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Longitudinal Cerebrospinal Fluid Biomarkers of Alzheimer Disease: Movement Toward the Diagnosis, Prognosis and Staging of Disease

Courtney Sutphen
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Neurosciences

Dissertation Examination Committee:
John Cirrito, Chair
Anne Fagan
David Holtzman
Paul Kotzbauer
Chengjie Xiong

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by

Courtney Sutphen

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Abstract

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John Cirrito, Chair

Alzheimer's disease (AD) is a devastating neurodegenerative disease that slowly claims the memories and experiences that comprise the life experiences of individuals that suffer from the disease. Despite a continually accelerating pace of research and discovery, a viable therapeutic intervention for AD has yet to be realized. There are a multitude of factors that may contribute to this difficulty including the challenge of separating the overall disease of Alzheimer's from the clinically recognizable memory loss that occurs in what is now known to be the end-stage of the disease. Efforts to treat AD have increasingly turned toward very early disease states, before clinical signs and symptoms become apparent, as a number of clinical trials have failed to meet cognitive endpoints over the last 5-10 years – potentially due to the sole recruitment of individuals already experiencing significant cognitive decline.

One important aspect of AD treatment is identification. It is now recognized that the disease begins more than a decade before the signature symptoms of cognitive impairment become apparent. Identifying individuals in this "preclinical" disease state has become a primary focus of

many investigators who believe that AD must be targeted and fought well before the clinical manifestations of memory impairment appear.

Biomarkers, indicators of normal biological or pathological processes that may be studied as a means to give individuals a disease diagnosis, prognosis, or theragnosis – provided a treatment is available for the disease in question – are of paramount importance in many diseases. AD has proved a difficult target to nail down reliable, sensitive, and specific biomarkers. This is in part due to analytical difficulties in major, core biomarkers of disease and in part due to setbacks in clinical trials of promising therapeutic candidates.

The current work begins with an overview of biomarker modalities used in AD; however, the primary focus is on protein biomarkers in cerebrospinal fluid (CSF). CSF provides an intimate window to the central nervous system that, in the case of AD, has shown the ability to identify and monitor disease progress over time in cohorts of cognitively normal and demented individuals. In an effort to pinpoint AD before clinical signs and symptoms manifest, biomarker research in preclinical AD has become a robust area of investigation. CSF biomarkers of amyloid pathology, neuronal damage, and neuroinflammation are discussed in two independent cohorts: the Adult Children Study (ACS) from Washington University in St. Louis and the Alzheimer's Disease Neuroimaging Initiative.

The ACS cohort is comprised of middle-aged, cognitively normal individuals recruited on a volunteer basis from community dwelling participants with and without a family history of AD. The ADNI cohort is comprised of older individuals also recruited on a volunteer basis from community dwelling participants, though participants are recruited with respect to clinical status and include cognitively normal individuals, individuals with mild cognitive impairment, and individuals with AD, in addition to being older than the ACS cohort.

In both cohorts, it was found that CSF markers of amyloid plaques – one of two required pathological hallmarks that indicate AD – changed earlier than those of tau tangles, the second required pathological hallmark.

Currently, examining biomarkers on a group-wide basis is the best way to get an accurate picture of biomarkers at baseline and followup lumbar punctures (LPs). As the goal is to be able to give individual people a diagnosis and prognosis of their disease, the behavior of biomarkers is particularly interesting because studies have found that CSF Aβ42 changes up to 15 or more years before cognitive signs and symptoms become apparent and, hopefully, beginning treatment in this period will be helpful not only for diagnosing for individuals with AD dementia, but also for individuals with very early disease.

Chapter 1: Introduction

Portions of this chapter were published in the April 2014 issue of Biological Psychiatry[1].

1.1 Background

Alzheimer disease (AD) is a chronic, progressive neurodegenerative disease that slowly strips individuals of their memories and other cognitive functions. In the United States alone, an estimated 5.5 million people are living with AD in 2017, and an estimated 253 billion dollars will be spent caring for individuals with AD or other dementias, with both estimates predicted to rise substantially as the population ages[2]. Unlike diseases such as heart disease or stroke, deaths from AD increased more than 89% between the year 2000 and 2014 - making development an intervention to slow or halt the disease has a paramount focus[2]. As AD research moves forward, recent proposals from some leaders in the field have trended toward defining AD on a continuum as the disease is multifactorial, quite variable between individuals, and a large body of research is still needed to fully define AD from onset to end of life[3], though AD is currently diagnosed in clinical stages[4]. These stages fit in to the proposed view of a continuum; preclinical AD is defined by the absence of clinical signs or symptoms but evidence of pathological amyloid and tau accumulation in the brain, mild cognitive impairment (MCI) (or prodromal AD) is defined by the addition of mild clinical symptoms such as a consistent inability to remember appointments, and mild, moderate, and severe dementia are marked by further dramatic cognitive impairment ultimately resulting in complete dependence on caregivers for all daily activities. Instead of strictly defining each stage, research has begun to indicate – particularly in preclinical disease – that stages may not be clearly demarcated, and some lenience when making diagnostic, prognostic, and eventually theragnostic judgments may be needed[3].

Currently, a definitive diagnosis of AD still requires postmortem identification of the pathological hallmarks of the disease: extracellular amyloid plaques composed mainly of aggregated amyloid-β (Aβ) peptides and neurofibrillary tangles composed mainly of hyperphosphorylated forms of the microtubule associated protein, tau (P-tau)[4]. Clinical diagnosis of AD is based on guidelines established by the National Institute of Neurological Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINDS-ADRDA), although the sensitivity and specificity of such a diagnosis is lower than desirable[5]. The addition of biomarkers to the diagnostic criteria for AD may increase the sensitivity and specificity of both the diagnostic and prognostic capabilities currently available through clinical and cognitive assessment. One goal of studying biomarkers is to reliably identify those with AD pathological changes (preclinical AD), as well as predict the odds that such individuals will clinically progress and at what rate.

Intensive research has propelled the field closer to finding a disease-modifying therapy, but setbacks in clinical trials and inherent difficulties in successfully tracking disease progress pre-mortem continue to be major roadblocks. The most promising clinical trials to date have focused on anti-Aβ antibodies that bind either aggregated or soluble forms of Aβ and encourage the removal or neutralization of these species from the brain. Phase III trials of the anti-Aβ antibodies bapineuzumab and solanezumab in mild to moderate dementia believed to be due to AD ended in late 2012. Two bapineuzumab trials showed no effect on the primary outcomes, the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) or Disability Assessment for Dementia (DAD), either in carriers of the Apolipoprotein ε4 (APOE ε4) allele or in noncarriers, but did show encouraging changes in two disease biomarkers in the APOE ε4 carrier group compared with placebo[6]. Two additional trials were halted early due to these

negative results and did not show the same changes in disease biomarkers[7]. Two trials involving solanezumab also failed to show efficacy in the primary outcome of ADAS-Cog and Alzheimer's Disease Cooperative Study – Activities of Daily Living (ADCS-ADL) scale, but did show changes in blood and cerebrospinal fluid (CSF) amyloid levels, as expected based on the application of an anti-amyloid antibody[8].

In part because clinical trials have been unsuccessful in reversing, halting, or slowing cognitive decline, the investigation of AD biomarkers has been propelled forward. A widely held belief is that some of this failure is due to the exclusive enrollment of individuals who already exhibit mild or moderate dementia, stages of AD that are accompanied by robust neuronal cell death. At even earlier stages of the disease (very mild dementia and MCI due to AD), neuron loss in certain critical brain regions is already significant[9]. Thus, it is important to diagnose individuals at the preclinical and MCI disease stages - and enroll them in clinical trials - in order to identify and apply therapies that have the best chance of preserving normal cognitive function.

A key roadblock is the development of robust, reliable biomarkers: as in many other medical conditions, the primary aim of biomarker development is to provide a diagnosis or prognosis to individuals with a disease or to track disease progress or severity. These are of particular importance because the underlying causative pathology of AD begins as many as 15-20 years before the appearance of cognitive symptoms[10], meaning the identification of disease must take place when there are no clinically identifiable signs of impairment. In AD, biomarkers are most widely used in research – focusing on identification of individuals with preclinical disease, in differential diagnosis with other dementias, and perhaps most interestingly to provide a downstream indicator of treatment efficacy in disease-modifying agents.

As the AD community moves toward a treatment, it seems certain that biomarkers will play a key role in the process. It is the goal of investigators to determine the most effective combination of biomarkers to enable identification, differentiation, and treatment of the disease in question. Cognitive measures can serve as a biomarker of disease; however, CSF biomarkers as measures of underlying pathology and disease progression are the focus of this work, along with relevant investigations in imaging biomarkers (magnetic resonance imaging [MRI] and positron emission tomography [PET]). Each modality presents its own challenges for identifying and/or developing viable markers from assay validation, intra- and inter-lab consistency in measurement to accurate identification of when and how biomarkers change during disease, and these challenges add to those in the overarching field of biomarker usage in the clinical diagnosis of AD such as how to best use biomarkers in regular clinical practice.

Nevertheless, within the last five years, groundbreaking clinical trials such as the Dominantly Inherited Alzheimer Network – Trials Unit (DIAN-TU)[11], the Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease (A4) trial[12], and others have begun enrolling preclinical individuals, as identified by genetic testing or biomarker status, and will use various biomarkers to track drug target engagement and/or as endpoint measures of drug efficacy. The DIAN-TU will hopefully be particularly informative as to the temporal ordering and efficacy of several multimodal biomarkers in a genetically defined population. The comparison of biomarkers in the DIAN-TU population vs. sporadic, or late onset, AD could eventually allow the identification of the optimal window for therapeutic intervention the sporadic population. A number of caveats apply, particularly the development of an efficacious AD intervention, but one of the primary underpinnings of this theory is that ADAD and sporadic AD are the same disease; therefore investigations concerning biomarker trajectories of all modalities are of paramount importance.

1.2 Biomarker Modalities

As outlined above, this work pertains largely to CSF biomarkers of AD, with relevant imaging biomarkers included as ancillary analyses. However, it is necessary to consider all biomarker modalities when attempting to identify a progression of disease indicators. Section 1.2 is dedicated to a brief literature review of current fluid (CSF, plasma, serum), imaging (MRI, PET), and cognitive biomarkers that aid investigators in better defining the spectrum of AD from preclinical through mild cognitive impairment and ultimate dementia. A brief summary of biomarkers covered in Chapter 1.2 can be found in **Table 1.1**.

1.2.1 Core CSF Biomarkers

CSF is considered a prime source for AD biomarkers because many proteins and metabolites in CSF directly reflect the internal milieu of the brain. A lumbar puncture (LP) is necessary for the collection of CSF which makes it somewhat more invasive than a blood draw. However, complications stemming from LP are not frequent[13,14], and when the procedure is performed by experienced clinicians, it is usually not painful.

Three proteins are typically considered the gold standard for AD CSF biomarkers – $A\beta_{42}$, total tau (Tau) and tau phosphorylated at threonine 181 (P-tau). Aβ and tau proteins are the most abundant components of amyloid plaques and neurofibrillary tangles, respectively. Each of these analytes has been extensively studied and validated in a variety of cohorts world-wide and, while absolute concentrations of each marker may vary, similar results have been reported in multiple studies.

Amyloid β_{42}

$A\beta_{42}$ is a 42 amino acid peptide created from the processing of the amyloid precursor protein (APP). There are multiple lengths of Aβ peptides; the 42 amino acid form is the most abundant

in amyloid plaques. Significantly reduced levels of CSF $A\beta_{42}$ in individuals diagnosed with AD, compared to cognitively normal, age-matched individuals, is one signature of amyloid plaques[15]. Studies have shown that CSF $A\beta_{42}$ levels correlate inversely with amyloid plaque load in the brain as determined by postmortem histology[16] and concomitant *in vivo* plaque measurement using amyloid imaging, regardless of clinical status[17–23]. CSF $A\beta_{42}$ is likely low in the presence of amyloid deposition due to its sequestration in plaques[24]. Continued investigation on the link between CSF and imaging Aβ levels indicates that CSF $A\beta_{42}$ begins changing prior to amyloid imaging[25,26], which replicates changes seen in autosomal dominant AD (ADAD)[27]. Low CSF $A\beta_{42}$ is useful as a marker that predicts future clinical disease progression and rate of cognitive decline, especially in the early clinical stages of the disease[28–30]. Most recently, longitudinal studies of AD biomarkers have shown CSF $A\beta_{42}$ is perhaps the most reliable indicator of preclinical AD[31–33]. $A\beta_{42}$ alone is not a sufficient biomarker for AD diagnosis and prognosis[34,35], nor does it mark the presence of other AD pathologies, however, more recent recommendations do support the use of CSF $A\beta_{42}$ to supplement clinical evaluation for differential diagnosis or to aid judgment in atypical or unclear cases[36,37].

Tau

Tau is a microtubule associated protein that, when quantified in the CSF, is considered to be a biomarker of neuronal injury in AD. High levels of tau in the CSF may reflect neuronal damage, as is suggested by increases in tau after acute neuronal injury such as stroke, traumatic brain injuries, and Creutzfeld-Jakob disease (CJD)[38,39]. Phospho-tau levels correlate with total tau and also correlate closely with neurofibrillary tangle load in AD[40]. Tau is normally released by neurons in the absence of cell death. Evidence from wild type and transgenic mice expressing mutant human Tau (P301S) suggests tau is continuously secreted from healthy neurons into the brain interstitial fluid space[41]. In addition, both soluble and aggregated forms of tau have been

shown to be secreted by cultured cells[42]. CSF tau and P-tau are significantly increased in AD and Mild Cognitive Impairment (MCI)[29(p200),40,43]. More recent studies indicate levels of both tau and P-tau are also increased in preclinical AD, though likely after Aβ42 levels have already dropped[44], which mirrors changes seen in ADAD[27]. Similar to CSF Aβ42 versus PET Aβ42, CSF Tau may also begin to increase prior to tangles being visualized with Tau PET imaging[45]. Levels of P-tau in CSF are also associated with disease progression in AD cases[46] and may aid in differential diagnosis between AD and other dementias[47]. Some longitudinal studies have also indicated that tau and P-tau change after indicators of amyloid abnormality[31]. Tau and P-tau are similar to Aβ42 in diagnostic and prognostic performance but are not sufficient biomarkers on their own.

Tau or Ptau$_{181}$ to Aβ42 Ratios

The Tau/Aβ42 ratio, when analyzed, has a history of good performance in identifying individuals with AD. The Geneva Task Force for the Roadmap of Alzheimer's Biomarker report on the clinical validity of CSF biomarkers in AD states that the ratio of Tau or Ptau to Aβ42 often is superior to Aβ42 alone when predicting AD in individuals with MCI[48]. This was seen both in biomarker meta-analysis reports as well as primary research reports, however, the Task Force recommended against the use of such ratios because they may be abnormal in individuals where CSF Tau alone increases (such as in CJD).

1.2.2 Non-Core CSF Biomarkers

Because it is likely that no single biomarker will perform satisfactorily on its own, identification and development of additional CSF biomarkers that do not directly reflect AD pathology (plaques and tangles), but instead reflect more general processes such as neurodegeneration and inflammation might be very useful. Unbiased approaches such as proteomics and multi-analyte profiling have been used to identify novel fluid biomarkers. A unique challenge presented by

these unbiased approaches, however, has been identifying biomarkers that have high enough sensitivity and specificity to warrant investigation in large cohorts. Five CSF proteins have proved useful in differentiating AD from cognitively normal individuals in large, independent cohorts: visinin-like protein 1 (VILIP-1); Neurogranin (Ng); synaptosomal associated protein 25 (SNAP-25); chitinase-3-like protein 1 (YKL-40); and neurofilament light (NfL).

Amyloid β40 and Aβ42 to Aβ40 Ratio

Aβ40 is the most abundant isoform of Aβ in the CSF at roughly ten times the concentration of CSF Aβ42, but relatively less reported in studies of AD biomarkers. One large meta-analysis in 2011 did not report on Aβ40 as a CSF biomarker[49], while a more recent meta-analysis from 2016 reported that levels of CSF Aβ40 did not differ significantly between individuals with MCI due to AD and MCI, but did report a "significant but minor" average effect size in distinguishing AD from control individuals, with Aβ40 levels being slightly lower in AD[50]. However, unlike CSF Aβ42 and the Tau(s), this result was not wholly consistent across all 25 papers analyzed; 7 of 25 studies showed almost no difference between AD and control individuals and one study showed higher levels. These meta-analyses reflect an interesting attitude in the research community of Aβ40 perhaps being less important than Aβ42 as a diagnostic or prognostic tool. A more recent study does report lower levels of CSF Aβ40 in individuals with preclinical AD as defined by low CSF Aβ42 levels and no cognitive impairment[44]. Reported in Chapter 2 of the current work, it was found that levels of Aβ40 decreased over time in middle-aged cognitively normal individuals, but findings were inconsistent between two assay platforms[31]. These inconsistencies likely contribute heavily to the relative dis-use of CSF Aβ40 as a common biomarker for AD.

Despite CSF Aβ40 not performing as well as CSF Aβ42 as a biomarker of AD, the Aβ42 to Aβ40 ratio has nonetheless been proposed as a useful CSF biomarker because, while CSF Aβ42

levels may also be reduced in individuals with subcortical white matter lesions, CSF Aβ40 is unaffected[51], resulting in better performance of the Aβ42 to Aβ40 ratio than Aβ42 alone in detecting amyloid pathology in MCI and AD. These and other studies indicate that the ratio may be a better predictor of AD pathology by adjusting for differences in overall amyloid production between individuals[48], which becomes particularly evident when investigating CSF amyloid compared with amyloid PET measures. In this case, when assessing both CSF and PET amyloid, the concordance between CSF Aβ42 alone and amyloid PET is improved by substituting the CSF Aβ42 to Aβ40 ratio[51–54].

VILIP-1

VILIP-1 is a neuron-specific intracellular calcium sensor protein. Particularly expressed in cortical and hippocampal neurons, VILIP-1 is found at high levels in the dendritic compartment mainly associated with cell membranes[55]. Levels of CSF VILIP-1 have been shown to be elevated in stroke[56]. Immunohistochemical and cell culture studies on VILIP-1 localization in AD revealed an association with dystrophic neurites as well as amyloid plaques and tau tangles[57,58]. Increases in CSF VILIP-1 have been observed in AD compared with cognitively normal controls as well as individuals with MCI[59,60]. Elevated VILIP-1 levels also perform as a strong predictor of future cognitive decline in individuals with MCI/very mild dementia and in cognitively normal controls[61–63]. Longitudinal studies on VILIP-1 have not yet been widely executed, though VILIP-1 and Tau correlate very well in the CSF, suggesting that VILIP-1 may increase after amyloid changes are evident in preclinical disease[31], and in ADAD may begin to decline after incipient cognitive impairment[64]. VILIP-1 may also aid in the differentiation between AD and other dementias such as Dementia with Lewy Bodies (DLB)[65,66]. When studied in conjunction with CSF Aβ42, Tau and P-Tau, VILIP-1 performs similarly to the Tau(s), indicating that it will not be a sufficient biomarker on its own.

