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SCHOOL OF THE USC

Marta
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PhD Thesis

Discriminating between mild
cognitive impairment and
Alzheimer's disease: Validation
of early Alzheimer's biomarkers
by combining advanced imaging
and in vitro diagnostic
techniques.

Santiago de Compostela, 2023

DOCTORAL THESIS

**Discriminating between mild cognitive impairment and
Alzheimer's disease: Validation of early Alzheimer's
biomarkers by combining advanced imaging and
In vitro diagnostic techniques**

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INTERNATIONAL DOCTORAL SCHOOL OF UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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Título da tese: **Discriminating between mild cognitive impairment and Alzheimer's disease: Validation of early Alzheimer's biomarkers by combining advanced imaging and *in vitro* diagnostic techniques.**

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The author, Marta Aramburu Núñez, declares no conflict of interest in relation to the Doctoral thesis named:

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ABBREVIATIONS

AD: Alzheimer's disease
ADAS-Cog: Alzheimer's disease Assessment Scale-Cognitive Subscale
ABCA1: ATP-binding cassette transporter A1
A β : Amyloid beta protein
APP: Amyloid precursor protein
Ach: acetylcholine
AChEIs: Acetylcholinesterase inhibitors
APOE: Apolipoprotein E
AGD: Argyrophilic grain disease
aCSF: Artificial cerebrospinal fluid
BBB: Blood brain barrier
BB: Blocking buffer
BNT: Boston naming test
BRSKs: Brain-specific kinases 1 and 2
Bs: Brainstem
BSA: Bovine serum albumin
 β -AR: β -adrenergic receptor
 β -CTF: β -C-terminal fragment
BD: Biodistribution
BACE1: β -secretase 1
CAMCOG: Cambridge Cognition Examination
CaMKII: Calmodulin-dependent protein kinase II
Cb: Cerebellum
CB: Coating buffer
CBD: Corticobasal degeneration
Cdk5: Cyclin-dependent kinase 5
Chk1: Checkpoint kinase 1
CLEIA: Chemiluminescent enzyme immunoassay
CMA: Chaperone-mediated autophagy
CSF: Cerebrospinal fluid
CNS: Central nervous system
CDR: Clinical dementia rating
COL: Cut-off level
Cx: Cortex
CK1: Casein kinase 1
CV: Coefficient of variation
DMT: Disease modifying treatment
DUBs: Deubiquitinases
ECL: Electro-chemiluminescence
ELISA: Enzyme-linked immunosorbent assay
EOAD: Early onset of Alzheimer's disease
FDA: Food and Drug Administration
FDG-PET: Fluorodeoxyglucose-PET
FERMT2: Fermitin family homolog 2
FTD: Frontotemporal dementia
FTDP-17: Parkinsonism linked to chromosome 17

FTLD: Frontotemporal lobar degeneration
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GSK3: Glycogen synthase kinase 3
GSK-3 β : Glycogen synthase kinase
GWAS: Genome-wide association studies
HDAC6: Histone deacetylase 6
Hc: Hippocampus
Hsp: Heat shock protein
htau: Human tau
IA: Intraarterial
IHC: Immunohistochemistry
ICV: Intracerebroventricular
IP: Intraperitoneal
IV: Intravenous
LMW: Low-molecular-weight
LOD: Limit of detection
LOAD: Late onset of Alzheimer's disease
LOQ: Limit of quantification
LTP: Long-term synaptic plasticity
LXR: Liver X nuclear receptors
mAb: monoclonal antibody
MCI: Mild cognitive impairment
MB: Methylene blue
MNPs: Magnetic nanoparticles
MR: Magnetoresistive
MRI: Magnetic resonance imaging
MS: Mass spectrometry
MSD: Meso Scale Discovery platform
MAPT: Microtubule associated tau protein
MMSE: Mini-Mental State Examination
MAPK: p38 mitogen-activated protein kinase
MARKs: Microtubule affinity-regulating kinases
MTBR: Microtubules binding repeat
MTL: Medial temporal lobes
MTBD: Microtubules binding domain
MTBR: microtubule-binding region of tau
NE: Neuronal outer envelope
NCT: Nucleocytoplasmic transport
NFTs: Neurofibrillary tangles
NPs: Nanoparticles
NMDA: N-methyl-D-aspartate
PET: Positron emission tomography
PSEN1: Presenilin 1
PSP: Progressive supranuclear palsy
PID: Pick's disease
PP: Protein phosphatases
PB: Phosphate buffer
PSEN2: Presenilin 2

PK: Pharmacokinetics
PKA: Cyclic AMP-dependent protein kinase
PEG: Polyethylene glycol
PHFs: Paired helical filaments
PKC: protein kinase C
p-tau: Phosphorylated tau
PTMs: Post-translational modifications
POC: Point-of-care
RT: Room temperature
ROS: Reactive oxygen species
RAGE: Advanced glycation end products
SCET: the symptomatic cognitive enhancing treatment
SIRT1: Sirtuin 1 deacetylase
SIMOA: Single molecule array
Sc: Spinal cord
SC: Subcutaneous
SUV: Standardized Uptake Value
SV: Spin-valve
TAs: therapeutic antibodies
TNF α : Tumor necrosis factor alpha
TREM2: Triggering receptor expressed on myeloid cells 2
t-tau: Total tau
TAIs: Tau aggregation inhibitors
TMT: Trail making test
TTK: Tau-tubulin kinases 1 and 2
TUNEL: Terminal Transferase dUTP Nick End Labeling
TMB: 3,3',5,5'-tetrametilbencidina
UPS: Ubiquitin- proteasome system
WB: Western Blot

RESUMO

A enfermidade de Alzheimer (EA) é o tipo máis común de demencia, comprendendo entre o 60 e o 80 % dos casos. É unha enfermidade neurodexenerativa que cursa cun deterioro cognitivo progresivo máis aló do xerado polo envellecemento habitual. Este deterioro cognitivo, a miúdo, vai asociado a trastornos emocionais e a un detrimento no comportamento social dos doentes. O avance da enfermidade conleva unha perda de autonomía e funcionalidade que limita a vida cotidiá dos doentes con EA.

Atendendo á súa incidencia, a magnitude desta enfermidade queda patente. Da cordo á Organización Mundial da Saúde (OMS), existen máis de 47 millóns de persoas que sofren de demencia, dos que ao redor duns 800 mil residen en España. Dos case 10 millóns de novos casos que se dan cada ano a nivel global, preto do 25% son diagnosticados en Europa. A prevalencia da enfermidade na poboación maior de 60 anos está próxima ao 6%. Debido ao envellecemento progresivo da poboación, estímase que globalmente haberá máis de 100 millóns de doentes con demencia no 2050.

En asociación co gran impacto negativo na vida cotidiá que ten a enfermidade durante os derradeiros anos de vida dos doentes, a mortalidade debida á EA pasa habitualmente a un segundo plano. Neste senso, estímase que a taxa de mortalidade da EA estaría infraestimada en 5 veces. Aínda así e a modo de exemplo, nun país como Estados Unidos, a EA suporía medio millón de mortes anuais. Debido ao aumento progresivo da incidencia e prevalencia da EA polo envellecemento da poboación, así como a falta de tratamentos modificadores do curso da enfermidade, hoxe en día a EA segue sendo unha das poucas causas de morte cuxa tendencia segue á alza.

A nivel socioeconómico, os costes derivados do manexo desta enfermidade supoñen máis de 1 billón de dólares anuais, estimándose que esta cifra será duplicada no 2030. Por outro lado, moitos dos doentes con EA precisan dun coidado constante que xeralmente recae en persoas do entorno familiar que non reciben unha remuneración. En consecuencia, esta enfermidade supón unha repercusión socio-sanitaria que transcende máis aló do propio doente. Estímase que este impacto socioeconómico e sanitario vai acentuarse nos vindeiros anos debido ás tendencias demográficas actuais. Deste xeito, en rexións altamente envellecidas coma Galicia, onde a poboación maior de 65 anos na próxima década acercarse ao 40%, pódese deducir a magnitude da situación, polo que compre a necesidade de desenvolver plans específicos de actuacións fronte a EA e outras demencias.

A etioloxía da EA é parcialmente descoñecida, xa que unicamente unha porcentaxe mínima dos casos (ao redor do 1%) débense a mutacións nos xenes da proteína precursora amiloide (APP) e nos xenes das encimas responsables do seu procesamento (PSEN1 e PSEN2). Na actualidade, a pesares de toda a investigación na EA, descoñécese a causa etiolóxica da maioría dos casos, máis a implicación de mecanismos asociados ao metabolismo enerxético, a dexeneración sináptica, o estrés oxidativo, a disfunción mitocondrial e a neuroinflamación foron amplamente descritos. Aínda que os procesos etiopatoxénicos primordiais non foron descritos con exactitude, as dúas hipóteses máis aceptadas pola comunidade biomédica fan foco sobre alteracións na proteína β amiloide e na proteína tau.

A hipótese máis estudada é a da proteína β -amiloide. Esta hipótese propón que existe un incorrecto procesamento da proteína β -amiloide, por parte da encima γ -secretasa, que xera uns fragmentos de 42 aminoácidos denominados péptidos amiloide (A β 1-42). Estes péptidos agréganse en estruturas de maior orde ata formar depósitos extracelulares insolubles denominadas placas amiloides ou seniles. Este proceso induce toxicidade a nivel sináptico, un

incremento do estrés oxidativo, un desequilibrio na neurotransmisión ou a activación de mecanismos neuroinflamatorios, entre outros.

A hipótese baseada na proteína tau propón que a súa hiperfosforilación aberrante induce unha agregación progresiva, conlevando a formación dos nobelos neurofibrilares intracelulares característicos da EA. A función da proteína tau está fortemente regulada a través de modificacións postraduccionais (PTMs) como a fosforilación, acetilación, glicosilación, ubiquitinación, glicación, nitración, SUMOilación, oxidación, metilación, escisión e truncamento. Especialmente, as fosforilacións ou acetilacións participan na regulación da expresión e funcionalidade de tau e, polo tanto, poden estar asociadas a patoloxía subxacentes. Neste senso, a hiperfosforilación de tau provoca a síntese de distintas isoformas, coma son os oligómeros de tau fosforilada que desestabilizan e desintegran os microtúbulos, impedindo o transporte axonal e causando, en último termo, disfunción sináptica ata finalmente provocar a atrofia de distintas rexións cerebrais. Estudos recentes evidencian unha maior correlación entre a progresión do deterioro cognitivo asociado EA cos depósitos cerebrais de tau fosforilada, que cos de β -amiloide, polo que, hoxe en día, a hipótese baseada na proteína tau é a máis estudada.

Na actualidade só están dispoñibles 4 fármacos sintomáticos empregados para paliar os síntomas que manifestan os doentes con EA. Pola contra, atoparíanse os tratamentos modificadores da enfermidade (TME), fármacos que actuarían sobre as bases biolóxicas podendo interromper ou enlentecer o curso evolutivo da enfermidade. Ata hai moi pouco tempo, todas as tentativas de encontrar un fármaco deste tipo fracasaron en diversos ensaios clínicos debido a súa ineficacia ou a problemas de seguridade, salvo os recentes fármacos biolóxicos, anticorpos monoclonais fronte a β -amiloide, que amosaron certo beneficio terapéutico en doentes especificamente seleccionados. Espérase que nos vindeiros meses as Axencias Regulatorias aprobaran algún destes fármacos contra a EA.

Os continuos fracasos dos múltiples ensaios clínicos puxeron o foco de atención na diagnose precoz da enfermidade. Dende o punto de vista clínico, a EA tende a ser unha enfermidade infradiagnosticada nun 20-25%, xa que o diagnóstico definitivo só é posible tras evidenciar placas seniles ou nobelos neurofibrilares intracelulares nunha análise *post-mortem*. Asimesmo, a diagnose baseada nos criterios clínicos e biomarcadores actuais ten lugar nun momento no que xa se comezaron observar fenotípicamente as manifestacións dos procesos fisiopatolóxicos subxacentes. Estímase que as acumulacións de proteína tau e β amiloide comezan a producirse entre 10 e 20 anos antes de que aparezan os primeiros síntomas neuropsicolóxicos ou clínicos. En consecuencia, é imperativo o desenvolvemento de avances enfocados cara á detección do Alzheimer en etapas temperás. Neste eido, na práctica clínica habitual establecéronse os denominados criterios ATN para a clasificación dos distintos estadios da EA, coa finalidade de contribuír a un mellor manexo e diagnóstico temperá posible. Esta iniciativa dos criterios ATN basea a clasificación dos estadios do Alzheimer en relación á identificación duns determinados biomarcadores (distintas isoformas das proteínas tau e β -amiloide) en mostras de Líquido Cefalorraquídeo (LCR); hipometabolismo cerebral medido por fluorodesoxiglicosa-PET, e un patrón de neurodexeneración e atrofia cerebral medido por imaxe de resonancia magnética (MRI). Se ben queda un longo camiño por percorrer cara acadar a diagnose precoz do Alzheimer, máis se cabe empregando mostras biolóxicas non invasivas, coma o sangue, coa finalidade de facer un screening en poboación de risco, semella imperativo potenciar as iniciativas cara á diagnose temperá do Alzheimer. Así, recentes estudos puxeron de manifesto a estreita relación entre os niveis plasmáticos de proteína tau fosforilada e o estadio da enfermidade. Todo isto, xunto cos fracasos nos ensaios

clínicos baseados na hipótese amiloide, fundamentan un enfoque no que a proteína tau é a chave para a diagnose e tratamento na EA.

En base ao anteriormente exposto, plantéxase un estudo para o desenvolvemento de técnicas avanzadas de diagnóstico *in vitro* (Enzyme-linked immunosorbent assay, ELISA) e *in vivo* (radiotrazador neuroimaxe PET) baseadas en modificacións específicas da proteína tau. Especificamente, o obxectivo principal deste estudo foi o desenvolvemento dun kit de ELISA de diagnóstico *in vitro* para a detección e cuantificación, en mostras de LCR, de isoformas específicas de tau (fosforilación da serina 208 (pS208) e a acetilación na lisina 174 (acK174)), empregando dous novos anticorpos monoclonais deseñados e desenvolto polo noso grupo, anti-ptau B6 e anti-actau E11, coa finalidade de facer unha proba de concepto clínica para testar a súa capacidade diagnóstica. As modificacións de pS208 e acK174 relacionáronse coa asociación microtubular, agregación e disfunción inicial na eliminación de tau, podendo estar involucradas en fases precoces da EA.

En paralelo, desenvolveuse unha proba de concepto preclínica *in vivo* cun novo radiofármaco para detección de p-tau intracerebral por PET. Para acadar este obxectivo, propúxose a combinación do anti-ptau B6 con Zirconio-89 (⁸⁹Zr), radiotrazador de gran vida media empregado en clínica para a marcaxe de anticorpos, para a determinación de depósitos cerebrais de p-tau mediante PET.

Finalmente, debido a que atopamos propiedades terapéuticas subxacentes ao anti-ptau B6, testamos a eficacia e seguridade nun estudio preclínico de inmunoterapia para determinar o efecto terapéutico da administración intracerebroventricular (ICV) do anti-ptau B6 nun modelo de rato transxénico humanizado con tauopatía (hTau.P301S) que expresa a isoforma mutante de tau humano (P301S) baixo control do promotor murino Thy1 (expresión específica en neuronas). Débese especificar que os estudos preclínicos *in vivo* de inmunoterapia e PET só se levaron a cabo co anticorpo anti-ptau B6, posto que non existen modelos animais que expresen a isoforma de tau acetilada (acK174) recoñecida polo anticorpo anti-actau E11.

Para o desenvolvemento dos kits de ELISA de diagnóstico *in vitro* para a detección e cuantificación, en mostras de LCR, de pS208 e acK174-tau, leváronse a cabo os seguintes pasos:

- Identificación de pares de anticorpos, que actuarán coma primarios ou de captura, compatibles cos anticorpos anti-ptau B6 e anti-actau E11 para o desenvolvemento dun ELISA tipo Sándwich.
- Optimización do protocolo para a detección e cuantificación de antíxenos sintéticos (péptidos de tau que conteñen pS208 e acK174) a través da validación dos distintos elementos do ELISA (péptidos sintéticos, anticorpos, reactivos, tampóns, placas, substratos, protocolos de lavado, etc.).
- Validación da funcionalidade do ELISA na detección e cuantificación de pS208 e acK174-tau en mostras biolóxicas, que son mostras complexas que poden afectar á especificidade ou comprometer o límite de detección do test ELISA.
- Validación destes novos kits de diagnóstico *in vitro* en mostras biolóxicas (LCR) de doentes en distintos estadios de EA. Asímesmo, compárase a capacidade diagnóstica destes kits de ELISA para a determinación de pS208 e acK174 tau co referente actual do diagnóstico *in vitro*, Sistema Lumipulse de Fujirebio.

O deseño final dos kits foi un ELISA tipo Sándwich, empregando os anticorpos monoclonais anti-ptau B6 e anti-actau E11 coma anticorpos de captura, conxuntamente cos pares seleccionados coma anticorpos de detección, BT2Biotin e BT2Biotin combinado con HT7Biotin, respectivamente. A razón de empregar anticorpos primarios biotinilados foi evitar

a necesidade de anticorpos secundarios, xa que tanto os anticorpos de captura como os de detección son murinos. Para o posterior revelado do sinal de captura, despois de distintos cribados con varios compostos conxugados HRP, procedemos cun conxugado encimático estreptavidina-HRP. A continuación, para definir o tipo de revelado óptimo do kit de ELISA, empregáronse métodos colorimétricos e de luminescencia, comparando a súa capacidade de detección pS208 e acK174 tau en mostras de LCR de doentes con deterioro cognitivo lixeiro (DCL) e EA (Protocolo aprobado polo CEIC de Galicia (Código: 2013/553) e as mostras biolóxicas incluídas na Colección C.0003517). Aínda que os datos obtidos confirmaron unha elevada relación sinal/ruído para ambos métodos de detección, o substrato de luminescencia demostrou un poder discriminativo máis amplo que o colorimétrico, polo que foi o finalmente seleccionado. En paralelo, optimizáronse secuencialmente todos os pasos adicionais do ELISA, coma os ciclos de lavado, as condicións de incubación e as concentracións dos diferentes reactivos (solución de lavado, solución de parada, diluínte, matriz de LCR para definición de patróns, etc.). Na medida do posible, empregáronse reactivos comerciais de alta calidade e dos mesmos lotes coa finalidade de reducir calquera variación entre ensaios. Os límites de detección acadados foron de 1,2 ng/mL para pS208-tau e 3,4 ng/mL para o en mostras de LCR. Os coeficientes de variación intra e inter-ensaio do ELISA de pS208-tau foron de 3,4 e 4,5%, respectivamente. No caso do ELISA de acK174-tau, os coeficientes de variación intra e inter-ensaio foron do 5,2 e 6,5%, respectivamente. Estes límites de detección e coeficientes de variación foron óptimos para a detección destas isoformas de tau (pS208 e acK174) en mostras de LCR de doentes con DCL e EA, confirmando a idoneidade destes ensaios para futuras aplicacións clínicas no diagnóstico precoz da EA.

Unha vez definida a configuración final dos kits de ELISA para a determinación e cuantificación de pS208 e acK174 tau en mostras de LCR, procedeuse a unha proba de concepto de validación clínica empregando o Biobanco de EA do Laboratorio de Investigación en Neurociencias Clínicas (LINC) do Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS). Realizamos un estudo clínico-molecular prospectivo e observacional de non intervención nunha poboación de doentes con DCL, EA e suxeitos control. A selección e inclusión de doentes e suxeitos control realizouse na Unidade de Neuroloxía Cognitiva do Servizo de Neuroloxía do Complexo Hospitalario Universitario de Santiago de Compostela (CHUS). Ao tratarse dun estudo de non intervención, o seguimento e control dos factores de risco realizouse nas mellores condicións posibles independentemente dos resultados das probas realizadas. Os suxeitos do estudo foron avaliados mediante un protocolo común de neuroimaxe, molecular e neurolóxico cognitivo profundo. O período de seguimento foi de case 4 anos con visitas anuais. O protocolo foi aprobado polo CEIC de Galicia (Código: 2013/553) e as mostras biolóxicas incluídas na Colección C.0003517. Para a diagnose de DCL debido a EA, EA prodrómica ou EA seguíronse as “Guías de Práctica Clínica en Demencias” da Sociedade Española de Neuroloxía (SEN) do 2018, aplicando os criterios AA-NIA (The National Institute on Aging and the Alzheimer’s Association) + firma biolóxica de biomarcadores neuropatolóxicos A β 1-40 y A β 1-42, tau total (t-tau) e pT181-tau en LCR determinados polo Sistema Lumipulse de Fujirebio.

Incluímos 89 doentes diagnosticados inicialmente como DCL. A idade media foi de 70 $\pm$ 7,1 anos e o 59,6% foron mulleres. O tempo medio de seguimento foi de 3,9 anos. Dos 89 pacientes, o 68,6% non progresaron á EA; o 20,2% (18) evolucionou a EA prodrómica; e o 10,2% (10) progresaron a EA moderada. Todos os biomarcadores de tau (pS208 e acK174 tau), así como os de t-Tau e p-Tau181 do sistema Lumipulse de Fujirebio (referencia) mostraron o mesmo comportamento, cun aumento dos seus niveis en LCR a medida que avanza a enfermidade, aínda que os resultados non foron estatisticamente significativos

debido ao tamaño da mostra e á dispersión dos datos. Polo tanto, son necesarios futuros ensaios clínicos multicéntricos para validar estes primeiros resultados que mostran a potencialidade dos kits de ELISA (pS208 e acK174 tau) para a diagnose precoz da EA.

Para poder facer un kit de screening poboacional é preciso determinar o biomarcador de interese nunha mostra biolóxica non invasiva coma pode ser a saliva, o sangue, ouriños ou as bágoas. Por iso, co fin de aumentar a sensibilidade da técnica para a detección dunha das isoformas de tau, especificamente a pS208, estableceuse unha colaboración internacional co Laboratorio Ibérico de Nanotecnoloxía (INL) de Braga, Portugal, adaptando o kit de inmunodiagnóstico desenvolvido ata ese momento a unha plataforma de diagnóstico co uso de Biochips Magnetoresistentes (MR). Nesta plataforma realizáronse diferentes técnicas de detección de pS208-tau, empregando nanopartículas magnéticas (MNP) biofuncionalizadas para axustar a sensibilidade dentro de rangos a nivel de pg/mL. A pesar de que os diferentes ensaios mostraron unha alta desviación estándar, o sistema de microfluídica da plataforma mostrou unha diferenza sinal/ruído suficiente para diferenciar a mostra do control negativo. O límite de detección obtido tras varias optimizacións mostrou un ruído de fondo baixo para mostras de LCR artificial cun sinal mínimo de 1 µg/mL de p-tauS208, maior ao límite de detección acadado polo ELISA Sándwich de 1,2 ng/mL.

En paralelo ao desenvolvemento de kits de inmunodiagnóstico *in vitro*, realizamos unha proba de concepto preclínica *in vivo* cun novo radiofármaco para detección de p-tau intracerebral por PET. Para acadar este obxectivo, empregamos a combinación do anticorpo anti-ptau B6 con ^{89}Zr , radiotrazador de gran vida media empregado en clínica para a marcaxe de anticorpos. Coma este tipo de radiofármacos adminístranse por vía sistémica, é preciso determinar a súa potencial toxicidade. En consecuencia, fixemos un estudo para avaliar a toxicidade *in vivo* da administración sistémica do anticorpo anti-ptau B6 en ratos transxénicos hTau.P301S e controis. A tinción con hematoxilina/eosina non revelou ningunha anomalía estrutural en mostras de tecido renal e hepático analizadas tanto en ratos transxénicos hTau.P301S coma controis. A marcaxe de caspasa-3 e a técnica Terminal Transferase dUTP Nick End Labeling (TUNEL), empregadas para a determinación de morte celular e apoptose respectivamente, mostraron que a inmunoterapia anti-ptau B6 non foi tóxica, ao revelarse a ausencia de células positivas para caspasa-3 e TUNEL en mostras de tecido renal, hepático e de parénquima cerebral. En canto á avaliación da toxicidade *in vitro*, levouse a cabo en neuronas, incubadas durante 24 horas con concentracións crecentes de anti-ptau B6 e sobredose de β -amiloide coma control positivo da apoptose neuronal mediante a activación da caspasa-3. Demostrouse que o anticorpo anti-ptau B6 non foi tóxico para as neuronas, xa que non se induciu unha apoptose neuronal significativa.

Unha vez demostrado que o anticorpo anti-ptau B6 non era tóxico, realizamos un estudo de proba de concepto *in vivo* baseado nun novo axente de contraste para a determinación de p-tau cerebral, radiomarcando tanto o anticorpo anti-ptau B6 completo coma o fragmento do anticorpo F(ab')₂, fragmentado mediante dixestión enzimática, e acoplado ao radiotrazador ^{89}Zr . Para iso, unimos tanto o anticorpo completo coma os seus fragmentos á deferoxamina, quelante que presenta altísima afinidade ao ^{89}Zr . O rendemento desta reacción foi cuantificado por iTLC, mostrando que máis do 90% do ^{89}Zr estaba unido ao anticorpo ou ao seu fragmento F(ab')₂. Tras purificación en HPLC, a porcentaxe dos conxugados ^{89}Zr -F(ab')₂ ou ^{89}Zr -anti-ptau B6 finalmente administrados *in vivo* sempre superaron ao 98%. Previa á radiomarcaxe e posterior administración nos modelos preclínicos, sempre se realizou un control de calidade, mediante ELISA directo, para demostrar a correcta funcionalidade do anti-ptau B6 e dos fragmentos F(ab')₂ libres ou quelados con deferoxamina e ^{89}Zr .

Os diferentes estudos realizados confirmaron que o complexo $^{[89]}\text{Zr}$ -anti-ptau B6 permanece en circulación ata a lo menos 7 días, garantindo a estabilidade do conxugado e a biodisponibilidade do anticorpo para chegar á periferia ou entrar no cerebro en función do perfil farmacocinético. Estudos de biodistribución do $^{[89]}\text{Zr}$ -anti-ptau B6 confirmaron a presenza ínfimo do anticorpo anti-ptau B6 no cerebro entre 0,055 e 0,114% ID/g, consistente con taxas de cruzamento limitado da Barreira Hematoencefálica (BHE) doutras IgG reportadas na literatura. Despois de 7 días, a maior parte do anticorpo permaneceu na circulación, o fígado e os riles (ademais do esqueleto, a columna vertebral e os músculos). De interés é o feito que o cerebro foi o único órgano onde os niveis de anticorpo anti-ptau B6 aumentaron ao longo do seguimento ata o 0,2%. Pola contra, o perfil farmacocinético do $^{[89]}\text{Zr}$ -F(ab')₂ mostra que está menos de 24 horas en circulación, o que podería afectar negativamente á biodisponibilidade necesaria para cruzar a BHE e alcanzar o tecido cerebral. Ademais, a biodistribución observada mediante PET, así coma a análise *ex vivo*, confirmou a rápida acumulación do fragmento F(ab')₂ de B6 principalmente nos riles, en contraste coa acumulación no fígado do anticorpo B6 completo. Ademais, a penetración cerebral do $^{[89]}\text{Zr}$ -F(ab')₂ en ratos controis é moi limitada, menor que a do anticorpo B6 completo, sendo inferior a <0,01 % ID/g. Sen embargo, en ratos transxénicos o $^{[89]}\text{Zr}$ -F(ab')₂ mostrou marcaxe a nivel cerebral durante a lo menos 48 horas en comparación con ratos controis. Estes resultados demostran que o anticorpo anti-ptau B6 ten potencialidade terapéutica, reconece especificamente p-tau, pode detectarse mediante metodoloxías de neuroimaxe non invasivas como o PET, non ten efectos tóxicos *in vitro* e *in vivo*, é estable na circulación, e ten capacidade terapéutica, aínda que a cantidade que alcanza o cerebro é inferior ao <0,2%.

Finalmente, debido ás intrínsecas propiedades terapéuticas subxacentes ao anti-ptau B6 e á ausencia de toxicidade, realizamos un estudo de proba de concepto preclínico *in vivo* para determinar o efecto terapéutico da administración ICV durante 4 semanas do anti-ptau B6 nun modelo de rato transxénico humanizado con tauopatía (hTau.P301S) (CEEA nº de procedemento:15011/2021/04). Decidiuse empregar a administración ICV ao ser traslacional clinicamente e solventar a limitación do cruce da BHE. Realizáronse diferentes probas de conduta e motricidade, como o Rotarod, Claspings e Grid Hanging, mostrando efectos terapéuticos positivos nos ratos tratados con anti-ptau B6. Tamén se evidenciou que os animais tratados con anti-ptau B6 tiveron unha menor perda de peso corporal. Cabe destacar que o tratamento con anti-ptau B6 diminuíu os depósitos de p-tau cerebral tanto a nivel de hipocampo, tronco cerebral, coma de corteza cerebral. Globalmente, os resultados demostran a capacidade terapéutica do anticorpo anti-ptau B6.

En global, este traballo demostra, dun xeito preliminar, o desenvolvemento de técnicas avanzadas de diagnóstico *in vitro* (ELISA) e *in vivo* (radiotrazador neuroimaxe PET) baseadas en modificacións específicas da proteína tau (pS208 e acK174), empregando dous novos anticuerpos monoclonais deseñados e desenvolto polo noso grupo, os anticorpos anti-ptau B6 e anti-actau E11. Asímesmo, demostrouse en estudos preclínicos *in vivo* as propiedades terapéuticas para o anticorpo anti-ptau B6. Estes resultados constitúen a base para a maduración da tecnoloxía para que alcance ao sector de diagnóstico e terapia da EA.

1. INTRODUCTION

1.1. ALZHEIMER'S DISEASE

1.1.1. History

The first characterization of Alzheimer's Disease (AD) neuropsychological symptoms was carried out by the German physician Aloysius "Alöis" Alzheimer in 1907 in a woman named Auguste Deter. At the age of 51 years old, she died and her brain was microscopically examined by using the histological technique of silver staining. He observed the molecular pathogenesis of the hallmarks of AD, the amyloid plaques composed of β -amyloid ($A\beta$) deposits, and the neurofibrillary tangles (NFTs) formed by hyperphosphorylated tau as a consequence of the neuronal neurodegeneration (Maurer & Maurer, 2003).

The term dementia associated to AD was adopted in 1980; nowadays, the diagnosis of AD was improved and the different dementia groups are differentiated through early disruption of episodic memory important in medial temporal lobes (MTL) structures such as the hippocampus (Hc) and the entorhinal cortex (Bondi et al., 2017).

AD has two main molecular hypothesis, the accumulation of $A\beta$ composed of amyloid plaques outside the neurons, and the microtubule-associated protein tau (MAPT) that forms intracellular deposits of NFTs. Currently, different isoforms of tau and $A\beta$, such as total tau (t-tau) and p-tau and $A\beta$ -42 or the ratio $A\beta$ -42/ $A\beta$ -40, have been used as diagnostic biomarkers, (**Figure 1**) (Atri, 2019).

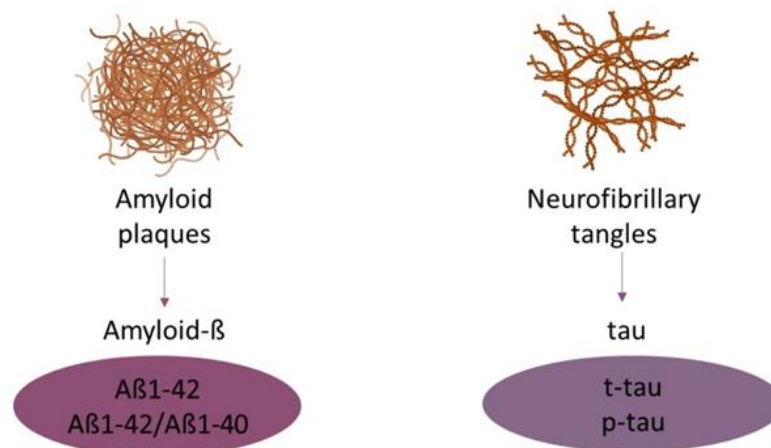


Figure 1. The two pathological molecules and their isoforms are involved in AD pathology. Self-created image using elements with Creative Common license.

1.1.2. Stages and pathophysiological pathways

The amyloid plaques or insoluble $A\beta$ peptides in the brain are formed through cleavage of the amyloid precursor protein (APP) by the sequential action of two enzymes: β - and γ -secretases. APP is an integral membrane protein composed of a long N-terminal extracellular domain, an intramembrane domain, and a short cytoplasmic C-terminal region. The physiological role of APP is unknown; however, it has been postulated that it has a range of roles, such as neurite outgrowth, controlling cell-to-cell or cell-to-matrix connections, and regulating cell adhesion (Vandersteen et al., 2012). APP can undergo two alternative pathways, one is through the non-amyloidogenic pathway where cleavage by α -secretase occurs within the amyloid peptide sequence and prevents the formation of the $A\beta$ peptides; and another amyloidogenic pathway initiated by cleavage from β -secretase 1 or BACE1

originating a 99 amino acid peptide, β -C-terminal fragment (β -CTF), which is then cleaved the transmembrane domain by γ -secretase to give rise to the A β 10 peptide (Vandersteen et al., 2012). In order to result in A β monomers, A β oligomers, and A β fibrils, that in turn form amyloid plaques, the enzyme γ -secretase is a protein complex, composed of two transmembrane proteins called presenilin 1 (PSEN1) and presenilin 2 (PSEN2), which produce a majority amount of the peptide A β 1-40 and smaller amounts of A β 1-42 (**Figure 2**) (Golde et al., 2012).

The tau protein has several potential sites for proteolytic cleavage that are accessible to a variety of proteases, including caspases and calpain. It has been demonstrated that tau's C-terminal end inhibits polymerization, that tau aggregates when its C-terminal is truncated *in vivo*, and that tau filament production is increased *in vitro* (H. Yin & Kuret, 2006). Caspase activation causes the tau protein to break down, which causes tau aggregates to form NFTS in the intraneuronal region (**Figure 2**) (Pritchard et al., 2011).

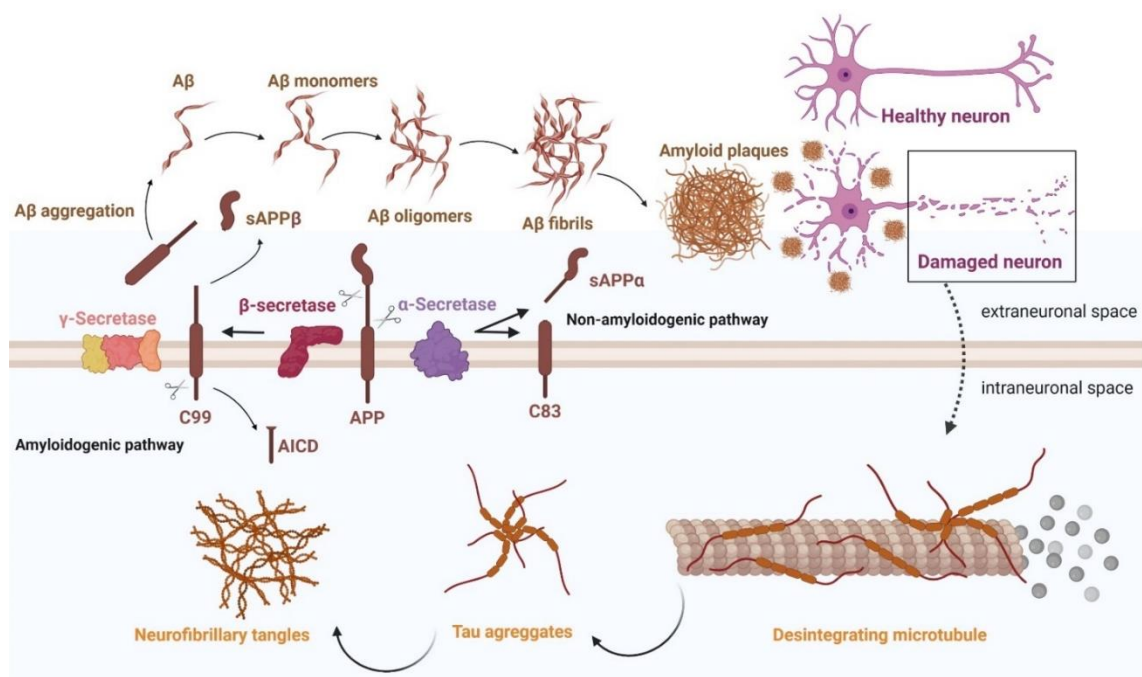


Figure 2. A β and Tau neuronal synthesis in AD pathology. The tau protein is cut off by caspase, which causes tau aggregation to form NFTS and A β monomers, A β oligomers and finally A β fibrils, which provoke the formation of amyloid plaques through the cleavage of the APP by the enzyme γ -secretase. Self-created image using Biorender license.

AD is the most common and prevalent neurodegenerative disease since more than 15 million people are affected worldwide (Blennow et al., 2006). AD represents the 60-80% of cases of dementia and is associated to cognitive degeneration that affects memory, language, and the capacity for self-management (Melzer et al., 2020).

At this time, up to 4 stages of progression to AD can be distinguished (**figure 3**). The first stage is the pre-clinical phase of AD where there are two states regarding the clinical symptoms, the asymptomatic AD risk state as well as the pre-symptomatic AD. In the first stage, at least one pathological biomarker of AD is present. It has been seen molecular alterations of A β concentrations in cerebrospinal fluid (CSF) or brain by positron emission tomography (PET) and to a lesser extent brain tauopathy can be identified because CSF tau can occur late in the preclinical phase where individuals did not show clinical signs and symptoms of AD (Dubois et al., 2016). In relation to the second state, pathophysiological

biomarkers such as increased levels of tau and decreased A β levels in CSF occurs 15 years and 10 years before symptoms respectively. Besides, A β deposits in the brain are showed by PET in pre-symptomatic phase of autosomal dominant mutation carriers (Bateman et al., 2012). This second stage is known as symptomatic AD, or prodromal AD. Prodromal AD is the early symptomatic phase, including hippocampal episodic memory loss, but does not affect the patient's autonomy in daily life. In terms of biomarkers or neuroimaging tests, alterations or pathological changes can be observed (Molinuevo et al., 2012). Specifically, it was observed that increased levels of t-tau and p-tau and low levels of A β 1-42 have a high predictive value for identifying cases of prodromal AD in patients who will degenerate to mild cognitive impairment (MCI) (Blennow et al., 2010). Importantly, increased CSF tau levels correlates with the presence of NFTs in the brain, and p-tau and t-total concentrations correlate with the degree of AD symptomatology (Jack et al., 2010).

In the third stage, episodic memory impairment, extensive brain pathology, and poorer executive functions are the main factors which leads to a high risk of developing AD dementia (Wada-Isoe et al., 2019). The diagnosis of MCI due to AD, pre-clinical AD, and AD dementia is nowadays performing by using core clinical and biomarker criteria. Core clinical criteria are defined as subjective or objective impairment of cognitive function that doesn't interfere with the patient's social functioning and doesn't result from any additional neurological, psychiatric, systemic, or metabolic impairment (Jongsiriyanong & Limpawattana, 2018). In the case of core biomarker criteria, there are three categories: i) neuronal damage biomarkers as A β and tau; ii) functional imaging by PET, and iii) structural magnetic resonance imaging (MRI). In this regard, lower levels of A β -42 and increased levels of p-tau and t-tau in CSF, as well as a positive of PET amyloid imaging, are biomarkers that indicate the presence of neurodegeneration. Also, a decreased metabolism in the parietal and temporal cortical lobe by ¹⁸fluorodeoxyglucose PET and atrophic change on MRI in the temporal and medial parietal cortical lobe are used as indicators of prodromal AD (Jack et al., 2013; Jongsiriyanong & Limpawattana, 2018).

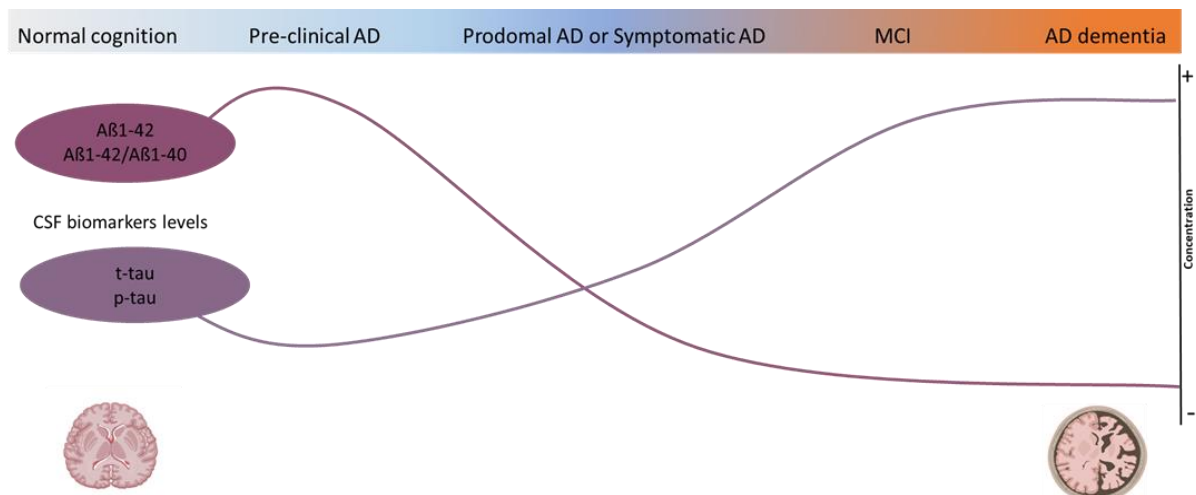


Figure 3. Stages of progression to Alzheimer's Disease with parallel biomarker levels in CSF and brain damage. At each stage from normal cognitive to AD dementia, a decrease in the levels of the different A β isoforms and an increase in the levels of t-tau and p-tau are shown. Self-created image using elements with Creative Common license.

As the disease progresses, the patient loses autonomy to perform certain daily tasks and the cognitive impairment worsens, progressing to the final stage of AD dementia. AD dementia is characterized by specific clinical symptoms, due to alterations and short-term

memory loss, followed subsequently by motor dysfunction and behavioral disturbance (“2021 Alzheimer’s Disease Facts and Figures,” 2021). The presence of pathological biomarkers is the same that those found in MCI but the levels in CSF of A β are lower and, by contrast, the levels of t-tau and p-tau higher. However, new biomarkers are needed to better predict deterioration due to AD. Therefore, a complementary MRI can be performed in order to identify atrophy of the medial temporal lobe. From this point onwards, the patient may suffer changes in behavior and motor impairment (Dubois et al., 2016; Lane et al., 2018).

The typical clinical phase of AD is a progressive decline of episodic memory and cognitive function, ultimately leading to a loss of independence. AD patients also show a deficiency of language, visuospatial abilities, and alterations of behavior such as apathy, aggressiveness, and depression (Silva et al., 2019).

Nowadays, histological techniques in post-mortem brains to analysis the presence of amyloid plaques and NFTs are definitive for the AD diagnosis (Tomlinson et al., 1970).

1.1.3. Impact: prevalence and incidence

According to global statistics from the Global Burden Disease Study, AD has become one of the fastest-growing neurodegenerative diseases due to the increase in the elderly population (Brookmeyer et al., 2007). It has increased significantly in recent years between 2000-2018 because the number of deaths has more than doubled, while heart disease has decreased (“2021 Alzheimer’s Disease Facts and Figures,” 2021). In 2006, the number of AD patients was 26.6 million worldwide, and it is predicted that in 2050 the number of AD patients were reached 106.8 million. It has been confirmed that these AD patients show an increase in the trend particularly in the European region between 2010-2050, affecting around 800000 people over 60 years in Spain (Alzheimer’s & Dementia Help | Spain | Alzheimer’s Association, n.d.); (Brookmeyer et al., 2007; Prince et al., 2013).

The improvement in quality of life has led to an increase in life expectancy and with it the elderly population, which is related to an increase in neurodegenerative diseases such as AD, especially in Asia (Wu et al., 2015). It is curious that in countries such as Japan with a high aging population, there is a higher prevalence but a lower incidence due to healthy lifestyle and dietary habits; low-fat dairy products and Mediterranean diet is related to a reduced risk of AD and the AD-associated mortality. These healthy dietary habits are also associated to a decrease of cardiovascular disease, a lower incidence of hypertension and diabetes, an improvement of insulin resistance, and reduced levels of inflammatory markers (Dhana et al., 2022; Ninomiya, 2015; Opie et al., 2013)

A higher educational level is linked to increased cognitive reserve, which would be a protective factor against cognitive loss in the context of neurodegeneration. This suggests that improved educational level may have impact in the reduction of AD risk (Hoenig et al., 2019). In particular, it has been shown that patients with higher levels of education have less neuronal dysfunction and lower tau pathology.

The prevalence and incidence of AD are higher in women because they have a longer life expectancy than men and is more pronounced in those with lower educational level, which is more reflected in older age groups (Hoenig et al., 2019).

In summary, life expectancy has increased due to improvements in quality of life including healthy diet and high level of education, leading to an increase in neurodegenerative diseases associated with ageing in the population, especially in Europe and developed countries (Niu et al., 2017).

1.1.4. Risk factors

As mentioned above, AD is a complex and multifactorial disorder that is determined throughout the patient's life by genetic and environmental risk factors (Zhang et al., 2021). In relation to the genetic factors of AD may be sporadic, familial and autosomal dominant subdivided into early onset (EOAD) and late onset (LOAD). Around 1-5% of AD are EOAD, which symptoms frequently appearing before the age of 60-65, while the majority of AD cases are LOAD, which clinical symptoms begin after that age (Bertram & Tanzi, 2005).

Firstly, autosomal dominant AD accounts for 5% of AD cases and is seen almost exclusively in families with EOAD, but has a variable component in terms of diagnostic criteria (Bertram & Tanzi, 2005). Secondly, familial AD represents approximately 47% of EOAD and 15–25% of LOAD cases (Brickell et al., 2006). Lastly, sporadic AD represents 75% of all AD cases of which are majority LOAD and 40% of EOAD that are associated with the mutations of three determinant genes such as *amyloid precursor (APP)*, *presenilin1 (PSEN1)* and *presenilin 2 (PSEN2)* and allelic variation in apolipoprotein E (APOE) in particular the polymorphism genetic risk factor (APOE ϵ 4 allele) (Table 1) (Armstrong, 2019; Bateman et al., 2012). It is well known that APOE acts as a potent A β remover by proteolytic degradation in the extracellular environment promoting A β production and accumulation in sporadic AD (Jiang et al., 2008). Moreover, APOE ϵ 4 alters cognitive function affecting memory and learning and increasing the development of early AD (Gomez-Isla et al., 1996; Oyama et al., 1995). Although it is not directly causal, the APOE ϵ 4 increases susceptibility to AD by 2 to 3-fold, while having 2 copies is linked to a 5-fold increase, seems to be responsible for 20% of elderly people's risk of acquiring AD (Slooter et al., 1998). Other gene involved in EOAD is *APP*, whose gene is encoded on chromosome 21. In people with AD is cut into A β fragments that accumulate into amyloid plaques in the brain (Goldman et al., 2011). The genes of *PSEN1* and *PSEN2* are encoded on chromosome 14 and 1 respectively, and are critical components of the γ -secretase complex and are responsible for the increase of the production of toxic A β peptides leading to NFTs, neuronal loss and finally AD dementia (Janssen et al., 2002). Animal models that have been helpful for understanding the pathophysiology of AD have been developed as a result of the finding of these genes (Slooter & van Duijn, 1997).

Table 1. Alzheimer's disease described genes in sporadic AD.

Gene	Protein name	Chromosome	Inheritance pattern
<i>APP</i>	Amyloid beta A4 Protein	21	Early-onset autosomal dominant
<i>PSEN1</i>	Presenilin 1	14	Early-onset autosomal dominant
<i>PSEN2</i>	Presenilin 2	1	Early-onset autosomal dominant
<i>APOE</i>	Apolipoprotein E	19	Early- and late-onset familial-sporadic

In addition, the genome-wide association studies (GWAS), involved several genes that functionally modulate the APP, with AD susceptibility loci. One of these genes studied is the *fermitin family homolog 2 gene (FERMT2)*, which is significantly associated with alterations of A β levels in CSF and has already been described as a modulator of tau toxicity in *Drosophila* (Armstrong, 2019; Chapuis et al., 2017).

Another gene involved in AD is *the ATP-binding cassette transporter A1 (ABCA1)*, which is thought to be a prospective therapeutic target since the liver X nuclear receptors (LXR) are in charge of the transcriptional regulation of ABCA1 and participates in the

aggregation and clearance of A β within the LXR-ABCA1-APOE axis (Koldamova et al., 2010).

Other genes as possible risk factors of AD include *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* and a rare variant of the triggering receptor expressed on myeloid cells 2 (TREM2) are genes involved in LOAD (M. Allen et al., 2012; Ruiz et al., 2014). In particular TREM2 has been linked to AD as a late reaction by lowering A β toxicity, hence having no early influence on the pathophysiology of AD and the onset of dementia (Perez et al., 2017).

It should be noted that epigenetic factors rather than altering DNA sequences cause heritable changes in gene expression. Instead, environmental factors like heavy metals and dietary folate intake affect how genes are expressed through epigenetic modifications like DNA methylation and histone modification (Kwok, 2010). As a result, by raising the amounts of APP and A β , the APP promoter's methylation pattern is altered, which encourages the formation of reactive oxygen species (ROS) that damage DNA. Additionally, this mechanism implies that the demethylation of cytosines influences the A β deposition in the aging brain (Tohgi et al., 1999). Therefore, a number of genes are significantly affected by DNA methylation, which is dynamically controlled in the human cerebral cortex over the lifetime, involves differentiated neurons, and is mostly influenced by an increase in aging (Siegmund et al., 2007).

In addition, there are several types of risk factors that contribute to the development of neurodegenerative diseases: cardiovascular risk factors such as diabetes, hypertension, obesity, high cholesterol levels, inflammation, previous stroke risk, lifestyle factors such as education level, smoking, physical activity, and others such as head injuries, infections, vitamin deficiencies and diet, and exposure to chemicals (Baumgart et al., 2015). The traumatic head injury and the time of education are the strongest evidence of increased risk to develop cognitive decline and dementia (Gardner & Yaffe, 2014; Sando et al., 2008). Smoking, in particular, raises the risk of dementia and cognitive decline, but this risk is higher in non-APOE ϵ 4 carriers. Since APOE ϵ 4 carriers have an increased risk of AD, this may be controversial. More studies are needed to conclude this, but the possible explanation is that the risk of AD is increased by carrying the APOE ϵ 4 allele and is not affected by other risk factors (Zhong et al., 2015). In conclusion, there is evidence that regular physical activity, a healthy diet, continuous cognitive stimulation over the years and reducing the possibility of suffering cardiovascular risk factors will mean a lower predisposition to suffer from cognitive decline and subsequent development dementia (Baumgart et al., 2015).

1.1.5. Hallmarks of AD: Amyloid plaque formation and tau hyperphosphorylation

The extracellular accumulation of A β forms amyloid plaques. These aggregates are accumulated in the walls of capillaries, arteries, and arterioles, causing cerebral angiopathy leading to a degeneration of the components of the vascular wall and worsening of blood flow, as well as predisposing to intraparenchymal hemorrhages (Shinohara et al., 2014).

Up today, the main research efforts in therapy and diagnostics of AD have focused on understanding the hypothesis of A β and combating the progression of amyloid plaques around neurons. Progress toward an effective therapy has not been satisfactory, so we have relied on the aggregation of the MAPT in NFTs within the cells (Chung et al., 2021). MAPT, is a key element of the microtubular system. It promotes microtubule assembly, and stability and might be involved in the establishment and maintenance of neuronal polarity and synaptic function, among others (Weingarten et al., 1975). The tau family is composed of six isoforms (**Figure 4**), that differ in chain length, due to the alternative splicing of exons 2 and 3 in the

N-terminal of the protein (2N, 1N, or 0N) and exon 10 of the tau gene. According to the number of repetition regions included, brain isoforms are referred to 3R as 0N3R (Tau352), 1N3R (Tau381) and 2N3R (Tau410) and to 4R as 0N4R (Tau383), 1N4R (Tau412) and 2N4R (Tau441) (Takashima et al., 2019). Whole-length tau protein can be divided into an N-terminal region, a proline-rich domain consisting of two separate parts, the repeat domain, and a C-terminal region (Goode et al., 1997). The C-terminal region binds axonal microtubules while the N-terminal region binds intracellular membranous components such as the mitochondria and the neuronal plasma membrane, which suggests a linker function of tau between both elements (Kolarova et al., 2012). Tau monomers are intrinsically disordered proteins that, upon ordered assembly, form structured amyloid filaments. Although it lacks a typical low-complexity domain, it has been reported that full-length tau can aggregate under certain conditions such as liquid-liquid phase separation (Wegmann et al., 2018).

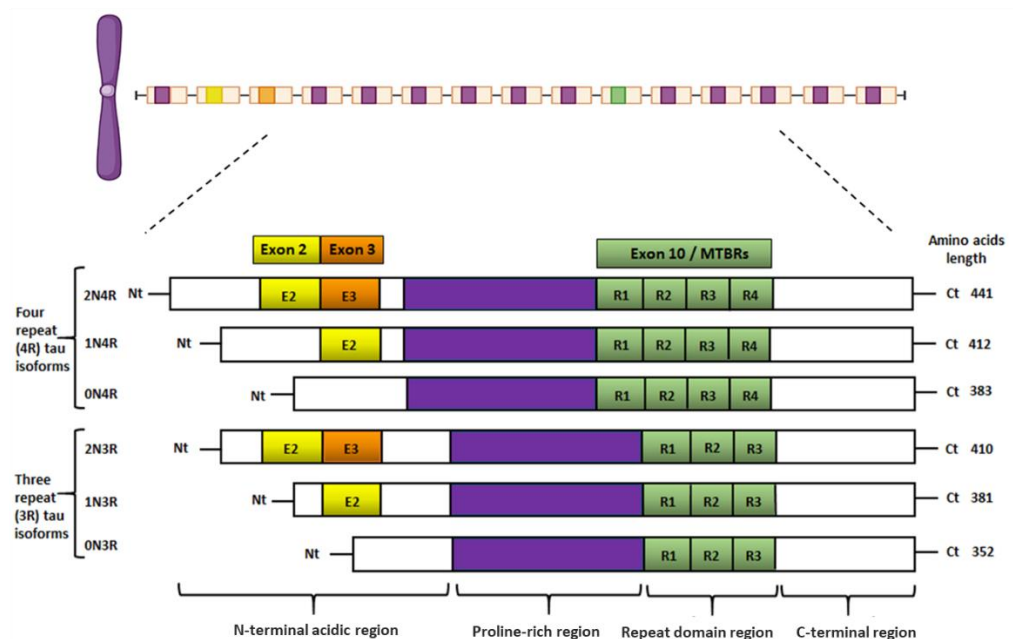


Figure 4. Representation of tau isoforms. The six different isoforms are based on the existence of 3 exons: N-terminal exons 2 and 3 (E2 and E3) of the projection domain and exon 10 encodes microtubules binding repeat (MTBRs, R1-R4) of the microtubules binding domain (MTBD). Self-created image using elements with Creative Common license.

Tau has been identified as a key driver element in several neurodegenerative diseases like AD, but also in primary tauopathies such as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PID) or argyrophilic grain disease (AGD) where NFTs are formed by the intraneuronal accumulation of p-tau, and molecules as A β have a role in the disease progression too (Jefferson-George et al., 2017) (Chung et al., 2021). Therefore, attention is being paid to other molecular mechanisms related to the disease in relation to the tau protein to form oligomers that act as a template for the conversion and coaggregation of endogenous proteins, which eventually form NFTs and have been shown to be relevant in the progression of AD (Yin et al., 2021).

Tau contributes to the stabilization and microtubular assembly and regulates the dynamic reorganization of the cytoskeleton. This regulation is done through the axonal transport by dynein and kinesin motor activity or through the function cargo, which regulates the release of

vesicles from kinesin and facilitates the dynactin association with microtubules, increasing the transport of dynein (Y. Wang & Mandelkow, 2016). In addition, tau also may interact with actin and induce aligned bundles of actin filaments, promoting the reorganization of the cytoskeleton (Biernat et al., 2002). However, other functions of tau have already been described, the insoluble tau filaments form "ghost tangles" in the extracellular space after NFTs containing neurons die. Along with the pattern of NFTs dispersion, neuropathic threads made of tau accumulation in dendrites and axons progress as well. Additionally, a subset of amyloid plaques that are linked to cognitive problems includes filamentous tau with dystrophic neurites around amyloid deposit (Yin et al., 2021). Additionally, tau is found in a number of cell organelles, including the nucleus, where it interacts with the neuronal outer envelope (NE) to cause deficits in the transport of RNA and proteins into and out of the nucleus. This defect in nucleocytoplasmic transport (NCT) has been linked to neurodegenerative diseases like frontotemporal dementia and AD (Eftekharzadeh et al., 2018). Furthermore, tau also appears to be associated with adult hippocampal neurogenesis in tau knockout mice models, since the glycogen synthase kinase 3 (GSK3), an essential tau kinase, was upregulated to cause a correlated increase in markers of immature neurons and tau phosphorylation in the Hc *in vivo*. Moreover, the increase in neurogenesis was brought on by increased proliferation rather than survival or differentiation of new neurons. The hippocampal neurogenesis was substantially compromised, and activating glycogen synthase kinase 3 β (GSK-3 β) in these mice did not make up for the deficiencies. In conclusion, tau phosphorylation is crucial for adult Hc neurogenesis (Hong et al., 2010).

1.5.1.1. Post-translational modifications

Tau function is strictly regulated by post-translational modifications (PTMs), such as phosphorylation, acetylation, glycosylation, ubiquitination, glycation, nitration, SUMOylation, oxidation, methylation, cleavage, or truncation. PTMs play a critical role in tau localization, protein-protein interactions, clearance, structure, and aggregation kinetics (Cohen et al., 2011; Sacco et al., 2012). Additionally, the clustering of PTMs within short sequence motifs and functional domains suggests that associated functions and subcellular localization are regulated by a multifactorial interplay between different PTMs. It has been observed that the dysregulation of these clustered PTMs can impact tau physiological functions and lead to pathological processes (Haj-Yahya & Lashuel, 2018). The change from functional healthy tau to pathological aggregated tau is probably not a consequence of a single PTM, but of a combination of intrinsic structural alterations and extrinsic cellular conditions leading to pathogenicity (Alquezar et al., 2021).

The following section discusses some PTMs that have impacted tau protein function. When ubiquitination occurs, other PTMs like phosphorylation and acetylation can upregulate the ubiquitin protein. This process can be reversed by a class of enzymes known as deubiquitinases (DUBs) that cleaves mono-ubiquitin or poly-ubiquitin chains of a target protein (Gopinath et al., 2016; Swatek & Komander, 2016). The causes of AD pathogenesis are predominantly carried out by the abnormal accumulation of misfolded peptides and proteins in the form of amyloid plaques and NFTs, respectively, by a malfunction of the degradation of misfolded proteins in the proteasome. Cellular mechanisms such as phosphatases, chaperones, and ubiquitin-proteasome system (UPS) are the main pathways of the removal through proteostasis of toxic misfolded proteins and aggregates, formed as amyloid plaques and NFTs in the case of AD (Ramesh et al., 2020).

In relation to the degradation of the proteins involved in AD pathology, proteasome function or dysfunction may impact A β generation since it has been demonstrated that the

(cdk5), casein kinase 1 (CK1), cyclic AMP-dependent protein kinase (PKA), p38 mitogen-activated protein kinase (MAPK), protein kinase C (PKC), calmodulin-dependent protein kinase II (CaMKII), the brain-specific kinases 1 and 2 (BRSKs), the tau-tubulin kinases 1 and 2 (TTK), and some microtubule affinity-regulating kinases (MARKs; also known as PAR1 kinases) (Hong et al., 2010; Ikezu & Ikezu, 2014; M. D. Martin et al., 2016). Tyrosine tau residues can be phosphorylated by kinases like the SRC family members (LCK, SYK, and FYN) and the ABL family members (ARG and ABL1). Alternatively, tau can be dephosphorylated by protein phosphatases (PP) 1, 2A, 2B, 2C, and 5 (Alquezar et al., 2021; Ferrer et al., 2005). Specifically, phosphorylation in serine 208 is generated by kinases like checkpoint kinase 1 (Chk1), casein kinase 1 delta (CK1δ), and TTK (Ikezu & Ikezu, 2014).

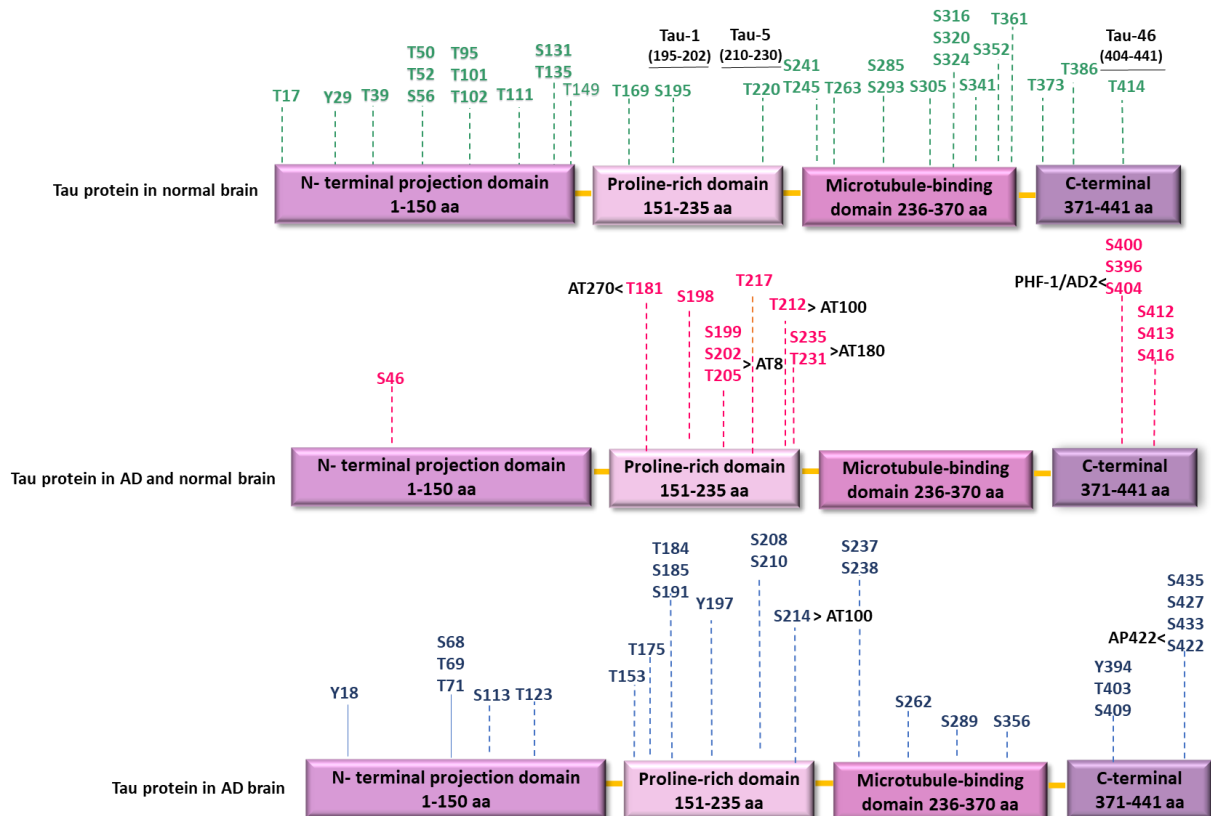


Figure 6. Phosphorylation sites on the tau protein and epitopes that are particular to the main tau antibodies. The phosphorylated tau epitopes identified in AD brain are indicated in red, green in those phosphorylations found for both AD and normal brain, and blue only in normal brain. Epitopes recognized by tau antibodies of phosphorylation sites on tau protein are shown in purple. Self-created image using elements with Creative Common license based on (Šimić et al., 2016).

