# Immunosignature of Alzheimer's Disease

by

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# ABSTRACT

The goal of this thesis is to test whether Alzheimer's disease (AD) is associated with distinctive humoral immune changes that can be detected in plasma and tracked across time. This is relevant because AD is the principal cause of dementia, and yet, no specific diagnostic tests are universally employed in clinical practice to predict, diagnose or monitor disease progression. In particular, I describe herein a proteomic platform developed at the Center for Innovations in Medicine (CIM) consisting of a slide with 10.000 random-sequence peptides printed on its surface, which is used as the solid phase of an immunoassay where antibodies of interest are allowed to react and subsequently detected with a labeled secondary antibody. The pattern of antibody binding to the microarray is unique for each individual animal or person. This thesis will evaluate the versatility of the microarray platform and how it can be used to detect and characterize the binding patterns of antibodies relevant to the pathophysiology of AD as well as the plasma samples of animal models of AD and elderly humans with or without dementia. My specific aims were to evaluate the emergence and stability of immunosignature in mice with cerebral amyloidosis, and characterize the immunosignature of humans with AD. Plasma samples from APPswe/PSEN1-dE9 transgenic mice were evaluated longitudinally from 2 to 15 months of age to compare the evolving immunosignature with non-transgenic control mice. Immunological variation across different time-points

was assessed, with particular emphasis on time of emergence of a characteristic pattern. In addition, plasma samples from AD patients and age-matched individuals without dementia were assayed on the peptide microarray and binding patterns were compared. It is hoped that these experiments will be the basis for a larger study of the diagnostic merits of the microarray-based immunoassay in dementia clinics.

To Dawn

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# Chapter 1

# INTRODUCTION

## Frequency of Alzheimer's Disease

Dementia literally means "losing the mind." It is a term used in clinical practice to describe the progressive loss of cognitive ability and related behavioral changes. AD is the principal cause of dementia and a frequent medical problem. World-wide, it is estimated that 24 million people are afflicted with AD [1]. In the United States, about 4.5 million people have diagnosis of AD, a figure that is projected to quadruple by the middle of the 21$^{st}$ century [2,3]. Although dementia can affect individuals of all ages, it is more frequent in the elderly, hence the term "senile" dementia. This term, however, has been abandoned; its clinical utility derived from the distinction between psychiatric diseases seen in young people, or "dementia praecox" (now called schizophrenia), and the organic psychosis seen in elderly individuals. AD affects 1/8 of people at age 65 and almost 1/3 of octogenarians [2,3]. The costs to American health care related to AD approach 100 billion dollars every year [4]. Disease progression is slow, inexorably advancing over the course of many years. It is believed that by the time of symptom onset a significant burden of neuropathology and irreversible neurological damage has already

occurred: these presymptomatic neuropathological changes are also thought to silently evolve over the course of over two decades [5]. Subtle cognitive problems are very common in elderly individuals without limitation of activities of daily living; in some instances, these symptoms may represent the prodromal stages of AD. Such cases of non-disabling memory and behavioral complaints without sufficient clinical criteria for the diagnosis of AD (referred to as mild cognitive impairment, or MCI) are at high risk of developing dementia, converting to frank AD at a rate of 15-17% per year [3,6-8]. The long prodromal stage of AD presents an opportunity to detect individuals who are pre-symptomatic or minimally impaired.

## Symptoms of Alzheimer's disease

AD can affect any part of the brain, although the most typical involvement is the hypoccampal formation, amygdala, nucleus basalis of Meynert, and entorhynal cortex [9]. This localization explains in part the initial symptomatology of the disease. The most typical presentation is an amnestic syndrome with subtle progression, reflecting —to a certain degree— a predominance of bilateral medial temporal lobe dysfunction [8,9]. Many patients and family members confuse these initial symptoms with the manifestation of normal aging. However, AD can sometimes have

a non-amnestic presentation, which may become a source of clinical confusion with other types of dementia. This presentation may feature language problems, particularly word-finding difficulties (called “anomia”), visuo-spatial deficits involving spatial cognition, (i.e., “agnosia”; this may involve impaired identification of things, places and even faces —which is called “prosopagnosia”) and difficulties reading or writing (called “alexia” and “agraphia”). Finally, AD can present with “executive dysfunction,” featuring difficulties making sound decisions (i.e., financially or personally) and solving problems. Subsequently, cognitive and behavioral changes, —which mainly reflect a more global frontal and temporal lobe dysfunction— may ensue, with apathy, change in interest in usual activities, poor judgment, derangement of introspection, and speech difficulties. Other common symptoms with advanced stages of AD are confusion, wandering, perceptual limitation (visual and auditory), depression, hallucinations and paranoid delusions, which greatly complicate the care and social life of AD patients and their families. Hence, AD eventually leads to a broad “organ failure,” with drastic intellectual deterioration, personality changes and severe limitation of activities of daily living, which render patients largely dependent on care-takers. During these stages, patients are also prone to accidents, infection (pneumonia and urinary tract) and may experience serious neurological side-effects from commonly-prescribed medications.

The global brain derangement caused by AD is illustrated by the case of a famous graphic artist, Carolus Horn [10]. Horn was active, preserving until the end of his life the discipline of daily painting. He used in his paintings recurrent themes that offer a glimpse into the sequential changes of visual perception and constructional praxis that he confronted when working on a familiar subject. Figure 1 shows six different depictions of a Horn‘s favorite theme, the old bridge in Venice. Strikingly, there is an initial change in the choice of colors, transitioning from dark hues to a more lively variety of yellows, as well as a progressive abandonment of detail and perspective that are hardly attributable to a change of style or taste. Horn’s last drawing displays the same artistic dexterity of a four year-old boy.

Figure 1: Gradual change in the quality of paintings by C. Horn.

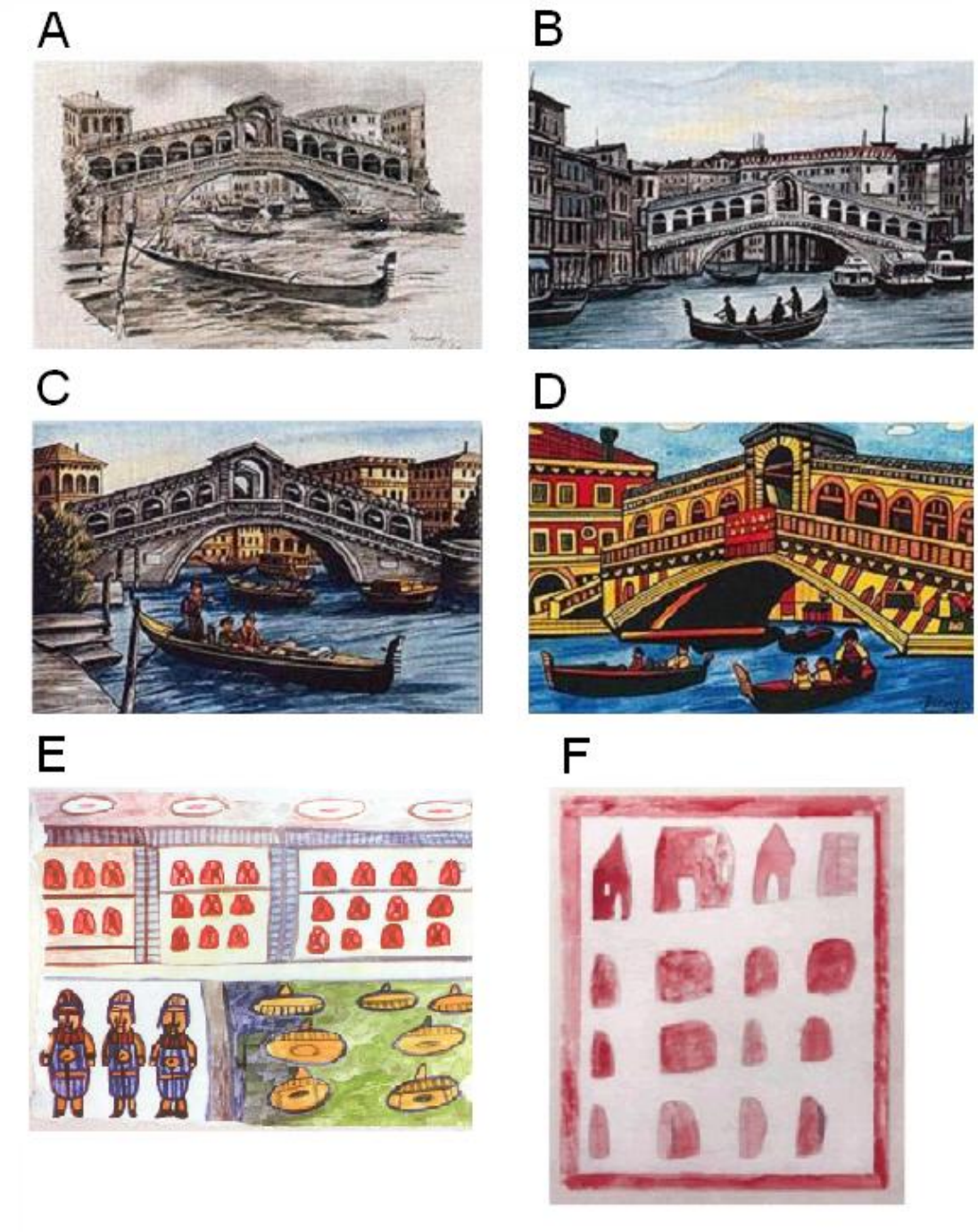


Fig. 1. Progression of Alzheimer's disease reflected on C. Horn's visual art.

Pathophysiology of Alzheimer's Disease.

AD is an age-related disorder characterized by the abundant deposition of the β-amyloid (Aβ) peptide in the brain parenchyma and cerebral vasculature. Aβ is generated from the cleavage of the amyloid precursor protein (APP) by the sequential processing of β-secretase cleaving enzyme 1 (BACE) and gamma-secretase. Studies of patients with trisomy 21 and families with pre-senile dementia suggest that Aβ plays a pivotal role in AD pathogenesis [11]. However, genetic mutations account for merely 1% of AD cases. The two most important risk factors associated with the disease are advanced age and the Є4 allele of apolipoprotein E (apo E) [5,8]. Apo E is a lipid transport protein produced in the nervous system predominantly by glia; humans possess a combination of two of the following alleles: 2, 3 and 4. However, the presence of Є4 allele by itself does not lead to AD, given that roughly one-third of AD patients lack the gene and some homozygotes may not develop AD. Put differently, the factors leading to AD are not understood in 99% of cases. Thus, the cause of sporadic AD is intricate and multi-factorial, with contributions from inherited and environmental factors.

Aβ deposition in the extracellular space of the brain is considered a fundamental feature of AD pathology. However, the production of Aβ is not

limited to the central nervous system, and the peptide circulates in plasma preferentially bound to several carrier proteins, including albumin, α-2 microglobulin, apo E, apolipoprotein J, Imunoglobulin G and fibrinogen. The predominant laboratory method used for Aβ detection in body fluids is an immunoassay, “double-sandwich” ELISA, in which plates are coated with anti-Aβ antibodies, then samples are added and finally, another antibody directed against Aβ is added; this can be detected with a secondary antibody labeled with horseradish peroxydase. This technology is not easy to interpret, as the immunological properties of Aβ change as it polymerizes.

Most studies suggest that $A\beta_{1-42}$ levels in the cerebro-spinal fluid (CSF) of AD patients detected (using double-sandwich ELISA techniques) are lower, on average, than those of control individuals [5]. This is attributed to the depletion of the monomeric form of Aβ as its polymerization occurs in the brain parenchyma. On the other hand, most reports show no differences in plasma Aβ levels between sporadic AD cases and non-demented controls, although total plasma Aβ and $A\beta_{1-42}$ levels are increased in cases of familial AD with presenilin or amyloid-precursor protein gene mutations, as well as in trisomy 21 [11]. However, it is presently unclear whether there is a predominant form of $A\beta_{1-40}$ and $A\beta_{1-42}$ that circulates in plasma (i.e., monomer, oligomer or fibrillary). It is

argued that the discrepancy across studies of Aβ plasma levels is due to the different populations tested, duration of studies, intensity of follow-ups, and variability of analytical tools used. In addition, differences in carrier protein expression appear to influence Aβ levels in plasma and their immunoreactivity, since the interaction between Aβ and transport proteins could potentially hide epitopes recognized by antibodies used in double-sandwich ELISAs [12]. Finally, Aβ oligomers have different immunological behavior compared to monomers and fibrils [13].

## Current challenges in the diagnosis of Alzheimer's Disease

No specific tests exist currently for the diagnosis of most types of dementia, including AD. The gold standard of AD diagnosis is its characteristic neuropathology described more than 100 years ago by Alois Alzheimer, which consists of senile plaques, neurofibrillary tangles and astrogliosis. This information is rarely available for treating physicians, who may corroborate or discard the diagnosis only through autopsy, when the information is hardly relevant. In the absence of a histopathology report, AD is a diagnosis of exclusion. Contrary to other medical conditions that are evaluated with specific tests (i.e., acute ischemic stroke is confirmed with diffusion-weighted magnetic resonance imaging, or MRI), the typical diagnostic work-up of dementia does not test directly for

AD. Instead, physicians test for other neurological diseases that can lead to dementia, including stroke, syphilis, hypothyroidism and vitamin $B_{12}$ deficiency, leaving AD as a *probability*. The recent revision of the 1984 criteria for AD diagnosis by the National Institute on Aging and the Alzheimer's Association still reflects tremendous diagnostic uncertainty, which is patent even at the semantic level [14]. For instance, the diagnostic categories include: "probable," "possible" and "probable AD with evidence of the AD pathophysiological process." This is in spite of the important advances in molecular and radiological diagnostics that we have discussed. Although AD is a reasonable assumption in suitable clinical scenarios, this judgment is prone to error. In fact, the diagnosis of "probable" AD during life is corroborated in 65-80% of cases submitted to autopsy under ideal conditions, i.e., when the diagnosis is made by a dementia specialist. In other words, under the best circumstances, 1 out of every 5 patients currently receives an incorrect diagnosis of AD.

Correct disease classification is imperative for many reasons: firstly, some dementias do not respond to the usual symptomatic treatment recommended for AD (i.e., cholinesterase inhibitors) or may even become worse with it. Secondly, the prognosis of several dementias is substantially different from AD; for instance, prion diseases have a rapid progression in a few weeks, whereas AD evolves over the course of years

or even decades. Lastly, clinical trials involving patients with AD can not be considered definitive considering that about 20-25% of enrolled subjects may not have the disease. On the other hand, correct prediction of AD in persons presenting with vague cognitive complaints (i.e., MCI) may present opportunities to slow the progression of neurological decline. Therefore, a diagnostic test that helps refine the classification of AD will have a very positive impact on patient care.

Many therapeutic strategies for AD have emerged recently, holding the promise of altering disease course. These include inhibitors of the amyloid precursor protein (APP) cleaving enzymes [16] or tau protein aggregation [7], as well passive immunization with specific anti-amyloid antibodies and pooled human gamma globulin [17], which will be discussed in more detail later on. As these approaches to treatment move forward to phase 3 clinical trials, the need for reliable tests to diagnose AD will only become more relevant. It is equally important that new diagnostic options are practical and inexpensive, particularly whenever pre-symptomatic diagnosis is concerned. In this regard, it is important to consider that positron-emission tomography (PET) is not universally available, MRI is expensive and has contraindications (such as the presence of implanted metal devices), while the measurement of proteins

in the cerebrospinal fluid requires a lumbar puncture, which is an invasive procedure.

There is a long list of potential biomarkers for AD; however, of many surveyed to date, none is used routinely in dementia clinics. Historically, AD biomarkers have derived from the amyloid cascade, cytokine signaling and neurotubule biology. These tests include: genetic testing on selected cases (i.e., Є4 allele), measurement of $A\beta_{1-42}$, total tau, and hyperphosphorylated tau (181p) in cerebrospinal fluid; assessment of cerebral glucose metabolism with FDG-PET; imaging cerebral Aβ deposition using PET with Pittsburgh-B compound (PIB, which binds to amyloid); estimating hippocampal volume using MRI; and standard memory performance tests [7,18]. The present section will briefly describe some of them.

Currently, PIB is the only amyloid imaging test. It has the drawback of a very short half-life (only 20 minutes), requiring that the compound be made on site with a cyclotron. This is hardly practical, as this is available in only 20 centers nation-wide. A new radiotracer with a longer half-life was recently developed by Eli Lily, $^{18}F$ florbetapir (Amyvid), and may become available for clinical use in 2012 [18]. The problems associated with PET are the exposure to radioactive tracers, which make the

technology unsuitable for frequent follow-up imaging; in addition, the anatomical definition of images is far from that obtained with MRI. It is also clear that many people with abnormal PET (PIB and FDG) do not have AD or may even lack symptoms of dementia, suggesting that PET techniques lack specificity for AD. Decreased total apo E plasma levels and low apo E4 have been detected in AD patients, particularly in individuals with the Є4 allele. These low plasma levels were inversely correlated with cerebral load of Aβ estimated by PIB [19].

Perhaps the most promising biomarker to date is the CSF signature of $A\beta_{1-42}$, total tau, and phosphotau (phosphorylated at threonine 181). The concentration of these biomarkers is done simultaneously with a multiplex immunoassay using the xMAX Luminex platform with 3 specific capture monoclonal antibodies. Using CSF from 56 autopsy-confirmed AD patients as gold standard, the measurement of $A\beta_{1-42}$, levels in CSF provided a diagnostic sensitivity of 96.4% on a cohort of 100 AD patients and 114 age-matched controls [5]. A very promising aspect of this set of biomarkers is that a profile consistent with AD (low $A\beta_{1-42}$, high tau) can be used to predict conversion of MCI to frank AD with a high degree of accuracy.

