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Detection of aberrant apolipoprotein E (apoE) complexes as an Alzheimer's biomarker

UNIVERSITAS Miguel Hernández

Javier Sáez-Valero

MINISTERIO DE CIENCIA, INNOVACIÓN Y UNIVERSIDADES
AGENCIA ESTATAL DE INVESTIGACIÓN
MEDICAMENTOS INNOVADORES Plataforma Tecnológica Española
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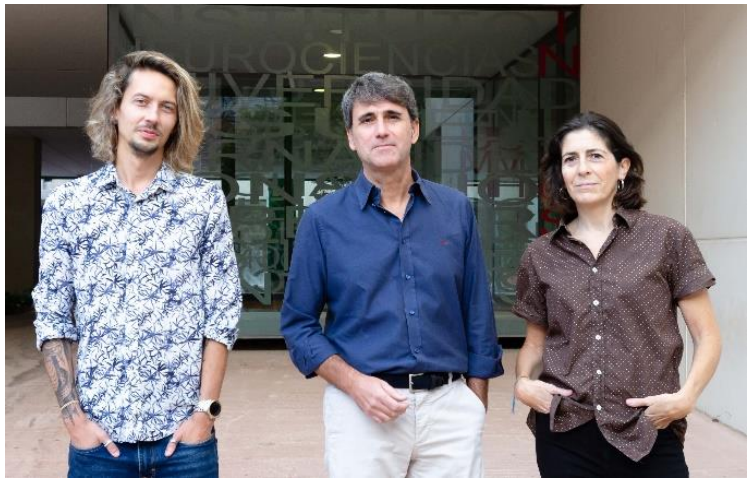
1. The Institution, the group



UNIVERSITAS Miguel Hernández

CSIC INSTITUTO DE NEUROCIENCIAS

ciberNed Centro Investigación Biomédica en Red Enfermedades Neurodegenerativas

ISABIAL INSTITUTO DE INVESTIGACIÓN SANITARIA Y BIOMÉDICA DE ALICANTE

Our group located at the Instituto de Neurociencias of the Miguel Hernández University is also member of **CIBERNED** (Center for Networked Biomedical Research focused in neurodegenerative diseases, an initiative of the Health Institute Carlos III) and of the Institute of Health and Biomedical Research of Alicante (**ISABIAL**).

CSIC UNIVERSITAS Miguel Hernández

INSTITUTO DE NEUROCIENCIAS

EXCELENCIA SEVERO OCHOA 2014-2022

Name of Research Group

Altered molecular mechanism in Alzheimer's disease and dementia

Group Leader, position and email

Javier Sáez Valero
UMH Professor
j.saez@umh.es

Group members

Other principal investigators

María Salud García Ayllón [Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunitat Valenciana (FISABIO) Research]

Postdocs

Inmaculada Cuchillo Ibañez (CIBERNED)

Rocío Pérez González [Instituto de Investigación Sanitaria y Biomédica de Alicante (ISABIAL); start January 2022]

PhD students

Matthew P Lennol
Sergio Escamilla Ruiz
María Ángeles Cortés Gómez
Adriana Gea González
Alba Marina Lucart Sánchez

Master students

Carlos Avilés Granados (2022-23)

Technicians

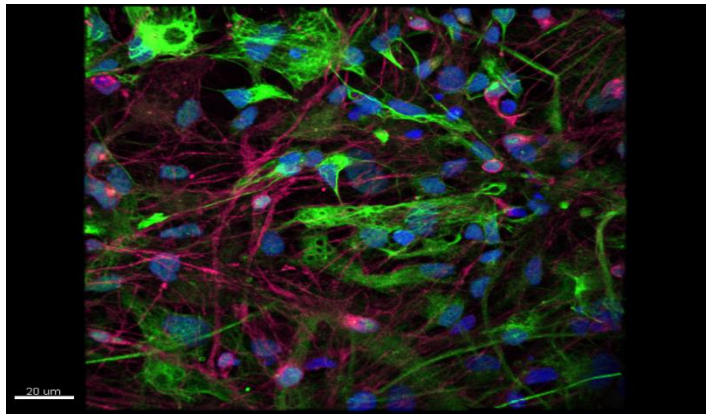
Manuel Javier Giner Pastor (part-time research support, UMH)

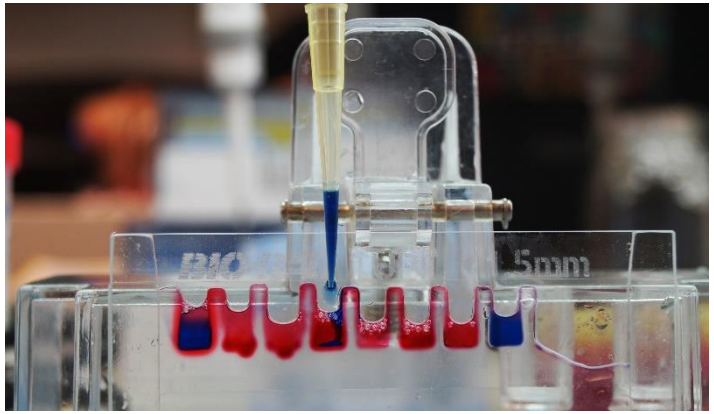
Edward Sellés Ciment. Generalitat Valenciana Primeras Experiencias award (start May 2022).

Henrik Zetterberg

Kaj Blennow

Our research focuses on **deciphering the molecular basis of impaired pathways involved in the etiopathogenesis of Alzheimer's disease** (AD), and other neurodegenerative disorders. The translational benefits of our research lie in the fact that we aim to clarify the pathological mechanisms behind the disease but also to define potential diagnostic tools and/or processes with therapeutic relevance.
We **aim to identify biomarkers that serve as a read-out of impaired brain function**. For this reason we **validate potential biomarkers in cellular models**; with an extensive experience in modeling the disease condition in primary cultures and cell line; now extending this experience to **iPS cells**.





We also are members of the **Society for CSF analysis** and clinical neurochemistry in the validation and standardization of CSF biomarkers. We are collaborate with the **Tear Research Network** for the implementation of Tear Fluid Biomarkers.

Current Research Interest

1. Relationship between **Aβ and presenilin-1 with acetylcholinesterase**
2. **ApoE** in Alzheimer's disease
   2.1. Influence of apoE in **ADAM10/α-secretase**
   2.2. Aβ and P-tau cross-talk, a role for **apoE/apoER2/reelin signaling**?
3. Mechanism behind the **failure in the therapy based in γ- and β-secretase inhibitors**
4. New Alzheimer's **CSF biomarkers**:
   4.1. **Glycoforms of proteins**
   4.2. **Aβ related proteins** (secretases and APP proteolytic fragments)
5. Neuronal and glia interplay: **Trem2 and SOCS3** microglial proteins.
6. Altered balance of synaptic/extrasynaptic N-methyl-D-aspartate receptors (**NMDARs**) in AD.
7. Prognostic biomarker in **COVID-19**, circulating levels of **ACE2** species, the host receptor of the SARS-CoV-2 virus, as a **read-out of infection progression**.

Our expertise comprises

i) **biochemical characterization of PTM for brain/CSF proteins**, including **glycosylation and phosphorylation** analysis, as characterization of **proteolytic processing**

ii) **characterization of ligand-receptor interaction** associated to signaling pathways

iii) assessment of **sustained inhibition of key enzymes** such as secretases.

iv) **cellular models**: iPSC, cell lines and primary cultures

2. The Product

VALUE PROPOSITION

- **AD diagnostic kits using electrophoresis technologies based on apolipoprotein E (apoE) protein alterations**. The methodology is based on the presence, quantity or concentration of apoE with a size of 34 kDa in an aggregate of apoE with a size of 100 kDa; the ratio of apoE dimers/monomers **detected in subjects with all APOE genotypes.**

- The test **showed high performance in terms of variability, precision, interferences, reagents stability and detectability.**

- **High diagnostic accuracy** for early diagnosis.

- The project **can be adapted to any clinical chemistry analyzer**, including the high throughput analyzers present in most hospitals and clinical laboratories.

- The project can be employed to **track response to therapy in AD patients for drug discovery validation.**

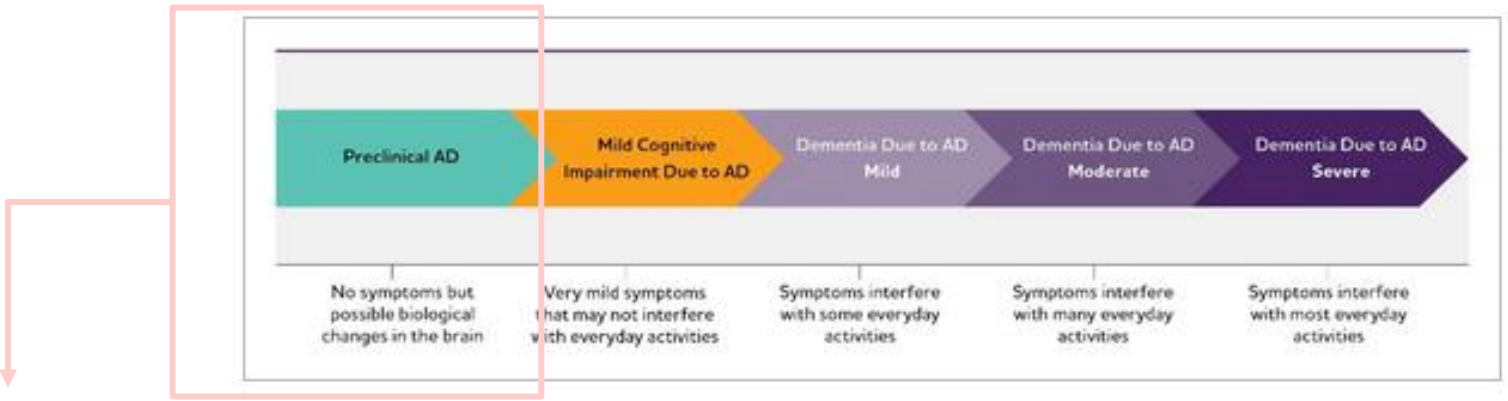
- **The project test versatility (qualitative and quantitative changes), low cost, and easiness** provides an **excellent solution for APOE ε4 carriers and non-carriers pathology identification using the same fluid sample drawn for biochemical diagnostic work-up of AD patients, which can have important advantages for patient stratification and follow-up in clinical trials, preventative strategies for AD, and pathology progression.**

2. The Product, Target Indications

MEDICAL NEEDS

- The progression of Alzheimer's disease from brain changes that are unnoticeable by the person affected to brain changes that cause memory problems and eventually physical disability is called the **Alzheimer's disease continuum**. On this continuum, there are **three broad phases**: **preclinical Alzheimer's disease, mild cognitive impairment (MCI) due to Alzheimer's disease and dementia due to Alzheimer's disease**, also called Alzheimer's dementia. The Alzheimer's dementia phase is further broken down into mild, moderate and severe dementia.

