Instituto de Neurociencias

Universidad Miguel Hernández-CSIC

Secretases as potential biomarkers and therapeutic target for Alzheimer's disease

Memoria de Tesis Doctoral

Aitana Sogorb Esteve

Director de Tesis:

Dr. Javier Sáez Valero

Codirectora de Tesis:

Dra. María Salud García Ayllón

Sant Joan d'Alacant, 2018

Programa de Doctorado en Neurociencias

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San Juan de Alicante, 8th of March 2018

DOCTORAL THESIS BY COMPENDIUM OF PUBLICATIONS

To whom it may concern:

The doctoral thesis developed by me, Aitana Sogorb Esteve, with title: "**Secretases as potential biomarkers and therapeutic target for Alzheimer's disease**", is a compendium of publications and includes the following publications in which I am the first author:

Cerebrospinal fluid Presenilin-1 increases at asymptomatic stage in genetically determined Alzheimer's disease

Aitana Sogorb-Esteve, María-Salud García-Ayllón, Juan Fortea, Raquel Sánchez-Valle, Alberto Lleó, José-Luis Molinuevo and Javier Sáez-Valero.

Molecular Neurodegeneration. 2016 11:66. doi: 10.1186/s13024-016-0131-2

Inhibition of γ-Secretase Leads to an Increase in Presenilin-1

Aitana Sogorb-Esteve, María-Salud García-Ayllón, Marta Llansola, Vicente Felipo, Kaj Blennow, Javier Sáez-Valero.

Molecular Neurobiology. 2017. doi: 10.1007/s12035-017-0705-1

Levels of ADAM10 are reduced in Alzheimer's disease CSF

Aitana Sogorb-Esteve, María-Salud García-Ayllón, Johan Gobom, Jordi Alom, Henrik Zetterberg, Kaj Blennow, Javier Sáez-Valero.

Submitted to Alzheimer's Research and Therapy. 2018. Submission code: AZRT-D-18-00071

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El Dr. **Javier Sáez Valero**, Catedrático del Departamento de Bioquímica y Biología Molecular de la Universidad Miguel Hernández (UMH) e Investigador adscrito al Instituto de Neurociencias de Alicante, centro mixto UMH-Consejo Superior de Investigaciones Científicas (CSIC), **y la Dra. María Salud García Ayllón,** Investigadora Miguel Servet de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana en el Hospital General Universitario de Elche declaran:

Que D[a]. Aitana Sogorb Esteve, ha realizado bajo su dirección el trabajo experimental que recoge en su Tesis Doctoral "**Secretases as potential biomarkers and therapeutic target for Alzheimer's disease**".

Que han revisado los contenidos científicos y los aspectos formales del trabajo y dan su conformidad para que se presente el borrador de tesis a la Comisión de Doctorado de la Universidad Miguel Hernández.

Para que así conste, y a los efectos oportunos, expiden y firman el presente Certificado en Sant Joan d'Alacant, 8 de Marzo de 2018.

Fdo.: Javier Sáez Valero
Director de Tesis

Fdo. : María Salud García Ayllón
Codirectora de Tesis

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INFORME DE LA COMISION ACADEMICA DEL PROGRAMA DE DOCTORADO EN NEUROCIENCIAS

Por la presente, la Comisión Académica del Programa de Doctorado en Neurociencias:

Informa FAVORABLEMENTE el depósito de la Tesis presentada por Dña. Aitana Sogorb Esteve,

Realizada bajo la dirección del Dr. Javier Sáez Valero y la codirección de la Dra. María Salud García Ayllón,

Titulada: Secretases as potential biomarkers and therapeutic target for Alzheimer's disease.

Presentada por compendio de publicaciones.

Sant Joan d'Alacant, 8 de marzo 2018

Dr. Miguel Valdeolmillos

Coordinador del programa de Doctorado en Neurociencias

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A Paquita y Alba

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A Salu, por no ser solo una jefa, por ser mi compañera, mi maestra y mi amiga; por enseñarme con paciencia, saber llevar mis errores y confiar siempre en mis capacidades; por no dejarme ir y contribuir a que pueda haber acabado esta tesis en condiciones y a que se quede tan bonita. Porque hiciste lo posible por mí y ha sido un placer trabajar contigo.

A Javier, por ser un jefe cercano e involucrarme en muchos de los proyectos del lab, ayudando así a mi formación como investigadora y como persona, y por intentar mantenerme formando parte de este equipo.

Por todo esto, os doy las gracias por haber depositado vuestra confianza en mí y haber luchado por mantenerme en el laboratorio y por conseguir que mi tesis saliese adelante, todo este trabajo y mis años en el laboratorio no hubiesen sido posibles sin vuestra ayuda. Soy muy consciente del esfuerzo que habéis hecho por mí y siempre os estaré agradecida.

Tampoco puedo olvidarme de las Inmas, gracias por vuestra amistad durante estos años, vuestra ayuda y vuestros innumerables consejos. Y también a todas las personas que han pasado por el 241 (Giulia, Mari, Guille, Claudia, Borja, Trini Maciá, Trini Mata, Mar, Esther…) y, de una forma u otra, habéis dejado huella en mí, ayudándome, enseñándome y dejando que os enseñe yo.

También querría dar las gracias a Josemi, Aída y Ana, por formar esta bonita familia conmigo, y a todos los del Viana's lab y agregados, que estuvieron (Silvia, Baptiste, Danny…) y están (Kat, Fede, Sempere…) para hacer siempre hueco a una bióloga molecular entre tanta electrofisiología. Por todos los asados y cervezas que nos han unido y nos quedan por celebrar. Y, por supuesto, no me olvidaré del apoyo que tuve de Javi y Adri en los comienzos de esta etapa.

AKNOWLEDGEMENTS

El més fort dels agraïments voldria dedicar-lo als meus pares, la meua germana i les meues ties. A vosaltres, papa i mama, per haver estat sempre de la banda de les vostres filles i haver donat el support necessari en totes les nostres decisions, encara que no foren les millors per a nostraltres; per haver-nos ensenyat que, amb la feina ben feta, es por arribar lluny, i que tot esforç paga la pena si arribes al final. Per no haver dubtat mai de la meua capacitat i ajudar-me a seguir endavant encara que parega que no hi ha eixida. Aquest treball també ha sigut possible per vosaltres.

Y como no todo en una tesis es ciencia, las gracias también van para aquellos que completan la otra parte de mi vida. A las niñas prodigio Maria, Inés, Laura, Cris, Irene y Raquel por acompañarme, aunque sea en la distancia, durante tantos años y hacer que me sienta feliz de teneros. A Julia, porque lo más bonito de Barcelona y de Granada, eres tú, gracias por hacer de mi tesis una obra de arte. Y a los gallegos naturales, Monchi, Pedrito, Maria, Nach, Ger, Giulia, Aitor, Maciá, Jaume… porque, aunque no os hayáis dado cuenta, también contribuís a esto.

Y, por último, no voy a olvidarme de ti, Ale. Porque has llegado en la etapa final, la más difícil, pero has sido un pilar fundamental para ella. Gracias por confiar en mí, y decírmelo, por apoyarme incondicionalmente y por aguantar los desquicios de una estudiante predoctoral en plena escritura de la tesis. Todo este trabajo lleva mucho de ti.

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ABREVIATIONS

AChEIs: Acetilcholinesterase Inhibitors

AEP: aspartyl endopeptidase

AD: Alzheimer Disease

ADAD or FAD: autosomal dominant AD or familial AD

ADAM: α-desintegrin and metalloprotease family

ADAM10: α-desintegrin and metalloprotease 10

ADAM10f: full length ADAM10

ADAM-17: tumour necrosis factor-K (TNFK)-converting enzyme

AICD: amyloid intracellular domain

Aph1: anterior pharynx-defective 1

ApoE: apolipoprotein E

APP: amyloid β-protein precursor

Aβ: amyloid-β peptide

Aβ40, Aβ42 and Aβ17: soluble amyloid beta peptide 1-40, 1-42 and 1-17

BACE1: Aβ-site APP cleaving enzyme 1

CNS: central nervous system

CSF: cerebrospinal fluid

CSF-PS1: PS1 in CSF

CTF: C-terminus fragment

Ctrl: control

DS: Down syndrome

dDS: DS subjects with Alzheimer's type dementia

ER: endoplasmic reticulum

eNC: elderly controls

FDG PET: 1^{8}F-fluoroseoxyglucose PET

GSAP: γ-secretase activating protein

GSI: γ-secretase inhibitor

ICD: intracellular domain

I-CLiPs: Intramembrane-cleaving proteases

IMR: immunomagnetic reduction

LDLR: low-density lipoprotein receptor

MCI: mild cognitive impairment

MRI: magnetic resonance imaging

NADC: non-AD controls

NDC: non-demented controls

ndDS: DS subjects without signs of dementia

NC: control subjects

NFTs: neurofibrillary tangles

NMDAR: N-metyl-d-aspartate receptor

NO: nitric oxide

NTF: N-terminal fragment

oAβ: oligomeric Aβ

P-tau: phospho-tau

PHF: paired helical filaments

Pen2: presenilin enhancer 2

PET: positron emission tomography

proADAM10: ADAM10 prodomain

PS1 and PS2: presenilin 1 and presenilin 2

psADAD: presymptomatic ADAD

sAD: sporadic AD

sADAM10: soluble ADAM10

sAPPα and sAPPβ: soluble APPα and APPβ

Simoa: single-molelule array

STEM: scanning transmission electron microscopy

syADAD: symptomatic ADAD

TGN: trans-Golgi network

TMD: transmembrane domain

T-tau: total amount of tau

yNC: youger non-disease controls


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ABSTRACT/RESUMEN/RESUM

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and cognition. The brain of AD patients is characterized by co-existence of amyloid plaques, extracellular protein deposits where the major component is the β-amyloid peptide (Aβ), and neurofibrillary tangles (NFTs), composed of paired helical filaments of the microtubule-associated protein tau abnormally hyperphosphorylated (P-tau). The Aβ peptide is a small polypeptide generated by the proteolytic processing of a much larger transmembrane protein, the β-amyloid precursor protein (APP) through the successive action of two proteolytic enzymes, β-secretase and γ-secretase. γ-Secretase is an intramembranous multi-protein complex that cleaves more than 90 substrates, some of them with critical roles in neuronal function. Presenilin-1 (PS1) is the catalytic component of the γ-secretase complex. Our group has previously demonstrated the presence of PS1 in human cerebrospinal fluid (CSF) samples.

Since the diagnosis for AD is mainly based on clinical symptoms, definition of an early biomarker for the disease is needed. Our present study further explores the potential of the levels of PS1 in human CSF as an early biomarker for AD. In this regard, we have analysed by Western blotting and sucrose gradients ultracentrifugation the levels of PS1 in CSF samples from symptomatic and asymptomatic genetically determined AD subjects (autosomal dominant AD: ADAD), from demented and non-demented Down Syndrome (DS) patients, from sporadic AD (sAD) and from mild cognitive impairment subjects (MCI), all compared to age-matched controls. We demonstrated an increase in high stable PS1 complexes and altered levels of CSF-PS1 in both symptomatic and asymptomatic ADAD subjects, in DS subjects with and without dementia and also in sAD and MCI subjects. We concluded that the occurrence of increased levels of high stable PS1 complexes in the CSF are more related to the brain pathological status than the occurrence of dementia and cognitive decline. Our results suggest that such increase is an early phenomenon associated to AD and may constitute an early and asymptomatic biomarker.

Moreover, APP can undergo alternative processing pathways. Indeed, in the main pathway, the non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ domain, precluding Aβ formation. We were also interested in characterize whether α-secretase ADAM10 (α-disintegrin and metalloprotease 10) is present in CSF. We found different ADAM10 species in human CSF. We identify by Western blotting two mature forms corresponding to the full length (ADAM10f) and the soluble form of ADAM10 (sADAM10), and also the immature form of the ADAM10 (proADAM10). In CSF samples from AD patients we found a significant decrease in mature forms, ADAM10f and sADAM10 compared to control CSF, while proADAM10 levels remained unaltered. Our data suggest the potential to explore decreased levels of mature forms of CSF-ADAM10 as a new and alternative biomarker for AD.

Finally, we also extended our research on understanding the failure of γ-secretase inhibitors (GSI) as a therapy for AD. Most of the recently assayed AD therapies have not been successful to improve the condition of the patients. Some of these therapies aim to decrease the production of Aβ by inhibition of γ-secretase/PS1. Specifically, we addressed the possibility that GSIs can provoke a rebound effect, elevating the levels of the catalytic γ-secretase subunit, PS1. We performed *in vitro* experiments in which we obtained augments in PS1 after treatment with DAPT, a well-known GSI, or avagacestat, one of the first GSI that undergone clinical trials. We also performed *in vivo* experiments in which rats were sub-chronically treated with avagacestat. PS1 was increased in brain extracts from treated rats. In all the conditions, we found a rebound effect in PS1 as consequence of the γ-secretase inhibition. These results indicate that the rebound increase in PS1 in response to GSIs must be taken into consideration for the design of future therapeutic drugs.

RESUMEN

La enfermedad de Alzheimer (EA) es un desorden neurodegenerativo caracterizado por la pérdida progresiva de memoria y capacidades cognitivas. El cerebro de los pacientes con EA se caracteriza por la presencia de depósitos extracelulares, las denominadas placas amiloides, cuyo principal componente es el péptido β-amiloide (Aβ), y ovillos neurofibrilares, formaciones intracelulares compuestas por filamentos de la proteína citoesquelética tau, anormalmente hiperfosforilizada (P-tau). El péptido Aβ es un pequeño polipéptido que se genera como resultado del procesamiento proteolítico de una proteína transmembrana de mayor tamaño, la proteína precursora del β amiloide (APP, del inglés amyloid precursor protein), a través de la acción sucesiva de dos enzimas proteolíticas, β-secretasa y γ-secretasa. γ-Secretasa es un complejo enzimático localizado en la membrana que procesa más de 90 sustratos diferentes, muchos de ellos con funciones importantes en la actividad neuronal. Presenilina-1 (PS1) es el componente catalítico de dicho complejo. Estudios previos de nuestro grupo han demostrado la presencia de PS1 en líquido cefalorraquídeo (LCR) humano.

Actualmente, el diagnóstico de la EA está basado en la sintomatología clínica, por ello, existe una necesidad de definir un biomarcador temprano para la enfermedad. Uno de los estudios incluidos en la presente Memoria de Tesis explora el potencial de los niveles de PS1 en LCR humano como un biomarcador temprano para la EA. En esta línea de la investigación hemos analizado mediante Western blot y ultracentrifugación en gradientes de sacarosa los niveles de PS1 en muestras de LCR de sujetos sintomáticos y asintomáticos que presentan la variante genéticamente determinada de la EA (EA dominante autosómica), de individuos demenciados y no demenciados con síndrome de Down (SD), de pacientes con EA esporádica y de pacientes con déficit cognitivo leve (DCL), todos ellos comparados con controles de edad pareada. Con estos estudios hemos demostrado un incremento en los denominados complejos de PS1 altamente estables, así como niveles alterados de la PS1 en LCR de pacientes con EA dominante autosómica sintomáticos y asintomáticos, en pacientes con SD con y sin demencia y en los pacientes de EA esporádica y DCL. Concluimos que el incremento en el LCR de los niveles de

complejos altamente estables de PS1 está más relacionada con el estado patológico del cerebro que con la progresión de la demencia y el declive cognitivo. Nuestros resultados sugieren que dicho aumento es un fenómeno temprano asociado con la EA y podría constituir un biomarcador asintomático temprano.

Dentro de la línea de búsqueda de nuevos biomarcadores de EA incluimos un segundo estudio en el que analizamos ADAM10, proteína identificada como la responsable de la actividad α-secretasa en LCR. APP es procesada por dos vías alternativas, la no amiloidogénica, que es la vía principal en situaciones normales, y la vía amiloidogénica, en la que se genera el Aβ. En la vía no amiloidogénica, la enzima α-secretasa corta a APP por el dominio Aβ, evitando así la formación del péptido Aβ. Demostramos mediante Western blot que en LCR están presentes diferentes variantes de ADAM10 que corresponden a una forma inmadura que mantiene el prodominio, una forma madura completa no procesada y una forma truncada secretada desde la membrana celular. No obstante, lo más relevante es que encontramos que los niveles de formas maduras estaban disminuidos en LCR de pacientes con EA, en comparación con pacientes control. Sin embargo, la cantidad de forma inmadura era semejante entre muestras control y patológicas. Nuestros resultados sugieren los niveles reducidos de las formas maduras de ADAM10 en el LCR como un potencial y alternativo biomarcador para la EA.

Por otro lado, también decidimos estudiar a que es debido el fracaso de los inhibidores de γ-secretasa (GSIs, del inglés gamma secretase inhibitors) como terapia para la EA. Hasta ahora, la mayoría de las terapias testadas para la enfermedad que han llegado a ensayo clínico no han tenido éxito alguno en la mejora cognitiva de los pacientes y los ensayos han tenido que ser suspendidos. Muchas de estas terapias son inhibidores de γ-secretasa o PS1 que tienen como fin reducir la producción de Aβ. Específicamente, en nuestro estudio abordamos la posibilidad de que el uso sostenido de GSIs pueda provocar un efecto rebote, elevando así los niveles de PS1, que contribuirían a un empeoramiento de la patología. Llevamos a cabo experimentos *in vitro* en los que realizamos tratamientos con dos GSIs, DAPT y avagacestat, este último probado en ensayos clínicos, y obtuvimos en ambos casos un aumento en PS1. Esto nos llevó a estudiar el efecto de avagacestat en experimentos *in vivo* en los cuales se realizó un

tratamiento sub-crónico con el GSI avagacestat en ratas. Obtuvimos un incremento de los niveles de PS1 en los extractos de cerebro de las ratas tratadas con el inhibidor. En resumen, encontramos un efecto rebote en PS1 como consecuencia de la inhibición de γ-secretasa. Es por ello que estos resultados deben tenerse en cuenta para el diseño de futuros fármacos para la EA.

RESUM

La malaltia d'Alzheimer (MA) és un desordre neurodegeneratiu caracteritzat per la pèrdua progressiva de memòria i capacitats cognitives. El cervell dels pacients amb MA es caracteritza per la presència de dipòsits extracel·lulars, les denominades plaques amiloides, el principal component dels quals és el pèptid β amiloide (Aβ), i cabdells neurofibrilars, formacions intracel·lulars compostes per filaments de la proteïna citoesquelètica tau, anormalment hiperfosforilitzada (P-tau). El pèptid Aβ és un petit polipèptid que es genera com a resultat del processament proteolític d'una proteïna transmembrana de major grandària, la proteïna precursora del β amiloide (APP, de l'anglès amyloid precursor protein), a través de l'acció successiva de dos enzims proteolítiques, β-secretasa i γ-secretasa. γ-Secretasa és un complex situat en la membrana que processa més de 90 substrats diferents, molts d'ells amb funcions importants en la funció neuronal. Presenilina-1 (PS1) és el component catalític de dit complex. Estudis previs del nostre grup han demostrat la presència de PS1 en líquid cefaloraquidi (LCR) humà.

Actualment, el diagnòstic de la MA està basat en la simptomatologia clínica, per això, existeix una necessitat de definir un biomarcador primerenc per a la malaltia. Un dels estudis inclosos en la present Memòria de Tesi explora el potencial dels nivells de PS1 en LCR humà com un biomarcador primerenc per a la MA. En aquesta línia de la recerca hem analitzat mitjançant Western blot i ultracentrifugació en gradients de sacarosa els nivells de PS1 en mostres de LCR de pacients simptomàtics i asimptomàtics que presenten la variant genèticament determinada de la MA (MA dominant autosòmica), de pacients demenciats i no demenciats amb síndrome de Down (SD), de pacients amb MA esporàdica i de pacients amb dèficit cognitiu lleu (DCL), tots ells comparats amb controls d'edat aparellada. Amb aquests estudis hem demostrat un increment en els denominats complexos de PS1 altament estables, així com nivells alterats de la PS1 en LCR de pacients amb MA dominant autosòmica simptomàtics i asimptomàtics, en pacients amb SD amb i sense demència i en els pacients de MA esporàdica i DCL. Concloem que l'increment en el LCR dels nivells de complexos altament

estables de PS1 està més relacionada amb l'estat patològic del cervell que amb la progressió de la demència i el declivi cognitiu. Els nostres resultats suggereixen que aquest augment és un fenomen primerenc associat amb la MA i podria constituir un biomarcador asimptomàtic primerenc.

Dins de la línia de cerca de nous biomarcadors de la MA incloem un segon estudi en el qual analitzem ADAM10, identificada com l'enzim responsable de l'activitat α-secretasa en LCR. APP és processada per dues vies alternatives, la no amiloidogènica, que és la via principal en situacions normals, i la via amiloidogènica, en la qual es genera l'Aβ. En la via no amiloidogènica, l'enzim α-secretasa talla a APP pel domini Aβ, evitant així la formació del pèptid Aβ. Vam demostrar mitjançant Western blot que en LCR estan presents diferents variants d'ADAM10 que corresponen a dues formes madures, identificades com la proteïna sencera i la seva forma soluble, així com una forma immadura. No obstant això, el més remarcable dels nostres resultats és que trobem que els nivells de formes madures estaven disminuïts en LCR de pacients amb MA, en comparació amb pacients control; mentre que la quantitat de forma immadura era semblant entre mostres control i patològiques. Els nostres resultats suggereixen els nivells reduïts de les formes madures d'ADAM10 en el LCR com un potencial i alternatiu biomarcador per a la MA.

D'altra banda, també decidim estudiar el fet a que és degut el fracàs dels inhibidors de γ-secretasa (GSIs, de l'anglès gamma secretase inhibitors) com a teràpia per a la MA. Fins ara, la majoria de les teràpies testades per a la malaltia que han arribat a assaig clínic no han tingut èxit algun en la millora cognitiva dels pacients i els assajos han hagut de ser suspesos. Moltes d'aquestes teràpies són inhibidors de γ-secretasa o PS1 que tenen com a fi reduir la producció de Aβ. Específicament, en el nostre estudi abordem la possibilitat que l'ús sostingut de GSIs puga provocar un efecte rebot, elevant així els nivells de PS1, que contribuirien a un empitjorament de la patologia. Duem a terme experiments in vitro en els quals realitzem tractaments amb dos GSIs, DAPT i avagacestat, que ha estat provat en assajos clínics i vam obtenir en tots dos casos un augment en PS1. Això ens va portar a estudiar l'efecte de avagacestat en experiments in vivo, en els quals es va realitzar un tractament sub-crònic amb aquest compost en rates. Vam obtenir un increment dels nivells de PS1 en els extractes de cervell de les rates

tractades amb l'inhibidor. En resum, trobem un efecte rebot en PS1 com a conseqüència de la inhibició de γ-secretasa. És per això que aquests resultats han de tenir-se en compte per al disseny de futurs fàrmacs per a la MA.

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1 - INTRODUCTION

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In 1901 Auguste Deter was admitted in the Municipal asylum of Frankfurt. She was described as a 51-year-old delusional, forgetful, disoriented, anxious, suspicious, unruly and disruptive woman. Her husband stated that in a 1-year period she had changed drastically: she became increasingly jealous, could not carry out her homemaking duties, and constantly expressed fears of being persecuted and bothered by the neighbours. She also described auditory hallucinatory experiences.

In 1907, in the article "Über eine eigenartige Erkrankung der Hirnrinde", the Bavarian psychiatrist Alois Alzheimer described for the first time the special disease of this patient, identified in the publication as Auguste D, who had shown progressive cognitive impairment. From 1901, Alzheimer followed Auguste D's case until her death in 1906. After that, using the newly developed Bielschowsky's silver staining method, Alzheimer observed and described the anatomical features of a new disease, different from all the others known at this time. It was named after his name in 1910 by Emil Kraepelin, Alois Alzheimer's superior (related in Ramirez-Bermudez, 2012). For this reason, he is considered to be the founding father of this neuropathology. He reported:

…She is entirely disoriented to time and place. Once in a while she makes comments that she does not understand anything going on; or has lost track of things…

…A single one or a few fibrils come to prominence on the inside of an otherwise still "normal" appearing cell. Then, during further progression, many such fibrils running next to each other show changes in the same way. They subsequently fold together into dense bundles and move towards the cell surface. Eventually the nucleus and the cell disintegrate, and only a tangled bundle of fibrils indicates the place which had formerly been occupied by a ganglion cell. Since these fibrils are stainable with other dyes than normal neurofibrils, a chemical transformation of the fibril substance must have taken place; which appears to be the cause for the fibrils' persistence after demise of the cell. The transformation of the fibrils seems to go hand in hand with the deposition of a not yet more closely examined pathological metabolic product into the ganglion cell… (Alzheimer et al., 1995).

An estimated 46.8 million people worldwide were living with dementia in 2015 and this number will reach 131.5 million in 2050. People living with dementia have poor access to appropriate healthcare, even in most high-income country settings, where only around 50% of people living with dementia receive a diagnosis. In middle- and low-income countries, less than 10% of cases are diagnosed (data from the World Alzheimer Report 2016).

Clinically, Alzheimer's disease (AD) is an irreversible, progressive brain disease that slowly impairs memory and cognitive skills. The inability to learn and remember new information (i.e., anterograde amnesia) is the clinical hallmark of AD pathology. At histological level, AD results in progressive spreading of specific pathological lesions in a non-random manner across various brain regions. Based on this evidence, six different stages of disease progression have been categorised (Braak and Braak, 1991): the clinically silent transenthorrinal stages I-II; the limbic stages II-IV of incipient AD; and the neocortical stages V-VI of fully developed AD. As dementia progress the condition worsens over time, and the cognitive decline is often seen by psychiatric features, including confusion, agitation, poor-judgement and behavioural disturbances, as well as by neurological symptoms, resulting in seizures, hypertonia, myoclonus, incontinence and mutism. At terminal illness, AD subject decease by infections, pneumonia, malnutrition, but not by the disorder itself.

1.1 PATHOGENESIS.

At the microscopic level, AD is characterized by the presence of senile plaques and neurofibrillary tangles (NFTs), together with a degeneration of neurons and synapses. Amyloid beta (Aβ) peptide is the compound of the senile plaques. The intracellular NFTs, are composed of paired helical filaments of the microtubule-associated protein tau abnormally hyperphosphorylated (P-tau).

1.1.1 Senile plaques.

At high concentrations, Aβ peptide forms a beta sheet-rich tertiary structure that aggregates to form amyloid fibrils that deposit outside neurons in senile plaques (Figure 1). Aβ can also be aggregated in less dense formations called diffuse plaques, which are considered more a product of senescence or biological ageing. These dense depositions can also be located in the vascular system producing amyloid angiopathy that, together with the extraneuronal deposition and the abundant microglia and astrocytes due to the inflammation, conform the degenerative structures of AD.

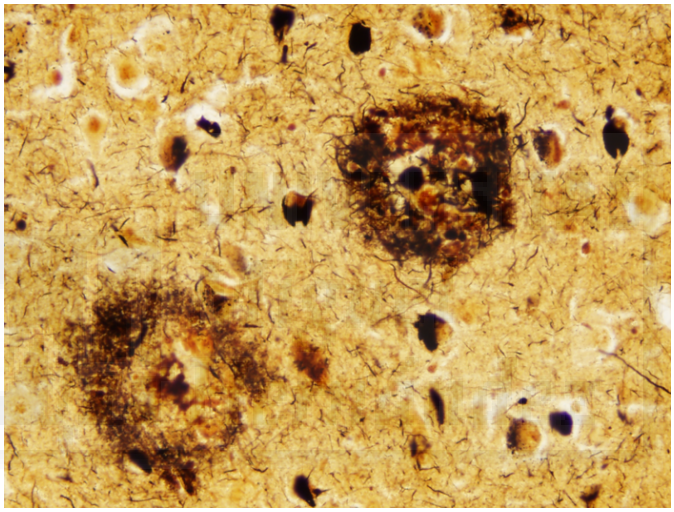

Figure 1. Human senile plaques*. Amyloid deposits from human brain staining using Bielchowsky method* (Agamanolis, 2016).

In human brain there are several species of Aβ produced by alternative sequential proteolysis of the largest β-amyloid precursor protein (APP). The most abundant form of this peptide is the Aβ40 (Aβ1-40, with 40 amino acids of length), whereas the Aβ42 specie (Aβ1-42, with 42 amino acids of length) is more associated to AD progression and pathology. An immunohistochemical study revealed that the longer (Aβ40 and Aβ42) and shorter (Aβ1-17 or Aβ17) Aβ peptides are differently distributed along the various types of amyloid deposits in AD. In fact, while the amyloid angiopathy and senile plaques are

constituted of both longer and shorter Aβ peptides, the principal component of the diffuse plaques is Aβ17 (Rabano et al., 2005).

APP was identified in 1987 as an approximately 700 amino acids type 1 transmembrane protein that has a large extracellular region, a single transmembrane domain, and a smaller cytoplasmic region. APP undergoes extensive alternative splicing such that APP transcripts encode eight different APP isoforms, being the most common the 695 amino acid form, which is expressed predominantly in the CNS, and the 751 and 770 forms, which are more ubiquitously expressed (Bayer et al., 1999). Since no other physiological function of this protein was known at the time of the identification, except as the precursor to Aβ, it was simply designated APP. APP is expressed widely in normal human tissues including in the heart, lung, liver and skin (Puig and Combs, 2013). The *APP* gene is located on chromosome 21, resulting in four-to-five folds overexpression of APP in patients with trisomy 21 (Beyreuther et al., 1993). This explains the Aβ overproduction and early development of AD in individuals with Down's syndrome (Kang et al., 1987).

In the alternative APP processing, we discriminate two principal pathways: the amyloidogenic pathway, which leads to Aβ generation; and the non-amyloidogenic pathway, which prevents Aβ generation (Figure 2). Other alternative proteolytic processing for APP have been described (Andrew et al., 2016). The non-amyloidogenic pathway cleavages APP within the Aβ domain and is performed by several members of membrane-bound disintegrin and metalloproteinase domain containing proteins (ADAM) family, proposed as α-secretases (Lichtenthaler, 2011). This cleavage results on a large APP soluble N-terminal fragment termed as soluble APPα (sAPPα), which is released to the extracellular space. The remaining fragment of APP, termed as C83, is further cleaved by the γ-secretase complex. γ-Secretase is an intramembranous protease complex, composed of four essential components: presenilin-1 (PS1), or its close homologue presenilin-2 (PS2), nicastrin, presenilin enhancer-2 (Pen2) and anterior pharynx-defective 1 (Aph1). Presenilins constitute the catalytic domain of γ-secretase. The processing by γ-secretase yields a non-fibrilar 3 kDa peptide (P3) and APP intracellular domain (AICD) fragments. The P3 peptides can be released into the extracellular environment in exosomes or it can be degraded by lysosomes (Kummer and Heneka, 2014). The P3 peptides (corresponding to residues Aβ17–40 and Aβ17–42) does not assemble into

soluble oligomers and devoid of any impact on synaptic function (Dulin et al., 2008).

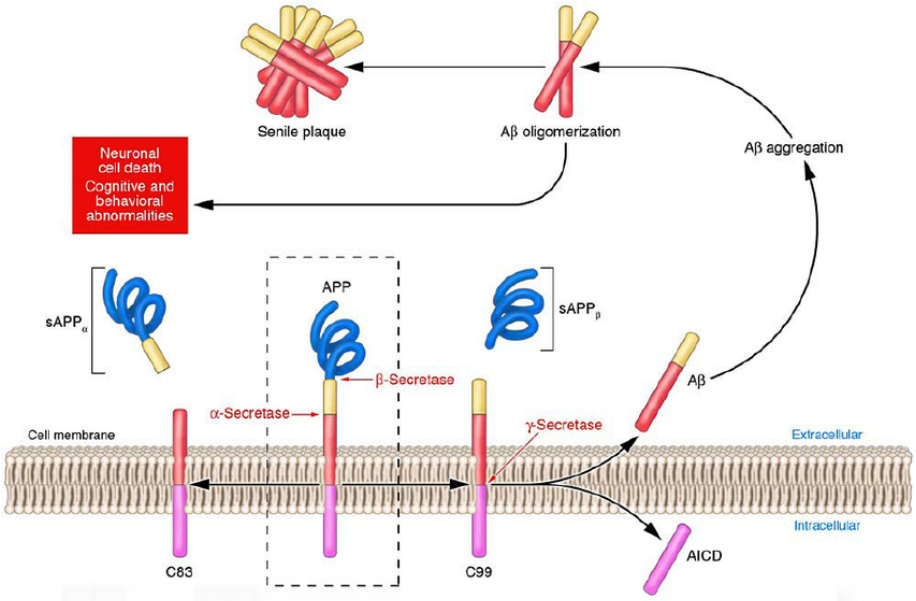

Figure 2. APP processing pathway. *A schema of the APP structure is shown with Aβ in red. APP transmembrane protein is cleaved in its non-amyloidogenic pathway by an α-secretase precluding the formation of an Aβ peptide. In the amyloidogenic pathway APP is sequentially processed by β- and γ-secretase. sAPPα: soluble APPα; sAPPβ: soluble APPβ; AICD: amyloid intracellular domain* (Gandy, 2005).

The APP amyloidogenic processing that produces Aβ peptide, has been described *in vitro* in several subcellular localizations, including the endoplasmic reticulum (ER), the trans-golgi network (TGN), the early and late endosomes, the recycling endosomes and the lysosomes (Choy et al., 2012; Rajendran et al., 2006). The amyloidogenic pathway of APP involves an initial cleavage by a β-secretase enzyme, termed β-site APP-cleaving enzyme 1 (BACE1) (Rajendran et al., 2006; Vassar et al., 2009). This cleavage generates the soluble APPβ (sAPPβ) peptide, which is released into the extracellular space (Hasebe et al., 2013). The large N-terminal sAPPβ fragment differs in length from sAPPα by 16 amino acids, which can result in different functions and signaling mechanisms. Following BACE1 cleavage, the remaining APP fragment, termed C99, is subsequently cleaved by the γ-secretase complex, which results in the generation of Aβ and AICD peptides. The AICD

produced via amyloidogenic pathway has the same peptide sequence than the AICD resulted in non-amyloidogenic pathway, however they appear to be functionally distinct. The AICD produced following β-secretase cleavage is transported to the nucleus and binds to several gene promoters, (Iwata et al., 2000); whereas the AICD produced following α-secretase cleavage appears mostly degraded in the cytosol (Belyaev et al., 2010). γ-Secretase cleaves the C99 at several sites in the transmembrane domain, "trimming" the Aβ peptide from the initial ε-cleavage sites to produce shorter, and relatively benign, Aβ species (Takami et al., 2009). However, inefficient trimming of the Aβ peptide at its C terminus results in the release of longer aggregation-prone Aβ species such as Aβ42, which are central to the production of the neurotoxic oligomeric Aβ (oAβ) assemblies (Figure 3) (Andrew et al., 2016; De Strooper and Karran, 2016; Jarosz-Griffiths et al., 2016). As commented before, the most common form of Aβ peptide is Aβ40, but the Aβ42 variant is the most amyloidogenic form (Steiner et al., 2008). Thus, the site of cleavage of the C99 fragment has important implications for the fate of the generated Aβ peptides.

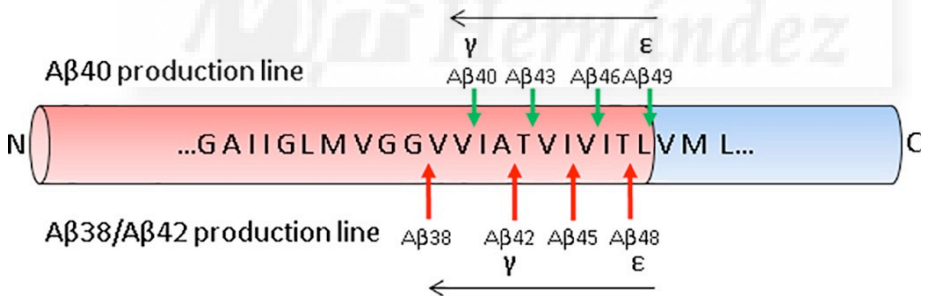

Figure 3. ***Formation of Aβ by γ-secretase cleavage of APP C99.*** *Cleavage of C99 by γ-secretase follows a "nibbling" pattern in the direction indicated by the black arrows, where the initial (ε) cleavage dictates the final (γ) cleavage* (Andrew et al., 2016) .

Under normal conditions, both amyloidogenic and non-amyloidogenic pathways co-exist and Aβ is found in appreciable amounts in the non-pathological human brain. The Aβ peptide can be degraded in the brain by several peptidases including the insulin-degrading enzyme, neprilysin, and the endothelin-converting enzyme (Finder, 2010; Miners et al., 2011). Moreover, Aβ is also cleared from the brain in a process balanced by

the afflux and the influx across the blood-brain barrier. It is a matter of controversy whether disturbance in Aβ clearing mechanism contribute to AD. Indeed, it has been proposed that Aβ accumulation in the brain, but not necessarily his production, is the event leading to neuronal degeneration and dementia.

The amyloid cascade hypothesis

The so-called "amyloid cascade hypothesis" was proposed in 1991 by John Hardy and David Allsop (Hardy and Allsop, 1991; Hardy and Higgins, 1992), and reformulated later to focus on oligomeric aggregates of AD (Hardy and Selkoe, 2002). Indeed, it describes the accumulation of Aβ within neural tissue as the initial event that triggers the disease. The accumulation is the result of an imbalance between Aβ production and clearance, which leads to the aggregation of Aβ and the formation of plaques which, in turn, cause the formation of neurofibrillary tangles (Hardy and Selkoe, 2002). The hypothesis was formed following two keystone observations: first, the identification of Aβ as the primary proteinaceous component of senile plaques (Glenner and Wong, 1984) and, second, the identification of several mutations in familial AD that lead to the accumulation of Aβ (Giasson et al., 2003; Hardy and Higgins, 1992; Selkoe, 1991). Thus, in the amyloid cascade hypothesis there are three tenets:

i) The parenchymal deposition of the Aβ peptide is important pathophysiologically. Thus, the presence of deposited Aβ peptide is a prerequisite. However, the role of deposited Aβ peptide as being the preeminent disease-causing Aβ species has been brought into question by the burgeoning literature on smaller molecular weight oAβ.
ii) Aβ peptide deposition occurs prior to the neuronal and synaptic loss that is the hallmark of AD.
iii) The evidence from mutations that cause autosomal dominant Alzheimer's disease (ADAD) is informative and relevant to sporadic AD.

Soluble Aβ peptides can also cause excitotoxicity at the pyramidal neurons inducing the over-activation of the N-methyl-d-aspartate receptor (NMDAR), the cationic channels

gated by the neurotransmitter glutamate (Götz et al., 2011). This over-activation results in neuronal damage and death due to the generation of excessive nitric oxide (NO) (Law et al., 2001). NO can mediate exotoxicity by triggering down-stream protein misfolding and aggregation, as well as mitochondrial fragmentation. Moreover the majority of transduction signal systems end on the activation or inactivation of enzymes responsible of tau phosphorylation and de-phosphorylation (Billingsley and Kincaid, 1997; Nuydens et al., 1997; Rapoport et al., 2002; Sindou et al., 1992).

A variant of the amyloid cascade hypothesis that is nowadays on the point of research is the Aβ oligomer hypothesis. It posits that small molecular weight oAβ represent neurotoxic agents that cause synaptic damage in AD. One of the facts supporting this new hypothesis is that amyloid plaques do not correlate in terms of their amount with AD symptomatology (Delacourte et al., 1999; Gómez-Isla et al., 1996). Thus, oAβ might act at a distance from plaques and mediate toxic effects (Karran and De Strooper, 2016).

As mentioned above, the amyloid cascade hypothesis is supported by the findings that the unique mutations identified in the early onset familiar form of AD (familiar AD, FAD, or autosomal dominant AD, ADAD), are present in the genes that encodes both the substrate (APP) and the proteolytic enzymes (presenilins) responsible of Aβ generation (Karran et al., 2011). Although the contribution of the canonical α-, β- and γ-secretases to APP proteolysis has been studied in depth, the proteolytic cleavage of APP, like many proteins, is more complex than originally described and there are also additional secretases implicated in the process. Asparagine endopeptidase (AEP), a pH-controlled soluble lysosomal cysteine protease, was previously linked to AD through its capacity to cleave tau. However, it was recently shown to cleave APP at two separate sites in the ectodomain. AEP could be a key player in the generation of toxic metabolites within the brain (Zhang et al., 2015). Another secretase that has been demonstrated to participate in APP processing is η-secretase. Identified as the matrix metalloproteinase MT5-MMP, η-secretase contributes to the Aβ production resulting on additional proteolytic fragments with the capacity to induce synaptic dysfunction (Baranger et al., 2016; Willem et al., 2015). A recent study has reported the presence of CTF peptides proceeding from the sequential cleavage of η-secretase and α- or β-secretase in human CSF, and also

accumulation of CTFη was observed in dystrophic neurites surrounding amyloid plaques in the brains of AD patients (Willem et al., 2015). In this regard, our group has been recently reported in human CSF samples the presence of CTFη of APP, probably resulting from proteolytic processing by η-secretase. Interestingly the levels of these CTFs appear increased in genetically determined AD, as well as in sporadic AD, compared to age-matched controls (García-Ayllón et al., 2017).

1.1.2 Neurofibrillary tangles.

Tau is a soluble microtubule-associated protein which has the ability to facilitate microtubule assembly, promote the stability of microtubules, and maintain neuronal cytoskeleton, so as to ensure the transport of axonal proteins (Scholz and Mandelkow, 2014; Sokolow et al., 2015). Tau has a repetitive microtubule combined sequence, which is encoded by 9 to 12 exons. The repetitive structure can combine with microtubules in the carboxyl terminal, and bind to other cytoskeleton or cell membrane in the dissociated N-terminal (Lv et al., 2017). Phosphorylation of serine and threonine residues, flanking the microtubule binding domain of tau, regulate its interactions with tubulin and influence its conformational state (Arendt et al., 2016; Spires-Jones et al., 2009)

In the normal human brain neurons, the phosphorylation and dephosphorylation of tau proteins are in a dynamic equilibrium. But in the neurons of subjects with AD the tau protein is always abnormally and excessively phosphorylated at certain residues and displays different solubility, as well tends to form clusters of paired helical filaments (PHF) (Wang et al., 2013). PHF are filamentous structures of a modified version of tau, highly stable to proteolysis, insoluble and toxic, able to aggregate to form the NFTs. The core of a PHF is composed of hyperphosphorylated tau that can be truncated at the C- and N-terminals. It has been proposed that such truncations favored tau polymerization and the subsequent NFTs formation (Fasulo et al., 2000; Guillozet-Bongaarts et al., 2005; Wischik et al., 1988a, 1988b). The PHF-core tau is not only unable to bind tubulin, but also binds normally-phosphorilated tau, sequestering it and blocking its physiological function (Alonso et al., 1994). Moreover, the formation of NFTs undergoes many

posttranscriptional modifications on tau such as phosphorylation, glycosylation and ubiquitination.

Tau aggregation to NFTs leads to microtubules depolymerisation, followed by the structural damage of neuronal microtubules, impairment of normal axonal transport, synapse loss, and neuron destruction (Rodríguez-Martín et al., 2013) (Figure 4).

