arachidonic acid levels concurrent to motor neuron loss. Further, we show that neuronal cell lines respond to oxidative stress by increasing DHA synthesis, a fact that will be focus of future studies. This result may be interpretated as a general response to neuron insult, as DHA is precursor of the potent resolvins and neuroprotectins implicated in the defence towards inflammation and oxidative damage (22). Present data show that TDP-43 phosphorylation, a consequence of oxidative stress, preceeds drebrin loss and overexpression of DHA synthetic enzymes. It is known that drebrin shows an inverse relationship with oxidative damage (23). Further, drebrin interacting proteins, such as Homer, are altered by oxidative stress, thereby potentially compromising drebrin function (24). Other data show that drebrin is phosphorylated by Cdk5(25). Thought other kinases may be quantitatively more important in TDP-43 phosphorylation (26), Cdk5 colocalizes with TDP-43 in granulovacuolar degeneration (27), suggesting that perhaps oxidative-stress driven Cdk5 activation (28) could impact both in drebrin and TDP-43 functions. In fact, the most sensitive enzyme to oxidative stress in N2A cells, SCP2, is the one that shows significant decreases in spinal cord. This suggests that peroxisomal function may be impaired in sALS, as previously indicated in transgenic models (29). The peroxisome-ALS connection is also suggested by the possitive effects of some drugs in the peroxisomal proliferator

agonist receptor family (30, 31) in these models, though these are challenged by negative results in clinical trials (32).

In mammals, the synthesis of DHA is a complex pathway, depending, among other, on the levels of the ω3 linolenic acid precursor, its conversion to eicosapentaenoic acid (EPA) (ω3), through elongases and desaturases. Later on, EPA(ω3) is elongated twice, leading to the ω3 24:5 and, via peroxisomal β-oxidation, to DHA(4). The evaluation of elongase and desaturase ratios -even accounting them only as estimative indexes- suggests that earlier steps from ALA to EPA (ω3) are diminished in the excitotoxic model. The best hypothesis explaining the low levels of linolenic acid and EPA (ω3) are the marked increase of enzymes of the later steps (those involving elongation and beta oxidation), which coincide with increased FADS2 and ACAA1,2 expression in ALS samples. This phenomenon may explain the protective role of L-carnitine (33) in ALS models. Thus, besides mitochondrial β-oxidation, L-carnitine is a cofactor needed for peroxisomal β-oxidation (34). All in all, our results demonstrating that both enzymes in peroxisomal and mitochondrial β-oxidation (ACAA1 and ACAA2) are increased in samples from ALS patients disclose a novel pathogenic pathway. Furthermore, the fact that mitochondrial (ACAA2) form is affected is in line of previously reported (14)and present findings showing mitochondrial impairment in ALS. Noteworthy, the fact that several steps of PUFA synthesis depend on enzymes located in endoplasmic reticulum and mitochondria, and the findings reported (present work and(14)) showing that those organelles are affected in ALS are not casual findings. These data support the hypothesis that endoplasmic reticulum and mitochondrial disturbances present in ALS could also impinge PUFA syntheses in nervous tissue.

In this sense, it has been suggested that, in neurons, $^{\triangle}6$ desaturation (mainly performed by *fads2*) may be the rate-limiting step for conversion from ALA to DHA(35). Despite it has been implicated in the pathogenesis of several psychiatric diseases(36, 37), no previous link between FADS2 and motor neuron disease was present. Interestingly, *in vitro* treatment of hippocampal cells with brain derived growth factor (a protective agent in several ALS models, reviewed in(38)) is able to increase *fads2* expression (39). Most importantly, data presented here suggest a hitherto unreported relationship between PUFA synthesis and ALS, again pointing to *fads2* as a novel player in the pathogenesis. Our data demonstrating specific increases in this desaturation key enzyme (both *in vitro* and *in vivo*) disclose a novel target of this disease validating the usefulness of organotypic spinal cord culture as a tool for pathogenic exploration of ALS molecular mechanisms.

Decreased indexes of ω6 family may be explained by a limit in common desaturase cofactors or a selective balance towards ω3 synthesis. It is known that in spinal cord injury models, while ω3 fatty acids (ALA and DHA) have protecting roles, through antioxidant and anti-inflammatory activities, ω6 AA acts in reverse way, worsening the outcome(40). Further, AA downregulate glutamate transporters in vitro(41), so decreased ω6 family synthesis may be a compensative response. Concerning the source of increased DHA in organotypic culture, despite it has been recently shown that hippocampal neurons *in vitro* could produce DHA from ALA(42), it may be a minor source when compared to circulating DHA produced by liver or the transference from glial cells. In this sense, *in vitro* data showed that astrocytes have a larger capacity for producing DHA and AA from their precursors (43, 44)than neurons. Data shown here evidence that isolated motor neuron-like cells behave slight different compared with whole organotypic slices, suggest that glial-neuron interaction in fatty acid metabolism could play a significant role in ALS lipid dearrangements.

The increased survival of motor neurons in spinal cord slices offered by LSFA, in front of pure DHA and fFA suggest that the introduction of precursors, rather than the final form of PUFA, could be an interesting therapeutic tool. In this line, recent data stresses the important neurophysiological differences between DHA and ALA(45). Our data, showing that drebrin was a common target in sALS and in chronic excitotoxicity, reinforce the importance of DHA-dependent processes in the pathogenesis of sALS. In this sense, it has been described that the lack of function of TDP-43, implicated in sALS, induces changes in a specific microRNA (let-7b), leading to impaired syntaxin-3 expression(46). Present findings, reporting cells with TDP-43 aggregations show also abnormal FADS2 immunostaining, support the notion of a pathogenically relevant interplay between TDP-43 and DHA-related processes, reinforced by findings with neuronal cell lines where TDP-43 pathological events (increased phosphorylation and cytosolic aggregation induced by H_2O_2) preceed changes in PUFA synthesis and drebrin depletion. Previous data have shown that lipid peroxidation derived 15-deoxy-δ(12,14)-prostaglandin J(2) is able to reproduce TDP-43 pathological findings(47), but no relationship between TDP-43 and fatty acid synthesis has been previously reported. On the other hand, ALA shares biological targets with riluzole, one of the few accepted treatments for excitotoxicity (reviewed in (48)). It is known that ALA and riluzole act on the 2P domain of the K channel familiy TREK/TRAAK(49), and that this common action could explain reduction of cytopathological treats of neuronal demise in focal ischemia models. It is also known that ALA is able to prevent neuronal cell death, Bax expression and, interestingly, increased chaperone protection exclusively in motor neurons in an ischemia model of the spinal cord, preventing necrosis and apoptosis, adding evidence to the previously described neuroprotective action of ALA(50, 51).

It can not be neglected that perhaps DHA -in contrast to LSFA□ is oxidized in chronic excitotoxicity conditions, contributing to loss of viability. The protective role of α-tocopherol, and its influence in mitochondrial function, supports this hypothesis. It has been previously demonstrated that DHA prevents neuronal cell death by decreasing responses to reactive oxygen species, increasing antiapoptotic protein expression and maintaining mitochondrial integrity and function(52). These results go in line with our data about increased expression of AIF in our samples (data not shown). On the other hand, DHA has been used with promising outcomes in models of spinal cord injury, where the inflammatory component is a clear mechanism of pathogenesis(53).

Despite mitochondrial (dys)-function has been previously invoked in ALS and ALS models, this is among the first direct evidences of loss of O_2 consumption by chronic excitotoxicity in lumbar spinal cords. Previous data ref? showing increased lipid peroxidation in this model, together with the preventive role of α-tocopherol, fully supports the hypothesis that chronic excitotoxicity leads, through loss of mitochondrial respiration, to motor neuron demise. Of note, it should be reminded that mitochondrial respiration is very sensitive to lipid peroxidation(54). The presented results are in line with previous data, suggesting that supplementation of DHA with α-tocopherol may be needed to conserve cellular bioenergetics into homeostasis(55) specially in neuronal tissues. Nevertheless, the lack of protective function of α-tocopherol towards oxidative stress induced demise in the motor-neuron like cells demonstrate the complexity of chronic excitotoxicity. Obviously, it may be argued whether H_2O_2 reproduces features of chronic excitotoxicity that may be prevented by antioxidant functions (e.g. mitochondrial anti-apopototic (56)) of α-tocopherol in the organotypic culture. Similarly, α-tocopherol antioxidant potential is lower than that of other, structurally related, lipophilic antioxidants, such as γ-tocopherol or the tocotrienolic compounds (57). An straightforward interpretation may be that the interplay between glia and motor neurons plays a key role in the protection of α-tocopherol. In this line, it has been previously demonstrated that both α-tocopherol and γ-

tocopherol inhibit oxidative and inflammatory responses in excitotoxicity, leading to diminished oxidative damage in the model of intracerebroventricular injection of kainate *in vivo*, with a clear interaction of glial cells (58). Interestingly, it has been described that preservation of mitochondrial function by α-tocopherol may enhance glutamate transport in an experimental stroke model (59).

It should be remarked that, in contrast to α-tocopherol, other antioxidants, such as hydroxytyrosol and p-HPEA-EDA act, in a fatty acid dependent fashion, as protective factors in the H_2O_2 induced loss of viability in NSC-34. These phenols, present in virgin olive oil phenolic fraction, have pleiotropic functions. They act as anti-inflammatory, antioxidants, anti-proliferative agents, through their interaction with still poorly unknown receptors. Interestingly, it has been recently shown that hydroxytyrosol acts *in vivo*, preventing lipid peroxidation and gluthatione depletion in brain induced by the neurotoxic drug 3-nitropropionate (60). Previous data revealed that, in different neuronal cell lines, hydroxytyrosol confers cytoprotection in a mechanism related to fatty acid uptake (61).

Globally, these results show that upregulation of DHA levels, initially a compensatory response, could contribute to increased peroxidiziability and consequently to damage spreading. It is also shown that DHA and other PUFA, when adequately protected from lipid peroxidation by other dietary available compounds (α-tocopherol or hydrophilic antioxidant polyphenols), could offer neuroprotection in chronic excitotoxicity. Further work is needed to evaluate its potential effect *in vivo*, but the present results may help to define the potential of neuroprotective diets based on adequate combinations of PUFA and selected phenolic antioxidants.

Materials and methods

Patient samples

Samples were from ten ALS cases and eight controls (see supplemental Table 2 for demographic characteristics). The post-mortem delay was between 2 and 16 h 30 min and did not differ between the groups. The brain and spinal cord were rapidly removed. Half of the brain and alternate sections of the brain stem and spinal cord were immersed in 4% buffered formalin for four weeks and then selected samples from 40 different areas embedded and paraffin and processed for current diagnostic neuropathological studies or stored until use. Selected samples of the other half of the brain, alternate sections of the brain stem and spinal cord were rapidly frozen on dry ice, kept on individual tag plastic bags and stored at -80ºC until use. All samples were obtained from the Institute of Neuropathology brain bank, a branch of the HUB-ICO-IDIBELL biobank, following the guidelines of the local ethics committees. Extensive pathological studies were done for ALS diagnosis as previously described (14, 62). Sample obtention and experiments have been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) .

Reagents

All reagents, unless stated otherwise, were purchased from Sigma-Aldrich Chemical Co. 2-(3,4-dihydroxyphenyl) ethyl alcohol (hydroxytyrosol) from Extrasynthese (Genay, France) and 4-formyl-3-(2-oxoethyl) 2-(4-hydroxyphenyl) ethyl ester (*p*-HPEA-EDA) was isolated from virgin olive oil phenolic extract by semi-preparative HPLC method according to the method of Artajo et al. (63). Stock solutions of commercial standards and phenolic compounds isolated from virgin olive oil were dissolved in MeOH/H_2O (80:20 v/v) and stored at -40º C before the incubation. Commercially available, fatty acid extracts Ecoflax Omega 3^r from linenseed (LSFA), and Biomegatech fish^r, from fish (fFA) were obtained from BTSA (Madrid, Spain). Both DHA or fatty acid extracts were used at a final concentration of 100 µM in culture media (either organotypic or cell culture media). Fatty acid composition from LSFA was α-linolenic acid 60%, oleic acid 23%, linoleic acid 12%, stearic acid 2% and palmitic acid 2%, and from fFA was eicosapentaenoic 60% and docosahexaenoic 40%. Peroxide index values of these products were below detectable limits.

Immunohistochemistry and western-blot

Immunohistochemistry (IF) and western blot (IB) analyses were performed and quantified as previously described (14, 62). Antibodies used for evaluation of enzymatic system for DHA synthesis by IB were: peroxisomal acyl-coenzyme A oxidase 1 (ACOX1, Abcam ab599964, 1:300), Acetyl CoA acyltransferase 1 (ACAA1, Abcam ab84635, 1:1000); Acetyl CoA acyltransferase 2 (ACAA2, Abcam ab58278, 1:1000); Fatty acid desaturase 1 (FADS1, Abcam ab124363, 1:300), Fatty acid desaturase 2 (FADS2, Abcam ab72189, IB 1:1000); Sterol carrier protein 2 (SCP2, Abcam ab 93175, 1:200), Peroxisomal multifunctional enzyme 2 (MFE2, Abcam ab97971, 1:500) and actin (Sigma a5441, 1:5000) for normalization of protein load. Other antibodies used were SMI- 32(Covance Inc., Princeton, NJ, USA) (IF at 1:200 dilution; IB at 1:1000 dilution), drebrin (Abcam Plc, Cambridge, UK; ab12350, IF, 1:100), syntaxin-3 (Abcam ab4113, IF, 1:50), neurofilament (Abcam, ab 72997, IF 1:200), TDP-43 (Abcam, ab 545002, IF 1:2000), phospho-TDP-43 (Cosmobio Ltd, Carlsbad, CA, USA, Tip PTD.P05, IF 1:250 or Millipore P-TDP-43 (Ser409/Ser410), clone 1D3 (MABN14), IB 1:1000) and neuronal specific tubulin antibodies (Abcam ab18207, IF, 1:200; IB 1:1000).

Immunoreactivity images were analyzed with the Image J software. For area distribution of immunoreactive anti-drebrin particles, images were binary converted and analyzed with the particle analyses algorithm of the software (http://rsbweb.nih.gov/ij/), in a double blinded fashion. Distributional analyses of the particle areas was produced using the Prism 5 software (Graphpad Software, La Jolla, CA, USA).

Neuro2a (N2A), motor-neuron like (NSC34) cell and spinal cord organotypic culture

Oxidative stress has been recently related to TDP-43 pathology in ALS [17] . Thus, to study the potencial link with DHA synthesis pathway in this condition, Neuro 2A (N2A) cells were incubated with 10 µM H_2O_2 for 2 h as previously described (62). TDP-43 plasmids and C-terminal fragments were obtained as indicated(18). Neuro 2A cells were transient transfected with the empty pEGFP-C1 vector (GFP) or vector expressing the wild type (WT) human TDP-43 cDNA (1-414) or C-terminal fragments 162-414 (162), 218-414 (218) and 274-414 (274) using Lipofectamine-2000 Reagent (Invitrogen-11668) according to the manufaturer's instructions for 24 hours (depending on the experiment). Under these experimental conditions, the efficiency of transfection with pEGFP-C1 vector was *ca* 80%.

Spinal cord cultures were prepared from lumbar spinal cord of postnatal day 8 rat pups as previously indicated and maintained in culture media. Ten days after plating, to induce excitotoxicity the slices were incubated with the glutamate transport inhibitor D,L-threohydroxyaspartate (THA) (Sigma, St. Louis, MO, USA) at 100 µM, inducing a selective motor neuron death after 3 weeks (19). Experiments for each condition (n=30 slices per experimental group) were repeated at least three times. All animal experiments were approved by the Institutional Animal Care and Experimentation Committee, and followed EU Directive 2010/63/EU for animal experiments.

To analyse the potencial neuroprotective role of antioxidants supplementation in combination of DHA in a cellular stress situation able to promote the ALS development, NSC-34 cells were incubated with fatty acids (as above) and antioxidants (α-tocopherol, hydroxytyrosol or *p*-HPEA□EDA at final concentrations 100 µM, 25 µM and 5 µM, respectively) were added 48 hours before incubation with 10 µM H_2O_2 for 2 hours. Cell viability was evaluated 8 hours after with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT test, Sigma) according manufacturer instructions.

Lipid analyses

Fatty acid profiles were established after whole lipid transesterification and gas chromatography analysis of methyl esters performed as previously described(64). The following indexes were calculated from the fatty acid composition: $\Delta^9$18:1/18:0 ratio between C18:1(n-9) and C18:0 % contents; $\Delta^9$16:1/16:0 ratio between C16:1 (n-9) and C16:0 % contents; $\Delta^5$20:4/20:3 ratio between C20:4 (n-6,9,12,15) and C20:3 (n-6,9,12) % contents; 18:1/16:1 Elongase from ratio between C18:1(n-9) and C16:1 (n-9) % contents; and 22:4/20:4 Elongase from ratio between C22:4(n-6,9,12,15) and C20:4(n-6,9,12,15) as previously described(65).

For LC-Q-TOF-based lipid molecular species analyses, lipid extracts were subjected to mass-spectrometry using a HPLC 1200 series coupled to an electrospray-Q-TOF MS/MS 6520 (Agilent Technologies, Barcelona, Spain), using a reverse phase column and a methanol:water to 1-propanol:hexane:water gradient, following the method described in (67). Lipid class representative internal standards were added to the samples and after analyses; ionized lipids were processed for targeted and untargeted analyses. Briefly, the MassHunter Mass Profiler Professional Software (Agilent Technologies, Barcelona, Spain) was used to perform a non-targeted lipidomic analysis over the extracted features. Only common features (found in at least 75% of the samples of the same condition) were taken into account to correct for individual bias. Principal component analyses (PCA) was obtained using this software. The masses representing significant differences by Student T-test (fold change ≥ 2, $p < 0.05$) were searched against the METLIN (metlin.scripps.edu), HMDB (www.hmdb.ca), LIPID MAPS (www.lipidmaps.org) and KEGG (www.genome.jp/kegg/) databases. Targeted lipidomic analysis was performed using the same chromatographic and spectrometric method as untargeted approach. MassHunter Qualitative Analysis Software (Agilent Technologies, Barcelona, Spain) was employed for integration and extraction of peak intensities of the different lipidic species. We looked for the following free fatty acids: docosahexaenoic acid, oleic acid, linolenic acid, stearic acid, arachidonic acid, lauric acid, palmitic acid, capric acid, myristic acid, palmitoleic acid and stearidonic acid. The m/z values used for quantification were: m/z 245.2486 [M-H]- for oleic acid, m/z 283.2643 [M-H]- for stearic acid, m/z 255.233 [M-H]- for palmitic acid, m/z 227.2017 [M-H]- for myristic acid and m/z 253.2173 [M-H]- for palmitoleic acid. We detected no differences induced by chronic excitotoxicity in those fatty acids. The following lipids were also searched: 10-hydroxy-docosahexaenoic, 17-hydroxy-docosahexaenoic, 8-iso-PGF2α, 13-hydroxyoctadecadienoic acid (HODE), 9-HODE, HODE-cholesteryl

ester, 1-palmitoyl-2-arachidonoyl-3-glycerophosphocholine, 1-palmitoyl-2-linoleyl-3-glycerophosphocholine, cholesterol, 4-hydroxynonenal, 10-nitrooleate, cholesterol-5α,6α-epoxide, 5-cholesten-3β-ol-7-one, 7β-hydroxycholesterol, resolving D1, cholesteryl linoleate hydroperoxide, cholesteryl linoleate. The m/z values used for quantification were: m/z 355.2479 [M+H]- for 8-isoprostaglandin F2α and m/z 377.2323 for resolvin D1. For d14 labelled linolenic acid (C18H16D14O2) and d5 labelled DHA (C22H27D5O2) the "Find by formula" (Agilent) algorithm was used.

Oxygen consumption

Oxygen flux of sets of 5 slices of organotypic spinal cord cultures was measured at 37 °C in Hank's balanced salt solution with 20 mM Hepes (pH 7.4) by high-resolution respirometry using the Oroboros® Oxygraph-2k with chamber volumes set at 2 mL and at a slow stirring speed (150 rpm) in order to avoid tissue disaggregation. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition (2 s time intervals) and analysis, which includes calculation of the time derivative of oxygen concentration and correction for instrumental background oxygen flux (68).The experimental regime consisted of routine respiration, which is defined as respiration in cell-culture medium without additional substrates or effectors, and calculated as Steady-state respiratory flux in the time interval between 5 and 10 min after closing the chamber. In order to avoid oxygen limitations, all the experiments were performed above 50% oxygen saturation. Oxygen consumption was normalized by actual protein content in the respirometer chambers and mitochondrial inhibitors were added as described (68).

DHA synthesis flux experiments

For evaluation of potential DHA synthesis under cell stress, N2A cells were seeded onto 6-well plates and grown in Advanced minimum essential media supplemented with 10% fetal bovine serum until 1 day postconfluence, the point at which fatty acid changes are maximized(69). Then, cells were incubated with medium containing 4.1 μM d_{14} labelled ALA (Cayman Chemical Company, 0000433) for 4 hours. After that, the medium was removed and cells were treated with H_2O_2 10 μM for 2 hours. Cells were washed and harvested in PBS using a cell scraper. Lipid extraction was carried out as described(70). Cells were pelleted by centrifugation (700 g, 4 °C, 5 min) and dissolved in chloroform-methanol (2:1, v/v). They were incubated on ice for 10 min, vortexed, and centrifuged (1100 g, 4ºC, 10 min). The lower organic phase was transferred to a new glass tube and dried completely under nitrogen, RT. Later, the fatty acid saponification was carried out by incubating lipid extracts in 2.5N KOH/MetOH (1/4, v/v) for 15 min at 72 ºC in a heating block, as indicated (71). The pH of the solution was acidified with one-eighth of the volume of formic acid. The free fatty acids were then extracted with an equal volume of chloroform and after drying, extracts were reconstituted in chloroform and stored at -20ºC until ready for LC-Q-TOF analysis. Levels of d_{14} labelled ALA, d_5 labelled DHA and oleic acid (used for normalization) were quantified as indicated above.

Statistical and bioinformatical analysis

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed by the Student's t tests, One way and Two way ANOVA analyses, with post-hoc analyses, once normality of variables was tested by Kolmogorov-Smirnov test. The 0.05 level was selected as the point of minimal statistical significance in every comparison. For interactomic analyses, we selected as input nodes the genes *tardbp* and *dbnl*, and we used the GeneMANIA software (http://www.genemania.org).

Acknowledgements.

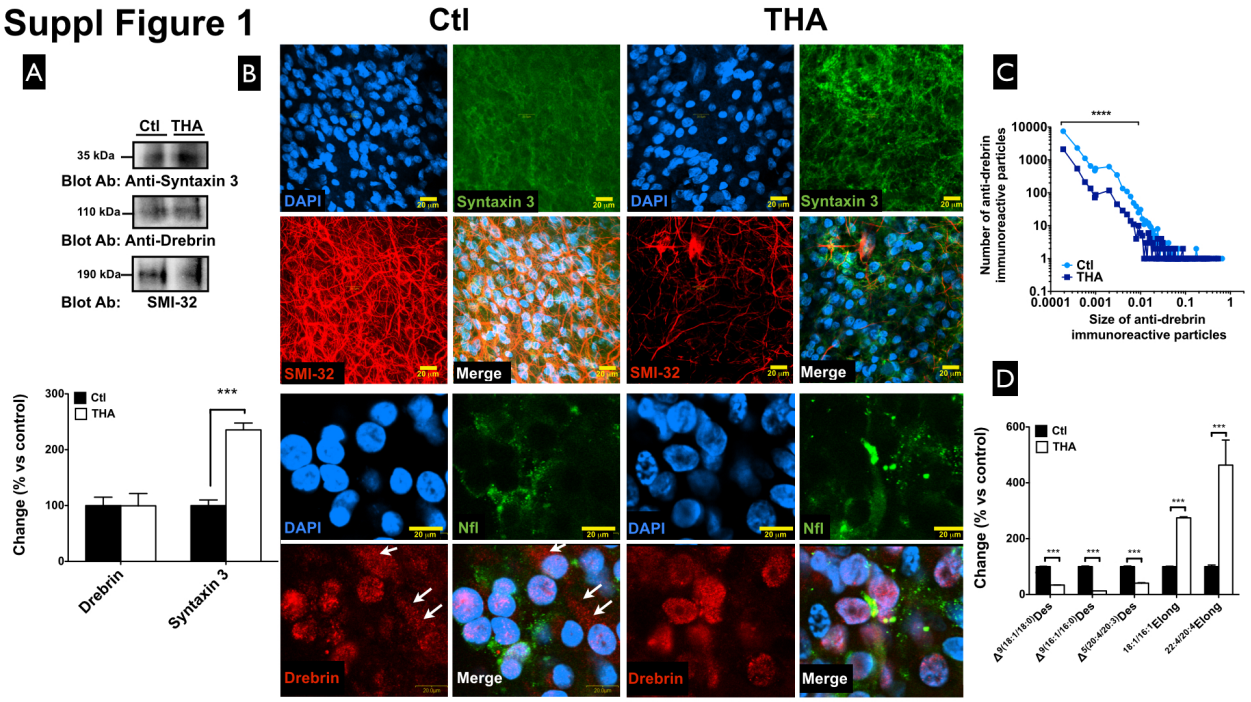
We are indebted to tissue donors and their families. We are grateful for the consortium of companies led by La Morella Nuts (Reus, Catalonia, Spain) with the following companies: BTSA, Selecció Batallé, Industrial Tècnica Pecuaria, Neuron BioPharma, Shirota Functional Foods, Grupo Leche Pascual, and Innaves. Supported by the Seventh Framework Programme of the European Commission, grant agreement 278486: DEVELAGE; by the Spanish Ministry of Science and Innovation [CENIT program, BFU2009-11879/BFI and AGL2006-1243] the Autonomous Goverment of Catalunya [2014SGR168] and the Spanish Ministry of Health [PI08/1843, 11/01532 and 13/00584]. DC is a predoctoral fellow from Spanish Ministry of Health [FI08-00707]. OR-N and RC are predoctoral fellows from the Autonomous Government of Catalonia. Supported also by the COST B-35 Action and the Fundació Miquel Valls.

1. Alessandri JM et al. (2004) Polyunsaturated fatty acids in the central nervous system: evolution of concepts and nutritional implications throughout life. *Reprod Nutr Dev* 44:509–538.
2. Darios F, Davletov B (2006) Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3 . *Nature* 440:813–817.
3. Calon F et al. (2004) Docosahexaenoic acid protects from dendritic pathology in an Alzheimer's disease mouse model . *Neuron* 43:633–645.
4. Qiu X (2003) Biosynthesis of docosahexaenoic acid (DHA, 22:6-4, 7,10,13,16,19): two distinct pathways . *Prostaglandins Leukot Essent Fatty Acids* 68:181–186.
5. Negre-Salvayre A et al. (2010) Pathological aspects of lipid peroxidation. *Free Radic Res* 44:1125–1171..
6. Broadhurst CL, Cunnane SC, Crawford MA (1998) Rift Valley lake fish and shellfish provided brain-specific nutrition for early Homo. *Br J Nutr* 79:3–21.
7. Chamberlain JG (1996) The possible role of long-chain, omega-3 fatty acids in human brain phylogeny. *Perspect Biol Med* 39:436–445.
8. Cunnane SC, Crawford MA (2003) Survival of the fattest: fat babies were the key to evolution of the large human brain. *Comp Biochem Physiol A, Mol Integr Physiol* 136:17–26.
9. Nair PP et al. (1993) Dietary fish oil-induced changes in the distribution of alpha-tocopherol, retinol, and beta-carotene in plasma, red blood cells, and platelets: modulation by vitamin E . *Am J Clin Nutr* 58:98–102.
10. Sanders TA, Hinds A (1992) The influence of a fish oil high in docosahexaenoic acid on plasma lipoprotein and vitamin E concentrations and haemostatic function in healthy male volunteers . *Br J Nutr* 68:163–173.
11. Gonzalez-Correa JA et al. (2008) Neuroprotective effect of hydroxytyrosol and hydroxytyrosol acetate in rat brain slices subjected to hypoxia-reoxygenation . *Neurosci Lett* 446:143–146.
12. Miljkovic D, Dekanski D, Miljkovic Z, Momcilovic M, Mostarica-Stojkovic M (2009) Dry olive leaf extract ameliorates experimental autoimmune encephalomyelitis . *Clin Nutr* 28:346–350.
13. Fitzgerald KC et al. (2014) Dietary ω-3 Polyunsaturated Fatty Acid Intake and Risk for Amyotrophic Lateral Sclerosis. *JAMA Neurol*.1-9.
14. Ilieva E V et al. (2007) Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. *Brain* 130:3111–3123.
15. Stark DT, Bazan NG (2011) Synaptic and extrasynaptic NMDA receptors differentially modulate neuronal cyclooxygenase-2 function, lipid peroxidation, and neuroprotection. *J Neurosci* 31:13710–13721.
16. Hunt WT, Kamboj A, Anderson HD, Anderson CM (2010) Protection of cortical neurons from excitotoxicity by conjugated linoleic acid. *J Neurochem* 115:123–130.
17. Heck MV et al. (2014) Dysregulated expression of lipid storage and membrane dynamics factors in Tia1 knockout mouse nervous tissue. *Neurogenetics* 15: 135-144.
18. Nonaka T, Kametani F, Arai T, Akiyama H, Hasegawa M (2009) Truncation and pathogenic mutations facilitate the formation of intracellular aggregates of TDP-43. *Hum Mol Genet* 18:3353–3364.
19. Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW (1993) Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity . *Proc Natl Acad Sci U S A* 90:6591–6595.
20. Quinlan CL, Perevoshchikova I V., Hey-Mogensen M, Orr AL, Brand MD (2013) Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. *Redox Biol* 1:304–312.
21. Pamplona R. et al. Maillard reaction versus other nonenzymatic modifications in neurodegenerative processes. *Ann New York Acad Sci* 1126:315-319.
22. Bazan NG, Molina MF, Gordon WC (2011) Docosahexaenoic Acid signalolipidomics in nutrition: significance in aging, neuroinflammation, macular degeneration, Alzheimer's, and other neurodegenerative diseases . *Annu Rev Nutr* 31:321–351.
23. Li L, Wang Z, Zuo Z (2013) Chronic Intermittent Fasting Improves Cognitive Functions and Brain Structures in Mice. *PLoS One* 8.
24. Nepliouev I, Zhang ZS, Stiber JA (2011) Effect of oxidative stress on Homer scaffolding proteins. *PLoS One* 6.
25. Worth DC, Daly CN, Geraldo S, Oozeer F, Gordon-Weeks PR (2013) Drebrin contains a cryptic F-actin-bundling activity regulated by Cdk5 phosphorylation. *J Cell Biol* 202:793–806.
26. Choksi DK et al. (2014) TDP-43 phosphorylation by casein kinase promotes oligomerization and enhances toxicity in vivo. *Hum Mol Genet* 23:1025–1035.
27. Nakamori M et al. (2012) Cyclin-dependent kinase 5 immunoreactivity for granulovacuolar degeneration. *Neuroreport* 23:867–872.
28. Sun KH et al. (2011) Glutathione-S-transferase P1 is a critical regulator of Cdk5 kinase activity. *J Neurochem* 118:902–914.
29. Ilieva HS et al. (2003) Age-related changes in peroxisomal membrane protein 70 and superoxide dismutase 1 in transgenic G93A mice. *Neurol Res* 25:423–426.
30. Benedusi V, Martorana F, Brambilla L, Maggi A, Rossi D (2012) The Peroxisome Proliferator-activated Receptor (PPAR) Controls Natural Protective Mechanisms against Lipid Peroxidation in Amyotrophic Lateral Sclerosis. *J Biol Chem* 287:35899–35911.
31. Kiaei M (2008) Peroxisome Proliferator-Activated Receptor-gamma in Amyotrophic Lateral Sclerosis and Huntington's Disease . *PPAR Res* 2008:418765.
32. Dupuis L et al. (2012) A randomized, double blind, placebo-controlled trial of pioglitazone in combination with riluzole in amyotrophic lateral sclerosis. *PLoS One* 7.
33. Kira Y, Nishikawa M, Ochi A, Sato E, Inoue M (2006) L-carnitine suppresses the onset of neuromuscular degeneration and increases the life span of mice with familial amyotrophic lateral sclerosis . *Brain Res* 1070:206–214.
34. Le Borgne F, Ben Mohamed A, Logerot M, Garnier E, Demarquoy J (2011) Changes in carnitine octanoyltransferase activity induce alteration in fatty acid metabolism . *Biochem Biophys Res Commun* 409:699–704.
35. Sprecher H, Luthria DL, Mohammed BS, Baykousheva SP (1995) Reevaluation of the pathways for the biosynthesis of polyunsaturated fatty acids . *J Lipid Res* 36:2471–2477.
36. Liu Y, Jandacek R, Rider T, Tso P, McNamara RK (2009) Elevated delta-6 desaturase (FADS2) expression in the postmortem prefrontal cortex of schizophrenic patients: relationship with fatty acid composition . *Schizophr Res* 109:113–120.
37. Liu Y, McNamara RK (2011) Elevated Delta-6 desaturase (FADS2) gene expression in the prefrontal cortex of patients with bipolar disorder . *J Psychiatr Res* 45:269–272.
38. Nagahara AH, Tuszynski MH (2011) Potential therapeutic uses of BDNF in neurological and psychiatric disorders . *Nat Rev Discov* 10:209–219.
39. Cazzin C, Mion S, Caldara F, Rimland JM, Domenici E (2011) Microarray analysis of cultured rat hippocampal neurons treated with brain derived neurotrophic factor. *Mol Biol Rep* 38:983–990.
40. King VR et al. (2006) Omega-3 fatty acids improve recovery, whereas omega-6 fatty acids worsen outcome, after spinal cord injury in the adult rat . *J Neurosci* 26:4672–4680.
41. Zerangue N, Arriza JL, Amara SG, Kavanaugh MP (1995) Differential modulation of human

glutamate transporter subtypes by arachidonic acid. *J Biol Chem* 270:6433–6435.
42. Kaduce TL, Chen Y, Hell JW, Spector AA (2008) Docosahexaenoic acid synthesis from n-3 fatty acid precursors in rat hippocampal neurons . *J Neurochem* 105:1525–1535.
43. Moore SA, Yoder E, Murphy S, Dutton GR, Spector AA (1991) Astrocytes, not neurons, produce docosahexaenoic acid (22:6 omega-3) and arachidonic acid (20:4 omega-6) . *J Neurochem* 56:518–524.
44. Moore SA (1994) Local synthesis and targeting of essential fatty acids at the cellular interface between blood and brain: a role for cerebral endothelium and astrocytes in the accretion of CNS docosahexaenoic acid . *World Rev Nutr Diet* 75:128–133.
45. Arsenault D, Julien C, Calon F (2011) Chronic dietary intake of alpha-linolenic acid does not replicate the effects of DHA on passive properties of entorhinal cortex neurons . *Br J Nutr*:1–13.
46. Buratti E et al. (2010) Nuclear factor TDP-43 can affect selected microRNA levels . *FEBS J* 277:2268–2281.
47. Zhang HX et al. (2010) Alteration of biochemical and pathological properties of TDP-43 protein by a lipid mediator, 15-deoxy-??12,14-prostaglandin J2. *Exp Neurol* 222:296–303.
48. Cheah BC, Vucic S, Krishnan A V, Kiernan MC (2010) Riluzole, neuroprotection and amyotrophic lateral sclerosis . *Curr Med Chem* 17:1199–1942.
49. Heurteaux C, Laigle C, Blondeau N, Jarretou G, Lazdunski M (2006) Alpha-linolenic acid and riluzole treatment confer cerebral protection and improve survival after focal brain ischemia. *Neuroscience* 137:241–251.
50. Lauritzen I et al. (2000) Polyunsaturated fatty acids are potent neuroprotectors . *EMBO J* 19:1784–1793.
51. Lang-Lazdunski L, Blondeau N, Jarretou G, Lazdunski M, Heurteaux C (2003) Linolenic acid prevents neuronal cell death and paraplegia after transient spinal cord ischemia in rats . *J Vasc Surg Off Publ Soc Vasc Surg & Int Soc Cardiovasc Surgery, North Am Chapter* 38:564–575.
52. Mayurasakorn K, Williams JJ, Ten VS, Deckelbaum RJ (2011) Docosahexaenoic acid: brain accretion and roles in neuroprotection after brain hypoxia and ischemia . *Curr Opin Clin Nutr Metab Care* 14:158–167.
53. Huang WL et al. (2007) A combination of intravenous and dietary docosahexaenoic acid significantly improves outcome after spinal cord injury . *Brain* 130:3004–3019.
54. Echtay KS et al. (2003) A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J* 22:4103–4110.
55. Infante JP, Huszagh VA (2000) Secondary carnitine deficiency and impaired docosahexaenoic (22:6n-3) acid synthesis: a common denominator in the pathophysiology of diseases of oxidative phosphorylation and beta-oxidation . *FEBS Lett* 468:1–5.
56. Kondo K et al. (2010) Poly(ADP-ribose) polymerase (PARP)-1-independent apoptosis-inducing factor (AIF) release and cell death are induced by eleostearic acid and blocked by alpha-tocopherol and MEK inhibition . *J Biol Chem* 285:13079–13091.
57. Sen CK, Khanna S, Roy S (2004) Tocotrienol: the natural vitamin E to defend the nervous system? *Ann N Y Acad Sci* 1031:127–142.
58. Milatovic D, VanRollins M, Li K, Montine KS, Montine TJ (2005) Suppression of murine cerebral F2-isoprostanes and F4-neuroprostanes from excitotoxicity and innate immune response in vivo by alpha- or gamma-tocopherol . *J Chromatogr Anal Technol Biomed life Sci* 827:88–93.
59. Hurtado O et al. (2003) Inhibition of glutamate release by delaying ATP fall accounts for neuroprotective effects of antioxidants in experimental stroke . *FASEB J* 17:2082–2084.
60. Tasset I, Pontes AJ, Hinojosa AJ, de la Torre R, Tunez I (2011) Olive oil reduces oxidative damage in a 3-nitropropionic acid-induced Huntington's disease-like rat model . *Nutr Neurosci* 14:106–111.
61. Young J, Wahle KW, Boyle SP (2008) Cytoprotective effects of phenolic antioxidants and essential fatty acids in human blood monocyte and neuroblastoma cell lines: surrogates for neurological damage in vivo . *Prostaglandins Leukot Essent Fatty Acids* 78:45–59.
62. Ayala V et al. (2011) Cell stress induces TDP-43 pathological changes associated with ERK1/2 dysfunction: implications in ALS. *Acta Neuropathol* 122:259–270.
63. Artajo LS, Romero MP, Morello JR, Motilva MJ (2006) Enrichment of refined olive oil with phenolic compounds: evaluation of their antioxidant activity and their effect on the bitter index. *J Agric Food Chem* 54:6079–6088.
64. Pamplona R et al. (2005) Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. *J Biol Chem* 280:21522–30.
65. Brenner RR et al. (2003) Desaturase activities in rat model of insulin resistance induced by a sucrose-rich diet . *Lipids* 38:733–742.
66. Mai K et al. (2011) Rosiglitazone increases fatty acid Delta9-desaturation and decreases elongase activity index in human skeletal muscle in vivo . *Metabolism*.
67. Sandra K, Pereira Ados S, Vanhoenacker G, David F, Sandra P (2010) Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry. *J Chromatogr* 1217:4087–4099.
68. Gnaiger E (2001) Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply . *Respir Physiol* 128:277–297.
69. Andersson C et al. (2008) Cell culture models demonstrate that CFTR dysfunction leads to defective fatty acid composition and metabolism. *J Lipid Res* 49:1692–1700.
70. Njoroge SW, Laposata M, Katrangi W, Seegmiller AC (2012) DHA and EPA reverse cystic fibrosis-related FA abnormalities by suppressing FA desaturase expression and activity. *J Lipid Res* 53:257–265.
71. Dillon R, Greig MJ, Bhat BG (2008) Development of a novel LC/MS method to quantitate cellular stearoyl-CoA desaturase activity. *Anal Chim Acta* 627:99–104.

Supplemental Figure 1

A) Representative western blot analyses reveal that chronic excitotoxicity leads to increased amount of syntaxin-3, but no major changes in drebrin amount, as quantified in the lower panel. B) Confocal microscopy revealed that chronic excitotoxicity induced increased syntaxin-3 immunoreactivity (green upper panels) concurrent with diminished SMI-32 immunoreactivity (red, upper panels). This was associated to a loss of drebrin compartimentalization (green, lower panels) since in control conditions anti-drebrin immunohistochemistry was distributed into several sizes with small granular-like particles (arrow), without apparent relationship with neurofilament marker (Nfl, green). C) The number of smaller particles was much lower in THA-treated spinal cord slices, compatible with a loss of drebrin compartimentalization. D) Estimation of desaturase and elongase activities in these samples suggest important changes in δ9, δ5 and elongase activities, according the ratios between products and precursors. Lower panels in A and D show the densitometric analyses of the above western-blots. Numbers in the left of blots show the approximate molecular weight. ***p<0,0001 and ****p<0,00001 between Ctl and THA-treated organotypic cultures (bars show means±S.E.M. for n=11-15 slices in each condition) by Student's t test.



Supplemental Table 1

Classification (Lipid Maps)	Compound	Regulation respect Ctl (at least p<0.05 by Student's T test)
Glycerophospholipids	PS(16:0/18:1)	down
	PS(17:0/20:4)	down
	PS(18:2/18:0)/ PS(18:1/18:1)	down
	PS(21:0/22:6)	down
	PE(36:1)/PENMe2(44:1)	down
	PE(41:0)	down
	PE(18:2/18:2)	down
	PE(38:0)/PENMe2(36:0)	down
	PE(38:1)	down
	PE(38:2)/PENMe(36:2)	down
	PE(36:0)	down
	PE(39:0)	down
	PE(44:1)	down
	PE(44:0)	down
	PE(43:0)	down
	PE(O-38:5)	down
	PENMe(32:0)/PE(33:0)	down
	PENMe(16:0/18:1)	down
	PENMe(34:0)/PE (36:0)	down
	PG(17:0/20:4)	down
	PG(18:1/18:1)	down
	PI(16:0/18:1)	down
Sphingolipids	Cer (d18:1/20:0)	down
	Cer(d18:1/22:0)	down
	Cer(d18:1/24:1)	down
	GlcCer (d18:1/25:0)	down
	GlcCer(d18:1/26:0)	down
	GlcCer(d18:1/20:0)	down
	GlcCer(d18:1/22:0)	down
	GlcCer(d18:1/24:0)	down
	GlcCer(d18:1/18:0)	down
	GlcCer(d18:1/24:1)	down
	LacCer (d18:1/20:0)	down
	LacCer(d18:1/24:0)	down
	SM(d18:1/24:0)	down
	SM(d18:1/25:0)	down
	C18 Sulfatide	down
	C20 Sulfatide	down
	C22 Sulfatide	down
	C24 Sulfatide	down
	C22-OH Sulfatide	down
	Ganglioside GM3 (d18:1/24:1)	down
	Trihexosylceramide (d18:1/26:1)	down

Supplemental Table 2. Demographic characteristics of patients studied

Diagnostic	Gender	Age	Post-mortem delay
ALS	F	79	2h 10 min
ALS	F	75	4h
ALS	M	57	4h
ALS	M	64	16h 30 min
ALS	F	76	13 h
ALS	M	76	12h 40m
ALS	M	54	4h 50min
ALS	F	59	14h 15m
ALS	M	71	8h 45m
ALS	F	65	4h 10 min
Control	F	75	6h 10 min
Control	M	76	6h 30 min
Control	M	70	2 h
Control	F	82	3h 5 min
Control	M	76	6h 30min
Control	M	75	3h 25min
Control	M	64	8h 35m
Control	M	68	10h 55 min

4.3 Article 3

Title: “Dietary Lipid Unsaturation Influences Survival and Oxidative Modifications of an Amyotrophic Lateral Sclerosis Model in a Gender-Specific Manner”.

Authors: Cacabelos D, Ayala V, Ramírez-Nuñez O, Granado-Serrano AB, Boada J, Serrano JC, Cabré R, Nadal-Rey G, Bellmunt MJ, Ferrer I, Pamplona R, Portero-Otin M.

Journal: Neuromolecular Medicine, 2014 Jul 1.
Received: 1 January 2014 / Accepted: 20 June 2014.
DOI 10.1007/s12017-014-8317-7

Neuromol Med
DOI 10.1007/s12017-014-8317-7

ORIGINAL PAPER

Dietary Lipid Unsaturation Influences Survival and Oxidative Modifications of an Amyotrophic Lateral Sclerosis Model in a Gender-Specific Manner

Daniel Cacabelos · Victoria Ayala · Omar Ramírez-Nunez · Ana Belen Granado-Serrano · Jordi Boada · Jose C. E. Serrano · Rosanna Cabré · Gisela Nadal-Rey · Maria Josep Bellmunt · Isidro Ferrer · Reinald Pamplona · Manuel Portero-Otin

Received: 1 January 2014 / Accepted: 20 June 2014

Abstract The implication of lipid peroxidation in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) derive from high abundance of peroxidation-prone polyunsaturated fatty acids in central nervous system and its relatively low antioxidant content. In the present work, we evaluated the effect of dietary changes aimed to modify fatty acid tissular composition in survival, disease onset, protein, and DNA oxidative modifications in the hSODG93A transgenic mice, a model of this motor neuron disease. Both survival and clinical evolution is dependent on dietary fatty acid unsaturation and gender, with high unsaturated diet, leading to loss of the disease-sparing effect of feminine gender. This was associated with significant increases in protein carbonyl and glycoxidative modifications as well as non-nuclear 8-oxo-dG, a marker of mitochondrial DNA oxidation. Comparison of these data with γH2AX immunostaining, a marker of DNA damage response, suggests that the highly unsaturated diet-blunted mitochondrial–nuclear free radical dependent crosstalk, since increased 8-oxo-dG was not correlated with increased DNA damage response. Paradoxically, the highly unsaturated diet led to lower peroxidizability but higher anti-inflammatory indexes. To sum up, our results demonstrate that high polyunsaturated fatty acid content in diets may accelerate the disease in this model. Further, these results reinforce the need for adequately defining gender as a relevant factor in ALS models, as well as to use structurally characterized markers for oxidative damage assessment in neurodegeneration.

Keywords Oxidative stress · Polyunsaturated fatty acid · DNA repair response · Gender dimorphism · Lipid peroxidation

Electronic supplementary material The online version of this article (doi:10.1007/s12017-014-8317-7) contains supplementary material, which is available to authorized users.

D. Cacabelos · V. Ayala · O. Ramírez-Nunez · A. B. Granado-Serrano · J. Boada · J. C. E. Serrano · R. Cabré · G. Nadal-Rey · M. J. Bellmunt · R. Pamplona · M. Portero-Otin (✉)
NUTREN-Nutrigenomics, Institut de Recerca Biomèdica de Lleida-Universitat de Lleida, Av. Rovira Roure 80, 25198 Lleida, Spain
e-mail: manuel.portero@mex.udl.cat

I. Ferrer
Institut de Neuropatologia, Hospital Universitari de Bellvitge - IDIBELL, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain

I. Ferrer
CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas), Instituto Carlos III, Spanish Ministry of Health, Madrid, Spain

Introduction

Amyotrophic lateral sclerosis (ALS, OMIM #105400) is a neurodegenerative, motor neuron-specific disease that causes death within 2–5 years after diagnosis. ALS sporadic forms account for most of the cases and despite much effort have been done, their causes remain unclear. However, familial forms of the disease (fALS) provide a valuable tool for the development of animal models to gain insight into disease mechanisms and therapy design, especially at preclinical stages. A breakthrough point in this line was the discovery by Rosen (1993), showing that mutations in Cu/ZnSOD were a primary cause for ALS. Since then, multiple mutations on Cu/ZnSOD (more than

Published online: 01 July 2014

100, reviewed in Gaudette et al. 2000) have been identified, thus leading to develop experimental models of fALS (Gurney et al. 1994; Oeda et al. 2001). In particular, transgenic mice bearing multiple copies of one of the fALS-linked mutations, a glycine for alanine substitution (G93A), has become the most used model to study the pathogenesis of ALS. Nevertheless, it is known that even a single mutation harbours an increasing disease complexity pointing novel roles of specific genes and proteins as disease modifiers (reviewed in Al-Chalabi et al. 2012).

Lipids account approximately for 50–60 % of the brain dry weight, from which polyunsaturated accounts around the 35 % (Lauritzen et al. 2001) being mostly long-chain polyunsaturated fatty acids (PUFAs). The majority of such lipids present in the central nervous system are structural, belonging to phospolipid bilayers in biomembranes. But apart from structural conformation in phospholipids and source of energy, lipids play key roles in different vital complex processes such as signalling. They act not only as direct messengers by signalling in paracrine communication but also in helping to correctly position cellular receptors (e.g. lipid rafts Simons and Toomre 2000) and also binding responsive elements to regulate DNA expression (e.g. PPARs; reviewed in Willson et al. 2000). They could also modulate reactive oxygen species (ROS) production and interact with those by generating reactive lipid species, which can be signalling intermediates (Niki 2012). Globally, PUFAs are essential for correct neuronal function. These data are behind the beneficial effects of an adequate intake of the n-3 series PUFAs, especially for DHA (C22:6, n-3). Accordingly, its deficiency leads to visual, cognitive and/or behavioural abnormalities in several animal models (Niu et al. 2004) as well as in humans (Deckelbaum and Torrejon 2012). DHA signalling is supposed to act: (1) through structural characteristics in lipid bilayers; (2) by DNA interaction through PPAR related genes enhancement (Willson et al. 2000); (3) also through its role as a source of signalling intermediates, since for instance, DHA is the metabolic source of docosanoids and resolvins, contrasted inflammatory resolution mediators (Serhan 2005); (4) and finally by oxidative signalling, since DHA oxidative derivatives also interact with PPARs family transcription factors (Itoh and Yamamoto 2008). Noteworthy, PUFA structure (n-3 vs n-6 families) is relevant, since they stimulate different genes (Barceló-Coblijn et al. 2003) as they share (and therefore compete) biosynthetic pathways for fatty acid itself, but also for its derivatives. This adds further complexity to the interaction of PUFA with central nervous system function.

Asides from these physiological roles, one potentially detrimental effect of PUFAs arises from their high susceptibility to oxidation, which is believed to play a major role in ALS pathogeny (as hypothesized 20 years ago; (Bowling et al. 1993). An excess of ROS could interact with any of the surrounding biomolecules within the cell, ranging from DNA and proteins to lipids or carbohydrates, amplifying ROS damage. Particularly, after oxidative protein damage, several oxidatively modified amino acids are generated, which can be used as specific markers of distinct types of oxidative stress: (1) for direct, metal-catalysed, carbonylation, glutamic and aminoadipic semi-aldehydes (GSA and AASA); (2) for glycoxidation, N$\sum$-carboxymethyl-lysine (CML) and N$\sum$-carboxyethyl-lysine (CEL) and finally, (2) for lipoxidation malondialdehyde-lysine (MDAL) (Portero-Otin et al. 2003; Thorpe and Baynes 2003). Similarly, ROS could modify DNA, by generating specific residues, such as 8-oxo-deoxyguanosine, between other (Weimann et al. 2001). In this line, dietary factors could modify 8-oxo-dG accrual (Zhu et al. 2000). In addition, it has not been studied whether PUFA intake could modify 8-oxo-DG accumulation in a neurodegeneration model, though positive role for dietary fish oil and vegetable PUFA was recently demonstrated related to a reduction of 8-oxo-dG accumulation both in colonic samples for a model of Crohn disease (Bancroft et al. 2003) or patients suffering type 2 diabetes (Müllner et al. 2013). Nonetheless, besides cytotoxic effects, a signalling role for ROS is known. Recent data show a crosstalk between mitochondrial-free radical production and nuclear DNA damage response (DDR), mediated by γH2Ax (Passos et al. 2010), involved in cell and tissue homeostasis.

In the present work, we hypothesized that if extensive lipid composition and its susceptibility to peroxidation could be modified, ALS progression could be partially modulated. In this sense, regarding dietary intervention, previous works from our group showed that the fatty acid profiles of several organs, including brain, can be modified as a result of dietary modification (Pamplona et al. 2004), leading to significant effects in the concentration of protein oxidative markers. For this reason, in this study, diets with different degree of unsaturation were designed to evaluate their effects in the survival of an ALS model. Several diet-related outcomes (weight gain and lipid composition) as well as clinical score, coordination/strength (stride length) and distinct markers for oxidative damage to proteins along the disease progression were also measured. We also analysed the potential effect of PUFAs in the crosstalk between mitochondrial ROS production (assessed by 8-oxo-dG immunostaining) and DDR in lumbar spinal cords, in order to ascertain the potential role of lipid peroxidation and dietary lipid composition in ALS pathogenesis.

Methods

Animals and Diets

A colony of the strain B6SJL-Tg (SOD1-G93A)1Gur/J (JAX catalogue stock number 002726; from now on hSODG93A) was purchased at The Jackson Laboratories (Bar Harbor, MN, USA) and maintained in the B6SJL background, by male founder crossing with B6SJLF1/J. All mice were genotyped by using PCR-according procedures stated by the providers. For tail DNA extraction a kit for tissue DNA extraction was used (XNAT, Sigma-Aldrich, Saint Louis, MO, USA). PCR amplification was performed in a 2740 Termal Cycler v2.08 (Applied Biosystems, Carlsbad, CA, USA) as follows: Pre-denaturation for 5 min at 95 °C, denaturation for 1 min at 94 °C, annealing for 45 s at 50 °C, elongation for 4 min at 72 °C and finally, after 35 cycles, elongation for another 7 min at 72 °C. The amplification products were stored at 4 °C before gel loading and electrophoresis, which was done in 1 % agarose gels and stained with Sybr safe (Molecular Probes, Carlsbad, CA, USA) following manufacture instructions. Products were electrophoresed for 30 min under constant 100 V and images were taken under (302 or 365 nm) UV light lamp from Alpha Innotech (Santa Clara, CA, USA) with a software acquisition Alpha Digidoc RT2 (Santa Clara, CA, USA).

After genotyping and weaning, animals were placed at 12:12 h dark/light cycle, at 22 ± 2 °C temperature, 50 % ± 10 relative humidity, in individual cages, using the experimental diets (at 21 days). Whenever was possible by litter size of transgenic animals, gender and litter-matched animals were randomly distributed across the three diets. Animals were weighted weekly and daily for those under stride length analysis.

All diets used for experiments were purchased at Harlan Tekland (Madison, WI, USA) and stored at 4 °C until use. Diet composition was designed to be isocaloric and essential fatty acids requirements were completed (Table S3). We randomly assigned individual mice to either high unsaturated diet (Harlan reference number TD06154) or low unsaturated diet (Harlan reference number TD06155), whose compositions were as previously described (Pamplona et al. 2004). Those diets have extreme differences for peroxidizability indexes (fivefold), content of n-3 fatty acids (sixfold), content of arachidonic and docosahexanoic acids (100-fold) and double-bond indexes (threefold), with control diet values in-between. Importantly, low unsaturated diet does not lead to essential fatty acid deficiency, as shown by content of the n-3 and n-6 precursors linolenic and linoleic acids. Food pellets were weighted (for intake calculations) and removed weekly. As a control group, 40 animals (20 male, 20 female) were maintained and fed with a regular rodent chow (AIN-93G, Harlan reference number TD94045) to ascertain survival homogeneity and standard growing curves. All experimental procedures were approved by the Institutional Animal Care Committee of Institut de Recerca Biomèdica de Lleida and were conformed to the Directive 2010/63/EU of the European Parliament.

Disability Score Assessment

Disability scores for both hind legs were assessed weekly for each mouse from 60 days of age to end point. The neurological score (Amyotrophic Lateral Sclerosis Therapy Development Institute and Jackson Laboratories) employed a scale where 0 points were given when full extension of hind legs away from lateral midline when mouse was suspended by its tail; one point when there was a collapse of leg extension towards midline or trembling of hind legs during this tail suspension; two points when toes

curled under at least twice during walking of 12 inch., or any part of foot was dragging along cage bottom/table; three points when it was present a rigid paralysis or minimal joint movement, foot not being used for forward motion and four points when mouse was not able to right itself within 30 s from either side (that was the endpoint of the measurement). Disease onset was considered the age at which an animal scored 2 in the above mentioned test. Observators for score were blinded to both diet and data analyses.

Stride Length Analysis

Stride length measurement was adopted from previously reported methods (Chiu et al. 1995). All animals were trained to walk freely along a U-shaped flat narrow corridor (5 cm wide, 70 cm long) three times per week before data collection (average age 70 days). Once trained, hindlimbs were stained with non-toxic poster children paints and their tracks were imprinted on paper (labelled with only with animal number and date) lining the floor of the corridor. Only continuous runs were measured among recorded and a minimum of 5–7 strides were measured per animal per day (three non-consecutive day measures were taken along week) to obtain daily mean. Since animals tended to walk faster when released and went slower at the end of the corridor, those areas were discarded for the stride measures, which were taken manually and were defined as the distance between successive right-to-right and left-to-left footprints. As the disease progresses, the painted footprints became increasingly smeared on the paper, which correlated with previous results (Chiu et al. 1995). Loss of stride length was quantified by determining the age at which

shortening of the stride length was lower than 10 % for two consecutive measures. Measurement of stride length was determined in a blinded manner since only number of the randomized animal and date identified the recorded tracks.

Analytical Measurements

Animals, after being fasted overnight, were anesthetised with 2.5 % isoflurane in air and finally sacrificed. Spinal cords and brain were rapidly excised, frozen in liquid N_2 and stored at −80 °C until further analyses. Samples were homogenized on ice in a buffer containing 180 mM KCl, 5 mM MOPS, 2 mM EDTA, 1 mM diethylenetriaminepentaacetic acid, and 1 μM of freshly prepared butylated hydroxyl toluene at pH 7.3 using a homogenizer device (T10 basic UltraTurraX, IKA, Staufen, Germany). Protein concentrations were measured by the Bradford method.

Measurement of Protein Oxidative (GSA, AASA), Glycoxidative (CML, CEL) and Lipoxidative (MDAL) Modifications

Gas chromatography–mass spectrometry (GC/MS) measurements of GSA, AASA, CEL, CML, and MDAL concentrations in total proteins from mice lumbar spinal cord homogenates were measured as routinely performed in our laboratory (Pamplona et al. 2005).Samples containing 0.5 mg of protein were delipidated using chloroform/methanol (2:1, v/v) (see below), and proteins were precipitated by adding 10 % trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM NaBH4 (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then precipitated by adding 1 ml of 20 % trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: [2H8]lysine (d8-Lys; CDN Isotopes); [2H4]CML (d4-CML), [2H4]CEL (d4-CEL), [2H8]MDAL (d8-MDAL), [2H5]5-hydroxy-2-aminovaleric acid (for GSA quantitation) and [2H4]6-hydroxy-2-aminocaproic acid (for AASA quantitation). The samples were hydrolyzed at 155 °C for 30 min in 1 ml of 6 N HCl, and then dried in vacuo. The *N*,*O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described (Pamplona et al. 2005). Then, GC/MS analyses were carried out on a Agilent model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Agilent model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at 275 °C; the temperature programme was 5 min at 110 °C, then 2 °C/min to 150 °C, then 5 °C/min to 240 °C, then 25 °C/min to 300 °C, and finally hold at 300 °C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and d8-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d5–5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and d4–6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and d4-CML, *m/z* 392 and 396, respectively; CEL and d4-CEL, *m/z* 379 and 383, respectively; and MDAL and d8-MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio micromolar of GSA, AASA, CML, CEL, or MDAL/mol of lysine.

Fatty Acid Compositional Analyses

Fatty acid analysis in tissue samples and diets was performed as previously described (Pamplona et al. 2005). Total lipids from homogenates were extracted with chloroform/methanol (2:1, v/v) (3 times) in the presence of 0.01 % butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2 ml of 5 % methanolic HCl for 90 min at 75 °C. The resulting fatty acid methyl esters were extracted by adding 2 ml of *n*-pentane and 1 ml of saturated NaCl solution. The *n*-pentane phase was separated, evaporated under nitrogen, re-dissolved in 80 μl of carbon disulphide, and 2 μl was used for GC analysis. Separation was performed by a DBWAX capillary column (30 m × 0.25 mm × 0.20 μm) in an Agilent GC System 7890A with a Series Injector 7683B and a FID detector (Agilent Technologies, Barcelona, Spain). The injection port was maintained at 220 °C, and the detector at 250 °C; the temperature programme was 2 min at 100 °C, then 10 °C/min to 200 °C, then 5 °C/min to 240 °C and finally hold at 240 °C for 10 min. Identification of fatty acid methyl esters was made by comparison with authentic standards (Larodan Fine Chemicals, Malmö, Sweden). Results are expressed as mol%.

From particular fatty acids, the following indexes were calculated as previously described (Pamplona et al. 2005). Saturated fatty acids (SFA) = Σ % of saturated fatty acids; unsaturated fatty acids (UFA) = Σ % unsaturated fatty acids; monounsaturated fatty acids (MUFA) = Σ % of monoenoic fatty acids; polyunsaturated fatty acids series n-3 (PUFAn-3) = Σ % of polyunsaturated fatty acids n-3

series; polyunsaturated fatty acids series n-6 (PUFAn-6) = Σ % of polyunsaturated fatty acids n-6 series; average chain length (ACL) = [(Σ % Total 14 × 14) + ⋯ + (Σ % total$n$ × n)]/100 (n = carbon atom number); peroxidizability index (PI) = [(Σ mol% monoenoic × 0.025) + (Σ mol% dienoic × 1) + (Σ mol% trienoic × 2) + (Σ mol% tetraenoic × 4) + (Σ mol% pentaenoic × 6) + (Σ mol% hexaenoic × 8)]; Double-bond index (DBI) = [(Σ mol% monoenoic) + (2 × Σ mol% dienoic) + (3 × Σ mol% trienoic) + (4 × Σ % mol tetraenoic) + (5 × Σ mol% pentaenoic) + (6 × Σ mol% hexaenoic); Antinflamatory index (AI) = (20:3n-6) + (20:5n-3) + (22:5n-3) +(22:6n-3)/(20:4n-6); ratio PUFAs = (PUFAn-3)/(PUFAn-6).

Immunohistochemistry

Selected animals were anaesthetized by intraperitoneal injection (pentobarbital and ketamine, 20 and 60 mg/kg respectively on PBS). Animals were therefore exsanguinated by perfusion, firstly with saline solution (4 °C) and thereafter with ice cold 0.4 % paraformaldeyde (Sigma-Aldrich, Saint Louis, MO, USA) solution (freshly prepared with pH 7.4 phosphate buffer). After tissue collection (spinal cord) samples were immersed for 24 h in the same fixative to assure preservation. Samples were thereafter immersed in 30 % sucrose, (made in pH 7.4 phosphate buffer) for 48 h to achieve cryopreservation. Tissue was encased in a cubic recipient (Peel-A-Way Disposable Embeding Molds-S-22, Polysciences Inc., Warrington, PA, USA), labelled and embedded in tissue freezing medium (Triangle Biomedical Sciences Inc., Newcastle, UK) for easily cutting and better preservation and finally frozen (−80 °C). For immunofluorescence, 16 µm wide sections of lumbar spinal cord were cut and carefully seeded along gelatine-coated slide. Selected sections were permeabilized with 0.4 % Triton ×-100 PBS. After blocking with 5 % normal horse serum in 0.4 % Triton ×-100 PBS for 2 h at room temperature (RT), the tissue was incubated for 24 h at 4 °C with the primary antibodies. Next day, the slices were washed with PBS three times for 10 min at RT. Then, the slices were incubated for 1 h at RT in darkness with corresponding secondary antibodies. Sections were finally counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 ug/ml) in PBS for 5 min at RT and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). In selected sections primary antibody was omitted to assure labelling specificity. Mounted slices were examined under a Fluoview 500 Olympus confocal laser scanning microscope (Olympus, Hamburg, Germany). For quantification of anti-ubiquitin, anti-syntaxin3 and anti-γH2Ax immunoreactivity images were analysed with the Image J software (http://rsbweb.nih.gov/ij/).

Statistical Analyses

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL, USA) or the Prism software (GraphPad Software, San Diego, CA, USA). Differences between groups were analysed by the Student's T tests or ANOVA with post hoc analyses, after normality of variable distribution was ensured by Kolmogorov–Smirnov test. Correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

Survival and Clinical Evolution of ALS G93A Mice is Modified by Dietary Fatty Acid Unsaturation in a Gender-Specific Fashion

When compared with a regular rodent chow, low unsaturation in diet diminishes gender-related influence on median survival (Fig. 1a and Supplemental Figure 1). Survival analyses taking diet into account show that under control diet females survived longer (Fig. 1b), a fact maintained under low unsaturated diets (Fig. 1c), but not under high unsaturated diets (Fig. 1d). For males, results (Supplemental Figure 1) show that survival was highest in animals under low unsaturated diets ($p < 0.05$ for Log rank test), while as differences in females were not significant.

Phenotypic analyses for disease onset (as assessed both by a clinical score and stride length loss) revealed a similar trend: global analyses show that dietary fats did not affect disease onset (Fig. 1e). In contrast with survival, where feminine gender showed an evident protective effect under control diets, these diets did not show differences in disease onset (Fig. 1f). In contrast, low unsaturated diet delayed significantly disease onset in females (Fig. 1g), in contrast with highly unsaturated diet (Fig. 1h). If performed separately, no major differences were evidenced neither in males nor in females (Supplemental Figure 1). When evaluating loss of stride length preservation (defined by a consecutive decay of at least 10 % in stride length), we found that males under highly unsaturated diet reached this stage faster than those under low unsaturated or control ones (Fig. 1i). However, this phenomenon was not present in females (Fig. 1j). Rather, it appeared that both low and high unsaturated diets enhanced stride length preservation significantly in females, in comparison with control diets.

Food intake calculations were similar between dietary groups and comparable with a control diets with no major statistical differences along the experiment (data not shown). Mice gained weight as expected (Fig. 1k, l), reaching a maximum value at 100 days for males,

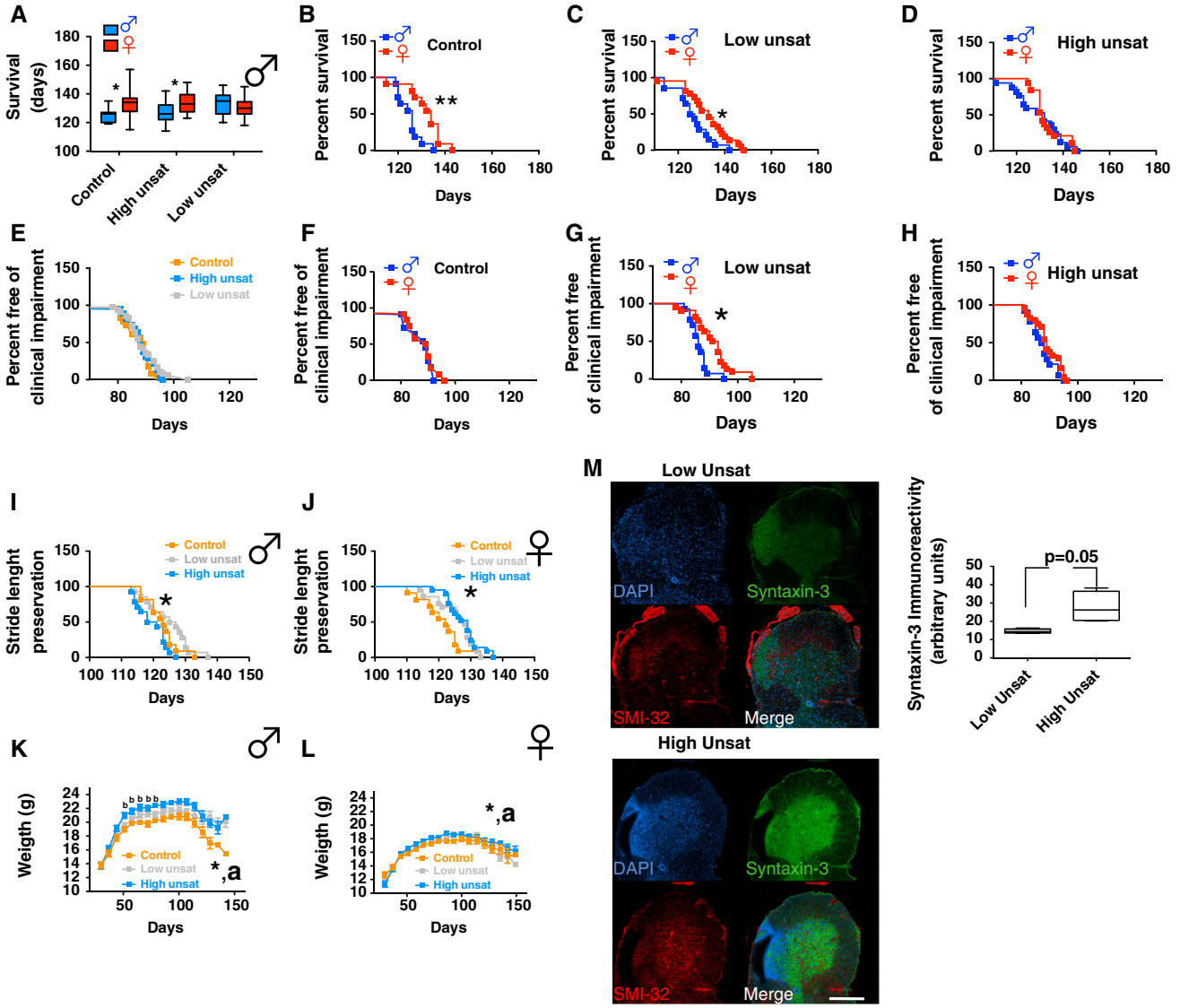


Fig. 1 Dietary fatty acid unsaturation induces a gender-specific effect in survival in the hSODG93A ALS mice model. **a** Low unsaturated fats (see text for description) maintained gender-related differences in median survival (seen under control conditions), while high unsaturated diet diminished this gender-induced difference. **b** Further, survival and gender-specific analyses demonstrated that under control (**b**) or low unsaturated diet (**c**), females lived longer than males, in contrast with the high unsaturation diet (**d**). Similarly, disease onset, as defined by changes in clinical score in the "Methods" section, was not affected by dietary fat unsaturation, as shown by global analyses (**e**). Disease onset in males was not affected by diets (**f**), in contrast with females (**g**) with significant differences ($p < 0.05$ by or that with high unsaturation (**h**). In line with this, disease progression proceeded faster in males under high unsaturation (**i**), while females were protected against this effect (**j**). Effects of dietary unsaturation in weight gain were similar for both genders (**k**, **l**). Males reached a maximum (23.04 ± 0.370 g, $n = 22$; 21.85 ± 0.559 g, $n = 22$; 20.83 ± 0.8 g, $n = 12$ for high, low and control unsaturation diets, respectively) at 100 days, meanwhile females did it at 100 days for ones under high unsaturation and control (18.69 ± 0.327 g, $n = 22$; 17.9 ± 0.62 g, $n = 13$) and at 97 days those on low unsaturated diet (18.70 ± 0.270 g, $n = 28$). **m** Confocal microscopy images in lumbar spinal cord demonstrating increased biological availability of dietary unsaturated fatty acids in male animals (endpoint: 130 ± 7 days) as suggested by increased syntaxin-3 immunostaining, quantified in the whiskers plot in the *left panel*. In **a** *asterisk* indicates statistical differences between males and females survival by Bonferroni multiple test post hoc analyses after 2-way ANOVA. For **b**–**j** significant differences between diets and/or genders were evaluated by Kaplan–Meier survival statistic (*$p < 0.05$; **$p < 0.001$). For **k** and **l** * and [a]significant differences by 2-way ANOVA of diet and age effects, respectively, while as [b]significant increases in weight at 50–80 days in males under high unsaturated diets by Bonferroni multiple test post hoc analyses after 2-way ANOVA. A total of 30 slices ($n = 3$ animals per treatment) were analysed for quantification in **m**, with differences induced by diet being evaluated by Student's *T* test. *Bars* in **m** are 500 μm

Table 1 Fatty acid composition (% of abundance) and derived indexes for whole brain homogenate (60 days)

	Male hSODG93A			Female hSODG93A		
	High unsat	Low unsat	*p* value	High unsat	Low unsat	*p* value
14:0	0.317 ± 0.092	0.542 ± 0.121	0.190	0.260 ± 0.027	0.420 ± 0.123	0.137
16:0	24.47 ± 0.592	16.32 ± 0.273	0.001	24.44 ± 0.239	13.05 ± 0.233	0.001
16:1n-7	0.593 ± 0. 038	0.535 ± 0.099	0.604	0.556 ± 0. 046	0.203 ± 0.114	0.024
18:0	22.67 ± 0.198	24.26 ± 1.177	0.230	23.21 ± 1.413	20.73 ± 1.701	0.352
18:1n-9	21.95 ± 0.848	22.00 ± 0.856	0.967	20.74 ± 0.521	18.82 ± 1.345	0.167
18:2n-6	0.690 ± 0.072	0.725 ± 0.053	0.732	0.621 ± 0.107	0.554 ± 0.077	0.698
18:3n-3	0.089 ± 0.010	0.035 ± 0.019	0.031	0.066 ± 0.085	0.446 ± 0.258	0.077
18:4n-6	0.905 ± 0.325	2.23 ± 0.130	0.009	0.685 ± 0.133	1.944 ± 0.385	0.015
20:0	0.365 ± 0.090	0.555 ± 0.048	0.100	0.330 ± 0.007	0.383 ± 0.008	0.384
20:1 n-9	1.142 ± 0.113	1.516 ± 0.140	0.084	1.203 ± 0.062	1.426 ± 0.520	0.538
20:2n-6	0.430 ± 0.145	0.726 ± 0.134	0.185	0.241 ± 0.015	0.948 ± 0.021	0.092
20:3n-6	0.094 ± 0.012	0.072 ± 0.018	0.175	0.081 ± 0.006	0.590 ± 0.329	0.066
20:4n-6	8.44 ± 0.796	10.09 ± 0.869	0.212	8.219 ± 0.255	8.965 ± 0.153	0.132
20:5n-3	0.276 ± 0.114	0.161 ± 0.073	0.440	0.314 ± 0.008	0.868 ± 0.427	0.103
22:0	1.522 ± 0.293	1.935 ± 0.197	0.289	0.478 ± 0.083	1.472 ± 0.122	0.002
22:4n-6	2.065 ± 0.339	2.976 ± 0.425	0.144	1.557 ± 0.065	2.567 ± 0.025	0.001
22:5n-6	0.365 ± 0.071	0.925 ± 0.186	0.030	0.363 ± 0.011	0.690 ± 0.011	0.001
22:5n-3	0.420 ± 0.018	0.680 ± 0.190	0.222	0.411 ± 0.005	1.177 ± 0.275	0.011
24:0	0.075 ± 0.015	0.152 ± 0.050	0.164	0.039 ± 0.006	0.769 ± 0.073	0.001
22:6n-3	12.741 ± 1.088	12.33 ± 1.807	0.654	15.77 ± 0.530	13.32 ± 0.619	0.039
24:5n-3	0.150 ± 0.049	0.485 ± 0.180	0.125	0.064 ± 0.016	0.928 ± 0.359	0.018
24:6n-3	0.290 ± 0.022	0.430 ± 0.115	0.279	0.325 ± 0.053	0.711 ± 0.175	0.045
SFA	49.46 ± 0.736	43.83 ± 1.402	0.012	48.77 ± 1.210	36.84 ± 1.625	0.004
UFA	50.06 ± 0.736	55.93 ± 1.345	0.013	51.22 ± 1.201	54.15 ± 0.128	0.130
MUFA	23.68 ± 0.760	24.07 ± 1.000	0.779	22.50 ± 0.576	20.45 ± 0.943	0.121
PUFA	26.87 ± 1.344	31.86 ± 2.238	0.105	28.72 ± 0.798	33.20 ± 1.071	0.016
PUFAn-3	13.98 ± 1.086	14.13 ± 1.049	0.917	16.95 ± 0.530	17.44 ± 0.901	0.637
PUFAn-6	13.10 ± 1.421	17.73 ± 1.221	0.045	11.77 ± 0.295	16.75 ± 0.169	0.001
ACL	18.43 ± 0.048	18.68 ± 0.798	0.209	18.46 ± 0.027	17.37 ± 0.247	0.005
DBI	156.97 ± 8.64	176.21 ± 10.10	0.133	168.88 ± 4.67	183.96 ± 2.85	0.105
PI	159.75 ± 11.6	179.91 ± 13.18	0.237	179.27 ± 4.40	192.71 ± 3.33	0.185
R/PUFAs	1.120 ± 0.155	0.795 ± 0.020	0.084	1.440 ± 0.023	1.071 ± 0.043	0.003
AI	163.04 ± 18.5	131.73 ± 9.19	0.192	200.74 ± 2.88	171.38 ± 2.18	0.001

p value reflects statistical significance between diets by Student's *T* test

irrespectively of diet; meanwhile, females reached a maximum at 100 days for those under high unsaturated and control diets and at 97 days on low unsaturated one. Males under high unsaturated diet weighted significantly more than those of control during preclinical stages (from day 50 to day 75) (Fig. 1k, l).

To confirm fatty acid bioavailability, we measured both lipid composition and surrogate markers of n-3 availability. Since the profile of fatty acids in control-fed animals resemble strongly those present in low unsaturation diets (Supplemental Table 1 and 2), we focused to differences between high and low unsaturation fed animals. Thus, lipid profiles in brain (Table 1) and spinal cord (Tables 2, 3) revealed that in this latter, the concentration of arachidonic acid (AA, 22:4n-6), under low unsaturation, was higher than those found in high unsaturated ones. In contrast, a major fatty acid (18:0) responsible for approximately 40 % of the total fatty acids measured was not affected by the diet.

Similarly, DHA levels were not severely altered. Anti-inflammatory index (AI) was always higher for animals under highly unsaturated diet independently of tissue or gender, including those from control-fed animals (Tables 1, 2, 3, Supplemental Tables 1 and 2). Double-bound (DBI) and peroxidizability (PI) indexes both reflecting susceptibility for lipid peroxidation were found

Table 2 Fatty acid composition (% of abundance) and derived indexes in spinal cord (lumbar region) for male hSODG93A

	60 days			90 days			120 days		
	High unsat	Low unsat	*p* value	High unsat	Low unsat	*p* value	High unsat	Low unsat	*p* value
14:0	0.830 ± 0.085	0.824 ± 0.035	0.954	0.171 ± 0.014	0.454 ± 0.064	0.006	0.202 ± 0.021	0.696 ± 0.10	0.003
16:0	21.411 ± 0.373	19.934 ± 0.403	0.022	22.33 ± 2.372	26.57 ± 0.391	0.087	22.65 ± 1.171	20.54 ± 0.624	0.130
16:1n-7	0.838 ± 0.124	0.842 ± 0.042	0.974	0.558 ± 0.076	0.715 ± 0.049	0.114	0.806 ± 0.195	1.011 ± 0.075	0.321
18:0	15.528 ± 0.504	16.160 ± 0.388	0.335	15.86 ± 1.056	16.59 ± 1.681	0.742	16.169 ± 0.885	15.61 ± 0.611	0.612
18:1n-9	37.065 ± 0.706	35.94 ± 0.480	0.204	38.76 ± 1.572	33.03 ± 1.865	0.058	38.665 ± 0.581	36.575 ± 0.401	0.014
18:2n-6	1.529 ± 0.244	1.429 ± 0.094	0.692	1.002 ± 0.302	0.978 ± 0.040	0.931	0.903 ± 0.031	1.296 ± 0.11	0.013
18:3n-3	0.198 ± 0.051	0.177 ± 0.027	0.712	0.101 ± 0.013	0.164 ± 0.033	0.141	0.137 ± 0.028	0.449 ± 0.063	0.003
18:4n-6	0.395 ± 0.067	0.456 ± 0.061	0.519	0.110 ± 0.050	0.205 ± 0.055	0.262	0.166 ± 0.046	0.521 ± 0.104	0.018
20:0	1.608 ± 0.107	1.69 ± 0.092	0.569	1.467 ± 0.042	1.528 ± 0.082	0.564	1.273 ± 0.125	1.198 ± 0.094	0.637
20:2n-6	0.683 ± 0.041	1.092 ± 0.076	0.001	0.447 ± 0.065	0.721 ± 0.045	0.010	0.557 ± 0.036	0.720 ± 0.107	0.219
20:3n-6	2.007 ± 0.371	2.269 ± 0.216	0.543	1.345 ± 0.189	1.199 ± 0.180	0.598	1.671 ± 0.096	1.968 ± 0.222	0.283
20:4n-6	4.394 ± 0.139	5.917 ± 0.127	0.001	4.738 ± 0.289	5.296 ± 0.126	0.098	4.419 ± 0.055	5.637 ± 0.327	0.009
20:5n-3	0.271 ± 0.021	0.094 ± 0.013	0.001	0.048 ± 0.013	0.094 ± 0.014	0.057	0.493 ± 0.062	0.622 ± 0.113	0.317
22:0	0.264 ± 0.070	0.151 ± 0.023	0.132	0.114 ± 0.018	0.389 ± 0.067	0.010	0.321 ± 0.034	0.635 ± 0.052	0.001
22:4n-6	1.616 ± 0.101	2.292 ± 0.042	0.001	1.395 ± 0.0884	2.260 ± 0.126	0.001	1.126 ± 0.050	2.234 ± 0.073	0.001
22:5n-6	0.415 ± 0.075	0.569 ± 0.044	0.094	0.249 ± 0.089	0.287 ± 0.041	0.694	0.273 ± 0.033	0.691 ± 0.112	0.010
22:5n-3	0.543 ± 0.097	0.412 ± 0.064	0.275	0.998 ± 0.121	0.289 ± 0.042	0.001	0.948 ± 0.062	0.487 ± 0.033	0.001
24:0	0.117 ± 0.011	0.209 ± 0.026	0.007	0.630 ± 0.017	0.055 ± 0.027	0.001	0.178 ± 0.024	0.039 ± 0.016	0.001
22:6n-3	7.930 ± 0.482	6.954 ± 0.233	0.083	7.587 ± 0.356	6.620 ± 0.278	0.066	6.362 ± 0.117	6.269 ± 0.173	0.683
24:5n-3	0.053 ± 0.004	0.054 ± 0.004	0.887	0.095 ± 0.047	0.098 ± 0.014	0.943	0.158 ± 0.027	0.377 ± 0.108	0.058
24:6n-3	2.300 ± 0.172	2.522 ± 0.101	0.275	2.151 ± 0.065	2.440 ± 0.134	0.120	2.542 ± 0.160	2.400 ± 0.134	0.512
SFA	39.756 ± 0.702	38.972 ± 0.636	0.425	40.58 ± 1.970	45.59 ± 1.754	0.099	40.796 ± 0.781	38.73 ± 0.458	0.042
UFA	60.243 ± 0.702	61.027 ± 0.636	0.421	59.59 ± 2.123	54.40 ± 1.754	0.098	59.203 ± 0.781	61.262 ± 0.458	0.051
MUFA	37.90 ± 0.674	36.78 ± 0.506	0.204	39.32 ± 1.49	33.75 ± 1.85	0.059	39.471 ± 0.659	37.586 ± 0.337	0.025
PUFA	22.33 ± 0.261	24.24 ± 0.394	0.003	20.27 ± 0.958	20.65 ± 0.274	0.512	19.75 ± 0.212	23.676 ± 0.71	0.001
PUFAn-3	11.29 ± 0.164	10.21 ± 0.084	0.123	11.62 ± 0.318	9.28 ± 0.109	0.005	10.83 ± 0.259	10.59 ± 0.169	0.413
PUFAn-6	11.04 ± 0.661	14.02 ± 0.512	0.004	9.289 ± 0.833	10.94 ± 0.144	0.063	9.116 ± 0.209	13.06 ± 0.632	0.001
ACL	18.27 ± 0.030	18.35 ± 0.010	0.029	18.31 ± 0.085	18.16 ± 0.008	0.081	18.22 ± 0.024	18.31 ± 0.017	0.019
DBI	142.37 ± 1.92	146.35 ± 1.17	0.095	136.93 ± 4.35	130.49 ± 1.69	0.172	133.32 ± 1.50	145.36 ± 2.08	0.001
PI	122.82 ± 3.86	125.64 ± 1.64	0.482	116.51 ± 3.74	113.40 ± 2.06	0.538	111.32 ± 1.60	123.56 ± 2.06	0.002
R/PUFAs	1.067 ± 0.141	0.735 ± 0.041	0.038	1.270 ± 0.131	0.877 ± 0.080	0.006	1.190 ± 0.022	0.872 ± 0.009	0.002
AI	245.35 ± 7.41	164.6 ± 2.91	0.001	211.54 ± 6.26	155.11 ± 3.12	0.001	214.50 ± 3.54	169.4 ± 13.34	0.016

p value reflects statistical significance between diets by Student's *T* test

Table 3 Fatty acid composition (% of abundance) and derived indexes in spinal cord (lumbar region) for female hSODG93A

	60 days			90 days			120 days		
	High unsat	Low unsat	*p* value	High unsat	Low unsat	*p* value	High unsat	Low unsat	*p* value
14:0	0.793 ± 0.068	0.761 ± 0.025	0.715	0.204 ± 0.012	0.364 ± 0.045	0.010	0.325 ± 0.040	0.246 ± 0.018	0.114
16:0	21.36 ± 0.514	19.82 ± 0.505	0.066	20.30 ± 0.706	24.96 ± 0.691	0.002	21.616 ± 0.462	21.730 ± 0.199	0.827
16:1n-7	0.904 ± 0.100	0.749 ± 0.071	0.278	0.583 ± 0.024	0.703 ± 0.043	0.043	0.920 ± 0.059	0.869 ± 0.037	0.488
18:0	15.90 ± 0.473	16.44 ± 0.174	0.379	18.960 ± 0.327	16.969 ± 0.273	0.002	16.476 ± 0.354	17.499 ± 0.287	0.055
18:1n-9	36.30 ± 0.485	36.89 ± 0.455	0.413	39.45 ± 0.384	34.03 ± 0.725	0.001	39.116 ± 0.555	37.338 ± 0.325	0.025
18:2n-6	1.401 ± 0.057	1.415 ± 0.048	0.862	0.817 ± 0.005	0.870 ± 0.033	0.427	0.816 ± 0.110	0.850 ± 0.037	0.783
18:3n-3	0.162 ± 0.022	0.219 ± 0.012	0.078	0.099 ± 0.009	0.104 ± 0.034	0.967	0.156 ± 0.013	0.100 ± 0.011	0.014
18:4n-6	0.461 ± 0.069	0.481 ± 0.042	0.830	0.129 ± 0.009	0.149 ± 0.035	0.590	0.189 ± 0.021	0.133 ± 0.009	0.041
20:0	1.581 ± 0.076	1.861 ± 0.089	0.039	1.329 ± 0.058	1.246 ± 0.111	0.529	1.115 ± 0.109	0.967 ± 0.100	0.348
20:2n-6	0.672 ± 0.057	0.927 ± 0.041	0.008	0.464 ± 0.014	0.673 ± 0.069	0.019	0.492 ± 0.025	0.435 ± 0.033	0.211
20:3n-6	2.193 ± 0.259	1.704 ± 0.130	0.169	1.183 ± 0.119	1.22 ± 0.128	0.810	1.537 ± 0.142	1.611 ± 0.067	0.655
20:4n-6	4.434 ± 0.200	5.254 ± 0.098	0.009	4.469 ± 0.103	5.508 ± 0.047	0.001	4.324 ± 0.155	6.366 ± 0.171	0.001
20:5n-3	0.154 ± 0.016	0.076 ± 0.008	0.005	0.096 ± 0.013	0.214 ± 0.033	0.011	0.552 ± 0.067	0.219 ± 0.011	0.001
22:0	0.110 ± 0.020	0.141 ± 0.042	0.488	0.202 ± 0.015	0.230 ± 0.034	0.485	0.269 ± 0.033	0.102 ± 0.004	0.001
22:4n-6	1.765 ± 0.086	2.635 ± 0.179	0.001	1.232 ± 0.060	2.636 ± 0.164	0.001	1.192 ± 0.089	2.100 ± 0.085	0.001
22:5n-6	0.557 ± 0.095	0.564 ± 0.044	0.960	0.214 ± 0.010	0.422 ± 0.090	0.053	0.297 ± 0.018	0.328 ± 0.026	0.365
22:5n-3	0.462 ± 0.078	0.440 ± 0.039	0.837	0.863 ± 0.012	0.269 ± 0.024	0.001	0.886 ± 0.030	0.522 ± 0.034	0.001
24:0	0.199 ± 0.021	0.237 ± 0.059	0.516	0.535 ± 0.024	0.025 ± 0.003	0.001	0.150 ± 0.036	0.031 ± 0.011	0.015
22:6n-3	7.916 ± 0.267	6.770 ± 0.203	0.010	6.819 ± 0.289	7.004 ± 0.079	0.557	6.724 ± 0.423	5.859 ± 0.201	0.102
24:5n-3	0.075 ± 0.017	0.041 ± 0.003	0.148	0.118 ± 0.016	0.137 ± 0.018	0.472	0.282 ± 0.036	0.106 ± 0.013	0.002
24:6n-3	2.572 ± 0.101	2.539 ± 0.096	0.862	1.922 ± 0.0466	2.252 ± 0.124	0.038	2.575 ± 0.057	2.581 ± 0.101	0.960
SFA	39.96 ± 0.901	39.27 ± 0.307	0.556	41.53 ± 0.735	43.79 ± 0.698	0.056	39.954 ± 0.598	40.577 ± 0.311	0.383
UFA	60.03 ± 0.901	60.71 ± 0.307	0.558	58.464 ± 0.735	56.200 ± 0.698	0.054	60.045 ± 0.598	59.422 ± 0.311	0.382
MUFA	37.20 ± 0.422	37.64 ± 0.517	0.508	40.034 ± 0.392	34.734 ± 0.699	0.001	40.036 ± 0.592	38.207 ± 0.292	0.024
PUFA	22.82 ± 0.643	23.03 ± 0.536	0.781	18.42 ± 0.469	21.46 ± 0.253	0.001	20.00 ± 0.153	21.21 ± 0.411	0.025
PUFAn-3	11.34 ± 0.297	10.09 ± 0.244	0.012	10.45 ± 0.309	10.00 ± 0.156	0.318	11.32 ± 0.378	9.42 ± 0.280	0.004
PUFAn-6	11.48 ± 0.552	12.98 ± 0.190	0.053	8.510 ± 0.206	11.47 ± 0.100	0.001	8.852 ± 0.297	11.85 ± 0.264	0.001
ACL	18.30 ± 0.033	18.34 ± 0.026	0.415	18.25 ± 0.033	18.21 ± 0.018	0.375	18.25 ± 0.019	18.24 ± 0.016	0.960
DBI	144.25 ± 2.83	143.06 ± 1.84	0.757	128.68 ± 2.85	135.73 ± 1.17	0.051	136.3 ± 1.280	136.6 ± 1.101	0.840
PI	125.74 ± 2.916	121.81 ± 2.413	0.379	105.58 ± 3.29	118.72 ± 1.71	0.008	114.92 ± 2.56	114.62 ± 2.72	0.946
R/PUFAs	1.007 ± 0.058	0.741 ± 0.008	0.006	1.227 ± 0.042	0.871 ± 0.013	0.001	1.281 ± 0.068	0.791 ± 0.010	0.001
AI	243.38 ± 6.03	171.88 ± 1.40	0.001	200.4 ± 2.818	158.2 ± 2.799	0.001	225.7 ± 14.42	129.23 ± 2.71	0.001

p value reflects statistical significance between diets by Student's *T* test

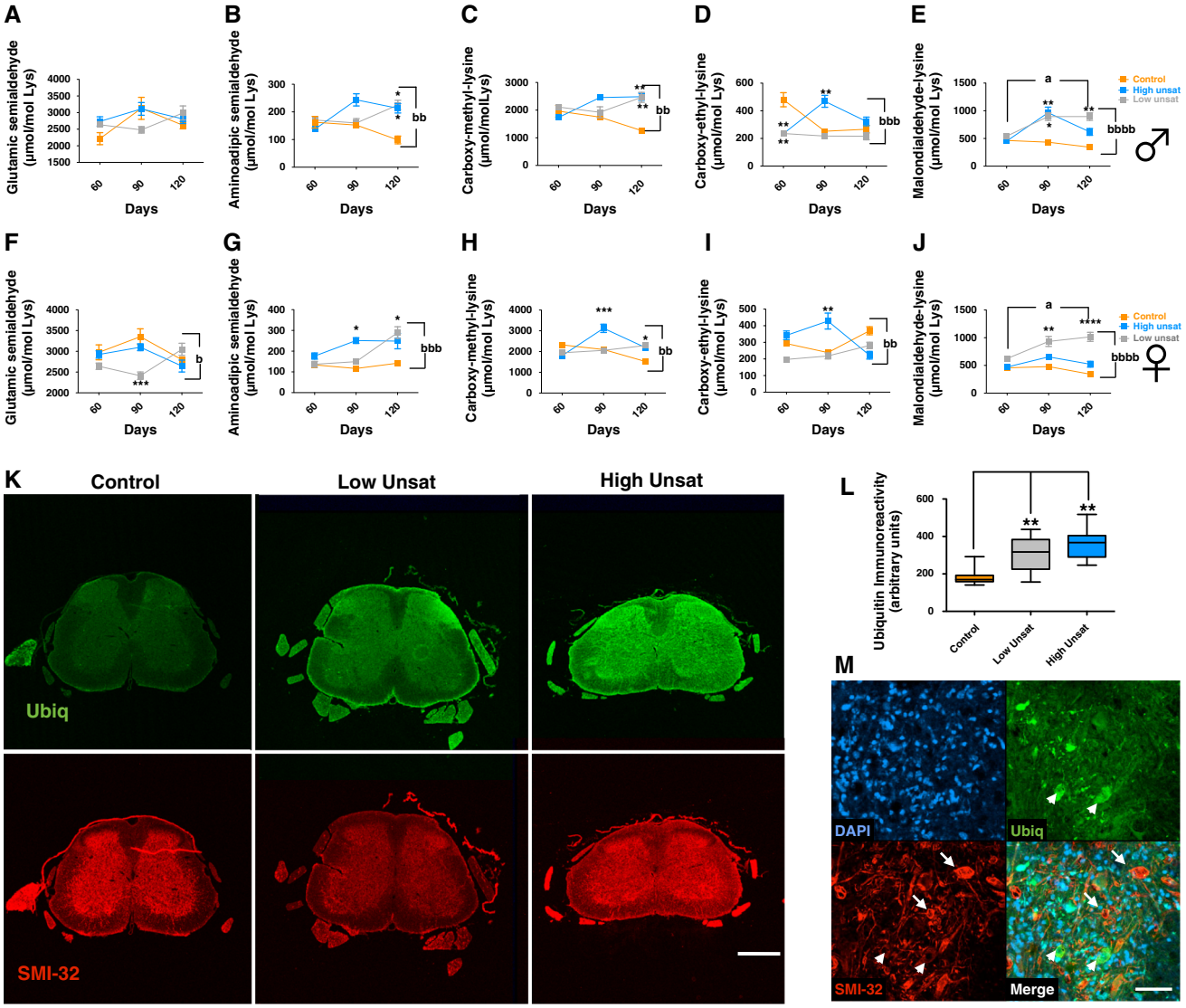


Fig. 2 Dietary unsaturation influences protein oxidative damage and ubiquitin aggregation in lumbar spinal cord. Protein carbonyls glutamic (**a**, **f**) and aminoadipic semi-aldehydes (**b**, **g**) were influenced by diet (but not GSA in males). Similarly, protein glycoxidative markers carboxy-methyl-lysine (**c**, **h**) and carboxy-ethyl-lysine (**d**, **i**) were influenced by age. A marker of protein lipoxidative modification, malondialdehyde-lysine showed a paradoxical increase in low unsaturation fed animals (**e**, **j**), specifically in females and was the only marker affected significantly by age. In accordance with faster rate of disease onset and proteotoxicity in highly unsaturated diets, males (endpoint: 130 ± 7 days) showed significantly higher ubiquitin immunostaining in lumbar spinal cords by confocal microscopy (**k**) as quantified (**l**) in comparison to control diet. Higher magnification images showed that ubiquitin immunostaining (*arrowheads*, **m**) was not restricted to SMI-32 positive neurons in anterior ventral horn (*arrows*, **m**). *, **, ***, and ****diet-related significant differences ($p < 0.05$, 0.01, 0.001 and 0.0001, respectively) by post hoc Bonferroni multiple comparisons after two-way ANOVA, while as [a]age-related ($p < 0.05$) and [b,bb, bbb and bbbb]diet-related ($p < 0.05$, <0.01, <0.001 and <0.0001, respectively) significant differences by two-way ANOVA. *Bars* in **k** and **m** are 500 and 50 µm, respectively. Number of animals used for each marker measure was $n = 7$ for each time point, both male and female. A total of 30 slices ($n = 3$ animals per treatment) were analysed for quantification in **l**, with differences evaluated by ANOVA post hoc Tukey's multiple comparison test

surprisingly high on the spinal cord of animals under low unsaturation diets, most probably due to an increase of 20:4 and 22:4, both from the n-6 series, specially in females.