- While we know the Alzheimer's disease continuum starts with **preclinical Alzheimer's disease (no symptoms)** and ends with severe Alzheimer's dementia (severe symptoms), how long individuals spend in each part of the continuum varies. The length of each part of the continuum is influenced by age, genetics, biological sex and other factors



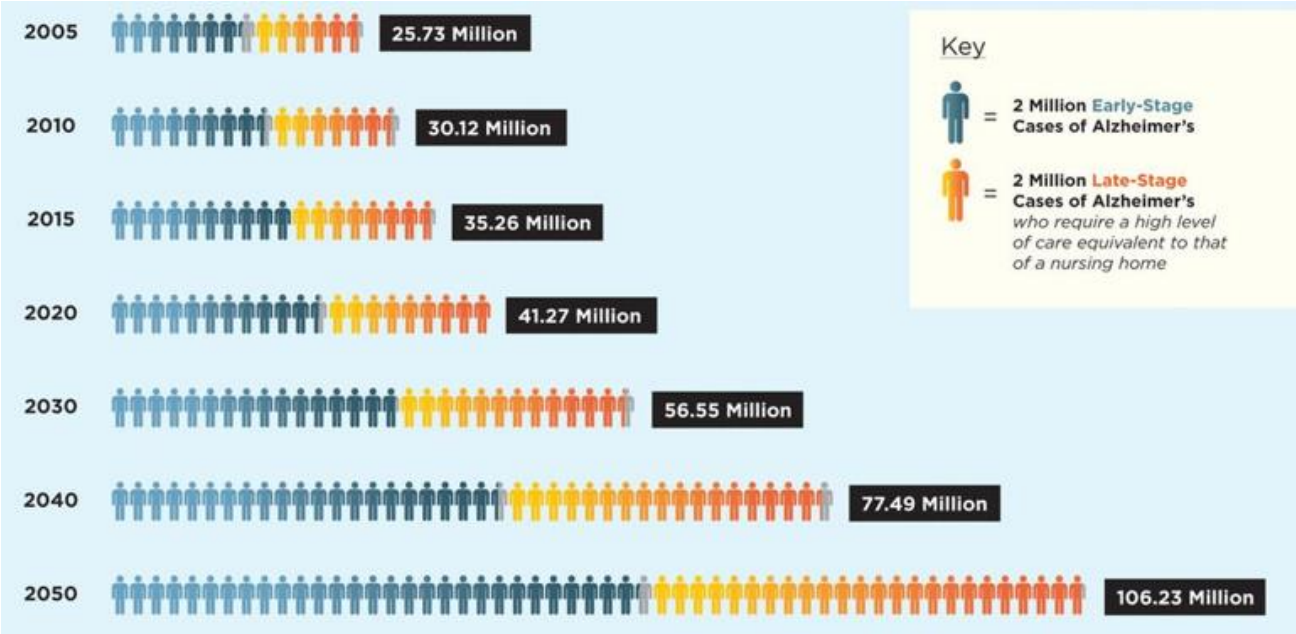
Pharmaceutical clinical trials

In this phase, individuals may have measurable brain changes that indicate the earliest signs of Alzheimer's disease (biomarkers), but they have not yet developed symptoms such as memory loss. Examples of Alzheimer's biomarkers include abnormal levels of amyloid as shown on positron emission tomography (PET) scans and in analysis of cerebrospinal fluid (CSF), changes in tau protein and Aβ in CSF and plasma, and decreased metabolism of glucose as shown on PET scans.

However**, there are not accurate and rapid cheap tools to identify some of the early brain changes of Alzheimer's and fine-tune the tools' accuracy before** they become available for widespread use in hospitals, doctors' offices and other clinical settings.
The ability to screen for AD in its **early stages would provide preventative therapeutic methods and reduce the economic burden that accompanies diagnosis,** such as treatment and patient care

MARKET OPPORTUNITY

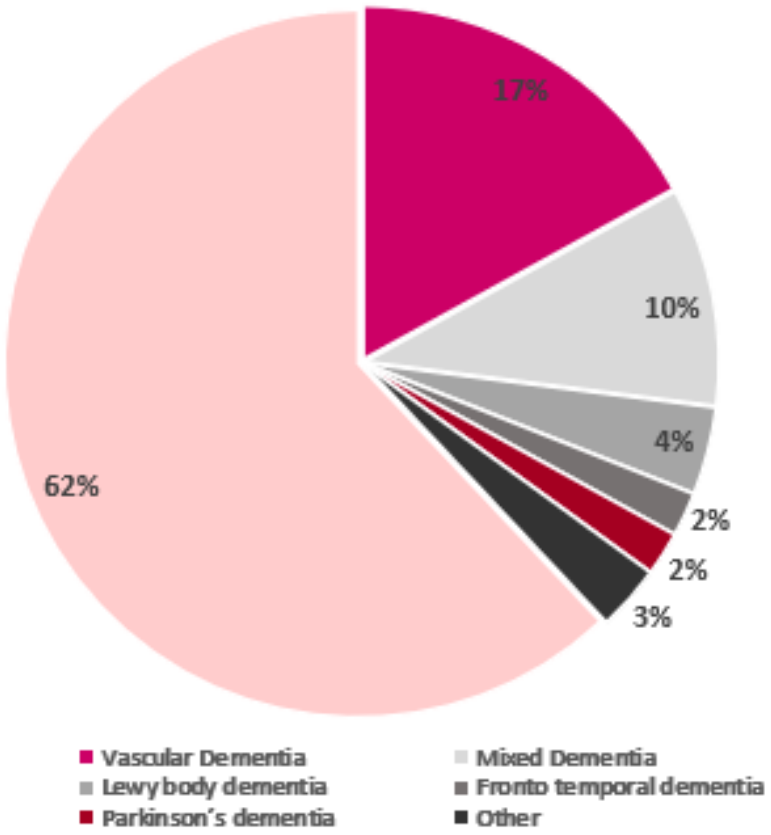
Alzheimer Disease's prevalence from 2005-50



- **In 2022, 50 million people will have been diagnosed with Alzheimer's worldwide** and it is predicted that in 2050 there will be 106.23M people with this disease.

- Dementia is the fifth leading cause of death worldwide and **Alzheimer's is the fourth leading cause of disability-adjusted life years (DALYs).**

Incidence of dementia-related diseases 2020



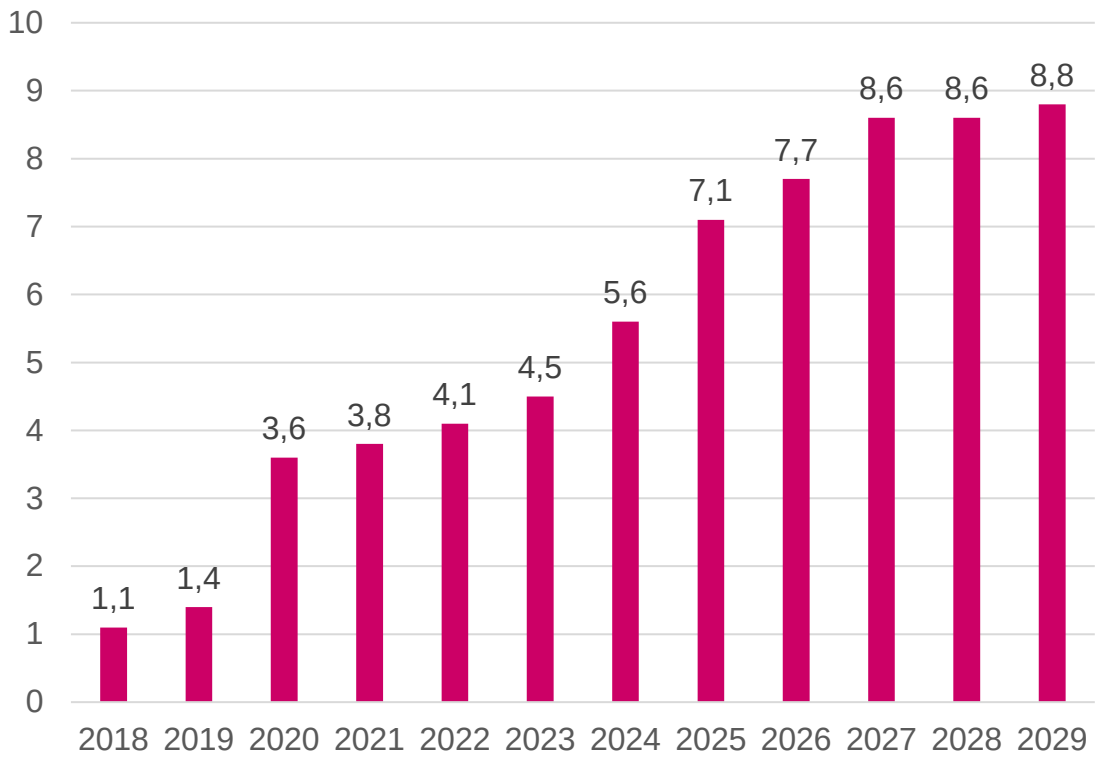
Among the diseases related to dementia, Alzheimer's is the most prevalent disease with 62%.

Sources: Colino S. *The Challenges of Alzheimer's and Dementia for Women*. AARP. 2020.