## Current Standard of Therapy

Several pharmaceuticals are approved by the FDA for AD treatment, although none of these can modify the course of the disease, and are mainly used to improve cognitive symptoms and functional scores [19,20]. No medications are routinely used to decrease neuroinflammation or decrease the bulk of cerebral amyloidosis. The principal drugs currently used in dementia patients are the acetyl-cholinesterase inhibitors, which increase the concentration of acetylcholine in the postsynaptic cleft in the central nervous system. Donepezil, rivastigmine, tacrine and galantamine are all acetyl-cholinesterase inhibitors [19,20]. Of these, donepezil is the most used agent, in part because is well tolerated and can be used once per day. N-methyl-D-aspartate (NMDA) antagonists are also frequently used in dementia. This type of drug is exemplified by memantine, which reduces glutamate-mediated neuro-toxicity. Many patients with AD require other psycho-tropic medications to modify behavior and improve affect. Many medications need be used in combination, in order to attain a desired effect. A common combination that has been proven beneficial in clinical trials is the addition of memantine to donepezil. Unfortunately, these medications do not alter neurological progression. Finally, careful modification of cardio-vascular risk factors and keeping overall a good state of health may also be relevant to slow disease progression. Several new drugs are being tested for safety and efficacy. To date, emphasis has

been on strategies to manipulate Aβ production, but other mechanisms targeted in phase III trials include: inhibition of Aβ aggregation, antioxidants, γ-Secretase modulation (including 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors, or statins), nerve growth factor mimics, and peroxisome-proliferator activated receptor γ (PPARγ) agonists [19].

## Inflammatory Changes in Alzheimer's Disease

Although AD is considered primarily a neurodegenerative disease, a constant finding in affected individuals is inflammation, which is demonstrable in the central nervous system as well as plasma [21-23]. It is well known that antigen-presenting cells such as astrocytes and microglia are recruited to areas of senile plaque deposition and various cytokines are upregulated in brain and plasma of AD cases [23]. The immune system's participation in AD pathophysiology has different facets that can be deemed either favorable or detrimental [21]. For instance, the phagocytic clearance of highly insoluble proteins from the extracellular space, as well as removal of cellular debris, can be construed as beneficial aspects of neuro-inflammation. On the other hand, cytokines released by activated microglia and complement activation can promote cytotoxicity and accelerate neuronal degeneration [21]. The importance of systemic inflammation in AD is such that a characteristic cytokine

expression pattern in plasma has been used as a potential diagnostic tool for this kind of dementia. Indeed, a study [22] showed that a 120-protein double-sandwich ELISA microarray of plasma cytokines can be used to classify blinded samples from patients with clinical diagnosis of AD, vascular dementia, and non-demented controls with almost 90% accuracy. Furthermore, 18 over-expressed cytokines identified MCI cases that converted to AD.

The premise of a beneficial neuroinflammatory response has promoted research aiming at harnessing the immune system for the sake of clearing cerebral amyloid deposits [17-19]. The aim of this strategy is to modify the natural course of AD. Several studies using transgenic mice bearing human mutations leading to AD show that active immunization with Aβ can indeed clear plaques from the brain of treated animals [17-19]. This information seemed compelling enough to justify a human trial in which AD patients were vaccinated with synthetic $A\beta_{1-42}$. [17]. This trial had to be stopped prematurely during its phase 2 because of a 6% incidence of T-cell mediated encephalitis in the active treatment arm. However, vaccinated individuals who developed measurable anti-Aβ immunoreactivity in plasma experienced clinical improvement. The post-mortem examination of a few vaccinated patients showed inconspicuous senile plaques within the brain parenchyma despite otherwise typical

findings of AD, including prominent amyloid angiopathy [17]. Alternative immunotherapeutic strategies have emerged, including passive immunization with amyloid-binding antibodies, which can also clear plaques in transgenic mice, as we will discuss later.

## Evidence of humoral auto-immunity in Alzheimer's Disease

Both plasma and cerebrospinal fluid contain naturally-occurring anti-Aβ antibodies in normal and pathological conditions [24-26]. However, many other antibodies targeting self-antigens are encountered in neurodegenerative diseases [27,28]. It is possible that the neurodegenerative process of AD offers a growing assortment of epitopes to the immune system, which may predate the symptomatic stage. The potential exposure of brain antigens to immune surveillance is facilitated by the progressive derangement of the blood-brain barrier that accompanies AD. Virus-transformed B cells from demented individuals have been shown to produce anti-Aβ antibodies [29], while sera from normal individuals contain antibodies that bind fibrils of amyloidogenic proteins, such as Aβ1-40, serum amyloid A, islet amyloid polypeptide, and transthyretin [30]. The latter antibodies do not cross-react with their respective monomers and inhibit fibrillogenesis in vitro. This may represent a physiological anti-amyloidogenic function (or misfolded protein

fail-safe mechanism) of immunoglobulins. In fact, anti-Aβ antibodies purified from immunoglobulin preparations can prevent amyloid fibril formation and thwart Aβ neurotoxicity [26]. This, however, is in contrast with a report showing that serum from APP transgenic mice augmented Aβ toxic effects on cultured hyppocampal neurons [31]. Pharmacological immunoglobulin preparations (IVIG) used for common neurological diseases can bind Aβ in vitro and decrease total Aβ and Aβ1-42 levels in the cerebrospinal fluid [24]. Studies using ELISA platforms with synthetic Aβ monomers have revealed that AD patients have anti-Aβ antibody titers that may be elevated [31,32], low [25], or similar [23] to those detected in controls without dementia. Another study using ELISA plates coated with oligomeric cross-linked β-amyloid protein species (CAPS) showed that anti-CAPS antibodies were reduced in AD patients compared to non-demented controls, suggesting that these antibodies may alter the susceptibility to developing AD [33]. It is unknown why normal individuals have anti-Aβ antibodies, although exposure to environmental mimotopes of Aβ, such as the potato virus Y has been proposed [34]. On the other hand, a recent report revealed that both AD patients and healthy elderly individuals possess circulating antibodies that react against tau protein (unphosphorylated and hyperphosphorylated) [35]. Some of these antibodies were of the IgM class, indicating an acute immune process.

As we mentioned, many auto-antibodies commonly detected in auto-immune diseases are frequently found both in patients with AD and seemingly normal elderly individuals. For instance, the anti-nuclear antibodies are found in about 30% of normal elderly individuals at low titers (i.e., about 1:80) [27]. Many patients with AD test positive for anti-nuclear, anti-parietal cell and anti-thyroid microsomal antibodies [27,28]. It is presently unclear whether the titer of circulating antibodies against Aβ, tau and other relevant antigens changes overtime and whether these changes, if any, may correlate with different clinical stages of the disease (for instance, the transition from normal cognition to MCI and finally, to frank dementia). Results of experiments carried out to evaluate the presence of anti-Aβ antibodies in human plasma samples are presented in Appendix 2.

## Immunotherapy for Cerebral Amyloidosis

Immunotherapy for cerebral amyloidosis can be divided into active and passive. The earliest report of active immunotherapy in mouse models of AD was by Schenk and colleagues [36], who vaccinated a group of transgenic mice with aggregated Aβ. The mice used in these experiments had a mutation in the APP gene leading to a phenotype that featured progressive cerebral amyloidosis. Aβ1−42 vaccination both

before and after the expected onset of cerebral amyloidosis resulted in extensive clearing of plaque pathology. Other investigations showed similar results, with pathological and neurological improvement of treated animals [37,38].

These results served as the basis for a phase 1 study in patients with AD [39]. The study enrolled 80 elderly individuals who were randomly assigned to either aggregated Aβ1−42 (called AN1792) or placebo delivered intramuscularly 4 times over 6 months. A phase 2 trial followed, which had to be stopped prematurely because of 18 / 298 instances of subacute meningoencephalitis (6% of patients), [40] a predominantly T-cell inflammatory disease that did not correlate with anti-Aβ antibody titers. Mirroring effects on vaccinated TG mice, antibodies developed by AD patients preferentially targeted Aβ's amino-terminus, binding monomers and fibrils alike [41]. However, significant antibody titers were detected in about 20% of patients, underscoring the technical difficulties of the employed detection system (ELISA) and overall lack of suitable biomarker availability in AD clinical trials. Although clinicopathological reports [42] of vaccinated subjects suggest some clearing or redistribution of senile plaques, this did not seem to translate into measurable cognitive improvement.

Passive vaccination is another strategy aimed at counteracting cerebral amyloidosis. Injection of monoclonal antibodies produce marked neurological improvement in mice, even if plaque pathology or brain Aβ levels were not significantly affected [43-45]. This apparent “dissociation” between effects on behavior and effects on plaques is not explained entirely by neutralization of Aβ in the brain, although it is possible that certain forms of Aβ deemed more toxic, such as oligomers, have not been always reported, or measured with techniques with debatable merit. Brody and Holzman, [46] on the other hand, aptly point out that “a fundamental issue that complicates interpretation of all these results is that we do not know whether behavioral abnormalities seen in TG mice are analogous to any of the cognitive deficits seen in humans with AD.” A potential problem with passive immunization is intra-parenchymal brain hemorrhages associated with amyloid angiopathy. It is possible that mobilization of Aβ out of the brain elicited by therapy may exacerbate amyloid deposition in arterioles, a finding that is common in mouse models and almost universal in AD patients. Notwithstanding these caveats, a single dose of a humanized monoclonal antibody (LY2062430, or solanezumab) as potential AD treatment did not result in significant side-effects in 19 patients [47]. Treatment was not associated with meningo-encephalitis, cerebral hemorrhage, or brain edema. Therapy led to dose-dependent increases in Aβ levels in both plasma and CSF. Solanezumab is undergoing two separate phase 3 trials: a placebo-controlled trial in Japan

(EXPEDITION, ongoing but no longer recruiting patients), and an open-label trial which is currently enrolling patients [48]. Table 1 summarizes current clinical trials of antibody-based therapies for AD.

Table 1

Antibody-based therapies for AD in on-going Clinical Trials

| Antibody | Company | Biomarker | Stage |
|---|---|---|---|
| Bapineuzumab | Pfizer | 11C-PIB | Phase 3 |
| Solanezumab | Eli Lilly/Elan | Plasma Aβ level | Phase 3 |
| Gantenerumab | MorphoSys/Roche | PIB | Phase 2 |
| Human IVIG | Baxter | None | Phase 3 |

## Animal models of Alzheimer's disease

Central to the understanding of any human disease is the creation of an animal model. Many animal models of AD have been developed to date, with variable degrees of success at replicating the histopathology and neurological impairment observed in humans. Needless to say, these

animal models are considered an approximation to the human problem. Careless extrapolation from animal models to the highly complex and often times messy circumstances of the average human patient, are recipes for confusion and disappointment. Indeed, laboratory animals are always raised and treated under controlled circumstances, offering a “pure” and replicable phenotype, while human’s phenotypes are the product of many synergistic processes, some of which are entirely fortuitous or unknown —in short, patients are impure models.

An important step in the development of animal models of AD was the application of recombinant technology to the creation of transgenic animals with genes from humans with inherited forms of early-onset AD. The first used gene was a mutant form of the APP [49,50]. However, these early transgenic animals failed to express a meaningful AD-like neuropathology. Subsequently, Games and colleagues [51] were able to express elevated levels of the V717F mutant form of APP using a platelet-derived growth factor (PDGF) mini-promoter. These mice, known as PDAPP transgenics, recapitulate many pathological features of AD, including broad Aβ extracellular accumulation (spreading from the hippocampus and increasing with age), astrocytosis and neuritic dystrophy. The PDAPP mice exhibit cognitive problems, although its correlation with observed neuropathology is unclear.

The second set of mutant genes used to create transgenic mice is the Presenilin-1 and -2. Deletion of Presenilin-1 in mice proved to be lethal immediately after birth, leading to a severe phenotype featuring gross skeletal deformities, impaired neurogenesis and intraventricular hemorrhage, all in part attributable to the important role of Presenilin-1 in embryo's Notch processing [52]. On the other hand, Presenilin-2 knockout mice are viable, although they develop pulmonary fibrosis [53].

To increase production and, more importantly, cerebral deposition of Aβ, some investigators pursued the idea of crossbreeding APP and Presenilin-1 mutant mice. Indeed, transgenic animals co-expressing a mutant Presenilin-1 gene (called A264E) together with a mutant APP gene from a Swedish family (APP Swe) had higher Aβ levels in brain and heavier plaque formation than mice carrying only one of the individual mutations [54]. A similar phenotype was obtained with mice carrying the Presenilin-1 M146L mutation and the APP Tg2576 mutation [55]. Although plaque formation begins at 6 months of age in these mice, cognitive problems are observed as early as 3 months of age.

Finally, transgenic mice have been engineered with 3 mutations involving Presenilin-1 (M146I gene), APP (SW) and tau (P301L),

controlled by the mThy1.2 promoter [56]. This triple transgenic mice model leads to overproduction of tau compared to mice with single tau mutations, developing amyloid-laden plaques at 6 months of age and subsequent neurofibrillary tangle formation scattered through the hippocampal and cortical regions, resembling human AD pathology. Interestingly, ApoE null mice do not have plaque deposition or a discernible neurological phenotype; these mice, however, develop severe atherosclerosis, particularly affecting the aorta [57]. Nevertheless, mutant APP expression on apoE knockout mice significantly reduced (but did not abolish) cerebral plaque formation [58].

Many animal species other than mice have been used for the study of AD. These include non-human primates, the fruit fly Drosophila, the sea lamprey, and the nematode Caenorhabditis elegans. Each of these animal models possesses a set of advantages, although ultimately, all share the same disadvantage: fundamental differences in anatomy, physiology and cognition compared to humans with dementia. For further details about animal models of neurodegenerative diseases, the reader is referred to the two extensive reviews by Götz and colleagues [49,50] As we will see in Chapter 1, we used TG mice bearing two mutations from patients with familial AD (APPswe/PSEN1-1dE9) to track age-related changes in their humoral immune repertoire.

## Proteomics and Protein Microarrays

Proteins, to use Virginia Espina's expression, are "the verbs of the cell" [59]. Proteomics, then, is the analysis of cellular grammar: the make-up of a biological system and the changes that occur not only in response to physiological and pathological conditions, but also as result of different manipulations (i.e., pharmacological, physical, etc.). It is expected that the knowledge derived from such analyses may lead to diagnostic tests, and that important insights into specific molecular mechanisms of disease may lead to novel therapies. The decoding of the genome of numerous species, including humans, has been the stepping stone of proteome mining, because the knowledge of species-specific gene sequences allows the projection of the amino acid constitution of peptidic chains [59-61]. Such wealth of information permits the identification of important components of cells, tissues, and body fluids. It is also hoped that proteomics may help select individuals who are likely to benefit from therapies or monitor response to therapy or disease course [60-61].

Traditional proteomic procedures rely on protein separation to facilitate analysis. These procedures include SDS-PAGE and two-dimensional gels, which can subsequently be analyzed with mass spectrometry. Needless to say, many limitations have become apparent

with these approaches; the main problem being the small amount of relevant protein available in a biological sample. It is clear that gene transcription and protein expression are not well correlated [60-61]. In addition, genomic arrays cannot convey information about post-translational protein modifications or protein–protein interactions. Many times, highly expressed proteins cloud the relative importance of other proteins that are present in samples at much smaller concentrations [60-61]. In addition, conventional analytical methods may be associated with considerable cost and time consumption. These limitations provoked the emergence of many new proteomic platforms, including protein microarrays.

Microarrays permit the simultaneous analysis of several molecules within the same experiment. Such molecules are spotted in parallel rows and columns onto a solid support, and then allowed to react with samples containing other binding molecules. The location and intensity of binding within each spot requires of sensitive detection systems, which are generally based on mass spectrometry, radioactivity, electrochemistry, chemiluminescence or fluorescence [62]. Since the location and composition of each spot is known beforehand, signals indicative of binding can be attributed to the interaction of a specific molecule in the array, an identification which can be facilitated by software.

Types of microarrays

The first microarrays employed parallel synthesis of nucleotide chains on cellulose discs contained in columns or plastic pins. An important milestone was the subsequent development of the SPOT method, in which peptides are synthesized in parallel on a solid platform such as cellulose panes using droplets on a porous membrane's surface, with an attached reactor for chemical synthesis. This became a popular method because the relative simplicity of microarray manufacture and detection. Nucleotide arrays enjoyed enthusiastic attention thereafter and experienced vigorous development during the eighties and nineties, being used for genotyping (of species, individuals, point mutations, single point mutations, and short tandem repeats) and gene expression studies. However, the development of protein arrays lagged for almost 2 decades, in part because of the greater chemical complexity of polypeptide chains, and also because of their greater structural frailty.

There are two broad approaches to protein microarray production: (a) the so-called "abundance"-based array, in which capture molecules (i.e., antibodies) are spotted on a solid phase; and (b) "function"-based arrays, in which proteins are generated from cell-free expression systems and printed on a slide's surface [63]. Several detection systems are

employed to detect the binding of relevant molecules to the arrays. For instance, the features printed on the array can be labeled directly with fluorescent molecules. Alternatively, “sandwich” immunoassays can be employed, in which analytes are captured by immobilized antibodies, which in turn are detected with a labeled secondary antibody [22]. Monoclonal, polyclonal and recombinant antibodies can be printed on a slide’s surface and used to detect proteins from any source. Evidently, antibodies will need to adhere preferentially to the microarray’s solid phase by the Fc portion, in order to have the hyper-variable regions available for epitope capture. Different antibodies targeting different proteins can be printed on pre-arranged spots with pre-set concentrations, to allow the correct identification and quantification of binding. This technology has been used to characterize the “signature” of neoplastic, autoimmune and infectious diseases [63].

In other microarrays, proteins are attached to the slide’s surface through a chemical linkage, i.e., a covalent bond, which is the predominant type of microarray used at the CIM. Reverse phase protein blot is another production strategy, in which a sample containing many molecules is printed on a slide’s surface and subsequently probed with a particular detection reagent. Some techniques entail the design of polypeptides featuring fusion tags (i.e., His or GST tags), which

respectively adhere to Nickel-coated slides or anti-GST antibodies. The protein used on these arrays can be produced using cell-free expression systems. Finally, “self-assembling” protein microarrays have been developed, in which proteins are produced in-situ [64]. This bypasses the tedious protein purification steps usually required for most arrays, and more importantly, helps prevent the decay of proteins prior to analysis (i.e., during storage or handling), which is almost universally expected in most endeavors involving proteomics. Self-assembling arrays are produced by printing complementary DNA onto glass slides, which is subsequently translated using eukaryotic retyculocyte lysates in situ. Newly formed proteins are immobilized in the slides thanks to tags that are captured by pre-spotted antibodies on the slides, and subsequently detected with another antibody.

Different from other methods of microarray development, the particle-based peptide array synthesis approach (or “PepperPrint”) uses chargeable aminoacids directed sequentially on a microchip surface using electric field patterns from separate pixel electrodes [65]. This type of microarray considerably increases the array density and may be used for high-throughput proteomic studies, such as humoral responses against a pathogen’s proteome.