Ng

Neurogranin is a postsynaptic calmodulin-binding protein expressed largely in neuronal dendrites that seems necessary for sensing calcium concentration and calmodulin activity, thus strengthening synapses[67]. In AD, Ng is elevated in the CSF compared to cognitively normal individuals and provides diagnostic and prognostic utility similar to that of the core AD CSF biomarkers[68,69]. In preclinical AD, Ng is also elevated and predicts cognitive decline[70,71], indicating it may be a useful biomarker across multiple disease stages. Longitudinally, levels of Ng in CSF increased in cognitively normal individuals at risk of developing dementia though did not change over time in individuals with MCI or AD[72]. Studies assessing Ng for differential diagnosis provided evidence that elevated Ng may aid in differentiation AD from behavioral variant frontotemporal dementia (bvFTD), LBD, Parkinson Disease (PD), progressive supranuclear palsy, and multiple system atrophy[73,74], and major depressive disorder[75]. In many of the studies cited above, other CSF proteins such as Tau were measured, with Ng correlating with Tau and P-Tau. Further longitudinal research is necessary to determine where and how Ng best fits in the staging, diagnosis, or prognosis of AD.

SNAP-25

SNAP-25 is a presynaptic t-SNARE protein involved in regulating neurotransmitter release through facilitation of synaptic vesicle release at presynaptic terminals[76]. Much of the work on SNAP-25 pertaining to AD has been done in postmortem tissue, where decreases in SNAP-25 protein levels are seen in AD, and other dementias, compared with control individuals[77–79]. Few studies in the CSF of demented compared with cognitively normal individuals have been reported. The first showed higher levels of SNAP-25 fragments in AD and prodromal AD compared with cognitively normal individuals[80]; the second showed increased levels of SNAP-25 in the CSF of individuals with advanced PD[81]. Much work remains to determine the temporal

ordering of changes in SNAP-25 compared with other CSF proteins in AD, including longitudinal cohort studies.

NfL

Neurofilament Light is the lowest weight member of the neurofilament family found in neurons. Heavily involved in axonal structure, NfL levels in CSF have been studied in a wide variety of neurodegenerative disorders and are considered a marker of white matter lesions[82,83]. In amyotrophic lateral sclerosis (ALS)[84], FTD[85],CJD[86], and Parkinson's Disease Dementia (PDD)[87], as well as vascular dementia (VaD)[88], CSF NfL is elevated compared with normal controls. A particular focus has been the differential diagnosis of AD and FTD using CSF NfL, in which consistently higher levels of NfL were seen in FTD compared to AD[89–91]. As with other non-core markers, significant work remains to determine the usefulness of NfL for differential diagnosis among neurodegenerative diseases[92,93]. In AD, NfL levels are increased at the MCI and dementia stages of clinical impairment and may indicate disease progression[94,95] but are not elevated in preclinical disease[44,96], suggesting a later stage of NfL involvement, though further research is needed.

YKL-40

YKL-40 is the one of two non-neuronal associated proteins to show promise as a CSF AD biomarker; it is an astrocytic protein upregulated in a variety of neuroinflammatory conditions[97–99], however, despite being a chitinase, YKL-40 has no chitinase activity and its exact function remains to be elucidated. Early reports on YKL-40 in CSF showed elevations in AD vs. control individuals, with diagnostic and prognostic performance on par with the core CSF biomarkers[100,101], though results were not universally replicated[102]. More recent studies, including longitudinal analyses, have shown that YKL-40 levels increase with advancing age which may complicate its analysis in older cohorts[31,103]. However, there is evidence that YKL-40

could be useful to differentiate between AD and other dementias such as VaD[104], DLB and PDD[103], but not FTD[105,106]. YKL-40 needs more research, particularly longitudinally, to determine its best application as a biomarker of neuroinflammation in AD.

TREM2

The other non-neuronal protein is soluble Triggering Receptor Expressed on Myeloid Cells 2 (sTREM2), associated primarily with microglia in the brain. Studies in DIAN and a LOAD cohort showed increases in sTREM2 in MCI and AD compared with control individuals[107,108], but a third study showed only an association with sTREM2 and age[109]. Though promising, TREM2 is in very early stages of investigation as an AD CSF biomarker.

1.2.3 Blood Biomarkers

Identification of blood biomarkers (plasma and serum) for AD has proved particularly challenging. Possible contributing factors include low expression of target biomarker proteins in the periphery that could make quantification of central nervous system (CNS)-derived analytes difficult, as well as the relatively higher levels of total protein in plasma and serum compared with CSF which could interfere with analyte detection.

Core and Novel Analytes in Blood Serum and Plasma

Findings from studies exploring blood-based biomarkers in AD have been largely inconclusive. The core biomarker Aβ42, as well as the non-core Aβ40, alone have historically shown no utility in differentiating AD from control individuals or assessing prognosis[110,111], though differences have been found between APOE ε4 carriers versus noncarriers[110,112].However, one study did report reductions in the plasma Aβ42/Aβ40 ratio in individuals with MCI and in those who transitioned from cognitively normal to MCI[113]. Two recent studies also found a predictive association between (1) the plasma Aβ42/Aβ40 ratio and cortical amyloid burden measure by

PET[114] and (2) the plasma Aβ42/Aβ40 an amyloid positivity defined by PET or mass spectrometry, in this case with high accuracy and precision[115].

Studies assessing plasma tau are at a similar uncertain stage due to the recent development of Tau PET agents[116,117], though one study states that plasma tau and NfL levels may aid in identifying or ruling out neurodegeneration in rapidly progressive neurological syndromes[118] and another identified elevated plasma $Ptau_{181}$ in AD and Down Syndrome (DS) compared to control individuals[119]. The non-core analyte NfL has had some success in plasma[120,121] and serum[122] in differentiating MCI and AD individuals from control individuals. Plasma Ng, however, was not able to differentiate MCI or AD from control individuals[123].

Much of the focus in blood-based fluid biomarkers has involved novel multi-analyte panels[124–127]. While results from these studies generally show efficacy in differentiating AD from control individuals, in some cases even longitudinally[128], there are important factors to consider: often the cohort size or composition makes it difficult to translate results to other cohorts; these studies are often very recent and therefore not replicated in additional cohorts; the use of general multi-analyte panels may result in lower success rates in differential diagnosis between AD and other dementias.

Overall, the state of blood biomarkers in AD has been held in its infancy for a number of years. Largely, this status hinges upon a lack of reproducibility and an unclear path to clinical utilization[129]. As these are very early stage, many manuscripts cited above use assays developed "in-house" and are different from cohort to cohort. Lessons learned from studying CSF biomarkers indicate that a common technique or assay would likely be beneficial to more clearly outlining biomarker proteins in blood.

1.2.4 Imaging Biomarkers

Imaging biomarkers capture a broad range of AD-associated processes, from brain size and structure to the presence of protein aggregates. Imaging biomarkers can be non-invasive or moderately invasive based on the modality used (ie, MRI vs. PET with radioactivity). One particular advantage for imaging biomarkers is the ability to image and track special patterns (e.g. regional atrophy, binding of PET tracers in regions with pathology). As with fluid biomarkers, inter-lab standardization is paramount but can be quite difficult due to the use of different makes and models of scanners, each with their own idiosyncrasies[130].

MRI Biomarkers

Volumetric MRI is one of the most studied imaging biomarkers. The measurement of the size of a brain region at a single time point and within individuals longitudinally allows for detection of atrophy in either whole brain or targeted areas[131–133]. In many studies, a marked decrease in volume is observed in AD – this is seen both in normalized whole brain volume and in specific areas such as the hippocampus and entorhinal cortex[134,135]. Volumetric MRI performs as well as the CSF gold standard biomarkers for diagnosis and prognosis, from preclinical through advanced disease states, and has a rich history of investigation using both single timepoint and longitudinal data[136–140]. What remains to solidify MRI volumetric biomarkers for clinical use is similar to CSF biomarkers[141] – reproducibility in large, independent cohorts; standardization of both imaging and image analysis throughout the field; longitudinal analysis; and identification of the most accurate and clinically useful brain regions for AD diagnosis and prognosis, particularly as regions such as caudate nucleus or hippocampus have not differentiated between AD and other dementias[142,143].

Both task-based and resting state functional MRI (fMRI) are promising imaging biomarkers for AD[144]. The difference in magnetization between oxygen-rich and oxygen-poor blood can be

measured using fMRI to detect changes in connectivity between areas of the brain while an individual is performing a task or resting. Some studies have shown fMRI differences between individuals with MCI vs. controls on task-based assessments[145]. Of particular interest in resting state fMRI assessments is the default mode network (DMN), a network of brain regions that is most active when a person is not engaged in a specific cognitive task and is deactivated when an individual is externally stimulated or is engaged in a specific task. One recent, large study of 500 individuals showed progressive decline in resting state functional connectivity across multiple networks with disease progression[146]. Whether fMRI will prove useful in differential diagnosis, prognosis, or in clinical trials awaits further studies, though it has shown promise in recent preclinical and longitudinal studies[147,148].

PET Biomarkers

PET biomarkers rely on radionuclide tracers specific to a molecule of interest within the body. Three radioligands are used regularly in AD research and, with amyloid PET, in clinical practice to aid in differential diagnosis of difficult or uncertain dementia cases[149]; ^{18}Fluorodeoxyglucose, or FDG PET; amyloid PET; and tau PET. FDG PET is a highly studied radioligand that acts as an indicator of glucose metabolism and, by proxy, neuronal activity. A number of studies have shown prognostic value for FDG PET[150] in identifying individuals who will progress from MCI to AD, but the usefulness of FDG PET as a diagnostic in differentiating cognitively normal from AD individuals is relatively less[151]. Studies using smaller cohorts have also reported longitudinal changes in FDG PET in individuals with MCI[152,153(p3)].

Specific to amyloid-aggregating diseases, the use of amyloid PET has increased dramatically since the development of Pittsburgh Compound B (PiB), the first radioligand specific to fibrillar Aβ[154]. There are now 3 FDA approved amyloid-imaging agents, with evidence pointing to

efficacy in diagnosis in both research and clinical settings[150], though extensive work remains to determine the relationship between amyloid PET and prognosis. Similar to CSF analytes, standardization of amyloid PET is a major point of concern[155], including for longitudinal study[156].

Tau imaging remains in the early stages of development but there are indications it may be useful in assessing the extent of tau pathology present in individuals with AD, as well as potentially aiding in differential diagnosis between AD and other tauopathies such as progressive supranuclear palsy (PSP)[157,158]. One very early longitudinal report indicates Tau PET may have prognostic value in MCI and AD[159], and yet another indicates it may have value in identifying high tau aggregation levels in preclinical AD[160].

Table 1.1 Summary of Biomarker Modalities and Characteristics

Biomarker	Changes Observed in AD vs Cognitively Normal Individuals	Disease Stage when Reported	Longitudinal Data Available	Major Obstacles to Validation as an AD Biomarker
CSF				
Aβ42	Decreased	Preclinical, MCI, Dementia	Yes	Assay validation, reduction in intra- and inter-lab variability
Aβ40	Small Decrease/Unclear	Preclinical, MCI, Dementia	Yes	May not be robust enough on its own
Aβ42/Aβ40	Decreased	Preclinical, MCI, Dementia	Yes	Validated assays, continued investigation in large, varied cohorts
Tau	Elevated	Preclinical, MCI, Dementia	Yes	Must be used in combination with other biomarkers
P-tau	Elevated	Preclinical, MCI, Dementia	Yes	Must be used in combination with other biomarkers
Tau/Aβ42	Elevated	Preclinical, MCI, Dementia	Yes	While reliable, Tau and Aβ42 may be better applied individually
VILIP-1	Elevated	Preclinical, MCI, Dementia	Yes	Continued investigation in large, varied cohorts
SNAP-25	Elevated	MCI, Dementia	No	Continued investigation in large, varied cohorts
Ng	Elevated	Preclinical, MCI, Dementia	Yes	Continued investigation in large, varied cohorts
NfL	Elevated	Preclinical, MCI, Dementia	No	Continued investigation in large, varied cohorts
YKL-40	Elevated/Unclear	Preclinical, MCI Dementia	Yes	May not be as robust as, e.g. sTREM2 due to age-associated elevation
sTREM2	Elevated	MCI, Dementia	No	Continued investigation in large, varied cohorts

Blood-Based Biomarkers				
Single Analyte (Aβ(s), Tau(s), NfL,)	Variable	Preclinical, MCI, Dementia	Yes	Continued investigation in large, varied cohorts
Multi-Analyte Panels	Variable	Dementia	Yes	May continue to have limited viability, though multi-analyte approaches may be useful in identifying novel single analytes
Imaging Biomarkers				
MRI	Increase in Atrophy	Preclinical, MCI, Dementia	Yes	Must be used in combination with other biomarkers
fMRI	Change in Connected Regions	Preclinical, MCI, Dementia	Yes	Must be used in combination with other biomarkers
FDG PET	Elevated	Preclinical, MCI, Dementia	Yes	Must be used in combination with other biomarkers
Amyloid PET	Elevated	Preclinical, MCI, Dementia	Yes	Continued investigation in large, varied cohorts
Tau PET	Elevated	Preclinical, MCI, Dementia	Yes	Continued investigation in large, varied cohorts

Table 1.1 summarizes the biomarker modalities covered in Chapter 1.2. Changes observed refers to the most commonly seen alterations in each biomarker in AD compared with cognitively normal individuals. Disease Stage when Reported refers to the stages of AD that a biomarker has published information available. Longitudinal data is available for the vast majority of the reported biomarkers, but further longitudinal research is required. The Major Obstacle column refers to only the most significant challenge for each biomarker. Continued investigation in large, varied cohorts is required to pinpoint the most useful diagnostic, prognostic or theragnostic characteristics of most biomarkers. Aβ42 and Tau(s) – are well recognized identifiers of AD pathology in living individuals but require further investigation as to the best application or technique for combinatorial analysis that will allow diagnosis or prognosis of AD on a within-person basis.

1.3 Biomarkers in the Diagnosis and Prognosis of AD

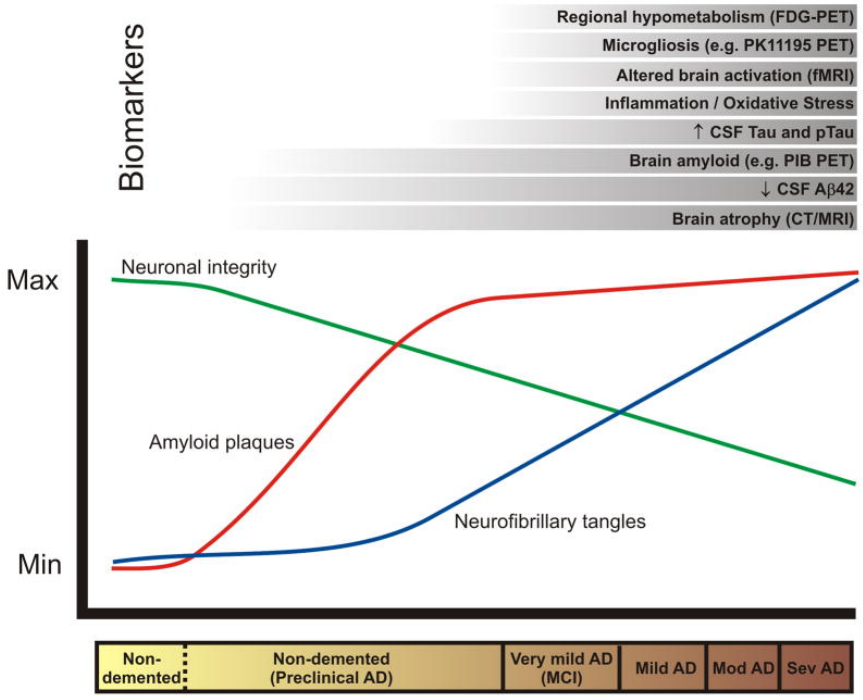
Clearly, a large body of work has been created in the study of biomarkers for AD. Despite this positive forward progress, an immense amount of work remains in defining high quality biomarkers that could be implemented worldwide. **Figures 1.1-1.4** present a brief glimpse in to the evolution of our broad understanding of AD in terms of biomarkers.

1.3.1 Hypothetical Biomarker Models

In an ideal scenario, AD biomarkers will identify individuals during preclinical disease, well before the neuronal death that drives cognitive impairments and within a window allowing for secondary, or even primary, prevention of the disease. In 2009, the first of a series of hypothetical models was published showing how markers such as CSF Aβ42 and tau(s), fMRI, or FDG PET might change along the course of AD[161]. Biomarker behavior as a whole was in an early stage of exploring preclinical AD, and most biomarkers are grouped together or shown as changing in the very mild stage of AD.

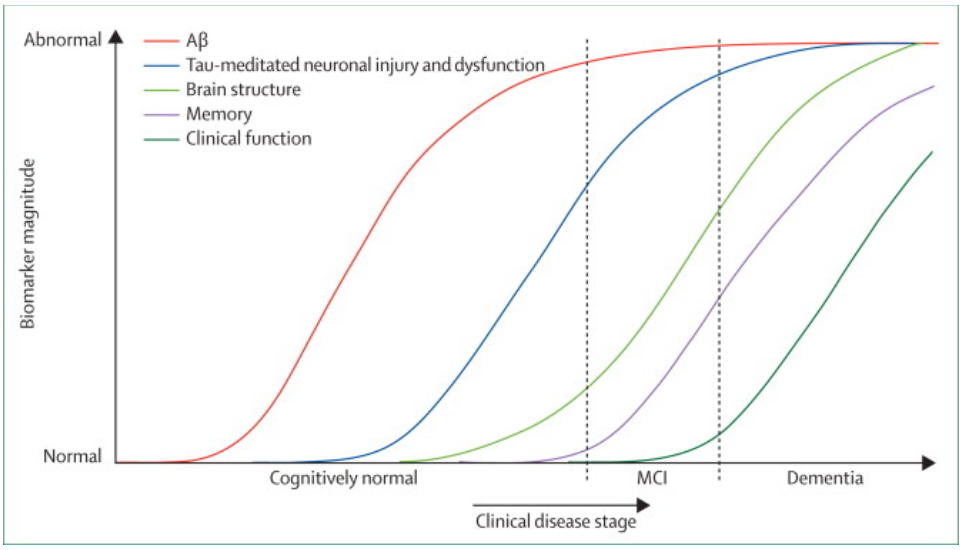
The second group of figures (**Figure 1.2 and 1.3)** were published in 2010 and 2013, respectively. The first of these figures is similar, grouping markers by Aβ, tau, brain structure, and clinical markers[162]. Both **Figures 1.1** and **1.2** propose a temporal ordering of biomarkers shifting from normal to abnormal along a disease continuum, but the level of detail was necessarily low given the state of the field at the time.

Figure 1.1 Hypothesized Relationship Between the Timecourse of Changes in Various Biomarkers in Relation to the Neuropathology and Clinical Changes of Alzheimer's Disease



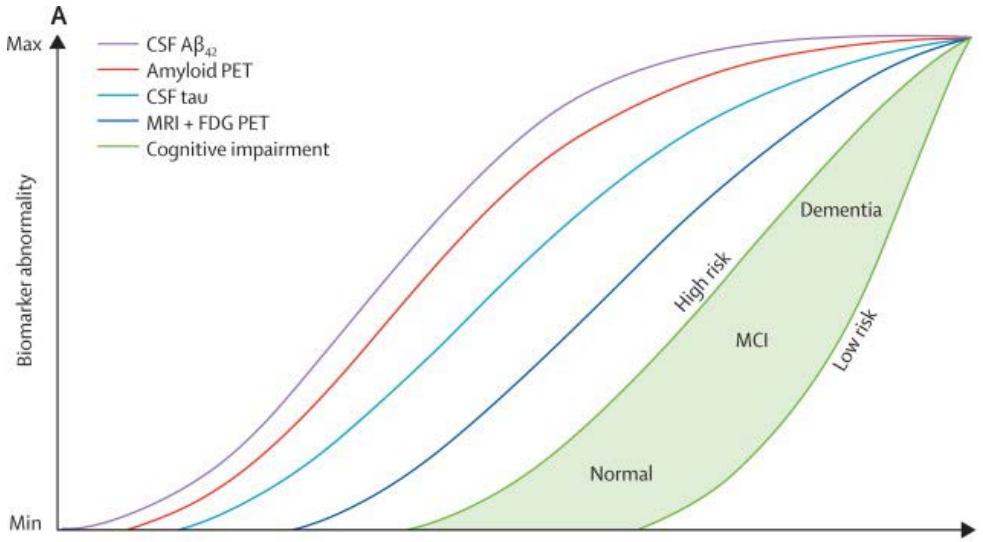
The first in a series of evolving proposed biomarker curves. From Craig-Schapiro et al., 2009[161].

