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Seeding of oligomers: new strategies for Alzheimer's diagnosis and possible implications for the progression of the disease.

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Lisbell Dianne Estrada Apablaza

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Director de Tesis Dr. Claudio Soto

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Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato:

Lisbell Dianne Estrada Apablaza

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Director de Tesis:

Dr. Claudio Soto .....

Comisión de Evaluación de la Tesis

Dr. Miguel Allende .....

Dr. Nibaldo Inestrosa .....

Dr. Christian González .....

Dedico esta Tesis con amor a mi padre,
quien hizo lo imposible para que obtuviera este título....

Realicé mis estudios básicos y la enseñanza media en Santiago, en el colegio Particular "Congregación Calasancia Divina Pastora" lugar en que me otorgó una educación que priorizaba los valores y donde hice amistades que hasta hoy perduran. En el año1999 ingresé a la Universidad de Chile para estudiar la Carrera de Ingeniería en Biotecnología Molecular, en la Facultad de Ciencias ubicada en la comuna de Macul. El paso por este campus marcó mi desarrollo como científica, con una educación que fomentaba el espíritu crítico, la rigurosidad científica y los problemas sociales que aquejan al país.

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Luego de finalizar mi tesis de pregrado en el área de los amiloides me centralicé en el estudio de la enfermedad de Alzheimer. Durante este período comencé mi Doctorado en Ciencias de la Universidad de Chile en conjunto con la University of Texas Medical Branch.

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Acronym List

aCSF : Artificial Cerebrospinal Fluid

Aβ : amyloid-beta peptide

AD : Alzheimer's disease

APP : Amyloid Precursor Protein

BCA : Bicinchoninic acid

CSF : Cerebrospinal fluid

FAD : Familial Alzheimer's disease

GDS: global deterioration scale

GFAP : Glial fibrilar acidic protein

HFIP: hexafluoroisopropanol

HRP : Horseradish peroxidase

i.c : Intracerebral

i.p. : Intraperitoneal

LTP : Long-term potentiation (Potenciación a largo plazo)

MMSE: Mini Mental State Examination

PBS : Phospate saline buffer

PS1 : Presenilin 1

PS2 : Presenilin 2

SDS : Sodium Dodecyl Sulfate

ThS : Thioflavin S

ThT : Thioflavin T

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1. ABSTRACT

Alzheimer's disease (AD) is a complex neurodegenerative condition which has become a major public health problem because of its increasing prevalence, long duration and high cost of care. It is estimated than more than 25 million people worldwide are affected to some degree by AD. Unfortunately, there is not effective treatment or accurate pre-clinical diagnosis for AD. The neuropathological hallmarks of AD are neuronal loss in regions related to memory and cognition, neurotransmitter depletion, synaptic alteration and the deposition of two types of abnormal protein aggregates: neurofibrillary tangles and amyloid beta plaques. Alzheimer's disease form part of a group of disorders called Protein Misfolding disorders (PMD). In general, proteins fold properly into their native conformation and, if they do not, the misfolding is corrected by chaperone proteins. In PMDs however, misfolding of a protein results in its aggregation and accumulation as amyloid deposits in diverse tissues. Compelling evidence suggest that the misfolding and aggregation of amyloid-beta peptide (Aβ), the major component of plaques, is the triggering factor of AD pathology. *In vitro* studies have shown that Aβ misfolding and aggregation follows a seeding-nucleation mechanism. Analogous to a crystallization process, in this model the limiting step is the formation of small oligomeric intermediates that act as seeds to catalyze the polymerization process. I hypothesize that oligomers are present in biological fluids of AD patients long before the clinical symptoms of

the disease appear. My experimental approach is to use the functional property of oligomers to catalyze (to seed) the polymerization of monomeric protein as a way to measure their presence in biological fluids. For this purpose I have developed a technology consisting in the cyclic amplification of seeds combined a sensitive detection of Aβ aggregates. Using this technique I was able to detect an increase of Aß oligomers in human biological fluids such as cerebrospinal fluid (CSF) and blood. The results of this research may help to design a biochemical test for Alzheimer's disease and other Protein misfolding disorders. Prion disease is the only member of the PMD group classified as transmissible. Compelling evidence supports the concept that the misfolded prion protein is the only component of the infectious agent and that it can 'replicate' in the brain in the absence of nucleic acid by converting the natively folded prion protein. Interestingly, the data available indicate that amyloid formation in all PMD disorders follows a seeding–nucleation mechanism. This suggests that disease propagation by a protein oligomer may perhaps be occurring in other Protein misfolding disorders of higher prevalence such as Alzheimer's disease. Indeed, recent developments in the field have demonstrated that misfolded proteins associated with some PMDs can initiate the conversion of the normal form of the protein into the misfolded form and propagate these changes to neighboring cells in experimental models. Taking these antecedents into account, I want to investigate in this thesis if misfolded Aβ oligomers implicated in Alzheimer's disease are capable to transmit the disease under experimental conditions. Another intersting aspect that misfolded aggregates composed of one protein may interact and promote the aggregation of another protein by a phenomenon known as heterologous-seeding. Evidence for this phenomenon has been found for a number of PMDs. Thus, I would like to determine if

exposure to Aβ misfolded protein may influence the development of another PMD, such as Prion disease, both *in vitro* and *in vivo*.

2. THEORETICAL BACKGROUND

Protein Misfolding Disorders

More than 20 human diseases are included in the group of Protein Misfolding Disorders (PMDs), including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), diabetes type II, transmissible spongiform encephalopathies (TSEs), serpin-deficiency disorders, Amyotrophic Lateral Sclerosis (ALS), dialysis-related amyloidosis and others (Carrell and Lomas, 1997; Soto, 2001; Stefani and Dobson, 2003). The common feature of PMDs is the accumulation of misfolded proteins in diverse tissues, forming protein deposits called amyloid. More than 200 years ago, physicians used the term amyloid to describe a substance present on disease organs that showed a black color reaction with iodine (Cohen, 1965). Originally, amyloid was thought to be a starch, a carbohydrate, and indeed, the very word means "starch-like". It has been only during the last decade that the composition and major role of these protein aggregates have been elucidated (Soto, 2003). Although the protein involved in the misfolding and aggregation process is different in each disease, the pathological structure in most cases is composed of β-sheet rich amyloid fibrils (Fig 1). The two major hallmarks in AD, amyloid plaques and neurofibrillary tangles are composed of aggregates of Aβ and hyperphosphorylated tau, respectively (Terry, 1994). Lewy bodies,

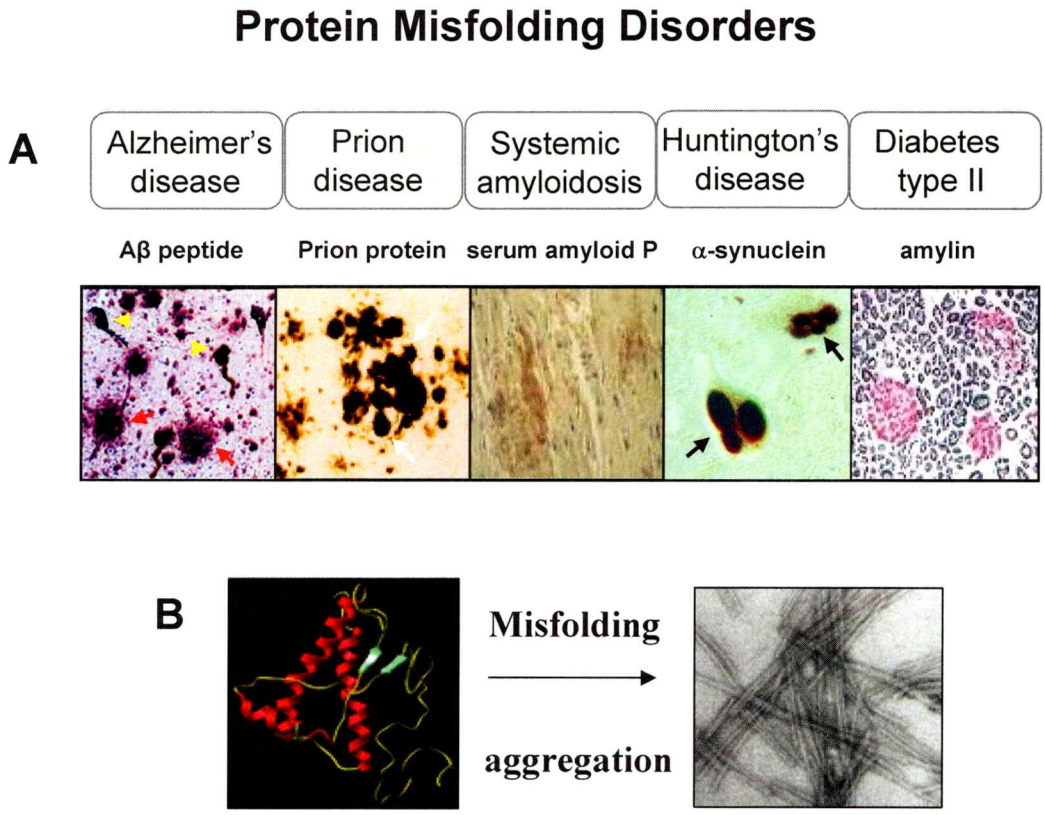


Figure 1: Protein Misfolding disorders.
A. Extracellular amyloid plaques (red arrows) and intracytoplasmic neurofibrillary tangles (yellow arrows) are the pathological signature of Alzheimer's disease. Extracellular prion deposits (white arrows) located in different brain regions are present in some cases of transmissible spongiform encephalopathy. Intracytoplasmic aggregates are typically present in the neurons of people affected by Parkinson's disease. In systemic amyloidosis there are aggregates in different organs, e.g deposition of serum amyloid P in kidneys. Intranuclear inclusions of huntingtin are observed in Huntington's disease patients. In diabetes type II, the pancreas accumulates aggregates of the protein amylin. **B** In spite of the different protein compositions, the hallmark is the presence of a protein, that after misfolding, deposits at the target organs. The ultra-structure of these deposits seems to be similar and composed mainly of a network of fibrillar polymers.

found in neurons of PD patients consist of aggregates of α-synuclein (Forno, 1996). Aggregates composed of the hormone amylin have been found in the majority of patients affected by diabetes type II (Hull et al., 2004). Intracellular aggregates composed of the huntingtin protein containing extended polyglutamine-repeats are characteristic of HD (Hull et al., 2004) and postmortem studies of patients with TSE show aggregates of protease-resistant prion protein (Bolton et al., 1982). Studies using X-ray fiber diffraction and solid-state NMR demonstrated the β-sheet rich nature for most of the misfolded aggregates (Serpell, 2000; Serpell et al., 2000a; Serpell et al., 2000b). Beta-sheets are composed of several extended motifs (β-strands) of a polypeptide that run alongside each other, linked by hydrogen bonding between the NH and CO groups of the peptide bond. Since β-sheets can also be formed by intermolecular bonding, misfolded proteins have a high tendency to form oligomers and large order aggregates. The structure of amyloid fibrils is usually called cross-β to refer to several β-sheets bound to each other with the β-strands running perpendicular to the long fiber axis (Blake et al., 1996). This is a very stable structure that can be adopted by diverse proteins regardless of their amino acids sequence (Stefani and Dobson, 2003). Neuropathological, biochemical and genetics data implicate protein misfolding and subsequent aggregation as the critical event in the development of PMDs. Amyloid deposits usually are present in diseased organs (Cohen and Connors, 1987; Glenner, 1981; Sipe and Cohen, 2000) and their accumulation is the end point in the majority of PMDs (Kelly, 1996; Soto, 2001). In addition, evidence from genetic studies demonstrated that genes linked to familial forms of PMD encode the protein constituent of aggregates (Singleton et al., 2004). Mutations in those genes may interfere with the normal protein folding, which may lead to protein aggregation. Indeed,

familial forms of the diseases are usually more severe than sporadic cases and have an earlier onset. The development of transgenic animals over expressing mutant human genes encoding misfolded proteins has supported the role of misfolding and aggregation in disease pathogenesis (Wong et al., 2002) . Finally, many *in vitro* studies have shown that misfolded oligomers and aggregates composed of different proteins acquire a cytotoxic activity, leading to cell death and tissue damage (Demuro et al., 2005; Soto, 2003).

Mechanism of protein misfolding and aggregation

Although the detailed mechanism of protein misfolding and aggregation is not entirely clear, kinetic studies have suggested that the critical event in PMDs is the formation of misfolding protein oligomers that act as seeds to further induce protein misfolding (Fig. 1A). This is the basis for the nucleation dependent polymerization model (Harper and Lansbury, 1997, Morgan et al., 2004). Diverse proteins have been shown to follow this crystallization-like process, including Aβ, huntingtin, amylin and α-synuclein (for references, see (Soto, 2003). According to this model, aggregation starts after the protein concentration exceeds a point known as the critical concentration (Harper and Lansbury, 1997). Unfavorable interactions between monomers determine a slow phase (termed lag phase) in which oligomeric species are formed providing an ordered nucleus to catalyze the further growth of the polymers (Fig 2). The growth phase continues until the equilibrium between aggregates and monomers is reached (steady state phase) (Jarrett and Lansbury, 1993). The addition of pre-formed nuclei (seeds) serves as templates for the reaction and, as a result, the initial, slow phase of primary nucleation is eliminated. Even though amyloid seeding is very selective because it needs a

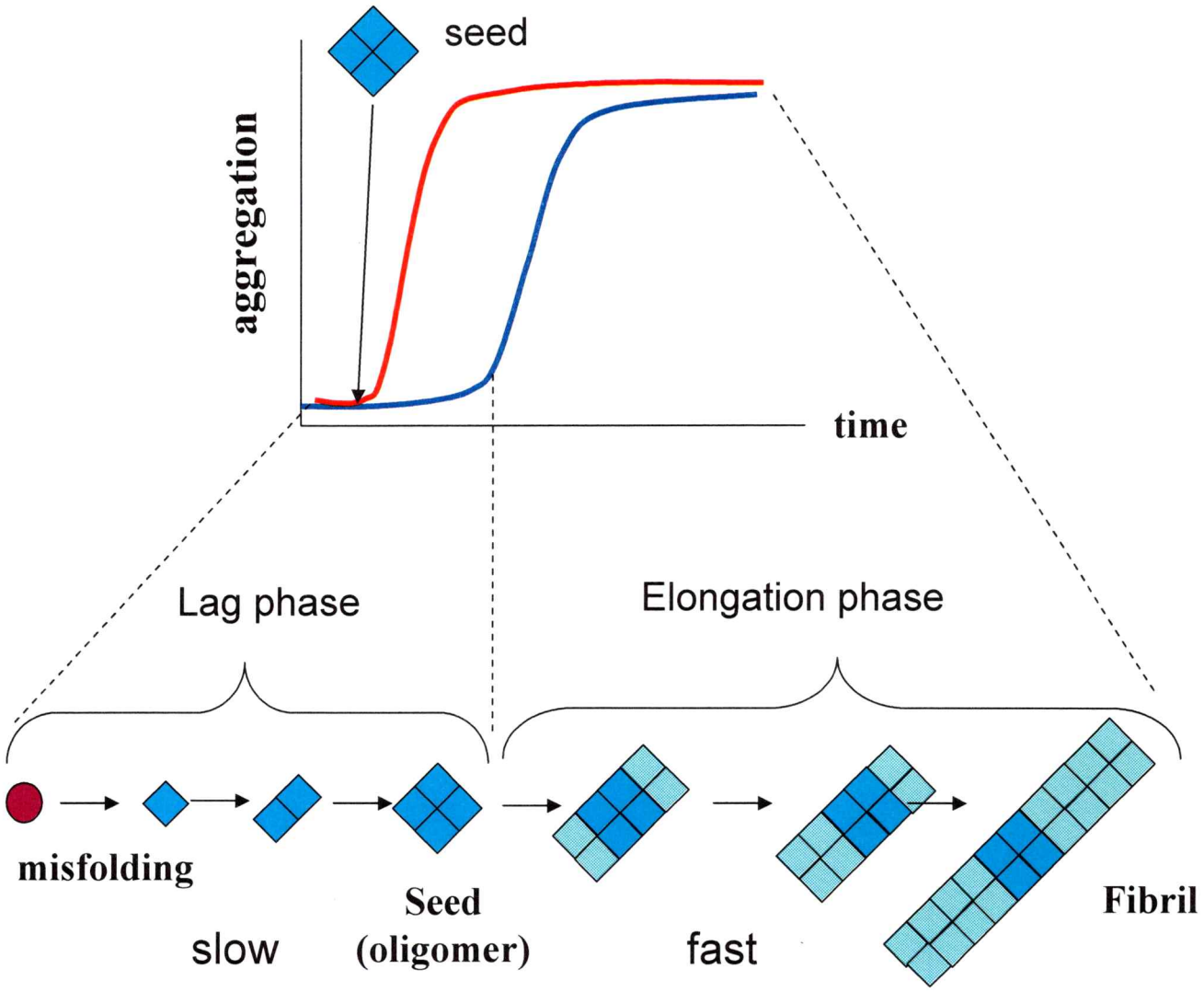


Figure 2: Seeding-nucleation model of amyloid polymerization.
In the amyloid polimerization model we can distinguish two kinetic phases. In the 'lag phase', oligomeric seeds (nuclei) are formed in a slow process that involves misfolding of the protein and unfavorable intermolecular interactions. The 'elongation phase' phase begins when the 'seeds' are formed, and culminates in the fibril formation. The limiting step in the seeding-nucleation process is the formation of seed to direct further aggregation. Fibril formation can be substantially accelerated by the addition of preformed seeds (red line).

match between the growing face and the monomer (Krebs et al., 2004), the addition of a different seed can bypass the lag phase, in a process known as heterologous seeding (O'Nuallain et al., 2004). Although it is clear that all proteins in PMDs undergo structural rearrangements during misfolding and aggregation, depending on the extent of the conformational changes and the thermodynamic forces driving the process, the diverse proteins can be classified in three different groups:

1) Proteins in which misfolding and aggregation result in a profound structural rearrangement of the polypeptide chain driven by hydrophobic interactions. The prototype proteins in this group are the Aβ peptide, Parkinson's α-synuclein and the prion protein.

2) Proteins in which misfolding and aggregation result in small changes in the secondary structure in a process driven by β-sheet instability. The prototype members of this group are transthyretin involved in systemic amyloidosis, SOD1 implicated in ALS and immunoglobulin light chain associated with primary amyloidosis. The characteristic of these proteins is that the native folding is rich in β-sheet and during aggregation, the intramolecular β–sheet interactions are replaced by intermolecular β-sheets (Kelly et al., 1997; Wall et al., 2004). Aggregation is dependent upon destabilization of the native β-sheets, usually by changes in the quaternary structure or by mutations. One characteristic of this group is that many different mutations spread all over the protein sequence can lead to aggregation, most likely by destabilization of the native oligomers and conversion into the amyloidogenic monomers (Damas and Saraiva, 2000; Saraiva, 2001).

3) Proteins in which misfolding and aggregation depend on glutamine/asparagine rich domains and protein aggregation is driven by hydrogen bonding involving both the peptide

bonds and the side-chains. The proteins in this group are implicated in several human diseases in which mutations result in an abnormal and inherited expansion of CAG (codon codifying for glutamine) repeats (Zoghbi and Orr, 2000). Huntingtin implicated in HD and ataxins associated to spinocerebellar ataxias as well as the yeast prions are some of the proteins in this group.

Alzheimer's disease

Neurodegenerative diseases are characterized by progressive neuronal loss. However, the mechanisms involved in neuronal death have not been completely elucidated. Alzheimer's disease is one of the most common neurodegenerative disorders, characterized by progressive neuronal death, loss of synaptic connections, cytoskeleton alterations, dystrophic neurites and axonal degeneration, which is associated to cognitive impairment and memory loss (Bossy-Wetzel et al., 2004; Mattson, 2004; Paradis et al., 1996). As we mentioned above, one of the pathological hallmarks of AD is the presence of protein aggregates (Hardy, 2006; Mattson, 2004). The intracellular deposits found in AD correspond to neurofibrillary tangles (NFT), which are composed of paired helical filaments of hyperphosphorylated Tau protein (Grundke-Iqbal et al., 1986). Extracellular deposits in AD are called senile plaques and the major protein component of the plaques is the amyloid-β peptide (Aβ), which is a 39-42 amino acid peptide, product of the proteolytic cleavage of a much larger transmembrane protein, the amyloid precursor protein (APP) (Reinhard et al., 2005; Selkoe, 1994). It has been described two origins for AD: (i) The familial type (FAD) which is hereditary and has an early onset (< 60-65 years) and (ii) Sporadic Alzheimer's which has a late onset (> 65 years). The FAD

correspond to 2% of the detected cases approximately, and there are three genetic loci involved in the development of the disease, which are located in the chromosomes 14, 1 and 21 encoding for presenilin 1 (PS1), presenilin 2 (PS2) and APP, respectively. In addition, there are other loci that has been identified as risk factors for AD: the apolipoprotein E (ApoE), the APP-binding protein APP (APP-BP1), the α2-macroglobulin and the LRP6 (De Ferrari et al., 2007).

Current Status of AD Diagnosis

Simple, noninvasive tests for an early detection of degenerative dementia by use of biomarkers are highly needed. However, up to the present, no accurate extracerebral diagnostic markers for the early diagnosis of AD are available.

Neuropsychological Diagnosis

Mental status examinations assess memory, concentration, and other cognitive skills. The most common mental status examination used in the evaluation of AD is the Mini-Mental State Examination (MMSE), a research-based set of questions that provides a score about a person's general level of impairment. The MMSE was designed to evaluate cognitive function in several domains including orientation, registration, attention, memory, language, and visual construction skills (Folstein et al., 1975). The MMSE is generally a reliable and valid measure of cognitive impairment. However, a number of studies have demonstrated that education, age, and ethnicity have an effect on MMSE scores (Blesa et al., 2001; Launer et al., 1993; Rosselli

et al., 2000). A study in five different cities in USA found that both age and educational level influenced scores (Crum et al., 1993). The MMSE is the most widely used brief screening measure of cognition, but it is not sensitive in detecting mild memory or other subtle cognitive impairments. The Short Test of Mental Status (STMS) was specifically developed for use in dementia evaluation and was intended to be more sensitive to problems of learning and mental agility that may be seen in mild cognitive impairment (MCI). Tang-Wai et al. showed that the STMS was slightly more sensitive than the MMSE in discriminating between patients with stable normal cognition and patients with prevalent MCI (Tang-Wai et al., 2003). Another widely accepted test for AD diagnosis is the Alzheimer's disease Assessment Scale-Cognitive Subscale (ADAS-cog). This test assesses a variety of functions including language ability (speech and comprehension), ability to copy geometric figures, orientation to current time and place and more importantly memory. Still, some weaknesses of the ADAS-cog are the subjective nature of several of the assessments; the fact that it fails to assess several core deficits of AD including attention, information processing and speed of retrieval of information held in memory, the time taken to administer it, and its relative insensitivity (for review see (Wesnes, 2008). A possible improvement could be to replace the ADAS-cog with a suitable automated alternative test. However, the major disadvantage of a computerized system is that clinicians are very familiar with the ADAS-cog and are often skeptical about automated tests.