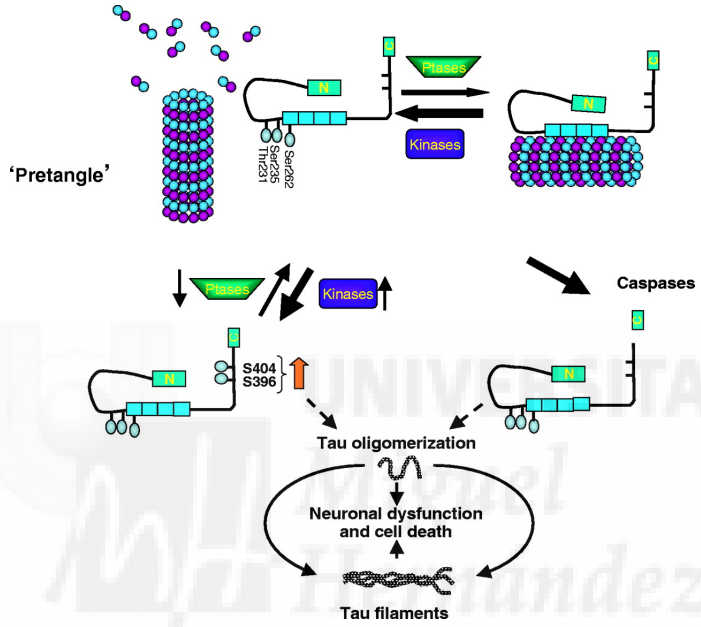

Figure 4. Tau pathology. *In the early stages of pathology, the phosphorylation probably decreases the ability of tau to bind microtubules. Subsequently, tau can be cleaved by caspase and/or phosphorylated increasing the propensity of tau to oligomerize and eventually form filamentous aggregates* (Johnson and Stoothoff, 2004).

Seminal studies by Braak and Braak first described a spatial and temporal pattern in the appearance of tangles in brain of AD patients that follow neuronal networks and correlate with cognitive decline. In the AD pattern, NFTs first appear in the transentorhinal region and progress along anatomical pathways to the hippocampus and eventually the neocortex (Braak and Braak, 1991) (Figure 5).

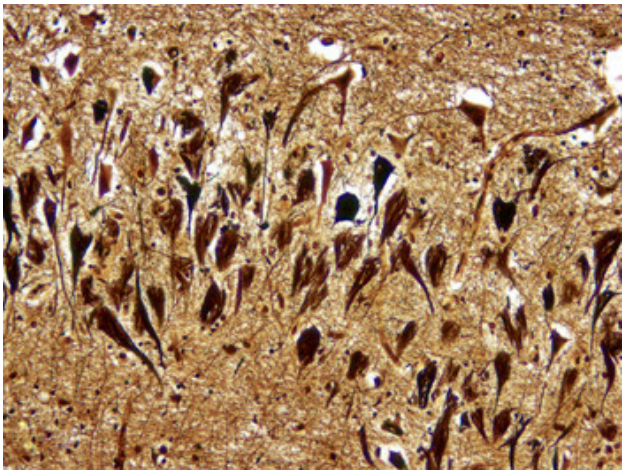

Figure 5. *Neurofibrillary tangles. A Bielschowsky stain of human brain NFTs* (Agamanolis, 2016)

1.2 GENETICS

From a genetic point of view, AD is divided in two forms: the early-onset genetic or familial Alzheimer's disease and the late onset sporadic Alzheimer's disease.

1.2.1 Autosomal dominant AD.

The ADAD accounts for less than 5-10% of all cases of AD. It is a rare form of AD characterized by an early-onset, a strong familial aggregation, and Mendelian transmission. ADAD patients typically develop symptoms of dementia in their 30s to 60s, depending on the specific gene mutation and the age of onset within their family. About 30-40% of patients with early symptom onset ADAD have an increased frequency of atypical presentations, such as impairments in non-memory domains, including executive, behavioural language and visuospatial (Balasa et al., 2011; Koedam et al., 2010; Mendez, 2012).

Those ADAD forms are most often caused by rare mutations, with complete penetrance, located in three genes, *APP*, *PSEN1*, and *PSEN2*, coding for amyloid precursor protein, presenilin 1 and 2, respectively. To date, 40 mutations in *APP*, 197 mutations in *PSEN1*, and 25 mutations in *PSEN2* have been identified that cause ADAD (Chouraki and Seshadri, 2014; Schindler and Fagan, 2015). The number of identified mutation is

increasing since some ADAD families are not yet characterized. Most of the mutations in *APP* gene occur around the putative γ-secretase cleaving site, which generates the Aβ, suggesting the critical implication of this proteolytic enzyme in the pathogenesis (Goate et al., 1991). Interestingly, a rare APP mutation (A673T) protects against AD, resulting in a reduction in the formation of amyloidogenic peptides by an amino acid substitution adjacent to the aspartyl protease β-site in APP (Jonsson et al., 2012).

In the case of presenilin mutations, most of them were determined to increase Aβ42 to Aβ40 ratio by modifying the way in which γ-secretase cuts APP; that means and increase in toxic function. However, AD-causing mutations in presenilin were found to have reduced proteolytic function (Wolfe, 2007), and are identified as a loss of function (De Strooper, 2007). In this context, several authors point that the current genetic terminology is misleading, and that all presenilin clinical mutations, indeed loss-of-function mutations, cause incomplete digestion of the Aβ and contribute to an increasing vulnerability of the brain (De Strooper, 2007; Wolfe, 2007). The unifying emerging hypothesis puts forward a biochemical mechanism by which slower less-efficient forms of the protease can result in a greater proportion of Aβ42-residue.

Regarding the other hallmark of the disease, mutations in tau that cause tau hyperphosphorylation lead also to dementia. However, in this scenario the result is a frontotemporal dementia without amyloid deposition, instead of AD (Hutton et al., 1998).

The discovery of the genetic mutations related to ADAD has allowed researchers to create transgenic animal models that display some important aspects of the disease and serve as a basis of AD research.

1.2.2 Sporadic AD.

Sporadic AD (referred hereafter as AD or sAD) is associated to aging and is usually diagnosed after age 65. The pathology develops over a long preclinical period of several decades. The sporadic is the major form of the disease and is not caused by a mutation in a single protein. In fact, in addition to aging, multiple genetic and environmental risk factors have been related with the progression of this disease.

The strongest genetic risk factor associated to AD is the ε4 allele of the apolipoprotein E (*APOEε4*) gene. In the brain, ApoE is predominantly secreted by glial cells and functions as a major transporter of lipoproteins between cells in the brain via ApoE receptors, which are members of the low-density lipoprotein receptor (LDLR) family (Liu et al., 2013).

ApoE has several potential effects on AD progression, decreasing Aβ turnover and clearance as well as by directly influencing Aβ aggregation (Castellano et al., 2011). ApoE has been shown to be also processed into neurotoxic fragments (Mahley and Huang, 2012). Additionally, reduced ability to suppress inflammatory stimuli and higher densities of NFTs have been reported in ApoE4 carriers (Leyns and Holtzman, 2017).

Genome-wide association studies have been used to identify more than 20 genetic loci associated with the risk of Alzheimer's disease. The newly identified genes point at pathways implicated in the immune system and inflammatory responses, cholesterol and lipid metabolism, and endosomal-vesicle recycling (Guerreiro and Hardy, 2014).

Alterations in other genes and in non-coding RNA, such as microRNA, might also have important roles in disease susceptibility (Lau et al., 2013; Wong et al., 2013). Novel-sequencing technologies have been used to identify rare mutations such as mutations in TREM2, a microglia receptor involved in Aβ clearance (Zhang et al., 2013). Genome-wide profiling of gene expression in the brains of patients with late onset AD supports the hypothesis of an upregulated immune-specific and microglia-specific module (Matarin et al., 2015; Zhang et al., 2013).

Increasing evidence suggests that many other lifestyle-related factors, including hypercholesterolemia, diabetes, obesity, physical and mental inactivity, depression, smoking, low educations attainment, and diet have a role in dementia, and the potential for primary prevention related to such modifiable risk is huge but yet to be fully explored (Norton et al., 2014).

1.3 DIAGNOSIS.

The main challenge of AD research is the discovery of reliable predictive markers to enable the diagnosis as early as possible before the loss of autonomy, setting the stage of dementia. With the advent of novel therapies to slow the progression of lesions in AD, the race for biomarkers' research nowadays is a top priority.

The first set of criteria proposed for diagnosis of AD was focused on clinical symptoms only. At the time, Alzheimer's pathological changes could not be measured *in vivo*, since brain biopsy is not appropriate, so disease could be definitively diagnosed only after death (Scheltens et al., 2016).

The diagnosis of AD requires careful evaluation of the patient medical history, mental status and physiological condition through validated test including amyloid and tau positron emission tomography (PET) and magnetic resonance imaging (MRI) measurements of brain volume and neuronal connectivity. In addition, a set of cerebrospinal fluid (CSF) tests reflecting key aspects of disease pathology (neurodegeneration, tau pathology and amyloid deposits) are available. Biomarkers for chronic neurodegenerative disorders such as AD are of great importance since the cognitive symptoms often are diffuse and overlap with other disorders. The clinical progression is slow and variable even between patients with the same disease; mild cognitive impairment (MCI) the earliest clinical phase of AD is a heterogeneous syndrome that may be caused by many disorders (only around a half of cases develop AD) (Blennow, 2017). Biomarkers reflecting different types of pathophysiology in the brain can be used for clinical diagnosis, especially in the early stages of the disease, to predict progression, to monitor effects of novel drug candidates in clinical trials, and lastly also in clinical research to deeper our understanding of the pathogenesis of the disease (Blennow, 2010). In the research field for new biomarkers for AD, ADAD provides a unique resource for characterizing changes in CSF biomarkers. More importantly, the study of ADAD patients would be a tool to monitor those changes in CSF that occur long before the onset of dementia, since mutations have 100% penetrance, thus allowing investigators to know with certainty that an individual will develop AD (Schindler and Fagan, 2015).

1.3.1 CSF biomarkers.

CSF provides a valuable diagnostic window into the brain for neurodegenerative diseases and has many advantages over other body fluids. It surrounds the brain and is in direct contact with the brain interstitial fluid. As a consequence, the molecular composition of CSF could reflect molecular pathogenic processes in the brain.

When assessing the diagnostic performance of AD biomarkers, it is essential to consider the unclear distinction in some pathological features between AD and elderly people without any cognitive alteration. Thus, it is unexpected that any biomarker reaches 100% diagnostic accuracy. Nonetheless, CSF biomarkers can help with diagnostic decision (Scheltens et al., 2016).

In addition to pre-analytical confounding factors, the variability in measurements between clinical laboratories has hampered the identification of uniform cutoffs for validate CSF biomarkers. However, there has been standardization efforts that provide the basis for the introduction to uniform cutoffs and more general use of CSF biomarkers for routine clinical diagnosis of AD (Scheltens et al., 2016).

Core CSF biomarkers for AD are Aβ42, which shows cortical amyloid deposition; total tau (T-tau), which reflects the intensity of neurodegeneration; and phosphorylated tau (P-tau), which correlates with neurofibrillary pathological changes (Blennow et al., 2010). Numerous laboratories have reported an increase in P-tau and T-tau levels in CSF, although tau alone lacks of specificity since it is also increased in other neurological processes (Rosen et al., 2013). Abnormal metabolism of Aβ is considered a more specific phenomenon related to AD. However, the increasing deposition of the Aβ peptide into the brain, especially of the Aβ42 form, determines that its level in CSF is decreased, while these pathological Aβ species are increased in the AD brain (Blennow, 2010).

The combination of Aβ42 and P-tau/T-tau leads to high (~80%) levels of sensitivity, specificity, and diagnostic accuracy. However, there is a continuing search for new CSF (or blood) biomarkers to improve the clinical diagnosis, especially on the early stages of AD, and the clinical trials (Cedazo-Minguez and Winblad, 2010). Other biomarkers, in addition to Aβ and tau, have been investigated for testing their ability to reflect further

pathophysiological processes implicated in AD. For example, CSF β-secretase and soluble APP fragments derived from α-secretase and β-secretase cleavages (sAPPα and sAPPβ, respectively) have been investigated in patients with AD (Lannfelt et al., 1995). The usefulness of sAPPα for diagnosis is unclear, but sAPPβ levels can aid clinical trials of β-secretase inhibitors in patients with AD, as this peptide can reliably reveal drug target engagement. ApoE levels in CSF have also been investigated (Cruchaga et al., 2012).

Other potential candidates are synaptic biomarkers, such as the dendritic protein neurogranin, which is involved in long-term potentiation and memory consolidation (Díez-Guerra, 2010). High CSF concentrations of neurogranin predict progression to AD in patients with MCI and correlate with rapid cognitive deterioration during clinical follow up (Kvartsberg et al., 2015).

Additional candidates for AD biomarkers are proteins implicated in the processing of APP and the production of Aβ, such as the mentioned β-secretase, since they participate in the altered pathway of the disease. In this line, our lab has previously determined the presence of PS1 in human CSF samples (García-Ayllón et al., 2013) as heteromeric complexes composed of N-terminal and C-terminal fragments. These CSF-PS1 complexes differ from active γ-secretase membrane-complexes, and may represent nonspecific aggregation of the PS1 protein. Interestingly, levels of PS1 complexes are increased in ventricular post-mortem CSF samples from autopsy-confirmed AD cases and are more stable than complexes in CSF from control subjects. This increment of highly stable PS1 complexes in AD is also observed in lumbar CSF samples from probable AD, although overall PS1 levels are similar to cognitively normal subjects. This increment on more stable complexes in AD could be the result of a change in the biochemical properties of PS1 complexes formed under amyloidogenic conditions. Taking account those results, part of my thesis project was focused on the interest of PS1 complexes as a potential biomarker of AD. The results of this research became a publication included in the present work.

To our knowledge, the presence of ADAM10/α-secretase has been not assessed in CSF. We also investigated the occurrence of ADAM10 in human CSF and if altered levels of this protein reflect the AD condition.

1.3.2 Imaging.

Imaging has a key role in the clinical assessment of patients with suspected AD. The diagnostic work-up of patients with cognitive impairment can be difficult during the early stage, when the differential diagnosis is still wide and includes normal ageing. 1^{8}F-fluorodeoxyglucose (FDG) PET measures glucose uptake of neurons and glial cells and is sensitive to synaptic dysfunction. A normal FDG PET virtually excludes a diagnosis of neurodegenerative disease (Perani et al., 2014).

Synaptic dysfunction in the posterior regions (the so-called default mode network) is captured also by network analysis of functional blood-oxygen-level-dependent MRI. However, standardisation and reproducibility issues mean that this technique is not yet useful on an individual level (Pievani et al., 2011).

The most innovative imaging marker for AD used clinically is PET with ligands for Aβ. Florbetapir, florbetaben, and flutemetamol ligands have very high accuracy for cortical amyloidosis. However, because brain amyloidosis is a necessary but not sufficient condition for diagnosis of AD, the diagnostic value of amyloid PET is more exclusionary than inclusionary.

Increasing evidence suggests that the combination of several markers has good positive and negative predictive value to differentiate AD from normal aging in patients with mild cognitive impairment.

Fluorinated ligands for tau have also been developed, and bind to fibrillary tau aggregates with remarkable accuracy (Villemagne et al., 2015). Tau ligands have shown binding topography that correlates well with the clinical syndrome in AD and show a better correlation with hypometabolism and atrophy than does amyloid PET (Ossenkoppele et al., 2015). Tau imaging is being used in clinical trials of drugs aiming to delay progression of AD, but its usefulness for clinical diagnosis remains to be confirmed (Scheltens et al., 2016).

1.3.3 Blood biomarkers.

Since blood is more accessible than CSF, blood biomarkers appear as advantageous for diagnostic purposes and use in clinical trials. Importantly, small amounts of brain-specific proteins and peptides are able to enter the blood, although these proteins are diluted and may undergo degradation by plasma proteases or are metabolized in the liver or through the kidneys. Thus, a major challenge in developing blood biomarkers is that brain-specific proteins reflecting the disease occurring at central nervous system (CNS), should be present at much lower concentrations in blood than in CSF (Blennow, 2017). Application of novel ultra-sensitive techniques, such as immuno-magnetic reduction (IMR) and Single-molecule array (Simoa) methods, could provide the analytic sensitivity needed to allow accurate measurement of CNS-specific proteins. To date, several candidate blood biomarkers have been evaluated, including Aβ42, but only marginal differences are found between AD patients and control individuals, with large overlaps, and the trend of change varies between studies. For plasma tau, the levels are elevated in AD, but with a much larger overlap to control levels than what it is seen in CSF studies.

1.4 THERAPIES.

Currently, the treatment of AD in dementia phases is largely based on cholinesterase inhibitors (AChEIs) donepezil, rivagstimine, and galantamine, which can be used alone or in combination with memantine, an antagonist of NMDA receptors that can also be used alone, depending on the stage of the disease. However, these pharmacological agents so far available for AD act as palliative drugs, only effective for symptomatic treatment, and have not been proven to be curative.

Since there were no drugs that would be able to combat the pathophysiology of AD, there is a continuous search of new AD therapies. Unfortunately, success rates among AD drugs in clinical development are disappointing, much lower than for cancer and other complex diseases. In the past 25 years, the search for therapies aimed at slowing or halting AD progression has been dominated by the straightforward rationale of

developing compounds targeting or disrupting Aβ formation. However, the failure of several Aβ-focused therapy development efforts has encouraged the development of other therapeutic approaches for AD, including tau targeted therapies.

1.4.1 Acetycholinesterase inhibitors.

The use of AChEIs as a treatment for AD derives from the hypothesis that deterioration in cholinergic neuron function causes part of the cognitive and behavioural impairments of AD. Thus, the administration of AChEIs provides a temporary increase of acetylcholine disposition and symptomatic relief. As a result, AChEIs tend to stabilise cognitive performance and daily functioning during a limited period of treatment (Sun et al., 2008).

Although these AChEIs treatments are still largely believed to provide "symptomatic" benefits only, there are evidences indicating that they may affect disease progression. In this regard, cholinergic mechanisms have proved capacity to modulate amyloid metabolism and AChEIs affect APP processing (reviewed in (García-Ayllón et al., 2011). In this context, AChEIs can activate the non-amyloidogenic pathway or inhibit the β-secretase activity (Fu et al., 2008; Racchi et al., 2004), and our group has reported that AChEIs are also able to modulate and interfere with PS1 (Garcia-Ayllon et al., 2007; Silveyra et al., 2012). However, this positive effects of AChEIs therapy on APP processing decrease after long-term inhibition, since long-term treatment with AChEIs results in a significant up-regulation of AChE protein levels (Darreh-Shori and Soininen, 2010; Garcia-Ayllon et al., 2007). This increased pool of AChE probably interacts with Aβ and increases its fibrillation and toxicity. Also, AChE up-regulation in response to inhibition is followed by PS1 increase (Silveyra et al., 2012). An increase of AChE may block γ-secretase activity and an increase of PS1 levels may result in an enhancement of Aβ generation. Interestingly, in animal models AChE up-regulation occurs after days/weeks of maintain inhibition, but PS1 up-regulation appears rapidly (Silveyra et al., 2012). In this complex scenario, conversely Aβ can also induce an increase of AChE (Sberna et al., 1998), which in turn binds the amyloid core closing an aberrant loop. In this way Aβ, PS1 and AChE

could establish a toxic triad, and probably new AChEIs are needed to prevent this undesirable loss of benefit during long-term treatment.

1.4.2 Aβ immunotherapy.

Immunotherapies for patients with AD are based either on active immunization with full-length Aβ or Aβ fragments, or on passive immunization with anti-Aβ antibodies. The goal in both cases is to produce/provide anti-Aβ antibodies that can bind to soluble or aggregated Aβ, thereby inducing Aβ clearance by microglia, or efflux of Aβ from the brain. In light of the adverse events observed with active immunization most immunotherapy studies in AD now use passive immunization, which is based on the administration of monoclonal antibodies directed towards different regions of Aβ. Examples are, Solanezumab, which was designed to bind to monomeric Aβ, thereby preventing oligomerization and deposition; and Bapineuzumab, a humanized N-terminus-specific monoclonal antibody that binds to Aβ (Russu et al., 2016). Neither compound was able to establish any signal of efficacy on measures of cognition (Gold, 2017). However, a promising compound is Aducanumab, another human humanized N-terminus-specific monoclonal antibody, that selectively reacts with Aβ aggregates, including soluble oligomers and insoluble fibrils (Sevigny et al., 2016). Aducanumab is currently on a phase 1b study (van Dyck, 2017).

Interestingly, it seems that antibodies directed against the N-terminus of Aβ may be most effective in clearing the toxic aggregated species of Aβ (Montoliu-Gaya and Villegas, 2016). Transgenic mouse models have demonstrated that these antibodies inhibit Aβ aggregation and disaggregate pre-existing Aβ fibrils (Bard et al., 2000; Bussière et al., 2004; Horikoshi et al., 2004). Using seeded fibril growth from brain extract and data from solid-state nuclear magnetic resonance and electron microscopy, it appears that Aβ40 monomers aggregate in oligomers and fibrils with multiples of three units, in which N-termini are exposed (Lu et al., 2013), whereas hydrophobic C-termini are inaccessible to antibodies (Montoliu-Gaya and Villegas, 2016). If a similar structure held true for Aβ42, antibodies targeting the N-terminus would likely be most efficient in clearing Aβ oligomers.

An often-cited explanation for the failure of anti-Aβ antibody trials is that they are set too late in the disease process, but new trials are indeed evaluating treatments at prodromal and preclinical stages (van Dyck, 2017).

1.4.3 Secretases inhibitors.

Because Aβ has such a crucial role, a rational way to treat or prevent AD would be to block the activity of the proteases that generate Aβ. The first cleavage step in the generation of Aβ is mediated by the β-secretase BACE1, which is highly expressed in neurons and cleaves many physiologically important substrates (Zhou et al., 2012). Inhibition of BACE1 induces alternative processing of APP and generation of synaptically active peptides that are different from Aβ. Inhibitors of β-secretase are being tested in clinical trials (Lleó et al., 2014), and side-effects so far seem surprisingly limited. The β-secretase inhibitors MK-8931, E2609, LY2811376 and LY2886721 are being investigated for the treatment of patients with AD. These compounds all markedly reduced CSF levels of Aβ and sAPPβ in phase I or phase II/III studies of healthy volunteers or patients with AD, indicating strong β-secretase inhibition (engagement) in the CNS (Bernier et al., 2013; Devos et al., 2014; Forman et al., 2013; Portelius, 2017). Unfortunately, clinical development of LY2811376 and LY2886721 was terminated owing to adverse toxicity profiles. A phase I trial of E2609 in patients with MCI or mild AD has been completed, and two phase III studies of MK-8931 in patients with prodromal AD or mild to moderate AD are ongoing. At this point, it remains unknown whether strong β-secretase inhibition in the CNS will be tolerable for extended periods of time in patients with AD, and whether this inhibition will provide clinical benefit.

Inhibitors of the γ-secretases, which are implicated in the second cleavage step, were unsuccessful in clinical trials because of important side-effects; but to rule out γ-secretases as drug targets for AD would be premature. Many γ-secretase inhibitors and modulators are being developed for AD therapy, with the goal of reducing Aβ generation without interfering with the cleavage of substrates other than APP, such as Notch a protein that is important for cell-to-cell communication and that has also been implicated in cancer (Wolfe, 2012).

Semagacestat (LY450139) is the most widely studied γ-secretase inhibitor in humans. Despite its evidence of target engagement in the CNS, its development was halted in August 2010 because of adverse effects in phase III trial (Doody et al., 2013). Semagacestat even caused unexpected aggravation of cognitive decline (Coric et al., 2013; Doody et al., 2013). In this context, it has been demonstrated that semagacestat does not inhibit but increases the levels of intracellular γ-byproducts and Aβ (Tagami et al., 2017). These effects are clearly different from those caused by a loss of function of presenilins.

Avagacestat (BMS-708163) is another potent oral γ-secretase inhibitor, which reached phase II clinical testing. Avagacestat reduced CSF Aβ38, Aβ40 and Aβ42 levels in healthy volunteers and in patients with AD (Tong et al., 2012). In the AD trial, CSF levels of Aβ14, Aβ15 and/or Aβ16 increased in a dose-dependent fashion, suggesting target engagement. In a separate phase II study of avagacestat, CSF biomarkers were used to select patients with prodromal AD. However, there was insufficient target engagement and due to the worsening cognition further development was terminated (Coric et al., 2013).

1.5 SECRETASES

In line with the amyloid hypothesis and together with the mutations found in ADAD, there is an interest on the knowledge of the processing pathway of APP and its alterations in the disease. For that, the study of the enzymes implicated in the processing has been in the point of interest on the actual research.

1.5.1 α-Secretase.

As mentioned before, the processing pathway of APP includes the cleavage by an α-secretase that produces the soluble extracellular domain of APP (sAPPα), which is presumed to have neuroprotective properties in a process called “ectodomain shedding” (Saftig and Lichtenthaler, 2015) and a C-terminal fragment of APP (C83), which is then

cleaved by γ-secretase to release a non-toxic P3 peptide (Nathalie and Jean-Noël, 2008). Cleavage by α-secretase occurs in both, regulated and constitutive manner. Regulated cleavage probably occurs in the Golgi complex under control of protein kinase C (PKC) (Furukawa et al., 1996; Lammich et al., 1999; Skovronsky et al., 2000), while constitutive cleavage occurs very rapidly at the cell membrane (Lammich et al., 1999). The first candidate enzyme proposed for α-secretase was the tumour necrosis factor-converting enzyme (TACE) or ADAM17 (Buxbaum et al., 1998), but the existence of other α-secretases was presumed. Thus, few years later, two more enzymes were shown to have α-secretase activity: ADAM9 and ADAM10 (Asai et al., 2003; Fahrenholz et al., 2000; Lammich et al., 1999). Like ADAM 17, both of them are part of the α-disintegrin and metalloprotease family (ADAM), a family of transmembrane and secreted metalloendopeptidases. Initial knockdown of the candidate α-secretases seemed to demonstrate that release of sAPPα was never fully abolished, and initially it was concluded that the α-secretase candidates showed significant functional overlap (Asai et al., 2003; De Strooper et al., 2010; Kuhn et al., 2010). However, ADAM10, but not ADAM9 or 17, is essential for the constitutive α-secretase cleavage of APP; indicating that ADAM10 is probably the most physiologically relevant α-secretase in neurons (Anders et al., 2001; Kuhn et al., 2010).

ADAM10 is synthesized via the rough endoplasmic reticulum (ER) and transported via the Golgi apparatus. Maturation includes removal of a prodomain, which keeps the enzyme in an inactive state. The mature form of ADAM10 of about 60-65kDa was found in the Golgi compartments, as well as in the ER/plasma membrane. The catalytic and the proximal disintegrin domain contain high-mannose as well as complex-type N-glycan attachment sites (Escrevente et al., 2008) (Figure 6).

ADAM10 is a multifunctional protease active throughout the life and its regulation is controlled at transcriptional, epigenetic, translational and post-translational levels (Hartmann et al., 2002). These different levels of regulation allow a cell to adapt ADAM10 levels rapidly to functional perturbations, as well as to slower changes induced by aging and/or differentiation. ADAM10 plays an essential role during development. Animals with a conventional ADAM10 knock-out die on E9.5 (Hartmann et al., 2002), which underlines the general importance of this protease. However, ADAM10 is probably best known for

its ability to process APP. Of note, the regional and cellular overlap of ADAM10 and APP, which is necessary for ADAM10 to process APP in tissues, is age-dependent. At early developmental stages the mRNA distributions of ADAM10 and APP are not fully congruent, but with aging the overlap increases (Marcinkiewicz and Seidah, 2000). This finding, but also the wealth of data on other substrates of ADAM10, suggests that main ADAM10 substrates may change during development phases. Thus, during development and in the young brain ADAM10 may preferentially cleave substrates other than APP, and the role of ADAM10 as α-secretase on APP processing may emerge with aging.

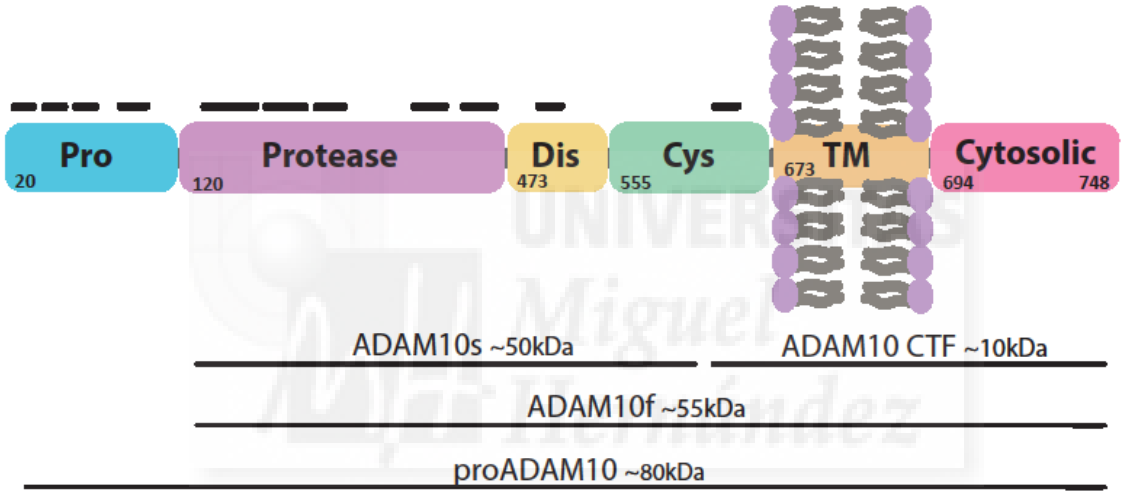

Figure 6. Scheme of ADAM10 protein. *Schematic representation of ADAM10 and its domain organization, which consists of a pro-domain (Pro), a zinc-binding metalloprotease (Protease) domain, a disintegrin domain (Dis), which binds to integrin cell adhesion molecules, a cysteine-rich domain (Cys), a variable stalk region, a transmembrane (TM) domain, and a cytosolic domain (not drawn in scale; adapted from* (Tousseyn et al., 2009)*).*

Alterations of ADAM10 exocytosis and endocytosis mechanism has been described in the earliest phases of the AD, suggesting that it can be considered a pathological synaptic feature of the disease. ADAM10 synaptic shedding activity is required for the implementation of activity-dependent synaptic plasticity events (Musardo and Marcello, 2017). In this regard, a decrease in ADAM10 synaptic localization and activity in AD could lead not only to an increase in Aβ levels, but also to an impairment in structural synaptic plasticity, affecting the plastic remodelling of the

synapses and contributing to AD pathology. This impairment on the synaptic plasticity regulated by ADAM10 is independent of Aβ-mediated synaptic toxicity (Colciaghi et al., 2002; Marcello et al., 2012). To support this hypothesis, several studies showed the involvement of ADAM10 in the so-called "synaptopathies" (Saftig and Lichtenthaler, 2015).

1.5.2 β-secretase.

It is known that β-secretase is widely expressed (Haass et al., 1992), but expression is most prominent in pancreas and brain, especially in neurons (Seubert et al., 1993; Zhao et al., 1996). Within neurons β-secretase is expected to be localized to endosomes, lysosomes and the Golgi complex (Haass et al., 1995; Koo and Squazzo, 1994) and to function optimally at an acidic pH (Knops et al., 1995).

In 1999 an enzyme that matched all these characteristics and showed proteolytic activity at the correct site on APP was discovered. The β-site APP cleaving enzyme (BACE1) was proposed as a likely candidate for β-secretase by four separate studies (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). As mentioned, BACE1 is a transmembrane aspartyl protease. It is about 500 residues in length with two active sites located on the lumenal side of the membrane. A sequence of an additional 82 amino acids extends C-terminally to the homologous pepsin carboxyl terminus, and includes a lumenal extension, a hydrophobic region containing the transmembrane domain, and a cytosolic domain. This allows the enzyme ready access to its substrate within the lumen of the Golgi, where it competes with α-secretase for APP, or within endosomes and lysosomes (El-Agnaf et al., 2000; Vassar et al., 1999).

BACE1 is present in endosomes and to a lesser extent in trans-Golgi network, compatible with its optimal activity in acidic environments (Kinoshita et al., 2003). It localizes to lipid rafts, possibly implicated in amyloidogenic pathway of APP processing (Ehehalt et al., 2003). Events in the compartmental transit of BACE1 and its association with APP are complex (Vassar et al., 2014). BACE1 reaches the plasma membrane after synthesis, and is internalized by cholesterol and lipid dependent pathways (Kalvodova et

al., 2005; Schneider et al., 2008). Recycling of BACE1 within endosomal compartments has been implicated in maintaining amyloidogenic activity (Buggia-Prévot and Thinakaran, 2014; Udayar et al., 2013), in opposition to lysosomal destination of BACE1 where it is degraded (Kang et al., 2010; Koh et al., 2005). Heritable molecular defects in secretory and endocytic pathways that regulate BACE1 processing of APP may increase Aβ production, further implicating BACE1 and amyloid in AD (Toh and Gleeson, 2016). Importantly, neuronal retrograde transport and somatic localization of BACE1 have been shown to be essential for limiting BACE1 activity and generation of Aβ in the synapse (Lee et al., 2005; Ye et al., 2017). BACE1 inhibition would be expected to impact the end result of Aβ independently of cellular trafficking dysfunction (Koelsch, 2017).

BACE1 activity has been shown to be elevated in cases of sporadic AD (Fukumoto et al., 2002; Yang et al., 2003). However, β-secretase is not exclusively active in individuals with AD. Instead it appears to be producing Aβ during normal cell metabolism (Haass et al., 1992). Therefore, the cause of AD is not simply the activation of β-secretase and its production of Aβ, but rather a change in the amount of APP processed by β-secretase. BACE2, a protease homologous to BACE1, is also expressed in many tissues including the brain (Ahmed et al., 2010). The potential contribution of BACE2 to neurodegenerative progression is still under discussion (Holler et al., 2012) Together the two enzymes define a new family of transmembrane aspartyl proteases (Vassar, 2004).

1.5.3 γ-Secretase.

γ-Secretase is an aspartyl protease that belongs to a diverse family of Intramembrane-cleaving proteases (I-CLiPs). To date, γ-secretase is also the unique intramembrane protease identified that functions as a multi-subunit protein complex, as we described before, γ-secretase is composed of 4 required subunits: PS1 (or PS2), Aph1, Pen2 and nicastrin (Li et al., 2014; Sato et al., 2007) (Figure 7). Consistent with the studies about γ-secretase stoichiometry, the absolute mass of the purified γ-secretase measured by scanning transmission electron microscopy (STEM) is ~230 kDa (Osenkowski et al 2009). PS1, or its close homologue PS2, are the catalytic subunits of γ-secretase. Both enzymes can be also found forming similar but independent γ-secretase complexes. Aph1

has 7 TMDs, with an N-terminus in the lumen/extracellular space and the C-terminus in the cytosol (Fortna et al., 2004). Pen2 spans the membrane twice, with the N- and C-termini facing the lumen space (Crystal et al 2003). In contrast, nicastrin has the typical topology of type I transmembrane protein, with a single TMD and an N-terminus spanning in the lumen/extracellular space with many potential glycosylation sites (Yu et al 2000).

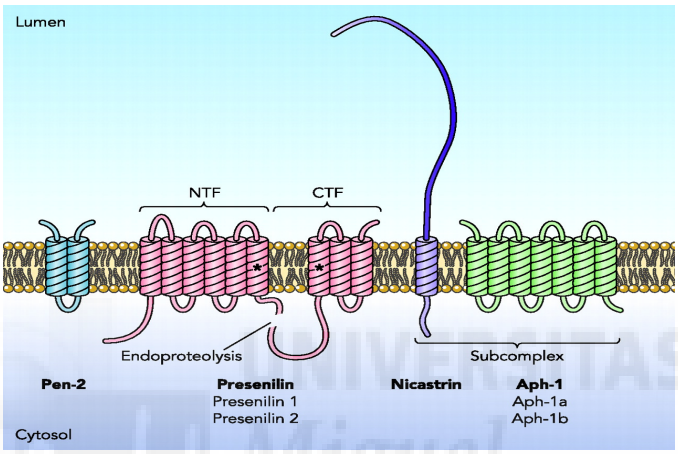

Figure 7. γ-Secretase complex. *The complex is formed by PS1 (or its homologue PS2), which is the catalytic subunit, Pen2, nicastrin and Aph1. The PS1 holoprotein is stabilised by the binding to Aph1-nicastrin complex. In the complex Aph1 and nicastrin are bound to the PS-CTF fragment while Pen2 binds to PS-NTF fragment (De Strooper, 2008).*

The correct assembly of the subunits is necessary for the proper function of the γ-secretase complex. This assemblage begins in the ER, soon after translation and membrane insertion with the interaction of Aph1 with the immature, hypoglycosylated form of nicastrin (Gu et al., 2003). The C-terminus of the nascent PS1 holoprotein binds Aph1 and nicastrin, forming a high molecular weight inactive complex where the proteins are stabilized. In this context, PS1 acts as a chaperone protein and facilitates nicastrin maturation inducing the transport of the complex to the medial Golgi compartments, where nicastrin is N-glycosylated (Fraering et al 2004, Kaether et al 2002, LaVoie et al 2003). Subsequently, nicastrin undergoes a major conformational change that involves

the entire ectodomain and becomes it selectively resistant to trypsin. This structural conformational change does not occur in absence of PS1, and is required for the γ-secretase assembly and activity (Shirotani et al 2003). The last step consists in the incorporation of Pen2 into the PS1-nicastrin-Aph1 trimeric intermediate. Indeed, it seems that Pen2 binds to the TMD 4 of PS1 and provokes its endoproteolysis into active PS1 formed by a N-terminal (NTF) and a C-terminal fragment (CTF), conferring the proteolytic activity (Fraering et al 2004, Watanabe et al 2005). The active complex is then shuttled to the Golgi where it is glycosylated (Takasugi et al., 2003). The mature γ-secretase complex is transported to the post-Golgi compartments including the plasma membrane where can be found in lipid rafts.

γ-Secretase cleaves more than 90 substrates in addition of APP and Notch within the membrane environment (Beel and Sanders, 2008; Hemming et al., 2008; Lleó and Saura, 2011; Wakabayashi and De Strooper, 2008). Although PS/γ-secretase substrates are diverse in their structure, localization, and physiological functions, the majority of these proteins share several common features: they are all type I transmembrane proteins with a small ectodomain (<300 amino acids), usually resulting from a prior shedding by a metalloprotease-like proteins. (Lleó and Saura, 2011). The previous shedding of the extracellular domain is usually mediated by specific proteases, α- or β-secretases (Brou et al 2000). However, the γ-secretase cleavage does not depend critically on a specific amino acid sequence or on endocytosis (Struhl & Adachi 2000). After that, the resulting C-terminal fragment is cleaved inside its TMD by the γ-secretase complex that executes an endopeptidase-like cleavage, followed by carboxypeptidase-like processive/successive cleavage. The transmembrane substrate is first proteolyzed at the border between the cytosol and membrane, which is called the ε-site (Kimberly et al 2003, Lichtenthaler et al 1999). This ε-cleavage allows the liberation of the intracellular domains (ICDs) of the substrates from the membrane. Some ICDs have been identified as signaling mediators in several pathways, including Notch signaling. The remaining hydrophobic sequence of the substrate is then processed by the γ-secretase carboxypeptidase activity, shedding shorter fragments (Qi-Takahara et al 2005, Takami et al 2009).

Evidence indicates that γ-secretase cuts APP initially at the ε-site and then progressively removes C-terminal residues until the γ-cleavage site has been reached (Kakuda et al., 2006) (Figure 3). In theory, the ε-cleavage yields the formation of Aβ peptides of 49 amino acids, but in practice, Aβ49 is extremely rare to find due to the progressive cleavages of PS1. In APP the recognition sequence for γ-secretase consists in 11 amino acids (Thr639-Lys649) inside the TMD at the C-terminal end. One time recognized, APP is presented to the catalytic domain of γ-secretase, which in turn recognizes many hydrophobic residues where it can acts (Barthet et al 2012, Tischer & Cordell 1996).

Remarkably, it seems that the whole known ADAD mutations, that shift the γ-secretase cleavage toward Aβ42 production, are within the small binding site region, probably affecting the presentation of APP to γ-secretase (Selkoe 1998).

γ-Secretase activity is controlled in the cell by a variety of mechanisms, in which one of the most important is regulation of active complex formation. γ-Secretase activity cannot be increased through the overexpression of PS alone in cellular models (Levitan et al., 2001) and can be reconstituted only when all four γ-secretase subunits are present (Edbauer et al., 2002). Indeed, the selective ablation of any one of the essential subunits leads to a loss of enzymatic activity (De Strooper, 2003). However, in studies performed in mouse models, overexpression of PS alone was able to increase γ-secretase activity (Li et al., 2011). These results suggested that γ-secretase regulation *in vivo* is much more complicated than originally anticipated. γ-Secretase is regulated from subunit composition to associated protein that may regulate the complex in specific tissues or disease situations. Thus, the γ-secretase complex can be associated with modulatory proteins, like a novel γ-secretase activating protein (GSAP), which complexes with γ-secretase and APP, giving preference to APP cleavage over Notch (Inoue et al., 2015). As a result, it is possible to modulate γ-secretase activity and therapeutic target by modification in the PS active site, but also in the subunits and associated proteins.

γ-Secretase, as regulator of many other substrates, is involved in a number of neuronal processes, such as cell adhesion, lateral inhibition, neurotrophin signaling, cell differentiation, ligand-receptor binding, calcium influx, NMDA receptor activation,

substrate recruitment and enzyme trafficking (Thinakaran and Parent 2004). The use of PS1 knock-out mice also showed that PS1 is not only important in the adult stage of the brain, but is also fundamental in somitogenesis, axial skeleton formation and neuronal population stabilization during development (Shen et al., 1997).

1.6 PS1

PS1 acts as a membrane-embedded aspartyl protease, in which the catalytic activity depends of two conserved and essential aspartates. The notion that PS1 is the γ-secretase active site was strongly supported by the observation that mutations of either of the two conserved Asp substantially reduced Aβ production, with a concomitant accumulation of its substrate APP-CTFβ (Kimberly et al., 2000; Steiner et al., 1999; Wolfe et al., 1999b); and by the fact that the γ-secretase activity was inhibited by aspartyl protease substrate-based peptidomimetic inhibitors (Esler et al., 2000; Wolfe et al., 1999a).

The crystallographic structure of PS1 has been in uncertainty along several years. Finally, it has been reported that PS1 is a 9 TMD protein (Laudon et al., 2005). It has the N-terminus and a large hydrophilic loop in the cytosol, and the C-terminus in the lumen/extracellular space (Figure 7). The two conserved Asp are located at the interface of the TMD 6 of PS-NTF and domain 7 of PS1-CTF. It seems that PS1, in presence of its substrate, is finally reorganized in a ring structure (Cao and Südhof, 2001).

Although the PS1 holoprotein is synthesized as a polypeptide with an apparent size of 42-43 kDa, as mentioned above, the mature and active PS1 undergoes an endoproteolysis that occurs at the amino acids 292 and 299. Endoproteolysis appears to be an intramolecular autocatalytic event that is carried out by the same γ-secretase activity (Brunkan et al., 2005; Wolfe et al., 1999), and results in a ~29 kDa NTF (containing TMD1-6) and a ~20 kDa CTF (with TMD 7-9). Thus, the NTF/CTF assembly is the biologically active form of PS1 and their NTF and CTF are the more abundant immunoreactive bands in brain extracts (Podlisny et al., 1997; Saura et al., 1999).

PS1 is ubiquitously expressed in peripheral tissue and in the CNS. Several studies have investigated the PS1 subcellular localization in neurons using biochemical methods, immunostaining and immunoelectron microscopy. It has been reported that PS1 resides principally in the ER and trans-Golgi network, but it is also present in small synaptic vesicles, synaptic plasma membranes, synaptic adhesion sites and neurite grown cone membranes (Annaert and De Strooper, 1999; Georgakopoulos et al., 1999). Mature forms of PS1 have been found at the cell surface in complex with other membrane associated proteins, like nicastrin (Chyung et al., 2005). Thus, despite the large proportion of PS1 localized within the endoplasmic reticulum and early Golgi, it is assumed that APP cleavage occurs on the cell surface and in endosomes/lysosomes compartments, where the proteolytic active PS1/γ-secretase is principally localized (Haass et al., 2012).