Figure 1.2 Dynamic Biomarkers of the Alzheimer's Pathological Cascade



The second AD biomarker curve evolution. From Jack et al. 2010[162]: "Aβ is identified by CSF $A\beta_{42}$ or PET amyloid imaging. Tau-mediated neuronal injury and dysfunction is identified by CSF tau or fluorodeoxyglucose-PET. Brain structure is measured by use of structural MRI. Aβ=β-amyloid. MCI=mild cognitive impairment."

Figure 1.3 Revised Model of Dynamic Biomarkers of the Alzheimer's Disease Pathological Cascade



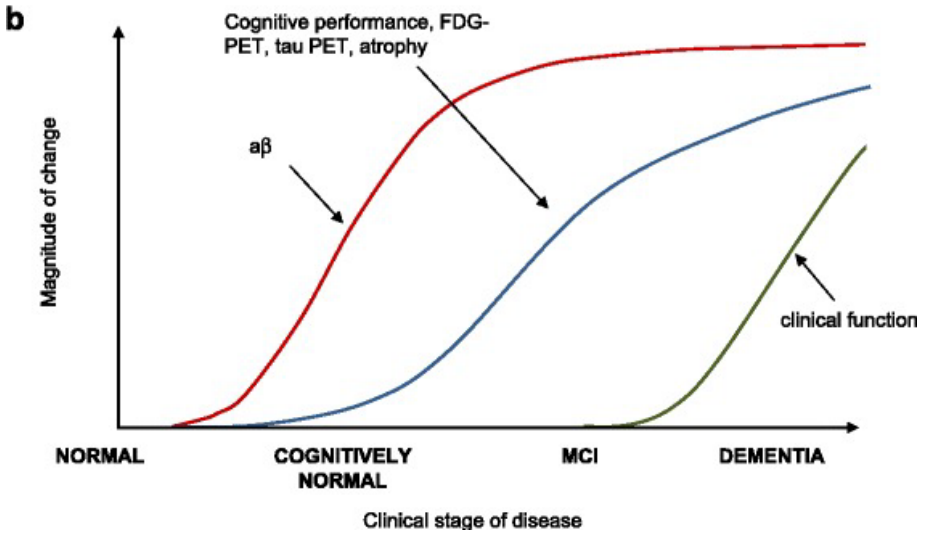
The third AD biomarker curve evolution. Adapted from Jack et al. 2013[163]: "Neurodegeneration is measured by FDG PET and structural MRI, which are drawn concordantly (dark blue). By definition, all curves converge at the top right-hand corner of the plot, the point of maximum abnormality. Cognitive impairment is illustrated as a zone (light green-filled area) with low-risk and high-risk borders." The bottom axis reflects time rather than disease stage.

The third iteration of hypothetical curves attempted to further incorporate known difficulties in representing a population through a single set of curves, as well as incorporated more recent findings for the temporal ordering of biomarkers[163]. For instance, CSF Aβ42 and amyloid PET reflect evidence of CSF amyloid abnormalities being detectable earlier than PET amyloid abnormalities. The sigmoidal curve shapes are also updated to reflect potential differing rates of change between the visualized biomarkers. Lastly, the uncertainty in individual cognitive reserve was acknowledged by adding a high- to low-risk development of cognitive impairment. As a reflection of this uncertainty, the x-axis was represented simply as "time" rather than "disease state". The premise was to define the temporal ordering of biomarkers for eventual application on a person-by-person basis. The last, most recently updated model (**Figure 1.4)** was published in 2017[3]. This model steps back from individual biomarkers to the combined groups of (1) amyloid, (2) cognitive performance, FDG-PET, tau PET, atrophy, and (3) clinical function,

reflecting the uncertainty in the application of biomarkers toward very specific disease-states or temporal orderings. The continued exploration of non-core CSF biomarkers such as VILIP-1, SNAP-25, Ng, YKL-40, and other non-CSF biomarkers has indeed introduced a higher level of uncertainty as to our ability to accurately pinpoint – if not diagnosis – prognosis in a single individual.

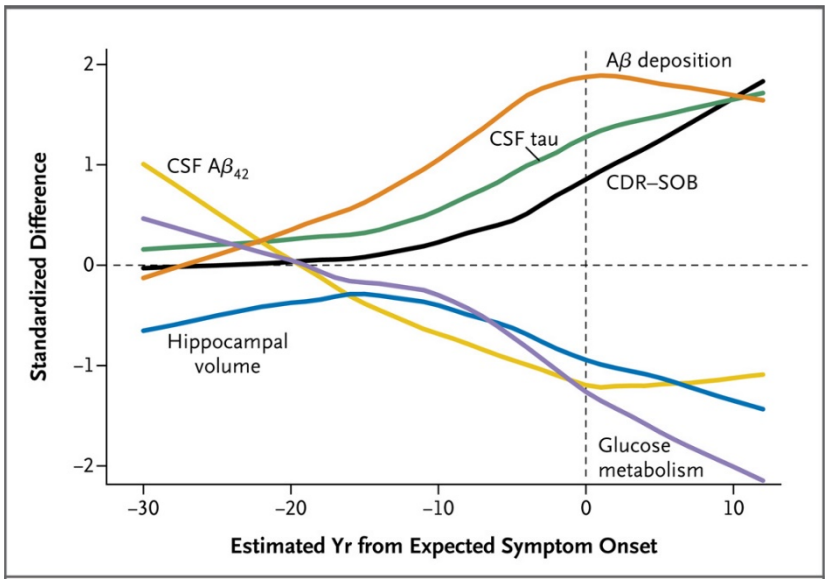
These models are incredibly useful in allowing researchers a common visual scale with which to compare biomarkers and biomarker modalities with respect to clinical status or time. However, each of these sets of curves is modeled off of cross-sectional studies, with relatively little input from within-person longitudinal studies of biomarker change – a caveat that has been clearly acknowledged[163]. Having a clear picture of the general longitudinal, temporal changes in AD, as well as their relationship with the clinical syndrome of AD, is an area of incredibly active research. Constant thought is given as to the best translation from hypothetical models such as those shown in **Figures 1.1-1.4** to functional clinical practice. The first example of data-driven biomarker curves was published using DIAN data, using cross-sectional data derive biomarker curves over the span of autosomal dominant AD (ADAD) by using the time difference in baseline biomarker measurements and each individuals parental age of AD onset as the basis for modeling biomarker changes over time, shown in **Figure 1.5**[27]. Data from the DIAN study was a step toward indicating temporal changes in biomarkers from CSF Aβ42 to amyloid imaging changes, to CSF Tau, hippocampal atrophy and hypometabolism, and finally to mild cognitive decline. What remains are studies of within-person longitudinal changes that will best showcase temporal biomarker trajectories for diagnostic, prognostic, or theragnostic utility.

Figure 1.4 Change in Biomarkers Over Time



The latest biomarker curve evolution. Adapted from Aisen et al. 2017[3]: "Modified graph showing that amyloid accumulation (measured as low CSF Aβ or elevated amyloid PET standard uptake value ratio) occurs first and functional decline occurs late in the continuum of AD (as before), bug cognitive performance, FDG-PET, tau PET, and MRI atrophy change along a common, gradually steepening curve".

Figure 1.5 Comparison of Clinical, Cognitive, Structural, Metabolic, and Biochemical Changes as a Function of Estimated Years from Expected Symptom Onset



Data derived biomarker curves from cross-sectional data using the DIAN cohort. From Bateman et al. 2012[27]: The normalized differences between mutation carriers and noncarriers are shown versus estimated years from expected symptom onset and plotted with a fitted curve. The order of differences suggests decreasing Aβ42 in the CSF (CSF Aβ42), followed by fibrillar Aβ deposition, then increased tau in the CSF (CSF tau), followed by hippocampal atrophy and hypometabolism, with cognitive and clinical changes (as measured by the Clinical Dementia Rating–Sum of Boxes [CDR-SOB]) occurring later. Mild dementia (CDR 1) occurred an average of 3.3 years before expected symptom onset.

1.3.2 Translation of Research Biomarkers to Clinical Settings

As AD research moves forward, different predictions and diagnostic or prognostic models are beginning to materialize using the wealth of biomarker information amassed in the last 10-20 years. Alongside the evolution of hypothetical models, working groups have formed, each tasked with aggregating the numerous and varied results from biomarker studies (which the hypothetical models do on a research-specific basis) and forming guidelines that may be applied in clinical settings.

In 2011, the National Institute on Aging (NIA) along with the Alzheimer Association (AA) set forth diagnostic criteria and guidelines for AD dementia in which magnetic resonance imaging (MRI) or positron emission tomography (PET), and cerebrospinal fluid (CSF) proteins were pinpointed as the strongest biomarkers for the disease, albeit with the caveat that extensive biomarker validation was still needed[164].

In 2013, the International Work Group (IWG) published an update on criteria developed in 2007 in which the presence of AD biomarkers indicates AD risk, while prodromal AD (MCI, by NIA-AA criteria) and AD dementia can be diagnosed if an individual also has biomarkers consistent with AD. The IWG considers low CSF Aβ plus high CSF tau or P-tau, or abnormal amyloid imaging as molecular biomarkers of AD and MRI or FDG PET as topographic biomarkers[165].

As a follow-up to the NIA-AA guidelines, in 2016 a further modification was proposed. Whereas the 2011 guidelines set forth 3 stages of disease encompassing preclinical disease (underlying pathology in the absence of overt cognitive changes), Mild Cognitive Impairment (MCI) due to AD, and AD, the 2016 modifications propose a move from disease- or syndrome-centric classifications toward unbiased indicators of pathology or damage as indicated by biomarkers[166]. This evolution may be considered as the merging of the IWG and NIA-AA guidelines in to a

single classification scheme – though these guidelines do not specify "Alzheimer Disease" as a diagnosis, rather, they simply allow classification of whether or not an individual exhibits biomarker changes indicative of underlying AD pathology. Dubbed "A/T/N", this classification scheme has three binary categories: amyloid pathology, as defined by CSF Aβ42 or amyloid imaging; tau pathology, as defined by CSF P-tau or tau PET; and neurodegeneration, as defined by CSF tau, FDG PET or MRI.

It may seem that these diagnostic or classification schemes are nebulous or far from implementation on a large scale. However, each iteration of guidelines provides important updates that either pinpoint critical issues (such as inter- and intra-lab variability in biomarkers) in need of improvement or adjust the expectations of "ideal" AD diagnostic scenarios to those that fit more accurately with data from AD biomarker studies. Recently, a strategic roadmap for using biomarkers to diagnose AD at early stages was proposed that may aid in bringing the concepts proposed by the IWG, NIA-AA, and 2016 update together[167]. Specific challenges in biomarker development and implementation were clearly outlined to encourage continued investigation in a framework of five phases of development, outlined in Table 1.1, that roughly follow the paradigm of (1) casting a wide net for potential biomarkers; (2) developing reliable assays to measure promising biomarkers from step 1; (3) longitudinal studies of identified biomarkers paired with clinical changes to develop a full picture of disease; (4) application of high-performing biomarkers in prospective cohorts to assess accuracy of biomarkers in clinical and research settings, and (5) assess applicability of biomarkers in providing positive value in a clinical setting.

Table 1.2 Barriers to the Widespread Clinical Usage of Biomarkers for AD Diagnosis and Prognosis

Phase	Status	Biomarkers Involved
Phase 1 – exploratory studies	Widely ongoing	Non-pathologic CSF markers (eg VILIP-1, SNAP-25, Ng, YKL-40, NfL); Imaging markers (tau PET, novel imaging analysis techniques); Blood-based biomarkers
Phase 2 – assay development	Currently developing	Core CSF biomarkers; Non-pathologic CSF biomarkers; Imaging biomarkers
Phase 3 – retrospective and longitudinal studies	Limited cohorts available	Ongoing in studies at large research centers and Alzheimer's Disease Research Centers
Phase 4 – prospective diagnostic accuracy studies	Widely ongoing	Promising biomarkers of all modalities have been tested in prospective cohorts with variable efficacy
Phase 5 – disease burden reduction studies	Minimal, in progress	Psychiatric effects of diagnosis can be investigated, but not outcomes during or after treatment

Information on research phase 1-5 drawn from Frisoni et al[167]. Information on Status and Biomarkers involved is drawn from information covered in Chapter 1.

1.3.3 Relation of the Current Work to the Challenges Outlined by the Field

The current work was initiated to explore CSF biomarker utility in a context specific to preclinical AD. The original hypothesis postulated that biomarkers of AD might change long before clinical symptoms manifest, and therefore that CSF protein levels in a subset of middle-aged, cognitively normal individuals would reflect such changes longitudinally; these data will be covered in Chapter 2 and could apply to Phase 3 as outlined in Table 1.1. A secondary

hypothesis postulated that CSF biomarkers would change longitudinally in a temporally distinct manner as individuals progressed through different disease stages from preclinical through symptomatic AD; these data will be covered in Chapter 3 and could apply to Phase 3. The gold standard being sought is a biomarker or, more likely, panel of biomarkers capable of indicating disease state and prognosis at the level of a single individual. While the current work does not provide anywhere near such a level of detail, it is certainly a step forward for a number of the obstacles set forth in Table 1.1 and allows for longitudinal, within-person case studies relevant to the primary hypotheses; these data will be covered in Chapter 4 and could apply to Phase 3 and 4. Lastly, as this work began in 2011, many topics in AD biomarkers have emerged necessitating further investigation of the analytic validation of CSF Aβ and tau which are widely considered the gold standard in pre-mortem diagnosis alongside amyloid and FDG PET and MRI measures; these data will be covered in Chapter 5 and could apply to Phase 2.

Chapter 2: Longitudinal Cerebrospinal Fluid Biomarker Changes in Preclinical Alzheimer Disease During Middle Age

This work was published in the July 2015 issue of JAMA Neurology[31].

2.1 Abstract

Importance

Individuals in the presymptomatic stage of Alzheimer disease (AD) are increasingly being targeted for AD secondary prevention trials. How early during the normal life span underlying AD pathologies begin to develop, their patterns of change over time, and their relationship with future cognitive decline remain to be determined.

Objective

To characterize the within-person trajectories of cerebrospinal fluid (CSF) biomarkers of AD over time and their association with changes in brain amyloid deposition and cognitive decline in cognitively normal middle-aged individuals.

Design, Setting, and Participants

As part of a cohort study, cognitively normal (Clinical Dementia Rating [CDR] of 0) middle-aged research volunteers (n=169) enrolled in the Adult Children Study at Washington University, St Louis, Missouri, had undergone serial CSF collection and longitudinal clinical assessment (mean, 6 years; range, 0.91-11.3 years) at 3-year intervals at the time of analysis, between January 2003 and November 2013. A subset (n=74) had also undergone longitudinal amyloid positron emission tomographic imaging with Pittsburgh compound B (PiB) in the same period. Serial CSF samples were analyzed for β-amyloid 40 (Aβ40), Aβ42, total tau, tau phosphorylated at threonine 181 (P-tau$_{181}$), visinin-like protein 1 (VILIP-1), and chitinase-3-like protein 1 (YKL-40). Within-person measures were plotted according to age and AD risk defined

by *APOE* genotype (ε4 carriers vs noncarriers). Linear mixed models were used to compare estimated biomarker slopes among middle-age bins at baseline (early, 45-54 years; mid, 55-64 years; late, 65-74 years) and between risk groups. Within-person changes in CSF biomarkers were also compared with changes in cortical PiB binding and progression to a CDR higher than 0 at follow-up.

Main Outcomes and Measures
Changes in Aβ40, Aβ42, total tau, P-tau$_{181}$, VILIP-1, and YKL-40 and, in a subset of participants, changes in cortical PiB binding.

Results
While there were no consistent longitudinal patterns in Aβ40, (P=.001-.97), longitudinal reductions in Aβ42 were observed in some individuals as early as early middle-age (P≤.05) and low Aβ42 levels were associated with the development of cortical PiB-positive amyloid plaques (area under receiver operating characteristic curve = 0.9352; 95% CI, 0.8895-0.9808), especially in mid middle-age (P<.001). Markers of neuronal injury (total tau, P-tau$_{181}$, and VILIP-1) dramatically increased in some individuals in mid and late middle-age (P≤.02), whereas the neuroinflammation marker YKL-40 increased consistently throughout middle age (P≤.003). These patterns were more apparent in at-risk ε4 carriers (Aβ42 in an allele dose-dependent manner) and appeared to be associated with future cognitive deficits as determined by CDR.

Conclusions and Relevance
Longitudinal CSF biomarker patterns consistent with AD are first detectable during early middle-age and are associated with later amyloid positivity and cognitive decline. Such measures may be useful for targeting middle-aged, asymptomatic individuals for therapeutic trials designed to prevent cognitive decline.

2.2 Introduction

Alzheimer disease (AD) is the most common cause of dementia in elderly individuals, accounting for up to 70% of all dementia cases, and is now estimated to be the third-leading cause of death after heart disease and cancer[168]. To date, clinical trials of potential disease-modifying therapies for AD have met with little success in halting or slowing cognitive decline in patients who already have cognitive symptoms or dementia[169]. However, clinicopathologic and more recent biomarker data suggest that AD pathology begins to accrue approximately 10 to 20 years before any cognitive signs or symptoms (termed *asymptomatic* or *preclinical AD*)[9,27,64,170–175], thus providing a window of opportunity for the initiation of secondary prevention trials that aim to prevent the development of symptoms in individuals while they are still cognitively normal[176]. How early during the normal life span such pathologies begin to develop, their patterns of change over time, and their relationship with future cognitive decline remain to be determined.

Because, by definition, preclinical AD eludes detection by current clinical measures, disease-specific biomarkers are necessary to identify individuals in this asymptomatic stage. To this end, the Adult Children Study (ACS) of the Knight Alzheimer's Disease Research Center at Washington University, St Louis, Missouri, was initiated. The ACS is a longitudinal clinical and biomarker research study of cognitively normal, middle-aged adults exhibiting different AD risk profiles including age, family history of AD, and *APOE* genotype (*APOE* ε4 carriers vs noncarrier)[177]. Participants undergo comprehensive, longitudinal clinical and psychometric assessments and evaluation of biomarkers in cerebrospinal fluid (CSF) and plasma, along with several imaging modalities. We hypothesized that biomarker patterns indicative of underlying AD pathology would be evident in a subset of cognitively normal individuals during middle age,

at a greater frequency in those at higher risk for AD (ie, older and/or carrying the ε4 allele of *APOE*), and would increase in severity over time, ultimately culminating in cognitive decline.