Electroencephalography

Electroencephalography (EEG) reflects cortical neuronal functioning and remains as an important tool in aiding the diagnosis of AD. The EEG is noninvasive, widely available, low-cost, and can be carried out rapidly. Although a normal EEG is found in many patients with mild AD, the vast majority of patients with moderate-to-severe AD have a pathological EEG profile. The feature of EEG abnormalities in AD is slowing of the rhythms and a decrease in coherence among different brain regions. An increase in theta and delta activities and a decrease in alpha and beta activities are usually observed (Brenner et al., 1986; Coben et al., 1983), as well as a reduced coherence of the alpha and beta bands (Leuchter et al., 1994; Leuchter et al., 1987; Locatelli et al., 1998). These abnormalities usually correlate with the severity of AD (Hughes et al., 1989; Kowalski et al., 2001). However, a direct interaction between EEG slowing and AD-associated cognitive dysfunction has not been definitively proven and the progressive EEG slowing is not always present in all AD cases. In addition, a commonly encountered problem in clinical practice during EEG recording is the blanking of the EEG signal due to blinking or movements of the patient's eyes.

Imaging

The value of structural MRI for AD diagnosis has been recently reviewed (Vemuri et al., 2008). Medial temporal lobe atrophy on MRI has been detected indicating degenerative hippocampal atrophy in old subjects, but is not specific for AD pathology (Barkhof et al., 2007). A PET scan provides both two- and three-dimensional pictures of brain activity by measuring radioactive isotopes injected into the bloodstream. Studies show that an elevated

uptake of the PET amyloid ligand-11C (Kemppainen et al., 2007) PIB in patients with MCI/mild AD could be indicative of an early AD process (Kemppainen et al., 2007; Rowe et al., 2007), even in non-demented individuals (Pike et al., 2007). However, at this time, the addition of neuroimaging to the usual diagnostic regimen at AD clinics has been declared not cost-effective given the effectiveness of currently available therapies.

AD Biomarkers

Biomarkers are required to improve the diagnostic sensitivity and specificity and to monitor the biological activity of AD in terms of disease progression. In view of the advancing scientific knowledge regarding biomarkers for AD, it was proposed to incorporate those biomarkers in revised diagnostic criteria in the future (Panza et al., 2007; Whitwell et al., 2007). Biomarkers will initially supplement our more traditional neuropsychological and imaging markers and may progress to useful surrogate measures to the pharmacological action of therapeutic compounds (Blennow, 2005; Blennow et al., 2007).

CSF Biomarkers

The cerebrospinal fluid (CSF) is in direct contact with the extracellular space of the brain, and therefore biochemical changes in the brain affect CSF composition. A β 42 is the major component of plaques (Masters et al., 1985). Therefore, it was postulated as a good candidate for AD diagnosis. Still the first reports of A β in CSF as a biomarker for AD were unsatisfactory (Farlow et al., 1992; van Gool et al., 1995; Van Nostrand et al., 1992). Recent

reports, however, have shown an inverse relation between in vivo amyloid load and CSF levels of A β 42 in humans (Fagan et al., 2006). On the other hand, Stefani et al. showed that soluble Aβ 42 was not related to the degree of cognitive impairment (Stefani et al., 2006). In familial cases of AD, extremely high CSF A β levels were found in early-onset AD and late-onset AD PSEN1 mutations (Kauwe et al., 2007)as well as in children with Down's syndrome (Englund et al., 2007). The relationships between plaque density in the brain and CSF A β reduction are not completely understood, but they may be due, at least in part, to depletion of the monomeric protein into oligomeric soluble and insoluble forms in the brain and increased Aβ deposition in plaques (Motter et al., 1995). Tau presence in CSF was initially proposed as a biomarker for AD by Vandermeeren et al. (Vandermeeren et al., 1993). It has been reported that antemortem CSF levels of Aβ 42, total tau (t-tau), and phosphorylated tau (p-tau) at Thr231 reflect the histopathological changes observed in the brains of AD patients (Buerger et al., 2007; Clark et al., 2003). Also, increase of t-tau CSF levels has been found from early to advanced stages of AD (Andersson et al., 2007). The CSF tau/A β 42 ratio has been correlated with cognitive decline in nondemented older adults (Fagan et al., 2006; Li et al., 2007; Stomrud et al., 2007) and patients with MCI (Hampel et al., 2004). The level of p-tau in CSF may reflect the phosphorylation state of tau in the brain. Several reports showed high concentrations of p-tau in CSF of AD patients (Blennow et al., 1995; Hu et al., 2002; Ishiguro et al., 1999; Kohnken et al., 2000; Vanmechelen et al., 2000). These studies suggested that CSF p-tau protein correlates with neocortical neurofibrillary pathology in severely demented AD patients and may serve as an in vivo marker of tau pathology in AD. Further studies showed no association of CSF biomarkers (Aβ 42, t-tau, and p-tau) with ApoE ε 4 or plaque and tangle burden in autopsy-confirmed AD (Buerger et al., 2007; Engelborghs et al., 2007).

Blood Markers

Blood inflammatory markers, like C-reactive protein or interleukin-6 (IL-6), are markers for vascular dementia (VaD) (Bibl et al., 2008; Ravaglia et al., 2007) or are increased before clinical onset of both AD and VaD (Engelhart et al., 2004). Motta et al. demonstrated that levels of IL-12, IL-16, IL-18, and tumoral growth factor- β 1 were higher in mild AD patients, but no significant difference was observed between AD-severe patients and nondemented age-matched subjects (Motta et al., 2007). Plasma total amyloid or Aβ 42 is increased in cases of familial AD and trisomy 21 (Kosaka et al., 1997; Schupf et al., 2001), but were not consistently related to diagnosis in clinical cross-sectional studies of typical late-onset AD (Assini et al., 2004; Mehta et al., 2000; Scheuner et al., 1996; Tamaoka et al., 1996; Vanderstichele et al., 2000). Amyloid precursor protein (APP) abnormalities in blood platelets also have been suggested as a biomarker of AD (Borroni et al., 2003; Tang et al., 2006), correlating with membrane fluidity and cognitive decline (Zainaghi et al., 2007). Recently, a novel gene/protein – ALZAS (Alzheimer-associated protein), has been discovered on chromosome 21 within the APP region. ELISA studies of plasma detected highest titers of ALZAS in patients with MCI (presymptomatic AD). Finally, recent proteomic studies led to

the discovery of various plasma signaling proteins altered in AD samples, which may allow the development of novel blood tests for AD (German et al., 2007; Ray et al., 2007).

Role of Aβ oligomers in AD

Aβ peptide assembles into a variety of structures of higher-order that include dimers, oligomers, protofibrils and fibrils. Compelling evidence support a role for soluble oligomeric and protofibrillar forms in synaptic alteration (Cleary et al., 2005; Tanzi, 2005; Walsh et al., 2002). Early studies clearly demonstrated that aggregation of Aβ was essential for toxicity and the evidence for the involvement of soluble, oligomeric Aβ in AD has been collected through several distinct experimental approaches such as: in vitro experiments using synthetic Aβ peptides; cell culture systems in which APP is over-expressed; APP transgenic mice; and human CSF and postmortem brain analysis.

In the case of human brain, it has long been recognized that amyloid plaque number does not correlate well with severity of dementia (Katzman, 1986; Terry et al., 1991), indeed this has been frequently cited as a critical flaw in the amyloid cascade hypothesis. However, further studies have shown a robust correlation between soluble Aβ levels and the extent of synaptic loss and severity of cognitive impairment (Lue et al., 1999; McLean et al., 1999; Wang et al., 1999). Moreover, in the transgenic mice Tg2576 which expresses human APP containing a mutation associated with a familial form of AD, the memory deficits start at 6 months of age, well before the presence of detectable Aβ deposits. As evidence of the role of oligomers in AD pathology, Ashe and colleagues demonstrated that a Aβ oligomer specie termed Aβ*56 (Aβ star), isolated from the brain of the Tg2576 mice, induce cognitive deficits when infused into the brains of naive rats (Lesne et al., 2006). Compelling evidence connects

Aβ oligomers with synaptic damage and the Wnt signaling pathway (for review see Cerpa et al., 2008). Indeed, recent research demonstrated that Aβ oligomers decrease synapses by affecting PSD-95 and glutamate receptors, which is prevented by Wnt-5a (Dinamarca et al., 2008).

Since Aβ oligomers and aggregates are directly involved in the pathogenic process, the presence of Aβ aggregates seem to be the most direct disease biomarker for AD and increasing effort is being made into the development of methods suitable for the detection of different Aβ aggregates in body fluids like CSF and plasma. Aβ peptide aggregates have been found in the CSF of AD patients using a seeded polymerization assay in combination with fluorescence correlation spectroscopy (Pitschke et al., 1998). An assay for the detection of oligomeric and fibrillar structures of Aβ using flow cytometry and fluorescence resonance energy transfer showed Aβ oligomers in human CSF (Santos et al., 2007). Aβ oligomeric species have been detected in plasma of human patients suffering AD, by immunoprecipitation and detection with magnetics beads (Santos et al., 2008). Moreover, Selkoe and co-workers showed that Aβ oligomers are increased in AD brain tissue, although plasma samples revealed a slight decrease (Xia et al.,2009).

Design of a diagnostic test for AD based on the detection of Aβ oligomers.

The challenge was how to detect the very minute amount of circulating oligomers and how to distinguish them from the more abundant normal soluble forms. To solve this problem I decided to use the functional property of oligomers to catalyze the polymerization of a soluble protein as a way to measure the presence of misfolded seeds in biological fluids (Fig 3). For this aim we used the concept behind the protein misfolding cyclic amplification technique

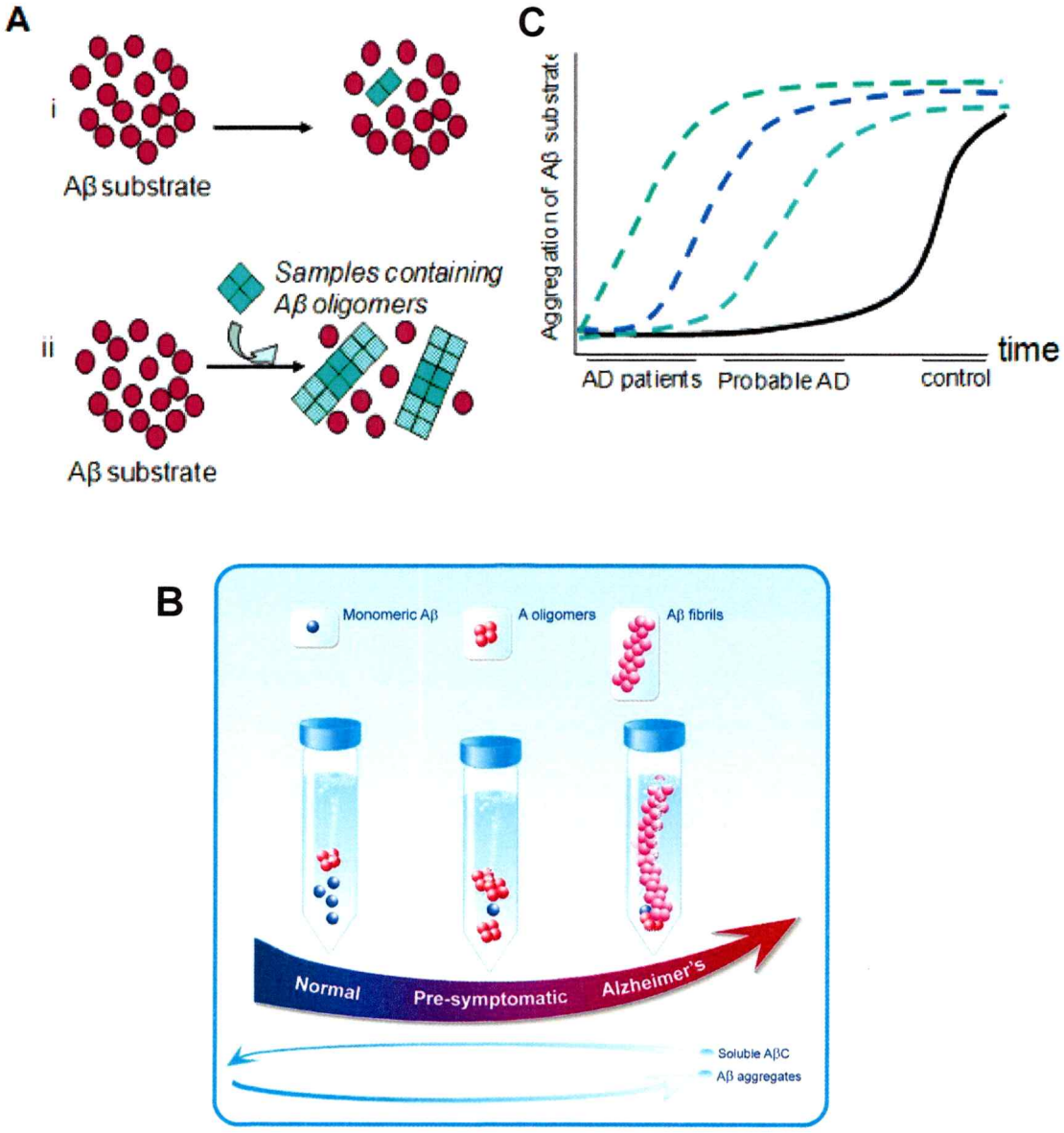


Figure 3: AD-PMCA is based on the seeding abilities of Aβ oligomers.
A Native monomeric Aβ polymerizes *in vitro* into big aggregates following a seeding-nucleation mechanism (i). Our hypothesis is that biological fluids from Alzheimer's patients contain Aβ oligomers and Aβ aggregation can be accelerated if these seeds are added to the system (ii). **B** Aβ oligomers may be circulating in the biological fluids of human individuals years before of the apparition of the disease symptoms. **C** *In vitro* Aβ aggregation follows a sigmoid kinetics. The addition of biological samples containing Aβ oligomers will short the lag phase in a concentration dependent manner, process that might be indicative of Alzheimer's pathology.

(PMCA), which was first applied in our laboratory to detect small amounts of misfolded prion protein in sick animals (Castilla et al., 2005). Even though the technique describe herein differs from the experimental approach used in the prion-PMCA, I named our diagnostic test AD-PMCA, given that we maintained the rationale of using the catalytic property of oligomers. Since misfolded Aβ oligomers are circulating in minute amounts, we planned to amplify their signal, by detecting the catalytic effect of Aβ oligomers over monomeric Aβ polymerization.

Transmissible spongiform encephalopathies

Transmissible spongiform encephalopathies (TSEs) also known as prion diseases are a group of devastating infectious neurodegenerative disorders affecting humans and animals (Collinge, 2001). Some of the diseases that belong to this group are Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals. Even though the etiology and pathogenesis are not fully understood, compelling evidence suggests that the disease is associated with the misfolding and aggregation of a normal cellular protein called prion protein (PrPc) (Prusiner et al., 1998). The disease associated conformation of PrP (PrPSc) seems to be the only component of the prion infectious agent and has the capability to transmit the disease by inducing an auto-catalytic conversion of PrPc into the misfolded form (Prusiner, 1998). An important feature of prions is the presence of PrPSc aggregates that sometimes accumulate as amyloid plaques very similar to the structures found in AD (Ghetti et al., 1996). In prion diseases, the conversion of PrPc into PrPSc also involves the

transformation of a α-helical/random coil soluble protein into a β-sheet rich aggregated structure (Pan et al., 1993).

Prion-like transmission of Protein Misfolding Disorders

Two series of experiments support the idea that Aβ seeds can initiate aggregate formation by recruiting additional oligomeric species of the same protein, contributing to the development of AD neuropathology. Gadjusek and co-workers injected brain homogenates from AD patients or from aged transgenic mice into the hippocampus of young transgenic mice. As a result both AD patient's extract and the mice brain extract induced a robust deposition of Aβ in the hippocampus compared to controls (Meyer-Luehmann et al., 2006). Besides, mutant human tau injected into mouse brains induces the aggregation of the endogenous wild-type tau mouse protein, and that pathology spreads from the injection site to neighbouring brain regions (Clavaguera et al., 2009). More recent experiments were done by Eisele et al. They induce cerebral β-amyloidosis in transgenic mice injecting dilute Aβ-containing brain extracts. In addition, intracerebral implantation of stainless steel wires previously coated with minute amounts of Aβ-containing brain extract succeed in inducing the disease, in a similar way to iatrogenic transmission of Prions (Eisele et al., 2009). However, they failed to induce cerebral-β-amyloidosis by peripheral and systemic inoculation. Nevertheless, it cannot be discarded the possibility that longer incubation times and/or more brain extract may be required to induce cerebral Aβ deposition with peripheral inoculations.

Against of the prion-like transmission of AD we can mention the experiments directed by Gadjusek in which brain extracts from AD patients were injected to non-human primates.

After more than 50 months of incubation, the experiment failed to produce any pathological changes.(Goudsmit et al., 1980). However, the pathological changes that Gadjusek investigated referred to spongiform degeneration instead of an Aβ like-phenotype. Another possible explanation for this result is a "species barrier" phenomenon that it has been well documented for Prion disorders. On the other hand, iatrogenic prion-like transmission of AD has been discarded by population-based studies in which no increased risk for AD has been found in patients with a history of blood transfusion (Bohen et al., 1994, O'meara et al., 1997). A possible prion-like transmission mechanism has been proposed for the spread and induction of α-synuclein from one cell to another (Desplats et al., 2009). These investigators further showed that normal stem cells transplanted into mutant α-synuclein transgenic mice also acquired the mutant protein, suggesting that cell-to-cell transfer occurs in vivo. This experiment might help explain why normal cell grafts transplanted into the brain of Parkinson's patients succumbed to Parkinson pathology years later. However, another possible explanation for this phenomenon is that chronically activated microglia/astrocytes at the affected brain sites progressively affect the grafted neurons. Neurotoxins produced chronically by these glia create a toxic microenvironment for neurons as has been proposed by Dr. Figueiredo (ARF comments, 2009).

Probably the most interesting data comes from studies with AA amyloidosis suggesting that transmitted by a prion-like infectious process through a seeding-nucleation mechanism. It has been shown that experimental murine AA amyloidosis can be enhanced by dietary ingestion of amyloid fibrils (Lundmark et al., 2002). Moreover, feces may be a transmission vehicle causing an increased incidence of AA amyloidosis in captive cheetah populations (Zhang et al., 2008). In addition, the incidence of visceral AA amyloidosis is unexpectedly high in

slaughtered cattle, and AA amyloid fibrils isolated from these cattle have amyloid-enhancing activity (Yoshida et al., 2009).

Heterologous seeding of amyloids

A typical signature of most PMDs is the accumulation of amyloid-like fibrils, folded in a β-cross conformation. A series of events of misfolding and protein–protein interaction occur to form oligomers and protofibrils by seeding-nucleation polymerization (Soto et al., 2006). Despite that the protein involved in each disease is different, the structure of the misfolded aggregates as well as the mechanism and intermediates in the process are similar (Glabe, 2006; Soto et al., 2006). Moreover, there have been reports of two different amyloid fibrils co-localizing in the same organ or tissue. Indeed, it has been reported extensively the coexistence of two PMDs in a single patient, including cases of AD, PD, TSEs, ALS, diabetes type 2, systemic amyloidosis, etc. (Brown et al., 1990; Fernandez-Alonso et al., 1994; Giasson et al., 2003; Moss et al., 1988; Popescu et al., 2004; Rajput et al., 1993). For example it has been shown previously that the two most prevalent PMDs (AD and diabetes type 2) are a risk factor for each other (Arvanitakis et al., 2004; Janson et al., 2004). Moreover, some PMDs involve the presence of more than one type of misfolded aggregated protein, the archetype case being AD, in which intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau are present simultaneously with extracellular Aβ amyloid plaques (Dickson, 2001). This colocalization of multiple amyloid fibrils may be due to the ability of preformed amyloid fibrils to accelerate conformational changes in other kinds of amyloid precursor protein (Fu et al., 2004; Lundmark et al., 2005). The heterologous-seeding (cross-seeding) model postulates

that there is a direct interaction between newly forming and pre-existing heterologous amyloid fibrils. Since the coexistence of PrPSc and Aβ in patients with clinical manifestation of both AD and TSE has been reported by several groups (Haraguchi et al., 2009; Yoshida et al., 2010; Yoshida et al., 2009) I we would like to study the interaction between Aβ aggregates and PrP, to evaluate the heterologous-seeding properties of Aβ. In addition, it was reported that the incidence of CJD in inherited AD patients is higher than the normal prevalence of the disease in the general population (Masters et al., 1981). These findings strongly suggest an interaction between prions and AD.

Final remarks

Protein misfolding and aggregation has an important role in the development of various human diseases. In Alzheimer's disease a crucial role of misfolded Aβ oligomers has been extensively reported. The lack of an effective non-invasive AD diagnosis is a growing problematic. Prion disease is the only member of the PMD group in which the pathology is naturally and experimentally transmitted by administration of the misfolded oligomers of a protein. The molecular mechanism underlying prion propagation is strikingly similar to the seeding-mechanism of amyloid formation. This suggests that disease propagation by a protein oligomer may perhaps be occurring in other Protein misfolding disorders of higher prevalence such as Alzheimer's disease. Misfolded Aβ oligomers have an inherent seeding capability. This feature can be used to indirectly detect minute amounts of Aβ oligomers as a diagnostic for AD. As a final point, an exciting challenge is to evaluate whether or not misfolded Aβ

oligomers might act in a prion-like manner, inducing Alzheimer's disease and affecting prion pathology under experimental conditions.

3. HYPOTHESIS

Oligomers of Aβ-peptide might accelerate Alzheimer's features and can be used to diagnose Alzheimer's disease. Also they could influence the progression of other PMDs.

4. THESIS GOAL

To study the ability of Aβ oligomers to seed amyloid fibrils and demonstrate that i) oligomers circulating in biological fluids could be used as biomarkers of AD and ii) might influence the development of other protein misfolding disorders.

SPECIFIC AIMS

Specific aim 1: To design a biochemical test for Alzheimer's disease based on the sensitive detection of Aβ misfolded oligomers.

Specific aim 2: To evaluate if a key feature of AD such as senile plaques can be induced through blood transfusion in AD animal models.

Specific aim 3: To evaluate the heterologous seeding phenomena using as example two cerebral amyloids (Aβ and prions) and one systemic amyloid (amyloid-enhancing factor).

5. METHODS

5.1 Reagents and supplies.

Acros: Acetonitrile, Sodium Chloride, Potassium Chloride, Magnesium Chloride, Calcium Chloride, Dimethylsulphoxide (DMSO), Methanol, Ethanol, Sodium Hydroxide, Tween 20.

Amersham Biosciences: HRP anti-mouse antibody, Ficoll plus.

Bachem: recombinant Aβ(1-42).

Burns Veterinary Supply, Inc: ketamine.

Dako North America: glial fibrillary acidic protein (GFAP) antibody.

Eppendorf: Thermomixer-R.

Fisher Scientific: phosphate buffer saline (PBS), proteinase K, Tris base, 10% formaldehyde solution.

GE Healthcare: Hybond-ECL nitrocellulose membrane, ECL Plus Western blotting detection system.

Invitrogen: loading buffer (NuPAGE LDS), denaturant buffer NuPAGE MES SDS (Running Buffer), polyacrilamide gradient gels NuPage Bis-Tris 4-12%.

Millipore: 4G8 antibody, Microcon Y10 KDa, Microcon Y30 KDa, Amicon 10KDa, Amicon 30 KDa.

Nalgene: Nunc maxisorp ELISA plates.

Pierce: ABC anti-mouse staining kit, ABTS 10X substrate.

Roche Diagnostics: Protease inhibitors cocktail.

Sigma- Aldrich: xilazine, congo red, thioflavin T, PIPES.