In addition to its γ-secretase-dependent functions, PS1 has been proposed for a number of γ-secretase independent roles in the regulation of protein functions. PS1 is involved in Wnt/β-catenin signalling (Soriano et al., 2001), calcium homeostasis forming calcium leak channels in the ER (Tu et al., 2006); protein glycosylation, trafficking and degradation (Barthet et al., 2012; Naruse et al., 1998). Some of this independent γ-secretase functions play a critical role in many events during development and aging (Parks and Curtis, 2007). In this regard, many reports showed, a large pool of resident PS1 in neurons in the early compartments of the biosynthetic pathway (Culvenor et al., 1997; Huynh et al., 1997). It has also been suggested that the over-expression of either the wild type or mutant PS1 disturbs glycoprotein processing within the Golgi (Farquhar et al., 2003; Silveyra et al., 2008). It has been also described the implication of PS1 protein in the regulation of neurotransmitter release during synaptic transmission. In fact, the presynaptic inactivation of PS1 decreases the probability of glutamate release (Zhang et al., 2009), probably due to its role in modulation of calcium release from intracellular stores. However, the participation of PS1 in those biological processes, independently from its proteolytic activity, is not clearly defined.

As mentioned above, more than 190 mutations in the *PSEN1* gene are currently known to cause the majority (probably more than 50%) of ADAD cases. Mutations in presenilins alter the cleavage of APP resulting in generation of distinct amyloidogenic Aβ peptides, decreasing total Aβ40 levels and resulting in unbalances and increase

proportion on the more amyloidogenic Aβ42 species (De Strooper, 2007). The balance of different Aβ species seems to be important for aggregation and their toxic effects in AD brain. Mutations in the presenilin genes accelerate the age of onset and cause earlier and severe progression of neurodegeneration than sporadic AD. The presence of some PS mutations results in quantitative differences in brain neuropathology compared to sporadic forms of AD (Shepherd et al., 2009).

Focused on the role of secretases in AD and the problems with AD diagnosis and therapy, during my PhD period I tried to investigate new biomarkers for an early diagnosis of AD and the insights on the failure of some GSI trials. All my research work will be summarized in the following chapters.

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2 - OBJECTIVES

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The aim of this Thesis is to explore the potential of secretases as biomarkers for Alzheimer's disease and to explain the failure in GSI therapies. Specifically, the main objectives of my study are:

1. In relation with the possibility that secretases constitute new biomarkers for Alzheimer's disease:
    a. To determine whether alterations in levels of PS1 complexes in the CSF might reflect the pathological state at early, even asymptomatic stages of Alzheimer's disease.
    b. To investigate the occurrence of ADAM10 in human CSF and the possible alteration of its levels in subjects with Alzheimer's disease in order to evaluate their potential as an alternative biomarker.

2. In relation to the failure of GSI therapies:
    a. To study whether prolonged inhibition of γ-secretase can initiate a feedback process that leads to a rebound effect, elevating PS1 levels, which may be relate with the reported failure of GSI compounds for an effective therapy.


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3 - GENERAL SUMMARY

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3.1 MATERIALS AND METHODS

In the present chapter we will summarize all the experimental procedures carried out in the time elapsed during this Thesis work.

3.1.1 Cellular models

For *in vitro* experiments, we employed the cellular lines of wild-type Chinese hamster ovarian cells (CHO cells), CHO cells stably overexpressing wild-type human PS1 and APP and the human neuroblastoma line SH-SY5Y. We also used cultures of primary cortical neurons. For some experiments, cells were transfected with PS1 and C99 constructs using Lipofectamine®. SH-SY5Y cells and cortical neurons were also treated with γ-secretase inhibitors DAPT, avagacestat or the dimethyl sulfoxide (DMSO) vehicle alone.

3.1.2 Patients

CSF samples were obtained from symptomatic and asymptomatic genetically determined AD subjects (ADAD), from Down syndrome (DS) patients with Alzheimer's type dementia (dDS) and DS subjects without signs of memory decline (ndDS) along with age-matched young non-demented controls (yNDC). CSF samples from patients with dementia due to sporadic AD (sAD), subjects with mild cognitive impairment (MCI) and age-matched elderly controls (eNC) were also collected.

3.1.3 Animal models

For *in vivo* experiments, Wistar male rats were used. The rats were orally administered with avagacestat (40 mg/kg) or vehicle alone (polyethylene glycol). CSF samples were collected by cisternal puncture. In addition, the rat’s cerebral cortices were dissected out and stored for Western blot and PCR analysis.

Alterations on memory and learning functions as well as motor coordination were analysed by the Y Maze alternation, active avoidance and Beam walking tests. The tests were performed 2-4 hours after the final administration of avagacestat.

3.1.4 Western blotting and immunoprecipitation

Cell brain extracts, and CSF samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under fully reducing conditions. The proteins separated were transferred to nitrocellulose membranes and probed with antibodies against PS1, ADAM10, Aβ, APP, ApoER2 and other γ-secretase components, as specified in each paper. Aβ peptides were resolved by 16% Tris-tricine SDS-PAGE. We also performed co-immunoprecipitation experiments for PS1 and Aβ in CSF and in conditioned media samples from CHO cells transfected with PS1.

3.1.5 Sucrose gradients

PS1 complexes were analysed in CSF samples by ultracentrifugation for 4 h at 4°C on a continuous sucrose density gradient (5-20%) at 250,000 × *g*.

3.1.6 Quantitative PCR analysis

The total RNA from rat brain hemi-cortices, SH-SY5Y cells and mouse cortical neurons was isolated. First-strand cDNAs were synthesized by reverse transcription and quantitative PCR amplification was performed using specific TaqMan Gene expression assays in a StepOne™ Real-Time PCR System.

3.1.7 Statistical analysis

All data were analysed using SigmaStat determining exact *p* values by applying a Student's *t* test (two-tailed) or a Mann-Whitney Rank Sum Test, when normality

was rejected. The results are presented as the means ±SEM.

3.2 RESULTS

Our group has previously reported the presence of heteromeric presenilin-1 complexes in human CSF (CSF-PS1) and serum. The proportion of stable CSF-PS1 levels served to discriminate sAD from non-disease controls. Supported by this finding, we aimed to determine CSF-PS1 complexes as a potential early biomarker for AD.

The diagnosis of sAD is based mainly on clinical symptoms and the final diagnosis of the disease cannot be determined until the autopsy. Genetically determined AD offers unique opportunities to analyse diagnostic biomarkers at asymptomatic stages, given that only in this group the diagnosis is guaranteed. Thus, we analysed samples from patients ADAD, sAD and MCI, which is considered as an early stage of AD. We found increased levels of PS1 complexes in symptomatic and asymptomatic ADAD and sAD cases, compared with age-paired controls, but not in MCI. Anyhow, since we discriminated between two types of complexes of PS1 (high stable complexes and unstable complexes), we propose that, rather to estimate the total PS1 in CSF, changes in the proportion of PS1 complexes may be a discriminating factor of the pathological state. We found that a quotient of PS1 (high-stable/unstable) complexes can discriminate all pathological groups from age-matched controls. Interestingly, we found significant increase in the proportion of high-stable PS1 complexes also in MCI cases.

We were also interested in what is the factor that contributes in the formation of high-stable complexes. Our first candidate was Aβ, so we tested the presence of Aβ in PS1 complexes and we found that Aβ oligomers are mainly associated to the highly stable complexes, but not within unstable PS1 complexes.

In this regard, we wanted to corroborate the influence of Aβ in relation with increased levels of PS1 in CSF. The APP gene is codified on chromosome 21; thus, a good model to study the effects of an excess of APP in CSF-PS1 is to analyse CSF samples from DS patients. It has been shown that almost all adults with DS over 40 years of age display

AD-like neuropathology. We found increased levels of PS1 in CSF from DS patients and also an increased quotient of PS1 complexes.

Following our interest in the study of secretases, we analysed the presence of ADAM10 in human CSF. We found in human CSF several species of ADAM10, an immature form retaining the prodomain (proADAM10), a mature unprocessed full-length form (ADAM10f), as well as a form lacking the C-terminal domain of ADAM10 that we attributed to a membrane cleaved large fragment (sADAM10). We demonstrated by gradient ultracentrifugation that sADAM10 and ADAM10f are present in the CSF as large complexes. We were also interested in the possible alteration of ADAM10 levels in CSF of AD patients. We showed in AD cases a decrease in the mature forms sADAM10 and ADAM10f, but not for the immature forms.

Some of the therapies that are on development for AD are based on the inhibition of the secretases implicated in Aβ formation, but none of them has outcome positive results to date. We focused our work on the inhibitors of gamma secretase (GSIs) analysing the possibility that γ-secretase inhibition could provoke a rebound effect, which increases the levels of PS1. We started analysing the effect of the well know GSI DAPT in cell cultures. SH-SY5Y cells and primary neurons were treated with DAPT and then, increase in PS1 levels were characterized in treated cells, as compared with vehicle treated controls. We corroborated the efficacy of the inhibition testing the levels of the substrate of γ-secretase, the CTF of APP, and, as expected, we found accumulation of the substrate in DAPT treated cells.

We decided to test other GSI that had reached clinical trials, avagacestat, which selectively blocks the processing of APP without notably affecting Notch. We treated primary cultures of cortical neurons with avagacestat for 4 days, and we also found an increase in PS1 levels in GSI treated cells. After these results, we were interested on the effects of avagacestat in an animal model. Rats were treated with a single dose of avagacestat per day along for 4 and 21 days. Increased PS1 levels were observed in brain extracts from rats treated for 21 days with avagacestat, which indicate a rebound effect. Interestingly, the sustained γ-secretase inhibition did not exert a long-term effect on PS1 activity, evident through the decrease in several γ-secretase substrates, the C-terminal

fragments (CTFs) of APP and ApoER2. Prolonged avagacestat-treatment of rats also produced a subtle impairment in anxiety-like behaviour. Surprisingly, an opposite effect with decreased CSF-PS1 levels was observed in avagacestat treated rats.

3.3 DISCUSSION

The goal of the following lines is to give an overview of the results obtained and, more importantly, to take advantage of the opportunity to be more speculative about the interpretation, as well to define future experiments and studies that could help improving the results already obtained. Even if the three different manuscripts contained in this Thesis correspond to different and independent objectives, they, altogether, try to explore the determination of CSF secretases, PS1 and ADAM10, as potential AD biomarkers, as well to dissect biochemically the rebound response during administration of GSIs that could serve to prevent adverse effect in the development of related therapies for AD.

The first published paper included in this Thesis corroborated the existence of different PS1 complexes in human CSF and demonstrated that highly stable CSF-PS1 complexes are increased in patients with ADAD, as well as in subjects with sAD. PS1 is the catalytic subunit of the γ-secretase complex, and mutations on PS1 have been related as cause of the genetic Alzheimer´s (Chouraki and Seshadri, 2014; Schindler and Fagan, 2015). However, the possibility to assess their levels in CSF was not affronted until an earliest study from our group (García-Ayllón et al., 2013), probably due that PS1 is a multipass transmembrane protein. Now, we demonstrated the association of the “highly” stable CSF-PS1 complexes with Aβ oligomers. Importantly, our data also indicated that the determination of these “highly” stable CSF-PS1 complexes could constitute an asymptomatic biomarker since their levels appeared altered in pre-symptomatic ADAD subjects, as well as in MCI patients.

In our last manuscript, we also investigated the presence of the main α-secretase, ADAM10, in human CSF. During the AD condition, it is presumable that the non-amyloidogenic proteolytic processing of APP diminished compared to the amyloidogenic

pathway, but there is not clear evidence. In our report, we demonstrated the decrease of mature species of ADAM10 in the CSF of sAD subjects, corroborating this possibility.

Our data, performing a biochemical characterization of the different ADAM10 species and PS1 complexes in CSF, could be useful not only to define new biomarkers for AD, but also to design appropriate strategies for further studies to define the real potential of these biomarkers for the diagnostic of AD.

In the current scenario, the discovery and validation of new biomarkers that will allow the early detection of AD is on the focus of research. To date, the criteria to diagnose AD is the examination of clinical symptoms, which is only about 80% accurate, and the disease could be only definitively diagnosed histologically, after autopsy. Moreover, it is important an earlier diagnosis, identifying and distinguishing AD from other neurodegenerative disorders. This would enable an earlier therapeutic intervention that could be the key to delay the onset of symptoms. The combined use of biochemical biomarker in CSF, blood or other fluid, and imaging techniques, such as PET and MRI measurements, are promising strategy for the future implementation of diagnosis. However, the current reality is a lack of verified and validated good biomarkers that allow differentiate between AD and other similar pathologies in early stages.

An important issue for the translation of our findings to real biomarker kits is the development of protocols for an easy, reliable and accurate determination; and of course, the validation of our findings for independent groups. In our study we analysed the proteins by Western blot including also fractionation by sucrose gradients. It becomes clear that to develop enzyme-linked immunoabsorbent assay (ELISA) kits would result in an easier approach for our further analysis, but also to contrast results from other laboratories and advance in the mentioned translation.

However, the development of efficient ELISA kits for CSF complexes of PS1 and ADAM10 species is challenging. In this regard, we propose that the most significant phenomenon related to the potential use of CSF-PS1 to discriminate the pathological state is the change in the proportion of PS1 complexes, rather than the estimates of the total PS1 levels. Since highly stable CSF-PS1 complexes co-exist with unstable complexes,

a potential diagnostic ELISA kit should include antibodies that discriminate between complexes, which is probably extremely difficult. Another possibility is that further biochemical characterization of the different complex served to design an appropriate strategy. For example, we found that Aβ oligomers are mainly associated to the highly stable PS1 complexes; thus, maybe a combination of PS1 and Aβ antibodies could serve to develop a specific ELISA kit. For ADAM10, only mature species, sADAM10 (truncated large soluble form lacking the C-terminal domain) and ADAM10f (full-length form), were found decrease in CSF from AD compared to control, while the levels of the proADAM10 specie (the immature form retaining the prodomain) remained unchanged. This situation add difficulty for the development of specific antibodies for an ELISA kit, since the only "exclusive" domain for a ADAM10 species present in CSF is the prodomain, but it is the specie that remained unchanged. A possibility is to develop custom pan-specific antibodies targeting the truncated C-terminal sequence of the sADAM10 specie present in CSF. This approach will discriminate only sADAM10 and not ADAM10f, but appears as the easier approximation.

In resume, more work is needed for the development of applicable ELISA kits for our biomarkers. Based on these future approaches or in existing protocols we also need to test the specificity of the changes, and whether stable PS1 complexes increase and ADAM10 decrease only in AD patients and not in subjects with other neurological conditions. A possible combination of both analysis, and existing AD biomarkers, may serve for a better discrimination and increasing specificity.

Furthermore, blood biomarkers will be a desirable outcome, however there is not any blood biomarker already defined. For the development of new biomarkers, it seems the most appropriate firstly to characterize changes in CSF, since CSF represents a "window" to the brain. Anyhow, it is important that in later development of the new biochemical biomarker to assess if levels in plasma also correlate with AD. In this regard, it is important to note that PS1 is also expressed in many peripheral organs as well as in brain (Lee et al., 1996; Nilsberth et al., 1999). In a previous publication, our group demonstrate that PS1 complexes are mostly absent in CSF from a conditional KO (cKO) mice in which PS1 is specifically silenced in neurons of the forebrain, while PS1 complexes appeared in the serum of these PS1 cKO mice (García-Ayllón et al., 2013). Those results

suggest that plasma and CSF PS1 may have distinct cellular origins, although a small contribution of brain PS1 to plasma levels cannot be discounted. Results also indicate a challenge to develop a test for PS1 detection in plasma, because probably, to be efficient, it should discriminate PS1 complexes with a CNS origin. Application of novel ultra-sensitive techniques, such as immuno-magnetic reduction (IMR) and Single-molecule array (Simoa) methods, could provide the analytic sensitivity needed to allow accurate measurement of CNS-specific proteins in peripheral fluids (Blennow, 2017). Regarding ADAM10, the presence of ADAM10 levels in human serum has been indicate only by ELISAs (Isozaki et al., 2017; Walkiewicz et al., 2017), thus a biochemical characterization of soluble ADAM10 species in serum is pending and necessary for defining the potential use as biomarker for AD. The cellular origin of these transmembrane proteins and how they reach the fluids are unknown, but could be also relevant in order to define their real potential as AD biomarkers. Indeed, a physiological mechanism which explain how and why PS1 or ADAM10 reached the CSF is uncertain and it seems more plausible that neuronal death or proteolytic release from exosomes will be the major contributing factors (Lopez-Font et al., 2015).

Finally, focusing in the secretases as a pharmacological target, we also addressed the problematic of the lack of an efficient treatment for AD. There are several avenues for the development of new and effective therapies in those days (commented in the Introduction chapter), but none of them have shown promising outcomes. Here, we have analysed the dynamics of the cellular target, PS1, during administration of GSIs, one of recent strategies developed to treat AD patients. We demonstrated that administration of GSIs result in a rebound increase in PS1 levels in cellular and animal models. In long-term avagacestat-treated rats we have observed a decrease in levels of two γ-secretase substrates, APP-CTF and ApoER2-CTF, indicating that the rebound in PS1 protein levels could derive in increased activity, at least in intermediate periods, between doses. Taking into account that γ-secretase has several substrates and is participating in several cellular pathways, the increase in its activity may have different affections in the normal cell function. These effects could be related to the reported failure of GSIs to achieve long-term Aβ regulation and their contribution to rather than the palliation of the AD pathology.

Unfortunately, negative results from clinical trials with GSIs in AD patients have severely dampened enthusiasm for the potential of pursuing γ-secretase research therapeutically, but clinical trials with these compounds could have been conducted in ways that would provide more guidance for future studies (discussed in De Strooper, 2014). In this regard, our study suggests that there is not a detailed examination of secondary effects in treatments with GSIs, and those effects may be dangerous for the patient and difficult to identify. Too often data that could have been used to guide future studies were not collected, and thus negative trials fail to fully inform the next generation of therapeutic development. Our data provide an important framework to evaluate results from completed human trials with these compounds and must be taken into consideration when using new GSIs or related drugs, such as γ-secretase modulators (GSM) and β-secretase inhibitors, in AD therapy. GSM, by definition only block the γ-secretase cleavage of APP to generate the Aβ42, without changing the production of total Aβ, but also noticed negative outcomes (Xia et al., 2012). Clinical development of BACE1 inhibitors is also being intensely pursued and several promising BACE1 inhibitors have entered human clinical trials (Yan and Vassar, 2014); but sign of toxicity forced to stop the earliest trial (discussed in Lahiri et al., 2014). Methods of assessment of the molecular target validation should include also better understanding of the cellular response to the sustained inhibition/modulation of secretases activities for a successful development of this class of drugs. Moreover, GSIs are in cancer clinical trials targeting Notch (Takebe et al., 2014; Yuan et al., 2015), and information about a potential parallel rebound in the target could be valuable in order to prevent adverse effect during long-term treatment.

In our study examining the *in vivo* effect of a GSI on brain PS1 levels we chose a wild-type rat model for the possibility to examine CSF-PS1 expecting to reproduce with this biomarker the changes detected in the brain. However, unexpectedly, the levels of the CSF-PS1 complexes diminished in 21 days avagacestat-treated rats, relative to the control rats. Although how PS1 reaches the CSF is unknown. Likewise, the mechanism by which PS1 levels are enhanced by GSI administration is also unknown, but we proposed in the Discussion of our manuscript that an excess of γ-secretase substrates (due to initial inhibition of γ-secretase activity) may result in transient stabilization of PS1/γ-secretase substrate complexes, interfering in the effective clearance/turnover of PS1. Indeed, the

compartmentalization of secretases and substrates should be considered for assessing the effect of secretase inhibitors (Beel and Sanders, 2008; Ben Halima et al., 2016), and our results in CSF may be indicative of decreased intracellular clearance of PS1 and/or changes in compartmentalization. The possibility to assess these aspects could increase the knowledge not only of the effects of GSI on cellular content of PS1, but also about the source and mechanism of CSF-PS1 thus, corroborating their potential as a biomarker.

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4 - PUBLICATIONS

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ARTICLE 1: Cerebrospinal fluid Presenilin-1 increases at asymptomatic stage in genetically determined Alzheimer's disease

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Published in Molecular Neurodegeneration. 2016 11:66.
doi: 10.1186/s13024-016-0131-2

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Sogorb-Esteve et al. Molecular Neurodegeneration (2016) 11:66
DOI 10.1186/s13024-016-0131-2

Molecular Neurodegeneration

RESEARCH ARTICLE Open Access

Cerebrospinal fluid Presenilin-1 increases at asymptomatic stage in genetically determined Alzheimer's disease

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Abstract

Background: Presenilin-1 (PS1), the active component of the intramembrane γ-secretase complex, can be detected as soluble heteromeric aggregates in cerebrospinal fluid (CSF). The aim of this study was to examine the different soluble PS1 complexes in the lumbar CSF (CSF-PS1) of individuals with Alzheimer's disease (AD), particularly in both symptomatic and asymptomatic genetically determined AD, in order to evaluate their potential as early biomarkers.

Methods: Western blotting, differential centrifugation and co-immunoprecipitation served to determine and characterize CSF-PS1 complexes. We also monitored the assembly of soluble PS1 into complexes in a cell model, and the participation of Aβ in the dynamics and robustness of the stable PS1 complexes.

Results: There was an age-dependent increase in CSF-PS1 levels in cognitively normal controls, the different complexes represented in similar proportions. The total levels of CSF-PS1, and in particular the proportion of the stable 100–150 kDa complexes, increased in subjects with autosomal dominant AD that carried *PSEN1* mutations (eight symptomatic and six asymptomatic ADAD) and in Down syndrome individuals (ten demented and ten non-demented DS), compared with age-matched controls ($n = 23$), even prior to the appearance of symptoms of dementia. The proportion of stable CSF-PS1 complexes also increased in sporadic AD ($n = 13$) and mild-cognitive impaired subjects ($n = 12$), relative to age-matched controls ($n = 17$). Co-immunoprecipitation demonstrated the association of Aβ oligomers with soluble PS1 complexes, particularly the stable complexes.

Conclusions: Our data suggest that CSF-PS1 complexes may be useful as an early biomarker for AD, reflecting the pathology at asymptomatic state.

Keywords: Presenilin-1, Cerebrospinal fluid, Biomarker, Pre-symptomatic, Autosomal dominant Alzheimer's disease, Down syndrome, Mild-cognitive impairment

Background

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that involves a gradual decline in memory and other cognitive functions, representing the most common cause of dementia in the elderly. Apart from the common late-onset forms of sporadic AD (sAD), rare mutations in the genes encoding the β-amyloid precursor protein (*APP*; chromosome 21q21), presenilin-1 (*PSEN1*; chromosome 14q24.3) and presenilin-2 (*PSEN2*; chromosome 1q31-q42) cause autosomal dominant AD (ADAD; also named as familial AD or FAD) [1]. ADAD exhibits similar phenotype as sAD but with an earlier clinical onset. The *APP* gene encodes a large type I transmembrane protein that upon proteolytic processing [2] can generate the β-amyloid peptide (Aβ), the major constituent of senile plaques and the triggering effector of AD. In the amyloidogenic pathway the Aβ peptide is generated by sequential cleavage of APP, starting with the cleavage of the large extracellular domain by the β-secretase cleaving enzyme (BACE1), which is followed by the successive action of γ-secretase at the membrane-spanning domain [3]. This γ-secretase is an intramembrane

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protease complex composed of presenilin-1 (PS1), nicastrin, APH1 (anterior pharynx-defective 1) and PEN2 (presenilin enhancer 2) [4]. PS1 is the catalytic subunit of the γ-secretase complex [5]. Duplications of APP and neighboring sequences are also linked to an early age of AD onset [6]. As such, Down's syndrome (DS) is also associated with the development of AD since the APP gene lies on chromosome 21, and the extra copy leads to Aβ over-expression. Accordingly, most DS patients who live beyond the age of 40 years develop typical brain neuropathology AD and a significant proportion develop additional cognitive decline [7–9]. Thus, both these disease conditions, ADAD and DS, can be considered as early-onset forms of genetically determined AD [10].

Classic biomarkers, total and phospho-tau, as well as Aβ42, have shown diagnostic accuracy for incipient AD [11]. However total and phospho-tau also increased as a result of other neurological processes; while levels of the pathological Aβ42 species, which increased in the AD brain, resulted decreased in CSF due to increasing deposition, hindering the interpretation of changes in their soluble levels in early stages. Thus, there is still a need to identify additional early biomarkers. We recently demonstrated the presence of heteromeric PS1 complexes in human CSF (CSF-PS1) and serum, and that increases in the proportion of stable CSF-PS1 complexes served to discriminate sAD from non-disease controls [12]. PS1 is known to undergo endoproteolytic cleavage as part of its maturation, generating N- and C-terminal fragments (NTF and CTF) of about 29 and 20 kDa, respectively [13]. Both, the NTF and CTF of PS1 contain several transmembrane domains [14]; and our earlier data indicated that PS1 fragments might be highly unstable in CSF and serum, and that they spontaneously form complexes due to the large number of hydrophobic regions. Indeed, we demonstrated the presence of stable 100–150 kDa heteromeric complexes in CSF that contained the NTF and CTF of PS1 (maybe also involving other γ-secretase components), as well other large complexes. Some of these complexes were unstable under denaturing conditions and resolved as ~50 kDa heterodimers upon electrophoresis [12]. Moreover, an increase in the proportion of stable 100–150 kDa complexes appears to be a good marker to discriminate pathological AD samples from controls.

As such, we set out to further characterize these soluble PS1 complexes and the involvement of oligomeric Aβ in the formation of these complexes. We also evaluated the possibility that the proportions and nature of the CSF-PS1 complexes may vary during aging. The main interest was to investigate the levels of CSF-PS1 complexes in ADAD, sAD and DS, particularly in AD and DS subjects who had not yet developed dementia, including also mild-cognitive impaired (MCI) subjects. Thereby, we attempt to determine whether alterations to the levels of these complexes might reflect the pathological state at early, asymptomatic stages. Using a collection of well-characterized CSF samples from sAD PS1 complexes were also analyzed. Genetically determined AD offers unique opportunities to analyze diagnostic biomarkers at asymptomatic stages, particularly given that only in this group is a diagnosis guaranteed for the early comparison of biomarkers.

Methods

Patients

Lumbar CSF samples were obtained from ADAD subject that were all carriers of *PSEN1* mutations and who were part of the Genetic Counseling Program (PICOGEN) at the Hospital Clínic, Barcelona [15]. This group included 14 subjects carrying PSEN1 mutations (including six asymptomatic mutation carriers), and eight age-matched non-mutation carriers from the same families (younger non-disease controls: yNC). The clinical and CSF data of some of these patients has been reported previously [16, 17]. We also included lumbar CSF samples from 10 DS subjects with Alzheimer's type dementia (dDS) and 10 DS subjects without signs of memory decline (ndDS) obtained at the Hospital Sant Pau, Barcelona, along with 15 additional age-matched yNC obtained from both hospitals. In addition, 15 patients with dementia due to sAD, 12 subjects with MCI and 17 age-matched elderly controls (eNC) were also obtained from the Hospital Sant Pau, Barcelona. See Table 1 for details of clinical and demographic data. All AD patients fulfilled the 2011 NIA-AA criteria for dementia or MCI due to AD [18, 19], while discrimination between the dDS subjects and those without dementia was assessed using the modified Cued Recall Test and the CAMDEX-DS battery [20, 21]. All the control subjects had no history or symptoms of neurological or psychiatric disorders, or memory complaints. This study was approved by the ethics committee at the Miguel Hernandez University and it was carried out in accordance with the Declaration of Helsinki.

PS1 over-expressing cells silencing by siRNA

CHO cells (400,000 cells/well) were grown in DMEM® (Gibco) containing 10 % Fetal Bovine Serum (Gibco) and 1 % Penicillin/Streptomycin (Sigma-Aldrich), and they were transfected with a construct encoding full-length PS1 (2 μg cDNA) [22] or with the pcDNA3 expression plasmid alone (Invitrogen), using Lipofectamine 2000® (Invitrogen). To reduce the PS1 gene expression we used CHO cells stably over expressing wild-type human PS1 and APP (CHO-PS1/APP) [23]. CHO-PS1/APP cells (350,000 cells/well) were grown in DMEM® containing 10 % Fetal Bovine Serum, 0,1 % Puromicin (Sigma-

Table 1 Clinical, demographic data and classic CSF biomarker levels

Group	Age (years)	n (Gender)	MMSE score	CSF Aβ42 (pg/mL)	CSF T-tau (pg/mL)	CSF P-tau (pg/mL)
yNC {yNC member of the same ADAD families}	45 ± 2 [25–60] {37 ± 3 [25–47]}	n = 23 (15 F/8 M) {6 F/2 M}	29 ± 1 [25–30] {29 ± 1 [28–30]}	809 ± 44 {791 ± 84}	207 ± 15 {231 ± 29}	43 ± 3 {45 ± 11}
syADAD	45 ± 3 [31–59]	n = 8 (5 F/3 M)	21 ± 2* [11–28]	300 ± 54*	899 ± 186*	164 ± 60*
psADAD	36 ± 3 [24–41]	n = 6 (4 F/2 M)	30 ± 1 [29, 30]	1120 ± 252	222 ± 26	49 ± 5
dDS	55 ± 2 [43–61]	n = 10 (5 F/5 M)	ND	411 ± 24*	788 ± 125*,[a]	106 ± 14*,[a]
ndDS	43 ± 2 [33–49]	n = 10 (5 F/5 M)	ND	570 ± 51*	232 ± 53	45 ± 8
eNC	67 ± 1 [61–80]	n = 17 (11 F/6 M)	29 ± 1 [26–30]	753 ± 30	197 ± 12	42 ± 2
sAD	68 ± 2 [54–83]	n = 13 (9 F/4 M)	20 ± 1** [18–24]	351 ± 17**	833 ± 87**	135 ± 18**
MCI due to AD	66 ± 1 [61–72]	n = 12 (5 F/7 M	26 ± 1** [20–30]	422 ± 31	618 ± 66**	81 ± 8**

In the yNC group (younger controls), the values for the control subgroup of non-mutation carriers from the same families as the carriers of *PSEN1* mutations are also indicated; the rest of cases correspond to subject without family history of ADAD. The *PSEN1* mutations included in this study from syADAD cases ("symptomatic" autosomal dominant AD subjects) corresponded to 3 carriers of L286P, and one of I439S, S169P, L173F, L235R and L282R. Those psADAD subjects (pre-symptomatic subjects carrying mutations in *PSEN1*) were 3 carriers of M139T, and one of I439S, R220G and K239N. Patients with (dDS) or without (ndDS) signs of clinical dementia were also compared with yNC; sporadic AD (sAD) and mild-cognitive impaired (MCI) subjects were compared with elderly controls (eNC). Levels of Aβ42, T-tau and P-tau were determined by ELISA; the intra-assay coefficient of variability (CV) was below 5 % and inter-assay CV below 15 % for all the classical AD biomarkers, in agreement with previous reports [36]. The number of samples "n" for female (F) and male (M) subjects is indicated. The data represent the means ± SEM, and for age and MMSE (Minimental State Examination), the range of values is also indicated. *Significantly different (p <0.05) from the yNC group, [a]and from the ndDS group; **Significantly different (p <0.05) from the eNC group

Aldrich) and 0,2 % G418 disulfate salt (Sigma-Aldrich), were transfected with BLOCK iT™ Alexa Fluor® Red Fluorescent Oligo (Invitrogen) as control, or siRNA (50 nM) targeting human PS1 (Santa Cruz Biotechnology, INC). Without removing the cell media, 24 h after the first transfection cells were transfected with the same siRNA (30 nM) and incubated for an additional 18 h.

Western blotting and immunoprecipitation

Although the denaturation temperature prior to electrophoresis has not been standardized, we found that high temperature sample preparation for electrophoresis (98 °C) produced an overall loss of CSF-PS1 immunoreactivity [24]. Hence, all analyses of in this study PS1 avoided freeze-thaw cycles (samples were aliquoted), and denaturation prior to electrophoresis was conducted at 50 °C.

Samples (30 μL for CSF) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience GmbH) that were probed with PS1 antibodies directed against the N-terminal amino acids 1–20 (antibody 98/1) [24]. GAPDH (Abcam) served as a loading control for cellular extracts. Membranes were incubated with the corresponding horseradish peroxidase conjugated secondary antibody and the immunoreactive signal was detected in a Luminescent Image Analyzer LAS-1000 Plus (FUJIFILM) using Super-Signal West Dura Extended Duration Substrate (Thermo Scientific). A control CSF sample was used to normalize the immunoreactive signal, and for semi-quantitative studies the intensity of the immunoreactive bands was measured by densitometry using Science Lab Image Gauge v 4.0 software provided by FUJIFILM. Aβ peptides in CSF immunoprecipitates (see below) were resolved by 16 % Tris-tricine SDS-PAGE and detected with the 6E10 antibody (Covance Research).

For immunoprecipitation, samples were precleared for 2 h at 4 °C by incubation with protein A-Sepharose (Sigma-Aldrich). Immunoprecipitations were performed at 4 °C by incubating 150 μL of CSF or cell media, overnight with the primary PS1 C-terminal antibody 00/2 (raised against residues 301–317) [23] previously coupled to protein A-Sepharose using Dimethyl pimelimidate dihydrochloride (Sigma-Aldrich Co). Precipitated proteins were washed with PBS and eluted with 0.1 M glycine buffer at pH 2.5. After pH neutralization, supernatants were denatured in Laemmli sample buffer at 50 °C for 15 min and subjected to SDS-PAGE. The membranes were then probed with anti-PS1 (98/1) and anti-Aβ (6E10) antibodies.

Sucrose gradients

PS1 complexes were analyzed by ultracentrifugation for 4 h at 4 °C on a continuous sucrose density gradient (5–20 %) at 250,000 × *g*. CSF aliquots (65 μL) were carefully loaded onto the top of the gradient containing 2 mL of 0.15 M NaCl, 50 mM $MgCl_2$ and 0.5 % Brij 97 in 50 mM Tris-HCl (pH 7.4). After centrifugation, ~14 fractions were collected gently from the top of the tubes. Enzyme markers of known sedimentation coefficient, β-galactosidase, catalase and alkaline phosphatase were used in the gradients to determine the approximate sedimentation coefficients. The sucrose fractions containing highly stable and unstable PS1 complexes were pooled separately, dialyzed against Tris buffer

and concentrated by ultrafiltration (Amicon Ultra 10,000 MWCO, Millipore Corporation, Bedford, MA). The PS1 complexes were then immunoprecipitated with anti-PS1 00/2 as described.

Measurement of T-tau, P-tau and Aβ42 by ELISA

The CSF levels of total tau (T-tau), phosphorylated tau (P-tau) and Aβ1-42 (Aβ42) were determined using specific enzyme-linked immunosorbent assays (ELISA: Fujirebio Europe, Ghent, Belgium).

Statistical analysis

All data were analyzed using SigmaStat (Version 3.5; Systac Software Inc.), applying a one-way analysis of variance or a Kruskal-Wallis test when the hypothesis of equality of sample variances was rejected. Pairwise group comparisons were then sustained using Student t test (two-tailed) or Mann-Whitney U test, and the exact p values determined. The results are presented as the means ± SEM, and correlations between the variables were assessed by linear regression analyses, with p values <0.05 considered statistically significant.

Results

The increase in CSF-PS1 with age

Since the main aim of the present study was to determine the changes in CSF-PS1 associated with ADAD and DS, and given that both ADAD and DS exhibits earlier clinical onset, we first assessed whether the amount and nature of the soluble PS1 complexes varies with age. The PS1 complexes in samples from control subjects (NC) from 25 to 80 years-of-age were detected with the 98/1 antibody, which predominantly recognized complexes of approximately 100 and 150 kDa, together with a less abundant 50 kDa band (Fig. 1a). The identity of these bands as complexes involving NTF- and CTF-PS1 was demonstrated in a previous study [12]. This soluble 50 kDa PS1 band may represent a NTF and CTF-PS1 aggregate, as the holoprotein had a mass of ~43 kDa and it differs in its electrophoretic migration [12]. PS1-NTF monomers are not detectable in human CSF samples. Since ADAD starts prior to 60 years of age [1], we sub-grouped young and elderly NC below and above this threshold. The sum of the immunoreactivity for the major 100 and 150 kDa PS1 complexes was significantly higher (~58 %) in the elderly NC (eNC; $n = 18$) than in the young NC samples (yNC; $n = 19$; $p < 0.001$: Fig. 1b). No differences were found between values obtained from the two center of sample collection. In all the NC samples, the major 100 and 150 kDa PS1 complexes were positively correlated with age ($r = 0.54$; $p < 0.001$: Fig. 1c). Therefore, this age-dependent increase in PS1 complexes must be taken into account when comparing the different pathological groups with non-disease subjects, defining appropriate age-matched controls.

We also attempted to assess potential differences in the class of the PS1 complexes in the NC sub-groups based on the direct analysis of the Western blots. As such, we defined the (100 + 150 kDa)/50 kDa quotient for each sample. No change was observed in the (100 + 150 kDa)/50 kDa quotient evaluated in CSF from yNC and eNC subjects (Fig. 1b).

Higher PS1 levels in symptomatic and asymptomatic ADAD

To assess whether the amount of CSF-PS1 is altered in ADAD, the levels in the age-matched yNC group were compared with those in the CSF from symptomatic (syADAD) and asymptomatic (pre-symptomatic: psADAD) subjects carrying mutations in *PSEN1* in Western blots (see Table 1 and Fig. 2a). Stronger immunoreactivity for the 100 and 150 kDa complexes was evident in syADAD (~119 %; $p < 0.001$) and in psADAD (~87 %; $p < 0.001$) subjects compared to the yNC, with no differences between the two pathological groups (Fig. 2b). Indeed, the levels in these AD subjects were significantly higher than in the yNC sub-group, composed by non-mutation carriers from the same ADAD families ($p < 0.001$). The previously defined quotient of CSF-PS1 complexes (see above) also discriminated between the yNC and the two ADAD groups, both individually ($p = 0.007$ for syADAD; $p = 0.027$ for psADAD) or when considered as a unique pathological group ($p = 0.007$). Thus, a higher proportion of 100 + 150 kDa CSF-PS1 complexes appears to be associated with ADAD even at pre-symptomatic stages (Fig. 2b).

PS1 complexes can be also characterized by gradient ultracentrifugation [24], followed by Western blotting under denaturing conditions, which served to illustrate the existence of different CSF-PS1 complexes [12]. When, CSF-PS1 complexes from yNC and syADAD subjects were characterized by sedimentation analysis on sucrose density gradients (Fig. 2c), 100–150 kDa PS1 complexes were identified close to the alkaline phosphatase marker (~140–160 kDa), along with larger complexes that sedimented in regions closer to the catalase marker (~232 kDa). These latter complexes were unstable and resolved as 50 kDa peptides by SDS-PAGE/Western blot analysis (Fig. 2c). In good agreement with results with the CSF-PS1 complex quotient obtained for direct Western blot analysis, samples separated by ultracentrifugation revealed higher abundance of the highly stable 100–150 kDa PS1 complexes in the syADAD samples than in the yNC samples, more so than the complexes of the 50 kDa fragments that sedimented in the denser fractions. This difference was clearly evident with the determination of a refined quotient, the "stability" quotient, reflecting the differences between the

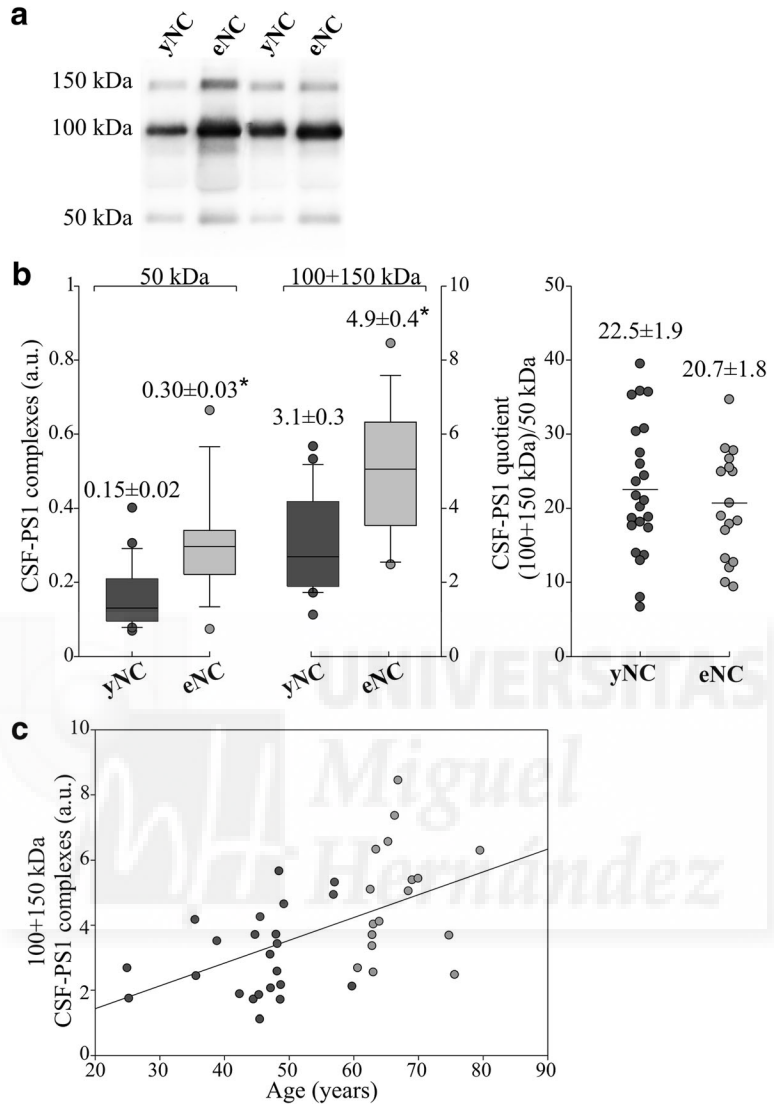

Fig. 1 Characterization of the CSF-PS1 complexes in younger and elderly NC subjects, and their correlation with age. **a** Representative Western blots of human CSF samples from non-demented control (NC) subjects arbitrarily categorized as young (yNC; ≤60 years; $n = 23$) and elderly (eNC; >60 years; $n = 17$), and probed with an anti-NTF-PS1 antibody. **b** Densitometric quantification of the major 100 and 150 kDa CSF-PS1 complexes (the sum of the 100 + 150 kDa CSF-PS1 bands) and the quotient derived from the immunoreactivity for the 100 and 150 kDa bands relative to that for the minor 50 kDa band in each sample [(100 + 150 kDa)/50 kDa]. The data represent the means ± SEM and they were compared using a paired Students *t* test: *p <0.001. **c** Correlation between the levels of the 100 + 150 kDa CSF-PS1 complexes with age

highly stable complexes (the 100–150 kDa heterodimers that sediment close to the internal marker of similar molecular mass) and the unstable complexes (the 50 kDa complexes that sediment closer to catalase), this quotient allowing us to discriminate syADAD ($p = 0.004$) from yNC samples (Fig. 2d).

Highly stable CSF-PS1 complexes are elevated in sAD and MCI

In sAD no notable differences in total PS1 were observed between patients with dementia due to sAD, MCI due to AD, or age-matched eNC subjects (Fig. 3a, b). However, the highly stable PS1 complexes were again more abundant in probable sAD cases compared to elderly eNCs when the CSF-PS1 complexes quotient was calculated ($p = 0.006$; Fig. 3b). Sucrose density centrifugation profiles (Fig. 3c) and the subsequent estimation of the "stability" quotient confirmed the greater abundance of highly stable PS1 complexes in sAD compared to eNC ($p = 0.02$; Fig. 3d), as well as indicating that the highly stable complexes were particularly increased in MCI subjects ($p = 0.008$; Fig. 3d).