As surrogate marker of n-3 availability, we chose syntaxin-3 immunoreactivity (Darios and Davletov 2006), which showed that high unsaturated diet led to more intense syntaxin-3 expression than low unsaturated one at similar ages (Fig. 1k).

All in all, these data demonstrated that highly unsaturated diets affected in a gender-dependent fashion the mean survival, the disease onset and the clinical evolution in the fALS model hSODG93A.

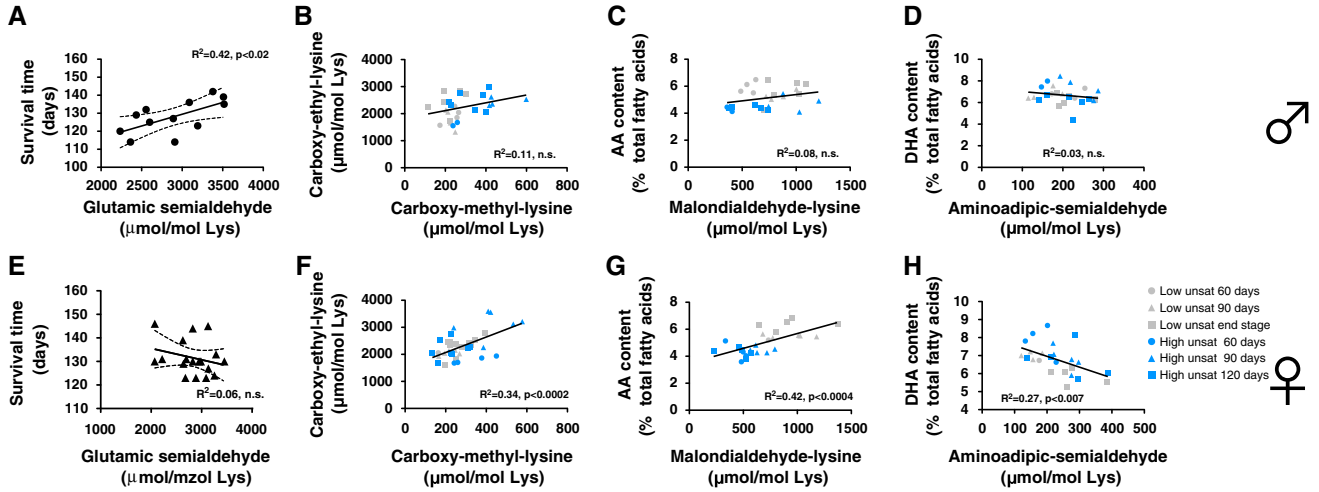


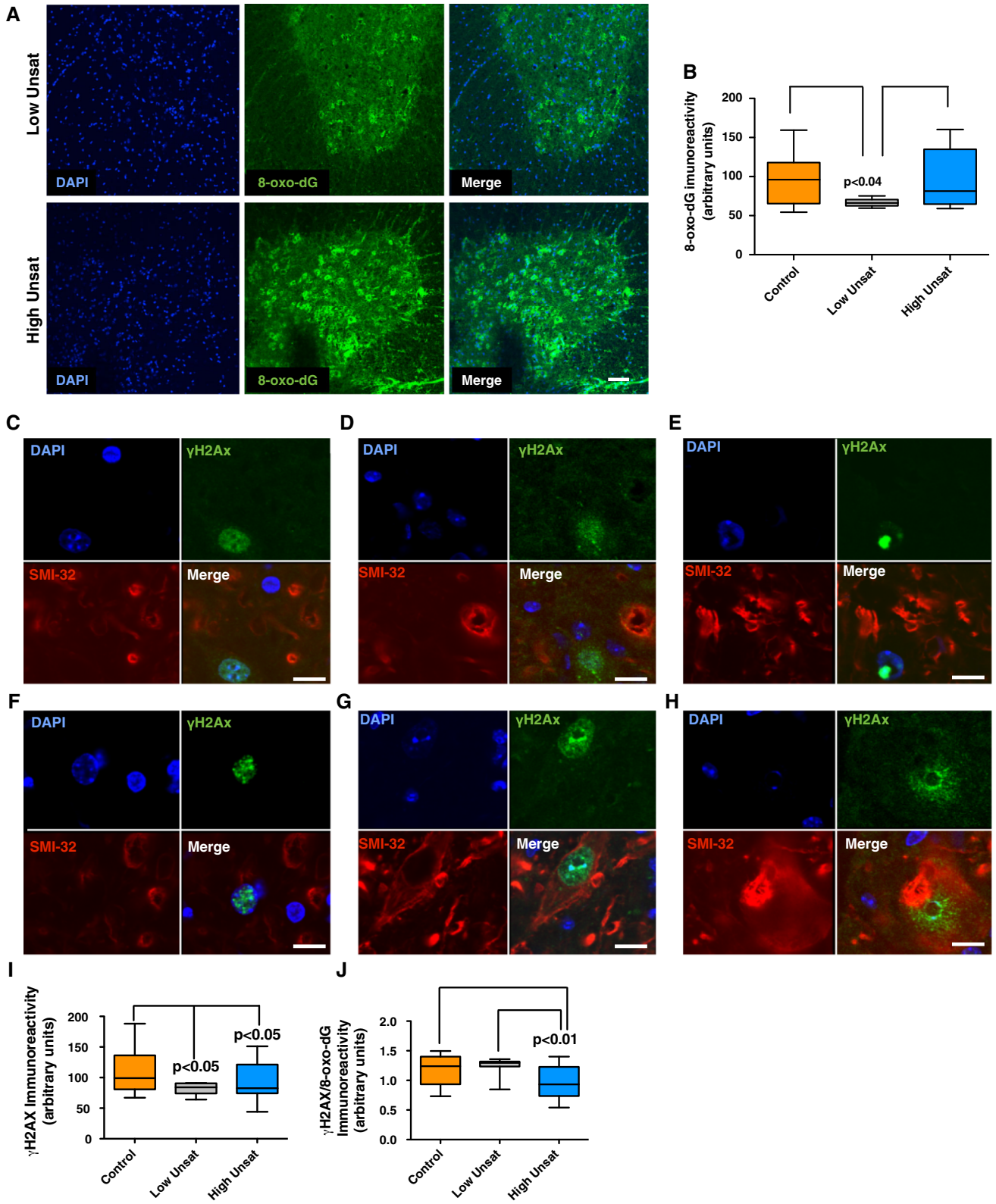
Fig. 3 Gender specific correlation between survival, protein oxidative damage and fatty acid contents. Glutamic semi-aldehyde is correlated with survival time in males (**a**) but not in females (**e**). Carboxy-ethyl-lysine is not correlated with carboxy-methyl-lysine modification in males (**b**) in contrast with females (**f**). Lipoxidative modification is not correlated with AA content in males (**c**) but it shows a positive correlation in females (**g**). Aminoadipic semi-aldehyde is inversely correlated with docosahexanoic acid content in spinal cord in females (**h**), but not in males (**d**). Inset show the linear regression statistic, with legend in **h** indicating the age and diets from individuals in the analyses from **c** to **h**

Protein Oxidative Damage in Neuronal Tissues of hSODG93A Transgenic Mice is Dependent on Dietary Fatty Acid Unsaturation

Potential causes of the deleterious effect of highly unsaturated diets comprise their high susceptibility to oxidative modification, though changes in DBI and PI suggested that highly unsaturated diets actually lowered PI. As shown in Fig. 2, the changes of unsaturation in diet induce significant changes in protein oxidative damage. For GSA, the most abundant marker among those detected belonging to direct oxidation pathway, changes in diet only induced significant effects in females, significantly lowering at early clinical stages. For AASA, both high and low unsaturated diets increased their concentrations, both in females and in males, being the changes more marked with high unsaturated diet. For CML, similar trends were seen in males and females, again showing that dietary unsaturation changes increased glycoxidative modifications at last stages, with reference to control diet. For CEL, significant increases were detected, induced by high unsaturated diet in males. Of note, at 90 days, levels of CEL were highest in males under high unsaturated diets, suggesting a key role for this modification in the determination of survival, as this is the group with faster disease progression and lower survival. Similarly, as females under highly unsaturated diets tended to have faster progression, significant CEL and CML increases at 90 days reinforce the role of glycoxidative modifications. Finally, for lipoxidative modifications (MDAL), significant increases induced by low unsaturated and high unsaturated diet were evident, in line with PI changes. Interestingly, age was only a significant factor for changes in MDAL concentrations. Importantly, protein oxidative modifications are associated with aggregate build-up and increased ubiquitination (Shang and Taylor 2011). Reinforcing the mass spectrometry findings indicating protein modification with changes in unsaturation of diets, confocal immunohistochemistry revealed increased ubiquitin immunoreactivity in motor neurons and glial cells (Fig. 2k–m) in comparison to control diets.

Correlation analyses of these variables revealed strong gender specificity. In order to study the relationship between fatty acid composition and protein oxidative damage, we clustered all the values, irrespectively of diets. Following these analyses, in males, protein oxidative modification was directly correlated with survival, but not in females (Fig. 3a, e). Similarly, the two glycoxidation markers, CEL and CML correlated in females, but not in males (Fig. 3b, f). Supporting a significant role of inflammatory potential in protein oxidative modifications, AA content correlated with MDAL modification in females (Fig. 3g). Finally, reinforcing the potential protective role of DHA, its level was inversely correlated with one of the markers of protein oxidative modification in females (Fig. 3h).

All in all, these results suggest a lack of a general relationship between protein oxidative modifications and survival or disease onset. Interestingly, under control fatty acid diets, lower lipoxidative damage (measured by MDAL concentration) is found, in line with fatty acid


A
Low Unsat
High Unsat
DAPI
8-oxo-dG
Merge
B
8-oxo-dG imunoreactivity (arbitrary units)
200
150
100
50
0
p<0.04
Control
Low Unsat
High Unsat
C
D
E
F
G
H
DAPI
γH2Ax
SMI-32
Merge
I
γH2AX Immunoreactivity (arbitrary units)
p<0.05
p<0.05
J
γH2AX/8-oxo-dG Immunoreactivity (arbitrary units)
2.0
1.5
1.0
0.5
0.0
p<0.01

◀**Fig. 4** Dietary fatty acid unsaturation changes 8-oxo-dG levels and its relationship with nuclear DNA damage response. The marker of oxidative DNA damage 8-oxo-dG was analysed by confocal immunohistochemistry in lumbar spinal cords from male mice in the preclinical stage (90 days) (**a**). Quantitative analyses demonstrated significantly decreased unsaturation in the low unsaturation group (**b**). Differential inmunohistochemical pattern for γH2Ax (90 days, male). Non-neuronal γH2Ax appears as discrete patterning (**c**), as heterogeneus foci (**d**) or as a single aggregate (**e**). Similarly, motor neuron, (SMI-32 positive cells) had discrete foci (**f**) or general nuclear staining (**g** and **h**). Quantitative analyses of γH2Ax and SMI-32 staining in whole sections are shown in the graph (**i**) and ratio between γH2Ax and 8-oxo-dG in the graph (**j**). *Bars* in **a** and **c** are 10 and 5 μm, respectively. A total of 30 slices (belonging to $n = 2$–3 animals per treatment) were analysed for quantification in **b** and **i**, with differences evaluated by ANOVA post hoc Tukey's multiple comparison test

compositional changes, when compared to diets extreme in unsaturation. Rather, a potential protective role of lipid peroxidative-derived modifications can be defined in females.

Dietary Fatty Acid Unsaturation Blunts the Mitochondrial–Nuclear Crosstalk Based on Free Radical Production and DNA Damage Response

To extend oxidative damage measurements to nucleic acids, we evaluated the presence of 8-oxo-dG, a direct marker of oxidative modification of DNA, focused to male group and extreme unsaturation diets. In line with previous data demonstrating that 8-oxo-dG levels are higher in mitochondrial DNA than in nuclear DNA (Barja and Herrero 2000), 8-oxo-dG immunoreactivity was mainly localized in non-nuclear locations (Fig. 4a, c). 8-oxo-dG immunoreactivity was significantly higher in spinal cord from animals fed with highly unsaturated diet, reinforcing the importance of mitochondrial DNA modification in this transgenic model (Fig. 4a, b), in comparison to low unsaturated diet. Recent data show the crosstalk between mitochondrial-free radical production and nuclear DNA damage response, mediated by γH2Ax (Passos et al. 2010). To evaluate whether this mechanism was affected by dietary fatty acid unsaturation, we evaluated by confocal microscopy the amount of γH2Ax foci, a surrogate for DNA damage response. The results showed the the presence of γH2Ax in both non-neuronal (Fig. 4c–e) and neuronal cells (Fig. 4f–h). Quantitative analyses of anterior horns of whole sections (Supplemental Figure 2) showed that dietary fatty acid unsaturation influenced the mitochondrial–nuclear crosstalk, as increased 8-oxo-dG immunostaining in animals fed with high unsaturated diet was not associated to an increase in γH2Ax staining in ventral horn of lumbar spinal cord (Fig. 4i), as reinforced by analyses of the γH2Ax to 8-oxo-dG immunostaining ratio (Fig. 4j).

Discussion

The influence of dietary components in ALS progression has been a focus of wide research. It is well accepted that malnutrition is a negative prognostic factor (Greenwood 2013). A recent clinical trial evidence the relevant role of nutrition in ALS (Wills et al. 2014). Furthermore, it has been suggested (ALS Untangled 2012) that saturated fats, such as those present in coconut oil, could alleviate the disease course by means of increasing energy bioavailability towards mitochondrial pathways. The favourable effect of low unsaturated diet in comparison to high unsaturated one might be attributed to enhanced calorie availability. However, the fact that, under low unsaturated diets, mice had lower weight gain, suggests a potential disease-accelerating effect, even if it was not reflected in mean survival (at least for males). Previous data have linked fresh fish intake to ALS development (Sienko et al. 1990), although the mechanisms behind might be related to intake of contaminants including cyanobacterial-derived neurotoxins or metals (Roos et al. 2013; Vinceti et al. 2012). Nonetheless, a recent review reveals that PUFAs, if appropriately protected from peroxidation (e.g. by vitamin E) can be protective (Veldink et al. 2007). Indeed, in a different population, epidemiological studies have revealed that high intake of PUFAs, with other dietary fats, may have a preventive effect in ALS (Okamoto et al. 2007).

Our data and a recent report (Yip et al. 2013) have shown that this potential protective role is not easily reproduced in the same murine model. Previous results, both in the sporadic form of the disease (Ilieva et al. 2007; Shibata et al. 2004), as well as in the murine model (Perluigi et al. 2005) have suggested the accrual of lipid peroxidation-derived aldehydes (such as 4-hydroxy-2-hexenal or 4-hydroxy-2-nonenal), as well as the resulting modification of specific proteins. However, our present data have shown that the relationship between lipid peroxidation and disease evolution is not lineal. Of note, despite diet was effective in changing the lipid profiles of several tissues (by comparison of previous data in liver (Herrero et al. 2001) using the same diets), central nervous system lipids were much more resistant to the introduced changes. Noteworthy, our low unsaturation diet was not intended to induce PUFA depletion. These formulations fulfil essential fatty acids requirements since it contained a sufficient amount of soybean oil. Interestingly, under the low unsaturation diet, we observed a paradoxical increase in the peroxidizability index, which is mainly attributable to the increased levels of AA. This increase is interpreted as an adaptive reaction to diet and disease, since diet composition for this particular fatty acid was in fact lower in the low unsaturated one. This may be related to the modulatory role of AA in inflammation. In any case, increased peroxidizability index

was associated with the increased load of protein lipoxidative damage (as evidenced by mass spectrometric measurements of MDAL). In contrast, the other protein oxidative modification markers measured (arising from direct oxidation or from glycoxidation) showed an increase under high unsaturated diets. This suggests that protein oxidative modifications such as GSA, AASA, CML and CEL could be more deleterious than MDAL. As a matter of fact, GSA shows a clear correlation with survival in males, a fact non present in females, suggesting than in these, protective mechanisms against protein oxidation could operate, as it has been suggested for ALS (Cervetto et al. 2013; Eschbach et al. 2013; Li et al. 2012) and in other diseases (Puertas et al. 2012).

Our results suggest that the relationship between lipid peroxidation and disease pathogenesis is not lineal; i.e. lipid peroxidation is not always noxious. Previous data have demonstrated the important adapting role induced by lipid peroxidation-derived aldehydes, such as 4-hydroxy-2-nonenal and 15-deoxy-delta-12,14-prostaglandin J(2) Thus, it is considered that lipid peroxidation may have a dual role in the pathogenesis of several diseases (Catalá 2009). In addition, protective roles of specific fatty acids can operate differently depending on diets and gender. The levels of a carbonyl damage marker (AASA), directly related to disease evolution, were inversely related to the content of DHA, supporting the potential protective role of this n-3 fatty acid. Docosahexanoic acid-derived molecules, such as resolvin D1, have been shown to be potentially protective in the process of phagocytosis which operates at later stages of ALS (Liu et al. 2012). Furthermore, previous data have revealed that while spinal cords with marked neuron loss showed diminished DHA contents, the cortex of the same individuals affected by ALS showed an increase in DHA (Ilieva et al. 2007). Interestingly, hSODG93A female transgenic mice at 90 d under low unsaturated diet (i.e. living longer than males) exhibited ca 6 % more DHA than males. However, DHA-dependent mechanisms could operate up to a certain point of pathological burden. Despite high unsaturated diet did not led to higher DHA content in males, biologically these diets allowed for higher accumulation of the DHA-dependent protein syntaxin-3 (Darios and Davletov 2006), supporting the notion than enhanced availability of DHA in high unsaturation diets may have some neurobiological impact. Our results support the notion that a trade-off for the high content of PUFAs in neuronal tissues, a high peroxidizability, may have a beneficial-adaptative role (i.e. accumulation of DHA-dependent proteins, enhanced responsiveness in terms of antioxidant mechanisms).

A similar trade-off is also evident from examining the potential implication of inflammation in the findings reported in our work. Neuroinflammation, just as lipid peroxidation, is recognized to play a dual role, being neuroprotective in some stages to become neurotoxic in others (Consilvio et al. 2004). Our data, demonstrating a significant correlation between arachidonic acid content and MDAL only in females, suggest that lipid peroxidation adaptative mechanisms also affect arachidonic acid levels, and, consequently, inflammatory potential. Interestingly, reinforcing the potential protective role of neuroinflammation in some stages of the disease, high unsaturated diets in males, associated with a poor outcome-diminished survival, earlier onset, enhanced aggregation of ubiquitin proteins in spinal cord were consistently associated with higher anti-inflammatory index, as suggested by fatty acid profiles.

Both inflammation and oxidative stress can be interplayers in ageing basic mechanisms. Being ageing a clear risk factor for ALS, we explored whether mechanisms linking ageing, oxidative stress and inflammation could explain part of our results. In this line, the mitochondrial–nuclear crosstalk is relevant in cellular senescence (Passos et al. 2010). A resulting unbalanced response includes sustained DNA damage response and senescence-associated secretory phenotype, fuelled by mitochondrial-free radical production dependent on p21 activation and TGFβ production. For this reason, we evaluated the presence of γH2Ax foci, related to DNA damage, in spinal cord in this model. The present results have shown markers of DNA damage repairing in motor neurons and glial cells. Despite it has been previously hypothesized that these phenomena could be present in various in vitro models of neurodegeneration (Avramovich-Tirosh et al. 2007), our results demonstrate an unreported variability in the immunostaining of γH2Ax. This morphological variety can be attributed to the several roles of this protein in cell homeostasis (Sharma et al. 2012) ranging from DNA replication to apoptosis, including also physiological neuronal activity (Suberbielle et al. 2013). Previous data have demonstrated that DNA in ALS is damaged in mitochondria (Kikuchi et al. 2002) and in the nuclei (Barbosa et al. 2010; Murata et al. 2008). Interestingly, recent data suggest a gender-specific difference in terms of DNA damage response/repair capacity (Trzeciak et al. 2008). The finding of increased mitochondrial DNA damage induced by high unsaturation, without a concomitant increase in γH2Ax suggests that the mitochondrial–nuclear crosstalk based on p21 and free radical production is influenced by diet, specifically by PUFAs. Previous work has related G93A mutation to increased 8-oxo-dG accumulation (Barbosa et al. 2010) and also data form our laboratory (Herrero et al. 2001), revealed higher free radical production in mitochondria from animals fed with similarly high unsaturated diets, a finding that fits with enhanced 8-oxo-dG present in this work. Therefore, a logical response to

enhanced free radical production would be increased DDR, thought data presented here suggest that this is not the case. Reasons for this blunted response on mitochondrial–nuclear crosstalk may arise from several facts. It is known that enhanced levels of n-6 fatty acids (such as those present in low unsaturated fed animals) can be linked to higher production of TGFβ, a key factor in the mitochondrial–nuclear crosstalk (Harbige and Sharief 2007). Similarly, it is known that docosahexanoic or eicosapentaenoic acids change the expression of p21 (Katakura et al. 2013; Slagsvold et al. 2010). When added to the fact that high unsaturated diets actually decreased lipid peroxidative modification in these experiments, our results reinforce a potentially protective role for lipid peroxidation. Thus, low unsaturated diets would sustain lipid peroxidation and reinforce mitochondrial–nuclear crosstalk via increased TGFβ secretion and γH2Ax foci formation and maintaining both an adequate antioxidant response—as evidenced by lower protein oxidative and glycoxidative damage—as well as diminished levels of mitochondrial DNA modification. In contrast, high unsaturated diets could act deleteriously in a double fashion: by increasing p21 expression, perhaps interfering with its activation and by increasing free radical production without an adequate response of cellular antioxidant systems, which will be focus of future studies.

As limitations of the present study, we recognize that other clinical measurements (e.g. Rotarod-based) may be also of interest. Further, establishment of standard methods for treatment testing is crucial in ALS models Ludolph et al. (2007, 2010). However, we hope that measurement of three indicators of disease evolution (i.e. weight changes, clinical score and stride lenght) could be helpful for global assessment of diet-induced changes. Importantly, these analyses show that values in animals fed with control diets were not always between the low unsaturated and high unsaturated groups: our suggestion is that survival, in relationship with changes in fatty acid composition present in spinal cord suggest the importance of an adequate lipid profile in neuronal resistance towards stressors. For instance, levels of AA are paradoxically lower in high unsaturated groups (irrespectively of the gender). A potential protective role of AA-derived bioactive compounds cannot be discarded based on these results. In line with this, fatty acids with a potential neuroprotective effects, such as DHA, are not altered in any of the groups, suggesting their importance and resilience towards dietary changes. We have also disclosed that in some terms, such as DNA modification and response to it, control animals behave differently to low unsaturated fed ones, demonstrating the complexity of the relationships behind.

Our data reveal that intervention on dietary fat unsaturation modulates the gender protective effect in females. Highly unsaturated diet apparently diminished survival in females, while as maintenance of a diet with a low unsaturation allowed to differentiate gender-specific effects in survival of this ALS model. Taking into account the several mechanisms studied, particularly oxidative stress, our data suggest that despite PUFAs could have some potential benefits in specific stages of the disease, through a surprisingly protective lipid peroxidation, later on, the disease-sparing mechanisms are overwhelmed by pathology burden. A similar mechanism could operate for neuroinflammation. Further, our data suggest that highly unsaturated diet could blunt a mitochondria–nuclear crosstalk, essential for cell homeostasis. Together, these results reinforce the need for adequately defining gender as a modifying factor in ALS models, as well as to use structurally characterized and variegated markers for oxidative damage assessment in neurodegeneration.

Acknowledgments We are grateful to J. Pairada for excellent technical assistance. We thank D. Argilés and M. Martí for their skilful assistance. Supported by the Spanish Ministry of Science and Innovation [CENIT Program, BFU2009-11879/BFI and AGL2006-1243] the Autonomous Goverment of Catalunya [2009SGR-735] and the Spanish Ministry of Health [FIS 08-1843, 11-01532 to M.P-O and FIS13-00584 to RP]. Supported also by the COST B-35 Action and the Fundació Miquel Valls. D.C. is a predoctoral fellow from Spanish Ministry of Health [FI08-00707]. O.R-N. and R. C. received a predoctoral fellowship from Autonomous Government of Catalonia.

Conflict of interest Daniel Cacabelos, Victoria Ayala, Omar Ramírez-Nunez, Ana Belen Granado-Serrano, Jordi Boada, Jose CE Serrano, Rosanna Cabré, Gisela Nadal-Rey, Isidro Ferrer, Reinald Pamplona and Manuel Portero-Otin have not financial relationship with the organizations that sponsored the research.

References

Al-Chalabi, A., Jones, A., Troakes, C., King, A., Al-Sarraj, S., & van den Berg, L. H. (2012). The genetics and neuropathology of amyotrophic lateral sclerosis. *Acta Neuropathologica, 124*(3), 339–352.

ALSUntangled Group. (2012). ALSUntangled 15: Coconut oil. *Amyotrophic Lateral Sclerosis: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases, 13*(3), 328–330.

Avramovich-Tirosh, Y., Reznichenko, L., Mit, T., Zheng, H., Fridkin, M., Weinreb, O., et al. (2007). Neurorescue activity, APP regulation and amyloid-beta peptide reduction by novel multifunctional brain permeable iron-chelating-antioxidants, M-30 and green tea polyphenol, EGCG. *Current Alzheimer Research, 4*(4), 403–411.

Bancroft, L. K., Lupton, J. R., Davidson, L. A., Taddeo, S. S., Murphy, M. E., Carroll, R. J., et al. (2003). Dietary fish oil reduces oxidative DNA damage in rat colonocytes. *Free Radical Biology & Medicine, 35*(2), 149–159.

Barbosa, L. F., Cerqueira, F. M., Macedo, A. F., Garcia, C. C., Angeli, J. P., Schumacher, R. I., et al. (2010). Increased SOD1 association with chromatin, DNA damage, p53 activation, and

apoptosis in a cellular model of SOD1-linked ALS. *Biochimica et Biophysica Acta, 1802*(5), 462–471.

Barceló-Coblijn, G., Kitajka, K., Puskás, L. G., Hogyes, E., Zvara, A., Hackler, L., et al. (2003). Gene expression and molecular composition of phospholipids in rat brain in relation to dietary n-6 to n-3 fatty acid ratio. *Biochimica et Biophysica Acta, 1632*(1–3), 72–79.

Barja, G., & Herrero, A. (2000). Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology, 14*(2), 312–318.

Bowling, A. C., Schulz, J. B., Brown, R. H., & Beal, M. F. (1993). Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *Journal of Neurochemistry, 61*(6), 2322–2325.

Catalá, A. (2009). Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and Physics of Lipids, 157*(1), 1–11.

Cervetto, C., Frattaroli, D., Maura, G., & Marcoli, M. (2013). Motor neuron dysfunction in a mouse model of ALS: Gender-dependent effect of P2X7 antagonism. *Toxicology, 311*(1–2), 69–77.

Chiu, A. Y., Zhai, P., Dal Canto, M. C., Peters, T. M., Kwon, Y. W., Prattis, S. M., et al. (1995). Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Molecular and Cellular Neurosciences, 6*(4), 349–362.

Consilvio, C., Vincent, A. M., & Feldman, E. L. (2004). Neuroinflammation, COX-2, and ALS—A dual role? *Experimental Neurology, 187*, 1–10.

Darios, F., & Davletov, B. (2006). Omega-3 and omega-6 fatty acids stimulate cell membrane expansion by acting on syntaxin 3. *Nature, 440*(7085), 813–817.

Deckelbaum, R. J., & Torrejon, C. (2012). The omega-3 fatty acid nutritional landscape: Health benefits and sources. *The Journal of Nutrition, 142*(3), 587S–591S.

Eschbach, J., Schwalenstöcker, B., Soyal, S. M., Bayer, H., Wiesner, D., Akimoto, C., et al. (2013). PGC-1α is a male-specific disease modifier of human and experimental amyotrophic lateral sclerosis. *Human Molecular Genetics, 22*(17), 3477–3484.

Gaudette, M., Hirano, M., & Siddique, T. (2000). Current status of SOD1 mutations in familial amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders: Official Publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases, 1*(2), 83–89.

Greenwood, D. I. (2013). Nutrition management of amyotrophic lateral sclerosis. *Nutrition in Clinical Practice: Official Publication of the American Society for Parenteral and Enteral Nutrition, 28*(3), 392–399.

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., et al. (1994). Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. *Science (New York, N.Y.), 264*(5166), 1772–1775.

Harbige, L. S., & Sharief, M. K. (2007). Polyunsaturated fatty acids in the pathogenesis and treatment of multiple sclerosis. *British Journal of Nutrition, 98*(Suppl 1), 46–53.

Herrero, A., Portero-Ot'in, M., Bellmunt, M. J., Pamplona, R., & Barja, G. (2001). Effect of the degree of fatty acid unsaturation of rat heart mitochondria on their rates of H_2O_2 production and lipid and protein oxidative damage. *Mechanisms of Ageing and Development, 122*(4), 427–443.

Ilieva, E. V., Ayala, V., Jové, M., Dalfó, E., Cacabelos, D., Povedano, M., et al. (2007). Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. *Brain: A Journal of Neurology, 130*, 3111–3123.

Itoh, T., & Yamamoto, K. (2008). Peroxisome proliferator activated receptor gamma and oxidized docosahexaenoic acids as new class of ligand. *Naunyn-Schmiedeberg's Archives of Pharmacology, 377*(4–6), 541–547.

Katakura, M., Hashimoto, M., Okui, T., Shahdat, H. M., Matsuzaki, K., & Shido, O. (2013). Omega-3 polyunsaturated Fatty acids enhance neuronal differentiation in cultured rat neural stem cells. *Stem Cells International, 2013*, 490476.

Kikuchi, H., Furuta, A., Nishioka, K., Suzuki, S. O., Nakabeppu, Y., & Iwaki, T. (2002). Impairment of mitochondrial DNA repair enzymes against accumulation of 8-oxo-guanine in the spinal motor neurons of amyotrophic lateral sclerosis. *Acta Neuropathologica, 103*(4), 408–414.

Lauritzen, L., Hansen, H. S., Jørgensen, M. H., & Michaelsen, K. F. (2001). The essentiality of long chain n-3 fatty acids in relation to development and function of the brain and retina. *Progress in Lipid Research, 40*, 1–94.

Li, R., Strykowski, R., Meyer, M., Mulcrone, P., Krakora, D., & Suzuki, M. (2012). Male-specific differences in proliferation, neurogenesis, and sensitivity to oxidative stress in neural progenitor cells derived from a rat model of ALS. *PLoS ONE, 7*(11), e48581.

Liu, G., Fiala, M., Mizwicki, M. T., Sayre, J., Magpantay, L., Siani, A., et al. (2012). Neuronal phagocytosis by inflammatory macrophages in ALS spinal cord: inhibition of inflammation by resolvin D1. *American Journal of Neurodegenerative Disease, 1*(1), 60–74.

Ludolph, A. C., Bendotti, C., Blaugrund, E., Chio, A., Greensmith, L., Loeffler, J. P., et al. (2010). Guidelines for preclinical animal research in ALS/MND: A consensus meeting. *Amyotrophic Lateral Sclerosis: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases, 11*(1–2), 38–45.

Ludoph, A. C., Bendotti, C., Blaugrund, E., Hengerer, B., Löffler, J. P., Martin, J., et al. (2007). Guidelines for the preclinical in vivo evaluation of pharmacological active drugs for ALS/MND: Report on the 142nd ENMC international workshop. *Amyotrophic Lateral Sclerosis: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases, 8*(4), 217–223.

Müllner, E., Brath, H., Pleifer, S., Schiermayr, C., Baierl, A., Wallner, M., et al. (2013). Vegetables and PUFA-rich plant oil reduce DNA strand breaks in individuals with type 2 diabetes. *Molecular Nutrition & Food Research, 57*(2), 328–338.

Murata, T., Ohtsuka, C., & Terayama, Y. (2008). Increased mitochondrial oxidative damage and oxidative DNA damage contributes to the neurodegenerative process in sporadic amyotrophic lateral sclerosis. *Free Radical Research, 42*(3), 221–225.

Niki, E. (2012). Do antioxidants impair signaling by reactive oxygen species and lipid oxidation products? *FEBS Letters, 586*(21), 3767–3770.

Niu, S.-L., Mitchell, D. C., Lim, S.-Y., Wen, Z.-M., Kim, H.-Y., Salem, N., et al. (2004). Reduced G protein-coupled signaling efficiency in retinal rod outer segments in response to n-3 fatty acid deficiency. *The Journal of Biological Chemistry, 279*(30), 31098–31104.

Oeda, T., Shimohama, S., Kitagawa, N., Kohno, R., Imura, T., Shibasaki, H., et al. (2001). Oxidative stress causes abnormal accumulation of familial amyotrophic lateral sclerosis-related mutant SOD1 in transgenic Caenorhabditis elegans. *Human Molecular Genetics, 10*(19), 2013–2023.

Okamoto, K., Kihira, T., Kondo, T., Kobashi, G., Washio, M., Sasaki, S., et al. (2007). Nutritional status and risk of amyotrophic lateral sclerosis in Japan. *Amyotrophic Lateral Sclerosis: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases, 8*(5), 300–304.

Pamplona, R., Dalfó, E., Ayala, V., Bellmunt, M. J., Prat, J., Ferrer, I., et al. (2005). Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. *The Journal of Biological Chemistry, 280*(22), 21522–21530.

Pamplona, R., Portero-Otín, M., Sanz, A., Requena, J., & Barja, G. (2004). Modification of the longevity-related degree of fatty acid unsaturation modulates oxidative damage to proteins and mitochondrial DNA in liver and brain. *Experimental Gerontology, 39*(5), 725–733.

Passos, J. F., Nelson, G., Wang, C., Richter, T., Simillion, C., Proctor, C. J., et al. (2010). Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Molecular Systems Biology, 6*, 347.

Perluigi, M., Fai Poon, H., Hensley, K., Pierce, W. M., Klein, J. B., Calabrese, V., et al. (2005). Proteomic analysis of 4-hydroxy-2-nonenal-modified proteins in G93A-SOD1 transgenic mice—A model of familial amyotrophic lateral sclerosis. *Free Radical Biology & Medicine, 38*(7), 960–968.

Portero-Otin, M., Bellmunt, M. J., Requena, J. R., & Pamplona, R. (2003). Protein modification by advanced Maillard adducts can be modulated by dietary polyunsaturated fatty acids. *Biochemical Society Transactions, 31*(Pt 6), 1403–1405.

Puertas, M. C., Mart'inez-Martos, J. M., Cobo, M. P., Carrera, M. P., Mayas, M. D., & Ram'irez-Expósito, M. J. (2012). Plasma oxidative stress parameters in men and women with early stage Alzheimer type dementia. *Experimental Gerontology, 47*(8), 625–630.

Roos, P. M., Vesterberg, O., Syversen, T., Flaten, T. P., & Nordberg, M. (2013). Metal concentrations in cerebrospinal fluid and blood plasma from patients with amyotrophic lateral sclerosis. *Biological Trace Element Research, 151*(2), 159–170.

Rosen, D. R. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature, 364*(6435), 362.

Serhan, C. N. (2005). Novel eicosanoid and docosanoid mediators: Resolvins, docosatrienes, and neuroprotectins. *Current Opinion in Clinical Nutrition and Metabolic Care, 8*(2), 115–121.

Shang, F., & Taylor, A. (2011). Ubiquitin-proteasome pathway and cellular responses to oxidative stress. *Free Radical Biology & Medicine, 51*(1), 5–16.

Sharma, A., Singh, K., & Almasan, A. (2012). Histone H2AX phosphorylation: a marker for DNA damage. *Methods in Molecular Biology, 920*, 613–626.

Shibata, N., Yamada, S., Uchida, K., Hirano, A., Sakoda, S., Fujimura, H., et al. (2004). Accumulation of protein-bound 4-hydroxy-2-hexenal in spinal cords from patients with sporadic amyotrophic lateral sclerosis. *Brain Research, 1019*(1–2), 170–177.

Sienko, D. G., Davis, J. P., Taylor, J. A., & Brooks, B. R. (1990). Amyotrophic lateral sclerosis. A case–control study following detection of a cluster in a small Wisconsin community. *Archives of Neurology, 47*(1), 38–41.

Simons, K., & Toomre, D. (2000). Lipid rafts and signal transduction. *Nature Reviews Molecular Cell Biology, 1*(1), 31–39.

Slagsvold, J. E., Pettersen, C. H., Storvold, G. L., Follestad, T., Krokan, H. E., & Schonberg, S. A. (2010). DHA alters expression of target proteins of cancer therapy in chemotherapy resistant SW620 colon cancer cells. *Nutrition and Cancer, 62*(5), 611–621.

Suberbielle, E., Sanchez, P. E., Kravitz, A. V., Wang, X., Ho, K., Eilertson, K., et al. (2013). Physiologic brain activity causes DNA double-strand breaks in neurons, with exacerbation by amyloid-β. *Nature Neuroscience, 16*(5), 613–621.

Thorpe, S. R., & Baynes, J. W. (2003). Maillard reaction products in tissue proteins: New products and new perspectives. *Amino Acids, 25*(3–4), 275–281.

Trzeciak, A. R., Barnes, J., Ejiogu, N., Foster, K., Brant, L. J., Zonderman, A. B., et al. (2008). Age, sex, and race influence single-strand break repair capacity in a human population. *Free Radical Biology & Medicine, 45*(12), 1631–1641.

Veldink, J. H., Kalmijn, S., Groeneveld, G.-J., Wunderink, W., Koster, A., de Vries, J. H. M., et al. (2007). Intake of polyunsaturated fatty acids and vitamin E reduces the risk of developing amyotrophic lateral sclerosis. *Journal of Neurology, Neurosurgery and Psychiatry, 78*(4), 367–371.

Vinceti, M., Fiore, M., Signorelli, C., Odone, A., Tesauro, M., Consonni, M., et al. (2012). Environmental risk factors for amyotrophic lateral sclerosis: Methodological issues in epidemiologic studies. *Annali di Igiene: Medicina Preventiva e di Comunita, 24*, 407–415.

Weimann, A., Belling, D., & Poulsen, H. E. (2001). Measurement of 8-oxo-2′-deoxyguanosine and 8-oxo-2′-deoxyadenosine in DNA and human urine by high performance liquid chromatography–electrospray tandem mass spectrometry. *Free Radical Biology & Medicine, 30*(7), 757–764.

Wills, A. M., Hubbard, J., Macklin, E. A., Glass, J., Tandan, R., Simpson, E. P., et al. (2014). Hypercaloric enteral nutrition in patients with amyotrophic lateral sclerosis: A randomised, double-blind, placebo-controlled phase 2 trial. *The Lancet*. doi:10.1016/S0140-6736(14)60222-1.

Willson, T. M., Brown, P. J., Sternbach, D. D., & Henke, B. R. (2000). The PPARs: From orphan receptors to drug discovery. *Journal of Medicinal Chemistry, 43*(4), 527–550.

Yip, P. K., Pizzasegola, C., Gladman, S., Biggio, M. L., Marino, M., Jayasinghe, M., et al. (2013). The omega-3 fatty acid eicosapentaenoic acid accelerates disease progression in a model of amyotrophic lateral sclerosis. *PLoS ONE*, *8*(4), e61626.

Zhu, C., Poulsen, H. E., & Loft, S. (2000). Inhibition of oxidative DNA damage in vitro by extracts of Brussels sprouts. *Free Radical Research, 33*(2), 187–196.

Table S1: Fatty acid composition (% of abundance) and derived indexes in spinal cord (lumbar region) from hSODG93A male mice (90days).

	Control	High Unsat	Low Unsat	*p* value (Hi/Lo)	*p* value (Hi/Ctl)	*p* value (Lo/Ctl)
18:0	17,72±0,11	15,86±1,05	16,59±1,68	0,676	0,297	**0,495**
18:1n-9	38,91±0,269	38,76±1,57	33,04±1,86	0,017	0,942	**0,011**
20:4n-6	5,44±0,100	4,73±0,289	5,29±0,126	0,048	0,017	**0,548**
22:4n-6	2,34±0,074	1,39±0,088	2,26±0,126	0,001	0,001	**0,543**
22:6n-3	6,79±0,117	7,40±0,490	6,62±0,278	0,101	0,189	**0,688**
SFA	38,91±0,17	40,58±1,97	45,59±1,75	0,037	0,443	**0,006**
UFA	60,92±0,25	59,41±1,97	54,40±1,75	0,037	0,489	**0,007**
MUFA	41,99±0,31	39,32±1,49	33,75±1,85	0,018	0,210	**0,001**
PUFA	18,93±0,16	20,08±0,86	20,65±0,27	0,416	0,116	**0,020**
PUFA n-3	7,31±0,11	10,79±0,42	9,70±0,33	0,031	0,001	**0,001**
PUFA n-6	11,62±0,11	9,28±0,83	10,94±0,33	0,020	0,003	**0,263**
DBI	125,74±0,76	135,83±3,85	130,49±1,69	0,121	0,009	**0,141**
PI	95,62±1,06	115,10±4,18	113,41±2,96	0,688	0,001	**0,001**
AI	140,31±3,77	207,84±8,00	155,11±3,13	0,001	0,001	**0,050**

P value for Bonferroni multiple comparison tests after ANOVA analyses

Table S2: Fatty acid composition (% of abundance) and derived indexes in spinal cord (lumbar region) from hSODG93A female mice (90days).

	Control	High Unsat	Low Unsat	*p* value (Hi/Lo)	*p* value (Hi/Ctl)	*p* value (Lo/Ctl)
18:0	17,78±0,14	18,70±0,36	16,95±0,27	0,001	0,023	**0,051**
18:1n-9	39,42±0,26	39,29±0,35	34,03±0,72	0,001	0,822	**0,001**
20:4n-6	5,40±0,07	4,51±0,09	5,50±0,04	0,001	0,001	**0,374**
22:4n-6	2,31±0,027	1,21±0,052	2,63±0,164	0,001	0,001	**0,020**
22:6n-3	6,78±0,049	6,83±0,263	7,00±0,079	0,441	0,815	**0,312**
SFA	38,97±0,23	41,35±0,063	43,79±0,69	0,006	0,005	**0,001**
UFA	61,07±0,23	58,67±0,63	56,20±0,69	0,006	0,005	**0,001**
MUFA	42,45±0,28	39,93±0,33	34,73±0,69	0,001	0,001	**0,001**
PUFA	18,56±0,08	18,73±0,49	21,46±0,25	0,001	0,698	**0,001**
PUFA n-3	7,34±0,04	10,17±0,39	9,97±0,18	0,591	0,001	**0,001**
PUFA n-6	11,21±0,08	8,56±0,17	11,48±0,10	0,001	0,001	**0,156**
DBI	125,54±0,27	130,22±2,79	135,73±1,17	0,046	0,063	**0,001**
PI	95,47±0,53	107,88±3,40	118,55±1,32	0,001	0,004	**0,005**
AI	140,28±2,42	201,46±2,52	158,20±2,79	0,001	0,001	**0,001**

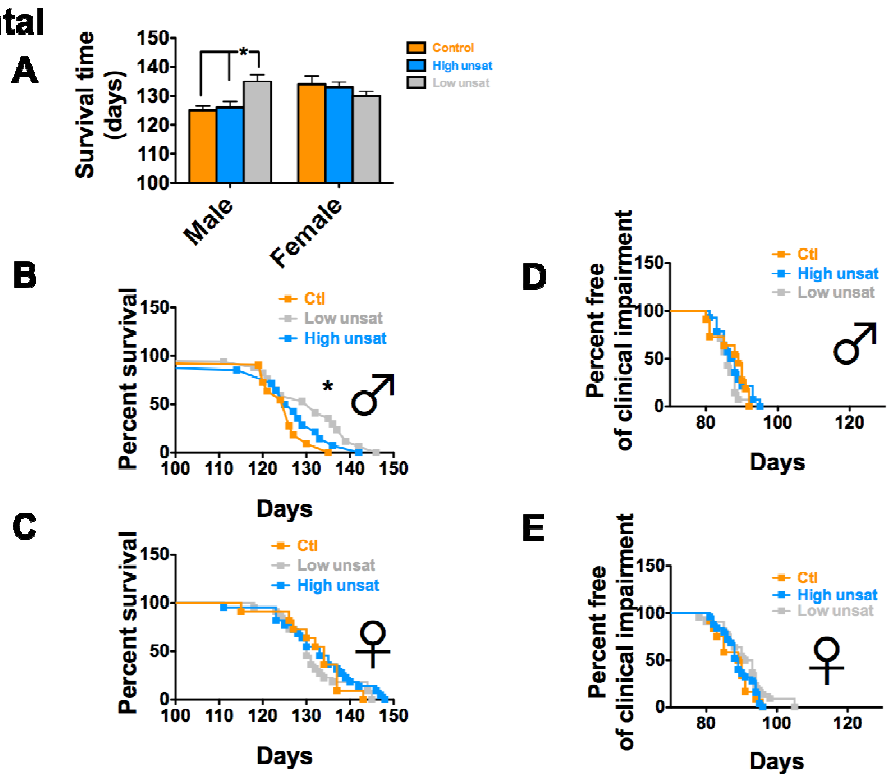
P value for Bonferroni multiple comparison tests after ANOVA analyses

Table S3. Fatty acid composition (mol%) of diets. Values are means ± SEM from n=3 samples.

	High Unsat	Low Unsat	Control	*p* value (Hi/Lo)	*p* value (Hi/Ctl)	*p* value (Lo/Ctl)
14:0	10,04±0,173	29,67±0,422	0,404±0,040	0,001	0,001	**0,001**
16:0	17,78±0,113	18,14±0,018	11,95±0,011	0,102	0,001	**0,001**
16:1n-7	10,78±0,085	0,096±0,003	0,245±0,028	0,001	0,001	**0,254**
18:0	4,184±0,021	18,75±0,333	4,430±0,046	0,001	0,381	**0,001**
18:1n-9	14,44±0,012	8,854±0,062	23,88±0,629	0,001	0,001	**0,001**
18:2n-6	12,71±0,217	20,64±0,034	49,73±0,122	0,001	0,001	**0,001**
18:3n-3	3,397±0,069	2,987±0,032	7,501±0,085	0,064	0,001	**0,001**
18:4n-6	n.d.	0,061±0,007	0,155±0,015	-	-	**0,047**
20:0	1,352±0,142	0,262±0,008	0,350±0,009	0,001	0,001	**0,604**
20:1n-9	1,775±0,077	0,083±0,004	0,214±0,013	0,001	0,001	**0,521**
20:2n-6	1,302±0,194	0,030±0,005	0,080±0,014	0,001	0,001	**0,061**
20:3n-6	1,200±0,189	0,020±0,002	0,054±0,009	0,001	0,001	**0,057**
20:4n-6	1,876±0,176	0,021±0,002	0,053±0,008	0,001	0,001	**0,060**
20:5n-3	8,532±0,429	0,038±0,005	0,101±0,005	0,001	0,001	**0,041**
22:0	0,917±0,073	0,130±0,010	0,318±0,014	0,001	0,001	**0,151**
22:4n-6	0,735±0,094	0,031±0,015	n.d.	0,001	-	**-**
22:5n-6	0,963±0,140	0,043±0,003	0,095±0,001	0,001	0,001	**0,031**
22:5n-3	1,871±0,170	0,028±0,001	0,068±0,009	0,001	0,001	**0,040**
24:0	0,990±0,098	0,053±0,001	0,154±0,011	0,001	0,001	**0,001**
22:6n-3	4,790±0,363	0,040±0,003	0,109±0,014	0,001	0,041	**0,080**
24:5n-3	0,340±0,083	n.d.	0,024±0,002	-	0,001	**-**
24:6n-3	n.d.	0,006±0,001	0,061±0,003	-	-	**0,001**
SFA	35,27±0,323	67,01±0,094	17,61±0,084	0,001	0,001	**0,001**
UFA	64,72±0,320	32,98±0,095	82,38±0,084	0,001	0,001	**0,001**
MUFA	25,22±0,068	8,95±0,062	24,13±0,065	0,001	0,001	**0,001**
PUFA	37,75±0,457	23,95±0,043	58,04±0,155	0,001	0,001	**0,001**
PUFA n-3	19,96±0,690	3,153±0,040	8,027±0,110	0,001	0,001	**0,001**
PUFA n-6	18,79±0,120	20,84±0,017	50,17±0,098	0,001	0,001	**0,001**
DBI	164,77±3,08	60,60±0,228	149,75±0,22	0,001	0,001	**0,001**
PI	142,9±4,491	28,40±0,270	69,49±0,002	0,001	0,001	**0,001**
AI	**893,6±109,6**	**617,9±76,3**	**659,9±117,2**	**0,001**	**0,001**	**0,812**

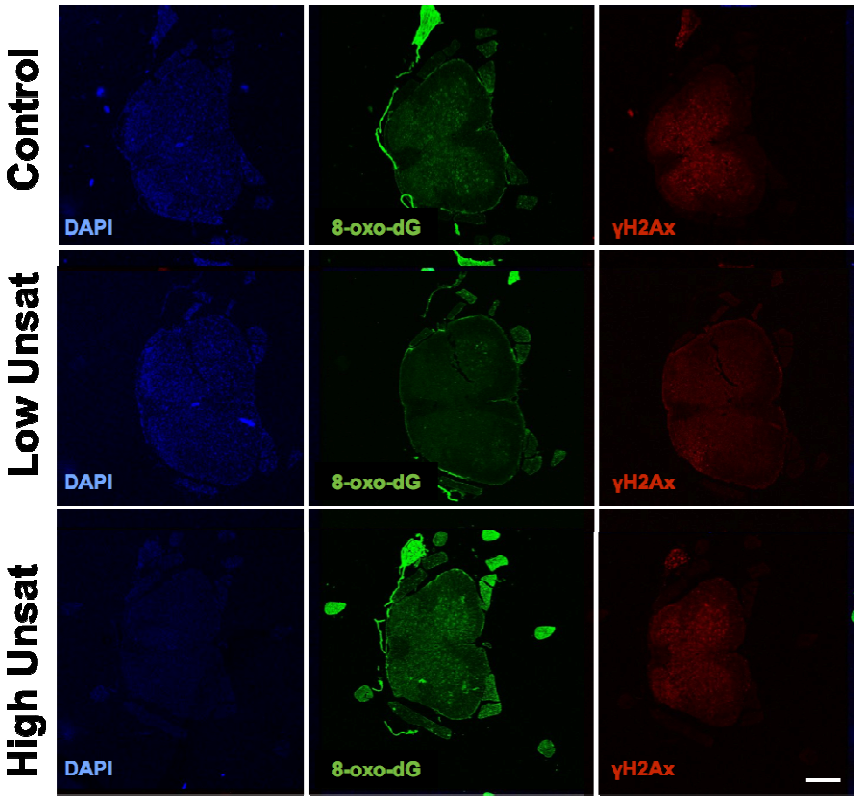
p value reflects statistical significance between diets by Bonferroni multiple comparison test after ANOVA analyses.

Supplemental Figure 1

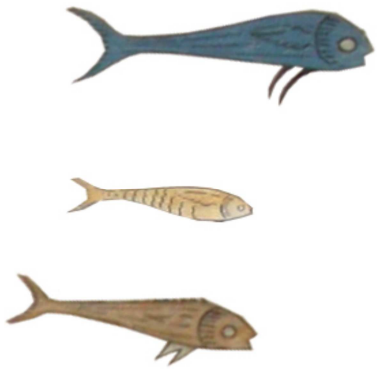


*Supplemental Figure 1. Effect of diet and gender in median survival (A). B and C show the effect of experimental diets in survival curves separated by gender (middle for male and lower for female gender). * In A indicates significant difference by Bonferroni post hoc after 2 way ANOVA ($p<0.05$ with reference to control or highly unsaturated diets). * In B indicates significant differences by Logrank test for trend between diets in males ($\chi^2=4.05$; $p<0.04$). D and E show the effect of experimental diets in clinical onset, according to gender*

Supplemental Figure 2



Supplemental Figure 2. Effect of dietary unsaturation in oxidative DNA modification (8-oxo-dG) and its relationship with nuclear DNA damage response (γH2Ax). Bar is 0.5 □m.



4.4 Article 4

Title: “Gender Specific Differences in Spinal Cord Mitochondrial Function and Oxidative Damage Markers in a Mouse Model of ALS in Early Stages of Disease”.

Authors: Cacabelos D, Boada J

Gender specific differences in spinal cord mitochondrial function and oxidative stress markers in a mouse model of ALS in the early stage of disease

Daniel Cacabelos, Omar Ramírez, Pascual Torres, Ana Belen Granado-Serrano, Victòria Ayala, Isidro Ferrer[1], Jordi Boada, Reinald Pamplona and Manuel Portero-Otín.

Running title: gender and spinal cord mitochondria in G93A mice

From:
Departament de Medicina Experimental, Facultat de Medicina, IRBLLEIDA, UdL, Lleida, and[1] IDIBELL-Ciberned, Bellvitge, Barcelona, Spain.

Correspondingauthor:
Manuel Portero
NUTREN-nutrigenomics,
Edifici Biomedicina I,
IRBLLEIDA UdL
Av. Alcalde Rovira Roure, 80
25198 Lleida
Catalonia
Tel 973702938; Fax: 973702426; e-mail: manuel.portero@mex.udl.cat

Abstract

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease with a gender bias towards major prevalence in male individuals. Several data suggest the involvement of oxidative stress and mitochondrial dysfunction in its pathogenesis, though differences between genders have not been evaluated by state-of-the art analytical approaches. This study aimed to compare mitochondrial function in male versus female in spinal cord of a mouse model of ALS the G93A-hSOD1 overexpressing mice, by using high-resolution respirometry. This was measured both in the early pre-symptomatic and pre-symptomatic stages, at onset and near endpoint of the disease. Subsequently, we analyzed mass-spectrometric markers of protein oxidative damage and tissue lipid profile. Mitochondrial respiratory chain protein expression and high resolution respirometry were also analysed in Neuro 2A cell cultures transfected to overexpress G93A-hSOD1 after pretreatment with17β-estradiol. Overexpression of G93A mutated human SOD1 in transgenic mice decreased efficiency of mitochondrial oxidative phosphorylation, revealing a temporal delay in females respect to males associated to a parallel increase in protein oxidative damage. Neuro 2A cells overexpressing G93A-hSOD1 showed a reduction *in situ* mitochondrial complex I O_2 consumption prevented by 17β-estradiol pretreatment. In conclusion, ALS associated SOD1 mutation leads to a delayed mitochondrial dysfunction in female mice in comparison with males partially attributable to the higher oestrogen levels of the former. This study may be important to further understand whether different degrees of spinal cord mitochondrial dysfunction are related to the earlier onset and faster disease progression in male ALS patients than in their female counterparts.

Introduction

Amyotrophic lateral sclerosis (ALS, OMIM #105400) is the motor neuron disorder with a higher occurrence in adult humans, characterized by a progressive loss of upper and lower motor neurons that leads to muscular atrophy, paralysis and death after median disease duration of 3 years (Leigh, 2007). The most common form of ALS is sporadic (sALS), having no apparent heritability, whereas the dominantly inherited familial ALS (fALS) accounts for only about 5 to 10% of all ALS patients (Al-Chalabi et al., 2012; Byrne et al., 2011). Among the genetic causes of ALS, Second to hexanucleotide repeat expansions in chromosome 9 open reading frame 72 (C9ORF72) (DeJesus-Hernandez et al., 2011; Renton et al., 2011), 15-20% of fALS cases and about 5% of sALS cases are associated to multiple mutations in the gene for Cu/Zn-superoxide dismutase (SOD1), which imparts a novel toxic function to this otherwise superoxide radical scavenging enzyme in normal condition (Rosen, 1993). Furthermore, mutated SOD1 forms appear misfolded, aggregated and accumulated primarily in spinal cord motor neurons and glial cells of ALS patients (Nagai et al., 2007). Transgenic mice overexpressing mutant forms of the human SOD1 (hSOD1) gene develop a progressive motor neuron syndrome similar to the human ALS phenotype (Gurney et al., 1994; Turner & Talbot, 2008) and have been extensively used as an experimental model to gain insight into ALS pathogenesis. Consequently, overexpression in animals and cultured cells of a mutated for of hSOD1 with a glycine for alanine substitution in amino acid 93 (G93A-hSOD1), has become the most used model to study pathophysiological ALS features.

Gender-related differences in many neurodegenerative diseases are observed across epidemiologic studies, pathophysiology and treatments (Crawford & Duncan, 2012; Członkowska, Ciesielska, Gromadzka, & Kurkowska-Jastrzebska, 2006). ALS not being an exception, its incidence (average male/female ratio 1.3) and prevalence are higher in men than in women, with a predominance of men with younger disease onset. Clinical phenotypes are also different in male and female patients, especially regarding to the site of onset of weakness, as well as cognitive impairment (Blasco et al., 2012; Portet, Cadilhac, Touchon, & Camu, 2001). The aforementioned gender differences have been reported in studies that included all ALS patients (sporadic and familial) but when fALS cases were studied separately, the gender ratio again was not equal to 1 (McCombe & Henderson, 2010), although according to some previous reports, fALS incidence was not significantly different between men and women, as theoretically expected with an autosomal dominant disease with complete penetrance (Orrell et al., 1999). However, supporting the higher occurrence of fALS in men than in women, data extracted from ALSoD web site (www.alsod.iop.kcl.ac.uk) revealed a c. 1.5 male/female ratio for most mendelian ALS-related mutant genes, including SOD1. Moreover, it has been reported that gender can account for variations in the course of disease in fALS (H. Y. Kim et al., 2007).

Most of the neurodegenerative disorders involve either causally or consequently mitochondrial abnormalities (Federico et al., 2012; Lin & Beal, 2006; Martin, 2012) (reviewed in (Lezi & Swerdlow, 2012)). Although the underlying causes of motor neuron degeneration in ALS remain largely unknown, ALS-causing SOD1 mutations result in mitochondrial dysfunction, among multiple pathogenic pathways also present in sALS, such as oxidative stress and endoplasmic reticulum stress (Ayala et al., 2011; Cozzolino, Ferri, Valle, & Carrì, 2013; Ilieva et al. 2007; Rothstein, 2009). A common trait of skeletal muscle biopsies of sALS and fALS patients, as well as transgenic animal models of ALS, is a mitochondrial electron transfer chain (ETC) dysfunction, leading to an impairment of ATP synthesis, metabolic stress, retrograde neurodegeneration and ultimately motor neuron cell death (Bowling, Schulz, Brown Jr, & Beal, 1993)(Vielhaber et al., 2000; Wiedemann et al., 1998). Being not restricted to

muscle mitochondria, similar deficits in the mitochondrial ETC have been reported in spinal cord of fALS patients, due to, among other causes, mitochondrial DNA mutations or binding of misfolded and aggregated SOD1 mutants to the voltage-dependent anion channel, dissipating the mitochondrial membrane potential (Jung, Higgins, & Xu, 2002; Mattiazzi et al., 2002). Moreover, besides its role in bioenergetics, buffering of intracellular calcium is one of the most important functions of the mitochondrion (Rizzuto, De Stefani, Raffaello, & Mammucari, 2012). Not surprisingly, mitochondrial dysfunction in spinal cord of ALS patients correlates with increased cytosolic calcium (Siklós et al., 1996). Likewise,SOD1-G93A transgenic mice exhibit a cytosolic calcium overload concomitant with a decrease in mitochondrial calcium loading capacity preceding neuromuscular alterations (Damiano et al., 2006) and a those changes are age-dependent (e.g. calpain activation and CaMKIV processing (Gou-Fabregas et al., 2014)).

Mitochondrial dysfunction may also directly provoke cell death by activating the apoptotic cascade, due to misfolded or aggregated SOD1 that results either in aberrant localization and release of proapoptotic factors, or binding to apoptotic inhibitors (Redler & Dokholyan, 2012). Studies in different species have reported that mitochondria in females are more differentiated, and, as a result, the mitochondria show a higher capacity and efficiency of substrate oxidation than in males. These features of female mitochondria have been described in nervous tissue, among other organs and tissues (Colom, Oliver, Roca, & Garcia-Palmer, 2007; Colom, Alcolea, et al., 2007; Guevara et al., 2009; Guevara, Gianotti, Roca, & Oliver, 2011; Justo et al., 2005). Results of several authors suggest a greater sensitivity of males to mitochondrial dysfunction compared to females. This includes higher permeability of the outer and inner mitochondrial membranes (whose occurrence is known to be affected by Ca^{+2} overload (Brustovetsky, Brustovetsky, Jemmerson, & Dubinsky, 2002)) with increased translocation of apoptosis-inducing factor (AIF) to the nucleus in neurons from immature rat brains submitted to hypoxia-ischemia (Weis et al., 2012). Moreover, male mitochondrial Ca^{+2}reuptakeis known to be increased respect to females (Arieli, Gursahani, Eaton, Hernandez, & Schaefer, 2004).

In brain, other mitochondrial abnormalities related to neurodegeneration and more pronounced in males than females are an inhibition of mitochondrial electron transport chain complexes activities, a diminished mitochondrial mass and a lower mitochondrial membrane potential (Renolleau, Fau, & Charriaut-Marlangue, 2008; Renolleau, Fau, Goyenvalle, & Charriaut-Marlangue, 2007; Xia, Zhou, Huang, & Xu, 2006). Further, male mesencephalic neurons revealed a higher vulnerability to 6-hydroxydopamine compared with female cells. This difference in viability could be due to higher ROS generation and a more pronounced down-regulation of mitochondrial gene transcription, accounting for a more diminished ETC activity and subsequently lower ATP production in the male compared with female cells (Misiak, Beyer, & Arnold, 2010). Finally, focusing in gender relevance in the mitochondrial permeability transition pore formation in the SODG93A mouse model researchers (H. J. Kim, Magranè, Starkov, & Manfredi, 2012) demonstrated that deletion of cyclophilin D (a protein structurally linked to the mitochondrial permeability membrane transition pore (Doczi et al., 2011)) completely abolished the phenotypic advantage of female SODG93A, but no effect was found in males.

Taking this background into account, the aim of this paper was to study gender-related differences in spinal cord mitochondrial function of a mouse model of ALS, including the early pre-symptomatic stage of the disease. Several features of mitochondrial oxidative metabolism, lipid profile and proteinoxidative stress markers have been analysed in SOD1-G93A transgenic mice and Neuro 2A cells overexpressing G93A-hSOD1. We hypothesized that differences in the

degree of spinal cord mitochondria dysfunction between male and female SOD1-G93A mice could explain, at least in part, their different clinical features. This study may be important to further understand whether different degrees of spinal cord mitochondrial dysfunction are related to the earlier onset and faster disease progression in male ALS patients than in their female counterparts. Moreover, understanding the causes of the sex differences in ALS could give clues to the pathophysiology of this devastating disease.

Materials and Methods

Chemicals

All reagents, unless stated otherwise, were purchased from Sigma Aldrich (St. Louis, MO). Media, sera, antibiotics and other media supplements and Lipofectamine 2000,were obtained from Invitrogen (El Prat de Llobregat, Catalonia). 17β-estradiol (E2) was dissolved in ethanol at a concentration of 1 mM, further diluted to 40 µM with culture medium, aliquoted and stored frozen up to one month at -20ºC. E2 stock solutions were used at a final concentration of 10 nM.

Animals

A colony of the strain B6SJL-Tg (SOD1-G93A)1Gur/J (JAX catalogue stock number 002726; from now on G93A mice) was purchased at The Jackson Laboratories (Bar Harbor, ME) and maintained in the B6SJL background, by male founder crossing with B6SJLF1/J females. All mice were genotyped as described in the Supplemental Methods section, and those non-transgenic littermates (from now on Control mice) where used as control. Animals were maintained under a constant 12h light–dark cycle in individual cages after weaning (day 21) with temperatures around 22±4 ºC and fed a standard rodent chow and water *ad libitum*. Animals were weighted weekly and daily for those under stride length analysis. A third-order polynomial curve was fitted to the age-body weight sets of data with Prism 6.0 software (GraphPad, La Jolla, CA) in order to calculate maximum weight and age of attaining maximum weight, and to interpolate age attaining a maximum weight minus 10%. Neurological scoring and paw print analysis were performed as previously reported (Cacabelos et al., 2014) in alternate days starting from day 60 and are further described in the Supplemental Methods section. All experimental procedures were approved by the Ethical Committee for Animal Testing of the Institut de Recerca Biomèdica de Lleida (IRBLleida) and were conformed to the Directive 2010/63/EU of the European Parliament.

Spinal cord sample preparation and homogenization

Animals were sacrificed by cervical dislocation at indicated times, after being fasted overnight. Spinal cord lumbar sections were rapidly excised, and kept in ice-cold saline, or frozen in liquid nitrogen and stored at -80ºC, to perform respirometry or for other analysis, respectively.

Frozen samples were thawed on ice and homogenized at 0ºC in a buffer containing 180mM KCl, 5 mM 3-[N-morpholino]propanesulfonic acid, 2mM ethylenediaminetetraacetic acid (EDTA), 1mM diethylenetriaminepentaacetic acid, 1µM freshly prepared butylated hydroxyl toluene (BHT), 10 µg/ml aprotinin, and 1mMphenylmethylsulfonyl fluoride (PMSF), at pH 7.3 using a Potter-Elvehjem motor-driven glass-Teflon homogenizer. After a brief centrifugation (500 x g, 5 min) to pellet cellular debris, protein concentrations were measured in the supernatants using the Bradford assay.

The spinal cord slice permeabilization protocol was a modification of a previously published method (Safiulina et al., 2004). Fresh spinal cords were rinsed with ice-cold normal saline and cut into slices with a tissue chopper adjusted to a cut width of 300 µm. About 12-15

slices of the lumbar region were collected and transferred quickly into individual wells of a 6-well tissue culture plate with 2 ml of ice-cold permeabilization medium (in mM: 7.23 potassium ethylene glycol-bis(b-aminoethyl ether)*N*,*N*,*N'*,*N'*-tetraacetate (K_2 EGTA), 2.77 CaK_2 EGTA, 60 *N*,*N*-bis[2-hydroxyethyl]- 2-aminoethanesulfonic acid (BES), 5.69 MgATP, 20 taurine, 3 K_2HPO4 , 0.5 dithiothreitol and 81 potassium methanesulfonate, pH 7.1 at 25ºC), rinsed and immediately transferred again into another well with the same medium containing 20 µl of saponin stock solution (5 mg/ml; final concentration 50 µg/ml). Lumbar spinal cord slices (LSCS) were shaken by gentle agitation at 4ºC for 30 min. Afterwards, all samples were quickly transferred from the saponin permeabilization medium into 2 ml of respiration medium (in mM: 7.23 K_2 EGTA, 2.77 CaK_2 EGTA, 100 potassium salt of 2-(N-morpholino)ethanesulfonic acid (K-MES), 1.38 $MgCl_2$, 20 taurine, 3 K_2HPO4 , 0.5 dithiothreitol, 20 imidazole and 5 mg/ml bovine serum albumine (BSA), pH 7.1 at 25ºC), and shaken by gentle agitation for 10 min at 4ºC before performing respirometry.

High resolution respirometry

Oxygen flux of sets of 5 mice LSCS (n=10-20 per genotype) or suspensions of Neuro-2A cells (100,000 cells/mL) was measured at 37 °C by high-resolution respirometry using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) with chamber volumes set at 2 mL. In order to avoid tissue disaggregation, LSCSC required setting a slow bar stirring speed (150 rpm), but not too low to compromise homogeneity of substrate and oxygen concentrations in the measuring chambers, and therefore signal stability. Stirring speed for suspensions of Neuro-2A cells was set to 400 rpm after a trypan blue exclusion assay showed cell viability was > 95% (data not shown) after 1 h of agitation. DatLab software (Oroboros Instruments, Innsbruck, Austria) was used for data acquisition (2 s time intervals) and it was also used for analysis, which includes calculation of the time derivative of oxygen concentration and correction for instrumental background oxygen flux (Gnaiger, 2001).

Respiration of LSCS and Neuro-2A cells was analysed following two standardized protocols. The experimental regime for intact LSCS and Neuro-2A cells started with routine respiration, which we defined as the oxygen flux in Hank's balanced solution and cell culture medium, respectively, without additional substrates or effectors. After observing steady-state respiratory flux in the time interval between 5 and 10 min after closing the chamber, respiration was inhibited by sequential addition of rotenone at 0.5 µM (to test for the effect of inhibiting complex I activity) and antimycin A at 2.5 µM (inhibiting complex III). This titration method was completed within 30 min. In order to avoid oxygen limitations, all the experiments were performed above 50% oxygen saturation. Oxygen consumption was normalized by actual protein content and citrate synthase activity in the respirometer chambers.

The second experimental regime required tissue or cell permeabilization before placing the sample in the measurement chamber. Initially, we measured endogenous respiration in the absence of additional substrates. For evaluation of relative contributions of mitochondrial complexes to oxygen consumption, several specific mitochondrial inhibitors and substrates added sequentially as routinely performed in our laboratory (López-Erauskin et al 2013) and calculated as steady-state respiratory flux in the time interval between 5 and 10 min after its addition. First, we added glutamate (10mM) and malate (5mM) to increase NADH levels in order to measure the complex I non-phosphorylative activity, or state 2. 10 mM ADP was added to quantify the complex I-dependent phosphorylative activity, or state 3. Immediately afterwards, we added succinate (10mM), which is the substrate for complex II. At this point, the level of oxygen consumption corresponded to complex I- and II-dependent phosphorylative activity. The addition of 0.5 µM rotenone inhibits complex I; therefore, oxygen consumption measured after the addition of rotenone only reflects complex II-dependent phosphorylative

activity (in absence of electron backflux to complex I). Then, complex III activity was inhibited with antimycin A (2.5 μM), and finally complex IV maximal activity was measured after addition of the non-physiological substrate *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) at 0.5 mM, ascorbate (2mM) and cytochrome c (10 μM). Maximal oxygen consumption rates of spinal cords were measured after addition of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). In order to avoid oxygen limitations, all the experiments were performed above 50% oxygen saturation. Oxygen measurements media for permeabilized tissue was Mir05 (Pesta & Gnaiger, 2012) Oxygen consumption was normalized by actual protein content in the respirometer chambers.

Western blot analysis

The protein levels of Neuro-2A cells mitochondrial respiratory chain complexes, were estimated using Western blot analysis. Equal amounts of protein (10-25 μg) were separated by SDS-PAGE gels. Proteins were transferred using a Mini Trans-Blot Transfer Cell (BioRad) to polyvinylidenedifluoride membranes (Immobilon-P, Millipore). Immunodetectionwas performed using specific antibodies for the 39kDa (NDUFA9; CI) subunit of complex I (1:1000), 70 kDa subunit (Flavoprotein) of complex II (1:500). (Ref. A21344 and A11142, respectively; Molecular Probes, Eugene, OR). Expression of mutant and wild type human SOD1 in mice spinal cords was immunodetected using a monoclonal antibody specific against human SOD1 (Abcam AB52950). An antibody to porin (1:5000, A31855, Molecular Probes) or or □-actin (1:5000, AB20272, Abcam, Cambridge, MA)as a control for total mitochondrial mass or total protein charge were also used in order to determine the proportion of protein levels referred to total mitochondrial mass or total protein content. Appropriate peroxidase-coupled secondary antibodies anti-rabbit (1:40000, Pierce, Rockford, IL, USA) or anti-mouse (1:10000, GE Healthcare, UK) and chemiluminescence horseradish peroxidase (HRP) substrate (Millipore, Billerica, MA) were used for primary antibody detection. Signal quantification and recording was performed with Chemi-Doc equipment (Bio-Rad Laboratories, Inc., Barcelona, Catalonia). Protein concentration was determined by the Bradford method. Data were expressed as arbitrary units.

Oxidation-derived protein damage markers measurements by GC/MS

Gas chromatography-mass spectrometry (GC/MS) measurements of glutamic semialdehyde (GSA), aminoadipicsemialdehyde (AASA), N^{ε}-(carboxyethyl)-lysine (CEL), N^{ε}-(carboxymethyl)-lysine (CML), and N^{ε}-(malondialdehyde)-lysine (MDAL) concentrations in total proteins from mice lumbar spinal cord homogenates were measured as routinely performed in our laboratory (Vigerust et al., 2012) (See Supplemental Methods for further information). Samples were homogenized in 10 volumes of ice-cold homogenization buffer (0.32 mol/L sucrose, 10 mmol/L HEPES pH 7.4, 2 mmol/L EDTA, containing the protease inhibitor cocktail (Sigma P8340) and homogenized using 10 to 15 strokes of a motor-driven glass-Teflon homogenizer. Protein concentrations were measured by the Bradford method, and samples were stored at -80°C. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS and the amounts of products were expressed in μmol of analyte per mol of lysine.

Lipid profile

Fatty acid analysis in tissue samples and diets was performed as previously described (Cacabelos et al., 2014). Total lipids from homogenates were extracted with chloroform/methanol (2:1, v/v) (3 times) in the presence of 0.01 % butylated hydroxytoluene. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by

incubation in 2 ml of 5 % methanolicHCl for 90 min at 75 ºC. The resulting fatty acid methylesters were extracted by adding 2 ml of n-pentane and 1 ml of saturated NaCl solution. The n-pentane phase was separated, evaporated under nitrogen, re-dissolved in 80 µl of carbon disulphide, and 2 µl was used for GC analysis. Separation was performed by a DBWAX capillary column (30 m × 0.25 mm × 0.20 lm) in an Agilent GC System7890A with a Series Injector 7683B and a FID detector (Agilent Technologies, Barcelona, Spain). The injectionport was maintained at 220 ºC, and the detector at 250 ºC; the temperature programme was 2 min at 100 ºC, then10 ºC/min to 200 ºC, then 5 ºC/min to 240 ºC and finally hold at 240 ºC for 10 min. Identification of fatty acidmethyl esters was made by comparison with authentic standards (Larodan Fine Chemicals, Malmö, Sweden). Results are expressed as % of analyte from the total % of FAMEs founded.

Cell culture and transfection

Neuro-2A cell line was obtained from ATCC (#CCL-131) and grown in MEM-Advanced medium without phenol red supplemented with 10% heat inactivated foetal bovine serum, 2 mM L-glutamine,20 U/ml penicillin and 20 µg/ml streptomycin. Cells were kept at 37 ºC in a humidified atmosphere with 5% CO_2. Neuro-2A cell permeabilization prior to respirometry and sample homogenization for protein expression and activity measurements were performed as described above for LSCS.

After 10 days of pre-treatment with 10nM E2 or vehicle, cells were harvested, counted, and subcultured in 6-well plates (200,000 cells per well) overnight. Transfection was performed by using Lipofectamine2000 (Invitrogen,El Prat de Llobregat, Catalonia) according to manufacturer instructions. Briefly, lipofectamine and selected DNA plasmids were mixed (1µg of DNA/1µL of lipofectamine) with Optimen medium (Invitrogen) for 20 min and the resulting mixture was dispensed to the cell cultures (10 µg DNA per well). The plasmids pEGFP-G93A-hSOD1 and pEGFP-wt-hSOD1, expressing G93A mutant or wild type human SOD1 tagged with enhanced green fluorescent protein (EGFP) were kindly provided by Dr Josep Esquerda (Lleida, Catalonia). The pEGFP expression vector was used for mock transfection.

Cytotoxicity Assay and ATP content

Viability of Neuro-2A cells was assessed with a LDH Cytotoxicity Assay Kit (Promega, Madison, WI) according to manufacturer's instructions. Briefly, the amount of lactate dehydrogenase (LDH) released into the culture medium is measured using an enzymatic reaction that results in a red formazan product which can be measured spectrophotometrically. Lactate dehydrogenase (LDH) is a cytosolic enzyme that is is an indicator of cellular toxicity. The cell viability was evaluated relative to the total LDH from whole cell lysate and the results were expressed as the percentage of viability versus treated with vehicle and/or mock transfected cells.

Adenosine triphosphate (ATP) content in Neuro 2A cell homogenates was measured with the ATP Bioluminiscent Assay Kit (Sigma) according to the manufacturer's instructions. Results are expressed as nanomole ATP per milligram protein.

Statistical analysis

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL) or the Prism software (GraphPadSoftware Inc.,La Jolla, CA). Differences between groups were analyzed by the Student's t tests or ANOVA with Post-Hoc analyses, after normality of variable distribution was ensured by Kolmogorov-Smirnov test. Correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results and discussion

Disease onset, clinical evolution and survival ofG93A mice

Our G93Amouse colony exhibited a phenotype consistent with the overexpression of the G93A-hSOD1 transgene (Gurney, 1997). G93A-hSOD1 protein expression in spinal cord of transgenic mice was verified by Western-blot analysis, using a monoclonal antibody specific against human SOD1, which has been shown to recognize distinct mutated human SOD1 forms, including G93A-hSOD1 (Sotelo-Silveira et al., 2009), Figure S0. G93A mice gained weight as expected, reaching a higher ($p < 0.05$) maximum value for males (21.1±0.2g, n=11) than for females (18.0±0.1g, n=12)(Figure S1A). Control and G93A mice body weight was significantly higher in males than in females from weaning day. However, in G93A animals this difference is faded out along disease, with no differences found from 107 days between sexes. In control animals, both sexes continued gaining weight until endpoint (Figure S1B). Food intake was not significantly different between G93A mice of both genders at any age, neither between male and female control mice (Figure S1C, Figure S1D, respectively). Onset of clinical symptoms occurred by approximately 70–90days of age, with body weight loss, abnormal gait, muscle weakness with decreased grip strength, and impaired coordination. As an estimation of disease onset, we used the interpolated value for age of attaining maximum weight after fitting a third-order polynomial curve to the weight evolution data. Maximum body mass was attained at a significantly younger age in males (87.9±1.2 days, n=11) than in females (95.9±1.0 days, n=12; $p < 0.05$; Figure 1A, bars on the left). Female G93A mice showed a significant delay in disease progression, as evidenced by the later age by which they dropped to 10% below their maximum weight (males, 120.2±2.2 days; females, 135.5±3.9 days, $P < 0.05$; Figure 1A, bars on the right).As a second estimation of disease progression, a paw print analysis was performed to measure stride length, indicating again a slower disease progression in female G93A mice, as previously reported (Cacabelos et al., 2014). Figure 1B shows that the median value of both right and left stride lengths in G93A females was attained at a significantly older age (116.2±3.2 days, on average of both hindlinbs) than in their male counterparts (107.3±4.1 days on average of both hindlinbs). Supporting this, overall clinical duration was greater in females (37.72±2.01 days n=11 and 45.5±2.52 days n=12 for male and female, respectively; $P < 0.001$, Figure 1D). However, the age reaching the different clinical score status (Figure S1E, S1F and S1G, respectively) was similar for both sexes.The animals were euthanized when it demonstrated hind limb paralysis or inability to right itself when placed on its side. Female G93A mice survived significantly longer than males ($P < 0.05$), with survival times of 134.1±2.9 days (n=11, range 119-135) and 124.9±1.5 days (n=12, range 115-157 days), respectively (Figure 1D).

Disease onset, neurological scores, disease progression and survival of our G93A mice were consistent with those reported in the literature (Gurney, 1997), so we considered validated our animal model of ALS. Moreover, gender differences in the aforementioned clinical features of the diseased mice were also confirmed. Indeed, sex appears to modify the course of disease and lifespan in animal models of ALS with mutations in the hSOD1 gene, with an earlier disease onset in male transgenic rats and mice overexpressing G93A-hSOD1 (Suzuki et al., 2007; Veldink et al., 2003), although sex appears to have no consistent effect on survival of ALS patients(Lee, Annegers, & Appel, 1995)

Preliminary experiments performed in our laboratory displayed a peak of G93A-hSOD1 protein expression at 60 and 90 days, and lower levels before and after (30 days and 120 days, respectively) in a mixed population of male and female G93A mice. After analysing separately male and female mice, the same pattern of mutated hSOD1 expression was observed (data not shown). Thus, we rule out different levels of aggregated or aberrant SOD1 activity between

male and female transgenic mice as the primary origin of gender differences in mitochondrial dysfunction and levels of protein oxidative modification markers.

Oxygen consumption of mice lumbar spinal cord slices

Respirometric analysis of mitochondrial function provides a tool to study changes in mitochondrial respiratory chain function and mitochondrial ATP production within tissue biopsies, cultured cells and isolated mitochondria (Hütter, Unterluggauer, Garedew, Jansen-Dürr, & Gnaiger, 2006). In order to assess gender-related differences in spinal cord mitochondrial function of a murine model of ALS both in presymptomatic and diseased stages, oxygen consumption in LSCS of male and female non transgenic and transgenic mice overexpressing G93A-hSOD1 were analysed in two standardized experimental regimes in 30, 60, 90 and 120 days old animals.