USA scientific: Low binding 500 µl microcentrifuge tubes, 200 µl-PCR striptubes.

Yale University: Aβ(1-42).

5.2 Animals

5.2.1 Wild type mice:

C57BL/6 mice were directly purchased to Harlan Sprague Dawley (USA).

5.2.2 Alzheimer's Models:

APPswe/PSEN1ΔE9 and PDGF-APPWT (hAPPwt) transgenic mice breeders were purchased to The Jackson Laboratory (USA). Tg2576 transgenic mice model were obtained from Taconic (USA). All three mice colony were expanded in a Level 2 animal house facility at the University of Texas Medical Branch (UTMB), USA. Transgenic mice specifications are detailed on Table I (Hsiao et al., 1996; Jankowsky et al., 2001; Mucke et al., 2000)

5.2.3 RML Prion animals:

C57BL/6 mice were inoculated with the RML prion strain and housed in a Level 3 Animal house facility at the University of Texas Medical Branch (UTMB), USA.

TABLE I

Trangenic line	Transgene/ Promoter and Regulatory Elements	Neuropathological characteristics	Primary reference	Source
Mo/Hu APPswe/PSEN1ΔE9	B6C3HF2 pronuclei coinjected with hPsen-1 and huAPP695/MoP rnp	β-amyloid deposits in the brains of transgenic animals by 6 to 7 months of age.	(Jankowsky et al., 2001).	The Jackson Laboratory
Tg2576 (APP swe)	Human APP 695 cDNA with KM670/671NL mutations. Hamster prion protein gene promoter	Numerous Aβ plaques, oxidative lipid and glycoxidative damages. Memory deficits seen in 9-10 month old tg mice.	(Hsiao et al., 1996).	Taconic
PDGF-APPWT (hAPPwt)	Human WT APP sequence. APP_{Ind} tg sequence converted to wildtype by PCR primer modification. PDGFB promoter	Transgene expression in cerebral neurons, neocortex and hippocampus, with total Aβ levels and 42 Aβ levels lower than in the APP SwInd mutant line. No amyloid plaques up to 24 months of age.	(Mucke et al., 2000).	The Jackson Laboratory

Table I: List of Alzheimer's animal models used in this study.
Transgenic mice breeders were purchased to commercial vendors. After genotyping, AD-transgenic mice colonies were in generated in a C57 background strain and neurological features were evaluated before utilizing the mice colony for experimentation.

5.3. METHODS

5.3.1. SF- Aβ protocol

Aβ(1-42) peptide was synthesized using solid-phase chemistry by the Protein Core at Yale University. Synthetic Aβ(1-42) was resuspended in acetonitrile (50% v/v), aliquoted, and lyophilized. Aliquots were resuspended in NaOH 10mM, pH 12, and filtrated using 30,000 kDa cutoff filters (Millipore). Filtrates containing soluble seed-free Aβ were stored at -80°C until use.

5.3.2. Generation of synthetic Aβ seeds

Aβ seeds used to seed aggregation were obtained by incubating the protein at 0.5g/ml in TrisHCl 10mM buffer at 37 °C with agitation for 15 h in order to generate mature fibrils. To break down the fibrils into small seeds, Aβ was sonicated for 20 s at 80% power using a Vibra-Cell ultrasonic processor (Sonics & Materials).

5.3.3. Thioflavin T Fluorescence

For spectral analysis, samples were diluted on Glycine 50 mM pH 9 buffer and added 200 μM of Thioflavin T (Aldrich). After 5 min of incubation at room temperature, fluorescence measurements were carried out on a Fluorolog 2 spectrofluorimeter (Spex Industries). The

excitation wavelength was set at 430 nm, emission wavelengths ranged from 440 to 700 nm, and slit widths were 6 mm for excitation and emission.

5.3.4. PK resistance assay

For PK resistance analysis, Aβ samples were treated with 2μg/ml PK and incubated at 37°C for 45 min. in a water bath. The reaction was stopped by quickly adding SDS sample buffer and heating at 95°C for 10 min. Samples were analyzed by Western blotting using 4G8 anti-Aβ antibody.

5.3.5. Western blot

Protein samples were denaturated using NuPAGE LDS Sample buffer (Invitrogen). Proteins were then fractionated by electrophoresis using 4–12% SDS-polyacrylamide gels (SDS-PAGE) (Invitrogen), electroblotted into Hybond-ECL nitrocellulose membrane (GE Healthcare), and probed with the 4G8 antibody (Millipore) (dilution, 1:1000). Then, blots were incubated with a horseradish peroxidase-linked anti-mouse antibody (1:5,000). The immunoreactive bands were visualized by ECL Plus Western blotting detection system (GE Healthcare) using a UVP Bioimaging system EC3 apparatus (UVP).

5.3.6. Alzheimer's Protein Misfolding assay (AD-PMCA)

Briefly, SF-Aβ samples at 2μM concentration were incubated at 25 °C with synthetic Aβ seeds, hCSF, mice blood fractions or human plasma. Samples were incubated for the indicated

times and centrifuged at 14.000 rpm for 10 min, at room temperature. Protein concentration in supernatants was measured By ELISA using an anti-Aβ antibody (4G8, Millipore). The amount of aggregated protein was obtained by subtracting this value from the total concentration of SF-Aβ and expressed as percentage of insoluble protein.

5.3.7. Electron Microscopy

Samples of Aβ fibrils or oligomers were placed onto 300-square mesh nickel grids (Maxtaform), negatively stained with 2% uranyl acetate for 90 s, and examined in a Philips EM410 electron microscope.

5.3.8. Human Samples

Cerebrospinal fluid samples

Cerebrospinal fluids (CSF) were kindly donated by the New York University School of Medicine (New York, NY) and the Instituto Nazionale Carlo Besta (Milan, Italy). CSFs were obtained by spinal tab from AD and non-AD individuals. Non-AD individuals suffered from hydrocephaly but herein we will refer as normal. Young CSF correspond to kids who suffered from hydrocephaly.

Blood samples

Human blood samples were donated by our collaborator Dr. Patricio Fuentes (Hospital Del Salvador). Fresh blood samples were obtained using K2 vacutainer tubes with EDTA and 22G

vacutainer needles from the right arm. Samples remain at room temperature until processing (-2 hours). All blood samples were taken following the exact same procedure by specialists under Dr. Patricio Fuentes supervision. Normal blood samples were obtained from healthy individuals younger than 29 years old following the same blood extraction protocol.

5.3.9. Blood transfusion in mice

Blood samples were obtained through cardiac puncture from old APPswe/PSEN1ΔE9 transgenic mice (12 months old) using a tuberculin syringe and K2 EDTA vacutainer tubes. Samples were diluted twice in sterile PBS and kept at room temperature until injection. 2 months old transgenic mice were anesthetized by i.p injection with 200 µl of Ketamine/Xilazine solution. Mice vein tails were warmed up with hot water and cleaned with sterile 70% isopropanol pads. 200µl of either AD-blood or C57 wild type blood were injected into 2 months-old transgenic mice through the vein tail, once a week, 3 times in total. An additional group of mice was injected with synthetic Aβ*56 KDa spiked on C57 non-AD blood. The same procedure was performed using hAPPwt transgenic mice.

5.3.10. Blood fractioning through Ficoll gradient.

6 ml of fresh blood were diluted with 10 ml of PBS and subjected to Ficoll separation (15 ml) by centrifuging at 2000 rcf for 25 minutes at room temperature. Blood fractions were separated, labeled and stored at -80°C in Nunc Cryotubes for biological samples. Human plasma samples were desalted using Amicon 30KDa cutoff filters and centrifuged at 3000 rcf

for 40 minutes at room temperature. Before using, concentrated plasma samples were reconstituted at their original volume in Tris-HCl 10mM.

5.3.11. AEF generation, purification and seeds preparation

Amyloid enhancing factor (AEF) was induced by injecting subcutaneously 500 uL of a 2% $AgNO_3$ solution into C57/BL6 mice. After 1-2 months, mice were euthanized and AEF was extracted from liver homogenates. Briefly, mouse liver was homogenized in a 10% NaCl 0.15M solution. Liver homogenizes were split into 20 tubes (1mL each) and subjected to 6 cycles of centrifugation (10,000 rpm for 30 min at 4°C) and resuspension in 10% NaCl 0.15M. The final pellets were reconstituted in nanopure water, centrifuged twice and stored at -80°C. AEF-water extracts (here named DW1, DW2 and DW3) were diluted 20 times in PBS to determine protein concentration (MicroBCA kit, Pierce) following the manufacturer specifications. To generate AEF-seeds, AEF-water extracts were diluted until a 1 μg/μL concentration and subjected to different sonication strengths (from 30 to 80% power) using a VibraCell sonicator.

5.3.12. Aβ and AEF heterologous seeding assay.

AEF was purified from the liver of mice as described before. Aβ samples were incubated with different amounts of AEF-seeds prepared by sonication of AEF-fibrills. After incubation at 25°C, samples were centrifuged at 14,000 rpm for 10 min at different time points, and the soluble Aβ peptide was quantified by ELISA, using 4G8 monoclonal antibody.

5.3.13. Aβ and Prion heterologous seeding assay.

PrPSc was highly purified from the brains of scrapie-affected animals produced by inoculation with RML prions, using previously described protocols (Hetz et al., 2003b) Briefly, Aβ samples were incubated with different amounts of PrPSc. After incubation for different times at 25°C, samples were centrifuged at 14,000 rpm for 10 min, and the soluble peptide was quantified by ELISA, using 4G8 monoclonal antibody.

5.3.14. Histopathological studies.

Brain samples were fixed in 10% formaldehyde solution, embedded in paraffin, and cut in sections. Six-micrometer-thick serial sections from each block were stained with hematoxylin–eosin, thioflavin S, or incubated with the anti-Aβ monoclonal antibody 4G8 (Millipore, Signet) or the glial fibrillary acidic protein (GFAP) antibody (Dako North America), using previously described protocols (Castilla et al., 2005; Permanne et al., 2002). Immunoreactions were developed using the peroxidase–antiperoxidase method, following the manufacturer's specifications. Antibody specificity was verified by absorption. Samples were visualized with a Nikon Eclipse 800 microscope. For co-immunolocalization, slides were incubated with fluorescently labeled 4G8 and 6H4 antibodies and visualized with a Leica microscope. Several images were taken from each animal using a Nikon Eclipse 800 microscope (100X magnification). Five zones of the cortex were submitted to image analysis using ImageJ. The area and number of Aβ plaques were assessed in brain cortex.

5.3.15. Prion infection assays

Prions were obtained from C57BL/6 mice affected by scrapie disease produced by inoculation with the RML prion strain. Brains containing prions were homogenized at 1.5% in PBS plus Complete mixture of protease inhibitors (Roche Diagnostics). Groups of 1.5 and 12 month-old Tg2576 mice were intraperitoneally inoculated with 50µl of PBS or 1.5% brain homogenate prepared from scrapie sick mice. As controls, age-matched wild-type (WT) mice (littermates) were inoculated i.p with the same prion preparation. Animals were checked three times every week to evaluate health condition and appearance of scrapie clinical symptoms. Left brain hemispheres were extracted and analyzed histologically.

5.3.16. Stereotactic surgery

hAPPwt transgenic mice were anesthetized by i.p injection of ketamine/xilazine. Mice were injected using an stereotactic apparatus (Scientific Instrument) at the following points: -2,3 mm before bregma, 2,0mm right, and 2,5 mm deep, which correspond to the hippocampus (Mouse brain atlas Paxinos & Watson, 1986). The injection was performed using a 10 µL Hamilton syringe.

5.3.17. Mice euthanasia

All mice used in this study were sacrificed by exposure to carbonic dioxide and posterior cervical dislocation.

5.3.18. Statistical analysis.

Aβ plaque burden was quantified by analysis of inmunohistochemistry and Thioflavin S straining in at least 6 pictures of 5 individuals using the ImageJ software (NIH). Non-paired T-student analysis was performed with the GraphPad Prism, version 4.0, software (GraphPad Software). The plaque number and plaque burden (area) are shown as average +/- the standard error (SE).

AD-PMCA assays and *in vitro* heterologous seeding assays and were analyzed using the GraphPad Prism software by either two-way ANOVA or T-student paired test. All time points are done in duplicate or triplicate if indicated. The results are representative of minimal 3 independent experiments, excepting AD-PMCA assays involving human samples. The results are shown as average +/- the standard error (SE).

6. RESULTS

6.1 Specific aim 1: To design a biochemical test for Alzheimer's disease based on the sensitive detection of Aβ misfolded oligomers.

The first aim was to detect small amount of oligomers circulating in biological fluids, using as experimental strategy the functional property of oligomers to catalyze the polymerization of a soluble protein. In this thesis I refer to the diagnostic assay as AD-PMCA since I use the same rationale that was used for prions detection by PMCA. As it's shown in Figure 3 I want to determine the amount of oligomers present in biological fluids by evaluating the differences in the lag phase of the Aβ substrate kinetic. From the different methods available to measure amyloid formation I evaluate 4 based on the resources available to perform them at our laboratory. Figure 4 summarizes the techniques I used to follow Aβ aggregation. Binding to specific dyes such as Thioflavin T and Congo red are well described methods to measure amyloid polymerization. I was able to follow Aβ aggregation using Thioflavin T binding. However, a high amount of peptide is required for this assay, making impossible the use of this method for high throughput screening of samples (Fig 4A). In the case of dot blot, it was very convenient in terms of rapid screening of large number of samples but did not allow a quantitative analysis of the Aβ aggregates (Fig 4B). Proteinase K (PK) resistance is a classic method used in prion disorders to differentiate the normal protein PrPc from the PK resistant

and infectious form PrPSc. Coupled to western blot this technique allows the determination of the molecular weight (MW) of the Aβ species. Aβ aggregates, although have resistance to low PK concentrations, do not show a shift in the MW after PK digestion like PrPSc. Instead, the 4-8KDa bands become weaker at the same MW (Fig. 4C). A final experimental strategy for AD-PMCA is described in Figure 4D. Here I combined Aβ sedimentation with detection of proteins by ELISA. This strategy was based on the different nature of the misfolded protein versus substrate protein. Big aggregates can be easily separated from small soluble forms of Aβ by centrifugation. In this way we separate the *newly formed misfolded protein* from the remaining *substrate* protein. Here, the term *soluble Aβ* will be use to describe any form of Aβ that is soluble in aqueous buffer and remains in solution following high speed centrifugation. Thus, although the assembly states of these Aβ species are unknown, their failure to pellet by centrifugation indicates that they are not fibrillar in nature. The advantages of this method are the high reproducibility, adequate cost and that can be performed in 96-well plate format allowing screening several samples at the time. This point is particularly important if automation of the technique is needed in the future to perform high throughput screening of biological samples.

Since we need monomeric Aβ as a substrate for the AD-PMCA assay, after the Aβ peptide is produced and purified, an essential part to enable efficient amplification is to remove the aggregates non-specifically formed during production and isolation of the Aβ peptide. Herein, I named this suitable substrate as seed-free Aβ (SF- Aβ). In the realization of this Thesis, a great part was spent to achieve this key step. A summary of different conditions that were used is summarized in table II. Otherwise; the non-specific aggregates may mask the effect of misfolded oligomers present in the sample. Figure 5 shows a schematic representation of the

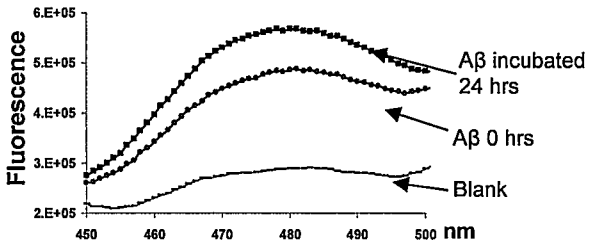
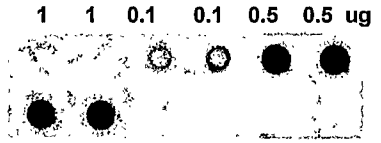
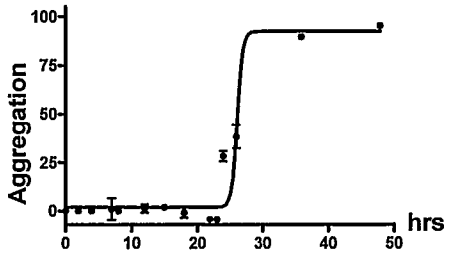
Technique	Detection limit	Advantages	Disadvantages	Results
Thioflavin T Binding	10 ug cuvette 2 ug 96-well plate	Specific for amyloid fibrills. Reliable.	Poor resolution between time points. Time consuming.	
Dot Blot 4G8 antibody	0.1 µg	Can be coupled to PK resistance or sedimentation. Several time points analyzed at once.	Poor resolution between different concentrations.	Different concentrations of Aβ peptide 
PK resistance Western Blot 4G8 antibody	0.05 µg	Molecular weight determination	High variability between PK replicates in Aβ samples. No "shift" in misfolded Aβ after PK.	
Sedimentation ELISA 4G8 antibody	0.01 µg	High through put Quantitative. Reproducible.	Do not differentiate between amyloid fibrills and oligomer clusters.	

Figure 4: Methods that were tested to measure Aβ aggregates over time.
We applied several techniques to discriminate between the new misfolded Aβ and the remaining Aβ substrate as part of the set-up of the AD-PMCA assay.

TABLE II

Solvents	**Concentration**
NaOH	10mM
Dimethylsulfoxide (DMSO)	100%
NaOH	100mM
Hexafluoroisopropanol (HFIP)	100%
Formic Acid	100%
Trifluoroactic acid (TFA)	1%
Acetonitrile (AN)	50%
Protocols	
Boiling	15 minutes
Size exclusion chromatography (SEC)	
Filtration using Microcon-10KDa	14,000 g x 15 min at RT.
Filtration using Microcon-30KDa	12,500 g X 12 min at RT
Freeze dry	In HFIP
Freeze dry	In Acetonitrile (50%) + TFA (1%)
AN+ TFA, Freeze-dry, NaOH + Filtration (30KDa).	Aliquot and store at -80°C.

Table 2: Protocols used to generate Aβ substrate.

methodology for the preparation of SF-Aβ substrate. The first step is to dissolve the Aβ lyophilized powder into an appropriate solvent to induce as much as possible the disassembly of preformed aggregates. Adequate solvents include various concentrations of sodium hydroxide, hexafluoroisopropanol, trifluoroacetic acid, dimethylsulfoxide, acetonitrile, and formic acid. The best yield, meaning the higher concentration of Aβ in the filtrate, was obtained with the treatment with 50% acetonitrile. Thus, I re-design the protocol and the Aβ peptide was dissolved in acetonitrile 50% (v/v), incubated for 10 min and lyophilized again. The lyophilized powder was resuspended in 10 mM sodium hydroxide, pH 12. The resulting Aβ aliquots were filtrated through a 30KDa cutoff filter and the material collected consists of SF-Aβ.

AD-PMCA protocol gives a reproducible and optimal lag phase.

The AD-PMCA technique described here contains a phase of incubation in which protein–protein interactions between the misfolded seeds and the SF-Aβ substrate are allowed, resulting in an optimal elongation of seeds. These elongated-seeds are high molecular weight species that can be separated from the remaining SF-Aβ. In order to clearly distinguish the early polymerization of SF-Aβ by exogenous seeds from the natural polymerization of the Aβ substrate we need a suitable lag phase that allow distinguishing between different amounts of oligomers. I established that an optimal lag phase must exceed 24 hrs. Several factors have been shown to modify the seeding-polimerization kinetics, including protein concentration, temperature, ionic strength and pH. I modified several of these variables in order to find an optimal protocol for controlled aggregation of SF-Aβ. Some of the conditions that I evaluated for AD-PMCA are described in Figure 6. First we tried 3 peptides as

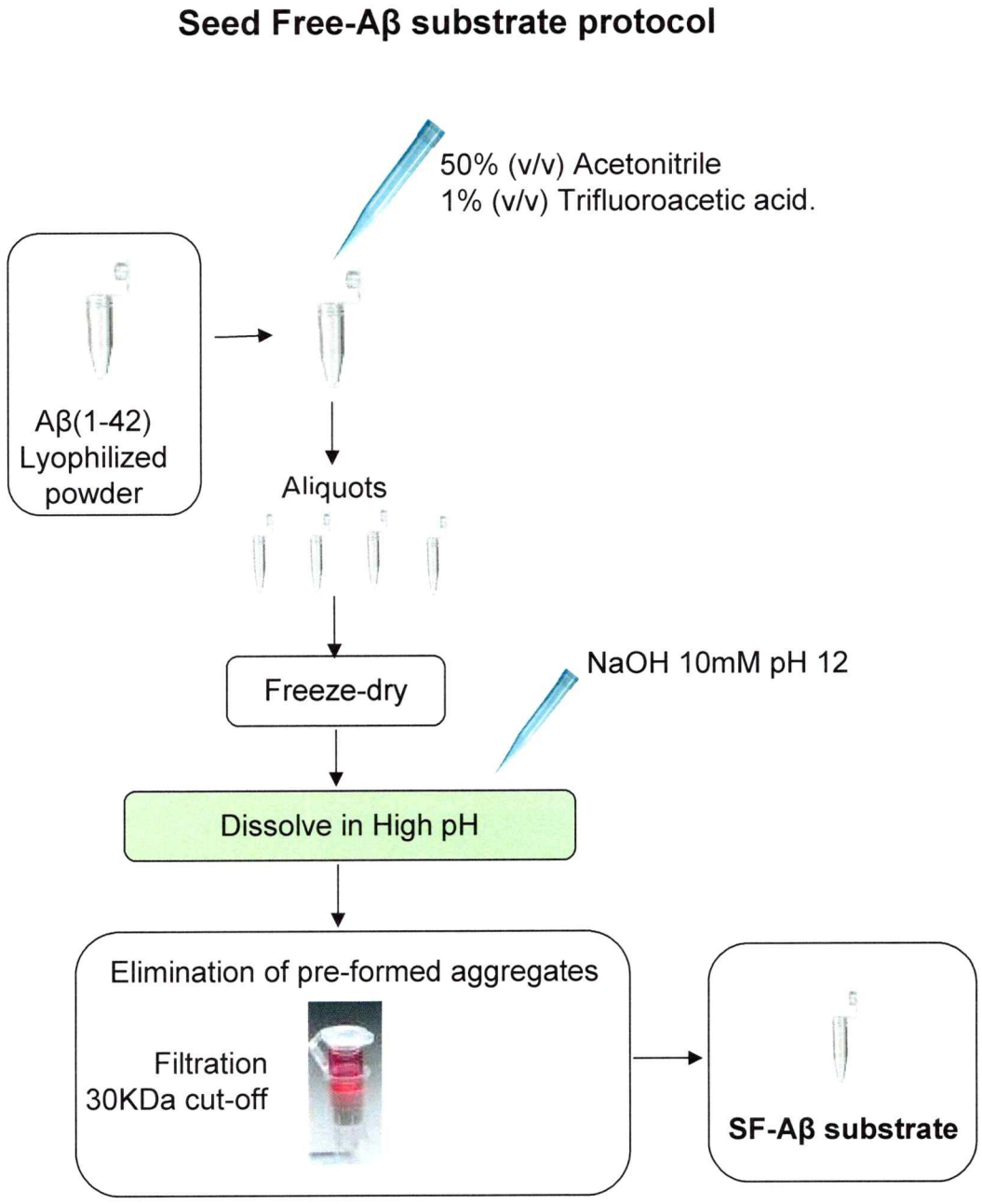


Figure 5: Schematic representation of the methodology for the preparation of SF-Aβ. SF fractions of Aβ represent the substrate for the amplification reaction. The first step is to dissolve the Aβ powder into an appropriate solvent to induce as much as possible the disassembly of preformed aggregates. The Aβ is dissolved in this buffer and lyophilized. Then, the lyophilized powder is resuspended in 10 mM sodium hydroxide, pH 12. The solution is passed through a 30KDa cutoff filter and the material collected consists of SF-Aβ. Protein concentration is determined by amino acid analysis or the BCA kit following manufacturer specifications. Samples are stored lyophilized at - 80°C.