GlobalData 2020

MARKET OPPORTUNITY

AD´s Diagnostic Market Size in USD Bn during 2018-29

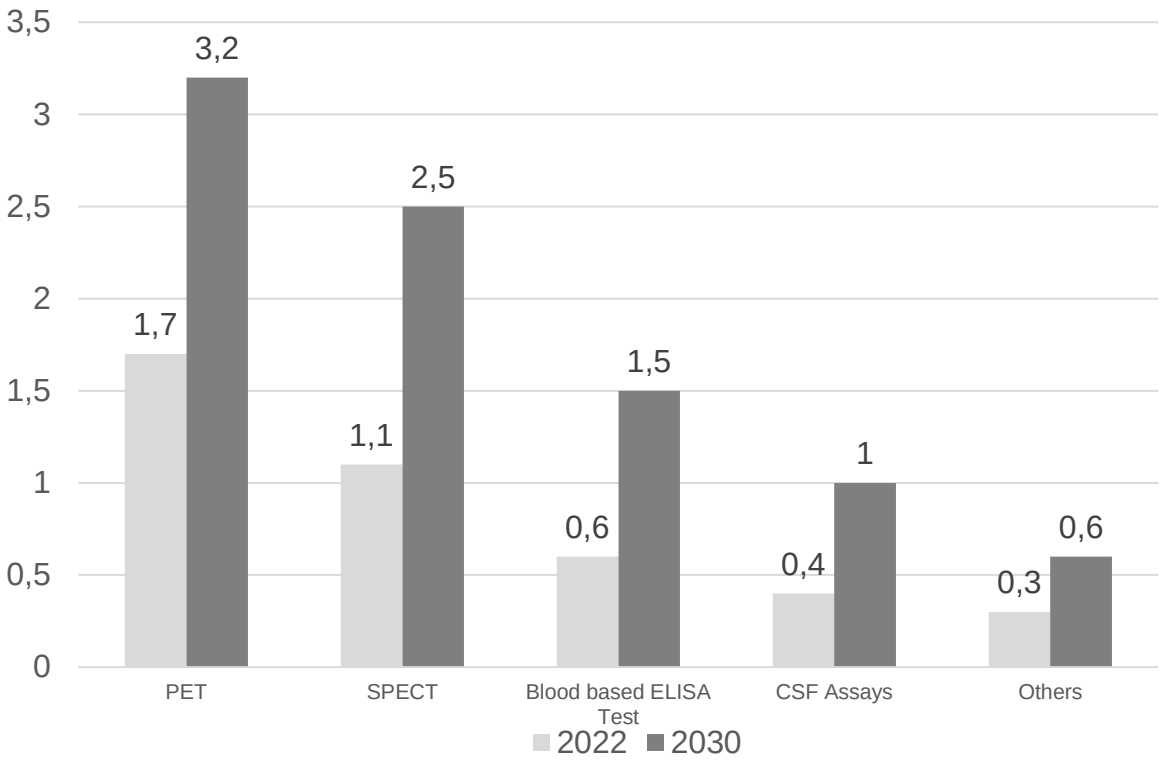


AD diagnostic market size was valued at USD 4.1B in 2022. The AD diagnostic market industry is projected to grow from USD 4.5B in 2023 to USD 8.8B by 2029, exhibiting a compound annual growth rate (CAGR) of 8.9% during the forecast period 2023-2029. Investment in biomarkers for drug research, an increase in pipeline drug development, and an increase in chronic diseases like dementia, are the key market drivers enhancing the market growth.

Sources: Market Research Future 2023.

MARKET OPPORTUNITY

AD´s Diagnostic Market by diagnostic test in USD Bn during 2022-2030



Based on Diagnostic Test, the market is bifurcated into PET, SPECT, Blood based ELISA Test, CSF Assays, and Others. PET is expected to dominated the Alzheimer's Disease Diagnostics market over the forecast period

- Several effective techniques commercialize for AD diagnosis, including PET, SPECT, ELISA and cerebrospinal fluid (CSF) assays.

- Whilst **cerebrospinal fluid (CSF)* and positron emission tomography (PET) biomarkers for amyloid-β (Aβ) and tau pathologies are accurate for the diagnosis of Alzheimer's disease (AD),** their broad implementation in clinical and trial settings are restricted by high cost and limited accessibility

- **Plasma phosphorylated-tau181 (p-tau181) is a promising blood-based biomarker that is specific for AD, correlates with cerebral Aβ and tau pathology**, and predicts future cognitive decline.

- The **use of plasma p-tau181 as a non-invasive diagnostic and prognostic tool for AD,** regardless of clinical stage, which **would be of great benefit in clinical practice and a large cost-saving in clinical trial recruitment.**

Sources: Verified Market Research 2022. Report Id: 30375. Global Alzheimer'S Disease Diagnostics Market Size By Diagnostic Test (PET, SPECT, Blood based ELISA Test, CSF Assays), By End-User (Hospitals, Diagnostic Centers, Pharmaceutical Companies, Clinics), By Geographic Scope And Forecast

2. The Product, Differential features facing the market

Current technologies and biomarkers in the AD diagnostic market: COMPETITOR ANALYSIS

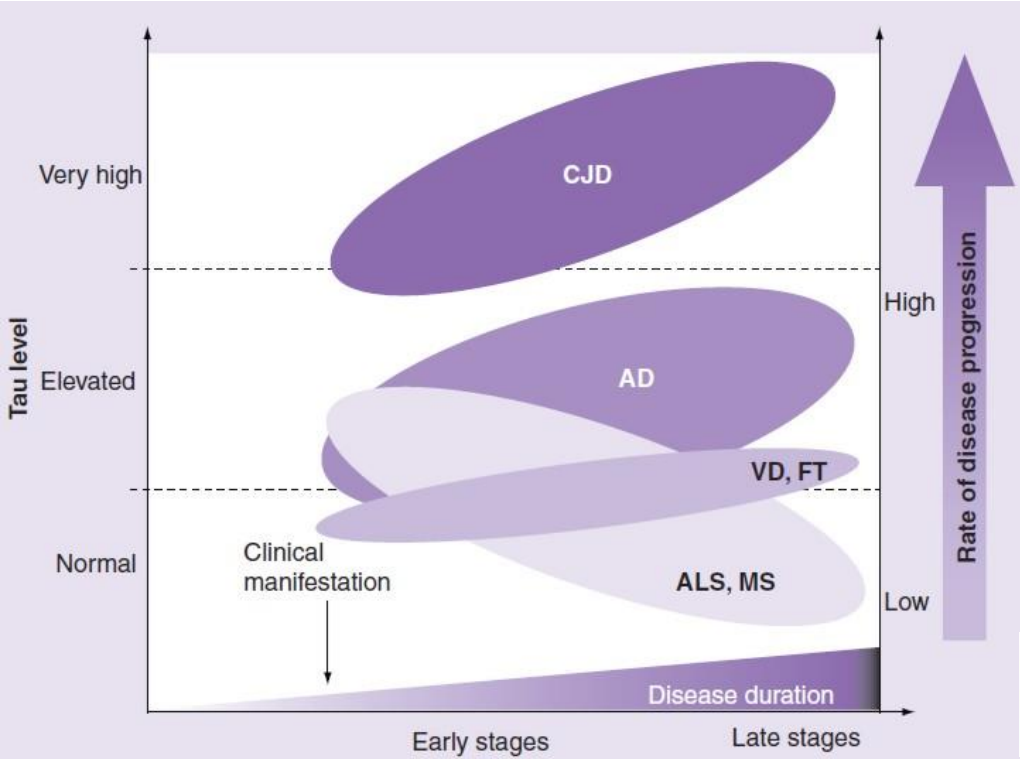
Fluid	Biomarkers	Methodology	Results
CSF	Aβ42	Commercial ELISA	CSF Aβ42 has an inverse correlation with amyloid burden, as measured by PIB-PET
		Commercial immunoassays	Ratios of CSF Aβ42 to other Aβ isoforms (Aβ40 or Aβ38) are strongly correlated with accurate AD diagnosis
	t-Tau	Commercial ELISA	*t*-Tau is associated with neurodegeneration and neuronal/axonal damage
	p-Tau	Commercial ELISA	*p*-Tau has a positive correlation with NFT regional distribution
Whole blood	120 proteins	Commercial cytokine antibody array assay	18 out of 120 proteins can be used to diagnose AD patients with 90% accuracy and predict MCI progression to AD with 91% accuracy
	30 proteins	Multiplexed immunoassay humanMAP	30 candidate markers can accurately diagnose AD with 88% sensitivity and 82% specificity
Plasma	Aβ40	Commercial sandwich ELISA	Plasma Aβ40 in AD patients increases (57–59), decreases (60), or is irrelevant (61,62)
		IP-MS with MALDI-TOF mass spectrometry	Ratios of plasma Aβ40/Aβ42 and APP/Aβ42 correlate with amyloid burden, as measured by PIB-PET
	Aβ42	Commercial sandwich ELISA	Plasma Aβ42 in AD patients increases (58), decreases (59, 61), or is irrelevant (57,62)
		Two-step immunoassay	Plasma Aβ42/Aβ40 ratios correlate with AD diagnosis
Oral	Aβ42	Commercial ELISA	Salivary Aβ42 is significantly elevated in AD patients
	Metabolites	NMR spectroscopy	There are several candidate AD biomarkers in saliva
Ocular	Aβ	FLES	Ocular Aβ levels correlate with quantitative PET and predict AD
Ocular—retina	Aβ plaques	Curcumin staining	Retinal Aβ plaques are present in AD patients and mice
	RNFL	Optical coherence tomography	AD patients tend to have a reduction in RNFL thickness
	Aβ42	Immunohistochemistry	Compared to the brains of AD mice, the retinas exhibit lower Aβ production
Ocular—lens	Aβ40, Aβ42	ESI-MS	Aβ42 and Aβ40 is found in the lenses of postmortem AD patients
	Aβ aggregates	Commercial ELISA	Aβ potentiates lens protein aggregation and can accumulate in the lens similar to how it accumulates in the cerebrum
Ocular—aqueous humor	Aβ40	SELDI-MS protein array chip	Aβ40 is present in the primary aqueous humor of AD patients
Olfactory	Aβ	Histopathology	Few neuritic plaques are present in the anterior olfactory nucleus; thus these plaques do not correlate with NFTs
	Tau	Histopathology	NFT/neuropil threads are present in the anterior olfactory nucleus and olfactory bulb (except the outer layer)
		Commercial olfactory test	*t*-Tau and *p*-tau are present in AD patient nasal secretions

1.Aβ amyloid-β, *t-tau* total tau, *p-tau* phosphorylated tau, *NFTs* neurofibrillary tangles, *ELISA* enzyme-linked immunosorbent assays, *PET* positron emission tomography, *humanMAP* human multi-analyte profile, *IP-MS* immunoprecipitation-mass spectrometry, *MALDI-TOF* matrix-assisted laser desorption ionization–time-of-flight, *NMR* nuclear magnetic resonance, *SELDI-MS* surface-enhanced laser desorption ionization mass spectrometry, *ESI-MS* electrospray ionization mass spectrometry, *FLES* fluorescent ligand eye scanning, *RNFL* retinal nerve fiber layer