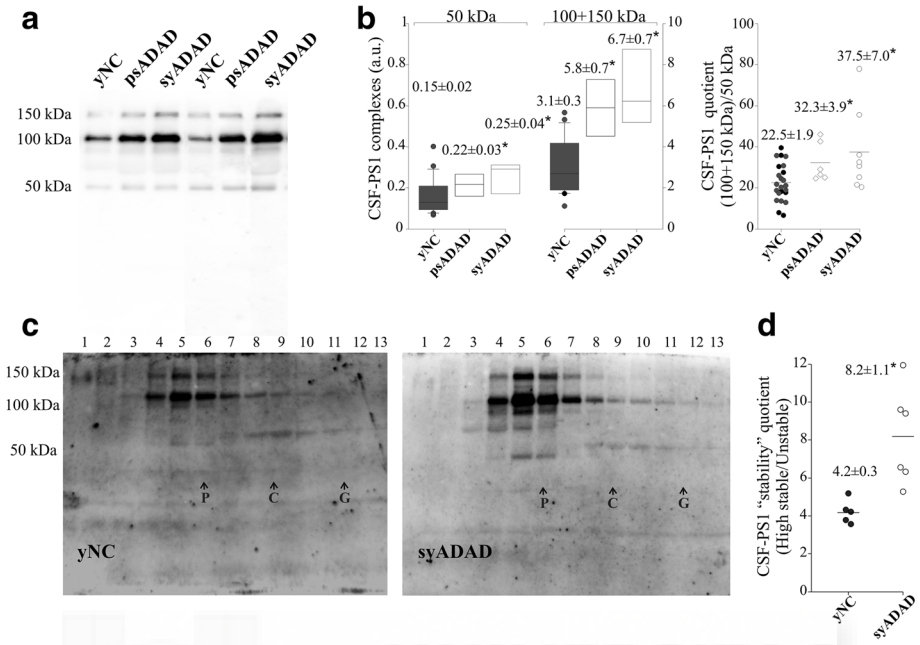

Fig. 2 The increase in the CSF-PS1 complexes in ADAD. **a** Representative blot of the PS1 complexes in the CSF samples from eight symptomatic ADAD (syADAD), six presymptomatic mutation carriers (psADAD) and 23 younger NC controls (yNC), eight of which were from the same families a the ADAD subjects but that did not carry mutations (black symbol; see also Table 1). **b** Densitometric quantification of the accumulative immunoreactivity from the sum of the higher molecular mass PS1 complex (100 + 150 kDa). A quotient was calculated for each sample defined as the sum of (100 + 150 kDa) immunoreactivity relative to the 50 kDa immunoreactivity: (100 + 150 kDa/50 kDa). **c** Six syADAD and five yNC samples were fractionated on 5–20 % sucrose density gradients to further characterize the PS1 complexes. The fractions (collected from the top of each tube) were immunoblotted under denaturing conditions and probed for PS1, as in (**a**). β-Galactosidase (G, 16.0S; ~540 kDa), catalase (C, 11.4S; ~232 kDa) and alkaline phosphatase (P, 6.1S; ~140–160 kDa) were used as internal markers. Representative blots are shown. **d** The "stability" quotient was defined as the sum of the stable immunoreactive bands that sediment close to alkaline phosphatase (~140–160 kDa; fractions 2–7), mainly the 100 and 150 kDa bands, relative to the large unstable complexes that sediment closer to catalase (~232 kDa; fractions 8–12), and resolve mainly as 50 kDa immunoreactive bands in Western blots. The data are the means ± SEM: *Significantly different (p <0.005) from the yNC group as assessed by the Student t or Mann-Whitney U tests

Higher PS1 levels in demented and non-demented DS

DS is considered a pre-symptomatic AD [10]. To assess whether an increase in the CSF-PS1 complexes is also associated with DS, we analyzed CSF samples from DS patients with (dDS) or without (ndDS) signs of clinical dementia, comparing these to age-matched yNC (Fig. 4a). The cumulative immunoreactivity of the major 100 and 150 kDa bands was significantly higher in both dDS (p <0.001) and ndDS (p = 0.007) CSF than in that from yNC subjects (Fig. 4b). Remarkably, the CSF-PS1 complexes quotient also revealed consistent changes in the proportion of the different complexes for both dDS (p <0.001) and ndDS subjects (p = 0.04) relative to yNC (Fig. 4b).

The formation of stable CSF-PS1 complexes is favored by β-amyloid

Although PS1 clearly forms native complexes in CSF, there is little knowledge about the dynamics of soluble PS1 fragment assembly into heteromeric complexes. Thus, we monitored the assembly of soluble PS1 into complexes in a cell model, CHO cells over-expressing wild-type human PS1. An increase in the 29 kDa NTF of PS1 in extracts from CHO cells transfected with human PS1 corroborated that these cells over-expressed the protein (Additional file 1: Figure S1A). Immunoblotting of the cell-conditioned medium revealed predominant bands of approximately 100 and 150 kDa, and a weaker ~70 kDa band. The amounts of these soluble PS1 complexes increased in conditioned media from CHO cells transfected with PS1 (Additional file 1: Figure S1A). CHO cells stably transfected with PS1 and APP showed similar soluble PS1 complexes with additional 50 kDa band and monomeric NTF (Additional file 1: Figure S1A). To ascertain the identity of the soluble PS1 complexes in the cellular model, we reduced PS1 expression in CHO cells stably over expressing wild-type human PS1 with siRNA PS1. Cells transfected with siRNA PS1 displayed decrease in cellular PS1-NTF, but also in soluble PS1 complexes identified in cell media (Additional file 1: Figure S1A).

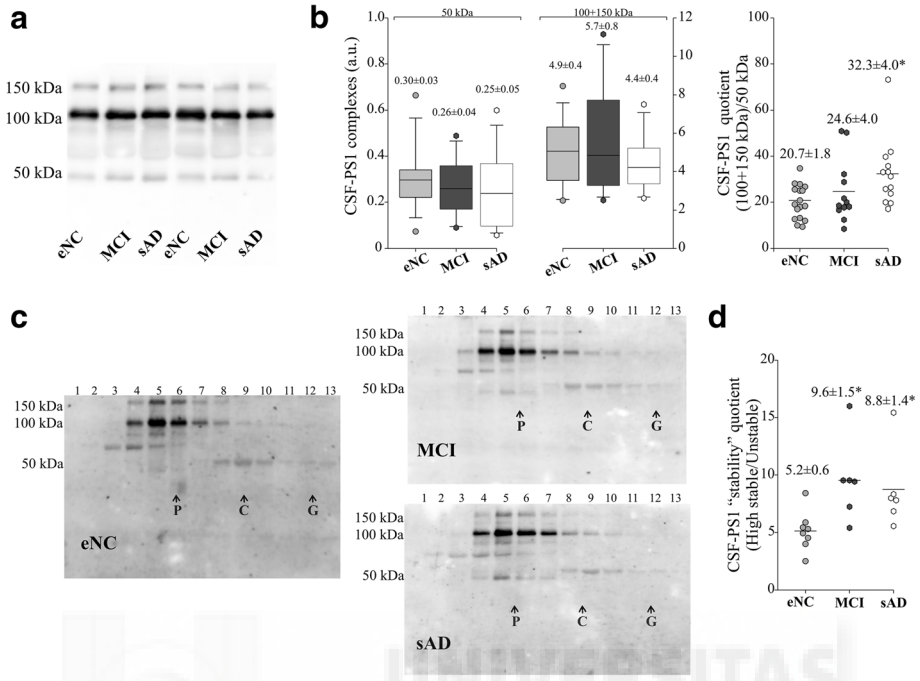

Fig. 3 Increase in the stable PS1 complexes in AD and MCI CSF. **a** Representative blot and (**b**) densitometric quantification of the accumulative immunoreactivity from the sum of stable higher molecular mass PS1 complexes (100 + 150 kDa) in CSF samples from 13 sAD, 12 MCI and 17 age-matched eNC subjects. A quotient calculated as the sum of (100 + 150 kDa) immunoreactivity relative to the 50 kDa immunoreactivity: (100 + 150 kDa/50 kDa) is also shown. **c** Six AD and MCI individuals, and 8 eNC subjects were fractionated on 5–20 % sucrose density gradients, and probed with the PS1 antibody under denaturing conditions. The internal markers were β-galactosidase (G), catalase (C) and alkaline phosphatase (P), as in Fig. 3. **d** The values for the "stability" quotient reflecting the highly stable complexes (100 + 150 kDa immunoreactive bands sedimenting in fractions 2–7) relative to the unstable complexes (50 kDa immunoreactive bands sedimenting in fractions 8–12) is also shown. *p <0.05, **p <0.01 as assessed by the Student t or Mann-Whitney U tests

We also analyzed the soluble PS1 complexes in the conditioned medium of PS1-transfected CHO cells and CHO cells over-expressing PS1 and APP, using sucrose-density gradient fractionation followed by Western blotting under denaturing conditions (Additional file 1: Figure S1B). The majority of the soluble PS1 in the CHO cell-conditioned medium accumulated close to the alkaline phosphatase marker (~140–160 kDa) and resolved as 70 kDa complexes after denaturation, with only faint bands at 100 kDa. However, some 29 kDa monomeric PS1 was also evident, probably released from the complexes (Additional file 1: Figure S1B). By contrast, in the medium of CHO cells over-expressing PS1 and APP there was virtually no 29 kDa NTF immunoreactivity, indicating that in the context of β-amyloid over-expression, most of the soluble PS1 is stably incorporated into complexes (Additional file 1: Figure S1B).

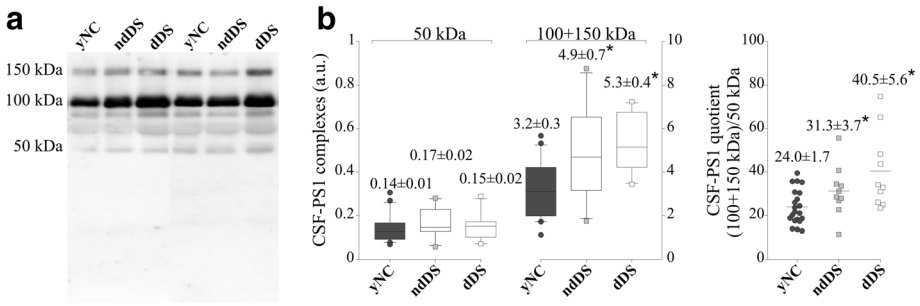

Fig. 4 An increase in PS1 stable complexes in DS CSF. **a** Representative blot of PS1 complexes in CSF from 10 DS subjects with dementia of the Alzheimer's type (dDS), 10 DS without any sign of memory decline (ndDS) and 23 yNC. **b** Densitometric quantification of the accumulated immunoreactivity from the sum of the higher molecular mass PS1 complex (100 + 150 kDa), and the quotient of the (100 + 150 kDa) immunoreactivity relative to the 50 kDa immunoreactivity (100 + 150 kDa/50 kDa). The means ± SEM are shown: *p <0.005, **p <0.005, Student t test

We further tested the possible interaction between soluble PS1 complexes and Aβ. PS1 was immunoprecipitated from the medium of CHO cells over-expressing PS1 and APP with the 00/2 antibody that recognizes the PS1 CTF. Immunoprecipitation of heteromeric PS1 complexes was confirmed in Western blots probed with the anti-N-terminal 98/1 antibody (Additional file 1: Figure S1C). Considerable amounts of Aβ oligomers were also detected in these immunoprecipitates by the 6E10 antibody (Additional file 1: Figure S1C), while no immunoreactivity was resolved by a C-terminal APP antibody (not shown); indicating that oligomers of Aβ, but not C-terminal fragments, interact with the soluble PS1 complexes.

To confirm that Aβ oligomers favors the formation of stable PS1 complexes in human CSF we examined the Aβ peptides in PS1 complexes immunoprecipitated from CSF samples from sAD and eNC subjects. Again, CSF samples immunoprecipitated with 00/2 antibody were probed in immunoblots with the 98/1 and 6E10 antibodies (Fig. 5a), demonstrating that Aβ oligomers co-immunoprecipitated with heteromeric PS1 complexes from both eNC and sAD CSF samples. We further tested the involvement of Aβ on the formation of the highly stable PS1 complexes. After CSF-PS1 complexes were fractioned by sucrose density gradients and the peak fractions of the highly stable and unstable complexes were isolated, they were immunoprecipitated with the 00/2 antibody (Fig. 5b). Aβ oligomers were clearly present in the fractions rich in stable 100–150 kDa complexes from both eNC and sAD samples, whereas

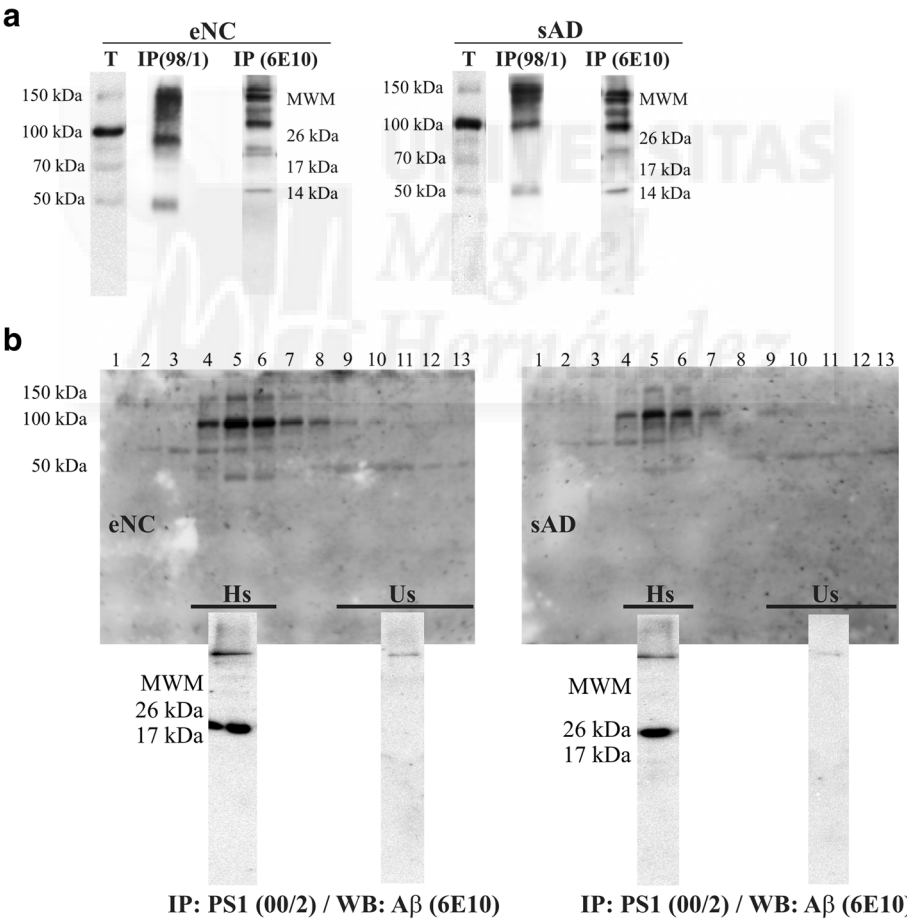

Fig. 5 Aβ oligomers are present in highly stable CSF-PS1 complexes. **a** CSF samples from eNC and sAD subjects were precleared with protein A-Sepharose (T: total), and then immunoprecipitated with the anti CTF-PS1 00/2 antibody. The precipitated proteins (IP) were probed in immunoblots with the antibody indicated (98/1 for NTF-PS1 and 6E10 for Aβ). Note that oligomeric Aβ species co-immunoprecipitate and interact with CSF-PS1 complexes in both eNC and sAD. None immunoreactivity was resolved in negative controls incubated with beads in the absence of antibody (not shown). **b** CSF-PS1 complexes were fractionated by sucrose gradient centrifugation, and the fractions containing highly stable (Hs) or unstable (Us) PS1 complexes were pooled, dialyzed and concentrated by ultrafiltration. Representative sedimentation profiles illustrate the fractions selected for peak isolation. The enriched CSF-PS1 complexes were then immunoprecipitated with the 00/2 antibody and assayed in immunoblots probed with the 6E10 antibody against Aβ (insert). Representative blots reveal that Aβ oligomers are mainly present in peak fractions containing highly stable PS1 complexes (illustrative examples from two different experiments)

virtually no Aβ immunoreactivity was detected in the pooled fractions of 50 kDa PS1 complexes (Fig. 5b). Hence, oligomers of Aβ appear to mainly associate with the highly stable PS1 complexes.

Discussion

The detection of soluble PS1 in CSF and serum [12] was a somewhat unexpected finding, particularly since PS1 is a multi-pass transmembrane protein with several hydrophobic regions [14]. Indeed, the presence of soluble PS1 has been reported in the medium of primary neurons [25] and confirmed in human serum [26]. Here, we corroborated the existence of different PS1 complexes in human CSF and we revealed their potential utility as a biomarker for AD. Like many membrane proteins, PS1 has a tendency to aggregate under non-native conditions [27, 28]. Thus, CSF-PS1 complexes probably represent non-specific aggregates of PS1 NTF and CTF distinct to the active γ-secretase membrane-complexes [12].

How PS1 complexes become soluble and appear in the CSF is yet to be determined. However, it appears that Aβ oligomers can probably contribute to the formation of stable CSF-PS1 complexes which are particularly abundant in AD. Indeed, it is remarkable that when we follow the formation of PS1 complexes in the cell-conditioned media, the co-expression of APP and PS1 favored the accumulation of complexes and not soluble monomeric PS1 is existent. We were able to pull down oligomeric Aβ species by PS1 immunoprecipitation from the medium, as well as from human CSF, in which Aβ oligomers are mainly associated to the highly stable PS1 complexes. Aβ peptides are chemically "sticky", gradually building up into fibrils and aggregates; although the mechanism of how can Aβ stabilize CSF-PS1 is yet to be determined. Also in this context, levels of soluble Aβ peptide assessed by ELISA determinations appear consistently decreased in AD CSF [11]. The possibility that some amounts of Aβ participate within stable protein complexes in CSF, resulting underestimated by conventional ELISA protocols, may deserve consideration.

In CSF samples from NC subjects we observe an age-related increase in the total amount of PS1, while the relative proportion of the different complexes remains unaltered. No changes were observed comparing NC samples from different center of sample collection or gender. However, the relative proportion of stable PS1 complexes does appear to increase in the AD condition.

We propose that the most significant phenomenon related to the potential use of CSF-PS1 to discriminate the pathological state is the change in the proportion of PS1 complexes, rather than the estimates of the total PS1 levels. Accordingly, we focused our analysis on the highly stable 100–150 kDa PS1 complexes in CSF. The highly stable CSF-PS1 complexes co-exist with unstable complexes, sedimenting after differential centrifugation in regions closer to 200–250 kDa, but mainly resolved as 50 kDa components by reducing SDS-PAGE. We found that a quotient of PS1 complexes can discriminate all pathological groups from age-matched controls. We suggest that these quotients reflect differences in the properties of the PS1 complexes formed under pathological conditions. Screening large numbers of samples by sucrose gradient ultracentrifugation is difficult. As a reliable alternative, we addressed the discrimination of samples using a complementary parameter, a quotient of CSF-PS1 complexes calculated directly from Western blot analysis [(100 + 150 kDa)/50 kDa], thereby simplifying the analysis. This alternative quotient is useful to discriminate ADAD and DS subjects from age-matched yNC, as well as sAD from eNC. In our analysis, this quotient of PS1 complexes only failed to adequately discriminate MCI subjects, maybe indicating a lack of sensibility with respect to the evaluation of the complexes after separation by ultracentrifugation in sucrose density gradients. The inherent uncertainty in clinical diagnosis may also account for these differences, particularly for MCI group in which some subjects maybe remained MCI stable or develop to other dementias.

Anyhow, large overlap is observed between groups when assessment of the relative amount of CSF-PS1 complexes is estimated by a quotient obtained directly from Western blot analysis, without fractioning by ultracentrifugation. It will be necessary to replicate these finding using other techniques, such as ELISA specific for stable CSF-PS1 complexes, to evaluate their true potential as biomarkers.

Interestingly, altered levels of CSF-PS1 are detectable in both symptomatic and asymptomatic ADAD subjects. Similarly, alterations to CSF-PS1 levels occur in DS subjects with and without dementia. The analysis of CSF samples from DS subjects is of particular interest since it is well known that almost all adults with DS over 40 years of age display AD neuropathology [29, 30], although the prevalence of dementia in these individuals varies considerably [31–34]. Thus, there is no association between the age of onset of AD neuropathology in DS subjects and the appearance of clinical dementia [35], and we cannot predict the number of ndDS that will develop future cognitive impairment. In the view of the consistent changes in CSF-PS1 in ndDS we assume that this biomarker is more related to the brain pathological status than the occurrence of dementia and cognitive decline.

Conclusions

In conclusion, our present findings demonstrate that CSF-PS1 complexes are altered in genetically determined AD, as well as in sAD. Together, our results indicate that the increase in stable PS1 complexes in CSF is an early phenomenon associated to AD pathology and may constitute an asymptomatic biomarker.

Additional file

Additional file 1: Figure S1. Aβ affects the dynamics and stability of the soluble PS1 complexes. (**A**) CHO cell were transfected with human PS1 or with the pcDNA3 expression plasmid as a control (Ø). CHO cells stably over-expressing PS1 and APP were also transfected with PS1 siRNA. PS1 in the cell extracts and soluble PS1 complexes in the medium were assayed in Western blots using a NTF-PS1 antibody (equivalent amounts of protein of the cell extracts and equal volumes of medium were loaded in each lane). GAPDH served as a loading control for cellular extracts. (**B**) Soluble PS1 complexes from the medium conditioned by CHO cells transfected with PS1 (CHO-PS1), or CHO cells over-expressing PS1 and APP (CHO-PS1/APP), characterized by ultracentrifugation on 5–20 % sucrose density gradients. Fractions were collected from the top of each tube and they were analyzed by SDS-PAGE under denaturing conditions. Enzymes of known sedimentation coefficient were used as internal markers: β-galactosidase (G, 16.0S; molecular mass ~540 kDa), catalase (C, 11.4S; ~232 kDa) and alkaline phosphatase (P, 6.1S; ~140–160 kDa). Note that both PS1 complexes and 29 kDa monomers were identified in CHO-PS1 cells, while the 29 kDa monomers are mostly absent from the CHO-PS1/APP cells. (**C**) Cell medium conditioned by CHO-PS1/APP cells was precleared with protein A-Sepharose and the soluble PS1 complexes were immunoprecipitated with the anti-PS1 antibody 00/2 raised against the CTF. The immunoprecipitated proteins (IP) were probed with the 98/1 antibody against the NTF of PS1 and the 6E10 antibody against Aβ (T: total). The PS1 antibody confirms the immunoprecipitation of heteromeric complexes of PS1 and the 6E10 confirms that these PS1 complexes contain or interact with small Aβ oligomers. Illustrative examples from three different experiments are shown. (TIF 2441 kb)

Abbreviations

AD: Alzheimer's disease; ADAD: Autosomal dominant AD; APP: β-amyloid precursor protein; Aβ: β-amyloid peptide; CSF: Cerebrospinal fluid; CTF: C-terminal fragment of PS1; dDS: DS patients with dementia; DS: Down syndrome; eNC: Elderly NC; MCI: Mild-cognitive impairment; NC: Normal control subjects; ndDS: DS patients without dementia; NTF: N-terminal fragment of PS1; PS1: Presenilin-1; psADAD: Pre-symptomatic ADAD; P-tau: Phosphorylated tau; sAD: Sporadic Alzheimer's disease; syADAD: Symptomatic ADAD; T-tau: Total tau; yNC: Young NC samples

Acknowledgements

We thank Drs J. Culvenor and G. Evin (Department of Pathology, The University of Melbourne, Australia) for the generous gift of the PS1 antibodies; and Dr D. Selkoe, Brigham and Women's Hospital, Boston for providing CHO cells over-expressing human PS1. We also thank Dr J. F. Hernández-Sánchez (Servicio de Análisis Clínicos, Hospital General Universitario de Elche) for assistance in the preliminary assays.

Funding

This study was funded in part by the EU BIOMARKAPD-Joint Programming on Neurodegenerative Diseases (JPND) project, by the Instituto de Salud Carlos III (ISCIII grants PI11/03026 to JSV, PI11/02425 and PI14/01126 to JF, PI11/03035 and PI14/1561 to AL, PI08/0036 and PI12/00013 to RSV, and PI11/03023 to JLM), co-financed by the Fondo Europeo de Desarrollo Regional, under the aegis of JPND, and through CIBERNED, ISCIII. This work was also supported by the Fundació Catalana de Síndrome de Down and by a "Marató TV3" grant (20141210 to JF) and a grant from the Griffols Foundation. The funding bodies played no role in the study design, data collection and analysis, the decision to publish, or the preparation of the manuscript. We also acknowledge the support for the publication fee to the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI).

Authors' contributions

JSV, MSGA, AL and JLM were involved with the conception, design, and interpretation of data. ASE and MSGA performed the experiments. JSV, ASE and MSGA were involved with data analysis. JLM, AL, RSV and JF collected the clinical material. JSV, JLM, AL, RSV and JF provided general overall supervision of the study, and acquired funding. All authors contributed to the drafting and critical revision of the manuscript and have given final approval of the version to be published.

Competing interests

All the authors contributed to this work and are in agreement with the findings presented. MSGA and JSV have submitted a patent application for the use of PS1 complexes as a biomarker for AD, as described herein. Appropriate procedures were followed to obtain approval from local ethics committees.

Ethics approval and consent to participate

This study was approved by the ethic committee at the Miguel Hernandez University and it was carried out in accordance with the Declaration of Helsinki. All patients (or their nearest relatives) and controls gave informed consent to participate in the study.

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Received: 30 April 2016 Accepted: 22 September 2016
Published online: 29 September 2016

References

1. Tanzi RE. The genetics of Alzheimer disease. Cold Spring Harb Perspect Med. 2012;2. doi: 10.1101/cshperspect.a006296.
2. Beyreuther K, Dyrks T, Hilbich C, Mönning U, König G, Multhaup G, Pollwein P, Masters CL. Amyloid precursor protein (APP) and beta A4 amyloid in Alzheimer's disease and Down syndrome. Prog Clin Biol Res. 1992;379:159–82.
3. Thinakaran G, Koo EH. Amyloid precursor protein trafficking, processing, and function. J Biol Chem. 2008;283:29615–9.
4. Kaether C, Haass C, Steiner H. Assembly, trafficking and function of gamma-secretase. Neurodegener Dis. 2006;3:275–83.
5. Yu G, Chen F, Levesque G, Nishimura M, Zhang DM, Levesque L, Rogaeva E, Xu D, Liang Y, Duthie M, St George-Hyslop PH, Fraser PE. The presenilin 1 protein is a component of a high molecular weight intracellular complex that contains beta-catenin. J Biol Chem. 1998;273:16470–5.
6. Guerreiro RJ, Gustafson DR, Hardy J. The genetic architecture of Alzheimer's disease: beyond APP, PSENs and APOE. Neurobiol Aging. 2012;33:437–56.
7. Glenner GG, Wong CW. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem Biophys Res Commun. 1984;122:1131–5.
8. Mann DM, Yates PO, Marcyniuk B. Alzheimer's presenile dementia, senile dementia of Alzheimer type and Down's syndrome in middle age form an age related continuum of pathological changes. Neuropathol Appl Neurobiol. 1984;10:185–207.
9. Wisniewski KE, Wisniewski HM, Wen GY. Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. Ann Neurol. 1985;17:278–82.
10. Dubois B, Feldman HH, Jacova C, Hampel H, Molinuevo JL, Blennow K, DeKosky ST, Gauthier S, Selkoe D, Bateman R, Cappa S, Crutch S, Engelborghs S, Frisoni GB, Fox NC, Galasko D, Habert MO, Jicha GA, Nordberg A, Pasquier F, Rabinovici G, Robert P, Rowe C, Salloway S, Sarazin M, Epelbaum S, de Souza LC, Vellas B, Visser PJ, Schneider L, Stern Y, Scheltens P, Cummings JL. Advancing research diagnostic criteria for Alzheimer's disease: the IWG-2 criteria. Lancet Neurol. 2014;13:614–29.
11. Blennow K, Hampel H. CSF markers for incipient Alzheimer's disease. Lancet Neurol. 2003;2:605–13.
12. García-Ayllón MS, Campanari ML, Brinkmalm G, Rábano A, Alom J, Saura CA, Andreasen N, Blennow K, Sáez-Valero J. CSF Presenilin-1 complexes are increased in Alzheimer's disease. Acta Neuropathol Commun. 2013;1:46.
13. Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G, Ratovitsky T, Davenport F, Nordstedt C, Seeger M, Hardy J, Levey AI, Gandy SE, Jenkins NA, Copeland NG, Price DL, Sisodia SS. Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron. 1996;17:181–90.

14. Laudon H, Hansson EM, Melén K, Bergman A, Farmery MR, Winblad B, Lendahl U, von Heijne G, Näslund J. A nine-transmembrane domain topology for presenilin 1. J Biol Chem. 2005;280:35352–60.
15. Fortea J, Lladó A, Clarimón J, Lleó A, Oliva R, Peri J, Pintor L, Yagüe J, Blesa R, Molinuevo JL, Sánchez-Valle R. PICOGEN: five years experience with a genetic counselling program for dementia. Neurologia. 2011;26:143–9.
16. Fortea J, Lladó A, Bosch B, Antonell A, Oliva R, Molinuevo JL, Sanchez-Valle R. Cerebrospinal fluid biomarkers in Alzheimer's disease families with PSEN1 mutations. Neurodegener Dis. 2010;8:202–7.
17. Pera M, Alcolea D, Sánchez-Valle R, Guardia-Laguarta C, Colom-Cadena M, Badiola N, Suárez-Calvet M, Lladó A, Barrera-Ocampo AA, Sepulveda-Falla D, Blesa R, Molinuevo JL, Clarimón J, Ferrer I, Gelpi E, Lleó A. Distinct patterns of APP processing in the CNS in autosomal-dominant and sporadic Alzheimer disease. Acta Neuropathol. 2013;125:201–13.
18. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jr Jack CR, Kawas CH, Klunk WE, Koroshetz WJ, Manly JJ, Mayeux R, Mohs RC, Morris JC, Rossor MN, Scheltens P, Carrillo MC, Thies B, Weintraub S, Phelps CH. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011;7:263–9.
19. Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, Gamst A, Holtzman DM, Jagust WJ, Petersen RC, Snyder PJ, Carrillo MC, Thies B, Phelps GH. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011;7:270–9.
20. Benejam B, Fortea J, Molina-López R, Videla S. Patterns of performance on the modified cued recall test in Spanish adults with down syndrome with and without dementia. Am J Intellect Dev Disabil. 2015;120:481–9.
21. Esteba-Castillo S, Dalmau-Bueno A, Ribas-Vidal N, Vilà-Alsina M, Novell-Alsina R, García-Alba J. Adaptation and validation of CAMDEX-DS (Cambridge Examination for Mental Disorders of Older People with Down's Syndrome and others with intellectual disabilities) in Spanish population with intellectual disabilities. Rev Neurol. 2013;57:337–46.
22. Culvenor JG, Maher F, Evin G, Malchiodi-Albedi F, Cappai R, Underwood JR, Davis JB, Karran EH, Roberts GW, Beyreuther K, Masters CL. Alzheimer's disease-associated presenilin 1 in neuronal cells: evidence for localization to the endoplasmic reticulum-Golgi intermediate compartment. J Neurosci Res. 1997;49:719–31.
23. Xia W, Zhang J, Kholodenko D, Citron M, Podlisny MB, Teplow DB, Haass C, Seubert P, Koo EH, Selkoe DJ. Enhanced production and oligomerization of the 42-residue amyloid beta-protein by Chinese hamster ovary cells stably expressing mutant presenilins. J Biol Chem. 1997;272:7977–82.
24. Evin G, Sharples RA, Weidemann A, Reinhard FB, Carbone V, Culvenor JG, Holsinger RM, Sernee MF, Beyreuther K, Masters CL. Aspartyl protease inhibitor pepstatin binds to the presenilins of Alzheimer's disease. Biochemistry. 2001;40:8359–68.
25. Benussi L, Alberici A, Mayhaus M, Langer U, Ghidoni R, Mazzoli F, Nicosia F, Barbiero L, Frisoni G, Zanetti O, Gasparini L, Nitsch RM, Binetti G. Detection of the presenilin 1 COOH-terminal fragment in the extracellular compartment: a release enhanced by apoptosis. Exp Cell Res. 2001;269:256–65.
26. Luciano R, Barraco GM, Muraca M, Ottino S, Spreghini MR, Sforza RW, Rustico C, Morino GS, Manco M. Biomarkers of Alzheimer disease, insulin resistance, and obesity in childhood. Pediatrics. 2015;135:1074–81.
27. Kovacs DM, Fausett HJ, Page KJ, Kim TW, Moir RD, Merriam DE, Hollister RD, Hallmark OG, Mancini R, Felsenstein KM, Hyman BT, Tanzi RE, Wasco W. Alzheimer-associated presenilins 1 and 2: neuronal expression in brain and localization to intracellular membranes in mammalian cells. Nat Med. 1996;2:224–9.
28. De Strooper B, Beullens M, Contreras B, Levesque L, Craessaerts K, Cordell B, Moechars D, Bollen M, Fraser P, George-Hyslop PS, Van Leuven F. Phosphorylation, subcellular localization, and membrane orientation of the Alzheimer's disease-associated presenilins. J Biol Chem. 1997;272:3590–8.
29. Mann DMA. Alzheimer's disease and Down's syndrome. Histopathology. 1988;13:125–27.
30. Teipel SJ, Hampel H. Neuroanatomy of Down syndrome in vivo: a model of preclinical Alzheimer's disease. Behav Genet. 2006;36:405–15.
31. Lai F, Williams RS. A prospective study of Alzheimer disease in Down syndrome. Arch Neurol. 1989;46:849–53.
32. Zigman W, Schupf N, Haveman M, Silverman W. The epidemiology of Alzheimer's disease in mental retardation: results and recommendations from an international conference. J Intellect Disabil Res. 1997;41:76–80.
33. Oliver C, Crayton L, Holland A, Hall S, Bradbury J. A four year prospective study of age-related cognitive change in adults with Down's syndrome. Psychol Med. 1998;28:1365–77.
34. Holland AJ, Hon J, Huppert FA, Stevens F, Watson P. Population-based study of the prevalence and presentation of dementia in adults with Down's syndrome. Br J Psychiatry. 1998;172:493–8.
35. Head E, Powell D, Gold BT, Schmitt FA. Alzheimer's disease in down syndrome. Eur J Neurodegener. 2012;1:353–64.
36. Alcolea D, Martínez-Lage P, Sánchez-Juan P, Olazarán J, Antúnez C, Izagirre A, Ecay-Torres M, Estanga A, Clerigué M, Guisasola MC, Sánchez Ruiz D, Marín J, Calero M, Blesa R, Clarimón J, Carmona-Iragui M, Morenas-Rodríguez E, Rodríguez-Rodríguez E, Vázquez Higuera JL, Fortea J, Lleó A. Amyloid precursor protein metabolism and inflammation markers in preclinical Alzheimer disease. Neurology. 2015;85:626–33.

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ARTICLE 2: Levels of ADAM10 are reduced in Alzheimer's disease CSF

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Submitted to Alzheimer's Research and Therapy. 2018.

Submission code: AZRT-D-18-00071

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Alzheimer's Research & Therapy

Levels of ADAM10 are reduced in Alzheimer's disease CSF

--Manuscript Draft--

Manuscript Number:	
Full Title:	Levels of ADAM10 are reduced in Alzheimer's disease CSF
Article Type:	Research
Funding Information:	Instituto de Salud Carlos III (PI11/03026) — Dr Javier Sáez-Valero Instituto de Salud Carlos III (PI15/00665) — Dr Javier Sáez-Valero Ministerio de Economía y Competitividad (SEV-2013-0317) — Dr Javier Sáez-Valero Torsten Söderbergs Stiftelse — Dr Kaj Blennow Knut och Alice Wallenbergs Stiftelse — Dr Henrik Zetterberg
Abstract:	Background: The disintegrin metalloproteinase 10 (ADAM10) is the main α-secretase acting in the non-amyloidogenic processing of the amyloid precursor protein. This study assesses whether ADAM10 is present in cerebrospinal fluid (CSF), and whether it has potential as a biomarker for Alzheimer's disease (AD). Methods: ADAM10 was characterized in human CSF samples by western blotting using antibodies specific for different domains of the protein and by ultracentrifugation in sucrose density gradients. Samples from AD patients (n= 20) and age-matched non-AD controls (n= 20) were characterized for classical CSF biomarkers, Aβ42, T-tau or P-tau, and assayed for sADAM10 levels with a mid-domain antibody (common to all the identified ADAM10 species). Results: We found that ADAM10 is present in human CSF as several distinct species: an immature form retaining the prodomain (proADAM10; ~80 kDa), a mature unprocessed full-length form (ADAM10f; ~55 kDa) and a truncated large soluble form released from the membrane (sADAM10; ~50 kDa). Fractionation by ultracentrifugation on sucrose density gradients showed that the ADAM10f and sADAM10 species form large complexes. Immunoblotting revealed a significant decrease in ADAM10f and sADAM10 in AD CSF compared to control CSF, while proADAM10 levels remained unaltered. Conclusions: Several forms of ADAM10 are present in CSF, mainly assembled as high molecular weight complexes. The determination of the levels of mature forms of CSF-ADAM10 may be useful as a biomarker for AD.
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Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the Word Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

Levels of ADAM10 are reduced in Alzheimer's disease CSF

Running title: *ADAM10 is decreased in AD CSF*

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Number of figures/tables: 3 figures + 1 tables + supplementary figure

Number of characters/words, Abstract: ~220 w / **Manuscript:** ~2900 w

Abstract

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Methods: ADAM10 was characterized in human CSF samples by western blotting using antibodies specific for different domains of the protein and by ultracentrifugation in sucrose density gradients. Samples from AD patients (n= 20) and age-matched non-AD controls (n= 20) were characterized for classical CSF biomarkers, Aβ42, T-tau or P-tau, and assayed for sADAM10 levels with a mid-domain antibody (common to all the identified ADAM10 species).

Results: We found that ADAM10 is present in human CSF as several distinct species: an immature form retaining the prodomain (proADAM10; ~80 kDa), a mature unprocessed full-length form (ADAM10f; ~55 kDa) and a truncated large soluble form released from the membrane (sADAM10; ~50 kDa). Fractionation by ultracentrifugation on sucrose density gradients showed that the ADAM10f and sADAM10 species form large complexes. Immunoblotting revealed a significant decrease in ADAM10f and sADAM10 in AD CSF compared to control CSF, while proADAM10 levels remained unaltered.

Conclusions: Several forms of ADAM10 are present in CSF, mainly assembled as high molecular weight complexes. The determination of the levels of mature forms of CSF-ADAM10 may be useful as a biomarker for AD.

Introduction

The amyloid-β peptide (Aβ) is a key pathological effector of Alzheimer's disease (AD) [1]. Aβ is a short polypeptide generated by processing of a larger type I transmembrane spanning glycoprotein, the amyloid precursor protein (APP), through the successive action of proteolytic enzymes called β-secretase and γ-secretase [2,3]. APP can undergo alternative proteolytic processing [4]; indeed in the main pathway APP is cleavage by α-secretase within the Aβ domain, precluding Aβ formation [5] . Several members of membrane-bound disintegrin metalloproteinase (ADAM) family have been proposed as α-secretases, mainly ADAM10, ADAM17 (TACE), and ADAM9 [6], but other ADAM family members, such as ADAM8, may also cleave APP [7]. However, convincing evidence, particularly data from *in vivo* studies [8,9], indicates that ADAM10 is the enzyme acting as the main physiologically relevant α-secretase [10].

The major neuronal β-secretase, the beta-site APP cleaving enzyme 1 (BACE1; [11] is present in CSF [12] in a soluble and truncated form, and increased β-secretase activity and BACE1 protein levels have been investigated as biomarkers for AD [13–16]. The presence in CSF of γ-secretase components, and particularly components of the catalytic subunit presenilin-1, have also been assessed recently as AD biomarkers [17,18]. However, to our knowledge, only ADAM17/TACE activity has been assessed in both CSF [19] and plasma [20,21]; while the potential of ADAM10 as an alternative AD biomarker has so far only been investigated in platelets [22,23], and other blood cells [24]. ADAM proteases, similar to BACE1, are type I transmembrane proteins, but also include secreted isoforms [6]. Indeed, ADAM10 and ADAM17 have been shown to be secreted outside cells in exosomes [25]. Recently, an in-depth analysis of the human CSF endopeptidome enabled identification of several ADAM-10 peptides [26].

In this study, we investigated the occurrence of ADAM10 in human CSF and whether altered levels of this protein occur in AD. We have characterized the full-length

and truncated forms of ADAM10 in CSF, as well as immature forms of the protein that need to be taken into consideration for the design of an appropriate strategy for development of further assay approaches. We report that the full-length and truncated forms of ADAM10, but no the immature forms, decrease in AD CSF compared to control CSF.

Material and methods

Patients

CSF samples were obtained from the Clinical Neurochemistry Laboratory (Mölndal, Sweden) from patients who sought medical advice because of cognitive impairment. In total, 27 patients with AD (7 men and 20 women, mean age 71 ± 1 years) and 26 age-matched non-AD controls (NADC; 18 men and 8 women, mean age 70 ± 2 years) were included. Patients were designated as normal or AD according to CSF biomarker levels using cutoffs that are >90% specific for AD: total tau (T-tau) >400 ng/L, P-tau >60 ng/L and Aβ42 <550 ng/L [27]. For more details, see Table 1. All AD patients fulfilled the 2011 NIA-AA criteria for dementia [28] The CSF samples used for the present study were de-identified leftover aliquots from clinical routine analyses, following a procedure approved by the Ethics Committee at University of Gothenburg. This study was also approved by the Ethics Committee at the Miguel Hernandez University.

Cell cultures

For obtaining conditioned cell-culture medium CHO cells (450,000 cells/well) were grown in six-well plates for 48 h in Dulbecco's modified Eagle's medium (DMEM) plus GlutaMAX™ (Gibco® Life Technologies, Paisley, UK) supplemented with 5% fetal bovine serum (FBS; Gibco) and 100 µg/mL penicillin/streptomycin (Gibco). After 48h, the cell medium was recollected, centrifuged for 15 min at 1500×*g* at 4ºC, and frozen for future analysis.

Western blotting

Samples of CSF (30 µL) and cell medium (20 µL) were denatured at 98ºC for 5 min and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Following electrophoresis, proteins were blotted onto

nitrocellulose membranes (Bio-Rad Laboratories GmbH, Munich, Germany). Bands of ADAM10 immunoreactivity were detected using an antibody specific for the mid-domain of ADAM10 (rabbit polyclonal; OAGA02442, Aviva Systems Biology, San Diego, USA) and an anti-C-terminal ADAM10 antibody (rabbit monoclonal; ab124695, Abcam, Cambridge, UK). Blots were then probed with the appropriate conjugated secondary antibodies, and imaged on an Odyssey Clx Infrared Imaging System (LI-COR Bioscences, Lincoln, NE, USA). Band intensities were analysed using LI-COR software (Image Studio Lite). A control CSF sample, resolved in all the blots, was used to normalize the immunoreactive signal between blots.

Sucrose density gradient ultracentrifugation

ADAM10 complexes were fractioned by ultracentrifugation at 250,000×*g* on a continuous sucrose density gradient (5–20%) for 4 h at 4°C in a Beckman TLS 55 rotor. CSF aliquots (65 µL) were carefully loaded onto the top of the gradient containing 2 mL of 0.15 M NaCl, 50 mM $MgCl_2$ and 0.5 % Brij 97 in 50 mM Tris-HCl (pH 7.4). After centrifugation, ~14 fractions were collected gently from the top of the tubes. Enzyme markers of known sedimentation coefficient, β-galactosidase, catalase and alkaline phosphatase were used in the gradients to determine the approximate sedimentation coefficients.

Measurement of T-tau, P-tau and Aβ42 by ELISA

Total tau (T-tau), phosphorylated tau (P-tau) and Aβ1-42 (Aβ42) concentrations in CSF were measured using INNOTEST ELISA methods (Fujirebio Europe, Gent, Belgium).

Statistical analysis

All the data were analyzed using SigmaStat (Version 3.5; Systac Software Inc.) using a Student's *t* test (two-tailed) or a Mann-Whitney U test for single pairwise comparisons, and determining the exact *p* values. The results are presented as means ±SEM and the correlation between variables was assessed by linear regression analyses.