possible substrates: synthetic Aβ(1-40), synthetic Aβ(1-42) and recombinant Aβ(1-42) (Fig 6A,B,C,E). From our experiments I determined that the optimal substrate is the synthetic Aβ(1-42) peptide when is treated as described in Table II, because it gives a better yield of monomeric SF-Aβ after filtration with 30-KDa cut off filters and has low batch-to-batch variation. The optimal concentration was close to 2 μM of SF-Aβ. Concentrations around 2 μM greatly reduced the lag phase, while concentrations below 2 μM difficult the ELISA detection and produce high dispersion between replicates (Fig 6D,6F). In addition, I determined that no seeding effect when adding spiked oligomers can be detected using substrate concentrations close to 0.5 μM, possible because the Aβ is below the critical concentration needed for fibril formation. Also, I modify the buffer in which the samples were incubated. First I tried TrisHCl pH 7.2 which is a common buffer for Aβ aggregation. When we vary this buffer to PIPES 10mM 6.5, I obtained a slightly better AD-PMCA performance, probably because PIPES presents less variation with the temperature. However, the experiments including human biological samples were performed with Tris-HCl 100mM since strong buffering was needed. Another variable that I modify was the temperature of reaction. Aggregation is favored by increase in temperature; therefore I vary the temperature from 37°C to 25°C which result in a decrease of the sample-to-sample variation and an increase of the lag phase (Compare Fig 6G and 6H). After establishing an optimal lag phase I have to determine the kinetic of aggregation of Aβ in AD-PMCA. For this, I incubated the peptide under the conditions described on Figure 6H and I then took samples at different times points. These samples were centrifuged at 14,000 rpm and an aliquot of the supernatant was taken to measure the quantity of remaining peptide by ELISA. I choose to measure soluble Aβ rather that the fibrillar pellet since antibody epitopes are usually hidden in compact

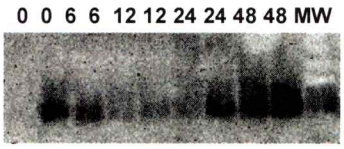
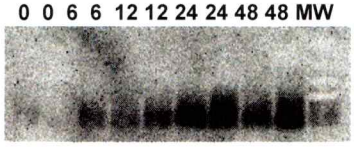
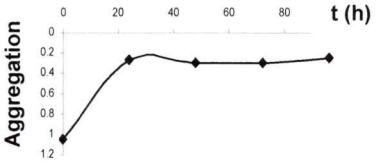
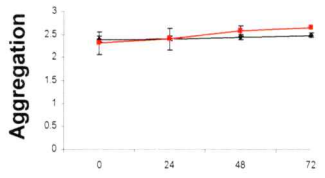
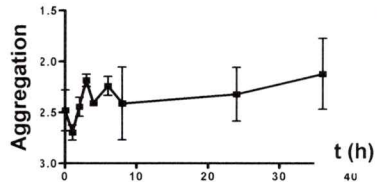
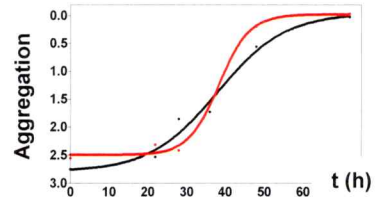
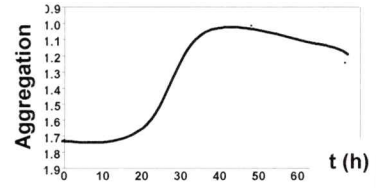
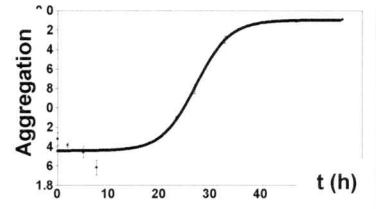
	Substrate	Buffer	pH	T °C	Result	Comments
A	Aβ(1-40) 1 μM	TrisHCl 10mM	7.2	37°C		Short Lag
B	Aβ(1-40) 0.2 μM	TrisHCl 10mM	7.2	37°C		Short Lag
C	Aβ(1-42) 2 μM	TrisHCl 10mM	7.2	37°C		Short Lag
D	Aβ(1-42) 0.065 μM	PIPES 10mM 0.5M NaCl	6.5	37°C		No-shaking No-seeding Seeds Control
E	Aβ(1-42) 2 μM Recombinant Seed-free	TrisHCl 10mM	7.2	25°C		Lag 40 hrs Low yield substrate Expensive
F	Aβ(1-42) 0.2 μM	TrisHCl 10mM	7.2	37°C		Lag 35 hrs. No-seeding Seeds Control
G	Aβ(1-42) 2 μM Seed-free	TrisHCl 10mM	7.2	37°C		Lag 24 hrs Not reproducible
H	Aβ(1-42) 2 μM Seed-Free Reproducible	PIPES 10mM	6.5	25°C		Lag 30 hrs

Figure 6: Schematic representation of the protocols used to set-up a reproducible and adequate Aβ aggregation kinetics. An extensive work as done to standardize the best conditions to develop an AD-diagnostics test based on Aβ seeding and aggregation. Different parameter were modified to obtain an otimal a reproducible lag phase. As we described on figure 3A, we need native monomeric Aβ to test the effect of putative seeds. **A** As a first approach we used Aβ(1-40) as substrate because it is less amiloidogenic than Aβ(1-42) but we did not obtain a reliable lag phase **(A,B).** We also change concentration of the substrate and buffer composition without good results **(C,D).** We tried recombinant Aβ(1-42) instead of synthetic Aβ(1-42), since some solvents used on the chemical synthesis may alter the aggregation kinetics. However, the yield of low-molecular weight Aβ obtained from recombinant peptide was too low **(E).** Decrease of substrate concentration was effective in extending the lag phase but it was not enough to generate any seeding effect **(F).** Several times we obtained promising results modifying buffer composition or ionic strength but that at the end the protocol was not reproducible **(G).** Once we standardized the **SF-Aβ** substrate protocol we obtained an optimal lag phase. However, we still observed a big dispersion between time points replicates. In order to solve this problem we introduce 2 modifications; we change to a stronger buffer (PIPES) and we decreased the temperature from 37C to 23C. Under these conditions we obtained high reproducibility, low variation between replicates and an optimal lag phase **(H).**

aggregates, which will cause an underestimation of their quantity by ELISA. Under these experimental conditions the curve of aggregation of SF- Aβ is optimal and reproducible (5 independent experiments), with a lag phase of ~30 hours and follows a sigmoideal kinetics which is typical of a seeding-polimerization process (Fig. 7).

To determine the detection limit of the diagnostic assay using synthetic oligomers spiked in CSF.

First I need to verify that our *in vitro* assay is able to detect minute amounts of misfolded seeds. Preliminary experiments using non-amyloid proteins such as BSA, showed no effect on decreasing the lag phase. For this aim I prepared synthetic misfolded seeds as described in METHODS. Small oligomers prepared by stirring on HFIP/water clearly accelerated the aggregation of Aβ but failed when used in concentrations below to 1% w/w or 7.5nM. As a second approach I prepare Aβ by strong sonication of Aβ amyloid fibrils (Fig. 8). The Aβ amyloid fibrils were prepared on the same aggregation buffer used for AD-PMCA, at 100 μM concentration (based on MW of the Aβ monomer). The fibrils were prepared on special low-binding microcentrifuge tubes to avoid fibril binding to the plastic, and incubated O/N at 37°C with strong shaking. In order to test the sensitivity of our AD-PMCA assay SF-Aβ samples were incubated in absence of presence of known amount of seeds.. Samples were taken at different time points and thereafter the quantity of peptide remaining soluble was determined by sedimentation assays (Fig. 9). The lag phase of the SF-Aβ substrate was notably shortened in presence of amyloid seeds. At the conditions used in this experiment we were able to detect as low as at 75 pM of seeds.

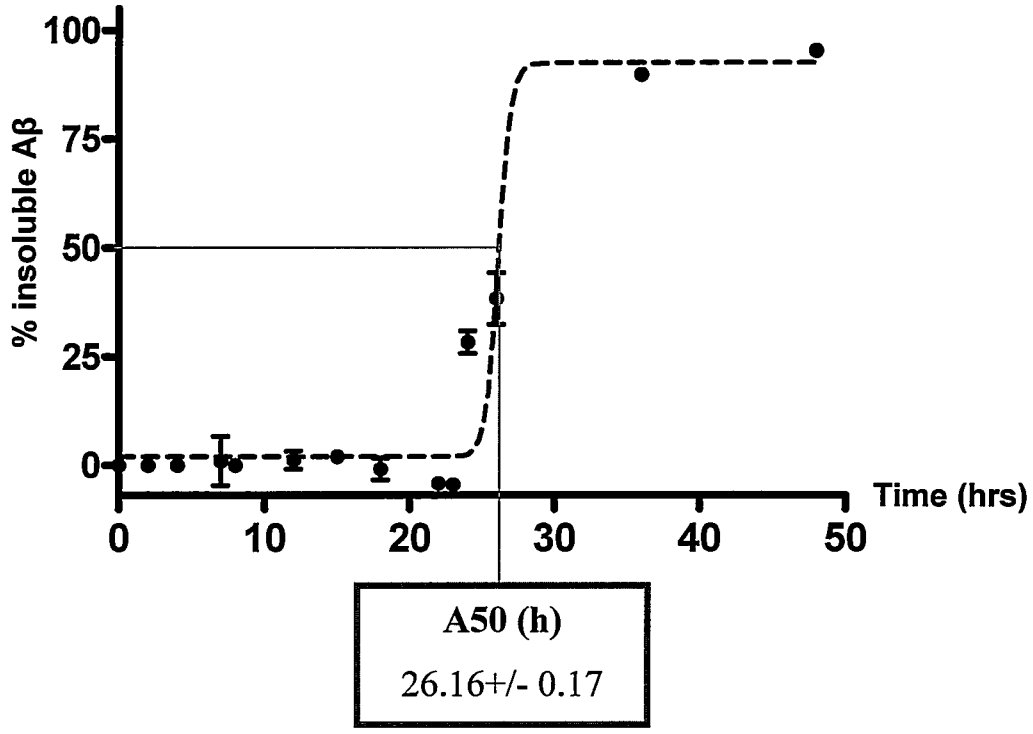


Figure 7: Seed free Aβ-substrate follows a seeding-polimerization kinetic. Aggregation kinetic of SF-Aβ peptide in TrisHCL pH 7.2. SF-Aβ(1-42) at 2μM concentration was incubated by 50 hours at 25°C with constant shaking. Percentage of insoluble peptide was calculate by subtracting the remaining soluble Aβ after centrifugation from total SF-Aβ substrate. Soluble Aβ was detected by ELISA using 4G8 antibody. The non-linear regression applied to all data points showed that SF-Aβ aggregation follows a sigmoid kinetic (dotted line), which is typical of a seeding-nucleation mechanism of aggregation. A50 parameter is defined as the time when 50% of the substrate peptide is aggregated and correspond to the logE50 from the best non-linear regression. Percentage of insoluble peptide is shown as average of 2 time points +/- Standard Error (SE). A50 is shown +/- the Standard Error. The A50 standard error is a function of the number of data points, the distance of the points from the curve, and the overall shape of the curve. Non-linear fit and A50 parameter were calculated using GraphPad Prism software. Best fit equation (curve-type) was done considering an $r^2>0.9$ as goodness of fit. This result is representative of 5 independent experiments.

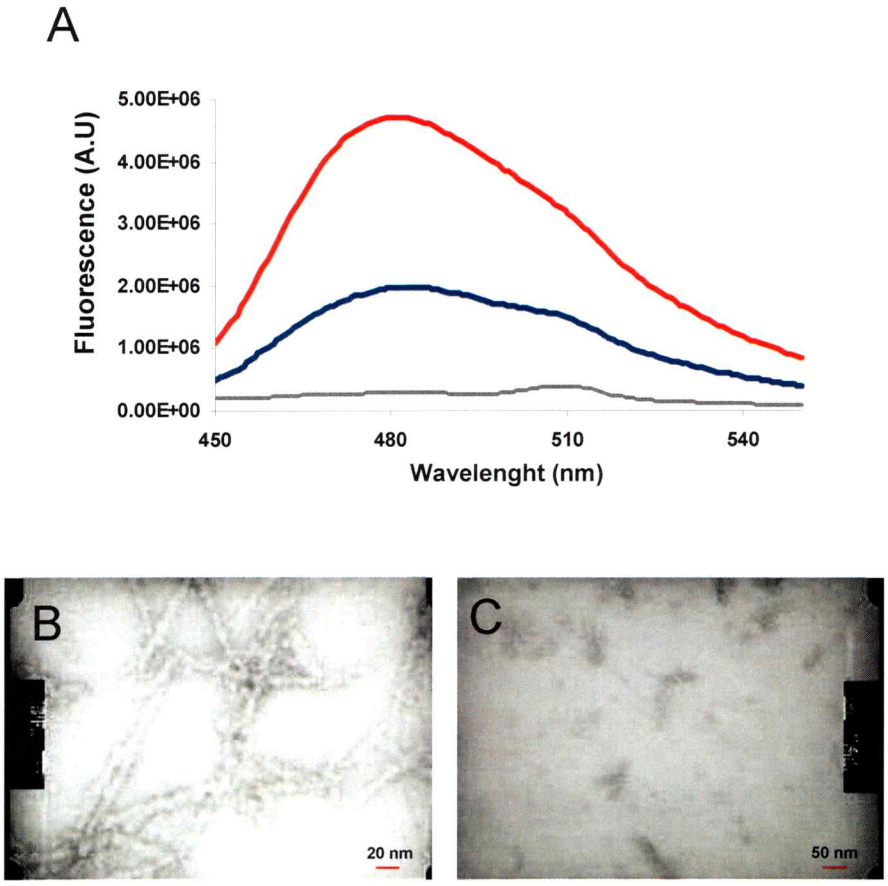
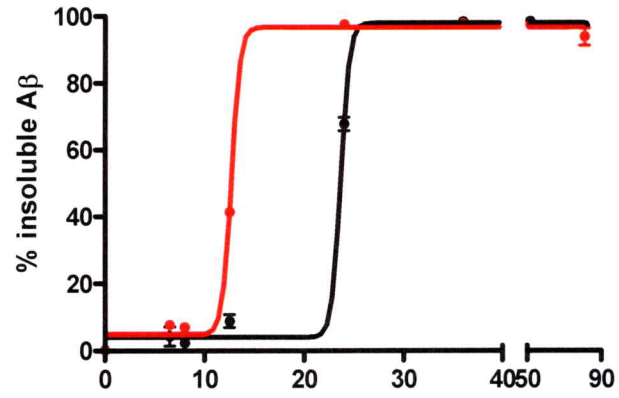


Figure 8: Aβ seeds generated *in vitro*.
Aβ fibrills were generated by incubating Aβ at 0.5 mg/mL concentration at 37C for 24 hrs. **A** Thioflavin T binding assay of Aβ preparations showed thioflavin T reactive-amyloid fibrils after 24 hrs of incubation. **B** Electron microscopy of Aβ fibrils showed long twisted fibrils after 24 hrs of incubation. In order to generate Aβ seeds, Aβ fibrils were break-down in small oligomers, by subjecting the samples to sonication by 20sec using 80% power (VibraCell sonicator). **C** Electron microscopy of Aβ seeds showed small protofibrils and oligomers.

Human cerebrospinal fluid does not interfere with oligomers detection by AD-PMCA.

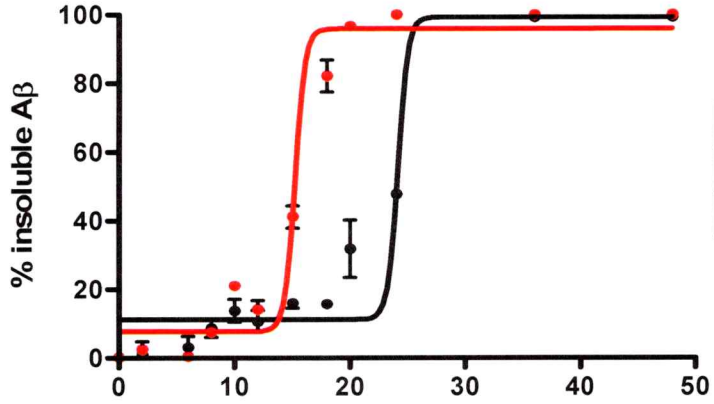
Because the translational aim of this thesis was to attempt the detection of misfolded seeds in biological samples, I first evaluate human cerebrospinal fluid (hCSF) since CSF is in direct contact with the brain, the pathological changes occurring in AD might be reflected on CSF composition. Human CSF samples are extremely valuable, especially those coming from non-demented and young individuals. Therefore, I first need to standardize the AD-PMCA for hCSF before testing any AD-sample. I optimized the AD-PMCA technique which allows to decrease the amount of sample required per time point from 75uL to 20uL. In order evaluate any unwanted effect of the CSF components (proteins, salts, etc) other than misfolded seeds on the aggregation kinetic of the SF-Aβ substrate, I compared artificial CSF (sCSF) with young non-AD hCSF samples. For this purpose I add an aliquot of either aCSF or hCSF to several SF-Aβ samples in AD-PMCA buffer. After incubation, no significant differences were observed between aCSF and hCSF, meaning that proteins or lipids contained on hCSF do not alter Aβ aggregation kinetics (Fig. 10). Although the lag phase and A50 diminish from ~27 hrs to 20 hrs in this experiment, this effect can be easily reverted if more buffer is added to the hCSF samples as it's shown in the next experiment. Since the AD-PMCA assay is based on the catalytic properties of Aβ-seeds, it was necessary to evaluate if the CSF components do not affect the ability of the Aβ oligomers to elongate SF-Aβ molecules. In the next experiment I spiked a minimal amount of oligomers into young normal CSF and we attempted to detect these misfolded oligomers by AD-PMCA. Figure 11 shows a decrease in both the lag phase and A50 of the hCSF samples containing exogenous seeds compared to the control. Therefore, hCSF does not affect the seeding capability of misfolded seeds. Thus, CSF is a suitable biological sample to be used for detection of misfolded oligomers through AD-PMCA.

A



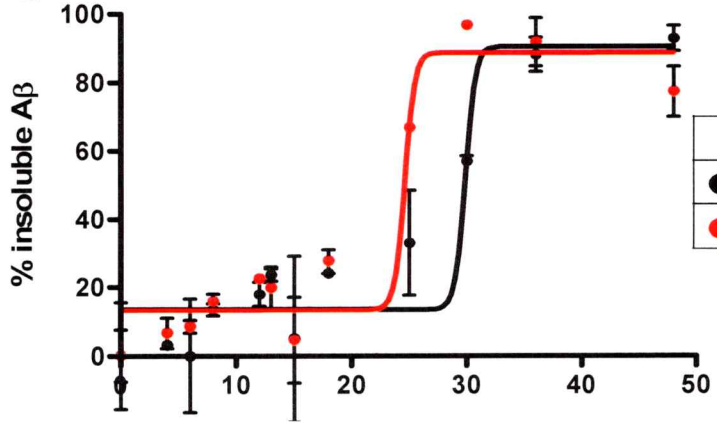
Condition	A50 (h)
Control	23.68
7.25 nM	12.68

B



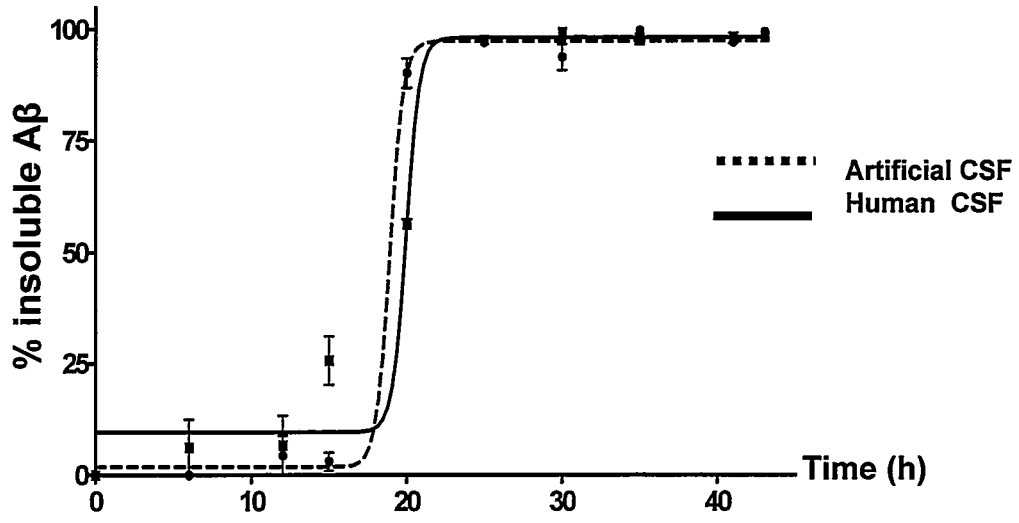
Condition	A50 (h)
Control	24.15
725 pM	15.21

C



Condition	A50 (h)
Control	29.88
72 pM	24.61

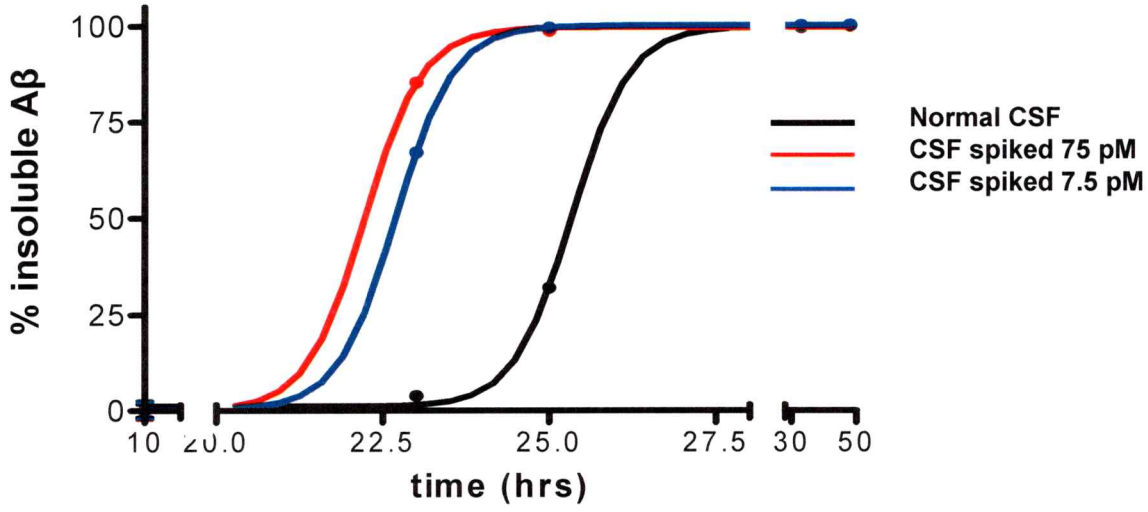
Figure 9: Detection of small quantities of seeds by AD-PMCA. Synthetic Aβ seeds were generated as shown on Figure 8. Decreasing amounts of Aβ seeds were subjected to AD-PMCA using SF-Aβ as substrate. After incubation soluble Aβ was detected by ELISA using 4G8 antibody. These 3 experiments were performed independently from each other. Seeding effect was determined as a shortening in the lag phase in samples containing 7.25nM (**A**), as well as in samples with 750 pM of seeds (**B**) and 72 pM (**C**) spiked samples. The non-linear regression showed that the 3 curves fit a sigmoid kinetic. A50 parameter was calculated from the best fit sigmoid curve using GraphPad Prism (logE50 +/- SE). The percentage of insoluble peptide was calculate by subtracting the remaining soluble Aβ after centrifugation from total SF-Aβ substrate. Results representative of 2 independent experiments per seed concentration.