The balance between kinase and phosphatase activities will determine the phosphorylated state of tau and thus its tendency to a physiological or pathological form that will trigger AD (Y. Wang & Mandelkow, 2016). It has been observed that depending on which tau epitope is phosphorylated has one effect or another on the tau protein. For example, phosphorylation of tau at Ser208 in the microtubule binding domain and/or in the proline-rich domain reduces the affinity of tau for negatively charged microtubules and has already been identified in NFTs isolated from patients (Biernat et al., 1993;). These have been linked to structural changes in the tau molecule that may promote its aggregation, supporting a relevant role of S208 phosphorylation in tau-related pathogenesis in AD brain (Figure 6) (H. H. Liu et al., 2021; Xia et al., 2020). There are numerous phosphorylated tau epitopes identified in AD brain,

normal brain and epitopes expressed by both types of brain, which are recognized by different antibodies as shown in **figure 6** (Šimić et al., 2016).

1.1.5.1.2. *Tau acetylations*

Acetylations are other important PTMs with pathological implications and are responsible for adding an acetyl group that neutralizes the positive charge of lysine residues, affecting the protein structure and protein-protein recognition. The presence of acetylations also prevents further modifications in the lysine residues, thus blocking further post-translational regulation (Portaleone, 2004). It should be noted that in the 2N4R tau protein, 10% of residues are lysine and thus, susceptible to being acetylated by p300 protein acetyltransferase because its inhibition causes the decrease of levels of t-tau, acetylation in the lysine 174 (acK174-tau), and phosphorylation in serine 202 (pS202-tau) and threonine 205 (pT205-tau) tau in neurons primaries, rescued memory deficits, and prevented hippocampal atrophy in PS19 transgenic mouse model of frontotemporal dementia (FTD). Particularly, tau acetylation at K174 has been linked to tau homeostasis and toxicity in mice, causing an early alteration in soluble tau in AD brains (S. W. Min et al., 2015). Further studies demonstrated that acK174-tau is also capable to induce tau aggregation without affecting tau-microtubule binding (Kamath et al., 2021). It has also been suggested that crosstalk of acetylation and phosphorylation patterns is likely and critical for the accumulation of hyperphosphorylation of tau and thus for the pathology progression of AD (Min et al., 2015). It has also been suggested that crosstalk of acetylation and phosphorylation patterns is likely and critical for the accumulation of hyperphosphorylation of tau and thus for the pathology progression of AD (Qin et al., 2006). These acetylations can be reversed by the histone deacetylase 6 (HDAC6) and the NAD⁺-dependent sirtuin 1 deacetylase (SIRT1) which are the major tau deacetylases identified so far (Ding et al., 2008; S. W. Min et al., 2010). Interestingly, several works have shown that dysregulation of these two deacetylases occurs in different states and impact tau pathology in a disease-dependent manner. Firstly, it has been demonstrated that SIRT1 can reduce γ -secretase activity, so lowering A β in embryonic neocortical primary neuronal cultures derived from transgenic mice and *in vivo*, in order to control aging and metabolic processes involved in AD pathogenesis (Qin et al., 2006). In a different investigation, they discovered that SIRT1 inhibition raised the levels of t-tau and ac-tau in a triple-transgenic animal model, most likely by preventing proteasome-mediated degradation. In conclusion, SIRT1 is closely associated with the accumulation of amyloid-beta and tau in AD, *in vivo* and *in vitro* (Julien et al., 2009). Secondly, actin and microtubule-dependent cell motility is controlled by HDAC6 through the regulation of the acetylation of α -tubulin and the proteins that bind to it, including cortactin (X. Zhang et al., 2007). Increased HDAC6 levels have been shown to remove ubiquitinated protein aggregates from tau protein through the recruitment of the autophagic machinery while decreasing protein phosphatase availability causes tubulin to be acetylated less and tau to be more phosphorylated. These effects are in contrast to each other. Therefore, whereas a rise in HDAC6 levels may have a protective effect at first, continued increases may have a deleterious impact on the survival of neuronal cells in AD and other tauopathies (Ding et al., 2008).

1.1.6. Pharmacological treatment

Nowadays, AD represents a global health problem. The current clinical trials of AD are focused in the potential disease-modifying therapies (DMTs) that constitute 83,2%, the symptomatic cognitive enhancing treatment (SCET) represents 9,8% and drugs for the treatment of associated neuropsychiatric symptoms comprises 6,9% of drugs used in clinical

trials (J. Cummings et al., 2022). Most therapeutic agents under development to find a cure for AD have failed to date, and this fact has a high economic and social impact on the population.

1.1.6.1. Symptomatic cognitive enhancing treatment (SCET)

SCET for AD is based on acetylcholinesterase inhibitors (AChEIs) and glutamate antagonists. SCET drugs act as palliative treatment to ameliorate the behavior and neuropsychiatric symptoms such as agitation, aggression, depression, and anxiety but do not slow down or prevent the progression of the disease (J. L. Cummings et al., 2019; Mendiola-Precoma et al., 2016). There are only four approved treatment options to treat the cognitive symptoms of AD in European Union, which are divided into two groups according to the mechanism of action. The first group include three cholinesterase inhibitors (donepezil, galantamine, and rivastigmine) and the second group, the N-methyl D-aspartate (NMDA) receptor antagonist (memantine) (J. L. Cummings et al., 2019). In addition, in the United States, the combination of AChEIs as donepezil and memantine is also approved in patients with moderate to severe AD (Matsunaga et al., 2018).

Regarding the first group, the AChEIs block the normal breakdown of acetylcholine (ACh) increasing it in synaptic levels. In the case of receptor antagonist serotonin (5-hydroxytryptamine [5-HT]) increase the cholinergic signal associated with AChEIs and it may improve the treatment of cognitive symptoms in patients with AD (J. L. Cummings et al., 2019). It has been seen that an abnormal accumulation of A β reduced cholinergic transmission effectiveness in AD patients (Garcia-Osta & Alberini, 2009).

Concerning the second group, Memantine is a non-competitive NMDA receptor antagonist which present a moderate affinity and it has the role of decreasing the excitatory neurotoxicity effect of glutamate improvement the cognition of the patients (*Alzheimer's Disease: Early Diagnosis and Treatment* | HKMJ, n.d.; Long & Holtzman, 2019). Also, the memantine can be a neuroprotector through the attenuation of tau phosphorylation, decreasing GSK-3 β activity (Mendiola-Precoma et al., 2016). This non-competitive glutamatergic NMDA receptor antagonist can be administered alone or in combination with an AChEIs but shows greater benefits in combined treatment (X. Wang et al., 2015).

1.1.6.2. Disease-modifying treatments (DMTs)

The DMTs are currently being tested in clinical trials as adjunctive therapies to symptomatic combination therapy such as AChEIs or memantine (J. L. Cummings et al., 2019).

In the present time, there are 143 drugs for the treatment of AD used in 172 clinical trials, among which 31 drugs in 47 clinical trials in phase 3, 82 drugs in 94 clinical trials in phase 2, and 30 drugs in 31 clinical trials in phase 1. The 2022 pipeline showed progress in A β therapies with the approval of the monoclonal antibody (mAb) aducanumab, including strategies focused on inflammation, alterations of tau, and synaptic dysfunction, therefore the biomarkers are increasing (J. Cummings et al., 2022).

DMTs were classified into small molecules, biomarkers, RNA-based, passive, and active immunotherapy. These DMTs are targeting essentially the two pathological features of AD: the A β and tau aggregates, but there are other targets such as kinases, vitamins, neurotransmitter receptors, inflammatory molecules, growth factors, and hormones, among others (J. Cummings & Fox, 2017). The main therapeutic strategies aimed at achieving DMTs targeting tau protein in clinical development are summarized in **Table 2**.

1.1.6.2.1. Active Tau immunotherapy (vaccination)

The role of active immunotherapy using anti-tau vaccines is increased to develop antibodies against conformational epitopes in the microtubule-binding region of tau (MTBR), to prevent tau aggregation and the removal of the tau protein, avoiding the spread of AD pathology (Novak et al., 2019).

AADvac1 was the first anti-tau vaccine tested in a study of AD in clinical trials of Phase 2. The treatment seemed to be safe and induced the production of IgG antibodies against the insoluble tau proteins for patients with mild-to-moderate Alzheimer's. The principal role of the treatment with AADvac1 was the decrease of the NFTs composed of highly phosphorylated forms of the MAPT, preventing dementia in patients with early symptomatic stages of AD (Kontseikova et al., 2014; Novak et al., 2019). Recently, another agent in Phase 2 is testing, named ACI-35. It is a liposome-based vaccine that carries a synthetic tau peptide acting as an antigen. It is able to activate the immune system to synthesize specific antibodies that target the misfolded and pathogenic forms of the tau protein (ACI-35 - Alzheimer's News Today, n.d.).

1.1.6.2.2. Passive Tau immunotherapy

The passive immunotherapy is directly injected a mAb against altered forms of tau protein, it is high epitope-specific, and therefore it usually has no side effects (Congdon & Sigurdsson, 2018). Different mAbs have failed in recent AD clinical trials in phase 2 as semorinemab, zagotenemab, gosuranemab, and ABBV-8E12, targeting extracellular tau NFTs or soluble tau oligomers (Mullard, 2021). At present, four anti-tau mAbs have reached AD clinical trials in phase 2 as JNJ-63733657, bepranemab, semorinemab, and E2814 and two continue in phase 1 as TB006 and Lu AF8790B, most with the function of to remove extracellular tau (J. Cummings et al., 2022).

1.1.6.2.3. Small molecules

Nowadays, there is a small molecule in phase 1 named ASN51, acting as a small molecule inhibitor of O-GlycNAcase (OGA), preventing aggregation, appearing to stabilize tau in a soluble form and reduce NFTs (Hastings et al., 2017).

However, Phase 2 clinical trials are testing three small molecules as Nicotinamide, LY3372689, and PU-AD. Firstly, Nicotinamide is an HDAC inhibitor, which is responsible for reducing tau-induced microtubule depolymerization and tau phosphorylation. As well as ASN51, the LY3372689 is another small molecule inhibitor of OGA (J. Cummings et al., 2022). Lastly, PU-AD is a brain-permeable inhibitor of the molecular chaperone heat shock protein 90 (HSP90) enhances the degradation of pathogenic tau proteins and promotes cell survival in animal models of tauopathies (A et al., 2013).

Tau aggregation inhibitors (TAIs) have the potential to prevent or reverse tau aggregation and consequently reduce tau pathology (Wischnik et al., 2014). Methylthioninium (MT) is the first identified as a selective TAI *in vitro* and it could have therapeutic utility because it is currently testing in phase 2 of the clinical trial in 321 subjects with mild or moderate AD (Wischnik et al., 2015).

1.1.6.2.4. Biomarkers and potential tau antibodies for new immunotherapies

At the present time, there are still no drugs available that can slow the progression of AD, let alone offer a cure. The preclinical assays have not been satisfactory for different reasons as unsuitable animal models, failed targets, and failure in clinical trials. Despite the development of new and more specific imaging techniques and biomarker analysis, the only current

definitive diagnosis of AD is the post-mortem histopathological analysis of brain tissue (Cummings et al., 2019). In this way, the use of novel antibodies, such as p-Tau B6 for the phosphorylation in serine 208 (pS208 tau), pS202-tau and pT205-tau, and ac-Tau E11 for acK174-tau, may allow the development of innovative diagnostic enzyme-linked immunosorbent assay (ELISA) kits to identify the relationship between their accumulation in various types of biological samples (CSF, serum, plasma, tears, etc.) in the early diagnosis of AD.

Currently, there are several Phase 2 and Phase 3 clinical trials of DMTs for AD with the target of tau, which are described on clinicaltrials.gov and are summarized in **Table 2**. Importantly, the inclusion criteria of these clinical trials are focused in biomarkers. Most of these DMT trials are in Phase 3, a total of 31 and CSF and other biomarkers are used as inclusion criteria. In Phase 3, five (16%) of DMT trials used both CSF-amyloid/tau ratio or amyloid positron emission tomography (PET), and one trial used plasma pT217-tau. In Phase 2, six (7%) of DMT trials used CSF-amyloid/tau ratio as an entry criterion, four (5%) used either CSF-amyloid/tau ratio or amyloid PET, three (4%) used tau PET and one trial (1%) used both amyloid PET or CSF-tau (J. Cummings et al., 2022).

Table 2. Disease-modifying therapies (DMTs) targeting tau protein currently in clinical phases for the treatment of AD. It summarizes the experimental pharmacological treatment targeting tau protein that are currently in clinical development in phases 1, 2 and/or 3 according to the information obtained in clinicaltrials.gov on November 25, 2022.

Agent	Target	FDA ID	Status	Spon sor	Start Date	End Date
Small Molecules						
<i>PU-AD</i>	Prevent aggregation and hyperphosphorylation of tau	NCT04311515	Phase 2	Samus therapeutics	06/2020	12/2022
<i>LY3372689</i>	O GlycNAcase inhibitor; promote tau glycosilation, prevent tau aggregation	NCT05063539	Phase 2	Eli Lilly	09/2021	06/2024
<i>Nicotinamide</i>	HDAC inhibitor; reduce tau-induced microtubule depolymerization and tau phosphorylation	NCT03061474	Phase 2	Univ California. Irvine	07/2017	07/2022
<i>ASN51</i>	O-GlycNAcase inhibitor	NCT04759365	Phase 1	Asceneuron	06/2021	01/2022
Passive Immunotherapy						
<i>Lu AF8790B</i>	Anti-tau monoclonal antibody	NCT04149860	Phase 1	Lundbeck	09/2019	07/2022
<i>Semorinemab (RO7105705)</i>	Anti-tau monoclonal antibody to remove extracellular tau	NCT03828747	Phase 2	Genentech	01/2019	06/2023
<i>Bepranemab</i>	Anti-tau monoclonal antibody	NCT04867616	Phase 2	UCB Biopharma	06/2021	11/2025
<i>E2814</i>	Anti-tau monoclonal antibody	NCT04971733	Phase 2	Esai	06/2021	04/2024
<i>JNJ-63733657</i>	Anti-tau monoclonal antibody	NCT04619420	Phase 2	Janssen	11/2020	03/2025
<i>TB006</i>	Anti-tau monoclonal antibody	NCT04920786	Phase 1	TrueBinding, Inc	06/2021	01/2023
Active Immunotherapy						
<i>ACI-35</i>	Targeting Tau	NCT04445831	Phase 2	Janssen	07/2019	10/2023
<i>AADvac1</i>	Targeting Tau	NCT02579252	Phase 2	Axon Neuroscience	03/2016	06/2019

SE						
RNA-Based						
<i>IONIS MAPTRx</i> (BIIB080)	Antisense oligonucleotide targeting tau expression; MAPT RNA Inhibitor	NCT03186989	Phase 2	IONIS Pharmaceutical	06/2017	05/2022
Biomarkers						
<i>CSF Aβ/tau ratio</i>	Targeting Tau and A β in CSF		Phase 2(6)			
<i>Plasma p-Tau</i>	Targeting pT217-tau		Phase 3(1)			
<i>CSF Aβ/tau ratio + amyloid PET</i>	Targeting Tau and A β in CSF		Phase 2 (4) Phase 3(5)			
<i>Tau PET</i>	Targeting Tau		Phase 2 (3)			
<i>Amyloid PET or CSF Tau</i>	Targeting Tau and A β		Phase 2 (1)			

1.1.7. Diagnostic tests and Biomarkers

1.1.7.1. Neuropsychological and clinical/neurological tests

There are several approved neuropsychological tests as Mini-Mental State Examination (MMSE), Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) and Cambridge Cognition Examination (CAMCOG) to assess global cognitive function in dementia, but these test lack of sufficient precision and accuracy in discriminating MCI due dementia from normal aging (Wouters et al., 2012). The most used, fastest (less than 10 min) and complete neuropsychological test is MMSE. It measures cognitive function mainly focused on language and memory, but also covers five cognitive domains such as time and spatial orientation, attention and calculation, language that embraces reading and writing and finally drawing. It uses scoring ranging from 0 which means severe impairment to 30 means no impairment (Kueper et al., 2018).

Another common test is the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog), which analyzes cognitively and functionally important impairments in milder stages of AD disease progression, namely pre-dementia stages (Wouters et al., 2012). The ADAS-Cog consists of 11 tasks of 30 min in total, which are divided into two parts: one part measures cognitive functions and the other one measures non-cognitive functions, such as mood and behavior. The tasks are word recall, naming objects and fingers, commands, ideational and constructional praxis, orientation, word recognition, comprehension of language and word-finding difficulty. Uses a score range of 0-70, where the higher score means severe the impairment (Kueper et al., 2018). Lastly, CAMCOG is designed to screen and assess dementia severity and the level of cognitive impairment. The measure assesses orientation, language, memory, praxis, attention, abstract thinking, perception, and calculation (Hon et al., 1999). The CAMCOG consists of 67 items, including the 19 items from MMSE. It is divided into 8 subscales: orientation, language (comprehension and expression), memory (remote, recent and learning), attention, praxis, calculation, abstraction and perception. The total score ranges are from 0 to 107 and scores lower than 80 are considered indicative of dementia (de Koning et al., 1998).

Other neuropsychological tests such as Trail making test A (TMT-A), B (TMT-B), and Boston naming test (BNT) correlate with decreased performance on episodic memory tasks due to impairment in the left Hc. There are other test scales, like the clinical dementia rating scale, that investigate different aspects of memory (Arlt et al., n.d.).

In this study, the clinical neurological and neuropsychological evaluation of the patients began when they arrive for medical consultation and it is initially diagnosed as MCI. The final diagnosis requires several complementary tests such as MRI and PET, the most usually neuropsychological tests such as MMSE and ADAS-Cog, and analysis of the biomarkers in CSF samples.

1.1.7.2. Diagnostic techniques and fluid Biomarkers

Some of the methods for classifying the degree of AD pathology at different stages include the Braak scale through the performance of an autopsy for the analysis of tau deposit in different regions of the brain (Lowe et al., 2018). Due to the scarcity of non-invasive tools to predict whether a patient with early or late-stage of MCI will develop AD or not, it is necessary to develop new diagnostic methods for early detection of patients in risk to develop AD. In this regard, the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) was established in 1986 by a grant from the National Institute on Aging (NIA), to standardize procedures for the evaluation and diagnosis of patients with AD (Morris et al., 1989).

A diagnostic biomarker is capable to detect or confirm the presence of a disease in an individual or may redefine the classification of this disease (Califf, 2018). There are 5 potential types of fluid biomarkers, blood/serum/plasma, CSF, urine, saliva, and tears; the last two are promising but little is known about them. These samples have advantages and disadvantages; in the case of CSF, there is a close relationship with the brain, it shows high accuracy in the diagnostic process and ability to test pathophysiological biomarkers. In contrast, the disadvantages are that lumbar puncture is invasive and the clinicians require training for the extraction, and in later stages of AD, CSF biomarkers consist in a confirmatory diagnostic method. On the other hand, plasma is obtained through a non-invasive and fast technique, less expensive and reproducible, the clinicians do not require complex training, and it can test a pathophysiological biomarker used as a diagnostic in the early stages of AD. Instead, the disadvantages are less accurate for detecting biomarkers from the brain, the blood has very low concentrations of the pathological biomarkers once they have crossed the blood-brain barrier (BBB). Consequently, the results are less consistent and it is susceptible to interference with another blood components. Lastly, the urine, tear and saliva are obtained by extremely non-invasive and cheap techniques, repeatable and their collections are easy to obtain, and it has also no risk of infection; but the main disadvantage is the lack of validated studies and replicated results (Ausó et al., 2020).

Regarding technology, nowadays, commercially available ELISA and their automated versions such as Lumipulse and Elecsys by Fujirebio and Roche Diagnostic, respectively, are the diagnostic techniques most used. Other potential techniques for the determination of biomarkers are the Meso Scale Discovery platform (MSD) and the Single molecule array (Simoa) platform. Multiplex analysis is also useful for the semiquantitative or quantitative analysis of neurodegenerative biomarkers as A β and tau in several samples as CSF, plasma, or another fluidic matrix (Janelidze et al., 2016; Olsson et al., 2016; Scheuer et al., 2009). In relation to the most validated methods such as Lumipulse and Elecsys, they use the chemiluminescent immunoassay (CLIA) detection method, which is a type of immunoassay that uses a luminescent molecule for detection. This type of assay has the advantage of

increased sensitivity and dynamic range, which allows the detection of lower analyte concentrations that is the goal for early diagnosis of AD (Dakterzada et al., 2021).

1.1.7.2.1. Current state of blood/serum/plasma biomarkers as diagnostic techniques

The minimally invasive sample as blood/serum/plasma would be preferable, but the major challenge is reaching the sensitivity for measuring the neuropathophysiological proteins as total tau, p-tau or the A β 1-42/40 ratio (Barthélemy et al., 2019). The detection of biomarkers in plasma is cheaper and easier than CSF, but also less accurate and requires technologies with high sensitivity in the detection of the neurological biomarkers (Blennow & Zetterberg, 2015; D. Li & Mielke, 2019). There are still limitations in the use of reliable blood biomarkers in clinical practice due to problems of standardization and reproducibility between samples. Currently, the most sensitive and less invasive method for determining blood biomarkers is the Meso Scale Discovery Electrochemiluminescence (MSD), which uses sandwich ELISA method coupled with electro-chemiluminescence (ECL) detection and plate array technology to provide highly sensitive and multiplexed detection of analytes in a complex biological matrix. MSD can be used for analyzing biomarkers especially most kinds of cytokines and chemokines, perform PK/PD analysis, and carry out immunogenicity testing in a broad range of sample types such as blood, serum, and tissue, etc. Notably, most of the platforms for quantifying blood biomarkers uses ECL as a detection technique since it provides high sensitivity and better efficiency, uses less volume, and shows a rapid throughput, as opposed to a colorimetric reaction employed by ELISA (Janelidze, Mattsson, et al., 2020; Thijssen et al., 2020). Unlike a conventional Elisa, when performing a sandwich-style MSD assay, a small plate is used which is an electrode with streptavidin and binds the capture antibody the biotinylated mouse monoclonal AT270 (anti- pT181-tau antibody, as an example of a biomarker with diagnostic potential in AD). In addition, the detection antibody is bound to a sulfo-TAG-conjugated ruthenium (Ru) metal ion (SULFO-TAG-RU), leading to light emission, as they are electro-chemiluminescent labels. If the secondary antibody binds to the antigen, the ruthenium ion will be close enough to the electrode to trigger an oxidation-reduction reaction that will eventually produce light at 620 nm (Kruse et al., 2012).

Recently, it has been reported that the plasma levels of pT181-tau have a diagnostic capacity because of differentiating AD from healthy controls, and differentiate MCI and AD relative to a variety of clinical Frontotemporal lobar degeneration (FTLD) phenotype (Thijssen et al., 2020). Likewise, it has been shown that high plasma levels of pT181-tau are associated with the development of AD dementia in cognitively unimpaired participants (Janelidze, Mattsson, et al., 2020). High plasma levels of pT181-tau were also found in AD dementia patients than those with cognitively unimpaired (Mielke et al., 2018). Therefore, these studies suggested that the levels of plasma pT181-tau may be a potential diagnostic and prognostic biomarker that can be used as a screening test in the population at risk.

Regarding the Simoa platform for determination of plasma levels of pT181-tau by using commercially available antibodies, it may be the most sensitive platform nowadays because it detected a steady rise in plasma pT181-tau marker levels as AD progressed. This fact is important since different studies has demonstrated that plasma pT181-tau levels are able to distinguish between AD patients from healthy controls and those with FTD and other primary tauopathies (Blennow & Zetterberg, 2015). Simoa platform is generally more sensitive than MSD, because it has been developed to detect simultaneously thousands of single protein molecules in a variety of different samples as serum, plasma, CSF, urine, cell extracts, etc. (de Meyer et al., 2020). Simoa uses the same reagents as a conventional ELISA but its sensitivity is close to femtomolar concentrations (fg/mL), offering an improvement of approximately

1000 times in sensitivity, unlike conventional ELISA limited to the picomolar range (pg/mL) (D. Li & Mielke, 2019).

Briefly, Simoa uses a combination of a capture antibody attached to the surface of paramagnetic beads which recognizes the target molecules. The beads are washed to remove non-specifically bound proteins and incubated with the biotinylated detection antibody and then with β -galactosidase-labelled streptavidin. If a target molecule has been captured and an immunocomplex is formed, the substrate will be converted into a fluorescent product by the bound enzyme and is measured through a digital technology (*Scientific Principle of Simoa (Single Molecule Array) Technology* | SelectScience, n.d.). The acquisition of images is through a digital array technology that allows the measurement of t-tau in plasma or serum but in the picomolar range (pg/mL). Elevated levels of t-tau measured with Simoa in plasma are associated with cognitive decline, but it does not differentiate between healthy subjects and among AD patients, which finally limits the diagnostic capacity of this technology (Fossati et al., 2019). Other studies that used Simoa platform have showed that plasma pT181-tau was a more sensitive and specific predictor of conversion to tau pathology in AD than plasma t-tau (**Figure 7**) (D. Li & Mielke, 2019; Zetterberg, 2019). Overall, pT181-tau has high accuracy to differentiate AD from healthy subjects and patients with other dementias. Importantly, there are platforms such as Simoa for its quantification in non-invasive biological samples, which may be useful in clinical practice as a rapid screening test. pT181-tau is a specific AD biomarker, better than t-tau and together with A β 1-42/40 ratio may be useful for the diagnosis of preclinical AD (Smirnov et al., 2022). In this regard, Karikari et al. also developed and validated an ultrasensitive blood immunoassay for detecting pT181-tau in plasma by Simoa in order to distinguish AD dementia from A β negative young adults and cognitively unimpaired older adults. In conclusion, the plasma pT181-tau assay has the potential to be incorporated into clinical practice as a rapid screening test for the diagnostic biomarker of AD pathophysiology (Karikari et al., 2020). In fact, plasma pT181-tau may be considering the main predictor of conversion to tau pathology in AD, but not in other tauopathies. (Zetterberg, 2019).

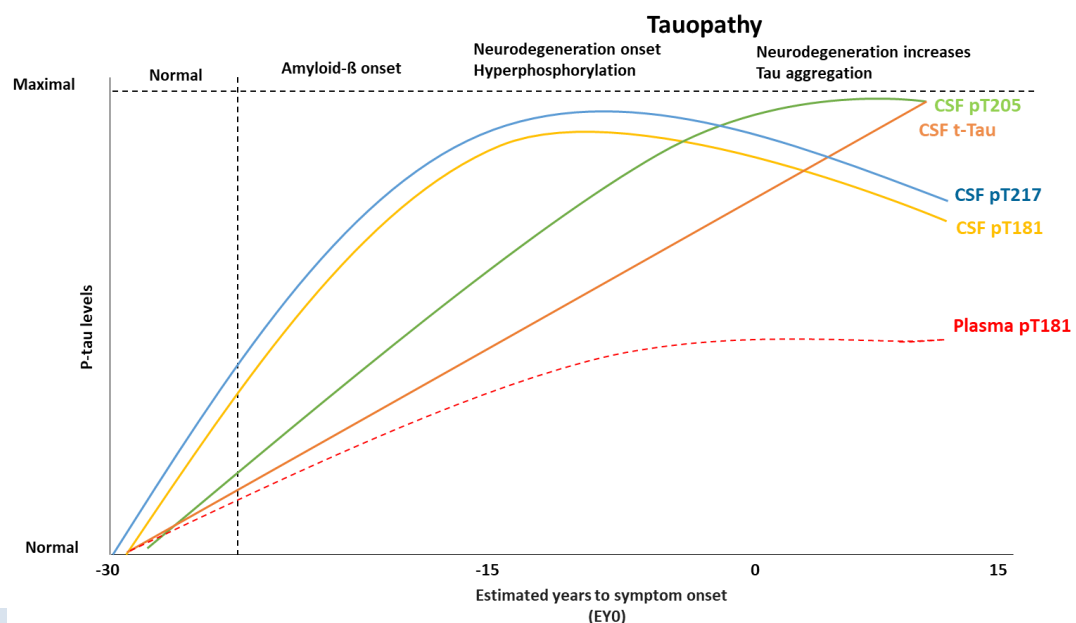


Figure 7. Sequentially changes of different tau species by stage of AD according to disease predictability (EYO). Firstly, the levels in CSF of pT217 (blue) and pT181 (yellow) start to increase with the development of amyloid pathology. Then, the levels of pT205 (green) are increased in CSF in neuronal dysfunction along with soluble t-tau (orange). Lastly, with the tau aggregation occur the cortical atrophy and cognitive decline, while pT217 and pT181 in CSF decrease and plasma pT181 increase. Self-created image using elements with Creative Common license based on (Barthélemy, Li, et al., 2020).

Moreover, higher levels of t-tau in plasma have been associated with greater cognitive decline and risk of MCI. T-tau was also found to be slightly increased in plasma in AD compared with MCI and healthy controls. These findings suggest that plasma t-tau levels may be useful prognostic risk factor or diagnostic biomarker for cognitive decline, but not an AD specific biomarker (Mielke et al., 2017).

There are other phosphorylations analyzed by Simoa in plasma samples, such as pT231-tau. but little research has been published (D. Li & Mielke, 2019). A recent study, published in 2021, compared the potential diagnostic capacity of and pT231-tau plasma levels showed that pT231-tau, but not pT181-tau, was able to differentiate AD patients at early stages of Braak I-II. Therefore, plasma pT231-tau may be also considered an early diagnostic biomarker of AD (Ashton et al., 2021). Barthélemy et al. characterized the presence of pT111, pS202, pT205 and pT217 tau in brain samples by mass spectrometry (MS). Interestingly, pT205 and pS208 tau are specific isoforms of CSF since they do not appear in the brain samples (**Table 3**). These results seem to indicate that AD pathology increases the cellular mechanisms associated with the release of these p-tau isoforms into the CSF (Barthélemy et al., 2019). Regarding the detection of pT217 tau in plasma, a higher discriminatory accuracy of pT217 tau was observed in AD compared to other neurodegenerative diseases. Overall, plasma p-tau assays are better than plasma t-tau assays for diagnosing AD (Palmqvist et al., 2020).

In conclusion, it is necessary to develop standardized protocol for plasma collection, reliable assays with low intra and inter-assays variability, and clinical trials to before clinical implementation of these p-Tau plasma biomarker (Andreasson et al., 2016). Prospective studies are now being conducted with pre-defined cut-off points of optimal combinations of biomarkers for the standardization and definition of diagnostic criteria (Altuna-Azkargorta & Mendioroz-Iriarte, 2018).

1.1.7.2.2. Current state of CSF biomarkers and diagnostic techniques

Nowadays the detection of tau in CSF is feasible and it has the advantage of being a biological sample nearer to the brain than serum or plasma with a relative enrichment of central nervous system (CNS) specific proteins, which are present in very low concentrations in serum or plasma (Barthélemy, Bateman, et al., 2020). A lumbar puncture to obtain CSF is an invasive procedure, but CSF biomarkers can differentiate AD from healthy controls and other dementias (Bateman et al., 2020; Paterson et al., 2018).

Despite the numerous biomarkers identified in CSF with the potential to track pathologies associated with AD and other neurodegenerative diseases, the only clinically validated biomarkers in CSF to date are the A β 1-42/40 ratio, t-tau and pT181-tau (Holtzman, 2011; Piccio et al., 2016). Particularly, it has been shown that pT181-tau levels begin to increase in early stages of AD, by contrast the A β 1-42/40 ratio decreases (Hu et al., 2013). Regarding, t-tau has been increases in AD dementia and prodromal AD in several immunoassays as ELISA and MSD applicated in clinical diagnosis (Blennow & Zetterberg, 2018). In recently studies, pT217-tau has been compared with pT181-tau (Barthélemy et al., 2019) using the latest quantitative MS approach, which achieves a great sensitive detection in CSF. The results showed that pT217-tau differentiated between AD patients and those with other

neurodegenerative diseases and control subjects much more specificity and sensitivity than pT181-tau. Therefore, both are highly specific biomarkers for detecting both the preclinical and advanced stages of AD (Bateman et al., 2020).

Barthélemy et al. used novel MS methods to identify tau phosphorylations in the CSF and brain samples from both AD patients and healthy subjects, finding several new phosphorylated sites in tau protein, as pT205-tau, exclusive of CSF. It has been also demonstrated that the tau isoforms are more hyperphosphorylated in CSF samples of AD patients. The degree of hyperphosphorylation is site dependent. In fact, pT111, pT205, pS208 and pT217- tau were more hyperphosphorylated than pT181-tau, which is the clinical p-tau biomarker most used for diagnosis of AD (**Table 3**). However, the most of hyperphosphorylated sites identified in CSF samples of AD patients were not detected in the brain samples, so the AD pathology seems to increase the release of p-tau isoforms in the CSF (**Figure 7**) (Barthélemy, Li, et al., 2020).

Table 3. Applications of fluid biomarkers for AD diagnosis. It shows the applications of each biomarker in each fluid such as plasma, CSF, saliva and tear, the potential role in AD diagnosis and the technique of development. Mass spectrometry (MS), Alzheimer disease (AD), Enzyme-linked immunosorbent assay (ELISA), Meso Scale Discovery platform (MSD), Single molecule array (Simoa).

Applications	Fluid	Biomarker	Outcome in AD	Stage of development	References
Clinical diagnostics	CSF	A β 1-42/40 ratio	The ratio decreases in early stages of AD	Immunoassays (ELISA, MSD) commercially available	(Bateman, Barthélemy, et al., 2020; Hu et al., 2013)
Clinical diagnostics	CSF	pT181-tau	Increase in early stages of AD, preclinical and advanced forms of AD	Immunoassays (ELISA, SIMOA, MSD) commercially available. Quantitative MS.	(Barthélemy et al., 2019; Bateman, Barthélemy, et al., 2020)
Research	CSF	pT217-tau	Increase in early stages of AD, preclinical and advanced forms of AD. It differentiated AD from other non-neurodegenerative diseases and control subjects. More specificity and sensitivity than pT181	Immunoassays (ELISA, MSD, SIMOA). Quantitative MS.	(Barthélemy et al., 2019; Bateman, Barthélemy, et al., 2020; Janelidze et al., 2018)
Research	CSF	pT181-tau/t-tau ratio	The ratio distinguishes FTLT-DTP from FTLT-tau	Immunoassays and quantitative MS.	(Hu et al., 2013)
Research	CSF	pT205-tau	Increase in neuronal dysfunction	Immunoassays and quantitative MS.	(Bateman, Barthélemy, et al., 2020)
Clinical diagnostics	CSF	t-tau	Increase in early stages of AD (prodromal AD)	Immunoassays (ELISA, MSD) commercially available.	(Blennow & Zetterberg, 2018)
Research	Plasma	pT181-tau	The main predictor of conversion to tau pathology in AD, but not in other tauopathies. Differentiates AD from healthy controls and MCI or FTLT phenotypes	Immunoassays (ELISA, MSD) commercially available.	(Janelidze, Mattsson, et al., 2020; Mielke et al., 2018; Thijssen et al., 2020)
Research	Plasma	pT231-tau	Increase in early stages of AD, preclinical and advanced forms of AD	Immunoassays (ELISA, SIMOA MSD)	(Ashton et al., 2021)
Research	Plasma	pT217-tau	Higher discriminatory accuracy of pT217-tau was observed in AD compared to other neurodegenerative diseases	Immunoassays (ELISA) Quantitative MS	(Palmqvist et al., 2020)
Research	Plasma	A β 1-42/40 ratio decreases	The ratio decreases in preclinical AD	Immunoassays and quantitative MS	(Smirnov et al., 2022)
Research	Plasma	t-tau	Elevated levels of t-tau in plasma are associated with cognitive decline, but it does not differentiate the patients with normal ageing among AD patients	Immunoassays (ELISA, MSD, SIMOA) commercially available	(Fossati et al., 2019)

Research	Tear	t-tau A β 40	and	The increase in t-tau and A β 40 was seen in MCI, for the discrimination of AD compared to controls	Multiplex immunoassays	(Gijs et al., 2021)
Research	Tear	t-tau A β 42	and	The increase of levels of t-tau and A β 42 are positively correlated with the AD stage	Sensitive Luminex assays	(Shi et al., 2011)

Janelidze, et al. showed the output of CSF pT217-tau as a biomarker of AD in comparison to pT181-tau. pT217-tau levels showed a higher increase in CSF compared to pT181-tau, and were better correlated with [^{18}F]flortaucipir uptake in patients with AD compared to those with non-AD neurodegenerative diseases (Janelidze, Stomrud, et al., 2020). Other studies also suggested that pT217-tau might be more useful than pT181-tau as biomarker in the early diagnosis of AD. Likewise, pT217-tau may be also considered a promising potential target for therapeutic applications as a linker between A β and tau pathology (Barthélemy, Li, et al., 2020; Janelidze et al., 2018).

In addition, it has been reported that certain species of p-tau have been detected in the CSF before symptom onset, and many years before tangles can be detected in the brain by PET imaging. Specifically, pT181-tau and pT217-tau, seems to begin to accumulate with the initial aggregation of A β , before the development of tau pathology. Furthermore, pT181-tau and pT217-tau levels increased with atrophy and hypometabolism at the beginning of symptoms. These studies may provide further knowledge into the pathways linking tau, A β and neurodegeneration, with the future development of tau-based treatments and clinical trials (Barthélemy, Li, et al., 2020).

It is known that tau pathology is evolved through different phases in AD. Four different tau species were measured sequentially through the different AD stages from the onset of amyloid plaques, neuronal dysfunction to neurodegeneration. From the development of fibrillary amyloid pathology, pT217-tau and pT181-tau levels begin to increase as shown in the **figure 7**. In the second stage, neuronal dysfunction increases with p205-tau and soluble t-tau levels. Finally, with the onset of neurodegeneration, NFTs begin to develop, while levels of pT217-tau and pT181-tau decrease (**Figure 7**)(Barthélemy, Li, et al., 2020).

Recently, the research of tau biomarkers has been focused in the identification of several new PTMs with different pathophysiological roles. For instance, several studies have found significant differences in the phosphorylation pattern of tau between CSF and brain from progressive MCI to stable MCI, AD, other neurodegenerative diseases and healthy subjects(Barthélemy et al., 2019; Hu et al., 2013; Meredith et al., 2013). For all these reasons, different tau isoforms are currently of special interest as diagnostic and therapeutic targets in AD.

1.1.7.2.3. Current state of biomarkers in other fluids (tears and saliva)

The composition of aqueous humor and tears may be comparable to CSF, as they are composed of molecules and potential neurodegenerative biomarkers (Romaus-Sanjurjo et al., 2022). Concerning the tear, it is an aqueous-lipid layer containing proteins, mucins, lipids, water, and electrolytes, where an increase in levels of t-tau and A β 42 are positively correlated with the AD stage (**Table 3**) (Gijs et al., 2021). Also, the aqueous humor is composed of proteins, electrolytes, solutes, and growth factors with an alteration in the ratio A β 40/42, in this case, tau is not increased. And in relation to the CSF, it shares some of the same components as the previous ones, as water, sodium, potassium, calcium, and organic and inorganic compounds with altered levels of p-tau, t-tau, and A β 40/42 (Romaus-Sanjurjo et al., 2022).

Regarding biomarkers in tear fluid, Gijs et al. demonstrated the presence of A β 38,40 and 42 peptides, t-tau, and p-tau in tear fluid for the first time using multiplex immunoassays. p-Tau was detected in different patient groups such as subjective cognitive decline (SCD), MCI, and dementia but not in healthy control subjects and it did not show differential expression between the different patient groups. In contrast, an increase in t-tau and A β 40 was seen in tears, for the diagnosis and discrimination of AD compared to healthy controls, especially in MCI (Gijs et al., 2020).

Another type of body fluid is saliva, as an exocrine secretion composed of 99% water and 1% protein and electrolytes (Gleerup et al., 2019). Quantitative assessment of salivary levels of t-tau, p-tau, and A β 42 by highly sensitive Luminex assays revealed that, A β 42 was not detectable and the ratio of phosphorylated p-tau/tau was significantly increased in AD patients compared to healthy controls. These results suggest that the saliva could be a promising stable biomarker of tau for the diagnosis of AD (Shi et al., 2011).

1.1.7.3. Our novel biomarkers (pS208 & acK174 tau)

In order to develop new diagnostic methods for early detection of AD, as well as novel therapies, the definition of etiopathogenic targets that occur with the evolution of the AD pathology is of great importance. In this regard, we have focused in different species of the tau protein; specifically, the acetylated tau in the lysine174 (acK174 tau) recognized by a novel mAb named E11; and p-tau in the serine 208 (pS208 tau), serine 202 (pS202 tau) and threonine 205 (pT205 tau) that are recognized by other novel mAb named B6.

Nowadays, there are no commercially available mAbs or ELISA that recognize any tau acetylation, although few research works have been published results on anti-acetylated tau antibodies. Therefore, the E11 antibody is the first to recognize the acK174 tau in human samples. However, it is necessary to emphasize the difficulty of developing research animal models expressing acetylated tau hardens the development and characterization of specific antibodies (Min et al., 2015; Tracy et al., 2016).

In contrast, there are antibodies commercially available for the recognition of the phosphorylation in several epitopes of tau protein as pT181, pS199, pS202, pT205, pT212, pS214, pT217, pT231, pS262, pS396, pS404, or pS422. Specifically, one of the most widely used specifically is the mAb AT8 (ref:MN1020, ThermoFisher), which recognizes pS202 and pT205 (Liu et al., 2021; Malia et al., 2016; Xia et al., 2020). Some of these phosphorylations in tau protein, such as pT181-tau, are validated for clinical diagnosis of AD or other tauopathies (Barthélemy, Bateman, et al., 2020).

The novel tau biomarkers developed in this study are pS208 and acK174. These residues are located in the proline-rich domain of human tau protein, which comprises two regions of residues from 114 to 230 and contains around 25 prolines (McKibben & Rhoades, 2019). However, more recent publications propose that tau's proline-rich domain would serve as a core tubulin-binding domain and relate it to the tau-mediated polymerization of tubulin (He et al., 2009). The pS208, mainly phosphorylated by the TTK, has been related to a reduced tau affinity for microtubules and was identified in CSF samples and tau aggregates of AD patients (Ikezu & Ikezu, 2014). Importantly, previous studies have characterized by MS several phosphorylations in the CSF and brain samples from both AD and non-AD human subjects, The results showed that the degree of hyperphosphorylation is site dependent. It was observed that pS208tau, among others, was more hyperphosphorylated than pT181-tau in CSF compared to the brain, suggesting that pS208 tau could be a more sensitive biomarker in CSF (Barthélemy et al., 2019). In contrast, it was recently published increased plasma levels of pT181-tau in pre-MCI subjects compared to healthy controls. Therefore, pT181-tau plasma

levels may be a cost-effective biomarker for the diagnosis of patients with preclinical AD (Qin et al., 2022).

In AD, tau is predominantly acetylated at K174, K274, K280, and K281 residues, mainly by the P300 acetyltransferase enzyme (Kingwell, 2015). Published data on acetylation-mimic mutations of K174 suggested reduced tau turnover, synaptic defects, neurodegeneration, and memory loss (S. W. Min et al., 2015). Specifically, acK174 has been linked to reduced ubiquitin-proteasome-mediated tau clearance (Min et al., 2010). Also, from a therapeutic point of view, a pharmacological modulation of a lysine acetyltransferase p300/CBP, altered the level of acK174-tau and affected hippocampal function and memory performance in tau transgenic mice models. The inhibition of p300/CBP reduces tau aggregation and cognitive deficits in tau pathology (S. W. Min et al., 2015). In another *in vivo* study, the overexpression of the deacetylase SIRT1 reduces the acK174-tau in the Hc attenuating the extent of tau pathology in hTau.P301S transgenic mice (S. W. Min et al., 2018). These findings suggest the functional importance of SIRT1 and p300/CBP in regulating the pathogenic tau acetylation and in suppressing the spread of tau pathology *in vivo*.

This results, together with other studies reporting that acK174 has an early change in AD brain of animal models expressing tau with the P301S mutation, suggesting that ac K174-tau could be an interesting biomarker of early tau pathology associated to AD (Min et al., 2015).

As shown in **Figure 8**, newly developed mAbs directed against epitopes including the pS208-tau and the acK174-tau proved to be potential diagnostic biomarkers. The B6 antibody, targeting the pS208-tau showed clear binding to intraneuronal and extracellular tau deposits in human AD cortex samples in a Braak stage-dependent manner, from I to VI. In Western Blot (WB) the B6 antibody showed again a band pattern correlated with the Braak stages, with special differences between phases I-II and ahead specially IV-V, from where it revealed the presence of other bands corresponding to hypothetical low-molecular-weight (LMW) oligomers. The E11 antibody, targeting acK174-tau, showed primary binding in samples from stages I -II in both immunohistochemistry (IHC) and WB, which may agree with the hypothesis of a very early presence of acK174-tau in AD pathology. Together these biomarkers could work as a robust differential diagnosis of AD, as they cover all Braak stages, from I-VI.

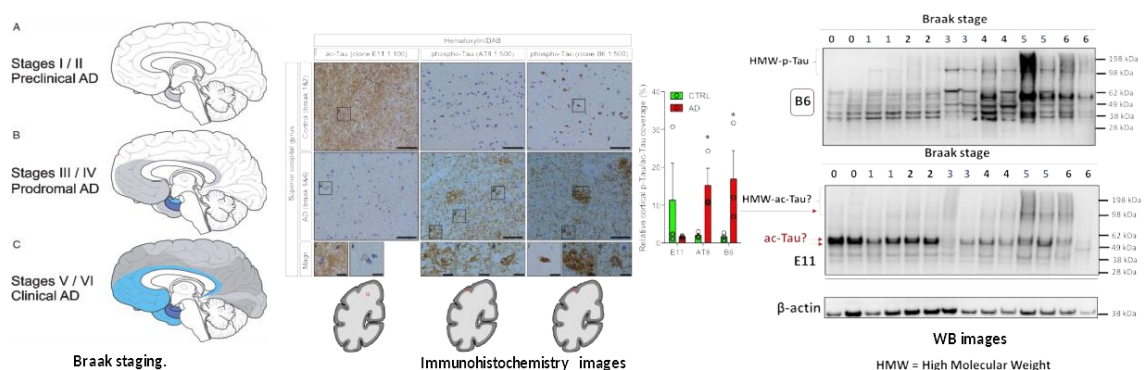


Figure 8. IHC (left) and WB (right) images showing that mAbs to phosphorylated (B6) and acetylated (E11) tau are capable of detecting p-tau and ac-tau oligomers in human brains with AD in Braak Stages IV- V and I-II, respectively. Note: a band of p-tau and/or ac-tau dimers is at 98 kDa. p-tau antibody (AT8) has been included in the immunohistochemical study as a control. Self-created image.

CSF sampling is a routine procedure in most hospitals around the globe, and the most close-up view of brain biochemistry we can have for now. The identification of new CSF biomarkers allowing a differential diagnosis of neurodegenerative diseases and even of different stages of a same disease is essential. In this regard, we propose two novel biomarkers, two PTMs of the same molecule, but related to very different pathologic mechanisms.

Firstly, the pS208-tau should allow to a better differential diagnosis of AD against other disorders, as its presence in CSF is much more specific than others such pT181-tau, t-tau or A β 42. The pS208-tau is the potentially better biomarker than pT181-tau, because it is phosphorylated by the TTK that is related to tau self-aggregation and paired helical filaments (PHFs), and it has a predominant presence in CSF of AD patients which if increase could indicate a potentially pathological decreasing of phosphatase activity. Lastly, the S208-tau is more hyperphosphorylated than T181-tau in CSF of AD patients compared to healthy subjects.

Secondly, the acK174-tau may be considered a new promise therapeutic target as a biomarker at ultra-early stages of AD. The presence of acK174-tau in CSF is practically uncharacterized today, and it has a key rol on tau clearance mechanism because it promotes the potentially neurotoxic tau dimers. Moreover, acK174-tau is related to cell cycle impairment, hippocampal atrophy and tau clearance impairment and it is acetylated by the P300 acetyltransferase.

In summary, these two novel biomarkers could allow to a significant improvement of a differential and early diagnosis of AD, as well as constitute a modifying treatment.

1.1.8. Animal models of Tauopathies

There are different types of transgenic mice with tauopathies that are used as preclinical models of AD. Although it is almost impossible to exactly reproduce human pathology in animals, these models have provided extensive resources for AD research.

The first animal model usually used is the triple transgenic mouse model of AD (3xTg-AD), which combine PS1(M146V), APP (Swe), and tau (P301L) human mutations and develops both amyloid plaques and NFTs in AD relevant regions on month 15. The animal model shows a synaptic dysfunction including long-term synaptic plasticity (LTP) levels, correlating with the accumulation of A β intraneuronal and deficit of memory and learning (Oddo et al., 2003).

In contrast, other most used animal models express human tau (htau) containing mutations in the tau isoform (4R/ON) with P301L or P301S (B. Allen et al., 2002). These mice develop over 4 months NFT, neurodegeneration, atrophy, and motor and cognitive deficits over 4 months (Drummond & Wisniewski, 2017). Specifically, the hTau P301S is the animal model that we use in our study and they are specifically hybrid mice of a cross between C57BL/6J females (B6) and CBA/J males (CBA) supplied by Remynd. The gene hTau [P301S] is expressed under the control of the neuron-specific Thy-1 promotor and is characterized by hyperphosphorylation of tau residues S202, T231, S235, S396, and S404 onsets at 3 months of age. The NFT-like pathology in the spinal cord (Sc), brainstem (Bs), cerebral cortex (Cx) and Hc from 4 months onwards (B. Allen et al., 2002). Starting at 2.5 months of age the problems in movement, memory, and vision. Particularly the loss of motor neurons in the Sc and some loss of superficial cortical neurons, produce an early motor impairment, including aberrant clasping and rotarod deficit at 4 months (Hampton et al., 2010). Also, they develop neuronal loss or neurodegeneration with a decline in spine density and length has been seen in the Hc at 3 months (Scattoni et al., 2010). Reactive astrogliosis

measured by GFAP reactivity have seen at 6 months and microgliosis in the Bs and Sc at 5 months by OX42 staining (Xu et al., 2014). Therefore, the hTau P301S transgenic mice have a median life expectancy of 5-6 months of age (B. Allen et al., 2002).

On the other hand, the PS19 transgenic mice (P301S), express the P301S mutant version of human MAPT under the control of the mouse prion protein promoter (Prnp), is a different model that is frequently utilized in research. Age-related accumulation of the hyperphosphorylated, insoluble mutant human MAPT protein in the brain reduces microtubule binding/density. The PS19 transgenic mice has a median life expectancy of nine months, with 80% of them passing away by 12 months. When hoisted by the tail, transgenic mice show clasping and limb retraction at three months of age, which develops into limb weakness. By the age of ten months, the mice display a bent back, paralysis, and then an inability to eat. The brain atrophy and neurodegeneration in the Hc are revealed by histological investigation (Yoshiyama et al., 2007).

The most particular case such as *Octodon degus* is the only animal model with natural aging associated with spontaneous AD (not included in the **table 4** because it does not express hTau). It is a moderate-sized rodent that is widely used in AD research because it is the only one that is not a transgenic animal. It shows age-related cognitive and white matter alterations and accumulates soluble A β oligomers and p-tau, leading to the neuropathology of cerebral vessels with consequent cerebral amyloid angiopathy (Castro-Fuentes & Socas-Pérez, 2013).

In summary, all these animal models of AD mentioned in **table 4** do not fully reproduce the pathology of AD as it occurs in humans, as they do not present all the molecular mechanisms that occur in the disease accurately at the level of the CNS. This is why it is so difficult to translate this disease from research to the clinic, but they are useful to study the specific molecular mechanism of the pathology and to use them as possible targets for the development of new diagnostic and therapeutic treatments for AD.

Table 4. Tau-related animal models available for AD research. All available animal models with tauopathies expressing native or mutated human tau (htau), some of them also combined with A β aggregates (Neuner et al., 2019). NFTs (neurofibrillary tangles).

Animal Model	Tau isoforms	Tau mutations	Tau accumulation		p-tau		Cognitive impairment		Bibliography	Supplier
			Type	Month	Residues	Month	Deficit	Month		
3xTg	hTau (P301L)	hTau (P301L)	PHF	15	202, 205, 235, 396, 404	12	learning & memory	4	(Oddo et al., 2003)	The Jackson Lab Stock# 004807.
AppNL-G-F/MAPT double knock-in	3R+4R	hTau	none	none	202, 205, 235, 396, 404, 422	6	Memory	12	(Saito et al., 2019)	Takaomi Saido, RIKEN Center for Brain Science
htau	All hTau (3R, 4R)	hTau	PHF	9	202, 231, 235, 396, 404	6-15	Spatial learning & memory	6-12	(Andorfer et al., 2003)	The Jackson Lab Stock# 005491
hTau.P301S	4R/0N (P301S)	hTau (P301S)	NFT	4	202, 231, 235, 396, 404	5-6	Memory/Motor+Visual	2.5/3	(B. Allen et al., 2002)	ReMYND
hTau-AT (hTau40-AT)	2N4R (A152T)	hTau (A152T)	NFT	3	p-tau	3	learning & memory	16	(Decker et al., 2016)	Eva Mandelkow
JNPL3 (P301L)	4R/0N (P301L)	hTau (P301L)	Pick-body like, NTF	4,5	180, 202, 205, 208, 396, 404, 422	4.5-6.5	motor & behavioral	8	(Lewis et al., 2000)	
mThy-1 3R Tau (line 13)	3R (L266V & G272V)	hTau (L266V), hTau (G272V)	Pick-body like, PHF	6-8	202, 205, 208, 235, 396, 404	8-10	memory	6-8	(Rockenstein et al., 2015)	
rTg(tauP301L)4510	4R/0N (P301L)	hTau (P301L)	NFT	2,5	202,205.2 31,409	4	Spatial memory/Motor	2.5/6	(Santacruz et al., 2005)	The Jackson Lab. Stock# 015815
tau35	187-441	hTau35	NFT	14	p-tau	2	Spatial memory	8	(Bondulich et al., 2016)	
Tau609 (Tau 10 + 16)	3R+4R	hTau (IVS10+1 6 C>T)	NFT	5	p-tau	6	Spatial memory	6	(Umeda et al., 2013)	Hiroshi Mori and Takami Tomiyama

TauΔK280	2N4R (K280del)	hTau (K280del)	NFT	24	262, 356	3	Spatial memory/passi ve-avoidance	16	(Eckermann et al., 2007)	Eva Mandelkow
Tau P301L	4R/2N (P301L)	hTau (P301L)	NFT	8	181, 202, 205, 212, 214, 231, 396, 404,	8	passive- avoidance/Me mory	5/9	(Terwel et al., 2005)	ReMYND
TauP301L-AAV	hTau (P301L)	hTau (P301S)	NFT	6	202, 396, 404	6	Fear conditioning	6	(Cook et al., 2015)	
Tau P301S (Line PS19)	4R/1N (P301S)	hTau (P301L)	NFT	6	p-tau	3	Spatial learning + Fear conditioning	6	(Yoshiyama et al., 2007)	The Jackson Lab: Stock# 024841
TauPS2APP	hTau (P301L)	hTau (P301L)	NFT	16	231,422	4	Spatial learning	4	(Grueninger et al., 2010)	Laurence Ozmen
Tau R406W	4R (R406W)	hTau (R406W)	NFT	18	199, 202, 205, 231, 396, 404, 413, 422,	18	Learning+ Memory	16-23	(Tatebayashi et al., 2002)	
TauRΔK280 ("Proaggregation mutant")	2N4R (ΔK280)	hTau (K280del)	NFT	2-3	262, 356	2	Learning+ Memory	10	(Mocanu et al., 2008)	
Tau V337M	hTau (V337M)	hTau (V337M)	NFT	4-11	199, 202, 205	11	Behavioral	11	(Tanemura et al., 2002)	
Tg2576/Tau(P301L) (APPSwe-Tau)	hTau 4R (P301L)	hTau (P301L)	NFT	3	202,205,2 31, 235,396, and 404	9	Visual and motor	6,5	(Lewis et al., 2001)	
THY-Tau22	4R (G272V & P301S)	hTau (G272V), hTau (P301S)	NFT, PHF	3-6	181, 202, 205, 208, 396, 422	4	Spatial memory	9	(Schindowski et al., 2006)	Luc Buée
TMHT	4R/2N (V337M, R406W)	hTau (V337M), hTau (R406W)	NFT	4	181, 199, 231, 235, 396, 404	2	Spatial memory	5	(Flunkert et al., 2013)	CRO QPS-Austria

2. HYPOTHESIS AND JUSTIFICATION

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative disorder that accounts for approximately 60% to 80% of all cases of dementia and is the fifth leading cause of death worldwide (“2021 Alzheimer’s Disease Facts and Figures,” 2021). Currently, an estimated 50 million people worldwide suffer from the disease, which has a significant impact on people's quality of life and independence. Moreover, as the population grows and life expectancy increases, the incidence and prevalence of AD are increasing (Sevigny et al., 2016).

Despite decades of research and some recent advances in clinical trials, there are still no drugs that can slow the progression of AD, let alone offer a cure. So far, promising preclinical results have repeatedly failed in clinical trials, possibly for a variety of reasons: unsuitable animal models, focusing on targets that have not yielded therapeutic results, conducting clinical trials in advanced stages of AD, poor clinical trial design, etc. (J. L. Cummings et al., 2014). Given this situation, there is a broad consensus on the need to develop new diagnostic methods to detect the disease in early stages and subsequent therapeutic approaches. The early stages of the disease, both the asymptomatic preclinical and the symptomatic prodromal stages, predecessor stages of mild cognitive impairment (MCI) and AD, are under constant study to better define them as they would be the basis for action to prevent the progression of AD disease. To date, the research efforts (in both therapy and diagnostics) have mainly focused on understanding and combating the progression of amyloid plaques around neurons, which is one of the main etiopathological characteristics of the disease. As progress in finding an effective therapy in this area has not yet crystallized, researchers are paying increasing attention to other molecular mechanisms related to the disease, such as the aggregation of the microtubule-associated protein tau into neurofibrillary tangles (NFTs) within cells (Molinuevo et al., 2012). Indeed, a feature of AD is the propensity of tau protein to form oligomers that act as a template for the conversion and co-aggregation of endogenous proteins, which eventually form NFTs and are relevant in the progression of the disease (Jahn et al., 2010).

Given the important role played by the tau protein in the progression of AD, the development of new biomarkers and subsequent antibodies specific for different residues of the tau protein, as acK174 tau recognized by E11 mAb and pS208, pS202 and pT205 tau recognized by B6 mAb, could contribute significantly to the fight against the AD in two ways:

1. Development of new diagnostic methods for early diagnosis of the disease.
2. Development of new modifying therapies based on targets directed at those species of the tau protein that may contribute to the development of the pathology.

3. OBJECTIVES

The first main objective of this study is the development of an *in vitro* diagnostic ELISA kit for detecting in CSF samples specific pathology-related forms of tau (p-tau and ac-tau), using the novel anti-ptau B6 and anti-actau E11 monoclonal antibodies (mAbs), in order to the identification of different stages of AD for early diagnosis and subsequent clinical validation.

The specific objectives were:

1. To develop an *in vitro* diagnostic ELISA kit for detecting in CSF samples specific pathology-related forms of tau (p-tau and ac-tau), using the novel anti-ptau B6 and anti-actau E11 mAbs, in order to the identification of different stages of AD for early diagnosis and subsequent clinical validation.
2. Characterization of anti-ptau B6 and anti-actau E11 mAbs defining their stability, functionality, affinity, and specificity.
3. The development of *in vivo* proof of concept based on a new contrast agent for p-tau compatible with MRI/PET. The combination of complete B6 antibody and F(ab')₂ B6 fragment with Zirconium-89 (⁸⁹Zr) radiotracer can be used for early detection of disease by neuroimaging.

The second main objective due to the potential therapeutic properties intrinsic to mAb, this study was also aimed to demonstrate the efficacy and safety of immunotherapy through the intracerebroventricular (ICV) administration of B6 antibody on behavior, brain morphology/functionality, and tau pathology in a preclinical study.

The specific objective was:

1. To test the efficacy and safety through an immunotherapy preclinical study to determine the therapeutic effect of the ICV administration of B6 antibody on behavior, brain morphology/functionality, and tau pathology of a transgenic mouse model of tauopathy (hTau.P301S).

4. MATERIAL AND METHODS

4.1. CHARACTERIZATION OF B6 AND E11 ANTIBODIES

The E11 and B6 mAbs were produced and developed within the PANA Project (H2020-NMP-2015 two-stage, Grant 686009), obtained through the BL57 strain wild-type mice (*Mus musculus*) after immunization with two immunogens including the targeted sequences and a KHL protein carrier. The resulting hybridomas were isolated through a limiting dilution technique and positive/negative selection against peptides with and without post-translational modifications.