Results

ADAM10 is present in human CSF as several species

ADAM10 is expressed as a 748 amino-acid-residue type I glycoprotein composed of an N-terminal signal sequence followed by a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, a transmembrane helix, and a cytoplasmic region (Fig. 1A). In a previous in-depth LC-MS analysis of human CSF peptides, we were able to identify 38 small peptides matching parts of the ADAM10 sequence, including the prodomain and the cysteine-rich region close to the transmembrane domain [26] (see also Fig. 1A). Analysis by SDS-PAGE and western blotting using an anti-ADAM10 mid-domain antibody revealed three immunoreactive species with apparent molecular masses of ~80 kDa, 55 kDa and 50 kDa (Fig. 1B). Immunoblotting with an anti-C-terminal ADAM10 antibody detected only the 80 and 55 kDa bands (Fig. 1B), suggesting that the 50-kDa form of CSF ADAM10 is C-terminally truncated. Immunoblotting of CHO cell-conditioned medium revealed a similar banding pattern for ADAM10 species (Fig. 1B). An additional ~70 kDa band was observed in CSF and cell-conditioned medium, but this band was not present in ADAM10 immunoprecipitates of CSF (blots not shown), and the immunoreactivity of the band from the cell culture media declined as the amount of FBS in the culture medium was lowered; thus, this band may represent nonspecific staining associated to albumin. Accordingly, based on the pattern of immunoreactivity with the different antibodies, and the apparent molecular mass of ADAM10 species reported previously [10,22,29,30], we attributed the 80-kDa band to the immature form of ADAM10 (proADAM10), and the 55-kDa form to the mature form (full-length: ADAM10f) derived from the proADAM10 form by removal of the prodomain (194 aa, [31,32]), and the 50-kDa form to a truncated ADAM10 (soluble: sADAM10), released from the membrane by metalloproteases (ADAM9/15; [29]).

ADAM10 species in CSF form complexes

Since ADAM10 exists at the plasma membrane as dimers [33], we characterized the occurrence of CSF-ADAM10 oligomers by gradient ultracentrifugation, which has previously served to illustrate the existence of different protein complexes in CSF [34,35]. Western blotting under denaturing conditions using the anti–mid-domain antibody, common to all species, showed that the proADAM10 species accumulated before to the alkaline phosphatase marker (molecular mass ~140-160 kDa), while the sADAM10 species were identified in denser fractions, between the alkaline phosphatase and (molecular mass ~232 kDa). Interestingly, the ADAM10f species were resolved in the denser fractions, close to β-galactosidase (molecular mass ~540 kDa) (Fig. 2). The particular sedimentation pattern for each ADAM10 species indicated that, at least, sADAM10 and ADAM10f can form large complexes in CSF.

Mature forms of ADAM10 are decreased in AD CSF

After assigning the different ADAM10 immunoreactive species present in CSF as full-length (~55 kDa: ADAM10f) or truncated (~50 kDa: sADAM10) mature species, as well immature forms (~80 kDa: proADAM10), we assessed whether the concentrations of these species are altered in AD. We analyzed CSF samples from 27 AD patients and 26 NADC. The core AD biomarkers were measured using ELISA, confirming elevated CSF T-tau and P-tau and low levels of Aβ42 in the AD samples (Table 1). Regarding ADAM10 immunoreactivities, we found that the 55 kDa species decreased in abundance (~40%; p= 0.005) in AD compared to NADC subjects (Fig. 3). A similar decrease was found for the truncated 50-kDa fragment (~36%; p= 0.004), whereas, the concentration of the 80-kDa immature ADAM10 form was unchanged (p= 0.44).

We further tested whether CSF-ADAM10 complexes are altered in AD cases. CSF-ADAM10 complexes were fractioned, from three AD and three NADC representative cases, using sucrose-density gradient fractionation, and then resolved by western blotting under denaturing conditions (Supplementary Fig. 1). Peaks of the CSF-ADAM10 complexes were identified in similar fractions for all the AD and ND cases tested, indicating that all complexes are present in AD CSF and the nature of the complexes is not affected by the pathological conditions.

Interestingly, levels of sADAM10 and ADAM10f were correlated, albeit weakly, in CSF from NADC subjects (R= 0.39; *p*= 0.048), but not in AD patients (R= 0.25; *p*= 0.20). In NADC subjects, no correlation was observed between proADAM10 and ADAM10f (R= 0.30; *p*= 0.13) or proADAM10and sADAM10 (R= 0.15 *p*= 0.46). In the AD subjects these correlations were not evident for both proADAM10 and ADAM10f (R= 0.05; *p*= 0.81) or for proADAM10 and sADAM10 (R= 0.12; *p*= 0.56). No correlations were observed with age or gender. Levels of the ADAM10 species did not correlate with the core AD biomarkers in NADC, while in AD samples, Aβ42 levels correlated with ADAM10f (R= 0.43; *p*= 0.027), but not with the levels of other ADAM10 species.

Discussion

There is a need to identify additional CSF biomarkers of AD. The knowledge that APP metabolism and Aβ production and aggregation are key steps in AD pathogenesis makes proteins involved in the pathological processing of APP, including secretases such as ADAM10, reasonable candidates for analysis in CSF. However, since secretases are transmembrane proteins, their assessment in CSF was not considered until recent years.

Previous studies have revealed that, in addition to proteins, CSF contains many endogenous peptides [36,37], including ADAM10 peptides [26]. In this study, we demonstrate the presence in human CSF of the mature and immature full-length ADAM10 protein, as well as a membrane cleaved large fragment (sADAM10). As sADAM10 can be released by proteolytic processing from the membrane [29], this suggests the potential for truncated isoforms to be present in CSF. Indeed, recent reports indicate the possibility that ADAM10 levels can even be measured in human serum by an enzyme-linked immunosorbent assay (ELISA; [38,39].

In our previous study [26] using LC-MS analysis, we identified several short peptide fragments of ADAM10 in human CSF, matching sequences located at the N-terminus of the protein as well peptide fragments located close to the transmembrane domain of the protein. In this study, several different molecular mass bands of ADAM10 were detected by western blot analysis using mid-domain and C-terminal anti-ADAM10 antibodies. Thus, in addition to a sADAM10 isoform attributed to the immunoreactive band of ~50-kDa molecular mass, other ADAM10 species retaining the intracellular C-terminal domain are present in the CSF. Moreover, as some of the sequences identified by LC-MS analysis were homologous to the N-terminal prodomain, this indicated that, unexpectedly, immature proADAM10 also reached the CSF. Thus, other full-length isoforms of the protein co-exist in the CSF with

sADAM10. The presence of proADAM10, together with ADAM10f, has been described at the cell surface [10].

The mechanisms by which these membrane-resident ADAM10 species reach the CSF are unknown, but neuronal death may be a contributing factor. Moreover, ADAM10 is abundant in exosomes of bovine endometrial stromal cells cultured at hypoxic conditions [40]. Thus, an exosomal contribution of ADAM10-CSF cannot be discounted. Interestingly, ADAM10 is also enriched in synaptic vesicles [41], being one of many synaptic proteins identified and measured in CSF [42]. In this context, we and others have reported evidence of the presence in the CSF of "unprocessed" forms of several transmembrane proteins, such as BACE1 [34], APP [35,43] as well the multi-pass presenilin-1 (PS1) [18,34]. Thus, the existence of a membrane-resident protein in CSF is not an unusual finding [44]. Recently, we also characterized in CSF the existence of C-terminal fragments of APP, which include the transmembrane domain [45].

The occurrence in CSF of proteins which still maintain their transmembrane and intracellular domains is also relevant for the development of strategies for their quantitative estimation. ADAM10, similar to many other transmembrane proteins exists as a dimer in the brain [33]. Both the transmembrane [46] and cytoplasmic [33] domains can participate in dimerization of ADAM10, a feature that may be is an inherent property of ADAM metalloproteinases. In the present study, we demonstrated by gradient centrifugation that sADAM10 and ADAM10f are present in the CSF as large complexes. Further studies will be necessary to clarify the biochemical properties of these homomeric complexes, but our preliminary analysis indicates that the species in NADC CSF are similar, if not identical to the species in the AD CSF. We have previously demonstrated the occurrence of APP heteromers in CSF, comprising both sAPPα/sAPPβ and also soluble full-length APP, and we have shown that these

heteromers affect the determination of sAPP by ELISA [35]. Given that the distinct ADAM10 species also form complexes, the development of an accurate ELISA protocol for the estimation of CSF-ADAM10 levels may require more knowledge about the potential variable stoichiometry and stability of these complexes. In fact, our early attempts to assess ADAM10-CSF levels by ELISA have resulted in poorly reproducible data (~60% intra-assay variability in CSF samples; ELISA kit from MyBioSource, Inc. San Diego, CA, USA). A previous study also reported difficulties in assessing ADAM10 in CSF, discarding their presence in the fluid [47]. In this study, to circumvent this issue, we analyzed ADAM10-CSF levels by SDS-PAGE.

Our determination of the different species of ADAM10 in CSF by western blotting indicated that in AD cases there is a decrease in sADAM10 and ADAM10f, but not in the immature forms. Since amyloidogenic processing of APP is expected to be altered in the Alzheimer brain, parallel changes in the levels of α-secretase and β-secretase might be expected. However, it is still unclear if α-secretase and β-secretase are inversely correlated during pathological progression, as the proteolytic products sAPPα and sAPPβ displayed similar trends in the CSF [43]. Data on ADAM10 in human bran are scarce, but the majority of the data indicate an overall decrease in ADAM10 mRNA, protein, and/or activity in the brain of AD patients compared to age-matched controls [48]. However, at least in platelets, the decrease of ADAM10 protein in AD patients is not caused by a reduction in ADAM10 mRNA [49]. Thus, the regulation of expression and activity of ADAM10 may be complex, being regulated by several pathways, epigenetically, and at translational and post-translational levels [48], and affected by normal aging [30]. In this context, it may be important to evaluate α-secretase activity in CSF. Enzymatic activity assays in CSF are usually based on the use of specific substrates (synthetic peptides) favourable for the assessment of a concrete activity, but as mentioned previously, other enzymes, in addition to ADAM10, display

α-secretase-like activity. Indeed, elevated activity levels for ADAM17/TACE have been found in both CSF [19] and plasma [20,21] from subjects with AD. Thus, it is questionable whether an enzymatic activity assay for ADAM10 in CSF based in the use of synthetic peptides (which can be cleaved by multiple proteases) should be used to measure changes in the CSF of AD patients. The general requirements for secretase cleavage are not strict and we cannot exclude the possibility that other CSF enzymes that may cleave the synthetic peptides also being detected. Therefore, only ELISA assays based on pan-specific antibodies for concrete ADAM10 species, and including pre-treatment methods designed to disaggregate complexes, may be a reliable approach to assess protein levels and enzymatic activity. Moreover, emerging evidence indicates that the plasma membrane with its unique dynamic properties may additionally play an important role in controlling sheddase function, as physicochemical properties of the lipid bilayer govern the action of ADAM-proteases [50]. Accordingly, determination of enzymatic activities does not appear to be the most adequate and sensitive molecular tool to evaluate ADAM10, and other secretases, as a potential CSF biomarkers.

Conclusions

Despite the limited precision of western blotting for quantitative analysis, we consider that mature forms of ADAM10 in CSF constitute potential new biomarkers of AD. Our present findings provide sufficient evidence to justify further studies focusing on the possibility of monitoring specific soluble forms of ADAM10, and to evaluate the progress and feasibility of developing molecular tools for this potential new CSF biomarker for AD.

List of Abbreviations

Aβ: amyloid-β peptide; AD: Alzheimer's disease; ADAM10: disintegrin metalloproteinase 10; ADAM10f: full-length form of ADAM10; APP: amyloid precursor protein; BACE1: beta-site APP cleaving enzyme 1; CSF : cerebrospinal fluid; NADC: non-AD controls; DMEM: Dulbecco's modified Eagle's medium; proADAM10: prodomain of ADAM10; PS1: presenilin-1; P-tau: phosphorylated tau; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sADAM10: soluble form of ADAM10 released from the membrane; T-tau: total tau.

Declarations:

Ethics approval and consent to participate

The CSF samples used for the present study were de-identified leftover aliquots from clinical routine analyses, following a procedure approved by the Ethics Committee at University of Gothenburg. This study was also approved by the Ethics Committee at the Miguel Hernandez University.

Consent for publication

"Not applicable"

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing interests

All the authors contributed to this work and are in agreement with the findings presented. The authors have no competing interests to disclose in connection with this article.

Funding

This study was funded in part by the EU BIOMARKAPD-Joint Programming on Neurodegenerative Diseases (JPND) project, by the Instituto de Salud Carlos III (ISCIII grants PI11/03026 and and PI15/00665), co-financed by the Fondo Europeo de Desarrollo Regional (FEDER, "Investing in your future"), under the aegis of JPND, and through CIBERNED, ISCIII. We also acknowledge financial support from the Spanish Ministerio de Economía y Competitividad, through the "Severo Ochoa" Programme for Centres of Excellence in R&D (SEV-2013-0317). We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). This work was also supported by a

grant from Torsten Söderberg Foundation, Sweden (to KB) and the Knut and Alice Wallenberg Foundation (to HZ).

Authors' contributions

ASE, MSGA, JG, JA, HZ, KB, and JSV were involved with the conception, design, and interpretation of data. ASE, MSG and JG performed the experiments. JSV, MSGA, JA, HZ and KB were involved with data analysis. KB and HZ were involved in the collection of the clinical material. JSV, MSGA, HZ and KB provided general overall supervision of the study, and acquired funding. All authors contributed to the drafting and critical revision of the manuscript and have given final approval of the version to be published.

Acknowledgements

"Not applicable"

References

1. Hardy J, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science. 1992;256:184–5.

2. Thinakaran G, Koo EH. Amyloid Precursor Protein Trafficking, Processing, and Function. J Biol Chem. 2008;283:29615–9.

3. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. Cold Spring Harb Perspect Med. 2012;2:a006270.

4. Andrew RJ, Kellett KAB, Thinakaran G, Hooper NM. A Greek Tragedy: The Growing Complexity of Alzheimer Amyloid Precursor Protein Proteolysis. J Biol Chem. 2016;291:19235–44.

5. Portelius E, Price E, Brinkmalm G, Stiteler M, Olsson M, Persson R, et al. A novel pathway for amyloid precursor protein processing. Neurobiol Aging. 2011;32:1090–8.

6. Vingtdeux V, Marambaud P. Identification and biology of α-secretase. J Neurochem. 2012;120 Suppl 1:34–45.

7. Naus S, Reipschläger S, Wildeboer D, Lichtenthaler SF, Mitterreiter S, Guan Z, et al. Identification of candidate substrates for ectodomain shedding by the metalloprotease-disintegrin ADAM8. Biol Chem . 2006;387:337–46.

8. Jorissen E, Prox J, Bernreuther C, Weber S, Schwanbeck R, Serneels L, et al. The disintegrin/metalloproteinase ADAM10 is essential for the establishment of the brain cortex. J Neurosci. 2010;30:4833–44.

9. Kuhn P-H, Wang H, Dislich B, Colombo A, Zeitschel U, Ellwart JW, et al. ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. EMBO J. 2010;29:3020–32.

10. Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, et al. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci U S A. 1999;96:3922–7.

11. Vassar R, Kovacs DM, Yan R, Wong PC. The beta-secretase enzyme BACE in health and Alzheimer's disease: regulation, cell biology, function, and therapeutic potential. J Neurosci. 2009;29:12787–94.

12. Holsinger RMD, McLean CA, Collins SJ, Masters CL, Evin G. Increased beta-Secretase activity in cerebrospinal fluid of Alzheimer's disease subjects. Ann Neurol. 2004;55:898–9.

13. Zetterberg H, Andreasson U, Hansson O, Wu G, Sankaranarayanan S, Andersson ME, et al. Elevated cerebrospinal fluid BACE1 activity in incipient Alzheimer disease. Arch Neurol. 2008;65:1102–7.

14. Barao S, Zhou L, Adamczuk K, Vanhoutvin T, van Leuven F, Demedts D, et al. BACE1 levels correlate with phospho-tau levels in human cerebrospinal fluid. Curr Alzheimer Res. 2013;10:671–8.

15. Perneczky R, Alexopoulos P, Alzheimer's Disease euroimaging Initiative. Cerebrospinal fluid BACE1 activity and markers of amyloid precursor protein metabolism and axonal degeneration in Alzheimer's disease. Alzheimers Dement. 2014;10:S425–S429.e1.

16. Shen Y, Wang H, Sun Q, Yao H, Keegan AP, Mullan M, et al. Increased Plasma Beta-Secretase 1 May Predict Conversion to Alzheimer's Disease Dementia in Individuals With Mild Cognitive Impairment. Biol Psychiatry. 2018;83:447–55.

17. Garcia-Ayllon M-S, Campanari M-L, Brinkmalm G, Rabano A, Alom J, Saura CA, et al. CSF Presenilin-1 complexes are increased in Alzheimer's disease. Acta Neuropathol Commun. England; 2013;1:46.

18. Sogorb-Esteve A, García-Ayllón M-S, Fortea J, Sánchez-Valle R, Lleó A, Molinuevo J-L, et al. Cerebrospinal fluid Presenilin-1 increases at asymptomatic stage in genetically determined Alzheimer's disease. Mol Neurodegener. 2016;11.

19. Jiang H, Hampel H, Prvulovic D, Wallin A, Blennow K, Li R, et al. Elevated CSF

levels of TACE activity and soluble TNF receptors in subjects with mild cognitive impairment and patients with Alzheimer's disease. Mol Neurodegener. 2011;6:69.

20. Bai L, Song N, Yu J, Tan L, Shen Y, Xie J, et al. Elevated plasma levels of soluble TNFRs and TACE activity in Alzheimer's disease patients of Northern Han Chinese descent. Curr Alzheimer Res. 2013;10:57–62.

21. Sun Q, Hampel H, Blennow K, Lista S, Levey A, Tang B, et al. Increased plasma TACE activity in subjects with mild cognitive impairment and patients with Alzheimer's disease. J Alzheimers Dis. 2014;41:877–86.

22. Colciaghi F, Borroni B, Pastorino L, Marcello E, Zimmermann M, Cattabeni F, et al. α-Secretase ADAM10 as well as αAPPs is reduced in platelets and CSF of Alzheimer disease patients. Mol Med. 2002;8:67–74.

23. Manzine PR, de França Bram JM, Barham EJ, do Vale F de AC, Selistre-de-Araújo HS, Cominetti MR, et al. ADAM10 as a biomarker for Alzheimer's disease: a study with Brazilian elderly. Dement Geriatr Cogn Disord. 2013;35:58–66.

24. Edwards D, Handsley M, Pennington C. The ADAM metalloproteinases. Mol Aspects Med . 2008;29:258–89.

25. Stoeck A, Keller S, Riedle S, Sanderson MP, Runz S, Le Naour F, et al. A role for exosomes in the constitutive and stimulus-induced ectodomain cleavage of L1 and CD44. Biochem J. 2006;393:609–18.

26. Hansson KT, Skillbäck T, Pernevik E, Kern S, Portelius E, Höglund K, et al. Expanding the cerebrospinal fluid endopeptidome. Proteomics. 2017;17:1600384.

27. Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol. 2006;5:228–34.

28. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Kawas CH, et al. The diagnosis of dementia due to Alzheimer's disease: Recommendations from the

National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimer's Dement. 2011;7:263–9.

29. Tousseyn T, Thathiah A, Jorissen E, Raemaekers T, Konietzko U, Reiss K, et al. ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. J Biol Chem. 2009;284:11738–47.

30. Schuck F, Wolf D, Fellgiebel A, Endres K. Increase of α-Secretase ADAM10 in Platelets Along Cognitively Healthy Aging. Cominetti M, editor. J Alzheimers Dis. 2016;50:817–26.

31. Lum L, Reid MS, Blobel CP. Intracellular maturation of the mouse metalloprotease disintegrin MDC15. J Biol Chem. 1998;273:26236–47.

32. Anders A, Gilbert S, Garten W, Postina R, Fahrenholz F. Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. FASEB J. 2001;15:1837–9.

33. Xu P, Liu J, Sakaki-Yumoto M, Derynck R. TACE activation by MAPK-mediated regulation of cell surface dimerization and TIMP3 association. Sci Signal. 2012;5:ra34.

34. García-Ayllón M-S, Campanari M-L, Brinkmalm G, Rábano A, Alom J, Saura C a, et al. CSF Presenilin-1 complexes are increased in Alzheimer's disease. Acta Neuropathol Commun. 2013;1:46.

35. Cuchillo-Ibañez I, Lopez-Font I, Boix-Amorós A, Brinkmalm G, Blennow K, Molinuevo J-L, et al. Heteromers of amyloid precursor protein in cerebrospinal fluid. Mol Neurodegener. 2015;10:2.

36. Hölttä M, Zetterberg H, Mirgorodskaya E, Mattsson N, Blennow K, Gobom J. Peptidome analysis of cerebrospinal fluid by LC-MALDI MS. Koomen JM, editor. PLoS One. 2012;7:e42555.

37. Wijte D, McDonnell LA, Balog CIA, Bossers K, Deelder AM, Swaab DF, et al. A

novel peptidomics approach to detect markers of Alzheimer's disease in cerebrospinal fluid. Methods. 2012;56:500–7.

38. Isozaki T, Nishimi S, Nishimi A, Saito M, Miwa Y, Toyoshima Y, et al. A disintegrin and metalloproteinase (ADAM)-10 as a predictive factor for tocilizumab effectiveness in rheumatoid arthritis. Mod Rheumatol. 2017;27:782–6.

39. Walkiewicz K, Nowakowska-Zajdel E, Strzelczyk J, Dzięgielewska-Gęsiak S, Muc-Wierzgoń M. Serum levels of ADAM10, ADAM12, ADAM17 AND ADAM28 in colorectal cancer patients. J Biol Regul Homeost Agents. 2017;31:929–34

40. Koh YQ, Peiris HN, Vaswani K, Reed S, Rice GE, Salomon C, et al. Characterization of exosomal release in bovine endometrial intercaruncular stromal cells. Reprod Biol Endocrinol. 2016;14:78.

41. Lundgren JL, Ahmed S, Schedin-Weiss S, Gouras GK, Winblad B, Tjernberg LO, et al. ADAM10 and BACE1 are localized to synaptic vesicles. J Neurochem. 2015;135:606–15.

42. Davidsson P, Puchades M, Blennow K. Identification of synaptic vesicle, pre- and postsynaptic proteins in human cerebrospinal fluid using liquid-phase isoelectric focusing. Electrophoresis. 1999;20:431–7.

43. Lopez-Font I, Boix CP, Zetterberg H, Blennow K, Sáez-Valero J. Alterations in the Balance of Amyloid-β Protein Precursor Species in the Cerebrospinal Fluid of Alzheimer's Disease Patients. Calero M, editor. J Alzheimers Dis. 2017;57:1281–91.

44. Lopez-Font I, Cuchillo-Ibañez I, Sogorb-Esteve A, García-Ayllón M-S, Sáez-Valero J. Transmembrane amyloid-related proteins in CSF as potential biomarkers for Alzheimer's disease. Front Neurol. 2015;6.

45. García-Ayllón M-S, Lopez-Font I, Boix CP, Fortea J, Sánchez-Valle R, Lleó A, et al. C-terminal fragments of the amyloid precursor protein in cerebrospinal fluid as potential biomarkers for Alzheimer disease. Sci Rep. 2017;7:2477.

46. Deng W, Cho S, Su P-C, Berger BW, Li R. Membrane-enabled dimerization of the intrinsically disordered cytoplasmic domain of ADAM10. Proc Natl Acad Sci U S A. 2014;111:15987–92.

47. Kieseier BC, Pischel H, Neuen-Jacob E, Tourtellotte WW, Hartung H-P. ADAM-10 and ADAM-17 in the inflamed human CNS. Glia. 2003;42:398–405.

48. Endres K, Deller T. Regulation of Alpha-Secretase ADAM10In vitroandIn vivo: Genetic, Epigenetic, and Protein-Based Mechanisms. Front Mol Neurosci. 2017;10:56.

49. Manzine PR, Marcello E, Borroni B, Kamphuis W, Hol E, Padovani A, et al. ADAM10 gene expression in the blood cells of Alzheimer's disease patients and mild cognitive impairment subjects. Biomarkers. 2015;20:196–201.

50. Reiss K, Bhakdi S. The plasma membrane: Penultimate regulator of ADAM sheddase function. Biochim Biophys Acta. 2017;1864:2082–7.

Figure Legends

***Figure 1*. Different ADAM10 species are present in human CSF.** (**A**) Schematic representation of ADAM10 and its domain organization, which consists of a pro-domain (Pro), a zinc-binding metalloprotease (Protease) domain, a disintegrin domain (Dis), which binds to integrin cell adhesion molecules, a cysteine-rich domain (Cys), a variable stalk region, a transmembrane (TM) domain, and a cytosolic domain (not drawn in scale; adapted from [29]). The potential species resulting from proteolytic removal of the prodomain that are further released from the membrane are indicated (immature form: proADAM10; mature full-length form: ADAM10f; truncated soluble form: sADAM10). The epitopes for the anti-ADAM10 antibodies used in this study are also indicated. (**B**) Western blot of human CSF samples from non-AD controls (NADC) subjects, and cell medium (cell med) by CHO cells, resolved with the indicated anti-ADAM10 antibodies. Arrow-head indicates a non-specific band.

***Figure 2*. Characterization of CSF-ADAM10 complexes by sucrose gradient ultracentrifugation.** CSF samples (NADC) were fractionated on 5-20% sucrose density gradients. The fractions (collected from the top of each tube) were immunoblotted using mid-domain and C-terminal antibodies specific for ADAM10. Enzymes of known sedimentation coefficient, alkaline phosphatase (P, 6.1S; ~140-160 kDa), catalase (C, 11.4S; ~232 kDa) and β-galactosidase (G, 16.0S; ~540 kDa) were used as internal markers.

***Figure 3*. Decreased levels of mature ADAM10 species in AD CSF samples.** (**A**) Representative blot of ADAM10 species immunoreactive to a mid-domain antibody in the CSF samples from 27 AD patients and 26 age-matched non-AD controls (NADC). (**B**) Densitometric quantification of ADAM10 immunoreactivity from the 55 kDa

species attributed to the mature form (ADAM10f), the truncated 50 kDa (sADAM10) and the 80 kDa immature form (proADAM10). Arrow-head indicates a non-specific band. Data are presented as means ± SEM: *$p \leq$ 0,005.

***Supplemental Figure 1*. Unaltered ADAM10 complexes in AD CSF.** (**A**) Representative blot of PS1 complexes in CSF from AD subjects and age-matched non-AD controls (NADC). Three representative AD and NADC cases were analyzed, in which the distribution of ADAM10 complexes displayed similar sedimentation patterns. Blots were resolved with an anti–mid-domain antibody (domain common to all the CSF-ADAM10 species).

Table 1: Demographic data and classic CSF biomarker levels.

Group	Age (years)	n (gender)	CSF Aβ42 (pg/mL)	CSF T-tau (pg/mL)	CSF P-tau (pg/mL)
NADC	70 ± 2 [55-88]	n=26 (8F/18M)	773 ± 29 [1010-561]	238 ± 13 [138-365]	36 ± 2 [21-51]
AD	71 ± 1 [55-86]	n=27 (20F/7M)	414 ± 15* [544-251]	689 ± 48* [1420-443]	88 ± 5* [164-61]

The data represent the means ±SEM. The ranges of values for each variable are also indicated. F= female; M= male. *Significantly different ($p< 0.001$) from the NADC group.

Figure 1

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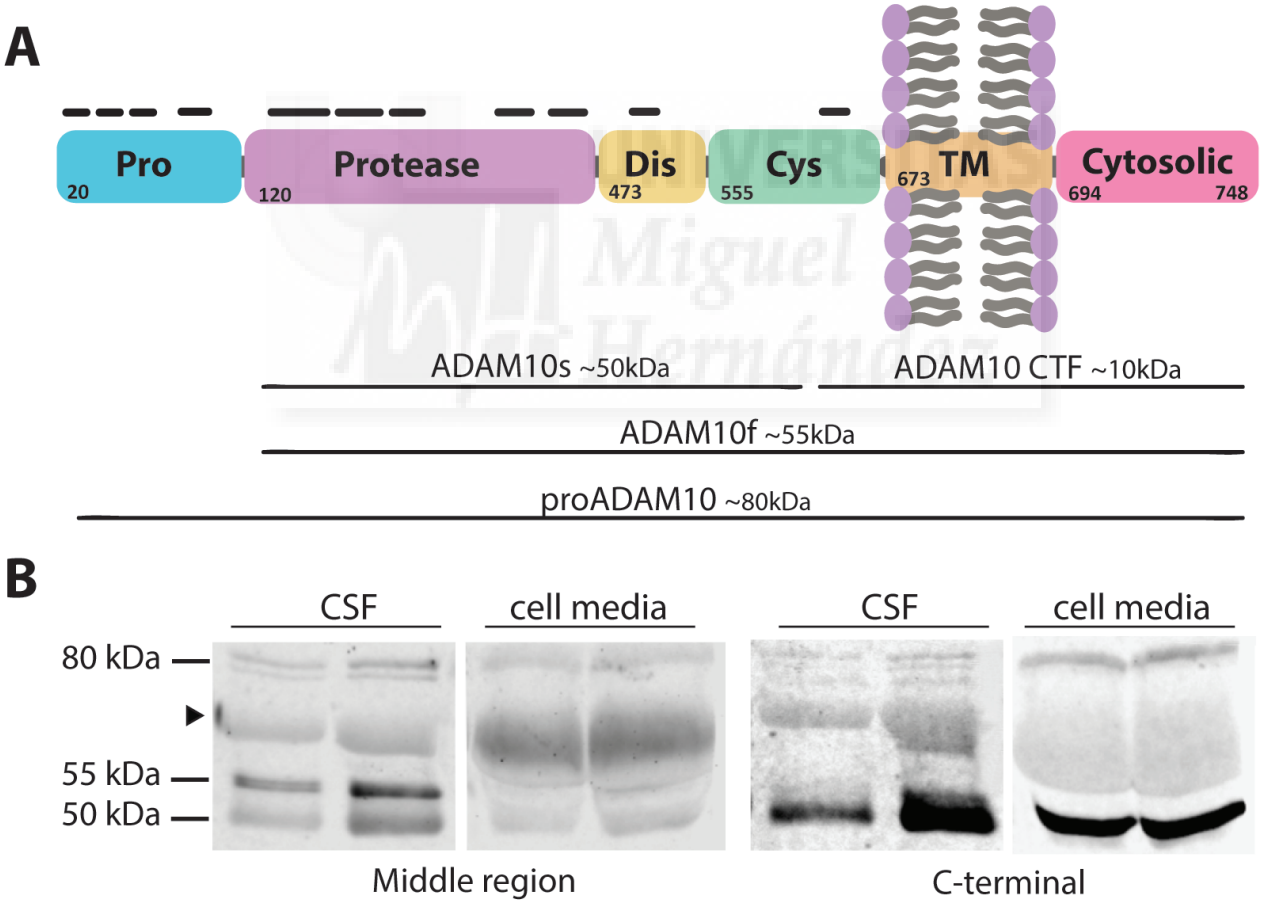

Figure 2

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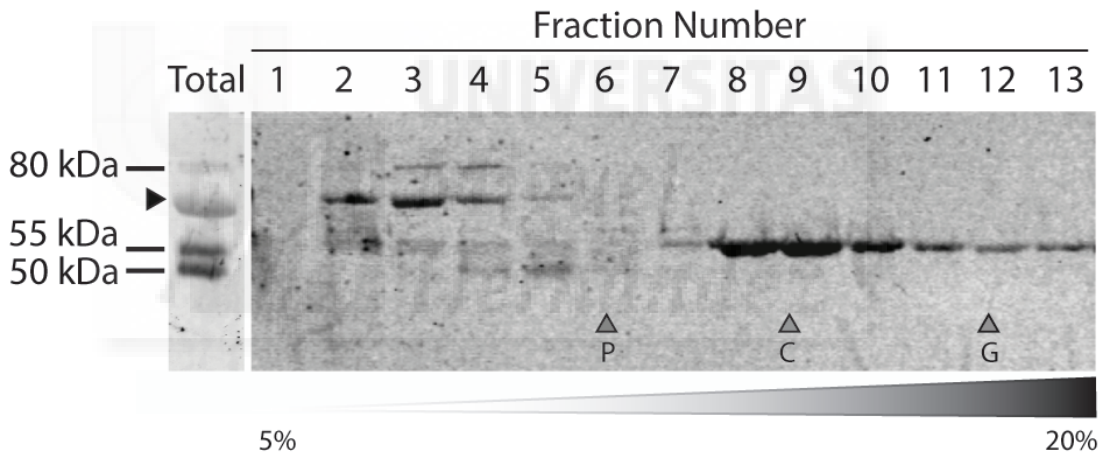

Figure 3

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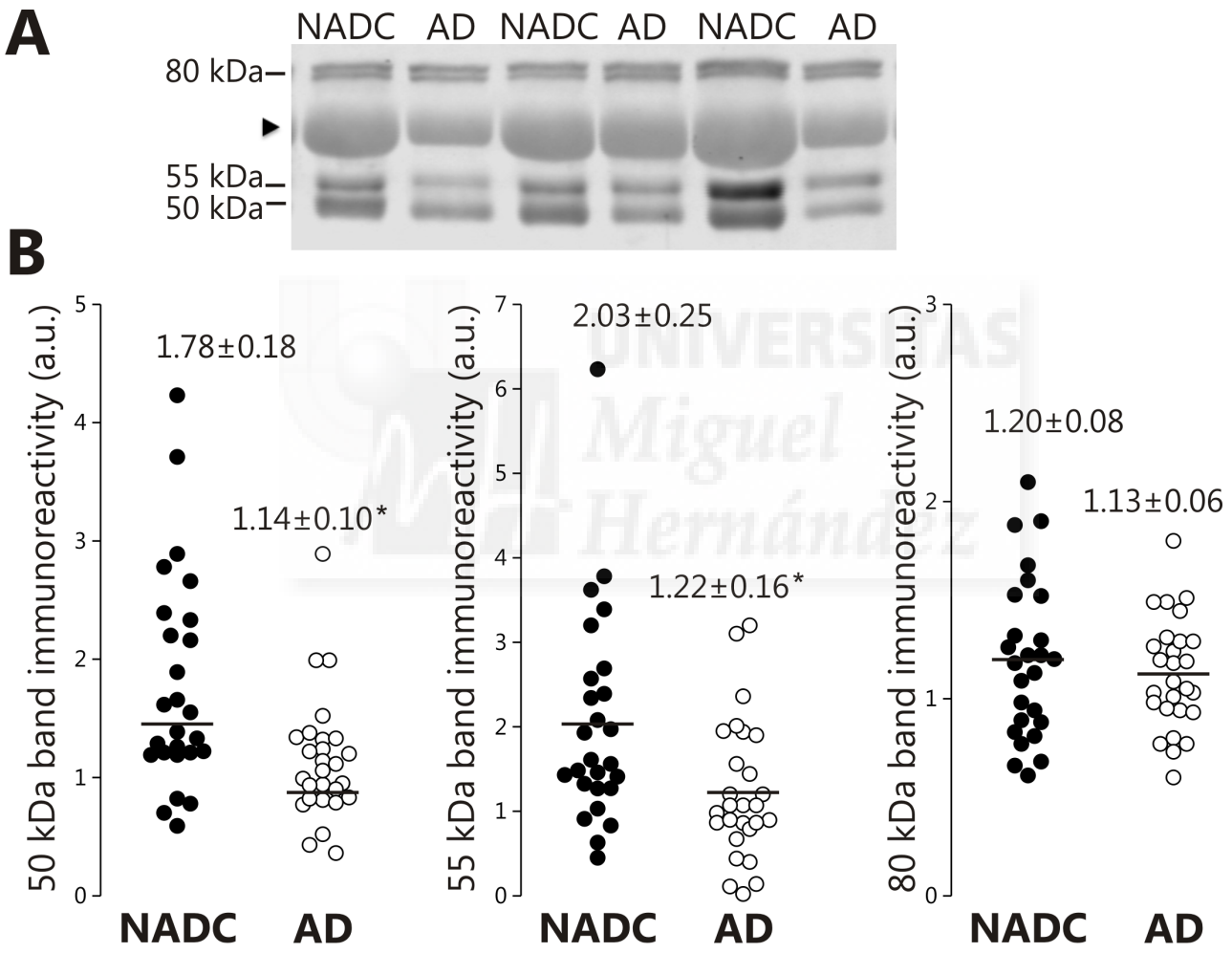
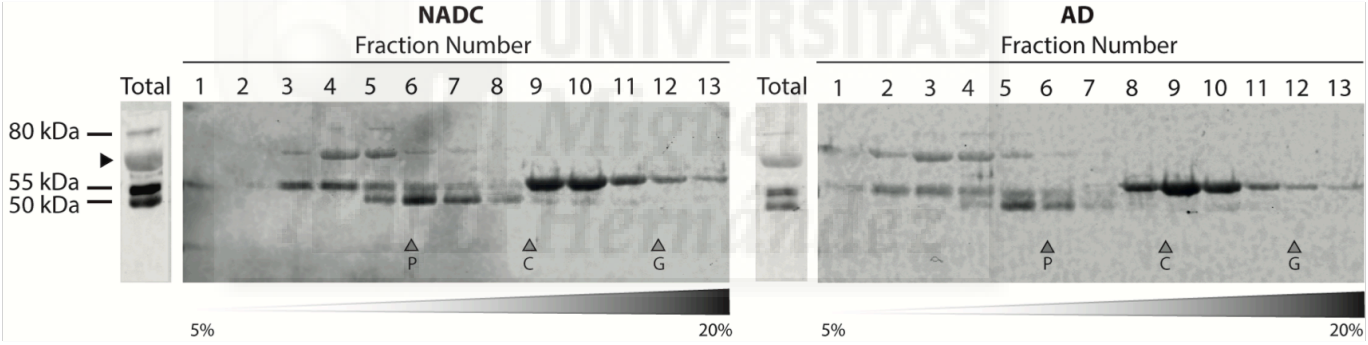


Figure Supplemental 1



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ARTICLE 3: Inhibition of γ-Secretase Leads to an Increase in Presenilin-1

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Published in **Molecular Neurobiology.** 2017.
doi: 10.1007/s12035-017-0705-1

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Mol Neurobiol
DOI 10.1007/s12035-017-0705-1

Inhibition of γ-Secretase Leads to an Increase in Presenilin-1

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Received: 30 May 2017 / Accepted: 1 August 2017

Abstract γ-Secretase inhibitors (GSIs) are potential therapeutic agents for Alzheimer's disease (AD); however, trials have proven disappointing. We addressed the possibility that γ-secretase inhibition can provoke a rebound effect, elevating the levels of the catalytic γ-secretase subunit, presenilin-1 (PS1). Acute treatment of SH-SY5Y cells with the GSI LY-374973 (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester, DAPT) augments PS1, in parallel with increases in other γ-secretase subunits nicastrin, presenilin enhancer 2, and anterior pharynx-defective 1, yet with no increase in messenger RNA expression. Over-expression of the C-terminal fragment (CTF) of APP, C99, also triggered an increase in PS1. Similar increases in PS1 were evident in primary neurons treated repeatedly (4 days) with DAPT or with the GSI BMS-708163 (avagacestat). Likewise, rats examined after 21 days administered with avagacestat (40 mg/kg/day) had more brain PS1. Sustained γ-secretase inhibition did not exert a long-term effect on PS1 activity, evident through the decrease in CTFs of APP and ApoER2. Prolonged avagacestat treatment of rats produced a subtle impairment in anxiety-like behavior. The rebound increase in PS1 in response to GSIs must be taken into consideration for future drug development.

Keywords Alzheimer's disease · Presenilin-1 · γ-Secretase inhibitor · Therapy

Electronic supplementary material The online version of this article (doi:10.1007/s12035-017-0705-1) contains supplementary material, which is available to authorized users.

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Introduction

Alzheimer's disease (AD) is the most common dementia in the elderly, and it is characterized by extracellular deposits of aggregated β-amyloid (Aβ) peptides and accumulation of intracellular tangles of the abnormally hyperphosphorylated microtubule-associated protein tau (P-tau) [1]. According to the amyloid cascade hypothesis, which is the most prevalent view on AD pathogenesis, the disease pathophysiology is triggered by an excess of neurotoxic Aβ peptides, potentially in combination with other genetics and risk factors [2]. Drug candidates targeting Aβ have dominated AD drug development programs for the past three decades [3], and accordingly, targets for each individual step in this cascade have been developed, with β/γ-secretase inhibitors representing one particular opportunity for front-line therapy.

Published online: 16 August 2017

The Aβ peptide is generated by successive proteolytic processing of the amyloid precursor protein (APP) by secretases. APP is a type I transmembrane spanning glycoprotein that is first processed by either α- or β-secretase, followed by γ-secretase cleavage. β- and γ-secretase cleavage generate Aβ peptides of variable amino acid length, being the most abundant the Aβ40 peptide [4] while Aβ42 appears to be the most amyloidogenic [5]. The major neuronal β-secretase is the beta-site APP-cleaving enzyme 1 (BACE1) [6], while the γ-secretase enzyme complex contains four essential subunits: presenilin-1 (or presenilin-2), nicastrin, anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2) [7]. γ-Secretase acts an aspartyl protease, which catalytic core is presenilin-1 (PS1), being its dysfunction associated with the pathological development of AD [8]. Thus, compounds that inhibit γ-secretase, targeting PS1, are potential therapeutic agents for AD.

Preclinical studies clearly established that γ-secretase inhibitors (GSIs) reduce brain Aβ in rodent models and also reverse Aβ-induced cognitive deficits in the AD Tg2576 mice [9]. However, the therapeutic effect of such drugs in humans has fallen below expectation, with no demonstrated efficacy in clinical trials and even impaired cognitive function in long-term treated subjects [10]. Problems of tolerability and dose-limiting effects during clinical trials with GSIs may have compromised target engagement for arriving to the minimum extent of Aβ lowering for significant cognitive benefit in AD patients (discussed in Toyn and Ahlijanian [11]). On the other hand, a paradoxical increase of plasma Aβ levels has been observed upon chronic treatment with a classical GSI in transgenic animal [12]. Treatment of transgenic mice and humans with other GSIs, including compounds involved in clinical trials, may cause late rebound effects on plasma Aβ levels [13–15]. These changes may be illustrative of a rebound effect in reaction to inhibition by a GSI-based therapy. To decipher why current GSIs fail to improve the disease state may help to optimize future drug development.

Upregulation of enzyme isoforms [16, 17], and also of the specific enzyme targeted by the drug [18–20], is not an uncommon phenomenon in reaction to inhibition, although to our knowledge, this possible effect remains unexplored in terms of GSI treatment. Interestingly, we recently reported that an increase in acetylcholinesterase could block γ-secretase activity and that this inhibition initiates a feedback process that leads to a rebound effect, elevating PS1 levels [21]. Here, we tested how GSIs affect PS1 levels in cellular and animal models. As such, we provide evidence that γ-secretase inhibition could provoke a rebound increase in PS1, which may be of particular importance for the design of specific AD therapies based on GSIs and related drugs.

Materials and Methods

Cell Cultures and Pharmacological Treatment with GSIs

SH-SY5Y cells (700,000 cells/well) were grown in six-well plates for 24 h in Dulbecco's modified Eagle's medium (DMEM) + GlutaMAX™ (Gibco® Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Gibco) and 100 μg/mL penicillin/streptomycin (Gibco). The cells were treated with 5 μM of γ-secretase inhibitor LY-374973: *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT; Calbiochem®, Merck KGaA) or the dimethyl sulfoxide (DMSO) vehicle alone. Following an 18-h treatment, the cells were washed twice with cold phosphate-buffered saline (PBS) and resuspended in 100 μL of ice-cold extraction buffer supplemented with a cocktail of protease inhibitors: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% (*w*/*v*) Nonidet P-40, and 0.5% (*w*/*v*) Triton X-100. Cell lysates were sonicated and centrifuged for 1 h at 70,000×*g* and 4 °C, and the extracts were frozen at −80 °C for future analysis.