The 3 CSF biomarker analytes that reflect the core neuropathologies in AD, β-amyloid 42 (Aβ42; the primary constituent of amyloid plaques), total tau (a marker of neuronal injury and/or death), and hyperphosphorylated tau (P-tau; forms intraneuronal neurofibrillary tangles), demonstrate excellent diagnostic and prognostic utility in research cohorts[64,178,179]. Other recently identified biomarkers, including visinin-like protein 1 (VILIP-1) and chitinase-3-like protein 1 (YKL-40) (markers of neuronal death and gliosis/neuroinflammation, respectively) have also demonstrated clinical utility in AD, especially when combined in an algorithm with CSF Aβ42[61,62,65,101,180]. This first report of longitudinal biomarker changes in the ACS cohort describes the within-person trajectories of these CSF biomarkers over time and their association with longitudinal changes on *in vivo* amyloid imaging and future cognitive decline as a function of risk conferred by *APOE* genotype.

2.3 Methods

Participants

Participants were cognitively normal, community-dwelling research volunteers enrolled in the ACS at the Knight Alzheimer's Disease Research Center at Washington University. Inclusion criteria include the following: (1) positive family history (≥1 biological parent with age at AD dementia onset <80 years) or negative family history (both biological parents living to age ≥70 years in the absence of AD dementia); (2) aged 45 to 74 years at study entry (1 enrollee was aged 43 years, 3 were aged 75 years, 3 were aged 76 years, and 1 was aged 81 years); (3) availability of an informant who knows the participant well; (4) normal cognition at study entry (defined as having a Clinical Dementia Rating [CDR][181] of 0; and (5) willingness in principle to complete all

study procedures at baseline and longitudinally. Exclusion criteria include the following: (1) presence of a neurological, psychiatric, or systemic illness that might affect cognition or interfere with longitudinal follow-up; (2) a known deterministic mutation for AD; and (3) medical contraindication to lumbar puncture for CSF collection or imaging.

Specific inclusion criteria for the present analysis included the availability of data from at least 2 serial clinical assessments and CSF collection procedures (mean [SD] interval between clinical assessment and CSF collection, 3.3 [3.8] years) as of September 2013; thus, this cohort represents a subset (n=169) of ACS participants to date. All procedures were approved by the Human Research protection Office at Washington University, and written informed consent was obtained from all participants and their informants.

Clinical and Cognitive Assessments

The presence or absence of dementia (and, when present, its severity) was operationalized with the CDR in accordance with standard protocols and criteria[182]. A CDR of 0 indicates cognitive normality, whereas CDRs of 0.5, 1, 2, and 3 are indicative of very mild, mild, moderate, and severe dementia, respectively[181].

Genotyping

Using standard procedures, DNA was extracted from peripheral blood samples. Genotyping of *APOE* was performed by the Knight Alzheimer's Disease Research Center Genetics Core as previously described[183].

CSF Collection and Processing

A sample of CSF (20-30 mL) was collected by routine lumbar puncture at 8 AM after overnight fasting as described[17]. Samples were processed into 500 uL aliquots and immediately frozen at -80^{o}C.

CSF Biomarker Analyses

The CSF samples were analyzed for Aβ and tau proteins using single-analyte enzyme-linked immunosorbent assays (ELISAs; research use only) from 2 different vendors. Samples were analyzed for Aβ1-40 (Aβ40), Aβ1-42 (Aβ42), total tau, and tau phosphorylated at threonine 181 ($P\text{-}tau_{181}$) using the Improved INNOTEST ELISA (Fujirebio Europe), a modified version of the assay most widely used in the field. In parallel, Aβ40, Aβ42, and total tau were measured at the same time (from the same sample aliquot) using a set of second-generation (precision-based and accuracy-based) EUROIMMUN ELISAs (EUROIMMUN). The Aβ42 to Aβ40 ratio was calculated to normalize the Aβ42 production concentrations to the total amount of Aβ (Aβ40 is the most abundant Aβ species in CSF)[184–186]. The ratio of total tau ($P\text{-}tau_{181}$) to Aβ42 was also evaluated because it has been shown to be a predictor of future cognitive decline in elderly cohorts[28,61,187,188]. It must be stated at the outset that the focus of this study is on the clinical utility of the biomarker and that conclusions drawn from one assay can be confirmed or qualified with data derived from another immunoassay. The well-studied INNOTEST ELISA was considered a priori to be the reference assay; therefore, INNOTEST data are shown.

The VILIP-1 concentration was measured using a 2-site immunoassay (Singulex)[61]. The YKL-40 concentration was measured with the MicroVue ELISA (Quidel)[101(p40)].

Longitudinal CSF samples from a given individual were run on the same assay plate (and same lot number) to minimize potential interpolate and interlot methodological variability. Samples underwent a single freeze-thaw cycle prior to assay, were thawed on wet ice (approximately 3 hours) prior to analysis, and were all run in duplicate. Values had to pass quality control criteria, including coefficients of variation of 25% or lower, kit controls within the expected range as

defined by the manufacturer (where applicable), and measurement consistency of 2 common pooled CSF samples that were included on each plate.

In Vivo Amyloid Imaging

A subset (n=74) of the 169 participants with longitudinal CSF analysis had also undergone longitudinal *in vivo* amyloid imaging via positron emission tomography (PET) with Pittsburgh compound B (PiB)[189–191] within approximately 12 months of CSF collection (mean [SD], 84.3 [92] days). The PiB PET imaging was conducted with a Siemens 962 HR+ Emission Computer-Aided Tomograph PET or Biograph 40 scanner (Siemens/CTI). Magnetic resonance imaing using magnetization-prepared rapid-acquisition gradient-echo T1-weighted imagine (1 x 1 x 1.25 mm) was obtained for anatomical reference.

Deposition of PiB in brain regions of interest was determined using FreeSurfer version 5.1 software (Martinos Center for Biomedical Imaging)[190,192,193], and a standardized uptake value ratio (SUVR) corrected for partial volume effects[194] was calculated for each region of interest. The mean cortical SUVR was calculated from FreeSurfer regions within the prefrontal cortex, precuneus, and temporal cortex. Cerebellar cortex served as the reference region. Based on a study of 77 symptomatic and asymptomatic Knight Alzheimer's Disease Research Center participants[190], PiB positivity was defined as an SUVR of 1.42, commensurate with a mean cortical binding potential of 0.18 defined previously for PiB positivity[189].

Statistical Analysis

Baseline demographic characteristics were summarized as mean (standard deviation) for continuous variables or number (percentage) for categorical variables. Demographic variables were compared across 3 age bins within the 2 *APOE* ε4 groups and between the ε4 carriers and noncarriers within each age bin using post hoc *t* tests within analysis of variance for continuous

variables or logistic regression for dichotomous variables. To quantify the within-person annual rate of change in CSF biomarkers, general linear mixed models with random intercepts and random time slopes at the participant level were used to regress the concentrations on time from study entry (baseline). These models incorporated baseline age category, *APOE* category, and time from study entry as fixed effects as well as all possible higher-order interactions among these factors. This facilitated the estimation of average baseline CSF biomarker concentrations as well as their change over time separately in each of the 6 participant groups (cross-classification of 3 baseline age categories by 2 *APOE* categories). The resulting estimated average within-person annual rates of change in CSF biomarkers were compared among the 6 groups with model-derived approximate *t* tests with the approximate denominator d*f* based on the Satterthwaite approximation[195]. Baseline comparisons between CSF biomarkers among the groups in **Table 2.1** were also carried out within these general linear mixed models by testing the estimated average concentrations when time from study entry was equal to 0. These CSF biomarker comparisons, at baseline and on the longitudinal rate of change, were also reexamined after adjusting for family history, sex, and education by including fixed effects for these factors and their interactions with time from study entry. The general linear mixed model assumptions were evaluated via analyses of residuals. Owing to the preliminary nature of hypotheses examined in this cohort, no adjustment was made for multiplicity. For exploratory purposes, an optimal CSF Aβ42 cutoff was determined using the Youden Index after receiver operating characteristic analysis for discriminating between PiB-positive and PiB-negative individuals at baseline. For each biomarker, baseline and longitudinal comparisons between PiB-positive (PiB SUVR $\geq$ 1.42) and PiB-negative individuals were performed using general linear mixed models with fixed effects included for PiB category, time from study entry, and their interaction. We

used SAS version 9.3 statistical software (SAS Institute, Inc.) for all statistical analyses, with statistical significance defined as $P < .05$.

2.4 Results

Baseline data are presented in **Table 2.1** and grouped into 6 bins: the absence (n=108) or presence (n=61) of at least 1 *APOE* ε4 allele (as an indicator of neutral and high AD risk, respectively) and middle-age bin at baseline (early [45-54 years], mid [55-64 years], or late [65-74 years]). Ninety-nine participants underwent 2 serial CSF collections, 65 underwent 3 serial CSF collections, and 5 underwent 4 serial CSF collections, at intervals of approximately 3 years. Forty-five of the 61 ε4 carriers (74%) and 49 of the 108 ε4 noncarriers (45%) reported a positive family history.

2.4.1 Comparison of the CSF Aβ40, Aβ42, and Total Tau Assays

Data for the ACS cohort was acquired using two assays for Aβ40, Aβ42, and Tau. The data presented in Chapter 2 are the "Improved" INNOTEST data. With the exception of EUROIMMUN data in **Table 2.**1, analyses comparing the INNOTEST and EUROIMMUN assays are located in Chapter 5.

Table 2.1 Demographic Characteristics and Baseline Cerebrospinal Fluid Biomarkers[a]

Variable	*APOE* ε4 Noncarriers (n=108)			*APOE* ε4 Carriers *(n=61)*		
	Early (n = 26)	Mid (n = 44)	Late (n = 38)	Early (n = 19)	Mid (n = 17)	Late (n = 25)
Baseline age, mean (SD), y	50.1 (3.0)	59.4 (2.9)[b]	69.9 (3.5)[b,c]	49.6 (2.9)	59.3 (3.1)[b]	69.4 (3.6)[b,c]
Female, No. (%)	17 (65)	32 (73)	22 (58)	14 (74)	11 (65)	16 (64)
Positive family history, No. (%)	12 (46)	22 (50)	15 (39)	15 (79)[d]	13 (76)	17 (68)[d]
APOE genotype, No.						
ε2/ε2	0	1	1	0	0	0
ε2/ε3	3	8	6	0	0	0
ε3/ε3	23	35	31	0	0	0
ε2/ε4	0	0	0	2	2	2
ε3/ε4	0	0	0	14	12	20
ε4/ε4	0	0	0	3	3	3
Education, mean (SD), y	16.1 (2.10)	16.9 (2.27)	15.6 (2.64)[c]	15.8 (1.95)	15.4 (3.45)[d]	16.3 (2.23)
Baseline MMSE score, mean (SD)[e]	29.5 (0.65)	29.3 (1.10)	28.8 (1.22)[b]	29.8 (0.38)	28.9 (1.52)[b]	28.9 (1.39)[b]
Received ≥1 CDR>0 at follow-up, No.[f]	0	1	4	1	3	5
Participants with 2/3/4 serial LPs, No.	12/13/1	21/19/4	25/13/0	11/8/0	12/5/0	18/7/0
LP interval, mean (SD), mo	3.3 (0.76)	3.3 (0.91)	3.1 (0.77)	3.3 (0.73)	3.6 (1.4)	3.2 (0.77)

Baseline biomarkers, mean (IQR)						
Improved INNOTEST ELISA						
Aβ40, pg/mL	12657 (10461-14480)	14319 (12185-16371)	15382[b] (12417-17906)	14555 (12984-16638)	13103 (10629-15838)	14343 (12199-16748)
Aβ42, pg/mL	1293 (1046-1525)	1340 (1132-1544)	1270 (1021-1608)	1306 (1193-1498)	936.9 [b,d] (671-1116)	969.7 [b,d] (733-1225)
Aβ42 to Aβ40 ratio	0.1052 (0.0900-0.1225)	0.0972 (0.0800-0.1100)	0.0871[b,c] (0.0700-0.1000)	0.0924 (0.0800-0.1000)	0.0719 [b,d] (0.0600-0.0850)	0.0709 [b,d] (0.0550-0.0900)
Total tau, pg/mL	202.3 (146.0-243.2)	259.0 (182.6-278.7)	324.3 [b,c] (205.2-389.3)	257.7 (194.4-314.6)	298.0 (210.2-391.6)	321.4 (198.6-413.2)
P-tau$_{181}$, pg/mL	39.8 (27.7-50.3)	51.2 (37.2-55.4)	58.8 [b] (41.7-68.5)	47.7 (38.5-55.4)	54.4 (37.9-67.8)	55.4 (38.2-69.8)
Tau to Aβ42 ratio	0.1541 (0.1200-0.1725)	0.1908 (0.1400-0.2200)	0.3054 [b,c] (0.1500-0.3100)	0.1986 (0.1600-0.2300)	0.4207 [b,d] (0.1900-0.4550)	0.3816 [b] (0.2150-0.5200)
EUROIMMUN ELISA						
Aβ40, pg/mL	4857 (3525-6101)	5408 (4305-6220)	5569 (4347-6224)	5535 (4816-6433)	5266 (3966-7043)	5257 (4119-5942)
Aβ42, pg/mL	616.1 (438.1-683.1)	616.1 (495.90741.8)	590.1 (459.4-701.1)	676.0 (462.3-797.6)	449.5 [b,d] (349.9-564.3)	487.5 b,d (365.3-601.8)

Total tau, pg/mL	254.7 (194.4-304.8)	310.3 (230.3-344.6)	362.5 [b,c] (255.6-430.3)	299.1 (234.1-351.6)	380.5 [d] (313.6-469.6)	395.8 [b] (274.1-487.4)
Tau to Aβ42 ratio	0.4050 (0.3341-0.4851)	0.5022 (0.3900-0.5192)	0.7023 [b,c] (0.4065-0.6874)	0.4563 (0.3675-0.5390)	1.073 [b,d] (0.4680-1.1100)	0.9342 [b,d] (0.5383-1.2250)
VILIP-1, pg/mL	140.8 (102.3-169.8)	154.4 (116.7-166.4)	179.8 [b,c] (133.6-218.9)	155.6 (128.8-175.4)	153.2 (105.4-193.8)	154.7 (117.0-180.2)
YKL-40, ng/mL	180.3 (124.2-220.3)	231.3 [b] (192.3-259.7)	301.1 [b,c] (221.7-368.2)	188.4 (135.3-238.7)	240.6 [b] (165.5-297.9)	281.5 [b] (201.8-353.8)

Abbreviations: Aβ, β-amyloid; CDR, Clinical Dementia Rating; ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range; LP, lumbar puncture; MMSE, Mini-Mental State Examination; P-tau$_{181}$, tau phosphorylated at threonine 181; VILIP-1, visinin-like protein 1; YKL-40, chitinase-3-like protein 1.

[a] Age groups indicate the ages within middle-age: early, ages 45-54; mid, ages 55-64; and late, ages 65-74 years

[b] Significantly different from early within the same *APOE* ε4 group ($P < .05$)

[c] Significantly different from mid within the same *APOE* ε4 group ($P < .05$)

[d] Significantly different from the same age group of the other *APOE* ε4 group ($P < .05$)

[e] The MMSE scores can range from 0 to 30, with 30 as a perfect score

[f] A CDR of 0 indicates cognitively normal; a CDR higher than 0, cognitively abnormal

2.4.2 Baseline and Slope Analyses: CSF Biomarker Changes Occur in Middle Age

Baseline biomarker levels (**Table 2.1**) and slopes of change within individuals (**Table 2.2**) were evaluated in the 6 bins defined earlier. Slopes were calculated as the representative mean of all annual individual slopes per age bin (extrapolated to 9 years for illustrative purposes) and superimposed on the spaghetti plots of the associated individual trajectories (**Figure 2.1** shows the INNOTEST assay data for Aβ40, Aβ42, Aβ42 to Aβ40 ratio, total tau, P-tau$_{181}$, and total tau to Aβ42 ratio; **Figure 2.2** shows the data for VILIP-1 and YKL-40; see **Figure 5.1** Chapter 5 for the EUROIMMUN assay data). Controlling for family history, sex, and education did not substantially influence the comparisons between age and ε4 categories.

Aβ40, Aβ42, Aβ42 to Aβ40 Ratio

Baseline levels of CSF Aβ40 (INNOTEST) were significantly higher in the late middle-aged group compared with the early middle-aged group in ε4 noncarriers (P = .004) (**Table 2.1)** but decreased significantly within individuals in the early (P = .04) and mid (P = .01) middle-aged groups over time (**Table 2.2** and **Figure 2.1A**). In contrast, no significant differences were observed in ε4 carriers at baseline or longitudinally (**Table 2.1** and **Table 2.2**).

In contrast to Aβ40, robust decreases within individuals in all age groups were observed for Aβ42 in both risk groups (**Figure 2.1B** and **Table 2.2**), and this pattern was detectable in many participants as early as 45 to 54 years of age. While baseline concentrations did not differ among the age groups in the ε4 noncarriers, levels in ε4 carriers were significantly lower in the mid (P < .001) and late (P < .001) middle-aged groups compared with the early middle-aged group and also significantly lower than the levels in the mid (P < .001) and late (P < .001) middle-aged ε4 noncarriers (**Table 2.1**).

Similar to the patterns observed for Aβ42 alone, the ratios of Aβ42 to Aβ40 were significantly lower in the mid (P = .02) and late (P = .005) middle-aged groups compared with the early middle-aged group in ε4 carriers (**Table 2.1**), and the within-person values significantly decreased over time in the 2 older age groups (both P < .001) (**Figure 2.1C** and **Table 2.2**). Although baseline ratios in the ε4 noncarriers were significantly lower in the late middle-aged group compared with the mid (P = .05) and early (P = .004) middle aged groups (**Table 2.1**), they did not change significantly within these low-risk individuals at any age (**Figure 2.1C** and **Table 2**).

Total Tau and P-tau$_{181}$

Baseline total tau was higher in late middle-aged participants compared with early middle-aged participants in both risk groups, with intermediate levels in the mid middle-aged participants, although differences were statistically significant only in the ε4 noncarriers (P < .001 and P = .02, respectively) (**Table 2.1**). Within ε4 noncarriers, total tau increased significantly over time during late middle age (P < .001), while increases were observed earlier (mid and late middle-age) in the higher-risk ε4 carriers (both p < .001) (**Figure 2.1D** and **Table 2.2**). Interestingly, the annual mean (SE) increase in total tau in mid middle-age was significantly higher in ε4 carriers (22.28 [4.45] pg/ml) compared with ε4 noncarriers (2.84 [2.68] pg/ml) (P < .001) (**Table 2.2**). Results for P-tau$_{181}$ were virtually identical to those for total tau, including more robust elevations in the ε4 carriers during mid middle-age (**Figure 2.1E** and **Table 2.2**).