The mechanism of action of an antibody will determine its therapeutic efficacy, thus it is essential to determine the antigen-antibody interaction dynamics in order to design future experiments and assess their potential. Epitope mapping, affinity, biochemical properties, stability, and functionality are key aspects to be analyzed.

4.1.1. Stability

The stability of B6 antibody were tested in an indirect ELISA in Nunc Maxisorp flat-bottom (Thermo Fisher Scientific, USA) plates coated with different concentrations of 3P-tau peptide. Briefly, the protocol followed was a simple indirect ELISA, where 96-well ELISA plates Nunc Maxisorp flat-bottom (Thermo Fisher Scientific, USA) were coated with 100 μ L of 3P-tau peptide in a carbonate buffer (BioRad, USA) at 4°C overnight. For blocking, 100 μ L of Protein-Free (TBS) Blocking Buffer(Thermo Fisher Scientific, USA) was added and incubated for 1h at 37°C. Primary antibodies (100 μ L) were diluted at the desired concentrations in 0.05% Tween 20 (ITW Reagents, USA) in TBS (Sigma Aldrich, USA) and incubated for 1 h at 25°C. Secondary HRP-conjugated antibodies (Dako Anti-Mouse-HRP, USA) were added (100 μ L) in a 1:2000 dilution in TBS and incubated again at 25°C for 1 h. Finally, 100 μ L of HRP substrate was added 1-Step Ultra 3,3',5,5'-tetrametilbencidina (TMB) (Thermo Fisher Scientific, USA). Following 5 min incubation at room temperature (RT), the reaction was stopped by the addition of 100 μ L Sulphuric Acid Solution,1M (Merckmillipore, USA). The plates were read in a Biotek spectrophotometer (Agilent, USA) and absorbance result at 450 nm are shown in form of bars histogram.

4.1.2. Epitope Mapping

In previous ELISAs have confirmed that the E11 antibody does not require the presence of both K163 and K174 acetylations for binding the synthetic peptides, but just the one in K174. However, we do not know the role of the K174 acetylation within the whole tau protein-E11 binding mechanism. In order to confirm such binding mechanisms and requirements, further NMR and peptidic mini-arrays experiments have been designed.

4.1.2.1. Mini-array Epitope Mapping: Method 1

4.1.2.1.1. Microarray synthesis

In order to reconstruct epitopes of the target molecule, a library of peptide-based peptide mimics was synthesized using Fmoc-based solid-phase peptide synthesis. An amino functionalized olypropylene support was obtained by grafting with a proprietary hydrophilic polymer formulation, followed by reaction with t-butyloxycarbonyl-hexamethylenediamine (BocHMDA) using dicyclohexylcarbodiimide (DCC) with N-hydroxybenzotriazole (HOBt) and subsequent cleavage of the Boc-groups using trifluoroacetic acid (TFA). Standard Fmoc-peptide synthesis was used to synthesize peptides on the amino-functionalized solid support by custom modified JANUS liquid handling stations (Perkin Elmer, USA).

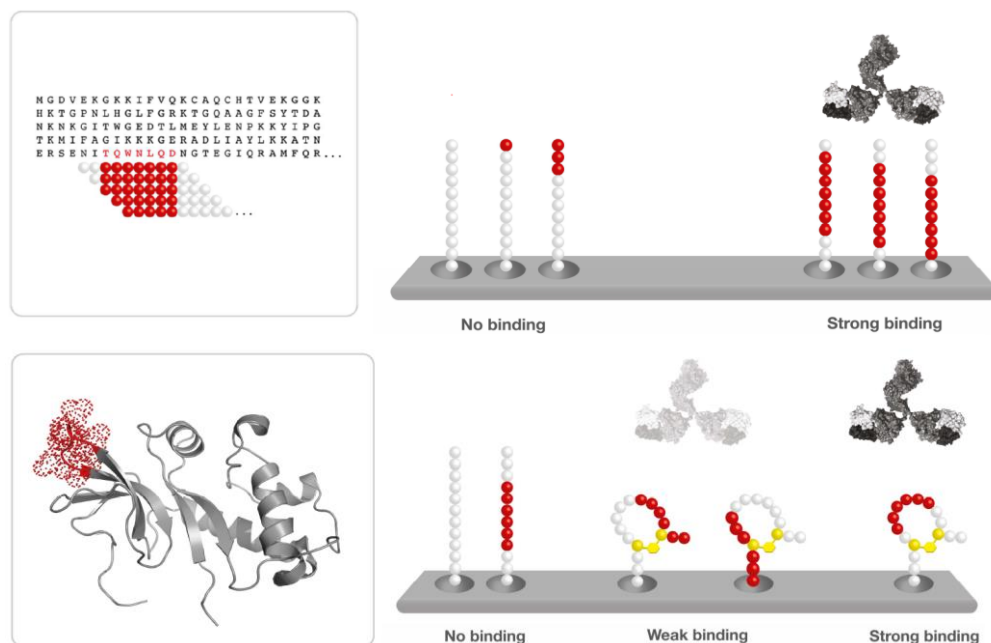


Figure 9. Pepscan's technology for constructing a linear (above) and a conformational (below) peptide library. The target linear sequence is converted into a library of all overlapping linear and conformational peptides directly synthesized on a proprietary solid surface. Self-created image using elements with Creative Common license based on Pepscan.

Synthesis of structural mimics was done using Pepscan's proprietary Chemically Linked Peptides on Scaffolds (CLIPS) technology (**Figure 9**). CLIPS technology allows to structure peptides into single loops, doubleloops, triple loops, sheet-like folds, helix-like folds, and combinations thereof. CLIPS templates to cysteine residues which the side-chains of multiple cysteines in the peptides are coupled to one or two CLIPS templates, which were bound to side-chains of two cysteines as present in the solid-phase bound peptides of the peptide-arrays (455 wells plate with 3 μ l wells). The peptide arrays are gently shaken in the solution for 30 to 60 minutes while completely covered in solution. Finally, the peptide arrays are washed extensively with excess of H₂O and sonicated in disrupt-buffer containing 1 % SDS/0.1 % 2,2' (Ethylenedioxy) diethanethiol in phosphate buffer saline (PBS) (Biowest, France) (pH 7.2) at 70°C for 30 minutes, followed by sonication in H₂O for another 45 minutes. The T3 CLIPS carrying peptides were made in a similar way but now with three cysteines.

4.1.2.1.2. Elisa screening

The binding of antibody to each of the synthesized peptides was tested in a pepscan-based ELISA. The peptide arrays were incubated with primary antibody solution at 4°C overnight. After washing, the peptide arrays were incubated with a 1/1000 dilution of an appropriate antibody peroxidase conjugate (Thermo Fisher Scientific, USA) for one hour at 25°C. After washing, the peroxidase substrate 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) (Sigma Aldrich, USA), and 20 μ l/ml of 3 percent H₂O₂ were added. After one hour, the color development was measured. The color development was quantified with a charge coupled device (CCD) camera and an image processing system.

4.1.2.1.3. Data processing

The values obtained from the CCD camera range from 0 to 3000 mAU, similar to a standard 96-well plate ELISA-reader. The results were quantified and stored into the Peplab

database. Occasionally, a well contains an air-bubble resulting in a false-positive value, the cards were manually inspected and any values caused by an air-bubble were scored as 0.

4.1.2.1.4. Synthesis quality control

To verify the quality of the synthesized peptides, a separate set of positive and negative control peptides were synthesized in parallel. These were screened with commercial antibodies 3C9 and 57.9 (Posthumus et al., 1990)

4.1.2.2. Mini-array Epitope Mapping: Method 2

4.1.2.2.1. Microarray synthesis

The sequence of MAPT (XP_005257419) and the acetyl lysine containing ac-tau immunogen were elongated with neutral GSGSGSG linkers at the C- and N-terminal to avoid truncated peptides. The elongated antigen sequence was translated into 15 amino acid peptides with a peptide-peptide overlap of 14 amino acids. The resulting MAPT peptide microarrays contained 913 different linear peptides printed in duplicate (1,826 peptide spots), and were framed by additional HA (YPYDVPDYAG, 98 spots) control peptides (**Figure 10**).

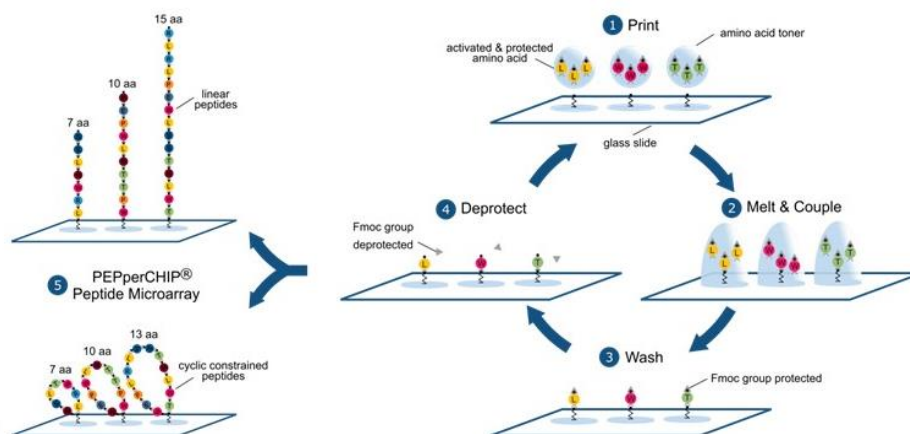


Figure 10. PEPperPRINT microarray preparation also implies the direct printing of the peptides in the chip, involving five steps. Once peptides are printed, some of them can be constrained in a cyclic way to mimic some conformational epitopes. Self-created image using elements with Creative Common license based on PEPperPRINT.

4.1.2.2.2. ELISA screening

Pre-staining of a linear MAPT peptide microarray was done with the secondary antibody in incubation buffer in order to investigate background interactions with the antigen-derived peptides that could interfere with the main assays. Subsequent incubation of another MAPT peptide microarray copy with mouse anti-actau E11 mAb at a concentration of 1 µg/ml in washing buffer with 10% (BB) for 16 h at 4°C, and shaking at 140 rpm, This step was precede by an incubation with BB (Rockland, USA) at 30 min before the first assay. Staining with secondary (Goat anti-mouse IgG (H+L) DyLight680 (Thermo Fisher Scientific, USA) (0.2 µg/ml); 45 min staining in incubation buffer at RT and using a control mouse monoclonal anti-HA (12CA5) DyLight800 (Thermo Fisher Scientific, Massachusetts, USA) (0.5 µg/ml); 45 min staining in incubation buffer at RT. The antibodies as well as read-out with a LI-COR Odyssey Imaging System at scanning intensities of 7/7 (red/green). The additional HA control peptides framing the peptide microarrays were subsequently stained with the control antibody as internal quality control to confirm the assay quality and the peptide microarray integrity. After each step, the microarray was washed with PBS, pH 7.4 with 0.05% Tween 20 (3 x 10 sec after each incubation step). Imaging was acquired with a LI-COR Odyssey Imaging

System; scanning offset 0.65 mm, resolution 21 μ m, scanning intensities of 7/7 (red = 700 nm/green = 800 nm).

4.1.2.2.3. *Data processing*

Quantification of spot intensities and peptide annotation were based on the 16-bit gray scale tiff files that exhibit a higher dynamic range than the 24-bit colorized tiff files. Microarray image analysis was done with PepSlide® Analyzer. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal, and calculates averaged median foreground intensities and spot-to-spot deviations of spot duplicates. Based on averaged median foreground intensities, an intensity map was generated and interactions in the peptide map highlighted by an intensity color code with red for high and white for low spot intensities. A maximum spot-to-spot deviation of 40% was tolerated; otherwise, the corresponding intensity value was zeroed. Averaged spot intensities of the assay with the mouse mAb against the antigen sequence from the N-terminal of MAPT to the C-terminal and the acetyllysine containing ac-tau immunogen were plotted to visualize overall spot intensities and signal-to-noise ratios. The intensity plot was correlated with peptide and intensity maps as well as with visual inspection of the microarray scan to identify the epitope of the antibody sample.

4.1.3. Specificity

4.1.3.1. *Cross reactivity test of E11*

As some inconsistent data were obtained in previous LC-MS/MS experiments with the E11 antibody, in order to discard non-specific interactions of the antibody with other proteins, it was performed a cross-reactivity test with a protein microarray HuProt™ Human Proteome Microarrays v3.1 (Cambridge Protein Arrays Ltd., Cambridge, UK).

4.1.3.1.1. *Microarray content*

The antibody profiling was done with HuProt™ Microarrays with over 20,000 individual proteins, representing more than 16,000 human genes. Proteins were expressed in yeast (*S. cerevisiae*) as GST fusions, purified and spotted in duplicate. The HuProt™ Human Proteome Microarray further contained several additional control proteins including rhodamine + Alexa Fluor 647 labeled IgG as landmarks for fluorescence detection at wavelengths of 532 nm/635 nm.

4.1.3.1.2. *ELISA screening*

After 15 min pre-swelling in washing buffer and 30 min incubation in BB (Rockland, USA), a HuProt™ Human Proteome Microarray was initially incubated with the secondary goat anti-mouse IgG (H+L) DyLight680 antibody (Thermo Fisher Scientific, USA) in incubation buffer to analyze background interactions with the >20,000 proteins of the microarray that could interfere with the main assays. Subsequent incubation of the HuProt™ Human Proteome Microarray with mouse anti-ac-tau E11 mAb at concentrations of 1 μ g/ml and 10 μ g/ml in incubation buffer was followed by staining with secondary goat anti-mouse IgG (H+L) DyLight680 antibody (Thermo Fisher Scientific, USA) and read-out with a LI-COR Odyssey Imaging System at a scanning intensity of 7 shown in red.

4.1.3.1.3. *Data processing*

Quantification of spot intensities and peptide annotation were based on the 16-bit gray scale tiff files that exhibit a higher dynamic range than the 24-bit colorized tiff files.

Microarray image analysis was done with Spotxel® and summarized in the Excel files listed in Material and Methods. A software algorithm breaks down fluorescence intensities of each spot into raw, foreground and background signal, and calculates averaged median foreground intensities and spot-to-spot deviations of spot replicates. We tolerated a maximum spot-to-spot deviation of 40%, otherwise the corresponding intensity value was zeroed.

In order to identify the top cross-reactions of the mouse mAb sample, the averaged and corrected intensity values were cleaned from background interactions of the secondary antibody and sorted by decreasing spot intensities. We further plotted the averaged spot intensities of the assay with the mouse anti-actau E11 mAb against the microarray content from left on top to right on bottom of the chip to visualize overall spot intensities and signal-to-noise ratios. Common interactions with the secondary antibody were not annotated in the intensity plot. For a better data overview, the baselines of the intensity plots were leveled and the Y-axis with the fluorescence intensities cut at an appropriate level to also highlight low intensity interactions.

A simple text search of the identified proteins in the HuProt Microarray in the raw data reported by the MS/MS analysis allowed to identify the presence or absence of each protein. All MS/MS data sets were organized in tables with the identified proteins, including their representation within the sample pool.

4.1.4. Functionality

4.1.4.1. ELISA

The functionality and specificity of E11 and B6 mAb were tested in an indirect ELISA (described in 4.1.1) in Nunc Maxisorp plates (Thermo Fisher Scientific, USA) coated with different concentrations of three synthetic peptides. The three synthetic peptides with 74 residues used as antigen are the following ones (**Figure 11**):

- p-tauL is phosphorylated in serine 208
- ac-tauL is acetylated in lysine 174
- un-tauL is not modified

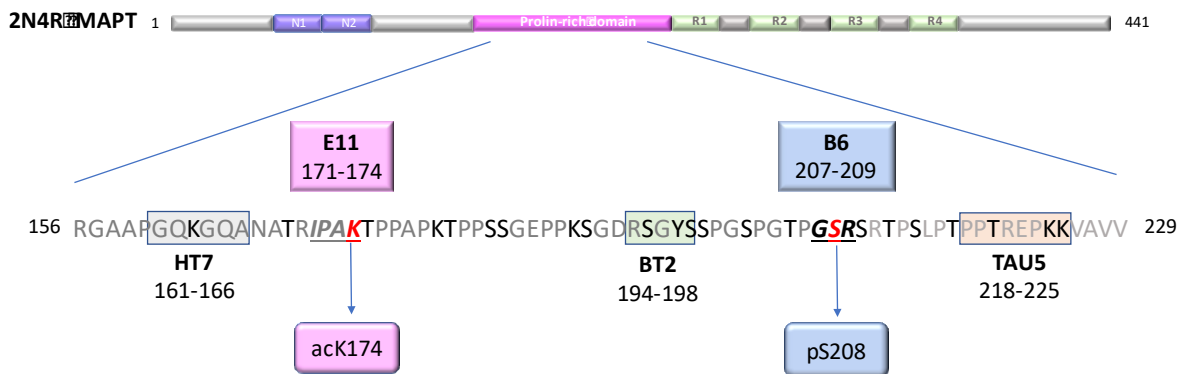


Figure 11. Peptides sequence and binding sites of the antibodies: E11, B6, HT7, BT2 and TAU5. The binding site for TAU1 antibody is 192-199 and it is not indicated in the figure because it is almost the same site as for BT2 antibody. Self-created image using elements with Creative Common license.

4.1.4.2. Western Blot

With the aim of assess the functionality and specificity of custom-made p-tau B6 antibody and ac-tau E11 antibody in the human brain tissue, WB was applied to

neurofibrillary pathology (Braak staging; 0-VI). Further comparison with other well-known commercially available antibodies was also performed (**Table 5**). The protocol is described as follow. The first step is the sample preparation, The media was removed with a vacuum, the cells were washed with TBST and lysed with 350 μ L IP lysis buffer with 1% NP-40 and Sigma Protease Inhibitor Cocktail 1:100 (Sigma, USA) or 350 μ L TNE lysis buffer with Sigma PIC 1:100 using rubber policeman (Sigma, USA). The lysate was transferred to an eppendorf tube and was incubated on ice for 30 min, vortexed thoroughly every 10 min. Then, it was centrifugated at 14,000 rpm for 15 min. After that, supernatant was transferred to a fresh eppendorf tube on ice. The Biorad Dc Protein Assay (Biorad, USA) was used to quantitate the amount of protein. A 2x tris-tricine sample buffer was added and boiled for 5 min. The sample was stored at -20° C until to proceed with WB.

In order to merge the gel, thoroughly clean glass plates before casting the gel with 70% Ethanol (EtOH). The glass plate sandwich in gel casting stand was placed. In a conical vial, all components were mixed for separating the gel and the TEMED (Thermo Fisher Scientific, USA) was added to polymerize the gel. The gel was pipetted into a glass sandwich and overlaid with about 1 mL dH₂O. The polymerization usually takes about 30-45 min. After the gel was polymerized, the water was dumped off, and was removed all water by placing a Kimwipe in the corner of the sandwich. 30% APS and TEMED (Thermo Fisher Scientific, USA) were mixed and added to the stacking gel. The comb was inserted until the gel reaches the very top of the glass plate and the stacking gel was polymerized for about 20-30 min. Running the gel was proceeded using the Bio-rad Mini PROTEAN 3 Electrophoresis System (Biorad, USA). The gel was wrapped in damp paper towels and was stored at 4° C in the fridge for up to one week.

The two gels were placed into a clamping frame and electrode assembly and were placed in a tank. The chamber with 1X Tris/tricine/SDS running buffer (Thermo Fisher Scientific, USA) was filled inner and was filled outer with 1X Anode buffer. After boiling samples, the samples and 5 μ L of Bio-rad Precision Plus All Blue (Biorad, USA). The Standard loaded equivalent amounts of protein/well for samples (20 μ L was fitted into each well of a 15-well comb. 30-35 μ L was fitted into each well of a 10-well comb). The gel was run at 30V constant until samples were completely run through the stacking gel and the separating gel about 1 hour and then the gel was run at 100V constant until the ion front reaches the bottom of the gel about 2 hours.

The gel was transferred to the holder cassette on a few paper towels. Thoroughly wash fiber pads with dH₂O. Using forceps, the gel was dipped into 1X transfer buffer and was placed one fiber pad into each side of the holder cassette. Two pieces of Whatman filter paper (Sigma, USA) were dipped into 1X transfer buffer, and was placed each piece on top of the fiber pad. One piece of nitrocellulose membrane was dipped into 1X transfer buffer and was placed on top of the Whatman paper (Sigma, USA) on the white side of the cassette. The running buffer from the gel tank was dumped and was removed one of the glass plates from your gel with a gel wedge. The gel was oriented over the membrane so that the marker was to the far left of the membrane and your samples were to the right of the marker. The gel was located from the glass plate onto the nitrocellulose membrane. Use a glass pipette dipped in the transfer buffer to smooth out the gel. Then the sandwich was placed into the electrode module with the white side of the gel cassette facing the red side of the electrode module, the black side of the cassette should be facing the black side of the electrode module. The electrode module was placed into the buffer tank. 1X transfer buffer was filled in the tank and was transferred at 4° C for 2 hours at 500 mA constant for two gels in one tank or 350 mA constant for one gel in a tank. After the transfer was completed, the cassette was removed

from the tank. Filter paper and the gel were thrown away. Any excess nitrocellulose membrane was cut off, but the outer edges that the gel did not touch. The ladder with a pencil was marked and placed into a small box with a lid.

The membrane was blocked for 1 hour at RT with 10 mL of 5% milk/TBST with gentle shaking. The primary antibody was added to the BB and was incubated at RT for 1 hour or at 4°C O/N. Then it was washed with TBST, 5 min each at RT with shaking. The secondary antibody 1:2000 was incubated in 5% milk/TBST for 30 min at RT with shaking. After, it was washed 3-4 times with TBST, 5 min each, at RT with shaking. In a fresh box, detection reagents were prepared by Amersham ECL Western Blotting Detection Reagents (Sigma Aldrich, Massachusetts, USA). 5 mL of reagent 1 to the far-left side of a box and 5 mL of reagent 2 to the far-right side of the box were pipetted and mixed. The entire surface of the blot is covered and incubated for 1 min with shaking. Remove blot from detection reagents with forceps. Some of the excess moisture from the membrane was removed by a piece of Whatman filter. The blot was into a sheet of saran wrap was placed and a glow-in-the-dark sticker to the top right corner of the saran wrap blot was added. The blot was exposed to film in the dark room with the lights off, for various lengths of time as 30s, 1 min, 2 min, and 5 min. The film was run through the processor, placed the blot behind the film, and lined up the stickers on the film and the blot.

Table 5. Antibodies used in WB analyses in total protein lysates extracted from human temporal cortex.

Antibody	Manufacturer	Specificity/Function	Dilution
AT8	ThermoFisher Scientific	p-tau (Ser 202, Thr 205)	1:500
ac-Tau E11	Custom ab	ac-tau(K163/K174)	1:500
ac-Tau F1	Custom ab	ac-tau(K163/K174)	1:100
β-actin	Abcam	Housekeeping protein	1:1000
p-tau B6	Custom ab	p-tau (Ser 202, Thr 205,S208)	1:500
Tau-5	Abcam	Human total tau protein	1:500
4R-Tau	RD4, Millipore	Human 4R-Tau protein	1:1000

4.1.4.3. Immunohistochemistry

IHC staining was used as confirmation of functionality of the antibodies in the human brain tissue. Firstly, the sections were deparaffinized and rehydrated and were induced antigen retrieval by heating. Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0), tri-sodium citrate (dihydrate) 2.94 g, distilled water 1000 ml, and 0.5 ml of Tween 20 were mixed in order to dissolve. The sections were stored at RT for 3 months or at 4°C for longer storage. The antigen retrieval buffer to the microwaveable vessel was added, and the slides from the tap water were removed and boiled for 20 minutes from this point. The vessel was removed and cold tap water was run into it for 10 minutes. In regard to the immunohistochemical staining protocol, first, the slides were washed twice in 5 minutes in TBS plus 0.025% Triton X-100 (Sigma, USA) with gentle agitation. Then they were blocked by 10% normal serum with 1% BSA (bovine serum albumin) (Sigma, USA) in TBS for 2 hours at RT. The slides were dried for a few seconds and were wiped around the sections with tissue. The primary antibody was diluted in TBS (Sigma, USA) with 1% BSA (Sigma, USA) and incubated overnight at 4°C. They have rinsed twice 5min in TBS 0.025% Triton with gentle agitation. The slides were incubated in 0.3% H2O2 in TBS for 15 min. Finally, the enzyme-conjugated secondary antibody was applied to the slide diluted in TBS with 1% BSA and was incubated for 1 hour at RT in dark. They were rinsed 3 times 5min in TBS. They were developed with chromogen for 10 min at RT and they were rinsed in running tap water for 5 min. Finally, were dehydrated, cleared, and mounted.

4.1.5. Toxicity of anti-ptau B6 antibody

4.1.5.1. Toxicity *in vitro*

The toxicity of anti-ptau B6 antibody was evaluated by the Caspase 3 activity measured according to the manufacturer's instructions Fluorimetric Caspase-3 Assay Kit (Sigma-Aldrich, USA). The absorbance of the solution was recorded at 460 nm using a microplate reader Varioskan Flash (Thermo Fischer, USA). Results were expressed as mean $\pm$ SEM from three different neuronal cultures.

Neurons were incubated in a culture medium in the presence or not (control) of anti-ptau B6 antibody, at the concentration contained in 10 (0,03 μ g) and 100 (0,3 μ g) μ g solid content/mL of p-tau, for 24 hours. Neurons were treated with amyloid- β (5 μ M A β ; 24 hours) as a positive control of caspase-3 activation. Fluorometric detection of caspase-3 activity was performed as an index of neuronal apoptosis. Values were expressed as mean $\pm$ SEM. $p < 0.05$ versus control (0 μ g) (one-way ANOVA followed by the Bonferroni post hoc test; $n = 3$ neuronal cultures).

4.1.5.2. Toxicity *in vivo*

The toxicity of immunotherapy with anti-ptau B6 antibody was done in kidney and liver samples from Tau.P301S Humanized p-tau transgenic mice (1.5-month-old) injected intraperitoneally with anti-ptau B6 antibody (group A) or IgG1 antibody (group B) for 4 months. Wild-type mice treated with IgG1 antibody (group C) were used as control. At the end of the treatment, mice were sacrificed and intracardially perfused with saline for 3 minutes. We received kidney and liver samples in a 4% formaldehyde solution and afterward the tissue was embedded in paraffin or cryopreserved for further analysis. The toxicity of immunotherapy was evaluated by the following procedures:

-Haematoxylin/Eosin staining. The tissue structure was assessed histologically using hematoxylin/eosin staining. Briefly, 3 μ m-thick tissue slices were deparaffinated and rehydrated through a series of ethanol dilutions from 100% to distilled water. The slices were stained with Harris's haematoxylin stain for 10 min. After a 10 min wash with distilled water and differentiation in acid alcohol, the slices were stained with eosin for 15 s. Then the sections were dehydrated with graded alcohol, cleared in xylene, and mounted. The structure of the tissue was assessed under a brightfield microscope by a researcher blinded to the experimental groups (Delgado-Baquerizo et al., 2016).

-Fast Green/Sirius Red staining. The tissue fibrosis was detected histochemically by an increase in collagen fibers. Fast green/Sirius red staining was used to assess collagen fiber content. Briefly, 3 μ m-thick tissue slices were deparaffinated and rehydrated through a series of ethanol dilutions from 100% to distilled water. Tissue slices were then incubated in 0.04% fast green for 30 min, washed with distilled water, and then incubated in 0.1% fast green and 0.04% Sirius red in saturated picric acid for 30 min. Then, sections were dehydrated through a series of ethanol dilutions ranging from 50% to 100%, cleared in xylene, and mounted with a water-free mounting medium (Entellan®). Collagen fibers appeared red, while the non-collagen proteins were stained green (Segnani et al., 2015). Quantitative estimations of histochemical staining were carried out independently by a researcher blinded to the experimental groups. Brightfield images were captured using a digital camera attached to an inverted microscope. Four animals per experimental group were analyzed. Four 20X random fields per slice were captured through an open-source bioimage analysis software for quantitative pathology, was used first to separate the Sirius red staining. The Sirius red channel was then exported to ImageJ, and a threshold method using the triangle algorithm was applied to the image to determine the area of the picture that contained collagen fibers. The

total area of the tissue was calculated using the same threshold method on the brightfield image. The relative area of collagen was then determined and the data were expressed as a percentage of the Sirius Red staining area (Bankhead et al., 2017).

-Immunofluorescence. Four months after intraperitoneal (IP) injections of anti -ptau B6 or IgG1, animals were sacrificed and then perfused intracardially with 0.9% NaCl for 3 minutes. Afterward, kidney and liver tissue were fixated by immersion in a 5% formaldehyde solution. After fixation, kidney and liver samples were dissected out sagittally in two parts. The blocks were then rinsed successively for 10 min, 30 min, and 2 h with 0.1 M phosphate buffer solution (PB; pH 7.4) and sequentially immersed in 10, 20, and 30% (wt/vol) sucrose in PB until they sank. After cryoprotection, 5, 10, and 20- μ m-thick sections were obtained with a freezing-sliding cryostat (Leica, USA). The 10 and 20- μ m sections were placed on microscope slides. Sections were rinsed in 0.1 M PB three times each for 10 min and then were incubated first in 1:100 anti-Cleaved Caspase-3 (Cell Signalling Technology, USA) in 0.2% Triton X-100 (Sigma-Aldrich, Massachusetts, USA) and 5% goat serum (Jackson ImmunoResearch Laboratories, USA) in 0.1 M PB for 24 h, at 4 °C. Secondly, were incubated in Alexa488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, USA) in 0.05% Triton X-100 and 2% goat serum in 0.1 M PB for 2 h at RT. Lastly, were used 0.5 μ g/mL DAPI in PB for 10 min at RT. After rinsing with PB, sections were mounted with Fluoromount aqueous mounting medium (Sigma-Aldrich, USA). Confocal images were taken with a scanning laser confocal microscope (“Spinning Disk” Roper Scientific Olympus IX81) with three lasers 405, 491 y 561 nm and equipped with 63 $\times$ PL Apo oil-immersion objective for high-resolution imaging and device digital camera (Photometrics, USA).

-TUNEL assay. This assay was performed in 20 μ m-thick kidney and liver sections, following the manufacturer’s protocol (Roche Diagnostics, Heidelberg, Germany). Kidney and liver sections, fixed as above, were preincubated in Terminal Transferase dUTP Nick End Labeling (TUNEL) buffer containing 1 mM CoCl₂, 140 mM sodium cacodylate, and 0.3% Triton X100 in 30 mM Tris buffer, pH 7.2, for 30 min. After incubation at 37 °C with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (800 U/ml) and nucleotide mixture (1 μ M) for 2 h, sections were rinsed with PB and counterstained with Cy3-streptavidin (Jackson Immunoresearch Laboratories, USA) (Lapresa et al., 2019).

4.2. DEVELOPMENT OF AN INNOVATIVE IN-VITRO DIAGNOSTIC KIT BASED ON ELISA

4.2.1. Selection of conditions

The development of an ELISA assay followed multiple steps, confirming several variables, from the type of ELISA, surface, plates, coating, blocking, washings, antibody pairs, incubation temperature and shaking or detection and amplification methods. An example of such steps for developing the ELISA kit can be summarized in the **Figure 12**.

Both indirect and sandwich approaches have been tested, choosing a final Sandwich setup (**Figure 13B**) assay considering the nature of the analytes to be detected and the protocol simplicity (**Figure 13A**). The advantages considered were the detection limits required and the possibility of future assay automation made the Sandwich ELISA more adequate. Under the premise of using a Sandwich ELISA for the kit, the main concern was to identify available working antibody pairs to work as trapping or detection antibodies within the ELISA scheme, in combination with the already selected B6 and E11 antibodies.

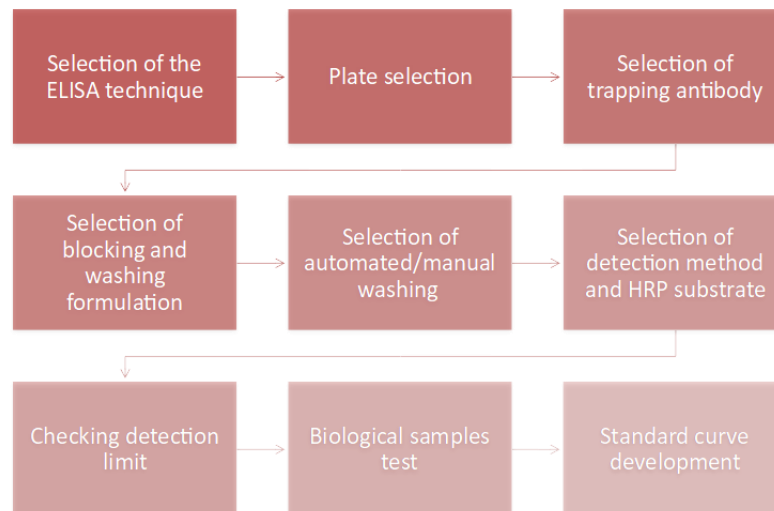


Figure 12. Example of a typical workflow of the steps involved in the development the diagnostic ELISA kit. Self-created image using elements with Creative Common license.

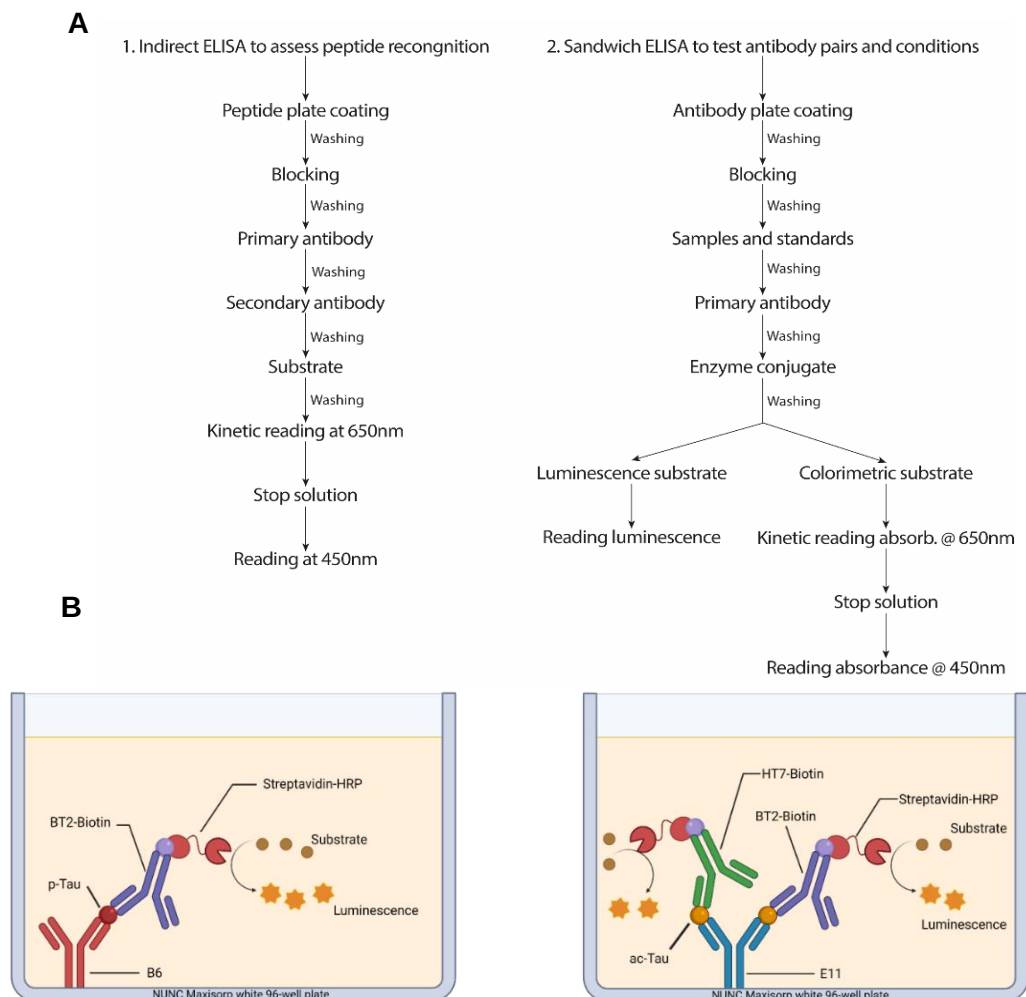


Figure 13. A. Schematic representation of the developed protocols for indirect and sandwich ELISAs. **B.** Scheme of the final sandwich ELISA setup. Tau PTMs-specific antibodies B6 and E11 function as trapping antibodies. Biotinylated BT2 and HT7 function as primary antibodies and a streptavidin-HRP polymer conjugate as detection element. Self-created image using elements with Creative Common license.

4.2.2. Sandwich Elisa Setting-Up Protocol

Serial dilution titration analyses were performed to determine the optimal concentrations of reagents to be used during the development of the ELISAs. In this protocol, we have always followed the following ELISA setup consisting of a capture antibody, an antigen that binds the capture antibody, and a detection antibody biotin-conjugated that binds to the antigen that was serially diluted and analyzed by a criss-cross matrix analysis. Once the optimal concentrations of different reagents to be used under particular assay conditions are determined, these variables were kept constant from experiment to experiment.

The first step was the definition of the experimental design that established the objective of the setup and the parameters to be tested. Briefly, well positions in the plate format were assigned. For accurate quantitation, samples should be run in duplicate. Reserve wells for controls for each plate as the negative control A (No antigen) were also defined.

The second step was the coated plate with a capture antibody. It was calculated the volume necessary (10% extra) for different concentrations of capture antibody to a final concentration of 10, 5, 2.5, and 1.25 $\mu\text{g}/\text{mL}$. An individually capture antibody solution for each concentration by serial dilution was prepared, and subsequently 100 μL capture antibody solution were dispensed into each well of the plate, and was incubated overnight at 4°C.

The next step was blocked the residual binding capacity of the plate. For it, 200 μL of BB was dispensed and incubated for 2 hours at 37°C shaking at 250 rpm. Then, the volume necessary (10% extra) for different concentrations of antigen to a final concentration of 1000, 500, 250, and 125 ng/mL were calculated. The aim of these experiments was to find the dynamic range of binding. 100 μL antigen solution to each well was added and afterwards incubated at different conditions overnight at 4°C shaking 250 rpm, overnight at 37°C shaking 250 rpm, and for 1 hour at 37°C shaking 250 rpm.

Next, the detection antibody was added to the plate at different concentrations of 500, 250, 125, and 62,5 ng/mL . After that, 100 μL of detection antibody solution was dispensed into each well of the plate and incubated for 1 hour at 37°C shaking at 250 rpm. Next, 100 μL of enzyme conjugate was added to each well following an incubation period for 1 hour at 37°C shaking at 250 rpm. Finally, 100 μL of substrate TMB was dispensed to each well and absorbance was measured. In dark at RT was incubated until the solution turns blue for monitoring the absorbance at 652 nm for 30 minutes in a spectrophotometer. The reaction was stopped by adding 100 μL of 2M sulfuric acid (Sigma, USA) to each well and was measured the absorbance of each well at 450 nm. The plate was washing five times between each step.

When the capture antibody is specific for a single determinant, the conjugate must be prepared from antibodies that recognize different determinants that remain available after the antigen is bound to the plate by the capture antibody.

4.2.2.1. Final Elisa Setting-Up Protocol

The chosen final protocol for the development of indirect and sandwich ELISAs is described below.

4.2.2.1.1. Indirect ELISA protocol

96-well ELISA plates Nunc Maxisorp flat-bottom (Thermo Fisher Scientific, USA) were coated with different concentrations of synthetic peptides diluted in a coating buffer (CB) of carbonate buffer (BioRad, USA), plated as 100 μL per well, and incubated overnight at 4°C without agitation. Wells were then blocked with Protein-Free (TBS) Blocking Buffer (Thermo Fisher Scientific, USA) for 1 hour at 37°C. After that, 100 μL of primary antibodies were added to the wells at 1000 ng/mL in TBS-Tween20 0.05% (Sigma-Aldrich, USA), and

incubated for 2 hours at 25°C under orbital agitation at 250 rpm. Secondary antibody anti-mouse-HRP (Dako, USA) was diluted in BB, added to the plate, and finally incubated for 1 hour at 25°C under orbital agitation at 250 rpm. Next, 100 µL of TMB substrate (Thermo Fisher Scientific, USA) was added per well to the plates immediately before starting a kinetic measurement at 650nm for 5 minutes in a Biotek microplate reader. Once optimized the TMB incubation time for assessing the best signal-to-noise ratio, 100 µL per well of Sulphuric Acid Solution, 1M (Merck millipore, Massachusetts, USA) was added to all wells for stopping the reaction. The last read of absorbance was done at 450nm. Data were then plotted as relative units per condition and concentration. Washes between all steps were done with 3 passes of 300 µL TBS-Tween 20 0.05% and 100 µL each step of the reagent.

4.2.2.1.2. Sandwich ELISA protocol

Microtiter plates were coated with the candidate trapping antibodies at a concentration of 5000 ng/mL in a CB of carbonate buffer (BioRad, USA). Wells were then blocked with protein-free blocking buffer (Thermo Fisher Scientific, Massachusetts, USA) for 1 hour at 37°C. Samples and peptides were added to the plate (100 µL per well) according to defined concentrations diluted in artificial cerebrospinal fluid (aCSF) (TOCRIS, UK) and they were incubated at 25 °C without agitation overnight (15-18h). After that, biotinylated primary antibodies were added to the wells at 1000 ng/mL in TBS-Tween20 0.05% (Sigma Aldrich, Massachusetts, USA), and incubated for 2 hours at 25°C under orbital agitation at 250 rpm. The secondary antibody as Streptavidin-HRP (R&D, Minneapolis, USA) was added to the plates at the recommended dilution in blocking solution and incubated again for 1 hour at 25°C under orbital agitation at 250 rpm. The TMB substrate (Thermo Fisher Scientific, Massachusetts, USA) was then added 100 µL per well to the plates immediately before starting a kinetic measurement at 650nm for 10 minutes in a microplate spectrophotometer. Once optimized the TMB incubation time for assessing the best signal-to-noise ratio, 100 µL per well of Sulphuric Acid Solution, 1M (Merck millipore, Massachusetts, USA), was added to all wells for stopping the reaction. The last read of relative absorbance was done at 450nm. In the case of the luminescence assay, both reagents of the SuperSignal™ ELISA Femto Substrate buffer (Thermo Fisher Scientific, Massachusetts, USA) were mixed, added 50 µL per well was to the plate. Luminescence generation was measured immediately with the microplate spectrophotometer using the TOP 440/60 method. When using samples instead peptides, wash cycles were increased from 3 to 5 washes of 300 µL of TBS-Tween20 0.05%.

The different protocols tested until reaching the optimal protocol are outlined below.

4.2.3. Protocol Steps

The different setups tested until reaching the optimal protocol are specified below:

First setup: The fixed variable was the Antigen at 1000 ng/mL.

	Detection ng/mL	500			250			125			62,5		
Capture ug/mL	Antigen 1000ng/mL	1	2	3	4	5	6	7	8	9	10	11	12
10	A												
5	B												
2,5	C												
1,25	D												
0	E	Control			Control			Control			Control		
Empty	F												
Empty	G												
10	H	Control			Control			Control			Control		

Second setup: Determination of the optimal concentration of the detection antibody. If the detection antibody has been established for example at 125 ng/mL it must kept as fixed variable.

	Capture	10			5			2,5			1,25		
Antigen ng/mL	Detection 125 ng/mL	1	2	3	4	5	6	7	8	9	10	11	12
100	A												
500	B												
250	C												
125	D												
0	E	Control A			Control A			Control A			Control A		
Empty	F												
Empty	G												
Empty	H												

Third setup: Determination of the optimal concentration of the capture antibody. If the detection antibody has been established for example at 125 ng/mL and capture antibody at 2,5 ug/mL them must kept as fixed variables.

	Capture	2,5			2,5		
Antigen ng/mL	Detection 125 ng/mL	1	2	3	4	5	6
1000	A				Control B		
500	B				Control C		
250	C				Control D		
125	D				Control E		
0	E	Control A			Control F		
	F	Control A			Control G		
1000	G	Unmodified peptide					
500	H	Unmodified peptide					

4.2.3.1. Control definition

The negative controls were defied as follows:

The control A is a detection-Capture Antibody Cross-Reactivity: No antigen. It was considered as blank.

The control B is a non specific binding of antigen: No capture antibody.

The control C is the capture Antibody-Conjugate Cross-Reactivity: No antigen, No detection antibody.

The control D is an Antigen-Conjugate Cross-Reactivity: No detection antibody.

The control E is a BSA-Detection Antibody Cross-reaction: No capture, No antigen.

The control F is a contamination of BSA with biotin: No capture, No antigen, No detection antibody.

The control G is an antigen-Capture Antibody Cross-Reactivity: peptide without modification.

The positive controls were the following:

The control H is an *Unconjugated antibody*: Indirect ELISA with antigen (modified and unmodified peptide) and Anti IgG mouse-HRP.

The control I is a *biotin conjugated antibody*: Indirect ELISA with antigen (modified and unmodified peptide) and streptavidin-HRP.

4.2.4. Materials and equipment: Reagents

Commercial coating, blocking, buffer solutions and reagents used for ELISA assays are shown in **Table 6**. Some of the first assays referred to in this report were performed with no commercial solutions, and they were used as they became available.

Table 6. List of solutions and reagents tested for ELISAs assays. The final reagents selected for the ELISA are shown in bold.

Name	Reference	Manufacturer	
5x ELISA Coating Buffer	BUF030C	Biorad	
PBS	L0616	Biowest	
BSA	A9647	Sigma	
Tween 20	28829.183	Thermo Scientific	Fisher
Protein-Free (TBS) Blocking Buffer	37570	Thermo Scientific	Fisher
Tris Buffered saline 10X	T5912-1L	Sigma	
TBS 20X Pierce™ Tween™-20	28360	Thermo Scientific	Fisher
Water DNase/Protease free	647016	Grifols	
Neutravidin-HRP	A2664	Molecular Probes	
Streptavidin Peroxidase Polymer, ultrasensitive	S2438-250UG	Sigma	
Streptavidin-HRP	DY998	R&D	
Reagent Dil. Conc 2 (for DY998)	DY995	R&D	
Pierce High Sensitivity Streptavidin-HRP, Pre-Diluted	21134	Thermo Scientific	Fisher
Pierce Streptavidin Poly-HRP	21140	Thermo Scientific	Fisher
Mouse PAP	223-005-024	Jackson Research	Immune
1-Step Ultra TMB	34029	Thermo Scientific	Fisher
1-Step Turbo TMB	34022	Thermo Scientific	Fisher
ELISA SuperSignal™ Femto	37075	Thermo Scientific	Fisher
Sulphuric Acid Solution, 1M	VL3161000	Fisher Chemical	
Artificial CSF Matrix	3525	Tocris	

References and batches of used antibodies are shown in **Table 7**.

Table 7. List of antibodies used for ELISAs assays.

Name	Reference	Manufacturer
E11	7/8/2019	StabVida
E11	24/2/19	StabVida
E11-Biot	13/5/2020	Immunostep
B6	29/3/2019	BiotechPharma
B6	14/12/2018	BiotechPharma

B6-Biot	B6-Biotin-097	Immunostep
BT2Biotin	MN1010B	Thermo Fisher Scientific
HT7Biotin	MN1000B	Thermo Fisher Scientific
Tau5Biotin	MA512805	Thermo Fisher Scientific

Synthetic Tau (195-224), Tau (155-230), and Tau (160-180) peptides were synthesized by Genecust. Synthetic peptides Tau (188-217) were synthesized by Genscript. All peptides utilized during the development of the ELISA are shown in **Table 8** and materials and equipment in the **Table 9**.

Table 8. Synthetic tau peptides used in the development of the ELISA assays. Numbering according to the longest 441-amino acid, brain isoform of tau, 2N,4R.

Peptide	Length	PTMs	Sequence	
un-tau short	195-224	None	SGYSSPGSPGTPGSRSRTPSLPTPPTREPKEPK	Genecust
3P-tau short	195-224	pS208, pT205, pS208	SGYSSPG(pS)PG(pT)PG(pS)RSRTPSLPTPPTREPKEPK	
unac-tau	160-224	None	PGQKGQANATRIPAKTPPAPK	
2ac-tau	160-224	acK163, acK174	PGQ(acK)GQANATRIPA(acK)TPPAPK	
ac-tauK163	160-224	acK163	PGQ(acK)GQANATRIPAKTPPAPK	
ac-tau K174	160-224	acK174	PGQKGQANATRIPA(acK)TPPAPK	
un-tauL	155-230	None	RGAAPPQGKGQANATRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKEKVVAVV	Genscript
ac-tauL	155-230	acK174	RGAAPPQGKGQANATRIPA(acK)TPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKEKVVAVV	
p-tauL	155-230	pS208	RGAAPPQGKGQANATRIPATPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPG(pS)RSRTPSLPTPPTREPKEKVVAVV	
unp-tau	188-217	None	PPKSGDRSGYSSPGSPGTPGSRSRTPSLPT	
p-tau S202	188-217	pS202	PPKSGDRSGYSSPG(pS)PGTPGSRSRTPSLPT	
p-tau T205	188-217	pT205	PPKSGDRSGYSSPGSPG(pT)PGSRSRTPSLPT	
p-tau S208	188-217	pS208	PPKSGDRSGYSSPGSPGTPG(pS)RSRTPSLPT	
3P-tau	188-217	pS202, pT205, pS208	PPKSGDRSGYSSPG(pS)PG(pT)PG(pS)RSRTPSLPT	

Table 9. Materials and equipment used in the development of the ELISA assays.

Materials/Equipment	Reference	Manufacturer
Multichannel pipet	-	-
Micropipet	-	-
Disposable pipet tips	Gilson	Gilson
protein Lo-bind 1.5 mL tubes	022431081	Eppendorf
protein Lo-bind 5 mL tubes	0030122356	Eppendorf
Nunc Maxisorp 96well plates	44-2404-21	NUNC
Strips Nunc Maxisorp	468667	NUNC

Plastic wrap	15036	Thermo Scientific	Fisher
Incubator with shaker	PST-60HL-4	Biosan	
Microplate Washer	Intelliwasher 3D-IW8	Biosan	
Microplate reader	Synergy 2	Biotek	

One of the first steps consisted of the verification of which microplate could be more suitable for our purposes. *Three microplates were chosen as candidates:* i) Clear Flat-Bottom Immuno Nonsterile 96-Well Plates MediSorp (Thermo Fischer, USA); ii) Clear Flat-Bottom Immuno Nonsterile 96-Well Plates PolySorp (Thermo Fischer, USA), and iii) 96-well Microlon High-binding plate Greiner (Sigma-Aldrich, USA) respectively referred as “MediSorp”, “PolySorp” and “Microlon”. These plates vary respect each other in terms of molecules adsorption properties.

In this experiment, we tested by means of a “Sandwich” type ELISA several plate coating formulations (varying from 0 µg/ml to 10 µg/ml), with the aim of discerning which microplate would be more suitable for the analysis of these antibodies. For details, see “ELISA Sandwich final Protocol” **Table 10**.

The used antibodies and peptides were the following; polyAb anti-A β (Ab2539, Abcam, UK) and polyAb anti TAU (ref. Ab75624, Abcam, UK) were used as capture antibodies. In the samples, we used A β , ac-tau, and p-tau peptides (the same that were used for the mAbs generation). The anti-A β , ac-tau, and p-tau were used as primary antibodies, and as a secondary antibody was used HRP conjugated polyclonal anti-mouse IgG1 antibody (Sigma-Aldrich, USA).

4.2.5. Antibodies pairing

4.2.5.1. Antibodies-peptides compatibility assessment

The Tau protein has a length of 1-441 aa, so long peptides were designed including residues from 155 to 230 for acetylated (ac-tauL) and phosphorylated (p-tauL) forms and unmodified peptide (unTauL) with the purpose of use for the calibration curve (**Figure 14**). These long synthetic peptides let test new antibodies as pairs for B6 and E11, allowing more flexibility to develop and optimize the test.

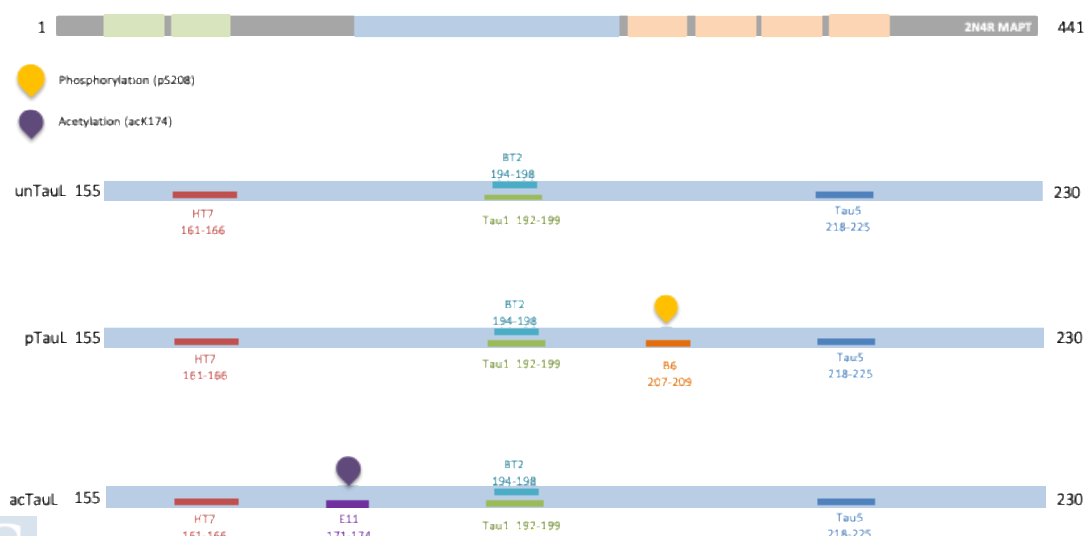


Figure 14. Schematic representation of 2N4R MAPT and the synthetic peptides used during the first phases of the ELISA development, showing the sequences targeting by several commercial antibodies. Some of these antibodies and others are used as trapping couple for the anti-tau B6 and anti-acTau E11 mAbs. Self-created image using elements with Creative Common license.

In previous assays with the anti-ptau B6 antibody and the p-tauL peptide (155-230) we did not obtain a good signal, so 5 new shorter peptides were designed, with a length of 188-217 aa that allows target with the antibody pairs BT2 and HT7. One peptide with 3 phosphorylations (S202, T205, S208), one without any, plus 3 more peptides with each other phosphorylation (**Figure 15**).

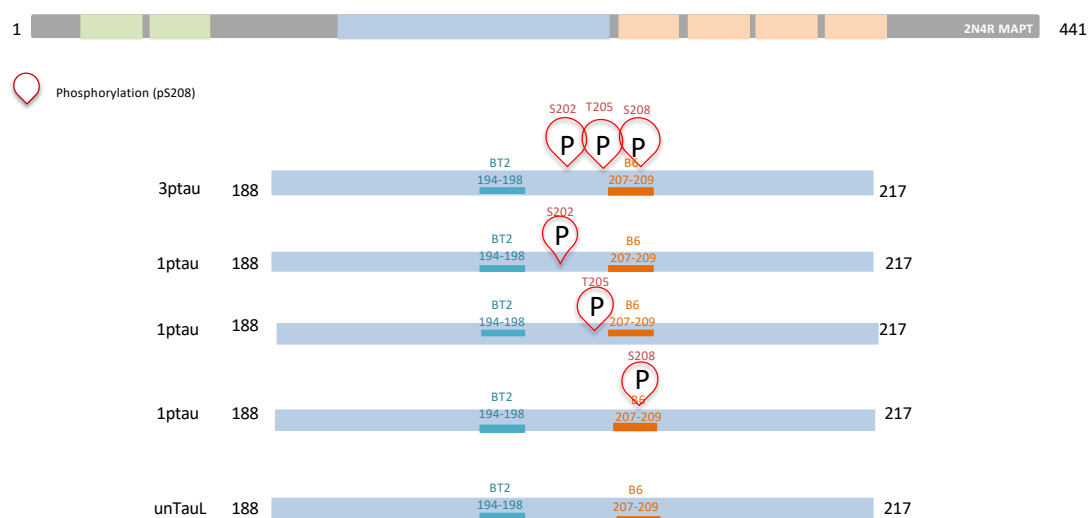


Figure 15. Phosphorylated tau peptides 188-217 for B6 antibody. Self-created image using elements with Creative Common license.

4.2.5.2. Antibody pair combination

With the aim of choosing the best pairs of antibodies for E11 and B6, several commercial antibodies were selected, such as Tau 5 (218-225), Tau1 (192-199), HT7 (161-166) and BT2 (194-198) for acting as trapping antibodies. These antibodies were biotinylated, except Tau1, for also using as detection antibodies. In addition, several secondary antibodies were also tested. All combinations of antibodies are described in the **Figure 16**. The ac-Tau E11 (171-174) and p-Tau B6 (207-209) antibodies were tested in both positions of Sandwich ELISA, as trapping and detection antibodies.

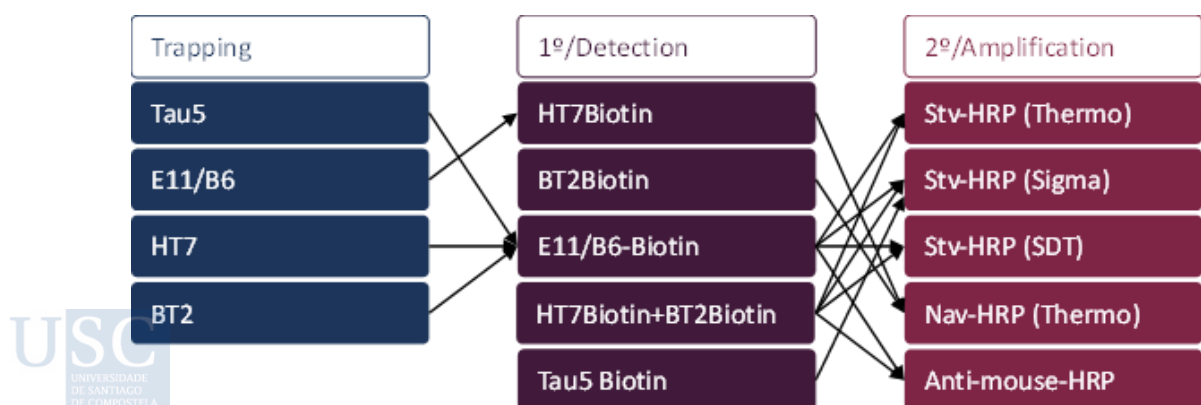


Figure 16. Scheme of all the combinations of antibody pairs tested so far during the development of the ELISAs for both ac-Tau and p-tau. Self-created image using elements with Creative Common license.

4.2.5.3. E11 antibody

The best performing identified antibody pairing for ac-Tau E11 mAb was the BT2+HT7 Biotin used simultaneously as 1st antibodies. The assay showed below includes one of the latest assays oriented to the identification of the Limit of Detection (LOD) of the E11-BT2+HT7 Biotin.

Microtiter plates were coated with the E11 trapping antibody at a concentration of 5.000 ng/mL in CB. Wells were then blocked with protein-free blocking buffer during 1 hour at 37°C. The peptides were added to the plate (100 µL per well) according to defined concentrations of 25 µl of ac-tauL peptides 10, 1, 0.1, 0.01 ng/mL diluted in 75 µl of TBS or a CSF matrix. Dilution factor was 1:4.

After that, biotinylated primary antibodies BT2+HT7 were added to the wells at 1000 ng/mL in TBS-Tween20 0.05%, and incubated for 1 hour at 25°C. HRP-conjugates as Streptavidin Poly-HRP 1:10.000 were added to the plates at the recommended dilution in blocking solution and incubated again for 1 hour at 25°C under orbital agitation at 250 rpm. Three washes with 300 µL of washing solution per well were done between all steps.

The TMB substrate, 100 µL per well, was then added to the plates immediately before starting a kinetic measurement at 650nm for 10 minutes in a microplate spectrophotometer. Once optimized the TMB incubation time for assessing the best signal-to-noise ratio, 100 µL per well of H₂SO₄ 1M was added to all wells for stopping the reaction. The last read of relative absorbance was done at 450nm.

In case of the luminescence assay, both reagents of the SuperSignal™ ELISA Femto Substrate (Thermo Fischer, USA) were mixed, added to the plate (50 µL per well) for 1 minute. Luminescence generation was measured immediately with the microplate spectrophotometer using the TOP 440/60 method.

4.2.5.4. B6 antibody

The best-performing identified antibody pairing for p-Tau B6 mAb was the BT2Biotin. The assay showed below includes one of the latest assays oriented to the identification of the LOD of the B6-BT2Biotin.

Microtiter plates were coated with the B6 trapping antibody at a concentration of 5.000 ng/mL in the CB. Wells were then blocked with a protein-free blocking buffer for 1 hour at 37°C. The peptides were added to the plate according to defined concentrations of p-tauL peptides 1.000, 500, 250, 125, and 62,5 ng/mL, diluted in a CSF matrix. After that, biotinylated primary antibody as B were added to the wells at 1.000 ng/mL in TBS-Tween20 0.05% and incubated for 1 hour at 25°C. HRP-conjugates as Streptavidin Poly-HRP 1:10.000 were added to the plates at the recommended dilution in blocking solution and incubated again for 1 hour at 25°C under orbital agitation at 250 rpm. Three washes with 300 µL of washing solution per well were done between all steps.

The TMB substrate, 100 µL per well, was then added to the plates immediately before starting a kinetic measurement at 650nm for 10 minutes in a microplate spectrophotometer. Once optimized the TMB incubation time for assessing the best signal-to-noise ratio, 100 µL per well of H₂SO₄ 1M was added to all wells for stopping the reaction. The last read of relative absorbance was done at 450nm.

In the case of the luminescence assay, both reagents of the SuperSignal™ ELISA Femto Substrate (Thermo Fischer, USA) were mixed, and added to the plate (50 µL per well) for 1 minute. Luminescence generation was measured immediately with the microplate spectrophotometer using the TOP 440/60 method.

4.2.5.5. ELISA signal development and substrate incubation optimization

The objective of this step was to unveil the kinetics of the signal development given by the so-far-used 1-Step Ultra TMB and the alternative 1-Step Turbo TMB, both from Thermo Fisher Scientific, USA.