Source: Lee, J.C., Kim, S.J., Hong, S. *et al.* Diagnosis of Alzheimer's disease utilizing amyloid and tau as fluid biomarkers. *Exp Mol Med* **51**, 1–10 (2019). https://doi.org/10.1038/s12276-019-0250-2

CSF Tau (P-tau) as an AD biomarker

*tau ↑+ (and P-tau) in AD CSF …
Numerous laboratories have reported an **increase in levels of T-tau and P-tau** in CSF

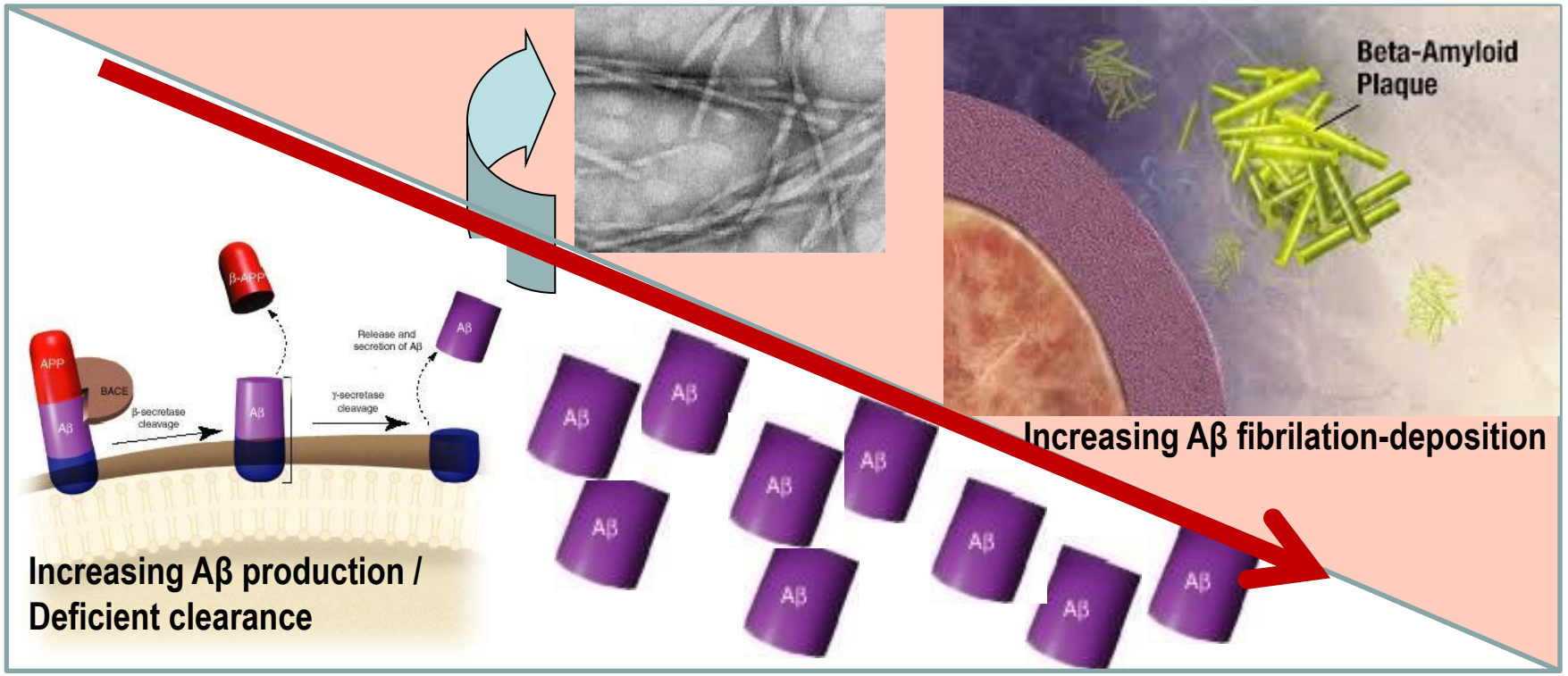


Suitable sensibility, but partially overlap with other neurological disorders

AD: Alzheimer's disease; ALS: Amyotrophic lateral sclerosis; CJD: Creutzfeldt–Jakob disease; FTD: Frontotemporal dementia; MS: Multiple sclerosis; t-tau: Total cerebrospinal fluid tau; VD: Vascular dementia.

CSF Aβ42 as an AD biomarker

*Aβ (**Aβ42**) ↓ in AD CSF…
While levels of the pathological Aβ42 species are increased in the AD brain, the **levels in CSF are decreased** due to increasing deposition



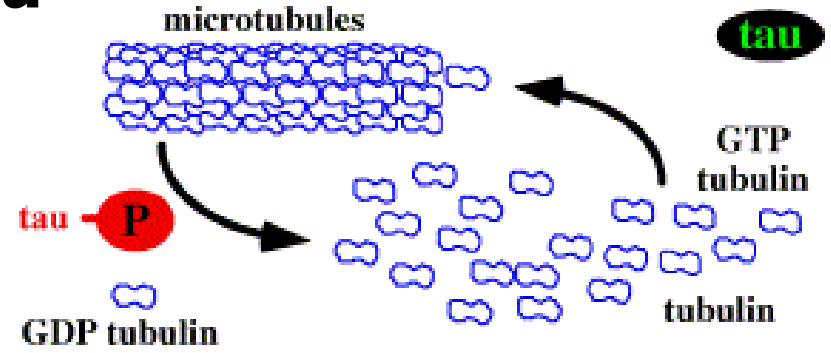
Failure of AD core biomarker within follow-up clinical trials

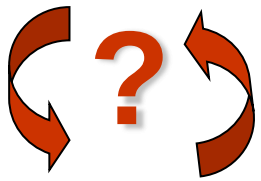
Significance of changes during clinicals trial designed to reducing Aβ?

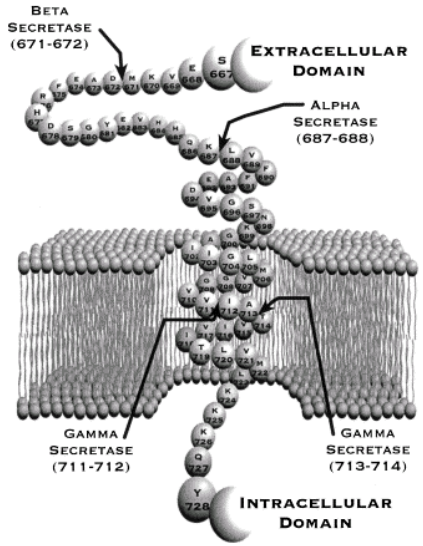
2. The Product, Innovative mechanisms of action

AD - Unresolved issues

tau



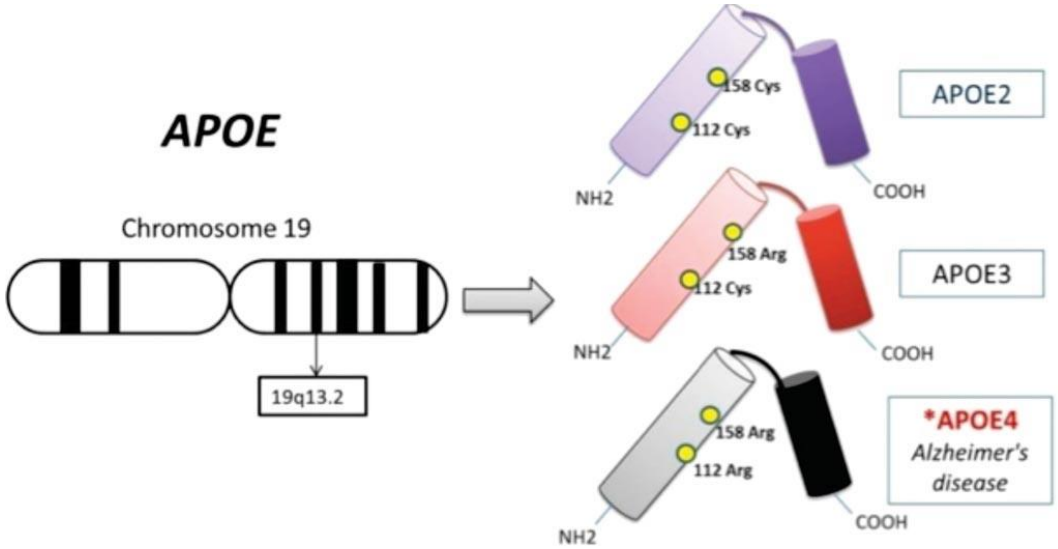




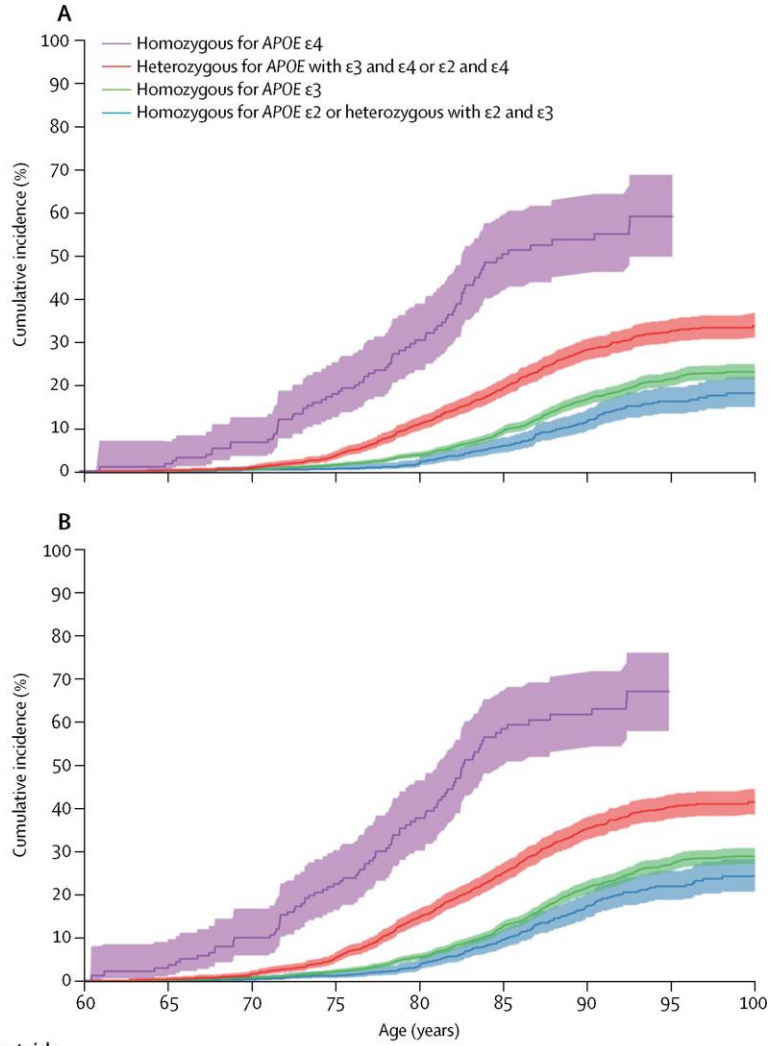
Aβ (APP)

apolipoprotein E (apoE)
Highly abundant in human fluids

apoE ε4 allele (apoE 4) is the strongest genetic risk factor for sporadic Alzheimer's disease (AD)