Conditions	A50 (h)
Artificial CSF	22.59 +/- 2.78
Human CSF	19.95 +/- 0.11

Figure 10: SF-Aβ aggregation kinetic in CSF samples.
Aggregation kinetic of SF-Aβ peptide in either artificial cerebrospinal fluid (aCSF) or human CSF. SF-Aβ(1-42) at 2μM concentration was incubated in CSF. No significant differences were observed between the aCSF and human CSF analyzed by two-way ANOVA (p= 0.92). The non-linear regression shows that SF-Aβ aggregation in both aCSF and human CSF follows a sigmoid kinetic (r^2=0.99, r^2=0.97). Percentage of insoluble peptide was calculate by subtracting the remaining soluble Aβ after centrifugation from total SF-Aβ substrate. Soluble Aβ was detected by ELISA using 4G8 antibody. Best fit and statistic were calculated using GraphPad software.

A



B

Condition	A50 (h)
Normal CSF	25.34 +/- 0.020
Spiked CSF 75pM	22.22 +/- 0.011
Spiked CSF 7.5pM	22.69 +/- 0.002

Figure 11: Detection of small quantities of oligomers spiked in CSF by AD-PMCA. Synthetic Aβ oligomers were spiked in human CSF samples. After incubation soluble Aβ was detected by ELISA using 4G8 antibody. **A** Seeding effect was observed in both spiked samples, as a significant shortening of the lag phase compared to the control ($p<0.0001$). All curves fit a sigmoid kinetic ($r^2=0.99$; $r^2=0.99$; $r^2=1$). **B** A50 parameter was calculated from best fit sigmoid curve using GraphPad Prism (logE50 +/- SE). The percentage of insoluble peptide was calculate by subtracting the remaining soluble Aβ after centrifugation from total SF-Aβ substrate. Results representative of 2 independent experiments. Statistical analysis by two-way ANOVA with Bonferroni test using as control column normal CSF.

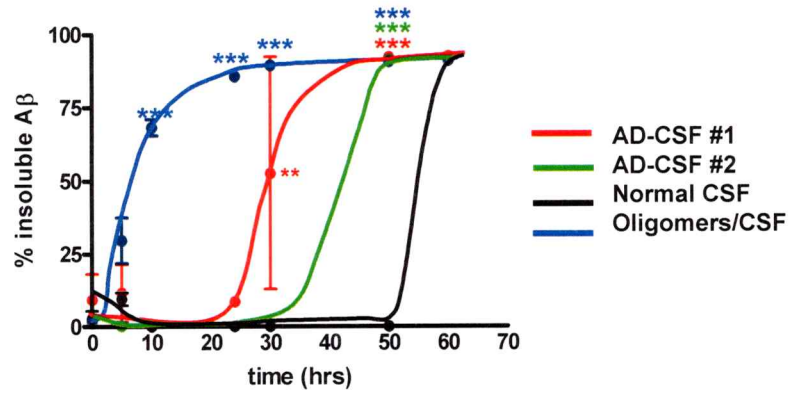
Detection of oligomers in CSF from Alzheimer's patients by AD-PMCA.

Once I was able to detect as little as 75 pM of spiked Aβ oligomers, I tested AD confirmed CSF samples (Gds 3,4) by AD-PMCA in order to determine if misfolded oligomers were circulating in the fluid of these patients. Since CSF samples are a very valuable fluid I performed a preliminary experiment using just two AD-confirmed samples and compared to a normal non-AD CSF. As a positive control I used non-AD cerebrospinal fluid spiked with 750pM of synthetic Aβ oligomers. I used around 150μL of human CSF for this assay, adding TrisHCl 200mM pH 7.2 to a final volume of 300 μL to subject the samples to AD-PMCA. I did not want to dilute more the CSF samples to assure a positive detection but unfortunately this small volume limited the number of time points and duplicates I can measure. Although these data is very preliminar I was excited to find a shorter lag phase in both AD patients compared to the control CSF. This effect might be due to endogenous misfolded oligomers present on the AD samples that induced the elongation of the SF-Aβ substrate (Fig 12).

Detection of Aβ oligomers in plasma from Alzheimer's patients by AD-PMCA.

The lumbar punction performed to extract cerebrospinal fluid is a risky, stressful and uncomfortable procedure for patients, especially aged-individuals. It must be performed by an experienced professional and cannot be done routinely on the same patient. Therefore, I aim the detection of Aβ oligomers on fluids with easier access like blood. While hCSF contains mostly salts and few peptides, blood is a more complex fluid, rich in protein and different cell types. Thus, my first obstacle was to maintain a suitable lag phase (>24hrs) when using blood samples. In previous experiments I observed a complete loss of the lag phase in hCSF

A



B

Condition	A50 (h)
Normal CSF	**57.27** +/- 1.23
AD#1 CSF	**25.70** +/- 1.61
AD# 2 CSF	**38.80** +/- $5.5x10^{-6}$
Spiked CSF	**8.20** +/- 1.7

Figure 12: Detection of oligomers in Alzheimer's CSF samples by AD-PMCA. Aβ aggregation kinetics in CSF from AD patients compared to a non-AD CSF sample. As positive control we add synthetic seeds (1%) to a normal CSF sample. **A** Seeding effect was observed in both AD-CSF samples. **B** A50 (logE50 +/- SE) was derived from best fit curves. The best fit found for AD#1, AD#2 and normal CSF data corresponds to a sigmoid curve (r^2=0.99; r^2=0.94; r^2 =0.98).The best fit for Spiked CSF correspond to an hyperbola with a r^2=0.98 against a r^2=0.74 from sigmoid kinetic. Statistical and best fit analysis were calculated using using GraphPad Prism. Percentage of insoluble peptide is shown as average +/- SE. Statistical analysis by two-way ANOVA with Bonferroni test using as control column normal CSF. ** $p< 0.01$ *** $p<0.005$.

contaminated with blood (needle touch vein during lumbar punction procedure). It's possible that blood cells interfere with the kinetics of SF-Aβ in AD-PMCA since they precipitate at low speed centrifugation; they pull-down the SF-Aβ even if it's not aggregated. To test this hypothesis I took fresh blood from wild type mice and separate it in different fractions after a Ficoll gradient. I observed that only the fractions containing the red blood cells prevented the lag phase, while plasma, PBL and ficoll fraction's do not shortened the lag phase, at the contrary, they seem to increase the lag period (Fig. 13). In a preliminary experiment, I mixed Aβ oligomers FITC-labeled with fresh blood and after min of incubation we separated the blood fractions using a Ficoll gradient. I detected a stronger fluorescent signal on Plasma and PBL fractions, which might be indicative of Aβ oligomers (data not shown). Also, previous data from our laboratory indicated that Prion seeds remained on PBL fractions after Ficoll separation. Taking this in consideration I took fresh blood from AD patients and normal individuals and after separation by Ficoll gradient I subjected the plasma samples to AD-PMCA. I compared samples from 3 normal healthy individuals with 3 patients diagnosed with dementia of the Alzheimer's type (MMSE < 20). Before AD-PMCA, the human plasma samples were desalted using a 10KDa cut-off filter and reconstituted them to their original volume in TrisHCl 100mM aggregation buffer. After incubation with the SF-Aβ substrate, I took centrifuged aliquots at several times points and measured the quantity of remaining soluble Aβ by ELISA. The young non-demented control showed a sigmoid kinetics with an average A50 of 79 hrs (Fig. 14A). In contrast, I observed that 2 out of 3 AD-plasma samples induced a significant shortening of the lag phase, compared to the controls, with an average A50 of 30 hrs (Fig. 14B,E). In addition, AD samples changed the kinetics of SF-Aβ substrate, from a sigmoid type of curve to an hyperbola. From this experiment I can predict

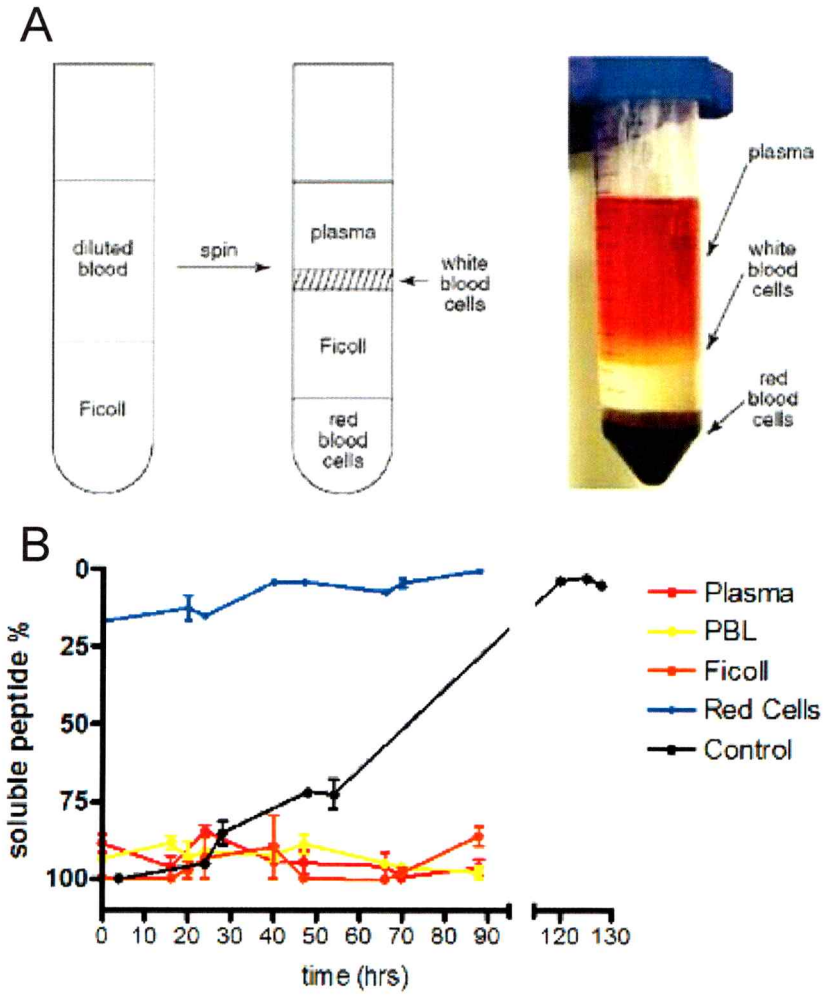
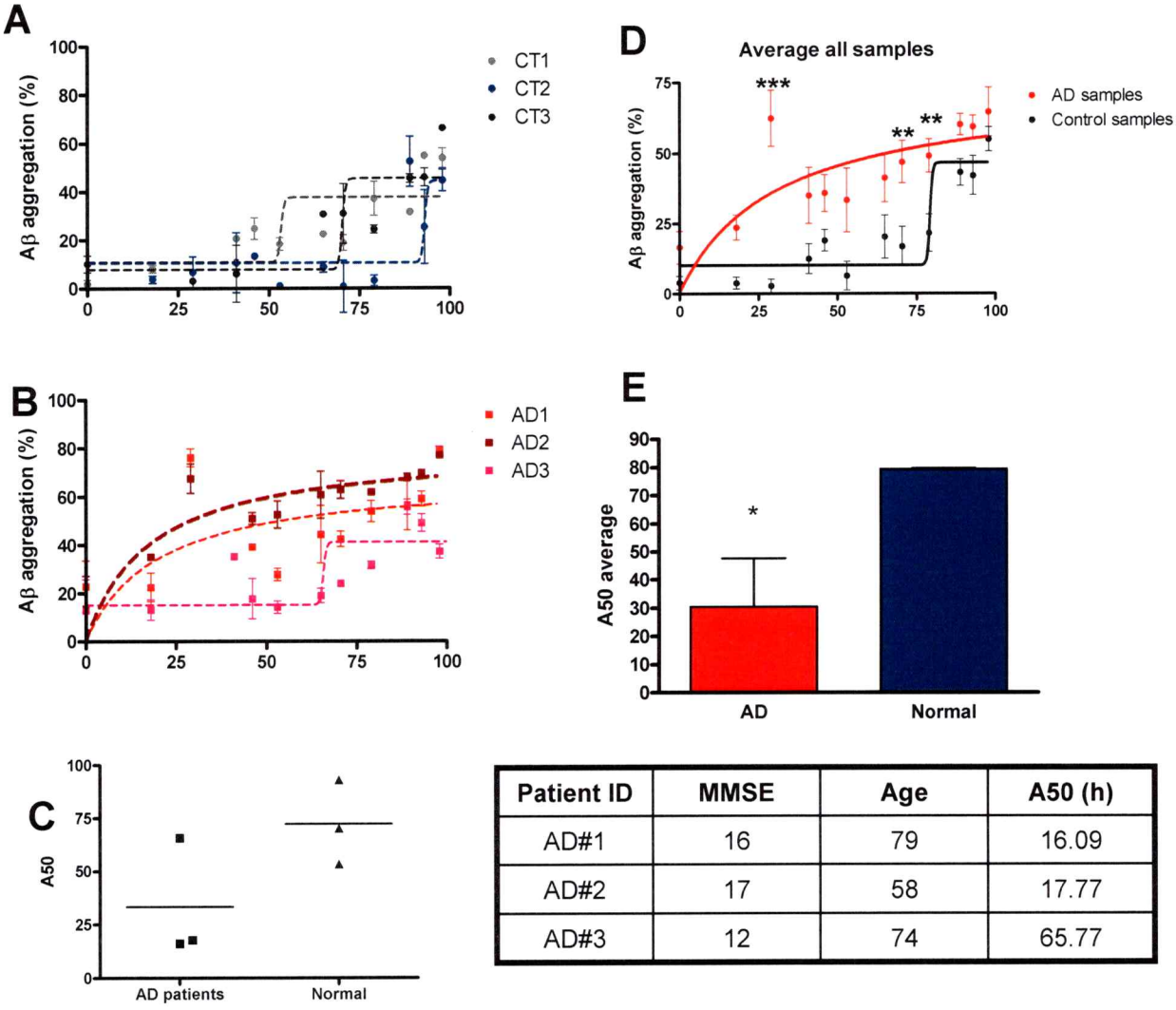


Figure 13: SF-Aβ aggregation kinetic in different blood fractions.
A. Blood from wild type mice was extracted by cardiac puncture. Blood samples were separated by Ficoll gradient as described in methods. Four different fractions were identified; Plasma, white blood cells (PBL), Ficoll and red blood cells. SF-Aβ(1-42) at 2μM concentration was incubated with non-diluted blood fractions. AD-PMCA buffer was used as control. Aggregation kinetic of SF-Aβ peptide in different blood fractions is shown in **B**. A slight increased in lag phase was observed in AD-PMCA when using plasma, PBL and Ficoll samples, possibly due to differences on pH and ionic strength. A complete loss of lag phase was observed in samples containing red blood cells. Soluble Aβ was detected by ELISA using 4G8 antibody. Percentage of soluble peptide is shown as average +/- SE.

that human plasma samples that induced a lag phase shorten than 47 hrs (A50 + SE) might be positive for dementia of the Alzheimer's type. These results suggest that i) Alzheimer's patients have Aβ oligomers circulating in peripherical blood and ii) AD-PMCA enables detection of Aβ oligomers in blood with 66.6% sensitivity (2/3 AD samples were positive) and 100% specificity (0/3 control samples were positive for AD). To validate this result we are currently testing a large number of AD-confirmed samples (50) and age-match controls (40). However, this part of the project, ambitious and time-consuming, was not reachable in the time frame of this doctoral thesis. Further analysis will include detection of Aβ oligomers in other blood fractions besides plasma and blood samples from patients afflicted by other neurodegenerative diseases.



Patient ID	MMSE	Age	A50 (h)
AD#1	16	79	16.09
AD#2	17	58	17.77
AD#3	12	74	65.77

Figure 14: Detection of oligomers in Alzheimer's blood samples by AD-PMCA. Blood from Alzheimer's patients and healthy individuals were separated and plasma fraction were analyzed by AD-PMCA. SF-Aβ substrate was mixed either with AD-plasma or control plasma samples and incubated at 25C with vigorous shaking. **A** Blood samples from normal individuals, average of 2 measurements +/- SE. **B**. Blood samples from AD patients, average of 2 measurements +/- SE Seeding effect was clearly observed in 2 out of 3 Alzheimer's blood samples. **C**. A50 of AD patients compared to normal controls. **D** Average of blood samples (n=3, per condition) +/- SE, best fit curve in dotted lines. AD samples fit a one binding site (hyperbola) curve while control samples follow a sigmoid kinetic. Average of AD samples is significantly different from control curve (Two-way ANOVA, $p<0.001$) **E** A50 calculated from best fit curves calculated on D (logE50 +/- SE) . Percentage of insoluble peptide was calculate by subtracting the remaining soluble Aβ after centrifugation from total SF-Aβ substrate. Soluble Aβ was detected by ELISA using 4G8 antibody. Best fit curves were generated using the GraphPad Prism software. Statistical analysis T Student's * <0.05.

6.2. Specific aim 2: To evaluate if a key feature of AD such as senile plaques can be induced through blood transfusion AD animal models.

The hypothesis of this work is that misfolded oligomers are present and circulating in biological fluids. From my point of view these small oligomers may act as seeds accelerating the process of Aβ aggregation and thus the progression of the disease, when they are transmitted to healthy individuals. This principle may explain the origin of some of the sporadic cases of AD and its high incidence.

As a proof of concept our team inoculated human AD-brain extracts into an AD transgenic mice (hAPPwt), by intracerebral injection (n=5-7). HAPPwt mice do not develop senile plaques although they express human APP and produce Aβ peptide (Mucke et al., 2000). Mice that were injected with AD-brain extracts developed Aβ deposits at 20 months of age, while control animals (injected with non-AD infant brain extracts) do not have any visible deposit (Fig. 15A). A semi-quantitative analysis of these deposits showed a tendency to increase the plaque number with the age on the mice that received the AD-brain extract (Fig. 15B) Taking in consideration the antecedents discussed in the literature (see theoretical background) and our preliminary experiments, I propose that under experimental conditions, oligomers present in old AD-transgenic mice might accelerate the formation of plaques when injected intravenously in young asymptomatic transgenic animals.

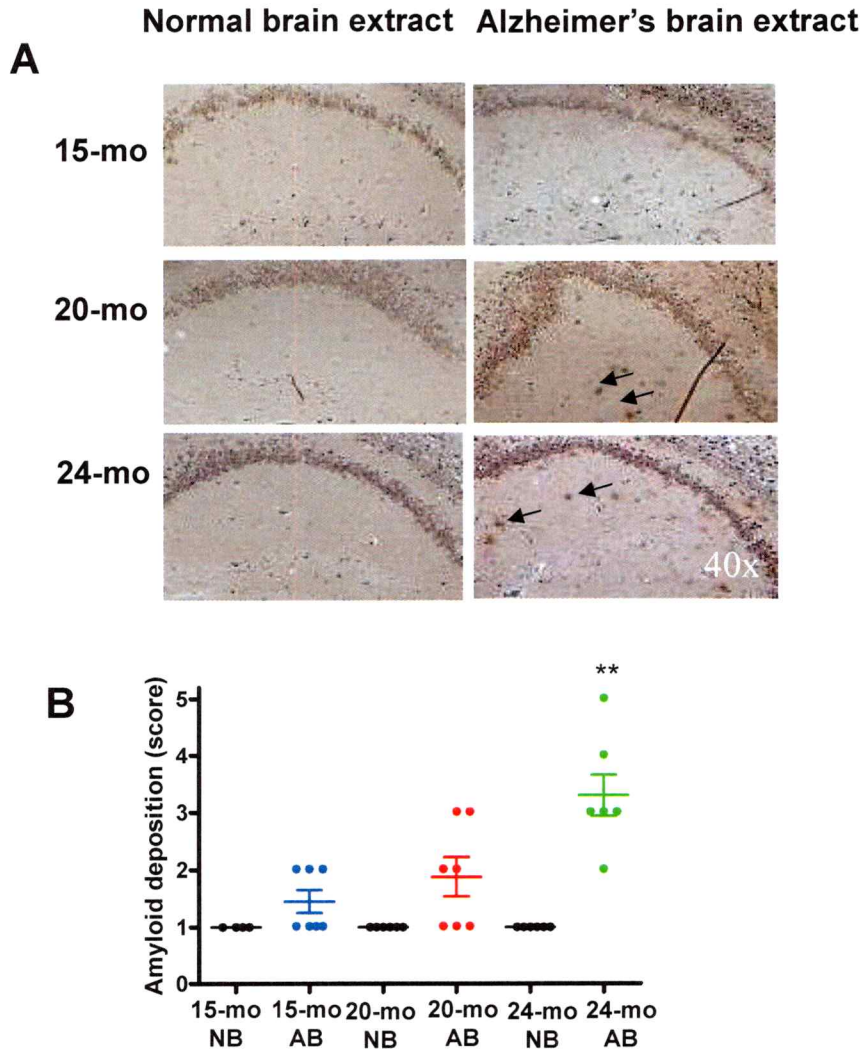


Figure 15: AD-brain extracts induces Aβ deposition in hAPPwt transgenic mice.

HAPPwt were injected i.c with AD-brain extracts and normal brain samples. **A** Inmunohistochemistry using 4G8 antibody against Aβ peptide (40x magnification). Mice were sacrificed at different time points. **B** Semi-quantitative analysis of Aβ deposits in huAPPwt mice. NB: mice injected with non-AD brain preparations. AB: mice inoculated with AD-brain derived extracts. One-way ANOVA with Newman-Keuls Multiple Comparison analysis was performed using GraphPad Prism; ** $p<0.01$. mo; months-old. n=8.

Transfusion of blood coming from AD-mice accelerates Aβ deposition in young APPswe/PSEN1Δ9.