The setup comprises an indirect ELISA, coating the plate with the 10.000, 1000, 100 ng/mL ac-tauL peptide, biotinylated E11-Biotin as 1st antibody at 10.000 ng/mL, StvPolyHRP (Thermo Fischer, USA) 1:10.000 and the two TMB reagents to compare. Kinetics were recorded just after 100 µL of 1-Step Ultra TMB/1-step Turbo TMB addition (Abs at 650nm) to the plate for 10 minutes at 25°C. After that, the reaction was stopped with 100 µL of 1M H₂SO₄ commercial STOP solution (Thermo Fischer, USA) and absorbance was measured again at 450nm.

4.2.6. Clinical Validation of ac-tau and p-tau ELISA kits in CSF samples

4.2.6.1. Final setup of Sandwich ELISAs

After optimizing the Sandwich ELISA scheme with the synthetic peptides, it was considered the setup was ready to be tested with the CSF samples of the SERGAS Biobank. Samples used were diagnosticated from MCI “without biomarkers” (MCI), MCI “with Biomarkers” (MCI+B), and AD, based on clinical criteria and previous biomarker assays as Aβ, t-tau and pT181-tau. The final protocol and reagents are shown in **the Table 10**.

Table 10. Setup and final protocol of Sandwich ELISA.

Steps of ELISA sandwich	Reagent	Batch	Stock (mg/mL)
<i>Capture antibody</i>	B6/E11	097 / 24.02.2020	5/3,96
<i>Antigen (peptide)</i>	ac-tauL	12/07/1905	1,5
<i>Detection antibody</i>	HT7 Biotin	UJ2855222	0,1
	BT2 Biotin	UE2757691	0,1
<i>Enzyme conjugate</i>	Streptavidin Poly-HRP Ref. 21140	VB293978	0,5

Protocol of ELISA Sandwich

Conditions

Capture ANTIBODY: 100 µl

Buffer Coating Buffer BUF030C

Dilution n.d.

Specificity pS208 Tau / acK174 Tau

Concentration 5 ug/mL

Temperature 4°C

Time Overnight

WASHES: 300 μl	<i>Buffer</i>	Washing solution Ref. 28360
	<i>Frequency</i>	3 times
Blocking BUFFER: 200 μl	<i>Buffer</i>	Protein-Free (TBS) Blocking Buffer Ref.37570 / BSA3%
	<i>Temperature</i>	37°C
	<i>Time</i>	1 h
WASHES: 300 μl	<i>Buffer</i>	Washing solution Ref. 28360
	<i>Frequency</i>	3 times
ANTIGEN: 100 μl	<i>Buffer</i>	Artificial CSF (75%) and TBS (25%)
	<i>Concentration</i>	100-0,00128 ng/mL / CSF sample (75%)
	<i>Temperature</i>	25°C
	<i>Time</i>	Overnight
WASHES: 300 μl	<i>Buffer</i>	Washing solution Ref. 28360
	<i>Frequency</i>	3 times
Detection ANTIBODY: 100 μl	<i>Buffer</i>	Tris Buffered saline 1X Ref. T5912
	<i>Concentration</i>	1000 ng/mL each
	<i>Time/Temperature</i>	2h at 25°C shaking 350 rpm
WASHES: 300 μl	<i>Buffer</i>	Washing solution Ref. 28360
	<i>Frequency</i>	3 times
Enzyme CONJUGATE:100 μl	<i>Buffer</i>	Protein-Free (TBS) Blocking Buffer Ref.37570
	<i>Concentration</i>	Dilution 1:10000
	<i>Time/Temperature</i>	1 hour at 25°C shaking 350 rpm (dark)
WASHES: 300 μl	<i>Buffer</i>	Washing solution Ref. 28360
	<i>Frequency</i>	3 times
SUBSTRATE: 100 μl/ 50 μl	<i>Buffer</i>	1-Step UltraTMB/ SuperSignal™ ELISA Femto Substrate
	<i>Time</i>	5 min (dark) /1 min (dark)
	<i>l</i>	-/ TOP 440/60
STOP solution: 100 μl	<i>Buffer</i>	H2SO4
	<i>Time</i>	-
	<i>l</i>	450nm

4.2.6.2. Protocol of the proof of concept clinical study

This clinical study was conducted in the Neurology Department – Cognitive Neurology Unit - Clinical Neuroscience Research Laboratory (LINC) of the University Clinical Hospital of Santiago de Compostela (CHUS). Prospective and observational molecular-clinical study of non-intervention in a population of patients with MCI and control subjects. The selection

and inclusion of patients and control subjects was done in the Cognitive Neurology Unit of the CHUS. Since this is a non-intervention study, the monitoring and control of risk factors was done in the best possible conditions, regardless of the results of the tests performed. Patients and control subjects were evaluated using a common neuroimaging, ultrasound, molecular and exhaustive cognitive neurological exploration protocol. The follow-up period was 3-5 years with annual visits. The protocol has been approved by the CEIC of Galicia (Code: 2013/553) and the biological samples included in the C0003517 Collection.

Assuming that the 30% of MCI patients were developed AD within 2-3 years of follow-up, for a confidence level of 95% and a power of 80%, a sample size of 90 patients with MCI are needed to test the hypothesis of the present project. Sample calculation was performed by using the EPIDAT software (<http://dxsp.sergas.es/ApliEdatos/Epidat/cas/default.asp>).

Criteria for inclusion of patients with MCI due to AD following the protocol of Management of Dementias published in 2008 by the Sociedad Española de Neurología (SEN):

1) Patients with MCI (criteria AA-NIA + biological signature (biomarkers LCR) of AD); 2) Patients of both sexes in follow-up in outpatient consultations; 3) Voluntary participation, with signed informed consent; 4) Age > 50 years; 5) Possibility of compliance with the protocol; 6) Baseline brain CT / MRI congruent with the diagnosis of AD; 7) Adequate auditory and visual capacity to perform cognitive and functional evaluations; 8) Reliable informer who can accompany the patient during visits.

Criteria AA-NIA for Dementia due to AD:

1) Dementia of insidious onset; 2) Impairment of clearly observed or referred cognition; 3) The initial and most prominent cognitive deficit is that of episodic memory (amnesic presentation); 4) Absence of concomitant brain disease that could have a substantial effect on cognition; 5) Criteria AA-NIA for LightMCI due to AD (Albert et al., 2011): 5.1) Perception of cognitive decline per patient, informant or medical doctor; 5.2) Objective evidence of impairment in at least 1 cognitive domain, including memory; 5.3) Preservation of independence in functional abilities; 5.4) No dementia.

Biological signature of EA following Lumipulse® System:

Lumipulse A β 42, A β 40, t-Tau and 181p-Tau are *in vitro* diagnostic immunoassays intended for the quantitative determination of the A β 42, A β 40, total Tau, and 181p-Tau protein concentration in human CSF. All these assays are intended to be used in adult subjects with cognitive impairment being evaluated for AD and other neurodegenerative diseases. The Lumipulse A β 1-42/A β 1-40 ratio in CSF is reduced in AD patients, and its assessment improves AD diagnostic accuracy. Furthermore, the Lumipulse A β 42 CSF assay is intended to be used in combination with Lumipulse 181p-Tau CSF or Lumipulse Total-Tau CSF assay as a ratio in adult subjects with cognitive impairment being evaluated for AD and other causes of cognitive impairment. Besides, A β 42 CSF assay is intended to be used alone or in combination with Lumipulse 181p-Tau CSF or Lumipulse Total-Tau CSF assay as a ratio in adult subjects with MCI as an aid to identify subjects who are at lower vs. higher risk of cognitive decline to AD. The values of Lumipulse A β 42, A β 40, t-Tau and 181p-Tau and their ratios respect to their cut-off levels (COLs) showed in the **Table 11**.

Table 11. COLs for the Lumipulse A β 42, A β 40, t-Tau and 181p-Tau for AD diagnosis.

Biomarker	COLs
$A\beta 1-42$	If $A\beta 1-42 < 599$ pg/mL test result positive.
$A\beta 1-42/A\beta 1-40$	If $A\beta 1-42/A\beta 1-40 < 0.069$ test result positive.
$tTau$	If $tTau > 404$ pg/mL test result positive.
pT_{au181}	If $pTau181 > 56.5$ pg/mL test result positive.
$A\beta 1-42/tTau$ ratio	If $A\beta 1-42/tTau$ ratio < 1.275 test result positive.
$A\beta 1-42/pTau181$ ratio	If $A\beta 1-42/pTau181$ ratio > 8.100 test result positive.

Criteria for exclusion

1) Neurological disease, apart from AD, that may affect cognition; 2) Atypical presentations of EA; 3) Vascular damage of small vessel superior to Fazekas II, ischemic or hemorrhagic stroke > 1cc, evidence of cerebral contusion, encephalomalacia, subdural / epidural hematoma, space-occupying lesions; 4) Concomitant major psychiatric disorder; 5) Symptomatic carotid or vertebrobasilar stenosis; 6) Treatment with drugs that enhance cognitive deterioration; 7) Inclusion in clinical trials with treatment *versus* placebo in the acute phase; 8) Severe systemic disease; 9) Pregnancy; 10) Other diseases or conditions that may cause interference with the study; 11) Impossibility of the patient to be seen in review consultations; 12) Illiteracy.

The study variables collected in each annual visit were the following:

- Demographics: Age; Gender; Educational level; Start date of the study.
- Anthropometric variables: Weight (Kg); Size (cm); Abdominal perimeter (cm);
- Vascular Risk Factors: Smoking habit: yes / no (consider if, in patients who have left it in the last 5 years); Consumption of alcohol (> 40 gr / day): yes / no; High blood pressure (> 5 years): yes / no; Diabetes Mellitus type II (> 5 years): yes / no; Dyslipidemia: yes / no.
- Other background: Open field variable.
- Habitual treatment: Open field variable.
- Functional/Neurological variables: Global Deterioration Scale (GDS); modified Rankin Scale (0-6).
- Neurophysiological variables: (MMSE); ADAS-COG; Blessed dementia scale; TAVEC (Spanish-Complutense verbal learning test); YESAVAGE geriatric depression scale; TMT A and B; Tower of Hanoi; Boston vocabulary test (evaluation of the denomination); Fluency; Verbal fluency; CDR (clinical assessment of dementia); CRC (Cognitive reserve questionnaire); NPI (Neuropsychiatric Inventory).
- Control of vascular risk factors: smoking: yes / no; alcohol consumption: yes/no; DM control (ADA criteria: HbA1c levels <7%, basal glycemia 90-130 mg / dL): yes/no; Good control of blood pressure figures <130/80 mmHg): yes/no; Good control of dyslipidemia (LDL <100 mg, TGL <150): yes/no; Incidents (descriptive variable); Has an event appeared that interrupted the follow-up? yes / no (include date and description).
- Neuroimage variables: Visual MTA rating Scale (Score 0,1,2,3,4); Koedam score for Parietal Atrophy (Grade 0, Grade 1, Grade 2 and Grade 3) (Eur Radiol 2011, 21: 2618-25); Scale of Fazekas (0,1,2 and 3) (Arch Intern Med. 2007; 167: 81-8). Number of silent infarcts (sequences FLAIR and T2); Number of microbleeds (sequences T2 * GRE); No. of intracranial stenosis; MRI permeability map.
- Biological variables: CSF biomarkers only for the diagnosis of patients with Alzheimer's disease (the Lumipulse system from Fujirebio was used): PHOSPHO-TAU (181P), β -AMYLOID (1-42) and hTAU Ag.

Endpoint: The endpoint of the study was to compare the CSF levels (p-Tau and ac-Tau) between patients with MCI who progress or not to AD.

The bivariate comparison of groups was carried out with Student-t test (normal continuous variables), U-Mann-Whitney (continuous non-normal variables) and chi-square (categorical variables). A value of $p < 0.05$ was considered significant in all tests. The statistical package SPSS 20.0 for Mac was used.

4.3. MAGNETORESISTIVE BIOSENSORS FOR BIOCHIP PLATFORM

This section of the study was conducted using a novel point-of-care (POC) diagnostic platform during the stay of the doctoral student at the International Iberian Nanotechnology Laboratory (INL), which was based on the use of magnetoresistive (MR) biosensors for the quantification of the pS208-tau levels in aCSF matrix. The MR biosensors (spin-valve) were functionalized with gold surface to create a bio-compatible surface for antibodies, which were immobilized to the biochip through a bifunctional linker. On this platform, different detection techniques were carried out for this analyte using biofunctionalized magnetic nanoparticles (MNPs) to adjust sensitivity within clinically relevant ranges at the pg/ml level.

4.3.1. Spin valve biochip design and characteristics

This platform is portable. This technology use MNPs of 250 nm, which allows adjusting the dynamic range and sensitivity of the quantification of different analytes through a microfluidic system that allows controlling the volume and position of the sample in a U-shaped channel, through which the sample passes. The platform was composed of a magnetic focus that allows the attraction of the magnetic nanoparticles, which increases the sensitivity of the assay. The Biochips were composed of an array of 30 U-shaped spin-valve (SV) sensors with gold pads ($12.9 \times 35.4 \mu\text{m}^2$), organized by 30 sensors divided into 6 groups, each group consisting of 5 sensors coated with a layer of gold (**Figure 17**).

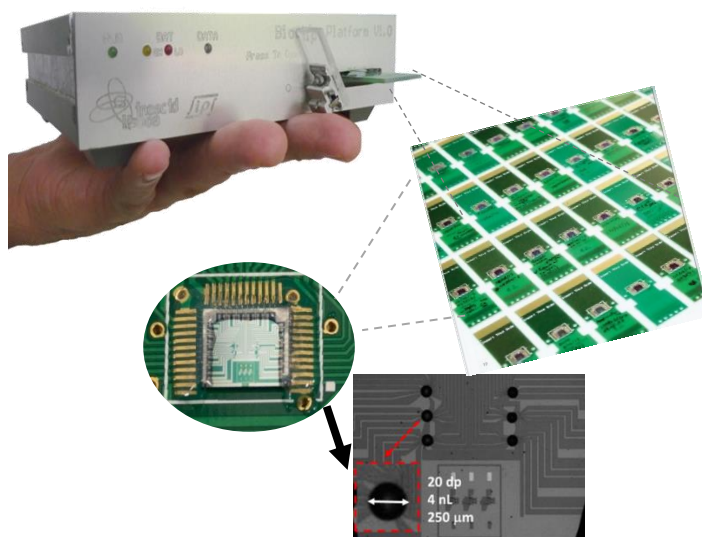


Figure 17. Scheme of Magnetoresistive (MR) Biochip Platform. Self-created image provided by the International Iberian Nanotechnology Laboratory (INL).

4.3.2. Biofunctionalization of MNPs

Firstly, in **Figure 18** can be seen the biofunctionalization of the biochip surface where the SV sensors were cleaned with isopropanol, deionized water, and UV-ozone for 5 min 55°C and were treated for 20 min with 1 mg/mL in phosphate buffer (PB) of sulfosuccinimidyl 6-[3'(2-pyridyldithio)-propionamido] hexanoate (Sulfo-LC-SPDP) (Thermo Fisher Scientific, USA) used as a heterobifunctional surface linker. After linker deposition, 250 µg/mL anti-tau B6 primary antibody was added in PB with 5% Glycerol and it was contacted for ON 4°C with the chip surface. 1 µL of BSA 5%, was added above the chip. Then, the biochip was washed with PB. 1% of BSA in PB was added and the negative control sensors were blocked in 5% BSA in PB for 1 h RT. Then, the biochip was washed in PB again.

The biofunctionalization of the MNPs began with a mix of 2 µL of streptavidin-conjugated with 250-nm MNPs (4.9×10^{11} mL⁻¹, Nanomag) (Micro mod, Germany) and 60 µL of 50 µg/mL of biotinylated BT2 antibody in PB with 0.05% Tween®20 (PB-T) and the mix was incubated for 1 h RT in solution.

After functionalization with the secondary antibody, the MNPs were washed in PB-T. The MNPs surface was blocked with 5% BSA in PB for 1 h RT. The MNPs have washed in PB-T again.

Several methods of detecting the analyte were tested; one of them was by incubating the sample separately from the MNPs named step by step, inside and outside the microfluidic circuit, to understand how the volume impacts the protein-antibody interaction, after passing over the MR sensors located on the biochips inside the platform. The other method was the pre-recognition step, where pS208-tau mAb was captured in solution by functionalized with MNPs. 2 µL of stock MNPs solution complex were added to 8 µL of PB-T with 60 µL of aCSF matrix and 100 ng/ml of p-tau protein was spiked in aCSF matrix for 1h at RT. The MNPs have washed in PB-T again and 10 uL of the sample was added to the biosensor. The presence of MNPs bounded in each region of the biosensor was validated by the NBI-Wide-Field Upright Fluorescence Microscope.

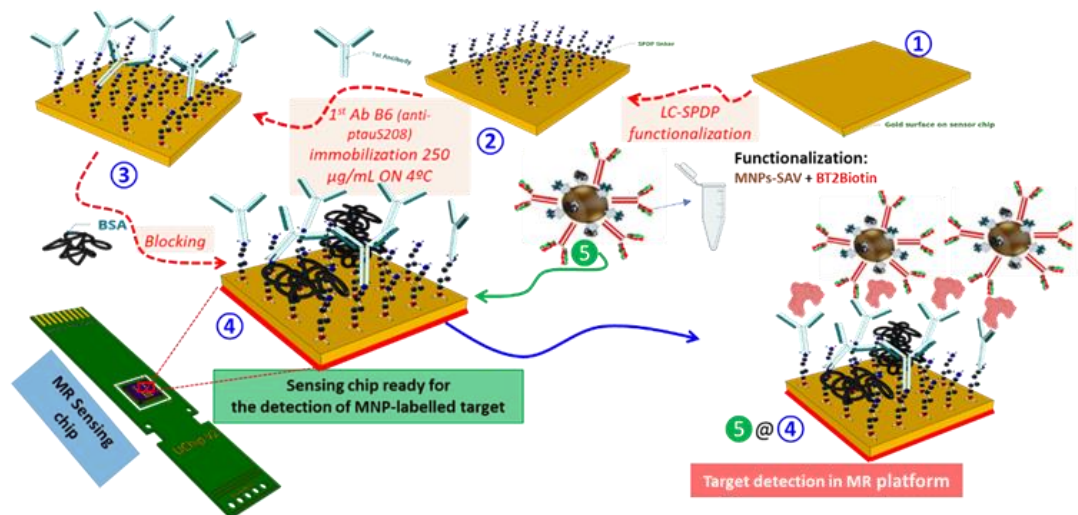


Figure 18. Experimental procedures of surface functionalization on Magneto-resistive (MR) sensing chip. Self-created image provided by the International Iberian Nanotechnology Laboratory (INL).

4.4. IMMUNOTHERAPY STUDY: INTRACEREBROVENTRICULAR ADMINISTRATION OF P-TAU B6 ANTIBODY

4.4.1. Animals and Protocol

In this study, the effect of therapeutic intervention with the continuous ICV administration of p-Tau B6 mAb on behavior, brain morphology, and tau pathology of a transgenic mouse model of tauopathy (hTau.P301S-Tg) was investigated. The aim was to evaluate the potential therapeutic effects of the administration of the p-Tau B6 mAb by infusing the drug directly into the ventricular system through an osmotic minipump.

The hTauP301S-Tg model, a clinical mutant of h-tau (P301S) is expressed under the control of the murine Thy1 promoter (neuron-specific expression) as described in section 1.1.8. It is characterized by an early onset of tau pathology in the brain and Sc, starting at the age of 3 months, where hyperphosphorylation of tau is already present in the superficial frontal cortex (Hampton et al., 2010). Furthermore, this model displays a progressive age-dependent increase in Tau hyperphosphorylation in the Sc, Bs (medulla + pons), cerebral Cx, and Hc from 3 months onwards. Together with tau pathology, neuronal loss associated with reactive astrogliosis is present in the superficial cortex and Sc from 3 months onwards. Concomitant to the pathology, these mice progressively develop motoric deficits like clasping, reduced general mobility, and weight loss and die prematurely around the age of 6.5 months.

The present study specifically aimed to determine the effect of p-Tau B6 antibody administration into the lateral ventricle by infusing the drug directly in the ventricular system through an osmotic pump (model 1004, Alzet, Thermo Fisher Scientific, USA) located under the skin of the upper back of the mice. A total volume of 100 μ L of 2.5 mg/ml of p-Tau B6 antibody was charged into the minipump and the infusion lasted 4 weeks at the rate of 0.11 μ L/h. Length 1.5 cm, diameter 6 mm, weight 0.4 g (**Figure 19** and **Table 12**). The protocol was approved by the Animal Experimentation Ethics Committee of Helath Research Institute of Santiago de Compostela (IDIS) (Procedure Number: 15011/2021/04).

Release rates and durations for all ALZET pump models.

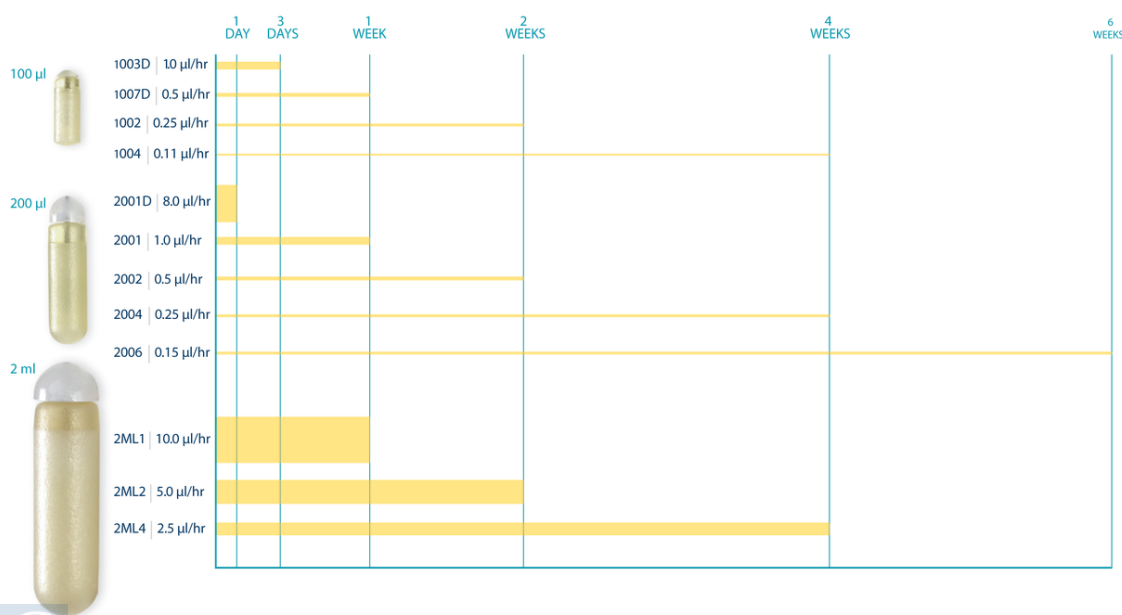


Figure 19. Example of the release rates and durations for all Alzet osmotic mini pumps available. Self-created image based on provided data by Alzet-Thermo Fisher Scientific.

Table 12. Treatment procedure and study set-up.

Group	N	Strain	Treatment [I]	Treatment duration and method	Administration route/ dose	Age at start (weeks)	Age at sacrifice (weeks)
1	20	hTauP301S-Tg	B6 mAb 2.5 mg/mL	4 weeks	ICV/100 μ l, 0.11 μ l/h	14	20
2	20	hTauP301S-Tg	IgG1 mAb 2.5 mg/mL	Alzet 1004 mini pump Alzet 0008851 brain infusion kit 3			
3	20	hTauP301S-Tg	Sham		-		

The mini pumps were implanted subcutaneously in the back of the animal and connected to the cannula through a 2.5 cm tubing (**Figure 20**). The coordinates of the infusion point were M/L -1.1 mm (right hemisphere), A/P -0.5 mm, and D/V -2.5 mm (length of the catheter).

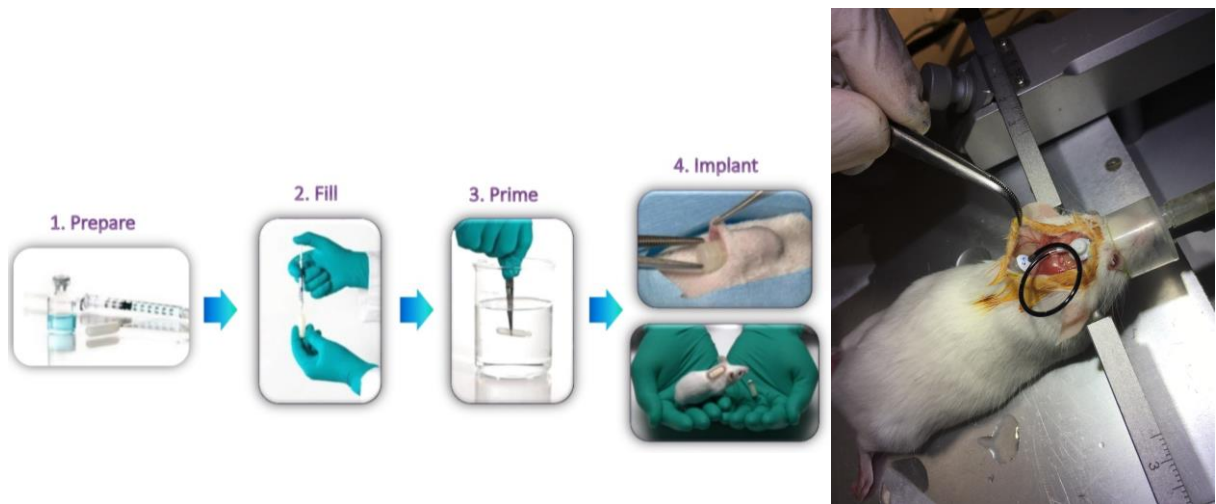
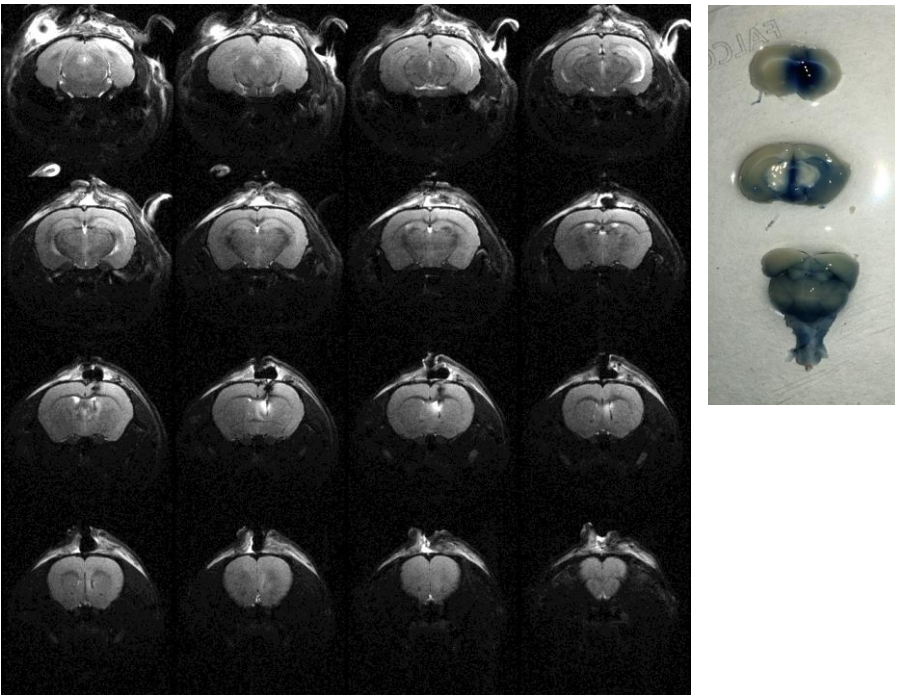


Figure 20. Example of the SC implantation procedure in the animal. Self-created image.

For confirming the adequacy of the infusion coordinates, several tests with gadolinium and color dyes as Evans blue served as contrast agents as shown in **Figure 21**. Notably, the



antibody stays active in the brain according to literature for 10-15 days after the pump has emptied.

Figure 21. Right; Brain coronal cuts of mice infused with Evans blue dyed almost all brain and cerebellum from the right lateral ventricle, where the intensity is higher. **Left;** MRI of several coronal sections of the administered animal confirming the presence of the gadolinium in the ventricles (clear greys).

The mini pump was implanted with a brain infusion kit (model 3, 0008851, Alzet, Thermo Fisher Scientific, USA) at week 14, so the treatment starts at 3.5 months and continues for 4 weeks and was removed at week 19 (**Table 12**).

Behavior and motor function, brain morphology and tau pathology in the brain of hTauP301S-Tg transgenic mice were analyzed. hTauP301S-Tg transgenic mice dosed with the same concentration of IgG1 isotype control were used as a negative control for potential therapeutic effects of the B6 antibody. hTauP301S-Tg transgenic mice with the same skin incisions and sutures were used as sham control group. To allow sufficient time for all in vivo readouts, the study was divided into two staggers of 20 mice with each treatment group represented equally in each staggering (**Table 12**).

Stagger 1 (months 3.5-4.5):

Group X: hTau female mice, B6 (n = 20)

Group Y: hTau female mice, Ctrl IgG1 (n= 20)

Group Z: hTau female mice, Sham surgery (n = 20)

Stagger 2 (months 3.5-4.5):

Group K: hTau female mice, B6 (n = 20)

Group L: hTau female mice, Ctrl IgG1 (n= 20)

Group M: hTau female mice, Sham surgery (n = 20)

Mice were received at 11.5 weeks, at this point animals were randomized into three groups and were habituated to the facilities. Baseline MRI was performed at 12 and 13 weeks, and weight and grid hanging test at 13 weeks. Weight was measured every week. At 14 weeks mini pump was implanted. Clasp test was performed three times every week from 14 to 18 weeks, both included. Grid hanging test was repeated at 17 and 18. And rotarod test was performed at 16 and 18. Between weeks 18 and 19 brain infusion kit and mini pump were removed just after MRI was run. Finally, at week 20 plasma was collected and the mice were perfused transcardially with ice-cold saline and subsequently, the brain was collected. The brain hemispheres were separated, one hemisphere was post-fixed in 4% paraformaldehyde, and the other hemisphere was microdissected into the Cx (frontal and posterior part), Hc, midbrain + Bs (medulla + pons), cerebellum (Cb), and a rest fraction. The experimental design of the study lasted 2 months, the maximum lifespan of the hTauP301S transgenic animals were 5 months as seen in the protocol showed in **Figure 22**.

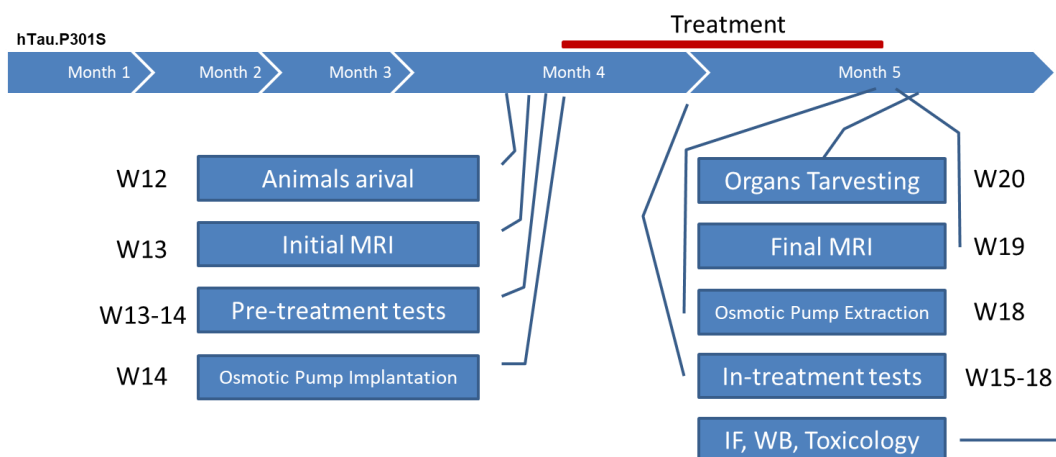


Figure 22. Timeline of the study during 5 months and the different work per week. Self-created image using elements with Creative Common license.

4.4.2. Health monitoring and behavioral tests

The mice were weighed weekly throughout the experiment.

4.4.2.1. Clasping

Clasping is a pathological reflex of the hindlimbs that is seen among other in damage of the Sc, Bs motor nuclei and cerebellar nuclei (**Figure 23**).



Figure 23. The two clasping positions. When held by the tail, a normal mouse reflexively extends its hindlimbs and spreads the toes (**left**). By contrast, a hTAuP301S mouse clasps its hindlimbs (**right**).

To score clasping behavior, mice were kept approximately 1.5 cm above their tail base for about 10 seconds (tail suspension test). Clasping of the limbs was scored for each limb separately, using the 4-point rating scale. Hind limb clasping was scored according to 0 = Hind limb folded backward and distant from the body, angle of the limb with body axis $>0^\circ$ (at least once during the screened time window); 0,5 = Any phenotype in between 0 and 1 (starting from 100% of time fully stretched, but in line ($=0^\circ$) with body axis) 1 = One hind limb partially retracted during more than 50% of the time; 3 = Limb completely retracted during more than 50% of the time; 4 = Mouse has died/has been prematurely sacrificed after reaching clasping stage wherein humane endpoint criteria are met (**Figure 24 and Table 13**). The mice showing severe clasping phenotype were sacrificed before death.

Figure 24. The four clasping scores. Self-created image using elements with Creative Common license.

Table 13. Clasping score conversion. Score 4 is assigned when the mouse is prematurely sacrificed or euthanized after reaching the ‘humane end point’ criteria.

		Right				
		0	0,5	1	3	
Left	0	0	0,5	1	2	4
	0,5	0,5	0,5	1	2	4
	1	1	1	2	2,5	4
	3	2	2	2,5	3	4
		4	4	4	4	4

4.4.2.2. Rotarod

On the other hand, the main method to assess the motor coordination and balance of the mice was the Rotarod test. Based on the established progression of the motor phenotype of hTauP301S mice the Rotarod test was performed at ages of 3.3, 4.2, and 4.7 months. At the younger age, Rotarod testing comprised four consecutive days of habituation, training, and test trials. At the age of 4.2 months and 4.7 months, Rotarod testing comprised only 3 days or 2 days. The test protocol is summarized in **Table 14**. The first Rotarod session, executed at an age of 3.3 months, was performed on four consecutive days. On day 1, each mouse was first familiarized two times with the Rotarod by leaving it to explore the rod for one minute. On day 2, each mouse was familiarized with the Rotarod once more. Next, it was subjected to four training trials, at a constant speed of 10 rpm for a maximum time of 120 s (minimum of 60 min inter-trial interval). After the 4th training trial, each mouse was subjected to a short test trial (10 rpm, 20 s) to evaluate whether it was sufficiently trained and motivated to participate in the test trials on a subsequent day. On day 3, mice received four consecutive test trials at a speed of 10 rpm for 120 s (minimum of 60 min inter-trial interval). On day 4, two acceleration tests were performed wherein the speed of the rod was increased from 4 to 40 rpm within 120 s.

Day 1	Day 2	Day 3	Day 4
Habituation 1 0 rpm 60 sec	Habituation 1 0 rpm 60 sec	Test 1 10 rpm 120 sec	1 Acceleration 4 – 40 rpm 120 sec
Habituation 2 0 rpm 60 sec	Training 1 10 rpm 120 sec	Test 2 10 rpm 120 sec	2 Acceleration 4 – 40 rpm 120 sec
	Training 2 10 rpm 120 sec	Test 3 10 rpm 120 sec	
	Training 3 10 rpm 120 sec	Test 4 10 rpm 120 sec	
	Training 4 10 rpm		

120 sec
Test 1
10 rpm
20 sec

Table 14. Rotarod protocol. Remark: Day 1 and 3rd and 4th training session and the test 1 of day 2 were not performed at an age of 4.2 months. Only day 3 and day 4 were performed at an age of 4.7 months.

The Rotarod performed at an age of 4.2 months was done on three consecutive days. On the first day, each mouse was familiarized with the Rotarod once again, whereafter it was subjected to two training trials, at a constant speed of 10 rpm for a maximum time of 120 s (minimum of 60 min inter-trial interval). Days 2 and 3 were similar to day 3 and 4 above, i.e. four consecutive test trials (fixed speed for 120 s) on day 2 and two acceleration tests on day 3.

At an age of 4.7 months, Rotarod comprises only two days, i.e. four consecutive test trials (fixed speed for 120 s) on day 1 and two acceleration tests on day 2.

During the test trials, the mean latency to fall off the Rotarod was recorded and used in the analysis.

4.4.2.3. Grid hanging

The inverted grid hanging test was used to evaluate the ability of the mouse to grasp an elevated horizontal grid and remain suspended for 2 min. The animal was placed on the grid (40 cm × 20 cm/0.5 cm meshes) and positioned 50 cm above a flat, soft surface. The latency for the animal to drop off was then measured (in seconds). A grid hanging test was performed with all groups, at an average age of approximately 2.3, 3.0, 3.9 and 5 months.

4.4.2.4. Statistical analysis

The statistical analysis was performed using two-way ANOVA for repeated measures followed by Tukey's post-hoc testing (hTauP301S-Tg isotype control vs hTauP301S-Tg antibody B6 groups and non-Tg isotype control vs hTauP301S-Tg isotype control, respectively).

4.4.3. Sacrifice and brain Sampling for Westetn Blot and Immunohystochemistry

4.4.3.1. Sacrifice

The mice were sacrificed after 4 weeks of treatment, one week after the last dosing, at an age of 20 weeks, following the sacrifice procedures showed in the **Table 15**.

The mice were sacrificed before general study termination (so-called premature sacrifice) when reaching the stage where clasping symptoms are resulting in weight loss below 20% of heaviest weight during study and temperature drops below 34°C, or when mice are not being able to get up within 5 seconds when put on either its left or right side in the home cage.

Table 15. Overview sacrifice procedures and sample recollection.

Body weight	Measured at sacrifice
Anaesthesia	Sevofurane
Perfusion	The thoracic cavity was accessed for perfusion of the systemic circulatory system via trans-cardiac perfusion with ice-cold saline for three minutes (10 mL/min via peristaltic pump) via the left ventricle. The right atrium was cut as an outflow route.
Left hemisphere	The Cx (separated in a frontal and a posterior part), Hc, Bs (pons + medulla) together with the midbrain, Cb and a rest fraction was micro-dissected, immersed in liquid nitrogen in separate tubes

	and stored in microfuge tube at -80°C for biochemical analysis.
Right hemisphere	It was post-fixed overnight in 1XPBS containing 4% paraformaldehyde and stored in 1XPBS containing 0.1 % (v/w) sodium azide at 4°C until further processing for immunohistochemistry.
Sample labelling	Tissue samples were identified with their respective mouse number to keep analysts blind to treatment.

4.4.3.2. Brain Sampling for Western Blot and Immunohistochemical analysis

The sample processing protocol began by fixing the animal to the heating pad at 37°C and the mice were anesthetized with a mixture of ketamine, xylazine 2%, and atropine and saline (4:2,5:1:2,5). The body temperature of mice under anesthesia was maintained by applying a heating blanket and monitored using a rectal thermometer. Temperatures were monitored using a rectal thermometer before anesthesia and before opening the body cavity. The thoracic cavity was opened and a blood sample was taken with a 1ml syringe with a 26G needle flushed with heparin by injecting into the right atrium and depositing the blood into heparin vacutainer tubes. The blood was centrifuged at 1650g for 2 min and the plasma phase was collected and kept on ice until ready and stored at -80°C.

The mice were flushed trans-cardially with ice-cold saline for three minutes (10 ml/min via peristaltic pump) via the left ventricle. The right atrium was cut as an outflow route. The brain was removed from the cranium and was divided evenly into the left and right hemispheres by a midline sagittal cut. The contralateral hemisphere or the left hemisphere was cryopreserved for biochemical analysis as WB and the ipsilateral hemisphere or the right hemisphere was fixed in 4% PFA for sectioning and immunostaining.

For the immunostaining protocol, **the ipsilateral hemibrain** or the right hemisphere was post-fixed in 4% PFA for 24 h at 4°C and then was moved to 30% sucrose with sodium azide, for at least 48 h at 4°C and longer if necessary until it sinks. Once sunk, the hemibrain was passed to optimal cutting temperature (O.C.T.) in cryomolds and frozen in liquid N₂. Sections fixed to the O.C.T. were attached to the cryostat stand for coronal sections. First, the hemibrain was cut for histology at the cross section near bregma (the dashed line through the 3rd ventricle). The frontal block was preserved at -80°C for eventual future use and cryosections were cut from the Hc toward the caudal direction. Started to make 40-micron sections with the cryostat, mounting the samples directly on a 25 mm x 75 mm x 1.0 mm slide, 2 coronal sections of dorsal Hc were done with 3 mice per slide, and 6 coronal sections were done in total (**Figure 25**). The mouse numbers on the slide were marked as long as the surface is dry and the slides were stored at -80°C.

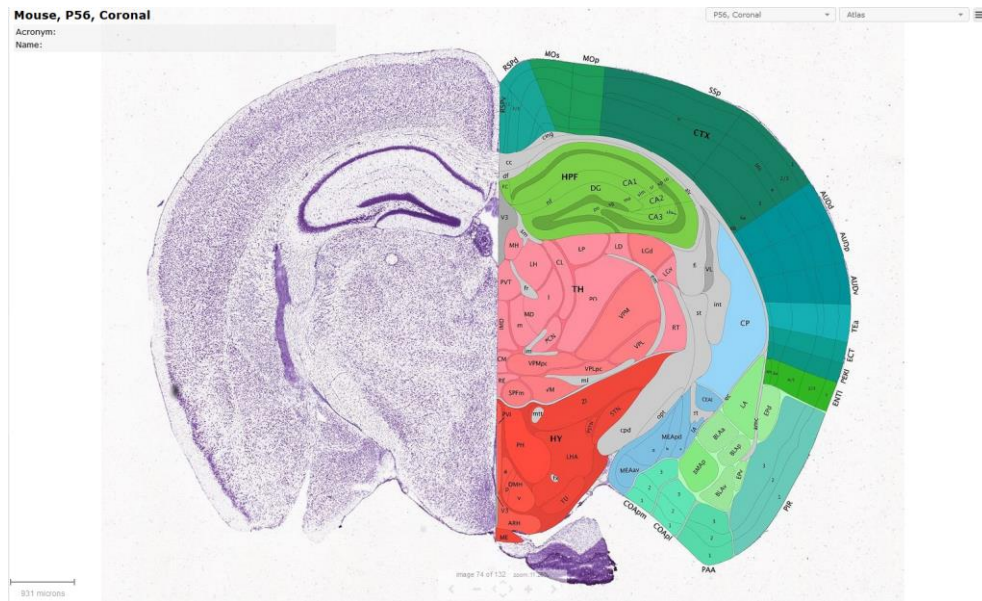


Figure 25. Coronal section cutting view of a mouse brain. Image supplied by the Allen Mouse Brain Atlas and Allen Reference Atlas – Mouse Brain.

In regard to **the contralateral hemibrain** microdissection protocol, the contralateral hemibrain or left brain was microdissected into Cb, Bs, Hc and Cx.

The brain half was cut and dissected at the frontal cross section line (through the third ventricle) and the frontal cortex as a block was dissected. The Hc, Bs and Cb were dissected out too. The remaining posterior cortical block into superior and inferior halves (= parieto-occipital and temporo-occipital blocks) were divided. All parts were snap frozen in liquid N₂, they were kept in individual microcentrifuge tubes labelled with mouse IDs and finally they were stored at -80°C. Location of the sections the orange arrow are seen in the **Figure 26**:

- From bregma: AP -2 mm.
- 2 mm from posterior cortex border.

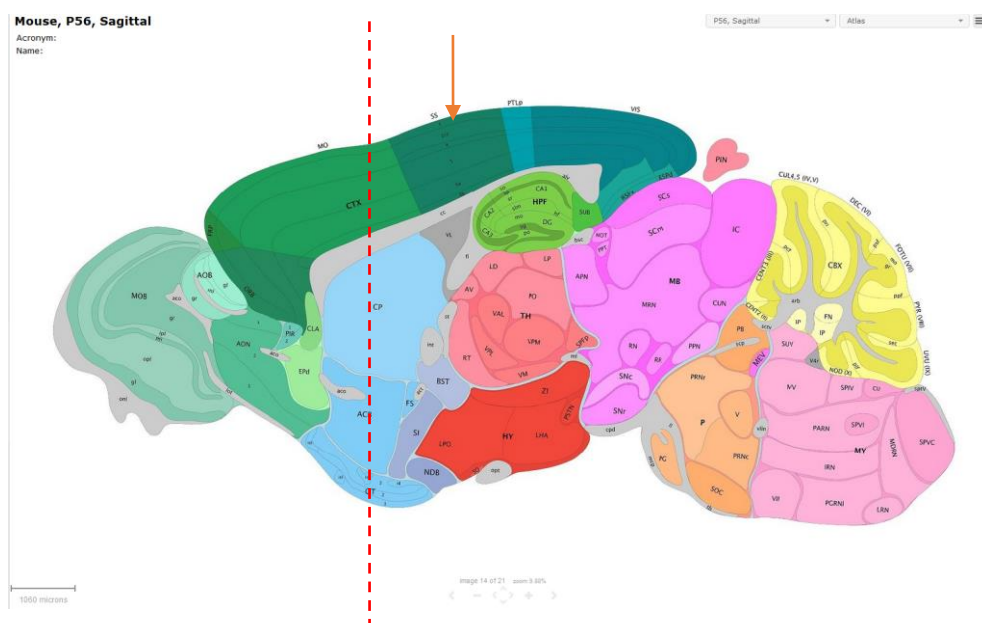


Figure 26. Sagittal section cutting view of a mouse brain. Image supplied by the Allen Mouse Brain Atlas and Allen Reference Atlas – Mouse Brain.

4.4.3.3. Western blot analysis of brain tissue samples of *Tau.P301S* transgenic mice.

4.4.3.3.1. Total protein extractions

Total protein lysates were extracted from the Hc and Bsof brain tissue samples as previously shown (Glasser et al., 2016). Briefly, tissue samples dissected from mouse brains were homogenized in 300 µl of ice-cold homogenization buffer, containing 20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA) (Merck, Sigma-Aldrich, Massachusetts, USA), 1 mM ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich, Massachusetts, USA), 10 mM 1,10-ortho-phenanthroline monohydrate (Sigma-Aldrich, Massachusetts, USA), a cocktail of proteinase inhibitors (Complete™, Roche, Germany) and halt protease and phosphatase inhibitor (Thermo Fisher Scientific, Massachusetts, USA). A fixed volume of approximately half of the total homogenates (TotH), was centrifuged at 136000xg, 60 min, 4°C to generate a Tris-soluble fraction (SF), while the remainder was stored at -80°C.

The supernatant (S1, further referred to as soluble fraction or SF) was separated from the pellet (P1), aliquoted, and stored at -80°C. The pellet (P1) was solubilized in 9, 19, and 19 weight-volumes high-salt (0.85 M NaCl) containing homogenization buffer for the Cx, Hc, and Bs, respectively. This fraction was centrifuged at 20000xg, 30 min, and 4°C, and the resulting high-salt pellet (P2) was stored at -80°C. The supernatant (S2) was brought to 1% Sarkosyl with 10% Sarkosyl and incubated at RT for 60 min in a top-over-top rotary tumbler and then was centrifuged at 136000xg, 60 min, 4°C. The supernatant (S3, further referred to as Sarkosyl-soluble fraction or SSF) was stored at -80°C. The pellet (P3) was resuspended in a homogenisation buffer and stored at -80°C (further referred to as insoluble fraction or IF).

The levels of total Tau (HT7 antibody) and phosphorylated Tau (AT100) were analyzed in the total homogenate, the Tris-soluble and sarkosyl insoluble fraction of the Cx, Hc, and the Bs (medulla + pons). Per tissue, an equal amount of total protein (determined by Lowry protein assay) was loaded.

4.4.3.3.2. Western blot analysis

Total protein lysates (30 µg) were subjected to SDS-PAGE and WB, samples were appropriately diluted using sample buffer (final concentrations 1X sample buffer: 1% (w/v) SDS and 2.5% (v/v) 2-mercaptoethanol) based on fresh weight concentration, denatured and reduced by heating at 95 °C for 10 min and separated on 4-12% Bis-Tris gels Criterion XT Precast Gel, 26 well comb, 15µl, 1.0 mm (Biorad,USA). After dry electrotransfer Trans-Blot Turbo™ Transfer System (Biorad,USA) to PVDF-membranes Trans-Blot Turbo™ Midi PVDF Transfer (Biorad,USA), the membranes were washed in Tris-buffered saline. Next the membranes were incubated in Tris-buffered saline (TBS, pH 7.6) containing 5 % (w/v) non-fat dry milk and 0.1 % (v/v) Tween-20 for 1 hour.

Blots were incubated with the primary antibodies overnight (**Table 16**). Primary mAbs used for detection were anti-p-tau (Thr212 and Ser214) AT100 (Thermo Fisher Scientific, Massachusetts, USA) and anti-t-tau HT7 (Thermo Fisher Scientific, Massachusetts, USA). After washing and incubation with an anti-mouse HRP-conjugated secondary antibody goat-anti-mouse or goat-anti-rabbit IgG (DAKO,USA) blots were developed by the ECL detection system SuperSignal West Pico or Femto Maximum Sensitivity Substrate (Thermo Scientific, Massachusetts, USA) and WB images were quantified using Quantity One software (BioRad,USA). p-tau and total human 4R-Tau protein levels were normalized to total human

4R-Tau or β -actin levels. As multiple gels were used for each antibody, reference samples of the total homogenate were run on each gel as an inter-gel reference.

Table 16. Antibodies, their respective specificity and dilutions used in WB analyses.

mAb	Supplier	Cat n°	Specificity	Host	Stock conc.	BC. []/Dilution
AT100	Thermo Scientific	MN1060	Human, mouse	Mouse	200 μ g/ml	0.8 μ g/ml/1:250
HT7	Thermo Scientific	MN1000	Human	Mouse	200 μ g/ml	0.2 μ g/ml/1:1000

4.4.3.3.3. Statistical analysis

Statistical analysis on data were performed using a Student's t-test comparing hTauP301S-Tg isotype control vehicle vs hTauP301S-Tg antibody B6 groups and a Student's t-Test comparing non-Tg isotype control vs hTauP301S-Tg isotype control). A Grubb's outlier test was applied; such statistical outliers were only be excluded from the analysis if there is a biological rational to support the outlier characteristic (e.g., lower exposure measured).

4.4.3.4. Immunohistochemistry analysis of brain tissue samples of Tau.P301S transgenic mice

4.4.3.4.1. Tissue preparation and immunohistochemistry analysis

From each right hemisphere, a total of about 40 sagittal sections (40 μ m) containing Cx, Hc and the Bs (medulla + pons) were collected. Brain sections of interest between bregma lateral 2.64 and 1.08 were selected based on a stereotaxic mouse brain atlas (Paxinos and Franklin). Three sets of five sections per mouse, collected at intervals of 200 μ m and comprising the regions of interest (ROI), were processed for AT100 immunohistochemical analysis (**Table 17**).

Table 17. Summary of antibody used for IHC.

mAb	Supplier	Cat n°	Specificity	Host	Stock conc.	Work conc.
AT100	Thermo Fisher Scientific	MN1060	Human	Mouse	200 μ g/ml	0.05 μ g/ml

The sections were washed three times in PBS and incubated for 20 minutes in hydrogen peroxide 1.5% in PBS and methanol (1:1) to remove endogenous peroxidase activity. After washing the sections three times in PBS containing 0.1% Triton X100 (PBST), the sections were blocked for 60 minutes in 10% Fetal Calf Serum (FCS) in PBST followed by an overnight incubation with primary antibody AT100 (Thermo scientific, Massachusetts, USA), using a concentration of 0.05 μ g/ml in PBST with 10% FCS. After rinsing, the sections were incubated with goat anti mouse peroxidase labelled (GAMPO) secondary antibody (DAKO, USA), 1/500 in PBST, 10% FCS and the signal were developed with 3,3' diaminobenzidine tetrahydrochloride (DAB, 1 tablet per 10 ml Tris-HCl with 3 μ l H₂O₂). Sections were counterstained with Mayer's hematoxylin, gently washed in streaming tap water for 4 minutes, dehydrated in five steps (50, 70, 95 and 2x100%) in ethanol and 2x xylene (Merck Eurolab) and mounted in Depex (Depex mounting medium, BDH Laboratory). Sections with too high a background staining, damage or artefacts were omitted.

4.4.3.4.2. Microscopy and image analysis

Images were acquired with an Olympus BX41 microscope equipped with a XC30 3Mpixel 1/1.8" cooled Olympus CCD camera and analyzed with a computer using AnalySIS

Five – CellSens software. Light intensity and condenser settings for the microscope were kept constant throughout the image acquisition process. All acquired images were subjected to the same computer subroutines to minimize investigator bias. Density slice thresholding was applied uniformly throughout analysis for the stained sections. The threshold was determined by varying the threshold in the blue channel while the other channels were set at full intensity range, and area was determined in different sections representative for the range of areas in the series. The threshold was from the range of thresholds that yields identical relative differences (as much as possible) between the areas in the different sections.

4.4.3.4.3. Statistical analysis

For each staining, at least four brain sections per mouse containing the ROI (motor cortex or Bs (medulla + pons) were included in the analysis and the median value were determined. Sections with too high background staining, damage or artefacts were omitted. Statistical analysis was based on the averages and were performed using either one-way ANOVA (P301S-Tg vehicle vs P301S-Tg compound groups) or Student's T-Test (non-Tg vehicle vs P301S-Tg vehicle). A Grubb's outlier test was applied; such statistical outliers were excluded from the analysis if there is a biological rational to support the outlier characteristic (e.g., lower exposure measured).

4.5. PET IMAGING, PHARMACOKINETICS AND BIODISTRIBUTION OF RADIOLABELED ¹⁸⁹ZR-B6 OR ¹⁸⁹ZR-B6 F(ab')₂ IN hTAUP301S AND C57/BL6 MICE.

Preliminary tests oriented to assess the biodistribution (BD), pharmacokinetics (PK) and brain uptake of radiolabeled B6 antibody in hTau.P301S and C67/BL6 (wt) mice. Several trials were completed in both animal models with either ¹⁸⁹Zr radiolabeled complete B6 or B6 F(ab')₂ fragment antibody through IP, SC, intravenous (IV, jugular or tail), and intraarterial (IA, carotid) administrations.

4.5.1. B6 enzymatic digestion and F(ab')₂ purification

The anti-ptau B6 antibody is a murine IgG1 produced in bioreactors from hybridoma cell cultures under GMP-like conditions. The antibody is purified by HPLC with G-protein affinity columns and dialized in PBS to the final formulation (5-8 mg/mL).

The enzymatic digestion of the B6 antibody was performed with the kit Pierce Mouse IgG₁ Fab and F(ab')₂ Preparation Kit (Ref. 44980). The kit uses Immobilized Ficin to prepare fragments from mouse IgG1. Ficin generates F(ab')₂ fragments exclusively in the presence of 1-4mM cysteine. Immobilized Ficin enables immediate cessation of the digestion by simply removing the resin from the antibody digest solution. The included Spin Columns allow easy manipulation of the resin and maximum F(ab')₂ recoveries. The prepacked, immobilized Thermo Scientific™ NAb™ Protein A Plus Spin Column and optimized binding buffer binds the intact Fc fragments and undigested IgG, allowing for efficient F(ab')₂ fragments purification.

4.5.2. ¹⁸⁹Zr-B6 mAb and F(ab')₂ radiolabeling

To follow the antibody in the mice bodies, the B6 antibody was radiolabelled with ¹⁸⁹Zr using p-isothiocyanatobenzyl-desferrioxamine (Df-Bz-NCS) as chelant and quantified by ITLC following the protocol described by (Vosjan et al., 2010). The positron emitter ¹⁸⁹Zr has very attractive properties for positron emission tomography (PET) imaging of intact mAbs using immuno-PET. The protocol followed allows facile radiolabeling of mAbs or other proteins with ¹⁸⁹Zr using p-isothiocyanatobenzyl-desferrioxamine (Df-Bz-NCS). First, Df-

Bz–NCS is coupled to the lysine–NH₂ groups of a mAb at pH 9.0 (pre-modification), followed by purification using centrifuge filters. Next, the pre-modified mAb is labeled at RT by the addition of [⁸⁹Zr]-oxalic acid solution followed by purification using centrifuge filters (**Figure 27**) (Vosjan et al., 2010).

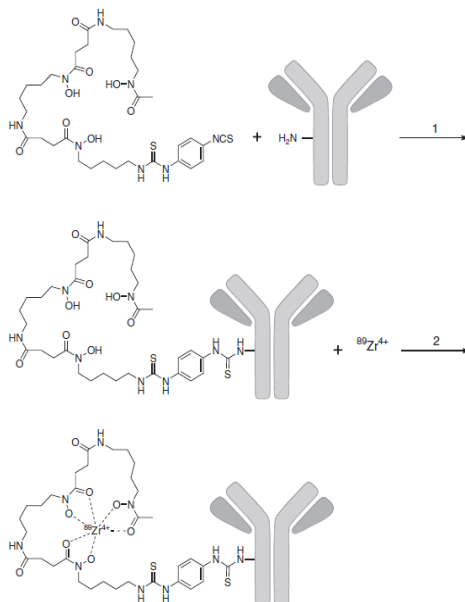


Figure 27. Schematic representation of the radiolabeling of the antibodies with [⁸⁹Zr]. Self-created image based on the method described by (Vosjan et al., 2010).

4.5.3. Mice administration

Animals were kept on a heating pad at 30 degrees from one hour before the PET injection in order to keep their body temperature. They were anesthetized with a gaseous mixture of 3% isoflurane in oxygen in an anaesthesia chamber before the injection of the radiolabeled antibodies and fragments with a 30G needle. Administrations included IP, SC, IV, jugular or tail, and IA, carotid injections to two animals per timepoint. All protocols were approved by the Animal Experimentation Ethics Committee of Helath Research Institute of Santiago de Compostela (IDIS) (Procedure Number: 15011/2021/04).

4.5.4. PET imaging acquisition

The acquisition was performed just after the injections with the animals kept asleep under a concentration of 2% zaisoflurane in oxygen. A single bed position was acquired during 20 minutes with the mouse in the center of the field of view. Whole-body PET/CT images were acquired with an Albira PET/CT Preclinical Imaging System (Bruker Biospin, Woodbridge, Connecticut, United States). The PET subsystem comprises three rings of eight compact modules based on monolithic crystals coupled to multi-anode photomultiplier tubes (MA-PMTs), which form an octagon with an axial FOV of 40 mm per ring and a transaxial FOV of 80 mm in diameter. The PET radial spatial resolution is 1.55 mm (full width at half-maximum) and the absolute sensitivity of the system is 2% for a 350-650 keV energy window and a 5 ns timing window. Image acquisition and reconstruction were performed by using the Bruker Albira Suite Software Version 5.0. Scatter and random coincidences were corrected for by using the algorithms provided by the manufacturer. Attenuation correction was not performed. Images were reconstructed by using the maximum likelihood expectation maximization (MLEM) algorithm: 12 iterations were performed with an output pixel size of 0.4×0.4×0.4 mm³.

4.5.5. Pharmacokinetics and biodistribution

The PK and BD studies were performed with both hTau.P301S transgenic and wt C57/BL6 mice. Mice were administrated with 15-100 μ Ci of radiolabeled antibodies and fragments. Blood samples were taken at several timepoints during 7 days and activity measured in a well counter for defining the PK profile. For ex vivo BD, mice were sacrificed at several timepoints for determining the activity present in brain, heart, spleen, liver, lungs, medulla, bone, muscle and kidneys in an activity well counter. Data was treated and represented as %ID/g (% of administered dose per gram), %ID/mL (% of administered dose per milliliter) or SUV (Standardized Uptake Value) using Excel, Graphpad Prism v5 and SPSS suites.

5. RESULTS

5.1. CHARACTERIZATION OF B6 AND E11 ANTIBODIES

5.1.1. Stability

The p-Tau B6 mAb showed long-term stability at 4°C in comparison to the lyophilized antibody at -80 °C. On the other hand, ac-Tau E11 mAb has showed long term stability and lack of aggregation at -20 °C tested by the company Biotechpharma (Vilnius, Lithuania) (Figure 28).

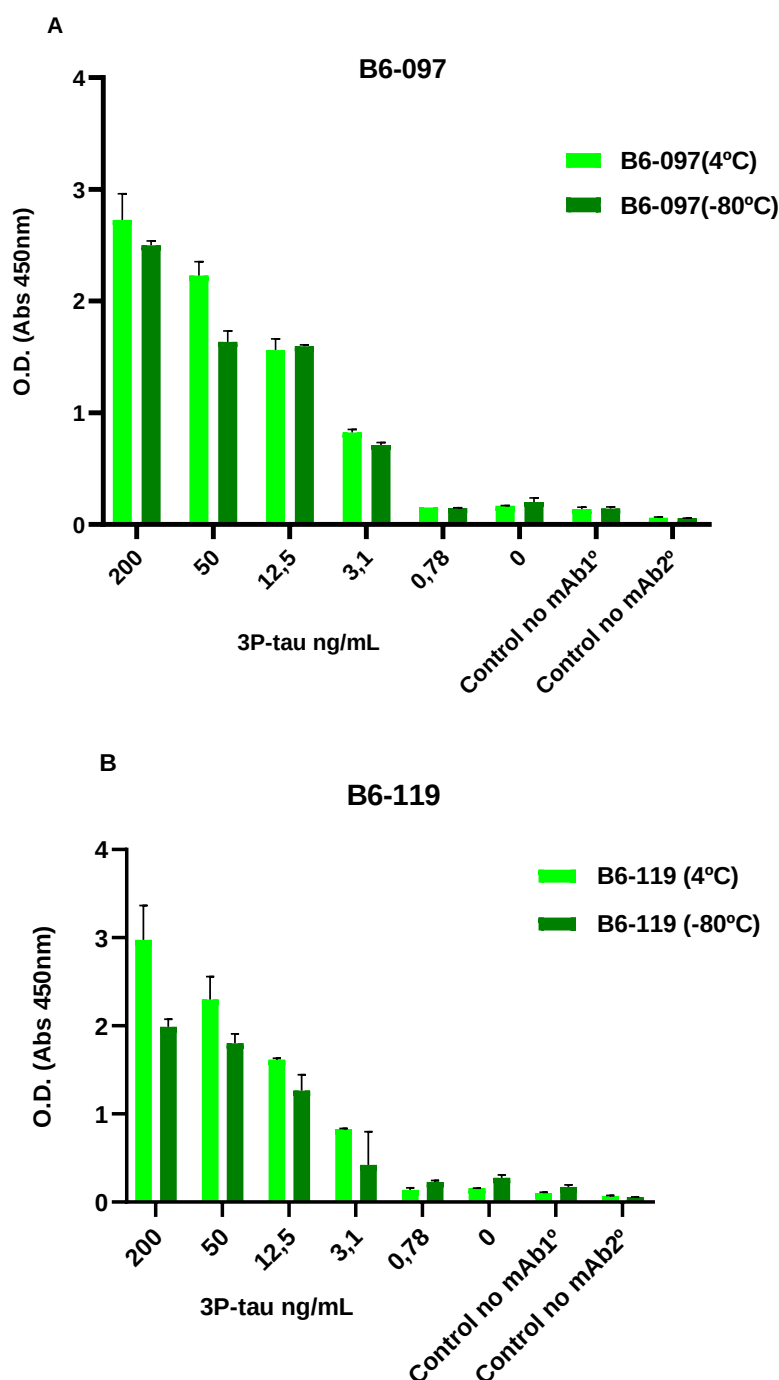


Figure 28. Indirect ELISA with B6 antibodies of different batches (A:097; B:119) tested lyophilized at -80°C vs not lyophilized at 4°C.