Profiling of humoral responses

The traditional approach to antibody detection entails the immobilization of a single antigen and subsequent probing with plasma or serum, followed by a secondary antibody. This can be accomplished using many techniques that are widely used, such as Western blot and enzyme-linked immunosorbent assay (ELISA). However, there are increasingly circumstances in which it becomes desirable to test the presence of different antibodies, particularly when it is unclear what is the cause of an individual's illness. In response to these needs, many antigens can be printed on the surface of a slide, providing a multiplex platform for high throughput analysis of complex biological samples, such as plasma [61].

Microarrays have been used for epitope mapping of auto-antibodies and allergen detection (called "antibodyome" by Andresen and Grötzinger) [66]. Protein microarrays have been used for the discovery of novel cancer antigens [67]. Some of these platforms detect autoantibodies at the presymptomatic stage. For instance, a study using serum samples from persons enrolled in the beta-Carotene and Retinol Efficacy Trial (CARET) showed that some antigens targeted by autoantibodies in patients with lung cancer (annexin I, PGP9.5, 14-3-3 theta and LAMR1) were bound by sera from presymptomatic donors [68].

## Future directions of protein microarrays

Since the human genome is composed by about 25.000 genes, an extensive human proteomic microarray should at the very least be able to accommodate as many individual proteins as possible on high-density arrays. Obviously, this is a daunting task considering the desirable dimensions of a practical platform. Some have proposed adopting nanotechnology-based solutions, in other words, switching from microarrays to nanoarrays. Promising designs include: planar, attovial-based and nanowire array designs. Fortunately, there are existing technologies for printing nanosized array features, including nanodispensing, nanoimprint litography and dip-pen nanolithography. On the other hand, there is interest in the development of label-free microarray systems, because the use of labels can alter protein-to-protein interactions and change protein structure and function. Some of these label-free systems include: single plasmon resonance, nanohole array, elipsometry, carbon nanotubes and nanowires, and interferometry [63].

## The CIM Random-Peptide Microarray Platform

The CIM microarrays consist of maleimide slides with 10,000 random-sequence 20-mers printed on their surface. Two prototypes,

CIM1.0 and CIM2.0 (each with a different set of 10,000 peptides), were tested in the experiments that I will describe in detail in subsequent chapters. The peptides become covalently-attached to the slides through the interaction of the amine-terminus of a Cysteine and the maleimide surface. Succinimidyl-4-(N maleimidomethyl) cyclohexane-1-carboxylate (SMCC), is used as amine-to-sulfhydryl cross linker. Peptide sequences and location in the microarray are known beforehand. The peptides were designed using a software that randomly picks 19 natural aminoacids (except Cysteine) to build stochastic sequences consisting of 17 residues. All peptides have Glycine-Serine-Cysteine linkers at either the carboxyl- (CIM1.0) or amino- (CIM2.0) terminus, to space the main aminoacid sequence from the slide.

# Chapter 2

# SIGNATURE OF AFFINITY-PURIFIED ANTIBODIES AND MURINE PLASMA

## Introduction

Why use an antibody assay for the assessment of a neurodegenerative disease? In the introduction of this thesis I discussed that currently, physicians have no accurate means to establish the diagnosis of AD, except when an autopsy is carried out [1-5]. This necessarily means that doctors base their diagnosis on the exclusion of other neurological disorders, rather than testing directly for AD, which misdiagnoses about 20% of patients [1-5]. Although new diagnostic techniques are promising, such as the profiling of Aβ and tau in CSF, they are not infallible and require a spinal tap, which is not a particularly pleasurable experience. On the other hand, amyloid imaging techniques such as PIB-PET are not universally available and may be abnormal in patients without dementia [6]. Hence, substantial interest exists in the development of alternative techniques that may help diagnosing AD. We also discussed earlier that the diagnostic merits of auto-antibodies are the focus of interest of recent research, because of the simplicity and wide availability of the involved analytical techniques and relatively stability of

target analytes, antibodies [7-14]. Clearly, immuno-globulins are present in senile plaques, and many individuals have circulating auto-antibodies targeting different molecules that are relevant in the pathophysiology of AD, including Aβ and tau [7-14]. It is also possible that the progressive destruction of the cerebral cortex caused by AD unveils novel epitopes to the immune system, and more importantly, this might actually predate the symptomatic stage of AD by many years [15-21]. The de novo exposure of brain antigens to immune surveillance is facilitated by the progressive failure of the blood-brain barrier that accompanies neurodegenerative processes, including AD [15]. Therefore, a test capable of assessing such humoral response may be harnessed as a diagnostic platform.

In this section of the doctoral thesis, I will describe an immunoassay that can be used to evaluate the signature of antibodies, called “Immunosignature,” which employs the random-peptide microarray described in the previous chapter. It will be my purpose to describe how the immunoassay works and show experiments with affinity-purified antibodies as well as plasma from mice.

Methods

Description of the microarray-based immunoassay: After the production of and storage of microarray slides, they are pre-washed for 5 minutes with a solution containing 33% isopropanol, 7.5% acetonitrile and 0.5% trifluroacetic acid in distilled water, then inserted in a TECAN HS4800-Pro automated incubator (Männedorf, Switzerland). This machine allows the programming of experiments with standardized incubation times and washes (TBST followed by water), as well as controlling temperatures. The first step of the process is blocking with 0.015% mercaptohexanol / 3% BSA / 0.05% Tween 20 in PBS (pH 7.4) for 1 hour at 20˚C, to decrease non-specific binding. Subsequently, the primary antibody is incubated (typical concentration varies was 10-50 nM or plasma at 1:500 dilution in 3% BSA / 0.05% Tween 20 in PBS) for 1 hour at 37˚C. Next, biotinylated, species-specific antibodies (targeted against rabbit, mouse, goat and human IgG, purchased from Bethyl, Montgomery, TX) are incubated on the slides at 5 µM, also for 1 hour at 37˚C, followed by Streptavidin conjugated to Alexa 647 or 555 (Invitrogen, Carlsbad, CA; concentration was also 5 µM). The TECAN dries out the slides after approximately 15 minutes and rings an alarm to indicate that the program is complete.

Slides are then scanned with a Surescan high-definition laser scanner (Agilent Technologies, Santa Clara, CA) to generate digital images (TIFF files) which are subsequently processed with GenePix Pro 6.0 (Axon Instruments, Union City, CA). This is a rather tedious process that requires the alignment of frames that convey the exact localization and identification of each peptide in the microarray. Saved files can then be used for data analysis.

Microarray analysis: Scanned data was loaded into GeneSpring 7.2.1 (Agilent Technologies, Santa Clara, CA) and analyzed. Signals were deemed present when intensities were >1 standard deviation from mean local background. Peptide identification was done using t-tests, Model I (fixed effects) 1-way or multi-way ANOVA, and correlation to specific expression patterns. Clustering techniques, including k-means, hierarchical clustering, and Self-organizing Maps were used for identifying antibody binding patterns. We screened for technically irreproducible values during data pre-processing. Each peptide array replicate provides a 1.5-fold minimum average detectable fold change at $\alpha=0.05$ and $\beta=0.20$. Appropriate false-positive corrections were used.

## Results

Binding patterns of affinity-purified antibodies against Aβ and tau: First, I endeavored to determine whether specific antibodies targeting peptides relevant to AD pathophysiology showed distinctive microarray binding patterns. I analyzed the signature of 11 monoclonal or affinity-purified antibodies: 7 against Aβ (4 monoclonal, 3 polyclonal) and 3 against tau (2 monoclonal, 1 polyclonal, summarized in Table 2). An anti-human albumin polyclonal antibody raised in goat (A 7544, Sigma) was used as control.

Each antibody bound different microarray peptides above median signal threshold (3-sigma). Binding intensity and order in which reactive peptides are ranked yielded specific information regarding each antibody. Peptides bound by each antibody were distinct and formed a distinctive pattern (Figure 2). The microarray segregated the signature of every individual antibody from the secondary biotylinated antibody by itself (anti-rabbit or anti-mouse) and from other monoclonal and polyclonal antibodies (Figure 3). The signature of the secondary antibody can be subtracted from the primary to enhance the specificity of patterns.

Table 2

Antibodies analyzed with the microarray platform

| Antibody | Antigen | Epitope | Type | Company |
|---|---|---|---|---|
| 4G8 | Aβ | residues 17-24 | M | Millipore |
| DE2 | Aβ | residues 1-16 | M | Millipore |
| 2B9 | Aβ | residues 1-17 | M | Santa Cruz |
| BAM-10 | Aβ | residues 1-12 | M | Sigma |
| α-tau 421 | tau | Asp residue 421 | M | Millipore |
| α-tau 210 | tau | residues 210-241 | M | Millipore |
| α-Aβ 1-40 | Aβ | carboxyl-terminus | P | Calbiochem |
| α-Aβ 1-42 | Aβ | carboxyl-terminus | P | Sigma |
| α-Aβ oligo | Aβ | Aβ octamers | P | Biosource |
| α-phos tau | tau | 210-threonine 231 | P | Millipore |

Abbreviations: M= monoclonal; P=polyclonal (all raised in rabbit).

Figure 2 shows a scanned microarray after completing the assay of 3 rabbit polyclonal antibodies against Aβ. The white boxes represent equivalent areas within the array, which are expanded above for greater detail. Spots represent individual peptides organized in the array; white,

red and black colors indicate strong, medium and low antibody binding, respectively.

Figure 2: Appearance of microarray after immunoassay

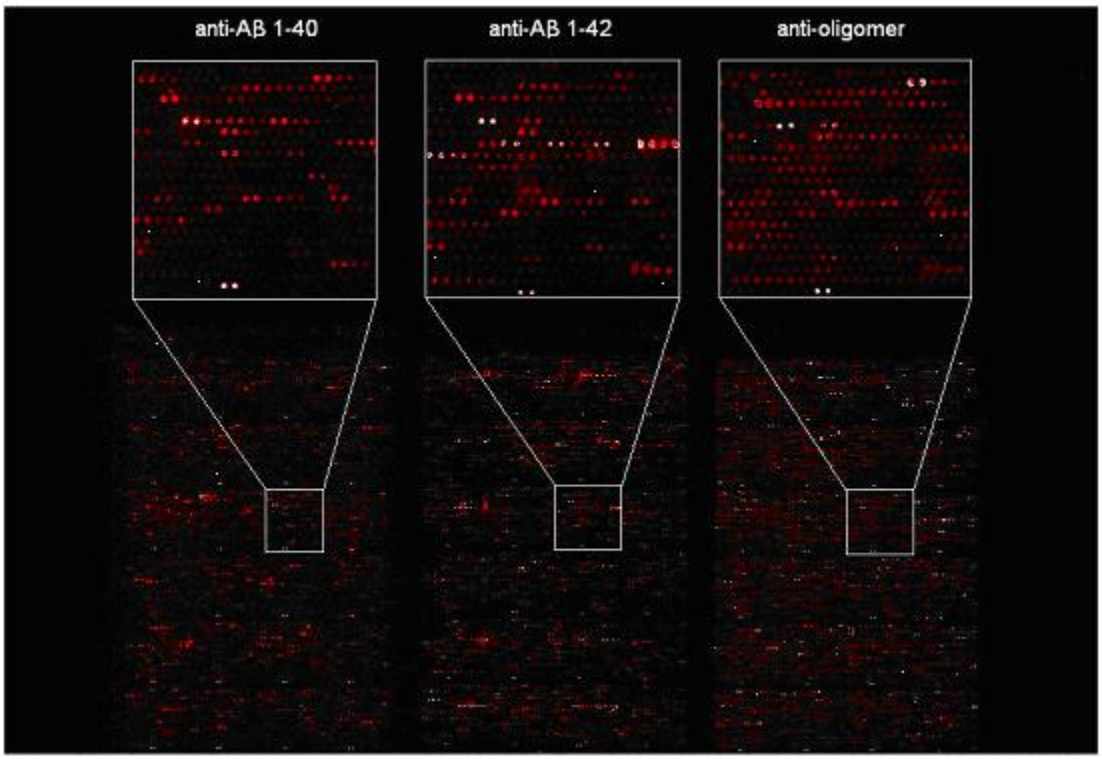


Fig. 2. Microarray signatures of anti-Aβ antibodies. Scanned image of peptide microarray hybridization of 3 rabbit polyclonal antibodies against Aβ. The white boxes represent equivalent areas within the array, which are expanded above for greater detail. Spots represent individual peptides organized in the array; white, red and black colors indicate strong, medium and low antibody binding, respectively.

Figure 3 (next page) shows a heatmap demonstrating high correlation between antibodies targeting the carboxyl-terminus of Aβ, amyloid oligomer and phosphotau. This particular heatmap features 93

peptides deemed informative by ANOVA. Polyclonal antibodies targeting the carboxyl-terminus of Aβ shared binding pattern similarities with an antibody that recognizes Aβ oligomers and an antibody raised against phosphorylated tau. Other antibodies, mainly monoclonal IgG targeting the amino-terminus of Aβ, shared no binding similarities.

Figure 3: Heatmap of different anti-Aβ and anti-tau antibodies.

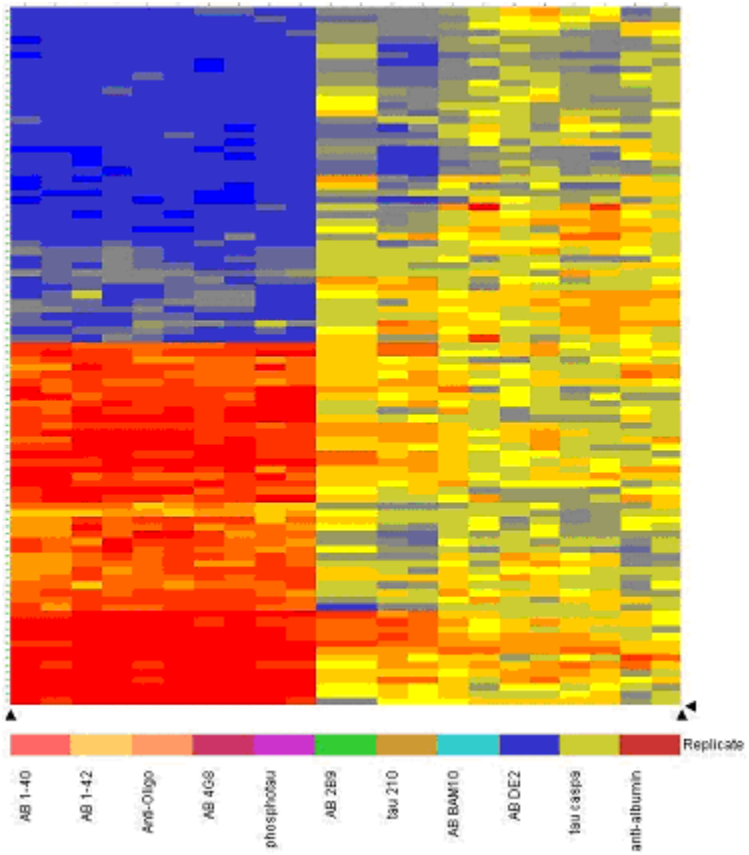


Fig. 3. The heatmap demonstrates high correlation between antibodies targeting Aβ's carboxyl-terminus and anti-oligomer and anti-phosphotau antibodies. This heatmap features 93 peptides deemed informative by ANOVA. Each pattern is represented in duplicate.

The carboxyl-terminus of Aβ is crucial for its polymerization, while additional amino acid residues in this region translate into greater aggregation potential, which provides a potential reason for the similarity between the Aβ antibody binding patterns. However, the similarity with the phosphotau antibody pattern is enigmatic. The phosphotau antibody used in this study reacts with a form of tau that is prone to aggregation within neurons. Although tau and Aβ do not share sequence similarity, it is conceivable that aggregated tau may share a conformational epitope with Aβ oligomers. Interestingly, the anti-Aβ oligomer used herein cross-reacts with several amyloidogenic proteins, including α-synuclein, islet amyloid polypeptide, prion protein, human insulin, lysozyme and polyglutamine, suggesting a common conformation-dependent structure, regardless of sequence.

In addition, I found differences between the signatures of the secondary anti-rabbit antibody, sera from a rabbit immunized with a control antigen (NMI), normal non-immunized rabbit sera and purified IgG from normal rabbits (Figure 4). Results were reproducible, with good agreement between duplicates run by the same individual ($r$=0.846-0.966) and different operators (r=0.95 for first slide, 0.94 for second one). Taken together, these experiments show that the microarray platform can detect distinctive patterns of antibody reactivity, and that these patterns are unique for each antibody, even if antibodies are raised against the same

target. Yet, some similarities are clearly noted, particularly if antibodies are raised against monomers or polymers.

Figure 4: Signatures of affinity-purified antibodies and plasma

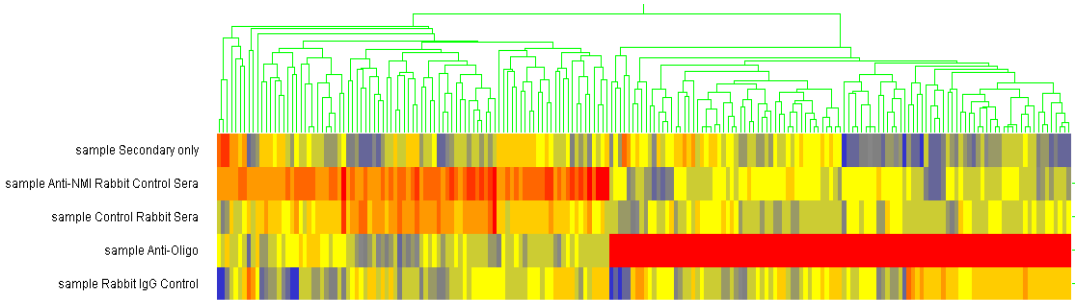


Fig. 4. Signature of anti-Aβ oligomer polyclonal antibody raised in rabbit. Heatmap of a select peptide array signature of anti-Aβ oligomer polyclonal antibody raised in rabbit, using hierarchical clustering. The heatmap sets apart the antibody signature from the secondary anti-rabbit antibody, sera from a rabbit immunized with a control antigen (NMI), normal non-immunized rabbit sera and purified IgG from normal rabbits.

Immunosignature of APPswe/PSEN1-1dE9 transgenic mice: As we discussed in previous sections of this thesis, APPswe/PSEN1-*1dE9* TG mice are engineered with 2 human mutations found in familial AD, affecting the amyloid precursor protein and presenilin-1 genes [22-27]. The resulting phenotype is well characterized, consisting of progressive amyloidosis involving cerebral cortex, astrocytosis, and neurodegene-

ration beginning at about 6 months of age, while cognitive impairment is noted around 9 months of age [22-27].