From the results obtained in our previous aim, I believe that Aβ oligomers leak from the brain in Alzheimer's disease and enter to the blood torrent, where they maintain their seeding capability. Thus I think that old symptomatic AD-transgenic mice have oligomers circulating in blood and that these oligomers may exert their seeding ability when introduced in other mice. To test this hypothesis I extract whole blood from APPswe/PSEN19 mice old mice (12 months) that already have extensive amyloid plaque accumulation since I believe that at this age their blood contains these putative oligomers (Fig 16). Accordingly, from now and on I will called it AD-blood. The whole blood was extracted by cardiac puncture using K2 vacutainers tubes with EDTA as anticoagulant, and injected into young APPswe/PSEN1d9 mice (2 months old) through the vein tail. The blood transfusion was performed three times in each animal (n=5), once a week with a maximum of 100 uL of blood to avoid drastic changes in blood pressure. A control group (n=5) was injected with non-transgenic blood of the same mouse strain and as a proof of concept young APPswe/PSEN1d9 mice were injected with synthetic oligomers spiked in non-transgenic blood (n=5). All three groups were euthanized at 5 months of age and the brains were analyzed by immunohistochemistry for Aβ load. As it's shown in Figure 17, animals that received AD-blood has more plaques in both hippocampus and motor cortex in comparison to control mice, and also a significant increase of the total Aβ burden in the cerebral cortex was determined (Fig 17C). In addition, mice that received synthetic oligomers spiked in blood, showed the same phenotype of the mice injected with AD-blood (increased Aβ deposition), supporting the hypothesis that the increase on Aβ deposition in the AD-blood group is due Aβ oligomers rather than to others factors present in

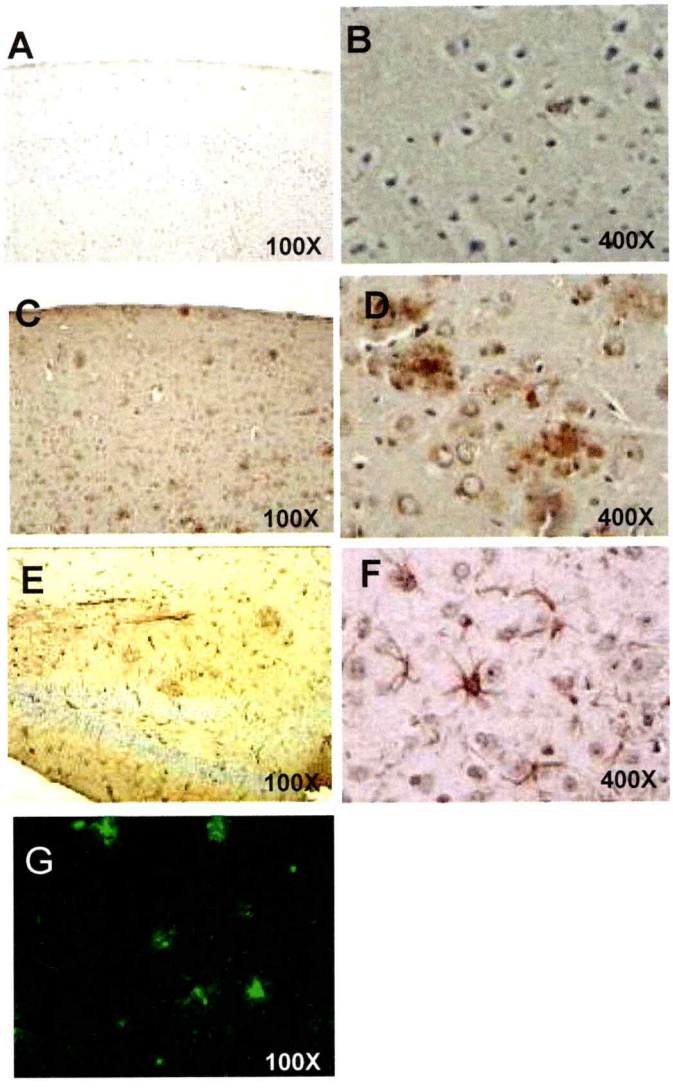


Figure 16: Old APPswe/PSEN1Δ9 transgenic mice showed AD-like features. Histological analysis of APPswe/PSEN1Δ9 transgenic mice older than 8-months of age. **A, B** APPswe/PSEN1Δ9 transgenic mice brain stained with H&E. **C, D** Inmunohistochemistry using 4G8 antibody against Aβ peptide (100x, 400x magnification). **E, F** Astrogliosis visualized through GFAP reactivity. (100x, 400x magnification). **G** Thioflavin S staining shows mature reactive senile plaques. (100x magnification). Mice n=4.

AD-blood. However, no quantitative analysis was possible due the high mortality that synthetic oligomer's injection caused to mice. This effect was probably due to the toxicity of solvent residues from the preparation of synthetic oligomers than Aβ *per se*.

Also, mice that receive AD-blood have extensive astrogliosis visualized by GFAP staining (Fig 17B). Interestingly, amyloid beta plaques detected on the AD-blood group have a different morphology than plaques found on control mice. In addition, mice injected with synthetic oligomers have 2 clear types of amyloid plaques different from controls; small condensate deposits and large and diffuse plaques.

Although I observed acceleration of the Aβ deposition process, we did not *transmit* a new characteristic (phenotype) to these mice, since the APPswe/PSEN1d9 mice will develop a full AD-like pathology (mature plaques and inflammation) around the 6 months of age regarding if they were injected with AD-blood or not. Therefore, the results obtained from this experiment in the APPswe/PSEN1d9 mice may well indicate a *seeding-effect* of Aβ rather than an AD-transmission, meaning that Aβ oligomers contained in the blood accelerated a process that is latent in the transgenic mice. Thus, in a second approach to test the blood transmission hypothesis we choose a transgenic mice line that do not develop AD pathology in its life-span (although synaptic dysfunction has been reported) (Mucke et al., 2000). We chose this transgenic (huAPPwt), which express the normal human APP, over the wild type mouse for the following reason. Despite its 96% similarity with the human sequence, murine Aβ peptide do not aggregate neither *in vitro* nor *in vivo*, possibly due to the three amino acid substitutions found in the mice: Arg→Gly, Tyr→Phe and His→Arg at the positions 5, 10 and 13, respectively (Dyrks et al., 1993). Thus, even if the exogenous Aβ oligomers reach the brain, they won't be able to convert (misfold) or catalyze the misfolding of the endogenous murine

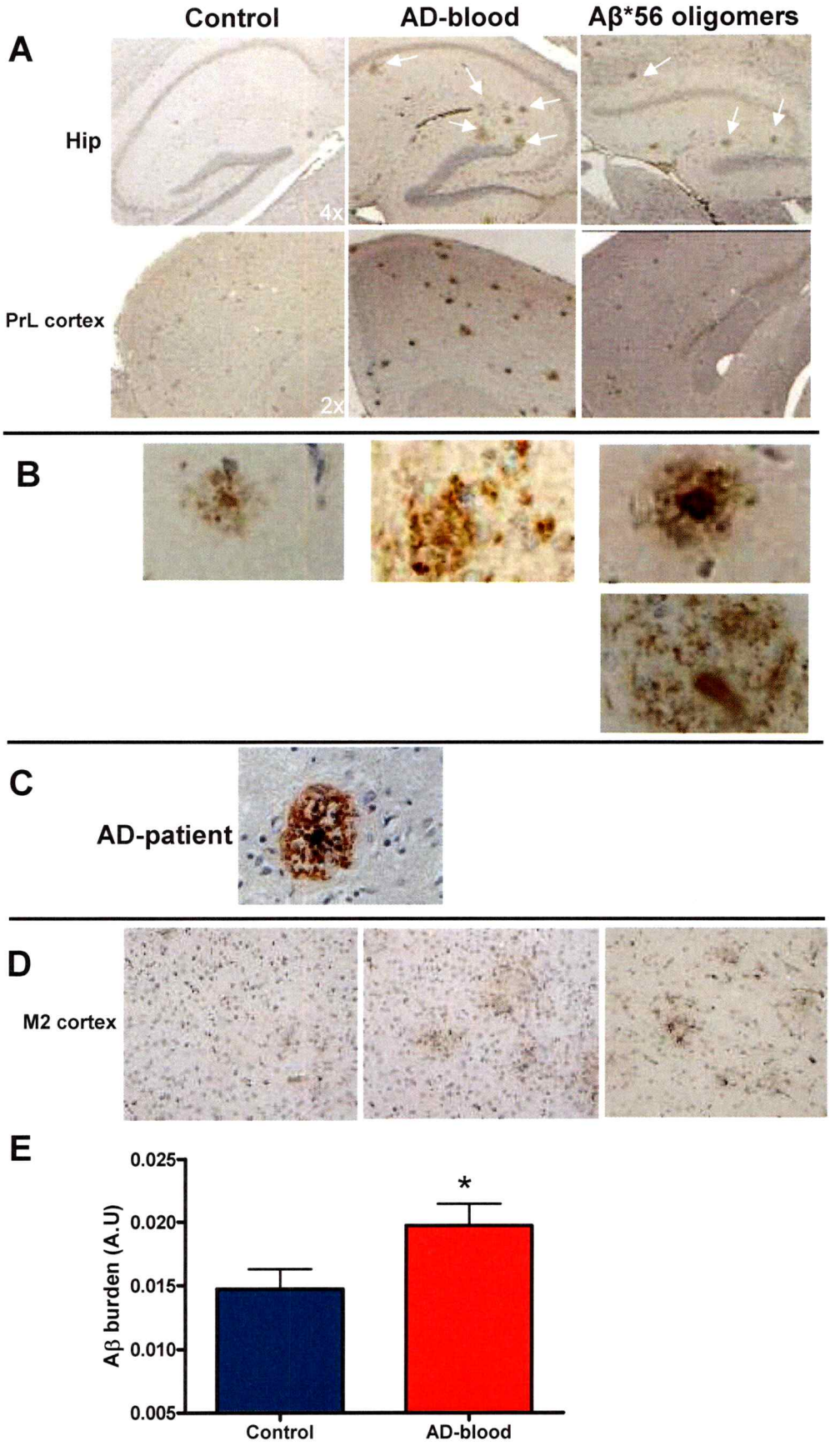

Control
AD-blood
Aβ*56 oligomers
A
Hip
4x
PrL cortex
2x
B
C
AD-patient
D
M2 cortex
E
0.025
0.020
0.015
0.010
0.005
Aβ burden (A.U)
*
Control
AD-blood

Figure 17: Blood transfusion using AD-blood accelerates amyloid deposition in APPswe/PSEN1Δ9 transgenic mice. A. Immunohistochemistry of mice brain against Aβ shows an increase of Aβ plaques in mice receiving blood transfusion from old-transgenic mice. **B.** Magnification of Aβ plaques showed that mice that received Ad-blood have bigger plaques, with stronger Aβ reactivity compared to the control group. Interestingly, in mice injected with synthetic Aβ*56 oligomers we found two clearly distinguishable types of plaques; small plaques with compact 4G8-reactive cores and big diffuses plaques. **C** Magnification of a representative Aβ plaque found on a 28-year old patient who died of iatrogenic CJD (and possible AD) after a dura graft. The strong Aβ reactive core is similar to those found of Aβ*56-injected mice. **D** Astrogliosis is abundant in mice that received AD-blood compared to the slight inflammation detected on control mice (visualized by immunohistochemistry against GFAP). Astrogliosis on mice that received synthetic Aβ*56 is slightly increased, with strong GFAP reactivity on discrete cells. **E** Quantification of 4G8 reactive plaques on brain hippocampus analyzed by ImageJ software. Aβ burden is significantly increased in mice injected with AD-blood. T-student analysis by Graph Pad Prism software; *$p<0.05$. Mice n=5.

Aβ. I injected hAPPwt mice of 17 months through the vein tail with AD-blood coming from old transgenic APPswe/PSEN1d9 mice as described before (n=4). The blood transfusion was performed three times in each animal, once a week with a maximum of 100 μL of blood to avoid drastic changes in blood pressure. A control group was injected with non-transgenic blood of WT C57 mice (n=3). The mice were euthanized 2 months later and the brains were analyzed by immunohistochemistry for Aβ load. I did not find a clear deposition of Aβ in these mice or Thioflavin S-positive plaques. However, in 3 out of 5 mice that were injected with AD-blood we found small Aβ deposits similar to those found in our preliminary experiment of intracerebral injections of AD brain extracts (Fig 18). In contrast none of our control mice showed any 4G8-reactive stain. Although this last experiment was not conclusive in showing the blood-transmission of AD, I believe that modifying the experimental conditions we can come closer to the understanding of the AD-transmission phenomena since I demonstrated at least a clear acceleration of the AD-pathology through blood-containing oligomers, in the APPswe/PSEN1d9 Alzheimer's model. These results might be of enormous impact in human Alzheimer's etiology because they showed the existence of a transmissible element, although non-infectious (Koch's postulates, see discussion) that may trigger Alzheimer's pathology in the AD-risk population.

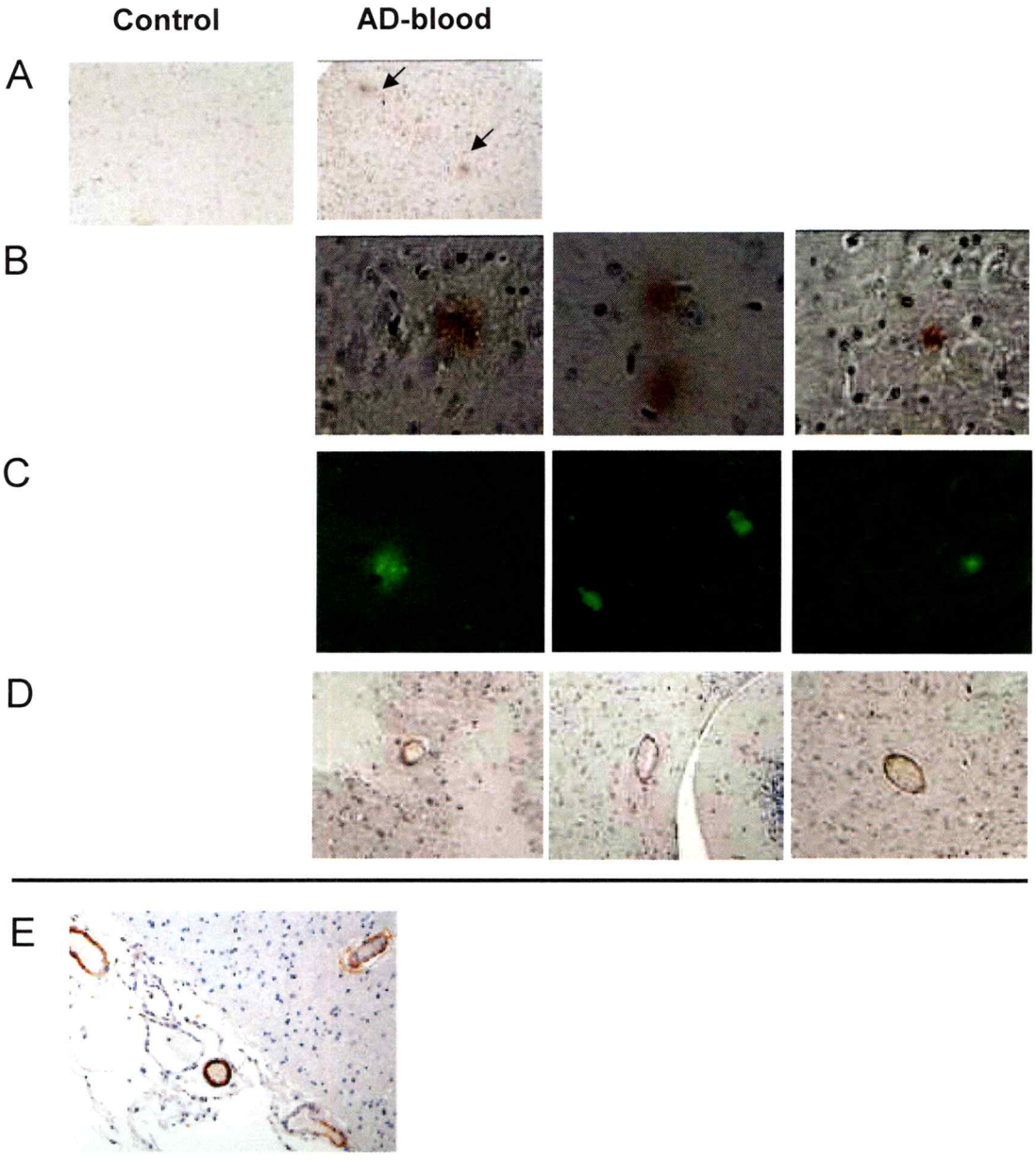


Figure 18: Blood transfusion using AD-blood accelerates amyloid deposition in hAPP mice. A. Immunohistochemistry of brain slides against Aβ shows few Aβ plaques in 2 out of 4 mice receiving blood transfusion from symptomatic transgenic mice while no deposits were detected on control hAPP mice. **B.** Magnification of Aβ deposits found on AD-blood mice, showed that correspond to small diffuse plaques in the basal ganglia. **C** Thioflavin-S staining showed an small plaque in the cortex of 2 mice treated with AD-blood. **D** Interestingly, Aβ-reactive deposits similar to those found on cerebral amyloidosis, were observed surrounding blood vessels only in 3 out of 4 hAPP mice that receive AD-blood. **E** Aβ-reactive blood vessels found on a 28-year old patient who died of iatrogenic CJD (and possible AD) after a dura graft. The size and shape are very similar to those found on Ad-blood-injected mice. Control mice n=3, AD-blood n=4.

6.3. Specific aim 3: To evaluate the heterologous seeding phenomena using as example two cerebral amyloids (Aβ and prions) and one systemic amyloid (amyloid-enhancing factor).

Heterologous seeding effect of AEF seeds over Aβ aggregation.

Amyloid protein A (AA) amyloidosis, one of the most common forms of life-threatening systemic amyloidosis, can be induced experimentally in mice injected for 2–3 weeks with silver nitrate or casein as the inflammatory stimulus (Ishihara, 1973; Skinner et al., 1977). This lag phase was dramatically shortened when mice received an intravenous, intraperitoneal or oral administration of cells or extracts from amyloid-containing tissue (Cui et al., 2002; Kisilevsky and Boudreau, 1983; Lundmark et al., 2002). These active components, termed amyloid-enhancing factors (AEF), are known to serve as a nucleus for fibril formation (Lundmark et al., 2002). Although AEF has never been characterized thoroughly, amyloid fibrils, amyloid-like synthetic fibrils and denatured silk were reported to have AEF activity (Johan et al., 1998; Lundmark et al., 2005; Niewold et al., 1991). In this experiment, I investigated amyloid-enhancing factor (AEF) activity of amyloid fibrils extracted from amyloid-loaded livers over Aβ peptide. I induced amyloidosis in wild type mice (n=2) using silver nitrate as the inflammation stimuli. After 2 months from the injection, the AEF accumulates on the mice livers. I euthanized the animals using $C0_2$ and extracted the AEF from the fresh livers as described in Methods. To probe that AEF-liver extracts are enriched in amyloid fibers I analyzed the amount of fibrils in different purification steps by Thioflavin T binding. Dw1, DW2 and DW3 are final water based extractions from the AEF-livers. DW2 fraction has the stronger signal Thioflavin T, which is indicative of amyloid fibrils (Fig 19A).

In contrast, no fluorescence was detected in liver extracts from non-treated mice (sp3 fraction).

Fibrils formed *in vivo* are usually bigger and stronger than fibrils made *in vitro* from synthetic peptide, since *in vivo* fibrils contain other proteins forming complexes and strong interaction. In general, large aggregates are not good seeds. For this reason I evaluate a range of different sonication intensity and time in order to break down AEF fibrils in smaller aggregates that can act as seeds. I follow fibril fragmentation using Thioflavin T; in protofibrills and large oligomers Thioflavin T molecules intercalates more easily than in big fibrils, thus reducing the quenching of Thioflavin molecules and increasing the fluorescence. As I shown in figure 19B, a strong sonication was needed (80 power) for more than 20 seconds to break down AEF fibrils. These small aggregates of AEF were used as seed for Aβ aggregation following the parameters used for AD-PMCA. As control I used Aβ mixed with 0.4% of non-AEF liver. We found that AEF seeds accelerated the deposition of Aβ in a concentration dependent manner. All curves follow a sigmoid kinetic, although the control curve seems slight different. These results suggest that AEF, which is found in a common type of systemic amyloidosis, may accelerate the process of Aβ deposition, which is a key feature of Alzheimer's pathogenesis.

Prion protein accelerates Aβ aggregation acting as a heterologous seed.

To test the possibility that PrP seeds accelerate Aβ misfolding and aggregation, we evaluated the seeding capability of purified PrPSc in the aggregation of SF-Aβ. To isolate PrPSc seeds wild type mice were inoculated i.c with RML, a mice strain of prion derived from scrapie.

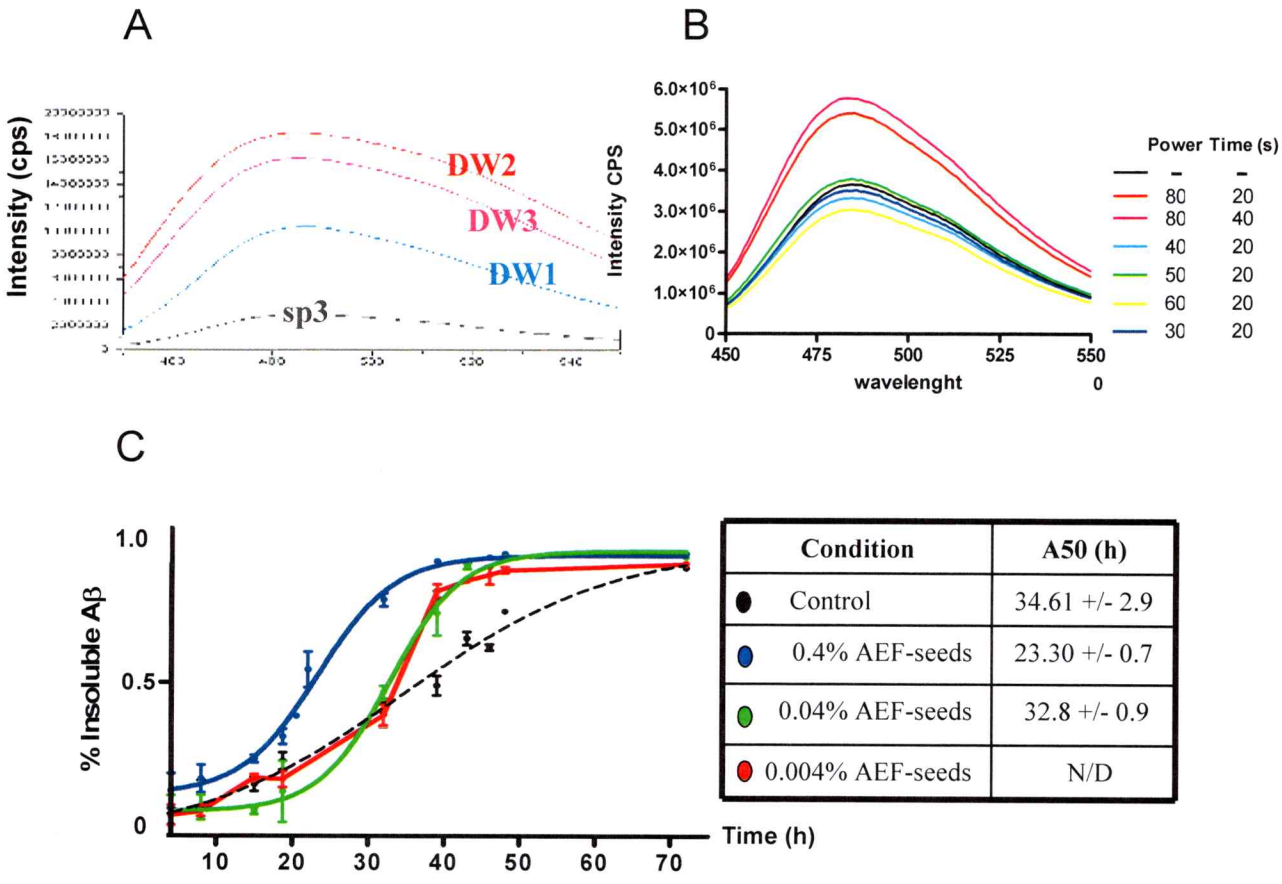


Figure 19 : Heterologous seeding effect of AEF from liver extracts over Aβ aggregation *in vitro*. C57 mice were injected with 2% AgNO3 to induce amyloidosis. Liver was collected and processed for AEF-extraction following the protocol described in methods. **A** Thioflavin T binding assay of AEF extractions showed that DW2 extraction has the higher concentration of amyloid fibrils. **B** AEF-fibrils from DW2 extraction were subjected to different sonication protocols, modifying both length and strength of sonication, to generate small AEF-seeds. **C** AEF-seeds were generated from DW2 extracts sonicated per 40sec at 80% sonication power. Soluble Aβ42 was incubated with different concentrations of AEF-seeds or non-amyloidogenic liver extract (control). Seeding effect was observed in AEF samples until 0.04% of AEF seeds. Aggregation of Aβ was measured overtime by sedimentation couple to sensitive ELISA. Aggregation of Aβ is shown as average +/- SE. A50 were derived from best fit curves using GraphPad software. DW1; first AEF-liver extraction. DW2; second AEF-liver extraction. DW3; Third AEF-liver extraction. Sp3; Non-AEF liver extraction. N/D; non-determined.