Table 2.2 Mean Annual Slopes of Within-Individual Longitudinal Change in Cerebrospinal Fluid Biomarkers During Middle Age[a]

Variable	*APOE* ε4 Noncarriers (n=108)			*APOE* ε4 Carriers (n=61)		
	Early (n = 26)	**Mid (n = 44)**	**Late (n = 38)**	**Early (n = 19)**	**Mid (n = 17)**	**Late (n = 25)**
Aβ40						
Estimated Annual Slope, mean (SE), pg/ml	-163.59 (80.50)	-153.82 (61.81)	-130.39 (77.20)	30.63 (102.86)	3.31 (107.75)	110.77 (97.06)
Different from zero, P value	.04[b]	.01[b]	.09	.77	.98	.26
APOE ε4 carriers vs noncarriers, P value	-	-	-	.14	.21	.05
Aβ42						
Estimated Annual Slope, mean (SE), pg/ml	-14.81 (5.83)	-19.34 (4.48)	-22.80 (5.56)	-14.99 (7.42)	-29.22 (7.79)	-26.76 (6.99)
Different from zero, P value	.01[b]	<0.001[b]	<0.001[b]	.045[b]	<0.001[b]	<0.001[b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.98	.27	.66
Aβ42 to Aβ40 ratio						
Estimated Annual Slope, mean (SE), pg/ml	0.00027 (0.00042)	-0.00023 (0.00032)	-0.00068 (0.00042)	-0.00090 (0.00055)	-0.00202 (0.00057)	-0.00220 (0.00052)
Different from zero, P value	.52	.47	.10	.10	<0.001[b]	<0.001[b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.09	.007[b]	.02[b]

Total tau						
Estimated Annual Slope, mean (SE), pg/ml	0.96 (3.44)	2.84 (2.68)	14.58 (3.08) [c,d]	5.40 (4.20)	22.28 (4.45) [c]	18.45 (3.85) [c]
Different from zero, P value	.78	.29	<0.001 [b]	.20	<0.001 [b]	<0.001 [b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.42	<0.001 [b]	.43
P-tau$_{181}$						
Estimated Annual Slope, mean (SE), pg/ml	0.23 (0.51)	0.32 (0.40)	1.84 (0.47) [c,d]	1.08 (0.63)	3.41 (0.67) [c]	1.92 (0.58)
Different from zero, P value	.66	.43	<0.001 [b]	.09	<0.001 [b]	.001 [b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.30	<0.001 [b]	.91
Total tau to Aβ42 ratio						
Estimated Annual Slope, mean (SE), pg/ml	0.0026 (0.0084)	0.0081 (0.0066)	0.0268 [c] (0.0071)	0.0076 (0.0100)	0.0538 [c] (0.0106)	0.0478 [c] (0.0088)
Different from zero, P value	.76	.22	<0.001 [b]	.45	<0.001 [b]	<0.001 [b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.70	<0.001 [b]	.07
VILIP-1						
Estimated Annual Slope, mean (SE), pg/ml	-0.18 (1.03)	-0.48 (0.80)	2.39 (1.01) [d]	0.79 (1.34)	5.17 (1.39) [c]	1.42 (1.27) [d]
Different from zero, P value	.86	.55	.02 [b]	.55	<0.001 [b]	.26
APOE ε4 carriers vs noncarriers, P value	-	-	-	.56	<0.001 [b]	.55

YKL-40						
Estimated Annual Slope, mean (SE), pg/ml	4.80 (1.29)	4.26 (0.99)	6.91 (1.27)	6.25 (1.68)	10.83 (1.75)	4.90 (1.60) [d]
Different from zero, P value	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]	.002[b]
APOE ε4 carriers vs noncarriers, P value	-	-	-	.50	.001[b]	.32

Abbreviations: Aβ, β-amyloid; P-tau$_{181}$, tau phosphorylated at threonine 181; VILIP-1, visinin-like protein 1; YKL-40, chitinase-3-like protein 1; dashes, not applicable.

[a] Age groups indicate the ages within middle-age: early, ages 45-54; mid, ages 55-64; and late, ages 65-74 years. Results for Aβ40, Aβ42, Aβ42 to Aβ40 ratio, total tau, P-tau$_{181}$, and total tau to Aβ42 ratio are from the improved INNOTEST enzyme-linked immunosorbent assay

[b] Statistically significant at $P < .05$

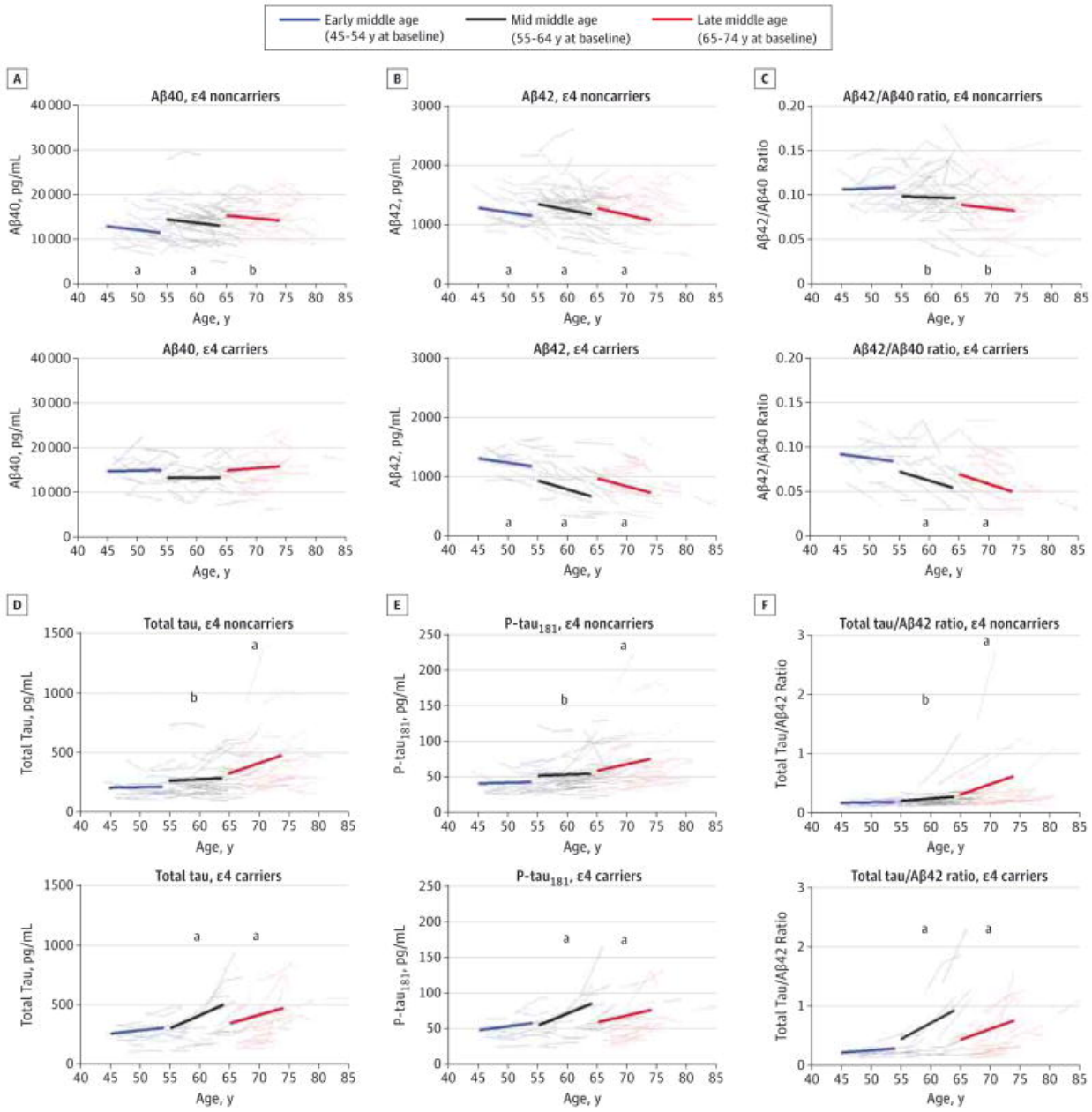
[c] Significantly different from early within the same *APOE* ε4 group ($P < .05$)

[d] Significantly different from mid within the same *APOE* ε4 group ($P < .05$)

Ratios of Total Tau and P-tau$_{181}$ to Aβ42

In ε4 noncarriers, the baseline total tau to Aβ42 ratio was significantly higher in late middle-age compared with both early (P = .005) and mid (P = .01) middle-age (**Table 2.1**). In at-risk ε4 carriers, significantly higher ratios were observed even earlier (mid [P = .002] and late [P = .004] middle-age) compared with early middle-age (**Table 2.1**). Longitudinal patterns for the total tau to Aβ42 ratio were virtually identical to those of total tau, with significant within-person increases in the late middle-aged group in ε4 noncarriers (P < .001) and even earlier (mid and late middle-age) in the ε4 carriers (both P < .001) (**Figure 2.1F** and **Table 2.2**). Patterns for the P-tau$_{181}$ to Aβ42 ratio were virtually identical to those of the total tau to Aβ42 ratio (data not shown).

Figure 2.1. Longitudinal Change in Cerebrospinal Fluid Biomarkers β-Amyloid 40 (Aβ40), Aβ42, Aβ42 to Aβ40 Ratio, Total Tau, Tau Phosphorylated at Threonine 181 (P-tau181), and Total Tau to Aβ42 Ratio During Middle Age



Estimated group slopes and within-person changes for Aβ40 (A), Aβ42 (B), Aβ42 to Aβ40 ratio (C), total tau (D), tau phosphorylated at threonine 181 (P-tau181) (E), and total tau/Aβ42 ratio (F) are shown in the 3 age bins for APOE ε4 noncarriers (top graph of each panel; n = 108 participants) and ε4 carriers (bottom graph of each panel; n = 61 participants). Annual slopes have been extrapolated to 9 years, and each slope begins at the mean baseline biomarker value from individuals in each age bin. Group baseline values and slopes represent the estimates reported in Table 1 and Table 2, respectively, for the different cohorts defined by baseline age in which biomarker concentrations were

regressed on time from study entry. Data are from the INNOTEST enzyme-linked immunosorbent assay (Fujirebio Europe).

[a]Slope significantly different from 0 (P < .05).

[b]Slope significantly different between *APOE* ε4 groups within a given age group (P < .05).

2.4.3 Other Biomarkers of Neuronal Injury and Gliosis/Neuroinflammation

VILIP-1

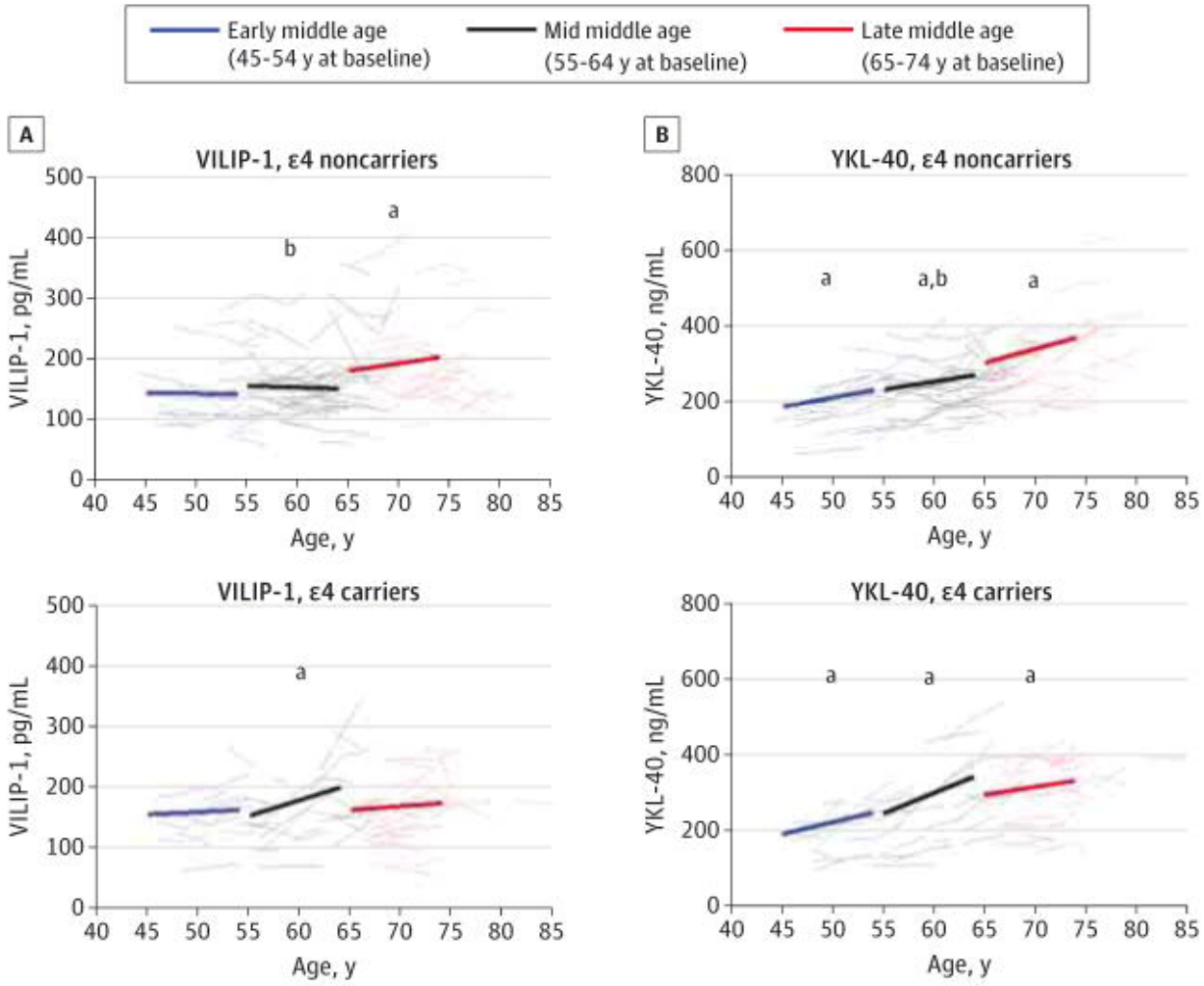
The concentration of VILIP-1 was positively correlated with total tau during middle age (INNOTEST total tau: n = 401, Pearson *r* = 0.763 [95% CI, 0.719-0.801], P < .001; EUROIMMUN total tau: n = 403, Pearson *r* = 0.743 [95% CI, 0.696-0.784], P < .001), consistent with earlier reports in elderly cohorts. Similar to total tau, mean baseline VILIP-1 concentration increased with age, with significantly higher levels in late middle-age compared with early (P = 0.008) and mid (P = .03) middle-age in the ε4 noncarriers (**Table 2.1**) and within-person increases over time in late middle-age (P = .02) (**Figure 2.2A** and **Table 2.2**). While baseline levels of VILIP-1 in the at-risk ε4 carriers at baseline were not significantly different among the age groups, (**Table 2.1**), they significantly increased longitudinally within individuals at an earlier age (mid middle-age [P < .001]) compared with the ε4 noncarriers (late middle-age [P = .02]) (**Figure 2.2A** and **Table 2.2**). Also similar to total tau, the annual mean increase in VILIP-1 concentration in mid middle-age was greater in ε4 carriers compared with ε4 noncarriers (P < .001).

YKL-40

Baseline CSF YKL-40 concentration was significantly higher in mid and late middle-age compared with early middle-age in both ε4 groups (all P ≤ .04) as well as in late middle-age compared with the mid middle-age in the ε4 noncarriers (P < .001) (**Table 2.1**). In both groups, YKL-40 concentration significantly increased within individuals over time in all age bins (P = .002 in late middle-age among ε4 noncarriers; all others, P < .001) (**Figure 2.2B** and **Table 2.2**).

In mid middle-age, YKL-40 concentration increased at a significantly higher rate in the ε4 carriers compared with ε4 noncarriers (P = .001) (**Table 2.2**), similar to what was observed for the injury markers.

Figure 2.2. Longitudinal Change in Cerebrospinal Fluid Biomarkers Visinin-Like Protein 1 (VILIP-1) and Chitinase-3-Like Protein 1 (YKL-40) During Middle Age



Estimated group slopes and within-person changes for VILIP-1 (A) and YKL-40 (B) are shown in the 3 age bins for APOE ε4 noncarriers (top graph of each panel; n = 108 participants) and ε4 carriers (bottom graph of each panel; n = 61 participants). Annual slopes have been extrapolated to 9 years, and each slope begins at the mean baseline biomarker value from individuals in each age bin. Group baseline values and slopes represent the estimates reported in Table 1 and Table 2, respectively, for the different cohorts defined by baseline age in which biomarker concentrations were regressed on time from study entry.

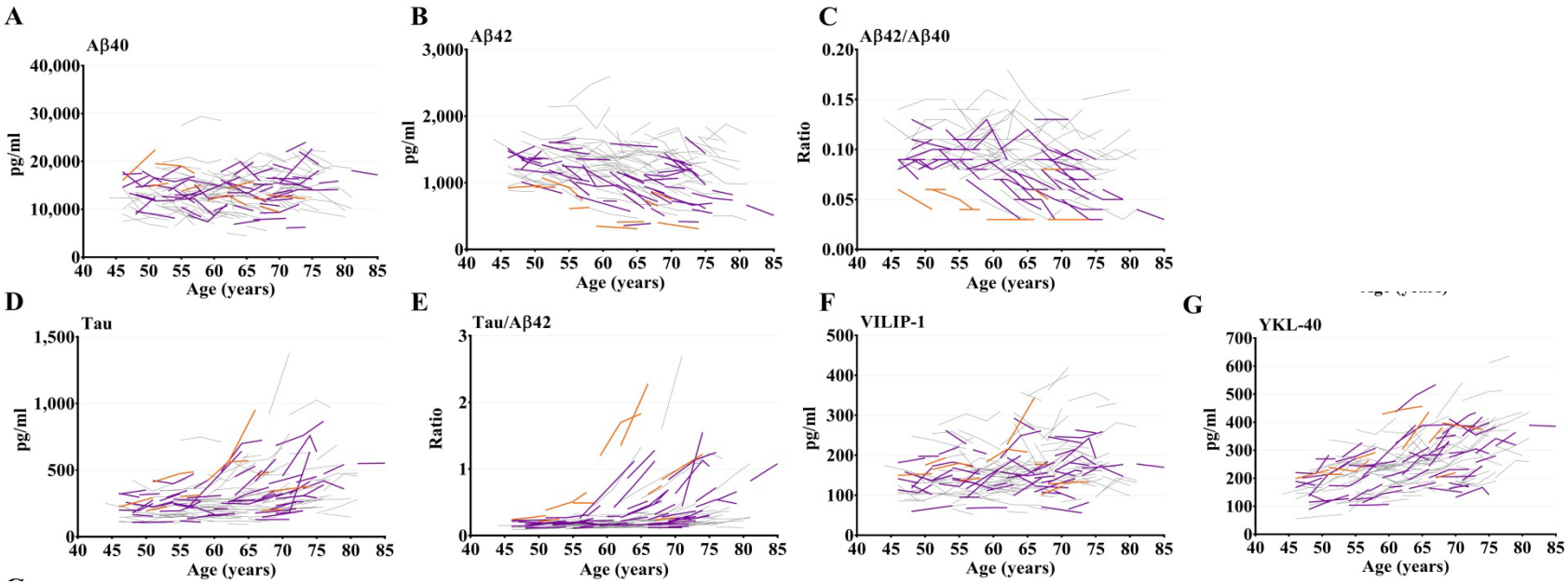
[a]Slope significantly different from 0 (P < .05).

[b]Slope significantly different between *APOE* ε4 groups within a given age group (P < .05).

2.4.4 *APOE* ε4 Gene Dose Influences CSF Biomarker Patterns Consistent with the Presence of Preclinical AD During Middle Age

Given the known *APOE* ε4 gene dosage effects on the risk of AD and age at dementia onset, we evaluated biomarker trajectories as a function of ε4 allele number. The majority (82%) of ε4 noncarriers had the ε3/ε3 genotype, whereas the majority (75%) of ε4 carriers had the ε3/ε4 genotype (**Table 2.1**). Nine participants were ε4 homozygotes (ε4/ε4 genotype). Trajectory patterns for Aβ40 did not differ as a function of ε4 allele dose (**Figure 2.3A**). In contrast, patterns differed dramatically for Aβ42 (**Figure 2.3B**) and the Aβ42 to Aβ40 ratio (**Figure 2.3C**) across the entire age range, with ε4 homozygotes falling among the lowest values, ε4 noncarriers typically falling among the highest, and heterozygotes falling in the middle range (although overlapping with many of the ε4 noncarriers). The longitudinal patterns for total tau, total tau to Aβ42 ratio, VILIP-1, and YKL-40 in ε4 carriers appeared to overlap to a greater extent with those for ε4 noncarriers (**Figure 2.3D-F**). However, the number of ε4 homozygotes is too small to perform rigorous statistical analyses in the current cohort.