First, using intact (not permeabilized) slices, and therefore resembling in vivo spinal cord cell conditions, we found significant differences in routine respiration (measured in Hank's balanced salt solution containing 10mM Hepes, pH 7.4, without additional substrates, inhibitors or uncoupling agents) between control and transgenic mice at 120 days of age, with a reduced oxygen uptake for both genders (control *vs.* transgenic; males, 37.0±3.8 *vs*. 22.5±2.7 pmol O_2/min×mg protein, $P < 0.01$; females, 40.1±1.5 *vs*. 31.9±2.8 pmol O_2/min×mg protein, $P < 0.01$; Figures S2A and S2B). Interestingly, when comparing 120 days old male and female G93A mice, routine respiration was 41.7% higher in females ($P < 0.05$), while no differences appeared between control mice of both sexes (Figure 2A and Figure S4A, respectively). Then, rotenone was added to the measurement chambers in order to inhibit the mitochondrial complex I, followed by the inhibitor of complex III, antimycin A. When rotenone-resistant respiration is subtracted to routine respiration, an estimation of the contribution of the mitochondrial chain complex I to cell or tissue respiration is obtained. Likewise, when antimycin-resistant respiration is subtracted to rotenone-resistant respiration, the resulting value is an estimation of the contribution of mitochondrial chain complex II to total respiration. According to the aforementioned calculation, the estimated complex I respiration was lower in male G93A mice respect to control animals by 26.0% ($P < 0.05$), 26.7% ($P < 0.05$) and 33.9% ($P < 0.01$) at 60, 90 and 120 days, respectively (Figure S2C), whereas in females, complex I respiration in transgenic mice was significantly reduced (25.0%, $P < 0.001$) only at an age of 120 days, and, intriguingly, augmented by 28.7% ($P < 0.01$) at 30 days (Figure 2SD). When we compared complex I respiration between male and female G93A mice, the decrease in oxygen consumption was more pronounced in the former in all age groups, with the exception of 30 days. Specifically, complex I respiration in G93A females was significantly higher than in males at 90 days (31.4%, $P < 0.05$) and 120 days (25.6%, $P < 0.05$), and also at 60 days (20.3%), although the difference was not significant ($P = 0.0760$) (Figure 2B). In control animals, complex I respiration was 39.6% higher ($P < 0.01$) in 30 day old males, whereas in older animals the values were similar in both genders, lacking significant differences (Figure S4B). Regarding complex II, its estimated oxygen uptake in control mice intact LSCS was significantly higher for females at 30 days (Figure S4C). However no gender differences were found for G93A animals (Figure 2C) along disease. Confirming this, no differences were found for G93A respect to control ones, neither globally, nor separated by gender (Figure S2E and S2F for male and female, respectively). Residual oxygen consumption is the respiration due to oxidative side reactions remaining after application of mitochondrial chain complex III inhibitors, such as antimycin A. Additionof antimycin A to the measurement chambers provoked a sharp fall in oxygen uptake that revealed a similar pattern for both genders expressing SOD1G93A, hence no differences were found between them (Figure 2D). Antimycin

A-resistant respiration was higher in control mice LSCS of both genders at 30 days (control *vs.* transgenic; males, 15.4±1.3 *vs.* 6.2±1.1 pmol O_2/min×mg protein, $P < 0.001$; Figure S2G; females, 15.6±0.7 *vs.* 9.8±2.0pmol O_2/min×mg protein, $P < 0.01$; Figure S2H) and 120 days (control vs. transgenic; males, 8.8±1.2 vs. 4.6±0.9pmol O_2/min×mg protein, $P < 0.01$; Figure S2G; females, 9.7±1.2 *vs.* 6.7±0.7pmolO_2/min×mg protein, $P < 0.05$; Figure S2H). Similar results were obtained when the total population of mice (males plus females) was analysed (data not shown). Interestingly, opposite results were obtained in residual respiration at 60 days and 90 days, displaying higher rates in male G93A mice at 60 days (control *vs.* transgenic; 8.0±1.8 *vs.* 13.7±1.0 pmol O_2/min×mg protein, $P < 0.01$; Figure 2G) and in female G93A mice at 90 days (control *vs.* transgenic; 7.2±1.2 *vs.* 12.0±1.0pmol O_2/min×mg protein, $P < 0.01$; Figure 2H). When comparing antimycin A-resistant respiration in G93A mice of both genders, this rate was 53.9% ($P < 0.05$) higher in males than in females at 60 days (data not shown).

Consistent with previous reports (Mattiazzi et al., 2002) routine (intact tissue, without any inhibitor) respiration only shows evident reduction of G93A animals at end stage of disease. This reduction was also verified when comparison between male and female was done in G93A overexpressors, but was abolish for control animals all studied points, suggesting that differences founded are attributable to G93A overexpression. Although the routine respiration of intact LSCS was not significantly different in males and female G93A mice (only at endpoint), the higher rotenone resistant respiration in males at 60 days suggests a lower Complex I contribution to overall respiration, as calculated by difference respect to routine respiration. Since routine respiration does not vary, a compensatory stimulation of Complex II respiration is plausible. Hence, we could demonstrate that G93A males consumed less oxygen than controls form day 60 (Figure S2), but females were protected (displaying even greater consumption than controls at 30 days) showing only a late reduction at endpoint.

Focusing in the G93A animals, females showed an upgraded Complex I oxygen consumption than males from 60 days (Figure 2). Nevertheless, this advantage was only significant for 90 and endpoint animals. Since rotenone mediated cytotoxicity was proved to be alleviated in female neuron primary cultures (Tao et al., 2012), this decreased respiration could lead cell survival demise in males. Studies from (Vinsant et al., 2013) showed that, in this mouse model, motor neuron abnormalities begins between 44 to 60 days. However, cytosol vacuolization and swollen and mega-mitochondrial were evident before (30 days, even P7). In line with this we also show in permeabilized slices early complex I dysfunction in males (Figure S3). On the contrary, females complex I–related reduced respiration was only evident under clinical symptoms. However, G93A-linked complex I malfunction was evident for early asymptomatic males respect to female's counterparts (Figure 2cont).

Underlying this gender dimorphism, studies from (Eschbach et al., 2013) show a male-specific protective mechanism of PGC-1α (a master metabolic regulator (Chaturvedi & Flint Beal, 2013)) in this mouse model. Further, since PGC-1α could enhance the transcriptional activities sex hormone receptors(e.g. androgen receptor (Shiota et al., 2010) or estrogen receptor (Tcherepanova, Puigserver, Norris, Spiegelman, & McDonnell, 2000)) this may ressult in a feed-back loop.

The second experimental regime required permeabilization of the LSCS with digitonin before the respirometry analysis in an artificial intracellular medium with sequential addition of mitochondrial substrates, uncouplers and inhibitors (SUIT protocol). Measurement of oxygen flux in permeabilized cells or tissues, in presence of saturating concentrations of substrates, ADP and P_i, provides an estimation of the in situ activities of the mitochondrial chain complexes, among other information, without the potential artefacts associated to the mitochondrial isolation procedure. Tissue permeabilization allows free diffusion of substances

through the cell membrane, while mitochondrial membrane integrity and permeability are not altered. Thus, the addition of substance to the measurement media allows direct manipulation of the chemical surround of the mitochondria. If the added substrates are added at saturating concentrations, its stimulating effect on its corresponding enzyme will be maximal. While this is not a physiological condition, it can give an estimation of the capacity of mitochondria to hold a metabolic challenge. Endogenous respiration is measured before addition of substrates, being supported by the remaining metabolites in the mitochondria, since the concentration of cytosolic metabolites has lowered to irrelevant levels due to its free diffusion through the permeabilized cell membrane. Under this situation no differences were found among male and female G93A overexpressors (Figure 2E), nor between control and transgenics for none sex (data not show) or even in control animals between sexes (Figure S4D). State 2 respiration (for Complex I) is achieved upon stimulation of those preparations with glutamate/malate. Hence, under this situation G93A females had higher oxygen consumption than G93A males only at early presimptomatic stages (30 days, Figure 2F). Differences between control and transgenic animals were also found for males (Figure S3A) and females (Figure S3E), but in this case, as expected, higher consumption was attributed to controls, only significant later in disease. Interestingly, male shows a dysfunction at 30 days which was not present in females. Finally, sexual dimorphism was confirmed for control animals, where at 60 and 90 days females displayed higher oxygen consumption (Figure S4E). Addition of ADP into the chambers reflects the oxygen consumption in state 3 (of complex I). Hence, in this situation, G93A females showed upgraded consumption compared with males all along disease (Figure 2G). Comparison of the overexpression separately between sexes shown G93A males (Figures S3B) underscored almost all along disease in contrast to females where decreased oxygen consumption was only evident at end stage (Figure S3F). For control animals, females showed increased complex I state 3 levels at 60 days when compared with males (Figure S4F). Succinate (substrate for the Complex II) addition serves us for complex I/II state 3 measurements. Thus, estimated measures showed early disruption in G93A females compared with males (Figure 2H) which was faded out along disease. No differences were found for males and (Figure S3C) only higher consumption was detected in controls females (Figure S3G) when compare the transgenic effect between sexes. Finally, as previously commented, rotenone addition blockades complex I and an estimation of complex II state 3 is obtained. In this situation, at 30 days there is an almost significant difference (P=0.065) between males and females overexpressing G93A which disappears along disease (Figure 2I). Splitting measures between sexes and comparison for G93A effect showed no differences for males and only endpoint G93A females had a reduced oxygen consumption compared with control littermates (Figures S3D and S3H for male and female, respectively).

Oxidative stress markers in spinal cord

To offer an accurate quantitative measurement of protein oxidation in spinal cord proteins, we measured structurally defined oxidation products by isotope-dilution GC/MS (Pamplona et al., 2005). Proteins from spinal cord samples contain oxidation products resulting from metal-catalysed oxidation (MCO), glycoxidation and lipoxidation. A general trend to decrease spinal cord levels of the five markers analyzed (GSA, AASA, CML, CEL and MDAL) with age can be observed in mice of both genders, either control or transgenic, with the exception of AASA and MDAL in female G93A mice and male control mice, respectively, at 120 days. (Figure 3) GSA and CML levels in transgenics of both genders were not different across disease (Figures S5A, S5B for males and Figures S5C and S5D for females). The comparison between male and female mice revealed that mutated hSOD1 overexpression was associated with differences in the steady-state levels of protein oxidative damage markers.

AASA, a MCO marker, displayed significantly 47.5% ($P < 0.01$; Figure 3A) and 39.5% ($P < 0.01$; Figure 3D) higher levels in spinal cord proteins of G93A mice at 60 days in males, and at 90 days in females, respectively, than in control mice. CEL is a glycoxidation marker whose levels were found surprisingly higher in spinal cord of female control mice at 90 days (Figure 3E). Finally, levels of the lipoxidation marker MDAL were augmented in spinal cord of males by 30.5% ($P < 0.01$) in G93A mice at 90 days, and, unexpectedly, by 23.5% ($P< 0.01$) in control mice at 120 days (Figure 3C).

ROS formation is particularly high when Complex I is inhibited (Votyakova & Reynolds, 2001). Therefore, its blockade may account for higher ROS production, and probably increased oxidative products accretion. Antimycin-resistant cell respiration has been used as an estimation of mitochondrial ROS production. In G93A mice spinal cord, the rise in AASA levels, a marker of metal-catalyzed oxidation (MCO) of proteins, occurred after the peak of antimycin-resistant respiration suggests the mitochondrion as the origin of the cellular protein oxidative damage. This delay in protein oxidative damage with respect to mitochondrial dysfunction in observed in mice of both genders. Interestingly, there is a shift of both mitochondrial-related and oxidative stress-related pathophysiological findings in female G93A mice respect to their male littermates. These results could explain, at least in part, the delayed clinical features observed in G93A female mice respect to male mice and, possibly, also in male patients respect to the female counterparts. In any case, higher oxidative damage was expected due to the G93A overexpression. Early studies already pointed for a nonlinear oxidative accretion in this animal model (accretion of MDA in spinal cord at 30 and 60 days, whole sections, by HPLC; greater MDA inmunoreactivity in lumbar spinal cord at 100 and 120 days (Hall, Andrus, Oostveen, Fleck, & Gurney, 1998); carbonylated products early accretion -30 days- for G93A animals (Andrus, Fleck, Gurney, & Hall, 1998)) and were more recently confirmed (decreased ROS production for SODG93A animals in isolated mitochondrial preparations respect to controls, 100 days age (Vinsant et al., 2013); increased spinal cord 4-HNE and MDA at 100 and 130 respect to controls by spectrophotometery; DNP-carbonylated products accumulation was similar to controls along disease; whole spinal cord sections, no gender consideration (Miana-Mena et al., 2011); MDA accumulation at 30 and 120 days in G93A respect to controls, no gender consideration, measured by colorimetry (Kirkinezos et al., 2005)). Even when our results do not completely fit this previous findings, our measures are lumbar specific (nor cervical or thoracic sections), we split by gender and methodology applied to the measurements were different. Hence, this could explain little variations with previous reports. This goes in line with our previous publication (Cacabelos et al., 2014) (focusing only in control diet) indicating that, among oxidative modification studied, most of them (but CEL in females) have greater accretion at early clinical stages (90-100 days), rather than end stage. And in any case, one should bear in mind that those animals are overexpressing an oxidant-scavenging enzyme (SOD1), which, even with less efficiency, is functional (Yim et al 1996).

Fatty acid profile in spinal cord

Since fatty acid profile strongly influences membrane peroxidizability, and consequently protein lipoxidative damage, we analysed fatty acid content in G93A mice spinal cord at early presymptomatic (30 and 60 days), disease onset (90 days) and symptomatic (120 days) ages as well as in control animals for both gender. Those analyses revealed significant differences in the G93A-hSOD1 colony between males and females (Figure 4) as well as differences among overexpressors and controls (Figure S6) along disease. The two most remarkable changes involve the highly peroxidizables docosahexaenoic acid (DHA) and arachidonic acid (AA). Levels of DHA decreased along time (Figures 4A, S6B, S6E and S6F),

with lower levels of this fatty acid at endpoint regardless sex or transgene. Focusing in gender dimorphism of G93A animals, interestingly DHA levels, despite being significantly higher in females early in disease, this difference is faded out along it. On the other hand, for non transgenic animals, even when decreasing along the experiment, AA and DHA levels were similar for males and females (Figure S6A and S6A, respectivelly). AA levels was severely affected by transgen, since for both sexes at preclinical ages (30, 60 days) non trangenic animals accumulated more than G93A ones, but this difference turned to be the opposite at endpoint (Figures S6A, S6C and S6D). Furthermore, focusing in G93A animals, AA levels turned from being significantly lower in males at 30 days, to be significantly higher at endpoint (Figure 4B), respect to females. This different FA handling goes in line with previous reports indicating a differential n-3 synthesis for both sexes. Studies from Aessandri and collaborators showed that females have a preference towards n-3 synthesis. Greater circulating DHA levels were reported for females plasma (Astorg et al., 2008)(Welch et al., 2006) and in tissues and neuronal cell lines (Alessandri et al., 2008). However, for our control animals, we could not observe any difference across the study for this particular FA (Figure S6B). Behind this differential lipid metabolism could rely an altered desaturase activity for males and females, as reported Extier and collaborators in a model of n-3 replenishment (Extier et al., 2010). Consistent with this DHA spinal cord depletion in this animal model, previous ressults form our group (Ilieva et al. 2007) had also reported decreased DHA accretion in spinal cord of patients (this study was carried out in ALS male patients, n=7). However in this report, even when levels are greater as in animals model, this deference was not significantly altered than controls.Finally,

Overexpression of G93A-hSOD1 in Neuro 2A cells: cell viability and ATP content, oxygen consumption and mitochondrial ETC complexes protein expression.

To evaluate the effect of estrogens on mitochondrial function of isolated neuronal cells overexpressing a mutated form of SOD1, Neuro-2A cells were treated with 17β-estradiol prior to transfection with aG93A-hSOD1 plasmid (for 10 days,10 nM final concentration). The G93A mutated form of hSOD tagged to an EGFP expression vector was transiently transfected in Neuro-2A cells using Lipofectamine (from now on G93A cells). Mock transfected cells expressing EGFP were used as a control (Control cells). Direct visualization of GFP fluorescence was analysed using fluorescence microscopy to ensure transfection (efficiency ≈80-90%, data not shown) or to denote differential expression between transfectants (Figure S7). To evaluate the cytotoxic effect of hSOD1 and mutant hSOD1 overexpression, alone and combined with E2 treatment in Neuro-2A cells, we compared the viability of WT and G93A cells *vs.* mock transfected cells, pre-treated during 10 days with 10 nM E2 or vehicle, after 24h of transfection. We found that there was no significant difference in the cell viability between the three transfectants either with or without exposure to estrogen (Figure 5A). The intracellular ATP content measurements neither revealed variations between transfections and E2 pretreatment or their mock and vehicle controls, respectively (Figure 5B).

As previously shown, we analysed oxygen consumption in two standardized experimental regimes. Neuro 2A cells transfected with an EGFP plasmid were included as a second control. First, using intact (i.e. not permeabilized) cells, we did not find significant differences in routine respiration (measured in standard culture medium without additional substrates, inhibitors or uncoupling agents) or in non-phosphorylating respiration (measured after inhibition of mitochondrial chain complex V F_0-ATP synthase with oligomycin). However, maximal respiratory capacity (measured after uncoupling with FCCP) in G93A cells was significantly reduced ($P < 0.05$) compared with control cells and wt cells by 27.6% and 18.2%,

respectively (Figure 5C). Respiration was inhibited by antimycin A to an equal extent in the three transfectants, pointing to a Complex I malfunction.

To further confirm this, the second experimental regime included cell permeabilization with saponin before the respirometry analysis in an artificial medium with sequential addition of mitochondrial substrates and inhibitors, quite similar to the protocol used to analyse respiration in permeabilized LSCS as described above. Neither endogenous respiration (before addition of substrates, data not shown) nor Complex I state 2 respiration stimulated by glutamate/malate, were significantly different between the three transfectants (data not shown). However, after addition of ADP, in order to achieve complex I state 3 respiration, oxygen uptake rose to a lower level in G93A cells than in wtSOD cells and GFP cells (Figure 5D, Complex I). This respiration difference was enhanced after addition of succinate (Figure 5D, Complex I+II), but disappeared when rotenone was injected in the measurement chambers (Figure 5D, Complex II). As in the non-permeabilized cell protocol, oxygen consumption was inhibited by antimycin A to an equal extent in the three transfectants (data not shown). Hence, all in all, similar to the respirometric measurements performed with intact cells, the aforementioned differences in oxygen uptake dependent on ADP and glutamate/malate, or ADP and glutamate/malate plus succinate, were abolished when cells were pre-treated with 10nME2 before the transfection.

In order to assess the origin of the reduced mitochondrial chain complex I state 3 respiration in G93A cells, we estimated the protein expression of the complex I (39KDa, NDUFA9) as well as Complex II (70KDa, Flavoprotein) subunits. Figure 5E shows that levels of expression of two peptides representative of mitochondrial chain complex I and complex II are not significantly different neither among transfects, nor E2 treatment.

Because the contribution of motor neurons, despite being large cells, in whole spinal cord is relatively minor, these results suggest that the decrease rate of respiration in G93A mice is present in different cell types, and not motor neuron specific. In fact previous result from our group (under revision) already reported a decreased respiration in an *in vitro* (tissular) model of ALS, particularly attributed to a Complex I malfunction. In any case, most functional experiments performed with nervous tissue provide a mixed result arising from its two major cell types. While much work showing glial involvement in the pathophysiology of the SOD1-G93A genetic mouse model has been done, the ultimate result is (motor) neuron dysfunction and death. On the other hand, the above-described differences in mitochondrial dysfunction between genders in SOD1-G93A mice could be attributable to the higher levels of estrogens in female mice. Taking these two points in mind, we aimed to investigate the effect of estrogen exposure on the mitochondrial dysfunction due to SOD1-G93A expression in neuronal cells. We found the mouse neuroblastoma N2A cell line a suitable model for this part of the study because: a) cells exhibit a differentiated neuronal phenotype (Graham, Gonatas, & Charalampous, 1974), b) expression of estrogen receptors (Mendez & Garcia-Segura, 2006)(Li, Hao, Cheng, & Li, 2014) , and c) it is a suitable transfection host. As a potential limitation of the present study, it may be argued that the Neuro-2A cell line is not derived from motor neuron. However, it has been demonstrated that not only spinal and upper motor neurons are degenerated in ALS, but also interneurons and glia. Here we show that SOD1 gene mutation did not induce harmful effects on cell viability of Neuro-2A cells under basal conditions, in accordance with a previous report by Pyo (Pyo et al., 2010) using stably transfected cells of the motoneuron–neuroblastoma hybrid (VSC 4.1) cell line. Although it has been reported that expression of mutant SOD1 (G37R) resulted in a time and dose-related death of differentiated neuroblastoma cells (Flanagan, Anderson, Ross, & Oberley, 2002; Gurney, 1997) and protein expression blockade (by RNAi against hSODG93A) resulted in disease alleviation (Ralph et al., 2005).Therefore, neuro-2A cells transfected transiently with mutated SOD1 for a relatively

short time previous to their analysis, could be considered a model comparable to spinal cord neurons of early pre-symptomatic hSOD1-G93A mice. Moreover exposure to 17-β-estradiol during 10 days did not alter significantly cell survival. Supporting this, G93A cells did not exhibit differences respect to their controls in viability or metabolism, measured as mitochondrial function and ATP content.

Finally, accounting the lesser extent of early oxidative damage in spinal cord of female G93A mice could be related to the higher levels of UCP4 protein expression compared to males. Thus, our results showing an increase in complex II state 3 respiration in female G93A mice, but not in males, and a higher efficiency in terms of ATP production in females (resulting form the subtraction of , altogether with the lack of difference in complex II peptide expression (at least in the cell line), suggest a role of UCP4 in the compensatory mechanisms against bioenergetic failure in spinal cord of G93A mice and possibly ALS patients. This would be a interesting question to be addressed since the neuroprotective effect of UCP4 and UCP5 has been reported in aged rat brain (Guevara et al., 2009). In this paper, researchers have found lower oxidative damage in proteins and lipids that correlated with higher protein levels of UCP4 and UCP5 in female rats compared with their age-matched male littermates. On the other hand, as reported by Ho and colleagues, UCP4 overexpression increased ATP synthesis by specifically interacting with mitochondrial complex II in SH-SY5Y with complex I deficiency induced by 1-methyl-4-phenylpyridinium (MPP(+)) treatment (Ho et al., 2012). However, these results could also be related to the fact that all complex II subunits are encoded in the nuclear genome, and therefore its DNA would be less exposed to the ROS generated by the dysfunctional mitochondria, contrary to complexes I, III and IV, all of them containing several subunits encoded in the mitochondrial DNA. But in any case, those results in terms of an upgraded mitochondrial physiology, concomitant to an increased UCP decoupling mechanism to reduce mitochondrial ROS should be revisited in this mouse model. In a recent publication model (Peixoto et al., 2013) researchers co-expressed hUCP2 into the SOD1G93A mouse model. Even when they demonstrated a protective effect in terms of ROS production with the co-overexpression, they also reported shortened lifespan and worst mitochondrial dysfunction (more sensitive to Ca^{+2}-induced mitochondrial depolarization, in purified brain mitochondria of 100 day). They also showed an accelerated disease progression when co-expressed in the SOD1G93A background. In line with our results (even taking in to account different methodology were used), their G93A animals showed a decreased peroxide production respect to controls, when complex I is in state 2 (Figures S2A and S3B).

However, besides UCPs, other proteins may contribute to the gender-related differences. In a recent publication (H. J. Kim et al., 2012) researchers focused in gender relevance in the mitochondrial permeability transition pore formation in the SODG93A mouse model. Hence, they demonstrated that ablation of cyclophilin D (a protein structurally linked to the mitochondrial permeability membrane transition pore (Doczi et al., 2011)) completely abolished the phenotypic advantage of female SODG93A, but no effect was found in males. In fact, they showed that 17β-estradiol protected SODG93A cortical neurons and spinal cord motor neurons against glutamate toxicity in brain mitochondria, but the protection was lost in neurons lacking cyclophilin D.

In summary, we have described an early mitochondrial defect during the pre-symptomatic stage associated to the expression of G93A mutated human SOD1 that appears at a younger age in males mice than in females, correlating with the delay in their clinical features. These data support a role for dysfunctional mitochondrial function control of spinal cord neurons in ALS pathogenesis and, in particular, the gender bias towards males observed in ALS. Thus, mitochondrial enzymes, such complex I or malate dehydrogenase, may represent viable

targets for novel treatments of a range of disorders affecting motor systems. This possibility underscores the importance of further studies investigating the role of sexual hormones or related molecules in the face of injury and disease as well as their potential contribution to motor recovery.

Figure Captions

Figure 1.- Disease onset and progression (A), stride length analysis (B), clinical progression (C) and survival curves (D) of the G93A colony, depicted by sex. Bars in (A) represented means ± SEM of the age at maximum weight, expressed in days. Bars in (B) represented means ± SEM of the age of clinical onset measured after stride length analysis, expressed in days. Dots in (C) represented disease duration among the G93A colony, depicted by sex, expressed in days. (D) represented a Kaplan-Meyer survival curve. *, P<0.05, between male and female mice of the same age by Student's t test. **, P<0.01 after a Kaplan-Meyer analysis.

Figure 2.- Oxygen flux of sets of 5 intact lumbar spinal cord slices from male and female G93A mice measured at 30,60, 90 and 120 (endpoint) days of age. Experimental procedures allows show basal (A), complex I (B), complex II (C) or residual (D) oxygen consumption differences between male and female. Measurements were taken at 37 ºC in Hank's balanced salt solution containing 10mM Hepes, pH 7.4, and corrected for instrumental background oxygen flux. Values were normalized by actual protein content in the respirometer chambers. Means ± SEM, expressed as pmol O_2/s·mg protein. *, P<0.05, between male and female mice of the same age by Student's t test.

Figure 2cont.- Oxygen flux of sets of 5 lumbar spinal cord slices from male and female G93A mice measured at 30,60, 90 and 120 (endpoint) days of age. Permeabilized LSCS measures on endogenous (E), Complex I state 2 (F), complex I state3 (G), complex I/II state 3 (H) and complex II state3 (I) oxygen consumption conditions between males and females overexpressing G93A. Measurements were taken at 37 ºC in Mir05 media. Values shown are means ± SEM expressed as pmol O_2/min×·mg protein for 4-6 independent measures. *, $p < 0.01$ respect to females of the same age by Student's t test.

Figure 3.- GC/MS analyses of protein oxidation products in LSCS from male and female G93A and control littermates measured at 60, 90 and 120 (endpoint) days. (A) and (D) represented levels of AASA, for male and female, respectively. (B) and (E) represented levels of CEL for male and female, respectively. (C) and (F) represented MDAL levels for male and female, respectively. Values shown are means ± SEM expressed as µmol of analyte per mol lysine. *, $p < 0.01$ respect to non transgenic group of the same age by Student's t test.

Figure 4.GC analyses of lipids in LSCS from male and female G93A measured at 30, 60, 90 and 120 (endpoint) days. (A) Shows DHA levels along disease. (B) Shows AA levels along disease course. Bars represented mean values ± SEM expressed as % of abundance of analyte among the total FAME identified. *, $p < 0.01$ respect to female group, of the same age by Student's t test.

Figure 5. Overexpression of mutated SOD1in Neuro-2A cells and treatment with E2. Neither cell viability (A) nor ATP content (B) were altered as a consequence of transfection or E2 treatment. (C) Oxygen flux measurements in intact N2A cells pretreated with E2 and transfected with pEGFP-G93A-hSOD1 and pEGFP-hSOD-wt showed a reduction on the maximal oxygen capacity in G93A transfected cells which was abrogated by E2 treatment. (D) Oxygen flux measurements in permeabilized-above mentioned cells showed a Complex I impairment for cells transfected with G93A respect to control cells which was abolished by the E2 treatment. In the same experimental regime, western blot analysis (E) for mitochondrial complex protein expression (complex I and complex II) confirmed an unaltered protein expression among the E2 treatment and transfects. Bars in (A) represent % of survival ± SEM

respect to control cells for 4-6 independent experiments. Bars in (B) represented ATP mean values ± SEM expressed as nmol/mg protein for 4-6 independent experiments. Oxygen consumption in (B) was measured at 37 °C in culture medium containing 10mM Hepes, pH 7.4, and corrected for instrumental background oxygen flux. In (D) culture medium was MIR05. Values were normalized by actual protein content in the respirometer chambers, expressed as pmol O_2/ min×mg protein. *, P<0.05; ** P<0.01;*** P<0.001 between control cells and G93A cells for 4-6 independent experiments. Bars in (E) represent means ±SEM densitometric analysis of the corresponding complexes relativized by actual porin content in each lane for 4-6 independent experiments

SUPLEMENTAL FIGURES

Figure S0.- Time course of G93A-hSOD1 protein expression in G93A mice spinal cord.

Western-blot analysis of transgenic male and female mice at 30 and 60 days, leads to the expression of a protein with an apparent MW of 16 kDa, whereas, as expected, the protein was not detected in control mice. Overespressors of non mutant SOD1 (WT) was used as control. These results provided evidence that the mutated form of the enzyme were expressed at similar levels in mice of both sexes, at least at preclinical ages.

Figure S1.- Clinical and metabolic features of G93A colony and control littermates, depicted by sex. (A) Weigth evolution across the study for G93A animals. (B) Weigth evolution across the study for controls. (C) Food intake calculations for G93A colony. (D) Food intake calculations for controls. Along disease, animals were studies every other day for clinical symptoms, been classified accordingly. As a result (E) shows the age at which animals reached clinical score 2, (F) shows the age at clinical score 3 and (G) shows the age at clinical score 4. Dots represented means ±SEM values in grams of weakly measurements in (A to D), n=at least, 11-12. In (E-F) boxes represented mean ±SEM expressed in days, n=11-12. *, P<0.05 between male and age matched females.

Figure S2.- Oxygen flux of sets of 5 intact LSCS from male and female G93A and control mice measured at 30,60, 90 and 120 (endpoint) days of age. Experimental procedures allows show basal (A-B), complex I (C-D), complex II (E-F) or residual (G-H) oxygen consumption differences between G93A and controls, depicted by sex. Measurements were taken at 37 °C in Hank's balanced salt solution containing 10mM Hepes, pH 7.4, and corrected for instrumental background oxygen flux. Values were normalized by actual protein content in the respirometer chambers. Means ± SEM, expressed as pmol O_2/s·mg protein. *, P<0.05, between male and female mice of the same age by Student's t test.

Figure S3.- Oxygen flux of sets of 5 LSCS from male and female G93A and control mice measured at 30,60, 90 and 120 (endpoint) days of age. Permeabilized LSCS measures on complexI state2 (A and E), Complex I state 3 (B and F), complex I/II state 3 (C and G) and complex II state3 (D and H) oxygen consumption conditions between G93A overexpresors and controls depicted by sex. Measurements were taken at 37 °C in Mir05 media. Values shown are means ± SEM expressed as pmol O_2/min×·mg protein for 4-6 independent measures. *, p < 0.01 respect to controls of the same age by Student's t test.

Figure S4.- Oxygen flux of sets of 5 LSCS from male and female control mice measured at 30,60, 90 and 120 (endpoint) days of age. In the upper panel, intact measures

showed basal (A), complex I (B) and complex II (C) oxygen consumption differences between males and age matched females in the control colony. Measurements were taken at 37 °C in Hank's balanced salt solution containing 10mM Hepes, pH 7.4, and corrected for instrumental background oxygen flux. On the lower panel, permeabilized LSCS measures on endogenous (D), Complex I state 2 (E), complex I state 3 (F) and complex I/II state3 (G) oxygen consumption conditions between male and age matched females in the control colony. Measurements were taken at 37 °C in Mir05 media. Values shown are means ± SEM expressed as pmol O_2/min×·mg protein for 4-6 independent measures. *, $p < 0.01$ respect to females of the same age by Student's t test.

Figure S5.- GC/MS analyses of protein oxidation products in LSCS from male and female G93A and control littermates measured at 60, 90 and 120 (endpoint) days. (A) and (C) represented levels of GSA, for male and female, respectively. (B) and (D) represented levels of CML for male and female, respectively. Values shown are means ± SEM expressed as µmol of analyte per mol lysine. *, $p < 0.01$ respect to non transgenic group of the same age by Student's t test.

Figure S6.-GC analyses of lipids in LSCS from male and female G93A and controls, measured at 30, 60, 90 and 120 (endpoint) days. Upper panel shows absence of sexual dimorphism in the control colony regarding DHA (A) or AA (B) levels in spinal cord. Lower panel indicates the differences founded among G93A colony and controls depicted by sex. (C) and (D) shows levels of AA along disease course for male and female, respectively. (E) and (F) shows levels of DHA along disease course in male and female, respectively. Bars represented mean values ± SEM expressed as % of abundance of analyte among the total FAME identified. *, $p < 0.01$ respect to female group in the upper panel and respect to the control littermate in the lower panel. All of them age-matched, by Student's t test.

Figure S6.- Phase contrast and fluorescence microscopy images of the N2A culture after pEGFP-hSOD-wt (A) and (C) or pEGFP-G93A-hSOD1 (B) and (D) transfection. Approximately, 80-90% trasfection efficiency was found.

Bibliography

Al-Chalabi, A., Jones, A., Troakes, C., King, A., Al-Sarraj, S., & van den Berg, L. H. (2012). The genetics and neuropathology of amyotrophic lateral sclerosis. *Acta Neuropathologica*, *124*(3), 339–52. doi:10.1007/s00401-012-1022-4

Alessandri, J.-M., Extier, A., Langelier, B., Perruchot, M.-H., Heberden, C., Guesnet, P., & Lavialle, M. (2008). Estradiol favors the formation of eicosapentaenoic acid (20:5n-3) and n-3 docosapentaenoic acid (22:5n-3) from alpha-linolenic acid (18:3n-3) in SH-SY5Y neuroblastoma cells. *Lipids*, *43*(1), 19–28. doi:10.1007/s11745-007-3117-6

Andrus, P. K., Fleck, T. J., Gurney, M. E., & Hall, E. D. (1998). Protein oxidative damage in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Journal of Neurochemistry*, *71*(5), 2041–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9798929

Arieli, Y., Gursahani, H., Eaton, M. M., Hernandez, L. A., & Schaefer, S. (2004). Gender modulation of Ca(2+) uptake in cardiac mitochondria. *Journal of Molecular and Cellular Cardiology*, *37*(2), 507–13. doi:10.1016/j.yjmcc.2004.04.023

Astorg, P., Bertrais, S., Laporte, F., Arnault, N., Estaquio, C., Galan, P., … Hercberg, S. (2008). Plasma n-6 and n-3 polyunsaturated fatty acids as biomarkers of their dietary intakes: a cross-sectional study within a cohort of middle-aged French men and women. *European Journal of Clinical Nutrition*, *62*(10), 1155–61. doi:10.1038/sj.ejcn.1602836

Ayala, V., Granado-Serrano, A. B., Cacabelos, D., Naudí, A., Ilieva, E. V, Boada, J., … Portero-Otin, M. (2011). Cell stress induces TDP-43 pathological changes associated with ERK1/2 dysfunction: implications in ALS. *Acta Neuropathologica*, *122*(3), 259–270. doi:10.1007/s00401-011-0850-y

Blasco, H., Guennoc, A.-M., Veyrat-Durebex, C., Gordon, P. H., Andres, C. R., Camu, W., & Corcia, P. (2012). Amyotrophic lateral sclerosis: a hormonal condition? *Amyotrophic Lateral Sclerosis : Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases*, *13*(6), 585–8. doi:10.3109/17482968.2012.706303

Bowling, A. C., Schulz, J. B., Brown Jr, R. H., & Beal, M. F. (1993). Superoxide dismutase activity, oxidative damage, and mitochondrial energy metabolism in familial and sporadic amyotrophic lateral sclerosis. *Journal of Neurochemistry*, *61*(6), 2322–2325.

Brustovetsky, N., Brustovetsky, T., Jemmerson, R., & Dubinsky, J. M. (2002). Calcium-induced cytochrome c release from CNS mitochondria is associated with the permeability transition and rupture of the outer membrane. *Journal of Neurochemistry*, *80*(2), 207–18. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11902111

Byrne, S., Walsh, C., Lynch, C., Bede, P., Elamin, M., Kenna, K., … Hardiman, O. (2011). Rate of familial amyotrophic lateral sclerosis: a systematic review and meta-analysis. *Journal of Neurology, Neurosurgery, and Psychiatry*, *82*(6), 623–7. doi:10.1136/jnnp.2010.224501

Cacabelos, D., Ayala, V., Ramírez-Nunez, O., Granado-Serrano, A. B., Boada, J., Serrano, J. C. E., … Portero-Otin, M. (2014). Dietary Lipid Unsaturation Influences Survival and Oxidative Modifications of an Amyotrophic Lateral Sclerosis Model in a Gender-Specific Manner. *Neuromolecular Medicine*. doi:10.1007/s12017-014-8317-7

Chaturvedi, R. K., & Flint Beal, M. (2013). Mitochondrial diseases of the brain. *Free Radical Biology & Medicine*, *63*, 1–29. doi:10.1016/j.freeradbiomed.2013.03.018

Chiu, A. Y., Zhai, P., Dal Canto, M. C., Peters, T. M., Kwon, Y. W., Prattis, S. M., & Gurney, M. E. (1995). Age-dependent penetrance of disease in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Molecular and Cellular Neurosciences*, *6*(4), 349–362. doi:10.1006/mcne.1995.1027

Colom, B., Alcolea, M. P., Valle, A., Oliver, J., Roca, P., & García-Palmer, F. J. (2007). Skeletal muscle of female rats exhibit higher mitochondrial mass and oxidative-phosphorylative capacities compared to males. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *19*(1-4), 205–12. doi:10.1159/000099208

Colom, B., Oliver, J., Roca, P., & Garcia-Palmer, F. J. (2007). Caloric restriction and gender modulate cardiac muscle mitochondrial H2O2 production and oxidative damage. *Cardiovascular Research*, *74*(3), 456–65. doi:10.1016/j.cardiores.2007.02.001

Cozzolino, M., Ferri, A., Valle, C., & Carrì, M. T. (2013). Mitochondria and ALS: implications from novel genes and pathways. *Molecular and Cellular Neurosciences*, *55*, 44–9. doi:10.1016/j.mcn.2012.06.001

Crawford, P., & Duncan, S. (2012). Gender issues. *Handbook of Clinical Neurology*, *108*, 759–81. doi:10.1016/B978-0-444-52899-5.00026-5

Członkowska, A., Ciesielska, A., Gromadzka, G., & Kurkowska-Jastrzebska, I. (2006). Gender differences in neurological disease: role of estrogens and cytokines. *Endocrine*, *29*(2), 243–56. doi:10.1385/ENDO:29:2:243

Damiano, M., Starkov, A. A., Petri, S., Kipiani, K., Kiaei, M., Mattiazzi, M., … Manfredi, G. (2006). Neural mitochondrial Ca2+ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *Journal of Neurochemistry*, *96*(5), 1349–61. doi:10.1111/j.1471-4159.2006.03619.x

DeJesus-Hernandez, M., Mackenzie, I. R., Boeve, B. F., Boxer, A. L., Baker, M., Rutherford, N. J., … Rademakers, R. (2011). Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron*, *72*(2), 245–56. doi:10.1016/j.neuron.2011.09.011

Doczi, J., Turiák, L., Vajda, S., Mándi, M., Töröcsik, B., Gerencser, A. A., … Chinopoulos, C. (2011). Complex contribution of cyclophilin D to Ca2+-induced permeability transition in brain mitochondria, with relation to the bioenergetic state. *The Journal of Biological Chemistry*, *286*(8), 6345–53. doi:10.1074/jbc.M110.196600

Eschbach, J., Schwalenstöcker, B., Soyal, S. M., Bayer, H., Wiesner, D., Akimoto, C., … Weydt, P. (2013). PGC-1α is a male-specific disease modifier of human and experimental amyotrophic lateral sclerosis. *Human Molecular Genetics*, *22*(17), 3477–84. doi:10.1093/hmg/ddt202

Extier, A., Langelier, B., Perruchot, M.-H., Guesnet, P., Van Veldhoven, P. P., Lavialle, M., & Alessandri, J.-M. (2010). Gender affects liver desaturase expression in a rat model of n-3 fatty acid repletion. *The Journal of Nutritional Biochemistry*, *21*(3), 180–7. doi:10.1016/j.jnutbio.2008.10.008

Federico, A., Cardaioli, E., Da Pozzo, P., Formichi, P., Gallus, G. N., & Radi, E. (2012). Mitochondria, oxidative stress and neurodegeneration. *Journal of the Neurological Sciences*, *322*(1-2), 254–62. doi:10.1016/j.jns.2012.05.030

Flanagan, S. W., Anderson, R. D., Ross, M. A., & Oberley, L. W. (2002). Overexpression of manganese superoxide dismutase attenuates neuronal death in human cells expressing mutant (G37R) Cu/Zn-superoxide dismutase. *Journal of Neurochemistry*, *81*(1), 170–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12067230

Gnaiger, E. (2001). Bioenergetics at low oxygen: dependence of respiration and phosphorylation on oxygen and adenosine diphosphate supply. *Respiration Physiology*, *128*(3), 277–97. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11718759

Gou-Fabregas, M., Ramírez-Núñez, O., Cacabelos, D., Bahi, N., Portero, M., Garcera, A., & Soler, R. M. (2014). Calpain activation and CaMKIV reduction in spinal cords from hSOD1G93A mouse model. *Molecular and Cellular Neurosciences*, *61*, 219–25. doi:10.1016/j.mcn.2014.07.002

Graham, D. I., Gonatas, N. K., & Charalampous, F. C. (1974). The undifferentiated and extended forms of C1300 murine neuroblastoma. An ultrastructural study and detection of concanavalin A binding sites on the plasma membrane. *The American Journal of Pathology*, *76*(2), 285–312. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1910860&tool=pmcentrez&rendertype=abstract

Guevara, R., Gianotti, M., Roca, P., & Oliver, J. (2011). Age and sex-related changes in rat brain mitochondrial function. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, *27*(3-4), 201–6. doi:10.1159/000327945

Guevara, R., Santandreu, F. M., Valle, A., Gianotti, M., Oliver, J., & Roca, P. (2009). Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress. *Free Radical Biology & Medicine*, *46*(2), 169–75. doi:10.1016/j.freeradbiomed.2008.09.035

Gurney, M. E. (1997). The use of transgenic mouse models of amyotrophic lateral sclerosis in preclinical drug studies. *Journal of the Neurological Sciences*, *152 Suppl* , S67–73. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9419057

Gurney, M. E., Pu, H., Chiu, A. Y., Dal Canto, M. C., Polchow, C. Y., Alexander, D. D., … Deng, H. X. (1994). Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science (New York, N.Y.)*, *264*(5166), 1772–1775.

Hall, E. D., Andrus, P. K., Oostveen, J. A., Fleck, T. J., & Gurney, M. E. (1998). Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. *Journal of Neuroscience Research*, *53*(1), 66–77. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9670993

Ho, P. W.-L., Ho, J. W.-M., Tse, H.-M., So, D. H.-F., Yiu, D. C.-W., Liu, H.-F., … Ho, S.-L. (2012). Uncoupling protein-4 (UCP4) increases ATP supply by interacting with mitochondrial Complex II in neuroblastoma cells. *PloS One*, *7*(2), e32810. doi:10.1371/journal.pone.0032810

Hütter, E., Unterluggauer, H., Garedew, A., Jansen-Dürr, P., & Gnaiger, E. (2006). High-resolution respirometry--a modern tool in aging research. *Experimental Gerontology*, *41*(1), 103–9. doi:10.1016/j.exger.2005.09.011

Ilieva, E. V, Ayala, V., Jové, M., Dalfó, E., Cacabelos, D., Povedano, M., … Portero-Otin, M. (2007). Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. *Brain*, *130*(Pt 12), 3111–3123. doi:10.1093/brain/awm190

Ilieva, E. V, Ayala, V., Jové, M., Dalfó, E., Cacabelos, D., Povedano, M., … Portero-Otín, M. (2007). Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. *Brain : A Journal of Neurology*, *130*(Pt 12), 3111–23. doi:10.1093/brain/awm190

Jung, C., Higgins, C. M. J., & Xu, Z. (2002). A quantitative histochemical assay for activities of mitochondrial electron transport chain complexes in mouse spinal cord sections. *Journal of Neuroscience Methods*, *114*(2), 165–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11856567

Justo, R., Boada, J., Frontera, M., Oliver, J., Bermúdez, J., & Gianotti, M. (2005). Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. *American Journal of Physiology. Cell Physiology*, *289*(2), C372–8. doi:10.1152/ajpcell.00035.2005

Kim, H. J., Magranè, J., Starkov, A. A., & Manfredi, G. (2012). The mitochondrial calcium regulator cyclophilin D is an essential component of oestrogen-mediated neuroprotection in amyotrophic lateral sclerosis. *Brain : A Journal of Neurology*, *135*(Pt 9), 2865–74. doi:10.1093/brain/aws208

Kim, H. Y., Ki, C.-S., Koh, S.-H., Park, K.-H., Sunwoo, I.-N., & Kim, S. H. (2007). Clinical characteristics of familial amyotrophic lateral sclerosis with a Phe20Cys mutation in the SOD1 gene in a Korean family. *Amyotrophic Lateral Sclerosis : Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases*, *8*(2), 73–8. doi:10.1080/17482960701223154

Kirkinezos, I. G., Bacman, S. R., Hernandez, D., Oca-Cossio, J., Arias, L. J., Perez-Pinzon, M. A., … Moraes, C. T. (2005). Cytochrome c association with the inner mitochondrial membrane is impaired in the CNS of G93A-SOD1 mice. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *25*(1), 164–72. doi:10.1523/JNEUROSCI.3829-04.2005

Lee, J. R., Annegers, J. F., & Appel, S. H. (1995). Prognosis of amyotrophic lateral sclerosis and the effect of referral selection. *Journal of the Neurological Sciences*, *132*(2), 207–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8543950

Leigh, P. N. (2007). Chapter 13 Amyotrophic lateral sclerosis. *Handbook of Clinical Neurology*, *82*, 249–78. doi:10.1016/S0072-9752(07)80016-9

Lezi, E., & Swerdlow, R. H. (2012). Mitochondria in neurodegeneration. *Advances in Experimental Medicine and Biology*, *942*, 269–86. doi:10.1007/978-94-007-2869-1_12

Li, X., Hao, X., Cheng, B., & Li, X. (2014). Acute blockage of voltage-gated K^+ currents by 17β-estradiol in mouse neuroblastoma N2A cells. *Neuroreport*, *25*(8), 574–9. doi:10.1097/WNR.0000000000000137

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787–95. doi:10.1038/nature05292

Martin, L. J. (2012). Biology of mitochondria in neurodegenerative diseases. *Progress in Molecular Biology and Translational Science*, *107*, 355–415. doi:10.1016/B978-0-12-385883-2.00005-9

Marx, J. (1994). Mouse model found for ALS. *Science (New York, N.Y.)*, *264*(5166), 1663–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8209242

Mattiazzi, M., D'Aurelio, M., Gajewski, C. D., Martushova, K., Kiaei, M., Beal, M. F., & Manfredi, G. (2002a). Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *The Journal of Biological Chemistry*, *277*(33), 29626–33. doi:10.1074/jbc.M203065200

Mattiazzi, M., D'Aurelio, M., Gajewski, C. D., Martushova, K., Kiaei, M., Beal, M. F., & Manfredi, G. (2002b). Mutated human SOD1 causes dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *The Journal of Biological Chemistry*, *277*(33), 29626–33. doi:10.1074/jbc.M203065200

McCombe, P. A., & Henderson, R. D. (2010). Effects of gender in amyotrophic lateral sclerosis. *Gender Medicine*, *7*(6), 557–70. doi:10.1016/j.genm.2010.11.010

Mendez, P., & Garcia-Segura, L. M. (2006). Phosphatidylinositol 3-kinase and glycogen synthase kinase 3 regulate estrogen receptor-mediated transcription in neuronal cells. *Endocrinology*, *147*(6), 3027–39. doi:10.1210/en.2005-1224

Miana-Mena, F. J., González-Mingot, C., Larrodé, P., Muñoz, M. J., Oliván, S., Fuentes-Broto, L., … García, J. J. (2011). Monitoring systemic oxidative stress in an animal model of amyotrophic lateral sclerosis. *Journal of Neurology*, *258*(5), 762–9. doi:10.1007/s00415-010-5825-8

Misiak, M., Beyer, C., & Arnold, S. (n.d.). Gender-specific role of mitochondria in the vulnerability of 6-hydroxydopamine-treated mesencephalic neurons. *Biochimica et Biophysica Acta*, *1797*(6-7), 1178–88. doi:10.1016/j.bbabio.2010.04.009

Nagai, M., Re, D. B., Nagata, T., Chalazonitis, A., Jessell, T. M., Wichterle, H., & Przedborski, S. (2007). Astrocytes expressing ALS-linked mutated SOD1 release factors selectively toxic to motor neurons. *Nature Neuroscience*, *10*(5), 615–22. doi:10.1038/nn1876

Orrell, R. W., Habgood, J. J., Malaspina, A., Mitchell, J., Greenwood, J., Lane, R. J., & deBelleroche, J. S. (1999). Clinical characteristics of SOD1 gene mutations in UK families with ALS. *Journal of the Neurological Sciences*, *169*(1-2), 56–60. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10540008

Pamplona, R., Dalfo, E., Ayala, V., Bellmunt, M. J., Prat, J., Ferrer, I., & Portero-Otin, M. (2005). Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. *The Journal of Biological Chemistry*, *280*(22), 21522–21530. doi:10.1074/jbc.M502255200

Peixoto, P. M., Kim, H.-J., Sider, B., Starkov, A., Horvath, T. L., & Manfredi, G. (2013). UCP2 overexpression worsens mitochondrial dysfunction and accelerates disease progression in a mouse model of amyotrophic lateral sclerosis. *Molecular and Cellular Neurosciences*, *57*, 104–10. doi:10.1016/j.mcn.2013.10.002

Pesta, D., & Gnaiger, E. (2012). High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods in Molecular Biology (Clifton, N.J.)*, *810*, 25–58. doi:10.1007/978-1-61779-382-0_3

Portet, F., Cadilhac, C., Touchon, J., & Camu, W. (2001). Cognitive impairment in motor neuron disease with bulbar onset. *Amyotrophic Lateral Sclerosis and Other Motor Neuron Disorders : Official Publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases*, *2*(1), 23–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11465929

Pyo, J.-S., Ko, Y. S., Kim, W. H., Kim, M., Lee, K.-W., Nam, S. Y., … Lee, B. L. (2010). Impairment of nuclear factor-kappaB activation increased glutamate excitotoxicity in a motoneuron-neuroblastoma hybrid cell line expressing mutant (G93A) Cu/Zn-superoxide dismutase. *Journal of Neuroscience Research*, *88*(11), 2494–503. doi:10.1002/jnr.22397

Ralph, G. S., Radcliffe, P. A., Day, D. M., Carthy, J. M., Leroux, M. A., Lee, D. C. P., … Azzouz, M. (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nature Medicine*, *11*(4), 429–33. doi:10.1038/nm1205

Redler, R. L., & Dokholyan, N. V. (2012). The complex molecular biology of amyotrophic lateral sclerosis (ALS). *Progress in Molecular Biology and Translational Science*, *107*, 215–62. doi:10.1016/B978-0-12-385883-2.00002-3

Renolleau, S., Fau, S., & Charriaut-Marlangue, C. (2008). Gender-related differences in apoptotic pathways after neonatal cerebral ischemia. *The Neuroscientist : A Review Journal Bringing Neurobiology, Neurology and Psychiatry*, *14*(1), 46–52. doi:10.1177/1073858407308889

Renolleau, S., Fau, S., Goyenvalle, C., & Charriaut-Marlangue, C. (2007). “Sex, neuroprotection, and neonatal ischemia”. *Developmental Medicine and Child Neurology*, *49*(6), 477; author reply 477–8. doi:10.1111/j.1469-8749.2007.00477.x

Renton, A. E., Majounie, E., Waite, A., Simón-Sánchez, J., Rollinson, S., Gibbs, J. R., … Traynor, B. J. (2011). A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD. *Neuron*, *72*(2), 257–68. doi:10.1016/j.neuron.2011.09.010

Rizzuto, R., De Stefani, D., Raffaello, A., & Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. *Nature Reviews. Molecular Cell Biology*, *13*(9), 566–78. doi:10.1038/nrm3412

Rosen, D. R. (1993). Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature*, *364*(6435), 362. doi:10.1038/364362c0

Rothstein, J. D. (2009). Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Annals of Neurology*, *65 Suppl 1*, S3–9. doi:10.1002/ana.21543

Safiulina, D., Kaasik, A., Seppet, E., Peet, N., Zharkovsky, A., & Seppet, E. (2004). Method for in situ detection of the mitochondrial function in neurons. *Journal of Neuroscience Methods*, *137*(1), 87–95. doi:10.1016/j.jneumeth.2004.02.027

Shiota, M., Yokomizo, A., Tada, Y., Inokuchi, J., Tatsugami, K., Kuroiwa, K., … Naito, S. (2010). Peroxisome proliferator-activated receptor gamma coactivator-1alpha interacts with the androgen receptor (AR) and promotes prostate cancer cell growth by activating the AR. *Molecular Endocrinology (Baltimore, Md.)*, *24*(1), 114–27. doi:10.1210/me.2009-0302

Siklós, L., Engelhardt, J., Harati, Y., Smith, R. G., Joó, F., & Appel, S. H. (1996). Ultrastructural evidence for altered calcium in motor nerve terminals in amyotropic lateral sclerosis. *Annals of Neurology*, *39*(2), 203–16. doi:10.1002/ana.410390210

Sotelo-Silveira, J. R., Lepanto, P., Elizondo, V., Horjales, S., Palacios, F., Martinez-Palma, L., … Barbeito, L. (2009). Axonal mitochondrial clusters containing mutant SOD1 in transgenic models of ALS. *Antioxidants & Redox Signaling*, *11*(7), 1535–45. doi:10.1089/ARS.2009.2614

Suzuki, M., Tork, C., Shelley, B., McHugh, J., Wallace, K., Klein, S. M., … Svendsen, C. N. (2007). Sexual dimorphism in disease onset and progression of a rat model of ALS. *Amyotrophic Lateral Sclerosis□: Official Publication of the World Federation of Neurology Research Group on Motor Neuron Diseases*, *8*(1), 20–5. doi:10.1080/17482960600982447

Tao, Q., Fan, X., Li, T., Tang, Y., Yang, D., & Le, W. (2012). Gender segregation in gene expression and vulnerability to oxidative stress induced injury in ventral mesencephalic cultures of dopamine neurons. *Journal of Neuroscience Research*, *90*(1), 167–78. doi:10.1002/jnr.22729

Tcherepanova, I., Puigserver, P., Norris, J. D., Spiegelman, B. M., & McDonnell, D. P. (2000). Modulation of estrogen receptor-alpha transcriptional activity by the coactivator PGC-1. *The Journal of Biological Chemistry*, *275*(21), 16302–8. doi:10.1074/jbc.M001364200

Turner, B. J., & Talbot, K. (2008). Transgenics, toxicity and therapeutics in rodent models of mutant SOD1-mediated familial ALS. *Progress in Neurobiology*, *85*(1), 94–134. doi:10.1016/j.pneurobio.2008.01.001

Veldink, J. H., Bär, P. R., Joosten, E. A. J., Otten, M., Wokke, J. H. J., & van den Berg, L. H. (2003). Sexual differences in onset of disease and response to exercise in a transgenic model of ALS. *Neuromuscular Disorders□: NMD*, *13*(9), 737–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14561497

Vielhaber, S., Kunz, D., Winkler, K., Wiedemann, F. R., Kirches, E., Feistner, H., … Kunz, W. S. (2000). Mitochondrial DNA abnormalities in skeletal muscle of patients with sporadic amyotrophic lateral sclerosis. *Brain : A Journal of Neurology*, *123 (Pt 7*, 1339–48. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10869047

Vigerust, N. F., Cacabelos, D., Burri, L., Berge, K., Wergedahl, H., Christensen, B. B., … Bjorndal, B. (2012). {F}ish oil and 3-thia fatty acid have additive effects on lipid metabolism but antagonistic effects on oxidative damage when fed to rats for 50 weeks. *J Nutr Biochem*, *23*(11), 1384–1393. doi:10.1016/j.jnutbio.2011.08.006

Vinsant, S., Mansfield, C., Jimenez-Moreno, R., Del Gaizo Moore, V., Yoshikawa, M., Hampton, T. G., … Milligan, C. (2013). Characterization of early pathogenesis in the SOD1(G93A) mouse model of ALS: part I, background and methods. *Brain and Behavior*, *3*(4), 335–50. doi:10.1002/brb3.143

Votyakova, T. V, & Reynolds, I. J. (2001). DeltaPsi(m)-Dependent and -independent production of reactive oxygen species by rat brain mitochondria. *Journal of Neurochemistry*, *79*(2), 266–77. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11677254

Weis, S. N., Pettenuzzo, L. F., Krolow, R., Valentim, L. M., Mota, C. S., Dalmaz, C., … Netto, C. A. (2012). Neonatal hypoxia-ischemia induces sex-related changes in rat brain mitochondria. *Mitochondrion*, *12*(2), 271–9. doi:10.1016/j.mito.2011.10.002

Welch, A. A., Bingham, S. A., Ive, J., Friesen, M. D., Wareham, N. J., Riboli, E., & Khaw, K. T. (2006). Dietary fish intake and plasma phospholipid n-3 polyunsaturated fatty acid concentrations in men and women in the European Prospective Investigation into Cancer-Norfolk United Kingdom cohort. *The American Journal of Clinical Nutrition*, *84*(6), 1330–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17158413

Wiedemann, F. R., Winkler, K., Kuznetsov, A. V, Bartels, C., Vielhaber, S., Feistner, H., & Kunz, W. S. (1998). Impairment of mitochondrial function in skeletal muscle of patients with amyotrophic lateral sclerosis. *Journal of the Neurological Sciences*, *156*(1), 65–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9559989

Xia, X., Zhou, H., Huang, Y., & Xu, Z. (2006). Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. *Neurobiology of Disease*, *23*(3), 578–86. doi:10.1016/j.nbd.2006.04.019

Yim MB, Kang JH, Yim HS, Kwak HS, Chock PB, Stadtman ER (1996).A gain-of-function of an amyotrophic lateral sclerosis-associated Cu,Zn-superoxide dismutase mutant: An enhancement of free radical formation due to a decrease in Km for hydrogen peroxide. *Proc Natl Acad Sci U S A.* Jun 11;93(12):5709-14.

Supplementary material

Mice Genotyping

Mice were identified by PCR with tail genomic DNA as templates. The REDExtract-N-Amp Tissue PCR Kit (XNAT; Sigma-Aldrich, St. Louis, MO) was used to extract genomic DNA from mouse tails and amplify targets of interest by PCR according to manufacturer's instructions. Four primers (a pair for wild type forward: 5´-CTA GGC CAC AGA ATT GAA AGA TCT-3´ and reverse: 5´-GTA GGT GGA AAT TCT AGC ATC ATC-3´ and for G93A forward: 5´-CAT CAG CCC TAA TCC ATC TGA-3´ and reverse: 5´-CGC GAC TAA CAA TCA AAG TGA-3´) were used at to amplify a bands from the inserted targeting vector. Briefly, the DNA is released from the tail tissue by incubating the sample with a mixture of the Extraction Solution and the Tissue Preparation Solution at room temperature for 10 minutes.After adding Neutralization Solution B, the extract is ready for PCR. A 4 □L aliquot of the neutralized extract is then combined with 10□L of 2x REDExtract-N-Amp PCR Reaction Mix (buffer, salts, dNTPs, Taq polymerase, JumpStart Taq antibody and REDTaq® dye) and adequate volumes of each primer and water (PCR grade; Sigma-Aldrich, St. Louis, MO). PCR conditions were: after 1 cycle of 5 minutes at 95°C, 35 cycles of 30 seconds at 94°C, 45 seconds at 50°C, and 4 minute at 72°C, with a final extension at 72°C during 7 min, using an Applied Biosystems 2740 v.2.08 (Carlsbad, CA) thermal cycler. As a control of effective PCR amplification, housekeeping gene interleukin 5 was used. The resulting PCR products were stored at 4˚C until direct loading onto an agarose gel. Products were electrophoresed for 30 minutes under constant voltage (100V) and imaged under UV light (302 or 365 nm) lamp from Alpha Innotech (Santa Clara, CA) and analysed with Alpha Digidoc RT2 software (Santa Clara, CA).

Neurological scoring

Neurological scoring was performed every other day for both hind legs for each mouse from 60 days of age. The neurological score employed a scale of 0 to 4 and was a slight variation of which was developed by observation at Amyotrophic Lateral Sclerosis Therapy Development Institute (ALSTDI). Criteria used to assign each score level were as following: score 0, full extension of hind legs away from lateral midline when mouse is suspended by its tail, and mouse can hold this for 2 seconds, suspended 2–3 times; score 1, collapse or partial collapse of leg extension towards lateral midline or trembling of hind legs during tail suspension; score 2, toes curl at least twice during walking a distance of 40cm, or any part of the foot is dragged along the cage bottom or table (if one hind leg is scored as 2, food pellets are left on bedding and a long sipper tube is placed on the water bottle); score 3, rigid paralysis or minimal joint movement, foot not being used for forward motion; and, score 4, mouse cannot right itself within 30 seconds from either side. When mice achieved a score of 4 for two alternate days, they were euthanized.

Paw print analysis

Mice were trained to walk freely along a U-shaped plastic corridor (5 cm wide, 70 cm long) three times per week before data collection (average age 70 days). Once trained, to perform stride length measurements, hind limbs were stained with non-toxic children paint and their tracks were imprinted on paper lining the floor of the corridor. Only continuous runs were selected among recorded paw prints and a minimum of 5-7 strides were measured per animal per day (measures were taken three times per week). Since animals tended to run when released and walk slowly at the end of the corridor, those sections of the paw print were discarded for the stride length measurements. Three stride length measurements were taken manually as described previously (Chiu et al., 1995) front and back stride, and front-to-back distance. 10 total measures were taken for each measurement. Front stride and back stride were collected as a straight line from paw print to the following paw print. Front-to-back distance was collected as a straight line from back paw print to corresponding front paw print. Correspondence was based on closest front footprint. The distance was recorded as length of line from paw to the stride line opposite the paw print. Since only number of the randomized animal and date identified the recorded tracks, measurement of stride length was determined in a blinded manner.As the disease progresses, the painted footprints became increasingly smeared on the paper, which correlated with previous results (Marx, 1994). The

onset of clinical weakness was quantified by determining the age at which shortening of the stride length was lower than 40% for two consecutive measures.

Oxidation-derived protein damage markers measurements by GC/MS.

Glutamic semialdehyde (GSA), aminoadipicsemialdehyde (AASA), N^{ε} -(carboxyethyl)-lysine (CEL), N^{ε} -(carboxymethyl)-lysine (CML), and N^{ε}-(malondialdehyde)-lysine (MDAL) concentrations in total proteins from spinal cord homogenates were measured by gas chromatography/mass spectrometry (GC/MS) as previously described (Cacabelos et al., 2014). Samples containing 0.75-1 mg of protein were delipidated using chloroform:methanol (2:1 v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM NaBH4 (final concentration) in 0.2M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: [2H8]Lysine (d8-Lys; CDN Isotopes); [2H4]CML (d4-CML), [2H4]CEL (d4-CEL), and [2H8]MDAL (d8-MDAL), prepared as described (8,26); [2H5] 5-hydroxy-2-aminovaleric acid (for GSA quantization) and [2H4]6-hydroxy-2-aminocaproic acid (for AASA quantization) prepared as described in (Pamplona et al., 2005). The samples were hydrolysed at 155oC for 30 min in 1ml of 6N HCl, and then dried in vacuo. The N,O-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared as previously described (Pamplona et al 2005). GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30m HP-5MS capillary column (30m x 0.25mm x 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Catalonia). The injection port was maintained at 275ºC; the temperature program was 5 min at 110ºC, then 2ºC/min to 150ºC, then 5ºC/min to 240ºC, then 25ºC/min to 300ºC, and finally hold at 300ºC for 5 min. Quantification was performed by external standardisation using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and d8-lysine, m/z 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d5-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), m/z 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and d4-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), m/z 294 and 298, respectively; CML and d4-CML , m/z 392 and 396, respectively; CEL and d4-CEL, m/z 379 and 383, respectively; and MDAL and d8-MDAL, m/z 474 and 482, respectively. The amounts of products were expressed as the ratio μmol GSA, AASA, CML, CEL or MDAL per mol lysine.

Figure 1

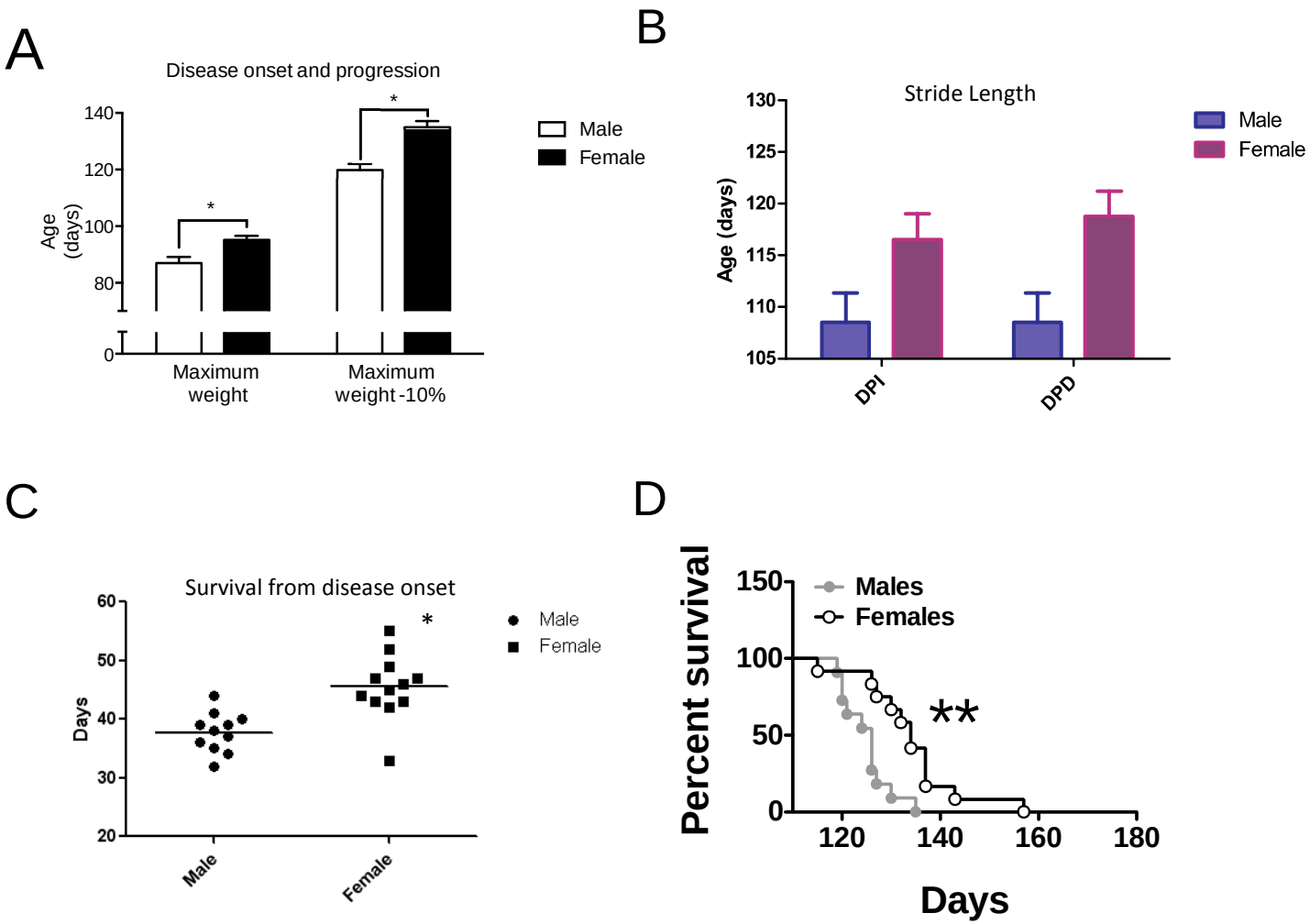
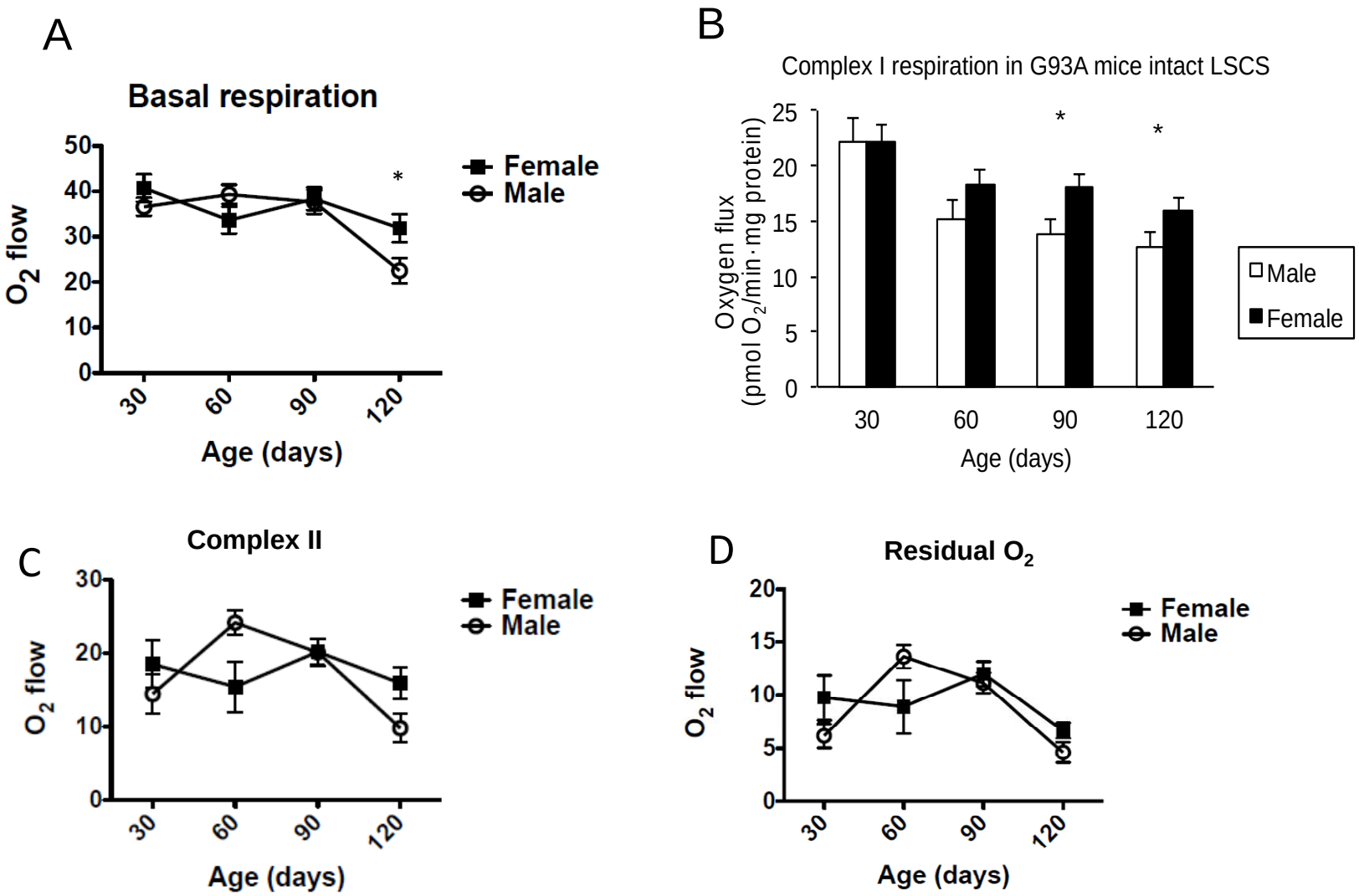

A
Disease onset and progression
Age (days)
140
120
100
80
0
*
*
Maximum weight
Maximum weight -10%
Male
Female
B
Stride Length
Age (days)
130
125
120
115
110
105
DPI
DPD
Male
Female
C
Survival from disease onset
Days
60
50
40
30
20
*
Male
Female
D
Percent survival
150
100
50
0
Males
Females
**
120
140
160
180
Days

Figure 2


A
Basal respiration
O_2 flow
50
40
30
20
10
0
30
60
90
120
Age (days)
*
Female
Male
B
Complex I respiration in G93A mice intact LSCS
Oxygen flux
(pmol O_2/min·mg protein)
25
20
15
10
5
0
30
60
90
120
Age (days)
*
*
Male
Female
C
Complex II
O_2 flow
30
20
10
0
30
60
90
120
Age (days)
Female
Male
D
Residual O_2
O_2 flow
20
15
10
5
0
30
60
90
120
Age (days)
Female
Male

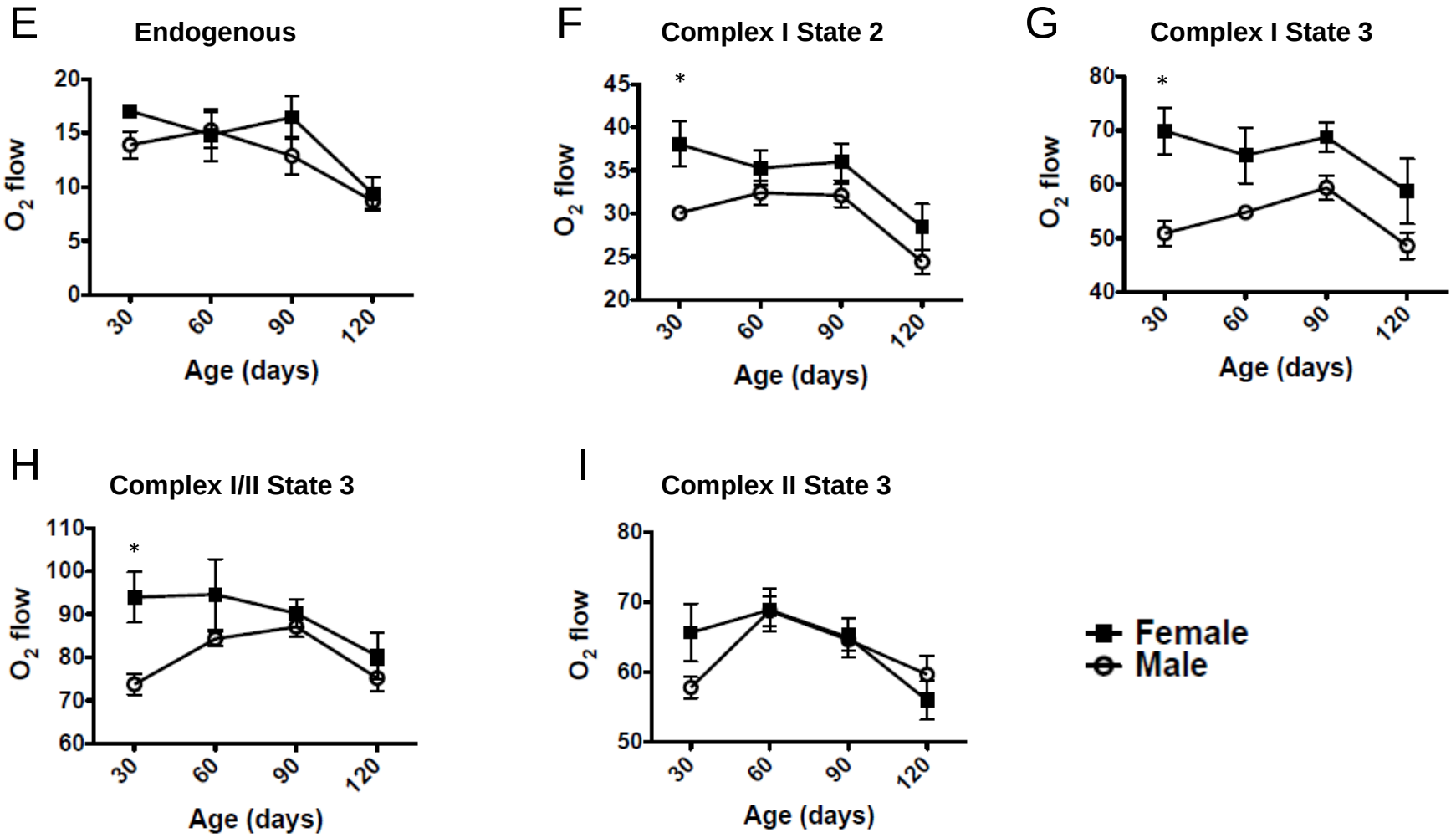
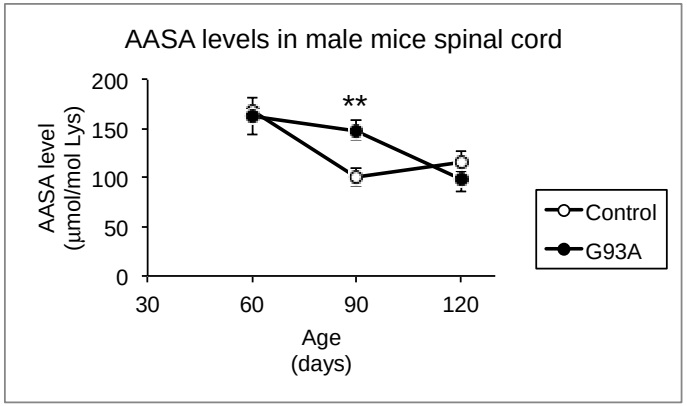


Figure 2 cont

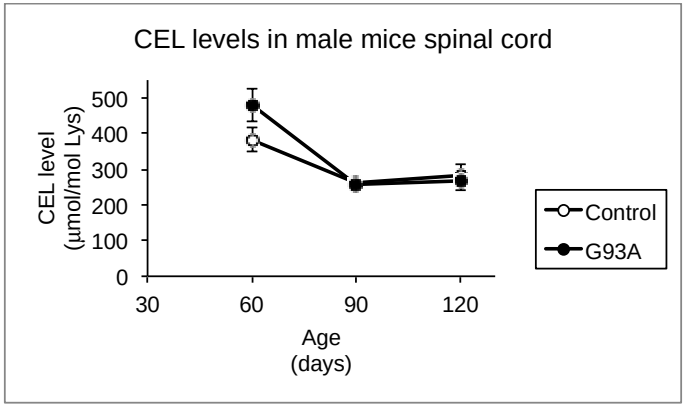
Figure 3

Male

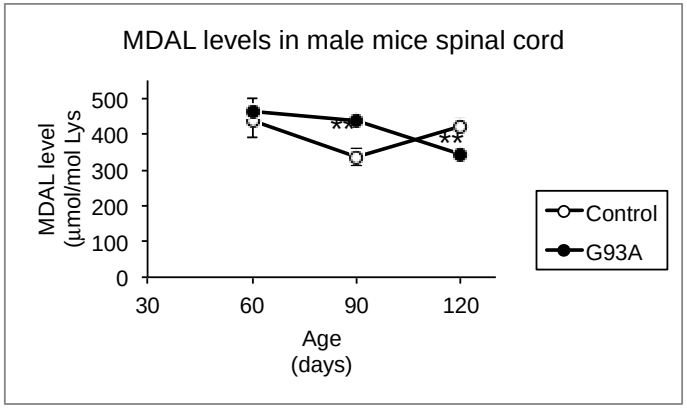
A



B



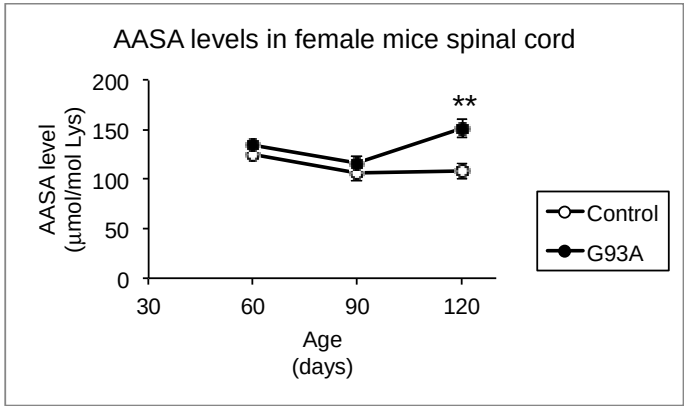
C



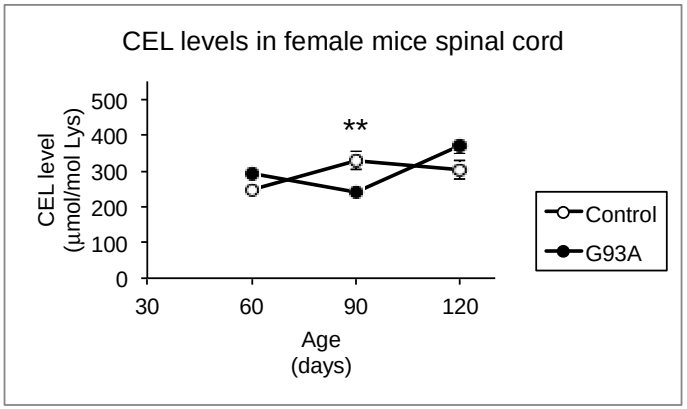
194

Female

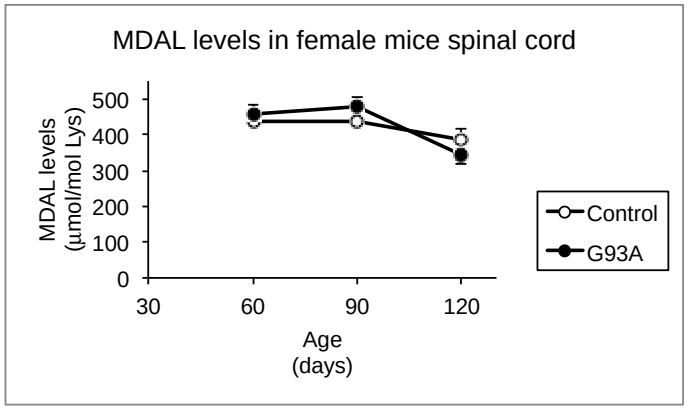
D



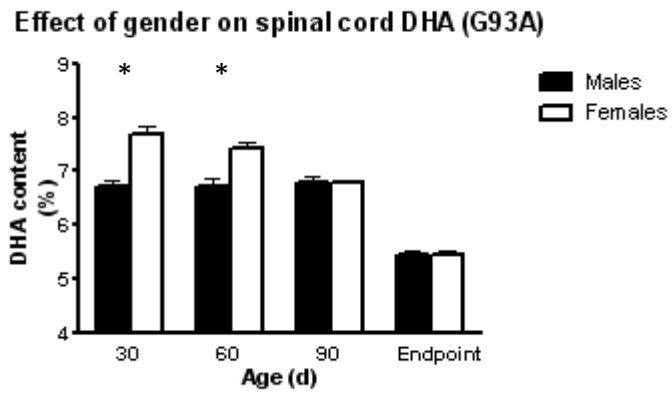
E



F



A


Effect of gender on spinal cord DHA (G93A)
DHA content (%)
9
8
7
6
5
4
*
*
30
60
90
Endpoint
Age (d)
Males
Females

B

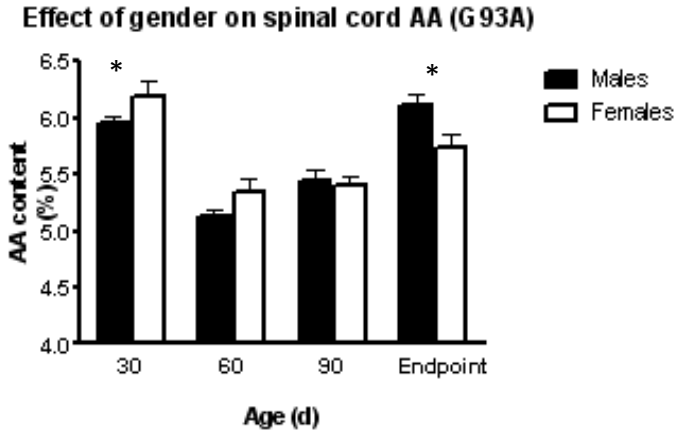
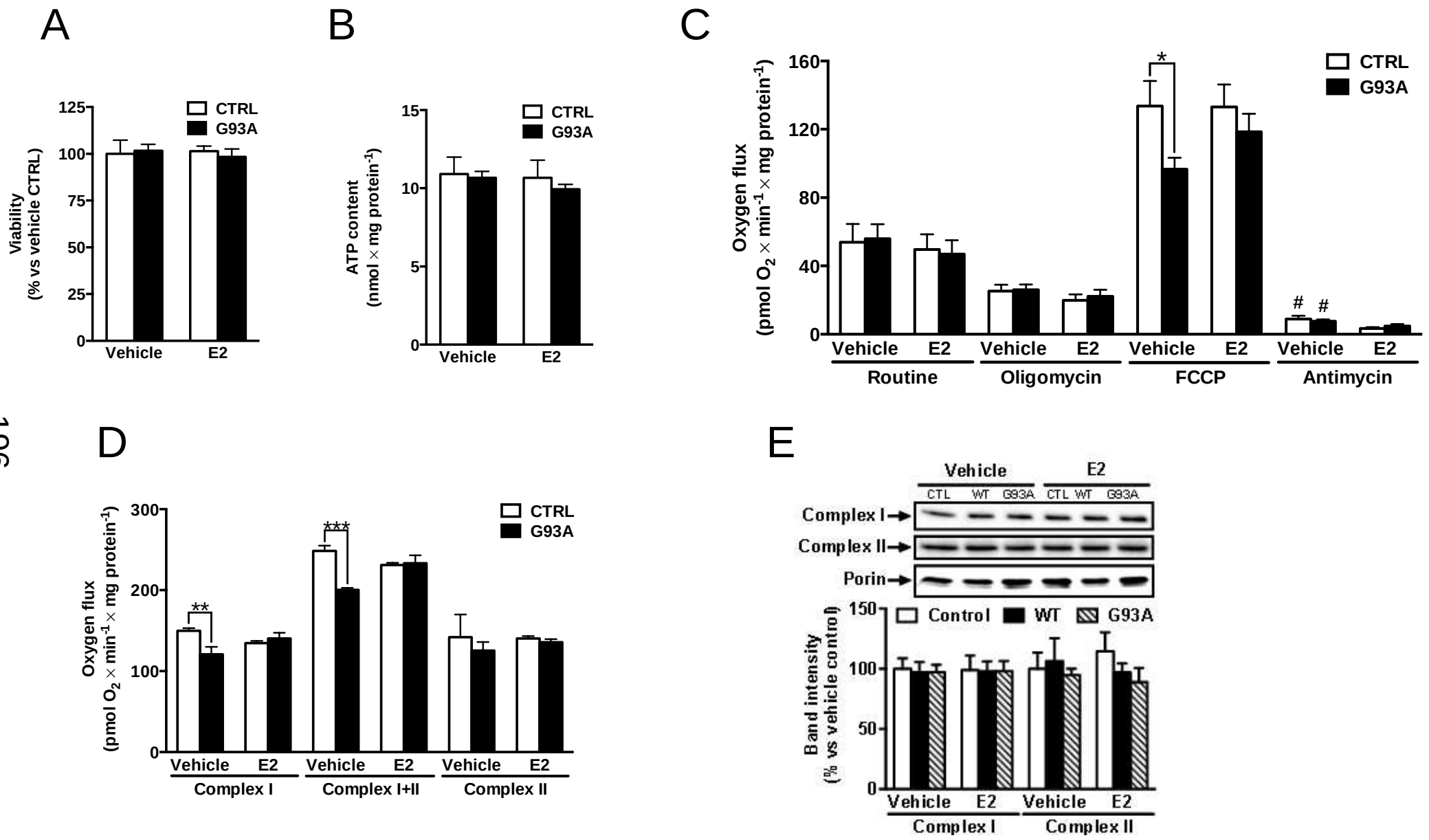

Effect of gender on spinal cord AA (G93A)
AA content (%)
6.5
6.0
5.5
5.0
4.5
4.0
*
*
30
60
90
Endpoint
Age (d)
Males
Females

Figure 4

Figure 5


A
Viability
(% vs vehicle CTRL)
125
100
75
50
25
0
CTRL
G93A
Vehicle
E2
B
ATP content
(nmol × mg protein-1)
15
10
5
0
CTRL
G93A
Vehicle
E2
C
Oxygen flux
(pmol O2 × min-1 × mg protein-1)
160
120
80
40
0
CTRL
G93A
*
# #
Vehicle
E2
Routine
Oligomycin
FCCP
Antimycin
D
Oxygen flux
(pmol O2 × min-1 × mg protein-1)
300
200
100
0
CTRL
G93A
**
***
Vehicle
E2
Complex I
Complex I+II
Complex II
E
Vehicle
E2
CTL
WT
G93A
Complex I
Complex II
Porin
Band intensity
(% vs vehicle control)
150
100
50
0
Control
WT
G93A
Vehicle
E2
Complex I
Complex II