5.1.2. Epitope Mapping

5.1.2.1. Mini-array Epitope Mapping: Method 1

A graphical overview of the complete dataset obtained from the ELISA is given in **Figure 9**. Here a box plot depicts each dataset and indicates the average ELISA signal, the distribution, and the outliers within each dataset. Depending on experiment conditions (amount of antibody, blocking strength, etc.) different distributions of ELISA data were obtained (**Figure 29**). The specific results for each mAb B6 and E11 are showed below.

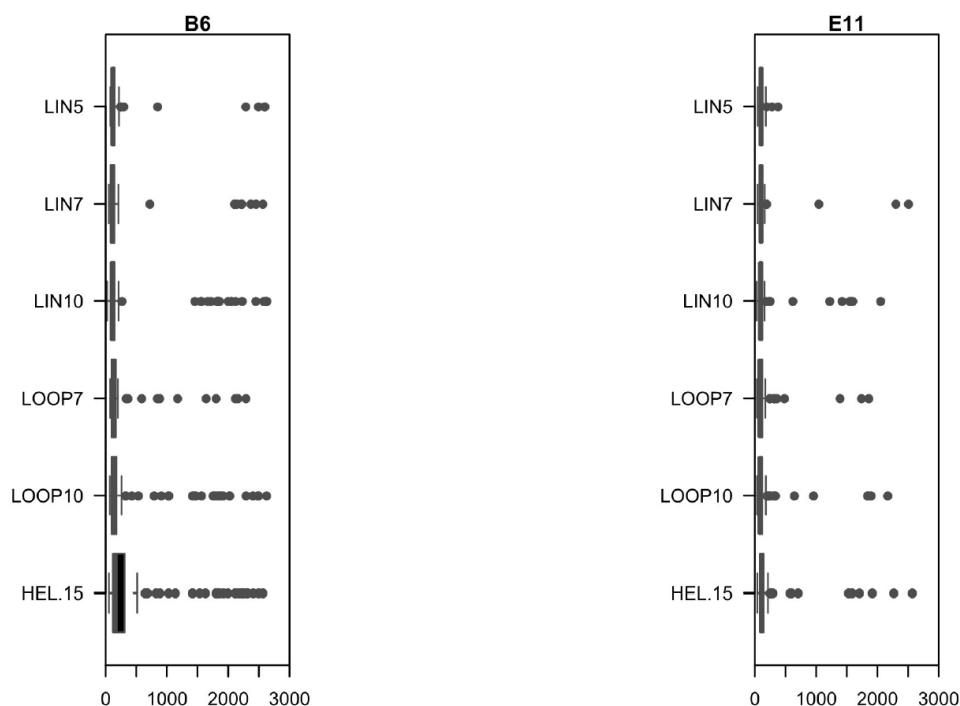


Figure 29. Box plot graphs of raw data of antibody screening. The bottom and top of the boxes are the 25th and 75th percentile of the data. The band near the middle of the box is the 50th percentile (the median). The whiskers are at 1.5 the inter-quartile range, an indication of statistical outliers within the dataset (*ref McGill et al. (1978) The American Statistician, 32: 12-16*).

5.1.2.1.1. B6 antibody

For the B6 antibody, a clear binding peak was found with linear and conformational mapping which focused on a region containing a single phosphorylation site. For the linear mapping, the linear 10-mer array showed a clear binding peak with multiple overlapping peptides. The conformational peptide array showed binding in similar regions (**Figure 30**). Within the different length loops and the helical mimics, similar regions are present. Binding was only observed with sequences which contained the common pS208-tau. Phosphorylation on other sites did not impact the specific binding for p-Tau B6 mAb. The overlapping peptides that showed binding are listed in **Figure30**. The core sequence was found to contain 207G(pS)R209.

Sample B6				
Type	Start	End	Epitope Candidate	value
LIN10	17	26	FG4RSRTPSL	2617
	11	20	PGSPGTPG4R	2611
	13	22	SPGTPG4RSR	2595
	14	23	PGTPG4RSRT	2565
	16	25	TPG4RSRTPS	2436
	15	24	GTPG4RSRTP	2214
	12	21	GSPGTPG4RS	1811
	18	27	G4RSRTPSLP	1548
LIN7	14	20	PGTPG4R	2551
	17	23	PG4RSRT	2441
	16	22	TPG4RSR	2356
	15	21	GTPG4RS	2093
	18	24	G4RSRTP	2089
LIN5	16	20	TFG4R	2585
	18	22	G4RSR	2480
	17	21	PG4RS	2273
LOOP10	13	22	CSPGTPG4RSRC	2612
	16	25	CTPG4RSRTPS	2481
	14	23	CPGTPG4RSRTC	2471
	15	24	CGTPG4RSRTPC	2392
	17	26	CPG4RSRTPSLC	2011
	11	20	CPGSPGTPG4RC	2007
	18	27	CG4RSRTPSLPC	1739
	12	21	CGSPGTPG4RSC	1549
LOOP7	16	22	CTPG4RSRC	2276
	17	23	CPG4RSRTC	2146
	14	20	CPGTPG4RC	2104
	18	24	CG4RSRTPC	1788
	15	21	CGTPG4RSC	1626
HEL.15	12	26	CSPGCPG4RSRTPSLP	2552
	11	25	CGSPCTPG4RSRTPSL	2482
	7	21	CYSSCGSPGTPG4RSR	2299
	13	27	CPGTCG4RSRTPSLPT	2201
	5	19	CSGYCSPGSPGTPG4R	2110
	9	23	CSPGCPGTPG4RSRTP	1908
	8	22	CSSPCSPGTPG4RSRT	1521

Figure 30. List of epitope candidates found for B6 mAb. The binding region, containing all overlapping peptides, is indicated by residue numbers. Peptides are listed by decreasing intensity. Peptides considered positive for recognition by B6 mAb were at least 2X the median value of the mimic type. Core epitopes (highlighted in yellow), are based on common sequences in overlapping peptides (overlapping sequences within 40% of the top peptide intensity in the peak).

5.1.2.1.2. E11 antibody

For ac-Tau E11 antibody, a clear binding peak was observed with linear and conformational mapping, which focused on a region including a single acetylation site. For this purpose, the full plots are shown for sequence 11 containing this site (sequences and numbering listed on **Figure29**). For the linear mapping, the linear 10-mer array showed a clear binding peak with multiple overlapping peptides. This is present also in the linear 7-mer peptide array. A minimal length appears to be required as linear 5-mers were not recognize properly. The conformational peptide array showed binding in similar regions. Within the different length loops and the helical mimics, similar regions are present. Binding was only observed with sequences which contained the common acetylation on Lys₁₇₄. Acetylation on Lys₁₆₃ did not impact the binding. The overlapping peptides that showed binding are listed in **Figure 31**. The core sequence was found to contain ₁₇₁IPA(acK)₁₇₄.

Sample E11					
Type	Start	End	Epitope Candidate	value	
LIN10	17	26	IPAA6TPPAPK	2039	
	15	24	TRIPA6TPPA	1583	
	16	25	RIPA6TPPAP	1551	
	12	21	ANATRIPA6T	1535	
	11	20	QANATRIPA6	1409	
	13	22	NATRIPA6TP	1209	
	14	23	ATRIPA6TPP	605	
LIN7	15	21	TRIPA6T	2494	
	14	20	ATRIPA6	2287	
	16	22	RIPA6TP	1029	
	13	19	NATRIPA	175	
	19	25	A6TPPAP	153	
LOOP10	15	24	CTRIIPA6TPPAC	2154	
	17	26	CIPA6TPPAPKC	1882	
	16	25	CRIPA6TPPAPC	1838	
	14	23	CATRIPA6TPPC	1825	
	12	21	CANATRIPA6TC	944	
LOOP7	15	21	CTRIIPA6TC	1845	
	16	22	CRIPA6TPC	1725	
	14	20	CATRIPA6C	1376	
HEL.15	10	24	CQANCTRIIPA6TPPAP	2555	
	9	23	CGQACATRIPA6TPPA	2253	
	11	25	CANACRIPA6TPPAPK	1902	
	7	21	CQKGCANATRIPA6TP	1573	
	6	20	CGQKCQANATRIPA6T	692	
	8	22	CKGQCANATRIPA6TPP	584	

Figure 31. List of epitope candidates found for E11mAb. The binding region, containing all overlapping peptides, is indicated by residue numbers. Peptides are listed by decreasing intensity. Peptides considered positive for recognition by E11 mAb were at least 2X the median value of the mimic type. Core epitopes (highlighted in yellow), are based on common sequences in overlapping peptides (overlapping sequences within 40% of the top peptide intensity in the peak)

5.1.2.2. Mini-array Epitope Mapping: Method 2

5.1.2.2.1. Linear mapping

The Linear Epitope Mapping Method 2 of mouse anti-actau E11 mAb was performed against MAPT and the acetyllysine containing ac-tau immunogen translated into linear 15 amino acid peptides with a peptide-peptide overlap of 14 amino acids. Pre-staining of a linear MAPT peptide microarray with the secondary antibody did not show any background interaction with the antigen-derived peptides that could interfere with the main assays. In contrast, incubation with mouse anti-actau E11 mAb highlighted a strong and clear mAb response against an epitope-like spot pattern formed by adjacent peptides with the consensus motif RIPA(acK)TP. The proposed epitope was located in the acetyllysine containing ac-tau immunogen. Since the corresponding lysine variant RIPA(K)TP did not show any response with mouse anti-actau E11 mAb, the acetyllysine has to be regarded as essential for antibody binding (**Figure 32**).

5.1.2.2.2. Conformational mapping

On the other hand, the Conformational Epitope Mapping Method 2 of mouse anti-actau E11 mAb was performed against MAPT and the acetyllysine containing ac-tau immunogen translated into cyclic constrained peptides with peptide lengths of 7, 10 and 13 amino acids and peptide-peptide overlaps of 6, 9 and 12 amino acids. Pre-staining of a conformational MAPT peptide microarray copy with the secondary antibody did not show any background interaction with the antigen-derived peptides that could interfere with the main assays (**Figure 33**). As with the linear epitope mapping, incubation of another conformational MAPT peptide microarray copy with mouse anti-actau E11 mAb highlighted a very strong and clear mAb

response against an epitope-like spot pattern formed by adjacent peptides with the consensus motif TRIPA (acK)TP with all three peptide lengths. The proposed epitope was located in the acetyllysine containing ac-tau immunogen. Again, since the corresponding lysine variant TRIPAKTP did not show any response with mouse anti-actau E11 mAb, the acetyllysine has to be regarded as essential for antibody binding.

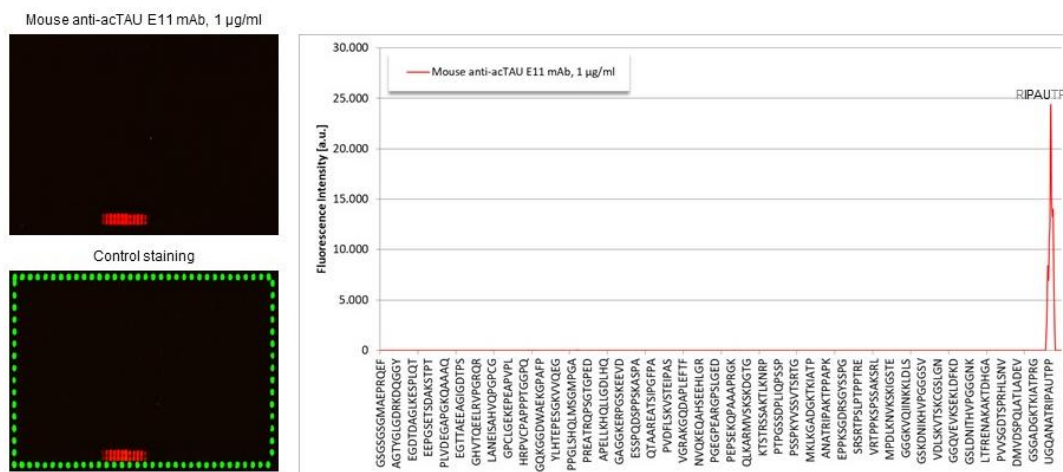


Figure 32. Pre-staining of a linear MAPT peptide microarray. Incubation of a linear MAPT peptide microarray copy with mouse anti-actau E11 mAb at a concentration of 1 µg/mL was followed by staining with secondary and control antibodies as well as read-out at scanning intensities of 7/7 (red/green). It was observed a strong and clear mAb response against an epitope-like spot pattern formed by adjacent peptides with the consensus motif RIPAUTP (acetyllysine was coded as U). Well-defined subsequent staining of HA control peptides in green.

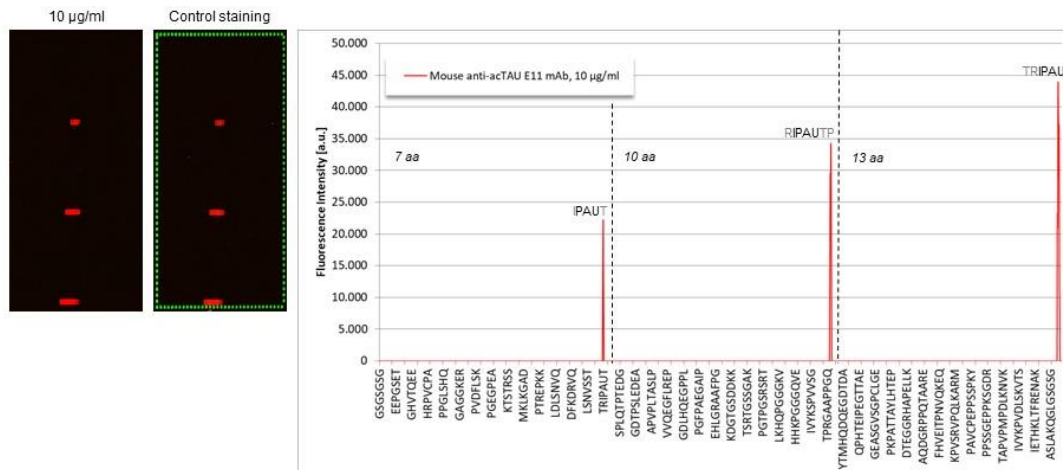


Figure 33. Pre-staining of a conformational MAPT peptide microarray. Incubation of a conformational MAPT peptide microarray copy with mouse anti-actau E11 mAb at a concentration of 10 µg/ml was followed by staining with secondary and control antibodies as well as read-out at scanning intensities of 7/7 (red/green). It was observed a very strong and clear mAb response against an epitope-like spot pattern formed by adjacent peptides with the consensus motif TRIPAUTP with all three peptide lengths (acetyllysine was coded as U). Well-defined subsequent staining of HA control peptides in green.

Both B6 and E11 antibodies showed clear binding signals in specific regions of the peptides in both epitope mapping experiments from Pepscan and PEPPERPRINT, confirming the robustness of the results.

The core sequence for B6 was $^{207}\text{G(pS)}\text{R}^{209}$, confirming the presence of the pS208-tau is essential for antibody binding. The phosphorylations in other sites did not affect in any way the binding of the B6 antibody.

For E11, a specific acetylation event on the lysine 174 was required for the binding, as well as a minimal peptide length. *The most constrained confirmed core sequence for E11 was ¹⁷¹IPA(acK)₁₇₄ based on different peptide mimics.* Data from PEPperPRINT both linear and conformational microarrays reported a slightly longer binding core sequence (RIPA(acK)TP and TRIPA(acK)TP, respectively) than the sequence from Pepscan. However, the only overlapping sequence among all positive signals were the ¹⁷¹IPA(acK)₁₇₄ motif.

Very similar affinity values were obtained indistinctively from linear and constrained peptides (loops, helical mimics) containing the same sequences in both experiments, which may suggest that antibodies bind a peptidic sequence, no matter the spatial conformation whatsoever.

5.1.3. Specificity

5.1.3.1. Cross reactivity test of E11

Because there are no commercial antibodies specific for Tau acetylation recognized by the ac-Tau E11 mAb, we performed a cross-reactivity study. Incubation of a HuProt™ Human Proteome Microarray with mouse anti-actau E11 mAb highlighted only weak interactions with multiple proteins at low to moderate signal-to-noise ratios. The top interactions were assigned to proteins CASC4 (cancer susceptibility 4), IGHG3 (immunoglobulin heavy constant gamma 3), GNAQ (G protein subunit alpha Q), CTNS (cystinosis, lysosomal cystine transporter), RBBP7 (Rb binding protein 7), GPR89A (G protein-coupled receptor 89A), IGHG1 (immunoglobulin heavy constant gamma 1), PHAX (phosphorylated adaptor for RNA export), SCGB1D1 (secretoglobin family 1D member 1) and ABCB6 (ATP binding cassette subfamily B member 6). In addition, we observed weak to moderate interactions with some of the human IgG control proteins (**Table 18**).

Table 18. Summary of the E11 cross-reacting proteins identified with the HuProt microarray chip.

CASC4	Cancer susceptibility 4
IGHG3	Immunoglobulin heavy constant gamma 3
GNAQ	G protein subunit alpha Q
CTNS	Cystinosis, lysosomal cystine transporter
RBBP7	Rb binding protein 7
GPR89A	G protein-coupled receptor 89A
IGHG1	Immunoglobulin heavy constant gamma 1
PHAX	Phosphorylated adaptor for RNA export
SCGB1D1	Secretoglobin family 1D member 1
ABCB6	Atp binding cassette subfamily B member 6

A simple text search of the identified proteins in the HuProt Microarray in the raw data reported by the MS/MS analysis allowed us to identify the presence or absence of each protein. The comparison of the data of the different results did not identify any commonalities between the two data sets from the HuProt microarray and the MS/MS analysis besides the IGHG1 (immunoglobulin heavy constant gamma 1), which is one of the components of the own E11 antibody.

The cross-reactivity test performed with the E11 antibody strongly indicated a low cross-reactivity against the >20,000 human proteins of the microarray. Compared to other mAbs

tested that way, the mouse mAb seemed to exhibit the clearly weakest overall response profile and hence cross-reactivity.

The B6 antibody has been shown to work well and detect band patterns in WB of animal models and human brain samples, indicating Braak stage-related bands in a similar way to the well-known AT8 antibody. Also, its functionality in immunofluorescence (IF) and ELISA has been confirmed with both biological samples and synthetic peptides.

5.1.4. Functionality

5.1.4.1. ELISA

Biotinylated antibodies were used for detecting coated peptides in Maxisorp plates. The B6 and E11 mAb were compared to the commercial BT2, HT7 and Tau5 antibodies with a concentration of 5 µg/mL (**Figure 34**).

High absorbance values were observed for the most of the biotinylated antibodies according to the binding site for each peptide. Lower values were observed with B6-Biotin antibody, where 0.1 µg/mL of p-tauL was not detected. The only antibody that showed high non-specific signal was Tau5-Biotin (0,46 AU). B6-Biotin and E11-Biotin antibodies showed scarce non-specific binding; however, the higher absorbance values correspond to specific recognizing for each case.

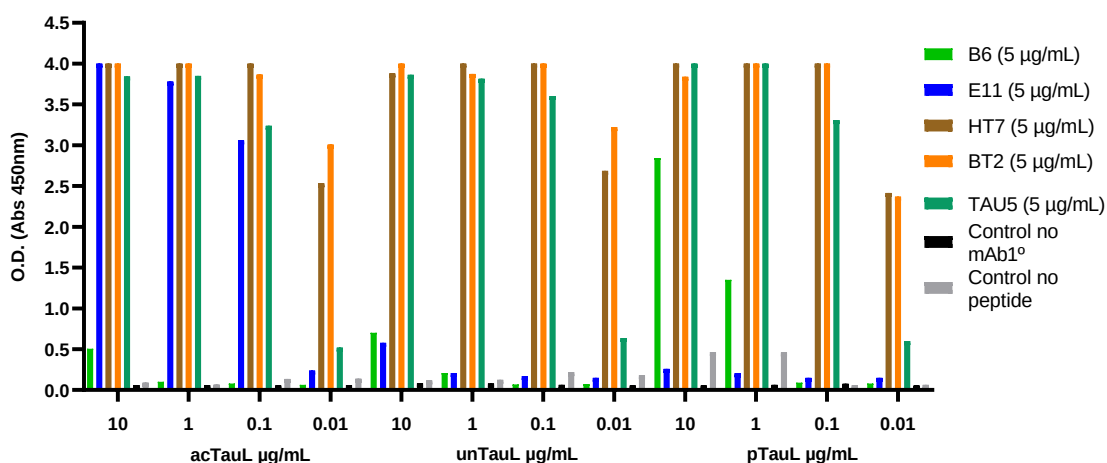


Figure 34. Indirect ELISA confirmation of biotinylated antibodies functionality. B6-Biotin, E11-Biotin and Tau5-Biotin were customized biotinylated by Immunostep SL (Spain). HT7-Biotin and BT2-Biotin were commercial antibodies. p-tauL, ac-tauL and un-tauL peptides were recognized by HT7-Biotin, BT2-Biotin and Tau5-Biotin antibodies. B6-Biotin and E11-Biotin antibodies were specific for p-tauL and ac-tauL, respectively. Bars reaching 4 units, exceeded the spectrometer maximum limit of detection.

5.1.4.2. Western Blot

Analysis of commercially available antibodies for p-Tau (AT8) and total Tau (Tau 4R and Tau5) (**Figure 35**) revealed the expected increase in the high molecular weight (HMW) representation of SDS-resistant p-tau oligomers (50 kDa) in WB with increasing AD-related neurofibrillary pathology in the total protein samples extracted from the human temporal cortex (**Figure 35A**). Consistent with this result, the p-tau B6 mAb showed a similar increase in the HMW representation of SDS-resistant p-Tau oligomers (50 kDa) and dimers (100 kDa) in WB with increasing AD-related neurofibrillary pathology (increased levels of Braak Stage) in the total protein lysates extracted from the human temporal cortex (**Figure 35B**). Importantly, the detection quality of p-tau oligomers in WB analysis was improved with the B6 antibody as compared to the AT8 one.

Moreover, the WB analysis of ac-tau (E11/F1) mAb showed the appearance of a prominent ~50kDa band (**Figure 36**), which decreased in relation to increased AD-associated neurofibrillary pathology in the total protein samples extracted from the human temporal cortex. In parallel, a ~100 kDa band appeared in the Braak stage IV, representing potentially the dimeric SDS-resistant ac-Tau form. Further analyses, such as MS-based analyses are needed to confirm whether the ~50 and ~100 kDa bands are mono and dimer forms of ac-tau.

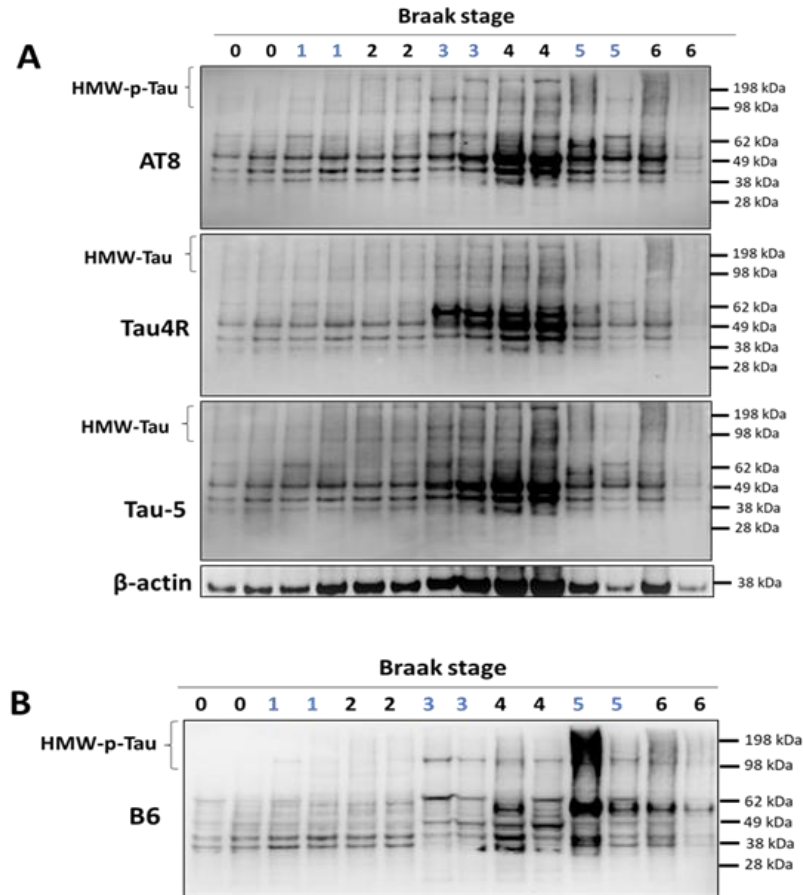


Figure 35. WB analysis of human temporal cortex samples classified by Braak Stage for p-Tau B6 (**B**) mAb compared with other commercial antibodies such as AT8, Tau4R and Tau5 (**A**). WB analysis showed that B6 mAb is able to detect both tau oligomers (50 kDa) and dimers (100 kDa).

5.1.4.3. Immunohistochemistry/Immunofluorescence

In addition, IHC analysis of paraffin-embedded tissue samples from the human temporal cortex (the same samples used for WB) revealed the expected staining characteristics with B6 p-tau antibody with respect to increased AD-associated neurofibrillary pathology (**Figure 37**). More specifically, B6 antibody stained the amyloid plaques, PHFs and NFTs as expected in the more advanced Braak stages IV-VI alongside with the activated microglia (IBA1 staining). As expected, there was no p-tau staining detected in the Braak stage 0 with B6 antibody.

IF stainings of hTau.P301S mice confirmed the functionality in this technique of the B6 antibody, reporting similar staining pattern than the AT8 antibody (**Figure 38**). The E11 antibody was not tested since there is no compatible animal model expressing acetylated tau.

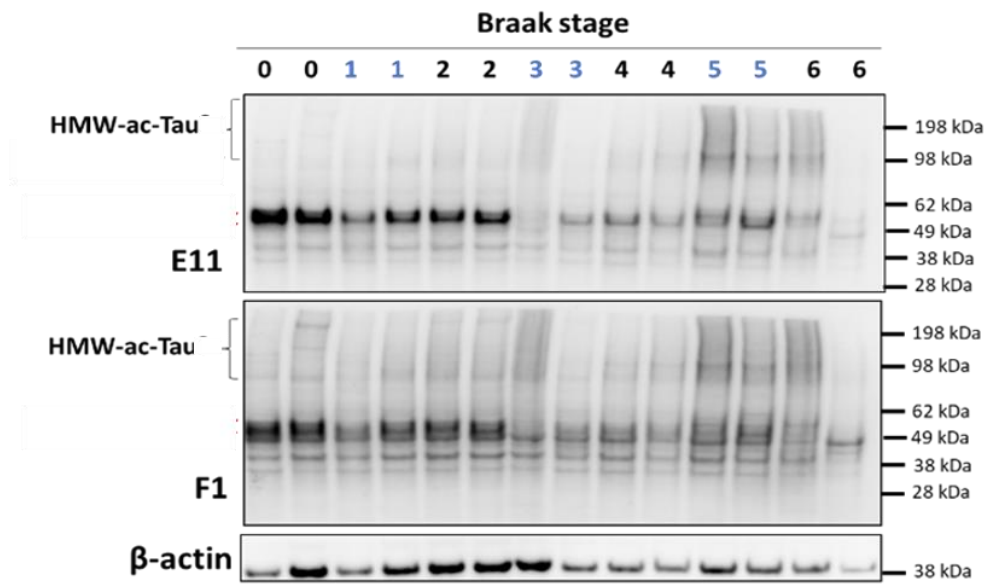


Figure 36. WB analysis of ac-tau (E11/F1) mAb in total protein samples extracted from human temporal cortex with respect to Braak staging. Both ac-tau (E11/F1) mAb showed the appearance of a prominent ~50kDa band (Tau oligomers) at very early Braak Stages (I &II).

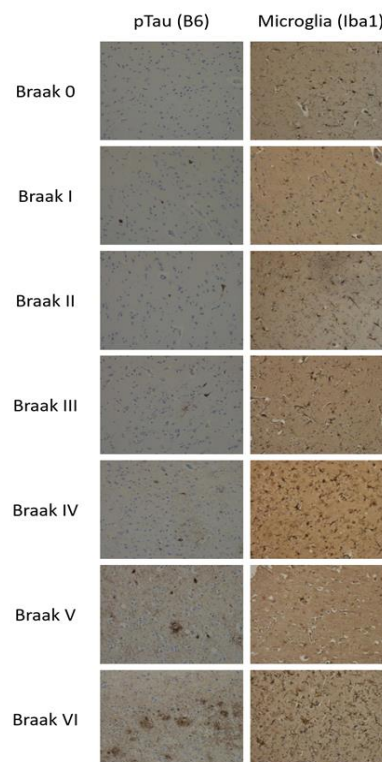


Figure 37. IHC analysis of human temporal cortex samples with B6 and Iba1 (microglia-specific) antibodies showing increased levels of p-tau and microglia as the Braak stage becomes more advanced.

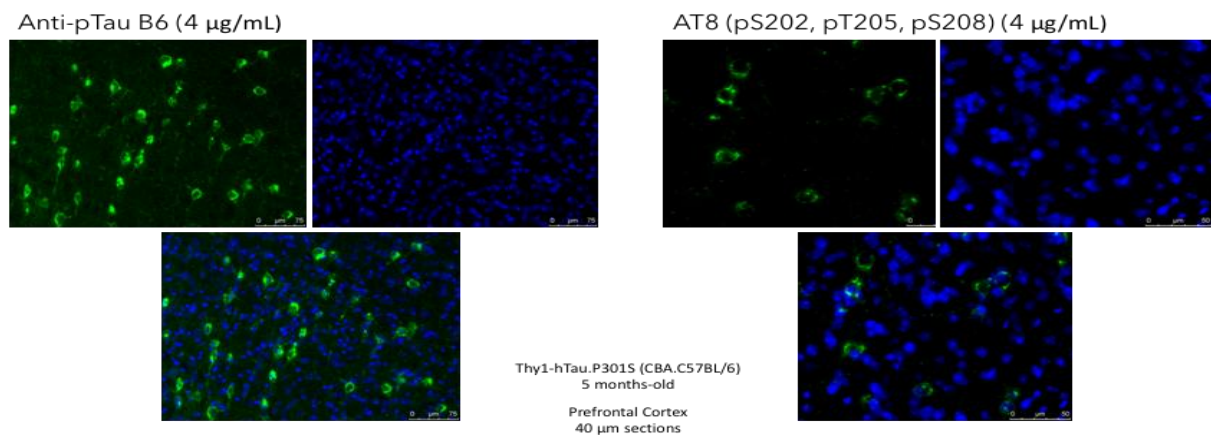


Figure 38. IF images of prefrontal cortex of hTau.P301S mice stained with B6 and AT8 for comparison. Green channel: p-tau. Blue Channel: DAPI-labeled Nuclei. Results showed that B6 mAB detects brain p-Tau deposits better than the AT8 antibody.

5.1.5. Toxicity of anti-ptau B6 antibody

5.1.5.1. Toxicity *in vitro*

Primary neurons were incubated for 24 hours with the concentration of anti-ptau contained in 10 (0.03 µg p-tau B6 antibody) and 100 (0.3 µg p-tau B6 antibody) µg /mL of p-Tau. β -amyloid was used as positive control of neuronal apoptosis through caspase-3 activation. *In vitro* experiments have shown that β -amyloid and its active fragment 25-35 (A β 25-35) possess direct neurotoxic effects. We found that the anti-ptau B6 antibody was not toxic for neurons, because there was no significant neuronal apoptosis as revealed by caspase-3 activity determination (**Figure 39**).

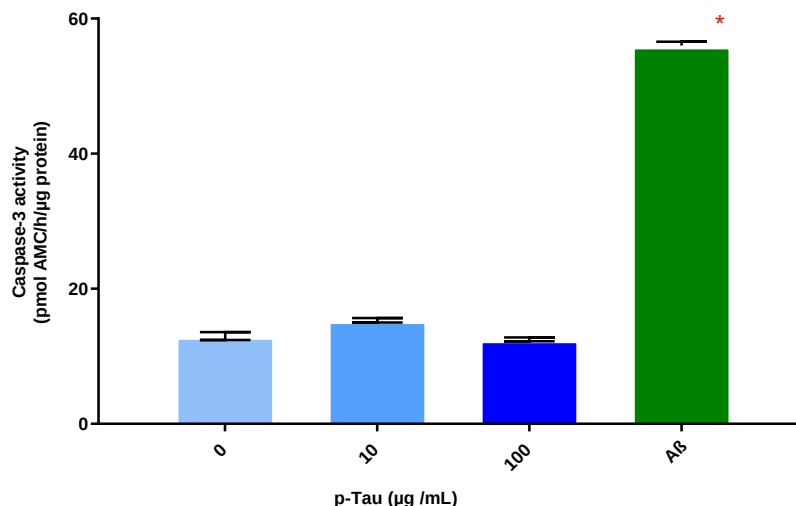


Figure 39. Toxicity of anti-ptau B6 antibody measured by caspase 3 activity showing no toxic effects in cultures of primary neurons. β -amyloid (A β) was used as positive control.

5.1.5.2. Toxicity in vivo

5.1.5.2.1. Anatomopathological evaluation of liver and kidney samples

Qualitative evaluation of haematoxylin/eosin staining revealed no structural abnormalities in the kidney and liver tissue samples (**Figure 40A**). Quantitative assessment of tissue fibrosis using Fast Green/Sirius Red staining, showed similar levels of collagen fibres in both control and treated animals, revealed by the Sirius Red staining area (**Figure 40B**).

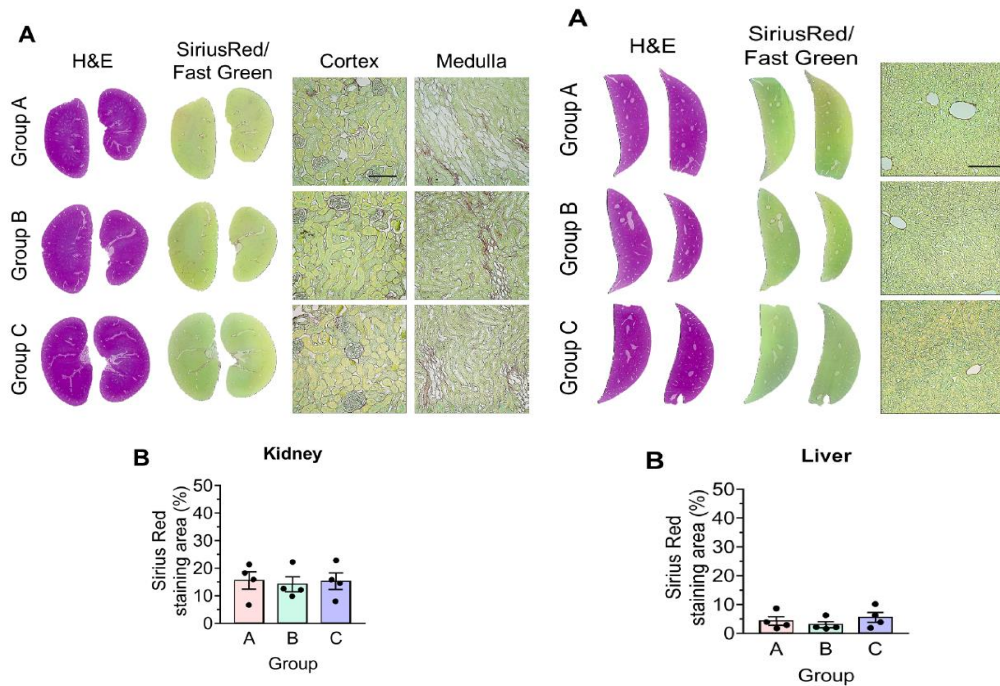


Figure 40. Anatomopathological evaluation of liver and kidney samples. A. 3 μ m-thick paraffin liver sections from Tau.P301S mice treated with anti-ptau B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C) were stained with both haematoxylin/eosin and fast green/Sirius red. Representative images are shown. Scale bar 100 μ m. B. Quantitative assessment of tissue fibrosis using Fast Green/Sirius Red staining was determined using QuPath-imageJ software. Values were expressed as Sirius red staining area percentage and are mean $\pm$ SEM. $p < 0.05$ versus control (one-way ANOVA followed by the Tukey post hoc test; $n = 4$ animals per group).

5.1.5.2.2. Cell death analysis

Determination of active caspase-3 staining revealed that the immunotherapy with p-Tau B6 mAb during 4 months was not toxic in both kidney and liver tissue samples, as revealed the absence of cleaved caspase-3 positive cells (**Figure 41A and B**).

Determination of cellular apoptosis by TUNEL assay revealed that the immunotherapy was not toxic for in both kidney and liver tissue samples, revealed by the absence of TUNEL positive cells (**Figure 42A and B**).

The safety of immunotherapy was confirmed in both kidney and liver tissue samples from 3 different mice treated intraperitoneally with anti-ptau B6 antibody or IgG1 antibody for 4 months, as revealed the absence of cleaved caspase-3 (**Figures 43 and 44**) and TUNEL (**Figures 45 and 46**) positive cells in all samples.

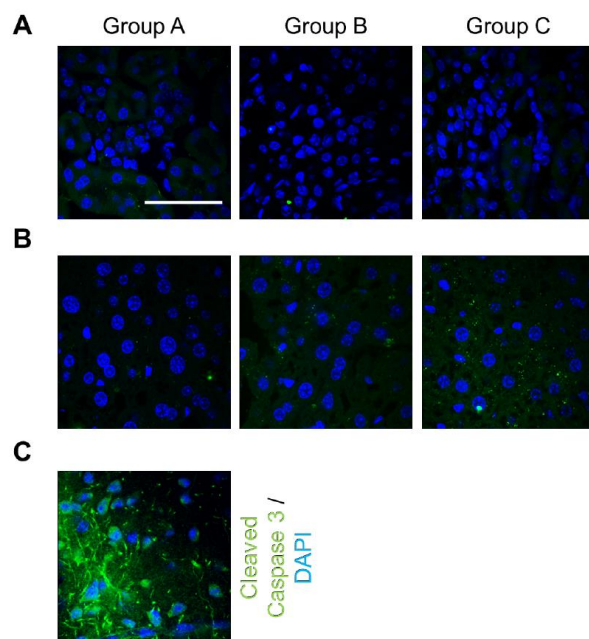


Figure 41. Apoptosis by cleaved caspase-3 determination. Representative images from 5 μm -thick. A. kidney and B. liver frozen sections from Tau.P301S mice treated with anti-ptau B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C) were immunostained with anti-Cleaved Caspase-3 (apoptosis marker, green) and DAPI (nuclear marker; blue). C. 5 μm -thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control. Scale bar 50 μm .

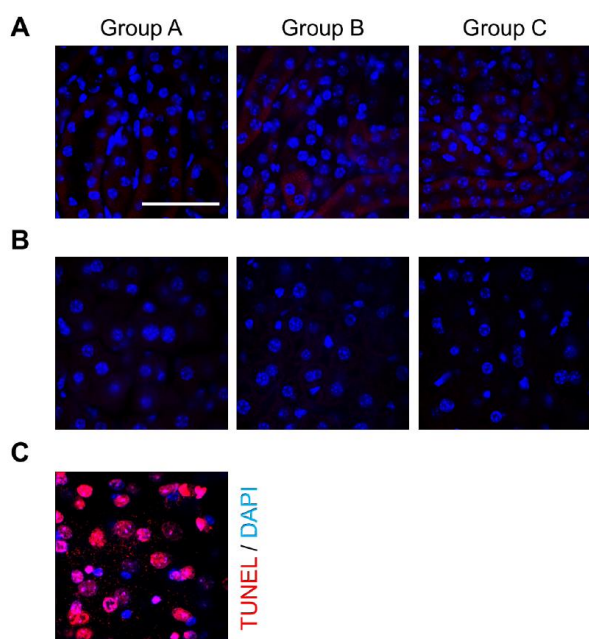


Figure 42. Apoptosis by TUNEL assay. Representative images from 20 μm -thick. A. kidney and B. liver frozen sections from Tau.P301S mice treated with anti-ptau B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C) were labelled with biotin conjugated dUTPs (DNAs break marker, red) and DAPI (nuclear marker; blue). C. 20 μm -thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control of toxicity (TUNEL positive cells, red staining). Scale bar 50 μm .

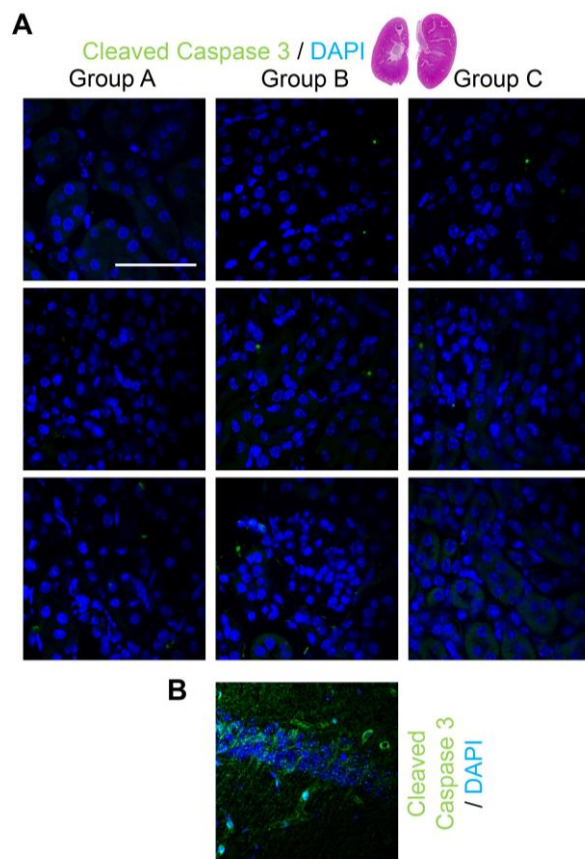


Figure 43. Confirmation of immunotherapy safety by using cleaved caspase-3 determination in kidney samples. Representative images from 5 μm -thick kidney. A. frozen sections from Tau.P301S mice treated with anti-ptau B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C). Sections from 3 different mice per condition were immunostained with anti-Cleaved Caspase-3 (apoptosis marker, green) and DAPI (nuclear marker; blue). B. 5 μm -thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control. Scale bar 50 μm .

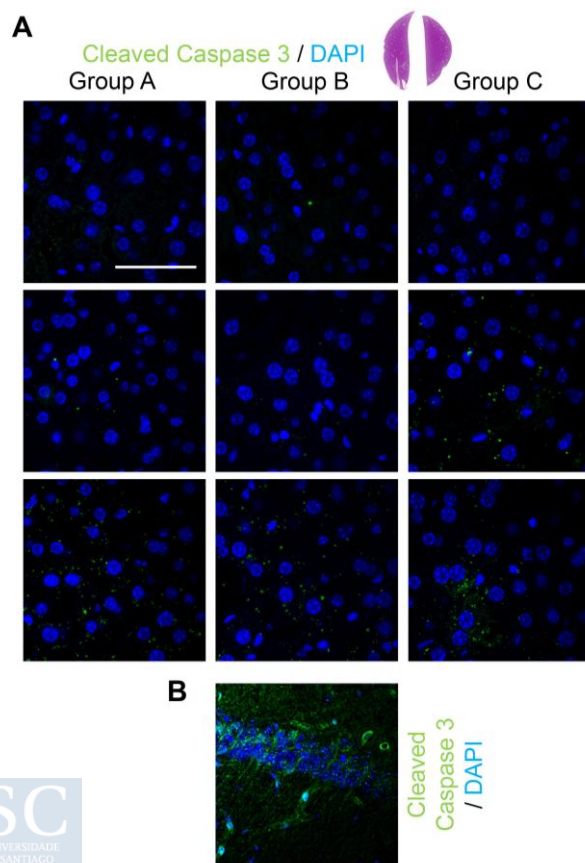


Figure 44. Confirmation of immunotherapy safety by using cleaved caspase-3 determination in liver samples. Representative images from 5 μm -thick liver A. frozen sections from Tau.P301S mice treated with anti-ptau B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C). Sections from 3 different mice per condition were immunostained with anti-Cleaved Caspase-3 (apoptosis marker, green) and DAPI (nuclear marker; blue). B. 5 μm -thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control. Scale bar 50 μm .

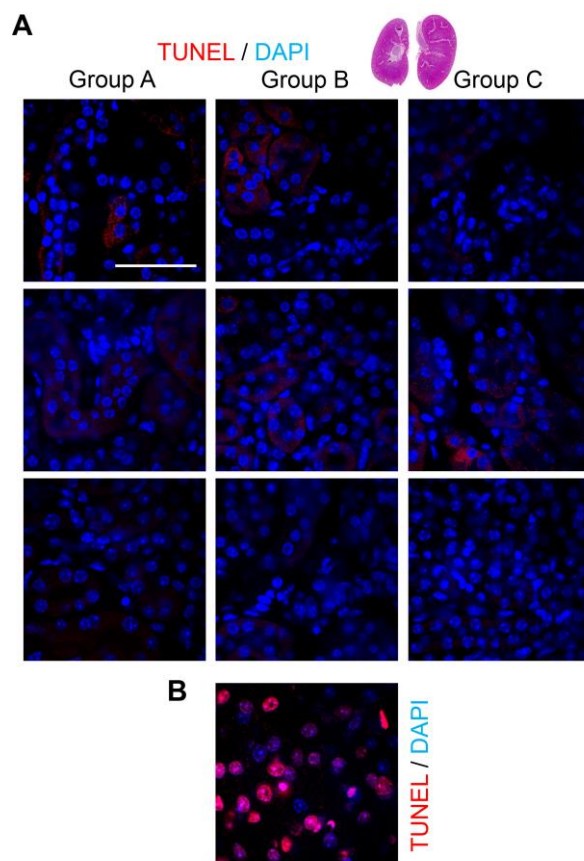


Figure 45. Confirmation of immunotherapy safety by using TUNEL assay in kidney samples. Representative images from 20 μ m-thick kidney. A. frozen sections from Tau.P301S mice treated with anti-PTAU B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C). Sections from 3 different mice per condition were labelled with biotin conjugated dUTPs (DNAs break marker, red) and DAPI (nuclear marker; blue). B. 20 μ m-thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control of toxicity (TUNEL positive cells, red staining). Scale bar 50 μ m.

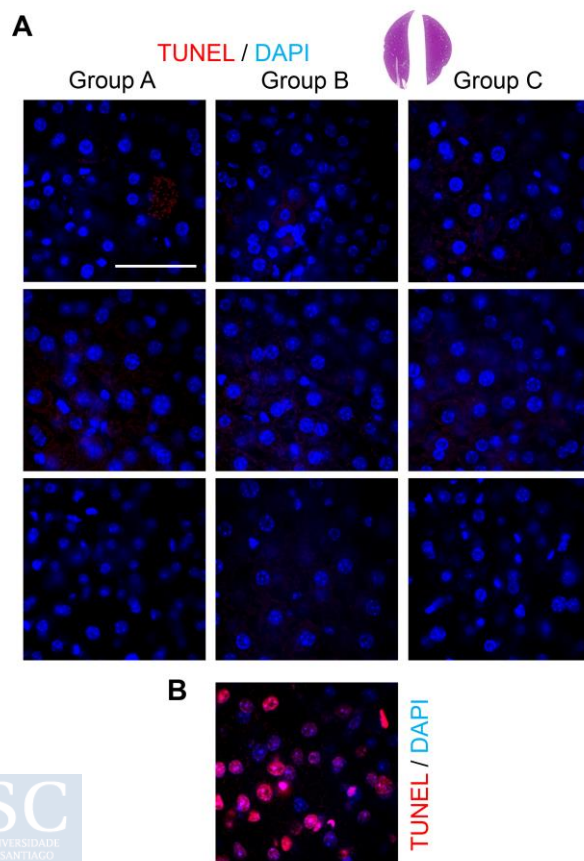


Figure 46. Confirmation of immunotherapy safety by using TUNEL assay in liver samples. Representative images from 20 μ m-thick liver. A. frozen sections from Tau.P301S mice treated with anti-PTAU B6 (group A), IgG1 antibody (group B) and wild-type mice treated with IgG1 antibody (group C). Sections from 3 different mice per condition were labelled with biotin conjugated dUTPs (DNAs break marker, red) and DAPI (nuclear marker; blue). B. 20 μ m-thick frozen sections from amyloid- β intracerebral injected mouse brain were used as positive control of toxicity (TUNEL positive cells, red staining). Scale bar 50 μ m.

5.2. DEVELOPMENT OF AN INNOVATIVE IN-VITRO DIAGNOSTIC KIT BASED ON ELISA

5.2.1. Selection of conditions

The summary of the selection of each condition in each step of the final setup of the Sandwich ELISA is summarized in **Table 19**.

Table 19. Summary of the conditions for the final setup of the ELISAs.

	Description	Impact	Additional comments
ELISA technique	Two different approaches were addressed for finding the best ELISA technique to use in our assay: Competitive and sandwich. The decision was to move ahead with sandwich technique, which is the most used and the simplest system, considering the levels of sensitivity we need to reach, and the potentially clinical use of the test.	The decision for one or another way to configure our ELISA test is something extremely basic and important, which determines all the components of the assay as well as the protocol to be followed.	Nothing to address in this regard
Plate selection	4 types of 96-well ELISA plates from two manufacturers were tested: Nunc Polysorp, Nunc MediSorp, Nunc MaxiSorp and Greiner Microton High Binding.	After testing the signal-to-noise ratio for each plate, the decision was to continue with the Nunc MaxiSorp. We need high sensitivity and CSF samples do not give so much background noise as serum or blood samples, thus a plate specially meant for reducing background noise does not seem necessary.	Nothing to address in this regard
Blocking selection	Several formulations using different concentrations of BSA, milk, fish gelatin and Ultrasorb were considered and tested. BSA, fish gelatin and Ultrasorb provided the best signal/noise ratio. Protein-Free (TBS) Blocking Buffer (Thermo Fisher Scientific 37570) was also tested and used as main blocking agent.	The commercial Protein-free Blocking buffer was chosen as the main solution. However, due to its good performance, the 3% BSA was the chosen solution for special cases, such functionalized NPs.	Nothing to address in this regard
p-Tau kit development status	The p-tau detection kit is currently formed by a B6-Pair 2. The detection limit reached so far varied from 50 to 200 ng/mL depending on the assay conditions.	The detection limit reached is quite poor, however, an issue with the p-tauL synthetic peptides was detected and values may vary significantly.	The use of nanoparticles could help to improve detection limit significantly. In indirect ELISA schemes, up to 25.6 pg/ml of p-tauL have been detected.
ac-Tau kit development status	The ac-tau detection kit is currently formed by a sandwich scheme with E11 and Pair2+3. The best detection limit reached with colorimetric reagent (1-Step UltraTMB) has been 300-500 pg/mL. With a luminescent reagent (ELISA SuperSignal™ Femto Thermo Fisher Scientific 37075) the LOD was reduced to around 10 pg/mL.	The LOD reached with the current scheme is sufficient to start CSF validation.	
Biological samples testing	A number of CSF human samples from SERGAS Biobank have been used along the development process, and validation purposes. Significant positive signals were detected for several CSF samples.	The identified positive samples were allowed to determine and compare the biomarker profile of each sample and potentially improve the diagnostic process.	

5.2.2. Plate selection

For the plate selection process, different types of plates were selected according to the type of biomolecule to be detected in the Elisa assay. For lipid detection they recommended the Nunc Polysorp plate type, Nunc Medisorp proteins and for antibodies two types of plates such as Nunc Maxisorp and Microton High Binding. **Table 20** shows the different surface plates tested within the development of the anti-ptau/actau ELISA kit.

First, we tested the PolySorp plates and noticed how the blank values were much higher than expected (O.D. (Abs450nm) = 0.4). On the other hand, we detected that most of the actau and tau groups did not show differences from the blank suggesting a binding problem between the trapping antibodies and peptide samples. Based on these results, MediSorp microplate was chosen for the next experiments, since it was the one with a wider range respect the blank (**Figure 47**). After some assays, and under recommendation by the manufacturer, the MediSorp plates were substituted by MaxiSorp plates, which use the same coating material, but with an improved absorption.

Table 20. Different surface plates tested within the development of the anti-ptau/actau ELISA kit.

Biomolecules	Molecule classification	Recommended plate surface	Key surface features
Lipids	Hydrophobic	Nunc PolySorp	These surfaces are typically used for the adsorption of hydrophobic molecules such as lipid-rich biomolecules.
Proteins	Slightly hydrophilic	Nunc MediSorp	The slight hydrophilicity of these plates enhances their ability to bind a diverse range of biomolecules, including glycoproteins, serum-containing samples, and amphoteric molecules such as lipopolysaccharides. With these plates non-specific adsorption of serum-containing samples are reduced and this was improved the signal-to-noise (S/N) ratio and consequently, sensitivity levels.
Antibodies (glycoproteins)	Hydrophilic	Nunc MaxiSorp	Optimized to bind high amounts of IgG (polyclonal), these plates are ideal for antibody sandwich assays (e.g., ELISAs). In addition, they show increased binding of many other proteins and biomolecules that possess hydrophilic/hydrophobic characteristics.
Antibodies	Hydrophilic	Greiner Microton High Binding	High binding plates are recommended for ELISAs. High binding denotes a binding capacity of 600 ng/cm ² and scatter of less than 10%. Optimal protein binding is variable and comparative tests between high and medium binding plates is suggested.

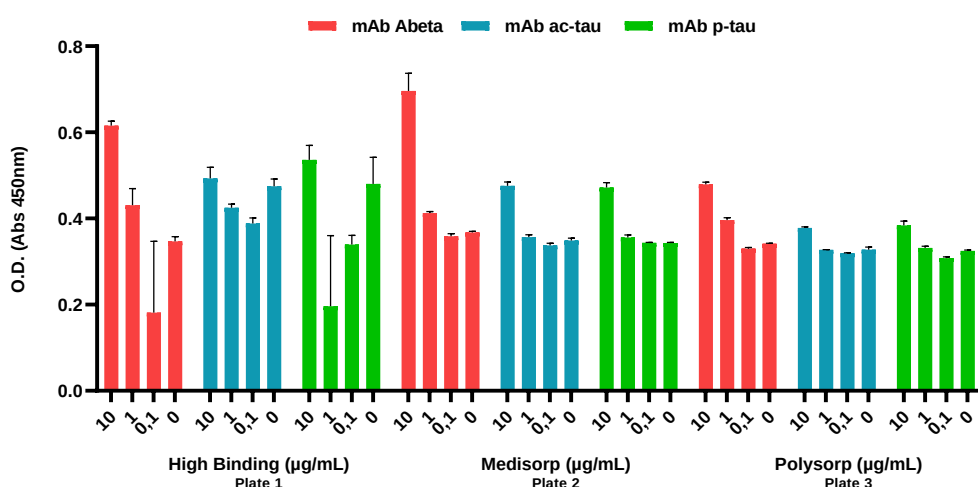


Figure 47. Microplate selection. Different microplates (Microton High Binding, MediSorp, Polysorp) and different coating antibody concentrations. It shows the difference between every sample and the respective blank. Orange for Aβ difference from Aβ blank and grey for the difference between TAU samples and blank.

5.2.3. Antibodies pairing

5.2.3.1. Antibodies-peptides compatibility assessment

The p-Tau B6 mAb was raised against a specific peptidic sequence within the proline-rich domain of the tau protein, including several phosphorylated residues. The specificity B6 antibody for the candidate phosphorylations was confirmed by indirect ELISA against serial concentrations of synthetic peptides including all possible targeted phosphorylations: one “native” peptide, three peptides wearing the three individual phosphorylations in serine 202, threonine 205, and serine 208, and two more peptides with different lengths wearing the three 202, 205 and 208 phosphorylations (*for details, see Table 8*).

The results showed that the 3 phosphorylations (S202, T205, and S208) conditioned the B6 binding because the strong binding is reached with pS208, pT205, and pS208-tau. Results confirmed the necessity of the phosphorylation in serine 208 for antibody binding (**Figure 49A**). However, the presence of the additional two phosphorylations (202 and 205) seemed to contribute to a higher binding affinity, especially in the indirect ELISA setup (**Figure 49B**). The differences observed between the indirect and sandwich performance of the B6 with pS208 and 3P-tau (188-217) peptides are represented in detail in **Figure 48**. Thus, the new 3P-tau peptide was used to confirm the calibration curves in the upcoming ELISA assays with CSF samples.

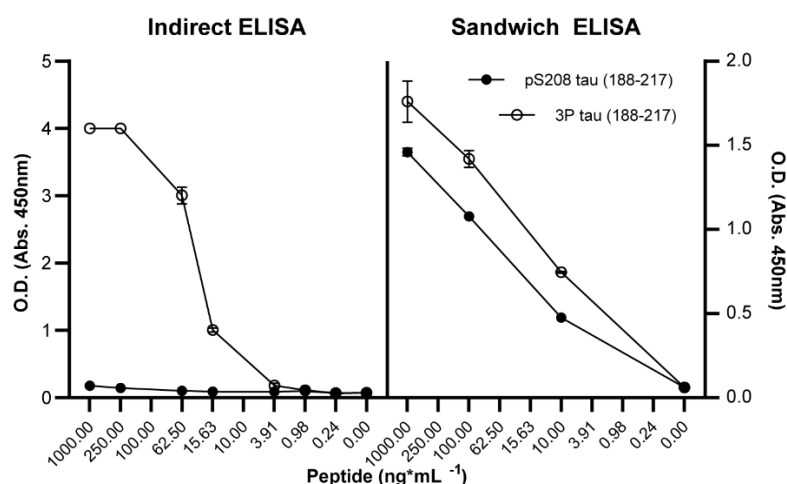


Figure 48. Direct comparison between indirect and sandwich (with BT2 as detection antibody) performance with B6 mAb and pS208 and 3P-tau (188-217) synthetic peptides. The best setup was Sandwich ELISA by using the 3P-tau peptide for calibration curves.

The E11 antibody was also raised against the proline-rich domain of tau protein, in this case including two acetylated residues, lysine 163 and lysine 174. For confirming specificity, E11 antibody was also tested against the two separate candidate tau acetylations. Synthetic peptides wearing single and double acetylations in lysine residues 163 and 174, and control peptides without any acetylation were coated in microtiter plates and incubated with the E11 antibody (*for details, see Table 8*). Results confirmed that the presence of the acetylation in lysine 174 is necessary for antibody recognition. The acetylation in lysine 163 does not affect the binding of the antibody whatsoever (**Figure 49C**).

Non-specific signals detected with both the commercial blocking buffer (BB) and BSA 3% were similar or lower than in previous assays (37°C vs 25°C incubation), but BB was used as the default blocking agent for the rest of the plate. Background signal still has some room for optimization. Other negative controls for both 1st antibodies and the StvPolyHRP remained within the expected values (<0.2) (**Table 21**).

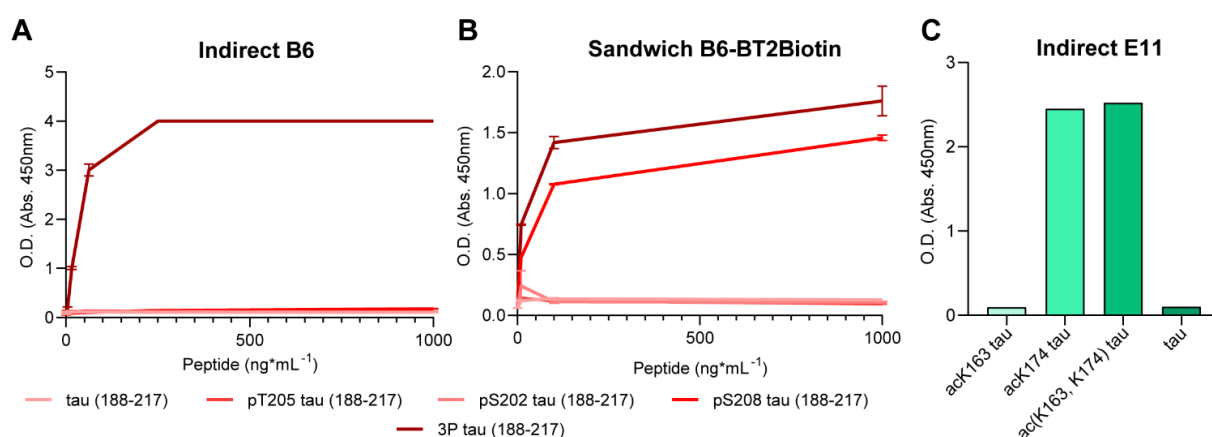


Figure 49. Titration of the B6 antibody in a colorimetric sandwich (**A**) and indirect (**B**) ELISAs against coated synthetic peptides including the different candidate phosphorylations. Concentrations of coated peptide range from 1000 ng mL⁻¹ to 6,1 pg mL⁻¹ in the indirect assay and from 1000 to 10 ng mL⁻¹ in the sandwich one. B6 antibody was used at a 1000 ng mL⁻¹ and the anti-mouse-HRP secondary antibody at 1:2000 dilution. 3P-tau carries the three phosphorylations: p(S202, T205, S208). **C.** Titration of the E11 antibody in indirect ELISA against coated synthetic peptides including the candidate acetylated residues. Concentration of coated peptide at 1000 ng mL⁻¹. E11 antibody was used at a 1000 ng mL⁻¹. The anti-mouse-HRP secondary antibody was used at 1:2000 dilution.