To investigate whether the immunosignature of TG mice differs from littermates, we purchased TG mice from Jackson Laboratories (Bar Arbor, ME), as well as non-transgenic controls (B6C3F1/J). In addition, plasma from vaccinated TG mice was provided by Dr Roger N. Rosenberg (Department of Neurology, University of Texas-Southwestern Medical School, Dallas, TX). At Dr Rosenberg's laboratory, 5 TG mice were vaccinated with a plasmid encoding $A\beta_{1-42}$, while 7 were vaccinated with mock DNA. All plasmids were delivered through gene gun for 10 doses. Two non-TG, non-immunized BALB/c mice were used as additional controls. Plasma samples were obtained at the time mice were sacrificed (15 months of age).

We used TG mice bearing two mutations from patients with familial AD (APPswe/PSEN1-1dE9) to track age-related changes in their humoral immune repertoire, which I will describe in detail later. A group of B6C3F1/J non-TG mice was used as control. These animals were used for regular plasma harvesting at monthly intervals until they were sacrificed at 15 months of age. All mice were female, in order to facilitate

handling and housing. A total of 5 TG and 5 non-TG mice were purchased (from Jackson Laboratories; Bar Arbor, ME) and housed with standard chow and water provided ad libitum. All murine experiments were conducted under a protocol reviewed and approved by the Arizona State University Institutional Animal Care and Use Committee. Mice were sacrificed at 15 months of age through intra-peritoneal injection of tribromoethanol (5 mg) followed by intra-cardiac ex-sanguination and cold PBS perfusion.

As we were interested in confirming the development of a characteristic neuropathology described in TG mice, brains were carefully dissected and removed from the skull after decapitation, rinsed sequentially in cold water (to lyse erythrocytes), soaked in cold PBS, and finally split across the mid-axial line. Samples were immediately fixed in cold PBS-buffered 10% paraformaldehyde for 12 hours and then embedded in paraffin for immuno-histochemistry. Every fifth section (with a thickness of 5-μm), was stained with hematoxylin and eosin. The Ventana automated slide preparation system was used for slide processing. In brief, the Ventana system heats slides and treats them with xylene, graded ethanols (100%, 95%, 75% and 50%), and distilled water. For immunostaining, slides were washed in full-strength formic acid for 2 minutes for antigen retrieval and dehydrated through graded alcohols.

Amyloid staining was attained with NovoCastra NCL anti-Aβ antibodies at 1:50 dilution. GFAP staining used anti-GFAP polyclonal antibodies from Athena Diagnostics, at 1:100 dilution. The secondary antibody was a biotin-conjugated rabbit antibody incubated for 30 minutes at room temperature, followed by incubation with streptavidin-peroxidase. Peroxidase activity was detected with diaminobenzidine tetrahydrochloride.

Although standardized cognitive tests were not performed, the TG mice were clearly different from the control group: the former were much more docile and easier to handle. TG mice had heavy cerebral amyloid deposition and astrocytosis as compared to B6C3F1/J controls, which was apparent on both Hematoxylin-Eosin staining and immunohistochemistry (Figure 5). The microarray signature of plasma from 10-month old TG mice (n=5) was different from 4 age-matched non-TG littermates (B6C3F1/J). Figure 6 shows the heatmap of 113 microarray peptides capable of discriminating between plasma signatures of APPswe/PSEN1-1dE9 transgenic (TG) mice (n=5) and non-TG B6C3F1/J littermates (n=4). In the heatmap, blue tones indicate low binding and red, avid binding (which occurs when more antibodies bind to the spotted random-peptide), whereas yellow designates intermediate binding. Plasma pools segregated with their constituting samples.

Figure 5: Histopathological changes in mice.

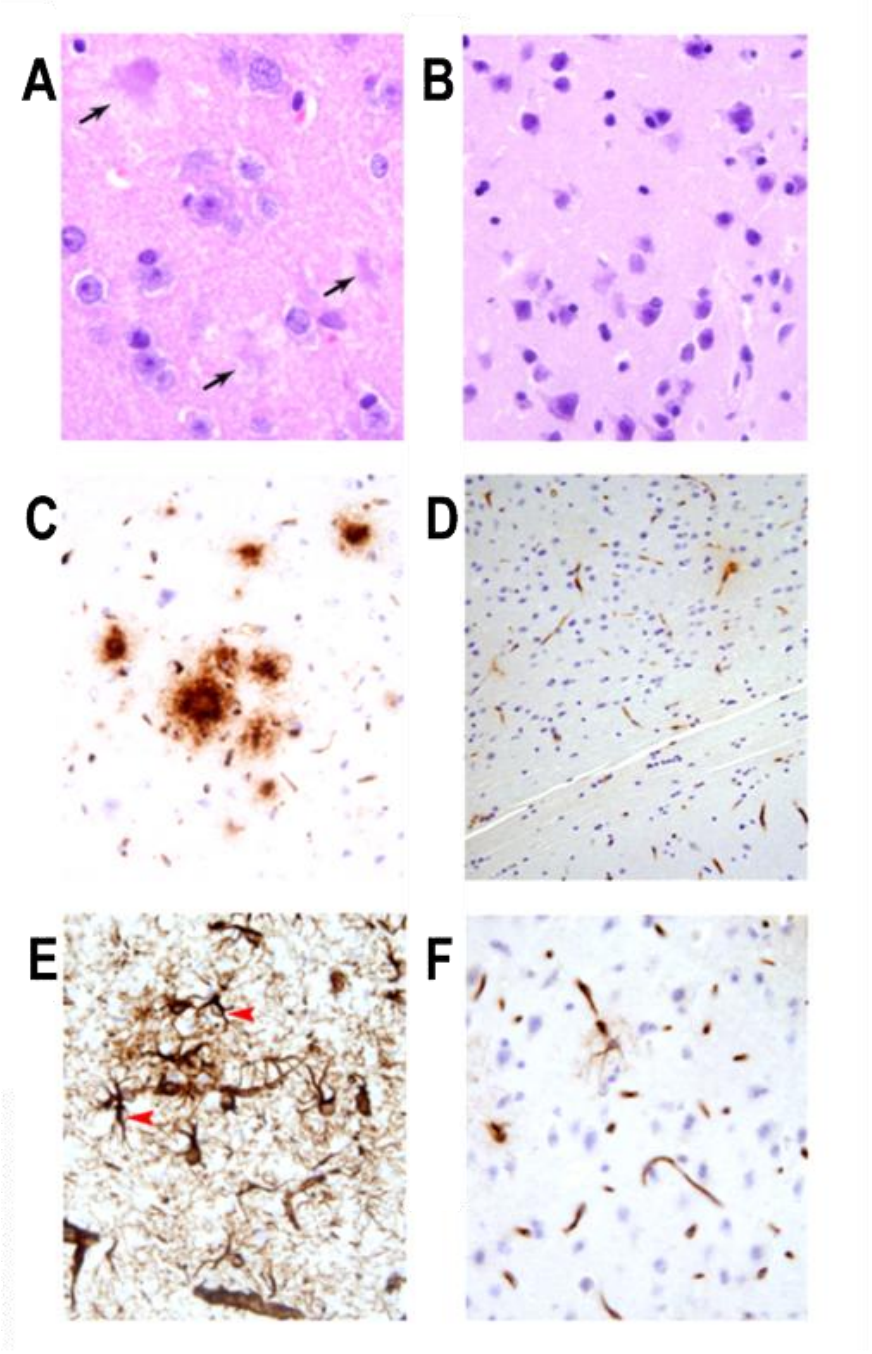


Fig. 5. Hematoxylin-Eosin staining shows widespread cortical senile plaque formation (arrows) and astrocytosis in TG mice (A) but not in B6C3F1/J controls (B). Staining with anti-Aβ antibodies reveals extensive amyloidosis in TG mice (C) but not in controls (D). Immunolabeling of glial fibrillary acidic protein (GFAP) showed dense astrocytosis. Stained cells were endowed with prominent fibrillary processes (red arrowheads). Magnification: 400X (except D, which is 200X).

Figure 6: Immunosignature of transgenic mice.

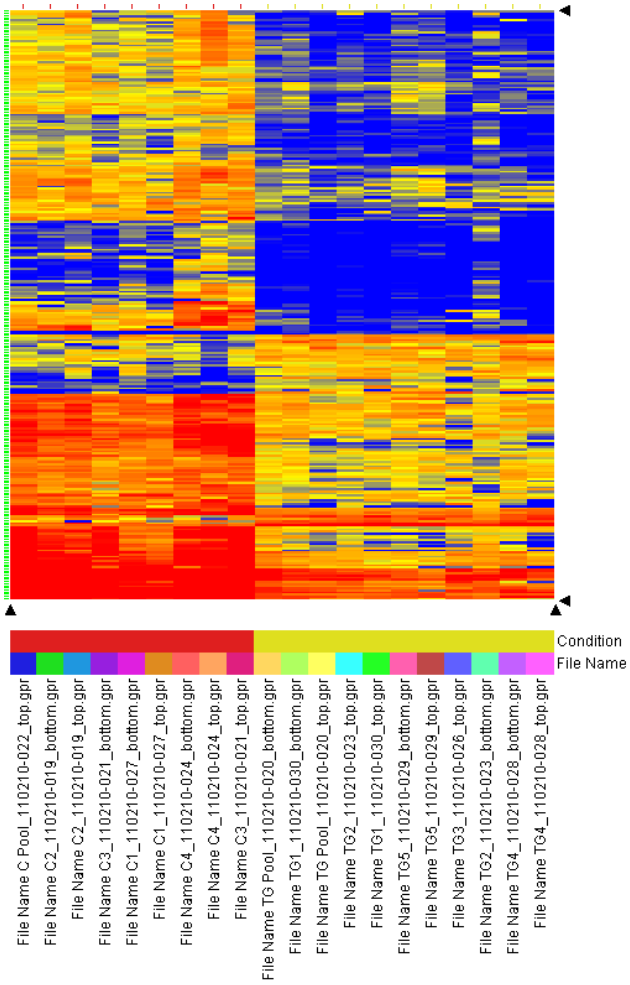


Fig. 6. Immunosignature of transgenic mice. Heatmap of 113 microarray peptides that can discriminate between plasma signatures of APPswe/PSEN1-dE9 transgenic (TG) mice (n=5) and non-transgenic B6C3F1/J littermates (n=4). Blue tones indicate low binding and red colors, avid binding (more antibodies bound per spot), whereas yellow hues designate intermediate binding. Notice that plasma pools segregate with individual samples.

A principal component scatter plot also proved useful to discriminate between the same mice plasma samples (Figure 7). Furthermore, the

microarray detected changes in the signature of TG mice immunized with a plasmid coding for human Aβ 1-42. A heatmap encompassing the entire 10,000 peptide array signature of serum samples from 15 month-old TG mice was generated (Figure 8), which sets apart 3 groups: on the far left, TG vaccinated with mock DNA; center-right, TG mice vaccinated with a plasmid coding for Aβ 1-42; and to the far right, serum samples from non-transgenic non-vaccinated C57 mice (NTG).

Figure 7: Principal component analysis of plasma signature in mice.

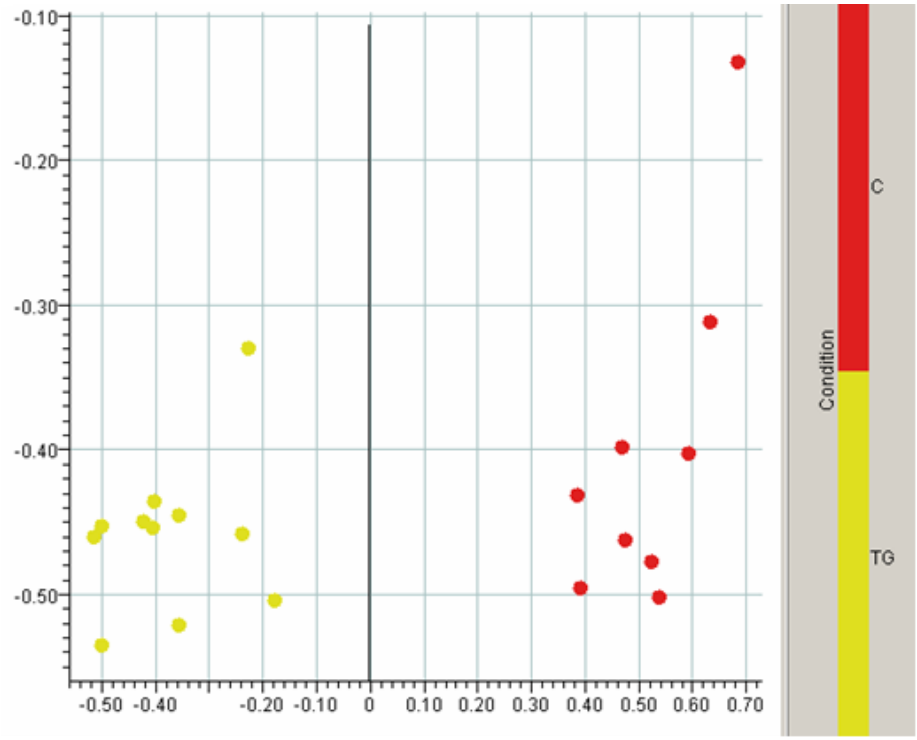


Fig. 7. PCA plot showing same mice plasma samples as in figure 5. Transgenic (TG) mice are represented in yellow and non-TG controls in red.

Another principal component scatter plot is shown in Figure 9, demonstrating segregation of plasma signature from mock DNA-treated, Aβ $_{1-42}$ plasmid-treated TG and non-TG mice. Aβ immuno-histochemistry revealed heavy amyloid deposition in the brain parenchyma of mock-vaccinated TG mice, whereas TG mice treated with Aβ plasmid had reduced amyloid deposits. Three microarray peptides avidly bound by plasma from mice vaccinated with Aβ also were among the top binders of the 7 commercial anti-Aβ antibodies that we discussed previously.

Figure 8: Immunosignature changes with $A\beta_{1-42}$ immunization.

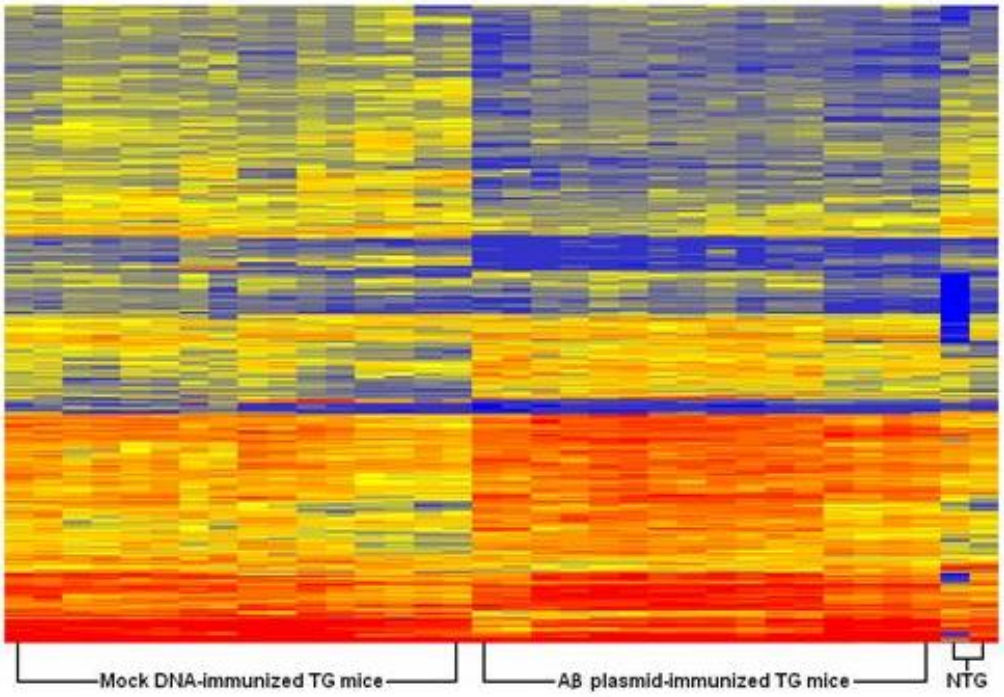


Fig. 8. Heatmap showing signature of plasma samples from 15 month-old TG mice. Three groups are noted: on the left, TG vaccinated with mock DNA; center-right, TG vaccinated with a plasmid coding for $A\beta_{1-42}$; and to the far right, serum samples from non-TG non-vaccinated C57 mice (NTG).

These experiments demonstrate that TG mice have a distinctive immunosignature that can be altered by genetic immunization, although a minimal component of the signature is shared with specific anti-Aβ antibodies. However, the animal model used has limitations in that it does not fully recapitulate all features of AD; in particular, APPswe/PSEN1-1dE9 mice do not develop neurofibrillary tangles.

Figure 9: Principal component analysis of mice signatures.

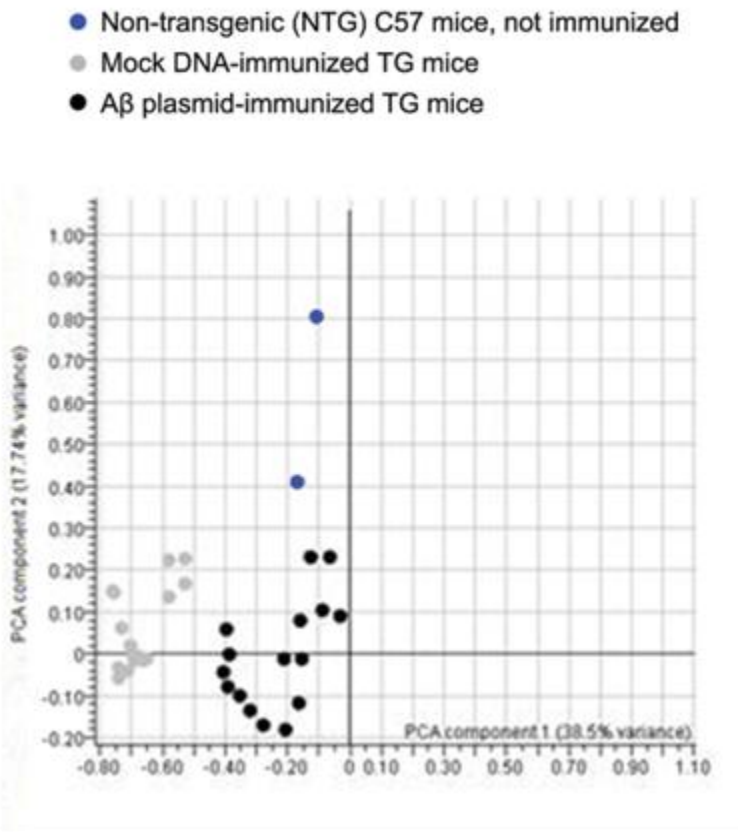


Fig. 9. Segregation of plasma signature from mock DNA-treated, Aβ $_{1-42}$ plasmid-treated TG and non-TG mice. Principal component scatter plot demonstrating segregation of plasma signature from mock DNA-treated, $Aβ_{1-42}$ plasmid-treated TG and NTG mice.