Figure 2.3 Longitudinal Biomarker Trajectories in Individuals with Different *APOE* Genotypes



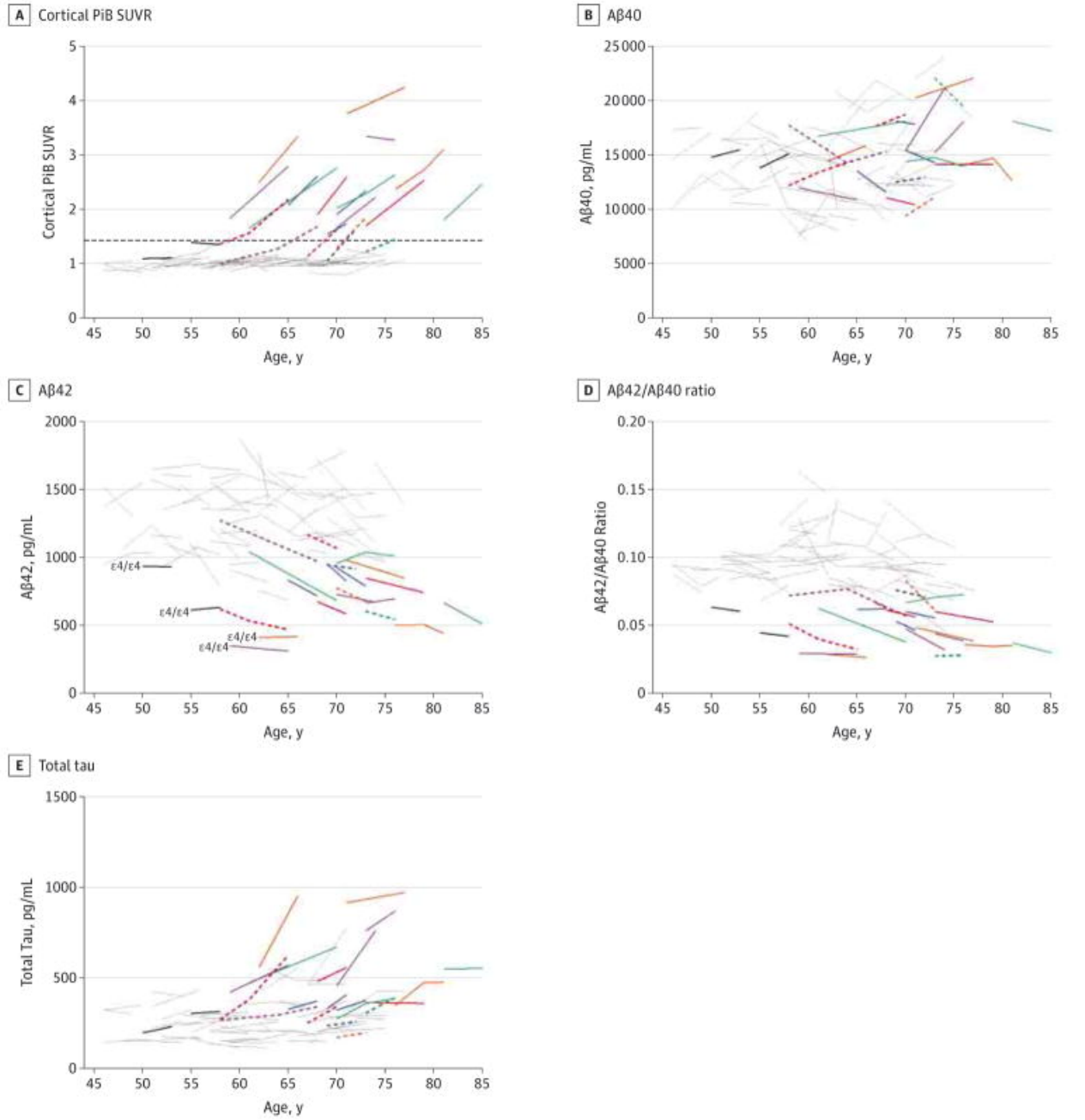
Within-person trajectories of CSF A) Aβ40, B) Aβ42, C) Aβ42/Aβ40 Ratio, D) Tau, E) Tau/Aβ42 Ratio, F) VILIP-1, and G) YKL-40 are plotted as a function of age. Aβ40, Aβ42 and tau were measured with the INNOTEST assay. Colors identify *APOE* genotype: no ε4 alleles (gray, ε4-/-; ε2/ε2, ε2/ε3, ε3/ε3), one ε4 allele (purple, ε4+/-; ε2/ε4, ε3/ε4) and two ε4 alleles (orange, ε4+/+; ε4/ε4).

2.4.5 Association of CSF Aβ42 and In Vivo Amyloid Imaging During Middle Age

Because studies to date evaluating the concordance of CSF Aβ42 concentrations with *in vivo* amyloid load have focused on elderly cohorts, it was of interest to characterize this association in middle-age, a time during which a subset of individuals are expected to be in the very earliest stages of preclinical AD. This analysis used data from a subset of 74 participants (n = 50 ε4 noncarriers; n = 24 ε4 carriers) within the longitudinal CSF cohort who had also undergone longitudinal *in vivo* PiB PET imaging within 376 days (mean [SD], 84.3 [92] days) of CSF collection. Twenty of these individuals were considered PiB positive (mean cortical SUVR ≥ 1.42) at baseline, follow-up, or both (**Figure 2.4A**). Of these 20 individuals, 10 (50%) were ε4 noncarriers and 10 (50%) were ε4 carriers. Although there was no significant association between the cross-sectional patterns (P = .12) or longitudinal trajectories (P = .65) of Aβ40 and cortical PiB binding (**Figure 2.4B**), PiB positivity was associated with low baseline levels of CSF Aβ42 (P < .001) but not longitudinal change (P = .37) (**Figure 2.4C**). However, 15 PiB-negative individuals (20%) had concentrations of Aβ42 that were as low as those who were PiB-positive. Because low Aβ42 values could conceivably reflect low production of all Aβ species rather than an amyloidosis-specific decrease in Aβ42, we also evaluated the relationship between PiB and the Aβ42 to Aβ40 ratio (**Figure 2.4D**). Twelve of the PiB-negative participants (16%) had Aβ42 to Aβ40 ratios at some point that were as low as those who were PiB-positive. Notably, all 4 ε4 homozygotes in this subcohort had a low Aβ42 concentration and a low Aβ42 to Aβ40 ratio at both baseline and follow-up (**Figure 2.4C** and **D**), including the 2 young participants (aged <55 years at baseline) who were PiB-negative (**Figure 2.4C** and **D**, solid black lines). The PiB-positive individuals typically had higher baseline (P < .001) and longitudinally increasing (P < .001) levels of total tau (and P-tau$_{181}$ [scatterplots not shown]) compared with

those who were PiB-negative (**Figure 2.4E**). The PiB associations with baseline (P = .04) and longitudinal (P = .004) VILIP-1 concentrations were similar to total tau but less concordant (**Figure 2.5A**). Being PiB-positive was not significantly associated with YKL-40 levels at baseline (P = .08) but was associated with greater longitudinal increases (P = .04) (**Figure 2.5B**). Overall, Aβ42, Aβ42 to Aβ40 ratio, total tau, and P-tau$_{181}$ appeared to be more strongly associated with PiB positivity than were Aβ40, VILIP-1, and YKL-40.

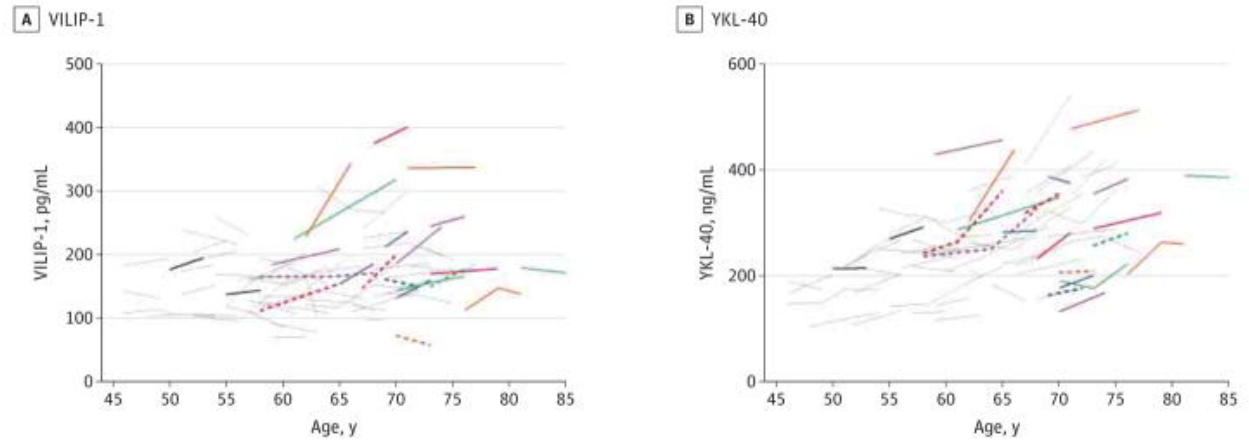
Figure 2.4. Association Between Longitudinal Patterns of Cerebrospinal Fluid Biomarkers β-Amyloid 40 (Aβ40), Aβ42, Aβ42 to Aβ40 Ratio, and Total Tau, Cortical Pittsburgh Compound B (PiB) Standardized Uptake Value Ratio (SUVR), and Age



A subset (n = 74) of Adult Children Study participants had undergone longitudinal amyloid imaging via PiB positron emission tomographic imaging within 376 days (mean [SD], 84.3 [92] days) of cerebrospinal fluid collection. Biomarker measures include cortical PiB SUVR (A), Aβ40 (B), Aβ42 (C), Aβ42 to Aβ40 ratio (D), and total tau (E). The Aβ40, Aβ42, and total tau were analyzed by INNOTEST enzyme-linked immunosorbent assay (Fujirebio Europe). Being PiB positive was defined as having a mean cortical PiB SUVR higher than 1.42 and is represented by the dashed horizontal line in panel A. Gray lines indicate PiB negative at baseline and follow-up (n = 52); solid colored lines, PiB positive at

both baseline and follow-up (n = 14); dashed colored lines, PiB negative at baseline but positive at follow-up (n = 6); and solid black lines, PiB negative with discordant (low) cerebrospinal fluid Aβ measures at baseline and follow-up (n = 2). Colored solid and dashed lines are each differently colored only to facilitate visual comparisons across all analytes for each PiB-positive individual.

Figure 2.5. Association Between Longitudinal Patterns of Cerebrospinal Fluid Biomarkers Visinin-Like Protein 1 (VILIP-1) and Chitinase-3-Like Protein 1 (YKL-40), Cortical Amyloid, and Age



A subset (n = 74) of Adult Children Study participants had undergone longitudinal amyloid imaging via Pittsburgh compound B (PiB) positron emission tomographic imaging within 376 days (mean [SD], 84.3 [92] days) of cerebrospinal fluid collection. Biomarker measures include VILIP-1 (A) and YKL-40 (B). Being PiB positive was defined as having a mean cortical PiB standardized uptake value ratio higher than 1.42 (see dashed horizontal line in Figure 3A). Gray lines indicate PiB negative at baseline and follow-up (n = 52); solid colored lines, PiB positive at both baseline and follow-up (n = 14); dashed colored lines, PiB negative at baseline but positive at follow-up (n = 6); and solid black lines, PiB negative with discordant (low) cerebrospinal fluid β-amyloid measures at baseline and follow-up (n = 2). Colored solid and dashed lines are each differently colored only to facilitate visual comparisons across all analytes for each PiB-positive individual.

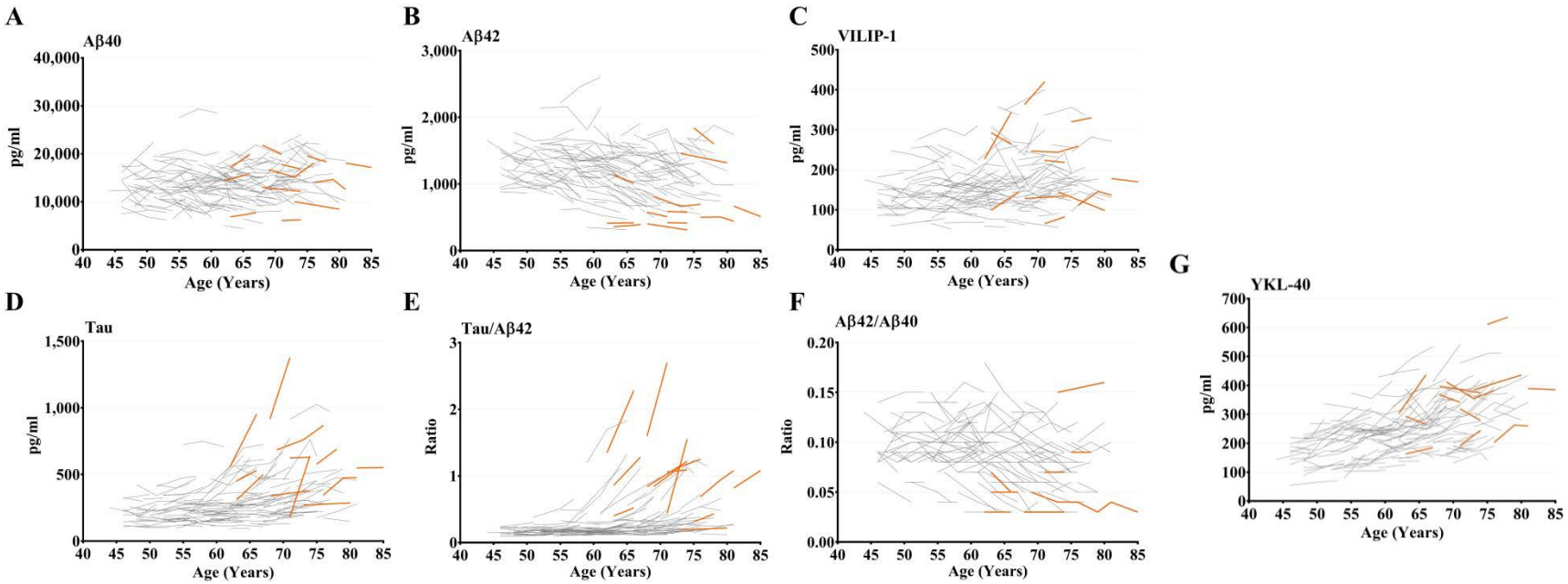
2.4.6 Aβ42 Cutoff as Estimated Using PiB at Baseline

Using only baseline CSF and PiB obtained within 376 days (mean [SD], 89.9 [95] days), a slightly larger subcohort of 105 participants was used to calculate a cutoff for CSF Aβ42 (INNOTEST) based on PiB positivity. The optimal cutoff in this cohort is 1041 pg/mL (sensitivity = 1; specificity = 0.82), with an area under the receiver operating characteristic curve of 0.9352 (95% CI, 0.8895-0.9808).

2.4.7 Case Study of Participants Who Received a CDR Higher Than 0 at Clinical Follow-up

Biomarker studies in cognitively normal elderly cohorts have demonstrated prognostic utility of baseline CSF measures for predicting future cognitive decline. To assess whether this relationship exists even earlier in the preclinical stages (during middle-age), as a preliminary analysis we compared the biomarker trajectories in participants who received a CDR higher than 0 at some point during clinical follow-up with those who retained a CDR of 0. Of the 169 participants evaluated, all of whom were cognitively normal (CDR of 0) at the time of baseline CSF collection, 14 received a CDR of 0.5 at some point during follow-up (mean [SD], 6.55 [1.94] years; median, 6.15 years; range, 4.21-10.28 years), and 3 of these progressed further to a CDR of 1. The remaining 155 participants had a CDR of 0 at all follow-up (mean [SD], 6.01 [1.94] years; median, 6.21 years; range 0.98-11.32 years). The duration of follow-up did not differ significantly between the groups ($P > .05$). All individuals who progressed to a CDR higher than 0 were older than 61 years at baseline. There was no apparent relationship between baseline or longitudinal trajectories of Aβ40 and cognitive status (**Figure 2.6A**). In contrast, the majority of progressors exhibited low Aβ42 (**Figure 2.6B**) and Aβ42 to Aβ40 ratio (**Figure 2.6C**) at baseline and follow-up and high total tau and total tau to Aβ42 ratio (**Figure 2.6D** and **E**). Patterns of VILIP-1 and YKL-40 did not appear to differ between the clinical groups (**Figure 2.6F** and **G**). However, the number of clinical progressors is too small to perform rigorous statistical analyses in the current cohort.

Figure 2.6 Cerebrospinal Fluid Biomarker Trajectories in Participants Receiving a Clinical Dementia Rating Higher Than 0 at Some Point During Clinical Follow-up



Within-person trajectories of cerebrospinal fluid β-amyloid 40 (Aβ40) (A), Aβ42 (B), total tau (C), total tau to Aβ42 ratio (D), visinin-like protein 1 (VILIP-1) (E), and chitinase-3-like protein 1 (YKL-40) (F) are plotted as a function of age. The Aβ40, Aβ42, and total tau were analyzed by INNOTEST enzyme-linked immunosorbent assay (Fujirebio Europe). Fourteen individuals received a Clinical Dementia Rating of 0.5 or 1 at some point during follow-up (mean [SD], 6.55 [1.94] years; range, 4.21–10.28 years). Orange lines indicate individuals who received a Clinical Dementia Rating higher than 0 at available follow-up visits; gray lines, individuals who did not receive a Clinical Dementia Rating higher than 0.

2.5 Discussion

Our results demonstrate the following: (1) levels of CSF Aβ42 in some cognitively normal individuals decrease over time, starting as young as early middle age (45-54 years); (2) in mid middle-age (55-64 years), reductions in Aβ42 are associated with the development of PiB-positive amyloid plaques; (3) elevations in neuronal injury markers total tau, P-tau$_{181}$, and (to a lesser extent) VILIP-1 increase dramatically in some individuals in mid and late (65-74 years) middle-age; (4) the gliosis/neuroinflammation marker YKL-40 increases throughout middle-age; (5) these biomarker changes are observed in both risk groups defined by *APOE* genotype but are more evident in ε4 carriers and (for amyloid-related measures) in an allele dose-dependent manner; and (6) these AD-consistent trajectories are not clinically benign but instead are associated with future cognitive decline. These observations were confirmed in both evaluated immunoassays for Aβ42 and total tau.

Reductions in CSF Aβ42 concentration within certain individuals throughout middle-age suggest an ongoing pathological process that for some people starts quite early (ages 45-54 years). Levels may begin to decrease even earlier, but additional investigation in younger cohorts is needed to test this hypothesis. During middle-age, the timing of this decrease is influenced by ε4 allele dosage, consistent with studies demonstrating a major influence of *APOE* genotype on Aβ aggregation and clearance[196,197]. Baseline and follow-up Aβ42 levels are among the lowest in ε4 homozygotes compared with heterozygotes and ε4 noncarriers, with reduction evident at earlier ages. Such effects are consistent with the ε4 dosage effects on age at dementia onset[198].