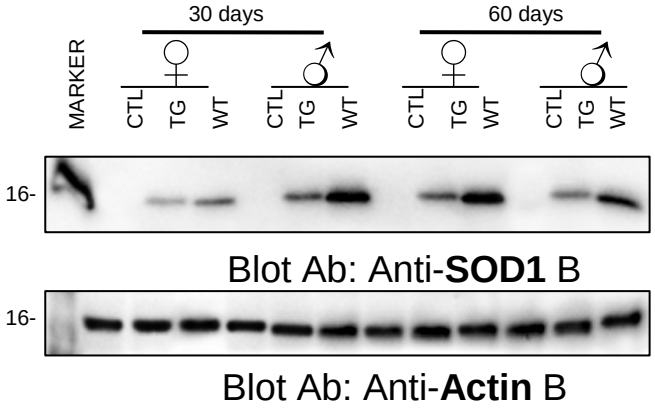
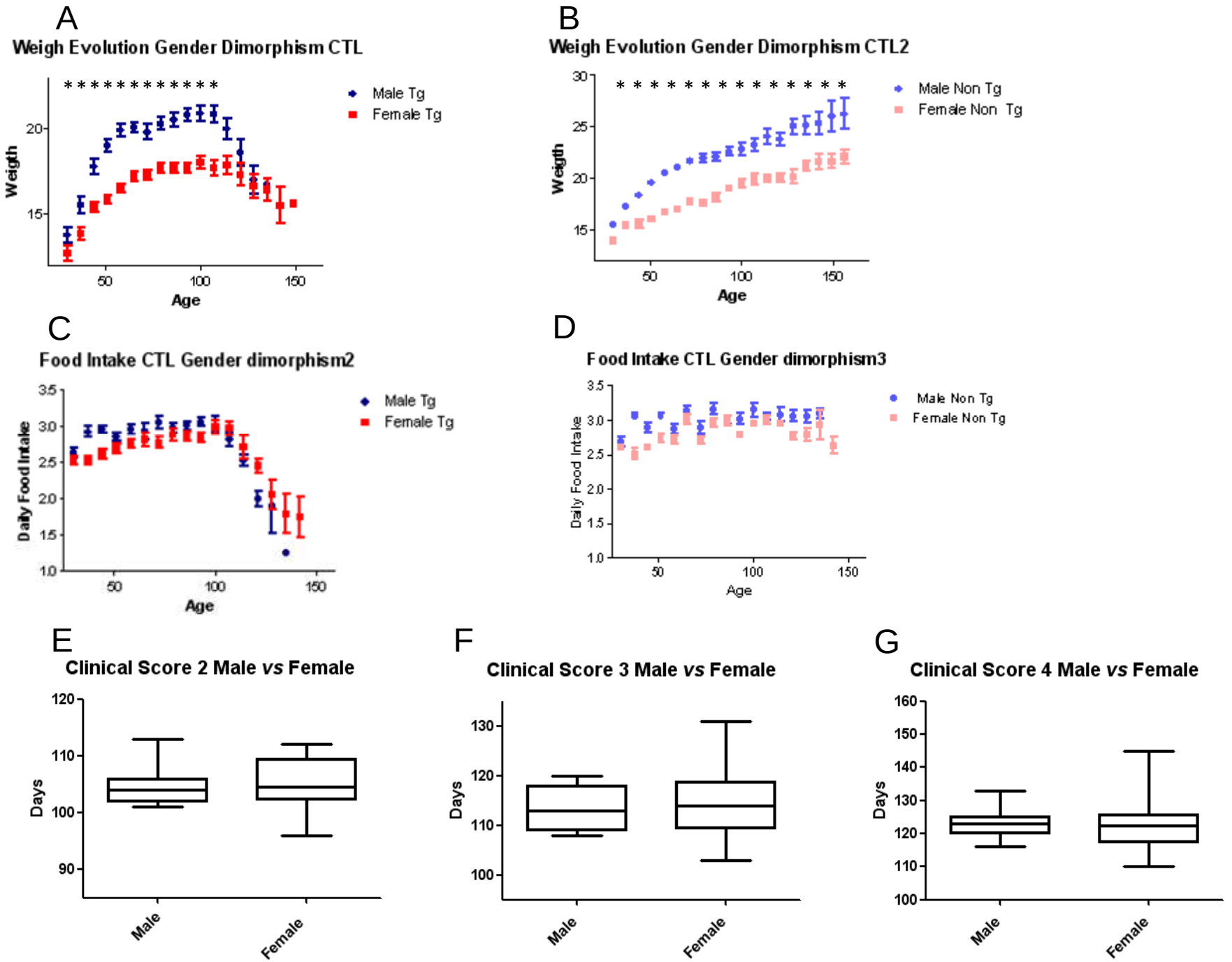


Figure S0 (supplemental)

Figure S1 (supplemental)



Male

Female

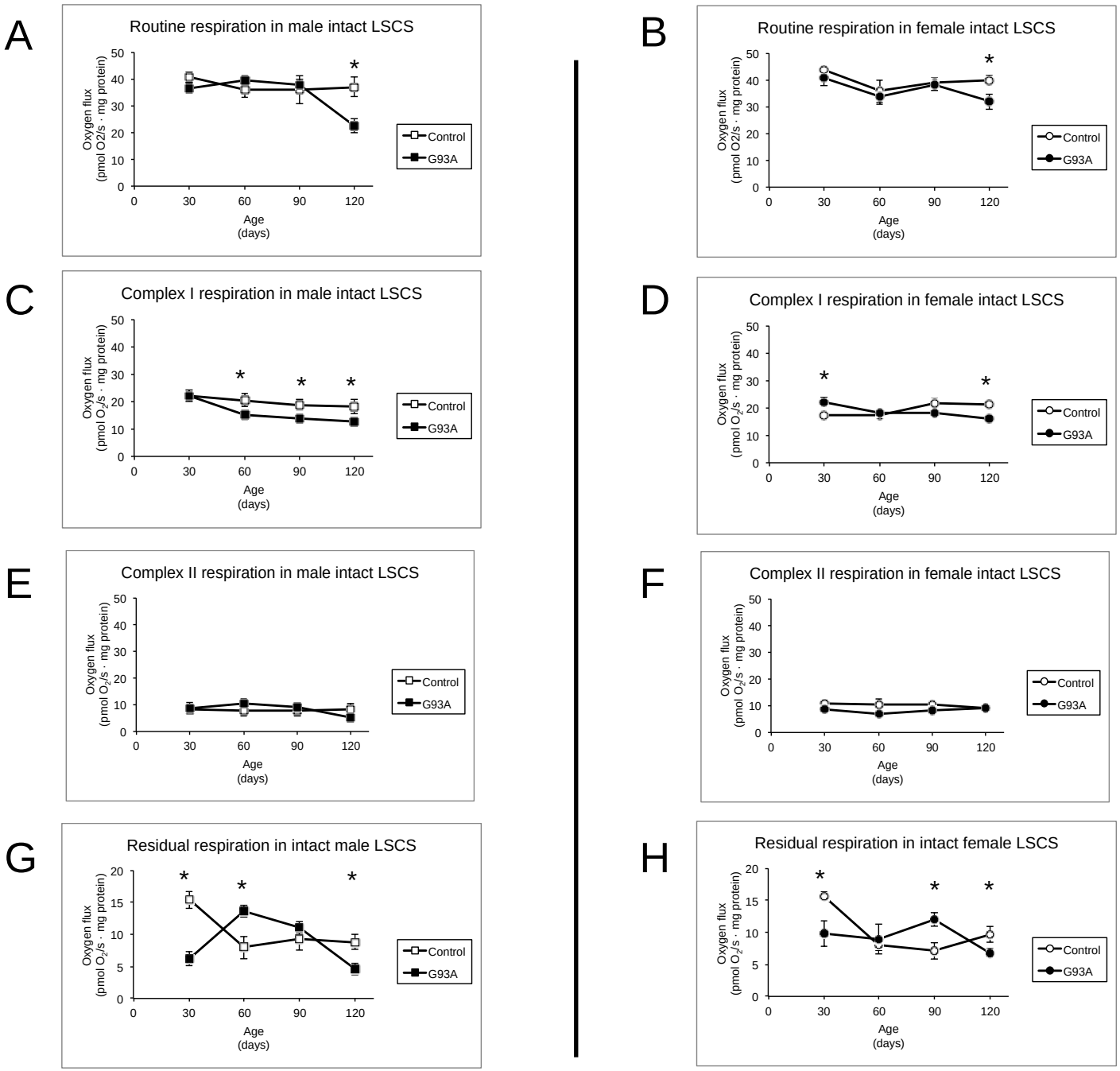
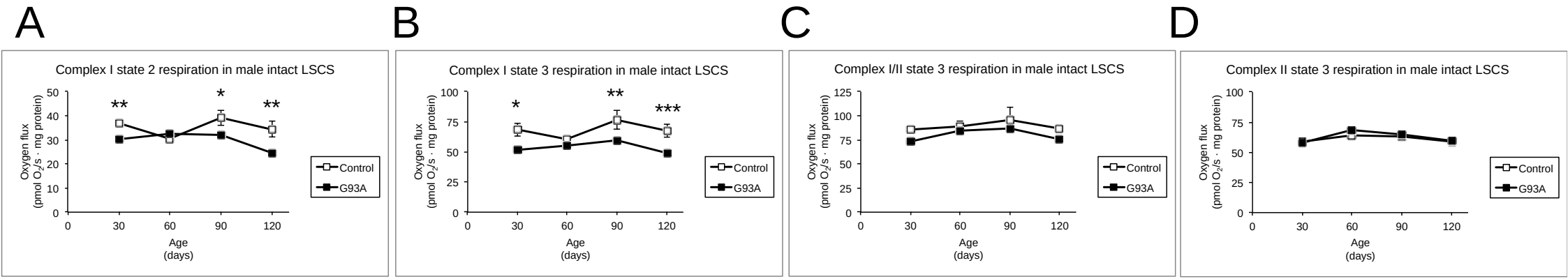


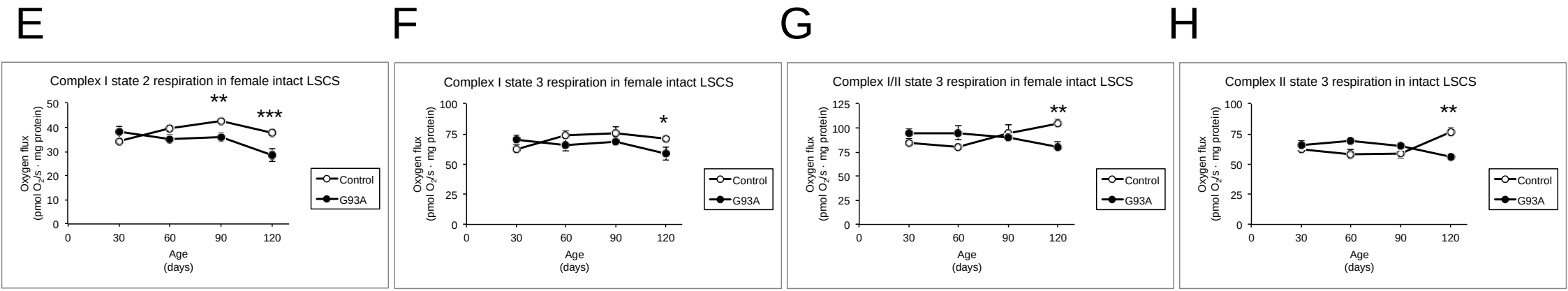
Figure S2 (supplemental)

Figure S3 (supplemental)

Male



Female



Control Animals

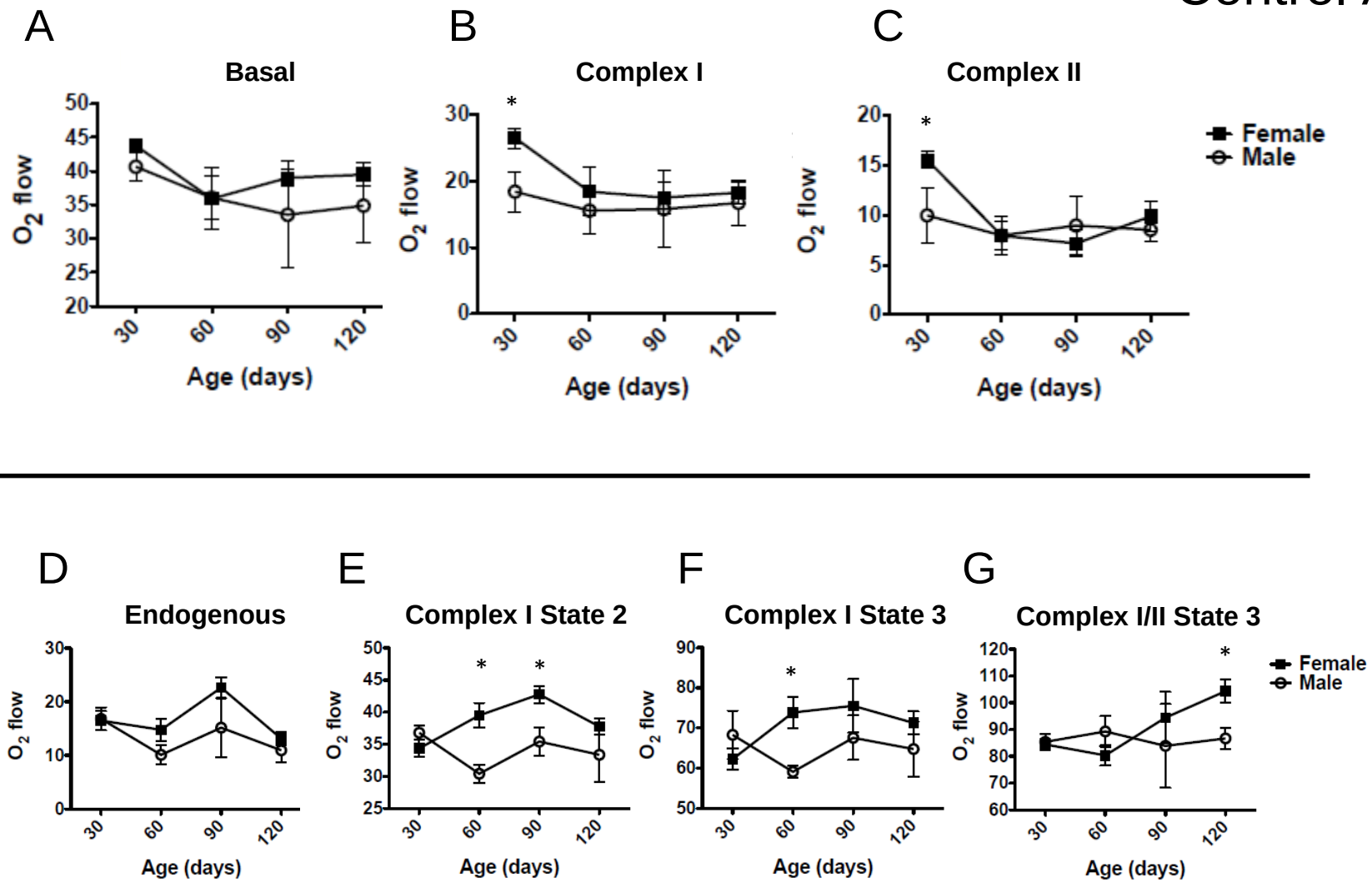
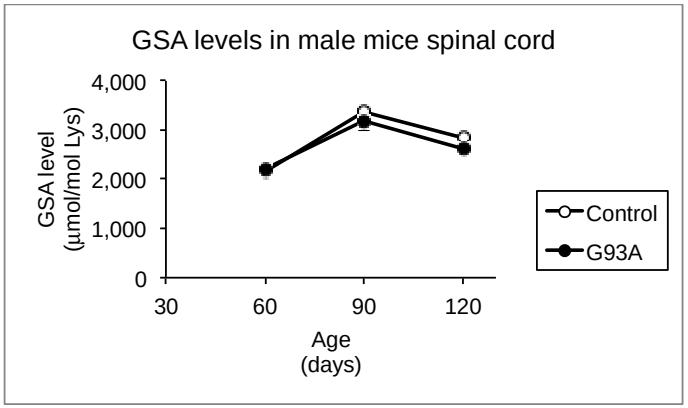
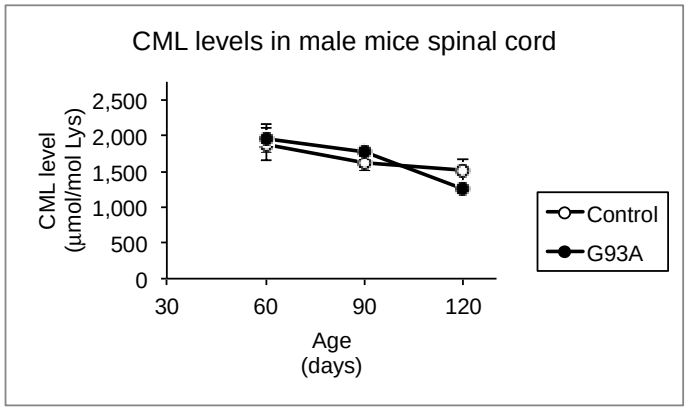


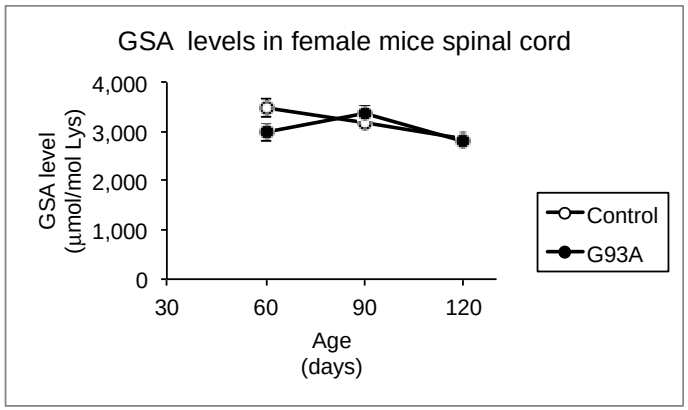
Figure S4 (supplemental)

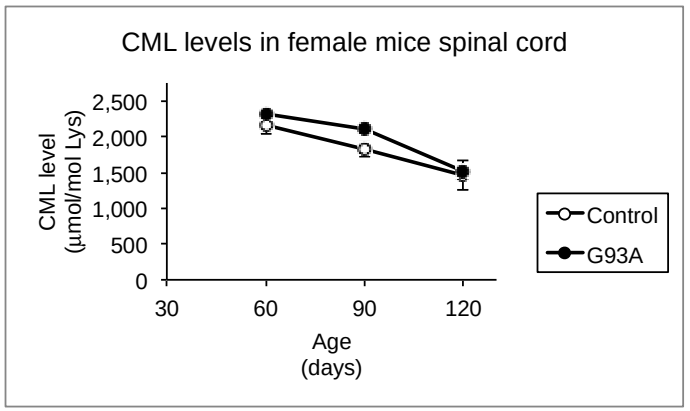
Figure S5 (supplemental)



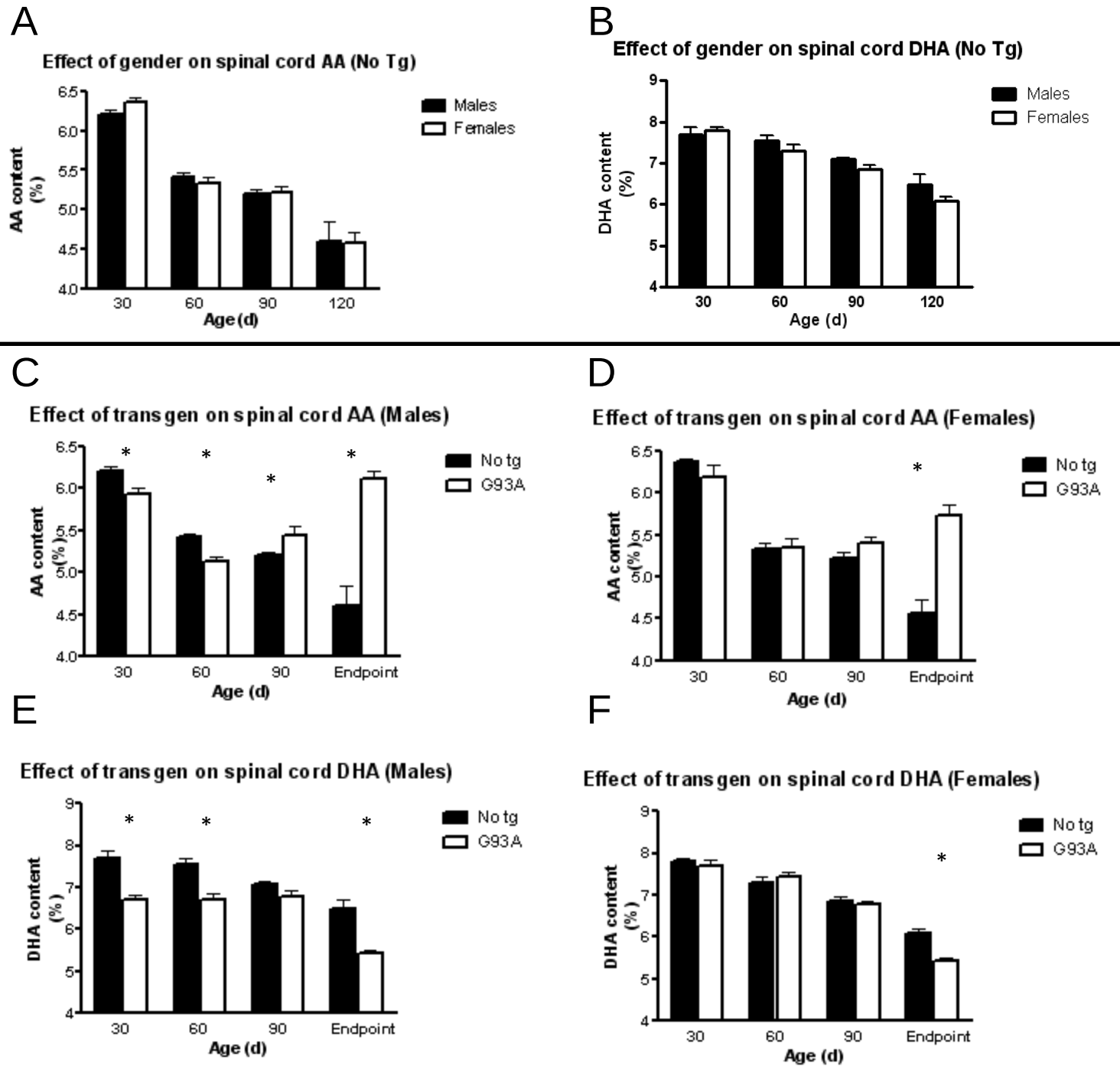


Male





Female

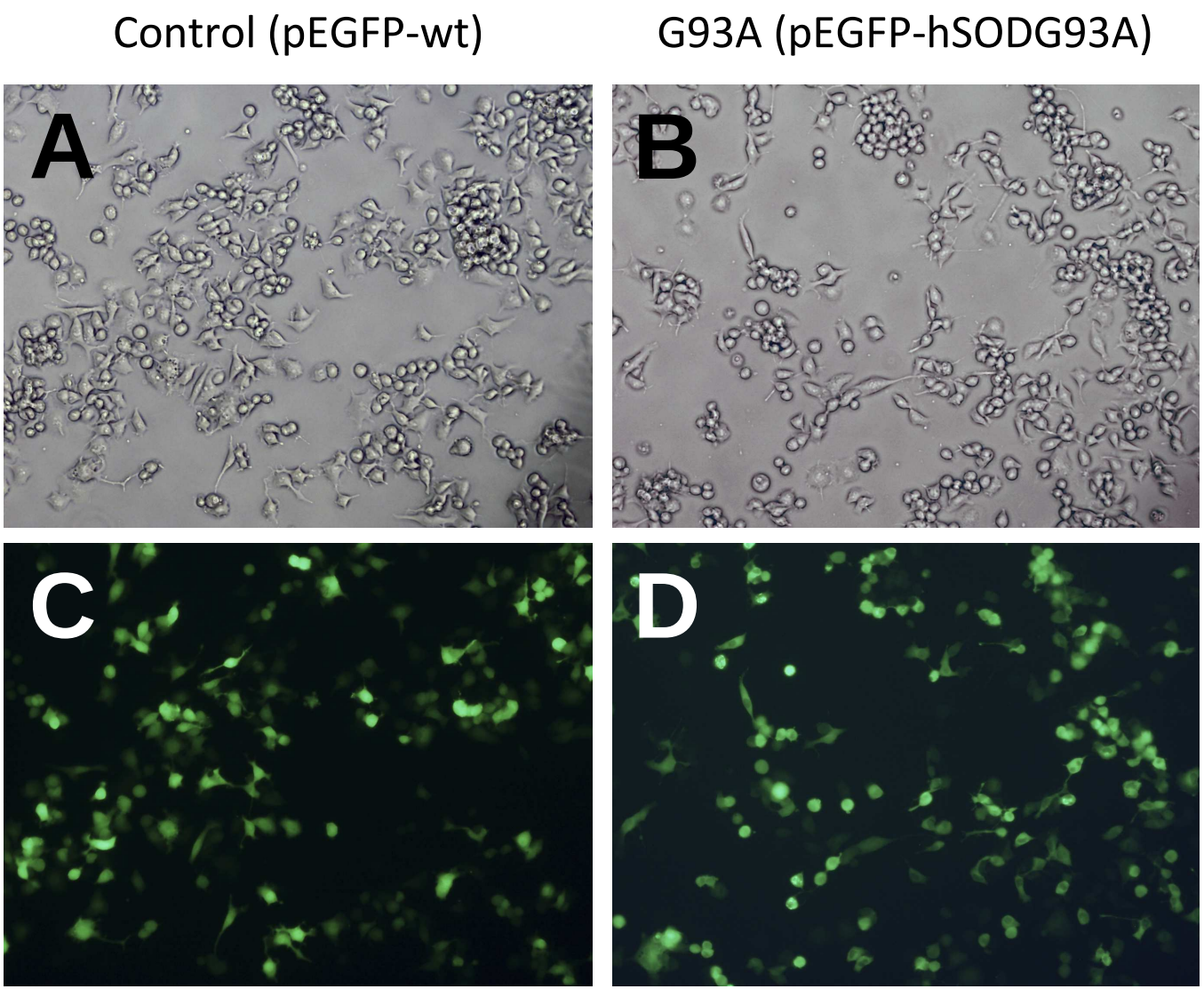


Male

Female

Figure S4 (supplemental)

Figure S7 (supplemental)



5. Discussion

5. Discussion

The work presented here summarized different methodological approaches aimed to shed some light on FA dietary interventions to better understand pathophysiology of ALS. Also relevant, we tried to increase the knowledge of a variety of well-established models of the disease (e.g. cell lines, OT, SODG93A), involving FA handling or sexual dimorphism, which were found altered in human patients.

For this purpose, we started with a very simplistic models of biomolecule substrates (lipid but also proteic) trying to define and characterize a suitable natural dietary antioxidant and its possible improved outcome (by means of reduced oxidative accretion and upgraded cell survival) when being co-treated against different oxidative stimuli. As many others had pointed before (reviewed in (Benzie and Choi 2014)), the heterogeneity encountered, which could be related to the different sources of oxidative stress, made the election of a single antioxidant substance very complex (to protect against those multiple situations). Those variable results could be therefore explained in terms of altered antioxidant properties of a specific compound as well as the different model involved. Thus, this capacity would greatly vary, sometimes based on antioxidant chemical structure. In fact, hyperconjugation and proton dislocation properties, like those obtained thanks to orto-dihydroxyl structures (e.g. those found in secoiridoids), phenol radical cation and phenoxyl radical delocalization (e.g. phenolic acids) (Joshi et al. 2004) and/or α/β unsaturated structures (e.g. flavonoids and lignans among others) (Brown et al. 1998) have been proved to counteract oxidative-damage spread.

Anyway, antioxidant potential would also rely on the possible interaction with the substrate (e.g. lipid or aqueous media) and, in a complete animal model, its inherent natural dietary bio-availability (e.g. the chance to be in the right time and location). Therefore, the election of a suitable antioxidant would depend on the specific mechanism of oxidation that intercepts and the particular environment where it takes place, for those reasons being very difficult to find a broad-field one.

In any case, in general, luteolin and hydroxytyrosol showed a better response to oxidative stress (by multiple oxidative methodological approaches), in terms of oxidative carbonylation accretion (as shown by WB against DNP adducts in ox LDL) and alleviated oxidative mediated-cell death in HEMC-1 and HepG2 cell lines (*tert*- butoxide and Cu^{+2} incubation, respectively). But in the other hand, luteolin failed to reduce PC-oxidative derivatives (chromatographically measured, e.g. PLPC, SGPC, PGPC) in oxLDL, when compared, for instance, with hydroxytyrosol. Former specie also failed to help improving the FA preservation in the LDL, as was shown thanks to lipid profile measurements, with a better behavior of hydroxytyrosol, pinoresinol or gallic acid under those conditions. Thus, luteolin action could be more relevant in terms of protein modification reducer than in truly lipoxidative prevention.

Interestingly, specific oxidative signature was found *in vivo* (as suggested by the lipidomic profile of hamsters' plasma and the oxLDL GC/MS data). Hence, lipidomic analysis of hamster plasma revealed lower accretion of oxidized PL (e.g. PLPC, OGPC, PGPC) when animals consumed a polyphenol extract diet, even when total antioxidant capacity (FRAP measured) was unchanged. Furthermore, DNP accretion was reduced *ex vivo* when plasma was incubated

under Fe^{+3}-Asc methodology and, similarly, human plasma was also protected with the luteolin incubation.

Either way, this first work established a noticeable relation between oxidative status, antioxidant action and specific lipid signature, which was useful in further research, even when, probably due to its intrinsic complexity, we failed to find a universal antioxidant.

Characteristic for ALS, as well as in other neurodegenerative diseases (Pamplona et al. 2008), it is known to have an increased oxidative accumulation (Shaw et al. 1995). Whether this fact is cause or consequence is still under debate, but strategies aimed reduce or control the spread of the damage showed positive outcomes, at least in some animal models (Gurney et al. 1996; Reinholz, Merkle and Poduslo 1999). Hence, for such a complex disease was worth to try to define a possible upgrade in terms of lipoxidative damage, both in cellular models (N2A, NSC34), tissular (organotypic) and animal models (SODG93A), before shifting to human scale. For this purpose, the election of GC/MS methodology, as well as WB against carbonylated adducts to assess the relevant accretion of damage to proteins was judged to be adequate. Moreover, we could depict the source of this damage, as we could relate it to specific metabolic damage *vias*, determining metal catalyzed -AASA and GSA- purely lipoxidative -MDAL-, glicoxidative -CEL-, mixed lipo-glycoxidative -CML- or even subcellular location (e.g. mitochondrial; 2-SC) as different origins protein oxidative damage. Furthermore, thanks to chromatography, whole lipidomic analysis as well as lipid percentage profiles of studied tissues/experimental situations we could ascertain whether "dietary" intervention is achieved (e.g. flux experiments, DHA accretion, AA depletion) and/or if an specific analyte is accumulated in response to a determined situation (e.g. NPD1, 8-iso-$PGF_{2\alpha}$). In a recent publication (Zhang et al. 2010) researchers showed an intriguing novel role of 15-deoxy PJ_2 (mostly derived from the AA) a PG derivative dercrived to exert anti-inflammatory actions (Shibata et al. 2002). They demonstrated that *in vitro* treatment of this PG in cell lines altered TDP-43 proteolysis, solubility and subcellular localizations, resulting in a general better status.

Hence, these two approaches (e.g. oxidative damage to proteins and lipid composition) were therefore very helpful for a better description of such situations and were completed with IHQ analysis. Expression of a determined protein in relation to others (which could be confirmed by WB) is relevant for a precise description, but more complete information could be obtained when the expression is also localized within a cell. Hence, for most enzymes is not sufficient to exist, but rather to exist in the right place and this is especially relevant for CNS where very different cell population (with completely different functions, thus protein expression) is found. Finally, measures of oxygen consumption (high resolution respirometry) may help to both, describe a particular malfunction situation along ETC and justify a greater ROS production. Therefore, all those techniques, plus animal behavioral test for precise clinical evolution measurement, were aimed to shed some light over this devastating disease and its relation with lipid status.

Admitting the multiple limitations of co-cultured experiments: FA/cell line; FA/primary cultures (e.g. multiple cell types, bioavailability, delivery, metabolic degradation, albumin crosslink), but aiming to minimize complexity, flux/incorporation experiments (exposure of FA or mixtures of them to single cell and/or organotypical cultures) were designed to ascertain PUFA supplementation effects in relation to various cellular functions (i.e. O_2 consumption, antioxidant activity potential, survival against oxidative stressors, excitotoxicity, synaptic

protein expression, among others). In those measures, lipidic products (and antioxidants) dosages were designed according to an homeostatic range. Similarly, in animal models (e.g. hypercholesterolemic-feed hamsters supplemented with a polyphenol extract and FA unsaturation degree of in ALS model) isocaloric diets fulfill essential FA necessities In both cases, particular compositions were aimed to, up to an extent, "stretch" the metabolic machinery, trying to observe subtle differences rather than big dietary alterations that would completely change the regular functioning. Anyway, in fact, accomplishing *in vivo* tissular modification is rather difficult, especially in CNS with exquisite nutrients necessities. Reinforcing this, only discrete modifications were found in response to the diet (WT animals) and few more due to the transgene expression (WT *vs.* SOD1G93A, data not shown).

Hence, since previous works claimed for an altered DHA homeostasis in ALS patients (Ilieva et al. 2007), it was interesting to pay special attention in its synthetic machinery. Interestingly, we found FASD1 and FASD2 altered expressions in spinal cord MNs. Further, thanks to IHQ, we showed a previously unreported FASD1, ACOX1 and AACA1 decrease in MN where pTDP-43 accumulates aberrantly. Surprisingly, those findings were not so evident when total protein extract (WB) was used. In fact, general increased AACA1 and FASD2 accumulation, indicating a potential higher DHA synthesis, was insufficient (Ilieva et al. 2007), pointing that cell specific mechanism may be operating. Thus, by switching to cellular models, our interest was in trying to define how this cellular relation could be altered in terms of DHA synthetic machinery and surrogates (STX-3, Drebrin) status. Undoubtedly, cell lines (N2A) transfected with aggregation prone TDP-43 fragment suffer from FASD2 depletion. Apart from the commented conversion of ALA to stearidonic acid (C18:4ω-3) along the ω-3 synthesis of DHA, this enzyme is also responsible for the generation of DGLA (C20:3ω-6, through the previous synthesis of γ-Linolenic -18:3ω-6) in the ω-6 series. Interestingly, DGLA derivatives (with just one double bound along the aliphatic residues, thus named 1 series PG, e.g. $PGF_{1\alpha}$, $PGE_{1,}$) even when less abundant, exerted mainly anti-inflammatory properties (Fan and Chapkin 1998; Kapoor and Huang 2006; Xu et al. 2014). Hence, FASD2 loss could result not only in less DHA synthesis (and consequently its anti-inflammatory derivatives depletion) but also for reduced non-DHA-related anti-inflammatory intermediates.

Also important, drebrin (but not STX-3) expression was reduced in these cells too (WB and IHQ measures) in response to TDP-43 aggregation, but again, a tissular variation was found in human samples. Spinal cord (where DHA is depleted) demonstrated drebrin and STX-3 reduction (the latter not significant), whereas brain cortex (with DHA accretion in patients) did not show those changes. However, MN drebrin reduction in patients was not linked with increased pTDP-43 accumulation, as shown by IHQ, even when drebrin function was altered in oxidative stress (Li, Wang and Zuo 2013). Furthermore, reduced mitochondrial Ca^{+2} buffering capacity was found under ω-3 supplementation (at least in in CD_4^+ T cells, (Yog et al. 2010)) although other reports showed no relation of dietary FA with the Ca^{+2} capacity, nor response to oxidative stimuli and, importantly, stress the importance of a correct dosage for supplements (Stavrovskaya et al., 2012). Nevertheless, altered lipid metabolism and TDP-43 have been recently linked (Heck et al. 2014), since deletion of the latter caused membrane alterations as well as fat storage disarrangements. And further, we could confirm in our model that TDP-43 phosphorylation and aggregation preceded PUFA's synthesis changes (measured after 2h) and drebrin loss, after an H_2O_2 challenge.

In more a complete model (in terms of intercellular relationships), the OT primary culture, increased DHA (concomitant with increased AA and a drastic depletion of ω-3 DPA, a DHA precursor) was found thanks to GC. Further, specific lipid signature (lipidomic measurements) and increased oxidative damage was confirmed under THA treatment. This is, significant accretion of 8-iso-$PGF_{2\alpha}$ and higher NPD1 (DHA derivative) as well as a positive correlation among them was confirmed. Several works showed increased NPD1 production in response to an insult (Bazan 2013; Bazan 2005; Mukherjee et al. 2007), but to our knowledge, this is among the first linking exictoxicity and NPD1 production (and excitoxicity and oxygen resepirometry reduction too). In addition, OT tissue composition (higher DHA under THA conditions) supports the possibility of this production, reinforced by the fact that in a neuronal cell line (N2A) chooses DHA synthesis as an initial response when confronted to oxidative stress (as show in flux experiments) and oxidative stress and mitochondrial dysfunction is known to be increased in excitotoxicity (Nguyen et al. 2011). Further explanation of an increased NDP1 production could be understood in terms of upgraded survival, which may involve PI3K/Akt signalling (Halapin and Bazan, 2010), know to be influenced by DHA (Akbar et al 2005) and by PLA_2 (Lukiw et al. 2005) action too. In any case, thanks to the previous studies (Ilieva et al. 2007), spinal cord DHA depletion was found, which is inconsistent with this increased DHA in cells and OT. However, this may be understood as an end point result. sALS samples are probably the most valuables ones since they are not a model, it's real life. But on the other hand, these patients have suffered for a long period of disease. Hence, their decreased levels could possibly indicate an insufficient synthesis or depletion due to disease-related-expenses. Beside this, models would reflect a more "acute" situation, whereas a possible correction to attain a new homeostasis may be achieved yet. Interestingly, co-culture with precursors (linen seed fatty acid extract) rather than final products (fish oil extract) showed better MN preservation. Altogether, these two facts (increased oxidative stress and better survival with lower peroxidability index -PRI- and double bound index -DBI- FA mixture -e.g. linenseed extract) could respond to an adaptative mechanism. Hence, this fact was reinforced by the upgraded MN survival when those explants are co-treated with DHA plus tocopherol (optimally preserved from oxidation), which goes in line with a recent publication demonstrating a reduced neuroinflammation in terms of decreased COX-2 activation and cell death prevention in a spinal cord cultures co-treated with a saturated FA (instead highly unsaturated DHA) and luteolin as antioxidant (Paterniti et al. 2013).

Finally, mitochondrial implication was proved, since lower (c.a. 30%) oxygen consumption was found under excitotoxicity. This may be explained in terms of complex I malfunction, because rotenone (complex I inhibitor) faded out this difference, but also due to oxidative modifications, since tocopherol treatment along the culture protected from this event. This is relevant because previous studies had link CI blockade with increased ROS production (Votyakova and Reynolds, 2001). Of note, interestingly mitochondrial permeability transition pore induction (discussed latter) and excitoxicity-ROS production was linked too (Hansson et al. 2008). This is supported by the upgraded MN survival when DHA and tocopherol are combined. Interestingly, UCP2 overexpression, which is been related to a ROS reduction in various systems (Andrews and Horvath 2009), shortened lifespan, worsens mitochondrial dysfunction and accelerates disease progression when co-expressed in the SOD1G93A mouse model (Peixoto et al., 2013). This surprising finding may indicate that a minimal oxidative damage is required and we probably need deeper studies for the understanding of ROS production and survival interdependences.

Gender dependence among neurodegenerative diseases is observed (Członkowska et al 2006), as well in ALS incidence and some animal models recapitulated this effect. Recent neuroimaging studies in patients showed significant gender differences in the anatomical patterns of cortical and subcortical pathology in ALS (mostly localizing to extramotor, frontotemporal and cerebellar regions; (Bedeet al. 2014)). However, specific description of this dimorphism was not deeply described in preclinical models. In particular, how this relation could be dietary modified. Similar to a recently published paper (Yip et al. 2013), we demonstrated that potential preventive and beneficial responses attributed to PUFA are not easily reproduced in the SODG93A mouse model. Hence, as previously shown (Herrero et al. 2001), FA had an influence on the specific oxidative stress markers accretion. We demonstrate that this dietary difference was linked to survival differences (with high unsaturation diet diminishing the feminine-gender life sparing extension effect). However, one of the more striking results was the surprisingly lower lipoxidative damage marker founded in animals under lower unsaturation diet. Those animals had similar values of DHA in their spinal cords along disease (with a clear reduction along its course for both sexes), but in the contrary higher levels of AA and one of its derivatives (C22:4ω-6) and those levels raised general higher PRI and DBI for animals under low unsaturation diet. In a detailed vision of this FA profile, males under low unsaturation diet were the ones with better DHA preservation along disease progression (estimated as % ratio of initial DHA content-60 days- respect to endpoint; 95,57% and 79,65% for males under low and high unsaturation diets respectively, 84.89% and 86,56% for females under low and high unsaturation diets respectively) and this group corresponds with the one with longer survival and lower UPR response.

Reinforcing this possible influence in survival with a correct DHA preservation, females, with general longer survival also conserve more tenaciously this FA. On the other hand, the AA accretion along disease of animals under low unsaturated diets should be viewed as a response to disease demands as well dietary influence, since this FA is in fact lower in this diet, compared with the high unsaturated one. Reinforcing this, results from animals under a control diet (the last paper) showed an increased AA accumulation latter in disease, in both genders, but more remarkable in males. Finally, correlation analysis showing a positive relation among spinal cord MDAL and AA content and a inverse relation of DHA and AASA, but just for females, emphasize the idea that lipoxidative damage could have a more deleterious effect in females even when they were preserved in terms of stride length preservation under high usaturation diet. Interestingly, correlation analysis of those variables in males only show a positive relation for GSA and survival, indicating again that deeper studies focusing in the oxidative modifications and survival interdependences should take gender as another variable.

Further, we showed an unreported relation of diet on UPR (e.g. ubiquitin), DNA oxidation (e.g. 8-oxo.dG) and DSB (e.g. γH2Ax) responses. Males under low unsaturation diet demonstrated lower DNA oxidative damage but an unaltered mitochondrial-nuclear crosstalk concomitant to a reduced UPR response and a slower disease progression (in terms of stride length preservation). All those findings could serve as an explanation for the upgraded survival of males under such diet.

For a deeper understanding of mitochondrial function, ROS generation and damage to different biomolecules focusing in the sex as a modulator character we performed experiments in intact tissue (previous work had described mitochondrial preparation (Mattiazzi et al. 2002) but not intact tissue) as well as permeabilyzed fibers of spinal cords. This helped to better describe a

natural occurring situation as well as what happened when mitochondria functioning at maximal capacity. Sample collection across all animal lifetime served us to show differential (late onset in females) respiration defects along it and, more importantly, how those changes were related with increased oxidative protein modification accretion. In fact a single sample collection, instead a cross-sectional study would only show a static frame and not those interesting outcomes.

Mitochondrial permeability transition pore is a protein "hole" in the inner mitochondrial membrane. It was originally discovered linked to neurodegeneration (Haworth and Hunter, 1979) and upon its formation, among others features, decreased membrane potential linked to cell death, mitochondrial swollen and mitophagy (reviewed in (Dagda et al. 2013) is found. Furthermore, its occurrence is known to be affected by Ca^{+2} overload (Brustovetsky et al. 2002), and to be induced by some FA derivatives (García-Ruiz et al. 2000). Therefore, excitoxicity severely affects its formation and better outcomes are expected when this pore is abolish (e.g. its blockade -by cyclosporin A- allows complete recovery of membrane potential and therefore prevents cell death in cultured neurons (Schinder, Olson and Spitze 1996)). Hence mitochondrial pore formation could serve as a link between decreased respiration founded in the OT model and the exitotoxicity, and gender could modulate this. However, gender implication in the OT model remains to be elucidated and could be an interesting point to be address which could account for differential survival of the MN and impinge many other outcomes and could also rely under the upgraded survival and decreased oxidative modification and better mitochondrial function founded in females overexpressing SOD1G93A. In fact there is controversy whether hormones could alter this survival. A recent publication shows progesterone neuroprotection after spinal cord injury (Labombarda et al. 2013) but previous works demonstrated androgen-related positives outcomes (Hauser and Toran-Allerand 1989 and Levy et al. 1996). However, male Ca^{+2} reuptake was showed increased respect to females, rendering them more sensible to overload, at least in heart mitochondria (Arieli et al. 2004)).

Thus, linking those issues, in a recent publication (Kim, Magranè and Manfredi 2012) researchers focused in gender relevance in the mitochondrial permeability transition pore formation in the SODG93A mouse model. Hence, they demonstrated that ablation of cyclophilin D (a protein structurally linked to the mitochondrial permeability membrane transition pore (Doczi et al. 2011)) completely abolished the phenotypic advantage of female SODG93A, but no effect was found in males. In fact, they showed that 17β-estradiol protected SODG93A cortical neurons and spinal cord motor neurons against glutamate toxicity in brain mitochondria, but the protection was lost in neurons lacking cyclophilin D. This could be under the positive outcome founded in terms of upgraded oxygen consumption in females, since our results showed that the mitochondrial dysfunction was not linked to specific mitochondrial complex protein expression (e.g. Complex I and II, at least in cell lines). Further research will focus in the role that uncoupling proteins and their previously commented ROS reducing potential.

In the animal model, we found a gender-shift of both mitochondrial-related and oxidative stress-related pathophysiological outcomes and clinical features in female G93A mice respect to their male littermates. This different mitochondrial dysfunction onset could be, at least in part, underneath the delayed clinical features observed in G93A female mice respect to males, and more importantly, in male patients respect to the female counterparts. From literature, a greater oxidative modification could be expected under G93A overexpression. However, in a closer

look, we are not among the first to unveil a non-linear progression in the oxidative damage for this model (Andrus et al. 1998; Hall et al. 1998; Vinsant et al. 2013; Miana-Mena et al. 2011; Kirkinezos et al. 2005; Cacabelos et al. 2014). Even when our results do not completely fit this previous findings, our measures are lumbar specific (nor cervical or thoracic sections), we split by gender and methodology applied to the measurements were different. Hence, this could explain little variations with previous reports.

Regarding lipid measurements in this model, the most remarkable feature was the reduction along disease founded for DHA. Animals expressing the G93A mutation demonstrated a DHA reduction in spinal cord content, concomitant to an increase of AA. This fact was also present in the dietary study previously commented, even when for those animals; the diet was in fact defective in this specific FA. Taking this two fact into account, and previous findings related to FA synthetic machinery, altogether seems clear that particular FA composition do play a role along disease and it would be hot topic for research in further works. In fact, even knowing that females displayed a differential FA regulation and metabolism (or probably due to this) any information on the gender-specific disease modification is welcome to try to apply a more personalized treatment.

Finally, trying to give some ligth regarding the estrogen interference, we used Neuro 2A cells overexpressing G93A-hSOD1 with a “feminization” treatment (This cell already express the estrogen receptor (Li, Hao and Li 2014)). Treatment with 17β-estradiol for long period of the culture (10 days) did not show to be detrimental in terms of survival. In the same line, GFPhSOD1G93A transfection do not comprise cell viability. However, estradiol treatment resulted in a similar oxygen consumption recovery, with “females” transfected with de G93A mutation showing an upgraded functionality. On the contrary, untreated “males” were unprotected against SODG93A-mediated mitochondrial malfunction. For this model, no protein expression modification was found between the mitochondrial complex subunits (I and II) to account for this reduced mitochondrial capacity, hence, we still miss a mechanisms to explain the altered functioning.

However, in a recent work (Tao et al. 2012) Tao and collaborators linked mitochondrial malfunctioning (rotenone mediated) and estradiol signaling in neuron primary culture. Hence, researchers showed altered gene induction in response to mitochondrial stress for male and female embryos. Furthermore, they detailed an upgrade survival of female primary cultured neurons rather than male when submitted to rotenome mediated cytotoxicity. Interestingly, they proved that the N2A cell line can be protected against oxidative stress through estrogen signaling (although they linked to a reduction of its protein receptor expression) and that under rotenome cytotoxicity, estradiol treatment both gender of primary-cultured neurons were protected. Altogether this results support our findings of feminine gender upgraded protection against mitochondrial dysfunction. Further analysis of these issues in relation to ALS and its potential therapeutical approaches should be considered in future works.

So globally, results in this thesis showed a relevant influence of sex on ALS outcome. FA metabolism seems to be altered (DHA and the enzymatic machinery) in patients, and cellular and tissular models could help to explain this difference. Furthermore, gender handling of FA is also different in animal models of the disease when confronted to saturated or unsaturated enrich diet, leading to differential survival and clinical features. Also relevant, oxidative modifications are dietary-altered and could help to understand survival differences

(lower ubiquitin inclusion, decreased 8-oxo-dG accretion upgraded DHA retention for males under low unsaturation diet). Finally, in line with previous works, mitochondrial implication was demonstrated also in intact tissue as well as in permeabilized tissue in the animal model (SODG93A), with a complex I deficiency, linked to a decreased oxidative accretion (MDAL) specially relevant in male and those reduced oxygen consumption was not attributed to a deficient protein expression nor activity of those.

… but may be related to a sexual-dependence Ca^{+2} excitotoxy ROS-interference … which would be further explored.

6. Conclusions

6. Conclusions

As a result of this research, we can consider the following conclusive statements:

One.- *In vitro* antioxidant capacity of a given nutritionally available substance is severely influenced by the oxidative methodology used and its definite action upon a substrate; however, a specific signature in terms of oxidative modification accretion and fatty acid protection preference in LDL and other biomolecules, could be described.

Two.- Endothelial and hepatocyte cell line cultures complement the above mentioned data for the biological relevance in terms of survival in front oxidative stress; hence, election of a single antioxidant to protect against those diverse situations can be challenging.

Three.- *Ex vivo* oxidative accumulation in hamsters serum under a grapeseed-derived, poliphenol-enriched diet showed an specific lipidomic (POVPC, PGPG) and proteic (DNP measured) signature, whereas overall antioxidant status (FRAP measured) remains steady.

Four.- sALS is associated with spinal cord specific changes in DHA synthetic machinery (FADS2, ACAA1, ACOX-1) and Drebrin. IHQ measurements show that these changes are related to TPD-43 aggregates in motor neurons.

Five.- Neuron cell culture with TDP-43 aggregates partially reproduced sALS findings (reduced FADS2 and Drebrin). However, sALS changes are cell and site specific since WB levels do not correlate to IHQ.

Six.- Drebrin depletion in sALS spinal cords is cell specific; *in vitro* experiments showed that oxidative-derived TDP-43 aggregates precede decreased Drebrin expression. Furthermore, DHA synthesis from its precursors could be trigger by H_2O_2 incubation.

Seven.- Chronic excitoxicity in organotypic spinal cord cultures promoted lipidomic and fatty acid profile changes compatible with altered DHA synthesis, highlighting the relevance of this model to mimic the pathophysiological features that lead to motorneuron death.

Eight.- Gender dimosphism is a relevant factor influencing lipid handling in the hSODG93A ALS animal model in terms of survival and clinical outcome.

Nine.- DHA-related synaptic machinery (syntaxin3) and UPR (Ubuquitin inclusions) responses in hSODG93A male mice is altered in response to high unsaturation degree diet.

Ten.- Dietary intervention aimed at modulating the FA composition by changes in FA sources evoked expected tissular FA modification and influenced protein and DNA oxidative damage with an altered nuclear-to-mitochondrial damage responses.

Eleven.- Mitochondrial dysfunction in hSODG93A mouse model (assessed by oxygen consumption measurements) is variable throughout disease. Females had a later oxygen consumption reduction.

Twelve.- Gender dimorphism in oxygen consumption correlated with female later onset and lower oxidative modifications as well as optimized lipid content.

Thirteen.- Overexpression of mutant SOD1 in cell cultures recapitulate oxygen consumption reduction in hSODG93A mouse model.

Fourteen.- Estrogen treatment (10nM) on those cells is able to reproduce female-outcome, protecting from CI dysfunction, without changes in expression of peptides belonging to CI or CII.

7. Bibliography

7. Bibliography

-Books

David L. Nelson and Michael M. Cox. Worth Publishers: New York, 2000. ISBN 1-57259-9316. 1255 p; p 343-364. Lipids. In: Principles of Biochemistry. New York: W.H. Freeman and Company; 2005.

Berne y Levy. "Fisiologia", 2000. Bruce M. Koeppen, Bruce A. Stanton (Ed.). Elsevier. ISBN-13 9788480864343.

Halliwell B, Gutteridge JMC (2000) "Free Radical Biology and Medicine". Oxford, science publications UK: Oxford University Press.

Mitochondrial Oxidative Phosphorylation, 2012. "Nuclear-Encoded Genes, Enzyme Regulation, and Pathophysiology. Series: Advances in Experimental Medicine and Biology", Vol. 748 Kadenbach, Bernhard (Ed.) 2012, XII, 372 p.; DOI 10.1007/978-1-4614-3573-0.

E Herrera 1993. "Elementos de Bioquimica". Mc Graw Hill 1993.

-Scientific Articles

Abbott, N Joan et al. 2010. "Structure and Function of the Blood-Brain Barrier." *Neurobiology of disease* 37(1): 13–25.

Abraki, Shahnaz Babaei, Leila Khalaj, Fatemeh Shaerzadeh, and Fariba Khodagholi. 2013. "Simultaneous Inhibition of COX-2 and Activation of PPAR-Γ Resulted in the Same Level and Pattern of Neuroprotection as They Were Targeted Separately." *Journal of molecular neuroscience : MN* 49(1): 116–29.

Adams, J, Y Collaço-Moraes, and J de Belleroche. 1996. "Cyclooxygenase-2 Induction in Cerebral Cortex: An Intracellular Response to Synaptic Excitation." *Journal of neurochemistry* 66(1): 6–13.

Agostoni, C et al. 1997. "Developmental Quotient at 24 Months and Fatty Acid Composition of Diet in Early Infancy: A Follow up Study." *Archives of disease in childhood* 76(5): 421–24.

Aïd, Sabah et al. 2003. "Effect of a Diet-Induced N-3 PUFA Depletion on Cholinergic Parameters in the Rat Hippocampus." *Journal of lipid research* 44(8): 1545–51.

Ajroud-Driss, Senda, and Teepu Siddique. 2014. "Sporadic and Hereditary Amyotrophic Lateral Sclerosis (ALS)." *Biochimica et biophysica acta*.

Akbar, Mohammed, Frances Calderon, Zhiming Wen, and Hee-Yong Kim. 2005. "Docosahexaenoic Acid: A Positive Modulator of Akt Signaling in Neuronal Survival." *Proceedings of the National Academy of Sciences of the United States of America* 102(31): 10858–63.

Akiba, Satoshi, and Takashi Sato. 2004. "Cellular Function of Calcium-Independent Phospholipase A2." *Biological & pharmaceutical bulletin* 27(8): 1174–78.

Al-Chalabi, A et al. 2012. "The Genetics and Neuropathology of Amyotrophic Lateral Sclerosis." *Acta Neuropathologica* 124(3): 339–52.

Alessandri, Jean-Marc et al. 2008. “Estradiol Favors the Formation of Eicosapentaenoic Acid (20:5n-3) and N-3 Docosapentaenoic Acid (22:5n-3) from Alpha-Linolenic Acid (18:3n-3) in SH-SY5Y Neuroblastoma Cells.” *Lipids* 43(1): 19–28.

Alessandri JM, Extier A, Al-Gubory KH, Langelier B, Baudry C, LePoupon C, Lavialle M, Guesnet P. 2011. “Ovariectomy and 17β-Estradiol Alter Transcription of Lipid Metabolism Genes and Proportions of Neo-Formed N-3 and N-6 Long-Chain Polyunsaturated Fatty Acids Differently in Brain and Liver.” *The Journal of nutritional biochemistry* 22(9): 820–27.

Alessandri JM, Extier A, Al-Gubory KH, Harbeby E, Lallemand MS, Linard A, Lavialle M, Guesnet P . 2012. “Influence of Gender on DHA Synthesis: The Response of Rat Liver to Low Dietary A-Linolenic Acid Evidences Higher ω3 Δ4-Desaturation Index in Females.” *European journal of nutrition* 51(2): 199–209.

Andrews, Zane B, and Tamas L Horvath. 2009. “Uncoupling Protein-2 Regulates Lifespan in Mice.” *American journal of physiology. Endocrinology and metabolism* 296(4): E621–7.

Andrus, P K, T J Fleck, M E Gurney, and E D Hall. 1998. “Protein Oxidative Damage in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis.” *Journal of neurochemistry* 71(5): 2041–48.

Anning, Peter B et al. 2005. “Elevated Endothelial Nitric Oxide Bioactivity and Resistance to Angiotensin-Dependent Hypertension in 12/15-Lipoxygenase Knockout Mice.” *The American journal of pathology* 166(3): 653–62.

Araque, A, V Parpura, R P Sanzgiri, and P G Haydon. 1999. “Tripartite Synapses: Glia, the Unacknowledged Partner.” *Trends in neurosciences* 22(5): 208–15.

Ariel, Amiram, and Charles N Serhan. 2007. “Resolvins and Protectins in the Termination Program of Acute Inflammation.” *Trends in immunology* 28(4): 176–83.

Arieli, Yehuda et al. 2004. “Gender Modulation of Ca(2+) Uptake in Cardiac Mitochondria.” *Journal of molecular and cellular cardiology* 37(2): 507–13.

Atkinson, Jeffrey et al. 2010. “The Location and Behavior of Alpha-Tocopherol in Membranes.” *Molecular nutrition & food research* 54(5): 641–51..

Atsumi, G et al. 1998. “Fas-Induced Arachidonic Acid Release Is Mediated by Ca2+-Independent Phospholipase A2 but Not Cytosolic Phospholipase A2, Which Undergoes Proteolytic Inactivation.” *The Journal of biological chemistry* 273(22): 13870–77.

Austen, K Frank, Akiko Maekawa, Yoshihide Kanaoka, and Joshua A Boyce. 2009. “The Leukotriene E4 Puzzle: Finding the Missing Pieces and Revealing the Pathobiologic Implications.” *The Journal of allergy and clinical immunology* 124(3): 406–14; quiz 415–6.

Ayala, Victòria et al. 2011. “Cell Stress Induces TDP-43 Pathological Changes Associated with ERK1/2 Dysfunction: Implications in ALS.” *Acta neuropathologica* 122(3): 259–70.

Azevedo, Frederico A C et al. 2009. “Equal Numbers of Neuronal and Nonneuronal Cells Make the Human Brain an Isometrically Scaled-up Primate Brain.” *The Journal of comparative neurology* 513(5): 532–41.

Bakewell, Lucy, Graham C Burdge, and Philip C Calder. 2006. “Polyunsaturated Fatty Acid Concentrations in Young Men and Women Consuming Their Habitual Diets.” *The British journal of nutrition* 96(1): 93–99.

Balazs, Zoltan et al. 2004. “Uptake and Transport of High-Density Lipoprotein (HDL) and HDL-Associated Alpha-Tocopherol by an in Vitro Blood-Brain Barrier Model.” *Journal of neurochemistry* 89(4): 939–50.

Bannenberg, Gerard L et al. 2005. “Molecular Circuits of Resolution: Formation and Actions of Resolvins and Protectins.” *Journal of immunology (Baltimore, Md. : 1950)* 174(7): 4345–55.

Barber, Siân C, and Pamela J Shaw. 2010. “Oxidative Stress in ALS: Key Role in Motor Neuron Injury and Therapeutic Target.” *Free radical biology & medicine* 48(5): 629–41.

Basu-Modak, S et al. 1999. “Peroxisome Proliferator-Activated Receptor Beta Regulates Acyl-CoA Synthetase 2 in Reaggregated Rat Brain Cell Cultures.” *The Journal of biological chemistry* 274(50): 35881–88.

Batthyany, Carlos et al. 2006. “Reversible Post-Translational Modification of Proteins by Nitrated Fatty Acids in Vivo.” *The Journal of biological chemistry* 281(29): 20450–63.

Bazan, N G. 2013. “The Docosanoid Neuroprotectin D1 Induces Homeostatic Regulation of Neuroinflammation and Cell Survival.” *Prostaglandins, leukotrienes, and essential fatty acids* 88(1): 127–29.

Bazan, Nicolas G. 2005. “Neuroprotectin D1 (NPD1): A DHA-Derived Mediator That Protects Brain and Retina against Cell Injury-Induced Oxidative Stress.” *Brain pathology (Zurich, Switzerland)* 15(2): 159–66.

Bazan NG. 2008. “Neurotrophins Induce Neuroprotective Signaling in the Retinal Pigment Epithelial Cell by Activating the Synthesis of the Anti-Inflammatory and Anti-Apoptotic Neuroprotectin D1.” *Advances in experimental medicine and biology* 613: 39–44.

Bede, Peter, Marwa Elamin, Susan Byrne, and Orla Hardiman. 2014. “Sexual Dimorphism in ALS: Exploring Gender-Specific Neuroimaging Signatures.” *Amyotrophic lateral sclerosis & frontotemporal degeneration* 15(3-4): 235–43.

Beers, David R et al. 2008. “CD4+ T Cells Support Glial Neuroprotection, Slow Disease Progression, and Modify Glial Morphology in an Animal Model of Inherited ALS.” *Proceedings of the National Academy of Sciences of the United States of America* 105(40): 15558–63.

Beghi, Ettore. 2013. “Are Professional Soccer Players at Higher Risk for ALS?” *Amyotrophic lateral sclerosis & frontotemporal degeneration* 14(7-8): 501–6.

Beghi E, Pupillo E, Bonito V, Buzzi P, Caponnetto C, Chiò A, Corbo M, Giannini F, Inghilleri M, Bella VL, Logroscino G, Lorusso L, Lunetta C, Mazzini L, Messina P, Mora G, Perini M, Quadrelli ML, Silani V, Simone IL, Tremolizzo L; Italian ALS Study Group. 2013. “Randomized Double-Blind Placebo-Controlled Trial of Acetyl-L-Carnitine for ALS.” *Amyotrophic lateral sclerosis & frontotemporal degeneration* 14(5-6): 397–405.

Beisiegel, U, and A A Spector. 2001. “Lipids and Lipoproteins in the Brain.” *Current opinion in lipidology* 12(3): 243–44.

Beltowski, Jerzy. 2010. “Statins and ALS: The Possible Role of Impaired LXR Signaling.” *Medical science monitor : international medical journal of experimental and clinical research* 16(3): RA73–78.

Bendotti, C et al. 2001. “Early Vacuolization and Mitochondrial Damage in Motor Neurons of FALS Mice Are Not Associated with Apoptosis or with Changes in Cytochrome Oxidase Histochemical Reactivity.” *Journal of the neurological sciences* 191(1-2): 25–33.

Benedusi, Valeria et al. 2012. “The Peroxisome Proliferator-Activated Receptor Γ (PPARγ) Controls Natural Protective Mechanisms against Lipid Peroxidation in Amyotrophic Lateral Sclerosis.” *The Journal of biological chemistry* 287(43): 35899–911.

Benzie, Iris F F, and Siu-Wai Choi. 2014. “Antioxidants in Food: Content, Measurement, Significance, Action, Cautions, Caveats, and Research Needs.” *Advances in food and nutrition research* 71: 1–53.

Berger, Joel, and David E Moller. 2002. “The Mechanisms of Action of PPARs.” *Annual review of medicine* 53: 409–35.

Berghuis, Paul et al. 2007. “Hardwiring the Brain: Endocannabinoids Shape Neuronal Connectivity.” *Science (New York, N.Y.)* 316(5828): 1212–16.

Bernardo, Antonietta, and Luisa Minghetti. 2006. “PPAR-Gamma Agonists as Regulators of Microglial Activation and Brain Inflammation.” *Current pharmaceutical design* 12(1): 93–109.

Bernoud, N et al. 1999. “Preferential Transfer of 2-Docosahexaenoyl-1-Lysophosphatidylcholine through an in Vitro Blood-Brain Barrier over Unesterified Docosahexaenoic Acid.” *Journal of neurochemistry* 72(1): 338–45.

Berridge, M J. 1984. “Inositol Trisphosphate and Diacylglycerol as Second Messengers.” *The Biochemical journal* 220(2): 345–60.

Bertucci, Micka C, and Christina A Mitchell. 2013. “Phosphoinositide 3-Kinase and INPP4B in Human Breast Cancer.” *Annals of the New York Academy of Sciences* 1280: 1–5.

Bhurruth-Alcor, Yushma et al. 2010. “Novel Phospholipid Analogues of Pan-PPAR Activator Tetradecylthioacetic Acid Are More PPAR Alpha Selective.” *Bioorganic & medicinal chemistry letters* 20(3): 1252–55.

Bjelakovic, Goran et al. 2007. “Mortality in Randomized Trials of Antioxidant Supplements for Primary and Secondary Prevention: Systematic Review and Meta-Analysis.” *JAMA : the journal of the American Medical Association* 297(8): 842–57.

Bjelakovic, Goran, Dimitrinka Nikolova, and Christian Gluud. 2013. “Meta-Regression Analyses, Meta-Analyses, and Trial Sequential Analyses of the Effects of Supplementation with Beta-Carotene, Vitamin A, and Vitamin E Singly or in Different Combinations on All-Cause Mortality: Do We Have Evidence for Lack of Harm?” *PloS one* 8(9): e74558.

Björntorp, P. 1993. “Visceral Obesity: A ‘Civilization Syndrome’.” *Obesity research* 1(3): 206–22.

Blais, V, and S Rivest. 2001. “Inhibitory Action of Nitric Oxide on Circulating Tumor Necrosis Factor-Induced NF-kappaB Activity and COX-2 Transcription in the Endothelium of the Brain Capillaries.” *Journal of neuropathology and experimental neurology* 60(9): 893–905.

Blaskovic, Sanja, Alexander Adibekian, Mathieu Blanc, and Gisou F van der Goot. 2014. “Mechanistic Effects of Protein Palmitoylation and the Cellular Consequences Thereof.” *Chemistry and physics of lipids* 180C: 44–52.

Boas, F. E., L. Forman, and E. Beutler. 1998. “Phosphatidylserine Exposure and Red Cell Viability in Red Cell Aging and in Hemolytic Anemia.” *Proceedings of the National Academy of Sciences* 95(6): 3077–81.

Bonizzi, G et al. 1999. “Reactive Oxygen Intermediate-Dependent NF-kappaB Activation by Interleukin-1beta Requires 5-Lipoxygenase or NADPH Oxidase Activity.” *Molecular and cellular biology* 19(3): 1950–60..

Brankatschk, Marko, and Suzanne Eaton. 2010. “Lipoprotein Particles Cross the Blood-Brain Barrier in Drosophila.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 30(31): 10441–47.

Brash, A R. 1999. “Lipoxygenases: Occurrence, Functions, Catalysis, and Acquisition of Substrate.” *The Journal of biological chemistry* 274(34): 23679–82.

Brown, J E, H Khodr, R C Hider, and C A Rice-Evans. 1998. “Structural Dependence of Flavonoid Interactions with Cu2+ Ions: Implications for Their Antioxidant Properties.” *The Biochemical journal* 330 (Pt 3: 1173–78.

Brown, M S, and J L Goldstein. 1997. “The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor.” *Cell* 89(3): 331–40.

Brüne, B, and V Ullrich. 1991. “12-Hydroperoxyeicosatetraenoic Acid Inhibits Main Platelet Functions by Activation of Soluble Guanylate Cyclase.” *Molecular pharmacology* 39(5): 671–78.

Brustovetsky, Nickolay, Tatiana Brustovetsky, Ronald Jemmerson, and Janet M Dubinsky. 2002. “Calcium-Induced Cytochrome c Release from CNS Mitochondria Is Associated with the Permeability Transition and Rupture of the Outer Membrane.” *Journal of neurochemistry* 80(2): 207–18.

Bunney, Tom D, and Matilda Katan. 2011. “PLC Regulation: Emerging Pictures for Molecular Mechanisms.” *Trends in biochemical sciences* 36(2): 88–96.

Burke, John E, and Edward A Dennis. 2009. “Phospholipase A2 Biochemistry.” *Cardiovascular drugs and therapy / sponsored by the International Society of Cardiovascular Pharmacotherapy* 23(1): 49–59.

Calandria, Jorgelina M et al. 2009. “Selective Survival Rescue in 15-Lipoxygenase-1-Deficient Retinal Pigment Epithelial Cells by the Novel Docosahexaenoic Acid-Derived Mediator, Neuroprotectin D1.” *The Journal of biological chemistry* 284(26): 17877–82.

Calandria JM, Mukherjee PK, de Rivero Vaccari JC, Zhu M, Petasis NA, Bazan NG. 2012. “Ataxin-1 poly(Q)-Induced Proteotoxic Stress and Apoptosis Are Attenuated in Neural Cells by Docosahexaenoic Acid-Derived Neuroprotectin D1.” *The Journal of biological chemistry* 287(28): 23726–39.

Calder, Philip C. “Immunomodulation by Omega-3 Fatty Acids.” *Prostaglandins, leukotrienes, and essential fatty acids* 77(5-6): 327–35.

Calderon, Frances, and Hee-Yong Kim. 2004. “Docosahexaenoic Acid Promotes Neurite Growth in Hippocampal Neurons.” *Journal of neurochemistry* 90(4): 979–88.

Calì, Tito, Denis Ottolini, and Marisa Brini. 2013. “Calcium and Endoplasmic Reticulum-Mitochondria Tethering in Neurodegeneration.” *DNA and cell biology* 32(4): 140–46.

Calon, F. 2011. “Omega-3 Polyunsaturated Fatty Acids in Alzheimer’s Disease: Key Questions and Partial Answers.” *Current Alzheimer research* 8(5): 470–78.

Camandola, S et al. 1996. “Nuclear Factor kB Is Activated by Arachidonic Acid but Not by Eicosapentaenoic Acid.” *Biochemical and biophysical research communications* 229(2): 643–47.

Capitanio, Daniele et al. 2012. “Molecular Signatures of Amyotrophic Lateral Sclerosis Disease Progression in Hind and Forelimb Muscles of an SOD1(G93A) Mouse Model.” *Antioxidants & redox signaling* 17(10): 1333–50.

Caraci, Filippo et al. 2012. “Metabotropic Glutamate Receptors in Neurodegeneration/neuroprotection: Still a Hot Topic?” *Neurochemistry international* 61(4): 559–65.

Carlen, P L et al. 1994. “Formation and Electrophysiological Actions of the Arachidonic Acid Metabolites, Hepoxilins, at Nanomolar Concentrations in Rat Hippocampal Slices.” *Neuroscience* 58(3): 493–502.

Carlson, S E et al. 1996. “Visual Acuity and Fatty Acid Status of Term Infants Fed Human Milk and Formulas with and without Docosahexaenoate and Arachidonate from Egg Yolk Lecithin.” *Pediatric research* 39(5): 882–88.

Catalá, Angel. 2012. “Lipid Peroxidation Modifies the Picture of Membranes from the ‘Fluid Mosaic Model’ to the ‘Lipid Whisker Model’.” *Biochimie* 94(1): 101–9.

Cha, Ji-Young, and Joyce J Repa. 2007. “The Liver X Receptor (LXR) and Hepatic Lipogenesis. The Carbohydrate-Response Element-Binding Protein Is a Target Gene of LXR.” *The Journal of biological chemistry* 282(1): 743–51.

Chapkin, Robert S et al. 2008. “Bioactive Dietary Long-Chain Fatty Acids: Emerging Mechanisms of Action.” *The British journal of nutrition* 100(6): 1152–57.

Chen, Qun et al. 2003. “Production of Reactive Oxygen Species by Mitochondria: Central Role of Complex III.” *The Journal of biological chemistry* 278(38): 36027–31..

Chen, Su, and Papasani V Subbaiah. 2007. “Phospholipid and Fatty Acid Specificity of Endothelial Lipase: Potential Role of the Enzyme in the Delivery of Docosahexaenoic Acid (DHA) to Tissues.” *Biochimica et biophysica acta* 1771(10): 1319–28.

Chen S, Subbaiah PV. 2013. “Regioisomers of Phosphatidylcholine Containing DHA and Their Potential to Deliver DHA to the Brain: Role of Phospholipase Specificities.” *Lipids* 48(7): 675–86.

Chernomordik, Leonid V, and Michael M Kozlov. 2003. “Protein-Lipid Interplay in Fusion and Fission of Biological Membranes.” *Annual review of biochemistry* 72: 175–207.

Childs, Caroline E., Meritxell Romeu-Nadal, Graham C. Burdge, and Philip C. Calder. 2008. “Gender Differences in the N-3 Fatty Acid Content of Tissues.” *Proceedings of the Nutrition Society* 67(01): 19–27.

Chiu, Isaac M et al. 2008. “T Lymphocytes Potentiate Endogenous Neuroprotective Inflammation in a Mouse Model of ALS.” *Proceedings of the National Academy of Sciences of the United States of America* 105(46): 17913–18.

Cho, H P, M Nakamura, and S D Clarke. 1999a. “Cloning, Expression, and Fatty Acid Regulation of the Human Delta-5 Desaturase.” *The Journal of biological chemistry* 274(52): 37335–39.

Cho, H P, M T Nakamura, and S D Clarke. 1999b. “Cloning, Expression, and Nutritional Regulation of the Mammalian Delta-6 Desaturase.” *The Journal of biological chemistry* 274(1): 471–77.

Choi, Chan-Il et al. 2008. “Effects of Estrogen on Lifespan and Motor Functions in Female hSOD1 G93A Transgenic Mice.” *Journal of the neurological sciences* 268(1-2): 40–47.

Chowdhury, Rajiv et al. 2012. “Association between Fish Consumption, Long Chain Omega 3 Fatty Acids, and Risk of Cerebrovascular Disease: Systematic Review and Meta-Analysis.” *BMJ (Clinical research ed.)* 345: e6698.

Chowdhury R, Warnakula S, Kunutsor S, Crowe F, Ward HA, Johnson L, Franco OH, Butterworth AS, Forouhi NG, Thompson SG, Khaw KT, Mozaffarian D, Danesh J, Di Angelantonio E. 2014.

"Association of Dietary, Circulating, and Supplement Fatty Acids with Coronary Risk: A Systematic Review and Meta-Analysis." *Annals of internal medicine* 160(6): 398–406.

Ciana, Paolo et al. 2007. "A Novel Peroxisome Proliferator-Activated Receptor Responsive Element-Luciferase Reporter Mouse Reveals Gender Specificity of Peroxisome Proliferator-Activated Receptor Activity in Liver." *Molecular endocrinology (Baltimore, Md.)* 21(2): 388–400.

Cimini, Annamaria, and Maria Paola Cerù. 2008. "Emerging Roles of Peroxisome Proliferator-Activated Receptors (PPARs) in the Regulation of Neural Stem Cells Proliferation and Differentiation." *Stem cell reviews* 4(4): 293–303.

Clària, J, and C N Serhan. 1995. "Aspirin Triggers Previously Undescribed Bioactive Eicosanoids by Human Endothelial Cell-Leukocyte Interactions." *Proceedings of the National Academy of Sciences of the United States of America* 92(21): 9475–79.

Clement, A M et al. 2003. "Wild-Type Nonneuronal Cells Extend Survival of SOD1 Mutant Motor Neurons in ALS Mice." *Science (New York, N.Y.)* 302(5642): 113–17.

Colman, Eric et al. 2008. "An Evaluation of a Data Mining Signal for Amyotrophic Lateral Sclerosis and Statins Detected in FDA's Spontaneous Adverse Event Reporting System." *Pharmacoepidemiology and drug safety* 17(11): 1068–76.

Conibear, Elizabeth, and Nicholas G Davis. 2010. "Palmitoylation and Depalmitoylation Dynamics at a Glance." *Journal of cell science* 123(Pt 23): 4007–10.

Conquer, J A, and B J Holub. 1998. "Effect of Supplementation with Different Doses of DHA on the Levels of Circulating DHA as Non-Esterified Fatty Acid in Subjects of Asian Indian Background." *Journal of lipid research* 39(2): 286–92.

Consilvio, C, A M Vincent, and E L Feldman. 2004. "Neuroinflammation, COX-2, and ALS--a Dual Role?" *Experimental neurology* 187(1): 1–10.

Corcia, Philippe et al. 2012. "Molecular Imaging of Microglial Activation in Amyotrophic Lateral Sclerosis." *PloS one* 7(12): e52941.

Couplan, Elodie et al. 2009. "Polyunsaturated Fatty Acids Inhibit PI3K Activity in a Yeast-Based Model System." *Biotechnology journal* 4(8): 1190–97.

Coussee, Evelyne et al. 2011. "G37R SOD1 Mutant Alters Mitochondrial Complex I Activity, Ca(2+) Uptake and ATP Production." *Cell calcium* 49(4): 217–25.

Cox, Paul Alan et al. 2009. "Cyanobacteria and BMAA Exposure from Desert Dust: A Possible Link to Sporadic ALS among Gulf War Veterans." *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 10 Suppl 2: 109–17.

Cozzolino, Mauro, Alberto Ferri, Cristiana Valle, and Maria Teresa Carrì. 2013. "Mitochondria and ALS: Implications from Novel Genes and Pathways." *Molecular and cellular neurosciences* 55: 44–49.

Crawford, Michael A et al. 2013. "A Quantum Theory for the Irreplaceable Role of Docosahexaenoic Acid in Neural Cell Signalling throughout Evolution." *Prostaglandins, leukotrienes, and essential fatty acids* 88(1): 5–13.

Crochemore, Cristophe et al. 2009. "Long-Term Dietary Administration of Valproic Acid Does Not Affect, While Retinoic Acid Decreases, the Lifespan of G93A Mice, a Model for Amyotrophic Lateral Sclerosis." *Muscle & nerve* 39(4): 548–52

Cronin, Simon, Orla Hardiman, and Bryan J Traynor. 2007. “Ethnic Variation in the Incidence of ALS: A Systematic Review.” *Neurology* 68(13): 1002–7.

Cunnane, S C et al. 1994. “Utilization of Uniformly Labeled 13C-Polyunsaturated Fatty Acids in the Synthesis of Long-Chain Fatty Acids and Cholesterol Accumulating in the Neonatal Rat Brain.” *Journal of neurochemistry* 62(6): 2429–36.

Cunnane, Stephen C, Mélanie Plourde, Kathy Stewart, and Michael A Crawford. “Docosahexaenoic Acid and Shore-Based Diets in Hominin Encephalization: A Rebuttal.” *American journal of human biology : the official journal of the Human Biology Council* 19(4): 578–81.

Członkowska, Anna, Agnieszka Ciesielska, Grazyna Gromadzka, and Iwona Kurkowska-Jastrzebska. 2006. “Gender Differences in Neurological Disease: Role of Estrogens and Cytokines.” *Endocrine* 29(2): 243–56.

Dagda, Ruben K, Tania Das Banerjee, and Elzbieta Janda. 2013. “How Parkinsonian Toxins Dysregulate the Autophagy Machinery.” *International journal of molecular sciences* 14(11): 22163–89.

Darios, F, and B Davletov. 2006. “Omega-3 and Omega-6 Fatty Acids Stimulate Cell Membrane Expansion by Acting on Syntaxin 3.” *Nature* 440(7085): 813–17.

Daum, G, and J E Vance. 1997. “Import of Lipids into Mitochondria.” *Progress in lipid research* 36(2-3): 103–30.

David, Samuel, and Antje Kroner. 2011. “Repertoire of Microglial and Macrophage Responses after Spinal Cord Injury.” *Nature reviews. Neuroscience* 12(7): 388–99.

Davies, M J, S Fu, H Wang, and R T Dean. 1999. “Stable Markers of Oxidant Damage to Proteins and Their Application in the Study of Human Disease.” *Free radical biology & medicine* 27(11-12): 1151–63.

Davletov, B, E Connell, and F Darios. 2007. “Regulation of SNARE Fusion Machinery by Fatty Acids.” *Cellular and molecular life sciences : CMLS* 64(13): 1597–1608.

DeJesus-Hernandez, Mariely et al. 2011. “Expanded GGGGCC Hexanucleotide Repeat in Noncoding Region of C9ORF72 Causes Chromosome 9p-Linked FTD and ALS.” *Neuron* 72(2): 245–56.

DeMar, James C et al. 2005. “Alpha-Linolenic Acid Does Not Contribute Appreciably to Docosahexaenoic Acid within Brain Phospholipids of Adult Rats Fed a Diet Enriched in Docosahexaenoic Acid.” *Journal of neurochemistry* 94(4): 1063–76.

DeMar, James C et al. 2006. “Brain Elongation of Linoleic Acid Is a Negligible Source of the Arachidonate in Brain Phospholipids of Adult Rats.” *Biochimica et biophysica acta* 1761(9): 1050–59.

Denic, Vladimir, and Jonathan S Weissman. 2007. “A Molecular Caliper Mechanism for Determining Very Long-Chain Fatty Acid Length.” *Cell* 130(4): 663–77..

Derosa, Giuseppe, and Pamela Maffioli. 2012. “Peroxisome Proliferator-Activated Receptor-Γ (PPAR-Γ) Agonists on Glycemic Control, Lipid Profile and Cardiovascular Risk.” *Current molecular pharmacology* 5(2): 272–81.

Dewil, M et al. 2007. “Vascular Endothelial Growth Factor Counteracts the Loss of Phospho-Akt Preceding Motor Neurone Degeneration in Amyotrophic Lateral Sclerosis.” *Neuropathology and applied neurobiology* 33(5): 499–509.

Dey, I et al. 1993. “Molecular and Structural Composition of Phospholipid Membranes in Livers of Marine and Freshwater Fish in Relation to Temperature.” *Proceedings of the National Academy of Sciences of the United States of America* 90(16): 7498–7502.

Dhe-Paganon, Sirano et al. 2002. “Crystal Structure of the HNF4 Alpha Ligand Binding Domain in Complex with Endogenous Fatty Acid Ligand.” *The Journal of biological chemistry* 277(41): 37973–76.

Doczi, Judit et al. 2011. “Complex Contribution of Cyclophilin D to Ca2+-Induced Permeability Transition in Brain Mitochondria, with Relation to the Bioenergetic State.” *The Journal of biological chemistry* 286(8): 6345–53.

Dolara, Piero, Elisabetta Bigagli, and Andrew Collins. 2012. “Antioxidant Vitamins and Mineral Supplementation, Life Span Expansion and Cancer Incidence: A Critical Commentary.” *European journal of nutrition* 51(7): 769–81

Domingues, Rosário M et al. 2013. “Lipoxidation Adducts with Peptides and Proteins: Deleterious Modifications or Signaling Mechanisms?” *Journal of proteomics* 92: 110–31.

Dongol, Bikesh et al. 2007. “The Acyl-CoA Thioesterase I Is Regulated by PPARalpha and HNF4alpha via a Distal Response Element in the Promoter.” *Journal of lipid research* 48(8): 1781–91.

Dorst, Johannes, Joschka Cypionka, and Albert C Ludolph. 2013. “High-Caloric Food Supplements in the Treatment of Amyotrophic Lateral Sclerosis: A Prospective Interventional Study.” *Amyotrophic lateral sclerosis & frontotemporal degeneration* 14(7-8): 533–36.

Drory, Vivian E, Tatiana Bronipolsky, Irena Artamonov, and Beatrice Nefussy. 2008. “Influence of Statins Treatment on Survival in Patients with Amyotrophic Lateral Sclerosis.” *Journal of the neurological sciences* 273(1-2): 81–83.

Dumas, D et al. 1997. “Membrane Fluidity and Oxygen Diffusion in Cholesterol-Enriched Erythrocyte Membrane.” *Archives of biochemistry and biophysics* 341(1): 34–39.

Duplus, Eric, and Claude Forest. 2002. “Is There a Single Mechanism for Fatty Acid Regulation of Gene Transcription?” *Biochemical pharmacology* 64(5-6): 893–901..

Dupuis, Luc et al. 2004. “Evidence for Defective Energy Homeostasis in Amyotrophic Lateral Sclerosis: Benefit of a High-Energy Diet in a Transgenic Mouse Model.” *Proceedings of the National Academy of Sciences of the United States of America* 101(30): 11159–64.

Dupuis L, Dengler R, Heneka MT, Meyer T, Zierz S, Kassubek J, Fischer W, Steiner F, Lindauer E, Otto M, Dreyhaupt J, Grehl T, Hermann A, Winkler AS, Bogdahn U, Benecke R, Schrank B, Wessig C, Grosskreutz J, Ludolph AC; GERP ALS Study Group. 2012. “A Randomized, Double Blind, Placebo-Controlled Trial of Pioglitazone in Combination with Riluzole in Amyotrophic Lateral Sclerosis.” *PloS one* 7(6): e37885.

Dupuis, Luc, Pierre-François Pradat, Albert C Ludolph, and Jean-Philippe Loeffler. 2011. “Energy Metabolism in Amyotrophic Lateral Sclerosis.” *Lancet neurology* 10(1): 75–82.

Echtay, Karim S et al. 2003. “A Signalling Role for 4-Hydroxy-2-Nonenal in Regulation of Mitochondrial Uncoupling.” *The EMBO journal* 22(16): 4103–10.

Eckert, Gunter P et al. 2011. “Liposome-Incorporated DHA Increases Neuronal Survival by Enhancing Non-Amyloidogenic APP Processing.” *Biochimica et biophysica acta* 1808(1): 236–43.

Edwards, I Ralph, Kristina Star, and Anne Kiuru. 2007. “Statins, Neuromuscular Degenerative Disease and an Amyotrophic Lateral Sclerosis-like Syndrome: An Analysis of Individual Case Safety

Reports from Vigibase." *Drug safety : an international journal of medical toxicology and drug experience* 30(6): 515–25.

Eerola, Leena I et al. 2006. "Analysis of Expression of Secreted Phospholipases A2 in Mouse Tissues at Protein and mRNA Levels." *Biochimica et biophysica acta* 1761(7): 745–56.

fremov, Rouslan G, Rozbeh Baradaran, and Leonid A Sazanov. 2010. "The Architecture of Respiratory Complex I." *Nature* 465(7297): 441–45.

Elbaz, Yael, and Maya Schuldiner. 2011. "Staying in Touch: The Molecular Era of Organelle Contact Sites." *Trends in biochemical sciences* 36(11): 616–23.

Ellis, Jessica M, G William Wong, and Michael J Wolfgang. 2013. "Acyl Coenzyme A Thioesterase 7 Regulates Neuronal Fatty Acid Metabolism to Prevent Neurotoxicity." *Molecular and cellular biology* 33(9): 1869–82.

Elstner, M et al. 2009. "The Mitochondrial Proteome Database: MitoP2." *Methods in enzymology* 457: 3–20.

Van Es, Michael A et al. 2007. "ITPR2 as a Susceptibility Gene in Sporadic Amyotrophic Lateral Sclerosis: A Genome-Wide Association Study." *Lancet neurology* 6(10): 869–77.

Van Es MA, Veldink JH, Saris CG, Blauw HM, van Vught PW, Birve A, Lemmens R, Schelhaas HJ, Groen EJ, Huisman MH, van der Kooi AJ, de Visser M, Dahlberg C, Estrada K, Rivadeneira F, Hofman A, Zwarts MJ, van Doormaal PT, Rujescu D, Strengman E, Giegling I, Muglia P, Tomik B, Slowik A, Uitterlinden AG, Hendrich C, Waibel S, Meyer T, Ludolph AC, Glass JD, Purcell S, Cichon S, Nöthen MM, Wichmann HE, Schreiber S, Vermeulen SH, Kiemeney LA, Wokke JH, Cronin S, McLaughlin RL, Hardiman O, Fumoto K, Pasterkamp RJ, Meininger V, Melki J, Leigh PN, Shaw CE, Landers JE, Al-Chalabi A, Brown RH Jr, Robberecht W, Andersen PM, Ophoff RA, van den Berg LH. 2009. "Genome-Wide Association Study Identifies 19p13.3 (UNC13A) and 9p21.2 as Susceptibility Loci for Sporadic Amyotrophic Lateral Sclerosis." *Nature genetics* 41(10): 1083–87.

Escriva, H, F Delaunay, and V Laudet. 2000. "Ligand Binding and Nuclear Receptor Evolution." *BioEssays : news and reviews in molecular, cellular and developmental biology* 22(8): 717–27.

European Food Safety Authority (EFSA) 2009 "Scientific opinion of the panel on dietetic products, nutrition and allergies on a request from European commission related to labelling reference intake values for n-3 and n-6 polyunsaturated fatty acids" .*The EFSA Journal* 2009, 1176:1-11.

Evans, M C, Y Couch, N Sibson, and M R Turner. 2013. "Inflammation and Neurovascular Changes in Amyotrophic Lateral Sclerosis." *Molecular and cellular neurosciences* 53: 34–41.

Extier, Audrey et al. 2010. "Gender Affects Liver Desaturase Expression in a Rat Model of N-3 Fatty Acid Repletion." *The Journal of nutritional biochemistry* 21(3): 180–87.

Fan, Y Y, and R S Chapkin. 1998. "Importance of Dietary Gamma-Linolenic Acid in Human Health and Nutrition." *The Journal of nutrition* 128(9): 1411–14.

Fang, Y et al. 2001. "Phosphatidic Acid-Mediated Mitogenic Activation of mTOR Signaling." *Science (New York, N.Y.)* 294(5548): 1942–45.

Farooqui, A A, L A Horrocks, and T Farooqui. 2000. "Deacylation and Reacylation of Neural Membrane Glycerophospholipids." *Journal of molecular neuroscience : MN* 14(3): 123–35.