Stability of murine immunosignature: the immunosignature platform offers the opportunity of tracking the immuno-reactivity to different peptides overtime. Looking for possible fluctuations of the signature over time, I assayed plasma pools from APPswe/PSEN1-dE9 mice and B6C3F1/J non-transgenic controls drawn monthly, starting at 2 months of age and ending 13 months later (2-15 months).

Figure 10: Changes in immunosignature across time.

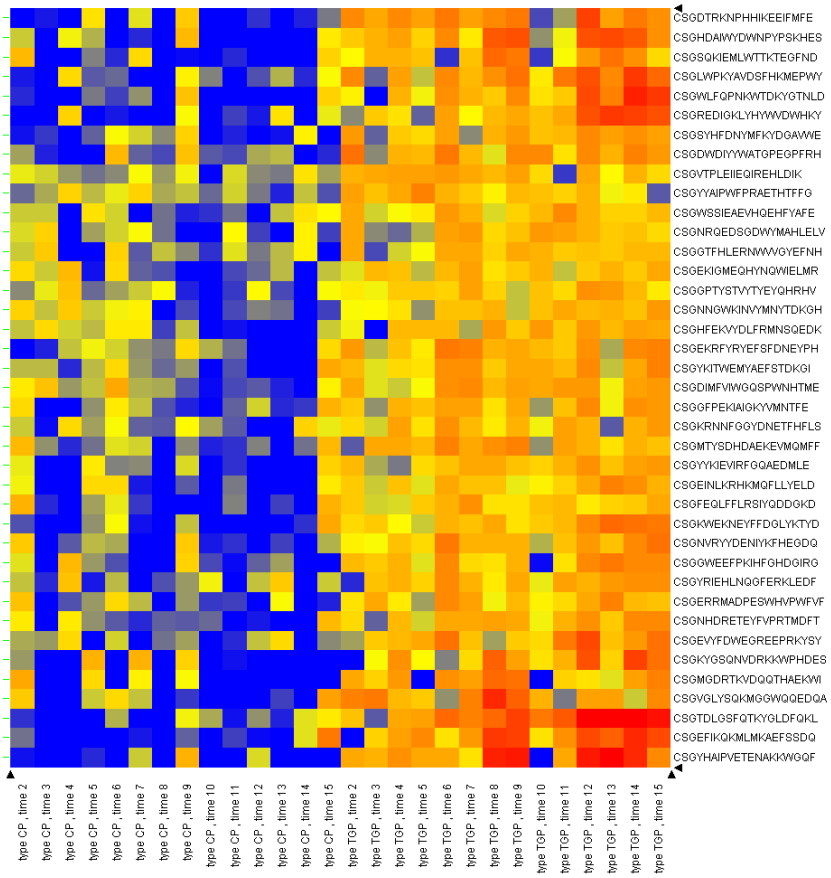


Fig. 10. Progressive build-up of signature in TG mice. Heatmap with 39 peptides with sustained immune-reactivity overtime in TG mice as compared with B6C3F1/J controls. The *y* axis lists the different peptides, whereas the *x* axis depicts plasma pools from TG mice and age-matched controls.

A two-tailed t-test (P=6.6 x10-7 to 6.7 x10-5) was used to find peptides that discriminated all TG mice from their non-TG controls, yielding a total of 39 peptides (listed in Table 3). Although this was a two-tailed t-test, these peptides showed higher binding in TG mice. The signature was evident even at 2 months of life (Figure 10, above). Notably, the immunoreactivity of these peptides became progressively stronger with TG mice plasma, remaining low or becoming fainter with B6C3F1/J plasma. Plasma samples highly correlated with replicates and other samples obtained at different time-points. Using the “Expression Profile” feature of Gene-Spring 7.3.1, which allows the detection of immuno-reactivity patterns that correlate to arbitrary patterns drawn by the operator, we noted that most microarray peptides have an intricate immuno-reactivity pattern which moderately fluctuates overtime. Such complexity is exemplified by the finding that only 2 out of 10.000 peptides had a reactivity profile that highly correlated to a traced flat line (Pearson’s correlation coefficient >0.7). The differences in the immunosignature of both mice groups changed at different time points, with the immune-reactivity of many peptides exhibiting high immune-reactivity at 2 months of age in TG mice (when cerebral amyloidosis first becomes apparent), but declining thereafter. In contrast, an unrelated set of 24 peptides had a similar trend in B6C3F1/J controls. The immune-reactivity of 17 additional peptides peaked at age 6 months to decline thereafter in TG mice (compared to 2 unrelated peptides in B6C3F1/J controls), whereas a

different set of peptides (n=77) had a steady reactivity decline in this mice group (42 unrelated peptides followed a similar trend in B6C3F1/J controls). These observations suggest that the plasma signature of TG mice can be distinguished from that of B6C3F1/J controls, and that the signature remains largely stable overtime or becomes better defined. However, some peptides seem more reactive at different times in life, suggesting that many possible epitopes are targeted by the immune system as the underlying pathological process evolves. The antibody signature of TG emerges early in life: incipient plasma reactivity against a set of peptides was detected in TG mice as early as 2 months after birth, before significant neuropathological or neurological signs are expected. Although these animal experiments cannot rigorously be extrapolated to humans, its relevance is that it is possible that an immunosignature, if present in humans, may be detectable during the early or even pre-symptomatic stages of disease, as humoral immune responses generally predate the onset of pathological and clinical signs of many diseases.

Classification of young mice using late immunosignatures. It is generally agreed in the literature that an effective AD therapy is likely to depend upon early detection and treatment [15]. In spite of recent advances [4-6], no specific tests are universally used to diagnose AD. As the pathology slowly progresses for decades before the initial symptoms

emerge [16], and since the initial manifestations are generally subtle [17-21], a potential diagnostic test for AD must be highly sensitive. Given that future treatments are likely to target people with mild or no symptoms [15,17,18], the test must also be highly specific. Considering the difficulties and time involved in obtaining enough samples from subjects with early AD stages, we used again the APPswe/PSEN1-dE9 mice, to explore the possibility of developing an early stage diagnostic. Specifically, I asked whether an immunosignature optimized to detect disease in older animals can be used to diagnose the early phases of the disease? This would be analogous to using late-stage AD human samples, to train a system to detect presymptomatic AD. To answer this question, mice were divided into three groups, according to age: early (2-5 months), mid (6-9 months) and late (10-15 months). These time-points are biologically relevant in APPswe/PSEN1-dE9 mice, considering that their neurocognitive function begins deteriorating at 8 to 9 months of age and their characteristic neuropathology (cortical senile plaque formation and astrocytosis) is first observed from 6 to 7 months of age [22-27], while no neurocognitive or pathological abnormalities are apparent before 4 months of age [22].

Figure 11 shows sequential heat maps separating TG and non-TG mice at the early, mid and late time-points. Only 35 peptides were selected in a t-test between APPswe/PSEN1-dE9 and B6C3F1/J mice at

each of the 3 time-points. This was done for 3 reasons: first, in all cases there were at least 35 peptides that survived multiple-testing correction (FWER=5%). Second, it is easier to demonstrate any overlap in peptides from one time-point to another when a fixed number of peptides are used. Third, the classifier we use (Linear Discriminant Analysis, LDA) works best when less than 100 features are used, and 35 features suits this algorithm well. The three 35-peptide sets chosen using a two tailed t-test (early $P<1.61\times10^{-5}$, mid $P<1.113\times10^{-4}$, late $P<8.73\times10^{-5}$) readily separated TG from non-TG mice at specific time-points, as is shown in Figure 10. Of these optimal peptides, there were only 3 that overlapped between early and mid-stages, and 8 that overlapped between mid and late signatures. No peptides overlapped between the early and late stages, suggesting differences in the ongoing pathological process through the different time-points.

Figure 11: Differences in the signature according to life stages.

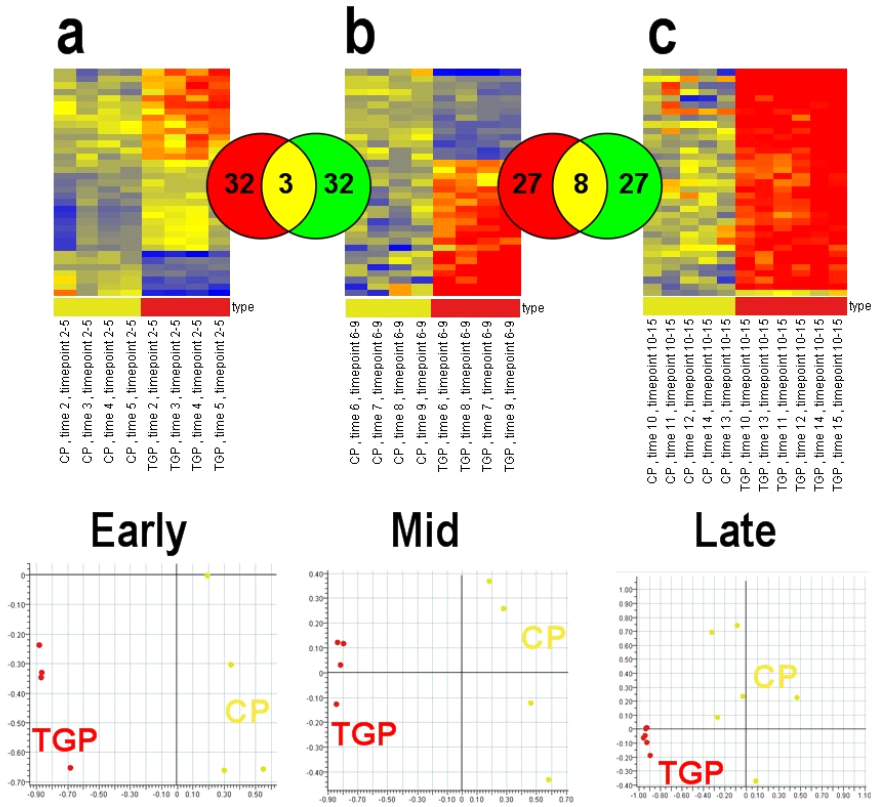


Fig. 11. Classification performance of immunosignatures obtained at different time-points, first looking at differences between age-matched TG and non-TG mice, then applying age-specific signatures to the classification of mice of different ages. (a) Heatmap depicting plasma pools obtained from APPswe/PSEN1-dE9 mice (TGP) and B6C3F1/J controls (CP) from 2-5 mos of life (early samples). Immunoreactivity of the 35 random-sequence peptides (early peptides) used here were significantly different between TGP and CP (peptides and sequences are listed on the Supplementary table); at the bottom of (a) is a PCA display of the same plasma pools using the same 35 peptides, showing relative differences between plasma obtained early in life when the early peptide set is used. (b) Heatmap (top) and principal component display (bottom) of plasma pools obtained at 6 to 9 months of age (mid group), showing another set of 35 peptides that can also distinguish between TGP and CP. (c) Same experiment using plasma pools obtained late in life (months 10-15 of age). The Venn diagrams show the number of peptides that overlap between each set. There was no overlap between peptides selected from early and late stages.

I evaluated whether the 35 peptides distinguishing TG from non-TG littermates late can differentiate the groups early. As shown in Figure 12, late peptides discriminated early disease with a 21% error rate (via LDA, Leave One Out Cross-validation). Mid stage peptides also separated groups using plasma obtained at early stages with 18% error, while late stage peptides distinguished the source of plasma drawn at mid stages with 8% error. None of the 39 peptides shown in Figure 9 that generally discriminate TG from non-TG mice across all time-points appeared in the list of 35 optimized for each stage, suggesting that there are antibodies specific to each disease stage. When asked to find antibodies present throughout the entire disease, the array may have identified lower affinity and lower specificity antibodies than the stage-specific ones.

Figure 12: Classification performance of late signatures.

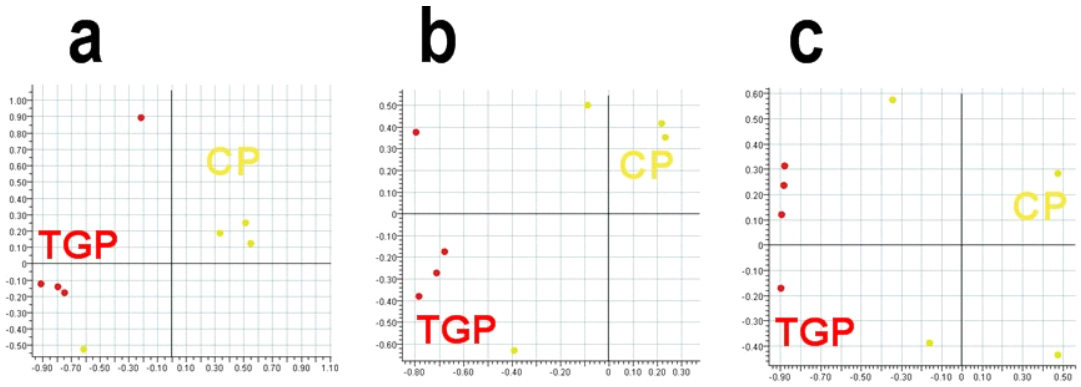


Fig. 12. Accuracy of mouse classification using stage-specific signatures. Optimal peptide sets from early, mid and late life stages were used to classify plasma obtained at different ages. (a) Late peptides discriminated early samples with 21% LDA error rate; (b) Mid peptides classified early samples with 18% LDA error rate; and (c) Late peptides classified mid samples with 8% error rate.

I also investigated whether the resolving power of late stage peptides on early stage samples could be improved by including more peptides that were informative against late stage disease. The top 130 predictive peptides ($p<0.000117$) for late stage discrimination included exactly all 35 early peptides. When these 130 peptides were used to distinguish transgenic mice at early stages, the error rate was zero (even so, the visual grouping by heatmap or PCA was noticeably worse than using only the 35 early peptides). Since the late stage peptides had some positive predictive power for early stage, we asked whether the 35 early peptides could predict when mixed with non-informative peptides. We added 95 randomly chosen peptides to the 35 early peptides to make a list of 130 peptides; the LDA misclassification rate was 10%, suggesting that the 35 early peptides could still perform fairly well even in the presence of random noise but also that there was some predictive power for early disease in the larger list of late-stage peptides.

Correlation between IgG purified from brain and plasma: finally, we asked the question whether IgG present in the brain has a similar signature to the one observed in plasma. Small amounts of IgG are normally found in the brain, reflecting leaking from plasma as well as local production. Additionally, IgG can be detected in senile plaques [28]. As we discussed, TG mice had heavy cerebral amyloid deposition and astrocytosis (Figure 5). We found a high correlation ($r$=0.96 to 0.998)

between binding patterns of brain-purified and plasma-purified IgG (n=4, 2 TG and 2 B6C3F1/J). The antibody signature was different for each individual, but similarities were again noted between TG and controls. There was a high correlation between the signatures of IgG and the whole plasma from which it was purified ($r$=0.99). This similarity between the IgG signature of murine brain and plasma suggests that the same assortment of antibodies is being detected by the microarray platform. While cross-contamination between blood and brain is possible during sample processing, it is well known that IgG can cross the blood brain barrier. In fact, the blood-brain barrier becomes more permeable to macromolecules as individuals age, particularly in the setting of chronic neurodegeneration.

## Discussion

Evaluating the potential of immunosignaturing as a diagnostic test for early AD, I and my mentors at CIM first looked at the stability of the signatures in mice. There appears to be a distinctive TG mouse signature, which remains stable over time with some variation. The signature has both a general group aspect and one that is individual, such that the samples from the same mouse over time were very similar. Employing the APPswe/PSEN1-dE9 mouse model and age-matched controls, blood was collected from individual mice from months 2 to 15 of life. When

considering only TG and non-TG groups without incorporating the time of collection, it was found that as few as 39 peptides could separate the two groups of mice, with a signature that increased over time (in Figure 9 there are ~8 peptides that seem to show a distinct increase in signal over time in the heatmap). The mice were then divided by age into early, mid and late stages. We selected 35 highly significant peptides using a standard t-test between TG and non-TG mice at each stage. There was little overlap between the sets of peptides characterizing each stage, and none at all between late and early stages. The late stage peptides separated TG and non-TG mice at early stages, but with a rather high error rate of 21%, while mid-stage peptides performed better (18% error). Increasing the p-value cutoff to 0.0001 for late peptides allowed 130 peptides to be selected. This set of 130 peptides included the 35 highly selective early peptides, and actually did classify the early peptides with 0% error. If translated to a clinical setting, one would not know which peptides would be best for early diagnostic, but since the early specific peptides were a subset of a large set of late peptides, and given that highly specific early peptides can still discriminate the disease state even when mixed with 95 randomly non-informative peptides, provides hope that a diagnostic for early diagnosis can be done using conventional patient selection (i.e., confirmed diagnosis at late AD stages).

The patterns formed with antibody binding to microarrays may have a diagnostic potential. However, the stability of signatures is important for two reasons: first, if the variation caused by time is small, then larger sample pools could be used without concerns about noise dampening the signature out over time. Second, if there is a personal component of the signature, it could be useful for monitoring disease progression or response to treatment. TG signatures were highly distinguishable from age-matched controls regardless of age. Relative to the second issue, there was clearly an individual component observed in mice. Mathematically, the two samples from the same individual were most similar to each other.

Ideally, AD should be detected at the pre-symptomatic or early symptomatic stages, when promising disease-modifying therapies are expected to exert greatest benefits [15]. Unfortunately, these stages are also the least understood aspects of the disease, and the most susceptible to diagnostic misclassification with current standards [16-22]. For these reasons, we are interested in knowing whether the late stage signatures can be used to guide an early stage diagnostic. We used the APPswe/PSEN1-dE9 mouse model to address this issue. While there are certainly concerns for the relevance of any mouse model to human disease [27], our perspective relative to technology development is that if

one cannot demonstrate the feasibility of an approach in a well-controlled model system, it is less likely to work in the complexity of humans.