Table 21. Negative Controls without peptide (Abs 450nm).

Control without peptide. Average O.D. (Abs 450nm)			Control without peptide. Average O.D.(Abs nm)	
	BSA 3%	BB	BT2	0,1585
E11	0,3465	0,255	BT2+HT7	0,20075
B6	0,323	0,3535	StvPoly-HRP	0,0755
	Cut off (3SD)	0,258		

5.2.3.2. Antibody pair combination

Combinations between B6, HT7, BT2, Tau5, and Tau1 antibodies were tested, but only the B6-BT2 seemed to be compatible and it was being optimized with -HRP conjugates. This setup was not improved by other combinations where the reached LOD and the non-specific signal were higher when using other antibodies such as HT7 or the biotinylated B6. This fact makes it difficult to find alternatives to improve the performance of the current setups. LOD reached for the different pairs for p-Tau B6 mAb were summarized in **Table 22**.

On the other hand, several combinations of E11, BT2, HT7, Tau5, and Tau1 antibodies were tested. More of the pairs for ac-Tau E11 mAb were compatible and capable of considerably low LOD, except Tau5 and Tau1 that were not compatible with E11 (*data not shown*). LOD reached for the different pairs for ac-Tau E11 mAb were summarized in **Table 22**.

In conclusion, the best pair selected for the detection of the peptide ac-tauL were the E11 antibody as the trapping antibody and the pair HT7biotin+BT2Biotin as the detection antibody. The B6 antibody is a trapping antibody and the BT2Biotin as detection antibody for the detection of p-tauL. The secondary antibody which reached the best signal for both pairs of antibodies was the Streptavidin Poly-HRP with conjugate stabilizer (SDT Reagents, Germany).

Table 22. A summary of LODs for the different ELISAs, antibody pairs and conditions. The final reagents selected for the ELISA are shown in bold.

Antibody pair combination	Values (LOD)
B6	
B6 Biotin (direct)	LOD 1,000-100,000 pg/mL. Low signal.
B6-BT2Biotin	LOD <62,5 pg/mL. Low signal.
BT2-B6Biotin	High noise/not working
B6-HT7	Not compatible
HT7-B6Biotin	High noise/not working
TAU5-B6Biotin	High noise/not working
TAU1-B6Biotin	High noise/not working
B6-BT2+HT7	200,000-250,000 pg/mL. High noise.
E11	
E11-BT2Biotin	Compatible good signal-to-noise ratio
E11-HT7Biotin	Compatible good signal-to-noise ratio
E11-BT2+HT7Biotin	300- 500 pg/mL
BT2-E11Biotin	1000-2000 pg/mL
HT7-E11Biotin	Compatible

5.2.3.3. E11 antibody

The best performing identified antibody pairing for ac-Tau E11 mAb was the BT2+HT7-Biotin used simultaneously as 1st antibodies (*for details, see LOD in Table 22*). The assay shown below includes one of the latest assays oriented to the identification of the LOD of the E11- BT2 +HT7 Biotin. The Streptavidin Poly-HRP and luminescent substrate allowed to detection of 10 picogram/mL ac-Tau peptide in TBS buffer (**Figure 50**). The LOD with artificial CSF matrix was between 10 and 100 pg/mL ac-tauL peptide. Under these experimental conditions, we have reached a LOD range that should allow us to proceed to the CSF samples validation phase. The control A with TBS and with the CSF matrix was according to expected; however, the control A in TBS using the E11 antibody stored at 4°C resulted higher as in the previous assay.

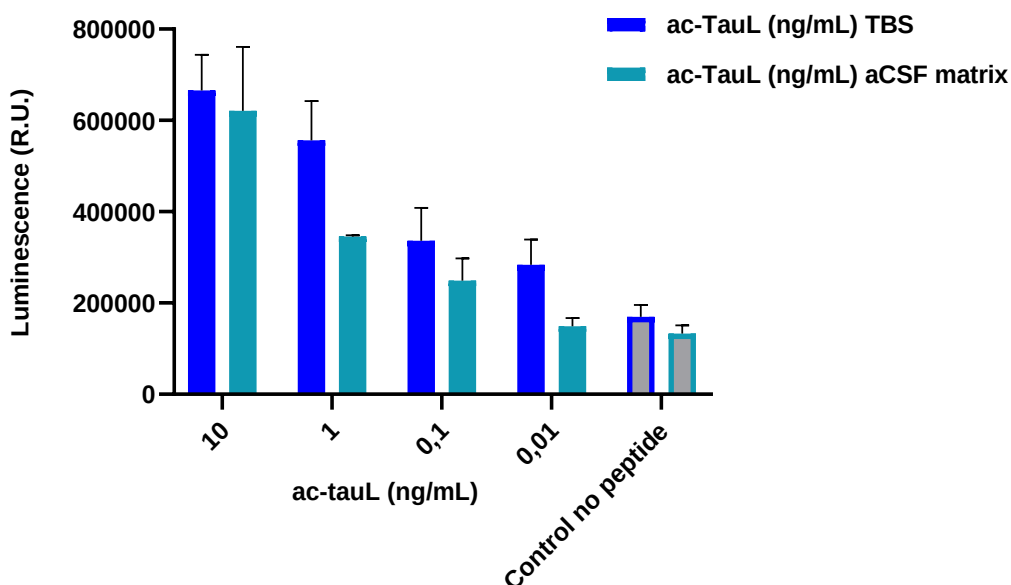


Figure 50. Sandwich ELISA E11-actauL-BT2+HT7Biotin luminescence. Results from the Sandwich Elisa with a range of peptide ac-tauL concentrations from 10 to 0,01 ng/mL in luminescent substrate.

5.2.3.4. B6 antibody

The best performing identified antibody pairing for p-Tau B6 mAb was the BT2-Biotin (for details, see LOD in **Table 22**). The assay showed below includes one of the latest assays oriented to the identification of the LOD of the B6-BT2Biotin. The B6-BT2Biotin pair seems the only compatible and all concentrations showed a similar signal, so we used the lowest concentration of the detection antibody (**Figure 51**).

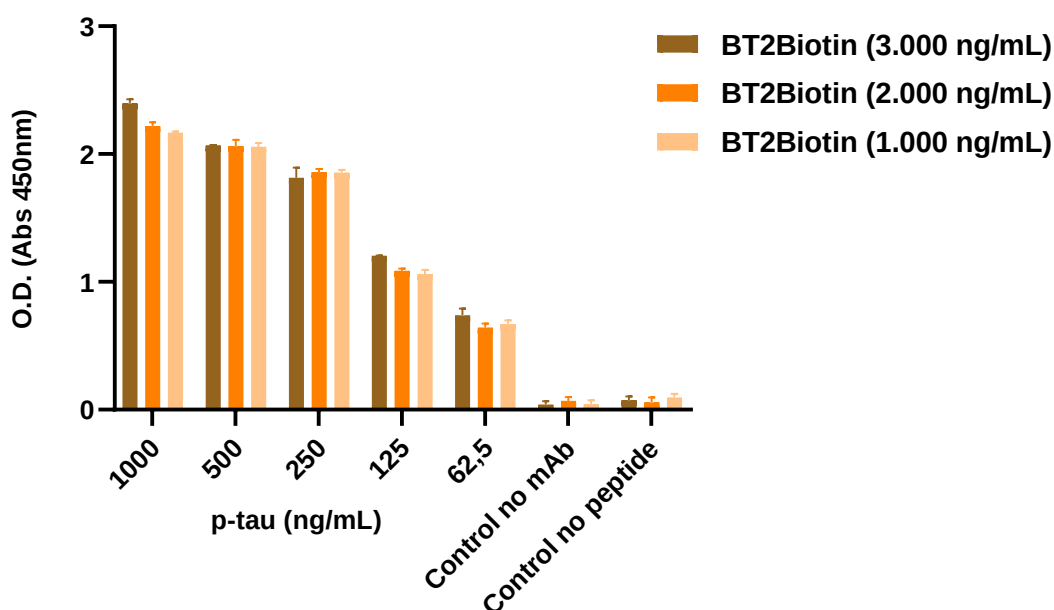


Figure 51. Colorimetric Sandwich ELISA with the following setup: B6119-p-tauL-BT2Biotin-tvPolyHRP. B6119 batch and commercial solutions were used in this assay.

5.2.3.5. ELISA signal development and substrate incubation optimization

The objective of this task was to unveil the kinetics of the signal development given by the so-far-used 1-Step Ultra TMB and the alternative 1-Step Turbo TMB. As shown in **figure 52**, significant differences were found between the two TMB reagents. The Ultra TMB gave overall higher maximum absorbance levels and was much faster compared to the Turbo TMB. Signal increase progression was longer as well with the Ultra TMB compared to the Turbo TMB. On the other side, Control A wells (without sample) with the Turbo TMB setup showed 50% less signal than wells with Ultra TMB. At peptide concentrations higher than 0.1 $\mu\text{g/mL}$ the maximum absorbance intensity was observed 6 minutes after TMB addition in both cases. However, at 0.1 $\mu\text{g/mL}$ peptide concentrations, absorbance intensity continuously increased until the end of the kinetics recording in both cases, although at a lower rate with the Turbo TMB. In overall conclusion, even though Turbo TMB showed lower Control A signal, the Ultra TMB showed a much higher signal and signal-to-noise ratio.

5.2.3.6. Luminescence vs Colorimetric development performance with CSF samples

Next, for confirming the correlation between CSF samples and how freeze-thawing cycles could affect the detection of the biomarkers of interest, samples without any thawing

and three thaw-cycles were analyzed. CSF samples used were taken from lumbar puncture of patients between the years 2019-2021. Samples were analyzed in 96-well plates and manual operation using the most optimized protocols for both luminescence and colorimetric development so far. Cut-off level (COL), LOD and coefficient of variation (CV) were determined for each of the assays to validate the results obtained.

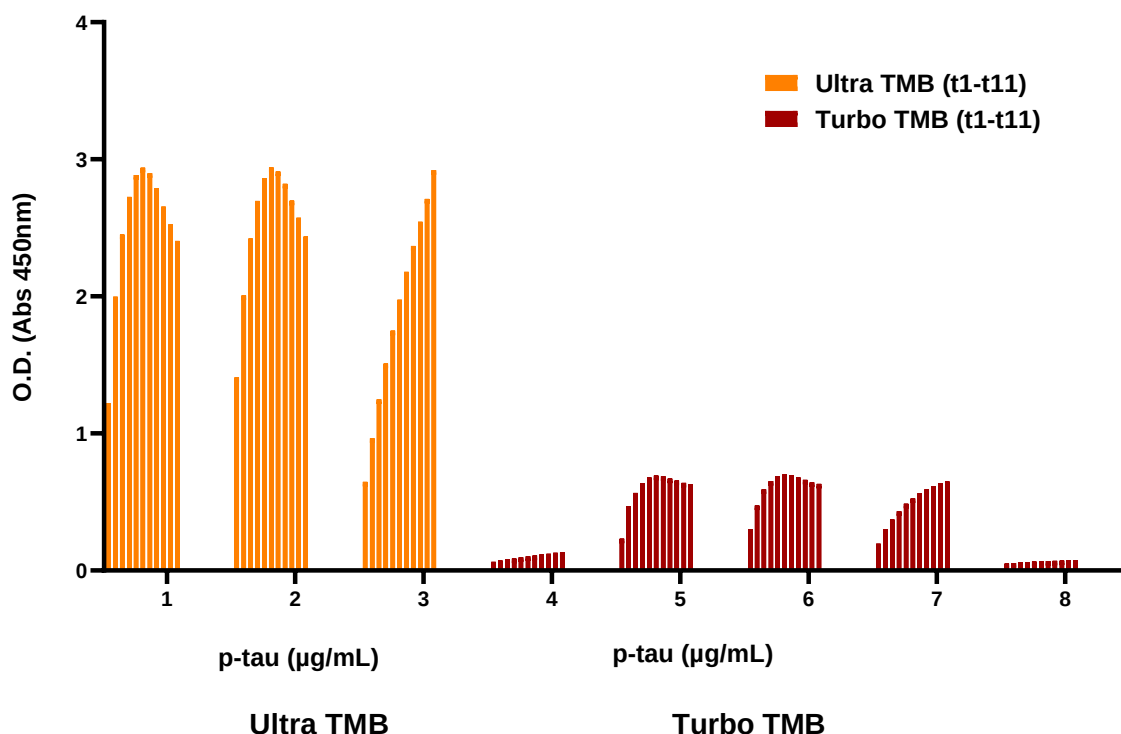


Figure 52. Indirect ELISA: actauL-E11Biotin-NAvHRP. Absorbance (450nm) values used to compare the performance of two TMB substrate solutions. Three concentrations of ac-tauL peptide (10; 1; and 0,1 µg/mL) and 10 µg/mL of E11-Biotin were used. The tested variable was the substrate reagent: 1-step Ultra TMB and the 1-step Turbo TMB. Values shows the maximum absorbance reached within the kinetic assay.

5.2.3.6.1. Luminescence method

To increase the sensitivity and signal-to-noise ratio of the ELISA assays, we performed a comparison between the luminescence substrate ELISA Femto SuperSignal Substrate (Thermo Fischer, USA) with the best colorimetric substrate used so far 1-step Ultra TMB (Thermo Fischer, USA). The use of the luminescence method does not require the stopping of the peroxidase enzymatic reaction, but it has a limited time window for reading the plate, as the reaction is much faster.

The obtained results showed that all tested CSF samples entered within the detection range, negative controls were within expected low values and the calibration curves were stable. Signals obtained from samples with 3 thaw cycles vs freshly thawed were lower, indicating that the freeze-thaw cycles could significantly affect the detection of the biomarkers of interest by our ELISAs (**Figures 53 and 54**).

The deviations between duplicates were acceptable for both E11 and B6 antibodies however, there were detected inconsistencies with the latter, where the used samples reported significantly higher CVs than the rest, and the ones obtained with the E11 antibody (**Tables 23 and 24, Figures 55 and 56**).

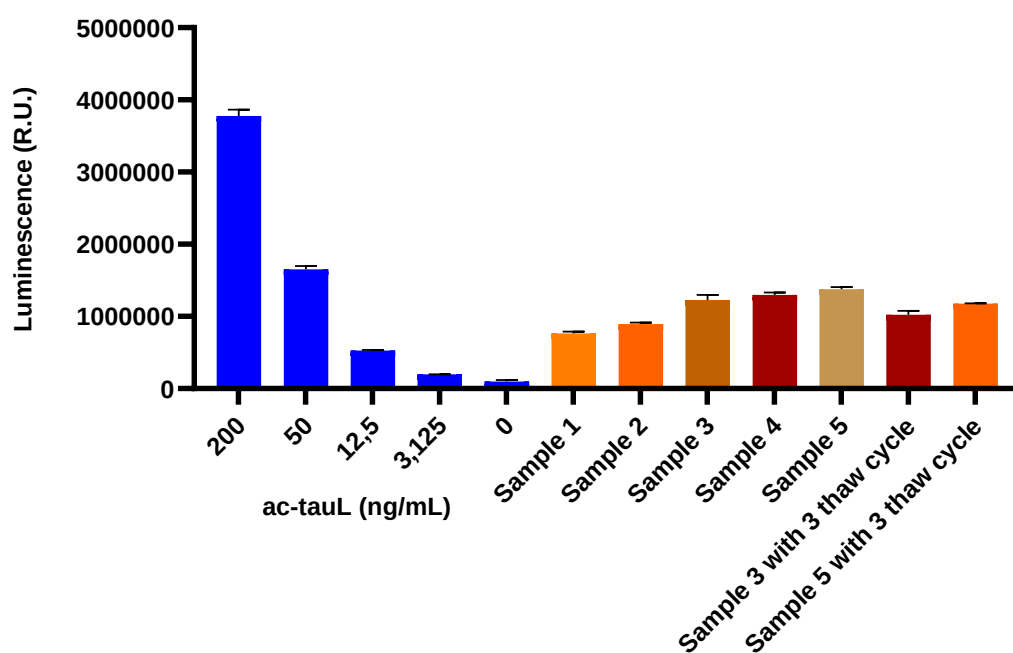


Figure 53. Luminescence Sandwich ELISA E11-actauL/BT2+HT7Biotin. Range of peptide ac-tauL concentrations from 200 to 3,125 ng/mL. Seven CSF samples were tested showing signals within the detection range. Orange columns represent the average of duplicates and error bars the standard deviation. R.U. relative units.

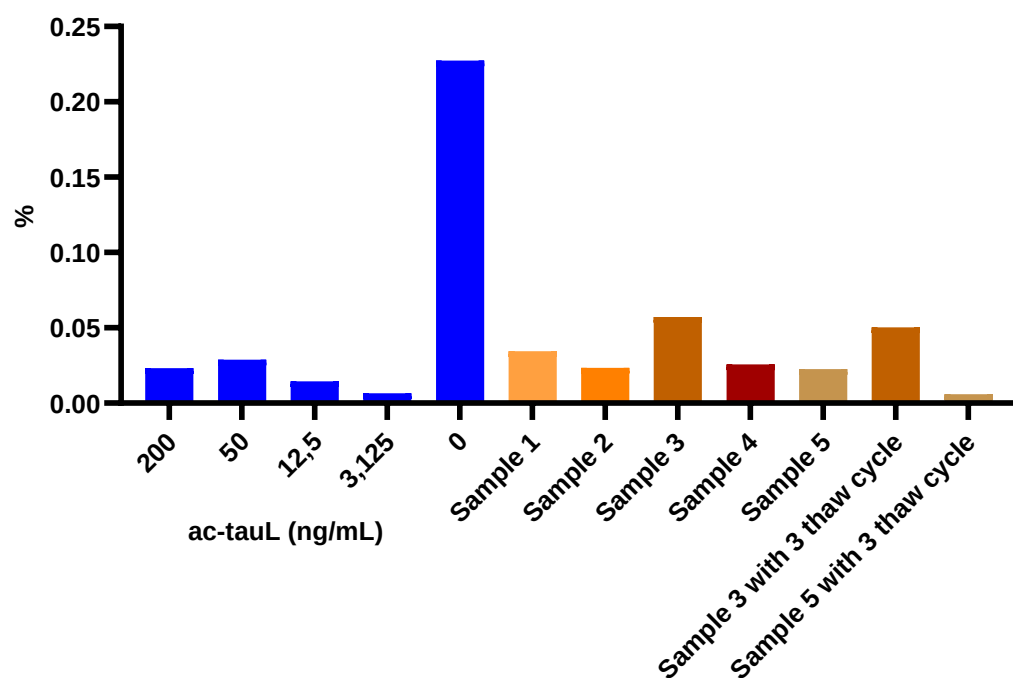


Figure 54. CV for the Sandwich ELISA E11-BT2+HT7Biotin. All CV, except for blank. Were lower than 5%.

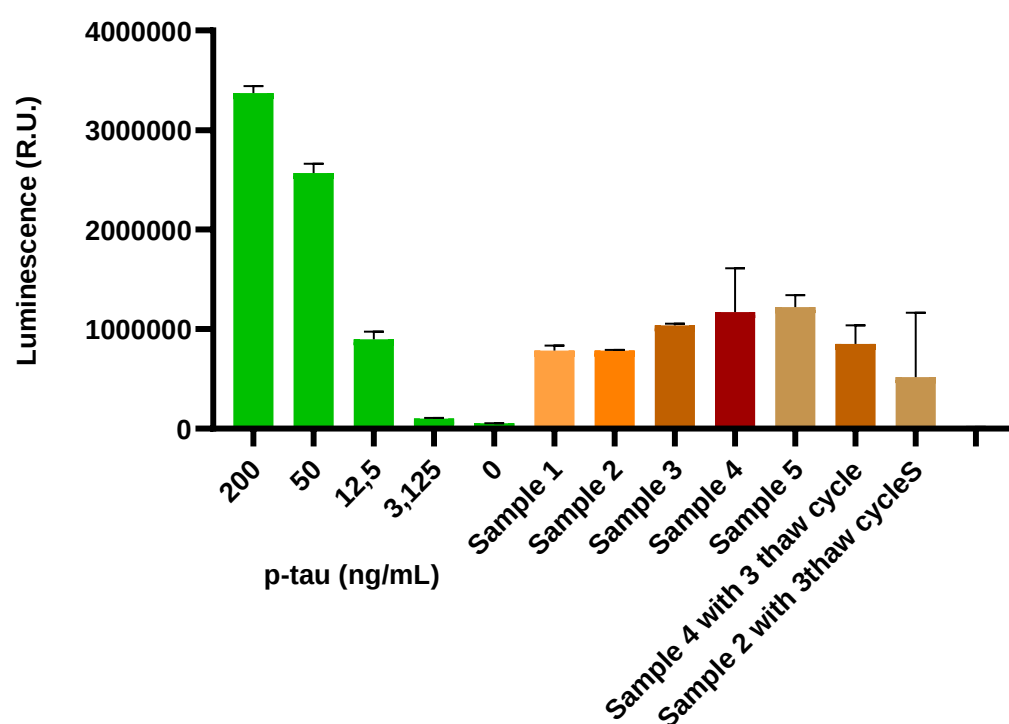


Figure 55. Luminescence Sandwich ELISA B6-BT2 Biotin. Range of 3P-tau peptide concentrations from 200 to 3,125 ng/mL. Seven CSF samples were tested showing signals within the detection range. Orange columns represent the average of duplicates and error bars the standard deviation. R.U. relative units.

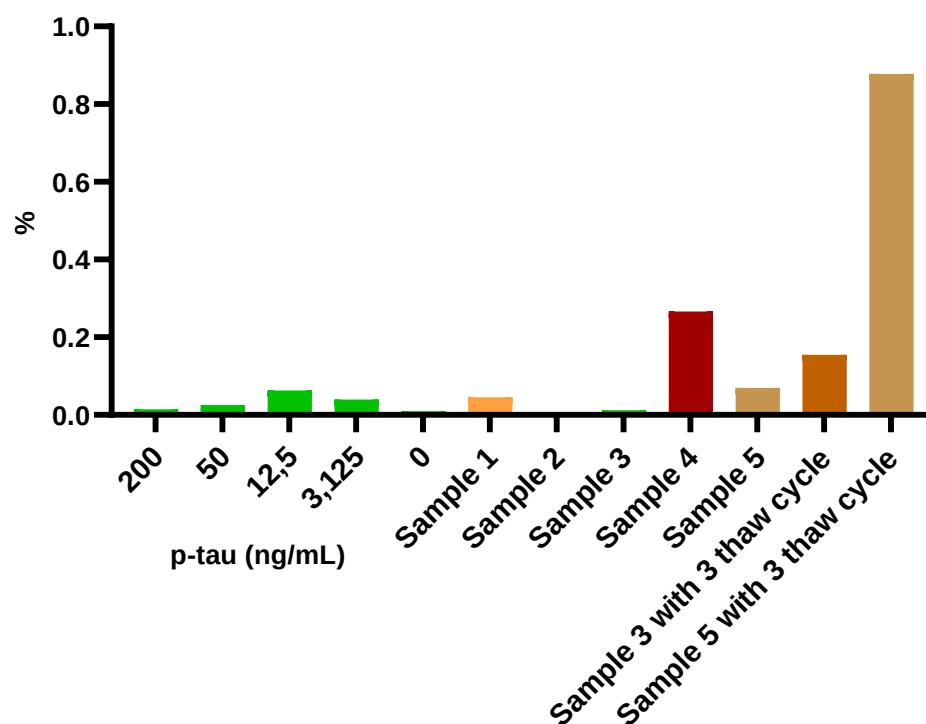


Figure 56. CV for Sandwich ELISA B6-BT2 Biotin. All CV, except for a sample with 3 thaw cycles, were lower than 5%.

Table 23. Details of COL, LOD, and CV of all single samples and the overall E11 luminescence assay.

COL	197000,5
LOD	2,87483478
E11-BT2+HT7Biotin Luminescence	CV
200	2,32%
50	2,88%
12,5	1,46%
3,125	0,66%
0	22,75%
Sample 1	3,45%
Sample 2	2,34%
Sample 3	5,71%
Sample 4	2,58%
Sample 5	2,25%
Sample 3 with 3 thaw cycles	5,03%
Sample 5 with 3 thaw cycles	0,58%
Average CV	4,33%

Table 24. Details of COL, LOD, and CV of all single samples and the overall B6 luminescence assay.

COL	58839
LOD	0,55323149
B6-BT2Biotin Luminescence	CV
200	1,44%
50	2,58%
12,5	6,27%
3,125	3,93%
0	0,96%
Sample 1	4,54%
Sample 2	0,69%
Sample 3	1,17%
Sample 4	6,62%
Sample 5	6,94%
Sample 3 with 3 thaw cycles	15,44%
Sample 5 with 3 thaw cycles	17,77%
Average CV	7,20%

5.2.3.6.2. Colorimetric method

The obtained results with the ELISA using colorimetric methods showed that the signal for all CSF samples entered within the detection range, negative controls within expected low values and the calibration curves were stable. While retained in the case of the B6 assay, only E11 signals from samples with 3 thaw cycles vs freshly thawed were lower compared to the fresh ones, indicating again that the freeze-thaw cycles could significantly affect the detection of the acK174Tau biomarker by ELISA (**Figures 57 and 58**).

CVs were overall low, except for some samples where the variation increased over 10%. However, none of them were coincident for both B6 and E11 (**Tables 25 and 26, Figures 59 and 60**).

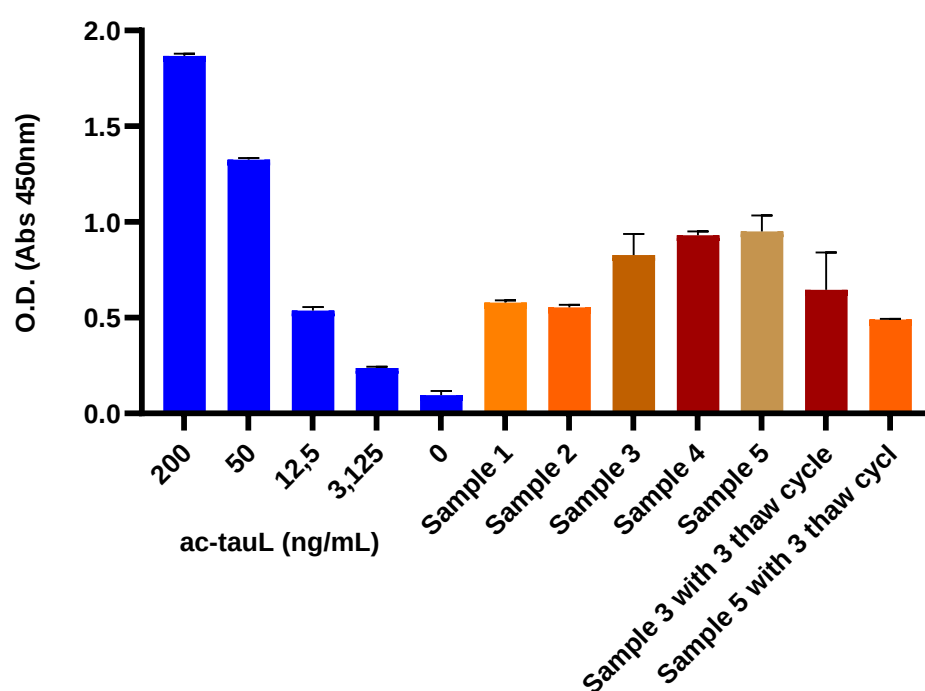


Figure 57. Colorimetric Sandwich ELISA E11- BT2+HT7Biotin. Range of peptide ac-tauL concentrations from 200 to 3,125 ng/mL. Seven CSF samples were tested showing signals within the detection range. Blue columns represent the average of duplicates and error bars the standard deviation.

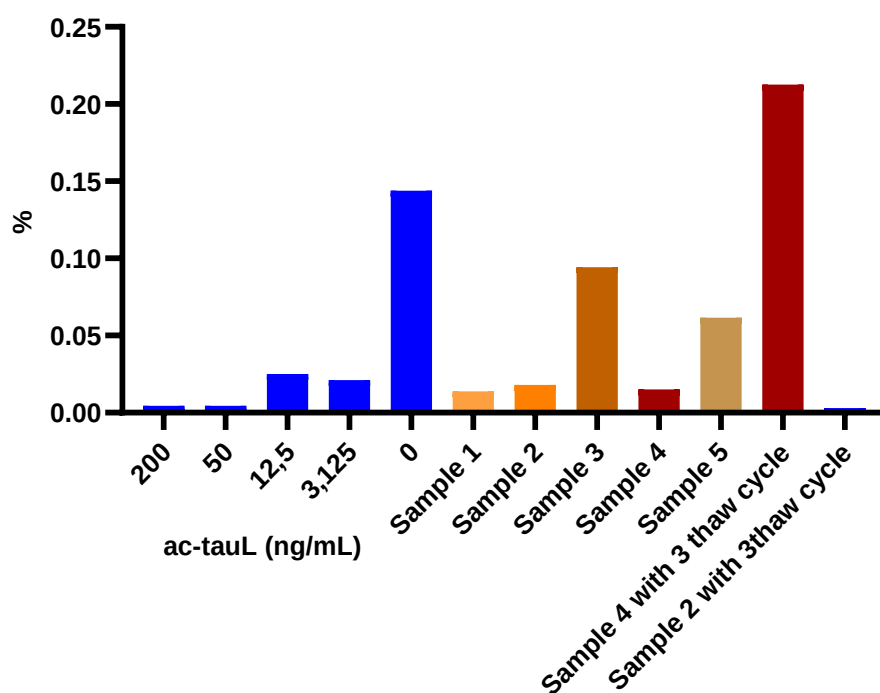


Figure 58. CV for Sandwich ELISA E11-BT2+HT7Biotin. All CV, except for blank and a sample with 3 thaw cycles, were lower than 10%.

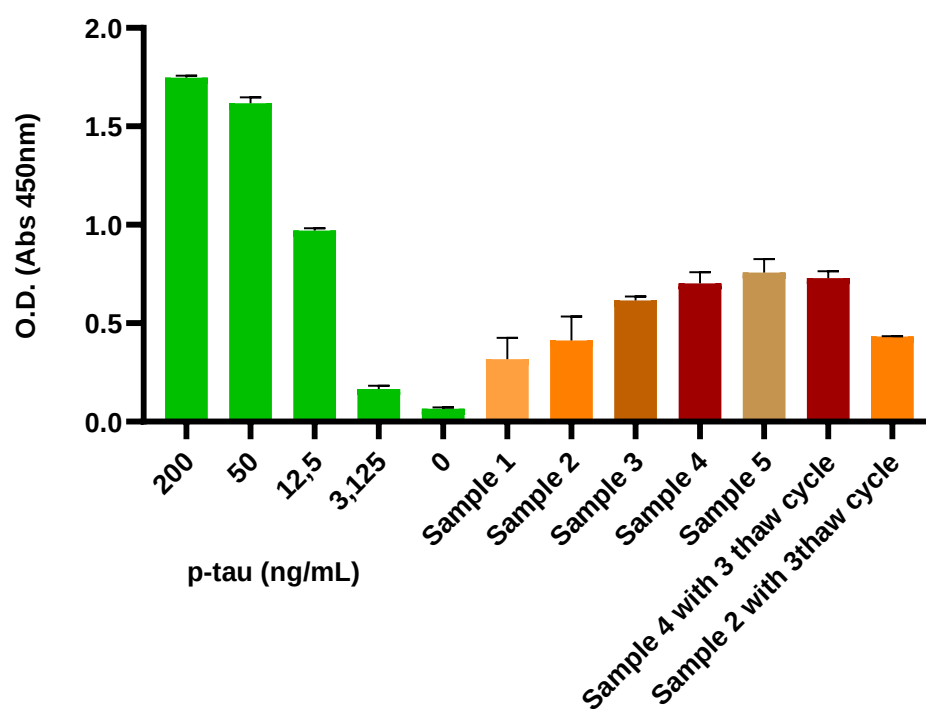


Figure 59. Colorimetric Sandwich ELISA B6-BT2 Biotin. Range of 3P-tau peptide concentrations from 200 to 3,125 ng/mL. Seven CSF samples were tested showing signals within the detection range. Orange columns represent the average of duplicates and error bars the standard deviation.

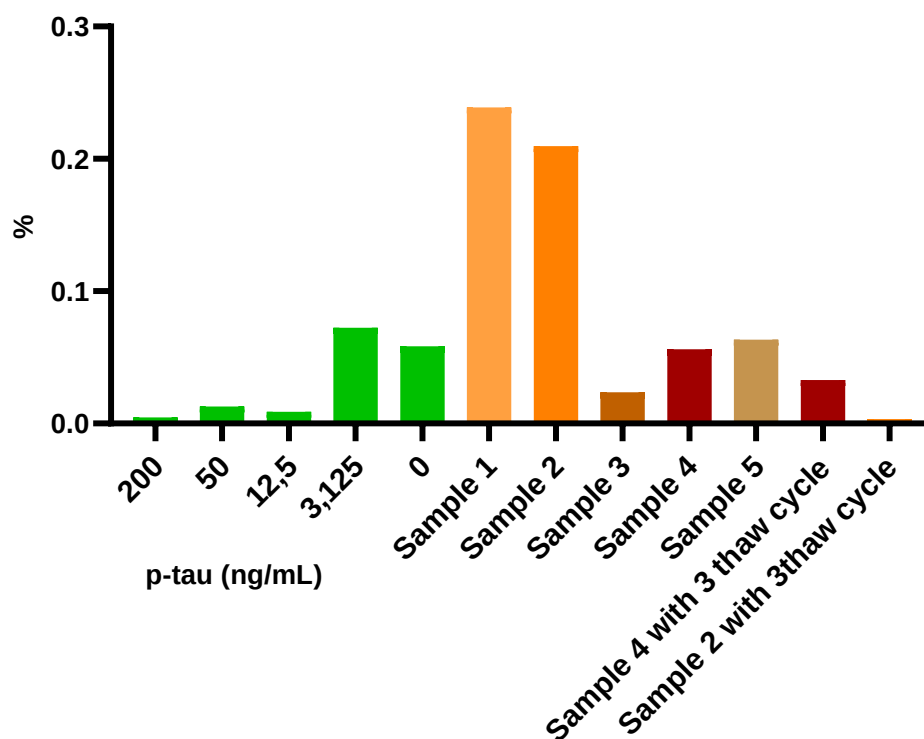


Figure 60. CV for Sandwich ELISA B6-BT2 Biotin. All CV were lower than 5%.

Table 25. Details of COL, LOD, and CV of all single samples and the overall E11 colorimetric assay.

COL	0,17325
LOD	0,487341772
E11-BT2+HT7Biotin colorim	CV
200	0,46%
50	0,45%
12,5	2,51%
3,125	2,11%
0	14,39%
Sample 1	1,38%
Sample 2	1,81%
Sample 3	9,43%
Sample 4	1,50%
Sample 5	6,15%
Sample 4 with 3 thaw cycles	21,27%
Sample 2 with 3 thaw cycles	0,31%
Average CV	5,15%

Table 26. Details of COL, LOD, and CV of all single samples and the overall B6 colorimetric assay.

COL	0,0995
LOD	1,12967914
B6-BT2 Biotin colorim	CV
200	0,46%
50	1,30%
12,5	0,88%
3,125	7,23%
0	5,84%
Sample 1	23,90%
Sample 2	20,97%
Sample 3	2,36%
Sample 4	5,62%
Sample 5	6,33%
Sample 4 with 3 thaw cycles	3,29%
Sample 2 with 3 thaw cycles	0,35%
Average CV	6,54%

5.2.3.7. Signal-to-Noise Ratio comparison

5.2.3.7.1. Luminescence vs Colorimetric

We have conducted a comparative study between the luminescence and colorimetric methods in order to determine which is the best in terms of signal-to-noise Ratio. For that, both pS208 and acK174 sandwich ELISAs were used for checking 5 selected human CSF samples by using colorimetric and luminescence development methods. Data confirmed high signal-to-noise ratios for both detection methods, but the luminescent substrate allowed to obtain wider ratios than the TMB in all cases (**Figure 61 and 62**). Although colorimetric methods allow easier procedures, we prioritized the higher sensitivity and signal-to-noise ratio of the luminescence method. The ratio was calculated by dividing the average of each sample by the average of the blank in both detection methods. All samples tested had a signal-to-noise ratio greater than 1 (the blank ratio). The luminescence method reported overall significantly higher signal-to-noise ratio than in the colorimetric method.

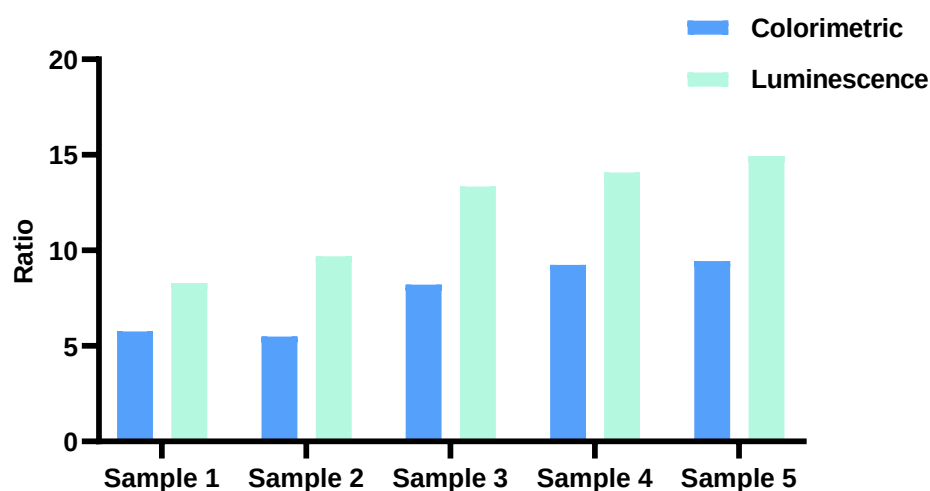


Figure 61. Signal-to-noise ratio of E11 in colorimetric and luminescence methods, showing best values for Luminescence.

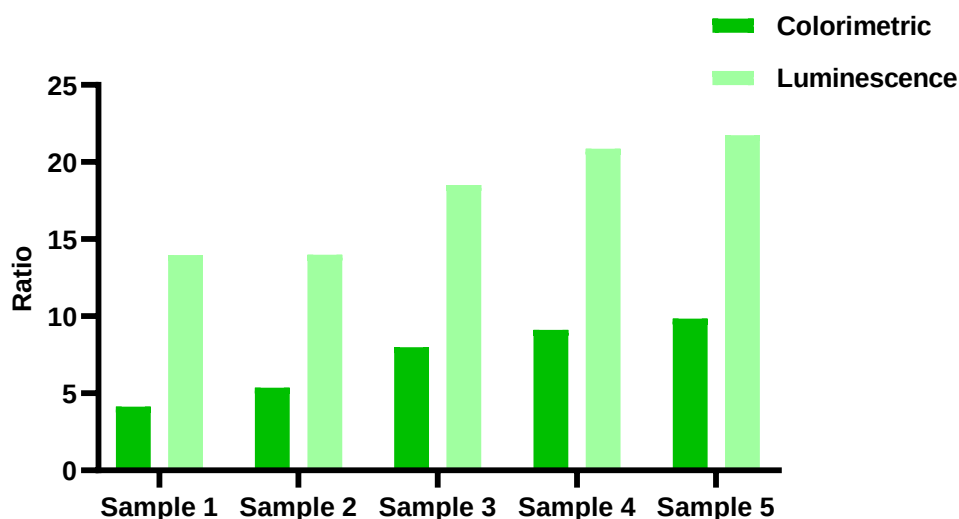


Figure 62. Signal-to-noise ratio of B6 in colorimetric and luminescence methods, showing best values for Luminescence.

Once the sandwich setup was confirmed, the performance of the test in terms of reproducibility, intra-assay COV, the LOD and the limit of quantification (LOQ) were assessed using the luminescence method. The LOD and LOQ of the assays were determined by adding 3.3 and 10 times the standard error of negative control respectively to the X1 variable of the linear regression of luminescence values ($n=4$). The specific synthetic peptides including the targeted tau PTMs were used for the setup refinement and subsequent semi-quantitative assays. B6-based sandwich ELISA reported good linearity up to 25 ng mL⁻¹ of 3P-tau (188-217) peptide (**Figure 63A**). LOD and LOQ values were 1.2 and 3.7 ng mL⁻¹, respectively for this antibody (**Table 27**). E11-based sandwich ELISA linear range was wider, reaching 200 ng mL⁻¹ of acK174 Tau (156-230) (**Figure 63B**). Identified LOD and LOQ were 3.4 and 10.4 ng mL⁻¹, respectively with E11.

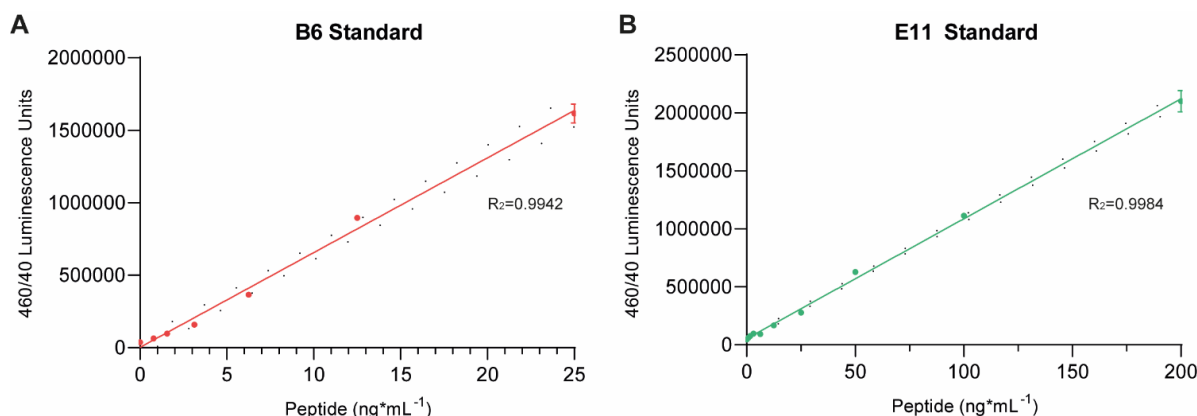


Figure 63. Standard curves of E11 and B6 ELISAs. **A)** Standard curve and R^2 values for p(S202, T205, S208) tau peptide (188-217) in the B6-based luminescence sandwich ELISA. **B)** Standard curve and R^2 values for acK174 tau peptide (155-230) in the E11-based luminescence sandwich ELISA. Dotted lines limit the dispersion of the linear regression values for each dataset.

When analyzing the stability of the test, CVs confirmed overall low both intra- and inter-assay variations. B6-based ELISA against pS208 tau reported a 3.4% and 4.5% intra- and inter-assay variation, respectively. Measured intra- and inter-assay CVs for E11-based ELISA against acK174 tau were 3.2% and 3.8%, respectively (**Table 27**).

Table 27. Control Quality parameters of the luminescence-based sandwich ELISA setup with 3P-tau (188-217) and acK174 tau (155-230) synthetic peptides, and with a set of 62 CSF samples from MCI and AD patients. LOD, limit of detection; LOQ, limit of quantification; CV, coefficient of variation.

	COL (RU)	LOD	LOQ	Intra-assay CV (%)	Inter-assay CV (%)	CSF Samples CV (%) (intra-assay)
B6	51,851	1.2	3.7	3.4	4.5	7.5
E11	61,216	3.4	10.4	3.2	3.8	6.1

5.2.4. Clinical Validation of ac-tau and p-tau ELISA kits in CSF samples

5.2.4.1. CSF Samples Validation of Sandwich Elisa

Firstly, initial data confirmed several signals from the analyzed 28 CSF samples above the COL (Control without antigen O.D. Average*3SD) and transformed to pg/mL using the resulting equation of the calibrators curve.

Calibrators only showed positive signals above Control without antigen from 800 pg/mL and above. Besides that, concentration values seem too high for the normal concentrations detected in these signals. This issue may have a relation with the state of the calibrators (degradation, aggregation, etc.) and/or the significant difference in molecular weight between the calibrators and the tau species within the biological samples. This could have led to a miscalculation of the actual “active antigen” in the wells. However, several CSF samples showed significant signals above the COL. Specifically, 18 CSF samples raised above the COL for Sandwich ELISA with E11, and 25 CSF samples for the B6 Sandwich ELISA. The robust positive signals obtained confirm that the ELISA setups are available for detecting the presence of the tau PTMs of interest (pS208 and acK174-tau). The differential distribution of these signals within the different clinical groups is also particularly interesting, especially when compared to the other analyzed biomarkers (hTau, pT181-tau and Amyloid beta) in the Lumipulse G600 equipment (**Figure 64 and 65**).

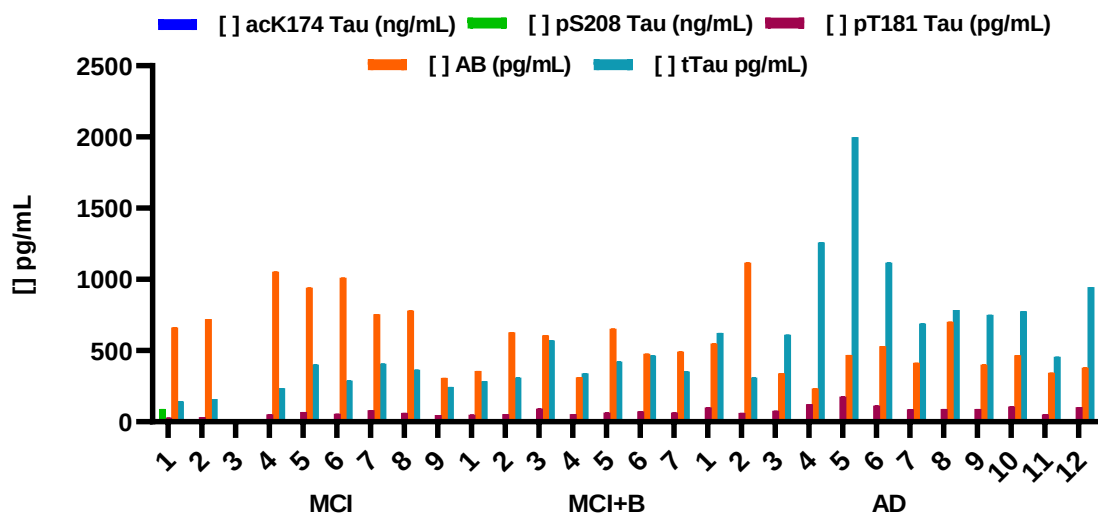


Figure 64. Randomized CSF samples analysis comparison of total tau (t-tau), amyloid beta (A β 1-42), pT181-tau, acK174-tau and pS208-tau biomarkers. Values for acK174-tau and pS208-tau in ng/mL, based on the small synthetic peptides prototype calibration curve. Values for pT181-tau, t-tau and A β 1-42, in pg/mL, according to Lumipulse G600 equipment calibration.

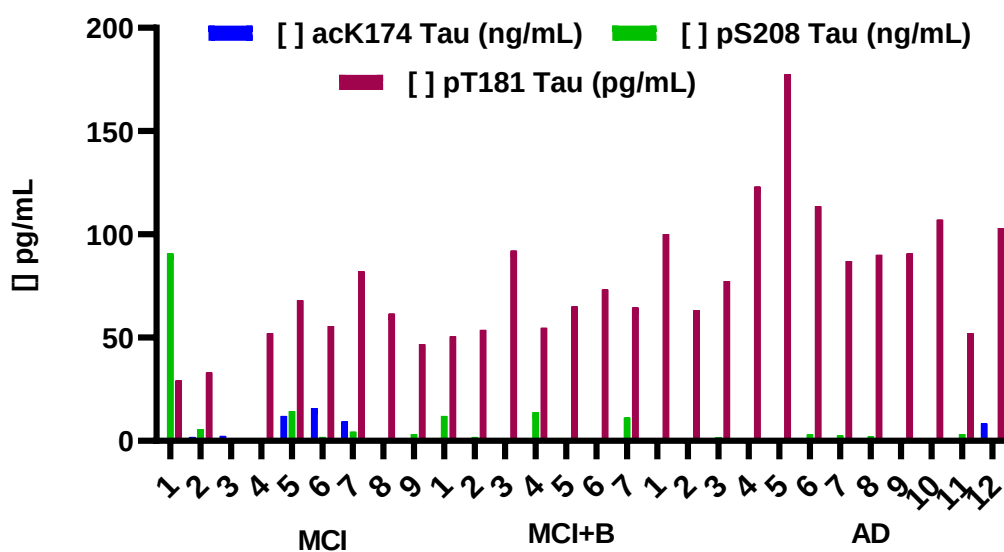


Figure 65. Randomized CSF samples analysis comparison of pT181-tau, acK174-tau and pS208-tau biomarkers. Values for acK174-tau and pS208-tau in ng/mL, based on the small synthetic peptides prototype calibration curve. Values for pT181-tau, t-Tau and A β in pg/mL, according to Lumipulse G600 equipment calibration.

After obtaining these results, we decided that CSF samples have to be analyzed without any dilution in our setup for pS208 and acK174-tau ELISAs. Standard curves of 3P-tau (188-217) and acK174 Tau (156-230) synthetic peptides served as a semi-quantification system (Figure 66).

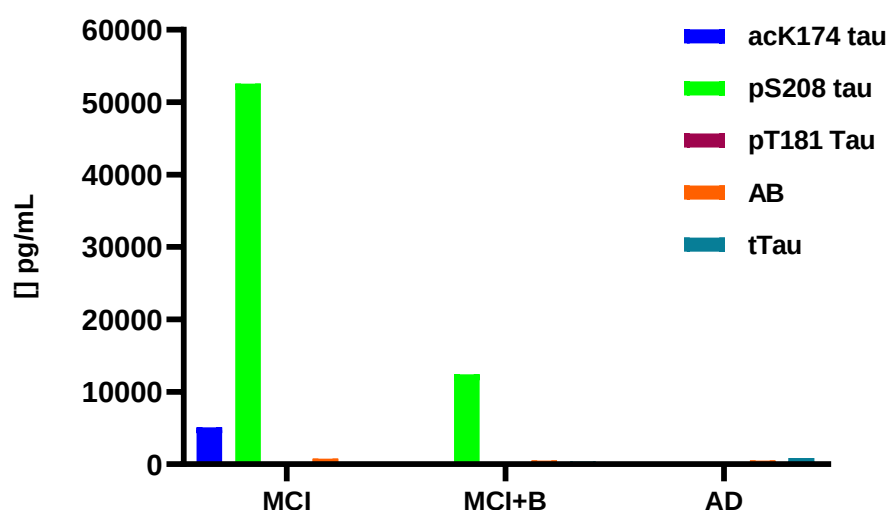


Figure 66. Comparison of signals for pT181-tau, t-Tau, Aβ1-42, acK174-tau and pS208-tau biomarkers in CSF samples of patients with MCI and/or AD. Values for acK174-tau and pS208-tau in ng/mL, based on the small synthetic peptides prototype calibration curve. Values for pT181-tau, t-tau and Aβ in pg/mL, according to Lumipulse G600 equipment.

Once the final Sandwich ELISA setup and basic parameters were defined, we proceeded with a proof-of-concept clinical study for the validation of these novel biomarkers in a total of 89 patients with MCI according to criteria AA-NIA.

Descriptive analysis

Age: 70.1 ± 7.8 years

Gender: 36 Males (40.4%) and 53 Females (59.6%).

Follow up period: 4.1 ± 1.2 years

Final Diagnosis:

- MCI non-AD 55 (61.8%)
- Prodromal AD 18 (20.2%)
- Dementia-AD 16 (18.0%)

MME score at visit 1: 27 [26, 28]

MME score at final visit: 26 [24, 27]

Total CAMCOG score at visit 1: 86 [77, 90]

Total CAMCOG score at final visit: 83 [72, 89]

The mean age was 70±7.1 years and the 59,6% were females. The mean time of follow-up was 4.9 years. Of the 89 patients, 61.8% (55) did not progress to AD; 20.2% (18) evolved to prodromic AD; and 18% (16) patients progressed to moderate AD. As shown in **Figure 67**, all the novel tau biomarkers (pS208 and acK174-tau), as well as those of t-Tau and p-Tau181 from the Fujirebio Lumipulse system (gold standard) show the same behavior with an increase in the levels of the isoforms of tau in CSF as the disease progresses, although the results are not statistically significant due to the small sample size and the dispersion of data. Therefore, based on these promising results future, larger, multicenter clinical trials are necessary to validate these first results.

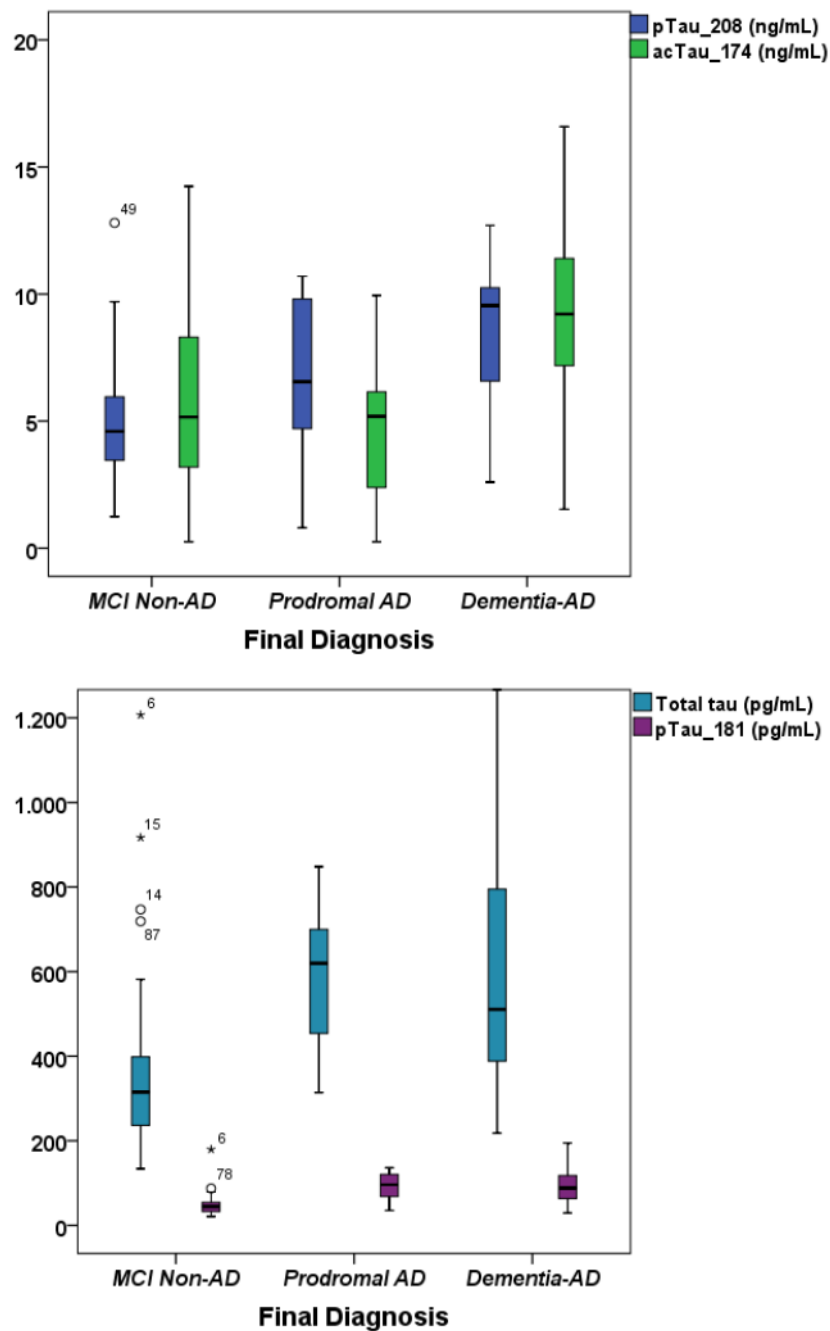


Figure 67. ELISA results of CSF levels of tau biomarkers (ac-Tau and pS208-tau), as well as those of t-tau and pT181-tau from the Fujirebio Lumipulse system (gold standard) showing the same behavior, an increase in the levels of the isoforms of tau in CSF as the disease progresses. The first group the patients not progress to AD (MCI), the second group represent AD prodromic and the third group the patients progressed to moderate AD.

In order to meet current market requirements for a successful commercialization, specifications for the ELISA kits have been defined in the **Table 28**.

Table 28. Specifications for the final Sandwich ELISA.

Product specifications (objective)	
Antigen	Tau (ac-tau y p-tau).
Reactivity	Human
Detection range	25-1.000 pg/mL
Minimum detection level	25 pg/mL
Method	ELISA Sandwich
Time	1,5 to 4 hours to obtain results
Test number	96
Use	Quantitative determination of the concentrations of h-tau (ac-Tau and p-Tau) proteins in cerebrospinal fluid (CSF).
Type of sample	LCR (Serum and tear variants will developed and tested).
Analytic method	Quantitative
Detection method	Colometry
Specificity	>80%
Sensitivity	5 ng/ml
Storage method	Antibodies and controls 2-8 ° C (1 year); solutions -20 ° C (1 year)

5.3. MAGNETORESISTIVE BIOSENSORS FOR BIOCHIP PLATFORM

5.3.1. Peptide p-Tau detection with the portable magnetoresistant platform

It started by optimizing a strategy for the detection of the pS208-tau using an assay that mimics the sandwich ELISA assay. Different incubation times of pS208-tau were tested to understand the impact on the binding affinity of this protein to specific antibodies. The incubation was also performed by using a pre-recognition step, where pS208-tau was captured in solution by functionalized MNPs, and by incubating the pS208-tau inside and outside a microfluidic circuit to understand how the volume impacts the protein-antibody interaction (**Figure 68 and 69**). Two sample conditions were tested only with aCSF matrix 1h and aCSF matrix with 1 µg/mL of p-tau peptide 1h in three different detection methods.

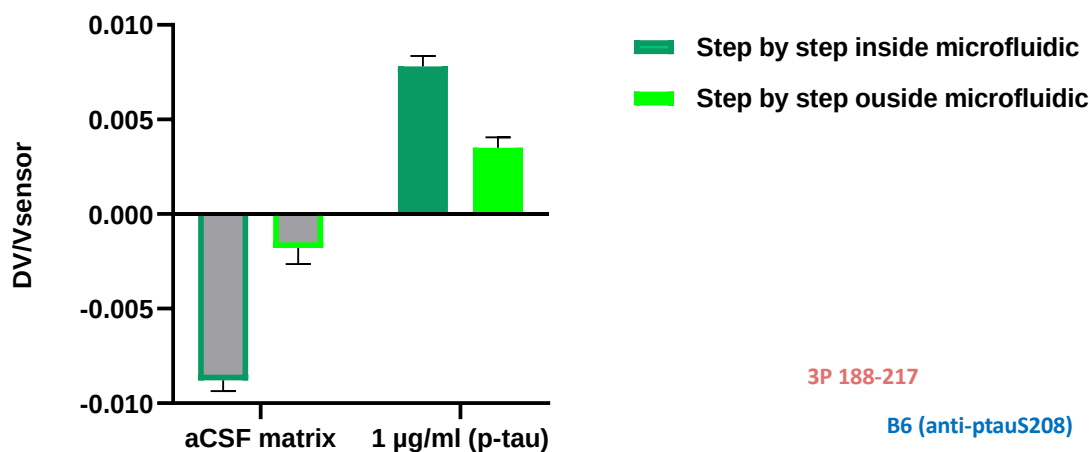


Figure 68. Step-by-Step: Incubate the sample inside and outside the microfluidic system separated of MNPs in aCSF matrix.

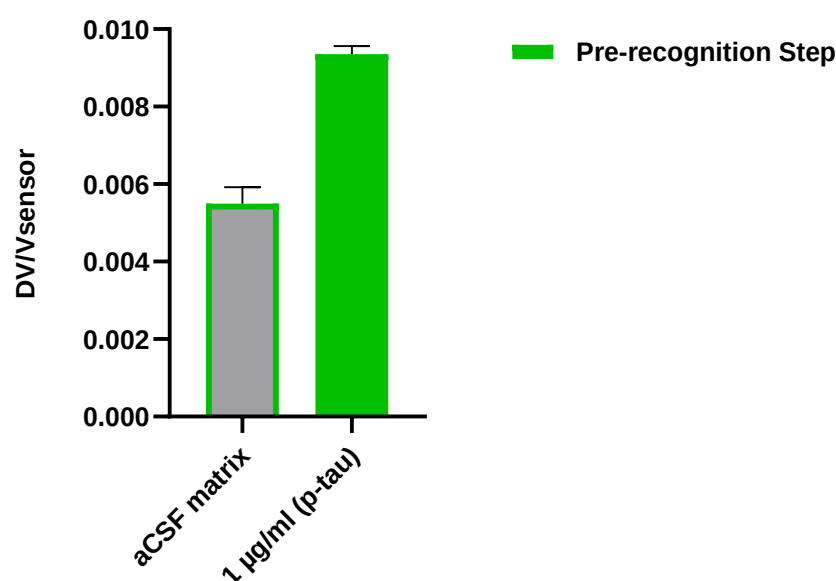


Figure 69. Pre-recognition step: Incubate the sample with MNPs+BT2Biotin in aCSF matrix.

Although all assays showed high standard deviations, the method of Step-by-Step inside microfluidic was the best signal/noise and deviation, because it allowed the sample to be differentiated from the negative control. **Figure 70** show the group of sensors of biochip with the method Step-by-Step inside microfluidic was seen in the NBI - Wide-Field Upright Fluorescence Microscope. The MNPs can be seen on the right side, which is where our target should bind more than on the left side, which does not have the target antibody. There was a lack of homogeneity of the MNPs in the sensors, although the last group of sensors showed more MNPs bound. The detection signal obtained after several optimizations showed a low background noise for aCSF and signal for 1 µg/mL of pS208-tau.