APOE genetic marker



Number at risk									
APOE ε4/ε4	82	136	128	108	64	23	10	2	0
APOE ε3/ε4 or ε2/ε4	875	1272	1370	1326	987	580	220	49	5
APOE ε3/ε3	1866	2874	3170	3035	2458	1500	648	178	30
APOE ε2/ε2 or ε2/ε3	437	678	743	730	573	387	183	58	9

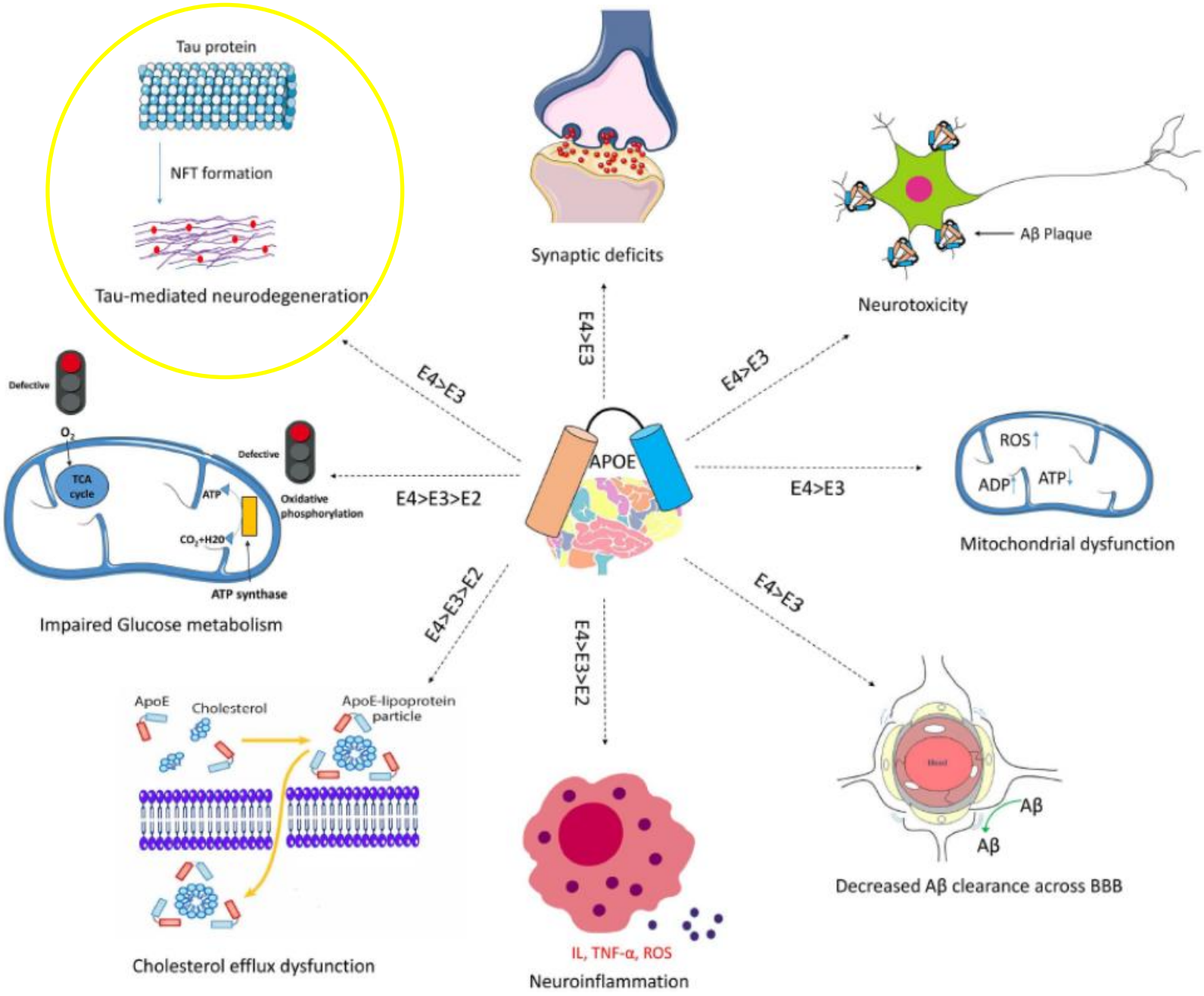
THE LANCET Neurology

To date in clinical trials apoE is only considered for *APOE* genotype

apoE
in Alzheimer's pathology

apoE "lipid-free"protein is a ligand of several membrane receptors…

Impaired function?



Normal condition
apoE 2/3

Aβ

Role for Aβ ??

Risk factor
apoE-4 carriers

Active apoE homodimers

Effective ApoER2 binding and processing

Less effective ApoER2 binding

ApoER2 soluble extracellular fragments

ApoER2 soluble Intracellular fragments

Dab1 phosphorylation (P) (P)

no Dab1 phosphorylation

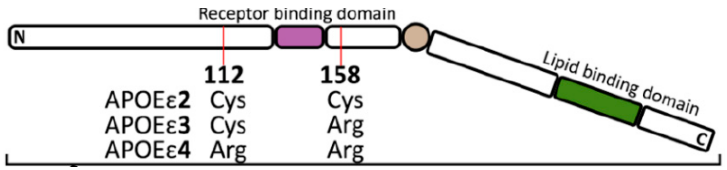
(P) -GSK3β Inactive

GSK3β *Active*

tau

(P) -tau *Increased tau phosphorylation*

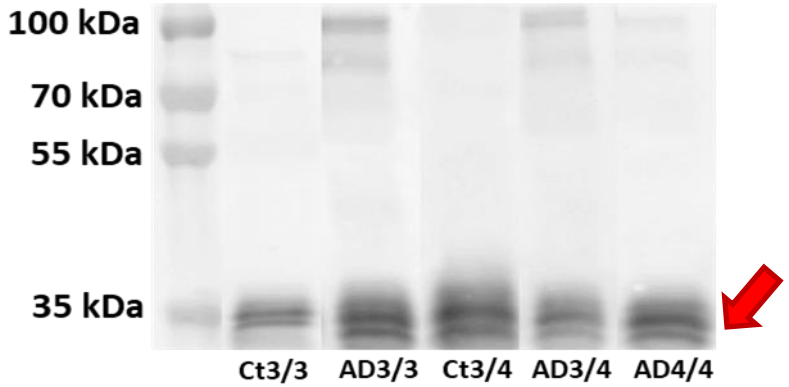
apoE3 and apoE2 form disulphide-linked dimers whereas apoE4 cannot form dimers as it lacks a cysteine residue



apoE-4 is less efficient to activate signaling

2. The Product, Current status of development

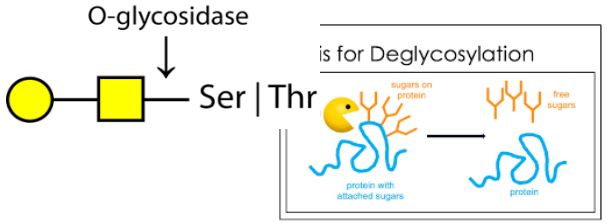
Biochemical characterization of apoE in human CSF

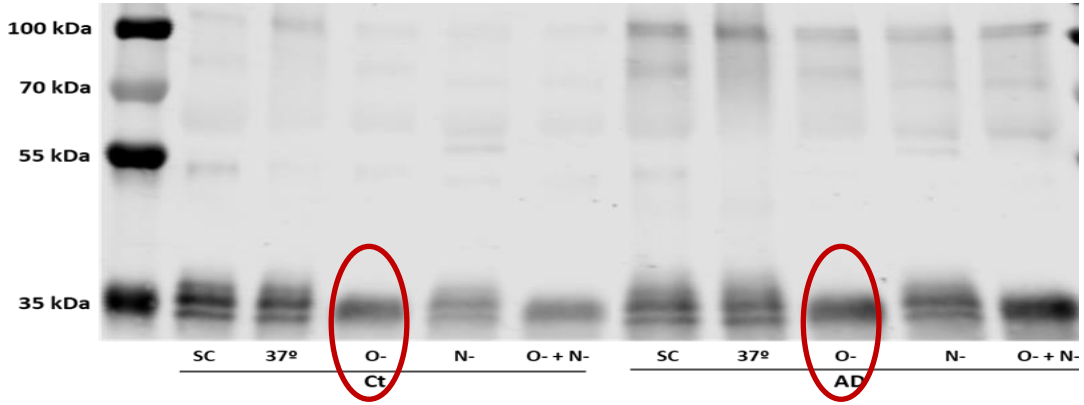


apoE monomers were identified as a **double band** around 354 and 36 kDa, showing two distinct species