Farooqui, Akhlaq A et al. 2007. "Comparison of Biochemical Effects of Statins and Fish Oil in Brain: The Battle of the Titans." *Brain research reviews* 56(2): 443–71.

"Fats and Fatty Acids in Human Nutrition. Report of an Expert Consultation." 2010. *FAO food and nutrition paper* 91: 1–166.

Ferrante, R J et al. 1997. "Evidence of Increased Oxidative Damage in Both Sporadic and Familial Amyotrophic Lateral Sclerosis." *Journal of neurochemistry* 69(5): 2064–74.

Fitzgerald, Kathryn C et al. 2014. "Dietary Ω-3 Polyunsaturated Fatty Acid Intake and Risk for Amyotrophic Lateral Sclerosis." *JAMA neurology*.

Florent-Béchard S, Koziel V. 2007 "Neuroprotective effects of DHA in Alzheimer's disease models". Book chapter *Oléagineux, Corps Gras, Lipides.* Volume 14, Number 3, 186-9, Mai-Août 2007, *PUFAs and aging disorders*. DOI : 10.1684/ocl.2007.0119

Forlenza, Orestes V, Camila T Mendes, Suely K N Marie, and Wagner F Gattaz. 2007. "Inhibition of Phospholipase A2 Reduces Neurite Outgrowth and Neuronal Viability." *Prostaglandins, leukotrienes, and essential fatty acids* 76(1): 47–55.

Frank-Cannon, Tamy C, Laura T Alto, Fiona E McAlpine, and Malú G Tansey. 2009. "Does Neuroinflammation Fan the Flame in Neurodegenerative Diseases?" *Molecular neurodegeneration* 4: 47.

Freeman, Marlene P et al. 2006. "Omega-3 Fatty Acids: Evidence Basis for Treatment and Future Research in Psychiatry." *The Journal of clinical psychiatry* 67(12): 1954–67.

Friedman, Jonathan R et al. 2010. "ER Sliding Dynamics and ER-Mitochondrial Contacts Occur on Acetylated Microtubules." *The Journal of cell biology* 190(3): 363–75.

Fruchart, Jean-Charles. 2013. "Selective Peroxisome Proliferator-Activated Receptor A Modulators (SPPARMα): The next Generation of Peroxisome Proliferator-Activated Receptor A-Agonists." *Cardiovascular diabetology* 12: 82.

Funalot, Benoit et al. 2009. "High Metabolic Level in Patients with Familial Amyotrophic Lateral Sclerosis." *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 10(2): 113–17.

Funk, Colin D, Xin-Sheng Chen, Eric N Johnson, and Lei Zhao. 2002. "Lipoxygenase Genes and Their Targeted Disruption." *Prostaglandins & other lipid mediators* 68-69: 303–12.

Furby, Alain et al. 2010. "Rural Environment and Risk Factors of Amyotrophic Lateral Sclerosis: A Case-Control Study." *Journal of neurology* 257(5): 792–98.

García-Martínez, Virginia et al. 2013. "Lipid Metabolites Enhance Secretion Acting on SNARE Microdomains and Altering the Extent and Kinetics of Single Release Events in Bovine Adrenal Chromaffin Cells." *PloS one* 8(9): e75845.

García-Ruiz, C, A Colell, R París, and J C Fernández-Checa. 2000. "Direct Interaction of GD3 Ganglioside with Mitochondria Generates Reactive Oxygen Species Followed by Mitochondrial Permeability Transition, Cytochrome c Release, and Caspase Activation." *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 14(7): 847–58.

Gaudette, M, M Hirano, and T Siddique. 2000. "Current Status of SOD1 Mutations in Familial Amyotrophic Lateral Sclerosis." *Amyotrophic lateral sclerosis and other motor neuron disorders : official publication of the World Federation of Neurology, Research Group on Motor Neuron Diseases* 1(2): 83–89.

Gawrisch, Klaus, Olivier Soubias, and Mihaela Mihailescu. “Insights from Biophysical Studies on the Role of Polyunsaturated Fatty Acids for Function of G-Protein Coupled Membrane Receptors.” *Prostaglandins, leukotrienes, and essential fatty acids* 79(3-5): 131–34.

Ghiasi, Parisa et al. 2012. “Mitochondrial Complex I Deficiency and ATP/ADP Ratio in Lymphocytes of Amyotrophic Lateral Sclerosis Patients.” *Neurological research* 34(3): 297–303.

Ghosh, Moumita, Dawn E Tucker, Scott A Burchett, and Christina C Leslie. 2006. “Properties of the Group IV Phospholipase A2 Family.” *Progress in lipid research* 45(6): 487–510.

Gilmore, T D. 2006. “Introduction to NF-kappaB: Players, Pathways, Perspectives.” *Oncogene* 25(51): 6680–84.

Giltay, Erik J, Louis J G Gooren, et al. 2004a. “Docosahexaenoic Acid Concentrations Are Higher in Women than in Men because of Estrogenic Effects.” *The American journal of clinical nutrition* 80(5): 1167–74.

Giltay, Erik J, Erik J J Duschek, et al. 2004b. “Raloxifene and Hormone Replacement Therapy Increase Arachidonic Acid and Docosahexaenoic Acid Levels in Postmenopausal Women.” *The Journal of endocrinology* 182(3): 399–408.

Di Giorgio, Francesco Paolo, Gabriella L Boulting, Samuel Bobrowicz, and Kevin C Eggan. 2008. “Human Embryonic Stem Cell-Derived Motor Neurons Are Sensitive to the Toxic Effect of Glial Cells Carrying an ALS-Causing Mutation.” *Cell stem cell* 3(6): 637–48.

Glaser, Claudia, Joachim Heinrich, and Berthold Koletzko. 2010. “Role of FADS1 and FADS2 Polymorphisms in Polyunsaturated Fatty Acid Metabolism.” *Metabolism: clinical and experimental* 59(7): 993–99.

Glass, Christopher K et al. 2010. “Mechanisms Underlying Inflammation in Neurodegeneration.” *Cell* 140(6): 918–34.

Gonatas, Nicholas K, Anna Stieber, and Jacqueline O Gonatas. 2006. “Fragmentation of the Golgi Apparatus in Neurodegenerative Diseases and Cell Death.” *Journal of the neurological sciences* 246(1-2): 21–30.

Gong, Y H et al. 2000. “Restricted Expression of G86R Cu/Zn Superoxide Dismutase in Astrocytes Results in Astrocytosis but Does Not Cause Motoneuron Degeneration.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 20(2): 660–65.

González-Núñez, D, J Claria, F Rivera, and E Poch. 2001. “Increased Levels of 12(S)-HETE in Patients with Essential Hypertension.” *Hypertension* 37(2): 334–38.

Greenwood, D I. 2013. “Nutrition Management of Amyotrophic Lateral Sclerosis.” *Nutrition in clinical practice : official publication of the American Society for Parenteral and Enteral Nutrition* 28(3): 392–99.

Groot, P H, H R Scholte, and W C Hülsmann. 1976. “Fatty Acid Activation: Specificity, Localization, and Function.” *Advances in lipid research* 14: 75–126.

Grosskreutz, Julian, Ludo Van Den Bosch, and Bernhard U Keller. 2010. “Calcium Dysregulation in Amyotrophic Lateral Sclerosis.” *Cell calcium* 47(2): 165–74.

Grossman, Lawrence I, Derek E Wildman, Timothy R Schmidt, and Morris Goodman. 2004. “Accelerated Evolution of the Electron Transport Chain in Anthropoid Primates.” *Trends in genetics : TIG* 20(11): 578–85.

Guatteo, Ezia et al. 2007. “Altered Calcium Homeostasis in Motor Neurons Following AMPA Receptor but Not Voltage-Dependent Calcium Channels’ Activation in a Genetic Model of Amyotrophic Lateral Sclerosis.” *Neurobiology of disease* 28(1): 90–100.

Guesnet, Philippe, and Jean-Marc Alessandri. 2011. “Docosahexaenoic Acid (DHA) and the Developing Central Nervous System (CNS) - Implications for Dietary Recommendations.” *Biochimie* 93(1): 7–12.

Guillou, Hervé, Damir Zadravec, Pascal G P Martin, and Anders Jacobsson. 2010. “The Key Roles of Elongases and Desaturases in Mammalian Fatty Acid Metabolism: Insights from Transgenic Mice.” *Progress in lipid research* 49(2): 186–99.

Gurney, M E et al. 1994. “Motor Neuron Degeneration in Mice That Express a Human Cu,Zn Superoxide Dismutase Mutation.” *Science (New York, N.Y.)* 264(5166): 1772–75.

Gurney ME, Cutting FB, Zhai P, Doble A, Taylor CP, Andrus PK, Hall ED. 1996. “Benefit of Vitamin E, Riluzole, and Gabapentin in a Transgenic Model of Familial Amyotrophic Lateral Sclerosis.” *Annals of neurology* 39(2): 147–57.

Hajjar, Toktam et al. 2012. “Omega 3 Polyunsaturated Fatty Acid Improves Spatial Learning and Hippocampal Peroxisome Proliferator Activated Receptors (PPARα and PPARγ) Gene Expression in Rats.” *BMC neuroscience* 13: 109.

Halapin, Natalie A, and Nicolas G Bazan. 2010. “NPD1 Induction of Retinal Pigment Epithelial Cell Survival Involves PI3K/Akt Phosphorylation Signaling.” *Neurochemical research* 35(12): 1944–47.

Hall, E D et al. 1998. “Relationship of Oxygen Radical-Induced Lipid Peroxidative Damage to Disease Onset and Progression in a Transgenic Model of Familial ALS.” *Journal of neuroscience research* 53(1): 66–77.

Hamadeh, Mazen J, and Mark A Tarnopolsky. 2006. “Transient Caloric Restriction in Early Adulthood Hastens Disease Endpoint in Male, but Not Female, Cu/Zn-SOD Mutant G93A Mice.” *Muscle & nerve* 34(6): 709–19.

Hamberg, M, and B Samuelsson. 1974. “Prostaglandin Endoperoxides. Novel Transformations of Arachidonic Acid in Human Platelets.” *Proceedings of the National Academy of Sciences of the United States of America* 71(9): 3400–3404.

Hamilton, James A. “New Insights into the Roles of Proteins and Lipids in Membrane Transport of Fatty Acids.” *Prostaglandins, leukotrienes, and essential fatty acids* 77(5-6): 355–61.

Han, Shuxin, and David E Cohen. 2012. “Functional Characterization of Thioesterase Superfamily Member 1/Acyl-CoA Thioesterase 11: Implications for Metabolic Regulation.” *Journal of lipid research* 53(12): 2620–31.

Hanada, Kentaro et al. 2003. “Molecular Machinery for Non-Vesicular Trafficking of Ceramide.” *Nature* 426(6968): 803–9.

Hansson, Magnus J et al. 2008. “Calcium-Induced Generation of Reactive Oxygen Species in Brain Mitochondria Is Mediated by Permeability Transition.” *Free radical biology & medicine* 45(3): 284–94.

Hao, C-M, and M D Breyer. 2007. “Physiologic and Pathophysiologic Roles of Lipid Mediators in the Kidney.” *Kidney international* 71(11): 1105–15.

Harman, D. 1956. “Aging: A Theory Based on Free Radical and Radiation Chemistry.” *Journal of gerontology* 11(3): 298–300.

Hashimoto, Michio, Shahdat Hossain, Toshio Shimada, and Osamu Shido. 2006. "Docosahexaenoic Acid-Induced Protective Effect against Impaired Learning in Amyloid Beta-Infused Rats Is Associated with Increased Synaptosomal Membrane Fluidity." *Clinical and experimental pharmacology & physiology* 33(10): 934–39.

Hastings, N et al. 2001. "A Vertebrate Fatty Acid Desaturase with Delta 5 and Delta 6 Activities." *Proceedings of the National Academy of Sciences of the United States of America* 98(25): 14304–9.

Hauser, K F, and C D Toran-Allerand. 1989. "Androgen Increases the Number of Cells in Fetal Mouse Spinal Cord Cultures: Implications for Motoneuron Survival." *Brain research* 485(1): 157–64.

Haworth, R A, and D R Hunter. 1979. "The Ca2+-Induced Membrane Transition in Mitochondria. II. Nature of the Ca2+ Trigger Site." *Archives of biochemistry and biophysics* 195(2): 460–67.

Heck, Melanie Vanessa et al. 2014. "Dysregulated Expression of Lipid Storage and Membrane Dynamics Factors in Tia1 Knockout Mouse Nervous Tissue." *Neurogenetics* 15(2): 135–44.

Helmkamp, G M. 1986. "Phospholipid Transfer Proteins: Mechanism of Action." *Journal of bioenergetics and biomembranes* 18(2): 71–91.

Henkel, Jenny S, David R Beers, Weihua Zhao, and Stanley H Appel. 2009. "Microglia in ALS: The Good, the Bad, and the Resting." *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology* 4(4): 389–98.

Henshall, David C et al. 2002. "Activation of Bcl-2-Associated Death Protein and Counter-Response of Akt within Cell Populations during Seizure-Induced Neuronal Death." *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22(19): 8458–65.

Herman, Mark A et al. 2012. "A Novel ChREBP Isoform in Adipose Tissue Regulates Systemic Glucose Metabolism." *Nature* 484(7394): 333–38.

Hermansson, Martin, Kati Hokynar, and Pentti Somerharju. 2011. "Mechanisms of Glycerophospholipid Homeostasis in Mammalian Cells." *Progress in lipid research* 50(3): 240–57.

Ho, Chia-Chi et al. 2008. "17-Beta Estradiol and Hydroxyestradiols Interact via the NF-Kappa B Pathway to Elevate Cyclooxygenase 2 Expression and Prostaglandin E2 Secretion in Human Bronchial Epithelial Cells." *Toxicological sciences : an official journal of the Society of Toxicology* 104(2): 294–302.

Hofacer, Rylon et al. 2011. "Omega-3 Fatty Acid Deficiency Selectively up-Regulates delta6-Desaturase Expression and Activity Indices in Rat Liver: Prevention by Normalization of Omega-3 Fatty Acid Status." *Nutrition research (New York, N.Y.)* 31(9): 715–22.

Holthuis, Joost C M, and Tim P Levine. 2005. "Lipid Traffic: Floppy Drives and a Superhighway." *Nature reviews. Molecular cell biology* 6(3): 209–20.

Hong, Song et al. 2003. "Novel Docosatrienes and 17S-Resolvins Generated from Docosahexaenoic Acid in Murine Brain, Human Blood, and Glial Cells. Autacoids in Anti-Inflammation." *The Journal of biological chemistry* 278(17): 14677–87.

Hopper, Rachel K et al. 2006. "Mitochondrial Matrix Phosphoproteome: Effect of Extra Mitochondrial Calcium." *Biochemistry* 45(8): 2524–36.

Hou, Xin et al. 2004. "Isomer-Specific Contractile Effects of a Series of Synthetic f2-Isoprostanes on Retinal and Cerebral Microvasculature." *Free radical biology & medicine* 36(2): 163–72.

Huang, Kun et al. 2009. “Neuronal Palmitoyl Acyl Transferases Exhibit Distinct Substrate Specificity.” *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 23(8): 2605–15.

Hunt, M C et al. 1999. “Peroxisome Proliferator-Induced Long Chain Acyl-CoA Thioesterases Comprise a Highly Conserved Novel Multi-Gene Family Involved in Lipid Metabolism.” *The Journal of biological chemistry* 274(48): 34317–26.

Igarashi, Miki, Kaizong Ma, et al. 2007a. “Dietary N-3 PUFA Deprivation for 15 Weeks Upregulates Elongase and Desaturase Expression in Rat Liver but Not Brain.” *Journal of lipid research* 48(11): 2463–70.

Igarashi, Miki, James C DeMar, et al. 2007b. “Docosahexaenoic Acid Synthesis from Alpha-Linolenic Acid by Rat Brain Is Unaffected by Dietary N-3 PUFA Deprivation.” *Journal of lipid research* 48(5): 1150–58.

Ihara, Yuetsu, Keigo Nobukuni, Hiroshi Takata, and Toshiyuki Hayabara. 2005. “Oxidative Stress and Metal Content in Blood and Cerebrospinal Fluid of Amyotrophic Lateral Sclerosis Patients with and without a Cu, Zn-Superoxide Dismutase Mutation.” *Neurological research* 27(1): 105–8.

Iizuka, Katsumi. 2013. “Recent Progress on the Role of ChREBP in Glucose and Lipid Metabolism.” *Endocrine journal* 60(5): 543–55.

Ilieva, Ekaterina V, Victòria Ayala, Mariona Jové, Esther Dalfó, Daniel Cacabelos, Mónica Povedano, Maria Josep Bellmunt, Isidre Ferrer, Reinald Pamplona, Manuel Portero-Otín, et al. 2007. “Oxidative and Endoplasmic Reticulum Stress Interplay in Sporadic Amyotrophic Lateral Sclerosis.” *Brain* 130(Pt 12): 3111–23.

Im, Dong-Soon. 2012. “Omega-3 Fatty Acids in Anti-Inflammation (pro-Resolution) and GPCRs.” *Progress in lipid research* 51(3): 232–37.

Innis, Sheila M. 2007. “Dietary (n-3) Fatty Acids and Brain Development.” *The Journal of nutrition* 137(4): 855–59.

Jacquier, Nicolas et al. 2011. “Lipid Droplets Are Functionally Connected to the Endoplasmic Reticulum in Saccharomyces Cerevisiae.” *Journal of cell science* 124(Pt 14): 2424–37.

Jakobsson, Tomas, Eckardt Treuter, Jan-Åke Gustafsson, and Knut R Steffensen. 2012. “Liver X Receptor Biology and Pharmacology: New Pathways, Challenges and Opportunities.” *Trends in pharmacological sciences* 33(7): 394–404.

Joshi, R. et al. 2004. “Phenol Radical Cations and Phenoxyl Radicals in Electron Transfer from the Natural Phenols Sesamol, Curcumin and Trolox to the Parent Radical Cations of 1-Chlorobutane.” *Journal of Physical Organic Chemistry* 17(8): 665–74.

Kabashi, Edor et al. 2013. “Investigating the Contribution of VAPB/ALS8 Loss of Function in Amyotrophic Lateral Sclerosis.” *Human molecular genetics* 22(12): 2350–60.

Kadamur, Ganesh, and Elliott M Ross. 2013. “Mammalian Phospholipase C.” *Annual review of physiology* 75: 127–54.

Kaduce, Terry L, Yucui Chen, Johannes W Hell, and Arthur A Spector. 2008. “Docosahexaenoic Acid Synthesis from N-3 Fatty Acid Precursors in Rat Hippocampal Neurons.” *Journal of neurochemistry* 105(4): 1525–35.

Kaji, Ryuji, Yishin Izumi, Yoshiki Adachi, and Shigeki Kuzuhara. 2012. “ALS-Parkinsonism-Dementia Complex of Kii and Other Related Diseases in Japan.” *Parkinsonism & related disorders* 18 Suppl 1: S190–1.

Kamp, F, and J A Hamilton. 1992. “pH Gradients across Phospholipid Membranes Caused by Fast Flip-Flop of Un-Ionized Fatty Acids.” *Proceedings of the National Academy of Sciences of the United States of America* 89(23): 11367–70.

Kamp, Frits, and James A Hamilton. 2006. “How Fatty Acids of Different Chain Length Enter and Leave Cells by Free Diffusion.” *Prostaglandins, leukotrienes, and essential fatty acids* 75(3): 149–59.

Kanekura, Kohsuke, Ikuo Nishimoto, Sadakazu Aiso, and Masaaki Matsuoka. 2006. “Characterization of Amyotrophic Lateral Sclerosis-Linked P56S Mutation of Vesicle-Associated Membrane Protein-Associated Protein B (VAPB/ALS8).” *The Journal of biological chemistry* 281(40): 30223–33.

Kang, Shin H et al. 2013. “Degeneration and Impaired Regeneration of Gray Matter Oligodendrocytes in Amyotrophic Lateral Sclerosis.” *Nature neuroscience* 16(5): 571–79.

Kapoor, Rakesh, and Yung-Sheng Huang. 2006. “Gamma Linolenic Acid: An Antiinflammatory Omega-6 Fatty Acid.” *Current pharmaceutical biotechnology* 7(6): 531–34.

Kastner, Daniel L, Ivona Aksentijevich, and Raphaela Goldbach-Mansky. 2010. “Autoinflammatory Disease Reloaded: A Clinical Perspective.” *Cell* 140(6): 784–90.

Kates, M, J Y Syz, D Gosser, and T H Haines. 1993. “pH-Dissociation Characteristics of Cardiolipin and Its 2’-Deoxy Analogue.” *Lipids* 28(10): 877–82.

Keller, J M et al. 2000. “Implications of Peroxisome Proliferator-Activated Receptors (PPARS) in Development, Cell Life Status and Disease.” *The International journal of developmental biology* 44(5): 429–42.

Kennedy, E P, and S B WEISS. 1956. “The Function of Cytidine Coenzymes in the Biosynthesis of Phospholipides.” *The Journal of biological chemistry* 222(1): 193–214.

Kiaei, Mahmoud et al. 2005. “Integrative Role of cPLA with COX-2 and the Effect of Non-Steriodal Anti-Inflammatory Drugs in a Transgenic Mouse Model of Amyotrophic Lateral Sclerosis.” *Journal of neurochemistry* 93(2): 403–11

Kiebish, Michael A, Xianlin Han, and Thomas N Seyfried. 2009. “Examination of the Brain Mitochondrial Lipidome Using Shotgun Lipidomics.” *Methods in molecular biology (Clifton, N.J.)* 579: 3–18.

Kim, Do Yeon, Jinkyung Kim, Hye Jin Ham, and Ryowon Choue. 2013. “Effects of D-A-Tocopherol Supplements on Lipid Metabolism in a High-Fat Diet-Fed Animal Model.” *Nutrition research and practice* 7(6): 481–87.

Kim, Hi C et al. “RNA Interference of Long-Chain Acyl-CoA Synthetase 6 Suppresses the Neurite Outgrowth of Mouse Neuroblastoma NB41A3 Cells.” *Molecular medicine reports* 2(4): 669–74.

Kim, Hyun Jeong, Jordi Magranè, Anatoly A Starkov, and Giovanni Manfredi. 2012. “The Mitochondrial Calcium Regulator Cyclophilin D Is an Essential Component of Oestrogen-Mediated Neuroprotection in Amyotrophic Lateral Sclerosis.” *Brain : a journal of neurology* 135(Pt 9): 2865–74.

Kim, Kwang S et al. 2013. “Transplantation of Human Adipose Tissue-Derived Stem Cells Delays Clinical Onset and Prolongs Life Span in ALS Mouse Model.” *Cell transplantation*.

Kim, Sung-Jo et al. 2010. “Omega-3 and Omega-6 Fatty Acids Suppress ER- and Oxidative Stress in Cultured Neurons and Neuronal Progenitor Cells from Mice Lacking PPT1.” *Neuroscience letters* 479(3): 292–96.

Kira, Yukimi et al. 2006. “L-Carnitine Suppresses the Onset of Neuromuscular Degeneration and Increases the Life Span of Mice with Familial Amyotrophic Lateral Sclerosis.” *Brain research* 1070(1): 206–14.

Kirichenko, Anna V et al. 2005. “Cytochrome c Oxidase as a Calcium Binding Protein. Studies on the Role of a Conserved Aspartate in Helices XI-XII Cytoplasmic Loop in Cation Binding.” *Biochemistry* 44(37): 12391–401.

Kirkby, Brenda et al. 2010. “Functional and Structural Properties of Mammalian Acyl-Coenzyme A Thioesterases.” *Progress in lipid research* 49(4): 366–77..

Kirkinezos, Ilias G et al. 2005. “Cytochrome c Association with the Inner Mitochondrial Membrane Is Impaired in the CNS of G93A-SOD1 Mice.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25(1): 164–72.

Kitson, Alex P et al. 2013. “Treatment of Ovariectomized Rats with 17β-Estradiol Increases Hepatic Delta-6 Desaturase Enzyme Expression and Docosahexaenoic Acid Levels in Hepatic and Plasma Phospholipids.” *Prostaglandins, leukotrienes, and essential fatty acids* 89(2-3): 81–88.

Kitson, Alex P, Tracy L Smith, Kristin A Marks, and Ken D Stark. 2012. “Tissue-Specific Sex Differences in Docosahexaenoic Acid and Δ6-Desaturase in Rats Fed a Standard Chow Diet.” *Applied physiology, nutrition, and metabolism = Physiologie appliquée, nutrition et métabolisme* 37(6): 1200–1211.

Knævelsrud, Helene, and Anne Simonsen. 2012. “Lipids in Autophagy: Constituents, Signaling Molecules and Cargo with Relevance to Disease.” *Biochimica et biophysica acta* 1821(8): 1133–45.

Knothe, Gerhard, and Robert O. Dunn. 2009. “A Comprehensive Evaluation of the Melting Points of Fatty Acids and Esters Determined by Differential Scanning Calorimetry.” *Journal of the American Oil Chemists’ Society* 86(9): 843–56. h

Kondo, Mitsuhiro et al. 2002. “15-Deoxy-Delta(12,14)-Prostaglandin J(2): The Endogenous Electrophile That Induces Neuronal Apoptosis.” *Proceedings of the National Academy of Sciences of the United States of America* 99(11): 7367–72.

Kostenis, Evi. 2004. “A Glance at G-Protein-Coupled Receptors for Lipid Mediators: A Growing Receptor Family with Remarkably Diverse Ligands.” *Pharmacology & therapeutics* 102(3): 243–57.

Kozak, K R et al. 2001. “Amino Acid Determinants in Cyclooxygenase-2 Oxygenation of the Endocannabinoid 2-Arachidonylglycerol.” *The Journal of biological chemistry* 276(32): 30072–77.

Kremmyda, Lefkothea Stella, Eva Tvrzicka, Barbora Stankova, and Ales Zak. 2011. “Fatty Acids as Biocompounds: Their Role in Human Metabolism, Health and Disease: A Review. Part 2: Fatty Acid Physiological Roles and Applications in Human Health and Disease.” *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia* 155(3): 195–218.

Krey, G et al. 1997. “Fatty Acids, Eicosanoids, and Hypolipidemic Agents Identified as Ligands of Peroxisome Proliferator-Activated Receptors by Coactivator-Dependent Receptor Ligand Assay.” *Molecular endocrinology (Baltimore, Md.)* 11(6): 779–91.

Krishnamoorthy, Sriram et al. 2010. “Resolvin D1 Binds Human Phagocytes with Evidence for Proresolving Receptors.” *Proceedings of the National Academy of Sciences of the United States of America* 107(4): 1660–65.

Kümpfel, Tania et al. 2007. “Late-Onset Tumor Necrosis Factor Receptor-Associated Periodic Syndrome in Multiple Sclerosis Patients Carrying the TNFRSF1A R92Q Mutation.” *Arthritis and rheumatism* 56(8): 2774–83.

Kurumbail, R G et al. “Structural Basis for Selective Inhibition of Cyclooxygenase-2 by Anti-Inflammatory Agents.” *Nature* 384(6610): 644–48.

Kwak, Shin, Takuto Hideyama, Takenari Yamashita, and Hitoshi Aizawa. 2010. “AMPA Receptor-Mediated Neuronal Death in Sporadic ALS.” *Neuropathology : official journal of the Japanese Society of Neuropathology* 30(2): 182–88.

Labombarda, Florencia et al. 2013. “Neuroprotection by Steroids after Neurotrauma in Organotypic Spinal Cord Cultures: A Key Role for Progesterone Receptors and Steroidal Modulators of GABA(A) Receptors.” *Neuropharmacology* 71: 46–55.

Lambeau, Gérard, and Michael H Gelb. 2008. “Biochemistry and Physiology of Mammalian Secreted Phospholipases A2.” *Annual review of biochemistry* 77: 495–520.

Lamptey, M S, and B L Walker. 1976. “A Possible Essential Role for Dietary Linolenic Acid in the Development of the Young Rat.” *The Journal of nutrition* 106(1): 86–93.

Lands, W E. 1958. “Metabolism of Glycerolipides; a Comparison of Lecithin and Triglyceride Synthesis.” *The Journal of biological chemistry* 231(2): 883–88.

Lang, Thorsten, Nagaraj D Halemani, and Burkhard Rammner. 2008. “Interplay between Lipids and the Proteinaceous Membrane Fusion Machinery.” *Progress in lipid research* 47(6): 461–69.

Larsson, P K, H E Claesson, and B P Kennedy. 1998. “Multiple Splice Variants of the Human Calcium-Independent Phospholipase A2 and Their Effect on Enzyme Activity.” *The Journal of biological chemistry* 273(1): 207–14.

Lattka, Eva, Thomas Illig, Berthold Koletzko, and Joachim Heinrich. 2010. “Genetic Variants of the FADS1 FADS2 Gene Cluster as Related to Essential Fatty Acid Metabolism.” *Current opinion in lipidology* 21(1): 64–69.

Lauritzen, L, H S Hansen, M H Jorgensen, and K F Michaelsen. 2001. “The Essentiality of Long Chain N-3 Fatty Acids in Relation to Development and Function of the Brain and Retina.” *Progress in lipid research* 40(1-2): 1–94.

Lee, Jong-Min, and Jeffrey A Johnson. 2004. “An Important Role of Nrf2-ARE Pathway in the Cellular Defense Mechanism.” *Journal of biochemistry and molecular biology* 37(2): 139–43.

Lee, Suzee E. 2011. “Guam Dementia Syndrome Revisited in 2011.” *Current opinion in neurology* 24(6): 517–24.

Lengqvist, Johan et al. 2004. “Polyunsaturated Fatty Acids Including Docosahexaenoic and Arachidonic Acid Bind to the Retinoid X Receptor Alpha Ligand-Binding Domain.” *Molecular & cellular proteomics : MCP* 3(7): 692–703.

Leonard, A E et al. 2000. “cDNA Cloning and Characterization of Human Delta5-Desaturase Involved in the Biosynthesis of Arachidonic Acid.” *The Biochemical journal* 347 Pt 3: 719–24.

Lev, Sima. 2010. “Non-Vesicular Lipid Transport by Lipid-Transfer Proteins and Beyond.” *Nature reviews. Molecular cell biology* 11(10): 739–50.

Lev, Sima. 2012. “Nonvesicular Lipid Transfer from the Endoplasmic Reticulum.” *Cold Spring Harbor perspectives in biology* 4(10).

Levy, A et al. 1996. “Action of Steroid Hormones on Growth and Differentiation of CNS and Spinal Cord Organotypic Cultures.” *Cellular and molecular neurobiology* 16(3): 445–50.

Levy, B D et al. 2001. “Lipid Mediator Class Switching during Acute Inflammation: Signals in Resolution.” *Nature immunology* 2(7): 612–19.

Li, Jia et al. 2012. “Reducing Systemic Hypermetabolism by Inducing Hypothyroidism Does Not Prolong Survival in the SOD1-G93A Mouse.” *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 13(4): 372–77.

Li, Liaoliao, Zhi Wang, and Zhiyi Zuo. 2013. “Chronic Intermittent Fasting Improves Cognitive Functions and Brain Structures in Mice.” *PloS one* 8(6): e66069.

Li, Xiaoqing, Xuran Hao, Bo Cheng, and Xiantao Li. 2014. “Acute Blockage of Voltage-Gated K^+ Currents by 17β-Estradiol in Mouse Neuroblastoma N2A Cells.” *Neuroreport* 25(8): 574–79.

Lim, Giselle P et al. 2005. “A Diet Enriched with the Omega-3 Fatty Acid Docosahexaenoic Acid Reduces Amyloid Burden in an Aged Alzheimer Mouse Model.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25(12): 3032–40.

Lindauer, Eva et al. 2013. “Adipose Tissue Distribution Predicts Survival in Amyotrophic Lateral Sclerosis.” *PloS one* 8(6): e67783.

Lino, Maria Maddalena, Corinna Schneider, and Pico Caroni. 2002. “Accumulation of SOD1 Mutants in Postnatal Motoneurons Does Not Cause Motoneuron Pathology or Motoneuron Disease.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 22(12): 4825–32.

Lio, Y C, and E A Dennis. 1998. “Interfacial Activation, Lysophospholipase and Transacylase Activity of Group VI Ca2+-Independent Phospholipase A2.” *Biochimica et biophysica acta* 1392(2-3): 320–32.

Liu, G et al. 2012. “Neuronal Phagocytosis by Inflammatory Macrophages in ALS Spinal Cord: Inhibition of Inflammation by Resolvin D1.” *American journal of neurodegenerative disease* 1(1): 60–74.

Liu, Han-Qiang et al. 2013. “A High Ratio of Dietary N-3/n-6 Polyunsaturated Fatty Acids Improves Obesity-Linked Inflammation and Insulin Resistance through Suppressing Activation of TLR4 in SD Rats.” *Nutrition research (New York, N.Y.)* 33(10): 849–58.

Liu, Nai-Kui et al. 2006. “A Novel Role of Phospholipase A2 in Mediating Spinal Cord Secondary Injury.” *Annals of neurology* 59(4): 606–19.

Liu, Yi et al. 2005. “The Antiinflammatory Effect of Laminar Flow: The Role of PPARgamma, Epoxyeicosatrienoic Acids, and Soluble Epoxide Hydrolase.” *Proceedings of the National Academy of Sciences of the United States of America* 102(46): 16747–52.

Liu, Zhihua et al. 2011. “Drosophila Acyl-CoA Synthetase Long-Chain Family Member 4 Regulates Axonal Transport of Synaptic Vesicles and Is Required for Synaptic Development and Transmission.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31(6): 2052–63.

Loll, P J, D Picot, and R M Garavito. 1995. “The Structural Basis of Aspirin Activity Inferred from the Crystal Structure of Inactivated Prostaglandin H2 Synthase.” *Nature structural biology* 2(8): 637–43.

López-Erauskin, J et al. 2013. “Impaired Mitochondrial Oxidative Phosphorylation in the Peroxisomal Disease X-Linked Adrenoleukodystrophy.” *Human molecular genetics* 22(16): 3296–3305.

Lukiw, Walter J et al. 2005. “A Role for Docosahexaenoic Acid-Derived Neuroprotectin D1 in Neural Cell Survival and Alzheimer Disease.” *The Journal of clinical investigation* 115(10): 2774–83.

Magnusardottir, Anna R et al. 2009. “Docosahexaenoic Acid in Red Blood Cells of Women of Reproductive Age Is Positively Associated with Oral Contraceptive Use and Physical Activity.” *Prostaglandins, leukotrienes, and essential fatty acids* 80(1): 27–32.

Mahipal, Suraneni V K et al. 2007. “Effect of 15-Lipoxygenase Metabolites, 15-(S)-HPETE and 15-(S)-HETE on Chronic Myelogenous Leukemia Cell Line K-562: Reactive Oxygen Species (ROS) Mediate Caspase-Dependent Apoptosis.” *Biochemical pharmacology* 74(2): 202–14.

Majounie, Elisa et al. 2012. “Frequency of the C9orf72 Hexanucleotide Repeat Expansion in Patients with Amyotrophic Lateral Sclerosis and Frontotemporal Dementia: A Cross-Sectional Study.” *Lancet neurology* 11(4): 323–30.

Mantz, J et al. 1994. “Riluzole, a Novel Antiglutamate, Blocks GABA Uptake by Striatal Synaptosomes.” *European journal of pharmacology* 257(1-2): R7–8.

Marszalek, Joseph R, Claire Kitidis, Ariya Dararutana, and Harvey F Lodish. 2004. “Acyl-CoA Synthetase 2 Overexpression Enhances Fatty Acid Internalization and Neurite Outgrowth.” *The Journal of biological chemistry* 279(23): 23882–91.

Marszalek, Joseph R, Claire Kitidis, Concetta C Dirusso, and Harvey F Lodish. 2005. “Long-Chain Acyl-CoA Synthetase 6 Preferentially Promotes DHA Metabolism.” *The Journal of biological chemistry* 280(11): 10817–26.

Martindale, Jennifer L, and Nikki J Holbrook. 2002. “Cellular Response to Oxidative Stress: Signaling for Suicide and Survival.” *Journal of cellular physiology* 192(1): 1–15.

Martínez, J, and J J Moreno. 2001. “Role of Ca2+-Independent Phospholipase A2 on Arachidonic Acid Release Induced by Reactive Oxygen Species.” *Archives of biochemistry and biophysics* 392(2): 257–62.

Mattiazzi, Marina et al. 2002. “Mutated Human SOD1 Causes Dysfunction of Oxidative Phosphorylation in Mitochondria of Transgenic Mice.” *The Journal of biological chemistry* 277(33): 29626–33.

Matus, Soledad, Vicente Valenzuela, Danilo B Medinas, and Claudio Hetz. 2013. “ER Dysfunction and Protein Folding Stress in ALS.” *International journal of cell biology* 2013: 674751.

McCombe, Pamela A, and Robert D Henderson. 2010. “Effects of Gender in Amyotrophic Lateral Sclerosis.” *Gender medicine* 7(6): 557–70.

McDermott, M F et al. 1999. “Germline Mutations in the Extracellular Domains of the 55 kDa TNF Receptor, TNFR1, Define a Family of Dominantly Inherited Autoinflammatory Syndromes.” *Cell* 97(1): 133–44.

McGuire, V et al. 1997. “Occupational Exposures and Amyotrophic Lateral Sclerosis. A Population-Based Case-Control Study.” *American journal of epidemiology* 145(12): 1076–88.

McNamara, Robert K et al. 2013. “Lower Docosahexaenoic Acid Concentrations in the Postmortem Prefrontal Cortex of Adult Depressed Suicide Victims Compared with Controls without Cardiovascular Disease.” *Journal of psychiatric research* 47(9): 1187–91.

Mead, J F. 1985. “The Metabolism of the Essential Fatty Acids.” *The American journal of clinical nutrition* 6(6): 656–61.

Méresse, S, C Delbart, J C Fruchart, and R Cecchelli. 1989. “Low-Density Lipoprotein Receptor on Endothelium of Brain Capillaries.” *Journal of neurochemistry* 53(2): 340–45.

Mereschkowsky, Konstantin 1910. "Theorie der zwei Plasmaarten als Grundlage der Symbiogenesis, einer neuen Lehre von der Ent-stehung der Organismen.". Biol Centralbl. 30: 353-367

Meruvu, Sunitha et al. 2005. “Sequence Determinants for the Reaction Specificity of Murine (12R)-Lipoxygenase: Targeted Substrate Modification and Site-Directed Mutagenesis.” *The Journal of biological chemistry* 280(44): 36633–41.

Metz, J G et al. 2001. “Production of Polyunsaturated Fatty Acids by Polyketide Synthases in Both Prokaryotes and Eukaryotes.” *Science (New York, N.Y.)* 293(5528): 290–93.

Miana-Mena, Francisco Javier et al. 2011. “Monitoring Systemic Oxidative Stress in an Animal Model of Amyotrophic Lateral Sclerosis.” *Journal of neurology* 258(5): 762–69.

Milne, Ginger L et al. 2011. “Isoprostane Generation and Function.” *Chemical reviews* 111(10): 5973–96.

Mitsumoto, Hiroshi et al. 2008. “Oxidative Stress Biomarkers in Sporadic ALS.” *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 9(3): 177–83.

Miyamae, Takako et al. 2013. “Increased Oxidative Stress and Coenzyme Q10 Deficiency in Juvenile Fibromyalgia: Amelioration of Hypercholesterolemia and Fatigue by Ubiquinol-10 Supplementation.” *Redox report : communications in free radical research* 18(1): 12–19.

Montine, T J et al. 1999. “Increased CSF F2-Isoprostane Concentration in Probable AD.” *Neurology* 52(3): 562–65.

Moolwaney, Anju S, and Orisa J Igwe. 2005. “Regulation of the Cyclooxygenase-2 System by Interleukin-1beta through Mitogen-Activated Protein Kinase Signaling Pathways: A Comparative Study of Human Neuroglioma and Neuroblastoma Cells.” *Brain research. Molecular brain research* 137(1-2): 202–12.

Moore, S A et al. 1991. “Astrocytes, Not Neurons, Produce Docosahexaenoic Acid (22:6 Omega-3) and Arachidonic Acid (20:4 Omega-6).” *Journal of neurochemistry* 56(2): 518–24.

Morrow, J D et al. 1990. “A Series of Prostaglandin F2-like Compounds Are Produced in Vivo in Humans by a Non-Cyclooxygenase, Free Radical-Catalyzed Mechanism.” *Proceedings of the National Academy of Sciences of the United States of America* 87(23): 9383–87.

Moustaqim-Barrette, Amina et al. 2014. “The Amyotrophic Lateral Sclerosis 8 Protein, VAP, Is Required for ER Protein Quality Control.” *Human molecular genetics* 23(8): 1975–89.

Mozaffarian, Dariush, and Eric B Rimm. 2006. “Fish Intake, Contaminants, and Human Health: Evaluating the Risks and the Benefits.” *JAMA : the journal of the American Medical Association* 296(15): 1885–99.

Mueller, Martin J. “Isoprostane Nomenclature: Inherent Problems May Cause Setbacks for the Development of the Isoprostanoid Field.” *Prostaglandins, leukotrienes, and essential fatty acids* 82(2-3): 71–81.

Mukherjee, P K, A Chawla, M S Loayza, and N G Bazan. 2007. “Docosanoids Are Multifunctional Regulators of Neural Cell Integrity and Fate: Significance in Aging and Disease.” *Prostaglandins, leukotrienes, and essential fatty acids* 77(5-6): 233–38.

Mukherjee, Pranab K, Victor L Marcheselli, Charles N Serhan, and Nicolas G Bazan. 2004. “Neuroprotectin D1: A Docosahexaenoic Acid-Derived Docosatriene Protects Human Retinal Pigment Epithelial Cells from Oxidative Stress.” *Proceedings of the National Academy of Sciences of the United States of America* 101(22): 8491–96.

Murakami, Makoto, and Gérard Lambeau. 2013. “Emerging Roles of Secreted Phospholipase A(2) Enzymes: An Update.” *Biochimie* 95(1): 43–50.

Murakami, Makoto, Yoshitaka Taketomi, Hiroyasu Sato, and Kei Yamamoto. 2011. “Secreted Phospholipase A2 Revisited.” *Journal of biochemistry* 150(3): 233–55.

Nadolski, Marissa J, and Maurine E Linder. 2007. “Protein Lipidation.” *The FEBS journal* 274(20): 5202–10.

Nakamura, Manabu T, Yewon Cheon, Yue Li, and Takayuki Y Nara. 2004. “Mechanisms of Regulation of Gene Expression by Fatty Acids.” *Lipids* 39(11): 1077–83.

Nefussy, Beatrice, Jonathan Hirsch, Merit E Cudkowicz, and Vivian E Drory. 2011. “Gender-Based Effect of Statins on Functional Decline in Amyotrophic Lateral Sclerosis.” *Journal of the neurological sciences* 300(1-2): 23–27.

Neuringer, M et al. 1986. “Biochemical and Functional Effects of Prenatal and Postnatal Omega 3 Fatty Acid Deficiency on Retina and Brain in Rhesus Monkeys.” *Proceedings of the National Academy of Sciences of the United States of America* 83(11): 4021–25.

Nguyen, D et al. 2011. “A New Vicious Cycle Involving Glutamate Excitotoxicity, Oxidative Stress and Mitochondrial Dynamics.” *Cell death & disease* 2: e240.

Nigam, Santosh, and Maria-Patapia Zafiriou. 2005. “Hepoxilin A3 Synthase.” *Biochemical and biophysical research communications* 338(1): 161–68.

Nishimura, Agnes L et al. 2004. “A Mutation in the Vesicle-Trafficking Protein VAPB Causes Late-Onset Spinal Muscular Atrophy and Amyotrophic Lateral Sclerosis.” *American journal of human genetics* 75(5): 822–31.

O’Banion, M K. 1999. “Cyclooxygenase-2: Molecular Biology, Pharmacology, and Neurobiology.” *Critical reviews in neurobiology* 13(1): 45–82. http://www.ncbi.nlm.nih.gov/pubmed/10223523 (April 9, 2014).

De Oliveira, Antonio C P et al. 2012. “Pharmacological Inhibition of Akt and Downstream Pathways Modulates the Expression of COX-2 and mPGES-1 in Activated Microglia.” *Journal of neuroinflammation* 9: 2.

Ong, W Y, L A Horrocks, and A A Farooqui. 1999. “Immunocytochemical Localization of cPLA2 in Rat and Monkey Spinal Cord.” *Journal of molecular neuroscience : MN* 12(2): 123–30.

Ou, J et al. 2001. “Unsaturated Fatty Acids Inhibit Transcription of the Sterol Regulatory Element-Binding Protein-1c (SREBP-1c) Gene by Antagonizing Ligand-Dependent Activation of the LXR.” *Proceedings of the National Academy of Sciences of the United States of America* 98(11): 6027–32.

Owada, Yuji. 2008. “Fatty Acid Binding Protein: Localization and Functional Significance in the Brain.” *The Tohoku journal of experimental medicine* 214(3): 213–20.

Pacelli, Consiglia et al. 2011. “Tight Control of Mitochondrial Membrane Potential by Cytochrome c Oxidase.” *Mitochondrion* 11(2): 334–41.

Paganoni, Sabrina et al. 2011. “Body Mass Index, Not Dyslipidemia, Is an Independent Predictor of Survival in Amyotrophic Lateral Sclerosis.” *Muscle & nerve* 44(1): 20–24.

Pamplona, Reinald et al. 2008. “Maillard Reaction versus Other Nonenzymatic Modifications in Neurodegenerative Processes.” *Annals of the New York Academy of Sciences* 1126: 315–19.

Park, Robert M et al. 2005. “Potential Occupational Risks for Neurodegenerative Diseases.” *American journal of industrial medicine* 48(1): 63–77.

Pasinelli, Piera et al. 2004. “Amyotrophic Lateral Sclerosis-Associated SOD1 Mutant Proteins Bind and Aggregate with Bcl-2 in Spinal Cord Mitochondria.” *Neuron* 43(1): 19–30.

Patel, Barkha P et al. 2010. “Caloric Restriction Shortens Lifespan through an Increase in Lipid Peroxidation, Inflammation and Apoptosis in the G93A Mouse, an Animal Model of ALS.” *PloS one* 5(2): e9386.

Paterniti, Irene et al. 2013. “A New Co-Ultramicronized Composite Including Palmitoylethanolamide and Luteolin to Prevent Neuroinflammation in Spinal Cord Injury.” *Journal of neuroinflammation* 10: 91.

Peixoto, Pablo M et al. 2013. “UCP2 Overexpression Worsens Mitochondrial Dysfunction and Accelerates Disease Progression in a Mouse Model of Amyotrophic Lateral Sclerosis.” *Molecular and cellular neurosciences* 57: 104–10.

Peters, J M et al. 2000. “Growth, Adipose, Brain, and Skin Alterations Resulting from Targeted Disruption of the Mouse Peroxisome Proliferator-Activated Receptor Beta(delta).” *Molecular and cellular biology* 20(14): 5119–28.

Peters-Golden, M, and T G Brock. “5-Lipoxygenase and FLAP.” *Prostaglandins, leukotrienes, and essential fatty acids* 69(2-3): 99–109.

Peviani, M et al. 2007. “Lack of Changes in the PI3K/AKT Survival Pathway in the Spinal Cord Motor Neurons of a Mouse Model of Familial Amyotrophic Lateral Sclerosis.” *Molecular and cellular neurosciences* 34(4): 592–602.

Peviani, Marco et al. 2014. “Specific Induction of Akt3 in Spinal Cord Motor Neurons Is Neuroprotective in a Mouse Model of Familial Amyotrophic Lateral Sclerosis.” *Molecular neurobiology* 49(1): 136–48.

Philips, T, and J D Rothstein. 2014. “Glial Cells in Amyotrophic Lateral Sclerosis.” *Experimental neurology*.

Philips, Thomas, and Wim Robberecht. 2011. “Neuroinflammation in Amyotrophic Lateral Sclerosis: Role of Glial Activation in Motor Neuron Disease.” *Lancet neurology* 10(3): 253–63.

Phillips, Scott E et al. 2006. “Specific and Nonspecific Membrane-Binding Determinants Cooperate in Targeting Phosphatidylinositol Transfer Protein Beta-Isoform to the Mammalian Trans-Golgi Network.” *Molecular biology of the cell* 17(6): 2498–2512.

Phillis, John W, Lloyd A Horrocks, and Akhlaq A Farooqui. 2006. “Cyclooxygenases, Lipoxygenases, and Epoxygenases in CNS: Their Role and Involvement in Neurological Disorders.” *Brain research reviews* 52(2): 201–43.

Piepers, Sanne et al. 2009. “Randomized Sequential Trial of Valproic Acid in Amyotrophic Lateral Sclerosis.” *Annals of neurology* 66(2): 227–34.

Pizzi, M et al. 2005. “Inhibition of IkappaBalpha Phosphorylation Prevents Glutamate-Induced NF-kappaB Activation and Neuronal Cell Death.” *Acta neurochirurgica. Supplement* 93: 59–63.

Planey, Sonia L, and David A Zacharias. 2009. “Palmitoyl Acyltransferases, Their Substrates, and Novel Assays to Connect Them (Review).” *Molecular membrane biology* 26(1): 14–31.

Porter, N A, and M O Funk. 1975. “Letter: Peroxy Radical Cyclization as a Model for Prostaglandin Biosynthesis.” *The Journal of organic chemistry* 40(24): 3614–15.

Pramatarova, A et al. 2001. “Neuron-Specific Expression of Mutant Superoxide Dismutase 1 in Transgenic Mice Does Not Lead to Motor Impairment.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 21(10): 3369–74.

Prell, T et al. 2014. “Endoplasmic Reticulum Stress Is Accompanied by Activation of NF-κB in Amyotrophic Lateral Sclerosis.” *Journal of neuroimmunology* 270(1-2): 29–36.

Prell, Tino, Janin Lautenschläger, and Julian Grosskreutz. 2013. “Calcium-Dependent Protein Folding in Amyotrophic Lateral Sclerosis.” *Cell calcium* 54(2): 132–43.

Prosser, Derek C et al. 2008. “FFAT Rescues VAPA-Mediated Inhibition of ER-to-Golgi Transport and VAPB-Mediated ER Aggregation.” *Journal of cell science* 121(Pt 18): 3052–61.

Prusakiewicz, Jeffery J, Philip J Kingsley, Kevin R Kozak, and Lawrence J Marnett. 2002. “Selective Oxygenation of N-Arachidonylglycine by Cyclooxygenase-2.” *Biochemical and biophysical research communications* 296(3): 612–17.

Pu, Jing et al. 2011. “Palmitic Acid Acutely Stimulates Glucose Uptake via Activation of Akt and ERK1/2 in Skeletal Muscle Cells.” *Journal of lipid research* 52(7): 1319–27.

Purdon, D, T Arai, and S Rapoport. 1997. “No Evidence for Direct Incorporation of Esterified Palmitic Acid from Plasma into Brain Lipids of Awake Adult Rat.” *Journal of lipid research* 38(3): 526–30.

Qiu, Xiao. 2003. “Biosynthesis of Docosahexaenoic Acid (DHA, 22:6-4, 7,10,13,16,19): Two Distinct Pathways.” *Prostaglandins, leukotrienes, and essential fatty acids* 68(2): 181–86.

Rapoport, S I, M C Chang, and A A Spector. 2001. “Delivery and Turnover of Plasma-Derived Essential PUFAs in Mammalian Brain.” *Journal of lipid research* 42(5): 678–85.

Rapoport, Stanley I, Epolia Ramadan, and Mireille Basselin. 2011. “Docosahexaenoic Acid (DHA) Incorporation into the Brain from Plasma, as an in Vivo Biomarker of Brain DHA Metabolism and Neurotransmission.” *Prostaglandins & other lipid mediators* 96(1-4): 109–13.

Reinholz, M M, C M Merkle, and J F Poduslo. 1999. “Therapeutic Benefits of Putrescine-Modified Catalase in a Transgenic Mouse Model of Familial Amyotrophic Lateral Sclerosis.” *Experimental neurology* 159(1): 204–16.

Reis, Ana, and Corinne M Spickett. 2012. “Chemistry of Phospholipid Oxidation.” *Biochimica et biophysica acta* 1818(10): 2374–87.

Rekling, J C et al. 2000. “Synaptic Control of Motoneuronal Excitability.” *Physiological reviews* 80(2): 767–852.

Renton, Alan E et al. 2011. “A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD.” *Neuron* 72(2): 257–68.

Repa, J J et al. 2000. “Regulation of Mouse Sterol Regulatory Element-Binding Protein-1c Gene (SREBP-1c) by Oxysterol Receptors, LXRalpha and LXRbeta.” *Genes & development* 14(22): 2819–30.

Requena, J R et al. 1997. “Carboxymethylethanolamine, a Biomarker of Phospholipid Modification during the Maillard Reaction in Vivo.” *The Journal of biological chemistry* 272(28): 17473–79.

Repa, Joyce J, and David J Mangelsdorf. 2002. “The Liver X Receptor Gene Team: Potential New Players in Atherosclerosis.” *Nature medicine* 8(11): 1243–48.

Richieri, G V, A Anel, and A M Kleinfeld. 1993. “Interactions of Long-Chain Fatty Acids and Albumin: Determination of Free Fatty Acid Levels Using the Fluorescent Probe ADIFAB.” *Biochemistry* 32(29): 7574–80.

Richter, C. 1997. “Reactive Oxygen and Nitrogen Species Regulate Mitochondrial Ca2+ Homeostasis and Respiration.” *Bioscience reports* 17(1): 53–66.

Rizos, Evangelos C et al. 2012. “Association between Omega-3 Fatty Acid Supplementation and Risk of Major Cardiovascular Disease Events: A Systematic Review and Meta-Analysis.” *JAMA : the journal of the American Medical Association* 308(10): 1024–33.

Roberts, L J et al. 1998. “Formation of Isoprostane-like Compounds (neuroprostanes) in Vivo from Docosahexaenoic Acid.” *The Journal of biological chemistry* 273(22): 13605–12.

Roberts, L J, and J D Morrow. 2000. “Measurement of F(2)-Isoprostanes as an Index of Oxidative Stress in Vivo.” *Free radical biology & medicine* 28(4): 505–13.

Rocha, Nuno et al. 2009. “Cholesterol Sensor ORP1L Contacts the ER Protein VAP to Control Rab7-RILP-p150 Glued and Late Endosome Positioning.” *The Journal of cell biology* 185(7): 1209–25.

Rocks, Oliver et al. 2005. “An Acylation Cycle Regulates Localization and Activity of Palmitoylated Ras Isoforms.” *Science (New York, N.Y.)* 307(5716): 1746–52.

Rogakou, E P et al. 1998. “DNA Double-Stranded Breaks Induce Histone H2AX Phosphorylation on Serine 139.” *The Journal of biological chemistry* 273(10): 5858–68.

Rosen, D R. 1993. “Mutations in Cu/Zn Superoxide Dismutase Gene Are Associated with Familial Amyotrophic Lateral Sclerosis.” *Nature* 364(6435): 362.

Rothstein, J D et al. 1995. “Selective Loss of Glial Glutamate Transporter GLT-1 in Amyotrophic Lateral Sclerosis.” *Annals of neurology* 38(1): 73–84. http://www.ncbi.nlm.nih.gov/pubmed/7611729 (July 26, 2014).

Rothstein, J D, L Jin, M Dykes-Hoberg, and R W Kuncl. 1993. “Chronic Inhibition of Glutamate Uptake Produces a Model of Slow Neurotoxicity.” *Proceedings of the National Academy of Sciences of the United States of America* 90(14): 6591–95.

Roussel, Benoit D et al. 2013. “Endoplasmic Reticulum Dysfunction in Neurological Disease.” *Lancet neurology* 12(1): 105–18.

Rowland, Ashley A, and Gia K Voeltz. 2012. “Endoplasmic Reticulum-Mitochondria Contacts: Function of the Junction.” *Nature reviews. Molecular cell biology* 13(10): 607–25.

Ryan, Aidan, and Catherine Godson. 2010. “Lipoxins: Regulators of Resolution.” *Current opinion in pharmacology* 10(2): 166–72.

Sabel, Karl-Göran et al. 2009. “Fatty Acid Patterns Early after Premature Birth, Simultaneously Analysed in Mothers’ Food, Breast Milk and Serum Phospholipids of Mothers and Infants.” *Lipids in health and disease* 8: 20.

Sako, Wataru et al. “Nuclear Factor K B Expression in Patients with Sporadic Amyotrophic Lateral Sclerosis and Hereditary Amyotrophic Lateral Sclerosis with Optineurin Mutations.” *Clinical neuropathology* 31(6): 418–23.

Sala-Vila, Aleix et al. 2011. “Determinants of the Omega-3 Index in a Mediterranean Population at Increased Risk for CHD.” *The British journal of nutrition* 106(3): 425–31.

Salminen, Antero et al. “Impaired Autophagy and APP Processing in Alzheimer’s Disease: The Potential Role of Beclin 1 Interactome.” *Progress in neurobiology* 106-107: 33–54.

Sánchez-Galán, Eva et al. 2009. “Leukotriene B4 Enhances the Activity of Nuclear Factor-kappaB Pathway through BLT1 and BLT2 Receptors in Atherosclerosis.” *Cardiovascular research* 81(1): 216–25.

Sandhya, T L, W Y Ong, L A Horrocks, and A A Farooqui. 1998. “A Light and Electron Microscopic Study of Cytoplasmic Phospholipase A2 and Cyclooxygenase-2 in the Hippocampus after Kainate Lesions.” *Brain research* 788(1-2): 223–31.

Sathasivam, Sivakumar, and Bryan Lecky. 2008. “Statin Induced Myopathy.” *BMJ (Clinical research ed.)* 337: a2286.

Saugstad, Letten F. 2006. “Are Neurodegenerative Disorder and Psychotic Manifestations Avoidable Brain Dysfunctions with Adequate Dietary Omega-3?” *Nutrition and health* 18(3): 203–15.

Schaeffer, Evelin L et al. 2010. “Differential Roles of Phospholipases A2 in Neuronal Death and Neurogenesis: Implications for Alzheimer Disease.” *Progress in neuro-psychopharmacology & biological psychiatry* 34(8): 1381–89.

Schäfer, G, M Engelhard, and V Müller. 1999. “Bioenergetics of the Archaea.” *Microbiology and molecular biology reviews : MMBR* 63(3): 570–620.

Schaloske, Ralph H, and Edward A Dennis. 2006. “The Phospholipase A2 Superfamily and Its Group Numbering System.” *Biochimica et biophysica acta* 1761(11): 1246–59.

Schinder, A F, E C Olson, N C Spitzer, and M Montal. 1996. “Mitochondrial Dysfunction Is a Primary Event in Glutamate Neurotoxicity.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 16(19): 6125–33.

Schlame, M, K Beyer, M Hayer-Hartl, and M Klingenberg. 1991. “Molecular Species of Cardiolipin in Relation to Other Mitochondrial Phospholipids. Is There an Acyl Specificity of the Interaction between Cardiolipin and the ADP/ATP Carrier?” *European journal of biochemistry / FEBS* 199(2): 459–66.

Schlame, Michael et al. 2005. “Molecular Symmetry in Mitochondrial Cardiolipins.” *Chemistry and physics of lipids* 138(1-2): 38–49.

Schmidt, Oliver, Nikolaus Pfanner, and Chris Meisinger. 2010. “Mitochondrial Protein Import: From Proteomics to Functional Mechanisms.” *Nature reviews. Molecular cell biology* 11(9): 655–67.

Schnorr, Carlos Eduardo et al. 2011. “The Effects of Vitamin A Supplementation to Rats during Gestation and Lactation upon Redox Parameters: Increased Oxidative Stress and Redox Modulation in Mothers and Their Offspring.” *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* 49(10): 2645–54.

Schulz, Tim J et al. 2007. “Glucose Restriction Extends Caenorhabditis Elegans Life Span by Inducing Mitochondrial Respiration and Increasing Oxidative Stress.” *Cell metabolism* 6(4): 280–93.

Schumann, Uwe, and Suresh Subramani. 2008. “Special Delivery from Mitochondria to Peroxisomes.” *Trends in cell biology* 18(6): 253–56.

Schütz, Burkhard et al. 2005. “The Oral Antidiabetic Pioglitazone Protects from Neurodegeneration and Amyotrophic Lateral Sclerosis-like Symptoms in Superoxide Dismutase-G93A Transgenic Mice.” *The Journal of neuroscience : the official journal of the Society for Neuroscience* 25(34): 7805–12

.Schwab, Jan M, Nan Chiang, Makoto Arita, and Charles N Serhan. 2007. “Resolvin E1 and Protectin D1 Activate Inflammation-Resolution Programmes.” *Nature* 447(7146): 869–74.

Serhan, Charles N et al. 2007. “Resolution of Inflammation: State of the Art, Definitions and Terms.” *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21(2): 325–32.

Serhan CN, Yang R, Martinod K, Kasuga K, Pillai PS, Porter TF, Oh SF, Spite M. 2009. “Maresins: Novel Macrophage Mediators with Potent Antiinflammatory and Proresolving Actions.” *The Journal of experimental medicine* 206(1): 15–23.

Serhan CN, Dalli J, Colas RA, Winkler JW, Chiang N. 2014. “Protectins and Maresins: New pro-Resolving Families of Mediators in Acute Inflammation and Resolution Bioactive Metabolome.” *Biochimica et biophysica acta*.

Serhan, Charles N, Nan Chiang, and Thomas E Van Dyke. 2008. “Resolving Inflammation: Dual Anti-Inflammatory and pro-Resolution Lipid Mediators.” *Nature reviews. Immunology* 8(5): 349–61.

Serhan, Charles N, Katherine Gotlinger, Song Hong, and Makoto Arita. 2004. “Resolvins, Docosatrienes, and Neuroprotectins, Novel Omega-3-Derived Mediators, and Their Aspirin-Triggered Endogenous Epimers: An Overview of Their Protective Roles in Catabasis.” *Prostaglandins & other lipid mediators* 73(3-4): 155–72.

Shaw, P J, P G Ince, G Falkous, and D Mantle. 1995. “Oxidative Damage to Protein in Sporadic Motor Neuron Disease Spinal Cord.” *Annals of neurology* 38(4): 691–95.

Shen, Yu-fei et al. 2013. “Adaptive Changes in Autophagy after UPS Impairment in Parkinson’s Disease.” *Acta pharmacologica Sinica* 34(5): 667–73.

Shi, C-h et al. 2011. “PLA2G6 Gene Mutation in Autosomal Recessive Early-Onset Parkinsonism in a Chinese Cohort.” *Neurology* 77(1): 75–81.

Shibata, N et al. 2001. “Morphological Evidence for Lipid Peroxidation and Protein Glycoxidation in Spinal Cords from Sporadic Amyotrophic Lateral Sclerosis Patients.” *Brain research* 917(1): 97–104.

Shibata, Noriyuki et al. 2010. “Increased Expression and Activation of Cytosolic Phospholipase A2 in the Spinal Cord of Patients with Sporadic Amyotrophic Lateral Sclerosis.” *Acta neuropathologica* 119(3): 345–54.

Shibata, Takahiro et al. 2002. “15-Deoxy-Delta 12,14-Prostaglandin J2. A Prostaglandin D2 Metabolite Generated during Inflammatory Processes.” *The Journal of biological chemistry* 277(12): 10459–66.

Shin, Jin Hee et al. 2012. “Concurrent Blockade of Free Radical and Microsomal Prostaglandin E Synthase-1-Mediated PGE2 Production Improves Safety and Efficacy in a Mouse Model of Amyotrophic Lateral Sclerosis.” *Journal of neurochemistry* 122(5): 952–61.

Shindo, K, S Tsunoda, and Z Shiozawa. 1995. “Increased Sympathetic Outflow to Muscles in Patients with Amyotrophic Lateral Sclerosis: A Comparison with Other Neuromuscular Patients.” *Journal of the neurological sciences* 134(1-2): 57–60.

Shindou, Hideo et al. 2009. “Recent Progress on Acyl CoA: Lysophospholipid Acyltransferase Research.” *Journal of lipid research* 50 Suppl: S46–51.

Siklós, L et al. 1996. “Ultrastructural Evidence for Altered Calcium in Motor Nerve Terminals in Amyotropic Lateral Sclerosis.” *Annals of neurology* 39(2): 203–16.

Simons, Kai, and Winchil L C Vaz. 2004. “Model Systems, Lipid Rafts, and Cell Membranes.” *Annual review of biophysics and biomolecular structure* 33: 269–95.

Simopoulos, A P, A Leaf, and N Salem. 1999. “Workshop on the Essentiality of and Recommended Dietary Intakes for Omega-6 and Omega-3 Fatty Acids.” *Journal of the American College of Nutrition* 18(5): 487–89.

Simopoulos, Artemis P. 2011. “Evolutionary Aspects of Diet: The Omega-6/omega-3 Ratio and the Brain.” *Molecular neurobiology* 44(2): 203–15

.Simpson, Claire L et al. 2009. “Variants of the Elongator Protein 3 (ELP3) Gene Are Associated with Motor Neuron Degeneration.” *Human molecular genetics* 18(3): 472–81.

Singer, Alan G et al. 2002. “Interfacial Kinetic and Binding Properties of the Complete Set of Human and Mouse Groups I, II, V, X, and XII Secreted Phospholipases A2.” *The Journal of biological chemistry* 277(50): 48535–49.

Singer, S J, and G L Nicolson. 1972. “The Fluid Mosaic Model of the Structure of Cell Membranes.” *Science (New York, N.Y.)* 175(4023): 720–31.

Smith, R G, Y K Henry, M P Mattson, and S H Appel. 1998. “Presence of 4-Hydroxynonenal in Cerebrospinal Fluid of Patients with Sporadic Amyotrophic Lateral Sclerosis.” *Annals of neurology* 44(4): 696–99.

Sofroniew, Michael V, and Harry V Vinters. 2010. “Astrocytes: Biology and Pathology.” *Acta neuropathologica* 119(1): 7–35.

Somerharju, P, J A Virtanen, and K H Cheng. 1999. “Lateral Organisation of Membrane Lipids. The Superlattice View.” *Biochimica et biophysica acta* 1440(1): 32–48.

Sovic, Andrea et al. 2005. “Regulated Expression of Endothelial Lipase by Porcine Brain Capillary Endothelial Cells Constituting the Blood-Brain Barrier.” *Journal of neurochemistry* 94(1): 109–19.

Spector, A A. 2001. “Plasma Free Fatty Acid and Lipoproteins as Sources of Polyunsaturated Fatty Acid for the Brain.” *Journal of molecular neuroscience : MN* 16(2-3): 159–65; discussion 215–21.

Spickett, Corinne M. 2013. “The Lipid Peroxidation Product 4-Hydroxy-2-Nonenal: Advances in Chemistry and Analysis.” *Redox biology* 1(1): 145–52.

Spite, Matthew et al. 2009. “Resolvin D2 Is a Potent Regulator of Leukocytes and Controls Microbial Sepsis.” *Nature* 461(7268): 1287–91.

Spite, Matthew, and Charles N Serhan. 2010. “Novel Lipid Mediators Promote Resolution of Acute Inflammation: Impact of Aspirin and Statins.” *Circulation research* 107(10): 1170–84.

Sprecher, H. 1981. “Biochemistry of Essential Fatty Acids.” *Progress in lipid research* 20: 13–22.

Staats, Kim A et al. 2013. “Genetic Ablation of Phospholipase C Delta 1 Increases Survival in SOD1(G93A) Mice.” *Neurobiology of disease* 60: 11–17.

Standing, Ariane, Ebun Omoyinmi, and Paul Brogan. 2013. “Gene Hunting in Autoinflammation.” *Clinical and translational allergy* 3(1): 32.

Stavrovskaya, Irina G et al. 2012. “Dietary Omega-3 Fatty Acids Do Not Change Resistance of Rat Brain or Liver Mitochondria to Ca(2+) And/or Prooxidants.” *Journal of lipids* 2012: 797105.

Stavrovskaya IG, Bird SS, Marur VR, Sniatynski MJ, Baranov SV, Greenberg HK, Porter CL, Kristal BS. 2013. “Dietary Macronutrients Modulate the Fatty Acyl Composition of Rat Liver Mitochondrial Cardiolipins.” *Journal of lipid research* 54(10): 2623–35.

Stefanatos, Rhoda, and Alberto Sanz. 2011. “Mitochondrial Complex I: A Central Regulator of the Aging Process.” *Cell cycle (Georgetown, Tex.)* 10(10): 1528–32.

Stefanyk, Leslie E et al. 2010. “Skeletal Muscle Type Comparison of Subsarcolemmal Mitochondrial Membrane Phospholipid Fatty Acid Composition in Rat.” *The Journal of membrane biology* 234(3): 207–15.

Stevens, J, E G Katz, and R R Huxley. 2010. “Associations between Gender, Age and Waist Circumference.” *European journal of clinical nutrition* 64(1): 6–15.

Stieber, A et al. 1998. “The Fragmented Neuronal Golgi Apparatus in Amyotrophic Lateral Sclerosis Includes the Trans-Golgi-Network: Functional Implications.” *Acta neuropathologica* 95(3): 245–53.

Stillwell, William, and Stephen R Wassall. 2003. “Docosahexaenoic Acid: Membrane Properties of a Unique Fatty Acid.” *Chemistry and physics of lipids* 126(1): 1–27.

St-Jacques, Bruno, and Weiya Ma. 2011. “Role of Prostaglandin E2 in the Synthesis of the pro-Inflammatory Cytokine Interleukin-6 in Primary Sensory Neurons: An in Vivo and in Vitro Study.” *Journal of neurochemistry* 118(5): 841–54.

Stribl, Carola et al. 2014. “Mitochondrial Dysfunction and Decrease in Body Weight of a Transgenic Knock-in Mouse Model for TDP-43.” *The Journal of biological chemistry*.

Stulnig, T M et al. 2001. “Polyunsaturated Eicosapentaenoic Acid Displaces Proteins from Membrane Rafts by Altering Raft Lipid Composition.” *The Journal of biological chemistry* 276(40): 37335–40.

Subczynski, W K, J S Hyde, and A Kusumi. 1991. “Effect of Alkyl Chain Unsaturation and Cholesterol Intercalation on Oxygen Transport in Membranes: A Pulse ESR Spin Labeling Study.” *Biochemistry* 30(35): 8578–90.

Sun, Fei et al. 2005. “Crystal Structure of Mitochondrial Respiratory Membrane Protein Complex II.” *Cell* 121(7): 1043–57.

Sun, Grace Y, Jianfeng Xu, Michael D Jensen, and Agnes Simonyi. 2004. “Phospholipase A2 in the Central Nervous System: Implications for Neurodegenerative Diseases.” *Journal of lipid research* 45(2): 205–13.

Suzuki, Masatoshi et al. 2007. “Sexual Dimorphism in Disease Onset and Progression of a Rat Model of ALS.” *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* 8(1): 20–25.

Swarup, Vivek et al. 2011. “Deregulation of TDP-43 in Amyotrophic Lateral Sclerosis Triggers Nuclear Factor κB-Mediated Pathogenic Pathways.” *The Journal of experimental medicine* 208(12): 2429–47.

Sydenham, Emma, Alan D Dangour, and Wee-Shiong Lim. 2012. “Omega 3 Fatty Acid for the Prevention of Cognitive Decline and Dementia.” *The Cochrane database of systematic reviews* 6: CD005379.

Taghiyar, Maryam et al. 2013. “The Effect of Vitamins C and E Supplementation on Muscle Damage, Performance, and Body Composition in Athlete Women: A Clinical Trial.” *International journal of preventive medicine* 4(Suppl 1): S24–30.

Takagi, Mitsuhiro, Fumitaka Suto, Tetsuya Suga, and Junji Yamada. 2005. “Sterol Regulatory Element-Binding Protein-2 Modulates Human Brain Acyl-CoA Hydrolase Gene Transcription.” *Molecular and cellular biochemistry* 275(1-2): 199–206.

Tamura, Kenji et al. 2009. “Novel Lipogenic Enzyme ELOVL7 Is Involved in Prostate Cancer Growth through Saturated Long-Chain Fatty Acid Metabolism.” *Cancer research* 69(20): 8133–40.

Tan, Wenzhi, Piera Pasinelli, and Davide Trotti. 2014. “Role of Mitochondria in Mutant SOD1 Linked Amyotrophic Lateral Sclerosis.” *Biochimica et biophysica acta*.

Tao, Qingqing et al. 2012. “Gender Segregation in Gene Expression and Vulnerability to Oxidative Stress Induced Injury in Ventral Mesencephalic Cultures of Dopamine Neurons.” *Journal of neuroscience research* 90(1): 167–78.

Tapia, Patrick C. 2006. “Sublethal Mitochondrial Stress with an Attendant Stoichiometric Augmentation of Reactive Oxygen Species May Precipitate Many of the Beneficial Alterations in Cellular Physiology Produced by Caloric Restriction, Intermittent Fasting, Exercise and Dietary P.” *Medical hypotheses* 66(4): 832–43.

Tateishi, Takahisa et al. 2010. “CSF Chemokine Alterations Related to the Clinical Course of Amyotrophic Lateral Sclerosis.” *Journal of neuroimmunology* 222(1-2): 76–81.

Tocher, D R, and J R Sargent. 1990. “Incorporation into Phospholipid Classes and Metabolism via Desaturation and Elongation of Various 14C-Labelled (n-3) and (n-6) Polyunsaturated Fatty Acids in Trout Astrocytes in Primary Culture.” *Journal of neurochemistry* 54(6): 2118–24.

Toit-Kohn, Joe-Lin du, Louise Louw, and Anna-Mart Engelbrecht. 2009. “Docosahexaenoic Acid Induces Apoptosis in Colorectal Carcinoma Cells by Modulating the PI3 Kinase and p38 MAPK Pathways.” *The Journal of nutritional biochemistry* 20(2): 106–14.

Tolosa, Laia, Víctor Caraballo-Miralles, Gabriel Olmos, and Jerònia Lladó. 2011. “TNF-A Potentiates Glutamate-Induced Spinal Cord Motoneuron Death via NF-κB.” *Molecular and cellular neurosciences* 46(1): 176–86.

Trojsi, Francesca, Maria Rosaria Monsurrò, and Gioacchino Tedeschi. 2013. “Exposure to Environmental Toxicants and Pathogenesis of Amyotrophic Lateral Sclerosis: State of the Art and Research Perspectives.” *International journal of molecular sciences* 14(8): 15286–311.

Trumbo, Paula, Sandra Schlicker, Allison A Yates, and Mary Poos. 2002. “Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids.” *Journal of the American Dietetic Association* 102(11): 1621–30.

Turrens, J F, and A Boveris. 1980. “Generation of Superoxide Anion by the NADH Dehydrogenase of Bovine Heart Mitochondria.” *The Biochemical journal* 191(2): 421–27.

Tvrzicka, Eva, Lefkothea-Stella Kremmyda, Barbora Stankova, and Ales Zak. 2011. “Fatty Acids as Biocompounds: Their Role in Human Metabolism, Health and Disease--a Review. Part 1: Classification, Dietary Sources and Biological Functions.” *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia* 155(2): 117–30.

Uchida, K, and E R Stadtman. 1992. “Modification of Histidine Residues in Proteins by Reaction with 4-Hydroxynonenal.” *Proceedings of the National Academy of Sciences of the United States of America* 89(10): 4544–48.

Uddin, Monica et al. 2008. “Molecular Evolution of the Cytochrome c Oxidase Subunit 5A Gene in Primates.” *BMC evolutionary biology* 8: 8.

Umemiya, M, and A J Berger. 1995. “Inhibition by Riluzole of Glycinergic Postsynaptic Currents in Rat Hypoglossal Motoneurones.” *British journal of pharmacology* 116(8): 3227–30.

De Urquiza, A M et al. 2000. “Docosahexaenoic Acid, a Ligand for the Retinoid X Receptor in Mouse Brain.” *Science (New York, N.Y.)* 290(5499): 2140–44.

Vaisman, Nachum et al. 2009. “Do Patients with Amyotrophic Lateral Sclerosis (ALS) Have Increased Energy Needs?” *Journal of the neurological sciences* 279(1-2): 26–29.

Veldink, J H et al. 2007. “Intake of Polyunsaturated Fatty Acids and Vitamin E Reduces the Risk of Developing Amyotrophic Lateral Sclerosis.” *Journal of neurology, neurosurgery, and psychiatry* 78(4): 367–71.

Vinsant, Sharon et al. 2013. “Characterization of Early Pathogenesis in the SOD1(G93A) Mouse Model of ALS: Part I, Background and Methods.” *Brain and behavior* 3(4): 335–50.

Voelker, D R. 1991. “Organelle Biogenesis and Intracellular Lipid Transport in Eukaryotes.” *Microbiological reviews* 55(4): 543–60.

Votyakova, T V, and I J Reynolds. 2001. “DeltaPsi(m)-Dependent and -Independent Production of Reactive Oxygen Species by Rat Brain Mitochondria.” *Journal of neurochemistry* 79(2): 266–77.

Wada, Masayuki et al. 2007. “Enzymes and Receptors of Prostaglandin Pathways with Arachidonic Acid-Derived versus Eicosapentaenoic Acid-Derived Substrates and Products.” *The Journal of biological chemistry* 282(31): 22254–66.

Wagey, R, S L Pelech, V Duronio, and C Krieger. 1998. “Phosphatidylinositol 3-Kinase: Increased Activity and Protein Level in Amyotrophic Lateral Sclerosis.” *Journal of neurochemistry* 71(2): 716–22.

Walker, Adam K, and Julie D Atkin. 2011. “Stress Signaling from the Endoplasmic Reticulum: A Central Player in the Pathogenesis of Amyotrophic Lateral Sclerosis.” *IUBMB life* 63(9): 754–63.

Wang, Yun et al. 2006. “Regulation of Hepatic Fatty Acid Elongase and Desaturase Expression in Diabetes and Obesity.” *Journal of lipid research* 47(9): 2028–41.

Watkins, P A. 1997. “Fatty Acid Activation.” *Progress in lipid research* 36(1): 55–83.

Watkins, Paul A. 2008. “Very-Long-Chain Acyl-CoA Synthetases.” *The Journal of biological chemistry* 283(4): 1773–77.

Watkins, Paul A, and Jessica M Ellis. 2012. “Peroxisomal Acyl-CoA Synthetases.” *Biochimica et biophysica acta* 1822(9): 1411–20.

Weber, T et al. 1998. “SNAREpins: Minimal Machinery for Membrane Fusion.” *Cell* 92(6): 759–72.

Whatmore, J et al. 1999. “Resynthesis of Phosphatidylinositol in Permeabilized Neutrophils Following Phospholipase Cbeta Activation: Transport of the Intermediate, Phosphatidic Acid, from the Plasma Membrane to the Endoplasmic Reticulum for Phosphatidylinositol Resynthesis Is Not .” *The Biochemical journal* 341 (Pt 2: 435–44.

White, Ursula A, and Yourka D Tchoukalova. 2014. “Sex Dimorphism and Depot Differences in Adipose Tissue Function.” *Biochimica et biophysica acta* 1842(3): 377–92.

Wills, Anne-Marie et al. 2014. “Hypercaloric Enteral Nutrition in Patients with Amyotrophic Lateral Sclerosis: A Randomised, Double-Blind, Placebo-Controlled Phase 2 Trial.” *Lancet* 383(9934): 2065–72.

Winkler, Ethan A et al. 2013. “Blood-Spinal Cord Barrier Breakdown and Pericyte Reductions in Amyotrophic Lateral Sclerosis.” *Acta neuropathologica* 125(1): 111–20.

Wisely, G Bruce et al. 2002. “Hepatocyte Nuclear Factor 4 Is a Transcription Factor That Constitutively Binds Fatty Acids.” *Structure (London, England : 1993)* 10(9): 1225–34.

Wissing, D et al. 1997. “Involvement of Caspase-Dependent Activation of Cytosolic Phospholipase A2 in Tumor Necrosis Factor-Induced Apoptosis.” *Proceedings of the National Academy of Sciences of the United States of America* 94(10): 5073–77.

Wu, Chu-Chiao, and Shawn B Bratton. 2013. “Regulation of the Intrinsic Apoptosis Pathway by Reactive Oxygen Species.” *Antioxidants & redox signaling* 19(6): 546–58.

Xiao, Xu, and Bao-Liang Song. 2013. “SREBP: A Novel Therapeutic Target.” *Acta biochimica et biophysica Sinica* 45(1): 2–10.

Xu, H E et al. 1999. “Molecular Recognition of Fatty Acids by Peroxisome Proliferator-Activated Receptors.” *Molecular cell* 3(3): 397–403.

Xu, J, M T Nakamura, H P Cho, and S D Clarke. 1999. “Sterol Regulatory Element Binding Protein-1 Expression Is Suppressed by Dietary Polyunsaturated Fatty Acids. A Mechanism for the Coordinate Suppression of Lipogenic Genes by Polyunsaturated Fats.” *The Journal of biological chemistry* 274(33): 23577–83.

Xu, Jianfeng, Sue Yu, Albert Y Sun, and Grace Y Sun. 2003. “Oxidant-Mediated AA Release from Astrocytes Involves cPLA(2) and iPLA(2).” *Free radical biology & medicine* 34(12): 1531–43.

Xu, Min-Xuan et al. 2013. “Resolvin D1, an Endogenous Lipid Mediator for Inactivation of Inflammation-Related Signaling Pathways in Microglial Cells, Prevents Lipopolysaccharide-Induced Inflammatory Responses.” *CNS neuroscience & therapeutics* 19(4): 235–43.

Xu, Yi et al. 2014. “Free Radical Derivatives Formed from Cyclooxygenase-Catalyzed Dihomo-Γ-Linolenic Acid Peroxidation Can Attenuate Colon Cancer Cell Growth and Enhance 5-Fluorouracil′s Cytotoxicity.” *Redox biology* 2: 610–18.

Yates, Clara M et al. 2011. “Docosahexaenoic Acid Inhibits the Adhesion of Flowing Neutrophils to Cytokine Stimulated Human Umbilical Vein Endothelial Cells.” *The Journal of nutrition* 141(7): 1331–34.

Yavin, Ephraim, Annette Brand, and Pnina Green. 2002. “Docosahexaenoic Acid Abundance in the Brain: A Biodevice to Combat Oxidative Stress.” *Nutritional neuroscience* 5(3): 149–57.

Yedgar, S, D Lichtenberg, and E Schnitzer. 2000. “Inhibition of Phospholipase A(2) as a Therapeutic Target.” *Biochimica et biophysica acta* 1488(1-2): 182–87.

Yin, Huiyong et al. 2007. “Identification of Novel Autoxidation Products of the Omega-3 Fatty Acid Eicosapentaenoic Acid in Vitro and in Vivo.” *The Journal of biological chemistry* 282(41): 29890–901.

Yin, Huiyong, Jason D Morrow, and Ned A Porter. 2004. “Identification of a Novel Class of Endoperoxides from Arachidonate Autoxidation.” *The Journal of biological chemistry* 279(5): 3766–76.

Yip, P K et al. 2013. “The Omega-3 Fatty Acid Eicosapentaenoic Acid Accelerates Disease Progression in a Model of Amyotrophic Lateral Sclerosis.” *PloS one* 8(4): e61626.

Yip, Ping K et al. 2013. “The Omega-3 Fatty Acid Eicosapentaenoic Acid Accelerates Disease Progression in a Model of Amyotrophic Lateral Sclerosis.” *PloS one* 8(4): e61626.

Yog, Rajeshwari, Rola Barhoumi, David N McMurray, and Robert S Chapkin. 2010. “N-3 Polyunsaturated Fatty Acids Suppress Mitochondrial Translocation to the Immunologic Synapse and Modulate Calcium Signaling in T Cells.” *Journal of immunology (Baltimore, Md. : 1950)* 184(10): 5865–73.

Yu, Michelle A et al. 2011. “Radical Formation in Cytochrome c Oxidase.” *Biochimica et biophysica acta* 1807(10): 1295–1304.

Zaldivar, T et al. 2009. “Reduced Frequency of ALS in an Ethnically Mixed Population: A Population-Based Mortality Study.” *Neurology* 72(19): 1640–45.

Zerangue, N, J L Arriza, S G Amara, and M P Kavanaugh. 1995. “Differential Modulation of Human Glutamate Transporter Subtypes by Arachidonic Acid.” *The Journal of biological chemistry* 270(12): 6433–35. h

Zhai, Jianjun et al. 2009. “Proteomic Characterization of Lipid Raft Proteins in Amyotrophic Lateral Sclerosis Mouse Spinal Cord.” *The FEBS journal* 276(12): 3308–23.

Zhang, Hai-Xin et al. 2010. “Alteration of Biochemical and Pathological Properties of TDP-43 Protein by a Lipid Mediator, 15-Deoxy-Delta(12,14)-Prostaglandin J(2).” *Experimental neurology* 222(2): 296–303.

Zhang, Kunxi et al. 2013. “Food Restriction-Induced Autophagy Modulates Degradation of Mutant SOD1 in an Amyotrophic Lateral Sclerosis Mouse Model.” *Brain research* 1519: 112–19.

Zhang, Wenting et al. 2011. “Omega-3 Polyunsaturated Fatty Acids in the Brain: Metabolism and Neuroprotection.” *Frontiers in bioscience (Landmark edition)* 16: 2653–70.

Zhang, Xiaojie et al. 2014. “MTOR-Independent, Autophagic Enhancer Trehalose Prolongs Motor Neuron Survival and Ameliorates the Autophagic Flux Defect in a Mouse Model of Amyotrophic Lateral Sclerosis.” *Autophagy* 10(4).

Zhao, Wei et al. 2012. “Caprylic Triglyceride as a Novel Therapeutic Approach to Effectively Improve the Performance and Attenuate the Symptoms due to the Motor Neuron Loss in ALS Disease.” *PloS one* 7(11): e49191.

Zhao, Yuhai et al. 2011. “Docosahexaenoic Acid-Derived Neuroprotectin D1 Induces Neuronal Survival via Secretase- and PPARγ-Mediated Mechanisms in Alzheimer’s Disease Models.” *PloS one* 6(1): e15816.

Zheng, Jun et al. 2011. “Latanoprost Promotes Neurite Outgrowth in Differentiated RGC-5 Cells via the PI3K-Akt-mTOR Signaling Pathway.” *Cellular and molecular neurobiology* 31(4): 597–604. h

8. Annexes

8. Annexes

For further immersion on PUFA's relevance and implications in different phatophysiological conditions (e.g. neurodegeneration and dietary interventions in chronic inflammatory models), we harvested relevant contributions for a better understanding of some hallmarks of oxidative reactions and its relevance in various neurodegenerative diseases in a paper entitled "*Maillard reaction versus other nonenzymatic modifications in neurodegenerative processes*", endorsed as an annex at the end of this book.

Furthermore, to increase the knowledge of PUFA's influence in various biological parameters (e.g. oxidative status, synthesis machinery, CHO status) in animals under a distorted diet (high fat diet). Various experiments were performed along my stay at the University of Bergen and were successfully published in "*Fish oil and 3-thia fatty acid have additive effects on lipid metabolism but antagonistic effects on oxidative damage when fed to rats for 50 weeks*" and could be also found below.

Finally, for a more comprehensive view of the pathophisiology of ALS, different models (cell lines confronted to oxidative and endoplasmic reticulum stressors and chronic exitotoxicity in OT spinal cord primary culture) as well as sALS samples were investigated trying to depict a new activation route which connects MN degeneration and TDP-43, together with and ER and oxidative stress and an unreported role calpain and CaMKIV along disease progression as relevant factors in ALS. The experiments performed were successfully published in "*Cell stress induces TDP-43 pathological changes associated with ERK1/2 dysfunction: implications in ALS*", "*Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis*" and "*Calpain activation and CaMKIV reduction in spinal cords from hSOD1G93A mouse model"* and could be found in the annex section.

Maillard Reaction versus Other Nonenzymatic Modifications in Neurodegenerative Processes

Reinald Pamplona, Ekaterina Ilieva, Victoria Ayala, Maria Josep Bellmunt, Daniel Cacabelos, Esther Dalfo, Isidre Ferrer, and Manuel Portero-Otin

Department of Experimental Medicine, School of Medicine, University of Lleida-IRBLLEIDA, Lleida 25008, Spain

Nonenzymatic protein modifications are generated from direct oxidation of amino acid side chains and from reaction of the nucleophilic side chains of specific amino acids with reactive carbonyl species. These reactions give rise to specific markers that have been analyzed in different neurodegenerative diseases sharing protein aggregation, such as Alzheimer's disease, Pick's disease, Parkinson's disease, dementia with Lewy bodies, Creutzfeldt-Jakob disease, and amyotrophic lateral sclerosis. Collectively, available data demonstrate that oxidative stress homeostasis, mitochondrial function, and energy metabolism are key factors in determining the disease-specific pattern of protein molecular damage. In addition, these findings suggest the lack of a "gold marker of oxidative stress," and, consequently, they strengthen the need for a molecular dissection of the nonenzymatic reactions underlying neurodegenerative processes.