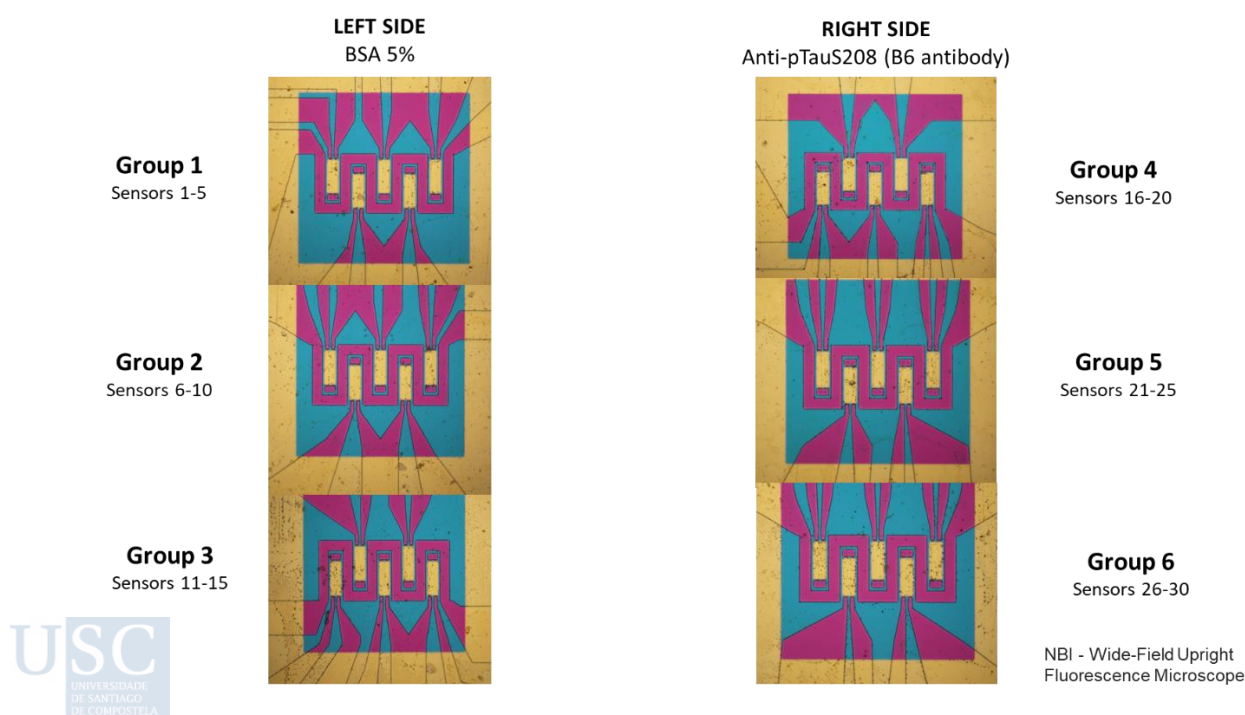


Figure 70. Step-by-Step inside the microfluidic system: Sample aCSF matrix (p-Tau 1 $\mu\text{g/mL}$) 1h RT.

Successfully, a sandwich assay based on two antibodies, anti-pS208-tau (primary antibody) and BT2-Biotin (secondary antibody) was performed using MNPs to detect the target of interest as pS208-tau. Once the analyte incubation method has been selected, the detection signal and sensitivity were amplified to get the calibration curve and achieved a dynamic range from pg/mL to ng/mL, with the aim to stratify different groups of patients with MCI and AD in non-invasive biological samples such as serum, plasma and tears samples. However, the detection signal obtained after several optimizations showed a low background noise for aCSF but a signal for only 1 $\mu\text{g/mL}$ of pS208-tau. This detection limit is much lower than what we have obtained for ELISAs. There are methods that can improve and achieve the proposed dynamic range needed to detect the target protein. One possible method is the improvement of signal amplification by changing the linker or the concentration of the antibody. Try another type of blocking as BSA or blocking buffer protein-free with the aim of avoiding unspecified unions or try another type of sample matrix as serum, because the aCSF could show interference with its own composition in the microfluidic system of the MR platform. However, as the results were not better than those obtained by ELISA, we decided to put aside this development based on magnetoresistive biosensors for biochip platform.

5.4. IMMUNOTHERAPY STUDY: INTRACEREBROVENTRICULAR ADMINISTRATION OF P-TAU B6 ANTIBODY

5.4.1. Results Summary

A summary of the results in behavioral test and p-tau brain levels are shown in **Table 29**.

Table 29. Results summary of test and techniques done in the immunotherapy study.

	Description	Impact
Body weight	Clear genotype difference, a modest beneficial treatment effect.	Evidence that the main effect of B6 treatment in the final age interaction were significant.
Rotarod and Claspings	Clear genotype difference, a modest beneficial treatment effect.	Evidence that immunotherapy can alleviate the symptoms caused by excessive p-tau aggregates.
Grid Hanging	Clear genotype difference, no treatment effect.	No evidence that immunotherapy can alleviate the symptoms caused by excessive p-tau aggregates. No differences between B6 vs. IgG treatment.
WB analysis	WB analysis of p-tau and t-tau of Hc and Bs.	ICV B6-antibody treatment for 4 weeks does not evoke measurable differences in p-tau protein levels in P301S mouse Bs or Hc based on the biochemical analyses as WB.
Immunohistochemistry	IHC analysis of p-tau levels in Hc and Cx, in ICV treatment groups.	ICV B6-antibody treatment for 4 or 8 weeks reduced p-tau protein levels in P301S mouse Cx and Hc based on the immunofluorescence analyses.

5.4.2. Therapeutic effect of a continuous ICV of B6 antibody on behaviour

5.4.2.1. Body weight

The body weight of sham group of hTauP301S mice kept increasing throughout the follow-up time as expected (**Figure 71**). In contrast, the groups of treatment of hTauP301S mice kept the same growth curve until 14 weeks of age, when the differences of the weight gain started between groups because that was when the pump was implanted. From week 14 onwards, the B6 and IgG1 group showed significantly higher weights than the Sham group. Despite the weight of both groups at week 17, the IgG1 versus Sham group was less significant than the B6 group, this is confirmed within the week 18, when the pump is removed, because the B6 group is significant with respect to the Sham and IgG1 group. Note, the sham group did not have a filled pump reservoir, therefore the statistical differences were limited to the most important comparison, B6 vs. IgG1, because at the 18 week the B6 group of treatment vs. IgG1 group ($p=0.002$) and vs. Sham group ($p=0.0051$) were significative. So, the main effect of B6 treatment ($p<0.0001$) and the treatment x age interaction were significant ($p<0.0001$).

5.4.2.2. Claspings reflex

In the hTauP301S mice appeared just before the age of 17 weeks and increased rapidly thereafter (the greater the degeneration of the mice), but at 14 weeks the B6 group starts to be somewhat significant with respect to the Sham group $p=0.0180$. At 17 weeks the B6 group starts to be significant with respect to the IgG1 group $p=0.0026$ and by 18 weeks it reaches double significance $p<0.0001$. The same happens with the IgG1 group with respect to the Sham group at 17 weeks had a significance of $p=0.0076$ and continues at 18 weeks $p=0.0032$ (**Figure 72**). This evolution was reflected in the ANOVA-RM as a significant group x age interaction for the three groups after mini pump implantation showed a treatment effect on the closure reflex ($p=0.0022$). Comparison between B6 and IgG revealed less severe claspings in B6 treated mice and also an age x treatment interaction $p<0.0001$, such that the treatment effect by B6 emerged only after two weeks from the treatment begin.

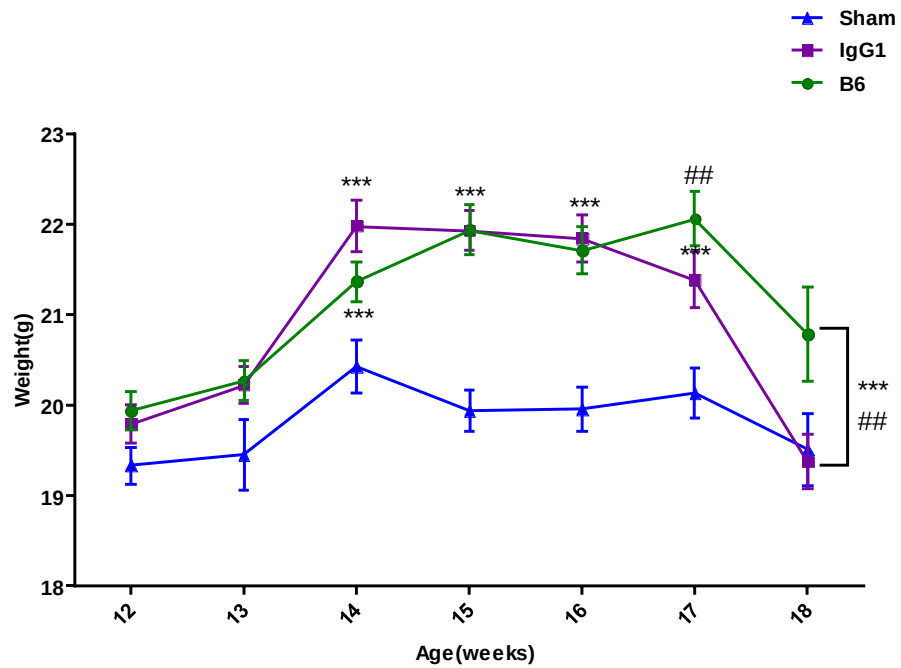


Figure 71. Change of body weight in sham group (blue), B6-treated hTauP301S mice (green) and hTauP301S mice receiving the control antibody (purple). Values represent the mean $\pm$ SEM, n=40: **p<0.01 and ***p<0.0001 compared to Sham; ##p<0.01 compared to IgG1. See text for details.

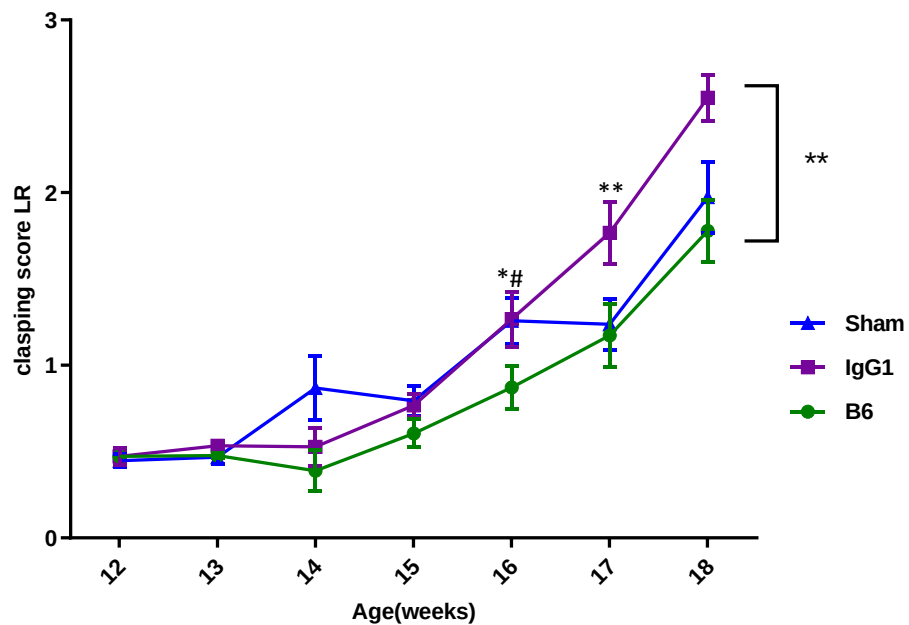


Figure 72. Clasping score as a function of age by study groups: sham group (blue), B6-treated hTauP301S mice (green) and hTauP301S mice receiving the control antibody (purple). Values represent the mean $\pm$ SEM, n=40: *p<0.05 and **p<0.001 compared to IgG1; #p<0.05 compared to Sham. See text for details.

5.4.2.3. Grid hanging

There was no overall treatment effect on grid hanging duration after the treatment onset (weeks 17 and 19) between the three groups ($p=0.4125$). On the other hand, it is significant with respect to the age of the animals, since the older they are, the less grip strength they show due to greater neuronal degeneration in mice ($p<0.0001$) (**Figure 73**). So, ANOVA-RM for three groups after mini pump implantation showed no overall treatment effect or age x treatment interaction on grid hanging duration. Neither was there any treatment effect in the B6 vs. IgG comparison.

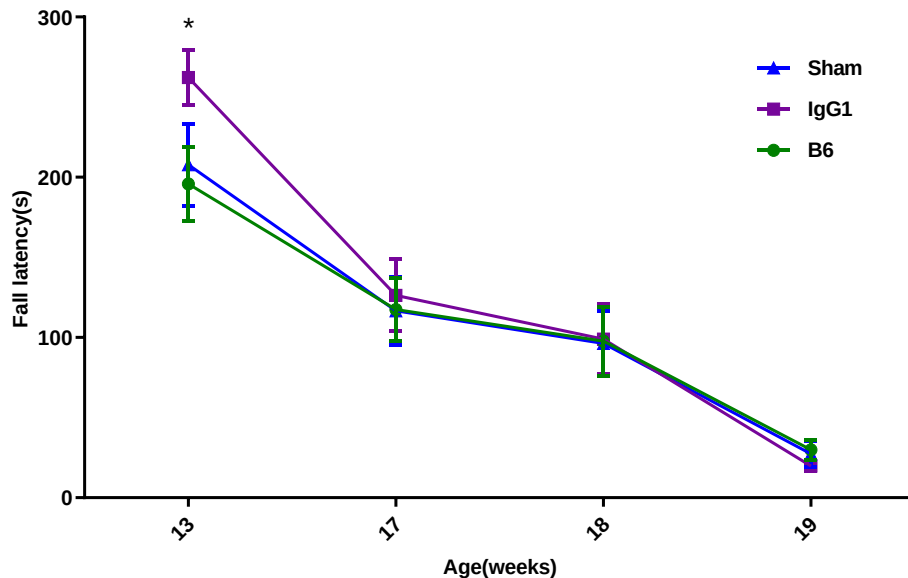


Figure 73. Grid hanging as a function of age in age by study groups: sham group (blue), B6-treated hTauP301S mice (green) and hTauP301S mice receiving the control antibody (purple). Values represent the mean $\pm$ SEM, $n=40$: * $p<0.05$ compared to B6-treated hTauP301S mice. See text for details.

5.4.2.4. Rotarod

ANOVA-RM for all the average of the fall latency (**Figure 74A**) of the three groups revealed a significant treatment effect respect to the time ($p<0.0001$). At week 16 the IgG1 group started to be significant compared to the Sham group ($p=0.0341$) and at week 18 the B6 group become more significant compared to the IgG1 group ($p=0.0028$).

In the case of acceleration (**Figure 74B**) was the same ANOVA-RM for all the average of the acceleration of the three groups revealed a significant treatment effect respect to the time ($p<0.0001$). At week 16 the B6 group started to be significant compared to the IgG1 group ($p=0.0214$) and at week 18 it increased ($p=0.001$).

Although the difference in the latency to fall and the acceleration between the B6 and IgG1 grew with age, the age x treatment interaction did not reach significance ($p=0.068$ and $p=0.2306$).

ANOVA-RM for the maximum value of the fall latency (**Figure 75A**) of the three groups revealed again a significant treatment effect with respect to the time ($p<0.0001$). In this case, it is only significant at 18 weeks, where the B6 group started to be significant compared to the IgG1 group ($p=0.0095$).

In the case of acceleration (**Figure 75B**) the same ANOVA-RM for all the averages of the acceleration of the three groups revealed a significant treatment effect with respect to the

time ($p < 0.0001$). At week 16 the B6 group started to be significant compared to the IgG1 group ($p = 0.0366$) and at week 18 it increased ($p = .0072$).

Although the difference in the latency to fall and the acceleration between the B6 and IgG1 grew with age, the age $\times$ treatment interaction did not reach significance ($p = 0.075$ and $p = 0.2306$). Therefore, the older age and the longer the treatment time, the better the B6 group's fall time and the longer they stayed in the rotarod compared to the other two groups. So, the treatment improves at the motor level in the hTauP301S mice from 16 weeks onwards.

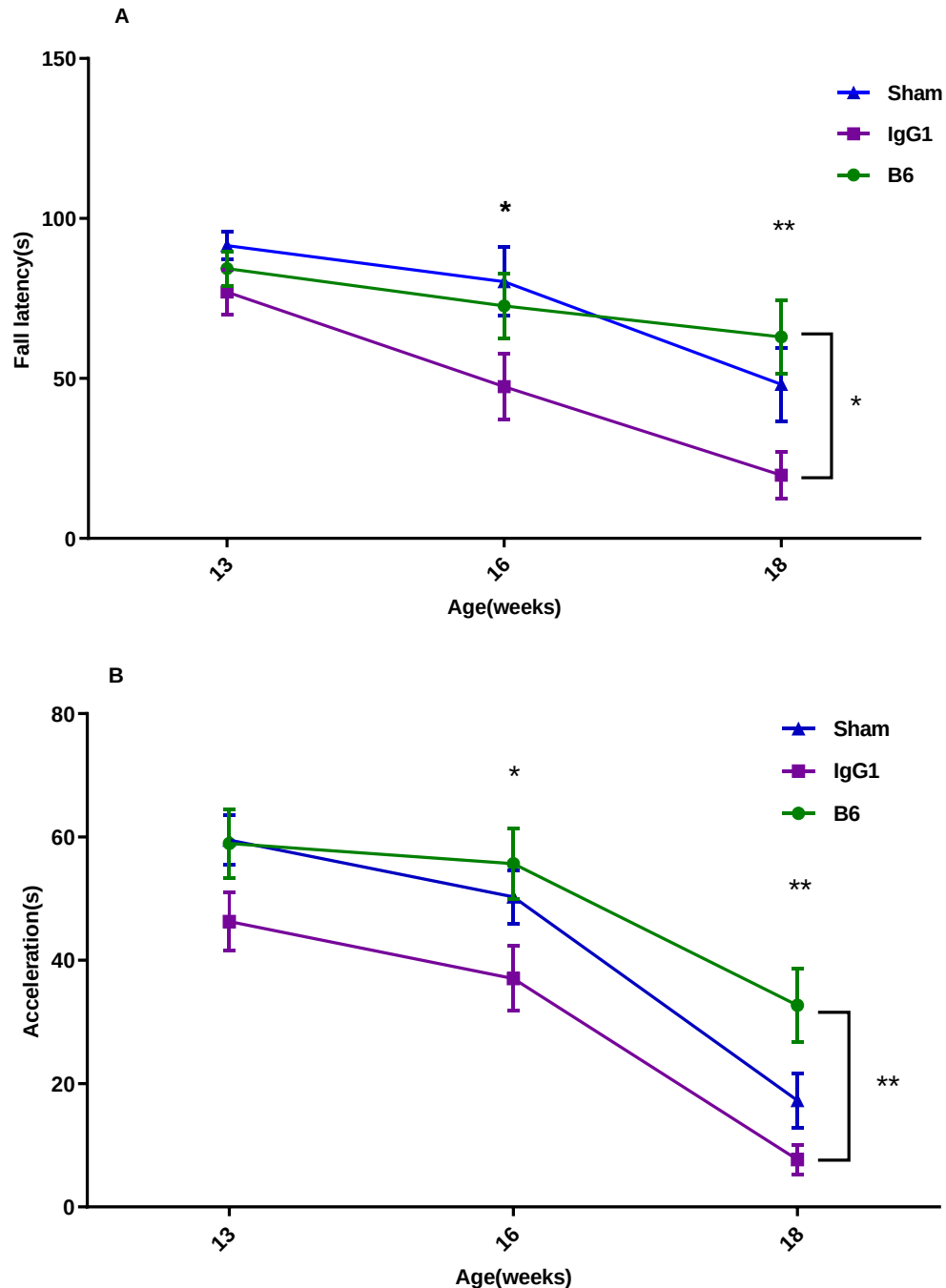


Figure 74. Rotarod performance at 16, 18 and 20 weeks of age in the average of the study groups: sham group (blue), B6-treated hTauP301S mice (green) and hTauP301S mice receiving the control antibody (purple). **A.** Fall latency to the maximum value of the 120 sec. **B.** Acceleration to the maximum value of the 120 sec. Values represent the mean $\pm$ SEM, $n = 40$; * $p < 0.05$ and $p < 0.001$ compared to IgG1-treated hTauP301S mice. See text for details.

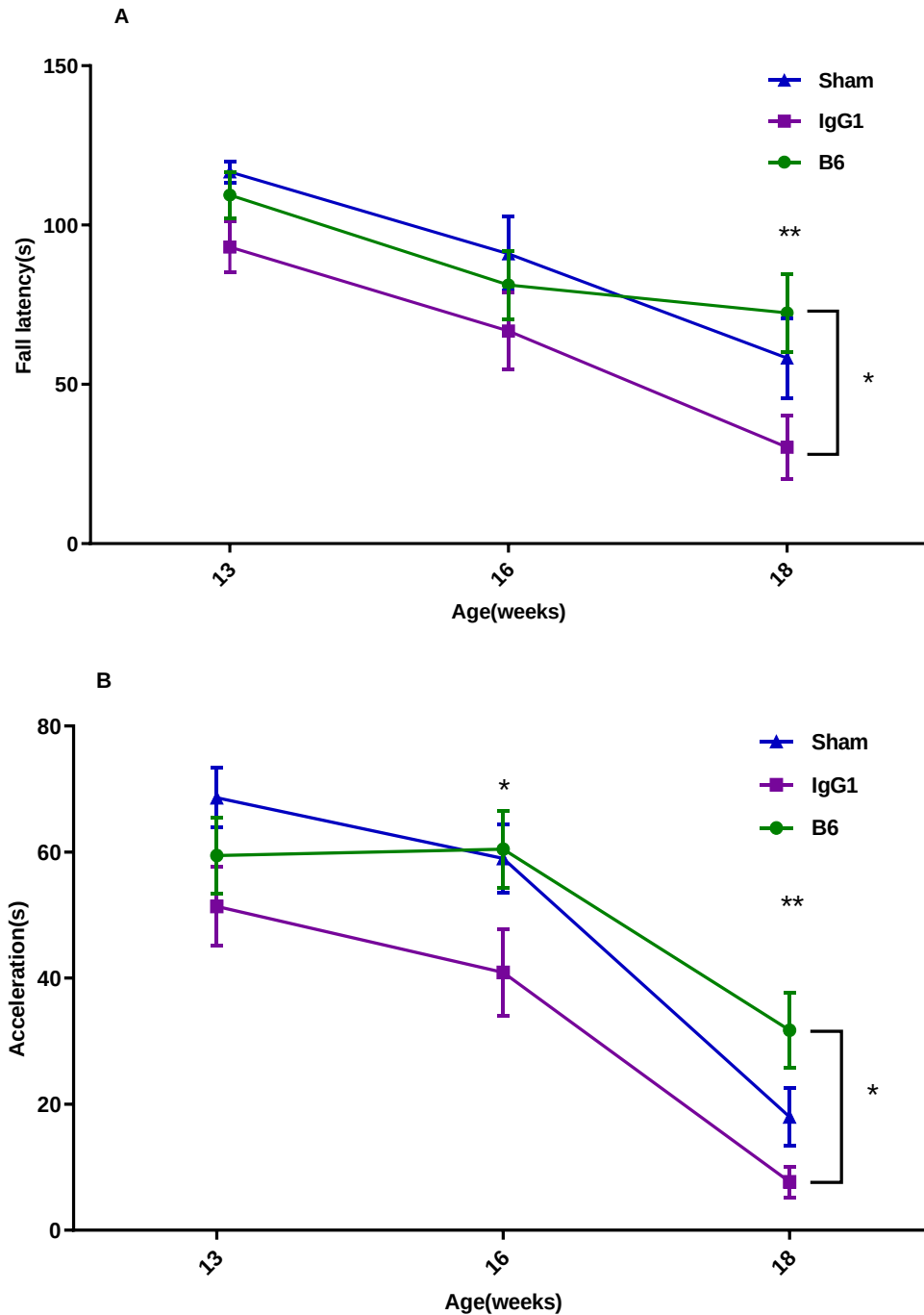


Figure 75. Rotarod performance at 16, 18 and 20 weeks of age in maximum value of of the study groups: sham group (blue), B6-treated hTauP301S mice (green) and hTauP301S mice receiving the control antibody (purple). **A.** Fall latency to the maximum value of the 120 sec. **B.** Acceleration to the maximum value of the 120 sec. Values represent the mean $\pm$ SEM, n=40: *p<0.05 and p<0.001 compared to IgG1-treated hTauP301S mice. See text for details.

5.4.3. Therapeutic effect of B6 antibody on Western Blot Analysis

At the age of 5.5 months there were no differences in p-tau levels in Bs or Hc, in any of the ICV treatment groups. ICV B6-antibody treatment for 4 or 8 weeks does not evoke

measurable differences in p-tau protein levels in P301S mouse Bs or Hc based on the biochemical analyses as WB (**Figure 76**).

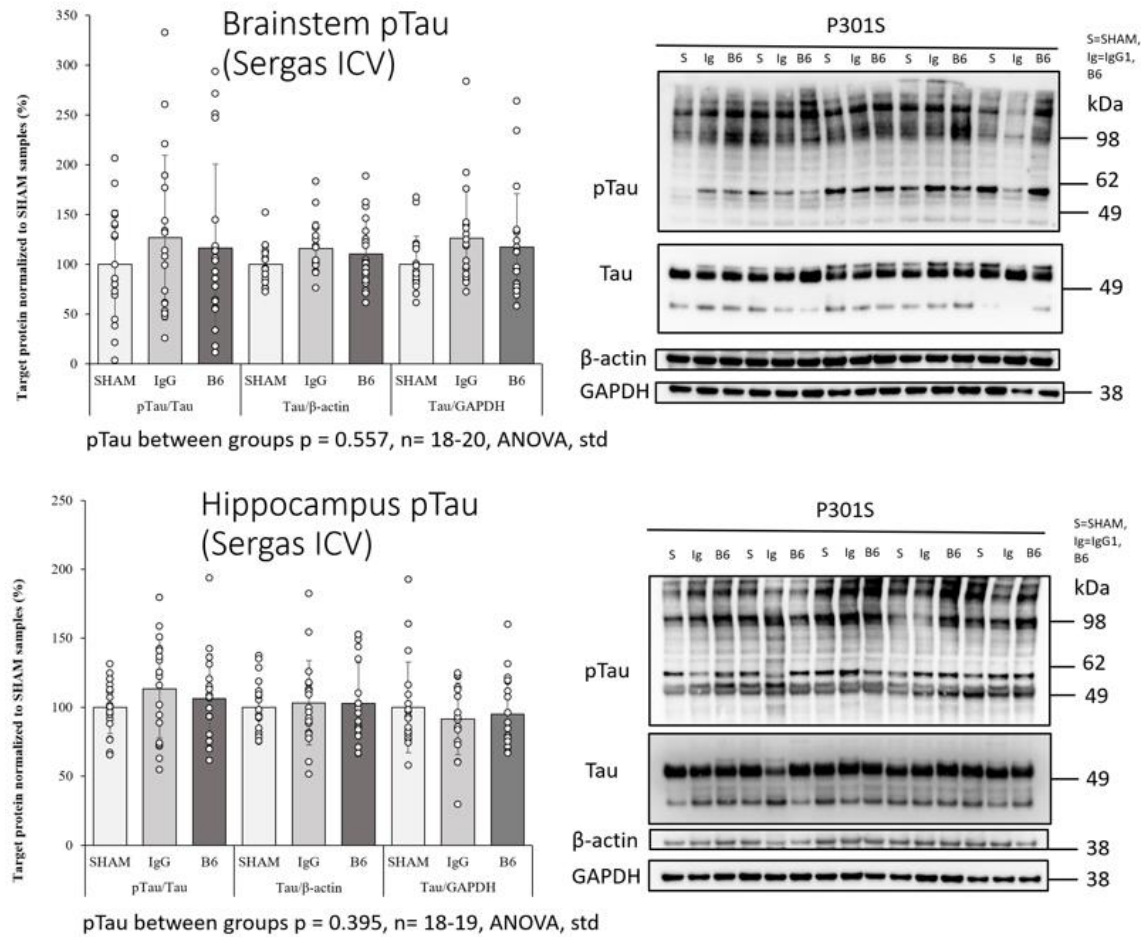


Figure 76. Biochemical analyses by WB of p-tau and total Tau levels in P301S mouse Bs or Hc showing no significant differences between treatment groups.

5.4.4. Therapeutic effect of B6 antibody on Immunohistochemical analysis

At the age of 5.5 months there were significant differences in p-tau levels in Hc and Cx, in intraventricular treatment groups. ICV B6-antibody treatment for 4 or 8 weeks reduced p-tau protein levels in P301S mouse cortex and Hc based on the IF analyses (**Figure 77**).

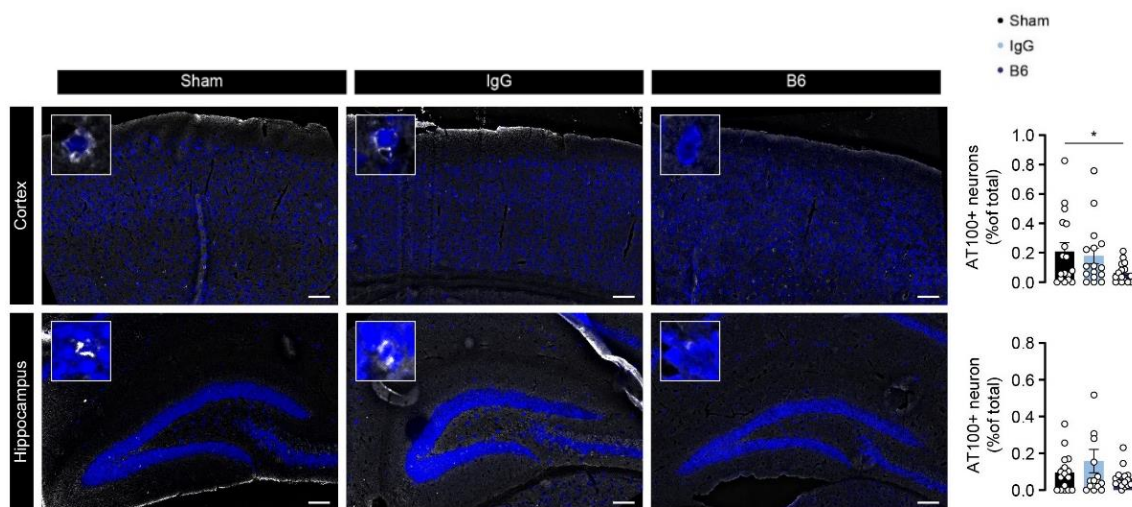


Figure 77. IF analyses of p-tau protein levels in P301S mouse Cx and Hc by treatment groups. Values represent the mean $\pm$ SEM, n=20: *p<0.05 compared to Sham and IgG1-treated hTauP301S mice. See tex for details.

5.5. PET IMAGING, PK AND BD OF RADIOLABELED ^{89}Zr -B6 IN hTAUP301S MICE

5.5.1. ^{89}Zr radiolabeling of B6 antibody

The protocol used for radiolabeling was adapted from the one described by Vosjan et al. (2010). The reaction yield was quantified by iTLC, right after chelation and after 3 washings with PBS pH 7.4 using Amicon 100K filters. Over 90% of the ^{89}Zr was chelated to the antibodies, and after three column washings, the percentage that finally was administrated to mice got up to 98% (**Figure 78**).

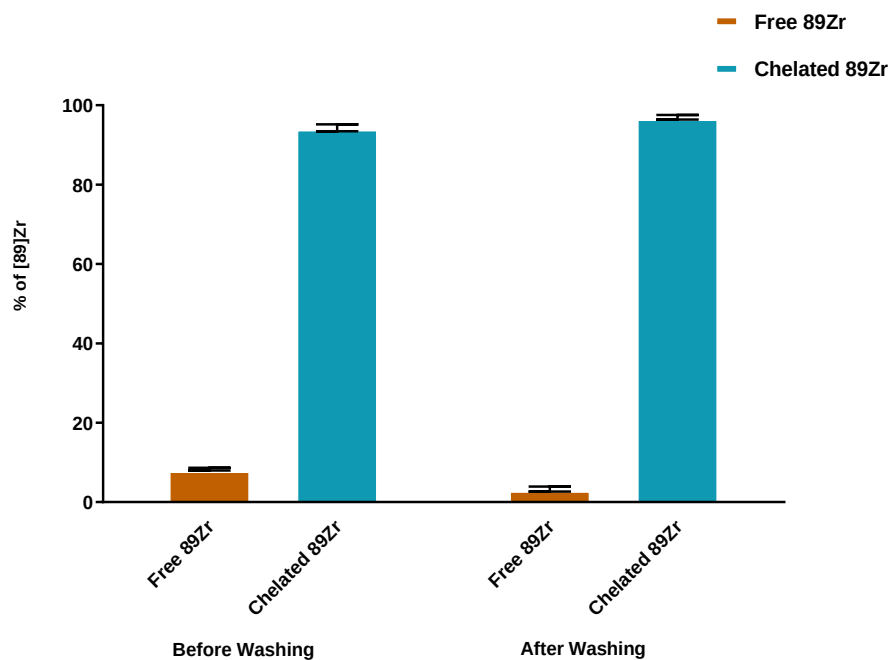


Figure 78. Histogram confirming the radiolabeling yield of the B6 antibody, before and after the washing through Amicon 100K filters.

5.5.2. PK and BD of differential administration of ^{89}Zr -B6 in C57/BL6 mice and hTauP301S.

5.5.2.1. Pharmacokinetics

Blood samples (between 10 and 30 μL) were taken from tail vein and immediately measured in a well-counter at 0.5, 1, 6, 24, 48, 72, 96 and 168h timepoints. The curves showed a typical long-life profile of the of the ^{89}Zr -B6 in circulation from $t=0.5\text{h}$ to $t=168\text{h}$. The activity decreased faster at the beginning and slower as time passes. The profile confirms a long-life in circulation of the antibodies. (**Figure 79**).

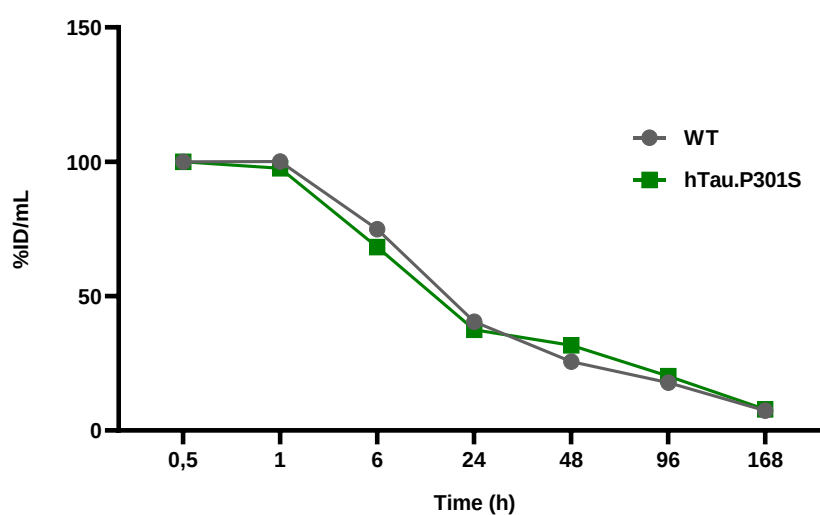


Figure 79. Pharmacokinetic profile of the ^{89}Zr -B6 in circulation from $t=0.5\text{h}$ to $t=168\text{h}$ after tail vein administration in C57/BL6 (wt) and hTau.P301S mice. Data represented as %ID/mL.

5.5.2.2. Biodistribution

5.5.2.2.1. Ex vivo

For assessing the BD profiles, 6 hTau.P301S animals (2 animals per timepoint) were IV administered through tail vein with ^{89}Zr -B6. Then, animals were perfused with saline for ex vivo BD on days 1, 3, and 7. Additionally, in order to investigate any possible differences between administration routes, 2 control (wt) mice per group were administered through SC, IP, IV, jugular and IA, carotid injections with ^{89}Zr -B6 and sacrificed after 7 days post-administration (**Figure 80B**). Animals were dissected, and the activity of the liver, spleen kidneys, heart, and brain were measured in a y-well-counter (**Figure 80A**). Data were normalized with the weight of the organ, the administered dose, and activity decay, and expressed as % of administered activity per gram (%ID/g).

BD of the radiolabeled B6 antibody showed little brain entry, and a vast presence in organs like liver, kidneys or spleen. However, brain was the only organ that significantly accumulates signal along the study besides liver, almost triplicating values from day 1 to day 7 (**Figure 80A**). Besides that, the accumulation in liver, spleen, kidneys and heart show significant variations depending on the administration route (**Figure 80B**).

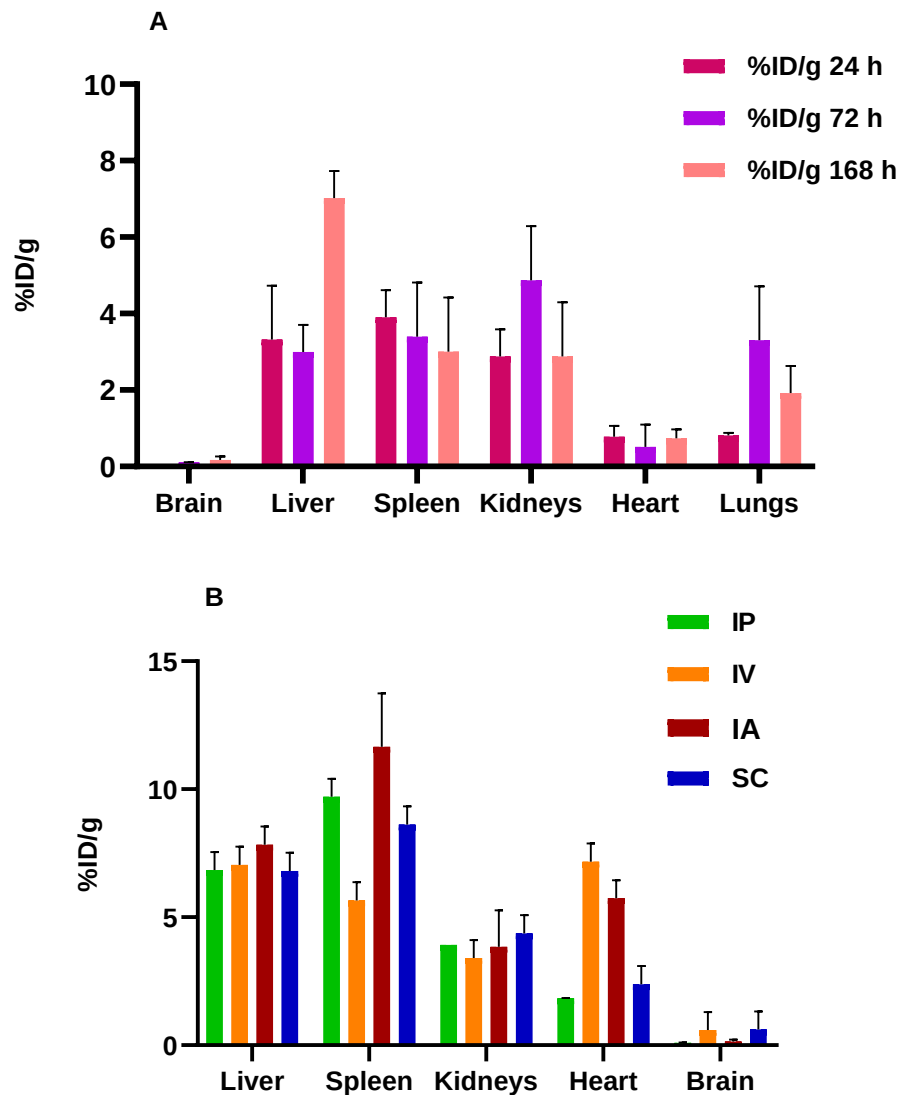


Figure 80. Histograms of ex vivo BD represented in % of injected dose (ID) per gram. **A.** Results of the 3-timepoints of ex vivo BD in hTau.P301S animal model after tail vein administration of ^{89}Zr -radiolabeled B6 antibody. **B.** Results of the comparative study after 4 distinct administration points (IP, intraperitoneal; IV, intravenous; IA, intraarterial; SC, subcutaneous) in C57/BL6 mice. Data normalized with ^{89}Zr activity decay (half-life 78.48h).

5.5.2.2.2. *In vivo*

In a first trial, one C57/BL6 control mouse was IV administrated with the same radioactivity load of free Oxalate- ^{89}Zr as a dosing control, while two hTau.P301S transgenic mice (33276BR and 332775TL) and one more C57/BL6 control mouse more were IV administrated with a 4MBq of radiolabeled ^{89}Zr -B6 antibody.

Mouse Control 2 (top) showed Oxalate- ^{89}Zr in circulation just after administration and bone BD at 1d and 6d time points, as expected (Fanrong et al. Am J Transl Res. 2016; 8(12): 5591–5600). On the other hand, 33276BR, as well as 33275TL, showed ^{89}Zr -Mab in circulation just after administration but it remained in circulation even after 7 days post-administration, thus ensuring the in vivo stability of the ^{89}Zr -Mab radiolabeling. There was a bit of extravasation of the radiolabeled antibody when administrated IV to the mouse 33275TL, however, during the next hours the antibody was posteriorly reabsorbed and distributed all over the organism as in the case of the second 33276BR mouse (**Figure 81**).

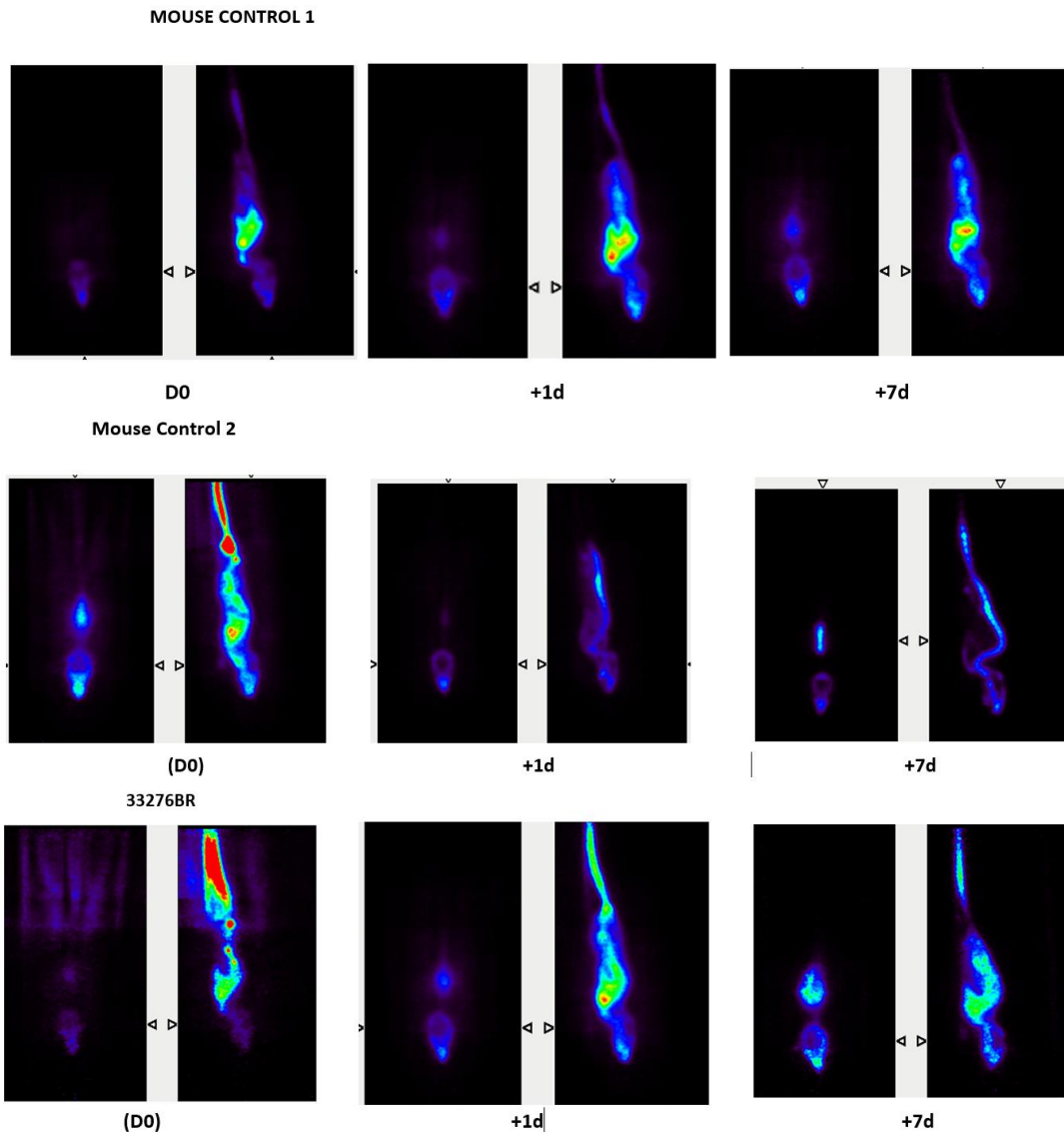


Figure 81. PET imaging of Oxalate- ^{89}Zr and ^{89}Zr -B6 administered through tail vein in CBA.C57/BL6 (wt) and hTau.P301S mice at $t=0\text{h}$, $t=1\text{ day}$ and $t=7\text{ days}$. From top to bottom: Control mouse administered with ^{89}Zr -B6, C57/BL6 mouse administered with Oxalate- ^{89}Zr , hTau.P301S mouse administered with ^{89}Zr -B6.

The drawback observed in the preliminary tests was the absence of a PET signal in the brain region. This may point to that the antibody does not seem to be able of crossing the blood-brain barrier (BBB) and entering the CNS. The PET BD observed in the second experiment comparing three different administration routes with the same amount of antibody did not report any significant differences for the first 24h. However, the presence of radiolabeled antibodies at the beginning of the experiment in the heart was higher after the IV and IA administrations. These results also confirmed the absence of detectable signals in the brain area by PET and the long-circulating life of the radiolabeled antibody (**Figure 82**).

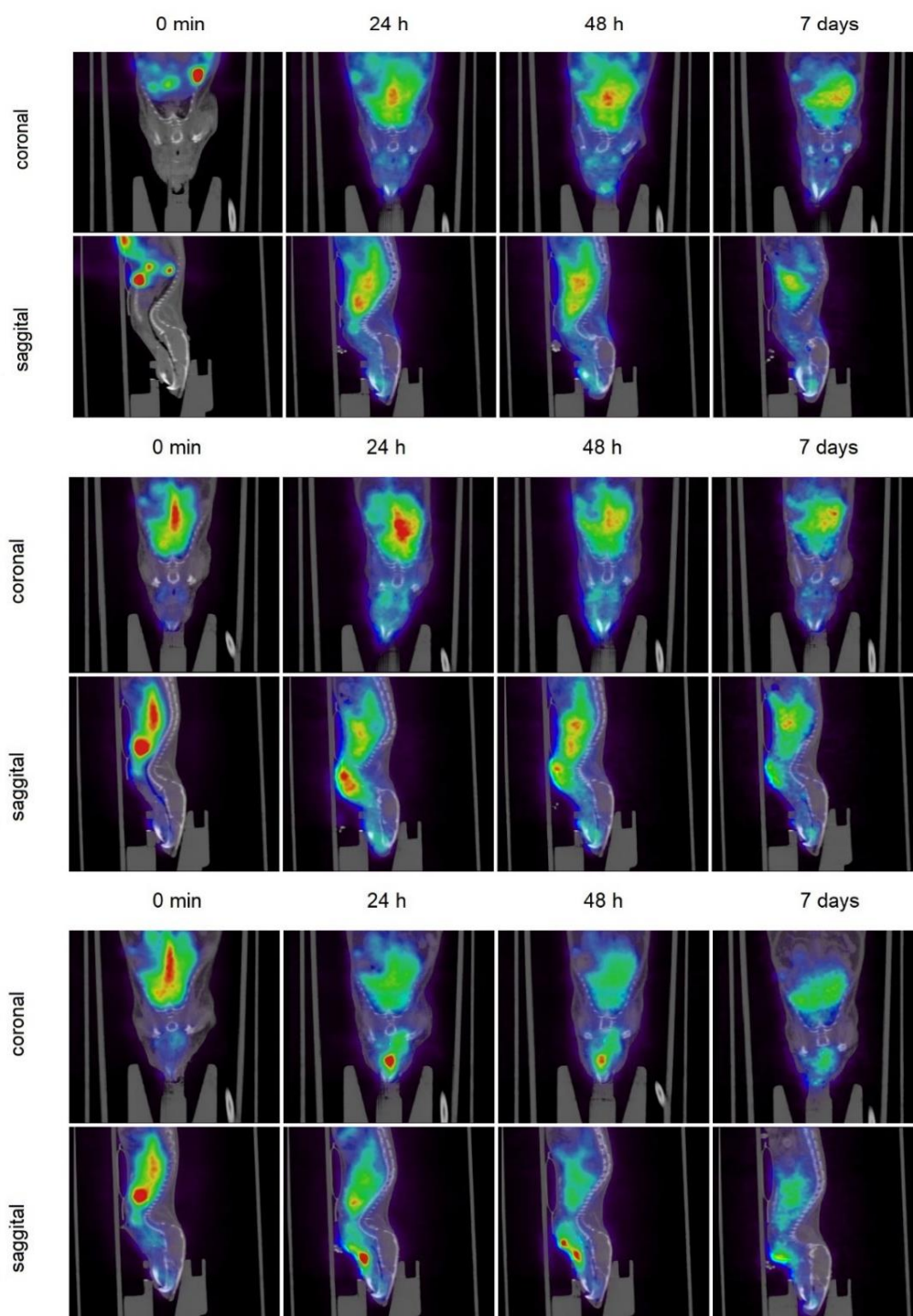


Figure 82. PET/CT imaging at times 0, 24, 48 and 168h after IP (top), IV (mid) and IA (down) administration of B6 in CBA.C57/BL6 (wt) animals.

5.6. PET IMAGING, PK AND BD OF RADIOLABELED ^{189}Zr - B6 F(ab')₂ FRAGMENTS IN C57/BL6 MICE.

For some applications, it is useful to study or make use of the activity of one portion of an immunoglobulin without interference from other portions of the molecule. It is possible to selectively cleave the immunoglobulin molecule into fragments that have discrete characteristics. Antibody fragmentation is accomplished using reducing agents and proteases that digest or cleave certain portions of the immunoglobulin protein structure. Although fragmentation of all immunoglobulin classes is possible, only procedures for fragmentation of mouse, rabbit, and human IgG and IgM have been well characterized.

Several types of antigen-binding fragments are possible, but each contains at least the variable regions of both heavy and light immunoglobulin chains (VH and VL, respectively) held together (usually by disulfide bonds) to preserve the antibody-binding site. Fc fragments consist of the heavy chain constant region (Fc region) of immunoglobulin and mediate cellular effector functions. Because of their smaller size as functional components of the whole molecule, antibody fragments offer several advantages over intact antibodies for use in certain immunochemical techniques and experimental applications:

- Reduced nonspecific binding from Fc interactions, because many cells have receptors that bind the Fc region.
- Ability to control Fc-binding to Protein A or Protein G in experiments involving immunoprecipitation and WB.
- More efficient penetration of tissue sections, resulting in improved staining in IHC.
- Potentially higher sensitivity in antigen detection in solid phase applications as a result of reduced steric hindrance from large protein epitopes
- Elimination of Fc-associated effector functions (e.g. complement fixation) in antigen-antibody binding studies.
- Simpler system for studying the structural basis for immune recognition using X-ray crystallography or NMR.
- Lower immunogenicity than intact antibody for experiments in vivo

The objective of this series of experiments is to confirm the possibility to conjugate the B6 F(ab')₂ fragments obtained through enzymatic digestion with NCS- desferrioxamine (DFO) for determining its pharmacokinetics and BD in mice.

5.6.1. B6 Ficin digestion and F(ab')₂ fragments purification

The enzymatic digestion of the B6 antibody was performed with the kit Pierce Mouse IgG₁ Fab and F(ab')₂ Preparation Kit (Ref. 44980). As a first approach with the Ficin Kit we used 0.25 mg of B6 antibody for each of the digestion conditions for obtaining Fab and F(ab')₂. All concentration changes and buffer exchanges were performed with 0.5 mL Amicon 30k and 100k filters, depending on the fragment/antibody.

F(ab')₂ analyzed by non-reducing and non-boiled SDS-PAGE migrates with an apparent molecular weight of 75 kDa. The IgG digest purified by Protein A was free of Fc fragments and contain Fab or F(ab')₂ (Lane 10, **Figure 83A**). Undigested IgG, Fc or Fc-containing fragments was in the elution fraction (Lane 11, **Figure 83A**).

The B6 F(ab')₂ fragments were successfully obtained and purified, as shown in **figure 83 B**. It was also confirmed its similar functionality to the complete B6 antibody in an indirect ELISA against the 188-217 p-tau (pS202, pT206, pS208) synthetic peptide.

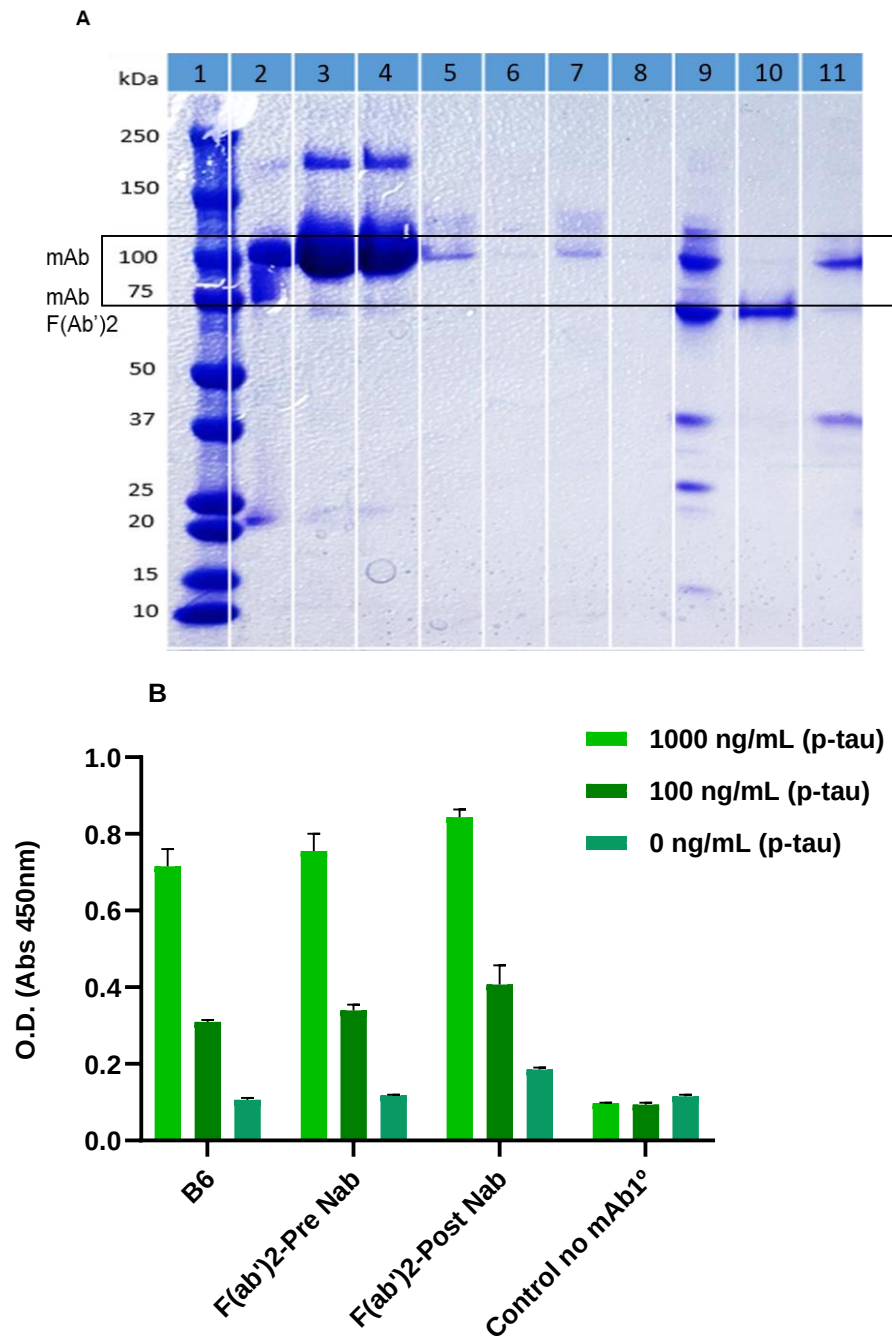


Figure 83. Quality control of the B6 mAb enzymatic digestion. **A.** SDS-PAGE non-reducing gel image displaying the products of B6 and B6 F(ab')₂ fragmentation and chelation. Lane 1: Molecular weight (MW); lane 2: B6 (110kDa); lane 9: B6 Ficin digestion pre-purification; lane 10: B6 F(ab')₂ purified fragment (75kDa) (Protein A column); lane 11: Eluted Fc and non-digested fractions. **B.** Quality Control of B6 and F(ab')₂ digested fragment by ELISA against the specific p-tau antigen. Concentrations used: 5 µg/mL of antibody and 1:1000 2° anti-mouse antibody.

5.6.2. B6 F(ab')₂ fragment conjugation with NCS-DFO

The antibody-DFO conjugation method is based on the same used for chelating the complete B6 antibody and described by Vosjan *et al.* (2010). The DFO crosslinker used in this case were complexed with Fe, allowing a finer control of the chelating process through HPLC and 430nm absorbance. The antibody-DFO molar ratio used was 1:2.

Again, ELISA quality control of the free and chelated fragments confirmed the retained functionality of the complete antibody and adequacy for radiolabeling with ^{89}Zr (**Figure 84**).

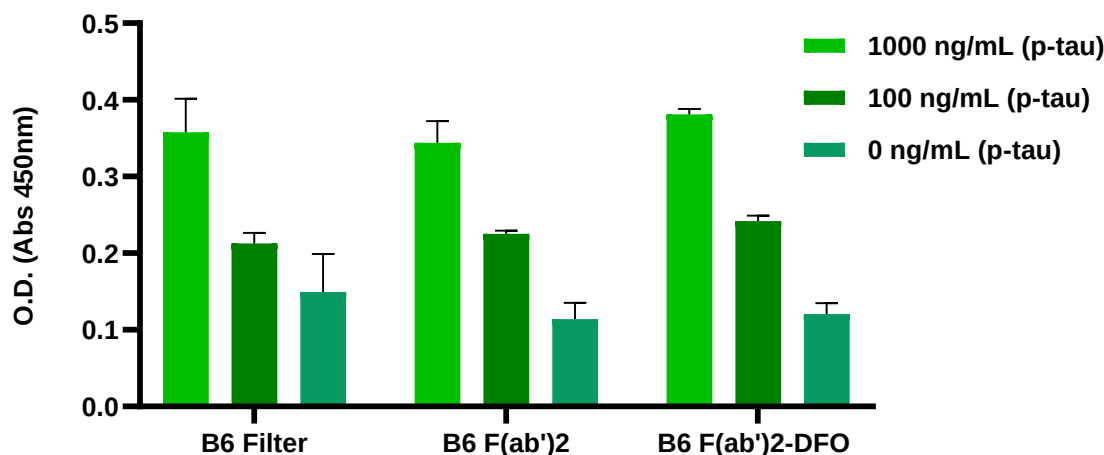


Figure 84. Quality Control of B6 and F(ab') digested fragment by ELISA. Against the specific p-tau antigen (1000, 100, 0 ng/mL). Concentrations used: 1 $\mu\text{g/mL}$ of antibody and 1:1000 2^o anti-mouse antibody.

5.6.3. B6 F(ab')₂ radiolabeling

The radiolabeling of the F(ab')₂ fragment was completed as indicated in methods section, following the method described by Vosjan et al. (2010). After the incubation with the free ^{89}Zr , the product was filtered by centrifugation to enrich the conjugate, achieving a 97.42% of conjugated ^{89}Zr (**Figure 85**). The product was then loaded and administered to the animals.

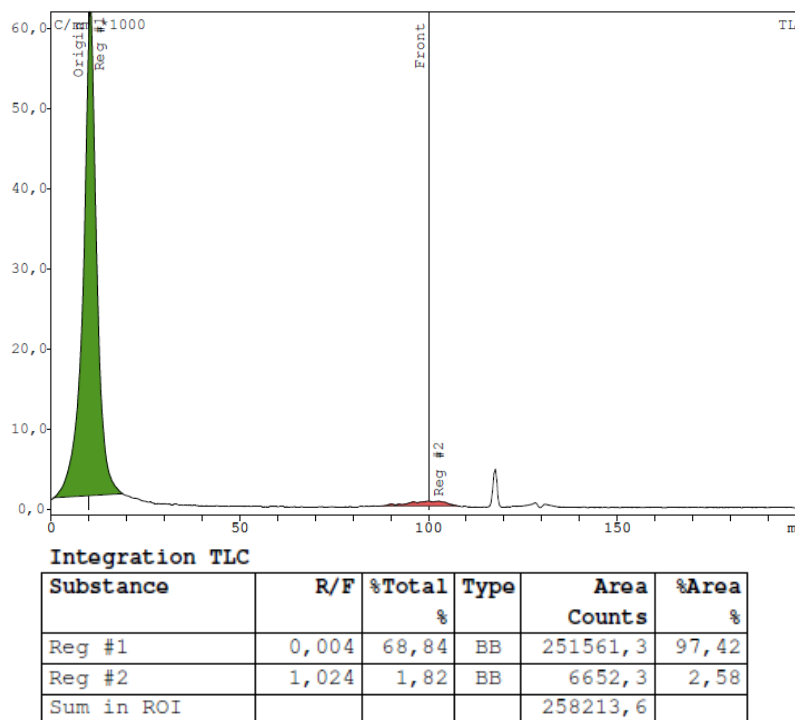


Figure 85. Radio-TLC chromatogram showing the purity of the radiolabeled B6 F(ab')₂ fragment. Green peak (Reg #1) represents the abundance of chelated ^{89}Zr and the red peak (Reg#2), the free ^{89}Zr . The table shows the integration of the chromatogram peaks.

5.6.4. BD and PK of B6 F(ab')₂-¹⁸⁹Zr in C57/BL6 mice.

5.6.4.1. Pharmacokinetics

Three C57/BL6 mice were IV, jugular administrated with the radiolabeled products. Two of them received a bolus of 15 microCi of product and a third one received a bolus of free Oxalate-¹⁸⁹Zr, as control of the presence of the radioisotope.

Blood samples (10 µL) were taken from tail vein and immediately measured in a well-counter at t= 0, 0.5, 1, 4, 24, 48, 96, 120 and 168h. The curves showed a faster profile of the of the ¹⁸⁹Zr-B6 F(ab')₂ in circulation compared to the complete B6 antibody (**Figure 86**). The activity decreases faster at the beginning, showing a multiphasic pharmacokinetics for all administered products.