The first issue is whether the TG mice are distinguishable by immunosignaturing at an early stage of disease. When the immunosignatures of all the TG mice were compared to the non-TG littermates, 39 peptides clearly separated the two groups regardless of age. Even mice at two months (when characteristic neuro-pathology is not expected), had a distinguishable signature, although noticeably weaker than in old mice. This signature became more intense over time, implying that there is more antigen driving the antibody response. Interestingly, 7 of the 39 peptides could also react with purified antibodies against Aβ, the concentration of which progressively increases with age in the brain and plasma of APPswe/PSEN1-dE9 TG mice. These changes in the antibody repertoire of TG mice illustrate the complexity of their pathological process, with amyloid overproduction setting off a cascade of events where additional epitopes become targeted by the immune system as animals grow older.

From the practical point of view of developing a human diagnostic signature, it will be challenging in the short term to acquire samples from

all stages of human AD. Using the mouse model does not circumvent this issue, but helped us to gain relevant insights. For instance, dividing the mice into early, mid and late stage groups, we found peptides from each life stage that separated transgenic from non-transgenic mice with 100% accuracy. Of note, there was no overlap between the 35 peptides in the late and early stages, and the late stage peptides classified the early stage mice with 21% error. However, based on the mouse data, there may be two solutions to this problem. We found that the informative 35 peptides for early stage were included in the top 130 late stage predictive peptides ($p<0.000117$). These 130 peptides had a 0% error rate in classifying early stage mice. Of course, this test is artificial in that we knew where to draw the cut-off in order to include the 35 early stage peptides. But it does indicate that an inclusive rather than exclusive strategy for choosing late stage peptides for a diagnostic would more likely succeed.

A second strategy may be based on using samples from people with mild cognitive impairment who progressed to autopsy-confirmed AD. While there was no overlap between early and late stage peptides in mice, there was a 23% overlap between late and mid stage peptides and 9% overlap between mid and early. Therefore, it may be useful to employ the

mild cognitive impairment samples to define the early stage peptides for the diagnostic. Of course these two strategies are not mutually exclusive.

What are the implications of this analysis for developing a diagnostic test for early stage AD? To the extent that the mouse model and its associated caveats can guide this effort, it is encouraging in implying that a signature of disease starts very early in life. However, this work also implies that optimizing the diagnostic test using late or minimally-symptomatic patients may not provide much overlap with the optimal early stage signature. Although the optimal 35 peptides selected in old mice were different from the 39 peptides that were useful at all the time-points and the 35 optimal early-stage peptides, the last 2 sets of peptides became again part of the signature when the p-value cutoff of the late-stage comparison was relaxed. The implication is that late stage immunosignatures should be used rather broadly when searching for an early AD diagnostic. This has the shortcoming of introducing non-informative peptides and subsequent noise in the analysis, but our analysis on the mouse samples indicates this may not be prohibitive in developing an accurate diagnostic test.

# Chapter 3

# ANTIBODY SIGNATURE OF PATIENTS WITH ALZHEIMER'S DISEASE

## Introduction

The major theme of this doctoral thesis is that post-mortem examination remains the gold standard of AD diagnosis, an option that is rarely feasible or desirable. This has important implications for medical practice and clinical trial design: to begin with, the prognosis of dementia varies according to the underlying etiology; secondly, pharmaceuticals used routinely for AD can exacerbate the symptoms of other types of dementia (for instance, donepezil can exacerbate the motor impairment of Progressive Supranuclear Palsy, or PSP) [1]; and lastly, clinical trials may be distorted if a substantial proportion of enrolled subjects are expected to have a wrong diagnosis. A pharmaceutical company conducting two phase 3 clinical trials for AD is using a blend of biomarkers to document disease progression, including neuro-imaging and Aβ measurements in plasma and cerebro-spinal fluid [2-3]. This underscores the necessity to develop alternative techniques to diagnose AD and monitor its course.

In the previous chapter, I described the application of our microarray platform to the study of the binding patterns of affinity-purified antibodies and plasma samples from transgenic mice with cerebral amyloidosis. In this chapter, I will describe my efforts to develop a diagnostic tool for AD based on immunosignatures. Some of these experiments have been peer-reviewed and published [4].

## Methods

Patient's Characteristics and plasma sample handling. Plasma from 12 patients with probable AD and 12 age-matched controls without cognitive derangement were provided by Alex Roher (Banner's Sun Health Research Institute, Phoenix, AZ). These patients were enrolled into a brain-bank program. Postmortem examination was performed by a neuropathologist on 9 patients (5 with and 4 without dementia). Samples were acquired after written consent and approval of the Banner Institutional Review Board (IRB). Profiling studies were approved by ASU's IRB (protocol # 0912004625). In addition to these patients, we obtained 100 plasma samples from the Alzheimer's Disease Neuroimaging Initiative (ADNI). ADNI is a comprehensive multi-institutional project funded in part by the NIH (P.I.: Dr Neil Buckholtz), aiming to identify neuroimaging and biomarkers of the cognitive changes

associated with MCI and AD [5]. Data acquired through ADNI are made available to the general scientific community and the entire repository of clinical and imaging data collected is accessible to authorized investigators after on-line application, which we submitted on 4-23-2009 and approved on 7-2-2009.

Microarray Platform and Immunoassay: the reader is referred to the description on Chapter 1. Regarding Microarray analysis, scanned data was loaded into GeneSpring 7.2.1 (Agilent Technologies, Santa Clara, CA) and analyzed. Signals were deemed present when intensities were >1 standard deviation from mean local background. Peptide identification was done using t-tests, Model I (fixed effects) 1-way or multi-way ANOVA, and correlation to specific expression patterns. Clustering techniques, including k-means, hierarchical clustering, and Self-organizing Maps were used for identifying antibody binding patterns. We screened for technically irreproducible values during data pre-processing. Each peptide array replicate provides a 1.5-fold minimum average detectable fold change at $\alpha=0.05$ and $\beta=0.20$. Appropriate false-positive corrections were used.

Blocking experiments with Aβ-coated beads: synthetic Aβ 1-40 covalently attached to TantaGel S NH2 polystyrene beads (Advanced

ChemTech, Louisville, KY) were used, carrying approximately 0.2 mmol antigen/gram. To decrease non-specific binding, various bead concentrations ranging from 1-0.01 mM were pre-blocked with 5%BSA-PBS. Beads were stored at 4°C overnight and rinsed with 3%BSA-PBS-0.05%Tween20 prior to mixture with plasma pools dissolved 1:500 in 3%BSA-PBS-0.05%Tween20. This mixture was incubated at 37°C, centrifuged, and the supernatant was assayed on microarray slides as previously described. Blank beads similarly treated were used as controls.

## Results

On average, patients with dementia were older than the cognitively-normal control (84.5±5.5 years old versus 72.6±7.8, respectively). This difference had a trend toward statistical significance ($p=0.08$) using a t-test. Most patients were women (7/12 in the AD group and 8/12 in the control group, Table 3).

Table 3

Clinical and Neuropathological characteristics of patients.

| P# | Age | Sex | Pathology Summary | PT | CERAD | Braak |
|---|---|---|---|---|---|---|
| 43 | 88 | F | Argyrophilic grains in mesial temporal lobe; Lewi bodies; white matter rarefaction. Dx: PSP | 14 | Prob AD | III |
| 44 | 81 | F | - | | | |
| 48 | 87 | M | - | | | |
| 53 | 83 | F | Many plaques and tangles; white matter rarefaction | 15 | Def AD | V |
| 57 | 85 | F | Many plaques and tangles; Lewi bodies | 11.2 | Def AD | V |
| 59 | 81 | F | - | | | |
| 4 | 77 | F | - | | | |
| 8 | 80 | M | - | | | |
| 11 | 73 | F | - | | | |
| 15 | 89 | M | Many plaques and tangles; severe white matter rarefaction; 3 small old infarcts and 6 old microinfarcts | 13 | Prob AD | V |
| 24 | 90 | M | Many plaques and tangles, white matter rarefaction | 11 | Prob AD | IV |
| 26 | 76 | M | - | | | |
| 39 | 86 | M | - | | | |
| 40 | 83 | F | - | | | |
| 41 | 77 | F | - | | | |
| 45 | 81 | F | Some senile plaques and occasional tangles | 10 | Pos AD | II |
| 49 | 70 | F | - | | | |
| 50 | 73 | M | Some plaques and tangles, insufficient for AD Dx; mild amyloid angiopathy; white matter rarefaction; 1 small old infarct; many old microinfarcts | 6.5 | Not AD | III |
| 1 | 82 | F | Not available | | | |
| 13 | 60 | F | - | | | |
| 16 | 79 | F | - | | | |
| 29 | 79 | F | - | | | |
| 52 | 90 | M | Occasional plaques and tangles | 4 | Not AD | III |
| 56 | 76 | M | - | | | |

P# is patient ID number; PT= total senile plaque count; CERAD= pathology diagnosis (Consortium to Establish a Registry for Alzheimer's Disease); Prob= “probable”; Pos= “possible”; Def= “definite”; Braak are the Braak scores.

Figure 13: Human immunosignature.

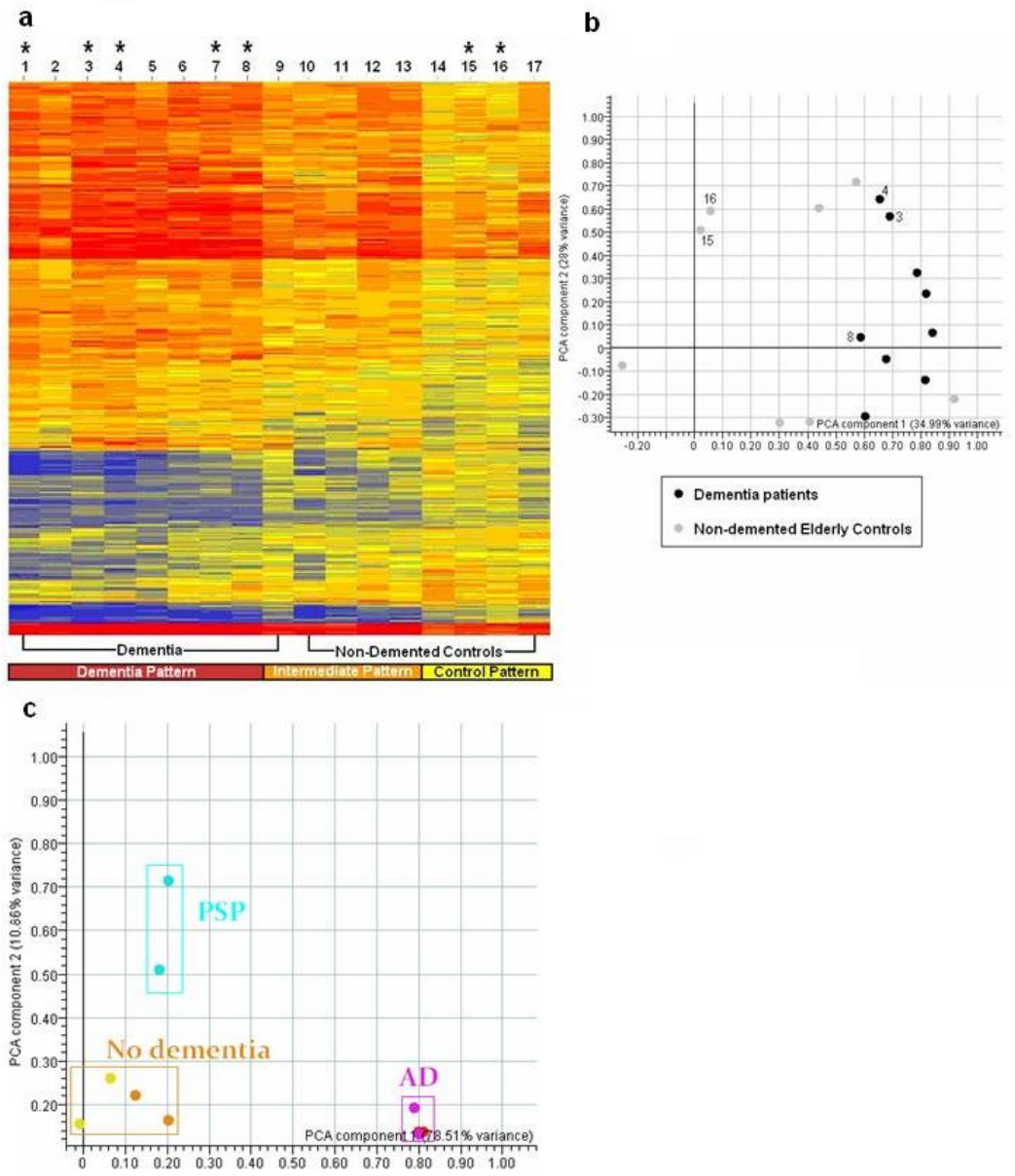


Fig. 13. (a) Heatmap of 169 peptides that distinguished AD plasma from age-matched controls. Patients cluster into 3 patterns: AD-type, intermediate, and non-demented. Asterisks denote individuals who had autopsy. (b) Principal component scatter plot analysis of same plasma samples, demonstrating that individual plasma samples from AD patients (red dots) cluster together, whereas samples from non-demented controls (yellow) are widely scattered. (c) Plasma pools (arrow heads) from AD patients and cognitively normal controls are also correctly discriminated by the platform. The signature of a patient with PSP on autopsy, migrated with the pattern of normal controls.

The immunosignatures of these patients formed three different patterns: one distinctive of AD, another representative of the non-demented controls and an intermediate pattern. The former pattern was noted on 9 individuals, all with AD (except 1 normal control and the PSP patient). The second pattern was seen in 4 cases, (3 controls, 1 AD). The intermediate pattern was seen in 4 cases (3 non-demented, 1 with AD). The asterisks in Figure 13 denote individuals who had autopsy, which confirmed AD in patients # 1, 3, 4, and 7; patient # 8 was diagnosed with PSP by the pathologist, whereas patients # 15 and 16 did not have significant AD pathology. Panel b shows a principal component scatter plot analysis, demonstrating that individual plasma samples from AD patients (black dots) cluster together, whereas samples from controls (grey) are widely scattered. The numbers near the dots represent patients from panel A. Next, we assayed plasma pools from 5 patient groups: autopsy-proven AD (n=4), clinical AD without autopsy (n=7), the PSP patient, cognitively normal elderly controls without definitive signs of AD on autopsy (n=4) and cognitively normal elderly controls without autopsy (n=8). The principal component plot shown in panel c of Figure 13 also demonstrates that the microarray platform can discriminate between different pools, and also that AD patients with or without autopsy cluster away from normal controls.

Using ClustalW 2.0, an automatic program for global multiple alignment of aminoacid sequences [6], we found that none of the 50 higher ranking peptides bound by the autopsy-proven AD plasma pool had sequence similarity with $A\beta_{1-40}$ or $A\beta_{1-42}$. Eleven microarray peptides highly bound by the AD autopsy plasma pool were also top binders of the 7 commercial anti-Aβ antibodies.

The predictive capacity of the immunosignature was assessed by re-testing 8 random samples (5 with AD and 3 controls) in a blinded fashion. Using the support vector machine algorithm of GeneSpring GX, we established a learning data set using known binding patterns exhibited by the complete sample set of human IgG. With this training set, blinded samples were assigned to any pattern, which correctly recognized 4 AD and 2 control cases but misclassified 2 samples (1 erroneously assigned to AD). While these are early results, the data supports the concept that different antibody binding patterns are detectable and reproducible, and that the immunosignaturing technique could be developed to assist in the classification of patients with dementia.

Blocking experiments with Aβ-coated beads: to determine whether the immunosignatures observed in humans are partly due to Aβ immunoreactivity, I carried blocking experiments using synthetic $A\beta_{1-40}$

covalently attached to polystyrene beads to pre-treat plasma pools before being assayed. Untreated plasma pools and pools treated with blank beads were used as controls. The overall signature of plasma pools did not change after blocking with the Aβ-coated beads. However, pre-treatment with Aβ beads decreased the reactivity of 4 microarray peptides as the concentration of Aβ $_{1\text{-}40}$ beads increased (Figure 14, panel a).

Figure 14: Blocking experiments with $A\beta_{1\text{-}40}$ beads.

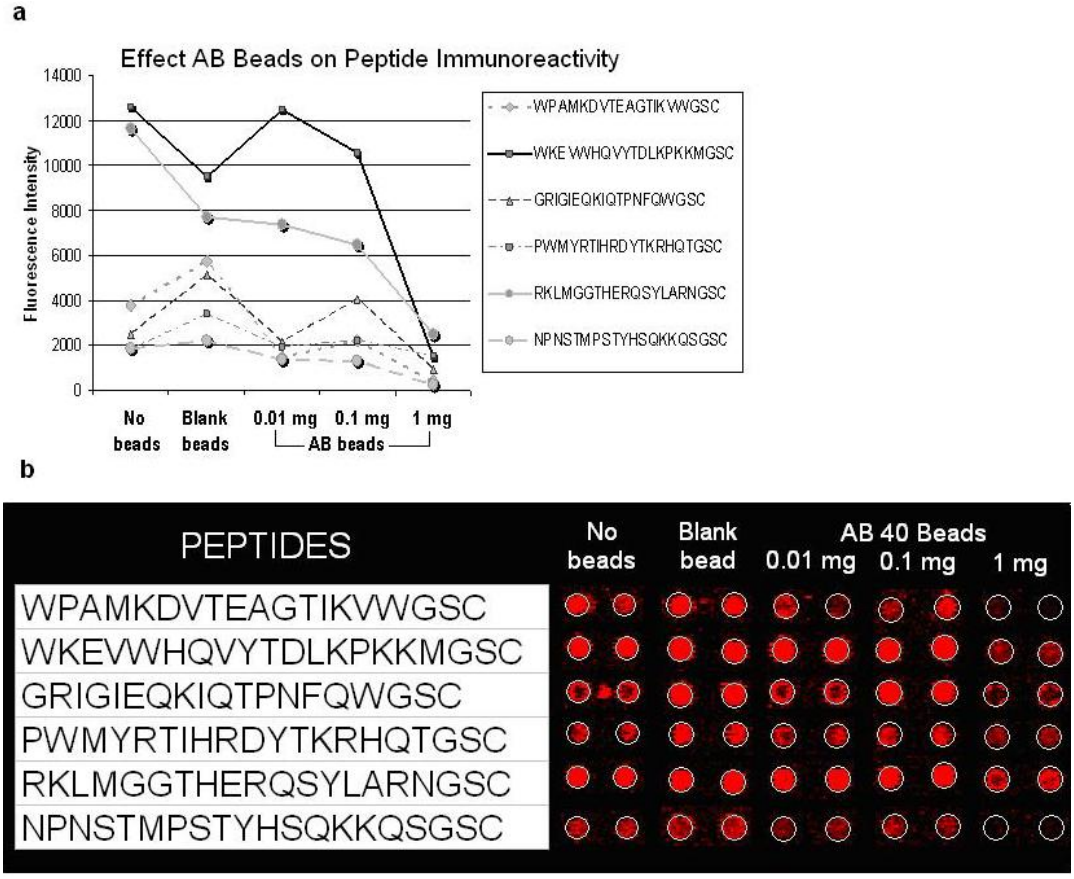


Fig. 14. Blocking of plasma immunoreactivity with Aβ-coated beads. Plasma pools from AD patients were treated with different concentrations of Tantagel beads. (a) Fluorescence declined for a few array peptides as the concentration of Aβ 1-40 beads increased. (b) Effects of Aβ 1-40 bead treatment on fluorescence intensity of the specific peptides shown above.