Key words: **advanced glycation end products; advanced lipoxidation end products; Alzheimer's disease; amyotrophic lateral sclerosis; Creutzfeldt-Jakob disease; energy metabolism; free radicals; mitochondria; oxidative stress; Parkinson's disease; Pick's disease; reactive carbonyl species**

Nonenzymatic Oxidative Protein Modification

As a rule, chemical reactions in living cells are under strict enzymatic control and conform to a tightly regulated metabolic program. One important factor implicit in evolution, from a biomolecular view, is the minimizing of unwanted side reactions. Nevertheless, uncontrolled and potentially deleterious reactions occur, even under physiological conditions. Free radicals (reactive oxygen species [ROS] and reactive nitrogen species [RNS]) are generated by both enzymatic and nonenzymatic sources and have been implicated in a multitude of physiological processes including aging and disease initiation and/or progression.[1] Oxidative stress occurs when the net flux of free radical production during normal aerobic metabolism exceeds the antioxidant defenses of the cell. Emerging evidence indicates that this stress causes specific protein modifications that may lead to a change in the structure and/or function of the oxidized protein.[2,3] Carbonylation is one of those changes, altering the conformation of the polypeptide chain and determining the partial or total inactivation of proteins. This can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage.

Structurally, carbonylation may arise from direct oxidation of amino acid side chains, mainly Pro, Arg, Lys, and Thr, resulting in the formation, among others, of glutamic semialdehyde (GSA) and aminoadipic semialdehyde (AASA), the main carbonyl products of metal-catalyzed oxidation of proteins. In addition, carbonyl groups may be introduced into proteins by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues with reactive carbonyl species (RCS) produced during lipid peroxidation (lipoxidation reactions) or generated as a consequence of the reaction with highly reducing sugars, such as glyoxal and methylglyoxal (an usual byproduct of glycolysis) or their oxidation products (glycation and glycoxidation reactions). Most of the biological effects of intermediate RCS are attributed to their capacity to react with the nucleophilic sites of proteins, forming advanced lipoxidation end products (ALEs) and advanced glycation end products (AGEs).[2] Compared to free radicals, RCS are stable and can diffuse within or even escape from the cell and attack targets far from the site of formation. Therefore, these soluble reactive intermediates are not only cytotoxic per se but

Address for correspondence: Manuel Portero-Otin, Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida-IRBLLEIDA; Carrer Montserrat Roig, 2; 25008 Lleida, Spain. Voice: +34-973702408; fax: +34-973702426.

reinald.pamplona@cmb.udl.cat, manuel.portero@cmb.udl.cat

Ann. N.Y. Acad. Sci. 1126: 315–319 (2008).
doi: 10.1196/annals.1433.014

also behave as mediators and propagators of oxidative stress and tissue damage, acting as second cytotoxic messengers.[4]

Oxidative decomposition of polyunsaturated fatty acids (PUFAs) initiates chain reactions that lead to the formation of a variety of RCS which, by reacting with nucleophilic sites in proteins, generate specific ALEs, such as MDA-Lys, HNE-Lys, and Nε-(hexanoyl)lysine, among others. These adducts have been detected by chemical and immunohistochemical methods in a broad range of tissues and species during physiological aging[4] and specific pathological states. The involvement of toxic RCS as products and propagators of oxidative damage in neurodegenerative diseases is currently under study.

The residual aldehyde group in some ALEs can further react to give protein cross-links and fluorescent products that are very similar to AGEs. Lipofuscin, the nondegradable intralysosomal fluorescent pigment that accumulates with age in postmitotic cells, is a recognized hallmark of aging.[4] Other important toxic products formed during nonenzymatic modification of proteins in aging and disease are referred to as "either advanced glycation or lipoxidation end products," so named because they may be formed from either carbohydrates or lipids. *N*ε-(carboxymethyl)lysine (CML) and *N*ε-(carboxyethyl)lysine (CEL) are, on a molar basis, the major modifications that have been measured in tissue proteins among these mixed-origin products, emphasizing the importance of the intersection between carbohydrate and lipid chemistry.[2]

Protein Damage and Neurodegenerative Diseases

The nervous system is potentially sensitive to oxidative modifications because i) the particular fatty acid composition of neuronal tissues that is rich in PUFA (hence easily peroxidizable); ii) the high O_2 consumption; and iii) the relatively poor expression antioxidant systems.[5] Also, nervous tissue is considered a postmitotic tissue and therefore highly susceptible to aging.[6] Cells in all regions of the nervous system are affected by aging, as indicated by the decline of sensory, motor, and cognitive functions with time. As this process is involved as a risk factor in most neurodegenerative diseases—there is a dramatic increase in the probability of developing a neurodegenerative disorder (e.g., Alzheimer's disease [AD], Parkinson's disease [PD], or amyotrophic lateral sclerosis [ALS], among others) during the sixth, seventh, and eighth decades of life—and oxidative modifications play a key role in aging, it is often accepted that these diseases should have increased oxidative damage.

Cells in the nervous system are affected by, and respond to, aging much as cells in other organ systems do, and so cells in the brain experience increased amounts of oxidative stress, impaired mitochondrial function and perturbed energy homeostasis, accumulation of damaged proteins, and lesions in their nucleic acids.[5,6] These changes during normal aging are exacerbated in vulnerable populations of neurons in neurodegenerative disorders. Therefore, some diseases might be viewed as a syndrome of accelerated aging in selected neurons.

The interest in the molecular dissection of each of these three pathways (i.e., direct oxidative modification, glycoxidation, and lipoxidation) clearly exceeds an academic context. An appropriate knowledge in this sense could help to rationally design therapeutic approaches aimed either at diminishing oxidative damage in a nonselective way (provided each oxidative pathway is increased in a similar extent) or at pinpointing those processes selectively increased. With this goal in mind, tissues from human tauopathies (AD and Pick's disease [PiD]), synucleopathies (PD and dementia with Lewy bodies [DLB]), and other neurodegenerative processes linked to protein misfolding and/or deposits (Creutzfeldt-Jakob disease [CJD] and ALS) were studied (TABLE 1). The concentration of selected markers of each pathway of protein oxidative damage was analyzed by gas chromatography coupled to mass spectrometry by using authentic deuterated internal standards, according to previously described procedures.[7] Tissues located in "target" zones of the diseases (showing pathological abnormalities) and "control" zones (without morphological changes) were evaluated in order to offer a biochemical correlate of the disease (TABLE 1). These diseases were chosen by the fact that all share accumulation or involvement of structurally modified protein deposits, which have been detected as modified with oxidation products using immunohistochemical procedures.

Tauopathies: Alzheimer's Disease and Pick's Disease

Alzheimer's disease is the more studied and prevalent neurodegenerative disease and is associated with β-amyloid deposits either in neurofibrillary tangles or hyaline bodies. The measurement of oxidative protein modifications[7] reveals that GSA and AASA contents are higher in the frontal cortex (area 8) of AD patients than in age- and sex-matched healthy controls. These increases (around 50% over control values) are larger than those present for well-known

TABLE 1. Changes in specific markers from oxidation-, glycoxidation-, and lipoxidation-derived reactions in human neurodegenerative diseases: a comparative molecular pathology approach

Disease	Number of cases	Location	GSA	AASA	CEL	CML	PI	DHA	MDAL
AD[7]	13	FC	↑	↑↑	↑	↑	↑	↑	↑↑
PiD[8] (1)	7	FC	↑	↑↑	↓	↓	↓	↓	↑
	7	OC	↑	↑	↑	↑	↑	↑	↑↑
PD[10]	7	SN	=	=	=	=	↓	↓	↑
	7	FC	=	=	↓	=	↑	↑↑	↑↑
DLB[10]	4	FC	=	=	↓	=	↑	↑	=
CJD (2)	10	FC	↑	↑	↑	↑	↓	↓	↑
ALS[12]	11	SC	↑	↑	↑	↑	↓	↓↓	↑↑
	11	FC	↑	↑	↑	↑	↑	↑	↑

Abbreviations: AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; CJD, Creutzfeldt-Jakob disease; DLB, dementia with Lewy bodies; PD, Parkinson's disease; PiD, Pick's disease. Location: FC, frontal cortex; OC, occipital cortex; SC, spinal cord; SN, substantia nigra. (1) unpublished results; (2) Pamplona, Naudi, Gavin, *et al.*, University of Lleída, Lleída Spain AASA, aminoadipic semialdehyde; CEL, *N*ε-(carboxyethyl)lysine; CML, *N*ε-(carboxymethyl)lysine; DHA, docosahexaenoic acid; GSA, glutamic semialdehyde; MDAL, Nε-(malondialdehyde)lysine; PI, peroxidizability index.

AGE markers (CEL and CML), which roughly increase 15% over control values. As suggested by changes in peroxidizability, which is significantly increased in these AD patients (basically because of changed contents of docosahexaenoic acid [DHA]), the most affected marker of protein oxidative modification is Nε-(malondialdehyde)lysine (MDAL), which doubles its content in samples from AD patients. By using a combination of two-dimensional electrophoresis, Western blot, and peptide fingerprinting with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), several cytoesqueletal proteins, metabolic enzymes, and heat shock proteins were identified as modified by MDAL.[7]

To ascertain whether those phenomena are specific to AD, we analyzed PiD (unpublished results and Ref. 8). PiD is another tauopathy, characterized by the specific involvement of the frontotemporal cortex. In common with AD, significant increases were found in AASA, GSA, and MDAL, suggesting increased direct oxidative and lipoxidative damage, although at a lower extent than in AD. However, concentrations of lipoxidative and glycoxidative protein modifications were decreased (both CEL and CML), a fact that can be related to the loss of glycolytic potential, well described in this disease.

The occipital cortex is usually viewed as a location without morphological evidence of involvement of the disease, and hence morphological evidence serves as controls for measurements. In this case, we evidenced increased oxidative, glycoxidative, and lipoxidative damage in this location, supporting the fact that oxidative stress may be an early-stage change in the pathogenesis of this disease. Most interestingly, there was a direct and significant correlation between CEL concentration and DHA levels, suggesting that DHA is increased in response to neuronal stress. This would involve both oxidative stress and increased glycolysis, leading to increased CEL through potentially increased methylglyoxal efflux in the occipital cortex, whereas in the frontal cortex, because of neuronal loss and consequent decreased glycolysis, decreased values in both DHA and CEL content are present. Concerning the targets of oxidative damage, five different proteins exhibit increased anti-DNP staining: reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) carbonyl-reductase, glial fibrillary acidic protein, heat shock protein 70, cathepsin D precursor, and vesicle-fusing ATPase (unpublished results).

Synucleopathies: Parkinson's Disease and Dementia with Lewy Bodies

Parkinson's disease is the most prevalent synucleopathy. Despite previous results describing increased immunoreactivity to anti-AGE and oxidative damage adducts,[9] no chemical evidence of protein oxidative damage was available. When evaluating the content of the above-mentioned markers in substantia nigra from incidental DLB,[10] MDAL was the only marker that was significantly increased in PD (approximately 100%). Similar increases were also found in the frontal cortex and amygdala, suggesting the importance of lipoxidation. In clear contrast, CEL levels were significantly decreased in both the amygdala and the frontal cortex, a fact that can be in accordance with a described loss of glycolysis in PD.[11] Targets of lipoxidative damage comprise several antioxidant enzymes, proteasome components, α-synuclein, and other proteins not shared with AD or PiD. To shed further light

on the potential relationship of oxidative damage in synucleopathies, we analyzed cortex samples from patients with DLB, which showed a lack of increased MDAL content but, again, significant decreases in CEL content.[10]

Other Neurodegenerative Processes Linked to Protein Misfolding and/or Deposits

Creutzfeldt-Jakob disease is a neurodegenerative spongiform disease, linked to transmissible prionopathies. The protein oxidative profile in the frontal cortex shows similarities to AD: increased direct oxidative, glycoxidative, and lipoxidative damage, but in this case a decrease in n-3 fatty acids is present. (Pamplona, Naudi, Gavin, *et al*., University of Lleída, Lleída, Spain) All increases are in the same range, suggesting a general change in the modified protein turnover. This would be compatible with reported alterations in proteasome present in related prion-induced diseases. Concerning targets of glycoxidative damage, we evidenced two key enzymes in glycolysis to be highly modified in CJD samples: glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphate aldolase. As the activity of this latter enzyme involves the formation of a Schiff's base in its active site, we hypothesize that some of those bases may be transformed, under increased oxidative conditions, to CEL.

Amyotrophic lateral sclerosis is characterized by the selective loss of motor neurons in spinal cord and in brain cortex, associated with highly ubiquitinated deposits of proteinaceous material. Spinal cord lysates from ALS patients showed significant increases in direct oxidative, glycoxidative, and lipoxidative damage.[12] Analogous to samples from the brain cortex in AD, the more sensible marker was MDAL, suggesting the importance of lipoxidative modification in this context. Similar to substantia nigra samples in PD and frontal cortex samples in PiD, lipoxidative damage was accompanied by a strong loss in the content of DHA. As observed from other locations without morphological evidence of pathology, samples from the brain cortex of ALS patients also showed increased oxidation, glycoxidation, and lipoxidation, associated with reactive increases in the content of n-3 fatty acids, particularly DHA. All these features are reproducible *in vitro* by the generation of chronic excitotoxicity—a mechanism linked to selective neuronal loss by disturbed intracellular Ca^{++} homeostasis—in a spinal cord organotypic culture, supporting the involvement of this neurodegenerative pathway *in vivo*.[12]

Final Remarks

In conclusion to these analyses, no single marker of protein oxidative modification (among those used here) can be viewed as a gold standard for assessment of oxidative damage in neurodegenerative processes. The same applies to targets of oxidative damage that show disorder-specific differences. Moreover, in some cases CEL levels decreased, supporting CEL relationship with glycolysis potential. There are significant associations between changes in fatty acid composition (especially DHA) and protein oxidative damage. Most interestingly, when tissue from pathologically preserved locations was available, the tissue analyses indicated that protein oxidative modifications take place before potential morphological and clinical changes appear, suggesting an early involvement of protein oxidative damage in neurodegenerative process.

Acknowledgments

This study was supported in part by I+D grants from the Spanish Ministry of Education and Science (BFU2006-14495/BFI), the Spanish Ministry of Health (ISCIII, Red de Envejecimiento y Fragilidad, RD06/0013/0012), and the Generalitat of Catalunya (2005SGR00101) to R.P.; the Spanish Ministry of Health (FIS 04-0355 and 05-2241), Spanish Ministry of Education and Science (AGL2006-12433), and "La Caixa" Foundation to M.P.O. and E.D., E.I. and D.C.; and Field-initiated Studies Program grants 020004 and 03-006, Spanish Ministry of Education and Science (CICYT SAF-2001-4681E), and European Union project Brain Net Europe II to I.F.

Conflict of Interest

The authors declare no conflicts of interest.

References

1. Sanz, A., R. Pamplona & G. Barja. 2006. Is the mitochondrial free radical theory of aging intact? Antioxid. Redox Signal. **8:** 582–599.
2. Thorpe, S.R. & J.W. Baynes. 2003. Maillard reaction products in tissue proteins: new products and new perspectives. Amino Acids **25:** 275–281.
3. Portero-Otin, M. & R. Pamplona. 2006. Is endogenous oxidative protein damage envolved in the aging process?. *In* Recent Research Developments in Pathological Biochemistry. Vol. 1 Protein Oxidation and Disease. J. Pietzsch, Ed.: 91–142. Research Signpost. Kerala, India.

4. HULBERT, A.J., R. PAMPLONA, R. BUFFENSTEIN & W.A. BUTTEMER. 2007. Life and death: metabolic rate, membrane composition and life span of animals. Physiol. Rev. doi: 10.1152/physrev.00047.2006.
5. MATTSON, M.P. & T. MAGNUS. 2006. Ageing and neuronal vulnerability. Nat. Rev. Neurosci. **7:** 278–294.
6. BARJA, G. 2004. Free radicals and aging. Trends Neurosci. **27:** 595–600.
7. PAMPLONA, R., E. DALFO, V. AYALA, *et al*. 2005. Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation: Effects of Alzheimer's disease and identification of lipoxidation targets. J. Biol. Chem. **280:** 21522–21530.
8. MUNTANE, G., E. DALFO, A. MARTINEZ, *et al*. 2006. Glial fibrillary acidic protein is a major target of glycoxidative and lipoxidative damage in Pick's disease. J. Neurochem. **99:** 177–185.
9. CASTELLANI, R., M.A. SMITH, P.L. RICHEY, *et al.* 1996. Glycoxidation and oxidative stress in Parkinson disease and diffuse Lewy body disease. Brain Res. **737:** 195–200.
10. DALFO, E., M. PORTERO-OTIN, V. AYALA, *et al*. 2005. Evidence of oxidative stress in the neocortex in incidental Lewy body disease. J. Neuropathol. Exp. Neurol. **64:** 816–830.
11. HU, M.T., S.D. TAYOR-ROBINSON, K.R. CHAUDHURI, *et al.* 2000. Cortical dys function in non-demented Parkinson's disease patients: a combined (31) P-MRS and (18) FDG-PET study. Brain **123:** 340–352.
12. ILIEVA, E., V. AYALA, M. JOVE, *et al*. 2007. Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. Brain, doi: 10.1093/brain/awm190.

Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 1384–1393

Journal of Nutritional Biochemistry

Fish oil and 3-thia fatty acid have additive effects on lipid metabolism but antagonistic effects on oxidative damage when fed to rats for 50 weeks☆

Natalya Filipchuk Vigerust[a,1], Daniel Cacabelos[b,1], Lena Burri[a], Kjetil Berge[a], Hege Wergedahl[c], Bjørn Christensen[d,e], Manuel Portero-Otin[b], Asgaut Viste[d,e], Reinald Pamplona[b], Rolf Kristian Berge[a,f,*], Bodil Bjørndal[a]

[a]Institute of Medicine, University of Bergen, N-5021 Bergen, Norway
[b]Department of Experimental Medicine, University of Lleida, E25008 Lleida, Spain
[c]Faculty of Education, Section for food and health, Bergen University College, N-5020 Bergen, Norway
[d]Department of Surgery, Haukeland University Hospital, N-5021 Bergen, Norway
[e]Department of Surgical Sciences, University of Bergen, N-5021 Bergen, Norway
[f]Department of Heart Disease, Haukeland University Hospital, N-5021 Bergen, Norway

Received 23 February 2011; received in revised form 5 August 2011; accepted 18 August 2011

Abstract

The 3-thia fatty acid tetradecylthioacetic acid (TTA) is a synthetic modified fatty acid, which, similar with dietary fish oil (FO), influences the regulation of lipid metabolism, the inflammatory response and redox status. This study was aimed to penetrate the difference in TTA's mode of action compared to FO in a long-term experiment (50 weeks of feeding). Male Wistar rats were fed a control, high-fat (25% w/v) diet or a high-fat diet supplemented with either TTA (0.375% w/v) or FO (10% w/v) or their combination. Plasma fatty acid composition, hepatic lipids and expression of relevant genes in the liver and biomarkers of oxidative damage to protein were assessed at the end point of the experiment. Both supplements given in combination demonstrated an additive effect on the decrease in plasma cholesterol levels. The FO diet alone led to removal of plasma cholesterol and a concurrent cholesterol accumulation in liver; however, with TTA cotreatment, the hepatic cholesterol level was significantly reduced. Dietary FO supplementation led to an increased oxidative damage, as seen by biomarkers of protein oxidation and lipoxidation. Tetradecylthioacetic acid administration reduced the levels of these biomarkers confirming its protective role against lipoxidation and protein oxidative damage. Our findings explore the lipid reducing effects of TTA and FO and demonstrate that these bioactive dietary compounds might act in a different manner. The experiment confirms the antioxidant capacity of TTA, showing an improvement in FO-induced oxidative stress.

Keywords: Reactive oxygen species; Oxidative damage; UCP; β-oxidation

Abbreviations: AASA, α-aminoadipic semialdehyde; *Acaca*, acetyl-coenzyme A carboxylase α; *Acadm*, acyl-coenzyme A dehydrogenase, medium chain; *Acadvl*, acyl-coenzyme A dehydrogenase, very long chain; ACOX1, acyl-CoA oxidase 1; *Arbp*, acidic ribosomal protein, P0; CPT, carnitine palmitoyltransferase; CEL, *N*ε-carboxyethyl-lysine; CML, *N*ε-carboxymethyl-lysine; DBI, double-bond index; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; GSA, γ-glutamic semialdehyde; *Hadha*, hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α-subunit; MDAL, *N*ε-malondialdehyde-lysine; PL, phospholipids; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TTA, tetradecylthioacetic acid; UCP, uncoupling protein.

☆ Grants and funding: This study was financially supported by grants from NordForsk, grant 070010, MitoHealth; the Research Council of Norway, grant 190287/110; and the European Community's Seventh Framework Program (FP7/2007-2013), grant 201668, AtheroRemo. Work carried out at the Department of Experimental Medicine was supported in part by R+D grants from the Spanish Ministry of Science and Innovation (AGL2006-12433 and BFU2009-11879/BFI), the Spanish Ministry of Health (RD06/0013/0012 and PI081843), the Autonomous Government of Catalonia (2009SGR735), and COST B35 Action of the European Union. D.C. received a fellowship from the Spanish Health Institute Carlos III. The authors declare no conflict of interest.

* Corresponding author. University of Bergen, Institute of Medicine, N-5021 Bergen, Norway. Tel.: +47 55973098; fax: +47 55973115.
E-mail address: rolf.berge@med.uib.no (R.K. Berge).

[1] Equal contribution.

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.08.006

1. Introduction

The body's inability to handle excess energy intake leads to metabolic abnormalities such as hyperglycemia and dyslipidemia. The coexistence of these risk factors with overweight and hypertension represents the metabolic syndrome [1,2]. Thus, the discovery and testing of dietary supplements that can improve lipid metabolism and maintain homeostasis, and consequently, prevent the development of risk factors of metabolic syndrome, is of great importance.

Both fish oil (FO) and tetradecylthioacetic acid (TTA) have been widely used in *in vivo* experiments. FO has been shown to inhibit lipogenesis and exert a hypolipidemic effect by lowering plasma cholesterol and triacylglycerol (TAG) levels [3], as well as having an anti-inflammatory effect beneficial against both atherosclerosis [4] and arthritis [5,6]. The effects of FO are attributed to ω−3 polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are thought to bind and activate peroxisome proliferator-activated receptors (PPARs) [7]. They act upon ligand activation by controlling networks of target genes and therefore serve as lipid sensors because they can be triggered by metabolic derivates of fatty acids in the body.

Tetradecylthioacetic acid, a structurally modified 16-carbon saturated fatty acid (SFA) with a sulfur atom inserted in the third position of the alkyl chain, has been documented as a pan-PPAR ligand in several cell lines [8–10]. Tetradecylthioacetic acid has a particularly high affinity for PPARα [9–11] and exerts its hypolipidemic effect by inducing gene expression of enzymes involved in hepatic fatty acid β-oxidation. It thereby reduces the availability of fatty acids for very-low-density-lipoprotein synthesis and secretion and lowers plasma TAG and cholesterol levels. In addition, TTA has been shown to have an important role in diminishing of inflammation [12–16].

Both physiological processes and externally induced oxidative stress reactions can lead to formation of reactive oxygen species (ROS) in the body. It is well-known that dietary FO supplementation can increase lipoxidative damage due to the high amount of unsaturated fatty acids [17–19]. Tetradecylthioacetic acid has a potent capacity to attenuate the oxidative stress and protect the cellular membrane lipids from oxidative damage. Based on this, we hypothesized that a dietary TTA supplementation to the high-fat diet would be able to reduce the oxidative stress induced by FO.

Thus, the main focus in this 50-week-long *in vivo* experiment was to investigate the effects of TTA and FO, given separately or in combination, on body weight gain, plasma and liver lipid levels, as well as fatty acid composition in plasma and several genes encoding important enzymes involved in fatty acid metabolism. Further, despite the similar ability to act through PPAR-activation, we revealed principal differences in the mechanisms of action of TTA and FO. Finally, we confirmed that TTA can act as an antioxidant and prevent the FO-caused oxidative damage.

2. Methods

2.1. Animals and diets

This animal study was conducted according to the Guidelines for the Care and Use of Experimental Animals, and the protocol was approved by the Norwegian State Board of Biological Experiments with Living Animals. Eight- to 10-weeks-old male Wistar rats, weighing 200–250 g, were obtained from Taconic Europe (previously Möllegaard and Bomholtgaard, Ry, Denmark). Throughout the experiment, the rats were housed in Makrolon III cages in an open system and kept under standard laboratory conditions with temperature 22°C±1°C, dark/light cycles of 12/12 h, relative humidity 55%±5% and 20 air changes per hour. The animals were housed five per cage and had free access to food and water during the study. They were acclimatized under these conditions with standard chow for 1 week before the experiment started. All rats were divided into four groups. The first group of animals (control group) was fed a high-fat diet with 25% fat, consisting of 23% lard and 2% soybean oil. The second group (TTA group) was fed a high-fat diet supplemented with TTA (0.375%). The third group (FO group) was fed a diet supplemented with 10% FO (EPAX 4020 TG) (12.6% lard and 2% soy oil). The fourth group (TTA+FO group) was fed both diet supplements. The amount of ω−3 fatty acids in FO-containing diets was 8% (where the EPA content was 4.5% and DHA was 2.3%). All diets were isocaloric in their energetic value (4900 kcal).

The animals were part of a larger study described elsewhere, and all animals underwent a jejunogastric reflux surgical procedure (manuscript in preparation). An additional 2-month feeding experiment on male Wistar rats with and without surgery was performed to determine if the procedure had an effect on the nutritional state of the animals. There was no difference in body weight or plasma lipids between the groups (data not shown), and thus it could be assumed that the surgery had no adverse effect on nutritional uptake.

Tetradecylthioacetic acid was synthesized as previously described [20]. The rats were anaesthetized with isofluorane (Forane; Abbott Laboratories, Abbott Park, IL) inhalation under nonfasting conditions. Blood was drawn by cardiac puncture and collected in BD Vacutainer tubes containing EDTA (Becton-Dickinson, Plymouth, UK), and the organs were immediately removed and frozen in liquid nitrogen.

2.2. Quantification of lipids and fatty acids

Plasma and liver lipids were measured on the Hitachi 917 system (Roche Diagnostics, Mannheim, Germany). Quantification of TAGs and total cholesterol in plasma and liver were obtained by using kits from Roche Diagnostics. Choline-containing phospholipids (PLs) in plasma and liver were measured by PAP150 from bioMérieux (Lyon, France). Hepatic lipids were analyzed in cytoplasmic extracts. Lipids from plasma and liver were extracted before the fatty acid composition was analyzed as described previously [21,22].

From the fatty acid profile, the double-bond index (DBI) of lipid susceptibility to oxidative modification [23] was calculated: DBI=[(1×∑mol% monoenoic)+(2×∑mol% dienoic)+(3×∑mol% trienoic)+(4×∑mol% tetraenoic)+(5×∑mol% pentaenoic)+(6×∑mol% hexaenoic)].

2.3. Enzyme activities

Fresh liver tissue samples were homogenized in ice-cold sucrose medium and centrifuged. The resulting three postnuclear fractions, a mitochondrial-enriched fraction (M), a peroxisome-enriched fraction (L) and a cytosolic fraction (S), were isolated as previously described and frozen at −80°C [24]. Then the activities of fatty acyl-CoA oxidase 1 (ACOX1) [25], carnitine palmitoyltransferase II (CPT-II) [26], 3-hydroxy-3-methylglutaryl-coenzyme A synthase (HMG-CoA synthase) [27] and fatty acid synthase (FAS) [28,29] were measured.

2.4. Gene expression analysis

Liver samples were frozen in liquid nitrogen immediately after dissection and stored at −80°C. Total cellular RNA was purified from 20- to 30-mg tissue using RNeasy Mini Kit (Qiagen). RNA was quantified spectrophotometrically (NanoDrop 1000; NanoDrop Technologies, Boston, MA), and the quality was evaluated by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies, Palo Alto, CA). For each sample, 400 ng total RNA was reversely transcribed in 20-μl reactions using Applied Biosystem's High Capacity cDNA Reverse Transcription Kit with RNase inhibitor according to the manufacturer's description. Real-time polymerase chain reaction was performed with custom-made 384-well microfluidic plates [Taq-Man

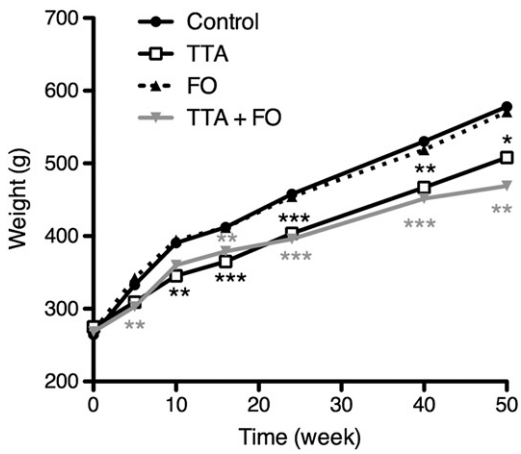


Fig. 1. Average weight of rats on high-fat diets supplemented with 3-thia fatty acid TTA and/or FO for 50 weeks. Data represent means (n=10). Values that were significantly different from control by t test are indicted by black (TTA) or gray (TTA+FO) asterisks (*P<.05, **P<.01, ***P<.001).

Low Density Arrays (TLDA), Applied Biosystems, Foster City, CA]. The genes selected were acyl-coenzyme A dehydrogenase, medium chain (*Acadm*), acyl-coenzyme A dehydrogenase, very long chain (*Acadvl*), carnitine palmitoyltransferase 1A and 2 (*Cpt1a* and *Cpt2*, respectively), acetyl-coenzyme A carboxylase α (*Acaca*), uncoupling protein 2 and 3 (*Ucp2* and *Ucp3*, respectively), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, α-subunit (*Hadha*). All probes and primers were obtained from Applied Biosystems. The reference gene was chosen according to the MIQE guidelines [30,31]. Three different control genes were included: *18s* (Kit-FAM-TAMRA, reference RT-CKFT-18s from Eurogentec, Seraing, Belgium), *Gapdh* (Rodent GAPDH Control Reagents, part 4308313) and *Arbp* (from Applied Biosystems). In a comparative analysis using the programs geNorm and Normfinder, *Arbp* was found to be the best. The expression value of each gene in each sample was normalized against this endogenous control.

2.5. Oxidative damage markers

Glutamic semialdehyde (GSA), aminoadipic semialdehyde (AASA), carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL) and malondialdehyde-lysine (MDAL) concentrations in total proteins from liver homogenates and mitochondrial fraction were measured by gas chromatography/mass spectrometry (GC/MS) as previously described [18]. Samples containing 0.5 mg of protein were delipidated using chloroform/methanol (2:1, v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM $NaBH_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an antifoam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labeled internal standards were then added: [$^2H_8$]lysine (d_8-Lys; CDN Isotopes); [$^2H_4$]CML (d_4-CML), [$^2H_4$]CEL (d_4-CEL) and [$^2H_8$]MDAL (d_8-MDAL), prepared as described [32,33]; [$^2H_5$]5-hydroxy-2-aminovaleric acid (for GSA) and [$^2H_4$]6-hydroxy-2-aminocaproic acid (for AASA) were prepared as described [34]. The samples were hydrolyzed at 155°C for 30 min in 1 ml of 6 HCl and then dried in vacuo. The *N,O*-trifluoroacetyl methyl ester derivatives of the protein hydrolyzate were prepared as previously described [34]. Gas chromatography/mass spectrometry analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30-m HP-5MS capillary column (30 m×0.25 mm×0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at 275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally held at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and nondeuterated standards. Analytes were detected by selected ion monitoring GC/MS. The ions used were lysine and d_8-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and d_5-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and d_4-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and d_4-CML, *m/z* 392 and 396, respectively; CEL and d_4-CEL, *m/z* 379 and 383, respectively; and MDAL and d_8-MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio of micromole of GSA, AASA, CML, CEL, or MDAL/mol of lysine.

2.6. Statistical analysis and presentation of data

The results are presented as mean values with their standard deviations for 7 to 10 rats per group. The data were evaluated by one-way ANOVA and Tukey test or by unpaired Student *t* test with the level of statistical significance set at $P<.05$ (GraphPad Prism version 4.01; GraphPad, San Diego, CA). Single outliers (significance level >0.05) were removed using Grubb test (www.Graphpad.com).

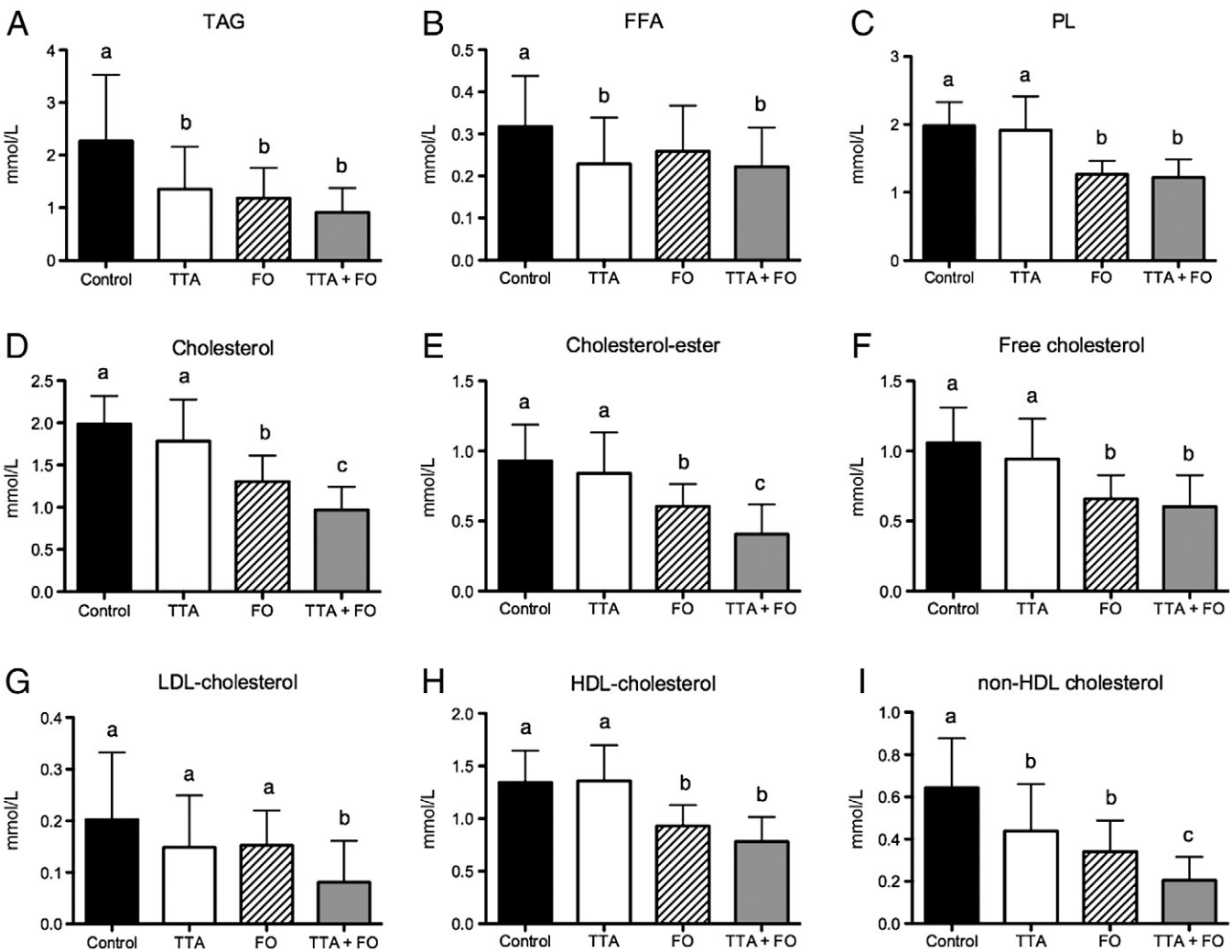


Fig. 2. Plasma lipid concentrations in male Wistar rats after 50 weeks of high-fat diets supplemented with 3-thia fatty acid TTA or FO. Values are means±S.D. (n=31–38). Results were analyzed by ANOVA, and means with the same letter are not significantly different from each other (Tukey–Kramer test, $P<.05$).

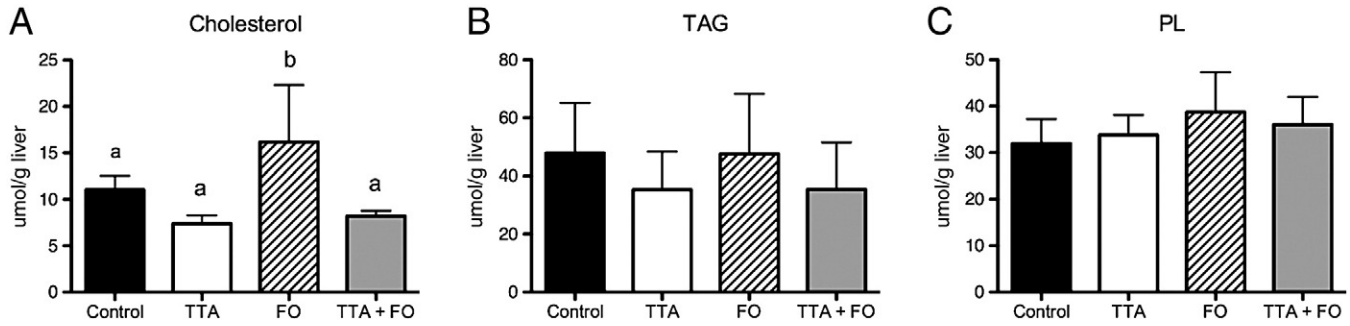


Fig. 3. Liver lipid concentrations in male Wistar rats after 50 weeks of high-fat diets supplemented with 3-thia fatty acid TTA or FO. Values are means±S.D. (n=10–11). Results were analyzed by ANOVA and means with the same letter are not significantly different from each other (Tukey–Kramer test, P<.05).

3. Results

3.1. Animal body weight

The addition of FO to a high-fat diet (25%) had no effect on the growth rate of rats, while the combined treatment led to a significant reduction in weight gain compared to the control group already after 5 weeks of treatment (Fig. 1). Moreover, the diet that contained both bioactive lipids seemed to reduce body weight more than TTA alone after 50 weeks of treatment but not significantly (P=.23). The weight loss caused by TTA stabilized over time. In this study, all groups demonstrated the same average feed intake. Thus, animals from TTA and TTA+FO groups had a reduced body weight compared to control group animals with the same energy intake over a 50-week period.

3.2. Plasma lipids

We studied the impact of TTA and FO on key parameters in lipid metabolism. Analysis of plasma lipids at the end point of the experiment demonstrated that all treatments had a substantial lipid-lowering potential (Fig. 2). The plasma TAG level was significantly improved by all the treatments (Fig. 2A). Only TTA and the combination of TTA and FO were able to lower the free fatty acids (FFAs, Fig. 2B). In contrast, FO and the combination with TTA were able to reduce plasma PL levels by 30%, while TTA had no effect compared to the control diet (Fig. 2C).

FO treatment alone or in combination with TTA was effective in lowering of the total plasma cholesterol, cholesterol esters and free cholesterol (Fig. 2D–F). While the FO-containing diet alone reduced cholesterol by 35% compared to the control high-fat diet, the cotreatment with TTA and FO reduced it by 49%, indicating an additive effect of the supplements (Fig. 2D). Likewise, a significant decrease in the cholesterol ester level was obtained after TTA+FO treatment compared to either treatment alone (Fig. 2E). The additive effect of TTA and FO was mainly on LDL cholesterol (Fig. 2G) and non-HDL cholesterol (Fig. 2I).

3.3. Liver lipids

The hepatic cholesterol concentrations had a tendency to decrease after administration of TTA and TTA+FO (Fig. 3) but increased significantly with FO treatment compared to control. In addition, in rats fed a TTA diet, the total hepatic TAG concentration was 26% lower than in control. In contrast, FO treatment did not influence the TAG concentration in liver and caused an insignificant rise in PL levels.

3.4. Fatty acid composition in plasma

The fatty acid composition in plasma is shown in Table 1. Compared to control animals, the plasma levels of SFAs and monounsaturated fatty acids (MUFAs) were marginally changed by TTA treatment alone, except for an increase in C20:3n−9 (P<.0005). In contrast to that, FO supplementation caused the significant reduction in amounts of SFAs (P<.0005), MUFAs (P<.0005) and n−6 PUFAs (P<.0005) in plasma from FO-treated animals. ω−3 PUFAs

Table 1
Plasma fatty acid composition of male Wistar rats after 50 weeks of feeding with high-fat (25% w/v) diets supplemented with TTA, FO or TTA+FO

	Dietary supplementation			
	Control	TTA	FO	TTA+FO
SFAs	35.20±0.88^{a}	34.98±1.36^{a}	30.82±2.06^{b}	32.66±1.65^{c}
C16:0	21.22±1.49^{a}	22.41±1.23^{b}	17.72±1.28^{c}	20.35±1.44^{a}
C18:0	12.12±1.43^{a}	10.78±0.81^{b}	11.27±1.26^{b}	10.40±0.96^{b}
MUFAs	24.65±4.75^{a}	25.87±3.73^{a}	16.79±1.62^{b}	20.28±2.74^{c}
C16:1n−7	1.05±0.29^{a,b}	1.20±0.65^{b}	0.77±0.13^{c}	0.91±0.18^{a,c}
C18:1n−7	1.96±0.28^{a}	1.65±0.29^{b}	1.49±0.14^{c}	1.50±0.17^{b,c}
C20:1n−7	0.17±0.08	0.13±0.08^{a}	0.19±0.09^{b}	0.16±0.07
C22:1n−7	0.03±0.01^{a}	0.02±0.01^{b}	0.04±0.02^{a}	0.03±0.01^{a}
C16:1n−9	0.26±0.06^{a}	0.36±0.07^{b}	0.24±0.03^{a}	0.32±0.06^{c}
C18:1n−9 (OA)	20.46±4.23^{a}	21.70±3.49^{a}	12.99±1.37^{b}	16.29±2.50^{c}
C22:1n−9	0.02±0.00^{a}	0.02±0.01^{a}	0.04±0.01^{b}	0.04±0.01^{b}
C24:1n−9	0.27±0.11^{a}	0.46±0.14^{b}	0.48±0.11^{b}	0.53±0.14^{b}
n−9 PUFAs				
C20:3n−9 (MA)	0.23±0.03^{a}	0.85±0.33^{b}	0.12±0.01^{c}	0.13±0.02^{c}
n−6 PUFAs	36.26±4.61^{a}	33.76±3.59^{b}	22.72±2.63^{c}	22.38±2.50^{c}
C18:2n−6 (LA)	17.94±1.76^{a}	15.16±3.22^{b}	12.32±1.03^{c}	13.07±0.97^{c}
C18:3n−6	0.22±0.05^{a}	0.46±0.10^{b}	0.11±0.02^{c}	0.22±0.04^{a}
C20:2n−6	0.26±0.04^{a}	0.20±0.04^{b}	0.16±0.03^{c}	0.16±0.03^{c}
C20:3n−6	0.56±0.12^{a}	1.11±0.22^{b}	0.37±0.08^{c}	0.74±0.18^{d}
C20:4n−6 (AA)	16.76±4.69^{a}	16.57±3.69^{a}	9.45±1.89^{b}	7.95±1.83^{b}
C22:4n−6	0.34±0.07^{a}	0.14±0.03^{b}	0.07±0.02^{c}	0.05±0.01^{c}
C22:5n−6	0.16±0.04^{a}	0.12±0.06^{b}	0.22±0.04^{c}	0.17±0.02^{a}
n−3 PUFAs	3.55±0.5^{a}	2.30±1.03^{a}	29.48±4.19^{b}	22.90±3.14^{c}
C18:3n−3 (ALA)	0.61±0.16^{a}	0.45±0.16^{b}	0.59±0.14^{a}	0.50±0.17^{b}
C18:4n−3	0.02±0.01^{a}	0.01±0.02^{a}	0.54±0.19^{b}	0.53±0.22^{b}
C20:4n−3	0.12±0.02^{a}	0.09±0.02^{b}	0.39±0.06^{c}	0.30±0.05^{d}
C20:5n−3 (EPA)	0.02±0.01^{a}	0.01±0.02^{a}	16.52±3.38^{b}	12.58±2.82^{c}
C22:5n−3 (DPA)	0.47±0.11^{a}	0.17±0.08^{b}	1.32±0.30^{c}	0.79±0.10^{d}
C22:6n−3 (DHA)	2.01±0.49^{a}	1.28±0.58^{a}	9.84±2.27^{b}	8.02±1.03^{c}
TTA	ND	1.36±0.38^{a}	ND	1.04±0.17^{b}
TTA:1n−8	ND	0.79±0.28^{a}	ND	0.55±0.25^{b}
n−3 PUFA/n−6 PUFA ratio	0.10±0.01^{a}	0.07±0.03^{a}	1.34±0.37^{b}	1.04±0.22^{c}
DBI	1.52±0.16^{a}	1.43±0.13^{b}	2.38±0.15^{c}	2.04±0.11^{d}
ω−3 index	2.33±0.52^{a}	1.58±0.94^{a}	26.36±3.83^{b}	20.59±2.85^{c}

Data are given as mol%±S.D. (n=31–38).
Results were analyzed by ANOVA, and means with the same letter are not significantly different from each other (Tukey–Kramer test, P<.05).
AA, arachidonic acid; ALA, α-linolenic acid, LA, linolenic acid; ND, not detectable; OA, oleic acid.

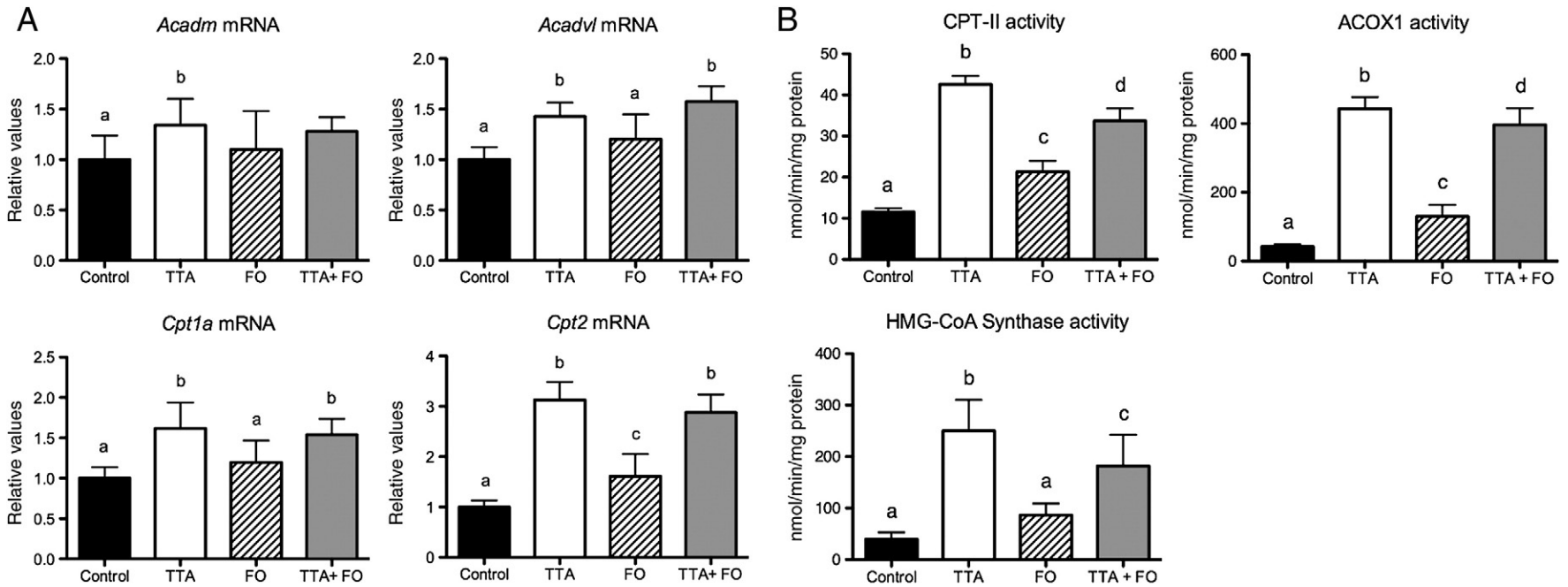


Fig. 4. Effect of 3-thia fatty acid TTA and FO on hepatic fatty acid β-oxidation. (A) Gene expression of *Acadm*, *Acadvl*, *Cpt1a*, and *Cpt2*. Data are shown as relative values. (B) Activity of ACOX1, measured in postnuclear fractions, CPT-II and HMG-CoA synthase, measured in crude mitochondrial fractions. Values are means±S.D. (n=8–10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

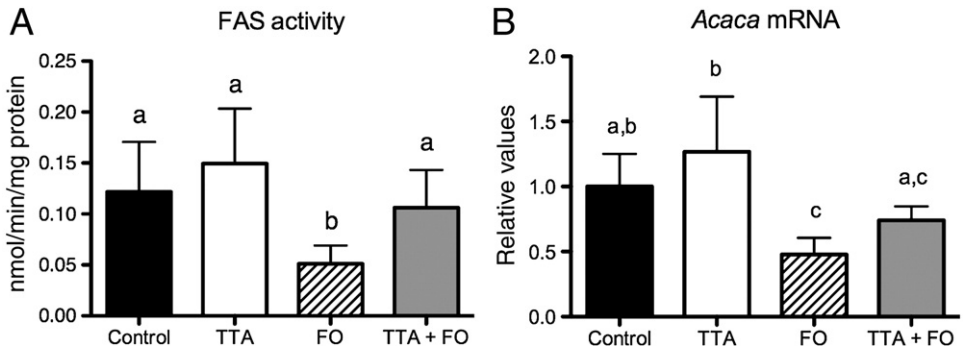


Fig. 5. Effect of 3-thia fatty acid TTA and FO on hepatic FAS activity (A) and hepatic gene expression of *Acaca* (B). Values are means±S.D. (n=8–10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

[C20:5n−3 (EPA), C22:5n−3 (docosapentaenoic acid, DPA), C22:6n−3 (DHA)] were decreased after TTA treatment [35,36], leading to a significantly reduced DBI. We also found a significant (P<.0005) reduction of ω−3 PUFA in TTA+FO-treated animals compared to FO treatment alone. However, the combined treatment increased the n−3/n−6 PUFA ratio, as well as the ω−3 index (DHA+EPA) (Table 1), by over 10-fold compared to TTA alone.

3.5. Genes and enzymes involved in fatty acid metabolism

Several of the PPAR-controlled genes involved in the mitochondrial fatty acid β-oxidation pathway, such as *Acadm*, *Acadvl*, *Cpt1a*, and *Cpt2* were induced in liver by the TTA diet (Fig. 4A). The FO diet seemed to give an induction of PPAR-activated genes; however, only *Cpt2* was significantly increased.

Hepatic ACOX1, CPT-II and HMG-CoA synthase activities indicated a high induction of fatty acid β-oxidation after 50 weeks of TTA diet (Fig. 4B). The addition of FO caused the less pronounced effect on hepatic enzyme activities in combination group compared to TTA group.

The activity of lipogenic enzyme FAS was significantly reduced by FO administration (Fig. 5A). Tetradecylthioacetic acid gave an insignificant increase in FAS activity and counteracted the FO effect in the combined treatment.

We also investigated the messenger RNA (mRNA) expression of *Acaca* (Fig. 5B). This gene encodes the enzyme acetyl-coenzyme A carboxylase, which catalyzes carboxylation of acetyl-CoA to malonyl-CoA. *Acaca* mRNA level in liver tended to be up-regulated by TTA compared to control group. The FO-supplemented group had a significantly lower *Acaca* mRNA expression than both TTA group and control group (P<.001). Also, in the TTA+FO group, *Acaca* expression was significantly lower than in the TTA group (P<.01).

3.6. Differential effects of TTA and FO on Ucp mRNA expression

In the liver samples, differential effects of TTA and FO were observed on the gene expression of *Ucp2* and *Ucp3* mRNA, which are suggested to act as regulators of mitochondrial energy metabolism and ROS. Both TTA and TTA+FO diets resulted in the down-regulation of hepatic *Ucp2* mRNA levels, in contrast to FO alone (Fig. 6A). We revealed a substantial up-regulation (1500×) of *Ucp3* in rats fed a TTA-supplemented diet (Fig. 6B). The combination of TTA with FO also led to an up-regulation of *Ucp3* mRNA levels, while the FO diet had no significant effect on this mitochondrial uncoupling protein.

3.7. Oxidative damage in homogenate of whole liver and mitochondria

We evaluated the relationship between different oxidative biomarkers (representing different oxidative damage pathways) between whole-liver *vs.* mitochondrial fraction (Fig. 7).

γ-Glutamic semialdehyde is a marker that is related to direct metal catalysed oxidation on proline and arginine residues in proteins. Interestingly, the diet supplementation with FO led to non-significantly higher levels of GSA than in the control group in both mitochondrial extract and whole-liver homogenate, which indicated an increase in protein oxidative damage (Fig. 7A). In contrast to this, the dietary supplementation of TTA significantly reduced protein oxidative damage in mitochondrial extracts obtained from TTA- and TTA+FO-fed animals.

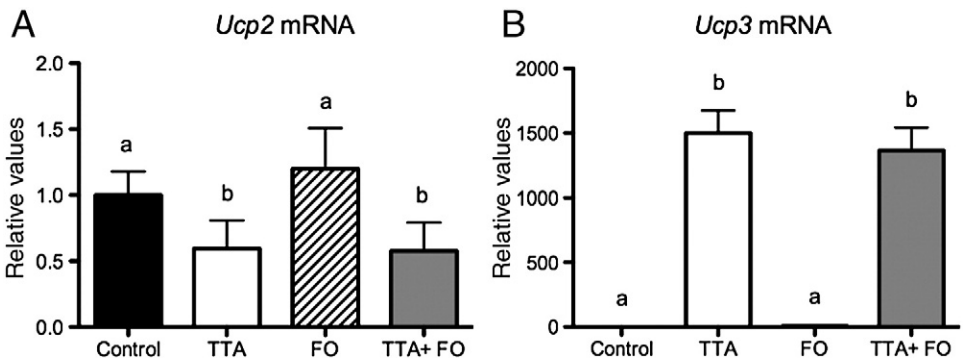


Fig. 6. Effect of 3-thia fatty acid TTA and FO on the hepatic gene expression of mitochondrial uncoupling proteins *Ucp3* (A) and *Ucp2* (B). Values are means±S.D. (n=10). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

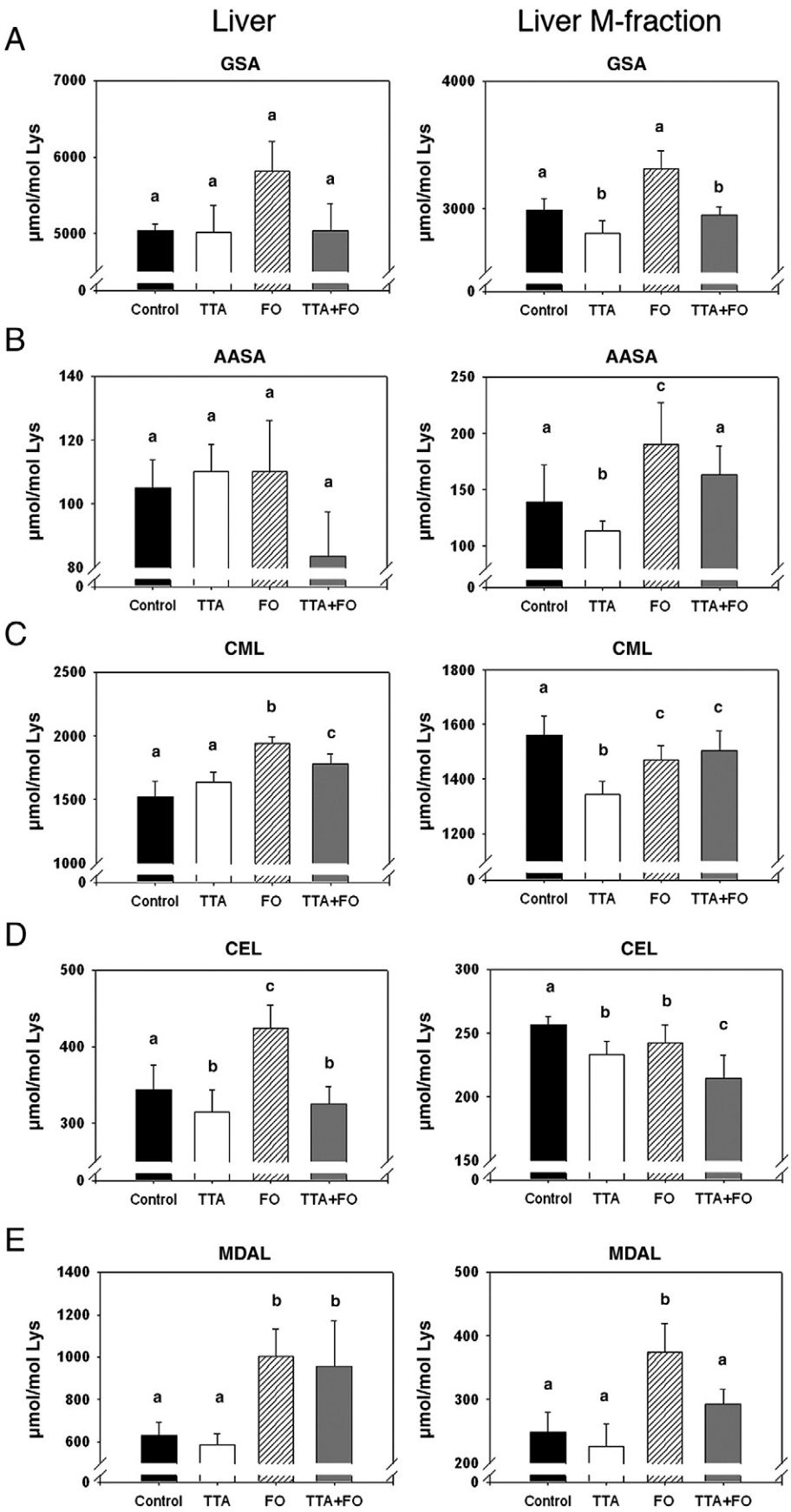


Fig. 7. Protein oxidative damage amongst different dietary interventions. GSA, AASA, CEL, MDAL and CML measured in liver (left) and liver mitochondrial homogenate (liver M-fraction, right). Values are means±S.D. (n=7 or 8 animals in each group). Results were analyzed by ANOVA, and means with different letters are significantly different from each other (Tukey–Kramer test, P<.05).

The other marker of protein oxidative damage, α-AASA, was also significantly higher in mitochondrial samples from the FO group than in animals from the control group and groups treated with TTA or the combination (Fig. 7B). The TTA supplementation led to reduction of protein oxidative damage mitochondrial extract.

The nonenzymatic glycation and subsequent irreversible oxidation of proteins cause formation of glycoxidation products. Nε-Carboxymethyl-lysine is known to be formed from the oxidation of both carbohydrates and lipids, making it a biomarker of general oxidative stress. The whole-liver samples from the FO group showed significantly increased lipoxidation and TTA diminished this potentially deleterious effect of FO in animals from the TTA+FO group (Fig. 7C). In mitochondrial extracts, both FO and TTA treatment resulted in reduced levels of CML compared to the control group.

Nε-Carboxyethyl-lysine is a homolog of CML, which is formed by the reaction of lysine residues in proteins with methylglyoxal as well as with triose phosphates and other sugars. Our results showed that FO increased oxidative (nonenzymatic) sources of methylglyoxal, while the addition of TTA into the diets reduced CEL levels in whole-liver homogenates (Fig. 7D). In mitochondrial extracts, both bioactive compounds resulted in lower levels of CEL compared to control group. The cotreatment with TTA and FO demonstrated significantly lowered CEL compared with control, TTA, and FO groups in mitochondrial extract.

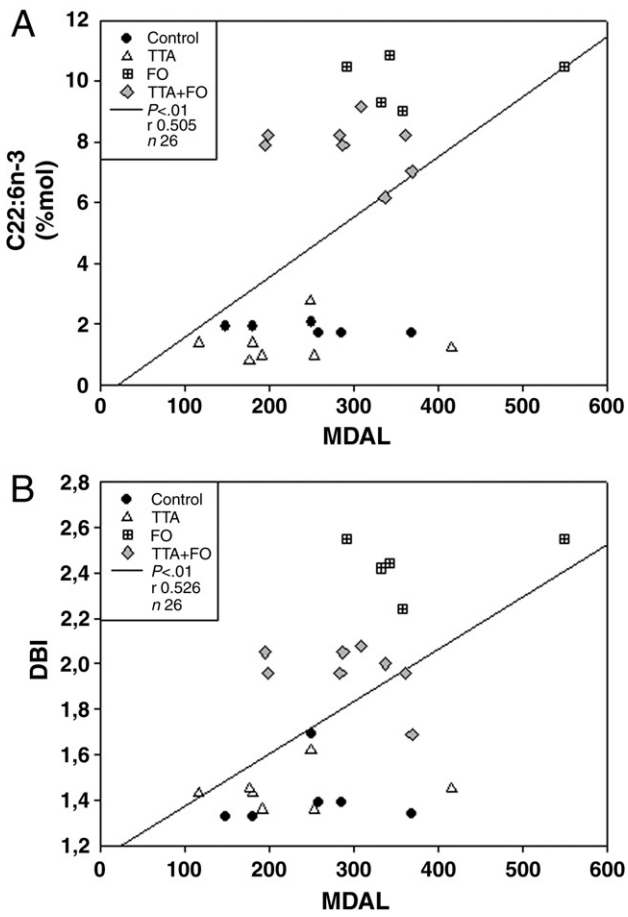


Fig. 8. Correlations for the fatty acid composition in plasma and the lipoxidative damage marker MDAL measured in mitochondrial extracts. (A) Correlation between DHA (C22:6n−3) and MDAL. C22:6n−3 values are shown as mole percent of the total lipid content. (B) Correlation between the DBI and MDAL. DBI values are expressed as ∑% of unsaturated fatty acids×number of double bonds per unsaturated fatty acid. MDAL values are expressed in μmol MDAL/mol Lys.

Nε-Malondialdehyde-lysine is a biomarker of lipoxidation. FO supplementation showed an increased oxidative damage compared to control group in both whole-liver and mitochondrial homogenate samples (Fig. 7E). We found that TTA cotreatment diminished the FO-induced lipoxidative damage in mitochondrial homogenates.

Furthermore, there is a significant positive correlation between MDAL concentration and the DHA content, as well as the DBI (Fig. 8).

4. Discussion

Contrary to earlier performed animal experiments on the effects of bioactive lipids such as TTA and ω−3 PUFAs, the distinguishing factor of our study was its duration of 50 weeks. The extended duration gave us the possibility to evaluate and compare the effectiveness of combined treatment *vs.* mono supplementation with TTA or FO. The prolonged beneficial effects observed were changes in expression of genes involved in fatty acid metabolism and activities of corresponding key enzymes, improvement of plasma lipid parameters and liver lipid composition and not the least weight reduction. A 50-week period of feeding with TTA resulted in a significantly reduced body weight gain during the whole study period, apparently reflecting increased energy expenditure due to TTA. In agreement with other studies, FO did not affect the overall body weight of male Wistar rats given a high-fat diet [37]. Further, the rise in activities of CPT-II, HMG-CoA synthase and ACOX1 (Fig. 4B) combined with the enhanced expression of hepatic *Cpt1*, *Cpt2*, *Acadm* and *Acadvl* (Fig. 4A) as well as *UCP3* (Fig. 6) suggests that TTA both increases the β-oxidation and induces the energy consumption via intensification of uncoupling in the liver (45).

The diets had a differential effect on the lowering of plasma lipid levels. While the TTA diet mainly lowered TAG, FFAs and LDL cholesterol in this long-term treatment, FO supplementation showed substantially more lowering of TAG, PLs, cholesterol and HDL cholesterol than TTA alone. The combined supplementation with TTA and FO demonstrated the additive effect of both components on the decrease in several plasma lipids and especially in a significantly enhanced reduction in plasma cholesterol.

In agreement with the results obtained by Duan at al. [38], our 50-weeks-long experiment showed that high-fat feeding can induce elevation in hepatic TAG levels (Fig. 3B). The accumulation of TAG was probably due to inability of the liver to handle the increased import of plasma lipids and indicated the development of liver steatosis. The diet with TTA showed a potential to improve the hepatic TAG and cholesterol levels, whereas the FO diet did not. This could be due to the high induction of hepatic β-oxidation by TTA that enhances lipid metabolism and consequently results in pronounced removal of lipids from both plasma and liver [13]. In addition, TTA has previously been shown to drain lipids from specific adipose tissue depots [39]. It might be possible that both FO and TTA diets are able to increase cholesterol and TAG transport to the liver, but animals given the FO diet are less able to metabolize the excess hepatic lipid levels. The stimulated activities of CPT-II and ACOX1 were much more pronounced with TTA than with FO feeding (Fig. 4B). Thus, TTA seemed to lower plasma lipid levels by an increased hepatic β-oxidation, while FO redistributed lipids without affecting the total lipid level and body weight.

We have revealed a tendency to decrease in the hepatic cholesterol concentrations after administration of TTA and TTA+FO, whereas FO supplementation alone gave a significant increase in hepatic cholesterol compared to controls. Consequently, the observed lowering effect of hepatic cholesterol by TTA+FO combination might be due to the TTA effect.

In this long-term experiment, TTA supplementation caused several changes in the plasma fatty acid composition, particularly, the decrease in ω−3 PUFA content (Table 1). The observed reduction in ω−3 PUFA levels can be explained by TTA-induced stimulation of

mitochondrial and peroxisomal fatty acid oxidation (36). Furthermore, the $n-9$ fatty acid, mead acid (MA, C20:3$n-9$), was up-regulated in the plasma of TTA-fed rats (approximately fourfold). Notably, both FO and the combined treatment led to a twofold reduction of MA compared to control. As an increased production of the nonessential MA may be linked to a poor PUFA status in humans [40], the FO-induced decrease in MA supports a similar regulation of MA in rats.

The addition of both TTA and FO to the high-fat diet induced genes linked to fatty acid metabolism and increased the corresponding enzyme activity levels. Tetradecylthioacetic acid was far more potent than FO, and the combination of TTA and FO did not further increase the effect. The pattern of enzyme activity implicated in fatty acid metabolism was similar to previous findings [19,36,39], demonstrating that the metabolic effects of FO and TTA supplementation are long-lasting.

Tetradecylthioacetic acid and FO seem to affect the factors involved in lipogenesis in a different manner. The significant reduction in hepatic FAS activity in rats fed the FO diet could contribute to the registered changes in plasma lipid content. In contrast, the TTA diet showed an insignificant increase in hepatic FAS activity ($P=.24$). Moreover, the FO diet significantly decreased *Acaca* expression in liver compared to TTA. Thus, comparing the effect of combination of both supplements with the effect of TTA treatment, the reduction of *Acaca* mRNA level could be a result of the competing modes of action of TTA and FO.

The study with Wistar rats fed a low-fat TTA-supplemented diet for 7 days showed an enhanced *Ucp2* mRNA expression in liver [41], and it has been suggested that this early, transient induction in hepatic *Ucp2* may contribute to increased energy expenditure. However, our long-term experiment and the 7-week study performed by Wensaas *et al.* [39] demonstrated a significant decrease in *Ucp2* expression caused by TTA-supplemented diets.

Ucp3 is usually not expressed in the liver, except in response to PPAR activation during situations with a high level of fatty acid metabolism [42]. The increase in hepatic *Ucp3* mRNA expression caused by TTA treatment alone (over 1500-fold) and the combined treatment (over 1300-fold) was the most striking results in our experiment. As discussed previously, the stimulated hepatic expression of *Ucp3* due to TTA administration could be related to an increased metabolism, possibly also through energy uncoupling (Fig. 6B). Altogether, these findings indicate that the induction of *Ucp2* becomes less pronounced over a longer period, while the induction of *Ucp3* is a prolonged effect by TTA.

A hypothesis about a possible protective role of endogenous UCP3 against oxidative damage by ROS in skeletal muscle mitochondria was proposed and tested by Brand *et al.* [43]. They discovered that down-regulation of *Ucp3* was associated with increased oxidative damage through higher ROS generation, while the *Ucp3* up-regulation was not. Based on findings from our study and in accordance with previous observations [44,45], we could propose that such pronounced effects might represent an adaptive response to protect the mitochondria and their host cells from oxidative stress in situations characterized by intensive lipid metabolism and consequently further support the theory of the defensive role of *Ucp3* against lipid-induced mitochondrial damage.

In agreement with previous data [46–48], we confirm that dietary FO supplementation increased lipoxidative damage. Our experiment revealed marked changes in CML and MDAL, which are markers related to lipoxidative status, in both liver homogenate and mitochondrial extracts. Since liver is a crucial organ in lipid metabolism, the fatty acid profile in liver is sometimes assumed to reflect the fatty acid composition in plasma. Therefore, it is not unreasonable to think that the profile might reflect the membrane lipid composition of liver cells. From that point of view and being demonstrated by a 1.5-fold increase of DBI for FO-treated animals compared with the control group (Fig. 8), one can postulate that the different degree of unsaturation in liver membranes may account for the increased damage observed and could achieve enough amplification without ROS production being raised. In other words, FO diet is not necessarily responsible for an increased ROS production but for a major ROS susceptibility, since membrane composition is slightly altered and the degree of unsaturation is increased. Finally, both CML and MDAL were reduced by addition of TTA into the diets. Thus, TTA was able to partially prevent those FO-induced changes. This fully agrees with the hypothesis of TTA as an antioxidant.

The results also demonstrate that a dietary manipulation comprising changes in the amount of fatty acids induce a significant change in the steady state levels of GSA and AASA, markers of direct oxidative damage. Reinforcing the implication of mitochondria in this change, only mitochondrial proteins show those changes, whereas liver homogenates did not. Fish oil effects could be due to increased free radical production as recently demonstrated in other tissues and experimental models, implicating Ca^{+2} transit between mitochondria and endoplasmic reticulum [49–51]. Most interestingly, the fact that TTA significantly lowers direct oxidative damage supports the notion that TTA increases mitochondrial efficiency (irrespectively of FO) and may reproduce beneficial effects of caloric restriction.

To conclude, this study provides evidence that the long-term effects of TTA are similar to those seen in experiments of shorter duration. We report that TTA and FO are efficient dietary supplements in improvement of plasma lipid profile, despite differences in their mechanisms of action. While TTA increased energy expenditure and lipid metabolism, FO seemed to lower plasma lipid levels mainly by a redistribution of lipids, as well as a reduced fatty acid synthesis. Through our experiment, we confirmed that, during long-term FO diets, the proper supplementation of antioxidants, such as TTA, is essential.

Acknowledgments

The authors are grateful to Kari Williams, Liv Kristine Øysæd, and Svein Krüger for excellent technical contribution and to the staff at the Laboratory Animal Facility, University of Bergen. We thank Professor Jon Skorve for helpful discussions.

References

[1] Crowley VE. Overview of human obesity and central mechanisms regulating energy homeostasis. Ann Clin Biochem 2008;45(Pt 3):245–55.
[2] Guri AJ, Hontecillas R, Bassaganya-Riera J. Peroxisome proliferator-activated receptors: bridging metabolic syndrome with molecular nutrition. Clin Nutr 2006;25(6):871–85.
[3] Connor WE. Importance of $n-3$ fatty acids in health and disease. Am J Clin Nutr 2000;71(1 Suppl):171S–5S.
[4] Yashodhara BM, Umakanth S, Pappachan JM, Bhat SK, Kamath R, Choo BH. Omega-3 fatty acids: a comprehensive review of their role in health and disease. Postgrad Med J 2009;85(1000):84–90.
[5] Adam O, Beringer C, Kless T, Lemmen C, Adam A, Wiseman M, et al. Anti-inflammatory effects of a low arachidonic acid diet and fish oil in patients with rheumatoid arthritis. Rheumatol Int 2003;23(1):27–36.
[6] Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 2010;68(5):280–9.
[7] Neschen S, Morino K, Dong J, Wang-Fischer Y, Cline GW, Romanelli AJ, et al. $n-3$ Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner. Diabetes 2007;56(4):1034–41.
[8] Berge K, Tronstad KJ, Flindt EN, Rasmussen TH, Madsen L, Kristiansen K, et al. Tetradecylthioacetic acid inhibits growth of rat glioma cells ex vivo and in vivo via PPAR-dependent and PPAR-independent pathways. Carcinogenesis 2001;22(11):1747–55.
[9] Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 1997;94(9):4312–7.
[10] Westergaard M, Henningsen J, Svendsen ML, Johansen C, Jensen UB, Schroder HD, et al. Modulation of keratinocyte gene expression and differentiation by PPAR-

selective ligands and tetradecylthioacetic acid. J Invest Dermatol 2001;116(5): 702–12.
[11] Raspe E, Madsen L, Lefebvre AM, Leitersdorf I, Gelman L, Peinado-Onsurbe J, et al. Modulation of rat liver apolipoprotein gene expression and serum lipid levels by tetradecylthioacetic acid (TTA) via PPARalpha activation. J Lipid Res 1999;40(11): 2099–110.
[12] Berge RK, Skorve J, Tronstad KJ, Berge K, Gudbrandsen OA, Grav H. Metabolic effects of thia fatty acids. Curr Opin Lipidol 2002;13(3):295–304.
[13] Berge RK, Tronstad KJ, Berge K, Rost TH, Wergedahl H, Gudbrandsen OA, et al. The metabolic syndrome and the hepatic fatty acid drainage hypothesis. Biochimie 2005;87(1):15–20.
[14] Dyroy E, Yndestad A, Ueland T, Halvorsen B, Damas JK, Aukrust P, et al. Antiinflammatory effects of tetradecylthioacetic acid involve both peroxisome proliferator-activated receptor alpha-dependent and -independent pathways. Arterioscler Thromb Vasc Biol 2005 Jul;25(7):1364–9.
[15] Muna ZA, Gudbrandsen OA, Wergedahl H, Bohov P, Skorve J, Berge RK. Inhibition of rat lipoprotein oxidation after tetradecylthioacetic acid feeding. Biochem Pharmacol 2002;63(6):1127–35.
[16] Muna ZA, Bolann BJ, Chen X, Songstad J, Berge RK. Tetradecylthioacetic acid and tetradecylselenoacetic acid inhibit lipid peroxidation and interact with superoxide radical. Free Radic Biol Med 2000;28(7):1068–78.
[17] Pamplona R. Membrane phospholipids, lipoxidative damage and molecular integrity: a causal role in aging and longevity. Biochim Biophys Acta 2008;1777(10):1249–62.
[18] Pamplona R, Portero-Otin M, Requena J, Gredilla R, Barja G. Oxidative, glycoxidative and lipoxidative damage to rat heart mitochondrial proteins is lower after 4 months of caloric restriction than in age-matched controls. Mech Ageing Dev 2002;123(11):1437–46.
[19] Totland GK, Madsen L, Klementsen B, Vaagenes H, Kryvi H, Froyland L, et al. Proliferation of mitochondria and gene expression of carnitine palmitoyltransferase and fatty acyl-CoA oxidase in rat skeletal muscle, heart and liver by hypolipidemic fatty acids. Biol Cell 2000;92(5):317–29.
[20] Spydevold O, Bremer J. Induction of peroxisomal beta-oxidation in 7800 C1 Morris hepatoma cells in steady state by fatty acids and fatty acid analogues. Biochim Biophys Acta 1989;1003(1):72–9.
[21] Wergedahl H, Liaset B, Gudbrandsen OA, Lied E, Espe M, Muna Z, et al. Fish protein hydrolysate reduces plasma total cholesterol, increases the proportion of HDL cholesterol, and lowers acyl-CoA:cholesterol acyltransferase activity in liver of Zucker rats. J Nutr 2004;134(6):1320–7.
[22] Gudbrandsen OA, Dyroy E, Bohov P, Skorve J, Berge RK. The metabolic effects of thia fatty acids in rat liver depend on the position of the sulfur atom. Chem Biol Interact 2005;155(1–2):71–81.
[23] Hulbert AJ, Pamplona R, Buffenstein R, Buttemer WA. Life and death: metabolic rate, membrane composition, and life span of animals. Physiol Rev 2007;87(4): 1175–213.
[24] Berge RK, Flatmark T, Osmundsen H. Enhancement of long-chain acyl-CoA hydrolase activity in peroxisomes and mitochondria of rat liver by peroxisomal proliferators. Eur J Biochem 1984;141(3):637–44.
[25] Asiedu DK, al-Shurbaji A, Rustan AC, Bjorkhem I, Berglund L, Berge RK. Hepatic fatty acid metabolism as a determinant of plasma and liver triacylglycerol levels. Studies on tetradecylthioacetic and tetradecylthiopropionic acids. Eur J Biochem 1995;227(3):715–22.
[26] Bremer J. The effect of fasting on the activity of liver carnitine palmitoyltransferase and its inhibition by malonyl-CoA. Biochim Biophys Acta 1981;665(3): 628–31.
[27] Clinkenbeard KD, Reed WD, Mooney RA, Lane MD. Intracellular localization of the 3-hydroxy-3-methylglutaryl coenzme A cycle enzymes in liver. Separate cytoplasmic and mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A generating systems for cholesterogenesis and ketogenesis. J Biol Chem 1975;250(8): 3108–16.
[28] Roncari DA. Fatty acid synthase from human liver. Methods Enzymol 1981;71(Pt C):73–9.
[29] Skorve J, al-Shurbaji A, Asiedu D, Bjorkhem I, Berglund L, Berge RK. On the mechanism of the hypolipidemic effect of sulfur-substituted hexadecanedioic acid (3-thiadicarboxylic acid) in normolipidemic rats. J Lipid Res 1993;34(7): 1177–85.
[30] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22.
[31] Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc 2006;1(3):1559–82.
[32] Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product, Nepsilon-(carboxymethyl)lysine, is a product of both lipid peroxidation and glycoxidation reactions. J Biol Chem 1996;271(17):9982–6.
[33] Requena JR, Fu MX, Ahmed MU, Jenkins AJ, Lyons TJ, Baynes JW, et al. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. Biochem J 1997;322(Pt 1):317–25.
[34] Requena JR, Chao CC, Levine RL, Stadtman ER. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. Proc Natl Acad Sci U S A 2001;98(1):69–74.
[35] Madsen L, Froyland L, Dyroy E, Helland K, Berge RK. Docosahexaenoic and eicosapentaenoic acids are differently metabolized in rat liver during mitochondria and peroxisome proliferation. J Lipid Res 1998;39(3):583–93.
[36] Gudbrandsen OA, Wergedahl H, Bohov P, Berge RK. The absorption, distribution and biological effects of a modified fatty acid in its free form and as an ethyl ester in rats. Chem Biol Interact 2009;179(2–3):227–32.
[37] Rokling-Andersen MH, Rustan AC, Wensaas AJ, Kaalhus O, Wergedahl H, Rost TH, et al. Marine $n-3$ fatty acids promote size reduction of visceral adipose depots, without altering body weight and composition, in male Wistar rats fed a high-fat diet. Br J Nutr 2009;102(7):995–1006.
[38] Duan XY, Zhao HP, Fan JG. Dynamic expression of hepatic thioredoxin mRNA in rats with non-alcoholic fatty liver disease. J Dig Dis 2010;11(2):94–100.
[39] Wensaas AJ, Rustan AC, Rokling-Andersen MH, Caesar R, Jensen J, Kaalhus O, et al. Dietary supplementation of tetradecylthioacetic acid increases feed intake but reduces body weight gain and adipose depot sizes in rats fed on high-fat diets. Diabetes Obes Metab 2009;11(11):1034–49.
[40] Decsi T, Koletzko B. Polyunsaturated fatty acids in infant nutrition. Acta Paediatr Suppl 1994;83(395):31–7.
[41] Grav HJ, Tronstad KJ, Gudbrandsen OA, Berge K, Fladmark KE, Martinsen TC, et al. Changed energy state and increased mitochondrial beta-oxidation rate in liver of rats associated with lowered proton electrochemical potential and stimulated uncoupling protein 2 (UCP-2) expression: evidence for peroxisome proliferator-activated receptor-alpha independent induction of UCP-2 expression. J Biol Chem 2003;278(33):30525–33.
[42] Camara Y, Mampel T, Armengol J, Villarroya F, Dejean L. UCP3 expression in liver modulates gene expression and oxidative metabolism in response to fatty acids and sensitizes mitochondria to permeability transition. Cell Physiol Biochem 2009;24(3–4):243–52.
[43] Brand MD, Pamplona R, Portero-Otin M, Requena JR, Roebuck SJ, Buckingham JA, et al. Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. Biochem J 2002;368(Pt 2):597–603.
[44] Hoeks J, Hesselink MK, Schrauwen P. Involvement of UCP3 in mild uncoupling and lipotoxicity. Exp Gerontol 2006;41(7):658–62.
[45] Bezaire V, Seifert EL, Harper ME. Uncoupling protein-3: clues in an ongoing mitochondrial mystery. FASEB J 2007;21(2):312–24.
[46] Pamplona R, Portero-Otin M, Sanz A, Requena J, Barja G. Modification of the longevity-related degree of fatty acid unsaturation modulates oxidative damage to proteins and mitochondrial DNA in liver and brain. Exp Gerontol 2004;39(5): 725–33.
[47] Portero-Otin M, Bellmunt MJ, Requena JR, Pamplona R. Protein modification by advanced Maillard adducts can be modulated by dietary polyunsaturated fatty acids. Biochem Soc Trans 2003;31(Pt 6):1403–5.
[48] Herrero A, Portero-Otin M, Bellmunt MJ, Pamplona R, Barja G. Effect of the degree of fatty acid unsaturation of rat heart mitochondria on their rates of H_2O_2 production and lipid and protein oxidative damage. Mech Ageing Dev 2001;122(4):427–43.
[49] Prasad A, Bloom MS, Carpenter DO. Role of calcium and ROS in cell death induced by polyunsaturated fatty acids in murine thymocytes. J Cell Physiol 2010;225(3): 829–36.
[50] Lemieux H, Bulteau AL, Friguet B, Tardif JC, Blier PU. Dietary fatty acids and oxidative stress in the heart mitochondria. Mitochondrion 2011;11(1): 97–103.
[51] Khairallah RJ, O'Shea KM, Brown BH, Khanna N, Des Rosiers C, Stanley WC. Treatment with docosahexaenoic acid, but not eicosapentaenoic acid, delays Ca2+-induced mitochondria permeability transition in normal and hypertrophied myocardium. J Pharmacol Exp Ther 2010;335(1):155–62.

Acta Neuropathol (2011) 122:259–270
DOI 10.1007/s00401-011-0850-y

ORIGINAL PAPER

Cell stress induces TDP-43 pathological changes associated with ERK1/2 dysfunction: implications in ALS

Victòria Ayala · Ana Belén Granado-Serrano · Daniel Cacabelos · Alba Naudí · Ekaterina V. Ilieva · Jordi Boada · Víctor Caraballo-Miralles · Jerònia Lladó · Isidro Ferrer · Reinald Pamplona · Manuel Portero-Otin

Received: 18 March 2011 / Revised: 2 June 2011 / Accepted: 9 June 2011 / Published online: 25 June 2011

Abstract TDP-43 has been implicated in the pathogenesis of amyotrophic lateral sclerosis and other neurodegenerative diseases. Here we demonstrate, using neuronal and spinal cord organotypic culture models, that chronic excitotoxicity, oxidative stress, proteasome dysfunction and endoplasmic reticulum stress mechanistically induce mislocalization, phosphorylation and aggregation of TDP-43. This is compatible with a lack of function of this protein in the nucleus, specially in motor neurons. The relationship between cell stress and pathological changes of TDP-43 also includes a dysfunction in the survival pathway mediated by mitogen-activated protein kinase/extracellular signal-regulated kinases (ERK1/2). Thus, under stress conditions, neurons and other spinal cord cells showed cytosolic aggregates containing ERK1/2. Moreover, aggregates of abnormal phosphorylated ERK1/2 were also found in the spinal cord in amyotrophic lateral sclerosis (ALS), specifically in motor neurons with abnormal immunoreactive aggregates of phosphorylated TDP-43. These results demonstrate that cellular stressors are key factors in neurodegeneration associated with TDP-43 and disclose the identity of ERK1/2 as novel players in the pathogenesis of ALS.

Keywords Oxidative stress · Proteasome stress · Endoplasmic reticulum stress · Excitotoxicity · ALS · TDP-43 · ERK1/2

V. Ayala and A. B. Granado-Serrano contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00401-011-0850-y) contains supplementary material, which is available to authorized users.

V. Ayala · A. B. Granado-Serrano · D. Cacabelos · A. Naudí · E. V. Ilieva · J. Boada · R. Pamplona · M. Portero-Otin (✉)
Grup de Fisiopatologia Metabòlica, Departament de Medicina Experimental, Universitat de Lleida-IRBLleida, Lleida, Spain
e-mail: manuel.portero@mex.udl.cat

V. Caraballo-Miralles · J. Lladó
Departament de Biologia, IUNICS, Universitat de les Illes Balears, Palma de Mallorca, Spain

I. Ferrer
Institut de Neuropatologia, Hospital Universitari de Bellvitge, IDIBELL, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain

I. Ferrer
CIBERNED (Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas), Instituto Carlos III, Spanish Ministry of Health, Madrid, Spain

Introduction

Transactive response DNA binding protein (TDP-43) is a 414-amino acid protein with two highly conserved RNA recognition motifs, a nuclear localization signal at the protein N-terminus, and a glycine-rich region mediating protein–protein interactions at the C-terminus [8, 9, 29, 31, 38]. TDP-43 was discovered as the gene encoding a 43-kDa protein that binds to the transactive response DNA sequence of human immunodeficiency virus type 1 [38]. Subsequently, it was demonstrated that TDP-43 regulates DNA transcription, RNA splicing and microRNA biogenesis in physiological conditions [9]. Recently, the discovery of TDP-43 cytosolic aggregates in sporadic amyotrophic lateral sclerosis (ALS), a progressive and deadly motor neuron disease, has prompted its research in neurodegeneration [2, 35]. TDP-43 ALS-associated forms characteristically exhibit ubiquitination, phosphorylation and fragmentation, together with abnormal subcellular localizations [35].

Neurodegeneration in ALS involves many different cellular stress mechanisms: oxidative stress, mitochondrial dysfunction, endoplasmic reticulum (ER) stress, proteasome stress and protein aggregation, glutamate excitotoxicity, cytoskeletal dysfunction, malfunctioning non-neuronal cells in the vicinity of motor neurons, and abnormal RNA processing [4, 14, 20, 22, 23, 25, 33, 47]. Some of them also originate after excitotoxicity caused by dysregulation of glutamate signaling [40]. In addition to ALS, there is a wide variety of neurodegenerative diseases (such as Alzheimer's, Parkinson's and Huntington's), that also show TDP-43 pathological forms, suggesting a potential association between TDP-43 and neurodegenerative processes [1, 15, 21, 34, 35, 42].

We have previously shown that ER stress, increased ubiquitination, protein oxidative damage and changes in fatty acid profile are associated with chronic excitotoxicity in organotypic spinal cord cultures [23], a well-supported model of the sporadic form of ALS [39]. However, it is not known if excitotoxicity or other ALS-involved cellular stressors induce TDP-43 pathological changes. Here, we report that oxidative, endoplasmic reticulum (ER) stress and proteasome dysfunction induce those changes. Furthermore, excitotoxicity leads to TDP-43 accumulation resembling the pathological aggregates shown in human disease. As oxidative stress impairs protein nuclear import, and this involves mitogen-activated protein kinase/extracellular signal-regulated kinases (ERK1/2) activation [11], we also investigated whether oxidative, ER and proteasome stress cause a ERK1/2 activation leading to cytosolic TDP-43 aggregation in neuronal cell lines. Paradoxically, the results indicate that inhibition of TDP-43 nuclear import associated with stress involves the inhibition of ERK1/2, which interestingly are also aggregated. Furthermore, we show the presence of abnormal phosphorylated ERK1/2 aggregates in motor neurons with p-TDP-43-positive inclusions in ALS.

Materials and methods

Cell culture

Neuro-2A and SH-SY5Y cell lines were incubated as detailed in the *Supplementary material*. To study the effects of oxidative stress, alteration of endoplasmic reticulum and inhibition of proteasome activity, cells were treated with 10 μM H_2O_2 (Sigma), 5 μM thapsigargin (Thp) (Sigma), or 2.5 μM epoxomicin (Epox) (Sigma), respectively, for 2 or 4 h. Similarly, the cells were incubated with 20 μM PD98059 (Sigma) for 2 h before any stress to specifically inhibit the ERK1/2 pathway. To avoid the influence of growth factors present in the fetal bovine serum on the results, the normal culture medium was replaced by Optimem (Invitrogen, Carlsbad, CA, USA) 12 h before all the assays. Cell viability was evaluated using the crystal violet assay as previously described [19] and detailed in the *Supplementary material* section.

Spinal cord organotypic cultures

Spinal cord cultures were prepared from lumbar spinal cord of postnatal day 8 rat pups as previously described [23, 39] and maintained in culture media as described in the *Supplementary material* section. One week after plating, to induce excitotoxicity the slices were incubated with the glutamate transport inhibitor D,L-threo-hydroxyaspartate (THA) (Sigma, St. Louis, MO, USA) at 100 μM. This treatment injures motor neurons with a morphology typical of excitotoxic degeneration after 3 weeks of treatment. Experiments for each condition ($n = 30$ slices per experimental group) were repeated at least three times. All animal experiments were approved by the Institutional Animal Care and Experimentation Committee.