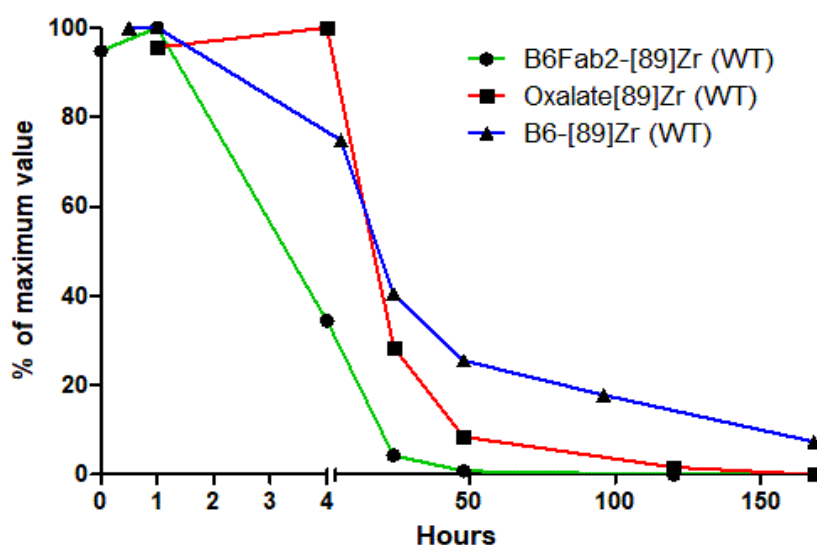


Figure 86. Pharmacokinetics profile of B6 F(ab')₂ fragment compared to complete B6 and Oxalate-¹⁸⁹Zr in C57/BL6 mice. Data from B6-¹⁸⁹Zr taken from a previous study. Data represented as % of measured activity per timepoint.

The rapid blood clearance of the fragment could be related to the smaller molecular weight and the fact that is not a “native” complete protein. This could expose it to a much rapid proteolytic activity. Also, the lower molecular weight compared to the complete antibody seems to lead to a much faster accumulation in kidneys, as observed in *ex vivo* BD (**Figure 80A**) and PET imaging (**Figure 81**). The much shorter half-life of the fragment compared to the antibody is a handicap in its bioavailability and could negatively impact its function as therapeutic agent.

5.6.4.2. Biodistribution

5.6.4.2.1. Ex vivo

After 7 days (168h) animals were sacrificed, perfused with saline, and dissected. The brain, heart, right lung, median liver lobe, spleen, right kidney, abdomen fat tissue, right femur, quadriceps femoris muscle, and Sc (from L1 to Co1) were weighed and measured in a well-counter.

Ex vivo BD analysis at t=168h confirmed the presence of the B6 F(ab')₂ fragment, being primarily accumulated in kidneys, and to a lesser extent in the liver and spleen, with residual presence in the brain and Sc (**Figure 87A**). Oxalate-¹⁸⁹Zr showed significantly different BD, accumulating mainly in the bone, as expected. The presence in the brain was again residual, but slightly higher than the radiolabeled B6 F(ab')₂ (**Figure 87B**).

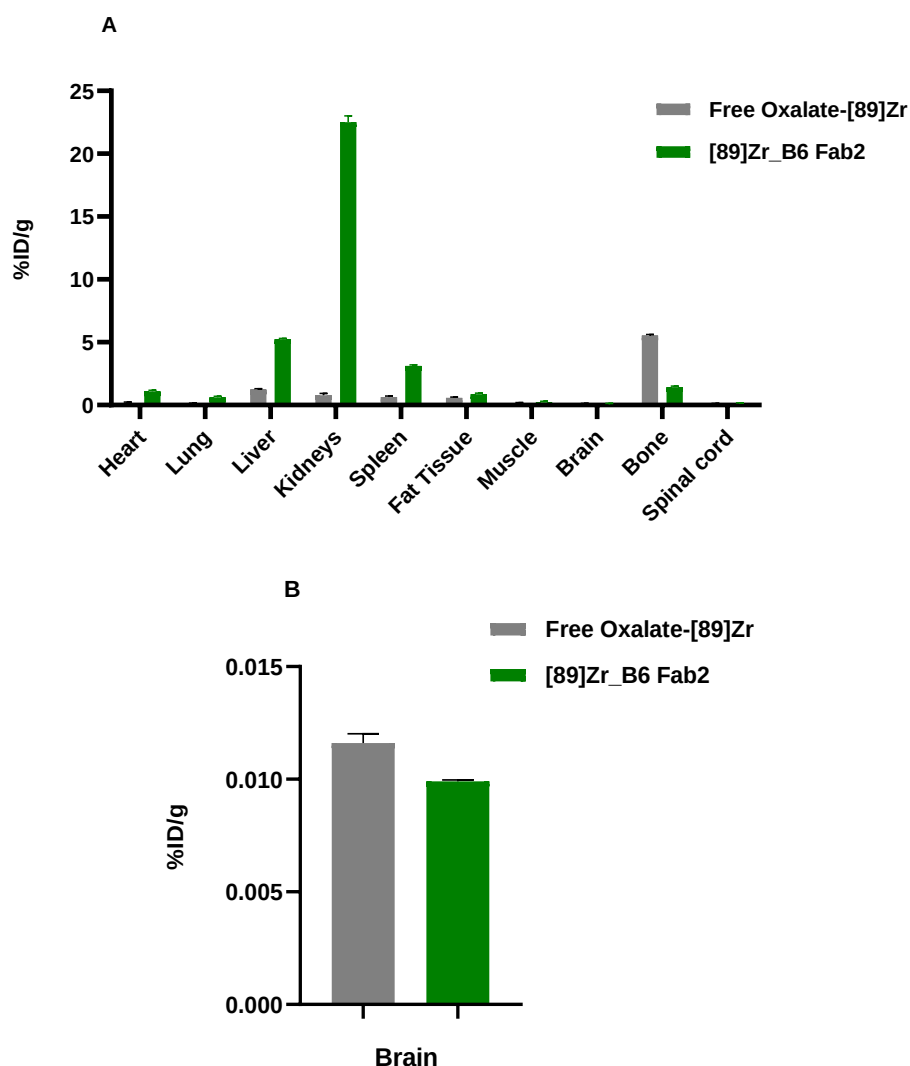


Figure 87. *Ex vivo* BD B6 F(ab')₂. **A.** *Ex vivo* BD measured in activity per gram of jugular administrated B6 F(ab')₂ fragment-⁸⁹Zr in C57/BL6 mice. **B.** In detail, brain values. Data normalized with ⁸⁹Zr activity decay (half-life 78.48h).

Compared to the complete B6 antibody which accumulated primarily in the liver, it seems the primary elimination or accumulation via the fragment is in the kidneys (**Figure 88A**). This differential behavior could be related to the lack of the Fc region, recognized by the ubiquitously expressed FcRn receptors, and the lower molecular weight of the fragment (about 70-80 kDa) compared to the native B6 (>110 kDa) (Pyzik et al., 2015). In fact, according to published data, normal mice glomerular molecular weight cut-off is roughly 70 kDa, which might explain the accumulation of the fragment in the kidneys, but scarce presence in urine (bladder), as observed in PET imaging (**Figure 89**) (Rieg, 2013).

Additionally, measured activity in the brain after radiolabeled B6 F(ab')₂ fragment administration was significantly lower than observed in previous studies, where brain accumulation was time-related and increased significantly along the 7 days follow-up (**Figure 88A**). While only 0.01 %ID/g of B6 F(ab')₂ was detected in the brain after 7 days in the C57/BL6 mice after jugular administration, after tail vein administration of complete ⁸⁹Zr-B6, levels reached roughly 0.07 %ID/g and 0.11 %ID/g in C57/BL6 and transgenic Tau.P301S mice, respectively (**Figure 88B**).

Although levels may slightly vary among studies, administration routes, and animals, it has been demonstrated that the PK and BD of the tested drugs is robust and distinctive.

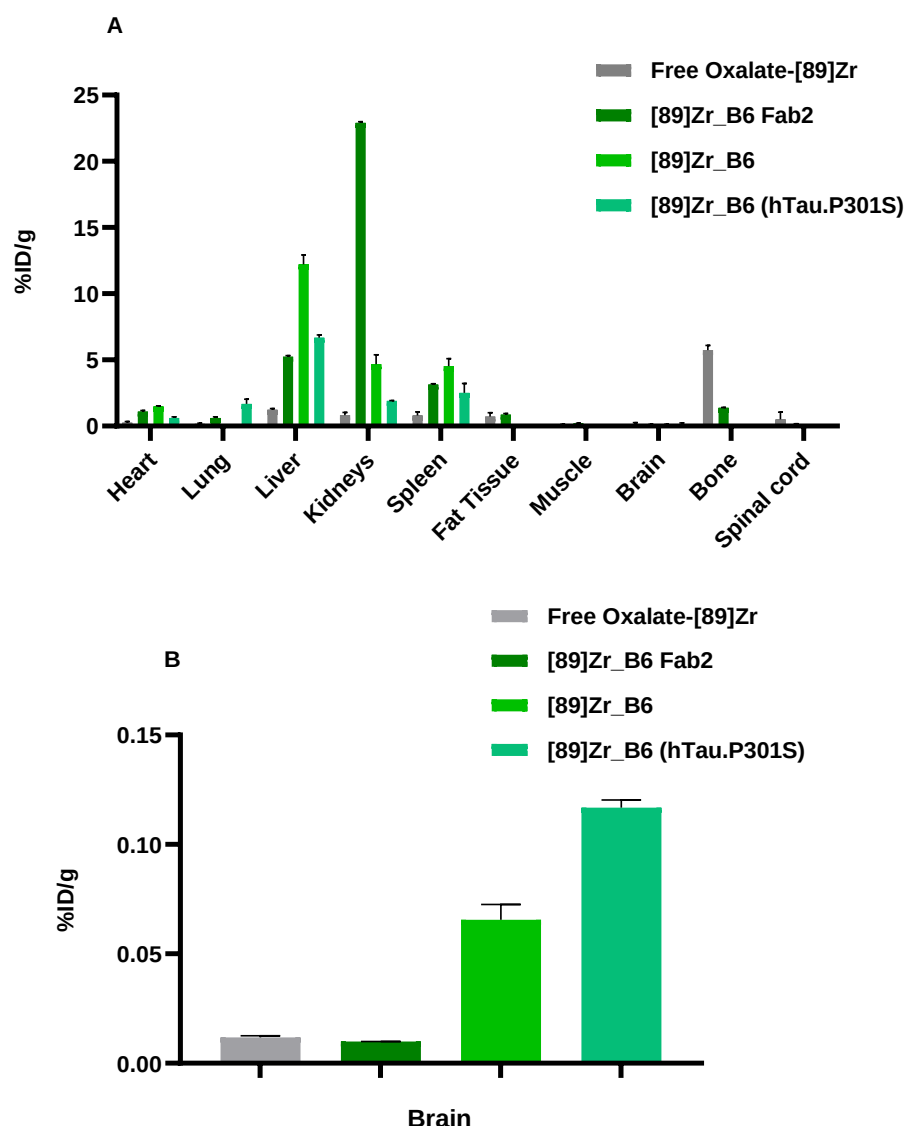


Figure 88. *Ex vivo* BD ^{89}Zr -B6 and ^{89}Zr -B6 F(ab')₂. A. *Ex vivo* BD after jugular administrated ^{89}Zr -B6 and ^{89}Zr -B6 F(ab')₂ fragment in both C57/BL6 and hTau.P301S mice represented in %ID/g. B. In detail, brain values. Data normalized with ^{89}Zr activity decay (half-life 78.48h).

5.6.4.2.2. *In vivo*

Animals were anesthetized at each timepoint and full-body PET-CT imaging was acquired at 0, 5, 24, 48, 120 and 168h after administration. **Figure 89** shows timepoints 0, 24, 48 and 168h of 1 animal after the administration of B6 F(ab')₂ plus another animal at timepoints 0h and 168h after the administration of Oxalate- ^{89}Zr , as representative control.

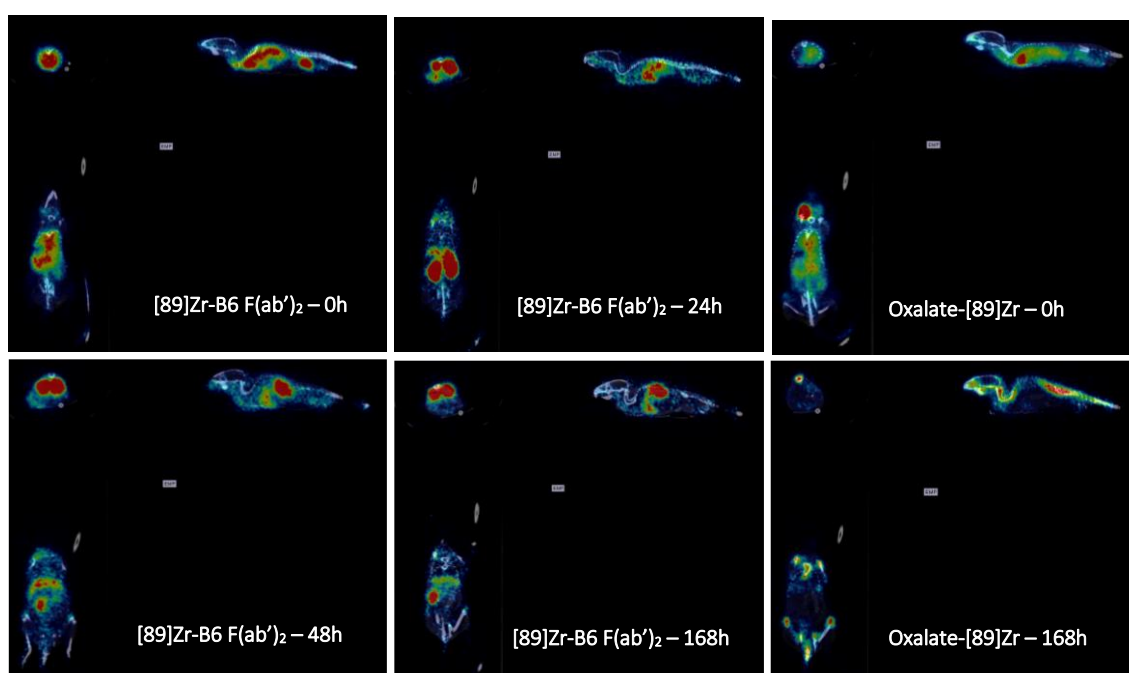


Figure 89. PET/CT imaging at times 0, 24, 48 and 168h after jugular (IV) administration of $[^{89}\text{Zr}]\text{-B6 F(ab')}_2$ fragment and Oxalate- $[^{89}\text{Zr}]$ to CBA.C57/BL6 animals. Animals represented: 102 $[^{89}\text{Zr}]\text{-B6 F(ab')}_2$, complete and 201 (Oxalate- $[^{89}\text{Zr}]$), only timepoints 0 and 168h.

Imaging confirms the pattern observed also in *ex vivo* BD, where the B6 fragment tends to accumulate in kidneys while Oxalate- $[^{89}\text{Zr}]$ chelates to bone, as expected. Quantification of the imaging is shown in **Figure 90** represented as SUV for each of the administered animals and several ROIs localized in the anatomical regions of brain, bladder, bone (femur), heart, kidneys, muscle (rear limb) and spleen.

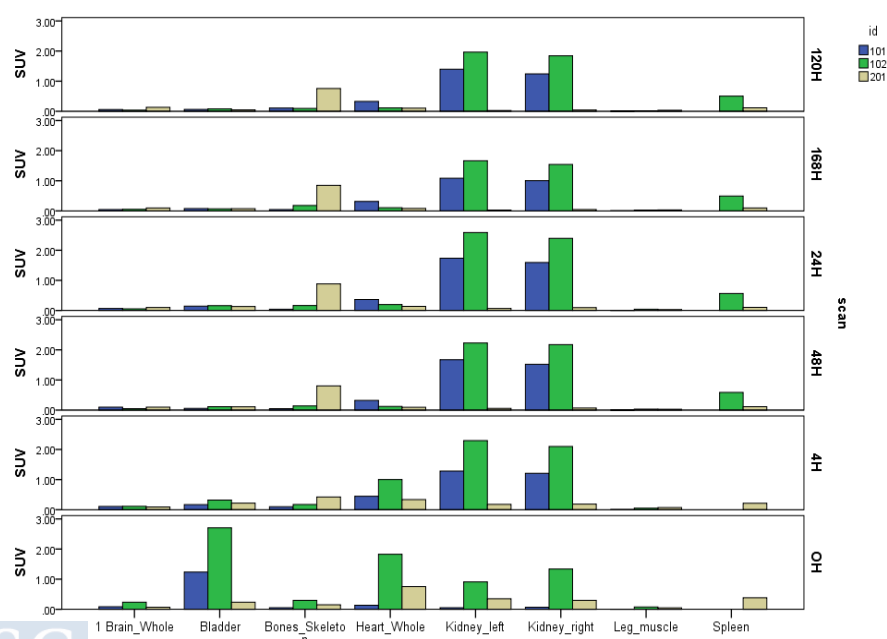


Figure 90. Quantification of PET imaging. Bars plot representing the Standardized Uptake Value (SUV) of each defined Region of Interest (ROI) corresponding to brain, bladder, bone (femur), heart, kidneys, muscle (rear limb) and spleen.

6. DISCUSSION

AD is a progressive and irreversible neurodegenerative disorder, which is the main cause of dementia and loss of autonomy in the elderly and is the fifth leading cause of death worldwide (“2021 Alzheimer’s Disease Facts and Figures,” 2021). In order to slow down the symptoms and effects related to AD, it is necessary to focus on early diagnosis and early treatment in the prodromal or asymptomatic phase of AD, before it develops into MCI that may lead to AD dementia (Long & Holtzman, 2019; Molinuevo et al., 2012).

Despite decades of research and some recent advances in clinical trials, there are still no drugs that can slow the progression of AD, let alone offer a cure. So far, promising preclinical results have repeatedly failed in clinical trials, possibly for a variety of reasons: unsuitable animal models, focusing on targets that have not yielded therapeutic results, conducting clinical trials in advanced stages of AD, poor clinical trial design, etc. (J. L. Cummings et al., 2014). Given this situation, there is a broad consensus on the need to develop new diagnostic methods to detect the disease in early stages and subsequent therapeutic approaches. The early stages of the disease, both the asymptomatic preclinical and the symptomatic prodromal stages, predecessor stages of AD, are under constant study to better define them as they would be the basis for action to prevent the progression of AD disease. To date, the research efforts (in both therapy and diagnostics) have mainly focused on understanding and combating the progression of amyloid plaques around neurons, which is one of the main etiopathological characteristics of the disease. As progress in finding an effective therapy in this area has not yet crystallized, researchers are paying increasing attention to other molecular mechanisms related to the disease, such as the aggregation of the microtubule-associated protein tau into NFTs within cells (Molinuevo et al., 2012). Indeed, a feature of AD is the propensity of tau protein to form oligomers that act as a template for the conversion and co-aggregation of endogenous proteins, which eventually form NFTs and are relevant in the progression of the disease (Jahn et al., 2010). In this regard, analysis of the different phosphorylated forms of tau in the brain that has been carried out in CSF and blood are good biomarkers for the detection and monitoring of AD, as tau protein levels and disease progression correlate (Janelidze, Mattsson, et al., 2020; Karikari et al., 2020; Smirnov et al., 2022).

Given the important role played by the tau protein in the progression of AD, the development of new biomarkers and subsequent antibodies specific for different residues of the tau protein, as acK174 tau recognized by E11mAb and pS208, pS202 and pT205 tau recognized by B6 mAb, could contribute significantly to the fight against the AD in two ways:

1. Development of new diagnostic methods for early diagnosis of the disease.
2. Development of new modifying therapies based on targets directed at those species of the tau protein that may contribute to the development of the pathology.

Moreover, as mentioned above, given that there is no clinical treatment available capable of resolving or slowing down the course of the disease, in the future, it could be a potential tool for early diagnosis by targeting hyperphosphorylated and acetylated tau protein. The toxic effect of tau aggregates would be avoided with an immunotherapy effect, as tau can have prion-like behavior, producing misfolded proteins involved in pathogenic mechanisms of different tauopathies including AD (Colin et al., 2020).

This can be considered a pioneering study in theranostics of AD, since two new mAbs against pTau and acTau were developed and characterized. Specifically, Epitope Mapping confirmed that pTau B6 mAb detects the core sequence that contains the phosphorylation 207G(pS)R209. The acTau E11 mAb specifically detects the core sequence that contains acetylation 171IPA(acK)174. Importantly, WB analysis showed a Braak stage-dependent

differential band pattern for both p-Tau B6 and ac-Tau E11 mAbs in human samples. It is noteworthy that the pTau B6 mAb detected both oligomers and dimers of p-Tau from early stages of the Braak Scale, indicating its potential *in vivo* therapeutic and diagnostic usefulness.

Two novel Sandwich ELISA kits with diagnostic purposes in CSF samples of AD patients were developed for detection of acetylated and phosphorylated tau by using the acTau E11 and pTau B6 mAbs, respectively. Notably, a clinical proof of concept study with patients with MCI who did or did not progress to early or advanced AD was performed. The results showed that all the tau biomarkers, including acTau and pTau208, as well as those of t-Tau and p-Tau181 from the Fujirebio Lumipulse system (gold standard) show the same behavior, with an increase in the levels of the isoforms of tau in CSF as the disease progresses, although the results are not statistically significant due to the small sample size and the dispersion of data. Therefore, future, larger, multicenter clinical trials are necessary to validate these promising results.

From a therapeutic point of view, *in vitro* toxicity studies demonstrated that pTau B6 mAb did not exert toxic effects for neurons. Furthermore, *in vivo* systemic administration of the pTau B6 mAb during 4 months did not cause toxicity in the liver and kidney, suggesting that it might be safe for both immunotherapy and radiotracer studies. In this sense, a 4-week treatment with ICV administration pTau B6 mAb demonstrated a clear beneficial therapeutic effect in terms of delayed appearance of hindlimb claspings and latency to fall from the rotarod. Moreover, this ICV administration pTau B6 mAb treatment for 4 weeks mediated a great reduction of p-Tau levels in Cx and Hc. Therefore, the most straightforward interpretation of the findings is that B6 immunotherapy is able to slow down the motor and neurological impairment in the hTauP301S mouse model due to reduction of tau hyperphosphorylation.

Finally, two new stable radiotracers have been developed based on ^{89}Zr radiolabeled pTau-B6 mAb or ^{89}Zr radiolabeled B6 F(ab')₂ fragment with potential utility for labeling brain p-Tau deposits through PET neuroimaging. The pharmacokinetic profile of confirmed that remains in circulation up to 7 days, ensuring the stability of the conjugate and availability of the antibody to reach the periphery or enter the brain. By contrast, the pharmacokinetic profile of the enzymatically digested F(ab')₂ B6 fragment radiolabeled with ^{89}Zr showed a rapid elimination in circulation that could impact negatively the bioavailability and thus the therapeutic potential. Moreover, the biodistribution studies of ^{89}Zr radiolabeled pTau-B6 mAb confirmed a presence of the B6 antibody in brain region between between 0.4 to 1.15% ID/g, according with other IgGs BBB crossing rates in the literature. After 7 days, most of the antibody remained in circulation, liver and kidneys (besides skeleton, spine and muscle), and brain was the only organ where the antibody levels got increased along the study. By contrast, the fast accumulation of ^{89}Zr radiolabeled B6 F(ab')₂ fragment was mainly in the kidneys and the brain uptake is very limited and much lower than the complete B6 antibody (<0.01 %ID/g).

Each particular utility of the acTau E11 and pTau B6 mAbs will be discussed below.

6.1. DEVELOPMENT OF AN INNOVATIVE *IN VITRO* DIAGNOSTIC KIT BASED ON ELISA

In this study, we successfully developed two new Sandwich ELISAs for specific quantification of pS208-tau and acK174-tau based on the B6 and E11 two newly developed antibodies. The B6 antibody demonstrated high specificity for the pS208-tau, requiring its presence for *in vitro* binding to the synthetic peptide. Nonetheless, the presence of the phosphorylations in residues S202 and T205 correlates with a higher binding affinity. The

same phenomenon has been also observed in the case of the well-known AT8 antibody (Malia et al., 2016). Recent studies have stressed the importance of pS208-tau mislocalization, structure, and aggregation dynamics (Xia et al., 2020).

Specifically, some studies have confirmed that the clustering of phosphorylations in residues 202, 205, and 208 strongly promotes the formation of a specific structure that further increases the aggregation of tau (Landrieu et al., 2006; H. Liu et al., 2021). Such conformational change might be also the reason for a more stable antigen-antibody complex with the B6 antibody. Further studies should clarify the mechanisms of such interaction.

On the other hand, the E11 antibody requires the acetylation in lysine 174 for recognition, and the presence of other acetylations, such as the one in lysine 163 does not affect binding affinity in this assay. Acetylation in lysine 174, has been related to reduced tau clearance, aggregation, and tau-induced toxicity (Kamah et al., 2014; S.-W. Min et al., 2010).

About the calibration curves, the standards needed for the quantification of the biological samples should have been recombinant tau proteins with phosphorylation in S208 or acetylation in K174. Initially, an indirect-type ELISA was designed, with the aim of using peptides or samples immobilized by adsorption on the plate, but it was not as sensitive as the Sandwich-type ELISA, as the former uses a detection or primary antibody and the latter uses a capture-type antibody. The Sandwich ELISA is the most widely used for rapid and specific antigen detection as it shows a higher sensitivity compared to the indirect ELISA and can be used to determine absolute antigen concentrations in samples provided purified antigen standards are available, although it requires the use of two different antibodies (Kohl & Ascoli, 2017). So, the indirect ELISA was used to see if the peptides worked and were recognized by the B6 or E11 antibody and their respective modifications and a Sandwich ELISA was designed as the use of an anti-tau trapping antibody adsorbed to the plate would increase the amount of target signal available for detection by both antibodies, because the intensity of the signal is directly proportional to the concentration of the antigen in the samples.

Although finding a compatible pair of antibodies with adequate performance was laborious, it was key to providing increased sensitivity in the detection of tau in biological samples. Detection of the synthetic peptides in aCSF was carried out before moving on to biological samples, as we first made sure that the signal, we had in an artificial matrix could then be reproduced in biological samples without wasting valuable CSF samples from patients with MCI or AD with the different proof-of-concept tests (Meredith et al., 2013).

Once we confirmed the specificity and binding affinity of the custom trapping antibodies B6 and E11 for the control peptides with the tau PTMs of interest, both antibodies were used as trapping elements in the Sandwich ELISA. Subsequent screening of different commercial antibodies (*for details, see Table 22*) allowed us to identify two candidates that finally confirmed the sandwich pairs as a detection element, the anti-t-tau BT2 and HT7. Both antibodies bind to the proline-rich domain of tau protein, close to the B6 and E11, but without competing for the binding site. The sandwich ELISA confirmed by B6-BT2 and E11-BT2/HT7 performed as expected, reaching nanomolar limits of detection with the synthetic peptides and keeping very low intra- and inter-assay CV (3.2-4.5%), similar to other commercially available anti-tau ELISA kits. As for the performance of the ELISAs with clinical samples, the proof-of-concept validation with 89 CSF samples from AD and MCI patients reported specific signals above the LOD for most of the analyzed individuals, while maintaining a low intra-assay CV (6.1-7.5%). (Knight et al., 2018). Notably, the results of the clinical proof of concept study with patients with MCI who did or did not progress to early or advanced AD showed that all the tau biomarkers, including acTau and pTau208, as well as

those of t-Tau and p-Tau181 from the Fujirebio Lumipulse system (gold standard) show the same behavior, with an increase in the levels of the isoforms of tau in CSF as the disease progresses, although the results are not statistically significant due to the small sample size and the dispersion of data. Therefore, future, larger, multicenter clinical trials are necessary to validate these promising results.

The worldwide continuous effort to find new biomarkers-based assays for precision diagnosis and treatment of neurodegenerative diseases is gathering a significant amount of resources and funding. In this study, the preliminary results of the development of two new ELISAs have analyzed two promising tau-related biomarkers, phosphorylation in serine 208 and acetylation in lysine 174. These PTMs have been related to key pathological tau processes in disease progression, either by conditioning tau structure or clearance. Future works should refine the assays for improving sensitivity and quantification range, and ultimately to clinical validation and implementation in automated multiplex platforms. The path to tackle neurodegenerative diseases were required precision medicine and earlier diagnostics, for which the combined use of sensitive assays and clinical imaging is critical (Pathak et al., 2021).

Overall, we can conclude that during this project we have successfully developed an innovative *in vitro* diagnostic kit based on Sandwich ELISA, which has exhibited high targeting affinity and specificity of AD-related phosphorylated and acetylated tau protein in biological samples as CSF.

6.2. CHARACTERIZATION OF B6 AND E11 ANTIBODIES

Epitope Mapping confirmed that despite the immunogens used to elicit the production of antibodies including all phosphorylations and acetylations of interest, the antibodies ultimately selected seem to require only one of them. The peptides used for the hybridomas screening and selection just included either peptides without any of the post-translational modifications, or peptides carrying all modifications, so there was no initial screening for each one of the acetylations or phosphorylations. This is the most common way to screen the hybridomas pool, so the inclusion of all combinations would have duplicated or triplicated the work, making it impossible to screen all 10 mice used in this project. A multiplex assay previously designed to screen and select the best cell lines could have to save workload.

In the case of one of the mAb studied, B6 identified the core sequence, it is quite short (207G(pS)R209) and includes just the phosphorylation in serine 208, which has been linked to a decreased affinity of tau for microtubules. The hyperphosphorylation of tau has been shown to decrease the binding or the affinity of axonal microtubule during axonal growth, which may contribute to tau self-association and the synthesis of neurofibrillary tangles (NFTs) one of the pathogenic molecular types of the AD (Takahashi et al., 2003).

Some works have shown that the serine 208 is phosphorylated by the TTK (Ikezu & Ikezu, 2014) and that is mainly present in CSF of amyloid-positive AD patients instead of brain parenchyma (Barthélemy et al., 2019). Nonetheless, we have already demonstrated that the B6 is capable to bind intraneuronal tau deposits both in hTau.P301S transgenic mouse model in an immunotherapy study.

This data could be both clinically and commercially interesting since there are no commercial antibodies specifically targeting such tau phosphorylation available. Antibodies against t-tau such as Tau-5, Tau-12, or Tau-46 (Chomiak et al., 2022) and against phospho-tau such as T231, the antibody AT100 that recognizes tau protein phosphorylated in the Thr212 and Ser214 and AT8 antibody that recognizes tau protein phosphorylated at both

serine 202 and threonine 205 are commercially available today for WB, IHC and IF (Anderson et al., 2008; Clinton et al., 2010).

In relation to the E11 binding core, the sequence encompasses more residues (171IPA(acK)174) and requires the presence of the acetylation in lysine 174, a post-transcriptional modification ultimately linked to a reduced Ubiquitin-Proteasome-mediated tau clearance. The longer binding core sequence points to a better specificity of the antibody, which is also in accordance with the cross-reactivity test using the HuProt™ Human Proteome Microarray, the weak response of mouse anti-actau E11 mAb strongly indicated a low cross-reactivity against the >20,000 human proteins of the microarray. Compared to other mAbs tested that way, the mouse mAb seemed to exhibit the clearly weakest overall response profile and hence cross-reactivity.

However, further studies with well-characterized samples should be needed to finally confirm the specificity and functionality of the E11 antibody. In any case, it is a promising starting point, thus there are no available mAb against any tau acetylation in the market so far.

Functionality tests have demonstrated the nice performance of the B6 and E11 antibodies in both WB, IF, IHC, and ELISA, with synthetic human and mouse samples, although with some important differences in sensitivity mainly observed by ELISA. Besides that, both antibodies have shown differential band patterns in human temporal cortex samples in a Braak stage dependent on I-VI in WB. The B6 antibody targeting pS208-tau, showed a band pattern with special differences between final Braak stages IV-V, in contrast to the E11 antibody, targeting acK174 tau, which showed primary binding in samples from early stages I to II. So, the E11 antibody detects the specific acetylation in the early stages of AD pathology and the B6 antibody detects the specific phosphorylation in the final stages of AD pathology.

In addition, the same samples of the human temporal cortex used for WB were analyzed by IHC analysis, and the same was corroborated because the B6 antibody stained the PHFs and NFTs as expected in the more advanced Braak stages IV-V. Together these biomarkers could function as a robust and pioneering differential diagnosis of AD, as they cover all Braak stages, from beginning in the stage I to the end in stage VI. Furthermore, the B6 mAb may also work as a nice candidate for immunotherapy studies since it is able to detect tau dimers in brain human samples. It has been shown that the immunotherapies, both *in vitro* and *in vivo*, can be successful in slowing down tauopathy using tau antibodies, which have been screened against relevant tau species responsible for AD progression in order to generate tau immunotherapy. In a recent study, brain regions collected from human cases ranging from Braak stages I-VI such as the Hc and entorhinal cortex were studied. The PHF-1 antibody was used in WB and IHC with the aim of targeting hyperphosphorylated tau, which was detected more in brain lysates such as the entorhinal and visual cortex with high Braak level scale (DeVos et al., 2018). Another study was also carried out with tau antibodies that reduced tau uptake by neurons such as Tau13, Tau46, and HT7 in brain extract from tau transgenic mice and postmortem AD brain samples, but did not obtain significant results in terms of blocking tau uptake at the neuronal level in AD (Nobuhara et al., 2017). More immunotherapy studies are needed but using antibodies specific to tau modifications such as phosphorylations and acetylations which, as we have seen, are more specific biomarkers in both the early and late stages of AD.

6.3. IMMUNOTHERAPY STUDY: INTRACEREBROVENTRICULAR ADMINISTRATION OF B6 ANTIBODY

As for the immunotherapy studies, the antibody was injected ICV administration *in vivo* in hTau P301S transgenic animals, in order to allow the antibody to pass through the BBB more easily by this route, as a previous study by a PANA project collaborator, such as UEF, carried out a 24 mg/kg intraperitoneally dose of B6 antibody in the same transgenic mice, during the same treatment time and study groups. The results showed that systemic administration of the B6 antibody relieved the motor symptoms associated with p-tau accumulation in the brain but only weakly. The most likely explanation for the modest treatment effect was insufficient penetration of the antibody across the BBB.

In regard to the change of the route of ICV administration, the 4-week treatment cohort demonstrated a clear beneficial treatment effect of the B6 antibody in terms of the delayed appearance of hindlimb claspings and latency to fall from the rotarod. The most straightforward interpretation of the findings is that the B6 immunotherapy is able to slow down the motor impairment due to tau hyperphosphorylation but not halt it. In the hTauP301S mouse model, the progression of the pathogenic process is so fast that it mitigates the reduction of p-tau aggregation by the antibody treatment over time. Importantly, in human tauopathies, the rate of tau hyperphosphorylation and aggregation is much slower leaving far more time for the antibody to act. In fact, the effect of B6-antibody treatment for 4 weeks does not evoke measurable differences in p-tau protein levels in the P301S mouse Bs or Hc based on the biochemical analyses. However, immunohistochemical analysis showed a great reduction of p-tau levels in the Cx and Hc in both ICV B6-antibody treatment for 4 or 8 weeks. A possible explanation for the difference found between the results obtained by WB and IHC is that the WB results contain other brain areas not relevant to tau pathology in these animals as the Bs.

Other published studies were conducted intraperitoneally in P301S tau transgenic mice, using another anti-tau antibody HJ8.5, for the same purpose as using the B6 antibody, to determine whether weekly administration of this antibody for 3 months would influence p-tau accumulation, tau insolubility, and neurodegeneration. HJ8.5 significantly reduced the loss of cortical and hippocampal tissue volumes and decrease motor/sensory deficits compared to control and vehicle-treated mice. Thus, they found that systemically administered anti-tau antibodies decreased p-tau accumulation, insoluble tau, and brain atrophy by improving motor/sensory function in a mouse model of tauopathy. But very high weekly doses of antibody were needed to achieve these treatment effects, 50 mg/kg, although they managed to lower the treatment to 10 mg/kg weekly intraperitoneally, they did not achieve the same effects as with the higher treatment (Yanamandra et al., 2015). In contrast, ICV administration we were able to release a dose of the antibody of 0,11 µl/hour as the pump volume was 100 µl, for 4 weeks and we saw in the same type of transgenic mice a great reduction of p-tau levels in cortex and Hc and improve the motor/sensory deficits.

The same authors published another study about passive immunization after peripheral administration of an anti-tau antibody to human patients with tauopathy and to mice expressing h-tau in the CNS increased the concentration of plasma tau which correlates with the concentration of extracellular and soluble tau in the brain. Because the administration of anti-tau antibody in tau transgenic mice, decreased brain soluble tau and interstitial fluid tau and produced an increase in plasma tau, but this increase was less than that observed in tau transgenic mice without these brain changes. These data support the idea that anti-tau antibodies should be evaluated as a potential treatment for tauopathy (Yanamandra et al., 2017). Recently study, used the IV administration of 77G7 tau mAb which inhibits the internalization of tau aggregates in the cells and ameliorated site-specific

hyperphosphorylation of tau, induced by AD O-tau in the Hc of 3xTg- AD mice. It is another promising immunotherapeutic antibody to decrease tau propagation (L. Li et al., 2022).

Another study, through ICV administration of a mAb specific for tau oligomers called TOMA, targets human mutant tau. Recent findings suggest that tau oligomers, which form upstream of NFTs, are the true neurotoxic forms of tau in neurodegenerative diseases such as tauopathies in AD and show a major role in behavioral and cognitive alterations. The dose of TOMA administered via ICV was sufficient to target and reduce tau oligomers, reversing locomotor and memory deficits in a P301L mouse model of tauopathy for 2 months, but did not target phosphorylated NFTs or monomeric tau (Castillo-Carranza et al., 2014).

In the case of amyloid- β , the same administration that our study the ICV passive immunization with anti-oligo amyloid- β antibody might be preventing permanent structural and functional damage in AD blocking early toxic changes in the brain of CRND8 transgenic mice (Chauhan, 2007). In another study recently, the A β levels, the WB showed that A β 42 oligomer and p-tau (pSer231), but not A β 40 levels and t-tau were decreased in brains by intraperitoneal administration of A8 mAb-treated APP/PS1 mice, inhibiting the amyloid cascade (Xiao et al., 2020).

In relation to the BBB crossing, the reversible opening of the BBB could be an option regarding the enhancement of the B6 antibody delivery to the brain after systemic administration. In this way, BBB opening mediated by focused ultrasound has been widely used in the last few years (Lipsman et al., 2018).

Overall, the positive results on the therapeutic properties of the pTau B6 mAb seem to indicate that further independent preclinical studies are needed in order to result confirmation and subsequent progress towards clinical trials.

6.4. PET IMAGING, PK AND BD OF RADIOLABELED ^{89}Zr -B6 AND ^{89}Zr -B6 F(AB')₂ IN HTAUP301S AND C57/BL6 MICE

In the last decade, several therapeutic antibodies (TAs), have been tested especially in the treatment of chronic inflammatory diseases and several oncological clinical trials, showing that the use of ^{89}Zr -based immuno-PET resulted in high spatial resolution images with a good signal-to-noise ratio (Jauw et al., 2016). In these studies, IgG molecules of the IgG1 type are commonly used for radiolabeling, which have an extremely high molecular weight of about 145-150 kDa and are also very hydrophilic, both properties complicating distribution and absorption (Martin, 1969; Reilly et al., 1995).

Drug elimination is usually determined by hepatic biotransformation and renal excretion, the elimination kinetics of TAs, due to their high molecular weight, renal elimination plays almost no role, as low molecular weight proteins such as peptides or amino acids that do not need to be degraded can be excreted renally and occur to a small extent in the liver, skin, and muscle (Espíe et al., 2009).

Another *in vivo* study was carried out, but labeling the antibody with ^{89}Zr , as it is a currently widely used radiotracer with a half-life of 78.41 hours, approximately 3 days (Perk et al., 2009). Currently, several mAbs and mAb fragments are in clinical development because of their excellent potential for systemic treatment of cancer and other pathologies. PET showed how the antibody behaves in circulation to confirm and quantify uptake and how the antibody is distributed in organs, in this case, we were interested in the brain to see what percentage of the antibody passed through the BBB and to see the variations in pharmacokinetics (Vosjan et al., 2010). A strategy to tag mAbs with ^{89}Zr was followed by a multi-step synthesis using a succinylated derivative of DFO as a bifunctional chelate where it was shown in clinical and preclinical studies to be a good chelator (Börjesson et al., 2009; Thom et al., 2017).

In regard to BD is a method of tracking where compounds of interest travel in an experimental animal or human subject, was observed by PET, and *ex vivo* analysis confirmed the fast accumulation of B6 F(ab')₂ fragment radiolabeled mainly in kidneys. It is corroborated by a recent study from 2021, where they studied molecules covered a wide molecular weight range 13–150 kDa and they observed that as the molecular size decreased, the degree of tissue distribution of proteins such as antibodies increased, indicating higher tissue penetration. Among all tissues, the kidney represented the most abundant site of disposition, especially for antibodies of smaller molecular sizes 27-13 kDa, where it is explained that the increase in biodistribution coefficient (BC) values in the kidney are due to glomerular filtration size (Z. Li et al., 2021).

In contrast the accumulation of the complete B6 antibody radiolabeled was in the liver and showed a completely different distribution to that found in mice injected with Oxalate-^[89]Zr (radiotracer alone) as it accumulated in bone. This relates to previous work in which ^[89]Zr alone accumulates preferentially in bone structures (Abou et al., 2011), while mAbs remains in circulation for several days as, compared to the blood which starts to clear after 24 h, in the organs where antibodies are processed, such as the spleen, liver and kidneys, they start to decrease after 24 h (K. J. H. Allen et al., 2018).

In this study, after 7 days, most of the B6 antibody remained in circulation, liver, and kidneys (besides the skeleton, spine, and muscle), and the brain was the only organ where the antibody levels got increased along the study. Additionally, brain uptake in the B6 F(ab')₂ fragment is very limited and much lower than the complete B6 antibody (<0.01 %ID/g), because the ^[89]Zr-B6 antibody confirmed presence in brain region between 0.07 to 0.11 %ID/g, according with other IgGs BBB crossing rates in the literature (Pyzik et al., 2015).

In regard to the PK profile determines the fate of substances administered in these case antibodies to a living organism. In recent decades, among all protein therapeutics, physiologically-based PK (PBPK) models of mAbs have made great progress. It has been shown that protein molecules of different sizes behave differently in speed and extent of tissue extravasation and are inversely related to the systemic clearance of antibody fragments of different sizes (Z. Li et al., 2016, 2017). So this study we have confirmed that the ^[89]Zr-B6 antibody remains in circulation for up to 7 days, ensuring the stability of the conjugate and availability of the antibody to reach the periphery or enter the brain. The enzymatically digested B6 F(ab')₂ fragment is much faster in circulation than the B6 complete antibody, which could impact negatively the bioavailability because it is eliminated before by the kidney because the weight is lower on improving ELISA amplification and thus the therapeutic potential (Z. Li et al., 2021). Unfortunately, no significant differences between the radiolabeled antibodies alone or fragments of B6 for crossing BBB with the respective controls were found. The insufficient accumulation of the B6 antibody in the brain was most likely due to their inability to cross the BBB. Therefore, the use of bispecific antibodies or peptides that help cross the BBB can be a great advance so that the developed radiotracers can bind to their target within the brain parenchyma (de Lucas, Á. G., et al., 2023).

7. CONCLUSIONS

1. Two new mAb against pTau and acTau were characterized. Specifically, Epitope Mapping confirmed that pTau B6 mAb detects the core sequence that contains the phosphorylation 207G(pS)R209. The acTau E11 mAb specifically detects the core sequence that contains acetylation 171IPA(acK)174. The cross-reactivity of the E11 antibody was low against the >20,000 human proteins of the microarray.
2. Western Blot analysis showed a Braak stage-dependent differential band pattern for both p-Tau B6 and ac-Tau E11 mAbs in human samples. It is noteworthy that the pTau B6 mAb detected both oligomers and dimers of p-Tau from early stages of the Braak Scale, indicating its potential *in vivo* therapeutic and diagnostic usefulness.
3. Two novel Sandwich ELISA kits with diagnostic purposes in CSF samples of AD patients were developed for detection of acetylated and phosphorylated tau by using the acTau E11 and pTau B6 mAbs, respectively.
4. The signal-to-noise ratio and detection range of the luminescence development system was the most suitable for detection pTau and acTau CSF levels. The intra- and inter-assay coefficients of variation, although dependent on samples storage and freeze-thaw cycles, were small and mostly less than 5%.
5. A clinical proof of concept study with patients with MCI who did or did not progress to early or advanced AD was performed. The results showed that all the tau biomarkers, including acTau and pTau208, as well as those of t-Tau and p-Tau181 from the Fujirebio Lumipulse system (gold standard) show the same behavior, with an increase in the levels of the isoforms of tau in CSF as the disease progresses, although the results are not statistically significant due to the small sample size and the dispersion of data. Therefore, future, larger, multicenter clinical trials are necessary to validate these promising results.
6. *In vitro* toxicity studies demonstrated that pTau B6 mAb did not exert toxic effects for neurons, as revealed by caspase-3 activity determination.
7. *In vivo* systemic administration of the pTau B6 mAb during 4 months did not cause toxicity in the liver and kidney, suggesting that it might be safe for both immunotherapy and radiotracer studies.
8. A 4-week treatment with intracerebroventricular administration of pTau B6 mAb demonstrated a clear beneficial therapeutic effect in terms of delayed appearance of hindlimb clasping and latency to fall from the rotarod.
9. Intracerebroventricular pTau B6 mAb treatment for 4 weeks mediated a great reduction of p-Tau levels in cortex and hippocampus as showed by immunohistochemical analysis. Therefore, the most straightforward interpretation of the findings is that B6 immunotherapy is able to slow down the motor and neurological impairment in the hTauP301S mouse model due to reduction of tau hyperphosphorylation.

10. Two new stable radiotracers have been developed based on ^{89}Zr radiolabeled pTau-B6 mAb or ^{89}Zr radiolabeled B6 F(ab')₂ fragment with potential utility for labeling brain p-Tau deposits through PET neuroimaging.
11. The pharmacokinetic profile of confirmed that remains in circulation up to 7 days, ensuring the stability of the conjugate and availability of the antibody to reach the periphery or enter the brain. By contrast, the pharmacokinetic profile of the enzymatically digested B6 F(ab')₂ fragment radiolabeled with ^{89}Zr showed a rapid elimination in circulation that could impact negatively the bioavailability and thus the therapeutic potential.
12. The biodistribution studies of ^{89}Zr radiolabeled pTau-B6 mAb confirmed a presence of the B6 antibody in brain region between between 0.4 to 1.15% ID/g, according with other IgGs BBB crossing rates in the literature. After 7 days, most of the antibody remained in circulation, liver and kidneys (besides skeleton, spine and muscle), and brain was the only organ where the antibody levels got increased along the study. By contrast, the fast accumulation of ^{89}Zr radiolabeled B6 F(ab')₂ fragment was mainly in the kidneys and the brain uptake is very limited and much lower than the complete B6 antibody (<0.01 %ID/g).

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9.3 ETHICAL CONSENTS



DICTAMEN DEL COMITÉ AUTONÓMICO DE ÉTICA DE LA INVESTIGACIÓN DE GALICIA

Paula M. López Vázquez, Secretaria del Comité Autonomico de Ética de la Investigación de Galicia

CERTIFICA:

Que este Comité evaluó en su reunión del día 22/12/15 :

Título: Evolución del deterioro cognitivo en el envejecimiento normal, deterioro cognitivo ligero y enfermedad de Alzheimer: estudio longitudinal con marcadores cognitivos, psicofisiológicos, biológicos y de neuroimagen

Promotor: Onésimo Juncos-Rabadan

Version: versión junio 2015

Código de Registro: 2013/553

Y, tomando en consideración las siguientes cuestiones:

- La pertinencia del estudio, teniendo en cuenta el conocimiento disponible, así como los requisitos legales aplicables, y en particular la Ley 14/2007, de investigación biomédica, el Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica, la ORDEN SAS/3470/2009, de 16 de diciembre, por la que se publican las Directrices sobre estudios Posautorización de Tipo Observacional para medicamentos de uso humano, y la Circular nº 07 / 2004, investigaciones clínicas con productos sanitarios.
- La idoneidad del protocolo en relación con los objetivos del estudio, justificación de los riesgos y molestias previsibles para el sujeto, así como los beneficios esperados.
- Los principios éticos de la Declaración de Helsinki vigente.
- Los Procedimientos Normalizados de Trabajo del CEIC de Galicia

Emite un **INFORME FAVORABLE** para la realización del estudio por el/la investigador/a del centro:

Centros	Investigadores Principales
Universidade de Santiago de Compostela CHU de Santiago	Onésimo Juncos-Rabadan José Manuel Aldrey



Y HACE CONSTAR QUE:

- 1 El CAEIG cumple los requisitos legales vigentes (R.D 223/2004 por el que se regulan los ensayos clínicos con medicamentos, y la Ley 14/2007 de Investigación Biomédica).
- 2 El CAEIG tanto en su composición como en sus PNTs cumple las Normas de Buena Práctica Clínica (CPMP/ICH/135/95).
- 3 La composición actual del CAEIG es:

Manuel Portela Romero. (Presidente). Médico Especialista en Medicina Familiar y Comunitaria.

Irene Zarra Ferro. (Vicepresidenta). Farmacéutica de Atención Especializada.

Paula M^a López Vázquez. (Secretaria). Médico Especialista en Farmacología Clínica.

Juan Vázquez Lago (Secretario Suplente). Médico Especialista en Medicina Preventiva y Salud Pública.

Jesús Alberdi Sudupe. Médico especialista en Psiquiatría.

Rosendo Bugarín González. Médico Especialista en Medicina Familiar y Comunitaria.

Juan Casariego Rosón. Médico Especialista en Cardiología.

Xoán X. Casas Rodríguez. Médico Especialista en Medicina Familiar y Comunitaria.

Juana M^a Cruz del Río. Trabajadora Social.

Juan Fernando Cueva Bañuelos. Médico Especialista en Oncología Médica.

José Álvaro Fernández Rial. Médico Especialista en Medicina Interna.

José Luis Fernández Trisac. Médico Especialista en Pediatría.

M^a José Ferreira Díaz. Diplomada Universitaria de Enfermería

Pablo Nimo Ríos. Licenciado en Derecho. Miembro externo

Pilar Gayoso Diz. Médico Especialista en Medicina Familiar y Comunitaria.

Agustín Pita Morandeira. Farmacéutico de Atención Primaria

Salvador Pita Fernández. Médico Especialista en Medicina Familiar y Comunitaria.

Carmen Rodríguez-Tenreiro Sánchez. Licenciada en Farmacia.

Susana María Romero Yuste. Médico Especialista en Reumatología.

M^a Asunción Verdejo González. Médico Especialista en Farmacología Clínica.

En Santiago de Compostela, a 05 de enero de 2015

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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTO DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 15011/2021/005

Data de inicio do expediente: 30.7.2021

Persoa solicitante: Isabel Lista García

Procedemento: resolución de autorización

Forma de inicio: solicitude da persoa interesada

ANTECEDENTES

A persoa solicitante, como representante da Fundación Instituto de Investigación Sanitaria de Santiago de Compostela (IDIS), presentou con data 30.7.2021 unha solicitude para a realización do proxecto de experimentación animal (entrada no Rexistro electrónico da Xunta de Galicia 2021/1855818). Posteriormente, achegou nova documentación o 24.9.2021 (entrada no Rexistro electrónico da Xunta de Galicia 2021/2243122), cuxos datos son os seguintes:

Denominación do proxecto: Desenvolvemento de novas solucións diagnósticas e terapéuticas para diferentes demencias relacionadas coa proteína Tau

Nome do centro usuario: Instituto de Investigación Sanitaria de Santiago de Compostela

Persoa responsable do proxecto: Tomás Sobrino Moreiras

Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Instituto de Investigación Sanitaria de Santiago de Compostela

Clasificación do proxecto: Tipo I ☐ Tipo II ☒ Tipo III ☐

CONSIDERACIÓNS LEGAIS E TÉCNICAS

1 O Real decreto 53/2013, de 1 de febreiro (BOE 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

2 O artigo 88 da Lei 39/2015, do 1 de outubro, do procedemento administrativo común das administracións públicas (BOE 236, do 2 de outubro de 2015) establece

CONSELLERÍA DO MEDIO RURAL
XEFATURA TERRITORIAL
SERVIZO DE GANDARÍA
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15071 A Coruña
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servizo.gandaria.a.coruna@xunta.gal
mediorural.xunta.gal

CVE: e8bMnFTm81
Verificación: <https://sede.xunta.gal/cve>





que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas deste.

3 O Servizo de Gandaría da Coruña revisou a documentación achegada na solicitude e o resultado favorable da avaliación do proxecto realizada polo órgano habilitado, o Comité de Ética de Investigación Animal do IDIS.

Esta xefatura territorial é competente para ditar unha resolución, de conformidade co Decreto 149/2018, do 5 de decembro, polo que se establece a estrutura orgánica da Consellería do Medio Rural e se modifica parcialmente o Decreto 177/2016, do 15 de decembro, polo que se fixa a estrutura orgánica da Vicepresidencia e das consellerías da Xunta de Galicia (DOG 235, do 11 de novembro).

De acordo con todo o indicado, RESOLVO:

- 1 Autorizar o proxecto solicitado.
- 2 O proxecto non precisa someterse a unha avaliación retrospectiva.
- 3 A autorización deste proxecto terá unha duración de tres anos e catro meses e unha vez transcorrido este tempo, esta autorización deberá ser renovada.

A citada autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandaría.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirada, previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpoñer un recurso de alzada ante o conselleiro de Medio Rural. O prazo comezará a contar dende o día seguinte ao da recepción desta resolución. Todo isto, segundo o disposto nos artigos 121 e 122 da citada Lei 39/2015.

Mediante este escrito notifícasele a Isabel Lista García esta resolución segundo o esixido no artigo 40.1 da antedita Lei 39/2015.

A Coruña, 30 de setembro de 2021

O xefe territorial

Jose Manuel Santos Maneiro
(asinado electronicamente)

Asinado por: SANTOS MANEIRO, JOSE MANUEL
Cargo: Xefe Territorial
Data e hora: 01/10/2021 09:29:20

CVE: e6b1mFTm81
Verificación: <https://sede.xunta.gal/cve>



9.4 ARRIVE GUIDELINES

Lista de verificación para Tesis con animales de experimentación. **EXPERIMENTACIÓN ANIMAL ARRIVE**

Sí/no/NA		Página
SÍ	Título	1
SÍ	Proporcionar una descripción tan exacta y concisa como sea posible sobre el contenido del trabajo.	1
SÍ	Resumen	2 4
SÍ	Proporcionar un resumen preciso de los antecedentes, los objetivos de la investigación, incluyendo detalles de la especie y cepa de los animales utilizados, métodos relevantes, hallazgos principales y conclusiones del estudio.	2 4-29
SÍ	Antecedentes	3 0
SÍ	Incluir suficientes antecedentes científicos (incluyendo las referencias pertinentes al trabajo anterior) a fin de comprender la motivación y el contexto para el estudio, y explicar las bases y el enfoque experimental.	3 0-54
SÍ	Explicar cómo y por qué la especie y el modelo animal utilizados permiten abordar los objetivos científicos y, cuando sea apropiado, la relevancia del estudio para la biología humana.	5 3-54
SÍ	Objetivos	6 0
SÍ	Describir claramente los objetivos primarios y secundarios del estudio, o las hipótesis específicas que se van a probar.	6 0
SÍ	Declaración Ética	2 12
SÍ	Indicar el permiso del comité ético, y las leyes o decretos pertinentes para el cuidado y uso de animales, bajo las que se realiza la investigación.	212- 213
SÍ	Diseño del estudio	8 5-88
SÍ	Número de grupos experimentales y control.	8 6
SÍ	Cualquier medida adoptada para minimizar los efectos de sesgo subjetivo al asignar los animales a los grupos de tratamiento (por ej., procedimiento aleatorio) y al evaluar los resultados (por ej., si se hace, describir quiénes eran ciegos y cuándo).	8 6
SÍ	La unidad experimental (por ej., un solo animal, grupos o jaulas de animales). Un diagrama cronológico o de flujo pueden ser útiles para ilustrar cómo se realizaron los diseños de estudio complejos.	8 8
SÍ	Procedimientos experimentales	8 5-88
SÍ	Cómo (por ej., formulación y dosis del tratamiento, el sitio y la vía de administración, anestesia y analgesia utilizadas [incluyendo la monitorización], procedimiento quirúrgico, el método de eutanasia). Proporcionar detalles de cualquier equipo especializado utilizado, incluyendo proveedor(es).	8 5-91
SÍ	Cuándo (por ej., la hora del día).	8 9-91
SÍ	Dónde (por ej., jaula de alojamiento, laboratorio, prueba del laberinto acuático).	8 9-91
SÍ	Por qué (por ej., fundamentos para la elección del anestésico específico, la vía de administración, dosis del fármaco utilizado).	8 6
SÍ	Animales de experimentación	8 5-86

SÍ	Proporcionar detalles de los animales utilizados, incluyendo especie, cepa, sexo, etapa de desarrollo (por ej., edad media o mediana de edad y rango) y peso (por ej., media o mediana más rango de peso).	8 5-86
SÍ	Proporcionar otra información pertinente, como la procedencia de los animales, nomenclatura internacional de la cepa, modificación genética (por ej., animal deficiente o transgénico), genotipo, estado de salud/inmune, si los animales han sido incluidos en estudios o recibido tratamientos anteriormente, procedimientos previos, etc.	8 5-86
SÍ	Animales	
SÍ	Alojamiento (tipo de instalación por ej., libre de patógenos específicos [SPF], tipo de jaula o habitáculo, material del lecho, número de animales por jaula, forma y material del tanque, etc. para peces).	9 1
NO	Condiciones de cría (por ej., programa de reproducción, ciclo de luz/oscuridad, temperatura, calidad de agua, etc. para peces, el tipo de alimentación, el acceso a los alimentos y al agua, enriquecimiento ambiental).	-
NO	Evaluaciones e intervenciones relacionadas con el bienestar que se llevaron a cabo antes, durante o después del experimento	8 4-90
SÍ	Tamaño de la muestra	8 6
SÍ	Especificar el número total de animales utilizados en cada experimento, y el número de animales en cada grupo experimental.	8 6
NO	Explicar cómo se determinó el número de animales. Proporcionar detalles del cálculo del tamaño muestral utilizado.	8 4-87
NO	Indicar el número de repeticiones independientes de cada experimento, si es pertinente.	8 7
SÍ	Asignación de animales a grupos experimentales	8 6
SÍ	Proporcionar detalles completos de la forma en que los animales fueron asignados a grupos experimentales, incluyendo distribución aleatoria o asignación específica, si así se hizo.	8 6
SÍ	Describir el orden en el que los animales en los diferentes grupos experimentales fueron tratados y evaluados.	8 6
SÍ	Resultados experimentales 12 Definir claramente los resultados primarios y secundarios evaluados (por ej., muerte celular, marcadores moleculares, cambios de comportamiento).	108-11
SÍ	Métodos estadísticos	9 1
SÍ	Proporcionar detalles sobre los métodos estadísticos utilizados para cada análisis.	9 1
SÍ	Especificar la unidad de análisis para cada grupo de datos (por ej., un solo animal, grupo de animales, neurona individual).	9 1
SÍ	Describir los métodos utilizados para evaluar si los datos corroboran las suposiciones del método estadístico.	9 1
SÍ	Datos basales	84-96
NO	Para cada grupo experimental, indicar las características relevantes y estado de salud de los animales (por ej., peso, estado microbiológico, si los animales han sido incluidos en estudios o recibido drogas anteriormente) antes de iniciar el tratamiento o prueba. (Esta información puede ser a menudo tabulada).	84-96
SÍ	Cantidades analizadas	86-95
SÍ	Indicar el número de animales en cada grupo incluido en cada análisis. Presentar números absolutos (por ej., 10/20, no 50 %2).	8 6
SÍ	Si alguno de los animales o datos no fue incluido en el análisis, explicar por qué.	9 5
SÍ	Resultados y Discusión	98-151
SÍ	Indicar los resultados de cada análisis llevado a cabo, con una medida de precisión (por ej., error estándar o intervalo de confianza).	132- 136
SÍ	Eventos adversos	136-

		138
NO	Dar detalles de todos los eventos adversos importantes en cada grupo experimental.	-
NO	Describir las modificaciones a los protocolos experimentales realizadas para reducir los eventos adversos.	-
SÍ	Interpretación /implicaciones científicas	154-156
SÍ	Interpretar los resultados, teniendo en cuenta los objetivos y las hipótesis del estudio, la teoría actual y otros estudios pertinentes en la literatura.	154-156
SÍ	Comentar las limitaciones del estudio incluyendo cualquier fuente potencial de sesgo, cualquier limitación del modelo animal y la imprecisión asociada con los resultados	154-156
SÍ	Describir cualquier implicación de los métodos experimentales o hallazgos para el reemplazo, refinamiento o reducción (las 3 R) del uso de los animales en investigación.	154-156
SÍ	Capacidad de generalización/aplicabilidad	154-156
SÍ	Comentar si, y de qué forma, los hallazgos de este estudio son aplicables a otras especies o sistemas, incluyendo cualquier relevancia para la biología humana.	154-156
SÍ	Financiación	1
SÍ	Listar todas las fuentes de financiación (incluyendo el número del proyecto) y el papel de la fuente(s) de financiación en el estudio.	1

Basado en The ARRIVE guidelines: Animal Research: Reporting of In Vivo Experiments.

Firma Doctorando



Alzheimer's disease (AD) is the leading cause of dementia, implying a progressive decline of cognitive performance and limitation of social activities. In this scenario, in parallel to current research of novel treatments, it is imperative to develop new strategies for an earlier diagnosis of AD. This study has focused on the importance of different isoforms of the Tau protein involved in early pathophysiological processes of AD. Specifically, two new monoclonal antibodies (mAb) against phosphorylated tau (p-Tau) and acetylated tau (ac-Tau) have been produced, with which a clinical immunodiagnostic kit and a preclinical radiotracer for PET 89-Zirconium (^{89}Zr)-pTau mAb have been developed for diagnostic purposes. In addition, a preclinical proof of concept of immunotherapy with p-Tau antibody has been also carried out.