For some experiments, SH-SY5Y cells were transfected with 4 μg of a construct that encodes the C-terminal 99 amino acids of APP (amino acids 597–695), extending from the β-secretase cleavage site to the C-terminus (a generous gift from David H. Small). A pCI *empty* vector (Promega) served as the negative control. These cells (7×10^5 cells/well) were then seeded on 35-mm tissue culture dishes and transfected using Lipofectamine® 2000 (Thermo Scientific™) according to the manufacturer's instructions. After 2 days in culture, the cells and culture supernatants were harvested separately, and the cell culture supernatants were cleared by centrifugation at 1000×*g* for 10 min at 4 °C. The cells were then washed with PBS and solubilized as described above. C-terminal fragment of APP (APP-CTF) levels were assayed in Western blots to determine transfection efficiency.

To culture primary cortical neurons, cortical lobes from E16.5 mice embryos were trypsinized and dissociated in Hank's balanced salt solution (Life Technologies). Neurons were plated onto 35-mm dishes (1.3×10^6 cells/dish) and maintained in Neurobasal medium (Invitrogen) containing B27 supplement (Gibco BRL), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. After 7 days in culture, the cortical neurons were treated with 2 μM of DAPT or the GSI avagacestat (BMS-708163; from Bristol-Myers Squibb) for four consecutive days and analyzed on day 5, 18 h after the last dose. The cells were washed with PBS and solubilized as described above.

Cell viability was measured using the tetrazolium assay (MTS; CellTiter 96® AQueous Assay, Promega) according to the manufacturer's instructions. Cells were cultured in 96-well plates and treated with GSIs as previously stated. MTS was added after GSI treatment, cells were incubated for 4 h,

and then viability was determined by measuring the absorbance at 490 nm in a microplate reader (Infinite M200, Tecan).

Animals and Tissue Preparation

All animal procedures were approved by the Animal Care and Use Committees at the Universidad Miguel Hernández and by Centro Principe Felipe (2016A/SC/PEA/00127). Wistar male rats that weighed 250–300 g at the beginning of GSI administration were used. The rats were orally administered the avagacestat (40 mg/kg) or vehicle alone (polyethylene glycol) using a single or once-a-day dose for 4 or 21 days ($n = 10$ for each group), and they were sacrificed ~4 h after the final administration of avagacestat. Cerebrospinal fluid (CSF) samples (50–60 μL) were collected by cisternal puncture with a needle inserted in the suboccipital region through the atlanto-occipital membrane, with a single incision into the subarachnoid space [22]. CSF samples were centrifuged at 1000×*g* for 10 min at 4 °C, and the supernatants were stored at −80 °C. In addition, the rat's brain was removed and their cerebral cortices were dissected out and stored at −80 °C. Hemi-cortices were thawed slowly at 4 °C and homogenized (10% *w*/*v*) in extraction buffer: 50 mM Tris-HCl (pH 7.4)/500 mM NaCl/5 mM EDTA/1% (*w*/*v*) Nonidet P-40/0.5% (*w*/*v*) Triton X-100, supplemented with a cocktail of protease inhibitors [23]. The homogenates were sonicated and centrifuged, as indicated above, and the supernatants were collected and frozen at −80 °C. Protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL, USA). The other hemi-cortices were reserved for messenger RNA (mRNA) analysis (see below).

Western Blotting

Cell (20 μg) and brain extracts (40 μg) and CSF samples (30 μL) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under fully reducing conditions. Samples were denatured at 50 °C for 15 min to analyze PS1 or, alternatively, at 98 °C for 5 min for other proteins. The proteins separated were transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience GmbH) and probed with a PS1 antibody raised against amino acids 1–20 (antibody 98/1; see Evin et al. [24]). Protein extracts from cell cultures were also probed for other γ-secretase subunits using the following antibodies: mouse anti-nicastrin (Millipore), rabbit anti-PEN2 (Sigma), and rabbit anti-APH1 (which recognizes both the APH1A and APH1B homologs; Sigma).

Brain extracts were also assayed for the CTF of APP or ApoER2 using the monoclonal anti-APP C-terminal antibody C1-6.1 (Covance) or a polyclonal antiserum against the C-terminal of ApoER2 (Abcam). Alternatively, the anti-APP monoclonal antibody 6E10 (Covance) was used. A rabbit anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Abcam) was used as a loading control. Western blots for different antibodies were performed separately to avoid re-probing the membranes. Antibody binding was detected with the corresponding conjugated secondary antibody (IRDye 680CW goat anti-mouse and IRDye 800RD goat anti-rabbit; LI-COR Biosciences) and visualized on an Odyssey CLx Infrared Imaging System (LI-COR Biosciences). Densitometric quantification of the signal from immunoreactive bands was obtained using LI-COR software (Image Studio Lite).

RNA Isolation and the Analysis of γ-Secretase Subunit Transcripts by qRT-PCR

The transcripts encoding PS1, nicastrin, PEN2, and two forms of APH1 (APH1A and APH1B) were assayed. The total RNA from rat brain hemi-cortices, SH-SY5Y cells, and mouse cortical neurons was isolated with the TRIzol® Reagent using the PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen™ Life Technologies), following the manufacturer's instructions. First-strand complementary DNAs (cDNAs) were synthesized by reverse transcription of 1.5 of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Life Technologies) according to the manufacturer's instructions. Quantitative PCR amplification was performed using a StepOne™ Real-Time PCR System (Applied Biosystems) and TaqMan PCR Master Mix with specific TaqMan Gene Expression Assays: Hs00997789 for PS1, Hs00950933_m1 for nicastrin, Hs00708570_s1 for PEN2, HS00211268_m1 for APH1A, and Hs0029911_m1 for APH1B on SH-SY5Y cell RNA; Mm00501184_m1 for PS1 on mouse cortical neuron RNA; and Rn00569763_m1 for PS1 on rat brain hemi-cortex RNA. GAPDH was amplified as a housekeeping marker (Hs03929097 for SH-SY5Y cells, Mm99999915_g1 for mouse cortical neurons, and Rn014626662_g1 for rat brain hemi-cortices), and the transcript levels were calculated relative to GAPDH using the comparative $2^{-\Delta Ct}$ method.

Behavioral Studies

The Y-maze alternation, active avoidance, and beam walking tests were performed to analyze memory and learning functions, as well as motor coordination. The tests were performed 2–4 h after the final administration of avagacestat.

Y-Maze Novel Spatial Recognition Memory This test is based on the rodents' natural curiosity to explore novel areas, and the rats were tested as described elsewhere [25]. Briefly, rats were placed into one of the arms of the Y-maze (start arm) and allowed to explore the maze with one of the arms closed for 3 min (training trial). After a 30-min inter-trial interval, the

rats were returned to the Y-maze, placing them in the start arm, and then the rats were allowed to freely explore all three arms of the maze for 3 min (test trial). The number of entries into and the time spent in each arm were registered manually by an observer blinded to the rat's treatment. The discrimination ratio is a measure of the preference for the novel arm over the familiar (old) arm, calculated as the Time spent in Novel / Time spent in the (Novel + Old).

Active Avoidance The active avoidance task is designed to test the ability of the rats to avoid an aversive event by first learning to perform a specific behavior in response to a stimulus. The test was performed on a single day and involved 50 trials per animal, as described previously [26].

Beam Walking Test The beam walking test assesses deficits in fine motor coordination [27], although it is also a useful assay to test for anxiety-like behavior [28] as it also causes some anxiety in the animal. Motor coordination was tested on a 1-m-long wooden stick (20 mm in diameter) situated approximately 1 m above the ground as described elsewhere [29]. The number of slips (foot faults) and the latency to cross (the time spent on the apparatus as an estimate of anxiety) are scored.

Statistical Analysis

All data were analyzed using SigmaStat (version 3.5; Systat Software, Inc.), determining exact p values by applying a Student's t test (two-tailed) or the Mann-Whitney rank-sum test, when normality was rejected. The results are presented as the means ± SEM.

Results

Inhibition of γ-Secretase by the GSI DAPT Increases the PS1 in SH-SY5Y and Primary Neuronal Cultures

We addressed whether DAPT, a well-known GSI that targets PS1 and reduces Aβ in vivo [30], alters PS1 expression and protein levels in SH-SY5Y neuroblastoma cells. Exposure to DAPT (5 μM) for 18 h did not affect cell viability ($p = 0.6$), as evaluated by the MTS assay and in agreement with a previous study [31]. We first corroborated the efficiency of an acute 18-h treatment with DAPT (5 μM) to inhibit γ-secretase activity by measuring the accumulation of the APP-CTF in cell extracts (Fig. 1a). PS1 undergoes endoproteolytic cleavage as part of its maturation, generating N-terminal fragment (NTF) and CTF [32], with very little full-length PS1 detectable in wild-type cultured cells [33]. As expected, a predominant band of ~29 kDa that corresponded to the PS1-NTF was evident when immunoblots were probed with an anti-PS1-NTF antibody, with little or no full-length PS1. The amount of PS1-NTF was significantly higher in extracts from DAPT-treated cells (32 ± 14%, $p = 0.03$) relative to the untreated controls (Fig. 1a). Similarly, there was a significant increase in the other γ-secretase components (nicastrin, PEN2, and APH1) in DAPT-treated SH-SY5Y cells (Supplemental Fig. 1A). However, there was no parallel increase in the mRNA encoding PS1 (Fig. 1a) or the other γ-secretase subunits (Supplemental Fig. 1B), which remained similar in DAPT-treated and untreated SH-SY5Y cells.

Likewise, repeated DAPT treatment of mouse primary neuronal cultures grown for 2 weeks and then treated daily with DAPT (2 μM) over 4 days also augmented the amount of PS1 protein (64 ± 11%, $p < 0.001$; Fig. 1b), with unaltered mRNA levels (Fig. 1b). Again, no cytotoxicity was observed during the treatment ($p = 0.4$, as compared with cell viability in cells treated with vehicle). Hence, the change in PS1 content persisted when γ-secretase inhibition was maintained.

Effects of APP-CTF Over-expression on PS1 in SH-SY5Y Neuroblastoma Cells

Since APP-CTF accumulation is a consequence of γ-secretase inhibition, we tested whether increasing APP-CTF mediated the change in PS1 levels by transfecting SH-SY5Y cells with APP-C99 cDNA, the β-secretase-derived CTF of APP. More APP-CTF was evident in these cells following transfection (48 h; Fig. 2a), with APP-C99 over-expression producing a significant increase in the cellular PS1 content (65 ± 21%, $p = 0.007$; Fig. 2b).

The GSI Avagacestat Alters the PS1 in Cultured Cells and Its Content In Vivo

Avagacestat is one of the first GSI that undergone clinical trials but discontinued development for AD because of a lack of efficacy at phase 2 trial [34–36]. Avagacestat selectively blocks the processing of APP substrates without notably affecting Notch processing [37, 38]. We analyzed the effect of avagacestat on PS1 in the primary neuronal cultures, where exposure to this GSI (2 μM) on four consecutive days increased the amount of PS1 relative to the controls exposed to the vehicle alone (41 ± 9%, $p = 0.007$; Fig. 3). There was no cell death in cultures treated with avagacestat, as evaluated by the MTS assay ($p = 0.5$).

Avagacestat was also administered orally to rats in a 40 mg/kg dose. In previous experiments in rats to which doses of 2–100 mg/kg avagacestat were used, a 40 mg/kg dose demonstrated significantly reduced Aβ in the brain, with no abnormalities detected [37, 39]. Acute treatment served to probe that avagacestat inhibits the processing of APP-CTF in treated rats, promoting their accumulation in animals treated with a single dose (Fig. 4a). We also tested whether avagacestat

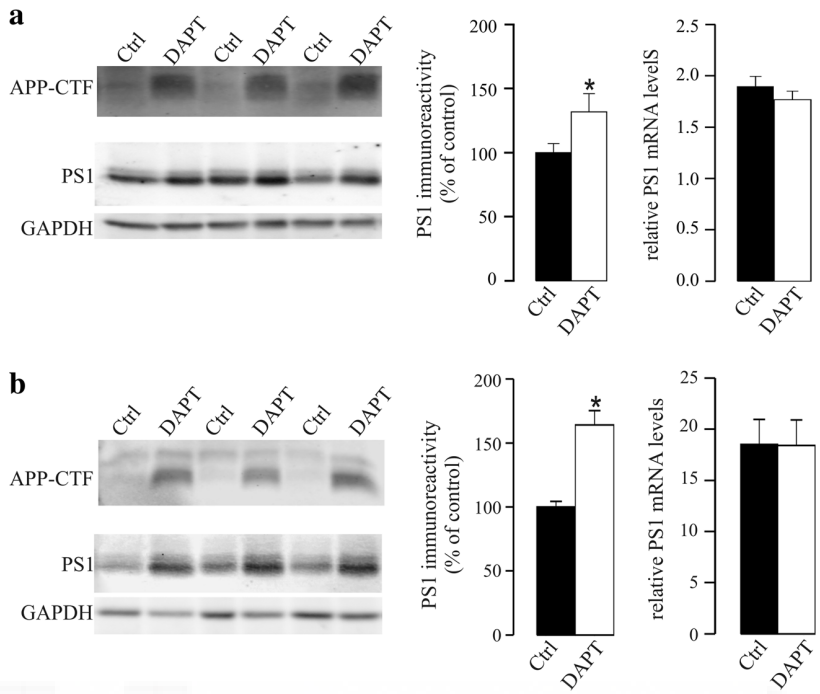

Fig. 1 GSI DAPT treatment augments PS1 in SH-SY5Y cells and in mouse primary neurons. **a** SH-SY5Y cells were treated for 18 h (acutely) with DAPT (5 μM) or the vehicle alone (control; Ctrl). Cell extracts were probed with antibody C1-6.1, against the APP C-terminal, to demonstrate the accumulation of the APP-CTF in treated cells as a result of the inhibition of γ-secretase processing. Cell extracts were also probed for PS1 with an anti-N-terminal antibody. Equivalent amounts of protein were loaded in each lane, and GAPDH was used as a loading control. Representative blots and densitometric quantification of the immunoreactivity are shown. Relative expression of PS1 mRNA was also analyzed by qRT-PCR. Transcript levels were calculated by the comparative $2^{-\Delta Ct}$ method with respect to GAPDH cDNA. **b** Primary neurons were treated with DAPT (2 μM) or the vehicle alone (Ctrl) for four consecutive days. Cell extracts were probed for APP-CTF and PS1 and for GAPDH as a loading control. The densitometric quantification for PS1-NTF is shown, as well the relative mRNA levels of the PS1 transcript. The data represent the means ± SEM of at least $n = 10$ independent determinations (obtained from two independent sets of experiments): $^*p < 0.05$

treatment increases PS1 in the brain of rats as part of a rebound effect, and we extended our analysis to include behavioral tests. When avagacestat (40 mg/kg) was administered orally to rats once daily for 4 days, there was apparently no effect on the amount of APP-CTF in the brain after treatment and PS1 levels remained unaltered (Supplemental Fig. 2). Conversely, treatment for 21 days significantly diminished the APP-CTF in the brain (79 ± 5%, $p = 0.005$; Fig. 4b). This unexpected decrease in APP-CTF, after prolonged GSI treatment, prompted the analysis of the levels of other γ-secretase substrates. ApoER2, a liporeceptor for ApoE/Reelin, is also a γ-secretase substrate [31], and a significant decrease in ApoER2-CTF (72 ± 9%, $p = 0.03$; Fig. 4b) was also detected in rats exposed to avagacestat, relative to the control rats. The increase in the rate of processing of γ-secretase substrates, APP-CTF and ApoER2-CTF, paralleled with an increase in PS1-NTF (29 ± 9%, $p = 0.008$: Fig. 4c). Again, the avagacestat-induced increase in PS1 protein was not paralleled by an increase in its mRNA transcripts (Fig. 4c).

We recently demonstrated the presence of heteromeric PS1 complexes in human and rodent CSF (CSF-PS1), the proportion of such stable, large molecular mass complexes being associated to AD status [40, 41]. In Western blots probed with an antibody against the PS1-NTF, predominant bands of approximately 100, 80, and 70 kDa were detected, corresponding to CSF-PS1 SDS-stable complexes previously characterized [40], as well a 29-kDa band corresponding to monomeric PS1-NTF. Unexpectedly, the immunoreactivity for the 100-kDa complexes diminished in 21-day avagacestat-treated rats relative to the control rats (57 ± 10%, $p = 0.03$; Fig. 5), whereas no notable changes were observed in rats treated for 4 days with avagacestat (Fig. 5).

Finally, we assessed potential behavioral, memory, and learning changes in rats treated for 21 days with avagacestat using the novel spatial recognition memory, the active avoidance, and the beam walking tests. Avagacestat-treated animals displayed no differences in the novel spatial recognition memory in the Y-maze, with similar discrimination between arms, nor delayed alternation, when compared to the control rats (Fig. 6a). We also observed similar abilities of avagacestat and vehicle-treated rats to learn the active avoidance task and avoid the aversive event (Fig. 6b). However, while

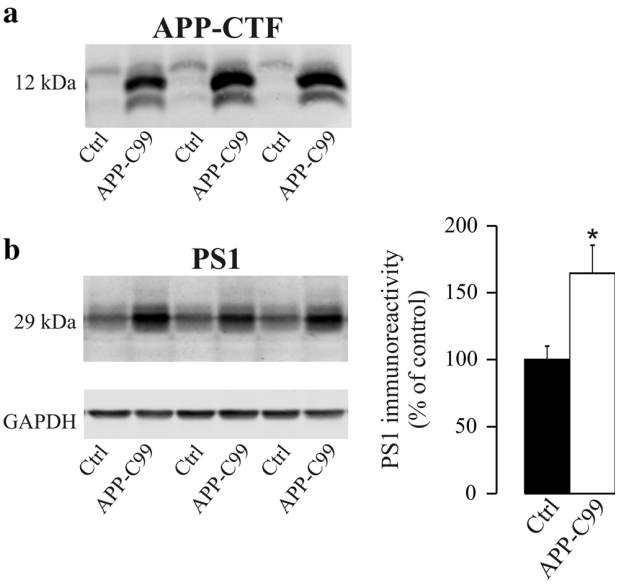

Fig. 2 Effects of the modulation of APP-CTF expression on PS1 levels. SH-SY5Y cells were transfected with APP-C99 cDNA, the β-secretase-derived CTF of APP, or with a control vector (Ctrl). **a** Immunodetection of APP-CTF with the anti-APP C-terminal antibody C1-6.1 served to assess the efficiency of over-expression. The identity of the increased immunoreactive band was also tested with the 6E10 antibody, which recognizes an epitope present in the N-terminal of APP-C99 (not shown). **b** The immunodetection and densitometric quantification of PS1 immunoreactivity in transfected cells are shown. The data are presented relative to control cells, expressed as the means ± SEM of at least 12 independent determinations (obtained from two independent sets of experiments): $*p = 0.007$

avagacestat-treated rats did not display any alterations in the ability to cross a round beam, revealing no gross motor deficits, significant differences were detected in the latency time to cross the beam, probably indicating higher levels of anxiety (Fig. 6c).

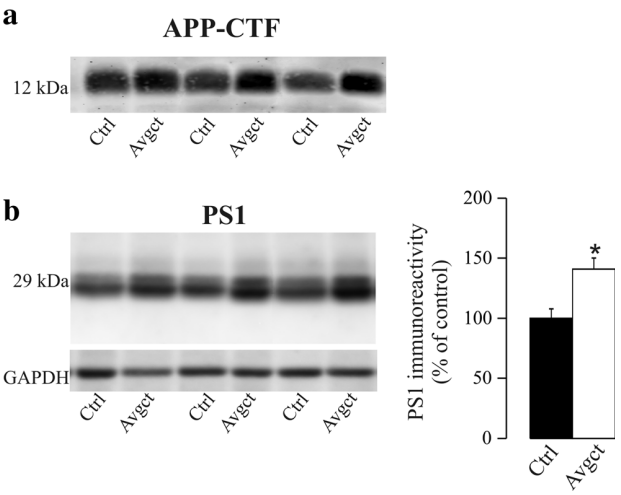

Fig. 3 Increased PS1 levels in neurons treated with the GSI, avagacestat. Primary neurons were treated with avagacestat (2 μM, Avgct) or the vehicle alone (Ctrl), and the cell extracts were probed for **a** APP-CTF and **b** PS1. Representative blots and their densitometric quantification are shown. The data presented are relative to the Ctrl cells, expressed as the means ± SEM of at least ten independent determinations (obtained from two independent sets of experiments): $*p = 0.007$

Discussion

The possibility that levels and activities of secretases are affected in the brain of AD subjects has been studied intensively [5, 8]. However, whether their inhibition by GSIs can induce persistent compensatory changes in the brain has yet to be addressed. It is known that neurotransmitter transporters potentially undergo alterations to gene transcription, mRNA translation/stability, post-translational, protein trafficking, cytoskeletal interactions, and oligomerization in response to chronic drug administration [42]. Indeed, an up-regulation of proteins targeted by pharmacological inhibition has also been documented [18–20]. Here, we demonstrate that GSIs can induce a feedback mechanism that results in accumulation of PS1 in different cell models. A similar elevation of brain PS1 was identified in 21-day avagacestat-treated rats, which also displayed an increasing rate of processing of the γ-secretase substrates APP-CTF and ApoER2-CTF, indicative of a rebound effect. These effects could be related to the reported failure of GSIs to achieve long-term Aβ regulation and their contribution to rather than the palliation of the AD pathology.

We found an increase in PS1 after a single day of DAPT administration to SH-SY5Y cells. Similar results were obtained in primary neuronal cultures treated for 4 days with DAPT and in rats treated for 21 days with avagacestat. The increase in PS1 protein was not paralleled by changes in PS1 mRNA content, indicating that this increase is not mediated by transcriptional upregulation. At present, the mechanism by which PS1 levels are enhanced by GSI administration remains unknown. Interestingly, over-expression of the β-secretase-derived APP fragment C99 could also mediate an increase in PS1. There is evidence that the accumulation of APP-C99 may be directly implicated in neurodegeneration and cognitive alterations [43]. Previous evidences indicate that excess in other γ-secretase substrates can compromise γ-secretase catalytic activity, being accompanied by an increase in PS1 levels [21]. Remarkably, it has been demonstrated that accumulation of APP-C99 can cause an impaired lysosomal-autophagic function [44]. Hence, it seems desirable to investigate whether an excess of γ-secretase substrates may result in transient stabilization of PS1/γ-secretase substrate complexes, interfering in the effective clearance of PS1. Similarly, the stabilization of PS1/GSI complexes during sustained γ-secretase inhibition could interfere in the clearance/turnover of PS1. Indeed, decreased intracellular clearance of PS1 may also reflect the reduction of the CSF-PS1 complex levels, although how PS1 reaches the CSF is unknown.

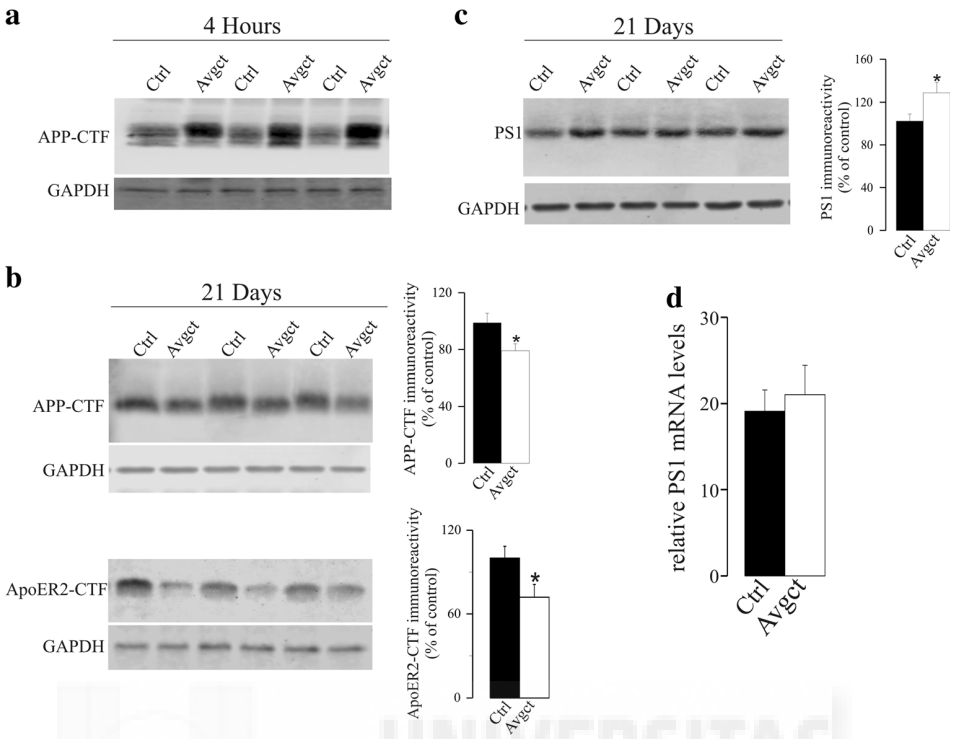

Fig. 4 Effect of prolonged inhibition of γ-secretase by avagacestat on γ-secretase substrates and PS1 in the cortex of rats treated for 21 days. Rats were treated daily with the GSI avagacestat (40 mg/kg, Avgct) or the vehicle alone (control; Ctrl) for 21 days, and they were sacrificed 4 h after the last dose. **a** As a control of the effective γ-secretase inhibition by the GSI in the brain, APP-CTF levels (probed with antibody C1-6.1) were firstly evaluated in rats sacrificed 4 h after a single dose of avagacestat (n = 6 per group). **b** The levels of APP-CTF and ApoER2-CTF were estimated in rats treated with avagacestat for 21 days; representative blots and densitometric quantifications are shown. **c** PS1 levels were also evaluated in Western blots of the same brain hemi-cortex extracts. GAPDH was used as a loading control. **d** Relative PS1 mRNA was analyzed by qRT-PCR in the other rat hemi-cortices obtained after 21 days of treatment (n = 10 per group). The data are presented relative to the control rats and expressed as the means ± SEM (n = 10 per group): * $p < 0.05$ significantly different from the controls

In this context, it has been demonstrated that changes in PS1 ubiquitination can alter cellular levels of PS1 and other γ-secretase subunits, leading to an alteration in the metabolism of APP [45, 46]. Therefore, a chronic treatment with GSIs may cause a sustained accumulation of PS1 leading a rebound effect with gain in γ-secretase activity. In this regard, although avagacestat has demonstrated effect in the accumulation of APP-CTF (acute treatment in rats), prolonged exposition to

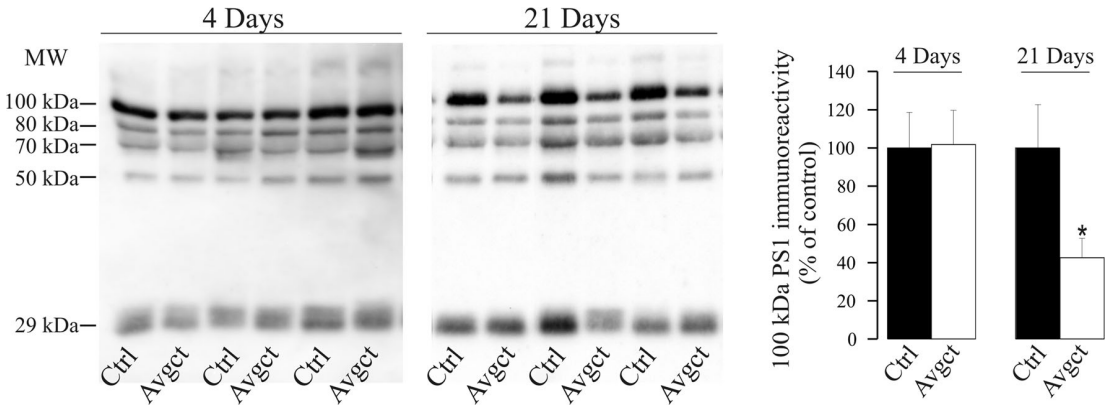

Fig. 5 Effect of avagacestat on PS1 levels in CSF of rats treated for 4 and 21 days. Rats were administered avagacestat (40 mg/kg, Avgct) or the vehicle alone (Ctrl) daily over 4 or 21 days. Soluble PS1 complexes were also evaluated in Western blots of CSF samples from Avgct-treated and control rats (n = 7 per group). CSF-PS1 complexes were detected with and N-terminal antibody, which predominantly recognized stable complexes of approximately 100 kDa, together with less abundant 80-, 70-, and 50-kDa complexes, as well monomers of 29 kDa. Previous studies indicated that these CSF-PS1 complexes represent aggregates of PS1-NTF and CTF [40, 41]. The densitometric quantification of the major CSF-PS1 100-kDa complex is shown. The data are presented relative to the control rats, expressed as the means ± SEM: *$p < 0.05$

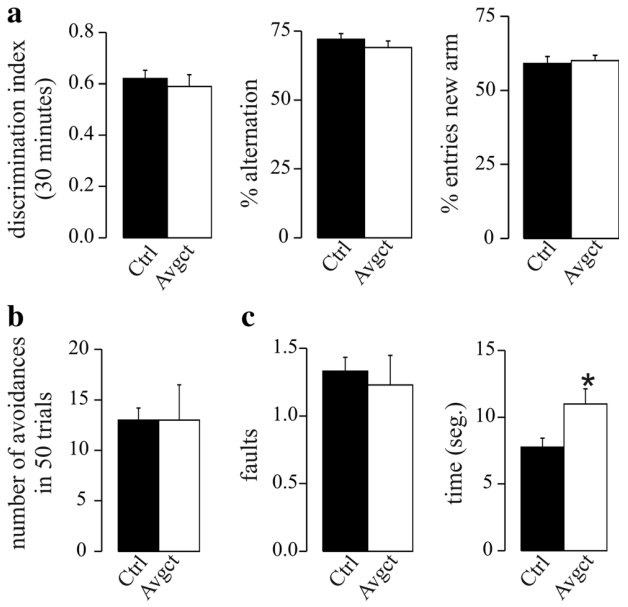

Fig. 6 Results of the behavioral tests in rats treated 21 days with avagacestat. **a** Novel spatial recognition memory in the Y-maze in rats treated with avagacestat for 21 days (Avgct) and in the vehicle-treated controls (Ctrl). The time spent in each arm was recorded in order to calculate the discrimination index after a 30-min inter-trial interval. **b** Result of the active avoidance test documenting the number of attempts made to avoid the foot shock. **c** Beam walking test in which the number of slips and the latency to cross were scored. The values are the means ± SEM ($n = 10$ for each group): $^*p < 0.05$

the GSI (21 days of treatment in rats) has lead to PS1 accumulation. An increase in PS1 levels, even maintained in GSI treatment, could result in an increasing rate of substrate processing during the oscillations in the effective inhibitory concentration of the drug, derived of the half-life and QD dosing.

Moreover, other alternatives are suitable. There are subtle differences in the subcellular accumulation of APP-CTF in PS1-deficient cells, with no obvious redistribution of the full-length protein [47]. Distinct subcellular locations of PSs have been shown to contribute to substrate specificity [48], and changes in the subcellular distribution of BACE1 induced by Aβ oligomers have been related to the pathogenesis of AD [49]. In brief, both post-translational and turnover/degradation mechanisms may participate in the pernicious response to GSI and deserve investigation. Moreover, we cannot discard that other enzymes distinct from PS1, or acting in parallel, could be involved in the rebound effect, with an increased rating of γ-secretase substrate processing during prolonged inhibition.

Chronic inhibition of PS1 with GSI has led to toxic side effects in clinical trials [37, 50, 51]. These adverse effects were thought to be related with the regulation of Notch activity by γ-secretase, a protein that is important for cell-to-cell communication and that has also been implicated in cancer [52]. Toxic side effects have been noted in clinical trials conducted with *Notch-sparing* GSIs as well as *non-selective* GSIs, although the true selectivity of the former is not clear [8]. Indeed, dozens of additional substrates for γ-secretase have been identified and, thus, non-selective GSIs would probably interfere with multiple cellular events [53, 54]. Currently, clinical trials with semagacestat (LY450139), an earlier-generation GSI that does not discriminate well between APP and Notch, have been discontinued, similar to clinical trials with avagacestat. Furthermore, the development of another Notch-sparing GSI, begacestat (GSI-953) [55], has also been discontinued for reasons that are not clear (discussed in De Strooper and Chávez-Gutiérrez [56]).

The therapeutic effect of GSIs appears to be transient, and the possibility of decelerating or halting cognitive deterioration also falls below expectations. At 2 years, no significant differences were observed in key clinical outcome measures in an avagacestat phase 2 trial, yet progression to dementia was more frequent in the prodromal AD cohort vs the observational cohort [36]. Similarly, semagacestat made AD patients cognitively worse in a phase 3 trial [57]. In Tg2576 mice, a 1-day treatment with two GSIs significantly ameliorated cognitive deficits (acute effects) but these effects disappeared when an 8-day treatment schedule was employed. Indeed, prolonged treatment with GSIs impairs spatial working memory and cognitive function [58]. In our study, an augmented latency time in the beam walking test in wild-type rats treated for 21 days with avagacestat suggests that some behavioral issues are affected by GSIs. This phenomenon is consistent with the dampening of initiative and the anxiety that are common neuropsychiatric features of AD [59, 60]. Interestingly, the conditional double presenilin knockout mice has observably altered anxiety-like behavior [61], and less anxiety is also displayed by transgenic mice expressing mutants PS1-A246E [62] and PS2-N141I [63]. An association of PS1 with altered anxiety-like behavior has been suggested [64] and is worthy of further investigation. The subtle alterations in behavioral tests in wild-type rats are inconclusive since we did not use an animal model with an impaired condition, and nor did we demonstrate a direct association between altered anxiety-like behavior and increased brain PS1 levels. However, we speculate that part of the impairment observed in clinical trials involving GSI use on humans and in chronically treated animals could be due to rebound increases in PS1.

Although simple in concept, the validation of amyloid drug targets, and specifically that of GSIs, has proved complex in practice. Earlier studies indicated that the acute oral administration of DAPT to APP_{V717F} transgenic mice reduces the Aβ in the brain [30]. The use of canine [65] and non-human primate [66] models also served to demonstrate that GSIs decrease the Aβ peptides in the CSF. However, it is well established that the levels of AD CSF diminish when there is an increase in brain deposition of Aβ. Thus, changes in CSF-Aβ are unlikely to provide significant information about

therapies aimed at reducing Aβ production, and a lowering of CSF-Aβ levels is unlikely to be a suitable measure of target engagement [67].

In this regard, acute administration of avagacestat robustly reduces CSF Aβ40 and Aβ42 levels similarly in rats and dogs [39]. Moreover, the administration of a single dose of avagacestat to healthy humans, as well over a 28-day schedule, also markedly decreases Aβ40 and Aβ42 concentrations in the CSF [68, 69]. However, exploratory CSF amyloid isoforms displayed a dose-dependent but not significant reduction in a small subset of patients in a phase 2 trial, and while well tolerated, lower doses did not affect the Aβ40 and Aβ42 levels in treated patients [35]. Similarly, earlier studies with semagacestat in volunteers indicated unchanged levels of CSF-Aβ [70], although in another study, single oral doses of semagacestat appeared to decrease Aβ levels in the CSF of healthy volunteers [71]. No significant reduction in CSF Aβ42 or Aβ40 level was detected in a phase 2 safety trial [72], a finding verified by mass spectrometry analysis of the same samples [73]. Instead, an increase in shorter Aβ peptides (Aβ1–14, Aβ1–15, and Aβ1–16) was identified, probably due to increased substrate availability (APP-C99) for α-secretase [73]. Interestingly, semagacestat produced a decrease in plasma Aβ concentrations in a 6-h interval following drug administration, returning to baseline and then transiently increasing the Aβ concentrations [13]. It was suggested that semagacestat might lower Aβ at high concentrations but cause Aβ elevation at low concentrations [15]. A structurally related γ-secretase inhibitor, LY-411575, also elevated plasma Aβ40 and Aβ42 in Tg2576 mice [14]. A biphasic activation-inhibition dose-response curve for GSIs was proposed to explain these changes in Aβ secretion [74]. However, these changes may also be indicative of a transient overshooting or rebound effect, since an increase in plasma Aβ40 and Aβ42 has been described in Tg2576 mice chronically treated with DAPT [12].

Here, we addressed the efficiency of GSIs to inhibit PS1 by assessing changes in the cellular γ-secretase substrate APP-CTF. As expected, the accumulation of APP-CTF served to assess the inhibitory effect of DAPT on PS1 in cellular models and also that of avagacestat. Accordingly, we were able to detect accumulation in the brain levels of APP-CTF in acutely treated rats (sacrificed 4 h after a single dose). However, sustained inhibition of γ-secretase activity over 21 days revealed decreased APP-CTF levels, suggesting that the consolidation of higher PS1 levels in reaction to chronic inhibition results in an increase in γ-secretase activity, at least in the intervals between GSI administration. The consolidation of higher levels of PS1 might indiscriminately affect all γ-secretase substrates, such as ApoER2 and others, further exacerbating the AD pathology. Interestingly, administration of GSIs increased APP-CTF in H4 cells over-expressing APP, although this increase was unexpectedly attenuated at high concentrations [58]. Elsewhere, APP-C99 levels increase in CHO cells co-expressing APP and PS1 relative to cells expressing APP alone, and PS1 can stabilize APP-CTF independent of γ-secretase activity [75]. Hence, the relationship between the substrate and the catalytic enzyme appears to be more complex than might at first appear.

PS1 also participates in other cell functions [76, 77], and therefore, the increase in PS1 after GSI administration may influence distinct cellular effects, even if this subunit is not-catalytically active. In this regard, PS1 has been implicated in the physiological maturation and glycosylation of several key proteins implicated in AD, such as nicastrin [78], BACE1 [79], acetylcholinesterase [80], and others, including APP [81]. Hence, the over-expression of either the wild-type or mutant PS1 disturbs glycoprotein processing [82]. Further research will be needed to clarify the influence of increased PS1, under prolonged GSI administration, in the role of PS1 in their non-proteolytic functions, and possible interference with the therapeutic response.

Conclusions

We show here that administration of GSIs result in a rebound increase in PS1 levels in cellular and animal models, which must be taken into consideration when using such compounds in AD therapy. Indeed, our results indicate that the effect of GSI inhibitors on APP processing failed to have a long-term effect in treated rats, possibly due to the persistent PS1 elevation in reaction to chronic inhibition.

The outcomes of the clinical trials with GSIs have been disappointing, although this may not represent the end of the development of these drugs to treat AD. The data presented here indicate that the therapeutic benefits of GSIs and related drugs should continue to be explored, or at least, we can extract information that will help understand the failure of GSIs in AD trials [83]. Hence, synthesizing new GSIs that distinguish strongly between APP and Notch may serve to lower the required dose, yet it still might not solve the unexplained and unexpected problem of the facilitation of toxic side effects and the AD-derived pathogenesis. The failure of GSIs in clinical trials highlights the need for a systematic re-examination of γ-secretase biology, including further characterization of the mechanisms related to the response to chronic inhibition. Elucidating the mechanisms involved, the complex self-regulation of γ-secretase is also important to optimize therapies based on γ-secretase modulation. A potent inhibition/modulation of secretase activities will result in the unbalanced generation of proteolytic fragments of APP (and fragments from other substrates), which could determine a self-regulatory response that will require further analysis for new secretase inhibitors/modulators designed to specifically inhibit the Alzheimer process.

In this regard, γ-secretase modulators (GSMs), which only block the γ-secretase cleavage of APP to generate the Aβ42, with no changes in the production of total Aβ, were also noticed to have negative outcomes [84]. The clinical development of BACE1 inhibitors is also being intensely pursued, and several promising BACE1 inhibitors have entered human clinical trials [85], but a sign of toxicity forced to stop the earliest trials (discussed in Lahiri et al. [86]). For a successful development of new secretase inhibitors/modulators, it is needed to better understand the cellular response to the sustained inhibition/modulation of the secretase activity.

Despite its enzymatic capacity, γ-secretase activity appears tightly regulated by many cellular components, including its own subunits, modulatory partners, and substrates, as well as by an array of cellular events [87]. Furthermore, GSIs are presently explored in clinical trials as potential therapeutic agents in cancer, targeting Notch, although a number of mechanism-based adverse events again emerge [88]. As the therapeutic benefits of GSIs and related drugs continue to be explored, a better understanding of the response of PS1 to chronic inhibition will become more necessary.

Acknowledgements We thank Dr. J. Culvenor and Dr. G. Evin (Department of Pathology, The University of Melbourne, Australia) for the gift of the PS1 antibodies and Dr. D. H. Small (Menzies Institute for Medical Research, University of Tasmania, Australia) for the advice and for providing the C99 cDNA. We also thank Bristol-Myers Squibb (Wallingford, CT, USA) for the generous gift of avagacestat. This study was funded in part by the EU BIOMARKAPD-Joint Programming on Neurodegenerative Diseases (JPND) project by the Instituto de Salud Carlos III (ISCIII grants PI11/03026 and PI15/00665 for JSV and PI14/00566 for MSGA), co-financed by the Fondo Europeo de Desarrollo Regional (FEDER) "Investing in your future," and through CIBERNED (ISCIII). We also acknowledge financial support from the Spanish Ministerio de Economía y Competitividad, through the "Severo Ochoa" Programme for Centres of Excellence in R&D (SEV-2013-0317). The funding bodies played no role in the study design, the data collection and analysis, the decision to publish, or the preparation of the manuscript.

Compliance with Ethical Standards All animal procedures were approved by the Animal Care and Use Committees at the Universidad Miguel Hernández and by Centro Principe Felipe (2016A/SC/PEA/00127).

Conflict of Interest The authors declare that they have no competing interests.