After the mice develop a scrapie-like phenotype they were euthanized and PrPSc was isolated and highly purified from the infected brains using previously described protocols (Hetz et al., 2003a). The purified Prion protein was subjected to a brief sonication (10 power, 1 sec) and diluted to the desired concentration in PBS. To evaluate if Prion seeds were able to accelerate Aβ aggregation, I mixed various small quantities of prion seeds with SF-Aβ and incubated at the same conditions than those used for AD-PMCA. Prion seeds have a strong effect over Aβ aggregation, measured as a shortening of the lag phase. The acceleration of Aβ polimerization was directly proportional to the amount of PrPSc seeds added to the sample (Fig. 20). The concentration of PrP Sc is expressed as a percentage of oligomers per Aβ monomer and was calculated assuming that a PrPSc oligomers has an average molecular weight of 7700 kDa.

Prion disease affects AD pathology increasing Aβ aggregation and deposition, *in vivo*. To assess the in vivo interaction between the pathological processes implicated in AD and TSEs, we inoculated prions intraperitoneally into Tg2576 mice at different stages of AD progression. One group of animals was inoculated at 1.5 month of age when Aβ accumulation is not yet detectable, and a second group was inoculated at the age in which amyloid deposition begin in these animals (12 months of age). Age matched wild-type mice (nontransgenic littermates) were treated in the same way (n=5). Histopathological analyses of brains from Tg2576 prion-infected mice showed the coexistence of both Prion and AD pathologies. The brain exhibited extensive spongiform degeneration and reactive astrogliosis (Fig 21), which are characteristic of PrPSc accumulation. Conversely, Aβ deposits were not detected in the brains of WT mice inoculated with prions and no vacuolation was seen in old non-infected Tg2576 mice (Fig. 21). Interestingly, brain inflammation was substantially higher on animals bearing the double pathology. The increase on brain inflammation may be

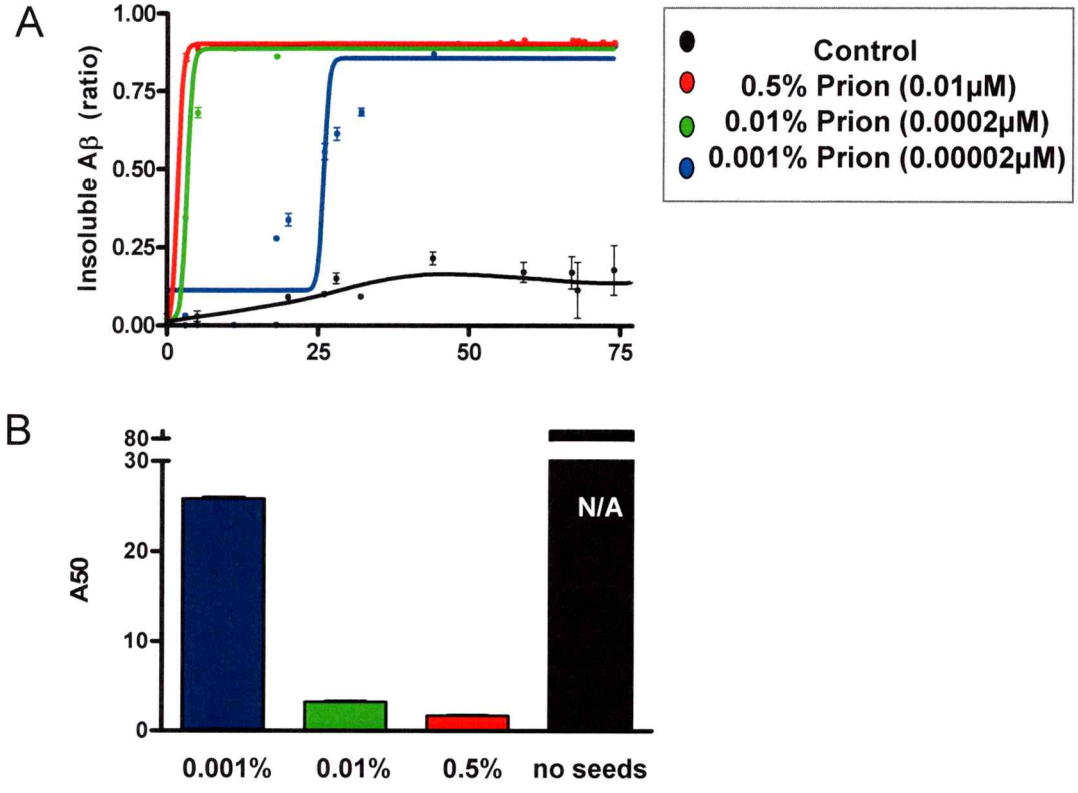


Figure 20 : Heterologous seeding effect of RML seeds on Aβ aggregation *in vitro*. **A** Soluble Aβ42 samples were incubated with different concentrations of Prion (RML strain) or PBS (control). **A** Soluble Aβ42 was incubated with different concentrations of Prion protein or PBS (control). Seeding effect was observed in Prion-containing samples until 0.001% of Prions. Time points in duplicate, average +/- SE. Best fit curves were calculated using GraphPad Prism software. All samples follow a sigmoideal kinetic. **B** A50 were derived from best fit curves using GraphPad software. A remarkably decreased on the A50 is observed at with 0.5% and 0.01% of seeds Aggregation of Aβ was measured overtime by sedimentation couple to sensitive ELISA. N/A=non applicable.

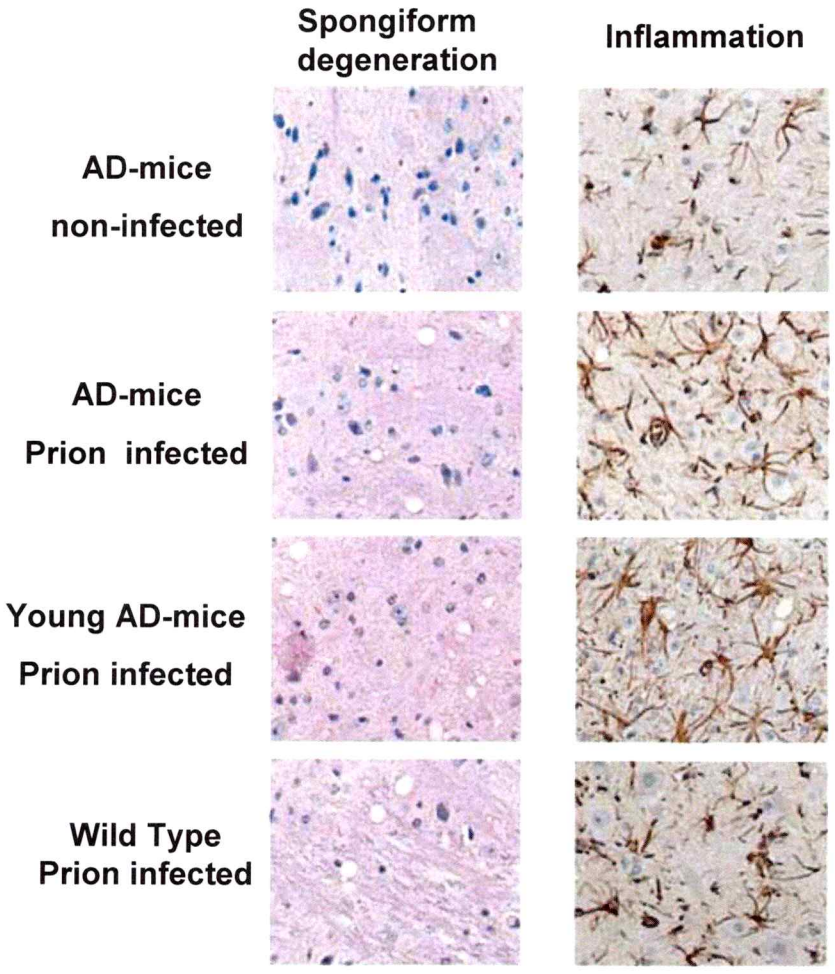


Figure 21: AD-trangenic mice have increased inflammation and spongiform degeneration when infected with prions. Sections from brains of transgenic mice and wildtype mice infected with prions were stained with H&E. Tg2576 mice brains exhibited extensive spongiform degeneration (white vacuoles), which is a hallmark of prion toxicity. Astrogliosis is augmented on AD-transgenic mice infected with prions, as seen with GFAP staining. Mice n=8.

an additive result, since both pathologies are associated with astrogliosis (Diedrich et al., 1987). More remarkably is the dramatic increase on Aβ deposition observed in the Prion-AD-mice compared with non-infected AD transgenics (Fig. 22). Indeed, some of the Tg2576 mice (2/8) inoculated at 45 d of age and killed when prion disease was evident 185 d later), showed Aβ diffuse amyloid deposits at an age when these animals do not have amyloid lesions (7 months) (Fig. 16). These deposits were detected using anti-Aβ specific antibodies in both hippocampus and cortex, but were negative for Thioflavin S staining (Fig. 22A). The latter is not surprising since early, diffuse, preamyloid deposits in Alzheimer's brain are usually not detectable by this amyloid-binding dye (Tagliavini et al., 1988). However, a more detailed study of Thioflavin S staining at higher magnification showed that some lesions were indeed slightly stained by thioflavin S (Fig 22B). Moreover, the size, number, and maturity of Aβ plaques in the AD-transgenic group group inoculated at 12 months of age was dramatically higher than in the age-matched control inoculated with PBS. In addition, the quantitative analysis showed that the total Aβ plaque area as well as the number of plaques at the cortex was significantly higher in AD-mice infected with prions than in non-infected AD-mice ($p<0.01$) (Fig 23). Indeed, infected animals have 2-fold higher number of plaques and strikingly 10-fold greater plaque area than non-infected AD transgenic mice. These data strongly support an interaction between the prion and AD pathologies, leading to a dramatic increase on the misfolding, aggregation, and cerebral accumulation of Aβ in the presence of PrPSc. Recently, our group made further analysis and studied the co-localization of both proteins in pathological aggregates. In AD-mice mice injected with prions, reactive signals against Aβ and PrP antibodies were detected in both compacted amyloid plaques, typical of AD, and large diffuse deposits, characteristics of prion-affected animals (Fig. 24). On the

contrary, no co-localization was observed in non-infected AD-mice or WT mice infected with prions. These results suggest that misfolded proteins interact in the brain when both pathological processes are occurring simultaneously.

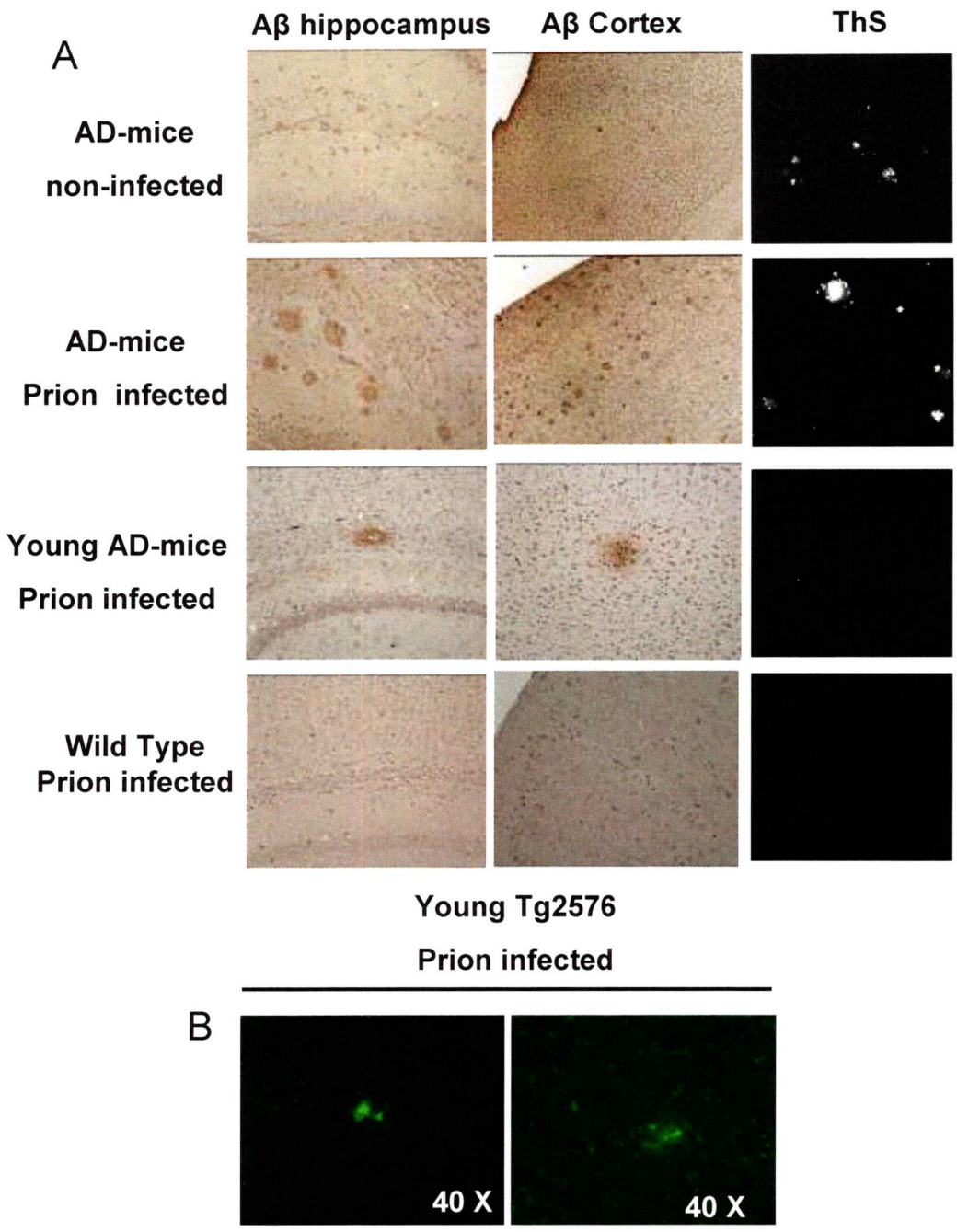


Figure 22: Prion disease affects AD pathology increasing Aβ aggregation and deposition, in vivo. A Sections from brains of transgenic mice and wild-type mice infected with prions were stained with 4G8 anti-Aβ antibody or Thioflavin S. Tg2576 mice brains exhibited an increase in Aβ plaque accumulation in both hippocampus and cortex. Thioflavin S-reactive Aβ plaques were increased in mice bearing both Alzheimer's and prions pathology. **B** Interestingly, young AD-mice showed mature thioflavin S positive plaques months before of what has been reported, when they are infected with prions.Mice n=8.

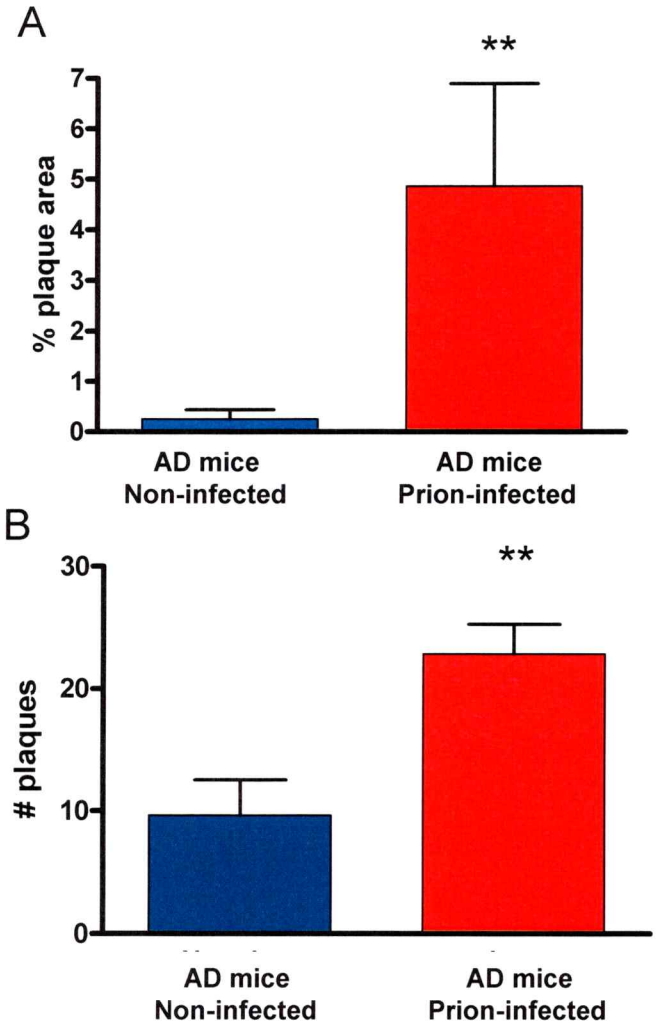


Figure 23: Prion infection increases plaque accumulation in AD-transgenic mice. Brain sections from transgenic mice infected or non-infected with prion were stained with Thioflavin S and microscopy images analyzed by ImageJ software. Tg2576 mice that were infected with prions, exhibited an increase in both brain area covered by plaques (**A**) and number of plaques in the cortex (**B**). Area of brain cortex covered by plaques is expressed as percentage of the total cortex area. 5 images were analyzed per mice. T-test statistical analysis was performed with GraphPad Prism software; ** $p<0.01$. Mice n=8.

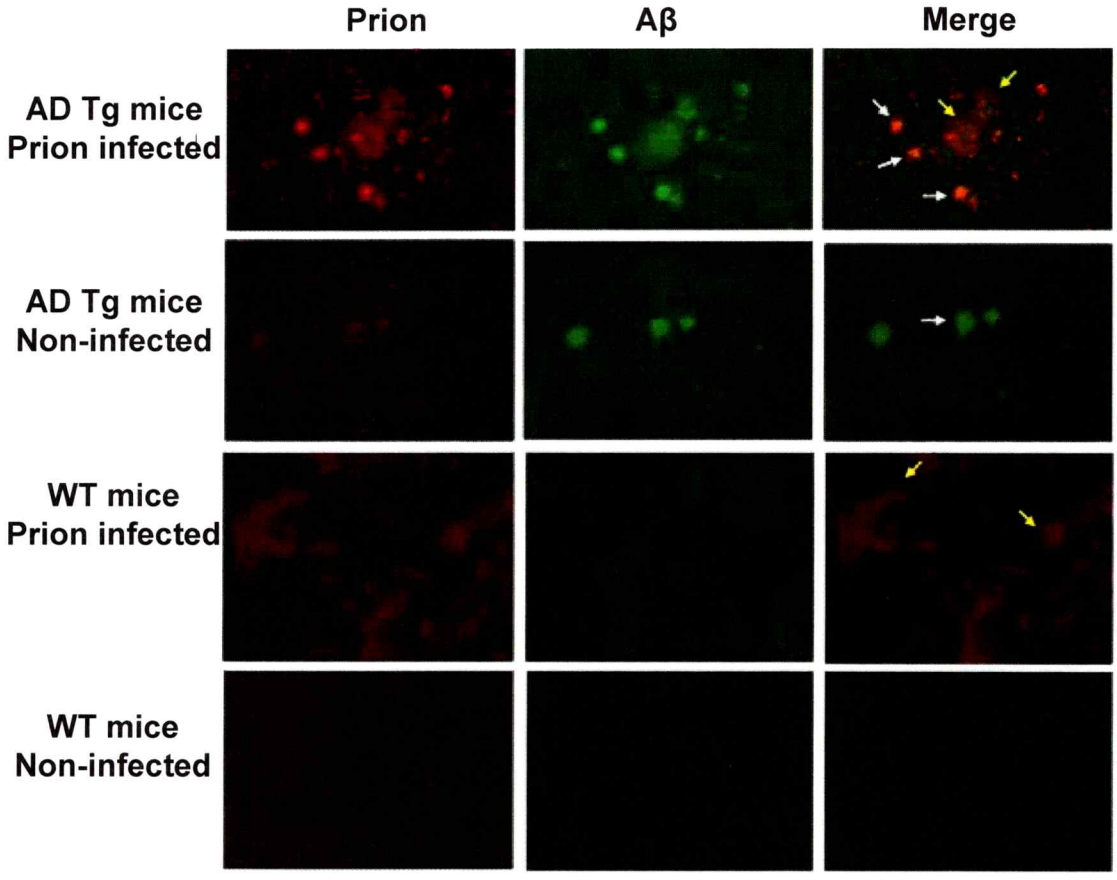


Figure 24: Aβ and PrP co-localized in the brain of Tg2576 infected with RML prions.

Sections from the cortex of animals in different groups were stained with fluorescent antibodies against Aβ (4G8; green) and PrP (6H4; red), and colocalization was evaluated by confocal microscopy. The white arrows point to the typical amyloid plaques seen in transgenic mice and AD patients while the yellow arrows indicates the typical diffuse accumulations of PrP Sc aggregates in the brain of TSE-affected individuals.

7. DISCUSSION

Misfolded aggregates present in amyloid fibrils are associated with various diseases known as 'protein misfolding' disorders. Included in this group is Alzheimer's disease which is characterized by the extracellular deposition of the Aβ peptide and intracellular accumulation of hyper-phosphorylated tau protein, both leading to synaptic dysfunction and neuronal death. Extensive evidence suggests that the cerebral accumulation of misfolded Aβ is the central event in the pathogenesis (Selkoe, 2000).

From all the diseases caused by protein misfolding, prion diseases are the only classified as infectious. Since the molecular bases of prion conversion have a striking resemblance to the process of amyloid formation occurring on AD, we propose in this thesis suggesting that misfolded Aβ aggregates might have an inherent ability to be "transmissible". In addition, this feature can be used to indirectly detect minute amounts of Aβ oligomers as a diagnostic for AD. The strategy consisted on detecting minute amounts of Aβ oligomers by their functional seeding capacity to induce and amplify the protein misfolding process at expenses of synthetic soluble Aβ used as substrate. As described in more details later, our findings show preliminary proof-of-concept demonstration of this strategy as well as the capability to use it to detect Aβ oligomers in human biological fluids, including CSF and blood.