Patients' information

The human spinal cord samples were from six males and five females aged between 54 and 76 years (mean age 62) affected with typical neurological and neuropathological characteristics of sporadic ALS. The post-mortem delay between death and tissue processing was between 3 and 13 h. Age- and gender-matched controls ($n = 11$) with no neurological disease and normal neuropathological study were processed in parallel. All samples were obtained from the Institute of Neuropathology and University of Barcelona Brain Bank following the guidelines of the local ethics committees. Extensive pathological studies were done for ALS diagnosis as previously described [23].

Immunohistochemical analysis

Detailed procedures, following previously described methods [23] for immunohistochemistry analysis of organotypic spinal cord cultures, cell lines and human samples are described in the *Supplementary material* section. Immunohistochemical controls, performed by omitting the primary antibodies, resulted in the abolition of the immunostaining in all cases. For quantitation of p-TDP-43 or p-ERK1/2 aggregates a minimum number of 1,000 cells were counted in each experimental situation.

Cell fractionation, protein electrophoresis and western blot

Tissue and cell lysis, protein electrophoresis and western blot were performed as previously described [23]. For details see the *Supplementary material* section.

Equal loading of western blot was ensured by β-actin or α-tubulin and blot quantification was carried out using the ChemiDoc XRS system and the softwares Quantity One (BioRad, Hercules, CA, USA) and Scion Image (Scion Corp., Frederick, MD, USA).

Statistical analysis

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed by the Student's *t* tests or ANOVA analysis, once normality of variables was tested by Kolmogorov–Smirnov test. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

TDP-43 pathogenic alteration is induced by different ALS-related stressors in different neuronal cell lines

Previous results from our group showed that sporadic ALS is associated with increased protein oxidation, loss of proteasome activity and ER stress contributing to motor neuron death [23]. ALS-associated TDP-43 changes include increased fragmentation, increased cytosolic localization and aggregate formation with concurrent phosphorylation. To validate the relationship between cell stress and TDP-43 changes we measured TDP-43 changes in accepted models of each of those stress types in neuronal cell lines. Neuro 2A cultures were treated with either H_2O_2 to induce oxidative stress, thapsigargin to induce endoplasmic reticulum stress, or with epoxomicin to inhibit proteasome activity. Treatments were sublethal (Fig. 1a), so the presented changes in TDP-43 are not directly related to immediate cell death. Furthermore, none of the stress conditions used here increased cleaved caspase-3 levels (data not shown).

Western blot using the polyclonal antibody anti-TDP-43 (Sigma ref HPA017284), extensively validated in the Human Protein Atlas (http://www.proteinatlas.org/), showed a transient increase of an anti-TDP-43 immunoreactive 37-kDa band (putatively a TDP-43 fragment) after each one of the stress inducers for 2 h (Fig. 1b, c). All the stressors induced a significant increase in TDP-43 phosphorylation versus control cells, measured by using an antibody against the phospho-Ser410/409 in TDP-43 (p-TDP-43). In the case of a 25-kDa phosphorylated fragment, its presence was sustained after 4 h (Fig. 1b, d). According to p-TDP-43 immunoreactivities a general increase was seen at fragments with apparent molecular weights between 32–37 kDa and 22–25 kDa, even comprising a very low molecular weight fragment of 15-kDa (Fig. 1b, d).

Subcellular fractionation and immunohistochemistry demonstrated that these stress inducers led to the accumulation of TDP-43 and p-TDP-43 in the cytosolic fractions (Figs. 1e–g, 2). Specifically, in the case of epoxomicin treatment, practically all TDP-43 were fragmented (37 kDa band) and localized in the cytosol after 4 h, as the TDP-43 immunoreactivity was practically lost in nuclear fractions of epoxomicin treated cells. Proteasome inhibition led to the highest accumulation of non-phosphorylated TDP-43 (Fig. 2a, b). In addition, both proteasome and ER stress inducers generated a high level of cytosolic p-TDP-43 aggregates (Fig. 2a, c). The highest labeling of epoxomicin is coincident with the suggestion that TDP-43 or some of its fragments are degraded by proteasome [37]. These results also show that TDP-43 phosphorylation is not always related to its cytosolic aggregation under cell stress. Most of these results were also observed in a human cell line (differentiated SHSY-5Y neuroblastoma cells), suggesting that oxidative, ER and proteasome stress-induced neuronal TDP-43 pathological changes are common mechanisms across different species (Fig. S1).

In summary, these data show that oxidative, ER and proteasome stress induce ALS-related TDP-43 pathogenic changes in neuronal cell lines. These include increased TDP-43 fragmentation, increased cytosolic localization and aggregate formation with concurrent phosphorylation.

Involvement of ERK1/2 in stress-induced ALS-related TDP-43 changes

ERK1/2 pathway is implicated in cellular death/survival signaling and is activated in response to certain situations of cellular stress [6]. Moreover, previous data have described that oxidative stress prevents nuclear import of transcription factors by activating ERK1/2 [11]. So this phenomenon could partially explain TDP-43 cytosolic accumulation observed after oxidative and ER stress, and proteasome inhibition. To demonstrate this hypothesis, the degree of ERK1/2 activation in all stress situations was analyzed and it was demonstrated that all cell stress—though at different rates—activated initially ERK1/2 (Fig. 3a–d). In fact, ERK1/2 inactivation by the specific inhibitor PD98059, not only failed to prevent TDP-43 abnormal distribution associated to stress, but it also increased cytosolic p-TDP-43 aggregation (Fig. 3e–h, S2) and it induced p-ERK1/2 aggregation (Fig. 3h, S2). Noteworthy, epoxomicin induced a cytosolic accumulation of p-ERK1/2 (Fig. 3h, Fig. S2), coincident (but not colocalizing) within cells showing p-TDP-43 aggregates. These

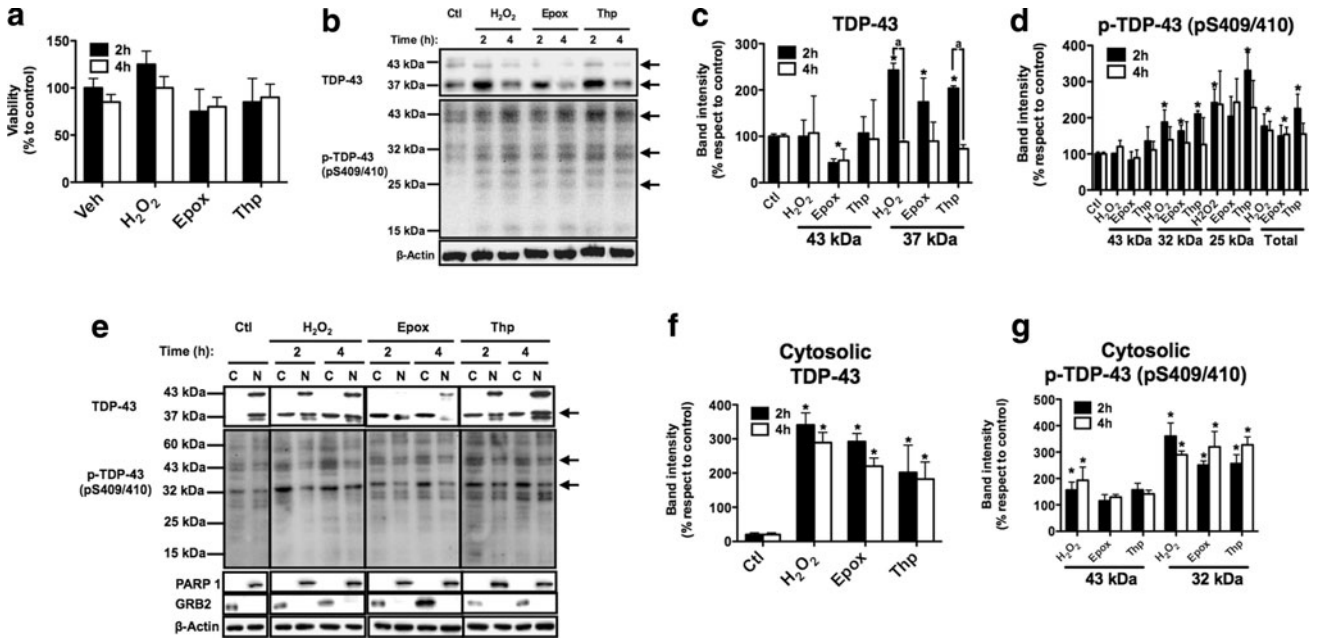


Fig. 1 TDP-43 pathogenic alteration is induced by different ALS-related stressors in neurons. **a** Treatments with oxidative stress, proteasome stress and ER stress did not induce a significant change in neuron cell line viability (neither at 2 nor at 4 h). **b** Representative immunoblots of TDP-43 and p-TDP-43 immunoreactivities of Neuro 2A cell lysates after induction of oxidative stress (10 μM H_2O_2), proteasomal inhibition (2.5 μM Epoxomicin, Epox) and endoplasmic reticulum stress (5 μM Thapsigargin, Thp). Densitometric analysis of the anti-TDP-43 (**c**) and anti-p-TDP-43 (**d**) bands, adjusted to actin content, being the differences analyzed with reference to unstressed cells. **e** Representative immunoblots of cytosolic (C) and nuclear (N) fractions of Neuro 2A cells after induction of the above referred stress. Anti-growth factor receptor-bound protein 2 (Grb2) immunoreactivity was used for ensure specificity of cytosolic isolation and anti-Poly (ADP-ribose) polymerase 1 (PARP1) immunoreactivity for nuclear isolation. Densitometric analyses of the anti-TDP-43 immunoreactivity (**f**) and anti-p-TDP-43 (**g**) in cytosolic fractions showed increased amount of TDP-43 protein (both phosphorylated and non-phosphorylated) after each stress with significant changes with reference to unstressed cells. Differences between control and H_2O_2, Epox and Thp-treated samples were analyzed by Student's t test being $^*p < 0.01$ (for different treatments) and a $p < 0.01$ (for differences between 2 and 4 h). *Arrows* at left of blots indicate bands whose intensity was quantified. Total in **d** indicates the summatory of individual immunoreactivities of anti-p-TDP-43 in **b**

data suggest that p-TDP-43 and p-ERK1/2 deposits may be present in different cellular compartments in the same cell. Importantly, those p-ERK1/2 cytosolic aggregates coincided with the lowest degree of ERK1/2 activation (Fig. 3a, b, h). These data suggest an association between aggregation/inactivation of ERK1/2 and TDP-43 abnormal localization. Noteworthy, cytosolic p-ERK1/2 did not colocalize with stress granules (anti-TIA immunoreactivity in cells treated with thapsigargin, Fig. S3).

To directly prove that ERK1/2 inactivation is sufficient for TDP-43 alteration, we submitted cells to pharmacological ERK1/2 inactivation without further stress, leading to a clear increase in nuclear TDP-43 fragmentation as well as an accumulation of total levels of cytosolic TDP-43 (Fig. 4a, b) and their appearance as perinuclear p-TDP-43 aggregates (Fig. 4c). Globally, these data demonstrate that ERK1/2 inactivation does not prevent the stress-induced changes in TDP-43. Furthermore, ERK1/2 inactivation is sufficient for ALS-related TDP-43 pathological changes in neurons (Fig. 4).

Chronic excitotoxicity leads to ALS-related TDP-43 pathogenic changes and ERK1/2 aggregation

Cell phenotype in neuronal lines is quite different from the spinal cord neurons. Moreover, these neuronal cell lines are grown in the absence of a natural cellular milieu (astrocytes, microglia), which plays a role in the development of ALS. Organotypic spinal cultures could solve these limitations, as glutamate excitotoxicity, a well-recognized mechanism of motor neuronal death in sporadic ALS, can be reproduced in vitro by exposing lumbar cord slices to the glutamate-uptake inhibitor THA [39]. We have previously shown that chronic excitotoxicity in this model induces ER stress, increased ubiquitination, protein oxidative damage and changes in fatty acid profile [23]. To evaluate whether cell stress-induced TDP-43 pathological changes were also present in chronic excitotoxicity, we measured potential fragmentation, cytosolic localization and aggregate formation of TDP-43 with concurrent phosphorylation with different antibodies, to avoid non-

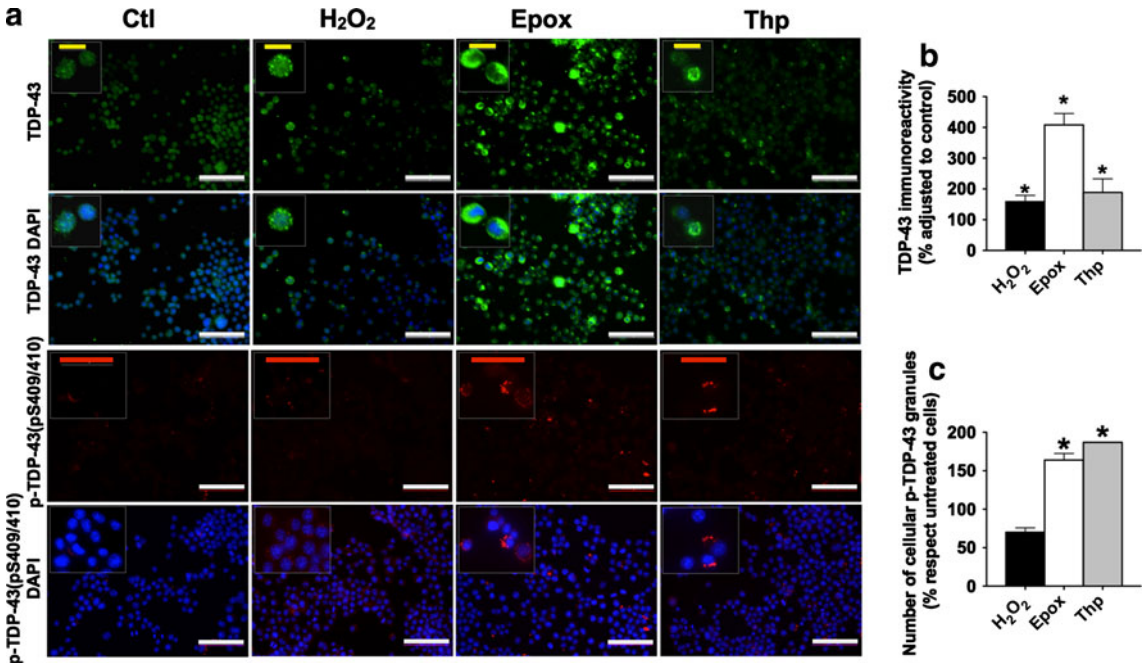


Fig. 2 TDP-43 cytosolic aggregation and phosphorylation is induced by different ALS-related stressors in neurons. **a** Both TDP-43 (*green*) and p-TDP-43 (*red*) aggregates were observed in Neuro 2A cells 2 h after induction of oxidative stress (10 μM H_2O_2), proteasomal inhibition (2.5 μM Epoxomicin, Epox) and endoplasmic reticulum stress (5 μM Thapsigargin, Thp). Nuclei were stained with DAPI (*blue*). **b** Evidences the effect of cell stress in anti-TDP-43 immunoreactivity and **c** shows the effect of stress inducers in the number of cellular anti-p-TDP-43 immunoreactive granules (number of aggregates for each 100 cells: 11.5 ± 1.6; 26.6 ± 5.8 and 30.3 ± 7.6, mean ± SD for H_2O_2, Epox and Thp-treated cells, respectively). Differences between control and H_2O_2, Epox and Thp-treated samples were analyzed by Student's *t* test being $*p < 0.01$ (for different treatments). *Scale bars*: *white* 50 μm; *yellow* 10 μm; *red* 25 μm

specific recognition. This approached revealed different patterns of TDP-43 distribution in organotypic spinal cord culture. The use of the polyclonal antibody anti-TDP-43 (Sigma ref HPA017284) revealed decreased TDP-43 immunostaining, specifically in the nucleus of neuronal cells, compatible with the above-mentioned nuclear loss of TDP-43 induced by cell stressors. These neurons, localized in the ventral horn, show a morphology and SMI-32 immunoreactivity compatible with its identity as motor neurons and they exhibited a near-complete depletion of TDP-43 (Fig. 5a, Fig. S4). In contrast, glial cells do not exhibit this TDP-43 loss (Fig. S4). The use of another antibody raised against a peptide comprising the N-terminal region (1-261 aminoacids, Abnova ref H00023435-A01) evidenced TDP-43 accumulation in the cytoplasm after THA treatment (Fig. 5b), also showing a nuclear loss. Interestingly, in those cells with nuclear TDP-43 depletion, TDP-43 immunoreactivity was present in the cytoplasm, both in perinuclear areas and dendritic terminals (small panels, Fig. 5b). Strikingly, while as TDP-43 nuclear loss was neuron specific, cytosolic immunoreactivity was extended to both neuronal and glial cells.

Western blot analysis disclosed two major bands: a 43-kDa (native) and the 37-kDa (Fig. 5c). Densitometric analysis demonstrated that excitotoxicity significantly increased the levels of this TDP-43 fragment while 43-kDa band tended to decrease, albeit non-significantly (Fig. 5d), a fact that might be explained by the contribution of non-neuronal TDP-43.

Chronic excitotoxicity also leads to increased p-TDP-43, specifically in the cytoplasm of cells in the ventral horn of spinal cord cultures (Fig. 6a–d). The resulting increased p-TDP-43 immunostaining showed two patterns: an aggregated and a diffuse one. Aggregates were not colocalized with ubiquitin (Fig. 6b upper panel), though they were restricted to cells showing ubiquitin inclusions. In contrast, the p-TDP-43 diffuse pattern was present in cells without increased protein ubiquitination (Fig. 6b lower panel). Western blot analysis evidenced that the presence of various immunoreactive bands, similar to neuronal cell lines (at apparent molecular weight 43, 37, 29, 25, 23 and 15 kDa), increased significantly after THA treatment, except the unfragmented 43-kDa band (Fig. 6c, d). P-ERK1/2 analyses revealed that chronic excitotoxicity increased extranuclear p-ERK1/2 immunoreactivity together with p-TDP-43 aggregates. p-ERK1/2 aggregates showed both fine granular and meshwork-like morphologies (Fig. 6e). In this specific case, and based on cell

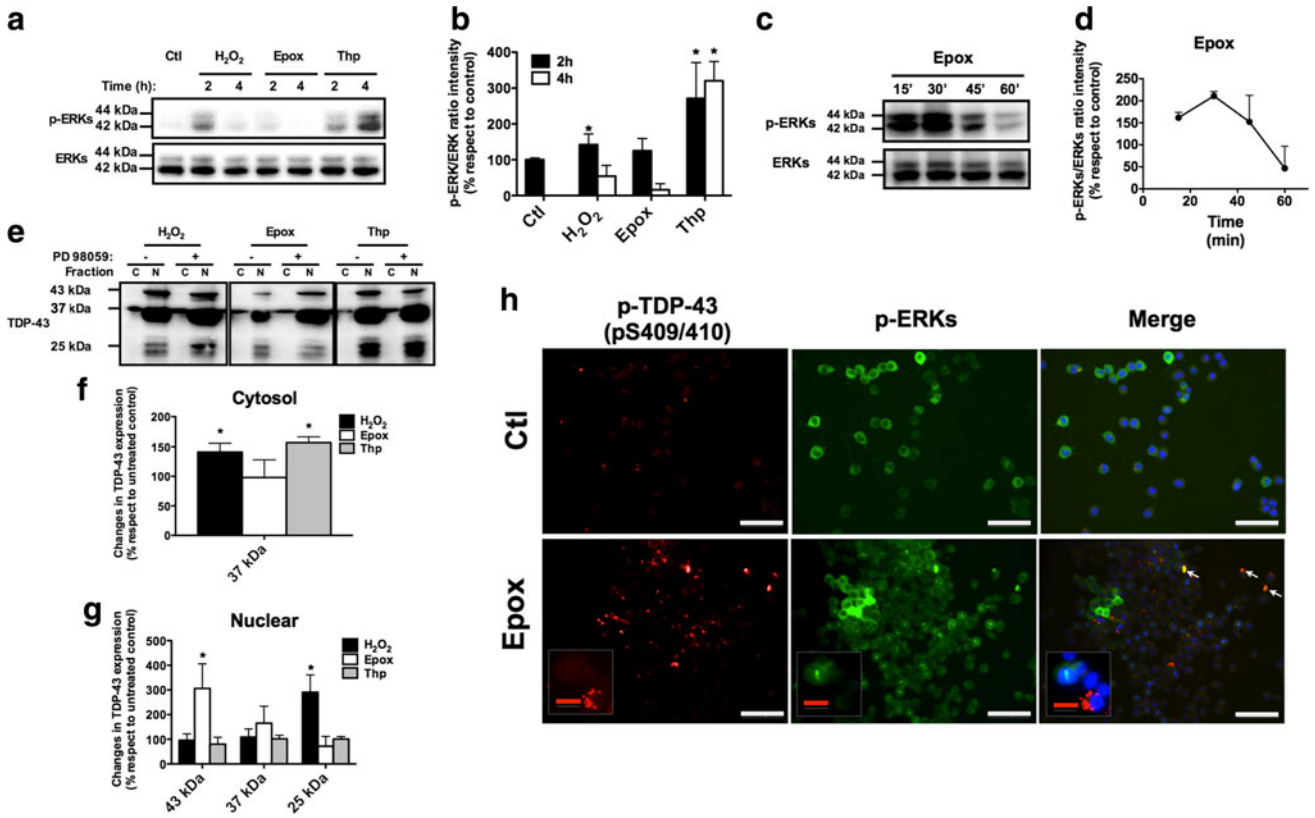


Fig. 3 Involvement of ERK1/2 in stress-induced ALS-related TDP-43 changes. **a** Representative immunoblots of total ERK1/2 and p-ERK1/2 immunoreactivities of Neuro 2A cell lysates after induction of oxidative stress (10 μM H_2O_2), proteasomal inhibition (2.5 μM Epoxomicin, Epox) and endoplasmic reticulum stress (5 μM Thapsigargin, Thp). **b** Densitometric analysis of p-ERK1/2 bands, adjusted to total ERK1/2 content, being the differences analyzed with reference to untreated cells. **c** Time course analyses show that in the case of epoxomicin, 2 h inactivation (shown in **a**) was preceded by a marked acute activation of p-ERK1/2, with a maximum at 30 min, as evidenced by the densitometric analysis (**d**). **e** Representative immunoblots of cytosolic (C) and nuclear (N) fractions of Neuro 2A cells after induction of the above referred stress under previous ERK1/2 inhibition induced by PD98059 treatment for 2 h. Densitometric analysis of the anti-TDP-43 immunoreactivity in cytosolic (**f**) and nuclear fractions (**g**). In the case of oxidative stress, PD98059 treatment increased nuclear TDP-43 fragmentation as evidenced by the accrual of a 25 kDa anti-TDP-43 reactive fragment (**e**, **g**). **h** Proteasome stress led to p-ERK1/2 aggregation (*green*) in association with p-TDP-43 aggregation (*red*), with some cells showing colocalization of both aggregates (*arrows*). Differences between control and H_2O_2, Epox or Thp-treated samples were analyzed by Student's *t* test being $*p < 0.01$ (for different treatments). *Scale bars*: *white* 50 μm; *red* 10 μm

abundance, p-ERK1/2 aggregates were present in both neuronal and non-neuronal cells in ventral horn (Fig. 6f). Importin beta, a protein implicated in nucleocytoplasmic transport, was studied by immunostaining, showing that this protein is absent in cells showing p-ERK1/2 aggregates in THA-treated organotypic cultures (Fig. S5). This supports the hypothesis that impaired nuclear import due to excitotoxicity may be related to p-ERK1/2 aggregation.

Globally, these results demonstrate that chronic excitotoxicity, which is known to induce ER, proteasome and oxidative stress, leads to TDP-43 pathogenic changes, namely increased fragmentation, increased cytosolic localization and aggregate formation with concurrent phosphorylation in cells of the ventral horn of lumbar spinal cord. Furthermore, increased p-ERK1/2 deposition is also present, coincident with p-TDP-43 aggregates.

Altered p-ERK1/2 deposition is restricted to motor neurons with p-TDP-43 immunoreactive deposits in ALS

As already reported, TDP-43 immunoreactivity in normal motor neurons of the spinal cord was characterized as fine granular precipitate localized in the nuclei. However, TDP-43 immunoreactivity was altered in many motor neurons in ALS as fine granular deposits, skein-like inclusions and globular inclusions in the cytoplasm, at the time that TDP-43 immunoreactivity vanished in the nuclei of the same neurons, reproducing what is observed in the spinal cord

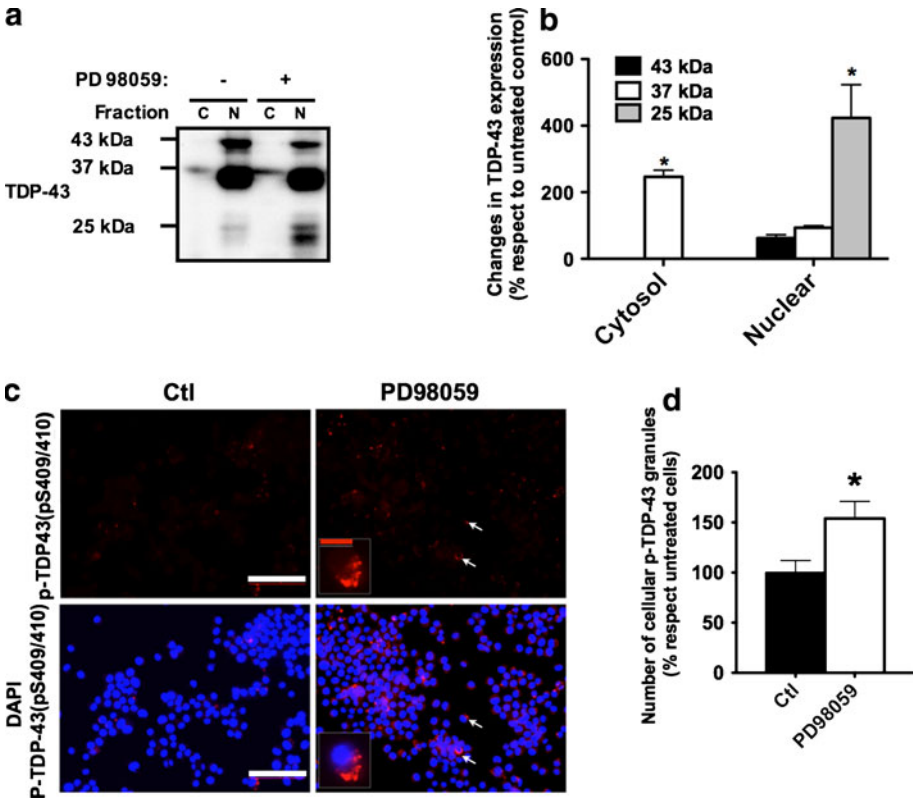


Fig. 4 ERK1/2 inactivation leads to ALS-related TDP-43 changes. **a** Representative immunoblots of cytosolic (C) and nuclear (N) fractions of Neuro 2A cells untreated after ERK1/2 inhibition by PD98059 treatment for 2 h. **b** Densitometric analysis of the anti-TDP-43 immunoreactivity in cytosolic and nuclear fractions. **c** ERK1/2 inactivation induces p-TDP-43 cytosolic aggregation, shown by *arrows*. Nuclei were stained with DAPI (*blue*). **d** Effect of ERK1/2 inhibition in the number of cell anti-p-TDP-43 immunoreactive granules (number of aggregates for each 100 cells: 16.1 ± 1.2 and 25.6 ± 5.8 for control and PD98059 treated cells, respectively). Differences between control and PD98059-treated samples were analyzed by Student's *t* test being $*p < 0.01$ *Scale bars*: *white* 50 μm, *red* 10 μm

organotypic cultures. Similar results were obtained with the two anti-TDP-43 antibodies used in samples from ALS patients (data not shown).

Phosphorylated form of TDP-43, as observed with both anti-p-TDP-43 antibodies, was only found in pathological inclusions whereas the normal nuclei were not stained. Thus, p-TDP-43 was a marker of altered TDP-43 localization in ALS motor neurons (data not shown). In those sections, p-ERK1/2 immunoreactivity was present in the nuclei of motor neurons in normal spinal cords and in apparently non-altered motor neurons in samples from ALS patients (Fig. 7a). Yet several motor neurons in the ALS showed abnormal p-ERK1/2 deposits in the form of dense nuclear aggregates (Fig. 7b, c) or dense (Fig. 7d–f), fine granular or rectilinear (Fig. 7g, h), skein-like (Fig. 7i, j) or meshwork-like (Fig. 7k, l) cytoplasmic inclusions. These were accompanied by a decrease or absence of nuclear p-ERK1/2 immunoreactivity.

Double-labeling immunofluorescence and confocal microscopy showed altered p-ERK1/2 deposition restricted to motor neurons with p-TDP-43 immunoreactive deposits in ALS cases (Fig. 7m–r). Subcellular colocalization of abnormal p-ERK1/2 and p-TDP-43 was also occasionally observed (Fig. 7s–x). These data reinforce a potential role for ERK1/2 dysfunction in ALS pathogenesis related to TDP-43 alterations, as the same motor neurons show both TDP-43 and p-ERK1/2 aggregates.

Discussion

Data presented here demonstrate that different paradigms of sporadic ALS, including organotypic culture of lumbar spinal cord slices under excitotoxic conditions [23, 39] reproduce TDP-43 abnormalities observed in sporadic ALS and frontotemporal lobar degeneration. In both diseases, TDP-43 shows increased fragmentation, hyperphosphorylation and ubiquitination [35]. Furthermore, its subcellular localization is changed from its physiological preferential nuclear localization to a cytosolic one, even showing aggregates [2, 3, 35]. Our data reinforces both the usefulness of organotypic culture as a valid model for sporadic ALS and the fact that chronic excitotoxicity could reproduce TDP-43 pathological findings in vitro.

Nuclear TDP-43 protein is decreased in ventral horn of glial and motor neurons of THA-treated cultures. ALS-associated extranuclear localization has been described in several subcellular fractions, including cytosol and ER [18, 41], concurrent with a loss in nuclear TDP-43 localization, potentially compatible with an impairment in its functions, mainly in the nucleus. Accordingly, in control conditions of organotypic cultures, nuclear TDP-43 seems considerably more accumulated in motor neurons than in other surrounding cells. This may be consistent with a neuron-selective functional dependency of TDP-43 [43]. Loss of nuclear TDP-43 in excitotoxic conditions may be related to

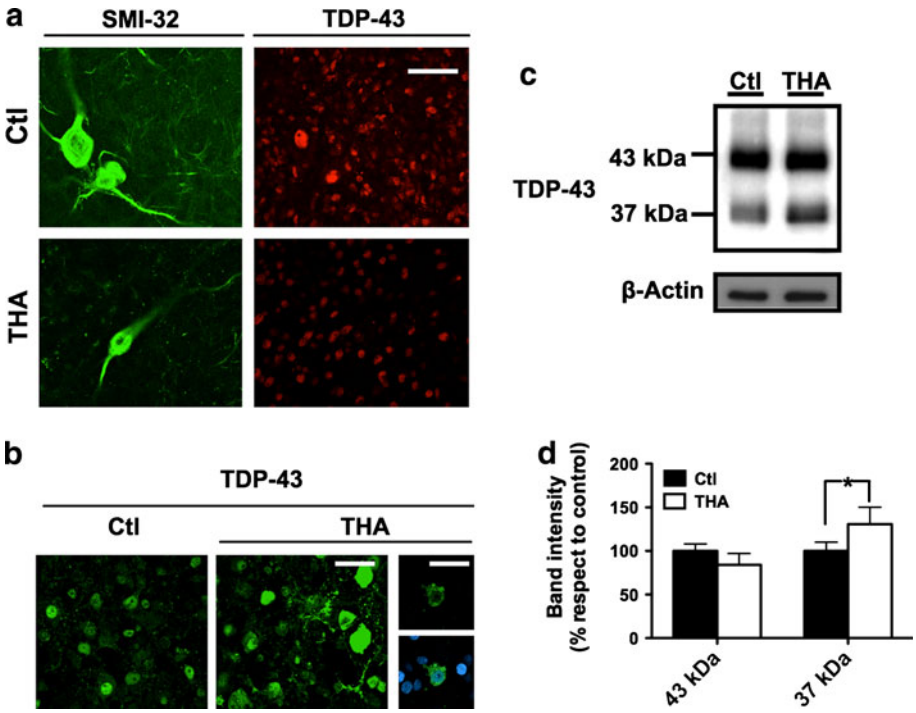


Fig. 5 Chronic excitotoxicity leads to ALS-related TDP-43 pathogenic changes. **a** Representative confocal microscope images showing decreased TDP-43 immunoreactivity in ventral horns of spinal cord slices cultured for 21 days with 100 μM D,L-threo-hydroxyaspartate (THA) (*lower panel*) or vehicle (*upper panel*) using rabbit anti-TDP-43 (*red*). Immunostaining was combined with anti-SMI-32 (*green*) to visualize motor neurons. **b** Using a mouse anti-TDP-43 antibody (*green*), immunoreactivity distribution was markedly changed by THA treatment. *Smaller panels* in **b** show that in some cells TDP-43 immunoreactivity is present in the cytoplasm, excluding DAPI stained nuclei (*blue*). **c** Representative immunoblots of spinal cord homogenates using rabbit anti-TDP-43 show that THA induced a significant increase in the levels of a 37-kDa band. **d** Densitometric analysis of the bands, adjusted to actin content, being the differences analyzed with reference to control values. *Scale bars* **a** 50 μm; **b** 25 μm. Differences between control and THA-treated samples were analyzed by Student's *t* test being $*p < 0.01$

TDP-43 fragmentation by excitotoxic phenomena, concurrently to both native TDP-43 (43-kDa) diminished levels and increased amount of lower molecular weight TDP-43 fragments. In this sense, recent data supported the pathogenic importance of TDP-43 fragments, specially those of 25-kDa in nuclei [7, 13, 46]. Most interestingly, these nuclear fragments were also present in neuronal cell cultures under the stressors evaluated here, including ERK1/2 inhibition. These results reinforce the potential interaction between cell stress and TDP-43 pathology, independently on the neurodegenerative paradigm chosen. Using other antibodies for p-TDP-43, chronic excitotoxicity was associated with increased diffuse and aggregate cytoplasmic p-TDP-43 immunoreactivity. Cytosolic p-TDP-43 aggregates did not show a constant colocalization with ubiquitin, another marker of ALS. However, only cells showing increased ubiquitin immunostaining demonstrated cytosolic p-TDP-43 aggregation, suggesting a close link between these two phenomena (e.g. by proteasomal dysfunction).

It is accepted that oxidative stress could influence proteasome activity [24]. In turn, proteasomal stress is one of the potential causes of ER stress [10]. In accordance with this, the increased TDP-43 aggregation linked to its fragmentation and cytosolic location which is observed in Neuro2A and SH-SY5Y cell lines could be explained by a combined effect of proteasomal dysfunction, oxidative and ER stress. Nevertheless, proteasomal activity impairment seems to be a key factor, as it induces the highest aggregation (in terms of immunohistochemical evidences) of both TDP-43 and its phosphorylated form, according to previous reports indicating TDP-43 fragmentation occurs mainly via proteasome [49], reproducing both the human disease and the organotypic culture under excitotoxicity.

The presented results demonstrate increased levels of p-TDP-43 induced by cell stress in several experimental models: chronic excitotoxicity (in spinal cord organotypic culture), and oxidative, ER and proteasome stress (in Neuro-2A and SH-SY5Y), suggesting that TDP-43 phosphorylation is a common feature in the potential for pathogenesis exhibited by this protein in stress-associated neurodegenerative disorders [17]. These results could be derived both from increased TDP-43 phosphorylating activity, depending on casein-kinase I or decreased

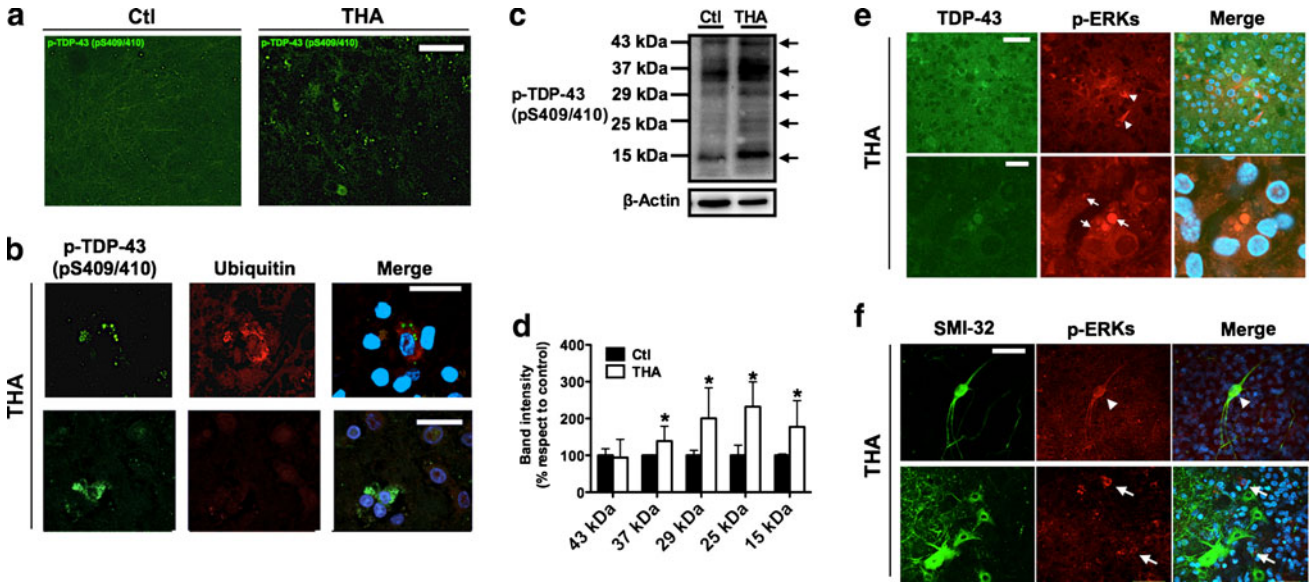


Fig. 6 Increased TDP-43 phosphorylation is associated with cytosolic p-ERK1/2 aggregation. **a** Representative confocal microscope images showing increased cytoplasmatic TDP-43 phosphorylation in ventral horns of spinal cord slices under chronic excitotoxicity. **b** Increased p-TDP-43 immunoreactivity was present both in aggregate form (*upper panel*, *green*) and in a diffuse pattern (*lower panel*, *green*), with no ubiquitin colocalization (*red*). Nuclei were stained with DAPI (*blue*). **c** Representative immunoblots of spinal cord homogenates using anti-p-TDP-43 shows that THA induced a significant increase in the levels of several bands. **d** Densitometric analysis of the bands, adjusted to actin content, being the differences analyzed with reference to control values. At least 30 hemi-slices from each condition were examined throughout the different experiments. **e** Increased p-ERK1/2 immunoreactivity after THA treatment was distributed following cytosolic location (*arrowheads*, *upper panels*) or granular-globular patterns (*arrows*, *lower panels*), some showing colocalization with TDP-43 immunoreactivity (**f**). Increased p-ERK1/2 immunoreactivity (*red*) after THA treatment was distributed among SMI-32 positive cells (identified as neurons, *arrowheads*) and preferentially in non SMI-32 positive cells (*arrows*). Nuclei were stained with DAPI (*blue*). *Scale bars*: **a** 50 μm; **b** 20 μm; (**e** *upper panel*) 25 μm; (**e** *lower panel*) 10 μm; **f** 50 μm. *Arrows* at left of blots indicate bands whose intensity was quantified. Differences between control and THA-treated samples were analyzed by Student's *t* test being $*p < 0.01$

phosphatase responses, depending on yet ill defined factors [26]. Furthermore, the relationship of TDP-43 aggregation–fragmentation and phosphorylation is still under evaluation, with recent data showing that p-TDP-43 (at Ser 409/410) is closely associated to aggregation [7]. However, we also evidence that increased phosphorylation is not always correlated with p-TDP-43 aggregation (e.g. while all stressors evaluated increased cytosolic p-TDP-43 levels, H_2O_2 failed to show increased aggregation by immunohistochemistry). This suggest the existence of stress-specific trends for TDP-43 pathogenetical changes. We recognize, as limitation of the cellular models used here, the fact that p-TDP-43 expression is present in "control" conditions. Nevertheless, in the tissular (organotypic) model and specially, in the human samples, p-TDP-43 expression is mostly restricted to the pathological conditions. Previous data show that SHSY-5Y cell lines using western blot reveal a very small, though visible, immunoreactivity over anti-p-TDP-43 in control conditions [5, 37, 48]. The mechanisms leading to this increased phosphorylation in cell lines are not currently known, but they may be related to the protecting role of TDP-43 over apoptosis in human cells, accounting the fact that TDP-43 deletion increases p-Rb-mediated cell death [5].

In any case, the present report linking neurodegeneration-basic mechanisms to TDP-43 abnormalities supports the notion that TDP-43 mutations are not necessary for its pathological role. Thus, in rodent models, TDP-43 overexpression leads to TDP-43 inclusions and neurodegenerative phenotypes compatible with sporadic ALS [45, 46]. Furthermore, previous results demonstrating colocalization of "stress granules" with mutated TDP-43 [12, 16, 30, 32] are extended in the present study, where it is clearly shown that cell stress leads to native TDP-43 pathogenical changes, without apparent participation of "stress granules". Thus, neither p-ERK1/2 nor TDP-43 aggregates were colocalized with TIA-1 immunostaining, a marker of stress granules, in any of the stress conditions examined. The relationship between those different aggregates linked to pathology will be the focus of future studies. Nevertheless, our results with chronic excitotoxicity in organotypic cultures of spinal cord and stress induction in different neuronal cell lines from different species allows to propose their use as

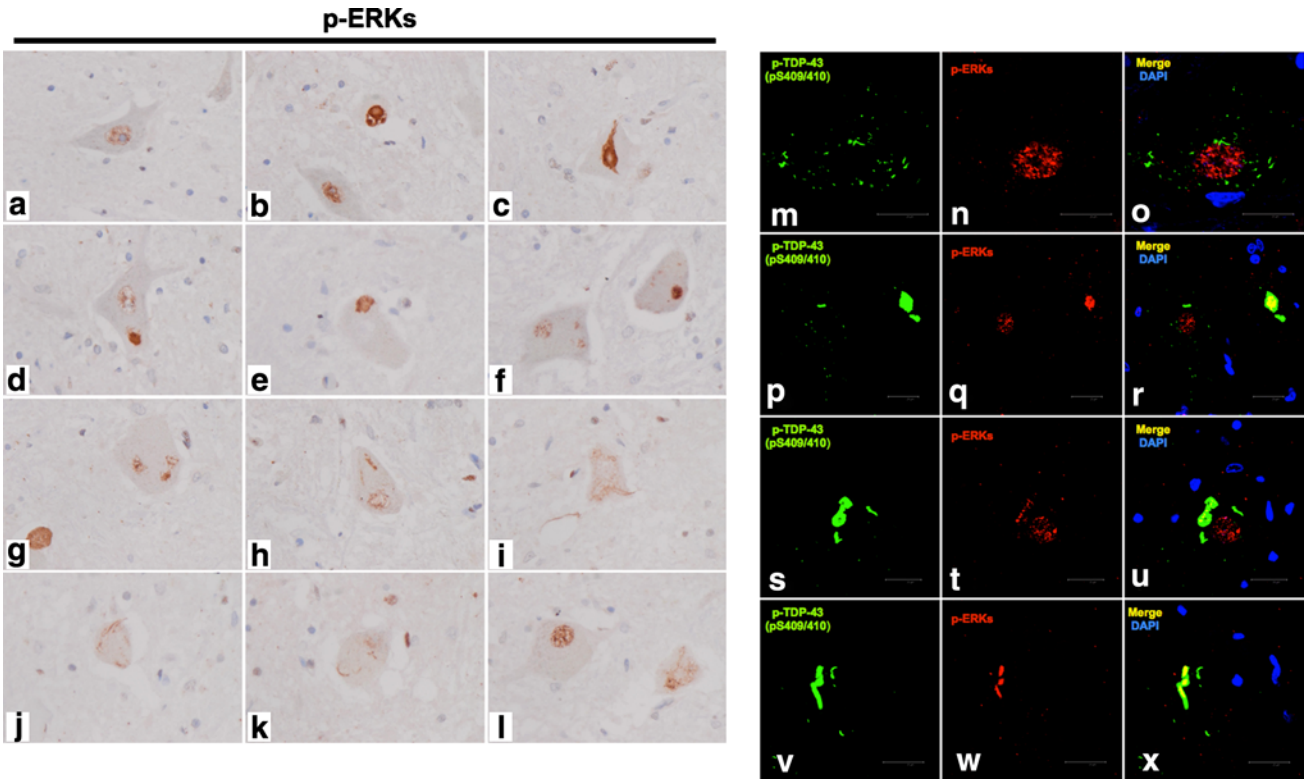


Fig. 7 Altered p-ERK1/2 deposition is restricted to motor neurons with p-TDP-43-immunoreactive deposits in ALS. *Left panel* p-ERK1/2 immunoreactivity in the nucleus of motor neurons in normal spinal cord (**a**) and abnormal, globular nuclear p-ERK1/2 inclusions in motor neurons in ALS (**b**, **c**), dense (**e**, **f**), fine granular or rectilinear (**g**, **h**), skein-like (**i**, **j**) or meshwork-like (**k**, **l**) cytoplasmic inclusions, accompanied by decreased or absence p-ERK1/2 immunoreactivity only in spinal cord motor neurons in ALS cases. Paraffin sections slightly counterstained with hematoxylin. *Right panel* Double-labeling immunofluorescence and confocal microscopy showing altered p-ERK1/2 deposition in ALS motor neurons with p-TDP-43 immunoreactive deposits (**m**–**r**). Sub-cellular co-localization of abnormal p-ERK1/2 and p-TDP-43 is also observed in altered ALS motor neurons (**s**–**x**). Nuclei were stained with DAPI (*blue*). *Scale bars* 30 μm

preclinical model in the screening of agents potentially affecting TDP-43 pathogenic changes.

An impairment of nuclear import mechanisms could play an important role in ALS pathogenesis, based on abnormal location of TDP-43 (a nuclear factor) [28, 36]. As it is known that oxidative stress impairs nuclear import by a mechanism depending on ERK1/2 activation [11], we tested whether ERK1/2 activation is related to TDP-43 pathogenic changes observed in our neuronal cell models exposed to different stress conditions. If this was the case, ERK1/2 pathway inhibition could relieve stress-associated TDP-43 pathogenicity. Oxidative and ER stress induced increased levels of p-ERK1/2 in a transient or sustained manner, in accordance with this hypothesis. However, inhibition of these kinases, far from avoiding these changes, exacerbated them, even in control conditions. In the case of proteasome stress, depletion of p-ERK1/2 is strongly associated with TDP-43 pathogenic phenomena. Our results suggest that ERK1/2 plays a previously unreported significant role in TDP-43 cellular homeostasis and cellular wellness, as specific ERK inhibition leads to TDP-43 fragmentation, cytosolic aggregation and increased phosphorylation. So the initial activation of ERK1/2 promoted by cell stress in both neuronal cell lines could be related to the first cellular response to a stress situation, since its modulation has been associated with protection mechanisms in others neuronal cell lines [44]. These results are also compatible with the known physiological role of ERK1/2 regulated pathways, acting to promote survival or death, depending upon the cellular context in which they are activated [27]. In fact, our results show that ERK1/2 activation is needed for cell survival under stress, as shown by viability assays. Several neurological insults linked to excessive release of glutamate and neuronal death result in tyrosine kinase activation, including ERK1/2. Furthermore, oxidative stress is a known activator of ERK1/2 pathway [27] as in our hands seems to be ER stress [2] and proteasome stress. In line with the observations in vitro, the present study has also evidenced, for the first time, aggregation of phosphorylated ERK1/2 in motor neurons in ALS, with a close relationship with TDP-43 inclusions. Thus, abnormal p-ERK1/2 is only found in motor neurons with altered TDP-43 deposition, further supporting an association but not necessarily a causal relationship

between abnormal TDP-43 expression and ERK1/2 signaling in this disease. Concerning a potential direct interaction between TDP-43 and ERK1/2, it has been recently shown that interactome of TDP-43 consists of proteins implicated in pre-mRNA splicing and RNA stability and transport, as well as other neuron-enriched proteins, methyl CpG-binding protein 2 and polypyrimidine tract-binding protein 2, besides others. Nevertheless, no direct interaction with ERK1/2 was reported [43]. These data do not exclude that these identified proteins may interact with TDP-43 and ERK1/2, an issue that merits further exploration.

In conclusion, the present work evidences interactions between TDP-43 and stress mechanisms involved in ALS development. Furthermore, a novel finding, implicating ERK1/2 inhibition as a potential contributor to TDP-43 pathogenetical changes, is presented for explaining p-ERK1/2 aggregates in human motor neuron disease.

Acknowledgments We are indebted to tissue donors and their families. This work was supported by grants from Spanish Ministry of Education and Science [grant numbers BFU 2009-11879/BFI, AGL2006-12433, BFU 2009-06427/E]; the Generalitat of Catalunya [grant number 2009SGR-735]; the Spanish Ministry of Health [grant number PI08-1843 to M.P.O., BESAD-P and PI08-0582 to I.F.]; the ALS Catalan Foundation [to I.F.]; and "La Caixa" Foundation. Supported also by the COST B-35 Action. V.C has been supported by a predoctoral fellowship from Govern Balear, Conselleria d' Economia Hisenda i Innovació and D.C. by a predoctoral fellowship from the Instituto de Salud Carlos III.

References

1. Amador-Ortiz C, Lin WL, Ahmed Z et al (2007) TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. Ann Neurol 61:435–445
2. Arai K, Lee SR, van Leyen K et al (2004) Involvement of ERK MAP kinase in endoplasmic reticulum stress in SH-SY5Y human neuroblastoma cells. J Neurochem 89:232–239
3. Arai T, Hasegawa M, Akiyama H et al (2006) TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Biochem Biophys Res Commun 351:602–611
4. Atkin JD, Farg MA, Walker AK et al (2008) Endoplasmic reticulum stress and induction of the unfolded protein response in human sporadic amyotrophic lateral sclerosis. Neurobiol Dis 30:400–407
5. Ayala YM, Misteli T, Baralle FE (2008) TDP-43 regulates retinoblastoma protein phosphorylation through the repression of cyclin-dependent kinase 6 expression. Proc Natl Acad Sci USA 105:3785–3789
6. Ballif BA, Blenis J (2001) Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 12:397–408
7. Brady OA, Meng P, Zheng Y et al (2011) Regulation of TDP-43 aggregation by phosphorylation and p62/SQSTM1. J Neurochem 116:248–259
8. Buratti E, Brindisi A, Giombi M et al (2005) TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing. J Biol Chem 280:37572–37584
9. Buratti E, Baralle FE (2001) Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9. J Biol Chem 276:36337–36343
10. Bush KT, Goldberg AL, Nigam SK (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem 272:9086–9092
11. Czubryt MP, Austria JA, Pierce GN (2000) Hydrogen peroxide inhibition of nuclear protein import is mediated by the mitogen-activated protein kinase, ERK2. J Cell Biol 148:7–16
12. Dewey CM, Cenik B, Sephton CF et al (2011) TDP-43 is directed to stress granules by sorbitol, a novel physiological osmotic and oxidative stressor. Mol Cell Biol 31:1098–1108
13. Dormann D, Capell A, Carlson AM et al (2009) Proteolytic processing of TAR DNA binding protein-43 by caspases produces C-terminal fragments with disease defining properties independent of progranulin. J Neurochem 110:1082–1094
14. Ferrante RJ, Browne SE, Shinobu LA et al (1997) Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem 69:2064–2074
15. Foulds P, McAuley E, Gibbons L et al (2008) TDP-43 protein in plasma may index TDP-43 brain pathology in Alzheimer's disease and frontotemporal lobar degeneration. Acta Neuropathol 116:141–146
16. Freibaum BD, Chitta RK, High AA et al (2010) Global analysis of TDP-43 interacting proteins reveals strong association with RNA splicing and translation machinery. J Proteome Res 9:1104–1120
17. Gendron TF, Josephs KA, Petrucelli L (2010) Review: transactive response DNA-binding protein 43 (TDP-43): mechanisms of neurodegeneration. Neuropathol Appl Neurobiol 36:97–112
18. Giordana MT, Piccinini M, Grifoni S et al (2010) TDP-43 redistribution is an early event in sporadic amyotrophic lateral sclerosis. Brain Pathol 20:351–360
19. Granado-Serrano AB, Martin MA, Haegeman G et al (2010) Epicatechin induces NF-kappaB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells. Br J Nutr 103:168–179
20. Henkel JS, Engelhardt JI, Siklos L et al (2004) Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. Ann Neurol 55:221–235
21. Higashi S, Iseki E, Yamamoto R et al (2007) Concurrence of TDP-43, tau and alpha-synuclein pathology in brains of Alzheimer's disease and dementia with Lewy bodies. Brain Res 1184:284–294
22. Hirano A, Nakano I, Kurland LT et al (1984) Fine structural study of neurofibrillary changes in a family with amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 43:471–480
23. Ilieva EV, Ayala V, Jove M et al (2007) Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis. Brain 130:3111–3123
24. Jung T, Engels M, Kaiser B et al (2006) Intracellular distribution of oxidized proteins and proteasome in HT22 cells during oxidative stress. Free Radic Biol Med 40:1303–1312
25. Kabashi E, Valdmanis PN, Dion P et al (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. Nat Genet 40:572–574

26. Kametani F, Nonaka T, Suzuki T et al (2009) Identification of casein kinase-1 phosphorylation sites on TDP-43. Biochem Biophys Res Commun 382:405–409
27. Kim EK, Choi EJ (2010) Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802:396–405
28. Kinoshita Y, Ito H, Hirano A et al (2009) Nuclear contour irregularity and abnormal transporter protein distribution in anterior horn cells in amyotrophic lateral sclerosis. J Neuropathol Exp Neurol 68:1184–1192
29. Kuo PH, Doudeva LG, Wang YT et al (2009) Structural insights into TDP-43 in nucleic-acid binding and domain interactions. Nucleic Acids Res 37:1799–1808
30. Liu-Yesucevitz L, Bilgutay A, Zhang YJ et al (2010) Tar DNA binding protein-43 (TDP-43) associates with stress granules: analysis of cultured cells and pathological brain tissue. PLoS One 5:e13250
31. Maris C, Dominguez C, Allain FH (2005) The RNA recognition motif, a plastic RNA-binding platform to regulate post-transcriptional gene expression. FEBS J 272:2118–2131
32. McDonald KK, Aulas A, Destroismaisons L et al (2011) TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1. Hum Mol Genet 20:1400–1410
33. Menzies FM, Ince PG, Shaw PJ (2002) Mitochondrial involvement in amyotrophic lateral sclerosis. Neurochem Int 40:543–551
34. Nakashima-Yasuda H, Uryu K, Robinson J et al (2007) Comorbidity of TDP-43 proteinopathy in Lewy body related diseases. Acta Neuropathol 114:221–229
35. Neumann M, Sampathu DM, Kwong LK et al (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. Science 314:130–133
36. Nishimura AL, Zupunski V, Troakes C et al (2010) Nuclear import impairment causes cytoplasmic trans-activation response DNA-binding protein accumulation and is associated with frontotemporal lobar degeneration. Brain 133:1763–1771
37. Nonaka T, Arai T, Buratti E et al (2009) Phosphorylated and ubiquitinated TDP-43 pathological inclusions in ALS and FTLD-U are recapitulated in SH-SY5Y cells. FEBS Lett 583:394–400
38. Ou SH, Wu F, Harrich D et al (1995) Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. J Virol 69:3584–3596
39. Rothstein JD, Jin L, Dykes-Hoberg M et al (1993) Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA 90:6591–6595
40. Rothstein JD, Tsai G, Kuncl RW et al (1990) Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. Ann Neurol 28:18–25
41. Sasaki S, Takeda T, Shibata N et al (2010) Alterations in subcellular localization of TDP-43 immunoreactivity in the anterior horns in sporadic amyotrophic lateral sclerosis. Neurosci Lett 478:72–76
42. Schwab C, Arai T, Hasegawa M et al (2008) Colocalization of transactivation-responsive DNA-binding protein 43 and huntingtin in inclusions of Huntington disease. J Neuropathol Exp Neurol 67:1159–1165
43. Sephton CF, Cenik C, Kucukural A et al (2011) Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes. J Biol Chem 286:1204–1215
44. Spencer JP, Rice-Evans C, Williams RJ (2003) Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J Biol Chem 278:34783–34793
45. Tatom JB, Wang DB, Dayton RD et al (2009) Mimicking aspects of frontotemporal lobar degeneration and Lou Gehrig's disease in rats via TDP-43 overexpression. Mol Ther 17:607–613
46. Wils H, Kleinberger G, Janssens J et al (2010) TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. Proc Natl Acad Sci USA 107:3858–3863
47. Wood JD, Beaujeux TP, Shaw PJ (2003) Protein aggregation in motor neurone disorders. Neuropathol Appl Neurobiol 29:529–545
48. Yamashita M, Nonaka T, Arai T et al (2009) Methylene blue and dimebon inhibit aggregation of TDP-43 in cellular models. FEBS Lett 583:2419–2424
49. Zhang YJ, Xu YF, Cook C et al (2009) Aberrant cleavage of TDP-43 enhances aggregation and cellular toxicity. Proc Natl Acad Sci USA 106:7607–7612

doi:10.1093/brain/awm190 *Brain* (2007), **130**, 3111–3123

Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis

Ekaterina V. Ilieva,[1,*] Victòria Ayala,[1,*] Mariona Jové,[1] Esther Dalfó,[2] Daniel Cacabelos,[1] Mónica Povedano,[3] Maria Josep Bellmunt,[1] Isidre Ferrer,[2,4] Reinald Pamplona[1] and Manuel Portero-Otín[1]

[1]Fisiopatologia Metabòlica, IRBLLEIDA, Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida, Lleida, Spain, [2]Institut de Neuropatologia, Servei Anatomia Patològica, IDIBELL-Hospital Universitari de Bellvitge, [3]Servei de Neurologia, Hospital Universitari de Bellvitge and [4]Facultat de Medicina, Universitat de Barcelona, Hospitalet de Llobregat, Spain

*These authors contributed equally to this work.

Correspondence to: Manuel Portero-Otín, MD, PhD, IRBLLEIDA, Departament de Medicina Experimental, Facultat de Medicina, Universitat de Lleida, C/Montserrat Roig, 2, 25008 Lleida, Spain
E-mail: manuel.portero@cmb.udl.es

The occurrence of endoplasmic reticulum (ER) stress in the sporadic form of amyotrophic lateral sclerosis (ALS) is unknown, despite it has been recently documented in experimental models of the familial form. Here we show that spinal cord from patients with sporadic ALS showed signs of ER stress, such as increased levels of ER chaperones such as protein-disulfide isomerase, and increased phosphorylation of eukaryotic initiation factor 2α (eIF2α). Among the potential causes of such ER stress proteasomal impairment was confirmed in the same samples by demonstrating increased ubiquitin immunoreactivity and increased protein lipoxidative (125%), glycoxidative (55%) and direct oxidative damage (62%) over control values, as evidenced by mass-spectrometry and immunological methods. We found that protein oxidative damage was strongly associated to ALS-specific changes in fatty acid concentrations, specifically of *n*-3 series (as docosahexaenoic acid), and in the amount of mitochondrial components as respiratory complexes I and III, suggesting a mitochondrial dysfunction leading to increased free radical production. Oxidative stress was also evidenced in frontal cortex, suggesting that this region is affected early in ALS. As those events were partially reproduced by threohydroxyaspartate exposure in organotypic spinal cord cultures, we concluded that changes in fatty acid composition, mitochondrial function and proteasome activity, which may be driven by excitotoxicity, lead to oxidative stress and finally contribute to ER stress in sporadic ALS.

Keywords: Proteasome; glycation; lipoxidation; mitochondria; motor neuron

Abbreviations: ER = endoplasmic reticulum; GSA = glutamic semialdehyde; AASA = aminoadipic semialdehyde

Received February 9, 2007. Revised July 5, 2007. Accepted July 20, 2007. Advance Access publication August 23, 2007

Introduction

Amyotrophic lateral sclerosis is a multifactorial disease whose pathophysiological mechanisms include decreased availability to neurotrophic factors, disturbances in calcium metabolism, increased neuroinflammatory status, cytoskeletal changes and oxidative stress (Dupuis *et al.*, 2004; Rao and Weiss, 2004; Strong *et al.*, 2005; Boillee *et al.*, 2006). Recent data indicate (Atkin *et al.*, 2006; Turner and Atkin, 2006) that endoplasmic reticulum (ER) stress may be also involved in the familial form of the disease. ER stress, a complex pattern involving highly specific signalling pathways, ensures through the so-called unfolded protein response that protein folding capacities of ER are not overwhelmed. However, prolonged ER stress could contribute to cell death, both by mitochondria-dependent and independent pathways (Lindholm *et al.*, 2006). In contrast with the familial form, no data was available on the occurrence of ER stress in the more common, sporadic form of the disease.

Some of the pathological hallmarks of ALS, such as increased ubiquitinated bodies, neuronal and astrocytic hyaline inclusions as well as axonal spheroids are protein aggregates that may be related to ER and oxidative stress. This fact is based on the relationship between protein oxidative damage and proteasomal activity following an inverted U shape, i.e. while moderate oxidative

modification of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity (Grune *et al.*, 1996; Sitte *et al.*, 2000; Grune *et al.*, 2004). Such a decreased proteasomal activity has been previously recognized in other neurodegenerative processes, and it may explain ALS-characteristic increased ubiquitination and presence of proteinaceous aggregates. The disruption of the ER-associated degradation, a pathway which helps to clear misfolded protein species from ER, is a potential consequence of such proteasomal impairment, finally contributing to ER stress (Marciniak and Ron, 2006; Oyadomari *et al.*, 2006; Zhang and Kaufman, 2006).

Despite these data suggesting the importance of protein oxidative damage in ALS, its study by using selective, chemically characterized markers has not been reported, though immunohistochemical evidences support ALS-induced increased oxidative damage (Ferrante *et al.*, 1997; Pedersen *et al.*, 1998; Shibata *et al.*, 1999; Kato *et al.*, 2000; Kikuchi *et al.*, 2000; Shibata *et al.*, 2000; Kikuchi *et al.*, 2002). Such molecular dissection, allowing quantitative analyses of oxidative pathways should be useful for diagnostic and therapeutic approaches. The use of highly selective mass spectrometry-based techniques could help to further delineate the potential pathogenic role of oxidative stress in ALS. Several markers could be used, such as glutamic semialdehyde (GSA), which derives from the metal-catalysed oxidation of proline and arginine; or aminoadipic semialdehyde (AASA) which results from lysine oxidation (Requena *et al.*, 1997, 2001, 2003; Dalfo *et al.*, 2005). Besides these direct modifications of protein structures, the effects of free radical efflux in proteins could also involve third-party molecules which also give rise to increased damage, such as carbohydrates and/or lipids, in processes termed glycoxidation and lipoxidation, respectively (Requena *et al.*, 1997; Baynes, 2003). Both carbohydrates and polyunsaturated fatty acids, when reacting with free radicals generate highly reactive dicarbonyl compounds, such as glyoxal, methylglyoxal, 4-hydroxynonenal and malondialdehyde, among others. These reactive carbonyl compounds can generate specific non-enzymatic adducts when reacting with proteins, such as N^{ε}-carboxymethyl-lysine (CML), N^{ε}-carboxyethyl-lysine (CEL) and N^{ε}-malondialdehyde-lysine (MDAL) (Baynes and Thorpe, 2000). The high content of polyunsaturated fatty acid in central nervous system and its elevated oxygen consumption support the possible significance of lipid peroxidation-derived processes in neurodegeneration, including ALS. However, there is no chemical evidence for lipoxidative or glycoxidative damage of proteins in sporadic or familial ALS based on structural identification and supported by mass-spectrometry.

For these reasons, in this work we have studied the development of ER stress in sporadic ALS. We examined ER stress causal factors such as proteasome function, protein oxidative damage, fatty acid composition and potential disturbances in mitochondrial respiratory complexes (as the major sources of free radical efflux). These changes have been evaluated in human samples and in lumbar spinal cord organotypic cultures under chronic excitotoxicity, a well-supported model of the sporadic form of ALS (Rothstein *et al.*, 1993).

Patients and methods

Human spinal cord and brain specimens

Brain and spinal cord samples were obtained from the Institute of Neuropathology Brain bank following the guidelines of the local Ethics Committee. The brains and spinal cords of seven men and five age-matched controls (four men and one woman) were obtained from 3 to 6 h after death, and were immediately prepared for morphological and biochemical studies. The agonal state was short with no evidence of acidosis or prolonged hypoxia. The pH of the post-mortem brain was between 7 and 7.4. All ALS patients had suffered from clinical signs and symptoms of lower and upper motorneuron disease, finally involving motor nuclei of the medulla oblongata. Importantly, none of these patients had cognitive impairment or dementia. Although variable from one case to another, the terminal stage of the disease was characterized by predominant bulbar failure manifested as impaired swallowing and usually complicated by aspiration pneumonia, or by dominant respiratory insufficiency. Age-matched controls did not show clinical and neuropathological anomalies. Frozen samples of the spinal cord (lumbar enlargement) and frontal cortex area 8 were used for biochemical studies. Samples of control and diseased spinal cords and brains were processed in parallel. Summary of the main clinical and neuropathological aspects is shown in Table 1.

Organotypic cultures

Spinal cord cultures were prepared from lumbar spinal cord of postnatal day 8 rat pups as previously described (Rothstein *et al.*, 1993) and maintained in 50% minimal essential medium, 25 mM Hepes, 25% Hanks balanced salt solution with D-glucose 25.6 mg/l, 25% heat-inactivated horse serum, 2 mM L-glutamine. Incubation at DIV7 of the slices with the glutamate transport inhibitor D,L-threo-hydroxyaspartate (THA) at 200 μM injures motorneurons with a morphology typical of excitotoxic degeneration after several weeks of treatment. In selected experiments, slices were also incubated with the ER stress inducers thapsigargin (32 ng/ml) and tunicamycin (500 and 5 ng/ml). After 15 or 30 days of treatment, cultures were harvested and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C and processed for immunocytochemistry. For western-blot and mass-spectrometric measurements slices were washed with PBS containing 1 mM diethylenetriaminepentaacetic acid and 1 μM butylated hydroxyl toluene, harvested and frozen at −80°C. Experiments for each condition ($n = 30$ slices per experimental group) were repeated at least three times.

Immunohistochemistry

De-waxed sections 5-μm-thick of the spinal cord were processed for immunohistochemistry following the streptavidin LSAB method (Dako). After incubation with methanol and H_2O_2 in PBS and normal serum, the sections were incubated with anti-phosphorylated eukaryotic initiation factor 2α (eIF2α) (1:100,

Table 1 Summary of clinical and pathological data in the present series

Case	Age	Gender	Diagnosis	Duration	Cause of death	p-m delay
1	41	M	ALS	3	Dysphagia, bronchopneumonia	3
2	68	M	ALS	5	Respiratory failure	3
3	76	M	ALS	4	Dysphagia, bronchopneumonia	3
4	69	M	ALS	4	Dysphagia, bronchopneumonia	2
5	40	M	ALS	3	Respiratory failure	2
6	73	M	ALS	4	Dysphagia, bronchopneumonia	4
7	71	M	ALS	4	Respiratory failure	6
8	75	M	Control		Neoplasia	6
9	76	M	Control		Cardiac infarction	4
10	67	F	Control		Cardiac infarction	3
11	51	M	Control		Bronchopneumonia	4
12	68	M	Control		Neoplasia	6

Note: ALS: amyotrophic lateral sclerosis; p-m delay: post-mortem delay (in hours); age and duration are in years.

Abcam, UK). Following incubation with the primary antibody, the sections were incubated with LSAB for 15 min at room temperature. The peroxidase reaction was visualized with diaminobenzidine and H_2O_2. Control of the immunostaining included omission of the primary antibody; no signal was obtained following incubation with only the secondary antibody. Sections were slightly counterstained with haematoxylin.

Confocal immunocytochemistry

The antibodies used are listed in the supplementary information (Table S1). Fluorescein-conjugated *Bandeiraea simplicifolia* lectin (type I) was used as a label for microglia. Appropriate secondary antibodies: Alexa Fluor 488 $F(ab)_2$ fragment of goat anti-mouse IgG (1:500, Molecular Probes, USA) and Alexa Fluor 546 goat anti-rabbit IgG (1:500, Molecular Probes, USA) were used for immunofluorescence. Image analyses were carried out by a single investigator who was blinded to the experimental conditions. Large (>25 μm in diameter) SMI-32-immunopositive neurons were identified as motorneurons. Immunocytochemical controls were performed by the omission of the primary antibodies, resulting in a negative immunostaining in all cases studied. Mounted slices were examined under a FluoView 500 Olympus confocal laser scanning microscope (Hamburg, Germany).

Protein electrophoresis and western blot

Samples (spinal cord, frontal cortex or organotypic spinal cord cultures) were homogenized in a buffer containing 180 mM KCl, 5 mM 3-[*N*-morpholino]propanesulfonic acid, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM diethylenetriaminepentaacetic acid and 1 μM butylated hydroxyl toluene, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, pH 7.3 with a Potter–Eljeveim device at 4°C. After a brief centrifugation (500 × *g*, 5 min) to pellet cellular debris, protein concentrations were measured in the supernatants using the Lowry assay (BioRad Laboratories, München, Germany).

For detection of protein carbonyls, and prior to electrophoresis, samples were derivatized with 2,4-dinitrophenylhydrazine (DNP) as previously described (Pamplona *et al.*, 2005). Briefly, to 15 μl homogenates adjusted to 3.75 μg/μl protein SDS was added to a final concentration of 6%, and, after boiling for 3 min, 20 μl of 10 mM DNP in 10% trifluoroacetic acid were added. After 7 min at room temperature, 20 μl of a solution containing 2 M Tris base, 30% glycerol and 15% β-mercaptoethanol were added for neutralization and sample preparation for loading onto SDS–PAGE gels.

For immunodetection, after SDS–PAGE, proteins were transferred using a Mini Trans-Blot Transfer Cell (BioRad) to PVDF membranes (Immobilon-P Millipore, Bedford, MA). Immunodetection was performed using as primary antibodies those listed in supplementary information (Table S1): (i) for ER stress and proteasome function assessment: anti-KDEL, which recognizes KDEL-sequence containing proteins such as ER chaperones as protein disulfide isomerase (PDI), GRP78 and GRP94; anti-eIF2*α* anti-GRP78, anti-phosphorylated eIF2*α* (S52), anti-PDI and anti-ubiquitin; (ii) for protein oxidative damage: anti-DNP antibody, anti-CML, anti-neuroketal and anti-MDAL; and (iii) for mitochondrial studies: anti-NDUFA 9 antibody for respiratory complex I, anti-core II antibody for respiratory complex III and anti-apoptosis inducing factor (AIF) antibody. For detection of primary antibodies, the following peroxidase-coupled secondary antibodies were used: sheep anti-mouse (1:30 000, Amersham, USA); anti-rabbit (1:40 000, Pierce, USA) and anti-goat (1:7500, Abcam, Cambridge, UK) antibodies. Luminescence was recorded and quantified in a Lumi-Imager equipment (Boehringer, Mannheim, Germany), using the Lumianalyst software. Control experiments showed that omission of primary or secondary antibody addition produced blots with no detectable signal.

Measurement of specific, protein-oxidation-derived markers: GSA, AASA, CML, CEL and MDAL

GSA, AASA, CML, CEL and MDAL concentrations in total proteins from spinal cord, frontal cortex or organotypic culture homogenates were measured by isotope-dilution gas chromatography/mass spectrometry (GC/MS) as previously described (Pamplona *et al.*, 2005). Samples containing 500 μg of protein were delipidated using chloroform:methanol (2:1 v/v), and proteins were precipitated by adding 10% trichloroacetic acid (final concentration) and subsequent centrifugation. Protein samples were reduced overnight with 500 mM $NaBH_4$ (final concentration) in 0.2 M borate buffer, pH 9.2, containing 1 drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 ml of 20% trichloroacetic acid and subsequent centrifugation. The following isotopically labelled internal standards were then added: $[^2H_8]$Lysine (*d8*-Lys; CDN Isotopes); $[^2H_4]$CML (d4-CML), $[^2H_4]$CEL (*d4*-CEL) and $[^2H_8]$MDAL (*d8*-MDAL); $[^2H_5]$ 5-hydroxy-2-aminovaleric acid (for GSA analysis) and $[^2H_4]$6-hydroxy-2-aminocaproic acid (for AASA analysis). The samples were hydrolysed at 155°C for 30 min in 1 ml of 6 N HCl, and then dried *in vacuo*. The *N*,*O*-trifluoroacetyl methyl ester derivatives of the protein hydrolysate were prepared and GC/MS analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) coupled to a Hewlett-Packard model 5973A mass selective detector (Agilent, Barcelona, Spain). The injection port was maintained at

275°C; the temperature program was 5 min at 110°C, then 2°C/min to 150°C, then 5°C/min to 240°C, then 25°C/min to 300°C and finally hold at 300°C for 5 min. Quantification was performed by external standardization using standard curves constructed from mixtures of deuterated and non-deuterated standards. Analytes were detected by selected ion-monitoring GC/MS. The ions used were: lysine and *d8*-lysine, *m/z* 180 and 187, respectively; 5-hydroxy-2-aminovaleric acid and *d5*-5-hydroxy-2-aminovaleric acid (stable derivatives of GSA), *m/z* 280 and 285, respectively; 6-hydroxy-2-aminocaproic acid and *d4*-6-hydroxy-2-aminocaproic acid (stable derivatives of AASA), *m/z* 294 and 298, respectively; CML and *d4*-CML, *m/z* 392 and 396, respectively; CEL and *d4*- CEL, *m/z* 379 and 383, respectively; and MDAL and *d8*-MDAL, *m/z* 474 and 482, respectively. The amounts of products were expressed as the ratio μmol GSA, AASA, CML, CEL or MDAL/mol lysine.

Fatty acid analysis

Distributional analysis of fatty acids was performed as previously described (Dalfo *et al.*, 2005; Pamplona *et al.*, 2005). Total lipids from spinal cord, frontal cortex or organotypic cultures were extracted with chloroform:methanol (2:1, v/v) in the presence of 0.01% butylated hydroxytoluene to avoid artifactual oxidation. The chloroform phase was evaporated under nitrogen, and the fatty acids were transesterified by incubation in 2.5 ml of 5% methanolic HCl for 90 min at 75°C. The resulting fatty acid methyl esters were extracted by adding 2.5 ml of *n*-pentane and 1 ml of saturated NaCl solution. The *n*-pentane phase was separated, evaporated under nitrogen, re-dissolved in 75 μl of hexane and 1 μl was used for GC/MS analysis. Separation was performed in a SP2330 capillary column (30 m × 0.25 mm × 0.20 μm) in a Hewlett Packard 6890 Series II gas chromatograph (Agilent, Barcelona, Spain). A Hewlett Packard 5973A mass spectrometer was used as detector in the electron-impact mode. The injection port was maintained at 220°C, and the detector at 250°C; the temperature program was 2 min at 100°C, then 10°C/min to 200°C, then 5°C/min to 240°C and finally hold at 240°C for 10 min. Identification of fatty acid methyl esters was made by comparison with authentic standards and based on mass spectra. Results are expressed as mol%.

From fatty acid composition, the following indexes were calculated: saturated fatty acids (SFA) = ∑% of saturated fatty acids; unsaturated fatty acids (UFA) = ∑% unsaturated fatty acids; monounsaturated fatty acids (MUFA) = ∑% of monoenoic fatty acids; polyunsaturated *n*-3 fatty acids (PUFAn-3) = ∑% of polyunsaturated fatty acids *n*-3 series; Polyunsaturated n-6 Fatty Acids (PUFAn-6) = ∑% of polyunsaturated fatty acids *n*-6 series; average chain length (ACL) = $[(\sum\%\text{Total}_{14} \times 14) + \cdots + (\sum\%\ \text{Total}_n \times n)]/100$ (n = carbon atom number); peroxidizability index (PI) = [(∑ mol% Monoenoic × 0.025) + (∑ mol% Dienoic × 1) + (∑ mol% Trienoic × 2) + (∑ mol% Tetraenoic × 4) + (∑ mol% Pentaenoic × 6) + (∑ mol% Hexaenoic × 8)].

Statistical analyses

All statistics were performed using the SPSS software (SPSS Inc., Chicago, IL). Once normality of distribution was assessed by Kolmogorov–Smirnov test, differences between groups (ALS samples versus control; THA treated versus vehicle) were analysed by the Student's t-tests and correlations between variables were evaluated by the Pearson's statistic. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

Samples from ALS patients evidence signs of ER stress: proteasomal impairment as a potential mechanism

Since ER stress has been implied in ALS experimental models we examined signs of ER stress by western-blot and immunohistochemistry in samples from ALS patients. The results demonstrated ER stress in ALS samples (Fig. 1). Thus, increased expression of chaperones PDI and KDEL-containing proteins were found in spinal cord from ALS patients (Fig. 1A), but not in frontal cortex samples (not shown). This was accompanied by a marked increase in eIF2α phosphorylation (a sign of protein synthesis control after ER stress), both in spinal cord lysates (Fig. 1A) and in remaining neuronal bodies in spinal cord (Fig. 1B).

As proteasome is responsible for degradation of ER misfolded proteins, we examined whether its function was preserved. Accounting that ubiquitin-modified proteins are degraded by the proteasome, its function can be inferred by western-blot and immunohistochemical analyses of ubiquitin-modified proteins. The results of such approach showing increased protein ubiquitination in spinal cord from ALS patients, but not in frontal cortex, are compatible with compromised proteasomal function (Fig. 1C). It should be also considered that increased protein turnover due to massive protein degradation could saturate the ubiquitin-proteasome system in spite of proteasome preserved activity.

Proteins from spinal cord and frontal cortex present structurally characterized oxidation products and the amount of these modifications increases with ALS, favoured by changes in fatty acid composition

While moderate oxidative modification of proteins increases their susceptibility for proteasome clearance, higher rates of oxidative modification actually inhibit proteasome activity. Therefore, we evaluated protein oxidative damage in spinal cord and frontal cortex samples, to ascertain whether proteasomal dysfunction could be related to increased protein oxidative damage. GC/MS analyses demonstrated that those proteins contained oxidation-derived products resulting from metal-catalysed oxidation, glycoxidation and lipoxidation. The more abundant products were those derived from metal-catalysed oxidation, AASA and GSA assuming almost 95% of measured markers. GSA stood as the more frequent modification, with levels being 30-fold higher than of those of AASA. The concentrations of both GSA and AASA were significantly higher in proteins from spinal cord ($P < 0.001$) and frontal cortex ($P < 0.01$) samples of ALS patients than in control age-matched individuals (Fig. 2A). Similarly to specific oxidation products, the concentrations of CEL and CML, glycoxidation products, were also significantly higher in

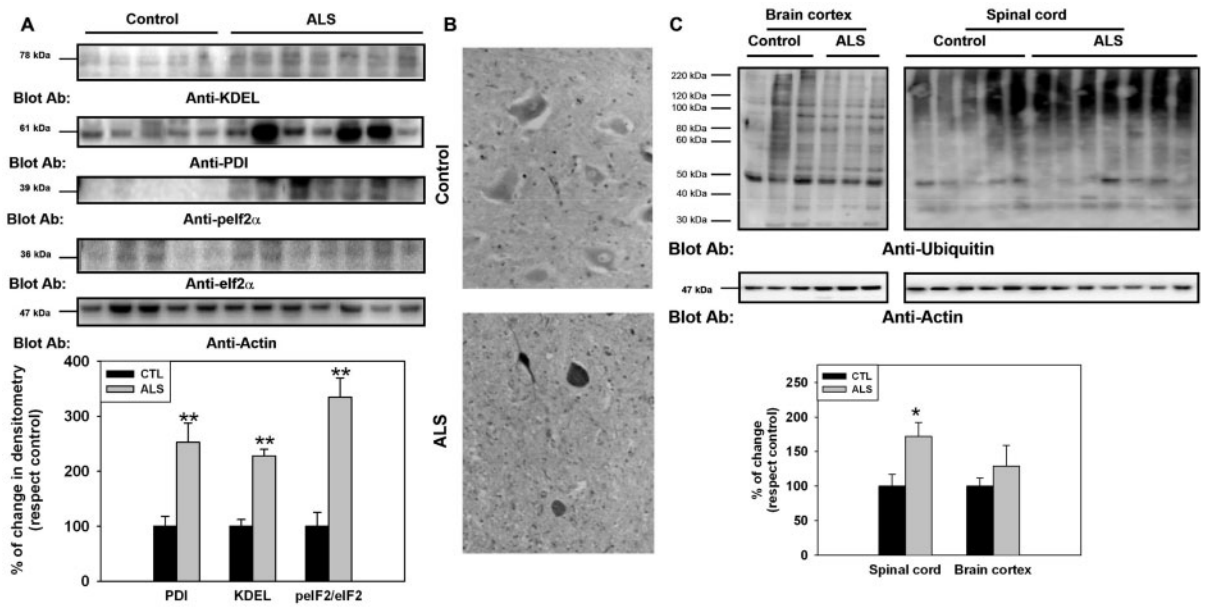


Fig. 1 ER stress and protein ubiquitination in spinal cords from ALS patients. (**A**) Representative western blot of spinal cord homogenates showing increased content of ER resident proteins containing KDEL motifs, PDI and phosphorylated eIF2α in samples from ALS patients. (**B**) Representative immunohistochemical image of phosphorylated eIF2α in the motor column of human lumbar spinal cords, showing increased staining in ALS samples. (**C**) Representative western blot of anti-ubiquitin, suggesting decreased ubiquitin degradation in spinal cord samples from ALS patients and control individuals, but not in frontal cortex. *Right numbers* of the blot indicate apparent molecular weight. Immunoblotting of actin is also shown. The *lower panels* shows the quantitation of those blots by densitometry, adjusted to actin content and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$ and $^{**}P < 0.001$.

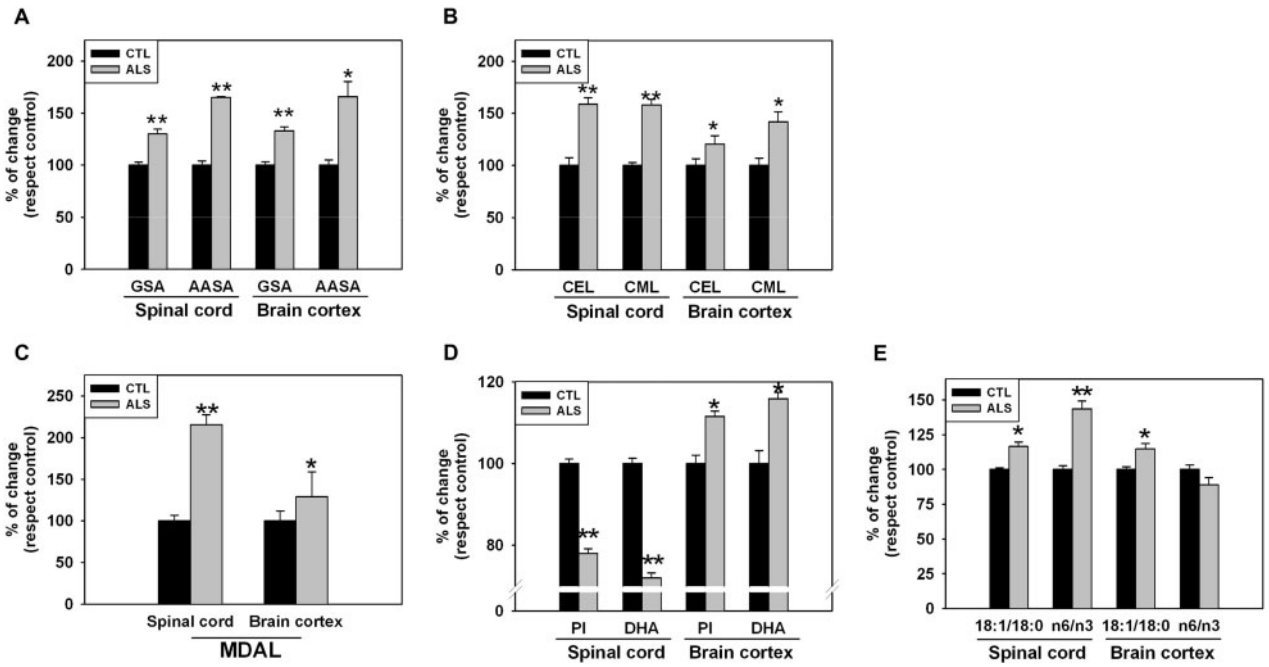


Fig. 2 Proteins from ALS samples show significant increases in the amounts of oxidation markers and changes in fatty acid composition. **A–C** show GC/MS analyses of GSA and AASA (markers of MCO), CML and CEL (arising from glycoxidation and lipoxidation) and MDAL, originated from lipoxidation. (**D**) Changes in PI and DHA levels associated with ALS in spinal cord differ from those present in frontal cortex. (**E**) ALS also leads to organ-specific changes in desaturation indexes (18:1/18:0) and *n*6/*n*3 ratios. Values shown are% changes of mean ± SE over values from control samples (in spinal cord GSA: 21498 ± 619 μmol/mol lysine; AASA: 102 ± 7 μmol/mol lysine; CEL: 385 ± 57 μmol/mol lysine; CML: 521 ± 30 μmol/mol lysine and MDAL: 258 ± 48 μmol/mol lysine; PI: 116.24 ± 4.23; DHA: 10.89 ± 0.20%; 18:1/18:0 ratio:1.36 ± 0.025; *n*6/*n*3 ratio: 0.67 ± 0.04; in frontal cortex GSA: 20512 ± 638 μmol/mol lysine; AASA: 82 ± 4 μmol/mol lysine; CEL: 249 ± 16 μmol/mol lysine; CML: 522 ± 37 μmol/mol lysine MDAL: 197 ± 6 μmol/mol lysine; PI: 155.31 ± 1.55; DHA: 12.35 ± 0.17; 18:1/18:0 ratio: 0.75 ± 0.01; *n*6/*n*3 ratio: 1.1 ± 0.034). $^{*}P < 0.01$ and $^{**}P < 0.001$ respect to control group by Student's *t*-test.

Table 2 Fatty acid composition (mol%) of total lipids from spinal cord and frontal cortex

	Frontal cortex			Spinal cord		
	CTL	ALS	P<	CTL	ALS	P<
14:0	0.50 ± 0.03	0.59 ± 0.05	0.234	0.49 ± 0.04	0.58 ± 0.01	0.112
16:0	24.57 ± 0.48	23.97 ± 0.35	0.352	14.61 ± 0.25	15.47 ± 0.23	0.02
16:1*n*-7	1.63 ± 0.15	1.64 ± 0.25	0.980	1 ± 0.07	1.53 ± 0.15	0.005
18:0	25.20 ± 0.40	22.92 ± 0.39	0.007	23.95 ± 0.23	22.34 ± 0.68	0.027
18:1*n*-9	19.09 ± 0.15	19.90 ± 0.45	0.141	30.15 ± 0.25	32.82 ± 0.25	0.001
18:2*n*-6	1.02 ± 0.28	0.77 ± 0.13	0.458	0.33 ± 0.03	0.62 ± 0.05	0.001
18:3*n*-3	n.d	n.d	–	0.24 ± 0.02	0.27 ± 0.001	0.254
20:0	0.88 ± 0.20	0.57 ± 0.04	0.190	7.05 ± 0.43	6.28 ± 0.25	0.117
20:1	n.d	n.d	–	0.54 ± 0.03	0.78 ± 0.07	0.016
20:2*n*-6	0.47 ± 0.16	0.29 ± 0.07	0.345	0.31 ± 0.03	0.31 ± 0.05	0.997
20:3*n*-6	0.36 ± 0.04	0.25 ± 0.05	0.210	0.6 ± 0.08	0.98 ± 0.25	0.137
20:4*n*-6	7.39 ± 0.23	8.70 ± 0.22	0.007	2.9 ± 0.17	3.15 ± 0.11	0.508
22:4*n*-6	4.16 ± 0.22	3.78 ± 0.42	0.460	2.86 ± 0.05	3.05 ± 0.14	0.289
22:5*n*-6	0.72 ± 0.14	0.66 ± 0.18	0.794	0.27 ± 0.03	0.22 ± 0.03	0.173
22:5*n*-3	0.17 ± 0.01	0.20 ± 0.01	0.170	0.14 ± 0.02	0.19 ± 0.03	0.118
24:0	0.61 ± 0.07	0.54 ± 0.04	0.466	0.91 ± 0.13	0.81 ± 0.15	0.600
24:1*n*-9	0.47 ± 0.14	0.56 ± 0.05	0.557	2.26 ± 0.10	2.23 ± 0.10	0.793
ACL	18.41 ± 0.02	18.49 ± 0.02	0.055	18.67 ± 0.01	18.51 ± 0.01	0.001
SFA	51.78 ± 0.58	48.60 ± 0.56	0.008	46.21 ± 0.37	44.55 ± 0.65	0.001
UFA	48.21 ± 0.58	51.39 ± 0.56	0.008	52.59 ± 0.35	54.92 ± 0.45	0.001
MUFA	21.20 ± 0.04	22.11 ± 0.73	0.263	33.84 ± 0.31	37.6 ± 0.46	0.001
PUFA	27.00 ± 0.58	29.28 ± 0.60	0.037	18.7 ± 0.22	16.61 ± 0.4	0.002
PUFA *n*-6	14.13 ± 0.33	14.47 ± 0.69	0.676	7.46 ± 0.18	8.39 ± 0.19	0.005
PUFA *n*-3	12.87 ± 0.40	14.81 ± 0.22	0.006	11 ± 0.15	8.63 ± 0.12	0.001
DBI	152.18 ± 2.68	166.92 ± 1.70	0.004	127.82 ± 1.58	119.69 ± 1.59	0.003

Note: Values: mean ± SEM. *N* × group: for brain cortex ($n = 4$); for spinal cord ($n = 5$ for control and $n = 7$ for ALS). ACL, average chain length; SFA, saturated fatty acids; UFA, unsaturated fatty acids; PUFA *n*-6/*n*-3, polyunsaturated fatty acids *n*-6 or *n*-3 series; MUFA, monounsaturated fatty acids; DBI, double bond index; Docosahexaenoic acid levels are shown in Fig. 1.

proteins from spinal cord ($P < 0.001$) and frontal cortex ($P < 0.01$) of ALS patients than in control individuals (Fig. 2B). The concentration of MDAL, a lipoxidation product, was also increased in samples from ALS patients, both in spinal cord ($P < 0.001$) and in frontal cortex ($P < 0.01$) compared to control individuals (Fig. 2C). Nonetheless, the magnitude of difference between ALS and control samples was considerably higher in spinal cord (120%) than in frontal cortex (50%).