References

1. Scheltens P, Blennow K, Breteler MM, De Strooper B, Frisoni GB, Salloway S et al (2016) Alzheimer's disease. Lancet 388:505–517
2. Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356
3. Schneider LS, Mangialasche F, Andreasen N, Feldman H, Giacobini E, Jones R et al (2014) Clinical trials and late-stage drug development for Alzheimer's disease: an appraisal from 1984 to 2014. J Intern Med 275:251–283
4. Portelius E, Price E, Brinkmalm G, Stiteler M, Olsson M, Persson R et al (2011) A novel pathway for amyloid precursor protein processing. Neurobiol Aging 32:1090–1098
5. Haass C, Kaether C, Thinakaran G, Sisodia S (2012) Trafficking and proteolytic processing of APP. Cold Spring Harb Perspect Med 2:a006270
6. Vassar R, Kovacs DM, Yan R, Wong PC (2009) The beta-secretase enzyme BACE in health and Alzheimer's disease: regulation, cell biology, function, and therapeutic potential. J Neurosci 29:12787–12794
7. Kaether C, Haass C, Steiner H (2006) Assembly, trafficking and function of gamma-secretase. Neurodegener Dis 3:275–283
8. Chávez-Gutiérrez L, Bammens L, Benilova I, Vandersteen A, Benurwar M, Borgers M et al (2012) The mechanism of γ-secretase dysfunction in familial Alzheimer disease. EMBO J 31:2261–2274
9. Barten DM, Meredith JE Jr, Zaczek R, Houston JG, Albright CF (2006) Gamma-secretase inhibitors for Alzheimer's disease: balancing efficacy and toxicity. Drugs R&D 7:87–97
10. Mikulca JA, Nguyen V, Gajdosik DA, Teklu SG, Giunta EA, Lessa EA et al (2014) Potential novel targets for Alzheimer pharmacotherapy: II. Update on secretase inhibitors and related approaches. J Clin Pharm Ther 39:25–37
11. Toyn JH, Ahlijanian MK (2014) Interpreting Alzheimer's disease clinical trials in light of the effects on amyloid-β. Alzheimers Res Ther 6:14
12. Sivilia S, Lorenzini L, Giuliani A, Gusciglio M, Fernandez M, Baldassarro VA, Mangano C et al (2013) Multi-target action of the novel anti-Alzheimer compound CHF5074: in vivo study of long term treatment in Tg2576 mice. BMC Neurosci 14:44
13. Siemers E, Skinner M, Dean RA, Gonzales C, Satterwhite J, Farlow M et al (2005) Safety, tolerability, and changes in amyloid beta concentrations after administration of a gamma-secretase inhibitor in volunteers. Clin Neuropharmacol 28:126–132
14. Lanz TA, Hosley JD, Adams WJ, Merchant KM (2004) Studies of Aβ pharmacodynamics in the brain, cerebrospinal fluid, and plasma in young (plaque-free) Tg2576 mice using the γ-secretase inhibitor N2-[(2S)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N1-[(7S)-5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7-yl]-l-alaninamide (LY-411575). J Pharmacol Exp Ther 309:49–55
15. Lanz TA, Karmilowicz MJ, Wood KM, Pozdnyakov N, Du P, Piotrowski MA et al (2006) Concentration-dependent modulation of amyloid-beta in vivo and in vitro using the gamma-secretase inhibitor, LY-450139. J Pharmacol Exp Ther 319:924–933
16. Säemann MD, Böhmig GA, Osterreicher CH, Burtscher H, Parolini O, Diakos C et al (2000) Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. FASEB J 14:2380–2382
17. Tanaka A, Hase S, Miyazawa T, Takeuchi K (2002) Up-regulation of cyclooxygenase-2 by inhibition of cyclooxygenase-1: a key to nonsteroidal anti-inflammatory drug-induced intestinal damage. J Pharmacol Exp Ther 300:754–761
18. García-Ayllón MS, Silveyra MX, Andreasen N, Brimijoin S, Blennow K, Sáez-Valero J (2007) Cerebrospinal fluid

acetylcholinesterase changes after treatment with donepezil in patients with Alzheimer's disease. J Neurochem 101:1701–1711
19. Xu L, Kappler CS, Mani SK, Shepherd NR, Renaud L, Snider P et al (2009) Chronic administration of KB-R7943 induces up-regulation of cardiac NCX1. J Biol Chem 284:27265–27272
20. Serfőző Z, Lontay B, Kukor Z, Erdődi F (2012) Chronic inhibition of nitric oxide synthase activity by N(G)-nitro-L-arginine induces nitric oxide synthase expression in the developing rat cerebellum. Neurochem Int 60:605–615
21. Campanari ML, García-Ayllón MS, Belbin O, Galcerán J, Lleó A, Sáez-Valero J (2014) Acetylcholinesterase modulates presenilin-1 levels and γ-secretase activity. J Alzheimers Dis 41:911–924
22. Sáez-Valero J, de Gracia JA, Lockridge O (2005) Intraperitoneal administration of 340 kDa human plasma butyrylcholinesterase increases the level of the enzyme in the cerebrospinal fluid of rats. Neurosci Lett 383:93–98
23. Sáez-Valero J, Tornel PL, Muñoz-Delgado E, Vidal CJ (1993) Amphiphilic and hydrophilic forms of acetyl- and butyrylcholinesterase in human brain. J Neurosci Res 35:678–689
24. Evin G, Sharples RA, Weidemann A, Reinhard FB, Carbone V, Culvenor JG et al (2001) Aspartyl protease inhibitor pepstatin binds to the presenilins of Alzheimer's disease. Biochemistry 40:8359–8368
25. Basavarajappa BS, Subbanna S (2014) CB1 receptor-mediated signaling underlies the hippocampal synaptic, learning, and memory deficits following treatment with JWH-081, a new component of spice/K2 preparations. Hippocampus 24:178–188
26. García-Ayllón MS, Cauli O, Silveyra MX, Rodrigo R, Candela A, Compañ A et al (2008) Brain cholinergic impairment in liver failure. Brain 131:2946–2956
27. Goldstein LB, Davis JN (1990) Beam-walking in rats: studies towards developing an animal model of functional recovery after brain injury. J Neurosci Methods 31:101–107
28. Hånell A, Marklund N (2014) Structured evaluation of rodent behavioral tests used in drug discovery research. Front Behav Neurosci 8:252
29. Gonzalez-Usano A, Cauli O, Agusti A, Felipo V (2014) Pregnenolone sulphate restores the glutamate-nitric oxide-cGMP pathway and extracellular GABA in cerebellum and learning and motor coordination in hyperammonemic rats. ACS Chem Neurosci 5:100–105
30. Dovey HF, John V, Anderson JP, Chen LZ, de Saint AP, Fang LY et al (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. J Neurochem 76:173–181
31. Balmaceda V, Cuchillo-Ibáñez I, Pujadas L, García-Ayllón MS, Saura CA, Nimpf J et al (2014) ApoER2 processing by presenilin-1 modulates reelin expression. FASEB J 28:1543–1554
32. Thinakaran G, Borchelt DR, Lee MK, Slunt HH, Spitzer L, Kim G et al (1996) Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. Neuron 17:181–190
33. Podlisny MB, Citron M, Amarante P, Sherrington R, Xia W, Zhang J et al (1997) Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. Neurobiol Dis 3:325–337
34. Tong G, Castaneda L, Wang JS, Sverdlov O, Huang SP, Slemmon R et al (2012) Multicenter, randomized, double-blind, placebo-controlled, single-ascending dose study of the oral γ-secretase inhibitor BMS-708163 (avagacestat): tolerability profile, pharmacokinetic parameters, and pharmacodynamic markers. Clin Ther 34:654–667
35. Coric V, van Dyck CH, Salloway S, Andreasen N, Brody M, Richter RW et al (2012) Safety and tolerability of the γ-secretase inhibitor avagacestat in a phase 2 study of mild to moderate Alzheimer disease. Arch Neurol 69:1430–1440
36. Coric V, Salloway S, van Dyck CH, Dubois B, Andreasen N, Brody M et al (2015) Targeting prodromal Alzheimer disease with avagacestat: a randomized clinical trial. JAMA Neurol 72:1324–1333
37. Gillman KW, Starrett JE Jr, Parker MF, Xie K, Bronson JJ, Marcin LR et al (2010) Discovery and evaluation of BMS-708163, a potent, selective and orally bioavailable γ-secretase inhibitor. ACS Med Chem Lett 1:120–124
38. Crump CJ, Castro SV, Wang F, Pozdnyakov N, Ballard TE, Sisodia SS et al (2012) BMS-708, 163 targets presenilin and lacks notch-sparing activity. Biochemistry 51:7209–7211
39. Albright CF, Dockens RC, Meredith JE Jr, Olson RE, Slemmon R, Lentz KA et al (2013) Pharmacodynamics of selective inhibition of γ-secretase by avagacestat. J Pharmacol Exp Ther 344:686–695
40. García-Ayllón MS, Campanari ML, Brinkmalm G, Rábano A, Alom J, Saura CA et al (2013) CSF presenilin-1 complexes are increased in Alzheimer's disease. Acta Neuropathol Commun 1:46
41. Sogorb-Esteve A, García-Ayllón MS, Fortea J, Sánchez-Valle R, Lleó A, Molinuevo JL, Sáez-Valero J (2016) Cerebrospinal fluid presenilin-1 increases at asymptomatic stage in genetically determined Alzheimer's disease. Mol Neurodegener 11:66
42. Zahniser NR, Doolen S (2001) Chronic and acute regulation of Na+/Cl−-dependent neurotransmitter transporters: drugs, substrates, presynaptic receptors, and signaling systems. Pharmacol Ther 92:21–55
43. Lauritzen I et al (2012) The β-secretase-derived C-terminal fragment of βAPP, C99, but not Aβ, is a key contributor to early intraneuronal lesions in triple-transgenic mouse hippocampus. J Neurosci 32:16243–16255
44. Lauritzen I, Pardossi-Piquard R, Bauer C, Brigham E, Abraham JD, Ranaldi S et al (2016) Intraneuronal aggregation of the β-CTF fragment of APP (C99) induces Aβ-independent lysosomal-autophagic pathology. Acta Neuropathol 132:257–276
45. Li J et al (2002) SEL-10 interacts with presenilin 1, facilitates its ubiquitination, and alters A-beta peptide production. J Neurochem 82:1540–1548
46. Massey LK, Mah AL, Monteiro MJ (2005) Ubiquilin regulates presenilin endoproteolysis and modulates gamma-secretase components, Pen-2 and nicastrin. Biochem J 391:513–525
47. Chen F, Yang DS, Petanceska S, Yang A, Tandon A, Yu G et al (2000) Carboxyl-terminal fragments of Alzheimer beta-amyloid precursor protein accumulate in restricted and unpredicted intracellular compartments in presenilin 1-deficient cells. J Biol Chem 275:36794–36802
48. Sannerud R, Esselens C, Ejsmont P, Mattera R, Rochin L, Tharkeshwar AK et al (2016) Restricted location of PSEN2/γ-secretase determines substrate specificity and generates an intracellular Aβ pool. Cell 166:193–208
49. Mamada N, Tanokashira D, Hosaka A, Kametani F, Tamaoka A, Araki W (2015) Amyloid β-protein oligomers upregulate the β-secretase, BACE1, through a post-translational mechanism involving its altered subcellular distribution in neurons. Mol Brain 8:73
50. Searfoss GH, Jordan WH, Calligaro DO, Galbreath EJ, Schirtzinger LM, Berridge BR et al (2003) Adipsin, a biomarker of gastrointestinal toxicity mediated by a functional gamma-secretase inhibitor. J Biol Chem 278:46107–46116
51. Wong GT, Manfra D, Poulet FM, Zhang Q, Josien H, Bara T et al (2004) Chronic treatment with the gamma-secretase inhibitor LY-411, 575 inhibits beta-amyloid peptide production and alters lymphopoiesis and intestinal cell differentiation. J Biol Chem 279:12876–12882
52. Wolfe MS (2012) γ-Secretase as a target for Alzheimer's disease. Adv Pharmacol 64:127–153
53. Wakabayashi T, De Strooper B (2008) Presenilins: members of the gamma-secretase quartets, but part-time soloists too. Physiology (Bethesda) 23:194–204

54. Lleó A, Saura CA (2011) γ-secretase substrates and their implications for drug development in Alzheimer's disease. Curr Top Med Chem 11:1513–1527
55. Martone RL, Zhou H, Atchison K, Comery T, Xu JZ, Huang X et al (2009) Begacestat (GSI-953): a novel, selective thiophene sulfonamide inhibitor of amyloid precursor protein gamma-secretase for the treatment of Alzheimer's disease. J Pharmacol Exp Ther 331: 598–6087
56. De Strooper B, Chávez-Gutiérrez L (2015) Learning by failing: ideas and concepts to tackle γ-secretases in Alzheimer's disease and beyond. Annu Rev Pharmacol Toxicol 55:419–437
57. Doody RS, Raman R, Farlow M, Iwatsubo T, Vellas B, Joffe S et al (2013) A phase 3 trial of semagacestat for treatment of Alzheimer's disease. N Engl J Med 369:341–350
58. Mitani Y, Yarimizu J, Saita K, Uchino H, Akashiba H, Shitaka Y et al (2012) Differential effects between γ-secretase inhibitors and modulators on cognitive function in amyloid precursor protein-transgenic and nontransgenic mice. J Neurosci 32:2037–2050
59. Frisoni GB, Rozzini L, Gozzetti A, Binetti G, Zanetti O, Bianchetti A et al (1999) Behavioral syndromes in Alzheimer's disease: description and correlates. Dement Geriatr Cogn Disord 10:130–138
60. Chung JA, Cummings JL (2000) Neurobehavioral and neuropsychiatric symptoms in Alzheimer's disease. Neurol Clin 18:829–846
61. Yan L, Li L, Han W, Pan B, Xue X, Mei B (2013) Age-related neuropsychiatric symptoms in presenilins conditional double knockout mice. Brain Res Bull 97:104–111
62. Lalonde R, Qian S, Strazielle C (2003) Transgenic mice expressing the PS1-A246E mutation: effects on spatial learning, exploration, anxiety, and motor coordination. Behav Brain Res 138:71–79
63. Yuk DY, Lee YK, Nam SY, Yun YW, Hwang DY, Choi DY et al (2009) Reduced anxiety in the mice expressing mutant (N141I) presenilin 2. J Neurosci Res 87:522–531
64. Luciano M, Houlihan LM, Harris SE, Gow AJ, Hayward C, Starr JM et al (2010) Association of existing and new candidate genes for anxiety, depression and personality traits in older people. Behav Genet 40:518–532
65. Borghys H, Tuefferd M, Van Broeck B, Clessens E, Dillen L, Cools W et al (2012) A canine model to evaluate efficacy and safety of γ-secretase inhibitors and modulators. J Alzheimers Dis 28:809–822
66. Cook JJ, Wildsmith KR, Gilberto DB, Holahan MA, Kinney GG, Mathers PD et al (2010) Acute gamma-secretase inhibition of non-human primate CNS shifts amyloid precursor protein (APP) metabolism from amyloid-beta production to alternative APP fragments without amyloid-beta rebound. J Neurosci 30:6743–6750
67. Lleó A, Cavedo E, Parnetti L, Vanderstichele H, Herukka SK, Andreasen N et al (2015) Cerebrospinal fluid biomarkers in trials for Alzheimer and Parkinson diseases. Nat Rev Neurol 11:41–55
68. Tong G, Wang JS, Sverdlov O, Huang SP, Slemmon R, Croop R et al (2012) Effects of single doses of avagacestat (BMS-708163) on cerebrospinal fluid Aβ levels in healthy young men. Clin Drug Investig 32:761–769
69. Dockens R, Wang JS, Castaneda L, Sverdlov O, Huang SP, Slemmon R et al (2012) A placebo-controlled, multiple ascending dose study to evaluate the safety, pharmacokinetics and pharmacodynamics of avagacestat (BMS-708163) in healthy young and elderly subjects. Clin Pharmacokinet 51:681–693
70. Siemers ER, Dean RA, Friedrich S, Ferguson-Sells L, Gonzales C, Farlow MR et al (2007) Safety, tolerability, and effects on plasma and cerebrospinal fluid amyloid-beta after inhibition of gamma-secretase. Clin Neuropharmacol 30:317–325
71. Bateman RJ, Siemers ER, Mawuenyega KG, Wen G, Browning KR, Sigurdson WC et al (2009) A gamma-secretase inhibitor decreases amyloid-beta production in the central nervous system. Ann Neurol 66:48–54
72. Fleisher AS, Raman R, Siemers ER, Becerra L, Clark CM, Dean RA et al (2008) Phase 2 safety trial targeting amyloid beta production with a gamma-secretase inhibitor in Alzheimer disease. Arch Neurol 65:1031–1038
73. Portelius E, Dean RA, Gustavsson MK, Andreasson U, Zetterberg H, Siemers E et al (2010) A novel Abeta isoform pattern in CSF reflects gamma-secretase inhibition in Alzheimer's disease. Alzheimers Res Ther 2:7
74. Svedružić ŽM, Popović K, Šendula-Jengić V (2013) Modulators of γ-secretase activity can facilitate the toxic side-effects and pathogenesis of Alzheimer's disease. PLoS One 8:e50759
75. Pitsi D, Octave JN (2004) Presenilin 1 stabilizes the C-terminal fragment of the amyloid precursor protein independently of gamma-secretase activity. J Biol Chem 279:25333–25338
76. De Strooper B, Iwatsubo T, Wolfe MS (2012) Presenilins and γ-secretase: structure, function, and role in Alzheimer disease. Cold Spring Harb Perspect Med 2:a006304
77. Stiller I, Lizák B, Bánhegyi G (2014) Physiological functions of presenilins; beyond γ-secretase. Curr Pharm Biotechnol 15:1019–1025
78. Leem JY, Vijayan S, Han P, Cai D, Machura M, Lopes KO et al (2002) Presenilin 1 is required for maturation and cell surface accumulation of nicastrin. J Biol Chem 277:19236–19240
79. Hébert SS, Bourdages V, Godin C, Ferland M, Carreau M, Lévesque G (2003) Presenilin-1 interacts directly with the beta-site amyloid protein precursor cleaving enzyme (BACE1). Neurobiol Dis 13:238–245
80. Silveyra MX, Evin G, Montenegro MF, Vidal CJ, Martínez S, Culvenor JG (2008) Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. Mol Cell Biol 28:2908–2919
81. Leem JY, Saura CA, Pietrzik C, Christianson J, Wanamaker C, King LT (2002) A role for presenilin 1 in regulating the delivery of amyloid precursor protein to the cell surface. Neurobiol Dis 11: 64–82
82. Farquhar MJ, Gray CW, Breen KC (2003) The over-expression of the wild type or mutant forms of the presenilin-1 protein alters glycoprotein processing in a human neuroblastoma cell line. Neurosci Lett 346:53–56
83. De Strooper B (2014) Lessons from a failed γ-secretase Alzheimer trial. Cell 159:721–726
84. Xia W, Wong ST, Hanlon E, Morin P (2012) γ-Secretase modulator in Alzheimer's disease: shifting the end. J Alzheimers Dis 31:685–696
85. Yan R, Vassar R (2014) Targeting the β secretase BACE1 for Alzheimer's disease therapy. Lancet Neurol 13:319–329
86. Lahiri DK, Maloney B, Long JM, Greig NH (2014) Lessons from a BACE1 inhibitor trial: off-site but not off base. Alzheimers Dement 10:S411–S419
87. Gertsik N, Chiu D, Li YM (2015) Complex regulation of γ-secretase: from obligatory to modulatory subunits. Front Aging Neurosci 6:342
88. Naoko Takebe, Dat Nguyen, Sherry X. Yang, (2014) Targeting Notch signaling pathway in cancer: Clinical development advances and challenges. Pharmacol Ther 141(2):140–149

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5 - CONCLUSIONS/CONCLUSIONES

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All the results presented and discussed in this Thesis are summarized in the following points:

1. Levels of highly stable PS1 complexes are increased in the CSF of symptomatic and asymptomatic genetically determined AD subjects, as well as in demented and non-demented Down syndrome individuals, and in sporadic AD and MCI patients. Thus, the increase in PS1 complexes in CSF is an early phenomenon associated to AD pathology and may constitute an asymptomatic biomarker.

2. Aβ oligomers favour the formation of stable PS1 complexes in human CSF.

3. We demonstrate the presence of different species of ADAM10 in human CSF, an immature form retaining the prodomain, a mature unprocessed full-length form and a truncated large soluble form released from the membrane.

4. The different forms of ADAM10 present in CSF are assembled in different types of high molecular weight complexes.

5. Mature species of ADAM10, both full-length and truncated forms, are decreased in sporadic AD CSF and may constitute potential new biomarker for AD.

6. *In vitro* treatment with γ-secretase inhibitors augments cellular protein levels of PS1 and other γ-secretase subunits without increasing mRNA expression.

7. Rats administered with the GSI avagacestat had more PS1 in cerebral cortex. This sustained γ-secretase inhibition fails to exert a long-term effect on PS1 activity proven by decrease in substrates such as the C-terminal fragments (CTFs) of APP and ApoER2.

8. Prolonged avagacestat-treatment of rats produced a subtle impairment in anxiety-like behaviour. The potential association of this phenomenon with the

increase in PS1 require further analysis to determine if it is related with the impaired condition of subjects enrolled GSIs clinical trials.

De los resultados presentados en la presente Memoria de Tesis se extraen las siguientes conclusiones:

1. Los niveles de los complejos altamente estables de PS1 en el LCR están aumentados en los sujetos con EA genéticamente determinada tanto para casos sintomáticos como los asintomáticos, en los individuos con Síndrome de Down con y sin demencia, así como en los pacientes con EA esporádica y deterioro cognitivo leve. Este aumento en los niveles de los complejos altamente estables de PS1 en LCR es un fenómeno que ocurre en un estadio temprano de la enfermedad y, por ello, podría postularse como un potencial biomarcador asintomático.

2. Los oligómeros del péptido Aβ favorecen la formación de los complejos estables de PS1 en LCR humano.

3. Hemos demostrado la presencia de diferentes especies de ADAM10 en LCR humano que corresponden a una forma inmadura que mantiene el prodominio, una forma madura completa no procesada y una forma truncada, probablemente secretada desde la membrana celular.

4. Las distintas formas de ADAM10 presentes en el LCR se encuentran formando complejos de elevado peso molecular.

5. Las formas maduras de ADAM10, tanto la entera como la truncada, están disminuidas en el LCR de pacientes con EA esporádica. De esta forma, las formas maduras de ADAM10 podrían ser un nuevo biomarcador de la EA.

6. El tratamiento *in vitro* con inhibidores de γ-secretasa aumenta los niveles celulares de la proteína PS1, así como de otros componentes del complejo. Sin embargo, esta inhibición en la actividad no produce un aumento en los niveles de RNA mensajero de ninguno de los componentes del complejo.

7. El tratamiento prolongado en ratas con el inhibidor de γ-secretasa avagacestat produce un incremento en los niveles de PS1 en la corteza cerebral. Este tratamiento sostenido falla en la inhibición de la actividad como lo demuestra el descenso en los niveles de sustratos de PS1, como los fragmentos C-terminales (CTFs) de APP y ApoER2.

8. El tratamiento prolongado con avagacestat en ratas resulta en un aumento en la ansiedad de los animales, determinada en test de comportamiento. La posible asociación de este fenómeno con el efecto rebote en PS1 requiere un estudio más completo para determinar si contribuye al deterioro cognitivo de los pacientes tratados en los ensayos clínicos con GSIs.

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6 - BIBLIOGRAPHY

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Agamanolis, D., 2016. Chapter 9, Neuropathology.

Ahmed, R.R., Holler, C.J., Webb, R.L., Li, F., Beckett, T.L., Murphy, M.P., 2010. BACE1 and BACE2 enzymatic activities in Alzheimer's disease. J. Neurochem. 112, 1045–53. https://doi.org/10.1111/j.1471-4159.2009.06528.x

Alonso, A.C., Zaidi, T., Grundke-Iqbal, I., Iqbal, K., 1994. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A. 91, 5562–5566.

Alzheimer, A., Stelzmann, R.A., Schnitzlein, H.N., Murtagh, F.R., 1995. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". Clin. Anat. 8, 429–431. https://doi.org/10.1002/ca.980080612

Anders, A., Gilbert, S., Garten, W., Postina, R., Fahrenholz, F., 2001. Regulation of the alpha-secretase ADAM10 by its prodomain and proprotein convertases. FASEB J. 15, 1837–9.

Andrew, R.J., Kellett, K.A.B., Thinakaran, G., Hooper, N.M., 2016. A Greek Tragedy: The Growing Complexity of Alzheimer Amyloid Precursor Protein Proteolysis. J. Biol. Chem. 291, 19235–44. https://doi.org/10.1074/jbc.R116.746032

Annaert, W., De Strooper, B., 1999. Presenilins: molecular switches between proteolysis and signal transduction. Trends Neurosci. 22, 439–43.

Arendt, T., Stieler, J.T., Holzer, M., 2016. Tau and tauopathies. Brain Res. Bull. 126, 238–292. https://doi.org/10.1016/j.brainresbull.2016.08.018

Asai, M., Hattori, C., Szabó, B., Sasagawa, N., Maruyama, K., Tanuma, S., Ishiura, S., 2003. Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. Biochem. Biophys. Res. Commun. 301, 231–5.

Balasa, M., Gelpi, E., Antonell, A., Rey, M.J., Sanchez-Valle, R., Molinuevo, J.L., Llado, A., Neurological Tissue Bank/University of Barcelona/Hospital Clínic NTB/UB/HC Collaborative Group, 2011. Clinical features and APOE genotype of pathologically

proven early-onset Alzheimer disease. Neurology 76, 1720–1725. https://doi.org/10.1212/WNL.0b013e31821a44dd

Baranger, K., Marchalant, Y., Bonnet, A.E., Crouzin, N., Carrete, A., Paumier, J.-M., Py, N.A., Bernard, A., Bauer, C., Charrat, E., Moschke, K., Seiki, M., Vignes, M., Lichtenthaler, S.F., Checler, F., Khrestchatisky, M., Rivera, S., 2016. MT5-MMP is a new pro-amyloidogenic proteinase that promotes amyloid pathology and cognitive decline in a transgenic mouse model of Alzheimer's disease. Cell. Mol. Life Sci. 73, 217–236. https://doi.org/10.1007/s00018-015-1992-1

Bard, F., Cannon, C., Barbour, R., Burke, R.-L., Games, D., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Lieberburg, I., Motter, R., Nguyen, M., Soriano, F., Vasquez, N., Weiss, K., Welch, B., Seubert, P., Schenk, D., Yednock, T., 2000. Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat. Med. 6, 916–919. https://doi.org/10.1038/78682

Barthet, G., Georgakopoulos, A., Robakis, N.K., 2012. Cellular mechanisms of γ-secretase substrate selection, processing and toxicity. Prog. Neurobiol. 98, 166–75. https://doi.org/10.1016/j.pneurobio.2012.05.006

Bayer, T.A., Cappai, R., Masters, C.L., Beyreuther, K., Multhaup, G., 1999. It all sticks together--the APP-related family of proteins and Alzheimer's disease. Mol. Psychiatry 4, 524–8.

Beel, A.J., Sanders, C.R., 2008. Substrate specificity of γ-secretase and other intramembrane proteases. Cell. Mol. Life Sci. 65, 1311–1334. https://doi.org/10.1007/s00018-008-7462-2

Belyaev, N.D., Kellett, K.A.B., Beckett, C., Makova, N.Z., Revett, T.J., Nalivaeva, N.N., Hooper, N.M., Turner, A.J., 2010. The transcriptionally active amyloid precursor protein (APP) intracellular domain is preferentially produced from the 695 isoform of APP in a {beta}-secretase-dependent pathway. J. Biol. Chem. 285, 41443–54. https://doi.org/10.1074/jbc.M110.141390

Ben Halima, S., Mishra, S., Raja, K.M.P., Willem, M., Baici, A., Simons, K., Brüstle, O., Koch, P., Haass, C., Caflisch, A., Rajendran, L., 2016. Specific Inhibition of β-Secretase Processing of the Alzheimer Disease Amyloid Precursor Protein. Cell Rep. 14, 2127–2141. https://doi.org/10.1016/j.celrep.2016.01.076

Bernier, F., Sato, Y., Matijevic, M., Desmond, H., McGrath, S., Burns, L., Kaplow, J.M., Albala, B., 2013. Clinical study of E2609, a novel BACE1 inhibitor, demonstrates target engagement and inhibition of BACE1 activity in CSF. Alzheimer's Dement. 9, P886. https://doi.org/10.1016/j.jalz.2013.08.244

Beyreuther, K., POLLWEIN, P., MULTHAUP, G., MÖNNING, U., KÖNIG, G., DYRKS, T., SCHUBERT, W., MASTERS, C.L., 1993. Regulation and Expression of the Alzheimer's β/A4 Amyloid Protein Precursor in Health, Disease, and Down's Syndromea. Ann. N. Y. Acad. Sci. 695, 91–102. https://doi.org/10.1111/j.1749-6632.1993.tb23035.x

Billingsley, M.L., Kincaid, R.L., 1997. Regulated phosphorylation and dephosphorylation of tau protein: effects on microtubule interaction, intracellular trafficking and neurodegeneration. Biochem. J. 323 (Pt 3, 577–591.

Blennow, K., 2017. A Review of Fluid Biomarkers for Alzheimer's Disease: Moving from CSF to Blood. Neurol. Ther. 6, 15–24. https://doi.org/10.1007/s40120-017-0073-9

Blennow, K., 2010. Biomarkers in Alzheimer's disease drug development. Nat. Med. 16, 1218–1222. https://doi.org/10.1038/nm.2221

Blennow, K., Hampel, H., Weiner, M., Zetterberg, H., 2010. Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. Nat. Rev. Neurol. 6, 131–144. https://doi.org/10.1038/nrneurol.2010.4

Braak, H., Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 82, 239–59.

Brunkan, A.L., Martinez, M., Walker, E.S., Goate, A.M., 2005. Presenilin endoproteolysis is an intramolecular cleavage. Mol. Cell. Neurosci. 29, 65–73. https://doi.org/10.1016/j.mcn.2004.12.012

Buggia-Prévot, V., Thinakaran, G., 2014. Sorting the role of SORLA in Alzheimer's disease. Sci. Transl. Med. 6, 223fs8. https://doi.org/10.1126/scitranslmed.3008562

Bussière, T., Bard, F., Barbour, R., Grajeda, H., Guido, T., Khan, K., Schenk, D., Games, D., Seubert, P., Buttini, M., 2004. Morphological characterization of Thioflavin-S-positive amyloid plaques in transgenic Alzheimer mice and effect of passive Abeta immunotherapy on their clearance. Am. J. Pathol. 165, 987–95.

Buxbaum, J.D., Liu, K.N., Luo, Y., Slack, J.L., Stocking, K.L., Peschon, J.J., Johnson, R.S., Castner, B.J., Cerretti, D.P., Black, R.A., 1998. Evidence that tumor necrosis factor alpha converting enzyme is involved in regulated alpha-secretase cleavage of the Alzheimer amyloid protein precursor. J. Biol. Chem. 273, 27765–7.

Cao, X., Südhof, T.C., 2001. A transcriptionally [correction of transcriptively] active complex of APP with Fe65 and histone acetyltransferase Tip60. Science 293, 115–20. https://doi.org/10.1126/science.1058783

Castellano, J.M., Kim, J., Stewart, F.R., Jiang, H., DeMattos, R.B., Patterson, B.W., Fagan, A.M., Morris, J.C., Mawuenyega, K.G., Cruchaga, C., Goate, A.M., Bales, K.R., Paul, S.M., Bateman, R.J., Holtzman, D.M., 2011. Human apoE Isoforms Differentially Regulate Brain Amyloid- Peptide Clearance. Sci. Transl. Med. 3, 89ra57-89ra57. https://doi.org/10.1126/scitranslmed.3002156

Cedazo-Minguez, A., Winblad, B., 2010. Biomarkers for Alzheimer's disease and other forms of dementia: Clinical needs, limitations and future aspects. Exp. Gerontol. 45, 5–14. https://doi.org/10.1016/j.exger.2009.09.008

Chouraki, V., Seshadri, S., 2014. Genetics of Alzheimer's Disease, in: Advances in Genetics. pp. 245–294. https://doi.org/10.1016/B978-0-12-800149-3.00005-6

Choy, R.W.-Y., Cheng, Z., Schekman, R., 2012. Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid beta (Abeta) production in the trans-Golgi network. Proc. Natl. Acad. Sci. U. S. A. 109, E2077-82. https://doi.org/10.1073/pnas.1208635109

Chyung, J.H., Raper, D.M., Selkoe, D.J., 2005. Gamma-secretase exists on the plasma membrane as an intact complex that accepts substrates and effects intramembrane cleavage. J. Biol. Chem. 280, 4383–92. https://doi.org/10.1074/jbc.M409272200

Colciaghi, F., Borroni, B., Pastorino, L., Marcello, E., Zimmermann, M., Cattabeni, F., Padovani, A., Di Luca, M., 2002. [alpha]-Secretase ADAM10 as well as [alpha]APPs is reduced in platelets and CSF of Alzheimer disease patients. Mol. Med. 8, 67–74.

Coric, V., Salloway, S., van Dyck, C., Kerselaers, W., Kaplita, S., Curtis, C., Ross, J., Richter, R.W., Andreasen, N., Brody, M., Sharma, S.K., Cedarbaum, J.M., Berman, R., 2013. A phase II study of the gamma-secretase inhibitor avagacestat (BMS-708163) in predementia Alzheimer's disease. Alzheimer's Dement. 9, P283. https://doi.org/10.1016/j.jalz.2013.05.569

Cruchaga, C., Kauwe, J.S.K., Nowotny, P., Bales, K., Pickering, E.H., Mayo, K., Bertelsen, S., Hinrichs, A., Alzheimer's Disease Neuroimaging Initiative, the A.D.N., Fagan, A.M., Holtzman, D.M., Morris, J.C., Goate, A.M., 2012. Cerebrospinal fluid APOE levels: an endophenotype for genetic studies for Alzheimer's disease. Hum. Mol. Genet. 21, 4558–71. https://doi.org/10.1093/hmg/dds296

Darreh-Shori, T., Soininen, H., 2010. Effects of cholinesterase inhibitors on the activities and protein levels of cholinesterases in the cerebrospinal fluid of patients with Alzheimer's disease: a review of recent clinical studies. Curr. Alzheimer Res. 7, 67–73.

De Strooper, B., Karran, E., 2016. The Cellular Phase of Alzheimer's Disease. Cell 164, 603–615. https://doi.org/10.1016/j.cell.2015.12.056

De Strooper, B., 2014. Lessons from a failed γ-secretase Alzheimer trial. Cell 159, 721–6. https://doi.org/10.1016/j.cell.2014.10.016

De Strooper, B., 2007a. Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. EMBO Rep. 8, 141–6. https://doi.org/10.1038/sj.embor.7400897

De Strooper, B., 2007b. Loss-of-function presenilin mutations in Alzheimer disease. Talking Point on the role of presenilin mutations in Alzheimer disease. EMBO Rep. 8, 141–146. https://doi.org/10.1038/sj.embor.7400897

De Strooper, B., 2003. Aph-1, Pen-2, and Nicastrin with Presenilin generate an active??-Secretase complex. Neuron 38, 9–12.

De Strooper, B., Vassar, R., Golde, T., 2010. The secretases: enzymes with therapeutic potential in Alzheimer disease. Nat. Rev. Neurol. 6, 99–107. https://doi.org/10.1038/nrneurol.2009.218

Delacourte, A., David, J.P., Sergeant, N., Buée, L., Wattez, A., Vermersch, P., Ghozali, F., Fallet-Bianco, C., Pasquier, F., Lebert, F., Petit, H., Di Menza, C., 1999. The biochemical pathway of neurofibrillary degeneration in aging and Alzheimer's disease. Neurology 52, 1158–65.

Devos, D., Moreau, C., Devedjian, J.C., Kluza, J., Petrault, M., Laloux, C., Jonneaux, A., Ryckewaert, G., Garçon, G., Rouaix, N., Duhamel, A., Jissendi, P., Dujardin, K., Auger, F., Ravasi, L., Hopes, L., Grolez, G., Firdaus, W., Sablonnière, B., Strubi-Vuillaume, I., Zahr, N., Destée, A., Corvol, J.-C., Pöltl, D., Leist, M., Rose, C., Defebvre, L., Marchetti, P., Cabantchik, Z.I., Bordet, R., 2014. Targeting Chelatable Iron as a Therapeutic Modality in Parkinson's Disease. Antioxid. Redox Signal. 21, 195–210. https://doi.org/10.1089/ars.2013.5593

Díez-Guerra, F.J., 2010. Neurogranin, a link between calcium/calmodulin and protein kinase C signaling in synaptic plasticity. IUBMB Life 62, 597–606. https://doi.org/10.1002/iub.357

Doody, R.S., Raman, R., Farlow, M., Iwatsubo, T., Vellas, B., Joffe, S., Kieburtz, K., He, F., Sun, X., Thomas, R.G., Aisen, P.S., Alzheimer's Disease Cooperative Study Steering Committee, E., Siemers, E., Sethuraman, G., Mohs, R., Semagacestat Study Group, 2013. A phase 3 trial of semagacestat for treatment of Alzheimer's disease. N. Engl. J. Med. 369, 341–50. https://doi.org/10.1056/NEJMoa1210951

Dulin, F., Léveillé, F., Ortega, J.B., Mornon, J.-P., Buisson, A., Callebaut, I., Colloc'h, N.,

2008. P3 peptide, a truncated form of A beta devoid of synaptotoxic effect, does not assemble into soluble oligomers. FEBS Lett. 582, 1865–70. https://doi.org/10.1016/j.febslet.2008.05.002

Edbauer, D., Winkler, E., Haass, C., Steiner, H., 2002. Presenilin and nicastrin regulate each other and determine amyloid beta-peptide production via complex formation. Proc. Natl. Acad. Sci. U. S. A. 99, 8666–8671. https://doi.org/10.1073/pnas.132277899

Ehehalt, R., Keller, P., Haass, C., Thiele, C., Simons, K., 2003. Amyloidogenic processing of the Alzheimer beta-amyloid precursor protein depends on lipid rafts. J. Cell Biol. 160, 113–23. https://doi.org/10.1083/jcb.200207113

El-Agnaf, O.M., Mahil, D.S., Patel, B.P., Austen, B.M., 2000. Oligomerization and toxicity of beta-amyloid-42 implicated in Alzheimer's disease. Biochem. Biophys. Res. Commun. 273, 1003–7. https://doi.org/10.1006/bbrc.2000.3051

Escrevente, C., Morais, V.A., Keller, S., Soares, C.M., Altevogt, P., Costa, J., 2008. Functional role of N-glycosylation from ADAM10 in processing, localization and activity of the enzyme. Biochim. Biophys. Acta 1780, 905–13. https://doi.org/10.1016/j.bbagen.2008.03.004

Fahrenholz, F., Gilbert, S., Kojro, E., Lammich, S., Postina, R., 2000. Alpha-secretase activity of the disintegrin metalloprotease ADAM 10. Influences of domain structure. Ann. N. Y. Acad. Sci. 920, 215–22.

Farquhar, M.J., Gray, C.W., Breen, K.C., 2003. The over-expression of the wild type or mutant forms of the presenilin-1 protein alters glycoprotein processing in a human neuroblastoma cell line. Neurosci. Lett. 346, 53–6.

Fasulo, L., Ugolini, G., Visintin, M., Bradbury, A., Brancolini, C., Verzillo, V., Novak, M., Cattaneo, A., 2000. The neuronal microtubule-associated protein tau is a substrate for caspase-3 and an effector of apoptosis. J. Neurochem. 75, 624–33.

Finder, V.H., 2010. Alzheimer's disease: a general introduction and pathomechanism. J.

Alzheimers. Dis. 22 Suppl 3, 5–19. https://doi.org/10.3233/JAD-2010-100975

Forman, M., Kleijn, H.-J., Dockendorf, M., Palcza, J., Tseng, J., Canales, C., Egan, M., Kennedy, M., Laterza, O., Ma, L., Scott, J., Tanen, M., Apter, J., Backonja, M., Ereshefsky, L., Gevorkyan, H., Jhee, S., Rynders, R., Zari, A., Bryan, E., Wagner, J., Troyer, M., Stone, J., 2013. The novel BACE inhibitor MK-8931 dramatically lowers CSF beta-amyloid in patients with mild-to-moderate Alzheimer's disease. Alzheimer's Dement. 9, P139. https://doi.org/10.1016/j.jalz.2013.04.083

Fortna, R.R., Crystal, A.S., Morais, V.A., Pijak, D.S., Lee, V.M.-Y., Doms, R.W., 2004. Membrane topology and nicastrin-enhanced endoproteolysis of APH-1, a component of the gamma-secretase complex. J. Biol. Chem. 279, 3685–3693. https://doi.org/10.1074/jbc.M310505200

Fu, H., Li, W., Luo, J., Lee, N.T.K., Li, M., Tsim, K.W.K., Pang, Y., Youdim, M.B.H., Han, Y., 2008. Promising anti-Alzheimer's dimer bis(7)-tacrine reduces beta-amyloid generation by directly inhibiting BACE-1 activity. Biochem. Biophys. Res. Commun. 366, 631–6. https://doi.org/10.1016/j.bbrc.2007.11.068

Fukumoto, H., Cheung, B.S., Hyman, B.T., Irizarry, M.C., 2002. Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch. Neurol. 59, 1381–9.

Furukawa, K., Sopher, B.L., Rydel, R.E., Begley, J.G., Pham, D.G., Martin, G.M., Fox, M., Mattson, M.P., 1996. Increased activity-regulating and neuroprotective efficacy of alpha-secretase-derived secreted amyloid precursor protein conferred by a C-terminal heparin-binding domain. J. Neurochem. 67, 1882–96.

Gandy, S., 2005. The role of cerebral amyloid β accumulation in common forms of Alzheimer disease. J. Clin. Invest. 115, 1121–1129. https://doi.org/10.1172/JCI25100

García-Ayllón, M.-S., Campanari, M.-L., Brinkmalm, G., Rábano, A., Alom, J., Saura, C. a, Andreasen, N., Blennow, K., Sáez-Valero, J., 2013. CSF Presenilin-1 complexes are increased in Alzheimer's disease. Acta Neuropathol. Commun. 1, 46. https://doi.org/10.1186/2051-5960-1-46

García-Ayllón, M.-S., Lopez-Font, I., Boix, C.P., Fortea, J., Sánchez-Valle, R., Lleó, A., Molinuevo, J.-L., Zetterberg, H., Blennow, K., Sáez-Valero, J., 2017. C-terminal fragments of the amyloid precursor protein in cerebrospinal fluid as potential biomarkers for Alzheimer disease. Sci. Rep. 7, 2477. https://doi.org/10.1038/s41598-017-02841-7

Garcia-Ayllon, M.-S., Silveyra, M.-X., Andreasen, N., Brimijoin, S., Blennow, K., Saez-Valero, J., 2007. Cerebrospinal fluid acetylcholinesterase changes after treatment with donepezil in patients with Alzheimer's disease. J. Neurochem. 101, 1701–1711. https://doi.org/10.1111/j.1471-4159.2007.04461.x

García-Ayllón, M.-S., Small, D.H., Avila, J., Sáez-Valero, J., 2011. Revisiting the Role of Acetylcholinesterase in Alzheimer's Disease: Cross-Talk with P-tau and β-Amyloid. Front. Mol. Neurosci. 4, 22. https://doi.org/10.3389/fnmol.2011.00022

Georgakopoulos, A., Marambaud, P., Efthimiopoulos, S., Shioi, J., Cui, W., Li, H.C., Schütte, M., Gordon, R., Holstein, G.R., Martinelli, G., Mehta, P., Friedrich, V.L., Robakis, N.K., 1999. Presenilin-1 forms complexes with the cadherin/catenin cell-cell adhesion system and is recruited to intercellular and synaptic contacts. Mol. Cell 4, 893–902.

Giasson, B.I., Lee, V.M.-Y., Trojanowski, J.Q., 2003. Interactions of Amyloidogenic Proteins. NeuroMolecular Med. 4, 49–58. https://doi.org/10.1385/NMM:4:1-2:49

Glenner, G.G., Wong, C.W., 1984. Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein. Biochem. Biophys. Res. Commun. 122, 1131–5.

Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704–706. https://doi.org/10.1038/349704a0

Gold, M., 2017. Phase II clinical trials of anti–amyloid β antibodies: When is enough, enough? Alzheimer's Dement. Transl. Res. Clin. Interv. 3, 402–409. https://doi.org/10.1016/j.trci.2017.04.005

Gómez-Isla, T., Price, J.L., McKeel, D.W., Morris, J.C., Growdon, J.H., Hyman, B.T., 1996. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. J. Neurosci. 16, 4491–500.

Götz, J., Eckert, A., Matamales, M., Ittner, L.M., Liu, X., 2011. Modes of Aβ toxicity in Alzheimer's disease. Cell. Mol. Life Sci. 68, 3359–75. https://doi.org/10.1007/s00018-011-0750-2

Gu, Y., Chen, F., Sanjo, N., Kawarai, T., Hasegawa, H., Duthie, M., Li, W., Ruan, X., Luthra, A., Mount, H.T.J., Tandon, A., Fraser, P.E., St George-Hyslop, P., 2003. APH-1 interacts with mature and immature forms of presenilins and nicastrin and may play a role in maturation of presenilin.nicastrin complexes. J. Biol. Chem. 278, 7374–7380. https://doi.org/10.1074/jbc.M209499200

Guerreiro, R., Hardy, J., 2014. Genetics of Alzheimer's Disease. Neurotherapeutics 11, 732–737. https://doi.org/10.1007/s13311-014-0295-9

Guillozet-Bongaarts, A.L., Garcia-Sierra, F., Reynolds, M.R., Horowitz, P.M., Fu, Y., Wang, T., Cahill, M.E., Bigio, E.H., Berry, R.W., Binder, L.I., 2005. Tau truncation during neurofibrillary tangle evolution in Alzheimer's disease. Neurobiol. Aging 26, 1015–22. https://doi.org/10.1016/j.neurobiolaging.2004.09.019

Haass, C., Kaether, C., Thinakaran, G., Sisodia, S., 2012. Trafficking and proteolytic processing of APP. Cold Spring Harb. Perspect. Med. 2, a006270. https://doi.org/10.1101/cshperspect.a006270

Haass, C., Lemere, C.A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., Selkoe, D.J., 1995. The Swedish mutation causes early-onset Alzheimer's disease by beta-secretase cleavage within the secretory pathway. Nat. Med. 1, 1291–6.