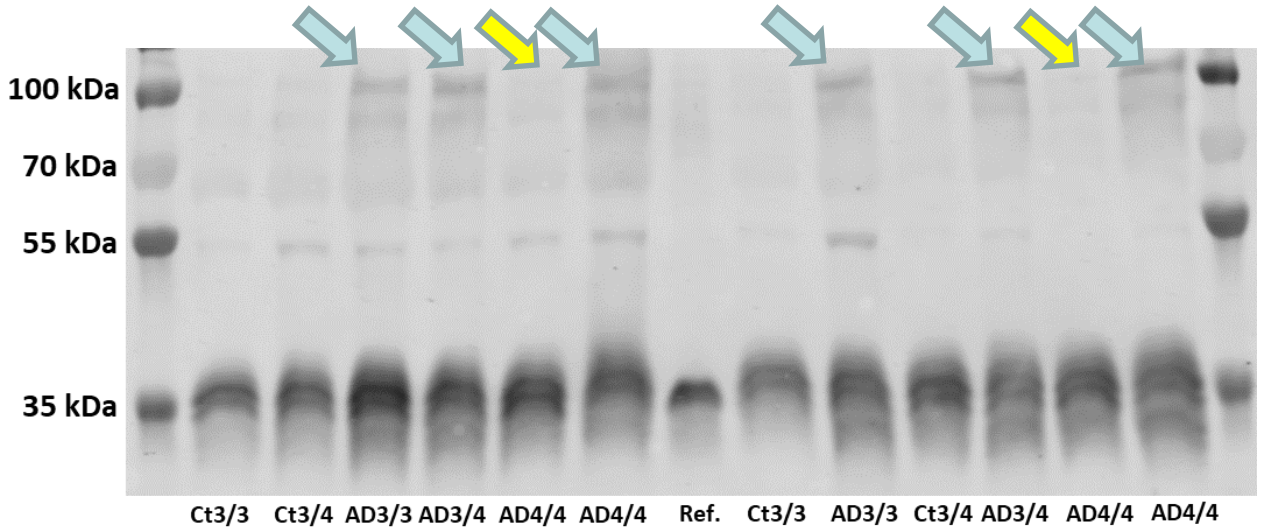
*Probably representing **mature-fully glycosylated** and **immature glycoforms***