There was minimal variation with blank beads, whereas minimal decline in fluorescence intensity was noted for a plasma pool from normal cognitive controls. Panel b depicts a microarray scan showing the effects of $A\beta_{1-40}$ bead treatment on fluorescence intensity of the specific peptides shown above. The immunoreactivity of 2 of these peptides exhibited marked decline after $A\beta_{1-40}$ treatment. Using ClustalW 2.0, I found no sequence similarity between these peptides and human Aβ. Figure 3 is a bar graph depicting the fluorescence intensity of the representative array peptides blocked by $A\beta_{1-40}$ when probed with specific commercial antibodies. Some of these peptides strongly bound polyclonal anti-$A\beta_{1-42}$, anti-Aβ oligomer and anti-phosphotau antibodies.

Figure 15: Fluorescence of representative peptides blocked by Aβ $_{1\text{-}40.}$

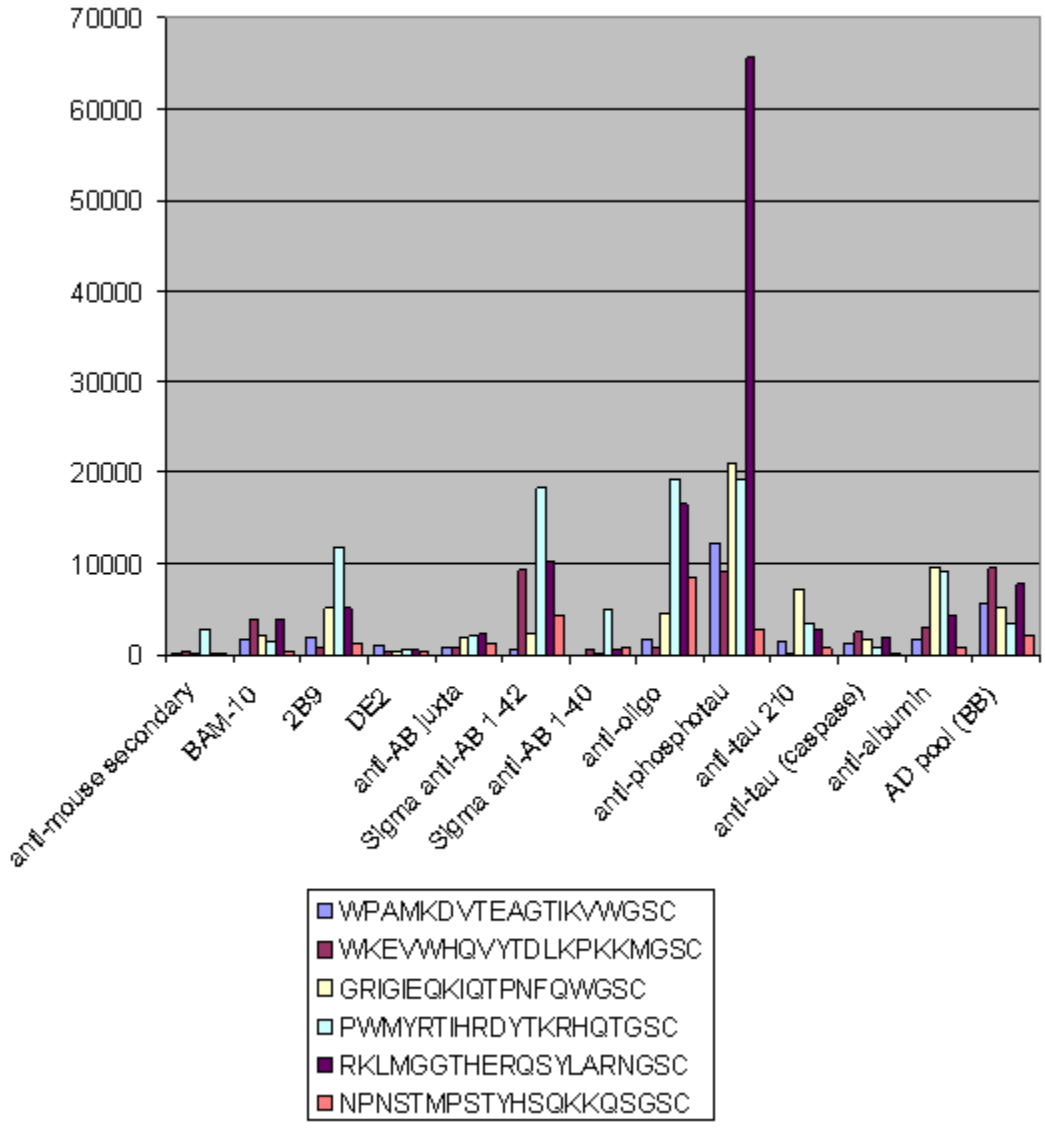


Fig. 15. Bar graph depicting the fluorescence intensity of the representative array peptides that were blocked by Aβ 1-40 when probed with specific commercial antibodies. Notice that only the anti-Aβ 1-42, anti-Aβ oligomer and anti-phosphotau bound well to some of these peptides.

These experiments suggest that only a small portion of the

signature is driven by anti-Aβ antibodies, and that blocked microarray

peptides may behave as epitope mimetics, given the lack of sequence homology with the blocking antigen. However, it is possible that an anti-Aβ antibody that conveyed a small portion of the signature or one whose removal was masked by binding of another antibody would not be detected.

Cross reactivity between AD, TG mice and anti-Aβ oligomer antibodies: 33 peptides were preferentially bound by anti-oligomer antibodies and AD plasma, whereas 19 peptides were specifically bound by plasma extracted from AD patients and TG mice (Figure 16).

Figure 16: Venn Diagram peptide overlap.

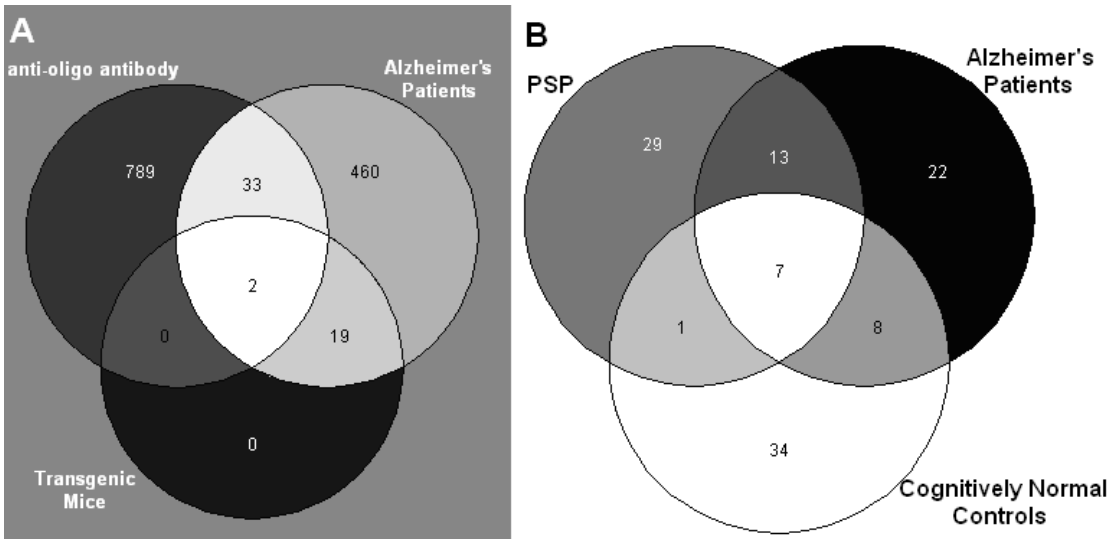


Fig. 16. Venn diagram representing cross reactivity between different sera. (a) highest-ranking peptides bound by plasma from APPswe/PSEN1-dE9 mice, AD patients, and the anti-Aβ oligomer antibody. (b) plasma pools from autopsy-proven AD, normal controls and plasma from a patient with PSP on autopsy.

Two random-sequence peptides were avidly bound by all groups: KKNFKTFGFDPLVTWSWGSC and GLPWTLYYLWMRPTYVRGSC. The probability of this occurring by chance is $8.894 \times 10^{-6}$. Panel (a) of Figure 16 shows the number of highest-ranking peptides from the microarray bound by sera from APPswe/PSEN1-*dE9* transgenic mice, AD patients' plasma, and the anti-Aβ oligomer antibody. Panel (b) of the same Figure shows a similar exercise using pools of plasma from autopsy-proven AD, cognitively normal controls without AD features on autopsy and plasma from a patient with neuropathological signs of PSP. Inquiry with ClustalW 2.0 found no sequence homology between these 2 peptides and human Aβ. Several peptides bound predominantly plasma from the PSP patient (29 peptides), the autopsy-confirmed AD plasma pool (22 peptides), and the plasma pool from elderly controls without signs of AD on autopsy (34 peptides). The probability of this occurring by chance is $1.25 \times 10^{-7}$.

Influence of print run variability: it was a significant problem during my experiments. This is partly explained by the fact that the microarray platform was modified while I was standardizing the immunoassay (i.e., my initial experiments were done with a microarray with 4.000 random-sequence peptides). Most of my experiments involved microarrays with a solid phase consisting of 2 different sets of 10,000 random-sequence 20-mers covalently attached to a glass slide's surface. The peptides on each

microarray were different, designed with Glycine-Serine-Cysteine linkers at either the carboxyl (CIM1.0) or amino (CIM2.0) terminus for slide adherence. Also, peptide synthesis and printing on the microarray was different for both microarrays: CIM1.0 peptides were synthesized by Alta Biosciences (Birmingham, UK) and spotted in duplicate using a NanoPrint LM60 microarray printer (ArrayIt, Sunnyvale, CA), while CIM2.0 peptides were synthesized by Sigma (St. Louis, MO) and printed by AMI (Tempe, AZ) using a piezo printer. In addition, problems were detected as the microarray was developed, including issues with peptide mixture, pH, concentration, printing, and handling. As a result, the reproducibility of results depended heavily on the print run. For instance, plasma sample replicates had a high correlation (i.e., >0.8) if the same print run was used, but less correlation (i.e., 0.4 or less) if different print runs were employed. Furthermore, when many print runs are compared, the described AD immunosignature became less defined or effaced altogether.

Similarly, if the training set of peptides that distinguished AD from controls with the Banner-Sun Health samples does not work if samples from the same or a different cohort (ADNI) are assayed on slides from different print runs. Figure 17 (below) demonstrates this variability when ADNI samples are run on slides printed at different times at our laboratory.

From these experiments, I learned that experiments required the same print run in order to avoid problems with reproducibility.

Figure 17: Print-run variability.

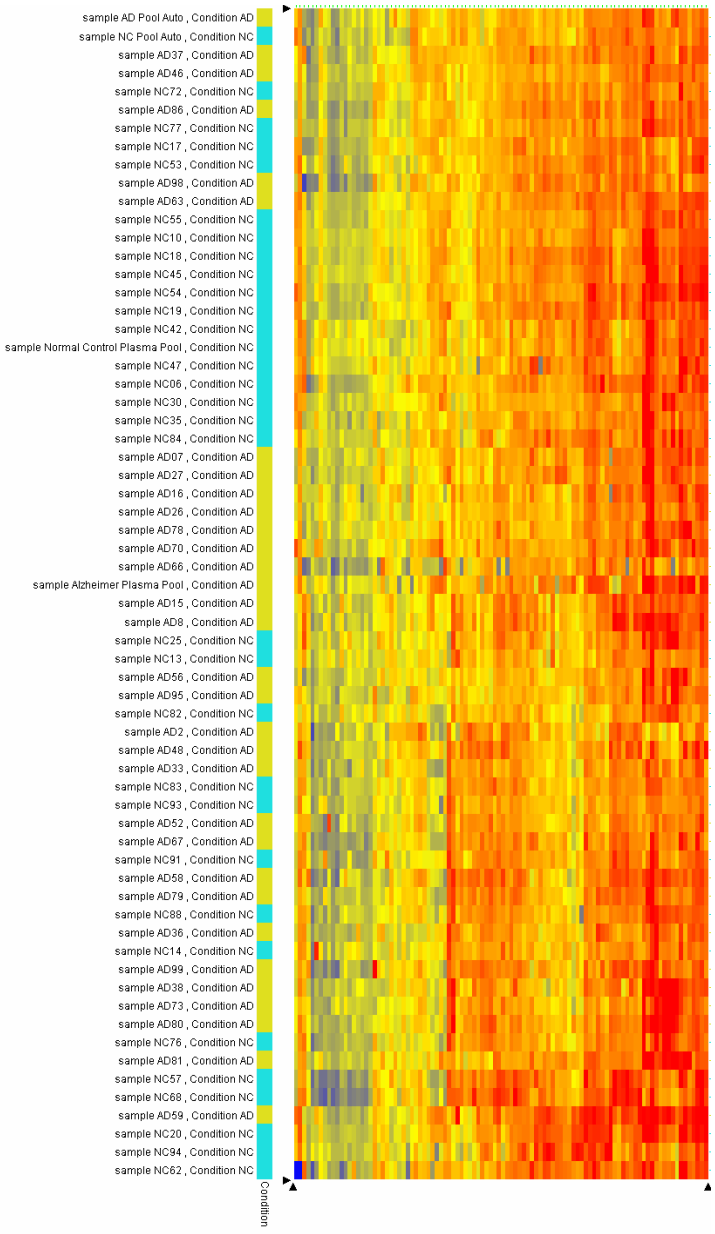


Fig. 17. Variability of results because of utilization of multiple print runs.

Stability of human immunosignature: as I described in the previous chapter, the immunosignature platform offers the opportunity of tracking the immuno-reactivity of plasma against different microarray peptides overtime. Finding whether a signature is stable overtime in humans is relevant for two practical reasons: firstly, if the overall immuno-signature is unstable, then the technique may not be suitable for future application as a diagnostic test; conversely, if the signature is stable, then it becomes pertinent to know at which point exactly it diverges from normal signature. In order to explore whether an antibody signature in humans remains constant overtime or disappears on follow-up, we assayed 2 plasma samples obtained several months apart from 5 patients with AD (including the 4 autopsy-proven cases), 6 normal elderly controls (including the 4 cases with autopsy) and the patient with diagnosis of PSP on post-mortem examination. Figure 18 shows that, using a single print-run, plasma samples taken at time zero strikingly align with their own follow-up samples. Moreover, 53 microarray peptides are capable of discriminating between AD and control plasma, whereas the PSP patient exhibits an intermediate pattern. On a Principal Component Analysis (Figure 19), AD plasma samples appear to aggregate away from controls, no matter if samples were taken at time zero or thereafter. Conversely, no discernible pattern was noted when time points (time zero versus follow-up) were used as the clustering paradigm. Similar results were noted when the

same print run of a second microarray platform with 10.000 different random-sequence peptides was used.

Figure 18: Stability of human immunosignature.

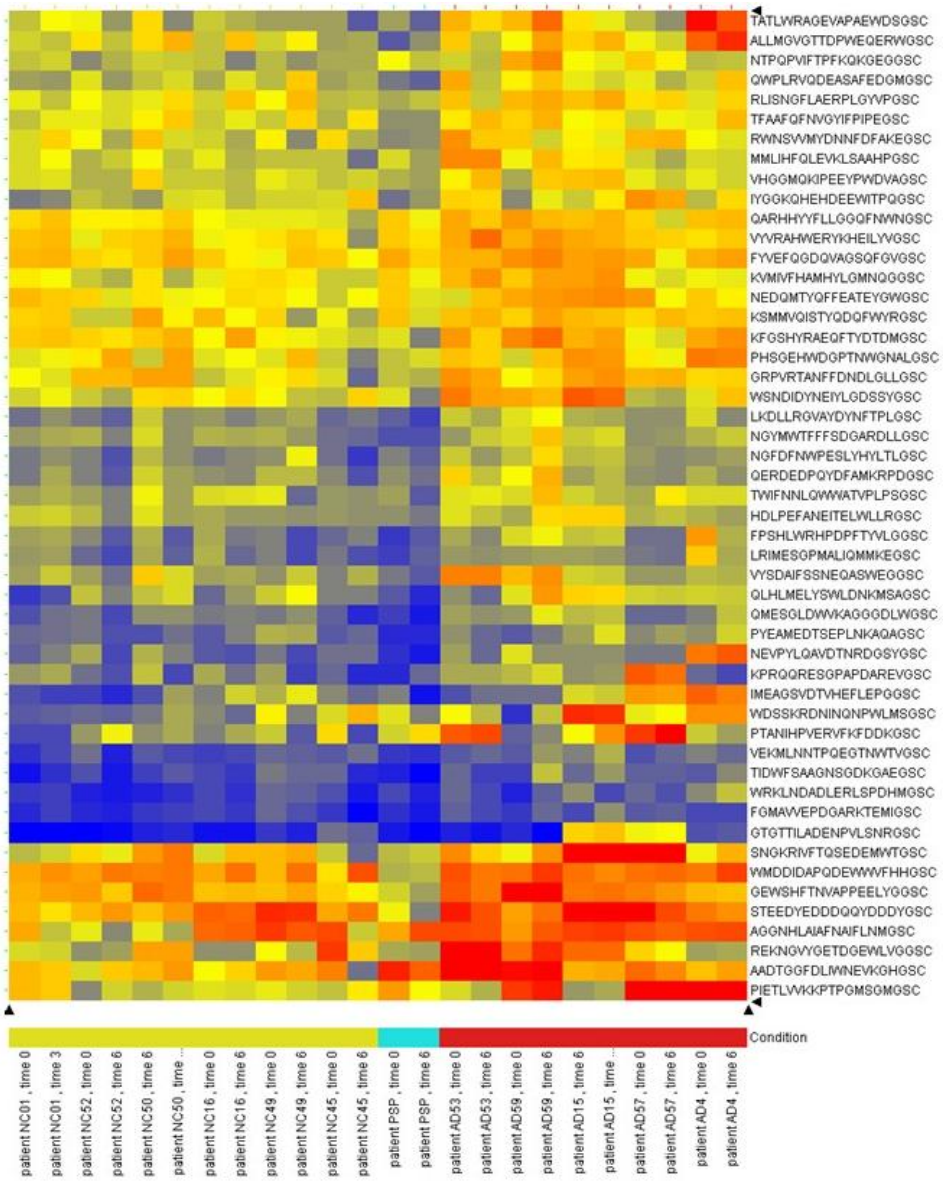


Fig. 18. Heatmap showing short-term stability of AD signature. Heatmap of plasma samples from AD and controls taken at time zero and follow-up (usually 6 mos).