Regardless of when Aβ42 levels begin to decrease during the preclinical period, these decreases did not coincide with the presence of amyloid detectable by PiB PET until mid middle-age. The Aβ42 level was stably low or beginning to decline in some individuals while cortical PiB binding

was still below the threshold of positivity, and PiB binding did not begin to increase until the CSF Aβ42 level was already relatively low. Thus, it seems likely that Aβ42 aggregation can be detected earlier with CSF analysis than with cortical PiB PET imaging, consistent with recent studies in autosomal dominant AD[27,199]. This is highlighted by 2 high-risk early middle-aged ε4 homozygotes who had stable, low Aβ42 levels (and Aβ42 to Aβ40 ratios) in longitudinal samples but were PiB negative. This observation may reflect sequestration of Aβ42 into oligomeric forms undetectable with the current assays or its deposition in nonfibrillar (PiB-negative) diffuse plaques. In support of the latter, low CSF Aβ42 concentration in the absence of PiB positivity has been reported in a case in which numerous diffuse plaques, but few neuritic plaques, were observed at autopsy[200]. However, the early middle-age bin of the longitudinal PiB subcohort is quite small; subregional PiB analyses and evaluation of future longitudinal PiB scan in ACS participants are necessary to rigorously evaluate PiB changes in early middle-age.

The calculated CSF Aβ42 cutoff in this cohort is quite high at 1041 pg/mL, higher than previously reported using the INNOTEST kit (typically 450-650 pg/mL)[17,20,201]. This apparent discrepancy may reflect the younger age of the ACS cohort. Most likely it reflects the fact that we used a newer modified, improved INNOTEST assay. This cutoff is not suggested for clinical use but was instead provided to evaluate amyloid positivity using CSF measures – similar to protocols being considered for enrollment in AD prevention trials. Using this cutoff, 51 of the 169 participants (30%) would be considered amyloid positive and eligible for clinical trial enrollment based on baseline CSF Aβ42 concentration alone. Further longitudinal follow-up is needed to determine what percentage of these individuals will present with cognitive decline, which will in turn enable analysis of the efficacy of CSF Aβ42 concentration at baseline for determination of preclinical AD.

In contrast to the early changes in Aβ42, increases in total tau, P-tau$_{181}$, and VILIP-1 are typically not apparent until later (ages ≥55 years). Notably, the rate of increase was significantly greater in the ε4-carrying at-risk group during mid middle-age, coincident with continuing, robust decreases in Aβ42 level. It was in this age range that many participants with the AD biomarker pattern began to exhibit cognitive decline. Interestingly, the absolute slopes (ie, rates of increase) of these neuronal injury markers in the ε4 carriers actually decreased from mid to late middle-age. This pattern is consistent with a potential slowing of an earlier robust phase of neuronal injury or perhaps reflects neuronal dysfunction that adversely affects the normal cellular secretion or release of these proteins. It will be interesting to determine whether this pattern is also observed in those at lower risk (ε4 noncarriers), albeit at older ages, how it compares with proposed early markers of synaptic function currently in development, and whether this proposed slowing continues into the symptomatic phase as has been reported in individuals with autosomal dominant AD[64] and late-onset AD dementia[202]. The rate accelerations in these markers at mid middle-age observed here in the at-risk group are consistent with the concept of an age-related transition between stage 1 (amyloid alone) and stage 2 (amyloid plus neuronal injury) of preclinical AD proposed by the National Institute on Aging-Alzheimer's Association Preclinical AD Working Group[203]. Although these proposed stages are currently defined by biomarker measures obtained at a single point in time, it is possible that a longitudinal biomarker metric may have more utility. This hypothesis awaits further investigation.

The consistent pattern of increases in YKL-40 level in all age bins suggests that neuroinflammation/gliosis (the hypothesized cause of the increase in YKL-40 level) is a process that occurs normally with aging. However, the particularly robust increases observed in at-risk ε4 carriers during mid middle-age suggest that this age-related process may be further exacerbated

in the presence of insults including amyloid deposition and neuronal injury. Whether this neuroinflammatory process contributes to the concomitant increase in neuronal injury remains to be determined.

This study is not without limitations. As by design the ACS cohort enrolls participants with and without family history of AD for longitudinal imaging and CSF biomarker studies, participants may not be representative of the general population. Despite the large number of participants in this unique cohort, there are fewer in the ε4-carrying group, and most participants at the time of analysis had only 2 longitudinal samples available. While some individuals had 10 years of clinical follow-up, others had only 4. Although the results provide support for a scenario in which changes in amyloid-related processes precede those of tau or other neurodegeneration-related processes, additional analyses during a longer period are required to determine the precise sequence of biomarker changes within a given individual. Furthermore, as expected in such a young, asymptomatic cohort, relatively few participants in this initial report had received a CDR greater than 0 during follow-up. Continued evaluation of longer clinical follow-up will provide an opportunity to better elucidate the biomarker patterns in middle-age that predict future cognitive decline.

2.5.1 Conclusions

The present groupwide analyses are supportive of a preclinical period of AD in which biomarker patterns consistent with underlying disease pathology are first detectable during middle-age, the timing of which is influenced by *APOE* genotype, with amyloid changes occurring prior to neuronal injury. However, proposals to use biomarkers in clinical settings require demonstration of their utility on a patient-by-patient basis. Importantly, our preliminary findings of an

association between CSF biomarker positivity in specific individuals who go on to develop cognitive deficits within a few years provide support for such potential use.

Chapter 3: Longitudinal Decreases in Multiple Cerebrospinal Fluid Biomarkers of Neuronal Injury in symptomatic Late Onset Alzheimer's Disease

This work is currently under review.

3.1 Abstract

Introduction

Individuals in early stages of Alzheimer's disease (AD) are a targeted population for secondary prevention trials aimed at preserving normal cognition. Understanding within-person biomarker(s) change over time is critical for trial enrollment and design.

Methods

Longitudinal cerebrospinal fluid (CSF) samples from the Alzheimer's Disease Neuroimaging Initiative were assayed for novel markers of neuronal/synaptic injury (VILIP-1, Ng, SNAP-25) and neuroinflammation (YKL-40) and compared with Aβ42, Tau, and P-Tau_{181}. General linear mixed models were used to compare within-person rates of change in three clinical groups (cognitively normal, mild cognitive impairment and AD) further defined by β-amyloid status.

Results

Levels of injury markers were highly positively correlated. Despite elevated baseline levels as a function of clinical status and amyloid-positivity, within-person decreases in these measures were observed in the early symptomatic, amyloid-positive AD group.

Discussion

Knowledge of within-person biomarker change will impact interpretation of biomarker outcomes in clinical trials that are dependent on disease stage.

3.2 Introduction

Clinical trials of potential disease-modifying therapies for Alzheimer's Disease (AD) have failed to slow cognitive decline in patients who have dementia or milder cognitive symptoms (e.g., mild cognitive impairment, MCI)[169]. Since AD pathology begins to develop ~20 years before cognitive decline (preclinical AD)[27,204], it is possible that trial participants were too far along in the disease process for such therapies to impact cognition. Therefore, individuals at earlier stages, including the asymptomatic, preclinical stage (defined by biomarkers), are now receiving intense focus for secondary prevention trials aimed at preserving normal cognitive function. Understanding the patterns of biomarker(s) change over time is critical for defining where individuals fall along the pathologic disease cascade.

Cross-sectional studies indicate that β-amyloid (Aβ)-related biomarkers become abnormal first, followed by markers of tau-related neuronal injury, both during the preclinical period[205]. Elevated injury markers in the presence of amyloid positivity then become a strong predictor of subsequent cognitive decline[44]. Interestingly, while regional brain atrophy then ensues, with abnormality increasing with symptomatic progression[206], a recent study of individuals with autosomal dominant AD (ADAD) reported longitudinal decreases in CSF levels of neuronal injury markers including Tau, P-Tau_{181} and visinin-like protein 1 (VILIP-1) in symptomatic mutation carriers[64], suggesting a slowing of acute neurodegenerative processes and/or a decrease in the number of viable neurons contributing to the pools of these markers in this later stage of the disease. Regardless of the mechanism, if confirmed in an independent cohort of persons developing late onset AD (LOAD), such a pattern will likely have an impact on interpretation of biomarker outcomes in clinical trials that is dependent on the disease stage. To this end, the present study evaluated the patterns of within-person longitudinal change in a variety of standard

(Tau, P-Tau$_{181}$) and novel (VILIP-1, neurogranin, SNAP-25) CSF neuronal injury biomarker levels in individuals spanning the full range of AD, including normal, preclinical AD, MCI due to AD and symptomatic AD, and a comparison of these changes with regional brain atrophy and cognitive decline.

3.3 Methods

ADNI Study Design

CSF Aβ42, total tau (Tau) and phospho-tau181 (P-Tau), demographic, imaging and cognitive data were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adniloni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. ADNI participants have been recruited from more than 50 sites across the USA and Canada. Regional ethical committees of all institutions approved of the study, and all participants provided written informed consent. For up-to-date information, see www.adni-info.org.

Study Participants

The ADNI cohort in the present study consisted of all cognitively normal (CN) participants and those with MCI or AD dementia (AD) with available CSF samples from at least two visits as of April 2012. This cohort included 152 individuals across ADNI1, ADNI GO and ADNI2 (n=56 CN, n=73 MCI, n=17 AD). Demographic and cognitive data were downloaded in August 2015 and were collected as described (adni.loni.usc.edu/methods/documents/). By definition, individuals in the CN group all had a clinical dementia rating (CDR) score of 0 at the time of lumbar puncture (LP) and a Mini-Mental State Examination (MMSE) score ≥ 24.

Individuals with MCI also scored ≥ 24 on the MMSE but exhibited subjective memory loss (>1 standard deviation below the normal mean of the delayed recall of the Wechsler Memory Scale Logical Memory II), received a CDR of 0.5, preserved activities of daily living and the absence of dementia. The AD group met the definition of probable AD according to criteria established by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[207], had MMSE scores of 20-26 and CDRs of 0.5 or 1. Groups were designated by clinical diagnosis at the time of initial available CSF sample in the longitudinal cohort (defined herein as baseline).

ADNI Clinical, CSF and Imaging Data

Scores for MMSE and Alzheimer's Disease Assessment Scale-cognitive 11 (ADAS11) and ADAS13 were downloaded from the LONI site in August 2015 via ADNIMerge R Package. Values for CSF Aβ42 (INNOBIA AlzBio3; Fujirebio, Ghent, Belgium) were downloaded at the same time from two datasets (UPENNBIOMK4 and UPENNBIOMK6) and were used to define amyloid-positivity based on a published, autopsy-confirmed cut-off value (<192 pg/ml)[208]. For statistical analyses, values for Aβ42, total Tau (Tau) and Ptau181 (P-Tau) generated by a single lot number of the novel, fully automated, electrochemiluminescent Elecsys® immunoassays (Roche Diagnostics, Basel, Switzerland) were downloaded from the LONI site in March 2017 from a single dataset (UPENNBIOMK9). The Elecsys® system aims to offer a fully automated CSF biomarker test for AD capable of achieving *In Vitro* Diagnostic (IVD) capability and offers some improvements over current Research Use Only (RUO) assays including: reduction in manual steps, improved precision and accuracy both within labs and between labs, and improved lot-to-lot reagent performance. The Elecsys® Aβ42 immunoassay in use is not a commercially available IVD assay. It is an assay currently under development and for investigational use only.

The measuring range of the assay is 200 (lower technical limit) – 1700 (upper technical limit) pg/ml. The performance of the assay beyond the upper technical limit has not been formally established. Therefore, values above the upper technical limit have been truncated at 1700 pg/ml. In the current study, baseline analyses excluded these data. Longitudinal statistical analyses were run with and without these truncated values and performed nearly identically**.**

Magnetic resonance imaging (MRI) data for the left and right hippocampal (HP) volume (white matter parcellation) and left and right entorhinal cortex (EC) thickness, two regions known to be affected early in AD, were also analyzed. EC thickness and HP volume were downloaded in November 2016 from the file UCSFFSL_02_01_16. Acquisition of 1.5 Tesla MRI and data processing methods are as described (adni.loni.usc.edu/methods/mri-analysis/). Data were processed with FreeSurfer v4.4, and only values that passed all quality control (QC) standards were included in the analyses. Values for left and right HP and EC thickness were added together to create a value for "total" HP volume and EC thickness.

Novel CSF Analytes

Samples were analyzed for YKL-40 (also known as chitinase 3-like 1, a marker of gliosis/neuroinflammation)[209], visinin-like protein 1 (VILIP-1, a neuronal calcium sensor protein and marker of neuronal injury)[210], neurogranin (Ng, a post-synaptic protein and marker of synaptic dysfunction)[106] and synaptosomal-associated protein 25 (SNAP-25, a pre-synaptic protein and marker of synaptic dysfunction) [19]. YKL-40 was measured with a plate-based enzyme-linked immunoassay (ELISA) (MicroVue ELISA; Quidel, San Diego, CA)[31]. VILIP-1[61,62], NG[63,69] and SNAP-25 were measured using microparticle-based immunoassays using the Singulex (now part of EMD-Millipore; Alameda, CA) Erenna system, and employed antibodies

developed in the laboratory of Dr. Jack Ladenson at Washington University. All samples (each on the same freeze/thaw cycle) were run in triplicate on a single lot number for VILIP-1, SNAP-25 and Ng and in duplicate for YKL-40. Within-person longitudinal samples were run on the same assay plate to reduce inter- and intra-plate variability. Quality control for VILIP-1, SNAP-25 and Ng included analysis of three internal standard CSF pools run on each plate and two internal pools for YKL-40. See **Appendix** for assay details.

Statistical Analysis

Since the study intent was to compare baseline biomarker levels and their longitudinal change over time in individuals who span the AD continuum (from no disease [normal], to preclinical AD, to MCI due to AD, to AD), participants in the three diagnostic categories (CN, MCI, AD) were further stratified into β-amyloid-positive (Aβ+) versus amyloid-negative (Aβ-) at baseline based on the published ADNI CSF Aβ42 cut-off of <192pg/mL[208]. Baseline characteristics for the five resultant groups (CN-, CN+, MCI-, MCI+, AD+) were summarized as mean (standard deviation [SD)]) for continuous variables or number (percentage) for categorical variables. Group differences among the various measures were assessed using one-way ANOVA and post-hoc Tukey tests. Correlations between measures were assessed via Spearman correlation.

Biomarker concentrations, cognitive performance and MRI measures within individuals over time were compared among the five groups (all AD individuals were Aβ+) by general linear mixed models with random intercepts/slopes at the subject level to allow estimation and comparison of within-person rates of change[211]. In addition to the mean intercept and slope for each group (unadjusted models), covariates including age at baseline, *APOE* ε4 carriage, sex, education, and their interactions with subject groups on the intercepts and slopes, were also included as fixed effects (see **Appendix)**. All general linear mixed models assumed a subject

level random effect on intercept and slope and were fitted using the maximum likelihood method. Statistical tests were based on the approximate F or t- tests with denominator degrees of freedom approximated by the Satterthwaite methods[212]. All analyses were performed with SAS version 9.4 (SAS Institute Inc.), with statistical significance defined as $p < 0.05$.

3.4 Results

3.4.1 Demographics

Of the 152 ADNI participants who met the criteria for having longitudinal CSF samples (range 2-7 LPs over 1-7 years of follow up [mean (SD) = 4.0 (1.62)] and a mean (SD) LP interval of 16 (8.6) months), four were omitted from the dataset due to missing values for CSF Aβ42 (via AlzBio3) required to define baseline amyloid status (Aβ+ vs Aβ-). Participants in the final dataset of n=148 were 38% female, between 58 and 90 years of age at the time of initial LP (mean [SD] = 75 [7.13]), and 68% were *APOE* ε4-positive (**Table 1**). As expected, baseline HP volume and EC thickness were different among the groups (CN>MCI>AD) ($p<0.0001$). Performances on MMSE, ADAS11 and ADAS13 were also as expected, with the MCI and AD groups performing worse than the CN group ($p<0.0001$).

When the clinical groups were dichotomized into amyloid-positive (Aβ+) and amyloid-negative (Aβ-)[208]), neuronal injury/inflammation biomarker levels were higher (more AD-like) in the Aβ+ compared to the Aβ- groups, both among and within each clinical group (**Table 2**). Positive correlations were observed among the injury markers at baseline, strongest among tTau, VILIP-1 and Ng (Spearman r= 0.798-0.853) (**Appendix Table 1**). SNAP-25 was moderately correlated with the other injury markers (r= 0.619-0.720), and as expected, Tau and P-tau exhibited the highest positive correlation (r= 0.975). Elecsys Aβ42 was positively correlated with

AlzBio3 Aβ42 (r=0.869) and negatively correlated with Tau, P-tau and SNAP-25 (r=-0.214, -0.324 and -0.240, respectively). YKL-40 was significantly, but weakly, correlated with the injury markers (r= 0.307-0.422) but not Aβ42.

Table 3.1 Study Demographics

	CN		MCI		AD
	Aβ-	Aβ+	Aβ-	Aβ+	Aβ+
N	35	21	18	58	16
Baseline age, mean (SD)	76 (5.7)	76 (3.7)	77 (7.3)	74 (6.5)	74 (6.7)
Gender, F/M (%F)	14/21 (40%)	10/11 (48%)	4/14 (22%)	18/40 (31%)	11/6 (65%)
Education, mean (SD), y	16 (3.1)	16 (3.4)	17 (1.8)	16 (2.8)	15 (3.0)
APOE ε4 allele, +/- (%+)	3/32 (9%)	9/12 (43%)	0/18 (0%)	40/18 (69%)	13/4 (77%)
# CDR 0/0.5/1, n	35/0/0	21/0/0	0/18/0	0/57/1	0/10/6
CDR-SB, mean (SD)	0.029 (0.12)[C-E]	0.024 (0.11)[C-E]	1.25 (0.55)[A,B,E]	1.61 (0.85)[A,B,E]	4.24 (1.49)[A-D]
MMSE, mean (SD)	29.1 (1.1)[C-E]	29.4 (0.9)[C-E]	27.6 (1.8)[A,B,E]	26.8 (1.8)[A,B,E]	23.7 (1.7)[A-D]
ADAS 11, mean (SD)	5.3 (2.2)[C-E]	7.1 (3.3)[D,E]	9.9 (4.1)[A,E]	11.7 (5.1)[A,B,E]	18.7 (6.1)[A-D]
ADAS 13, mean (SD)	8.4 (3.5)[C-E]	10.5 (3.9)[D,E]	15.5 (5.9)[A,E]	19.5 (7.1)[A,B,E]	28.9 (7.4)[A-D]
# LP's 2/3/4/5/6/7, n	0/15/7/8/5/0	0/8/6/4/3/0	0/5/10/2/1/0	2/26/18/5/6/1	1/9/5/1/0/0
LP interval, mean (SD), mo	17.01 (9.44)[E]	17.55 (10.40)[E]	16.92 (8.98)	15.90 (7.92)	12.73 (2.86)[A,B]
LP follow-up, mean (SD, Range), mo	52.9 (19.7, 23-86)[E]	55.0 (17.0, 26-85)[E]	49.8 (17.9, 24-87)[E]	45.0 (18.9, 16-86)[E]	30.2 (10.2, 12-50)[A-D]
Total EC thickness, mean (SD), mm	6.88 (0.84)[C-E]	6.88 (0.95)[C-E]	6.32 (0.96)[A,B,E]	6.44 (0.87)[A,B,E]	5.26 (0.82)[A-D]
Total HP volume, mean (SD), mm^3	6577 (815)[C-E]	6553 (886)[C-E]	5818 (978)[A,B,E]	5861 (880)[A,B,E]	5117 (848)[A-D]

A – Significantly different from CN Aβ-
B – Significantly different from CN Aβ+
C – Significantly different from MCI Aβ-
D – Significantly different from MCI Aβ+
E – Significantly different from AD Aβ+

Abbreviations: Aβ, amyloid-β status; AD, Alzheimer disease; ADAS 11; Alzheimer's Disease Assessment Scale-cognitive test, version 11 (higher score is worse performance; ADAS 13, Alzheimer's Disease Assessment Scale-cognitive test, version 13 (higher score is worse performance); APOE, apolipoprotein E; CDR, Clinical Dementia Rating score; CDR-SB, CDR sum of boxes; CN, cognitively normal; EC, entorhinal cortex; HP, hippocampus; LP, lumbar puncture; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination

3.4.2 Patterns of Neuronal Injury and Neuroinflammatory Markers

Participant-level CSF biomarker trajectories were plotted for each of the five amyloid-defined clinical groups (**see Appendix Figure 1 for spaghetti plots**). General linear mixed models (with random intercepts/slopes at the subject level) were then used to estimate and compare baseline biomarker levels and within-person rates of change in the five groups. Results adjusting for sex, *APOE* ε4 status, education, baseline age, and total ventricular volume are provided in the **Appendix**.