SDS-PAGE





A SDS-PAGE-resistant apoE band of ~100kDa appeared almost exclusively in AD patients (also in apoE4/4)




```
  1 MKVLWAALLV  TFLAGCQAKV  EQAVETEPEP  ELRQQTEWQS  GQRWELALGR
 51 FWDYLRWVQT  LSEQVQEELL  SSQVTQELRA  LMDETMKELK  AYKSELEEQL
101 TPVAEETRAR  LSKELQAAQA  RLGADMEDVC  GRLVQYRGEV  QAMLGQSTEE
151 LRVRLASHLR  KLRKRLLRDA  DDLQKRLAVY  QAGAREGAER  GLSAIRERLG
201 PLVEQGRVRA  ATVGSLAGQP  LQERAQAWGE  RLRARMEEMG  SRTRDRLDEV
251 KEQVAEVRAK  LEEQAQQIRL  QAEAFQARLK  SWFEPLVEDM  QRQWAGLVEK
301 VQAAVGTSAA  PVPSDNH
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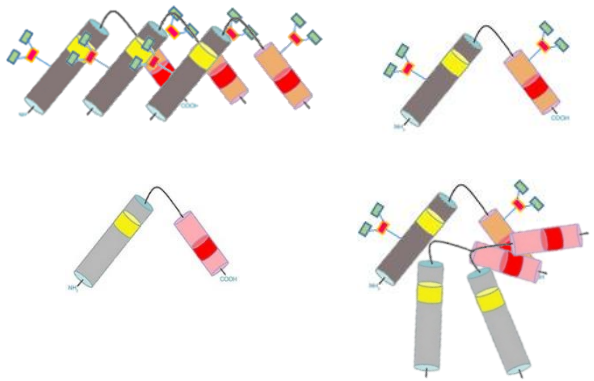
Mass spectrometry validatio

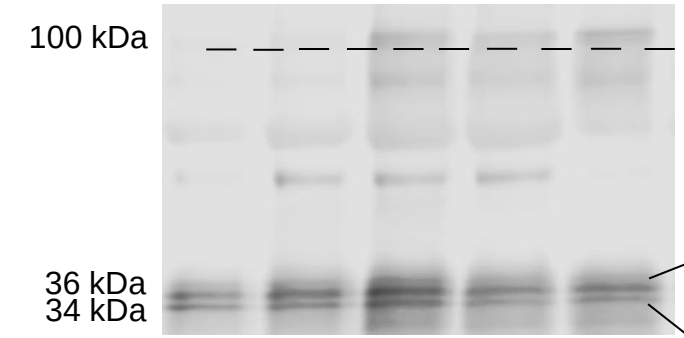
Mature apoE
(36 kDa)

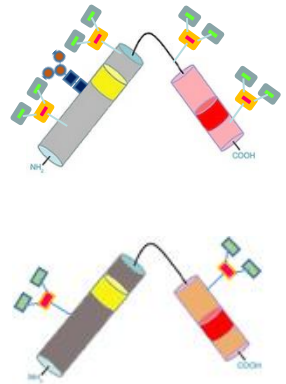
Immature apoE
(34 kDa)



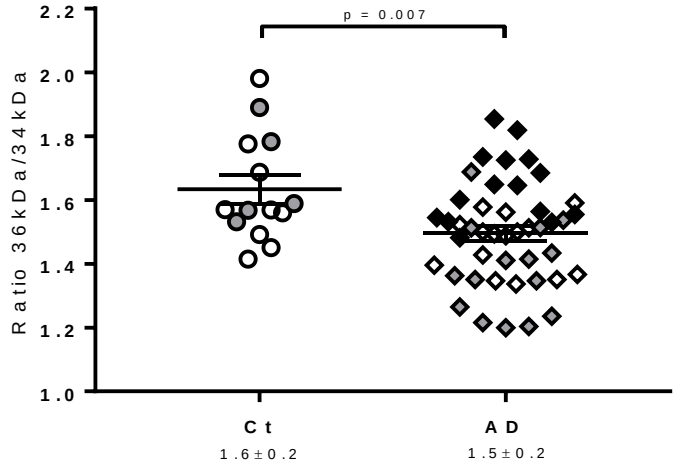
apoE aberrant aggregates
mature/immature species

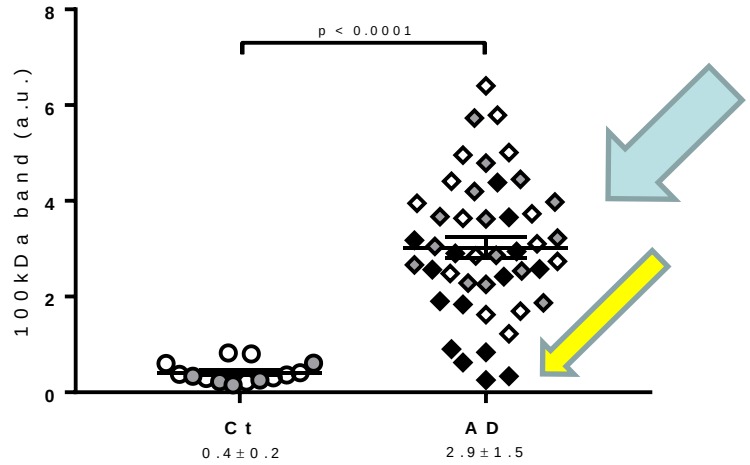
Ct 3/3
Ct 3/4
AD 3/3
AD3/4
AD 4/4





ApoE aberrant complexes, enriched in immature species, as an AD biomarker





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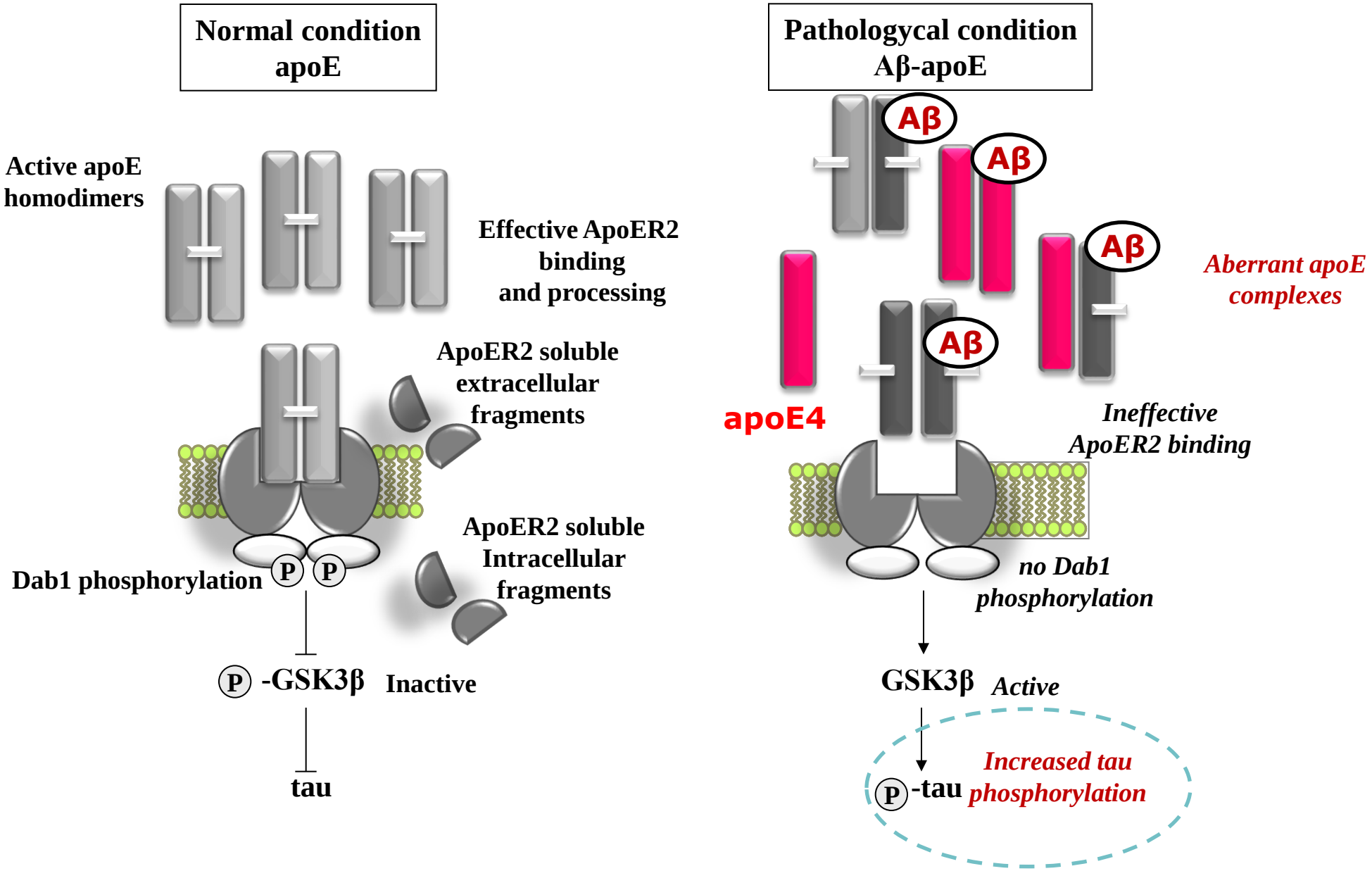
Alzheimer's
Research & Therapy

RESEARCH Open Access

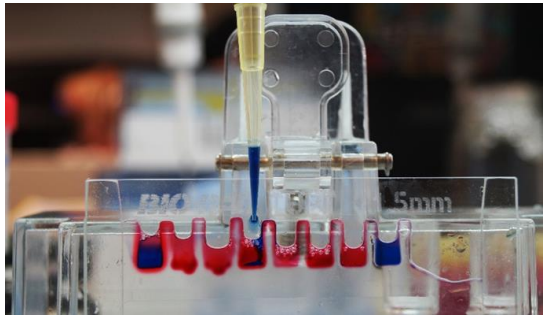
Apolipoprotein E imbalance in the cerebrospinal fluid of Alzheimer's disease patients

Matthew Paul Lennol[1,2], Irene Sánchez-Domínguez[3,4], Inmaculada Cuchillo-Ibañez[1,2,5], Elena Camporesi[6], Gunnar Brinkmalm[6], Daniel Alcolea[2,7], Juan Fortea[2,7,8], Alberto Lleó[2,7], Guadalupe Soria[4,9], Fernando Aguado[3,4], Henrik Zetterberg[6,10,11,12,13], Kaj Blennow[6,10] and Javier Sáez-Valero[1,2,5*]

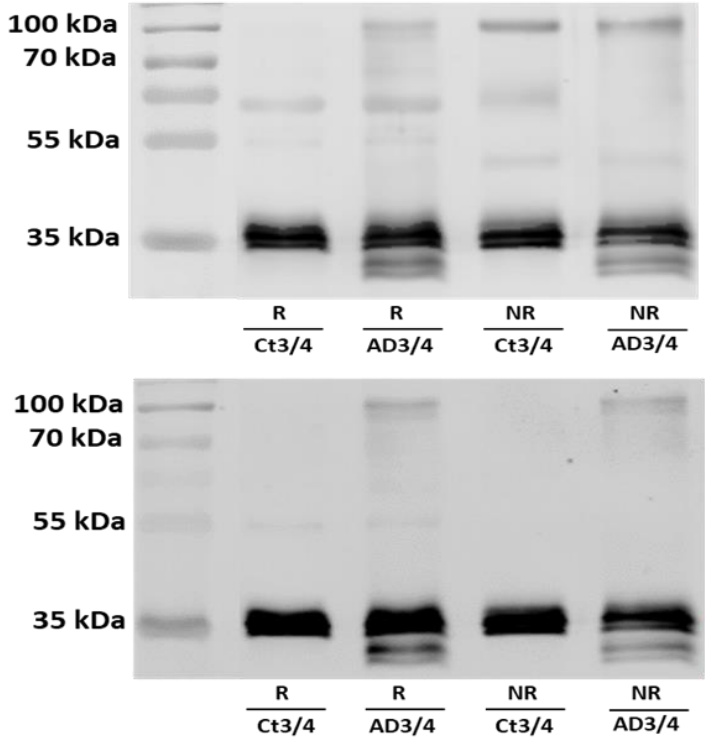


Altered Aβ-apoE may sequester active apoE impairing apoE function

Present apoE kit based in electrophoretical/western bloting determination of aberrant dimers



REDUCING
vs
NON-REDUCING
ELECTROPHORESIS



WB apoE antibody (common to all isoforms)

CSF samples immunoblotted following non-reducing (NR) or reducing (R) SDS-PAGE with apoE antidoides

WB: apoE4 (specific antibody)

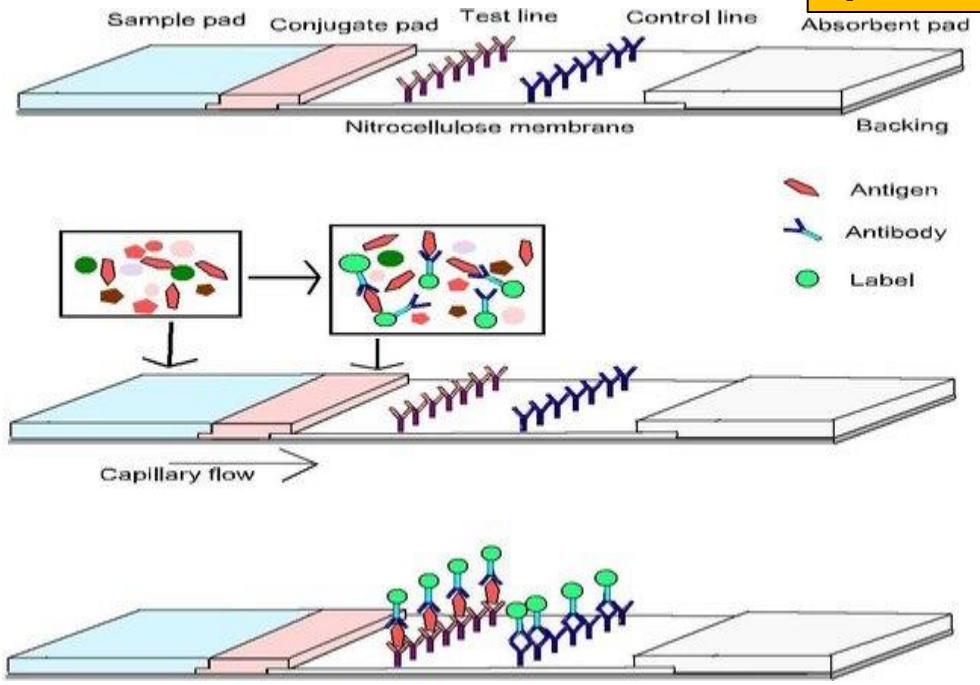
Optimization:

-analysis in reducing/non-reducing condition for all patients

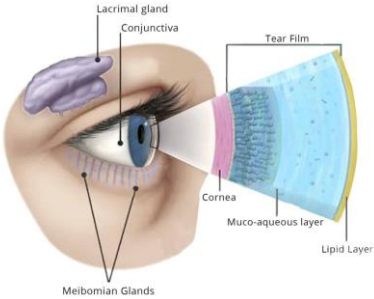
-use of anti-apoE4 antibodies for apoE4 carriers **and adapt to an Immunochromatographic membrane or ELISA**

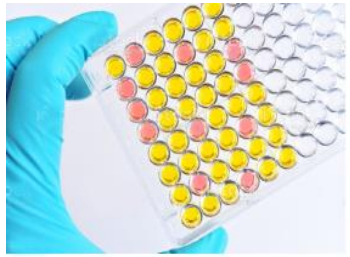
Future apoE kits based in determination aberrant dimers+ immature apoE

qualitative and quantitative

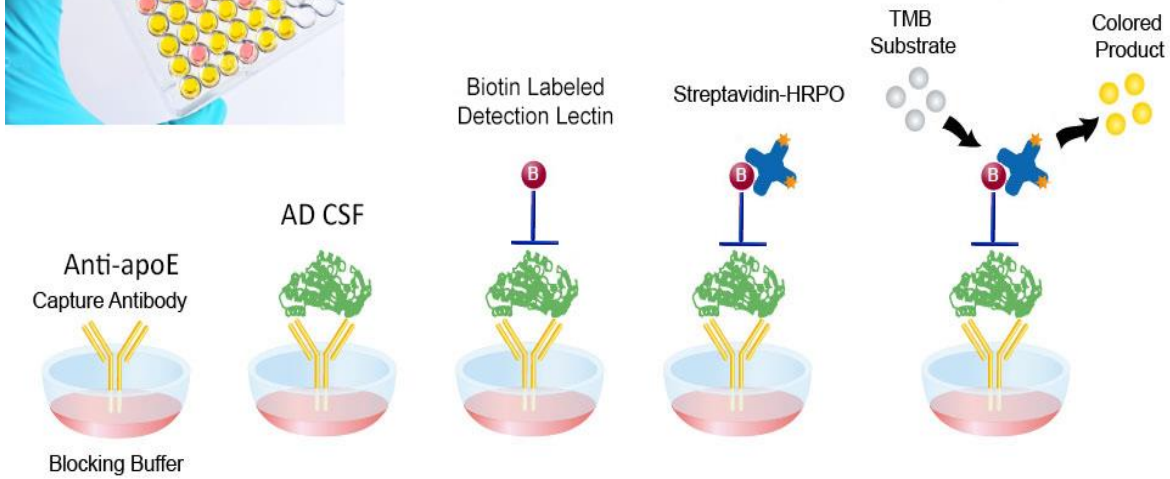


Immunochromatographic membrane assay that uses a system to separate dimers from monomers, and identify aberrant complexes combining highly sensitive anti-apoE antibody and lectin





LECTIN ELISAS



ELISA combining highly sensitive conformational anti-apoE antibody and lectin

Early biomarker and follow-up biomarker within AD Clinical trials

Monoclonal antibodies (aducanumab, lecanemab, donanemab… active vaccination) that targets Aβ (aggregates) are useful tools for the treatment of AD, but are also associated with amyloid-related imaging abnormalities (ARIA) due to edema (ARIA-E) or hemorrhage (ARIA-H) from blood vessels in the brain.

apoE4 carriers have an increased risk of ARIA-E or -H

2. The Product, IPR protection

Spanish Patent 202230224 “Method and kit for diagnosing Alzheimer’s Disease based on the detection of Apolipoprotein E”	
Co-ownwership	UMH (85%) CIBER (15%)
International Application	**WO 2023/175225 A1** (publication number)
National Phases	**EEUU Application** 18/88,336 - filed 09/17/2024 **Europea Application** 23769971,5 - filed 09/16/2024

2. The Product, Pitfalls & Risks to be considered

ENTRY BARRIERS

- **TECHNICAL BARRIERS:**
  - Need to validate the technology with more patients
  - Not obtaining sufficient sensitivity in tears, blood or other non-invasive fluids
  - Not obtaining, at least, the same sensitivity and specificity as the gold standard in AD diagnosis.
  - Failure to obtain approval of the CE Marking of the program as a medical device. Then main hurdle will be the clinical trial, if the aim is to use it in medical diagnostics
  - High investment is required to obtain CE marking. However, this responsibility will be within the scope of activity of licensee.
  - Failure to achieve validation and approval by the FDA and EMA.

- **REGULATORY BARRIERS**:
  - The implementation of ISO 13485 guideline is required to validate the test.
  - In Europe, the regulatory framework is Regulation (EU) 2017/745 that modified Directive 2001/83/EC, Regulation (EC) No. 178/2002 and Regulation (EC) No. 1223/2009. The new regulations have tightened the framework and the requirement of a clinical trial , but it is still simpler than the FDA's.