Interestingly, one of these 53 peptides cross-reacts with an antibody that binds Aβ oligomers, while 7 peptides cross-react with plasma from TG mice vaccinated with a plasmid coding for Aβ $_{1-42}$. These observations suggest that AD plasma has a signature that can be distinguished from that of cognitively normal controls, and that the signature remains largely stable overtime.

Figure 19: Principal Component Analysis.

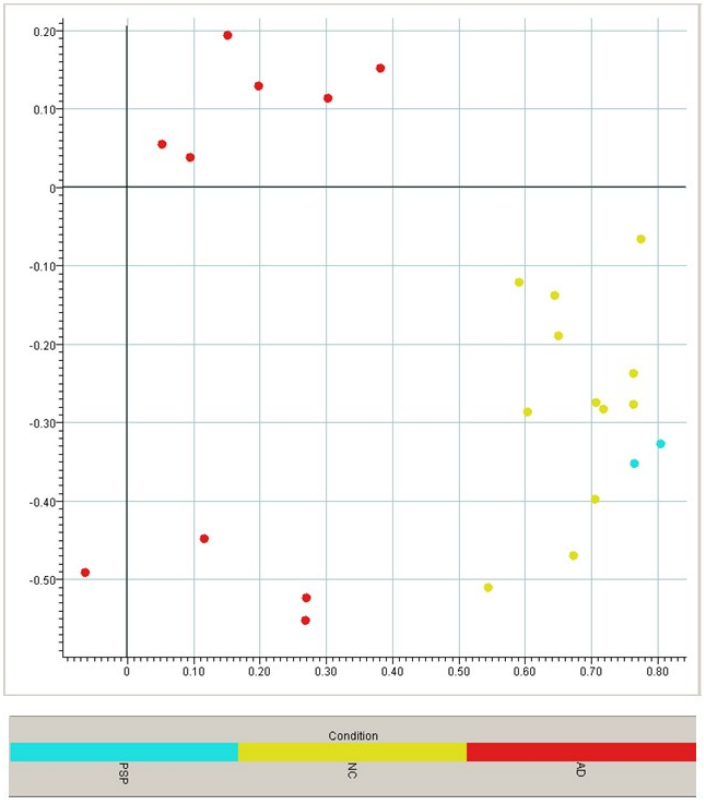


Fig. 19. Principal component analysis of plasma samples and their follow-up. Same plasma samples from Figure 6 are separated topographically in this representation. The AD is depicted in red, age-matched non-demented controls in yellow and the PSP patient in blue.

Alternative methods of data-mining: several microarray peptides are preferably bound by AD plasma, even if slides from different print runs were used. Table 4 (below) shows a ranking of the top-10 peptides from a total of 25 that were useful to distinguish AD plasma from elderly controls, regardless of print-run.

Table 4

Top peptides predicting AD using other statistical techniques

| Rank | ES | PEPTIDE SEQUENCE | *p* Value |
|---|---|---|---|
| 1 | 1.2731 | KIAMFKWLMGDNFNWKKGSC | 7.2e-006 |
| 2 | 1.2361 | RRSVQQYNFYLSQMNQYGSC | 1.2e-005 |
| 3 | 1.2018 | HKEAWREPWEGKYPFMTGSC | 1.9e-005 |
| 4 | 1.1841 | HFGAWRFFGTAWYARNPGSC | 2.5e-005 |
| 5 | 1.174 | ITEETMVQYEYVRIKQDGSC | 2.8e-005 |
| 6 | 1.1729 | MWKFQPRSNDNPARWNDGSC | 2.9e-005 |
| 7 | 1.172 | GFHGPGMLGKTGRLSYGGSC | 2.9e-005 |
| 8 | 1.1665 | KIGKNIHHQQRTMTYTWGSC | 3.1e-005 |
| 9 | 1.1597 | ISYLKTALALYFIVQESGSC | 3.4e-005 |
| 10 | 1.157 | KDRFLQKGKQMFVPPWKGSC | 3.6e-005 |

Where ES equals effect size (intensity threshold of ≥1.1).

Using a multiple variable Receiver Operating Characteristic (ROC) technique, 25 peptides (Table 2) provided efficient means to predict AD, regardless of the print-run used, when the effect size (ES) threshold was ≥1.1. The combined partial least square (PLS) showed a highly significant difference between AD and controls ($p=0.000002$). Using all of the top-10 selected peptides with Jackknife technique, an overall sensitivity and specificity of 83% was found. Using the highest ranking peptide by itself, sensitivity and specificity were 80%. When the 3 most significant peptides were used, 87% sensitivity 87% and 77% specificity was attained. Using the top 5 most significant peptides, the sensitivity was 90% and specificity 77%. Using the 8 most significant peptides, the sensitivity and specificity were 87%.

## Discussion

I have described herein a novel method to assess the immunoreactivity patterns of humans with or without AD. The used microarray platform features 10,000 random-sequence peptides that appear to behave as mimetics of the original targets of tested antibodies. I demonstrated that plasma of elderly patients with or without dementia reacts with microarray peptides, and that this reaction takes the form of different patterns that allowed us to discriminate, to certain degree,

between patients with or without disease. Furthermore, I showed that the bulk of the immunosignature is independent of Aβ.

I also identified a set of random peptides from the array with the highest binding by particular plasma samples, allowing plans for development of arrays with reduced number of peptides, or individual ELISAs using random peptides as antigen. This high-throughput screening platform has been used for identifying surface-immobilized peptides which specifically bind bacterial lipopolysaccharides [7-8], guiding production of synthetic antibodies [9] and characterizing humoral response to infections and vaccination [10], but not until now employed until now to evaluate a chronic disease such as dementia.

In a different proteomic approach to the assessment of dementia, a double-sandwich ELISA microarray featuring plasma cytokines was used to classify blinded samples from patients with clinical diagnosis of AD with almost 90% accuracy [11]. Compared to such platform, our microarray has 3 advantages: (a) it multiplies by 83.3 the number of analytes, (b) it assays antibodies, as opposed to cytokines, which are very stable, and (c) it is inexpensive, with average slide cost of about $50.

As previously said, AD diagnosis is an imprecise process of exclusion of other neurological entities, as illustrated by the misdiagnosis of the PSP patient. The gold standard of AD diagnosis is its characteristic neuropathology, which is rarely available to clinicians. Correct disease classification is imperative for obvious reasons; therefore, a simple test that helps refine the classification of dementia is needed. Also, many auto-antibodies, including anti-Aβ and anti-tau are found in normal elderly individuals at low titers. However, it is unclear whether titers change overtime or correlate with different clinical stages. I speculate that autoantibodies react to the microarray peptides, accounting in part for the observed signatures. This assertion is based on our finding of microarray peptides that bound commercial anti-Aβ antibodies and AD plasma, while a small portion of the AD immunosignature was blocked with Aβ.

Finally, I demonstrated that the antibody signature exhibited by elderly human subjects with or without AD remains mostly stable over time. Such antibody-binding pattern is different for each individual, in effect resembling a fingerprint, but sharing commonality with other individuals from the same group, an important effect when attempting to classify disease status. This particular property of the microarray platform, combined with its stability, suggests use as a diagnostic tool.

Without doubt, my studies have many limitations. Given the limited patient cohort, these results should be considered preliminary, but a proof of principle. I am currently assaying more plasma samples from AD patients and normal elderly controls to answer whether our microarray platform can be used to assist in the clinical classification of dementia. I also wish to confirm with larger numbers whether an immunosignature precedes the onset of cognitive impairment in humans. Given the slow progression of AD pathology (thought to develop many years in advance of symptom onset), an emerging humoral immune response, if any, could be detected and tracked in plasma.

## Closing remarks

The patterns formed with antibody binding to microarrays may have potential as a diagnostic tool for many diseases, including AD. Understanding the stability of these signatures over time is important because if time-point variations are small, then larger sample pools could be used without concerns about noise dampening the signature out over time. Furthermore, if there is a personal component of the signature, it could be useful for monitoring disease progression or response to treatment. Relative to the first issue, AD signatures seem distinguishable from age-matched controls regardless of whether they were early or late

samples. Relative to the second issue, there was clearly a personal component. The two samples from the same individual, including the PSP patient, were most similar to each other. This was also true for the non-AD samples, indicating that each person may have a distinctive immunosignature that is stable, analogous to a fingerprint.

The ability to create a signature for AD could have value in several ways including confirmation of standard diagnosis, enrollment in clinical trials and monitoring responses to treatment. Lacking practical tests to diagnose AD is not only problematic for patient care, it also represents a barrier for clinical trials, since many enrolled subjects will not have the disease of interest and therefore would not expect benefit from the studied intervention. Antibody-based diagnostic tests have experienced renewed interest with the development of microarrays featuring plasma cytokines, random-sequence peptides or peptoids. Surveying the antibody repertoire of individuals with or without a disease has many advantages. There are ~$10^9$ estimated different antibody specificities, reflecting a history of exposure to a variety of antigens [10]. Antibodies are produced early in the course of diseases, amplify a signal, and are easily retrieved from body fluids, including blood. Finally, antibodies are durable and can be easily stored, making them suitable for retrospective analysis. Until recently, immunoassays were limited by the traditional view that the

eliciting antigen needs to be known and immobilized to detect an antibody response. However, we developed unbiased platforms to evaluate AD using random-sequence peptides, which principally behave as mimetics of unknown antigens.

Ideally, AD should be detected at the pre-symptomatic or early symptomatic stages, when promising disease-modifying therapies are expected to exert greatest benefits. Unfortunately, these stages are also ill-defined aspects of the disease, susceptible to diagnostic misclassification with current standards. In summary, the evaluation of immunosignatures using random-sequence peptide arrays is a promising technique that can be applied to AD research. Future studies with more patients are needed to appraise the merits of immunosignaturing as a potential diagnostic test.

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# APPENDIX A

# EXPLORING THE PREDOMINANT FORM OF Aβ IN PLASMA

Using SDS-PAGE followed by western blotting of plasma samples from normal donors, I found that the predominant forms of circulating Aβ 1-40 and Aβ 1-42 are oligomers, constituted mainly by dodeca- and hexamers (Figure 1). These oligomers can be detected with 4 different mono- and polyclonal antibodies raised against Aβ 1-40 and Aβ 1-42 and quantified using a densitometry software. The relevant bands are also recognized by a specific anti-oligomer antibody. The pattern of band immunoreactivity was replicated in 9 normal donors. I did not detect circulating monomers or dimers, even after separating plasma fractions using size-exclusion high performance liquid chromatography (HPLC).

Figure 1: Circulating forms of Aβ.

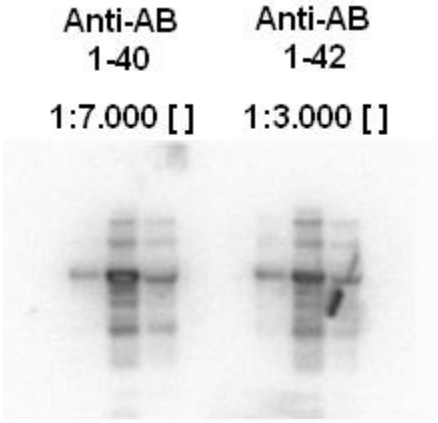



Fig. 1. Western blot of 3 plasma fractions separated using size-exclusion chromatography. There is a predominant 50 kDa band which roughly corresponds to the Aβ dodecamer.

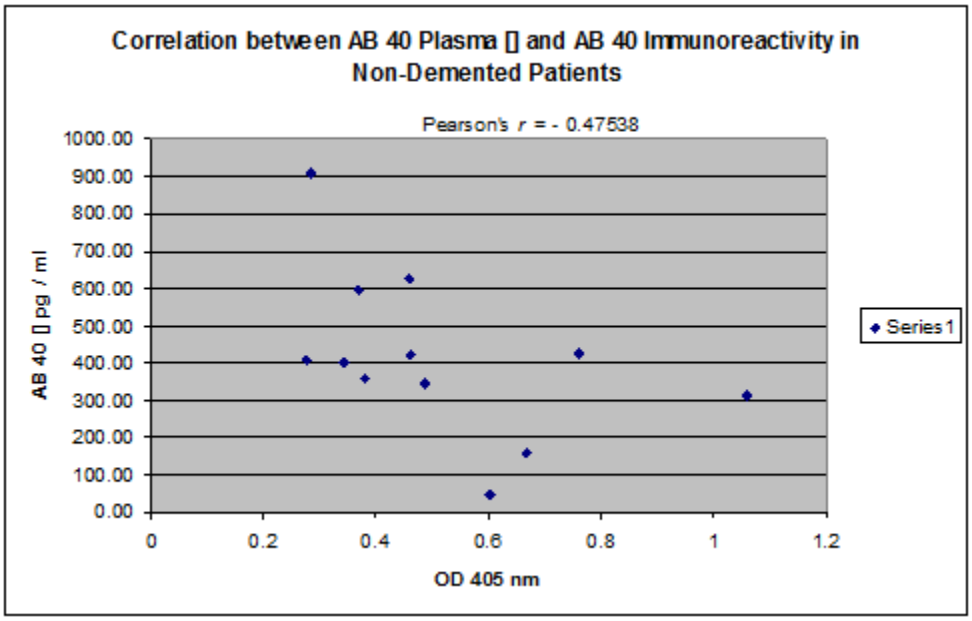
Based on this experiment, I postulate that individuals with AD (in particular, those with mutations leading to cerebral amyloidosis) may have circulating oligomers of different molecular weight compared to those in normal donors. Furthermore, AD cases may exhibit circulating monomers or dimers, while normal individuals do not. In other words, this very simple and widely available technology could be used as a diagnostic tool, if AD patients turn out to have a distinct pattern of immunoreactivity that sets them apart from normal individuals.

# APPENDIX B

# TESTING ANTI-Aβ ANTIBODIES IN HUMAN PLASMA

To confirm whether Aβ antibodies are present in human plasma samples, I developed an Enzyme-linked immunosorbent assay (ELISA) in which polysterene plates were coated with Aβ (1-40 or 1-42), with a concentration of 10 uM in Sodium Carbonate / Bicar-bonate buffer (pH=11). Synthetic Aβ was purchased from AnaSpec Inc (San Jose, California). The plates were blocked with 5% Bovine Serum Albumin in PBS and 0.05% Tween 20 for 1 hour, followed by plasma from patients dissolved at 1:100 in PBS. The primary antibody was detected with anti-human antibodies conjugated to HRP (Pierce) and then a colorimetric reaction was elicited by the addition of ABTS. Optic density was read at 405 nm with a spectrophoto-meter. The tested plasma came from patients from the Brain-Bank at Sun Health Institute; Aβ levels were reported by Dr Alex Roher, who measured levels using a double-sandwich ELISA standardized in his laboratory.

Figure 2: Relationship between Aβ levels and anti-Aβ antibody titer



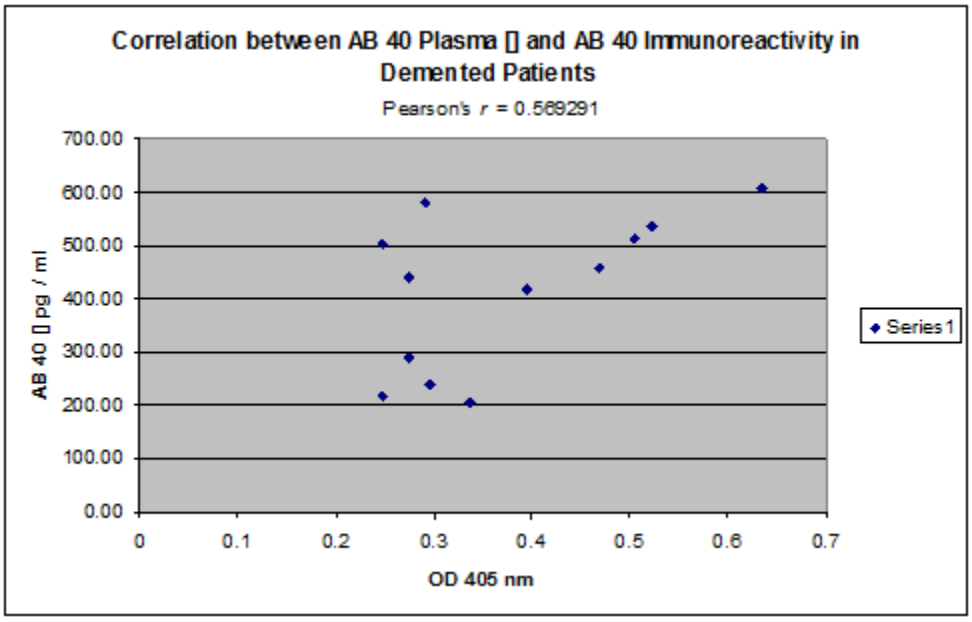




Fig. 2. Aβ levels decreased as anti-Aβ antibody titers rose in non-demented elderly subjects (i.e., negative correlation between Aβ levels and anti-Aβ antibody titers; Parson's r= -0.475), whereas AD patients had a contrary trend (r=0.569). Although the number of samples is small (n=12 for each patient group), this illustrates the point that in spite of testing the same antigen, the Aβ-binding antibodies may have different biological properties, depending on the selected population.