Haass, C., Schlossmacher, M.G., Hung, A.Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B.L., Lieberburg, I., Koo, E.H., Schenk, D., Teplow, D.B., Selkoe, D.J., 1992. Amyloid β-peptide is produced by cultured cells during normal metabolism. Nature 359, 322–325. https://doi.org/10.1038/359322a0

Hardy, J., Allsop, D., 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol. Sci. 12, 383–8.

Hardy, J., Higgins, G.A., 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science 256, 184–5.

Hardy, J., Selkoe, D.J., 2002. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. Science (80-.). 297, 353–356. https://doi.org/10.1126/science.1072994

Hartmann, D., de Strooper, B., Serneels, L., Craessaerts, K., Herreman, A., Annaert, W., Umans, L., Lübke, T., Lena Illert, A., von Figura, K., Saftig, P., 2002. The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum. Mol. Genet. 11, 2615–24.

Hasebe, N., Fujita, Y., Ueno, M., Yoshimura, K., Fujino, Y., Yamashita, T., 2013. Soluble β-amyloid Precursor Protein Alpha binds to p75 neurotrophin receptor to promote neurite outgrowth. PLoS One 8, e82321. https://doi.org/10.1371/journal.pone.0082321

Hemming, M.L., Elias, J.E., Gygi, S.P., Selkoe, D.J., 2008. Proteomic profiling of gamma-secretase substrates and mapping of substrate requirements. PLoS Biol. 6, e257. https://doi.org/10.1371/journal.pbio.0060257

Holler, C.J., Webb, R.L., Laux, A.L., Beckett, T.L., Niedowicz, D.M., Ahmed, R.R., Liu, Y., Simmons, C.R., Dowling, A.L.S., Spinelli, A., Khurgel, M., Estus, S., Head, E., Hersh, L.B., Murphy, M.P., 2012. BACE2 expression increases in human neurodegenerative disease. Am. J. Pathol. 180, 337–50. https://doi.org/10.1016/j.ajpath.2011.09.034

Horikoshi, Y., Mori, T., Maeda, M., Kinoshita, N., Sato, K., Yamaguchi, H., 2004. Aβ N-terminal-end specific antibody reduced β-amyloid in Alzheimer-model mice. Biochem. Biophys. Res. Commun. 325, 384–387. https://doi.org/10.1016/j.bbrc.2004.10.039

Hussain, I., Powell, D., Howlett, D.R., Tew, D.G., Meek, T.D., Chapman, C., Gloger, I.S.,

Murphy, K.E., Southan, C.D., Ryan, D.M., Smith, T.S., Simmons, D.L., Walsh, F.S., Dingwall, C., Christie, G., 1999. Identification of a novel aspartic protease (Asp 2) as beta-secretase. Mol. Cell. Neurosci. 14, 419–27. https://doi.org/10.1006/mcne.1999.0811

Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R.C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J.M., Nowotny, P., Che, L.K., Norton, J., Morris, J.C., Reed, L.A., Trojanowski, J., Basun, H., Lannfelt, L., Neystat, M., Fahn, S., Dark, F., Tannenberg, T., Dodd, P.R., Hayward, N., Kwok, J.B., Schofield, P.R., Andreadis, A., Snowden, J., Craufurd, D., Neary, D., Owen, F., Oostra, B.A., Hardy, J., Goate, A., van Swieten, J., Mann, D., Lynch, T., Heutink, P., 1998. Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702–5. https://doi.org/10.1038/31508

Inoue, M., Hur, J.-Y., Kihara, T., Teranishi, Y., Yamamoto, N.G., Ishikawa, T., Wiehager, B., Winblad, B., Tjernberg, L.O., Schedin-Weiss, S., 2015. Human brain proteins showing neuron-specific interactions with γ-secretase. FEBS J. 282, 2587–2599. https://doi.org/10.1111/febs.13303

Isozaki, T., Nishimi, S., Nishimi, A., Saito, M., Miwa, Y., Toyoshima, Y., Inagaki, K., Kasama, T., 2017. A disintegrin and metalloproteinase (ADAM)-10 as a predictive factor for tocilizumab effectiveness in rheumatoid arthritis. Mod. Rheumatol. 27, 782–786. https://doi.org/10.1080/14397595.2016.1256025

Iwata, N., Tsubuki, S., Takaki, Y., Watanabe, K., Sekiguchi, M., Hosoki, E., Kawashima-Morishima, M., Lee, H.J., Hama, E., Sekine-Aizawa, Y., Saido, T.C., 2000. Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: suppression leads to biochemical and pathological deposition. Nat. Med. 6, 143–50. https://doi.org/10.1038/72237

Jarosz-Griffiths, H.H., Noble, E., Rushworth, J. V, Hooper, N.M., 2016. Amyloid-β Receptors: The Good, the Bad, and the Prion Protein. J. Biol. Chem. 291, 3174–83.

https://doi.org/10.1074/jbc.R115.702704

Johnson, G.V.W., Stoothoff, W.H., 2004. Tau phosphorylation in neuronal cell function and dysfunction. J. Cell Sci. 117, 5721–9. https://doi.org/10.1242/jcs.01558

Jonsson, T., Atwal, J.K., Steinberg, S., Snaedal, J., Jonsson, P. V, Bjornsson, S., Stefansson, H., Sulem, P., Gudbjartsson, D., Maloney, J., Hoyte, K., Gustafson, A., Liu, Y., Lu, Y., Bhangale, T., Graham, R.R., Huttenlocher, J., Bjornsdottir, G., Andreassen, O.A., Jönsson, E.G., Palotie, A., Behrens, T.W., Magnusson, O.T., Kong, A., Thorsteinsdottir, U., Watts, R.J., Stefansson, K., 2012. A mutation in APP protects against Alzheimer's disease and age-related cognitive decline. Nature 488, 96–9. https://doi.org/10.1038/nature11283

Kakuda, N., Funamoto, S., Yagishita, S., Takami, M., Osawa, S., Dohmae, N., Ihara, Y., 2006. Equimolar Production of Amyloid β-Protein and Amyloid Precursor Protein Intracellular Domain from β-Carboxyl-terminal Fragment by γ-Secretase. J. Biol. Chem. 281, 14776–14786. https://doi.org/10.1074/jbc.M513453200

Kalvodova, L., Kahya, N., Schwille, P., Ehehalt, R., Verkade, P., Drechsel, D., Simons, K., 2005. Lipids as modulators of proteolytic activity of BACE: involvement of cholesterol, glycosphingolipids, and anionic phospholipids in vitro. J. Biol. Chem. 280, 36815–23. https://doi.org/10.1074/jbc.M504484200

Kang, E.L., Cameron, A.N., Piazza, F., Walker, K.R., Tesco, G., 2010. Ubiquitin regulates GGA3-mediated degradation of BACE1. J. Biol. Chem. 285, 24108–19. https://doi.org/10.1074/jbc.M109.092742

Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., Müller-Hill, B., 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325, 733–736. https://doi.org/10.1038/325733a0

Karran, E., De Strooper, B., 2016. The amyloid cascade hypothesis: are we poised for success or failure? J. Neurochem. 139, 237–252. https://doi.org/10.1111/jnc.13632

Karran, E., Mercken, M., De Strooper, B., 2011. The amyloid cascade hypothesis for Alzheimer's disease: an appraisal for the development of therapeutics. Nat. Rev. Drug Discov. 10, 698–712. https://doi.org/10.1038/nrd3505

Kinoshita, A., Fukumoto, H., Shah, T., Whelan, C.M., Irizarry, M.C., Hyman, B.T., 2003. Demonstration by FRET of BACE interaction with the amyloid precursor protein at the cell surface and in early endosomes. J. Cell Sci. 116, 3339–3346. https://doi.org/10.1242/jcs.00643

Knops, J., Suomensaari, S., Lee, M., McConlogue, L., Seubert, P., Sinha, S., 1995. Cell-type and amyloid precursor protein-type specific inhibition of A beta release by bafilomycin A1, a selective inhibitor of vacuolar ATPases. J. Biol. Chem. 270, 2419–22.

Koedam, E.L.G.E., Lauffer, V., van der Vlies, A.E., van der Flier, W.M., Scheltens, P., Pijnenburg, Y.A.L., 2010. Early-Versus Late-Onset Alzheimer's Disease: More than Age Alone. J. Alzheimer's Dis. 19, 1401–1408. https://doi.org/10.3233/JAD-2010-1337

Koelsch, G., 2017. BACE1 Function and Inhibition: Implications of Intervention in the Amyloid Pathway of Alzheimer's Disease Pathology. Molecules 22, 1723. https://doi.org/10.3390/molecules22101723

Koh, Y.H., von Arnim, C.A.F., Hyman, B.T., Tanzi, R.E., Tesco, G., 2005. BACE is degraded via the lysosomal pathway. J. Biol. Chem. 280, 32499–504. https://doi.org/10.1074/jbc.M506199200

Koo, E.H., Squazzo, S.L., 1994. Evidence that production and release of amyloid beta-protein involves the endocytic pathway. J. Biol. Chem. 269, 17386–9.

Kuhn, P.-H., Wang, H., Dislich, B., Colombo, A., Zeitschel, U., Ellwart, J.W., Kremmer, E., Roßner, S., Lichtenthaler, S.F., 2010. ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. EMBO J. 29, 3020–3032. https://doi.org/10.1038/emboj.2010.167

Kummer, M.P., Heneka, M.T., 2014. Truncated and modified amyloid-beta species. Alzheimers. Res. Ther. 6, 28. https://doi.org/10.1186/alzrt258

Kvartsberg, H., Duits, F.H., Ingelsson, M., Andreasen, N., Öhrfelt, A., Andersson, K., Brinkmalm, G., Lannfelt, L., Minthon, L., Hansson, O., Andreasson, U., Teunissen, C.E., Scheltens, P., Van der Flier, W.M., Zetterberg, H., Portelius, E., Blennow, K., 2015. Cerebrospinal fluid levels of the synaptic protein neurogranin correlates with cognitive decline in prodromal Alzheimer's disease. Alzheimer's Dement. 11, 1180–1190. https://doi.org/10.1016/j.jalz.2014.10.009

Lahiri, D.K., Maloney, B., Long, J.M., Greig, N.H., 2014. Lessons from a BACE1 inhibitor trial: off-site but not off base. Alzheimers. Dement. 10, S411-9. https://doi.org/10.1016/j.jalz.2013.11.004

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., Fahrenholz, F., 1999. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc. Natl. Acad. Sci. U. S. A. 96, 3922–7.

Lannfelt, L., Basun, H., Wahlund, L.O., Rowe, B.A., Wagner, S.L., 1995. Decreased alpha-secretase-cleaved amyloid precursor protein as a diagnostic marker for Alzheimer's disease. Nat. Med. 1, 829–32.

Lau, P., Bossers, K., Janky, R., Salta, E., Frigerio, C.S., Barbash, S., Rothman, R., Sierksma, A.S.R., Thathiah, A., Greenberg, D., Papadopoulou, A.S., Achsel, T., Ayoubi, T., Soreq, H., Verhaagen, J., Swaab, D.F., Aerts, S., De Strooper, B., 2013. Alteration of the microRNA network during the progression of Alzheimer's disease. EMBO Mol. Med. 5, 1613–1634. https://doi.org/10.1002/emmm.201201974

Laudon, H., Hansson, E.M., Melen, K., Bergman, A., Farmery, M.R., Winblad, B., Lendahl, U., von Heijne, G., Naslund, J., 2005. A nine-transmembrane domain topology for presenilin 1. J. Biol. Chem. 280, 35352–35360. https://doi.org/10.1074/jbc.M507217200

Law, A., Gauthier, S., Quirion, R., 2001. Say NO to Alzheimer's disease: the putative links

between nitric oxide and dementia of the Alzheimer's type. Brain Res. Brain Res. Rev. 35, 73–96.

Lee, E.B., Zhang, B., Liu, K., Greenbaum, E.A., Doms, R.W., Trojanowski, J.Q., Lee, V.M.-Y., 2005. BACE overexpression alters the subcellular processing of APP and inhibits Abeta deposition in vivo. J. Cell Biol. 168, 291–302. https://doi.org/10.1083/jcb.200407070

Lee, M.K., Slunt, H.H., Martin, L.J., Thinakaran, G., Kim, G., Gandy, S.E., Seeger, M., Koo, E., Price, D.L., Sisodia, S.S., 1996. Expression of presenilin 1 and 2 (PS1 and PS2) in human and murine tissues. J. Neurosci. 16, 7513–25.

Levitan, D., Lee, J., Song, L., Manning, R., Wong, G., Parker, E., Zhang, L., 2001. PS1 N- and C-terminal fragments form a complex that functions in APP processing and Notch signaling. Proc. Natl. Acad. Sci. 98, 12186–12190. https://doi.org/10.1073/pnas.211321898

Leyns, C.E.G., Holtzman, D.M., 2017. Glial contributions to neurodegeneration in tauopathies. Mol. Neurodegener. 12, 50. https://doi.org/10.1186/s13024-017-0192-x

Li, T., Li, Y.-M., Ahn, K., Price, D.L., Sisodia, S.S., Wong, P.C., 2011. Increased Expression of PS1 Is Sufficient to Elevate the Level and Activity of γ-Secretase In Vivo. PLoS One 6, e28179. https://doi.org/10.1371/journal.pone.0028179

Li, Y., Bohm, C., Dodd, R., Chen, F., Qamar, S., Schmitt-Ulms, G., Fraser, P.E., St George-Hyslop, P.H., 2014. Structural biology of presenilin 1 complexes. Mol. Neurodegener. 9, 59. https://doi.org/10.1186/1750-1326-9-59

Lichtenthaler, S.F., 2011. Alpha-secretase in Alzheimer's disease: molecular identity, regulation and therapeutic potential. J. Neurochem. 116, 10–21. https://doi.org/10.1111/j.1471-4159.2010.07081.x

Liu, C.-C., Kanekiyo, T., Xu, H., Bu, G., 2013. Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy. Nat. Rev. Neurol. 9, 106–118.

https://doi.org/10.1038/nrneurol.2012.263

Lleó, A., Cavedo, E., Parnetti, L., Vanderstichele, H., Herukka, S.K., Andreasen, N., Ghidoni, R., Lewczuk, P., Jeromin, A., Winblad, B., Tsolaki, M., Mroczko, B., Visser, P.J., Santana, I., Svenningsson, P., Blennow, K., Aarsland, D., Molinuevo, J.L., Zetterberg, H., Mollenhauer, B., 2014. Cerebrospinal fluid biomarkers in trials for Alzheimer and Parkinson diseases. Nat. Rev. Neurol. 11, 41–55. https://doi.org/10.1038/nrneurol.2014.232

Lleó, A., Saura, C.A., 2011. γ-secretase substrates and their implications for drug development in Alzheimer's disease. Curr. Top. Med. Chem. 11, 1513–27.

Lopez-Font, I., Cuchillo-Ibañez, I., Sogorb-Esteve, A., García-Ayllón, M.-S., Sáez-Valero, J., 2015. Transmembrane amyloid-related proteins in CSF as potential biomarkers for Alzheimer's disease. Front. Neurol. 6. https://doi.org/10.3389/fneur.2015.00125

Lu, J.-X., Qiang, W., Yau, W.-M., Schwieters, C.D., Meredith, S.C., Tycko, R., 2013. Molecular structure of β-amyloid fibrils in Alzheimer's disease brain tissue. Cell 154, 1257–68. https://doi.org/10.1016/j.cell.2013.08.035

Lv, Z.-Y., Tan, C.-C., Yu, J.-T., Tan, L., 2017. Spreading of Pathology in Alzheimer's Disease. Neurotox. Res. https://doi.org/10.1007/s12640-017-9765-2

Mahley, R.W., Huang, Y., 2012. Apolipoprotein E Sets the Stage: Response to Injury Triggers Neuropathology. Neuron 76, 871–885. https://doi.org/10.1016/j.neuron.2012.11.020

Marcello, E., Epis, R., Saraceno, C., Gardoni, F., Borroni, B., Cattabeni, F., Padovani, A., Di Luca, M., 2012. SAP97-mediated local trafficking is altered in Alzheimer disease patients' hippocampus. Neurobiol. Aging 33, 422.e1-422.e10. https://doi.org/10.1016/j.neurobiolaging.2010.09.015

Marcinkiewicz, M., Seidah, N.G., 2000. Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain. J. Neurochem. 75, 2133–43.

Matarin, M., Salih, D.A., Yasvoina, M., Cummings, D.M., Guelfi, S., Liu, W., Nahaboo Solim, M.A., Moens, T.G., Paublete, R.M., Ali, S.S., Perona, M., Desai, R., Smith, K.J., Latcham, J., Fulleylove, M., Richardson, J.C., Hardy, J., Edwards, F.A., 2015. A Genome-wide Gene-Expression Analysis and Database in Transgenic Mice during Development of Amyloid or Tau Pathology. Cell Rep. 10, 633–644. https://doi.org/10.1016/j.celrep.2014.12.041

Mendez, M.F., 2012. Early-onset Alzheimer's Disease: Nonamnestic Subtypes and Type 2 AD. Arch. Med. Res. 43, 677–685. https://doi.org/10.1016/j.arcmed.2012.11.009

Miners, J.S., Barua, N., Kehoe, P.G., Gill, S., Love, S., 2011. Abeta-degrading enzymes: potential for treatment of Alzheimer disease. J. Neuropathol. Exp. Neurol. 70, 944–959. https://doi.org/10.1097/NEN.0b013e3182345e46

Montoliu-Gaya, L., Villegas, S., 2016. Aβ-Immunotherapeutic strategies: a wide range of approaches for Alzheimer's disease treatment. Expert Rev. Mol. Med. 18, e13. https://doi.org/10.1017/erm.2016.11

Musardo, S., Marcello, E., 2017. Synaptic dysfunction in Alzheimer's disease: From the role of amyloid β-peptide to the α-secretase ADAM10. Eur. J. Pharmacol. 817, 30–37. https://doi.org/10.1016/j.ejphar.2017.06.018

Naruse, S., Thinakaran, G., Luo, J.J., Kusiak, J.W., Tomita, T., Iwatsubo, T., Qian, X., Ginty, D.D., Price, D.L., Borchelt, D.R., Wong, P.C., Sisodia, S.S., 1998. Effects of PS1 deficiency on membrane protein trafficking in neurons. Neuron 21, 1213–21.

Nathalie, P., Jean-Noël, O., 2008. Processing of amyloid precursor protein and amyloid peptide neurotoxicity. Curr. Alzheimer Res. 5, 92–9.

Nilsberth, C., Luthman, J., Lannfelt, L., Schultzberg, M., 1999. Expression of presenilin 1 mRNA in rat peripheral organs and brain. Histochem. J. 31, 515–23.

Norton, S., Matthews, F.E., Barnes, D.E., Yaffe, K., Brayne, C., 2014. Potential for primary prevention of Alzheimer's disease: an analysis of population-based data. Lancet Neurol. 13, 788–794. https://doi.org/10.1016/S1474-4422(14)70136-X

Nuydens, R., Dispersyn, G., de Jong, M., van den Kieboom, G., Borgers, M., Geerts, H., 1997. Aberrant tau phosphorylation and neurite retraction during NGF deprivation in PC12 cells. Biochem. Biophys. Res. Commun. 240, 687–91. https://doi.org/10.1006/bbrc.1997.7721

Ossenkoppele, R., Schonhaut, D.R., Baker, S.L., O'Neil, J.P., Janabi, M., Ghosh, P.M., Santos, M., Miller, Z.A., Bettcher, B.M., Gorno-Tempini, M.L., Miller, B.L., Jagust, W.J., Rabinovici, G.D., 2015. Tau, amyloid, and hypometabolism in a patient with posterior cortical atrophy. Ann. Neurol. 77, 338–42. https://doi.org/10.1002/ana.24321

Parks, A.L., Curtis, D., 2007. Presenilin diversifies its portfolio. Trends Genet. 23, 140–150. https://doi.org/10.1016/j.tig.2007.01.008

Perani, D., Della Rosa, P.A., Cerami, C., Gallivanone, F., Fallanca, F., Vanoli, E.G., Panzacchi, A., Nobili, F., Pappatà, S., Marcone, A., Garibotto, V., Castiglioni, I., Magnani, G., Cappa, S.F., Gianolli, L., EADC-PET Consortium, 2014. Validation of an optimized SPM procedure for FDG-PET in dementia diagnosis in a clinical setting. NeuroImage Clin. 6, 445–454. https://doi.org/10.1016/j.nicl.2014.10.009

Pievani, M., de Haan, W., Wu, T., Seeley, W.W., Frisoni, G.B., 2011. Functional network disruption in the degenerative dementias. Lancet. Neurol. 10, 829–43. https://doi.org/10.1016/S1474-4422(11)70158-2

Podlisny, M.B., Citron, M., Amarante, P., Sherrington, R., Xia, W., Zhang, J., Diehl, T., Levesque, G., Fraser, P., Haass, C., Koo, E.H., Seubert, P., St George-Hyslop, P., Teplow, D.B., Selkoe, D.J., 1997. Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue. Neurobiol. Dis. 3, 325–337. https://doi.org/10.1006/nbdi.1997.0129

Portelius, E. et al., 2017. The Aβ5–40/Aβ1–34 ratio reflects BACE1 inhibition in human cerebrospinal fluid., in: 11th International Conference on Alzheimer's & Parkinson's Diseases.

Puig, K.L., Combs, C.K., 2013. Expression and function of APP and its metabolites outside the central nervous system. Exp. Gerontol. 48, 608–611. https://doi.org/10.1016/J.EXGER.2012.07.009

Rabano, A., Jimenez-Huete, A., Acevedo, B., Calero, M., Ghiso, J., Valdes, I., Gavilondo, J., Frangione, B., Mendez, E., 2005. Diversity of Senile Plaques in Alzheimers Disease as Revealed by a New Monoclonal Antibody that Recognizes an Internal Sequence of the Aβ Peptide. Curr. Alzheimer Res. 2, 409–417. https://doi.org/10.2174/156720505774330500

Racchi, M., Mazzucchelli, M., Porrello, E., Lanni, C., Govoni, S., 2004. Acetylcholinesterase inhibitors: novel activities of old molecules. Pharmacol. Res. 50, 441–51. https://doi.org/10.1016/j.phrs.2003.12.027

Rajendran, L., Honsho, M., Zahn, T.R., Keller, P., Geiger, K.D., Verkade, P., Simons, K., 2006. Alzheimer's disease beta-amyloid peptides are released in association with exosomes. Proc. Natl. Acad. Sci. U. S. A. 103, 11172–7. https://doi.org/10.1073/pnas.0603838103

Ramirez-Bermudez, J., 2012. Alzheimer's Disease: Critical Notes on the History of a Medical Concept. Arch. Med. Res. 43, 595–599. https://doi.org/10.1016/j.arcmed.2012.11.008

Rapoport, M., Dawson, H.N., Binder, L.I., Vitek, M.P., Ferreira, A., 2002. Tau is essential to beta -amyloid-induced neurotoxicity. Proc. Natl. Acad. Sci. U. S. A. 99, 6364–9. https://doi.org/10.1073/pnas.092136199

Rodríguez-Martín, T., Cuchillo-Ibáñez, I., Noble, W., Nyenya, F., Anderton, B.H., Hanger, D.P., 2013. Tau phosphorylation affects its axonal transport and degradation. Neurobiol. Aging 34, 2146–2157. https://doi.org/10.1016/j.neurobiolaging.2013.03.015

Rosen, C., Hansson, O., Blennow, K., Zetterberg, H., 2013. Fluid biomarkers in Alzheimer's disease - current concepts. Mol. Neurodegener. 8, 20. https://doi.org/10.1186/1750-1326-8-20

Russu, A., Samtani, M.N., Xu, S., Adedokun, O.J., Lu, M., Ito, K., Corrigan, B., Raje, S., Liu, E., Brashear, H.R., Styren, S., Hu, C., 2016. Biomarker Exposure-Response Analysis in Mild-To-Moderate Alzheimer's Disease Trials of Bapineuzumab. J. Alzheimer's Dis. 53, 535–546. https://doi.org/10.3233/JAD-151065

Saftig, P., Lichtenthaler, S.F., 2015. The alpha secretase ADAM10: A metalloprotease with multiple functions in the brain. Prog. Neurobiol. 135, 1–20. https://doi.org/10.1016/J.PNEUROBIO.2015.10.003

Sato, T., Diehl, T.S., Narayanan, S., Funamoto, S., Ihara, Y., De Strooper, B., Steiner, H., Haass, C., Wolfe, M.S., 2007. Active gamma-secretase complexes contain only one of each component. J. Biol. Chem. 282, 33985–33993. https://doi.org/10.1074/jbc.M705248200

Saura, C.A., Tomita, T., Davenport, F., Harris, C.L., Iwatsubo, T., Thinakaran, G., 1999. Evidence that intramolecular associations between presenilin domains are obligatory for endoproteolytic processing. J. Biol. Chem. 274, 13818–13823.

Sberna, G., Saez-Valero, J., Li, Q.X., Czech, C., Beyreuther, K., Masters, C.L., McLean, C.A., Small, D.H., 1998. Acetylcholinesterase is increased in the brains of transgenic mice expressing the C-terminal fragment (CT100) of the beta-amyloid protein precursor of Alzheimer's disease. J. Neurochem. 71, 723–731.

Scheltens, P., Blennow, K., Breteler, M.M.B., de Strooper, B., Frisoni, G.B., Salloway, S., Van der Flier, W.M. aria, 2016. Alzheimer's disease. Lancet (London, England) 388, 505–517. https://doi.org/10.1016/S0140-6736(15)01124-1

Schindler, S.E., Fagan, A.M., 2015. Autosomal Dominant Alzheimer Disease: A Unique Resource to Study CSF Biomarker Changes in Preclinical AD. Front. Neurol. 6, 1–7. https://doi.org/10.3389/fneur.2015.00142

Schneider, A., Rajendran, L., Honsho, M., Gralle, M., Donnert, G., Wouters, F., Hell, S.W., Simons, M., 2008. Flotillin-dependent clustering of the amyloid precursor protein regulates its endocytosis and amyloidogenic processing in neurons. J. Neurosci. 28, 2874–82. https://doi.org/10.1523/JNEUROSCI.5345-07.2008

Scholz, T., Mandelkow, E., 2014. Transport and diffusion of Tau protein in neurons. Cell. Mol. Life Sci. 71, 3139–3150. https://doi.org/10.1007/s00018-014-1610-7

Selkoe, D.J., 1991. The molecular pathology of Alzheimer's disease. Neuron 6, 487–98.

Seubert, P., Oltersdorf, T., Lee, M.G., Barbour, R., Blomquist, C., Davis, D.L., Bryant, K., Fritz, L.C., Galasko, D., Thal, L.J., Lieberburg, I., Schenk, D.B., 1993. Secretion of beta-amyloid precursor protein cleaved at the amino terminus of the beta-amyloid peptide. Nature 361, 260–3. https://doi.org/10.1038/361260a0

Sevigny, J., Chiao, P., Bussière, T., Weinreb, P.H., Williams, L., Maier, M., Dunstan, R., Salloway, S., Chen, T., Ling, Y., O'Gorman, J., Qian, F., Arastu, M., Li, M., Chollate, S., Brennan, M.S., Quintero-Monzon, O., Scannevin, R.H., Arnold, H.M., Engber, T., Rhodes, K., Ferrero, J., Hang, Y., Mikulskis, A., Grimm, J., Hock, C., Nitsch, R.M., Sandrock, A., 2016. The antibody aducanumab reduces Aβ plaques in Alzheimer's disease. Nature 537, 50–56. https://doi.org/10.1038/nature19323

Shepherd, C., McCann, H., Halliday, G.M., 2009. Variations in the neuropathology of familial Alzheimer's disease. Acta Neuropathol. 118, 37–52. https://doi.org/10.1007/s00401-009-0521-4

Silveyra, M.-X., Evin, G., Montenegro, M.-F., Vidal, C.J., Martínez, S., Culvenor, J.G., Sáez-Valero, J., 2008. Presenilin 1 interacts with acetylcholinesterase and alters its enzymatic activity and glycosylation. Mol. Cell. Biol. 28, 2908–2919. https://doi.org/10.1128/MCB.02065-07

Silveyra, M.-X., García-Ayllón, M.-S., Serra-Basante, C., Mazzoni, V., García-Gutierrez, M.-S., Manzanares, J., Culvenor, J.G., Sáez-Valero, J., 2012. Changes in acetylcholinesterase expression are associated with altered presenilin-1 levels. Neurobiol. Aging 33, 627.e27-627.e37. https://doi.org/10.1016/j.neurobiolaging.2011.04.006

Sindou, P., Couratier, P., Barthe, D., Hugon, J., 1992. A dose-dependent increase of Tau immunostaining is produced by glutamate toxicity in primary neuronal cultures. Brain Res. 572, 242–246.

Sinha, S., Anderson, J.P., Barbour, R., Basi, G.S., Caccavello, R., Davis, D., Doan, M., Dovey, H.F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S.M., Wang, S., Walker, D., Zhao, J., McConlogue, L., John, V., 1999. Purification and cloning of amyloid precursor protein beta-secretase from human brain. Nature 402, 537–40. https://doi.org/10.1038/990114

Skovronsky, D.M., Moore, D.B., Milla, M.E., Doms, R.W., Lee, V.M., 2000. Protein kinase C-dependent alpha-secretase competes with beta-secretase for cleavage of amyloid-beta precursor protein in the trans-golgi network. J. Biol. Chem. 275, 2568–75.

Sokolow, S., Henkins, K.M., Bilousova, T., Gonzalez, B., Vinters, H. V, Miller, C.A., Cornwell, L., Poon, W.W., Gylys, K.H., 2015. Pre-synaptic C-terminal truncated tau is released from cortical synapses in Alzheimer's disease. J. Neurochem. 133, 368–79. https://doi.org/10.1111/jnc.12991

Soriano, S., Kang, D.E., Fu, M., Pestell, R., Chevallier, N., Zheng, H., Koo, E.H., 2001. Presenilin 1 negatively regulates beta-catenin/T cell factor/lymphoid enhancer factor-1 signaling independently of beta-amyloid precursor protein and notch processing. J. Cell Biol. 152, 785–94.

Spires-Jones, T.L., Stoothoff, W.H., de Calignon, A., Jones, P.B., Hyman, B.T., 2009. Tau pathophysiology in neurodegeneration: a tangled issue. Trends Neurosci. 32, 150–159. https://doi.org/10.1016/j.tins.2008.11.007

Steiner, H., Fluhrer, R., Haass, C., 2008. Intramembrane proteolysis by gamma-secretase. J. Biol. Chem. 283, 29627–31. https://doi.org/10.1074/jbc.R800010200

Sun, Y., Lai, M.-S., Lu, C.-J., Chen, R.-C., 2008. How long can patients with mild or moderate Alzheimer's dementia maintain both the cognition and the therapy of cholinesterase inhibitors: a national population-based study. Eur. J. Neurol. 15, 278–83. https://doi.org/10.1111/j.1468-1331.2007.02049.x

Tagami, S., Yanagida, K., Kodama, T.S., Takami, M., Mizuta, N., Oyama, H., Nishitomi, K., Chiu, Y.-W., Okamoto, T., Ikeuchi, T., Sakaguchi, G., Kudo, T., Matsuura, Y., Fukumori,

A., Takeda, M., Ihara, Y., Okochi, M., 2017. Semagacestat Is a Pseudo-Inhibitor of γ-Secretase. Cell Rep. 21, 259–273. https://doi.org/10.1016/j.celrep.2017.09.032

Takami, M., Nagashima, Y., Sano, Y., Ishihara, S., Morishima-Kawashima, M., Funamoto, S., Ihara, Y., 2009. gamma-Secretase: successive tripeptide and tetrapeptide release from the transmembrane domain of beta-carboxyl terminal fragment. J. Neurosci. 29, 13042–13052. https://doi.org/10.1523/JNEUROSCI.2362-09.2009

Takasugi, N., Tomita, T., Hayashi, I., 2003. The role of presenilin cofactors in the g -secretase complex 422, 2114–2117.

Takebe, N., Nguyen, D., Yang, S.X., 2014. Targeting notch signaling pathway in cancer: clinical development advances and challenges. Pharmacol. Ther. 141, 140–9. https://doi.org/10.1016/j.pharmthera.2013.09.005

Toh, W.H., Gleeson, P.A., 2016. Dysregulation of intracellular trafficking and endosomal sorting in Alzheimer's disease: controversies and unanswered questions. Biochem. J. 473, 1977–93. https://doi.org/10.1042/BCJ20160147

Tong, G., Castaneda, L., Wang, J.-S., Sverdlov, O., Huang, S.-P., Slemmon, R., Gu, H., Wong, O., Li, H., Berman, R.M., Smith, C., Albright, C., Dockens, R.C., 2012. Effects of Single Doses of Avagacestat (BMS-708163) on Cerebrospinal Fluid Aβ Levels in Healthy Young Men. Clin. Drug Investig. 32, 761–769. https://doi.org/10.1007/s40261-012-0006-4

Tousseyn, T., Thathiah, A., Jorissen, E., Raemaekers, T., Konietzko, U., Reiss, K., Maes, E., Snellinx, A., Serneels, L., Nyabi, O., Annaert, W., Saftig, P., Hartmann, D., De Strooper, B., 2009. ADAM10, the rate-limiting protease of regulated intramembrane proteolysis of Notch and other proteins, is processed by ADAMS-9, ADAMS-15, and the gamma-secretase. J. Biol. Chem. 284, 11738–47. https://doi.org/10.1074/jbc.M805894200

Tu, H., Nelson, O., Bezprozvanny, A., Wang, Z., Lee, S.-F., Hao, Y.-H., Serneels, L., De Strooper, B., Yu, G., Bezprozvanny, I., 2006. Presenilins form ER Ca2+ leak channels, a function disrupted by familial Alzheimer's disease-linked mutations. Cell 126, 981–

93. https://doi.org/10.1016/j.cell.2006.06.059

Udayar, V., Buggia-Prévot, V., Guerreiro, R.L., Siegel, G., Rambabu, N., Soohoo, A.L., Ponnusamy, M., Siegenthaler, B., Bali, J., AESG, M., Simons, M., Ries, J., Puthenveedu, M.A., Hardy, J., Thinakaran, G., Rajendran, L., Brás, J., Sassi, C., Gibbs, J.R., Hernandez, D., Lupton, M.K., Brown, K., Morgan, K., Powell, J., Singleton, A., Hardy, J., 2013. A paired RNAi and RabGAP overexpression screen identifies Rab11 as a regulator of β-amyloid production. Cell Rep. 5, 1536–51. https://doi.org/10.1016/j.celrep.2013.12.005

van Dyck, C.H., 2017. Anti-Amyloid-β Monoclonal Antibodies for Alzheimer's Disease: Pitfalls and Promise. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2017.08.010

Vassar, R., 2004. BACE1: the beta-secretase enzyme in Alzheimer's disease. J. Mol. Neurosci. 23, 105–114. https://doi.org/10.1385/JMN:23:1-2:105

Vassar, R., Bennett, B.D., Babu-Khan, S., Kahn, S., Mendiaz, E.A., Denis, P., Teplow, D.B., Ross, S., Amarante, P., Loeloff, R., Luo, Y., Fisher, S., Fuller, J., Edenson, S., Lile, J., Jarosinski, M.A., Biere, A.L., Curran, E., Burgess, T., Louis, J.C., Collins, F., Treanor, J., Rogers, G., Citron, M., 1999. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. Science 286, 735–41.

Vassar, R., Kovacs, D.M., Yan, R., Wong, P.C., 2009. The beta-secretase enzyme BACE in health and Alzheimer's disease: regulation, cell biology, function, and therapeutic potential. J. Neurosci. 29, 12787–94. https://doi.org/10.1523/JNEUROSCI.3657-09.2009

Vassar, R., Kuhn, P.-H., Haass, C., Kennedy, M.E., Rajendran, L., Wong, P.C., Lichtenthaler, S.F., 2014. Function, therapeutic potential and cell biology of BACE proteases: current status and future prospects. J. Neurochem. 130, 4–28. https://doi.org/10.1111/jnc.12715

Villemagne, V.L., Fodero-Tavoletti, M.T., Masters, C.L., Rowe, C.C., 2015. Tau imaging:

early progress and future directions. Lancet Neurol. 14, 114–124. https://doi.org/10.1016/S1474-4422(14)70252-2

Wakabayashi, T., De Strooper, B., 2008. Presenilins: members of the gamma-secretase quartets, but part-time soloists too. Physiology (Bethesda). 23, 194–204. https://doi.org/10.1152/physiol.00009.2008

Walkiewicz, K., Nowakowska-Zajdel, E., Strzelczyk, J., Dzięgielewska-Gęsiak, S., Muc-Wierzgoń, M., 2017. Serum levels of ADAM10, ADAM12, ADAM17 AND ADAM28 in colorectal cancer patients. J. Biol. Regul. Homeost. Agents 31, 929–934.

Wang, J.-Z., Xia, Y.-Y., Grundke-Iqbal, I., Iqbal, K., 2013. Abnormal hyperphosphorylation of tau: sites, regulation, and molecular mechanism of neurofibrillary degeneration. J. Alzheimers. Dis. 33 Suppl 1, S123-39. https://doi.org/10.3233/JAD-2012-129031

Willem, M., Tahirovic, S., Busche, M.A., Ovsepian, S. V., Chafai, M., Kootar, S., Hornburg, D., Evans, L.D.B., Moore, S., Daria, A., Hampel, H., Müller, V., Giudici, C., Nuscher, B., Wenninger-Weinzierl, A., Kremmer, E., Heneka, M.T., Thal, D.R., Giedraitis, V., Lannfelt, L., Müller, U., Livesey, F.J., Meissner, F., Herms, J., Konnerth, A., Marie, H., Haass, C., 2015. η-Secretase processing of APP inhibits neuronal activity in the hippocampus. Nature 526, 443–447. https://doi.org/10.1038/nature14864

Wischik, C.M., Novak, M., Edwards, P.C., Klug, A., Tichelaar, W., Crowther, R.A., 1988a. Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A. 85, 4884–8.

Wischik, C.M., Novak, M., Thøgersen, H.C., Edwards, P.C., Runswick, M.J., Jakes, R., Walker, J.E., Milstein, C., Roth, M., Klug, A., 1988b. Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. Proc. Natl. Acad. Sci. U. S. A. 85, 4506–10.

Wolfe, M.S., 2012. γ-Secretase as a Target for Alzheimer's Disease, in: Advances in Pharmacology (San Diego, Calif.). pp. 127–153. https://doi.org/10.1016/B978-0-12-394816-8.00004-0

Wolfe, M.S., 2007. When loss is gain: reduced presenilin proteolytic function leads to increased Abeta42/Abeta40. Talking Point on the role of presenilin mutations in Alzheimer disease. EMBO Rep. 8, 136–40. https://doi.org/10.1038/sj.embor.7400896

Wolfe, M.S., Xia, W., Ostaszewski, B.L., Diehl, T.S., Kimberly, W.T., Selkoe, D.J., 1999. Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity. Nature 398, 513–517. https://doi.org/10.1038/19077

Wong, H.-K.A., Veremeyko, T., Patel, N., Lemere, C.A., Walsh, D.M., Esau, C., Vanderburg, C., Krichevsky, A.M., 2013. De-repression of FOXO3a death axis by microRNA-132 and -212 causes neuronal apoptosis in Alzheimer's disease. Hum. Mol. Genet. 22, 3077–92. https://doi.org/10.1093/hmg/ddt164

Xia, W., Wong, S.T., Hanlon, E., Morin, P., 2012. γ-Secretase modulator in Alzheimer's disease: shifting the end. J. Alzheimers. Dis. 31, 685–96. https://doi.org/10.3233/JAD-2012-120751

Yan, R., Bienkowski, M.J., Shuck, M.E., Miao, H., Tory, M.C., Pauley, A.M., Brashier, J.R., Stratman, N.C., Mathews, W.R., Buhl, A.E., Carter, D.B., Tomasselli, A.G., Parodi, L.A., Heinrikson, R.L., Gurney, M.E., 1999. Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity. Nature 402, 533–7. https://doi.org/10.1038/990107

Yan, R., Vassar, R., 2014. Targeting the β secretase BACE1 for Alzheimer's disease therapy. Lancet. Neurol. 13, 319–29. https://doi.org/10.1016/S1474-4422(13)70276-X

Yang, L.-B., Lindholm, K., Yan, R., Citron, M., Xia, W., Yang, X.-L., Beach, T., Sue, L., Wong, P., Price, D., Li, R., Shen, Y., 2003. Elevated β-secretase expression and enzymatic activity detected in sporadic Alzheimer disease. Nat. Med. 9, 3–4. https://doi.org/10.1038/nm0103-3

Ye, X., Feng, T., Tammineni, P., Chang, Q., Jeong, Y.Y., Margolis, D.J., Cai, H., Kusnecov, A., Cai, Q., 2017. Regulation of Synaptic Amyloid-β Generation through BACE1

Retrograde Transport in a Mouse Model of Alzheimer's Disease. J. Neurosci. 37, 2639–2655. https://doi.org/10.1523/JNEUROSCI.2851-16.2017

Yuan, X., Wu, H., Xu, H., Xiong, H., Chu, Q., Yu, S., Wu, G.S., Wu, K., 2015. Notch signaling: an emerging therapeutic target for cancer treatment. Cancer Lett. 369, 20–7. https://doi.org/10.1016/j.canlet.2015.07.048

Zhang, B., Gaiteri, C., Bodea, L.-G., Wang, Z., McElwee, J., Podtelezhnikov, A.A., Zhang, C., Xie, T., Tran, L., Dobrin, R., Fluder, E., Clurman, B., Melquist, S., Narayanan, M., Suver, C., Shah, H., Mahajan, M., Gillis, T., Mysore, J., MacDonald, M.E., Lamb, J.R., Bennett, D.A., Molony, C., Stone, D.J., Gudnason, V., Myers, A.J., Schadt, E.E., Neumann, H., Zhu, J., Emilsson, V., 2013. Integrated Systems Approach Identifies Genetic Nodes and Networks in Late-Onset Alzheimer's Disease. Cell 153, 707–720. https://doi.org/10.1016/j.cell.2013.03.030

Zhang, C., Wu, B., Beglopoulos, V., Wines-Samuelson, M., Zhang, D., Dragatsis, I., Südhof, T.C., Shen, J., 2009. Presenilins are essential for regulating neurotransmitter release. Nature 460, 632–6. https://doi.org/10.1038/nature08177

Zhang, Z., Song, M., Liu, X., Su Kang, S., Duong, D.M., Seyfried, N.T., Cao, X., Cheng, L., Sun, Y.E., Ping Yu, S., Jia, J., Levey, A.I., Ye, K., 2015. Delta-secretase cleaves amyloid precursor protein and regulates the pathogenesis in Alzheimer's disease. Nat. Commun. 6, 8762. https://doi.org/10.1038/ncomms9762

Zhao, J., Paganini, L., Mucke, L., Gordon, M., Refolo, L., Carman, M., Sinha, S., Oltersdorf, T., Lieberburg, I., McConlogue, L., 1996. Beta-secretase processing of the beta-amyloid precursor protein in transgenic mice is efficient in neurons but inefficient in astrocytes. J. Biol. Chem. 271, 31407–11.

Zhou, L., Barão, S., Laga, M., Bockstael, K., Borgers, M., Gijsen, H., Annaert, W., Moechars, D., Mercken, M., Gevaert, K., Gevaer, K., De Strooper, B., 2012. The neural cell adhesion molecules L1 and CHL1 are cleaved by BACE1 protease in vivo. J. Biol. Chem. 287, 25927–40. https://doi.org/10.1074/jbc.M112.377465

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Aunque no lo recuerdes, aunque olvides,

no permitas que la oscuridad oculte lo único que es cierto:

existes porque te quieren, existes porque los quieres.

Aunque tu no lo sepas.

Poesía para el Alzheimer. Elvira Sastre.

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