  - In the US, very extensive and expensive clinical validation is needed. The only wat of avoiding this process is to apply for a 510(K) Premarket Notification. But in order to follow this process, there must be an equivalent product on the market.
  - If the product is not approved for medical diagnostics, it can only be sold as a reagent for research us only.

ENTRY BARRIERS

- **MARKET BARRIERS:**
  - Not being able to license the technology to a top-level company.
  - Not being able to implement this technology in the market.
  - Even though **SDS PAGE and Western blotting are standard methodologies, they are quite labor intensive**.
  - As AD diagnostics is multi parametric, the project may be more attractive if integrated into a multi marker automatic diagnostic platform. ELISA es the recommended platform.
  - The market is highly concentrated in international IVD companies that commercialize the discoveries of a large number of start ups.
  - **The launching of a new diagnostic kit is costly and time consuming**. Ideally it should be carried out by one of the largest players
  - **Access to Key opinion Leaders (KoL) is essential** as they can prescribe the diagnostic kit is medical symposia and meetings, and hence helping in the promotion of the product.

- **INTELECTUAL PROPERTY**:
  - A search in Patentscope of the terms apoE and Alzheimer yields 140 families of patents. It is essential that a Freedom to Operate study is carried out.
  - The patent submission protects a methodology and the use of polymers of the biomarker apoE. However, **the methodology uses commercial antibodies**. If proprietary antibodies monoclonal antibodies are not developed, a substantial license royalty will have to be paid to the originator of the antibody.
  - Unless proprietary antibodies are obtained, all the regulatory approvals will be obtained for a third-party antibody.

2. The Product, Pitfalls & Risks to be considered

*apoE does not cross the blood-CSF barrier
*glycosylation is tissue-specific

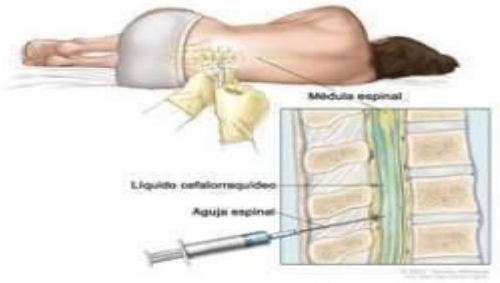
apoE monomers (mature and immnature)
are **present in tears**

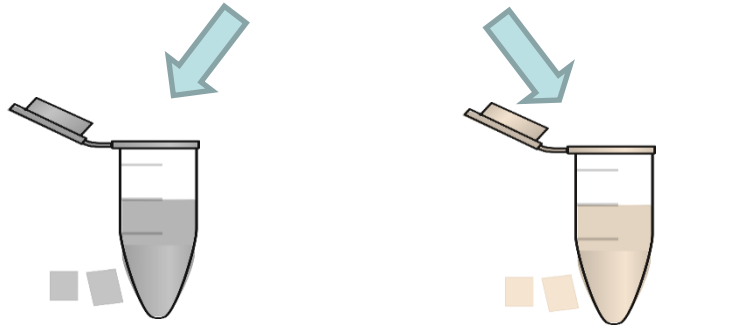
Glycosylation of apoE monomers in AD CSF

¿Are the hypoglycosylated monomers in the aggregates?

Do we have a specific lectin?

Asparia Glycomics

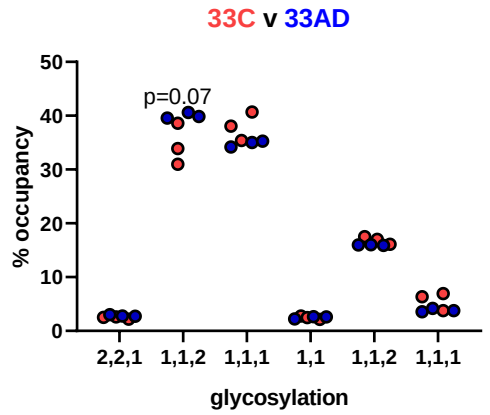




CONTROL GROUP AD GROUP

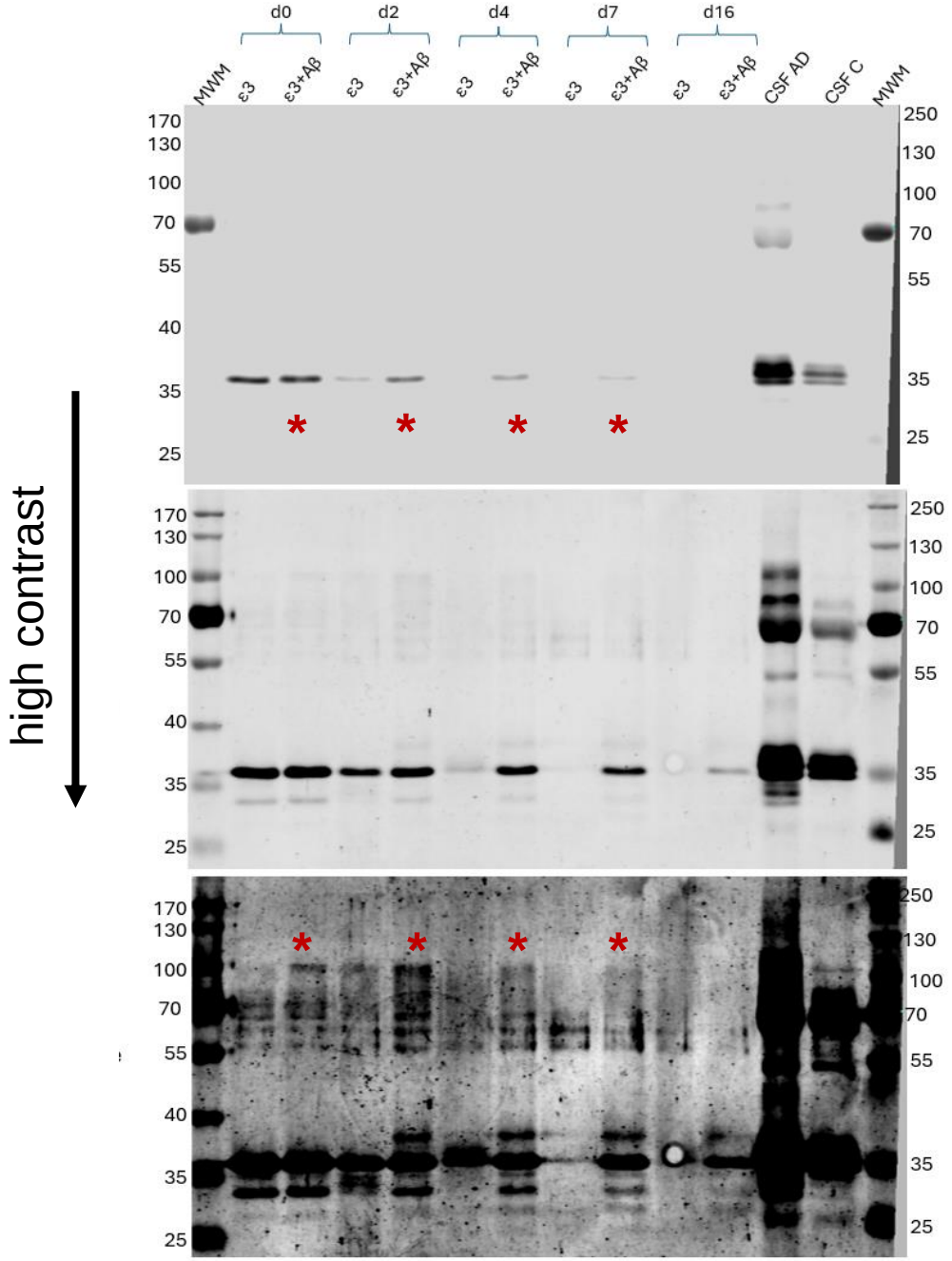
O-glycosylation of APO-E is detected after trypsin digestion of the protein. This method is more selective as it analyzes the glycans still attached to a specific peptide sequence (instead of releasing the O-glycans from the protein through reductive beta-elimination). The samples were analyzed using UHPLC with QTOF MS detection as follows: peptides and glycopeptides were separated using a C18 reversed-phase column, and the latest generation of Q-TOF mass spectrometer (Synapt XS) was used for detection.

(HexNAc(1)Hex(1),
HexNAc(1)Hex(1)NeuAc(1),
HexNAc(1)Hex(1)NeuAc(2),
HexNAc(2)Hex(2)NeuAc(1))



Aβ triggers stability of apoE and formation of aberrant complexes

Recombinant (non-glycosylated) apoE and Aβ42 were incubated at 37ºC (cell incubator)



3. Partnering Opportunities

►License agreement (collaboration in the development)

►Lead further patent development (*co-development with adaptation to specific needs*)

* Funding opportunities

** Collaboration for patent validation to approach it more to market

- Human resources are needed (full dedication to kit development)
- Support/Collaboration with a biotechnology company to develop a specific conformational antibody (anti-apoE)
- Collaboration to finish glycosylation characterization (lectin)

CONCLUSIONS

- There is a **clear medical need** for screening with AD patients who are not yet symptomatic (in early stages), particularly for Clinical trials recruitment.

- The project provides a **differential solution** that allows the diagnosis **by qualitative and quantitative approaches**.

- The business model determined as the option for inventors is **licensing the technology (participating in co-development)**.

- The promoters are well aware that that they need a proprietary antibody and a selective lectin to **increase project** value.

- There is a clear intellectual **protection strategy**, although it is important to carry out a Freedom to Operate study

- It is a rapidly **growing market since from 2023 to 2029 it will go from 4.5 to 8,8 Billion US dollars**