In the second aim I wanted to evaluate if Aβ oligomers might act in a prion-like manner under experimental conditions. As a proof of concept we intracerebrally injected brain extracts form AD patients into hAPPwt mice, a model that don't develop amyloid plaques in its life span. Interestingly we found, for the first time in this animal model, few discrete amyloid deposits in mice that receive AD-brain extracts (Fig. 15). To evaluate a more relevant route of "transmission", I designed an experimental approach of blood transfusion based on the hypothesis that AD-mice models have Aβ oligomers circulating in their blood. I showed that transfusion of blood coming from mice with a full AD-like pathology into young non-symptomatic AD mice accelerates the Aβ deposition in the brain, an important hallmark of AD (Fig. 17). Because the APPswe/PSEN1Δ9 transgenic mice 'spontaneously' develop AD-pathology later in life, it is not possible to conclude whether the AD-blood acted as an real infectious agent or as an accelerator of a process that was genetically programmed to occur. For this reason I performed a second experiment in which we used the hAPPwt model which won't develop AD during the animal life span. Interestingly, I observed few diffuse plaques on some of the mice that receive blood from old APPswe/PSEN1Δ9 transgenic mice (Fig. 18). Although exciting, this result was not statistically significant since the small area of the Aβ deposits that were found.

The third aim of this thesis was to analyze the interaction of the protein misfolding processes implicated in AD and other PMDs, taking as example systemic amyloidosis and prion disease. *In vitro* experiments combining non-misfolded Aβ and misfolding seeds from Amyloidosis and prions showed that Aβ protein misfolding can be enhanced by a heterologous-seeding mechanism (Figs 19, 20). To evaluate the seeding mechanism *in vivo* we inoculated prions in an AD-transgenic mouse model. We found a remarkable increase of

amyloid plaque deposition was in prion-infected mice compared with non-inoculated controls (Fig 20). Also, the disease onset of prions in transgenic mice appeared significantly faster (Fig 21) .These results suggest a molecular interaction between AD and prion pathologies, indicating that one protein misfolding process may be an important risk factor for the development of a second one.

The following sections discuss in deeper details the interpretation of our findings and the conclusions obtained in the studies in each of the specific aims.

Detection of misfolded Aβ oligomers by AD-PMCA as a biochemical test for Alzheimer's disease (specific aim 1).

Currently the diagnosis of AD is based on clinical examination and ruling out other causes of dementia (Nestor et al., 2004). Definitive diagnosis is done post-mortem by brain histological analysis and identification of amyloid plaques and neurofibrillary tangles. No pre-clinical diagnosis is yet possible, and remains one of the highest priorities in the field. Longitudinal studies have shown that the process of protein misfolding and aggregation begin several years or even decades before substantial brain damage and clinical symptoms appear (Mann and Jones, 1990). In this thesis I propose that the sensitive detection of misfolded Aβ oligomers in biological fluids may lead to a novel diagnosis of AD. The measurement of total Aβ in CSF and blood had failed has a biomarker for AD diagnosis due to the lack of a good correlation with the severity of disease and reproducibility in the results. One explanation to the latter findings may be that biological fluids contain low quantities of Aβ, which are composed of many different species within the aggregation pathway. For this reason I proposed the specific biochemical detection of some of the precursors of amyloid plaques, in

particular soluble Aβ oligomers, which might be circulating in biological fluids decades before the onset of AD.

The difficulty of this approach is that the quantity of Aβ oligomers is very small and it is not easy to distinguish them from other Aβ species. To overcome this problem I adapted and applied a procedure previously developed for the biochemical detection of misfolded prions (Saborio et al., 2001; Soto et al., 2002). This technology, termed protein misfolding cyclic amplification (PMCA), reproduce in an accelerated manner the misfolding and aggregation process *in vitro*, enabling amplification of the misfolded prions in the test tube. One of the first obstacles that we had to overcome was the generation of a suitable, reproducible "substrate" for the reaction mixture.

AD-PMCA consists on the incubation of the sample we wanted to evaluate for Aβ oligomers, with a substrate protein to make a reaction mixture. The substrate protein refers to a preparation of protein (or homogenates containing a protein) that is in a non-misfolded form but is able to misfold and aggregate when exogenous oligomers are added to the reaction mix. I used monomeric Aβ as a substrate, but unlike prion protein, purified Aβ tends to spontaneously misfold and aggregate *in vitro*. Thus, the synthetic Aβ peptide commercially available usually contains several aggregated species of different molecular weight when is reconstituted at the laboratories. Therefore I established a protocol (summarized on Figure 5) that gives a homogenous and reproducible Aβ substrate, which contains mainly monomers and dimmers of Aβ peptide.

Misfolded proteins can be detected by different standard methodologies. I tried different approaches described on Figure 4. From all of them the most adequate for the test conditions was the sedimentation, coupled to detection of the soluble remaining substrate by ELISA. I

detect the soluble fractions instead of the pellet because aggregated species usually mask the antibody epitopes. A second problematic was the establishment of an optimal lag phase, meaning:

1) A lag phase that is long enough to differentiate between the spontaneous aggregation of the Aβ substrate and the acceleration of this aggregation by exogenous seeds. I established that an optimal lag phase should be longer than 24 hrs. To achieve this aim I tried different aggregation conditions, such as buffer type, pH, substrate concentration and temperature (Fig 6). The final conditions for our AD-PMCA assay are detailed on Figure 6H.
2) An optimal lag phase must be reproducible. After setting an optimal lag phase I worked hardly on improving the experimental conditions to make this assay highly reproducible. At the end I obtained a simple, well define protocol to perform a very reproducible AD-PMCA. Moreover, I demonstrated the reproducibility of our assay when I implemented the AD-PMCA that we set-up at the Protein Misfolding Laboratory (UTMB, USA) in Dr. Alvarez's Laboratory at Chile, obtaining the same yield of SF-Aβ substrate and a similar lag phase range described at UTMB (Fig 7).

Some improvements that can be done to optimize the current AD-PMCA include using an incubator capable of being programmed and robotic probes for sample and reaction mix manipulation. Also, instead of manually taking and centrifuging the samples at different time points, the separation of substrate from aggregates and further detection of misfolded aggregates in the reaction mix may be automated. For example we can fluorescently label the substrate and measure the soluble peptide by fluorescence emission instead of ELISA.

The amount of Aβ oligomers or even if they exist on human biological fluids has not been yet determined, although some efforts has been done in trying to find AD oligomers. For this reason I hypothesize a possible range of oligomers that may be present in human CSF. The total Aβ concentration on CSF from AD patients has been largely debated. If I consider that total Aβ concentration range from 0.1 nM to 5nM, and that from the pool of Aβ species that compose total Aβ in CSF a 10% correspond to oligomers, I need to set-up our AD-PMCA test to detect at least 0.01nM-0.5nM. In other words if I want to detect oligomers on CSF, first I should observed a distinguishable lag phase when I add 10pM-500pM of Aβ seeds. I demonstrated that I can clearly detect until 75pM of Aβ seeds, concentration that significantly shorten the lag phase (Fig 9). In addition I observed a shortening of the lag phase with as little as 75 pM of seeds when spiked in normal CSF (Fig 11).

After I optimized our AD-PMCA to detect picomolar concentrations of oligomers I test CSFs samples coming from patients in early or mild stage of AD (Gds 3-4). I found a significant difference in the lag phase and A50 parameter in CSF samples coming from patients with probable AD, when compared to a "normal" control sample. As I pointed out in METHODS, normal CSF corresponds to a patient of 54 years and suffering of hydrocephaly. When I used other non-AD samples as a "controls" I obtained a shortening of the lag phase that mask the effects of the AD-samples. Unfortunately, besides the Gds (1-2) I did not have more antecedents over the clinical history of these individuals. To deeply investigate the utility of CSF for Aβ oligomer detection, we plan to test a larger number of CSF samples and age-matched controls.

Attempting blood detection of Aβ oligomers in AD patients was a much intricate aim. CSF composition very much differs from blood composition. For this reason I choose a more

similar fluid such as plasma. Plasma composition has similar concentrations of ions, glucose and water. However, plasma has 200-fold more proteins than CSF (Table II). From these proteins one the most abundant is albumin. Another important protein component are the lipoproteins, which are carriers of Aβ and possibly of Aβ oligomers in blood. In a preliminary experiment I found that addition of plasma to SF-Aβ does not shorten the lag phase of the substrate (Fig 13). Then I tested samples coming from AD patients and compared with blood from control individuals. Interestingly, I found a significant decrease in the lag phase in 2 out of 3 AD samples. Calculating the average of insoluble peptide of all AD samples at each time point and comparing with the control's average I observed 2 important features:

1) AD samples have in average a shorter lag phase with a concomitant decreased on the A50 which is statistically different from the control groups.
2) The AD samples (patient #1 and patient#2) in which I observed a shorter lag phase, do not follow a sigmoid kinetic but an hyperbola, which is typical for high seeds concentrations in our AD-PMCA assay (>10nM). Then, when I plot the average of all AD samples and compare to the control I also observed that the average of AD samples fits a hyperbola-type curve and not a sigmoid curve like controls. This suggests that the kinetic of aggregation can also be a parameter to evaluate seeds concentration.

The AD patients included in this study were diagnosed with dementia of the Alzheimer type having MMSE scores lower than 20. These patients were under medical treatment, taking drugs such as Trazodone (anti-depressant), Zoloft (anti-depressant), Omeprazole (hydrogen-pump inhibitor) and Enalapril (anti-hypertensive). From the control group the medications include Migranol (analgesic + ergotamine), Venaflaxina (anti-depressant) and ibuprofen.

Interestingly, 3 out of 3 control individuals have a family member who suffered from sporadic Alzheimer's. Antidepressants, anti-inflammatory drugs and pain killers are common medications taken by AD and young individuals, therefore is very important to investigate whether these medications affects Aβ oligomers detection by AD-PMCA.

I found that Aβ oligomers have a seeding capability that can be used to indirectly detect their concentration in biological fluids. More importantly, I had shown that biological fluids from AD patients have seeding properties that accelerate the aggregation of monomeric Aβ *in vitro.* These results suggest that Aβ seeds are circulating in biological fluids of AD patients which may help in designing new therapeutic approaches for AD.

A key feature of AD such as senile plaques can be induced through blood transfusion AD animal models (specific aim 2).

Decades ago the unorthodox prion hypothesis was proposed to explain the transmission mechanisms of prion diseases (Griffith, 1967; Prusiner, 1982).The Prion hypothesis was a milestone in biology since it implicates that proteins can behave like micro-organisms to transmit a disease and also inherit information (in this case protein conformation) in absence of genetic material.

The prion hyphothesis postulated the prion as an infectious agent, accomplishing the Koch's postulates like bacteria, virus, fungi and parasites. Prions have the typical characteristics of valid infectious agents, including: exponential multiplication in an appropriate host, transmission between individuals by various routes of infectious, titration by infectivity bioassays, resistance to biological clearance mechanisms, penetration of biological membrane barriers, transmission controlled by species barriers and the generation of new

strains. In this last point is important to remark that appearance of new Prion strains is caused by a change in the conformation of the protein instead of a change in the genetic material.

In Protein Misfolding Disorders the conformational change in a protein results in the formation of β-sheet rich oligomers with a high tendency to form amyloid aggregates. The process by which conformational change (misfolding) and aggregation occurs in PMDs follows a seeding-nucleation mechanism. This process is initiated by the slow interaction between protein monomers to form a stable seed (oligomer) around which a faster phase of fibril elongation occurs. In prion diseases the key element that makes PrPSc infectious is its ability to act as a seed to induce the conversion of PrPC into PrPSc. In PMDs the acceleration of protein aggregation by the addition of seeds has been convincingly reported *in vitro* for several proteins. Extrapolating the *in vitro* results to the *in vivo* situation suggests that the correct administration of a pre-aggregated, stable, misfolded seed should substantially accelerate the misfolding, aggregation and tissue accumulation of the proteins involved in PMDs. In this thesis work I wanted to study if the oligomers circulating in the blood of Alzheimer's models induce the misfolding of normal Aβ peptide. I observed a clear increase in the plaque burden of mice that receive blood coming from symptomatic AD-animals (Fig. 17E) This result strongly suggest that old transgenic mice have seeds in their blood that are capable to induce the misfolding of Aβ in a similar way than PrPSc induces the misfolding of PrPc. However, other properties are needed to classify this phenomenon as a Prion-like transmission.

When an amyloid is a Prion?

Yet the central question remains: are other PMDs transmissible beyond the experimental setting and the misfolded proteins bona-fide infectious agents? First I should analyze the properties of prion protein that make it an infectious agent and compared to misfolded Aβ. i) The infectious protein must self-propagate. I showed that the seeding-nucleation mechanism of misfolding and aggregation allows Aβ oligomers to replicate its folding characteristics at expenses of native monomeric Aβ (Fig. 7) ii) The infectious protein must survive the biological clearance mechanisms; the accumulation of misfolded aggregates depends on a delicate equilibrium between new aggregate formation and their elimination through normal biological processes such as degradation, metabolism and correction of protein misfolding (e.g chaperons). iii) The infectious protein must trigger a phenotypic change such as cell damage, organ dysfunction, or disease. In my experiment the blood of AD-symptomatic mice (that contain putative Aβ seeds) was able to accelerate a hallmark phenotype of AD meaning the appearance of senile plaques (Fig. 17). iv). The infectious protein must reach the site of replication and pathology. Since I injected AD-blood through the vein tail, the putative Aβ oligomers contained in the AD-blood must enter into the brain, thus crossing the blood brain barrier in quantities enough to trigger the misfolding and aggregation of native Aβ.

In addition, efficient infectious proteins must form aggregates stable enough to not spontaneously disassemble yet not so stable to permit frequent fragmentation to multiply seeds. These considerations will limit the ability of amyloids to behave like "Prions", preventing the transmission of some amyloids *in vivo*.

After the prion discovery, Gajdusek's group investigated the transmissibility of AD. Non-human primates were inoculated intracerebrally with brain tissue from 52 patients with

confirmed Alzheimer disease (AD) (Goudsmit et al., 1980). Seventeen cases on test for more than 50 months failed to produce similar changes, and 33 cases have not been incubating for a sufficient period of time to ascertain the presence of a transmissible agent. One point that the author did not consider in this study is the "species-barrier phenomena". In the prion-transmission the species-barrier mechanism implicates that the misfolding of the prion protein is impaired when the infectious occurs between 2 different species. Still, in some cases the species-barrier can be trespassed, like in the case of variant CJD affecting human, which comes from BSE infected cows (Bruce et al., 1997; Hill et al., 1997). In the second aim of this thesis I also have to overcome a species-barrier mechanism since we injected human Aβ on transgenic mice. Trying to minimize the species-barrier I preferred to utilize a human misfolded "seeds" (such as human brain homogenate or blood coming from human-APP expressing mice) over human Aβ "substrate" (human-APP transgenic mice). However, other factors in the host (mice) may participate in the generation of a species-barrier. For example cellular factors, have been implicate in as "helpers" in the conversion of normal Prion into infectious protein. It could be possible than in my blood-transfusion experiments, the Aβ oligomers with the human sequence needed an special factor to induced brain Aβ deposition, that is absent in the rodents. Indeed, one of the characteristics of the species-barrier phenomena is the increase of the incubation periods, the *in vivo* analogous of the lag phase, Therefore, it could be that in my experiment using the hAPPwt mice I observed the beginning of the disease, that's why few deposits were observed and not in all the mice. Indeed, I found plaques in the basal ganglia were it has been described that plaques and tangles first appear in human AD (Braak and Braak, 1991; Delacourte et al., 2002; Pearson et al., 1985). Unfortunately, I cannot probe the increase in the incubation time hyphothesis since hAPPwt

were very old at the time I observed the plaques, and probably a full Aβ accumulation was out of the life span of the mice.

The putative infectivity of sporadic Alzheimer's has not been proved, but the lack of epidemiological data supporting disease transmission is often used to rule out an infectious origin. Epidemiological tracking of an infectious origin can be complicated by variable or extended time between exposure to the infectious agent and the onset of clinical symptoms, especially when this interval can be decades, as is typical for human TSEs. Indeed, epidemiological studies of relatives and people in contact with Creutzfeldt-Jakob disease (CJD) patients consistently produce negative results. Thus, infectious disease transmission of PMDs cannot be ruled out by a lack of epidemiological support alone. In this thesis I proposed that prion-like transmission mechanisms could explain the origin of a number of idiopathic PMDs, such as some cases of sporadic Alzheimer's disease, but also the prion-like transmission of Aβ might contribute to the gradual spreading of the pathology from cell-to-cell in the brains of Alzheimer's patients (Brundin et al., 2010) or even allow the interaction with other amyloid proteins, as in the third aim of this thesis.

Aβ peptide interacts with other misfolded proteins through a heterologous seeding mechanism and AD influence the pathology of a second protein misfolding disease (specific aim 3).

The events of misfolding, protein–protein interaction and the cellular consequences of the accumulation of misfolded aggregates are similar in diverse diseases, including extensive tissue inflammation, cellular stress, and activation of the unfolded-protein response (Rutkowski and Kaufman, 2004).

The mechanistic and pathological similarities among these diseases imply that protein-misfolding processes occurring simultaneously may synergistically interact among each other leading to an acceleration of the disease. Indeed, I showed that seeds coming from one sick animal (AEF) accelerated the aggregation of a second amyloid (Aβ), at least *in vitro*. The implications of this experiment to an *in vivo* situation are restricted by the same parameters described before for prion-like proteins; resistance of the heterologous-seeds to the biological clearance, ability to reach the target site and propagation ability, but in the case of heterologous seeding I proposed that additional circumstances must be considered, such as:

i) A spatial (conformational) match between both misfolded proteins is needed, which will allow interaction of the protein and further misfolding and polymerization.

ii) In the case that heterologous seeds are incorporated into the aggregate forming a "hetero-fibril", the new aggregate must be toxic and if not, at least must seed the formation of toxic "homo-aggregates" to induce a disease.

iii) In the case of diseases caused by loss-of-function, the "hetero-fibril" does not need to be toxic, but must sequester the target protein to obstruct the protein function, thus inducing the disease.

iv) An interesting output of the heterologous-seeding phenomena is the formation of new diseases if the "hetero-aggregates" acquire new conformations with increased toxicity, ability to attack new target organs (e.g to cross the BBB and enter to the brain) or modifying the incubation periods of an existing condition.

v) In contrast, we can imagine a scenario in which "hetero-aggregates" impede the development of a disease by sequestering toxic "homo-aggregates" into non-toxic

"hetero-aggregates". Also "hetero-aggregates" may decrease the rate of misfolding and aggregation of a toxic aggregate by binding to the "polymer-growing-end" and preventing the further addition of monomers, stopping the fibril polymerization, thus slowing the onset of the disease.

The findings from this work suggest that AD and prion pathologies synergistically interact to accelerate the onset of both diseases. Prion clinical signs appeared much faster after infection of animals that were simultaneously developing Alzheimer's disease (Tg2576 mice model). Moreover, AD transgenic mice infected with prions had a significant higher load of amyloid plaques than non-infected mice. These results may be explained by a direct interaction between Aβ and prion misfolded proteins that results in the acceleration of protein misfolding and aggregation through a heterologous-seeding mechanism. This event leads to higher and faster accumulation of toxic misfolded aggregates. Our *in vitro* heterologous-seeding experiment supports this hypothesis, in which purified prion protein strikingly accelerates the aggregation of SF-Aβ, in a prion concentration dependent manner. In addition, further studies from our group showed that preformed Aβ aggregates can induce the formation of misfolded PrPSc-like protein. The direct interaction hypothesis is also supported by co-immuno-colocalization studies, which showed a direct interaction between Aβ and PrP in the brain of transgenic mice injected with prions.

Several studies have demonstrated the cross-seeding of misfolded aggregates both *in vitro* and *in vivo* (Johan et al., 1998; O'Nuallain et al., 2004). These data, added to the now well accepted idea that seeding is the general mechanism by which these proteins aggregate and the basis for prion infectivity, determine that cross-seeding between diverse misfolded proteins is a feasible mechanism.

However, I cannot completely rule out the contribution of other factors to the synergistic effect observed in the experiment. For instance, the cellular clearance mechanisms may be impaired by the first misfolded protein and then will be even weakened by the appearance of a second misfolded aggregate. This may cause saturation of the proteasome system, stress of the endoplasmic reticulum and activation of apoptotic pathways, leading to the faster and higher accumulation of misfolding proteins, cellular dysfunction and death. Other possible explanation is that neurons are already stressed and injured by exposure to one misfolded aggregated. Thus, the recovery of the cell to respond to a second misfolded protein can be seriously impaired. This will cause accelerated the cellular dysfunction and death. Other described modes of interaction between Aβ and prion may also contribute to explain in part my results. A previous report showed that PrP regulates APP cleavage by β-secretase cleavage, thus altering the levels of Aβ peptide (Parkin et al., 2007). It has also been proposed that PrPc acts as a receptor for Aβ oligomers, mediating Aβ-induced synaptic dysfunction but this interaction does not require the infectious PrPSc conformation (Lauren et al., 2009). However controversial results have been lately reported regarding this interaction (Balducci et al., 2010).

Finally, a synergic inflammation process may also contribute to the effects observed in our experiment. Indeed, we observed an augmented astrogliosis process in AD-transgenic mice that were injected with prions. However, we cannot differentiate if this phenomenon was the cause or a consequence of both diseases occurring simultaneously.

Regardless of which is the mechanism underlying the acceleration and exacerbation of AD and prion diseases in our experiments, our results suggest that one Protein misfolding disorder constitute a risk factor for the development of a second disease. Whether this conclusion can

be extrapolated to human diseases in humans will require additional epidemiological studies. Human prion disorders have very long incubation periods, which can span several decades (Collinge et al., 2006). Thus, asymptomatic prion infection may increase the risk for developing AD.

In conclusion, the findings from this thesis may have important implications in understanding the consequences of protein misfolding and aggregation, especially regarding the transmission and progression of protein misfolding diseases.

Implications and future projections of this work

This thesis is a preliminary proof-of-concept demonstration of a new strategy to detect misfolded Aβ oligomers as well as the capability to use it to detect Aβ oligomers in human biological fluids.

As a projection of this thesis we are currently testing a large number of AD-confirmed samples that will be compared with age-match controls. Luckily we have access to the complete clinical history of the patients and also access to urine and CSFs samples as well as fresh blood. In addition, it will be very interesting to dissect whether Aβ oligomers binds to lipoproteins in plasma, since I did not include in this assay the lipid-fraction of plasma. The physiological significance and implications of my findings is still under analysis. Whether Aβ oligomers found in the blood of AD patients comes from brain leakage is unknown and currently under investigation. If true, alternative AD treatments may be directed to remove circulating Aβ oligomers. Thus, periphery-aimed therapies won't have to deal with brain-barrier penetration and targeting as the current AD drugs.

Evidence has grown that within an individual organism, misfolded forms of proteins spread through cells and tissues, corrupting normal proteins and seeding protein aggregation as they go. In this thesis I showed that misfolded Aβ spreads their conformation inducing the accumulation of amyloid plaques through blood transfusion. If this is a common mechanism for the spread of pathogenic proteins in a given person, it would have profound implications for the study and treatment of neurodegenerative diseases.

8. CONCLUSION

In this thesis work I showed that Aβ oligomers circulating in biological fluids might act as seeds to accelerate the misfolding and aggregation of misfolding prone proteins.

I found that Aβ oligomers have a nucleating capability that can be used to indirectly detect their concentration as biological fluids. Moreover, I had shown that biological fluids from AD patients have seeding properties that accelerate the aggregation of monomeric Aβ *in vitro.*

I shown that blood from symptomatic animal models of Alzheimer's disease contain seeding factors, most probably Aβ oligomers, which accelerates Aβ deposition *in vivo.*

Finally, I showed that one misfolding disease can be a risk factor for the development of a second misfolding disease, as we shown when we infected prions in a mice model of Alzheimer's disease.

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