As fatty acid profile strongly influences membrane peroxidizability, and consequently protein lipoxidative damage, we analysed fatty acid content in ALS samples. Those analyses revealed significant differences associated with ALS in spinal cord and frontal cortex, both in individual fatty acids and in global indexes (Table 2). The most remarkable change involves the highly peroxidizable docosahexaenoic acid (DHA), which showed a significant decrease in spinal cord samples with ALS ($P < 0.01$), contrasting with the significant increase observed in frontal cortex ($P < 0.01$; Fig. 2D). With reference to the fatty-acid-derived indexes, spinal cords from ALS patients showed significant decreases in the content of PUFA of the *n*-3 family ($P < 0.001$; Table 2), while increases of this parameter were detected in frontal cortex ($P < 0.006$; Table 2). Changes in fatty acid profile led to significant changes in double bond index ($P < 0.004$; Table 2), PI ($P < 0.003$; Fig. 2D), Δ9-desaturase estimation ($P < 0.01$; Fig. 2E) and *n*6/*n*3 ratio ($P < 0.001$; Fig. 2E). Overall, these indexes could reflect the potential vulnerability of membranes to peroxidative damage. Since changes in double bond and PI in spinal cord were inverse to those observed in frontal cortex, these data suggest that spinal cord membranes are actively producing substrates for peroxidative modification of proteins, while as frontal cortex are not under this circumstance. Accordingly, analysis of MDAL/PI ratio, suggest that for a given PI, rates of MDAL formation are 3-fold higher in spinal cord than in frontal cortex from ALS patients.

Different kinds of protein oxidative damage are correlated together and are associated to changes in fatty acid content

After quantitations of protein oxidation and fatty acid analyses, several significant correlations were present among anatomically different locations (Fig. 3). GSA levels correlated significantly with AASA ($r = 0.918$; $P < 0.0001$; Fig. 3A), with MDAL ($r = 0.865$; $P < 0.0001$; Fig. 3B), with CEL ($r = 0.716$; $P < 0.002$) and with CML ($r = 0.878$; $P < 0.0001$). This suggests that protein carbonyl formation

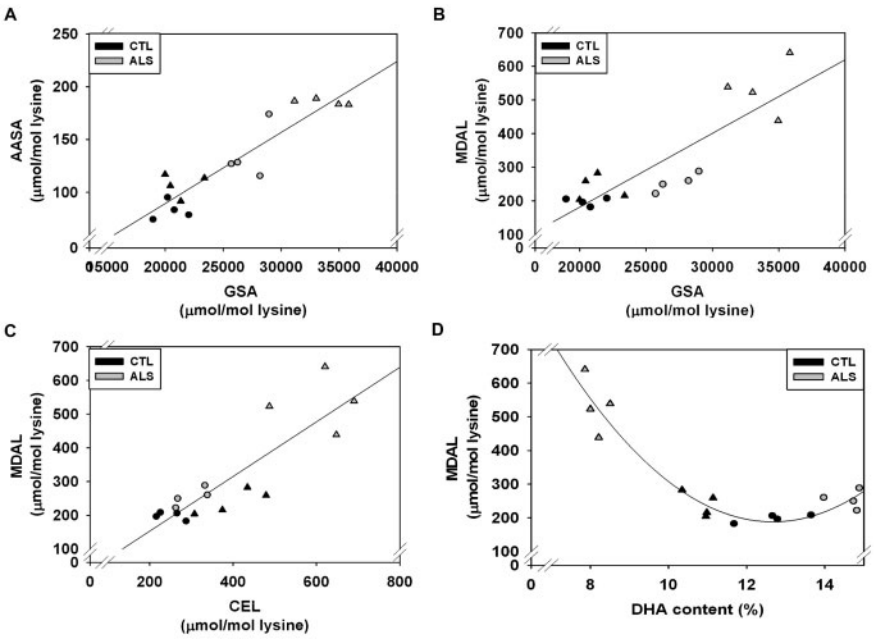


Fig. 3 Changes in protein oxidation indexes and fatty acid contents are strongly correlated. Indexes of protein carbonyl GSA and AASA are strongly correlated (upper panel, left; $r = 0.92$; $P < 0.0001$). Protein oxidative damage is also correlated with protein lipoxidative damage (upper panel, right; $r = 0.865$; $P < 0.0001$). Protein lipoxidative damage is correlated to glycoxidative modifications (lower panel, left; $r = 0.865$; $P < 0.0001$). DHA content shows a quadratic relationship with protein lipoxidative damage (lower panel, right; $r = 0.95$; $P < 0.0001$; model:[MDAL] = 17.3*[DHA]^2 - 440.3*[DHA] + 2977). Triangles (%) represent values from spinal cord samples and circles () represent values from frontal cortex. Results are only shown for those samples were both frontal cortex and spinal cord were available ($n = 4$ for each group).

is also associated to glycoxidative and lipoxidative modifications in spinal cord and frontal cortex. Furthermore, peroxidizability index was inversely correlated with CEL ($r = -0.816$; $P < 0.0001$) and MDAL ($r = -0.685$; $P < 0.003$) concentrations, suggesting an association between lipid peroxidizability, glycoxidation and lipoxidation modifications. Interestingly, levels of MDAL display a second-order relationship with DHA levels ($r = 0.961$; $P < 0.0001$; Fig. 3D), suggesting an intimate interplay between these two factors.

Protein oxidative damage, showing preferential targets in ALS spinal cord proteins can be associated to mitochondrial disturbances

It is known that an important determinant for oxidative damage of proteins, besides fatty acid content, is the mitochondrial free radical production. Therefore, we analysed the expression of representative subunits of mitochondrial respiratory complexes I and III, whose activity is counted among the major sources for mitochondrial free radical production (Herrero and Barja, 2000; Chen *et al.*, 2003), as well as the levels of AIF, shown recently to enhance the functional stability of complex I (Vahsen *et al.*, 2004). These analyses demonstrated that both complex I and III concentrations are significantly decreased in spinal cord samples from ALS patients ($P < 0.01$; Fig. 4A) while AIF expression is not changed in ALS (Fig. 4A). Reinforcing an apparently different pace of ALS-induced changes between frontal cortex and spinal cord, these effects were not present in frontal cortex from ALS patients (data not shown).

Western-blot analyses of frontal cortex and spinal cord proteins showed differences in the distribution of oxidation (DNP-reactive), glycoxidation (anti-CML) and lipoxidation-derived (anti-neuroketal) protein modifications (Fig. 4B) supporting both diversity and specificity of protein oxidative damage. These findings agree with quantitative analyses by GC/MS as densitometric measurements revealed increased oxidative damage in ALS samples. Major targets of glycoxidation were proteins with apparent molecular weights ranging from 35 to 55 kDa, partially coincident with anti-DNP immunoreactivity (which was also evident for high-molecular weight bands). This pattern differs from targets of neuroketal formation (Fig. 4B), which showed more discrete targets (being targets of 40 and 60 kDa the more prominent). These differences were not present in frontal cortex samples (data not shown).

Chronic excitoxicity in organotypic spinal cord cultures leads ER stress, ubiquitin alterations, protein oxidative damage and changes in fatty acid profile

Chronic excitotoxicity has been implied in the pathogenesis of ALS (Rothstein *et al.*, 1993; Boillee *et al.*, 2006). This can be reproduced *in vitro* by treatment with the pre-synaptic

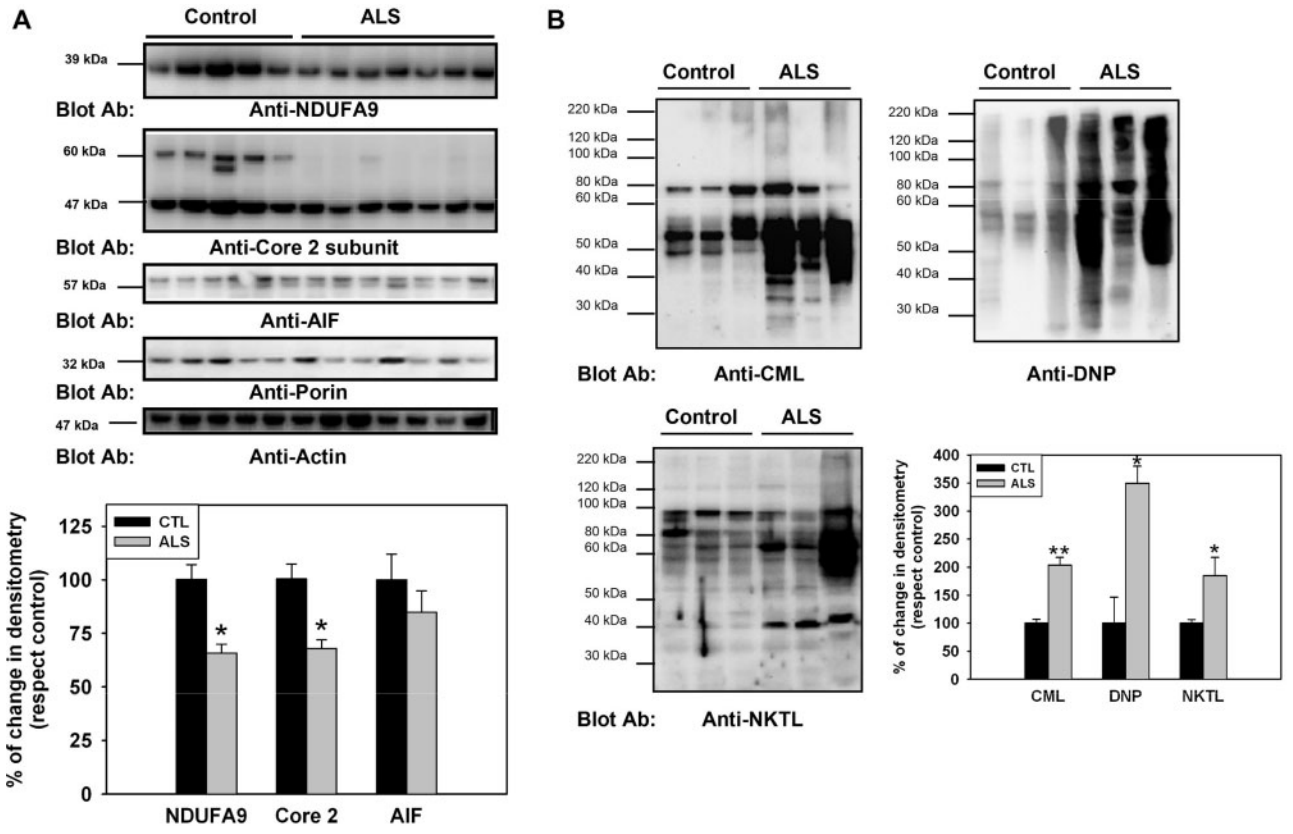


Fig 4 ALS-induced mitochondrial changes and protein-oxidation-specific targets in spinal cord. (**A**) Representative western blot of peptides NDUFA9 and core 2 subunits of mitochondrial respiratory chain complexes I and III, respectively, suggesting decreased complex III content in spinal cord samples from ALS patients compared to control individuals. This was not associated with increased amount of apoptosis-inducing factor (AIF). The *lower panel* shows the quantitation of these blots by densitometry, adjusted to porin density and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$. (**B**) Western blot for mixed glycoxidation/lipoxidation (CML), MCO (DNP) and lipoxidation [neuroketal (NKTL)] revealed differential targets for each of these oxidative pathways. *Right numbers* of the blots indicate apparent molecular weight. The *lower panels* show the quantization of these blots by densitometry, after densities of bands (ranging between 30 and 220 kDa) or actin (not shown, for B), and differences were analysed respect to control group by Student's *t*-test being $^{*}P < 0.01$ and $^{**}P < 0.001$.

glutamate transport inhibitor THA, leading to losses in the number of motorneurons, as assessed by SMI-32 immunoreactivity (data not shown). In accordance to findings in ALS samples, signs of ER stress were also found in spinal cord slices under chronic excitotoxicity after a 30-day period (Figs. S1A and S2A). Although eIF2α phosphorylation was present in neuronal bodies of vehicle-treated slices, increased immunoreactivity was found along neuritic processes as well as in small cellular populations morphologically compatible with glia on THA-treated slices. Furthermore, demonstration of a markedly increased intracellular ubiquitin immunoreactivity excluding nucleus (Fig. S1B) suggest a role of proteasomal dysfunction in this phenomena at an earlier stage. Furthermore, incubation with the ER stress inducing agents tunicamycin and thapsigargin led to increases in chaperone expression (Fig. S2A) and to a marked decrease in the number of motorneurons, suggesting its preferential sensitivity to ER stress (Fig. S2B).

Since those analyses revealed ER stress and proteasome dysfunction in chronic excitotoxicity, we analysed whether protein oxidative damage was also increased in these conditions. The results demonstrated that protein oxidative damage is increased by excitotoxicity (Fig. S3). All measured markers of oxidation, glycoxidation and lipoxidation, increased significantly after THA treatment ($P < 0.001$, Fig. S3A). As in the ALS cases, these increases in protein oxidative modifications were associated to changes in fatty acid composition (Table S2, Fig. S3B). Noteworthy, THA-induced changes resembled those present in frontal cortex, with 2-fold increases in the DHA content ($P < 0.0001$, Fig. S3B), PI increases—150% respect vehicle-treated slices—($P < 0.0001$, Fig. S3B) and decreased *n*6/*n*3 ratios ($P < 0.0001$, Fig. S3B). Confocal microscopy of the most THA-sensitive marker, CEL, revealed that proteins modified with this product were present, in increased amounts, throughout glial and neuronal populations (Fig. S3C). Neuropil and star-shaped cells resembling microglia were major distribution sites of CEL immunoreactivity in THA-treated slices. Co-localization studies with *Bandairaea Simplicifolia* lectin supported the microglial origin of some of those cells (Fig. S3C).

Nevertheless, chronic excitotoxicity also led to increased AIF immunoreactivity in a non-nuclear distribution, suggesting common basis for mitochondrial dysfunction between sporadic ALS and chronic excitotoxicity both in neuronal and glial cells (Fig. S1C).

Discussion

Cells facing protein misfolding in the ER initiate the so-called unfolded protein response. As a part of this unfolded protein response, the initiation process in global protein synthesis is repressed by eIF2α phosphorylation via PERK kinase. There is also increased expression of ER chaperones and, if ER stress cause is not corrected, apoptosis is induced (Marciniak and Ron, 2006; Zhang and Kaufman, 2006). The results reported here, showing both increased phosphorylation of eIF2α and increased expression of ER chaperones (PDI and KDEL-containing proteins), strongly support the participation of ER stress in ALS pathogenesis. The relevance of this pathway was also evident in the chronic excitotoxicity paradigm, where both motorneurons and glial cells were stained for anti-phosphorylated eIF2α. Noteworthy, the importance of this pathway is also demonstrated by immunohistochemical analyses, where remaining neuronal bodies of spinal cord ventral horns in ALS show intense anti-phosphorylated eIF2α. Our data suggest that ER stress arises from oxidative stress and from a mitochondrial disturbance. Based on correlative data, we propose that this is a late phenomenon, when compared with protein oxidative damage, as we are able to detect increased protein oxidative damage, increased DHA amount and other fatty acid changes in brain cortex, without increased ubiquitination or any other noticeable change. Recent data demonstrates the occurrence of ER stress in familial ALS paradigms, associating PDI and mutated SOD in motorneurons (Atkin *et al.*, 2006; Furukawa *et al.*, 2006), showing also caspase-12 activation (Wootz *et al.*, 2004; Turner and Atkin, 2006). More interestingly, chronic excitotoxicity leads to protein aggregation in ER from motorneurons (Tarabal *et al.*, 2005), without inducing an upregulation of heat-shock proteins, as those cells have a characteristically high threshold for heat-shock proteins induction (Batulan *et al.*, 2003). This fact, when added to the increased chaperone expression and immunohistochemical data on organotypic cultures, strongly suggest that ER stress is also taking place in glial cells. Due to the important role of the proteasome in ER-directed disposal of misfolded proteins, ER stress could be caused by decreased ER-associated degradation (Bush *et al.*, 1997; Obeng *et al.*, 2006; Yamamuro *et al.*, 2006).

In agreement with our findings, previous data indicate a potential loss of proteasome activity in ALS (Urushitani *et al.*, 2002; Cheroni *et al.*, 2005; Ahtoniemi *et al.*, 2006; Basso *et al.*, 2006; Kabuta *et al.*, 2006; Koyama *et al.*, 2006; Mendonca *et al.*, 2006), specially in the familial forms of the disease. *In vitro* experiments and *in vivo* data show that mutated SOD is associated with decreased amount of specific proteasome subunits, particularly LMP7 (Allen *et al.*, 2003). Furthermore, proteasome inhibition leads to the reproduction of the abnormal solubility properties shown by mutated SOD *in vivo* (Koyama *et al.*, 2006). However, the role of proteasome in sporadic ALS has received less attention. Recent data demonstrate increased proteasome immunoreactivity both in glia and motorneurons from spinal cords in sporadic ALS (Mendonca *et al.*, 2006). Accordingly, findings reported here, demonstrating increased amount of ubiquitinated proteins in spinal cord samples, is compatible to such proteasomal involvement. It should be recalled that ubiquitinated lesions are prominent in ALS morphology. Noteworthy, this increased ubiquitination was also evident under chronic excitotoxicity, being present both in neuronal and non-neuronal populations. This agrees with the previous works, showing that proteasome inhibition in organotypic cultures induce selectivity damage to motorneurons, as chronic excitotoxicity does (Tsuji *et al.*, 2005). As chronic excitotoxicity leads to increased oxidative damage (Rao and Weiss, 2004; Rival *et al.*, 2004), our data suggest that this condition could contribute to proteasome inhibition. Relating differences with frontal cortex, western-blot analyses did not reveal such changes in this latter location. Once a given protein is modified by oxidation, it is degraded by 20S proteasome in an ATP and ubiquitin-independent fashion (Shringarpure *et al.*, 2003; Grune *et al.*, 2005), as recently shown for metal-free SOD (Di Noto *et al.*, 2005). Nevertheless, a high degree of oxidative modification could even decrease proteasome activity (Sitte *et al.*, 2000).

For these reasons we evaluated whether, by using novel mass spectrometry measurements, ALS samples presented increases in protein oxidative damage, both in spinal cord and in frontal cortex. Nonetheless, the magnitude of changes with reference to control was higher in spinal cord than in frontal cortex. These results suggest an early selective involvement of oxidative stress in spinal cord during ALS pathogenesis. In addition, the involvement of frontal cortex agrees with previous reported data in other neurodegenerative diseases, such as Alzheimer disease or Parkinson disease, where pathologically preserved locations of nervous system show incipient increases in protein oxidative damage, thought at a lower extent than classical targets of disease (Dalfo *et al.*, 2005; Pamplona *et al.*, 2005). Cognitive dysfunction and dementia have been reported as complications in a subgroup of patients with ALS, the majority of them presenting atrophy, neuronal loss and reactive gliosis in the frontal and temporal lobes (Kato *et al.*, 2003). Although the present study is not focused on ALS cases with frontotemporal dementia, these results point to the suggestion that frontal cortex is a vulnerable region to ALS. As a working hypothesis it can be proposed that modifications in the levels of certain lipids are predisposing factors to further cellular damage. Nevertheless, data presented here support the notion that

lipid peroxidation-associated processes seem to be the more sensible cellular oxidative phenomenon, based on MDAL values. Previous immunohistochemical evidences demonstrate increased lipid peroxidation in ALS (Hall *et al.*, 1998; Pedersen *et al.*, 1998). Furthermore, recent data showing accumulation of 4-oxo-2-nonenal DNA etheno adduct in brain cortex from ALS patients support the importance of lipid peroxidation-derived pathways (Shibata *et al.*, 2006). Interestingly, while PI is still increased in frontal cortex, basically due to increases in DHA content, a potential defensive response of nervous tissue (Akbar *et al.*, 2005), these indexes were decreased in spinal cord, suggesting a functional collapse and/or a lower content of neurons. Thus, while brain cortex could produce *n*-3-derived anti-inflammatory resolvins and docosatrienes (Hong *et al.*, 2003), spinal cord neurons would have decreased DHA availability due to increased lipoxidative consumption. Rather than being a general phenomena, the selectivity for changes in *n*-3 strongly suggest specific mechanisms of the disease depending on these fatty acids, maybe affecting biosynthetic pathways and/or membrane remodelling systems that deserve further studies. Nonetheless, long-chain unsaturated fatty acids contribute to the formation of cytotoxic aggregates of ALS-linked superoxide dismutase-1, thereby stressing the importance of fatty acid changes in ALS pathogenesis (Kim *et al.*, 2005). To shed further light on these issues we analysed a chronic excitotoxicity paradigm. The results demonstrated—at an early stage of the excitotoxic paradigm—changes partially resembling those present in frontal cortex, e.g. increases in all oxidative markers and increases in DHA content and PI. This was present at a time when motorneuron death is still not evident. In line with this, DHA induces resistance against excitotoxic degeneration of cholinergic neurones *in vivo*, leading to higher survival, lower dendritic involution and decreased axon degeneration (Hogyes *et al.*, 2003). Globally, these results suggest a pathological spectrum, driven by excitotoxicity, ranging between overt pathological manifestation (present in spinal cord) and more subtle changes, with variations in fatty acids as reactive changes and protein oxidative modifications as important signals of disease.

The significant correlations found between different types of protein oxidative damage suggest that ALS could influence general mechanisms determining protein oxidative modifications, such as free radical production (Pamplona and Barja, 2006). In most cells, the major sources of free radical production are mitochondrial respiratory complexes I and III (Herrero and Barja, 2000) although in brain some mitochondrial matrix enzymes also contribute to free radical production (Starkov *et al.*, 2004; Tretter and Adam-Vizi, 2004). The analyses performed in spinal cords suggest decreases in both respiratory complexes, as well to qualitative changes in complex III distribution. Due to the role of complex I and III as free radical generators (Chen *et al.*, 2003), the results may be compatible with increased free radical efflux by incorrect assembly of complexes or to metabolic reprogramming (Iuso *et al.*, 2006). These data complements previous reports demonstrating decreased activities of respiratory chain complexes I + III, II + III and IV, suggesting a loss of mitochondria in spinal cords from ALS patients (Wiedemann *et al.*, 2002). Moreover, specific decreases in the activities of complexes II are observed in a mutated SOD transgenic model (Jung *et al.*, 2002). Furthermore, losses of complex IV activity are present in the *Wobbler* mouse (Xu *et al.*, 2001). It may be suggested that ALS samples presented a defect in the assembly of mitochondrial respiratory complexes, that would lead to changes in free radical production (Rana *et al.*, 2000; Sellem *et al.*, 2005). It may be also hypothesized that this scenario could contribute to a more reduced state of Fe-S clusters, leading to increased free radical efflux (Herrero and Barja, 2000). In this line, the amount of AIF, a bifunctional flavoprotein with NADH oxidase activity involved in mitochondrial respiration and caspase-independent apoptosis, was unchanged in spinal cords. Besides an ALS-induced increased apoptotic rate in this tissue (Oh *et al.*, 2006), AIF could be also considered as a part of an adaptation response to increased free radical production (Zhu *et al.*, 2003; Cande *et al.*, 2004) or to decreased respiratory activities. Changes in AIF are not evident in ALS samples when compared to data in the *in vitro* paradigm: AIF presenting increased non-nuclear staining in THA-treated slices suggests an important role of this protein in the response to excitotoxicity, that may be focus of future studies. Therefore, it could be suggested that increased free radical production, arising from complex I and III suboptimal assembly, together with unchanged or even increased AIF expression, contributes to protein oxidative damage specifically in spinal cords, being those changes absent in frontal cortex. Despite such an increased mitochondrial leak could lead to potentially extensive protein modifications, western-blot analyses revealed that there are specific targets for oxidative, glycoxidative and lipoxidative damage that will be the focus of future studies.

To summarize, the present data demonstrate that there is increased protein oxidative modification in spinal cords and frontal cortex from ALS cases, together with changes in lipid composition. Concerning cellular targets of those phenomena, we have demonstrated a motorneuron involvement, but it should be remarked that glial cells are the major contributors to protein mass in spinal cords. Therefore, it is feasible to assume that many of the changes observed here respond to changes in both (or mainly) glial cell and neuronal populations. In this line, it should be recalled the importance of glial support and trophic environment for motorneurons, so any given change to glial population could contribute to motorneuron loss. In spinal cords, where loss of motorneurons was evident, build up of oxidatively damaged proteins was linked to changes in mitochondrial respiratory complexes and to increased ubiquitination, potentially linked to impaired

proteasome function. More importantly, this was associated to changes in ER proteins suggesting the occurrence of ER stress. Since those changes were reproducible by an excitotoxic paradigm at an early stage, it can be suggested that excitotoxicity leads to increased protein oxidation, proteasomal dysfunction and ER stress in neuronal and non-neuronal cells, potentially contributing to motor-neuron death in ALS.

Supplementary material

Supplementary material is available at *Brain* online.

Acknowledgements

We are fully indebted to tissue donors and their families. We thank David Argiles for excellent technical assistance. We are deeply thankful to Dr Jesus Requena (from the Universidad de Santiago de Compostela, Spain) for providing us with GSA and AASA standards and to Dr Josep Esquerda for thoughtful discussion.

This study was supported in part by I+D grants from the Spanish Ministry of Education and Science (BFU2006-14495/BFI) and the Generalitat of Catalunya (2005 SGR00101) to R.P.; the Spanish Ministry of Health (FIS 04-0355, 05-2214 and 05-2241), Spanish Ministry of Education and Science (AGL2006-12433), the ALS Association (USA) and "La Caixa" Foundation to M.P.O. and E.D., E.I. (a predoctoral fellow from the Generalitat) and D.C.; and FIS grants PI04-0184 and PI05-1570, and support by the European Commission under the Sixth Framework Programme (BrainNet Europe II, LSHM-CT-2004-503039) to I.F. Supported by the COST B-35 Action.

References

Ahtoniemi T, Goldsteins G, Keksa-Goldsteine V, Malm T, Kanninen K, Salminen A, et al. Pyrrolidine dithiocarbamate inhibits induction of immunoproteasome and decreases survival in a rat model of amyotrophic lateral sclerosis. Mol Pharmacol 2006.

Akbar M, Calderon F, Wen Z, Kim HY. Docosahexaenoic acid: a positive modulator of akt signaling in neuronal survival. Proc Natl Acad Sci USA 2005; 102: 10858–63.

Allen S, Heath PR, Kirby J, Wharton SB, Cookson MR, Menzies FM, et al. Analysis of the cytosolic proteome in a cell culture model of familial amyotrophic lateral sclerosis reveals alterations to the proteasome, antioxidant defenses, and nitric oxide synthetic pathways. J Biol Chem 2003; 278: 6371–83.

Atkin JD, Farg MA, Turner BJ, Tomas D, Lysaght JA, Nunan J, et al. Induction of the unfolded protein response in familial amyotrophic lateral sclerosis and association of protein-disulfide isomerase with superoxide dismutase 1. J Biol Chem 2006; 281: 30152–65.

Basso M, Massignan T, Samengo G, Cheroni C, De Biasi S, Salmona M, et al. Insoluble mutant SOD1 is partly oligoubiquitinated in amyotrophic lateral sclerosis mice. J Biol Chem 2006; 281: 33325–35.

Batulan Z, Shinder GA, Minotti S, He BP, Doroudchi MM, Nalbantoglu J, et al. High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. J Neurosci 2003; 23: 5789–98.

Baynes JW. Chemical modification of proteins by lipids in diabetes. Clin Chem Lab Med 2003; 41: 1159–65.

Baynes JW, Thorpe SR. Glycoxidation and lipoxidation in atherogenesis. Free Radic Biol Med 2000; 28: 1708–16.

Boillee S, Vande Velde C, Cleveland DW. ALS: a disease of motor neurons and their nonneuronal neighbors. Neuron 2006; 52: 39–59.

Bush KT, Goldberg AL, Nigam SK. Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J Biol Chem 1997; 272: 9086–92.

Cande C, Vahsen N, Metivier D, Tourriere H, Chebli K, Garrido C, et al. Regulation of cytoplasmic stress granules by apoptosis-inducing factor. J Cell Sci 2004; 117: 4461–8.

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: Central role of complex III. J Biol Chem 2003; 278: 36027–31.

Cheroni C, Peviani M, Cascio P, Debiasi S, Monti C, Bendotti C. Accumulation of human SOD1 and ubiquitinated deposits in the spinal cord of SOD1G93A mice during motor neuron disease progression correlates with a decrease of proteasome. Neurobiol Dis 2005; 18: 509–22.

Dalfo E, Portero-Otin M, Ayala V, Martinez A, Pamplona R, Ferrer I. Evidence of oxidative stress in the neocortex in incidental lewy body disease. J Neuropathol Exp Neurol 2005; 64: 816–30.

Di Noto L, Whitson LJ, Cao X, Hart PJ, Levine RL. Proteasomal degradation of mutant superoxide dismutases linked to amyotrophic lateral sclerosis. J Biol Chem 2005; 280: 39907–13.

Dupuis L, Gonzalez de Aguilar JL, Oudart H, de Tapia M, Barbeito L, Loeffler JP. Mitochondria in amyotrophic lateral sclerosis: a trigger and a target. Neurodegener Dis 2004; 1: 245–54.

Ferrante RJ, Browne SE, Shinobu LA, Bowling AC, Baik MJ, MacGarvey U, et al. Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. J Neurochem 1997; 69: 2064–74.

Furukawa Y, Fu R, Deng HX, Siddique T, O'Halloran TV. Disulfide cross-linked protein represents a significant fraction of ALS-associated cu, zn-superoxide dismutase aggregates in spinal cords of model mice. Proc Natl Acad Sci USA 2006; 103: 7148–53.

Grune T, Reinheckel T, Davies KJ. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. J Biol Chem 1996; 271: 15504–9.

Grune T, Jung T, Merker K, Davies KJ. Decreased proteolysis caused by protein aggregates, inclusion bodies, plaques, lipofuscin, ceroid, and 'aggresomes' during oxidative stress, aging, and disease. Int J Biochem Cell Biol 2004; 36: 2519–30.

Grune T, Merker K, Jung T, Sitte N, Davies KJ. Protein oxidation and degradation during postmitotic senescence. Free Radic Biol Med 2005; 39: 1208–15.

Hall ED, Andrus PK, Oostveen JA, Fleck TJ, Gurney ME. Relationship of oxygen radical-induced lipid peroxidative damage to disease onset and progression in a transgenic model of familial ALS. J Neurosci Res 1998; 53: 66–77.

Herrero A, Barja G. Localization of the site of oxygen radical generation inside the complex I of heart and nonsynaptic brain mammalian mitochondria. J Bioenerg Biomembr 2000; 32: 609–15.

Hogyes E, Nyakas C, Kiliaan A, Farkas T, Penke B, Luiten PG. Neuroprotective effect of developmental docosahexaenoic acid supplement against excitotoxic brain damage in infant rats. Neuroscience 2003; 119: 999–1012.

Hong S, Gronert K, Devchand PR, Moussignac RL, Serhan CN. Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation. J Biol Chem 2003; 278: 14677–87.

Iuso A, Scacco S, Piccoli C, Bellomo F, Petruzzella V, Trentadue R, et al. Dysfunctions of cellular oxidative metabolism in patients with mutations in the NDUFS1 and NDUFS4 genes of complex I. J Biol Chem 2006; 281: 10374–80.

Jung C, Higgins CM, Xu Z. Mitochondrial electron transport chain complex dysfunction in a transgenic mouse model for amyotrophic lateral sclerosis. J Neurochem 2002; 83: 535–45.

Kabuta T, Suzuki Y, Wada K. Degradation of amyotrophic lateral sclerosis-linked mutant cu,zn-superoxide dismutase proteins by macroautophagy and the proteasome. J Biol Chem 2006; 281: 30524–33.

Kato S, Shaw PJ, Wood-Allum C, Leigh PN, Shaw C. Motor neuron disorders. In: Dickson DW, editor. Neurodegeneration: the molecular pathology of dementia and movement disorders. Basel: ISN Neuropath Press; 2003. p. 349–77.

Kato S, Horiuchi S, Liu J, Cleveland DW, Shibata N, Nakashima K, et al. Advanced glycation endproduct-modified superoxide dismutase-1 (SOD1)-positive inclusions are common to familial amyotrophic lateral sclerosis patients with SOD1 gene mutations and transgenic mice expressing human SOD1 with a G85R mutation. Acta Neuropathol (Berl) 2000; 100: 490–505.

Kikuchi S, Ogata A, Shinpo K, Moriwaka F, Fujii J, Taniguchi N, et al. Detection of an amadori product, 1-hexitol-lysine, in the anterior horn of the amyotrophic lateral sclerosis and spinobulbar muscular atrophy spinal cord: evidence for early involvement of glycation in motoneuron diseases. Acta Neuropathol (Berl) 2000; 99: 63–6.

Kikuchi S, Shinpo K, Ogata A, Tsuji S, Takeuchi M, Makita Z, et al. Detection of N epsilon-(carboxymethyl)lysine (CML) and non-CML advanced glycation end-products in the anterior horn of amyotrophic lateral sclerosis spinal cord. Amyotroph Lateral Scler Other Motor Neuron Disord 2002; 3: 63–8.

Kim YJ, Nakatomi R, Akagi T, Hashikawa T, Takahashi R. Unsaturated fatty acids induce cytotoxic aggregate formation of amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutants. J Biol Chem 2005; 280: 21515–21.

Koyama S, Arawaka S, Chang-Hong R, Wada M, Kawanami T, Kurita K, et al. Alteration of familial ALS-linked mutant SOD1 solubility with disease progression: its modulation by the proteasome and Hsp70. Biochem Biophys Res Commun 2006; 343: 719–30.

Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. Cell Death Differ 2006; 13: 385–92.

Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. Physiol Rev 2006; 86: 1133–49.

Mendonca DM, Chimelli L, Martinez AM. Expression of ubiquitin and proteasome in motorneurons and astrocytes of spinal cords from patients with amyotrophic lateral sclerosis. Neurosci Lett 2006; 404: 315–9.

Obeng EA, Carlson LM, Gutman DM, Harrington WJ, Jr, Lee KP, Boise LH. Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells. Blood 2006; 107: 4907–16.

Oh YK, Shin KS, Kang SJ. AIF translocates to the nucleus in the spinal motor neurons in a mouse model of ALS. Neurosci Lett 2006; 406: 205–10.

Oyadomari S, Yun C, Fisher EA, Kreglinger N, Kreibich G, Oyadomari M, et al. Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. Cell 2006; 126: 727–39.

Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. Biochim Biophys Acta 2006; 1757: 496–508.

Pamplona R, Dalfo E, Ayala V, Bellmunt MJ, Prat J, Ferrer I, et al. Proteins in human brain cortex are modified by oxidation, glycoxidation, and lipoxidation. Effects of Alzheimer disease and identification of lipoxidation targets. J Biol Chem 2005; 280: 21522–30.

Pedersen WA, Fu W, Keller JN, Markesbery WR, Appel S, Smith RG, et al. Protein modification by the lipid peroxidation product 4-hydroxynonenal in the spinal cords of amyotrophic lateral sclerosis patients. Ann Neurol 1998; 44: 819–24.

Rana M, de Coo I, Diaz F, Smeets H, Moraes CT. An out-of-frame cytochrome b gene deletion from a patient with parkinsonism is associated with impaired complex III assembly and an increase in free radical production. Ann Neurol 2000; 48: 774–81.

Rao SD, Weiss JH. Excitotoxic and oxidative cross-talk between motor neurons and glia in ALS pathogenesis. Trends Neurosci 2004; 27: 17–23.

Requena JR, Levine RL, Stadtman ER. Recent advances in the analysis of oxidized proteins. Amino Acids 2003; 25: 221–6.

Requena JR, Chao CC, Levine RL, Stadtman ER. Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. Proc Natl Acad Sci USA 2001; 98: 69–74.

Requena JR, Fu MX, Ahmed MU, Jenkins AJ, Lyons TJ, Baynes JW, et al. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. Biochem J 1997; 322 (Pt 1): 317–25.

Rival T, Soustelle L, Strambi C, Besson MT, Iche M, Birman S. Decreasing glutamate buffering capacity triggers oxidative stress and neuropil degeneration in the drosophila brain. Curr Biol 2004; 14: 599–605.

Rothstein JD, Jin L, Dykes-Hoberg M, Kuncl RW. Chronic inhibition of glutamate uptake produces a model of slow neurotoxicity. Proc Natl Acad Sci USA 1993; 90: 6591–5.

Sellem CH, Lemaire C, Lorin S, Dujardin G, Sainsard-Chanet A. Interaction between the oxa1 and rmp1 genes modulates respiratory complex assembly and life span in podospora anserina. Genetics 2005; 169: 1379–89.

Shibata N, Hirano A, Kato S, Nagai R, Horiuchi S, Komori T, et al. Advanced glycation endproducts are deposited in neuronal hyaline inclusions: a study on familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation. Acta Neuropathol (Berl) 1999; 97: 240–6.

Shibata N, Nagai R, Miyata S, Jono T, Horiuchi S, Hirano A, et al. Nonoxidative protein glycation is implicated in familial amyotrophic lateral sclerosis with superoxide dismutase-1 mutation. Acta Neuropathol (Berl) 2000; 100: 275–84.

Shibata T, Iio K, Kawai Y, Shibata N, Kawaguchi M, Toi S, et al. Identification of a lipid peroxidation product as a potential trigger of the p53 pathway. J Biol Chem 2006; 281: 1196–204.

Shringarpure R, Grune T, Mehlhase J, Davies KJ. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. J Biol Chem 2003; 278: 311–8.

Sitte N, Huber M, Grune T, Ladhoff A, Doecke WD, Von Zglinicki T, et al. Proteasome inhibition by lipofuscin/ceroid during postmitotic aging of fibroblasts. FASEB J 2000; 14: 1490–8.

Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, et al. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci 2004; 24: 7779–88.

Strong MJ, Kesavapany S, Pant HC. The pathobiology of amyotrophic lateral sclerosis: a proteinopathy? J Neuropathol Exp Neurol 2005; 64: 649–64.

Tarabal O, Caldero J, Casas C, Oppenheim RW, Esquerda JE. Protein retention in the endoplasmic reticulum, blockade of programmed cell death and autophagy selectively occur in spinal cord motoneurons after glutamate receptor-mediated injury. Mol Cell Neurosci 2005; 29: 283–98.

Tretter L, Adam-Vizi V. Generation of reactive oxygen species in the reaction catalyzed by alpha-ketoglutarate dehydrogenase. J Neurosci 2004; 24: 7771–8.

Tsuji S, Kikuchi S, Shinpo K, Tashiro J, Kishimoto R, Yabe I, et al. Proteasome inhibition induces selective motor neuron death in organotypic slice cultures. J Neurosci Res 2005; 82: 443–51.

Turner BJ, Atkin JD. ER stress and UPR in familial amyotrophic lateral sclerosis. Curr Mol Med 2006; 6: 79–86.

Urushitani M, Kurisu J, Tsukita K, Takahashi R. Proteasomal inhibition by misfolded mutant superoxide dismutase 1 induces selective motor neuron death in familial amyotrophic lateral sclerosis. J Neurochem 2002; 83: 1030–42.

Vahsen N, Cande C, Briere JJ, Benit P, Joza N, Larochette N, et al. AIF deficiency compromises oxidative phosphorylation. EMBO J 2004; 23: 4679–89.

Wiedemann FR, Manfredi G, Mawrin C, Beal MF, Schon EA. Mitochondrial DNA and respiratory chain function in spinal cords of ALS patients. J Neurochem 2002; 80: 616–25.

Wootz H, Hansson I, Korhonen L, Napankangas U, Lindholm D. Caspase-12 cleavage and increased oxidative stress during motoneuron degeneration in transgenic mouse model of ALS. Biochem Biophys Res Commun 2004; 322: 281–6.

Xu GP, Dave KR, Moraes CT, Busto R, Sick TJ, Bradley WG, et al. Dysfunctional mitochondrial respiration in the wobbler mouse brain. Neurosci Lett 2001; 300: 141–4.

Yamamuro A, Yoshioka Y, Ogita K, Maeda S. Involvement of endoplasmic reticulum stress on the cell death induced by 6-hydroxydopamine in human neuroblastoma SH-SY5Y cells. Neurochem Res 2006; 31: 657–64.

Zhang K, Kaufman RJ. The unfolded protein response: a stress signaling pathway critical for health and disease. Neurology 2006; 66: S102–9.

Zhu C, Qiu L, Wang X, Hallin U, Cande C, Kroemer G, et al. Involvement of apoptosis-inducing factor in neuronal death after hypoxia-ischemia in the neonatal rat brain. J Neurochem 2003; 86: 306–17.

Molecular and Cellular Neuroscience 61 (2014) 219–225

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Calpain activation and CaMKIV reduction in spinal cords from hSOD1G93A mouse model

Myriam Gou-Fabregas [a,1], Omar Ramírez-Núñez [b], Daniel Cacabelos [b], Nuria Bahi [a], Manuel Portero [b], Ana Garcera [a,2], Rosa M. Soler [a,*,2]

[a] Unitat de Senyalització Neuronal, Dept Ciencies Mediques Basiques, Facultat de Medicina, Universitat de Lleida, IRBLLEIDA, Rovira Roure, 80, 25198 Lleida, Spain
[b] Dept Medicina Experimental, Facultat de Medicina, Universitat de Lleida, IRBLLEIDA, Rovira Roure, 80, 25198 Lleida, Spain

ARTICLE INFO

Article history:
Received 24 October 2013
Revised 17 July 2014
Accepted 21 July 2014
Available online 22 July 2014

Keywords:
ALS
Neurodegeneration
Intracellular calcium
CaMKIV
Calpain
hSOD1G93A

ABSTRACT

Amyotrophic Lateral Sclerosis (ALS), a severe neurodegenerative disease, affects the upper and lower motor neurons in the brain and spinal cord. In some studies, ALS disease progression has been associated with an increase in calcium-dependent degeneration processes. Motoneurons are specifically vulnerable to sustained membrane depolarization and excessive elevation of intracellular calcium concentration. The present study analyzed intracellular events in embryonic motoneurons and adult spinal cords of the hSOD1G93A ALS mouse model. We observed activation of calpain, a calcium-dependent cysteine protease that degrades a variety of substrates, and a reduction in calcium–calmodulin dependent protein kinase type IV (CaMKIV) levels in protein extracts from spinal cords obtained at several time-points of hSOD1G93A mice disease progression. However, in cultured embryonic motoneurons these differences between controls and hSOD1G93A mutants are not evident. Our results support the hypothesis that age-dependent changes in calcium homeostasis and resulting events, e.g., calpain activation and CaMKIV processing, are involved in ALS pathogenesis.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal, adult-onset, neurodegenerative disease causing the degeneration of cranial and spinal cord motor neurons (MNs), which leads to muscle atrophy and paralysis. Numerous ALS studies have analyzed subgroups of familial cases originating from mutations in the SOD1 gene (Rosen et al., 1993). The precise mechanisms whereby mutant SOD1 is toxic to MNs are not defined. However, studies in mutant hSOD1 transgenic mice have revealed many pathogenic changes in degenerating MNs, including hyperexcitability, disturbed calcium homeostasis, SOD1 aggregation, and activation of cell death signals (Bento-Abreu et al., 2010; Cleveland and Rothstein, 2001; Pasinelli and Brown, 2006). Several of these pathogenic changes are related to calcium deregulation in neurons. Unfortunately, whether these are primary or secondary events or represent compensatory mechanisms remains unknown.

MNs are specifically vulnerable to membrane depolarization and increased intracellular calcium concentration (Arakawa et al., 2002). Levels of intracellular calcium determine neuronal dependence on neurotrophic factors and susceptibility to cell death, although how calcium induces MN cell death is not fully understood. Diverse molecular mechanisms that lead to neuronal degeneration and death in response to excessive calcium influx are being elucidated, among them the activation of specific enzymes such as protein phosphatases, endonucleases, or proteases. One of these enzymes is calpain, the calcium-sensitive protease that mediates cell death when intracellular calcium is increased (Das et al., 2005).

Previous results from our group and others have shown that chronically depolarizing conditions induces cell death in mouse MNs through increased intracellular calcium and calpain activation (Gou-Fabregas et al., 2009; Kaiser et al., 2006). This protease can become overactivated under extreme conditions — for example, as a consequence of sustained elevation of cytosolic calcium levels, which in turn is associated with apoptotic or non-apoptotic cell death (Wang, 2000). In the central nervous system, calpain activation is related to neuronal damage in ischemia, stroke, and Alzheimer and Huntington diseases (Cowan et al., 2008; Shields et al., 2000; Yamashima, 2013). One of these calpain substrates is the calcium–calmodulin dependent protein kinase type IV (CaMKIV). This kinase is highly expressed in the nervous system and is

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; MN, spinal cord motor neurons; hSOD1G93A, mutant human superoxide dismutase 1; CaMKIV, calcium–calmodulin dependent protein kinase IV; NTF, neurotrophic factor; BDNF, brain derived neurotrophic factor; GDNF, glial cell line-derived neurotrophic factor; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin I; HGF, hepatocyte growth factor; WT, wild type.

* Corresponding author at: Edif. Biomedicina I. Lab 4.1, Universitat de Lleida-IRBLLEIDA, Rovira Roure, 80, 25198-Lleida, Spain.
E-mail address: rosa.soler@cmb.udl.cat (R.M. Soler).
[1] Present address: Dept of Morphology & Biomedical Research Institute, Hasselt University, Diepenbeek BE3590, Belgium.
[2] AG and RMS are senior co-authors.

http://dx.doi.org/10.1016/j.mcn.2014.07.002
1044-7431/

involved in neuronal survival and development (Bok et al., 2007; Perez-Garcia et al., 2008; See et al., 2001). Under conditions of sustained calcium influx, CaMKIV and associated signaling molecules can be processed by activated calpain (McGinnis et al., 1998; Tremper-Wells and Vallano, 2005).

In the present study, we investigated the hypothesis that processes resulting from intracellular calcium increase, such as calpain activation and CaMKIV processing, are involved in ALS pathogenesis. We studied CaMKIV levels under culture conditions that are known to activate calpain protease activity in MNs. Results obtained indicate that membrane depolarization activates calpain and reduces CaMKIV protein level. We further analyzed calpain activation in cultured hSOD1G93A embryonic MNs and their vulnerability in response to membrane depolarization. No differences were found, compared to the control cultures. However, analysis of protein extracts from adult SOD1G93A mutant spinal cords revealed calpain activation and CaMKIV reduction, in support of our hypothesis.

Results

Effect of high-potassium medium on CaMKIV protein level in cultured mouse MNs

Addition of high potassium to the culture medium induces chronic depolarization of the neuronal plasma membrane, resulting in calcium influx and elevation of intracellular calcium concentration. Mouse spinal cord MNs are specifically vulnerable to this calcium increase and high-potassium treatment induces cell death. This cell death process is dependent on calpain activation (Gou-Fabregas et al., 2009). Under certain depolarizing conditions, calpain induces CaMKIV proteolysis (Tremper-Wells and Vallano, 2005), and CaMKIV is involved in neuronal survival (Hansen et al., 2003; Perez-Garcia et al., 2008; See et al., 2001).

To test whether membrane depolarization treatment induces changes in CaMKIV protein level, isolated MNs from E12.5 CD1 mouse embryos were cultured in the presence of neurotrophic factors with or without high potassium (see Experimental methods section). Three days after treatment, samples were collected and submitted to western blot. To determine calpain activation, we analyzed the presence of the calpain-specific 150/140 kDa α-fodrin fragment using an anti-α-fodrin antibody. As we reported previously (Gou-Fabregas et al., 2009), under depolarizing conditions the level of 140/150 kDa product was 1.7 times higher than observed in the NTF-treated control condition, indicating the activation of calpain. CaMKIV protein was also analyzed using an anti-CaMKIV antibody. The levels of CaMKIV protein in depolarized cultures were significantly lower than in controls (Fig. 1A). Together, these results suggest that depolarizing conditions in MNs induce an increase of calpain activity that can lead to CaMKIV proteolysis. Fodrin proteolysis and CaMKIV protein reduction are indicators of calcium dysregulation.

Effect of high-potassium treatment on cultured hSOD1G93A mutant MNs

In hSOD1 transgenic mice, intracellular calcium increase contributes to MN degeneration (Kaiser et al., 2006). High potassium treatment causes intracellular calcium increase and cell degeneration in cultured embryonic CD1 MNs. In order to establish whether there are differences between control and hSOD1 MNs, we decided to analyze the response of hSOD1-mutated MNs to depolarization. We obtained E12.5 embryos by crossing B6SJL-Tg (SOD1*G93A)1Gur/J (hSOD1G93A) mutants and B6SJL females. After genotyping, spinal cords from WT and hSOD1G93A mutants were dissected and MNs were isolated as described in the Experimental methods section. Cells were plated in 4-well dishes. Culture medium was supplemented with NTFs with or without 30 K. Three days after treatment, cell survival was evaluated. The percentage of surviving cells in 30 K conditions (WT and hSOD1G93A) was significantly lower than in NTFs' controls (51.1 ± 5.5% 30 K-treated WT and 44.8 ± 4.6% 30 K-treated hSOD1, $p < 0.005$; Fig. 1B). However, there were no significant survival differences between WT and hSOD1G93A cells under 30 K-treated conditions. This result shows that hSOD1G93A embryonic MNs are vulnerable to extracellular potassium increase in culture, but this vulnerability is comparable to cells from WT controls.

We also sought to analyze whether high-potassium treatment induces calpain activation and CaMKIV decrease. To this end, MNs were isolated from WT and hSOD1G93A mutants and cultured in the presence or absence of 30 K. Three days later, protein extracts were obtained and submitted to a western blot analysis using anti-α-fodrin and anti-CaMKIV antibodies. Results shown in Fig. 1C demonstrate an increase of 150/145 kDa-specific fodrin breakdown products and a reduction of CaMKIV protein levels in 30 K-treated hSOD1G93A MNs. Both parameters are also altered in the WT condition. These results together indicate that embryonic WT and hSOD1G93A mutant MNs show the same vulnerability in response to depolarizing conditions.

Fodrin cleavage and CaMKIV reduction in spinal cords from adult hSOD1G93A mice

Because isolated embryonic hSOD1G93A MNs did not show differences compared to WT embryonic MNs, we decided to analyze changes in calpain activity and CaMKIV protein level in adult spinal cords from hSOD1G93A mice. As described in the Experimental methods section, we obtained two sets of pooled protein extracts from spinal cord fragments, dissected from 30, 60, 90, and 120-day-old WT and hSOD1G93A mice. Protein extracts were obtained and submitted to western blot analysis using antibodies against α-fodrin or CaMKIV. We observed a significant increase of 150/145 kDa fodrin product in 90- and 120-day-old hSOD1G93A mutant samples compared to the WT condition (Fig. 2). The increase of fodrin products is time-dependent. Sixty-day-old samples show a slight but non-significant increase of 140/150 kDa product and no differences were observed in 30-day-old samples. The same protein extracts were probed using the CaMKIV antibody. Decreases in CaMKIV protein level are time-dependent. Accordingly, no differences were observed in 30-day-old samples, 60-day-old samples showed a slight but non-significant reduction, and 90-day-old and 120-day-old samples showed a significant reduction.

To determine whether calpain activation and CaMKIV measures differed by spinal cord section, 90-day-old WT or hSOD1G93A spinal cords were dissected in cervical or lumbar fragments and protein extracts were submitted to western blot. As observed in protein extracts obtained from the whole spinal cord, both cervical and lumbar hSOD1G93A samples showed increased fodrin products and reduced CaMKIV protein levels compared to WT controls (Fig. 3). However, there were no significant differences between cervical and lumbar samples, suggesting that these sections of the spinal cord undergo similar changes in fodrin and CaMKIV parameters at this period of disease evolution.

Discussion

The results obtained from the present work allow us to make two important contributions to the hSOD1G93A ALS mouse model. First, we report that embryonic cultured MNs did not differ from controls in their response to high-potassium treatment, and second, we propose that CaMKIV protective effects may be impaired during disease progression.

The increased presence of calpain-specific degradation products in protein extracts from adult spinal cords of SOD1 mutant mice indicates increased calpain activity. The observed calcium-dependent protease activation can be a consequence of either neuronal hyperexcitability or cytotoxic pathological conditions. We previously demonstrated that increased calpain activity induces MN cell death as a consequence of the specific vulnerability of these cells to high extracellular potassium

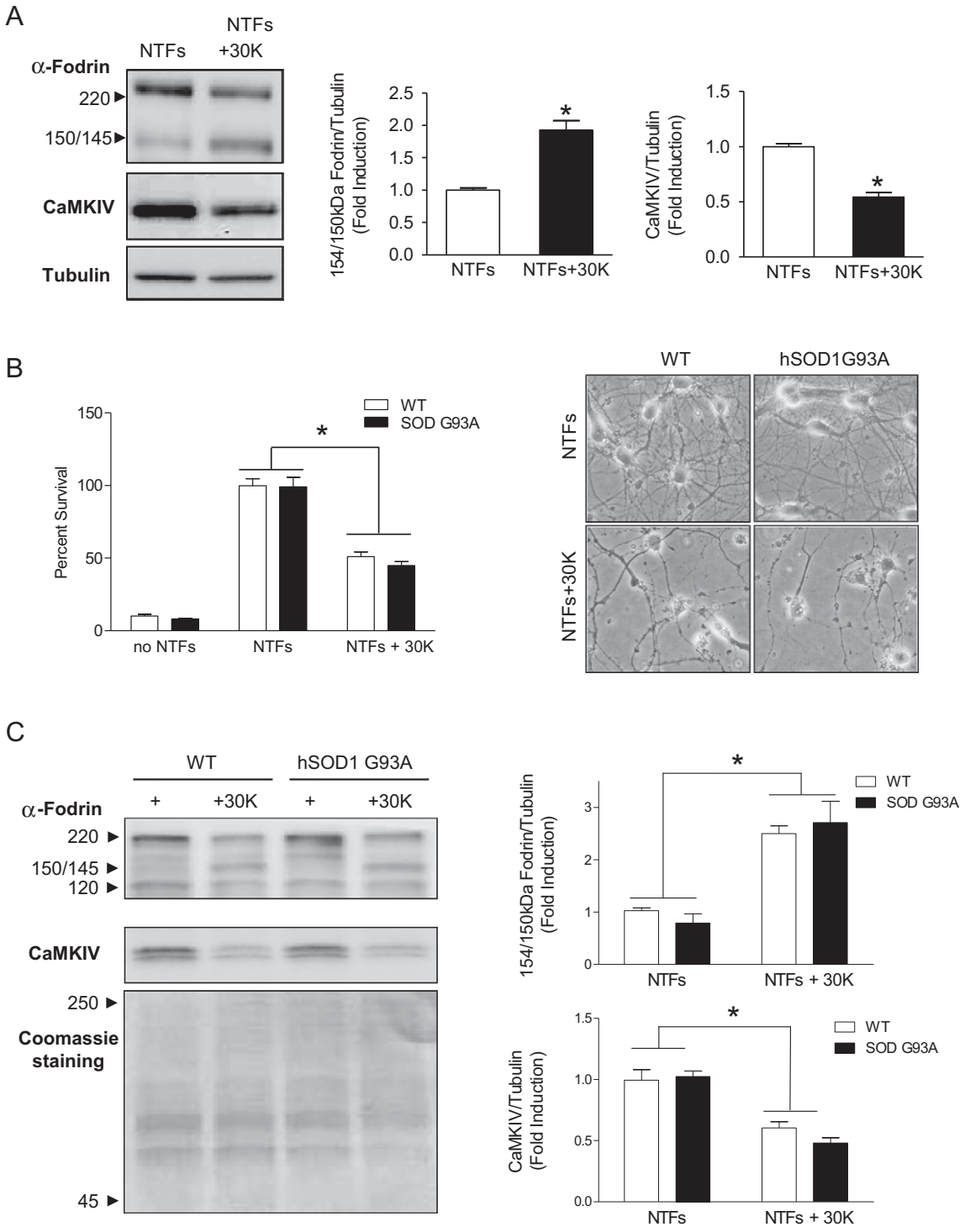


Fig. 1. Effect of 30 K treatment on embryonic MN survival, calpain-dependent α-fodrin cleavage, and CaMKIV protein level. MNs were cultured in the presence of NTFs (1 ng/mL BDNF; 10 ng/mL of GDNF, CNTF, CT-1, and HGF) or in the presence of NTFs plus 30 mM potassium (NTFs + 30 K). A) Total cell lysates of NTFs and NTFs + 30 K treated cultures were analyzed by western blot using anti-α-fodrin or CaMKIV antibodies. Membranes were re-blotted with α-tubulin antibody used as a loading control. Graphs represent the quantification of the 145/150 kDa band and CaMKIV from 3 or more independent experiments. *Asterisk* indicates significant differences between the 3 independent experiments using Student *t* test (*$p < 0.0007$ for 145/150 and $p < 0.0005$ for CaMKIV). B) The graph shows survival levels of MNs from WT or hSOD1G93A in the absence of NTFs or in the presence of NTFs with or without 30 K. Values are the mean of 3 independent wells for each condition ± SEM (error bars) of a representative experiment repeated at least 3 times, with comparable results. *Asterisk* indicates significant differences between NTFs treatment in the presence or absence of 30 K of 3 independent experiments using two-way ANOVA test (*$p < 0.005$). Representative microscope images of WT and hSOD1G93A mouse MNs' cultures under control (NTFs) and high-potassium (NTFs + 30 K) conditions. C) Representative western blot images from protein extracts of embryonic WT or hSOD1G93A MN cultures using antibodies against α-fodrin and CaMKIV. Coomassie staining was used as a loading control. The graphs show relative protein quantification levels of the 145/150 kDa α-fodrin fragment and CaMKIV levels. *Asterisk* indicates significant differences between NTFs treatment in the presence or absence of 30 K of 3 independent experiments using two-way ANOVA test (*$p < 0.005$).

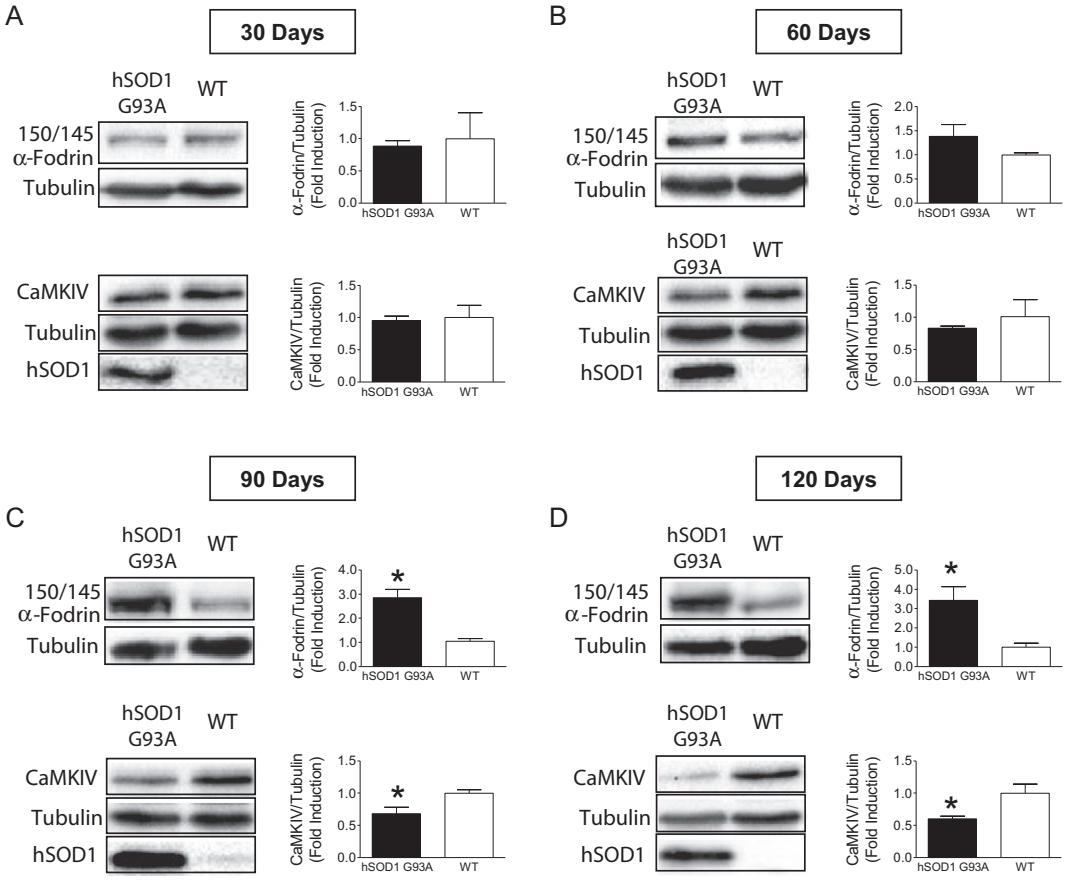


Fig. 2. Calpain-specific cleavage of α-fodrin and CaMKIV reduction in spinal cords from adult WT and SOD1G93A mice. Representative western blot images of protein extracts were obtained from pooled spinal cord fragments of WT or SOD1G93A transgenic mice. Each pool contains fragments of 5 spinal cords dissected from 30-, 60-, 90- or 120-day-old animals. Membranes were probed with an anti-α-fodrin antibody or anti-CaMKIV antibody and reprobed with an anti-α-tubulin antibody, used as a loading control. The graphs show the relative measures of the α-fodrin 145–150 kDa fragment or the CaMKIV levels from 3 separate experiments obtained from 3 independent WT or hSOD1G93A spinal cord pools. *Asterisk* indicates significant differences when comparing the data from the 3 independent experiments using Student *t* test ($^*p < 0.05$).

concentration, which becomes elevated during neuronal activity; if uncorrected, this causes continued neuronal depolarization and hyperexcitability (Gou-Fabregas et al., 2009).

Although it is technically difficult to directly determine the potassium concentration in local MN compartments of the spinal cord, some information from mouse models is available. For example, in the SOD1 transgenic model, the glial inwardly rectifying potassium channels (Kir 4.1) are decreased in the brain and ventral horn of the spinal cord of SOD1 mutants (Kaiser et al., 2006). These observations strongly support that potassium accumulation in perineuronal compartments of the spinal cord is a likely mechanism of MN excitotoxicity in the SOD1 transgenic model of ALS. Clearance of potassium from the extracellular space (potassium spatial buffering) is considered an important function of astrocytes. A number of studies have suggested that astrocyte maintenance of extracellular potassium levels is mediated by potassium uptake through the Kir4.1 channels. Thus, the loss of these channels impairs perineuronal potassium homeostasis and may contribute to MN degeneration by excitotoxic mechanisms (Kaiser et al., 2006; Kofuji et al., 2000, 2002; Neusch et al., 2001). The consequence of extracellular potassium accumulation is continued membrane depolarization, which induces an increase in intracellular calcium concentration.

It is well-known that calpain regulates cell survival in response to calcium signals and can be over-activated under extreme conditions as a result of sustained elevation of cytosolic calcium levels (Das et al., 2005; Stifanese et al., 2010; Wang, 2000). Calpain protease has several substrates (Wang, 2000). One of them, α-fodrin, has been extensively used as a marker of calpain protease activity. The specific degradation products (150/145 kDa) of α-fodrin have been attributed to the activation of calpain, whereas the 120 k Da fragment indicates caspase activation (Nath et al., 1996). Our previous work showed that sustained intracellular calcium increase causes calpain activation, elevation of 150/145 kDa fragments and MN cell death. This cell-death effect can be counteracted using calpain inhibitors or calpain gene silencing (Gou-Fabregas et al., 2009). Other calpain substrates have been related to the ALS pathology. It has been recently reported that calpain activation generates carboxy-terminal-cleaved TDP-43 fragments and causes mislocalization of TDP-43 in the MNs of a mouse model of ALS. The calpain-dependent TDP43 fragments have been found in the spinal cord and brain of ALS patients, supporting the crucial role of calpain-dependent cleavage of TDP43 in the ALS pathology (Yamashita et al., 2012).

Our results also demonstrate that CaMKIV protein levels are reduced in spinal cord protein extracts of adult SOD1 mutant mice. CaMKIV, a protein kinase involved in MN survival and a calpain substrate, has been reported to protect NSC34 MN-like cells from degeneration caused by transgenic SOD1 over-expression (Chiba et al., 2004). CaMKIV is also known to associate with the p85 subunit of PI 3-kinase and regulate PI 3-kinase/Akt pathway activity (Perez-Garcia et al., 2008), and a recent study reported that specific expression of constitutively active Akt3 in MNs prevents neuronal loss induced by hSOD1G93A (Peviani et al., 2013). A decade ago, Chiba and collaborators reported that activity-dependent neurotrophic factor protection (ADNF) against cell death caused by mutant SOD1 overexpression depends on CaMKIV and certain tyrosine kinase activity (Chiba et al., 2004). This suggests CaMKIV involvement in ALS pathogenesis through its role in regulating the PI 3-kinase/Akt survival pathway. Even though CaMKIV knockout mice

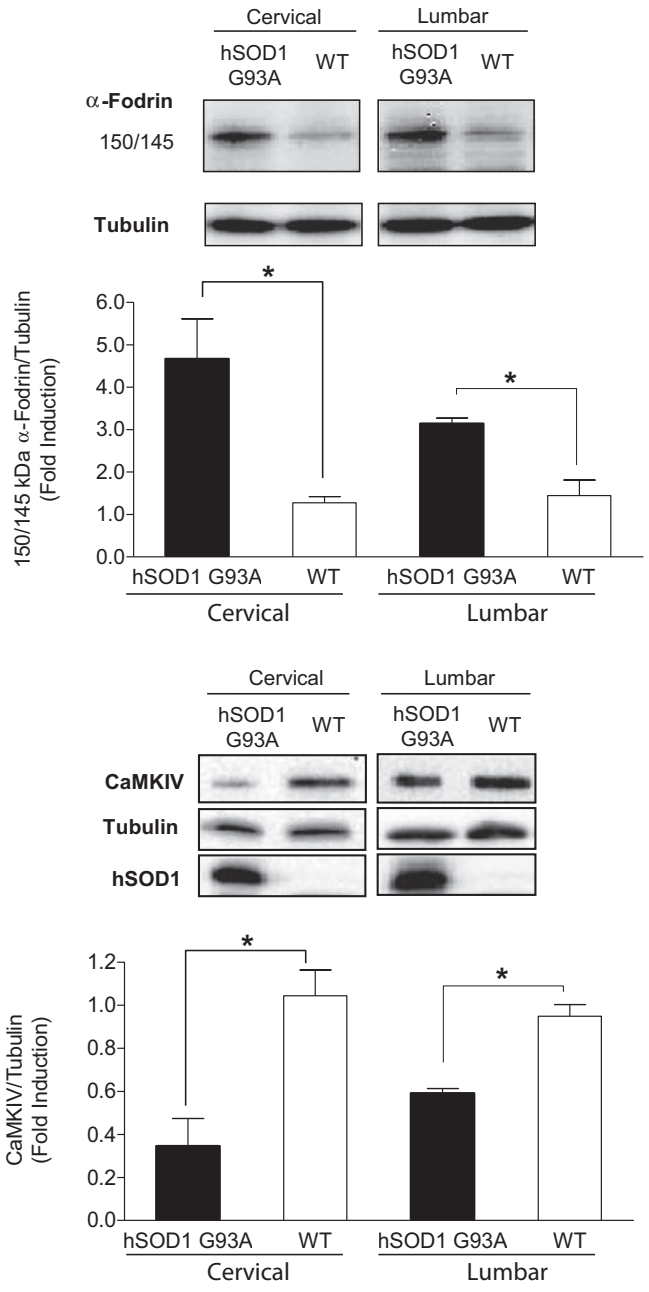


Fig. 3. α-Fodrin and CaMKIV levels in cranial and lumbar fragments of spinal cords of WT and hSOD1G93A mice. Representative western blot images of protein extracts obtained from pooled cervical or lumbar spinal cord fragments of WT or SOD1G93A transgenic mice. Each pool contains fragments of 5 spinal cords dissected from 90-day-old animals. Membranes were probed with an anti-α-fodrin antibody or anti-CaMKIV antibody and reprobed with an anti-α-tubulin antibody, used as a loading control. The graph shows the relative measures of the α-fodrin 145–150 kDa fragment or the CaMKIV levels from 2 separate experiments obtained from 2 independent WT or SOD1G93A spinal cord pools. *Asterisk* indicates significant differences when comparing the data from the 2 independent experiments using one-way ANOVA test ($^*p < 0.05$).

do not show specific MN defects under healthy conditions (Ho et al., 2000; Ribar et al., 2000), under pathologic conditions it is probable that deficiency or reduced activity of the protein could exacerbate degeneration processes that result from excitotoxic mechanisms or mutant SOD1 over-expression.

Our results from in vitro studies showed no specific differences in embryonic hSOD1G93A MNs compared to embryonic WT cells. This observation suggests that cell vulnerability to high potassium treatment is not affected by mutant SOD1 overexpression in embryonic MNs. Cell death, increased α-fodrin products and reduced CaMKIV were observed after high potassium treatment, but differences between embryonic hSOD1G93A MNs and control cells were not significant. However, significant differences were observed in proteins related to high calcium toxicity in spinal cords from symptomatic mice. Our results do not show whether these differences are located specifically in MNs, but do clearly demonstrate a deregulation of the level of these proteins, supporting the hypothesis that calcium homeostasis can be altered during disease progression (Grosskreutz et al., 2010; Kawamata and Manfredi, 2010). Further research is needed to determine whether CaMKIV reduction is directly caused by calpain activation in MNs and/or other spinal cord cells. The use of calpain inhibitors and a comparative analysis between events occurring in dorsal vs ventral spinal cord protein homogenates could provide valuable information.

Earlier theories suggested that MN degeneration during disease progression may be caused by alterations in the surrounding cells and MNs environment (Ilieva et al., 2009; Liao et al., 2012; Phatnani et al., 2013; Wang et al., 2011). This observation is further supported by the reduction of glial Kir4.1 channel observed in ALS, which contributes to developing an environment that is toxic to the MNs (Bataveljic et al., 2012; Kaiser et al., 2006). Thus, modification of calcium homeostasis in ALS MNs and surrounding cells is also supported by calpain activation and CaMKIV reduction in the adult symptomatic SOD1 mutant.

In summary, our study demonstrates calpain activation and reduced levels of CaMKIV protein in damaged MNs. We report calpain activation – assessed as calpain-specific processing of the cytoskeleton substrate α-fodrin (145/150 kDa fragment) – and decreased CaMKIV levels after 30 K treatment in WT and hSOD1G93A mutant mouse MN cultures. Protein extracts of adult hSOD1G93A spinal cord analysis revealed similar signs of calcium dysregulation, increased calpain-processed substrates, and reduced CaMKIV levels. These observations suggest the role of intracellular calcium changes in ALS pathogenesis and of calpain and CaMKIV mediation in this process (Fig. 4). Therefore, calpain and CaMKIV can be considered therapeutic targets in future ALS intervention strategies.

Experimental methods

Animals

Care and use of rodents followed the Spanish Council on Animal Care guidelines and was approved by the University of Lleida Advisory Committee on Animal Services. Transgenic mice expressing high copy numbers of the mutated (glycine 93 to alanine) form of human SOD1 (hSOD1), B6SJL-Tg(SOD1*G93A)1Gur/J (hSOD1-G93A), were purchased from The Jackson Laboratories (Bar Harbor, Maine, USA). The colony was maintained by breeding male hemizygous carriers with non-transgenic B6SJL females. Offspring were identified by PCR and non-transgenic G93A littermates were used as wild-type (WT) controls. For PCR analysis, genomic DNA was extracted from mice tails (adults) or heads (embryos) by proteinase K overnight digestion or REDExtract-N-Amp Tissue PCR Kit (Sigma), respectively. Primer pairs were the following: forward 5′-CTAGGCCACAGAATTGAAAGATCT-3′ and reverse 5′-GTAGGTGGAAATTCTAGCATCATCC-3′, both of which amplify a product of 324 bp from the endogenous interleukin2 gene, used as positive control for DNA amplification, and forward 5′-CATCAGCCCTAATCCATCTGA-3′ and reverse 5′-CGCGACTAACAATCAAAGTGA-3′, which amplify a product of 236 bp of exon4 from the hSOD1 gene within the transgene.

Spinal cords of 30, 60, 90, or 120-day-old genotyped mice were dissected and protein extracts were obtained. At the 90-day-old stage, hSOD1-G93A mutants show clinical symptoms. Protein extracts from 5 genotyped animals (hSOD1-G93A or WT) of each age were pooled and submitted to western blot using several antibodies. Two or three separate pools of hSOD1-G93A or WT protein extracts were analyzed.

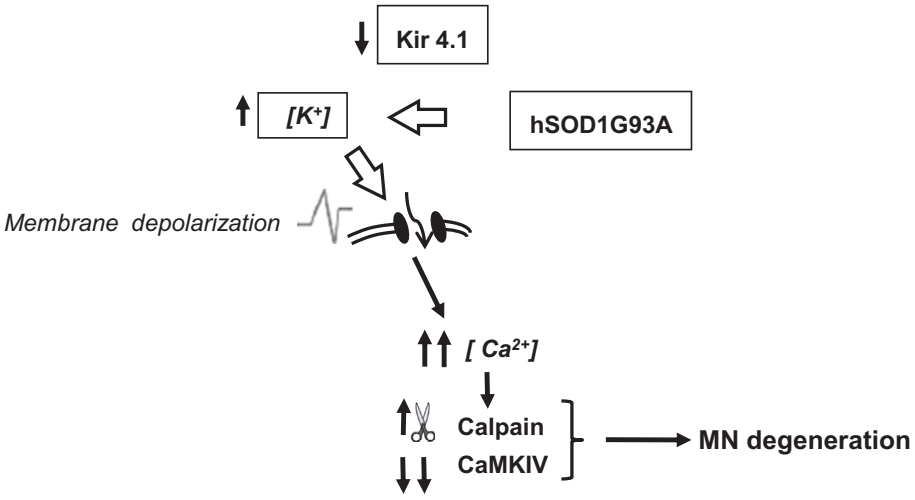


Fig. 4. Schematic model showing the proposed intracellular events of extracellular potassium increase and/or expression of mutated SOD1. Both mechanisms induce MN intracellular calcium overload, calpain activation, and reduction of CaMKIV protein levels. In ALS disease model, extracellular potassium increase can be a consequence of the reduction of the inward rectifying potassium channel, Kir 4.1. These mechanisms together or separately can contribute to MN hyperpolarization and degeneration.

Spinal cord MN isolation and culture

MN cultures were prepared from embryonic 12.5-day (E12.5) CD1 or transgenic mice spinal cord, essentially as described (Arce et al., 1999; Gou-Fabregas et al., 2009). After genotyping, WT and hSOD1-G93A embryos were submitted to spinal cord dissection and MN isolation following the same purification protocol. The isolated cells were collected in a tube containing culture medium and distributed in the tissue culture dishes. Enriched cultured MNs are clearly identified by morphological criteria (Fig. 1) and more than 80% of the cells are MNs identified by immunofluorescence using Islet-1/2 antibodies (Gou-Fabregas et al., 2009).

Isolated MNs were plated in 4-well tissue-culture dishes (Nunc, Thermo Fisher Scientific, Madrid, Spain) for survival experiments (1500 cells per well) and western blot (50,000 cells per well). Culture medium was NBMc (Neurobasal (Gibco, Invitrogen, Paisley, UK) supplemented with B27 (Gibco; Invitrogen), horse serum (2% v/v), L-glutamine (0.5 mM), and 2-mercaptoethanol (25 μM)). Cells were plated with complete medium containing a cocktail of recombinant NTFs (1 ng/mL BDNF; 10 ng/mL GDNF, 10 ng/mL CNTF, 10 ng/mL CT-1, and 10 ng/mL HGF; Peprotech, London, UK).

Western blot analysis

Western blots were performed as described (Gou-Fabregas et al., 2009; Perez-Garcia et al., 2004). Total cell lysates were resolved in sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride Immobilon-P transfer-membrane filters (Millipore, Billerica, MA, USA) using an Amersham Biosciences (Piscataway, NJ, USA) semidry Trans-Blot. The membranes were blotted with anti-SOD1 antibody (1:2000) (Millipore, Billerica, MA, USA), anti-alpha-Fodrin antibody (1:2000) (Biomol International Inc., Exeter, UK), and anti-CaMKIV antibody (1:1000) (BD, Transduction Laboratories, Franklin Lakes, NJ, USA). To control the specific protein content per lane, membranes were reprobed with a monoclonal anti-α-tubulin antibody (Sigma) or Coomassie stain. Blots were developed using the Super Signal chemiluminiscent substrate (Pierce, Rockford, IL, USA).

Statistical analysis

All experiments were performed at least 3 times. Values were expressed as mean ± SEM. The data obtained from the independent experiments were used for statistical analysis. We used one-way or two-way ANOVA to assess differences between treated and untreated groups and for transgenic and control cultures. If the ANOVA test was statistically significant, we performed post-hoc multiple comparisons using Bonferroni test; p values below 0.05 were considered statistically significant.

Acknowledgments

This work was supported by grants from the GENAME (Defining Targets for Therapeutics in SMA) to RMS; from the Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias [PI11-01047], Generalitat de Catalunya [SGR740], and Consolider-Ingenio 2010 [CSD2007-00020] to RMS. ORN holds a fellowship from the Comissionat de Universitats i Recerca, Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya, and Fons Social Europeu. We thank Elaine Lilly, Ph.D. (Writer's First Aid), for the English language revision of the manuscript.

References

Arakawa, Y., Nishijima, C., Shimizu, N., Urushidani, T., 2002. Survival-promoting activity of nimodipine and nifedipine in rat motoneurons: implications of an intrinsic calcium toxicity in motoneurons. J. Neurochem. 83, 150–156.
Arce, V., Garces, A., de Bovis, B., Filippi, P., Henderson, C., Pettmann, B., deLapeyriere, O., 1999. Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique. J. Neurosci. Res. 55, 119–126.
Bataveljic, D., Nikolic, L., Milosevic, M., Todorovic, N., Andjus, P.R., 2012. Changes in the astrocytic aquaporin-4 and inwardly rectifying potassium channel expression in the brain of the amyotrophic lateral sclerosis SOD1(G93A) rat model. Glia 60, 1991–2003.
Bento-Abreu, A., Van Damme, P., Van Den Bosch, L., Robberecht, W., 2010. The neurobiology of amyotrophic lateral sclerosis. Eur. J. Neurosci. 31, 2247–2265.
Bok, J., Wang, Q., Huang, J., Green, S.H., 2007. CaMKII and CaMKIV mediate distinct prosurvival signaling pathways in response to depolarization in neurons. Mol. Cell. Neurosci. 36, 13–26.
Chiba, T., Hashimoto, Y., Tajima, H., Yamada, M., Kato, R., Niikura, T., Terashita, K., Schulman, H., Aiso, S., Kita, Y., Matsuoka, M., Nishimoto, I., 2004. Neuroprotective effect of activity-dependent neurotrophic factor against toxicity from familial amyotrophic lateral sclerosis-linked mutant SOD1 in vitro and in vivo. J. Neurosci. Res. 78, 542–552.
Cleveland, D.W., Rothstein, J.D., 2001. From Charcot to Lou Gehrig: deciphering selective motor neuron death in ALS. Nat. Rev. Neurosci. 2, 806–819.
Cowan, C.M., Fan, M.M., Fan, J., Shehadeh, J., Zhang, L.Y., Graham, R.K., Hayden, M.R., Raymond, L.A., 2008. Polyglutamine-modulated striatal calpain activity in YAC transgenic huntington disease mouse model: impact on NMDA receptor function and toxicity. J. Neurosci. 28, 12725–12735.
Das, A., Sribnick, E.A., Wingrave, J.M., Del Re, A.M., Woodward, J.J., Appel, S.H., Banik, N.L., Ray, S.K., 2005. Calpain activation in apoptosis of ventral spinal cord 4.1 (VSC4.1) motoneurons exposed to glutamate: calpain inhibition provides functional neuroprotection. J. Neurosci. Res. 81, 551–562.
Gou-Fabregas, M., Garcera, A., Mincheva, S., Perez-Garcia, M.J., Comella, J.X., Soler, R.M., 2009. Specific vulnerability of mouse spinal cord motoneurons to membrane depolarization. J. Neurochem. 110, 1842–1854.

Grosskreutz, J., Van Den Bosch, L., Keller, B.U., 2010. Calcium dysregulation in amyotrophic lateral sclerosis. Cell Calcium 47, 165–174.
Hansen, M.R., Bok, J., Devaiah, A.K., Zha, X.M., Green, S.H., 2003. Ca^{2+}/calmodulin-dependent protein kinases II and IV both promote survival but differ in their effects on axon growth in spiral ganglion neurons. J. Neurosci. Res. 72, 169–184.
Ho, N., Liauw, J.A., Blaeser, F., Wei, F., Hanissian, S., Muglia, L.M., Wozniak, D.F., Nardi, A., Arvin, K.L., Holtzman, D.M., Linden, D.J., Zhuo, M., Muglia, L.J., Chatila, T.A., 2000. Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca^{2+}/calmodulin-dependent protein kinase type IV/Gr-deficient mice. J. Neurosci. 20, 6459–6472.
Ilieva, H., Polymenidou, M., Cleveland, D.W., 2009. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J. Cell Biol. 187, 761–772.
Kaiser, M., Maletzki, I., Hulsmann, S., Holtmann, B., Schulz-Schaeffer, W., Kirchhoff, F., Bahr, M., Neusch, C., 2006. Progressive loss of a glial potassium channel (KCNJ10) in the spinal cord of the SOD1 (G93A) transgenic mouse model of amyotrophic lateral sclerosis. J. Neurochem. 99, 900–912.
Kawamata, H., Manfredi, G., 2010. Mitochondrial dysfunction and intracellular calcium dysregulation in ALS. Mech. Ageing Dev. 131, 517–526.
Kofuji, P., Ceelen, P., Zahs, K.R., Surbeck, L.W., Lester, H.A., Newman, E.A., 2000. Genetic inactivation of an inwardly rectifying potassium channel (Kir4.1 subunit) in mice: phenotypic impact in retina. J. Neurosci. 20, 5733–5740.
Kofuji, P., Biedermann, B., Siddharthan, V., Raap, M., Iandiev, I., Milenkovic, I., Thomzig, A., Veh, R.W., Bringmann, A., Reichenbach, A., 2002. Kir potassium channel subunit expression in retinal glial cells: implications for spatial potassium buffering. Glia 39, 292–303.
Liao, B., Zhao, W., Beers, D.R., Henkel, J.S., Appel, S.H., 2012. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. Exp. Neurol. 237, 147–152.
McGinnis, K.M., Whitton, M.M., Gnegy, M.E., Wang, K.K., 1998. Calcium/calmodulin-dependent protein kinase IV is cleaved by caspase-3 and calpain in SH-SY5Y human neuroblastoma cells undergoing apoptosis. J. Biol. Chem. 273, 19993–20000.
Nath, R., Raser, K.J., Stafford, D., Hajimohammadreza, I., Posner, A., Allen, H., Talanian, R.V., Yuen, P., Gilbertsen, R.B., Wang, K.K., 1996. Non-erythroid alpha-spectrin breakdown by calpain and interleukin 1 beta-converting-enzyme-like protease(s) in apoptotic cells: contributory roles of both protease families in neuronal apoptosis. Biochem. J. 319 (Pt 3), 683–690.
Neusch, C., Rozengurt, N., Jacobs, R.E., Lester, H.A., Kofuji, P., 2001. Kir4.1 potassium channel subunit is crucial for oligodendrocyte development and in vivo myelination. J. Neurosci. 21, 5429–5438.
Pasinelli, P., Brown, R.H., 2006. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat. Rev. Neurosci. 7, 710–723.
Perez-Garcia, M.J., Cena, V., de Pablo, Y., Llovera, M., Comella, J.X., Soler, R.M., 2004. Glial cell line-derived neurotrophic factor increases intracellular calcium concentration. Role of calcium/calmodulin in the activation of the phosphatidylinositol 3-kinase pathway. J. Biol. Chem. 279, 6132–6142.
Perez-Garcia, M.J., Gou-Fabregas, M., de Pablo, Y., Llovera, M., Comella, J.X., Soler, R.M., 2008. Neuroprotection by neurotrophic factors and membrane depolarization is regulated by calmodulin kinase IV. J. Biol. Chem. 283, 4133–4144.
Peviani, M., Tortarolo, M., Battaglia, E., Piva, R., Bendotti, C., 2013. Specific induction of Akt3 in spinal cord motor neurons is neuroprotective in a mouse model of familial amyotrophic lateral sclerosis. Mol. Neurobiol. 49, 136–148.
Phatnani, H.P., Guarnieri, P., Friedman, B.A., Carrasco, M.A., Muratet, M., O'Keeffe, S., Nwakeze, C., Pauli-Behn, F., Newberry, K.M., Meadows, S.K., Tapia, J.C., Myers, R.M., Maniatis, T., 2013. Intricate interplay between astrocytes and motor neurons in ALS. Proc. Natl. Acad. Sci. U. S. A. 110, E756–E765.
Ribar, T.J., Rodriguiz, R.M., Khiroug, L., Wetsel, W.C., Augustine, G.J., Means, A.R., 2000. Cerebellar defects in Ca^{2+}/calmodulin kinase IV-deficient mice. J. Neurosci. 20, Rc107.
Rosen, D.R., Siddique, T., Patterson, D., Figlewicz, D.A., Sapp, P., Hentati, A., Donaldson, D., Goto, J., O'Regan, J.P., Deng, H.X., et al., 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362, 59–62.
See, V., Boutillier, A.L., Bito, H., Loeffler, J.P., 2001. Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons. FASEB J. 15, 134–144.
Shields, D.C., Schaecher, K.E., Hogan, E.L., Banik, N.L., 2000. Calpain activity and expression increased in activated glial and inflammatory cells in penumbra of spinal cord injury lesion. J. Neurosci. Res. 61, 146–150.
Stifanese, R., Averna, M., De Tullio, R., Pedrazzi, M., Beccaria, F., Salamino, F., Milanese, M., Bonanno, G., Pontremoli, S., Melloni, E., 2010. Adaptive modifications in the calpain/calpastatin system in brain cells after persistent alteration in Ca^{2+} homeostasis. J. Biol. Chem. 285, 631–643.
Tremper-Wells, B., Vallano, M.L., 2005. Nuclear calpain regulates Ca^{2+}-dependent signaling via proteolysis of nuclear Ca^{2+}/calmodulin-dependent protein kinase type IV in cultured neurons. J. Biol. Chem. 280, 2165–2175.
Wang, K.K., 2000. Calpain and caspase: can you tell the difference? Trends Neurosci. 23, 20–26.
Wang, L., Gutmann, D.H., Roos, R.P., 2011. Astrocyte loss of mutant SOD1 delays ALS disease onset and progression in G85R transgenic mice. Hum. Mol. Genet. 20, 286–293.
Yamashima, T., 2013. Reconsider Alzheimer's disease by the 'calpain–cathepsin hypothesis'—a perspective review. Prog. Neurobiol. 105, 1–23.
Yamashita, T., Hideyama, T., Hachiga, K., Teramoto, S., Takano, J., Iwata, N., Saido, T.C., Kwak, S., 2012. A role for calpain-dependent cleavage of TDP-43 in amyotrophic lateral sclerosis pathology. Nat. Commun. 3, 1307.