Elecsys® tTau

Baseline Tau levels were significantly elevated in the AD+ group compared to all other groups (all $p \leq 0.01$) and the MCI+ compared to the MCI- and CN- ($p<0.0001$) and CN+ groups ($p=0.02$) (**Table 2**). Longitudinally, Tau levels significantly increased in both CN (both $p<0.05$) and the MCI+ groups ($p<0.0001$) (**Figure 1, Table 2**). Tau levels decreased longitudinally in the AD+ group, but this change did not reach statistical significance ($p=0.095$).

Elecsys® pTau

P-tau levels at baseline were significantly elevated in the AD+ compared to all other groups (all $p<0.01$), MCI+ compared to MCI- and CN- (both $p<0.0001$) and CN+ groups ($p=0.02$), and the CN+ compared to the MCI- and CN- groups (both $p<0.03$) (**Table 2**). Longitudinally, P-tau levels significantly increased in the CN+ ($p=0.001$) and trended toward increase in the MCI+ group ($p=0.055$). Strikingly, P-tau levels significantly declined in the AD+ group ($p \leq 0.0001$) (**Figure 1, Table 2**), with rate of change greater than the change in all other groups ($p<0.001$).

VILIP-1

Levels of baseline VILIP-1 were significantly higher in the MCI+ and AD+ compared to both the MCI- and CN- groups (all $p \leq 0.01$) (**Table 2**). The amyloid-positive groups did not differ from one another (all $p>0.05$). Longitudinally, as with P-tau, VILIP-1 levels strongly and

significantly decreased in the AD+ group (p=0.006), whereas no significant changes were observed in the other groups (**Figure 1, Table 2**).

SNAP-25

SNAP-25 values at baseline were significantly higher in the AD+ and MCI+ compared to the CN- (both p<0.0003), CN+ (p=0.001 and p=0.01, respectively), and MCI- groups (both p<0.0001) (**Table 2**). Longitudinally, SNAP-25 levels declined significantly in the AD+ group (p=0.05), whereas no significant changes were observed in the other groups (**Figure 1, Table 2**).

Ng

Baseline levels of Ng were significantly higher in the AD+ group than the CN- (p=0.003), CN+ (p=0.02), and MCI- (p=0.0006) groups, although not between the MCI+ and AD+ groups (p=0.10) (**Table 2**). Levels were also higher in the MCI+ compared to the CN- (p=0.004) and MCI- (p=0.02) groups. Longitudinally, Ng markedly and significantly decreased in the AD+ group (p<0.0001), whereas no significant changes were observed in the other groups (**Figure 1, Table 2**).

YKL-40

In contrast to the markers of neuronal injury, baseline levels and longitudinal patterns of change in the neuroinflammatory marker, YKL-40, exhibited a large degree of within-group variability. Baseline YKL-40 was significantly higher in the AD+ compared to the MCI- (p=0.04) but not the other groups (**Table 2**). Longitudinally, all groups showed an increase in mean levels over time, but this increase was statistically significant only in the MCI+ group (p=0.03) (**Figure 1, Table 2**), perhaps due to less variability (smaller SD) within that group.

Elecsys® Aβ42

Although CSF Aβ42 (as measured in ADNI by AlzBio3) was used *a priori* to define amyloid status in the clinical groups, we were also interested in evaluating the patterns of this biomarker

using the novel Elecsys® platform. As expected, baseline Aβ42 levels (via Elecsys®) were significantly lower in all Aβ+ compared to Aβ42- groups (all p<0.0001) (**Table 2**). Longitudinally, levels decreased in all groups (and at similar rates), although only the AD+ and CN- groups reached statistical significance (p=0.04 and p=0.0004, respectively) (**Figure 1, Table 2**).

Table 3.2 Baseline CSF biomarker levels and estimated within-person annual change over time

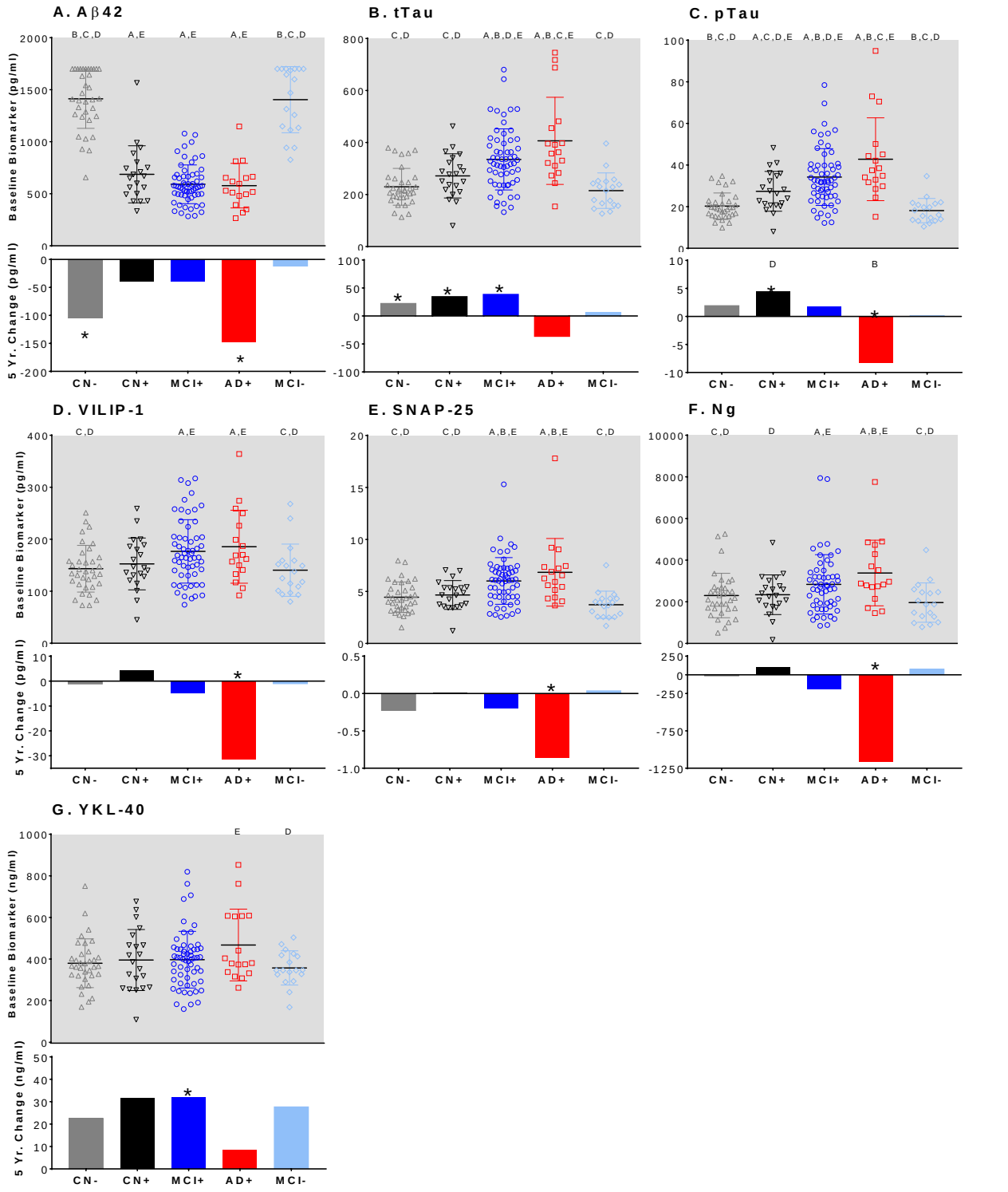
	CN		MCI		AD
	Aβ-	Aβ+	Aβ-	Aβ+	Aβ+
N	35	21	18	58	16
Baseline CSF Biomarkers					
Elecsys Aβ42, mean (SD), pg/ml	1413 (284)[B,D,E]	687 (274)[A,C]	1404 (318)[B,D,E]	590 (187)[A,C]	578 (214)[A,C]
Elecsys tTau, mean (SD), pg/ml	230 (70.8)[D,E]	272 (84.9)[D,E]	215 (68.2)[D,E]	331 (117.5)[A-C,E]	407 (167.5)[A-D]
Elecsys pTau, mean (SD), pg/ml	20.3 (6.30)[B,D,E]	27.4 (9.56)[A,C-E]	18.1 (5.83)[B,D,E]	33.7 (13.62)[A-C,E]	42.8 (19.90)[A-D]
VILIP-1, mean (SD), pg/ml	143.3 (44.9)[D,E]	152.6 (49.8)	140.5 (50.2)[D,E]	176.7 (61.0)[A,C]	185.6 (70.1)[A,C]
SNAP-25, mean (SD), pg/ml	4.45 (1.5)[D,E]	4.66 (1.4)[D,E]	3.72 (1.3)[D,E]	6.01 (2.2)[A-C]	6.84 (3.3)[A-C]
Ng, mean (SD), pg/ml	2302 (1066)[D,E]	2339 (953)[E]	1962 (945)[D,E]	2836 (1426)[A,C]	3383 (1576)[A-C]
YKL-40, mean (SD), ng/ml	384.1 (20.08)	399.6 (19.4)	361.6 (19.4)[E]	401.3 (17.87)	471.9 (41.86)[C]
CSF Biomarker Estimated Annual Slope					
Elecsys Aβ42, pg/ml (SE)	**-20.91 (5.6)**	-7.96 (7.27)	-2.38 (8.4)	-7.82 (5.17)	**-29.48 (14.2)**
p value	***0.0004***[F]	*0.28*	*0.78*	*0.13*	***0.039***[F]
Elecsys tTau, pg/ml (SE)	**4.29 (2.1)**[E]	**6.75 (2.7)**[E]	1.10 (3.1)	**7.55 (1.8)**[E]	-7.11 (4.2)[A,B,D]
p value	***0.048***[F]	***0.015***[F]	*0.72*	***<0.0001***[F]	*0.095*
Elecsys pTau, pg/ml (SE)	0.39 (0.2)[E]	**0.88 (0.3)**[C,E]	0.028 (0.3)[B,E]	0.35 (0.2)[E]	**-1.65 (0.4)**[A-D]
p value	*0.069*	***0.0013***[F]	*0.93*	*0.055*	***<0.0001***[F]
VILIP-1, pg/ml (SE)	-0.23 (1.0)[E]	0.89 (1.2)[E]	-0.21 (1.4)[E]	-0.96 (0.9)[E]	**-6.31 (2.3)**[A-D]
p value	*0.81*	*0.48*	*0.88*	*0.27*	***0.006***[F]
SNAP-25, pg/ml (SE)	-0.0453 (0.042)	0.00279 (0.053)	0.00715 (0.060)	-0.0387 (0.037)	**-0.172 (0.088)**
p value	*0.28*	*0.96*	*0.91*	*0.29*	***0.05***[F]
Ng, pg/ml (SE)	-2.74 (26.1)[E]	19.88 (33.6)[E]	15.5521 (38.2)[E]	-38.6334 (23.6)[E]	**-232.43 (58.9)**[A-D]
p value	*0.92*	*0.56*	*0.68*	*0.10*	***<.0001***[F]
YKL-40, ng/ml (SE)	4.51 (3.5)	6.29 (4.3)	5.54 (4.9)	**6.37 (3.0)**	1.68 (7.1)
p value	*0.20*	*0.15*	*0.26*	***0.035***[F]	*0.81*

A – Significantly different from CN Aβ-
B – Significantly different from CN Aβ+
C – Significantly different from MCI Aβ-
D – Significantly different from MCI Aβ+
E – Significantly different from AD Aβ+
F – Statistically significant slope

Bold – Slope that is statistically different from zero
All significance at least $p<0.05$

Abbreviations: Aβ, amyloid-β status; AD, Alzheimer disease; CN, cognitively normal; MCI, mild cognitive impairment; Ng, neurogranin; P-tau, phospho-tau181; SNAP-25, synaptosomal associated protein-25; Tau, total tau; VILIP-1, visinin-like protein 1

Figure 3.1 Baseline Biomarker Values and Estimated Within-person annual Change in CSF Biomarkers



Baseline biomarker values (top panel) and estimated group slopes for Aβ42 (A), tTau (B), pTau (C), VILIP-1 (D), SNAP-25 (E), Ng (F), and YKL-40 (G). Baseline is shown for each individual, estimated group slopes are shown as average annual change in 5 bins defined by diagnostic group and amyloid status, extrapolated to show 5 years of change.

[A] Different from CN- group

[B] Different from CN+ group

[C] Different from MCI+ group

[D] Different from AD+ group

[E] Different from MCI group

* Different from 0.

3.4.3 Cognitive Measures

Cognitive Measures

As expected, cognitive performance differed with clinical diagnosis, particularly in the Aβ+ symptomatic groups. Furthermore, Aβ+ individuals exhibited longitudinal changes in MMSE and ADAS11/13 that are consistent with a worsening of cognitive performance and often at a faster rate than the Aβ- groups. See **Appendix Figure 2** for spaghetti plots.

MMSE

Baseline MMSE was lower (indicative of worse performance) in the AD+ group than any other group (all $p<0.0001$), lower in the MCI+ compared to the MCI- ($p=0.03$) and both CN groups (both $p<0.0001$), and in the MCI- compared to both CN groups (both $p<0.03$) (**Table 3**). In the AD+ and MCI+ groups, MMSE was decreasing significantly (both $p<0.0001$) and at a faster rate in the AD+ compared to the MCI+ group ($p<0.0001$) (**Table 3**).

ADAS11 and ADAS13

At baseline, ADAS11 was significantly elevated (indicating worse performance) in the AD+ compared to both CN groups (both $p<0.0001$), both MCI groups compared to both CN groups (both $p<0.02$), and in the AD+ compared to both MCI groups (both $p<0.0001$) (**Table 3**).

Longitudinally, ADAS11 score significantly increased in the AD+ and MCI+ groups (both $p<0.0001$) and at a significantly faster rate in the AD+ versus the MCI+ group ($p<0.0001$) (**Table 3**).

Baseline ADAS13 performance was similar to ADAS11 except that the MCI+ group was also significantly elevated (worse performance) compared to the MCI- group ($p=0.05$) (**Table 3**). Longitudinally, ADAS13 was significantly increasing in all three Aβ+ groups (all $p<0.004$), at a faster rate in the AD+ compared to the MCI+ ($p=0.0005$) and CN+ ($p<0.0001$) groups, and at a faster rate in the MCI+ than the CN+ group ($p=0.02$) (**Table 3**).

3.4.4 Volumetric MRI Measures

As expected, HP volume and EC thickness were smaller at baseline in the AD+ compared to the other groups. However, all but the CN- group exhibited significant atrophy over time, albeit at different rates. See **Appendix Figure 3** for spaghetti plots.

Hippocampal Volume

HP volume at baseline was significantly smaller in the AD+ compared to all other groups ($p<0.001$ for both CN groups; $p=0.03$ for both MCI groups) and in both MCI groups compared to the CN groups (MCI- vs CN- [$p=0.003$] and CN+ [$p=0.01$]; MCI+ vs CN- and CN+ [both $p\leq0.0007$]) (**Table 3**). Longitudinally, all groups exhibited significant HP shrinkage over time (all $p\leq0.0001$) (**Table 3**). Volume in the AD+ and MCI+ groups decreased at a significantly faster rate than in both CN groups ($p\leq0.003$ and $p\leq0.001$, respectively) and the MCI- group ($p=0.04$ and $p=0.003$, respectively). The rate of atrophy in the MCI- group was faster than the CN- group ($p=0.0009$) and in the CN+ compared to the CN- group ($p=0.03$).

Entorhinal Cortex Thickness

At baseline, EC thickness was significantly smaller in the AD+ compared to all other groups ($p \leq 0.0003$), in the MCI+ compared to the CN groups ($p=0.0004$ for CN- and $p=0.01$ for CN+) (**Table 3)**. MCI-was also significantly thinner than the CN- group ($p=0.03$) and at the significance level compared to the CN+ group ($p=0.05$). Longitudinally, EC thickness was declining in all but the CN- group (all $p \leq 0.0003$) and at a faster rate in the AD+ compared to the CN+ ($p=0.005$) and MCI- ($p=0.007$) groups (**Table 3**). The EC in the MCI+ group was also shrinking more quickly than the CN+ and MCI- groups (both $p \leq 0.0001$).

Table 3.3 Baseline cognitive performance and imaging measures and estimated within-person annual change over time

	CN		MCI		AD
	Aβ-	Aβ+	Aβ-	Aβ+	Aβ+
N	35	21	18	58	16
Baseline Cognitive and Imaging Biomarkers					
MMSE, mean (SD)	29.1 (1.1)$^{C\text{-}E}$	29.4 (0.9)$^{C\text{-}E}$	27.6 (1.8)^{A,B,D,E}	26.8 (1.8)$^{A\text{-}C,E}$	23.7 (1.7)$^{A\text{-}D}$
ADAS 11, mean (SD)	5.3 (2.2)$^{C\text{-}E}$	7.1 (3.3)$^{C\text{-}E}$	9.9 (4.1)^{A,B,E}	11.7 (5.1)^{A,B,E}	18.7 (6.1)$^{A\text{-}D}$
ADAS 13, mean (SD)	8.4 (3.5)$^{C\text{-}E}$	10.5 (3.9)$^{C\text{-}E}$	15.5 (5.9)^{A,B,D,E}	19.5 (7.1)$^{A\text{-}C,E}$	28.9 (7.4)$^{A\text{-}D}$
Total EC thickness, mean (SD), mm	6.88 (0.84)$^{C\text{-}E}$	6.88 (0.95)$^{C\text{-}E}$	6.32 (0.96)^{A,B,E}	6.44 (0.87)^{A,B,E}	5.26 (0.82)$^{A\text{-}D}$
Total HP volume, mean (SD), mm^3	6577 (815)$^{C\text{-}E}$	6553 (886)$^{C\text{-}E}$	5818 (978)^{A,B,E}	5861 (880)^{A,B,E}	5117 (848)$^{A\text{-}D}$
Cognitive and Imaging Estimated Annual Slope					
MMSE, points (SE)	-0.051 (0.2)^{D,E}	-0.22 (0.2)^{D,E}	-0.039 (0.2)^{D,E}	**-1.26 (0.1)$^{A\text{-}C,E}$**	**-2.49 (0.3)$^{A\text{-}D}$**
p value	*0.76*	*0.30*	*0.87*	***<0.0001^{F}***	***<0.0001^{F}***
ADAS 11, points (SE)	0.20 (0.3)^{D,E}	0.75 (0.4)^{D,E}	0.30 (0.4)^{D,E}	**2.06 (0.3)$^{A\text{-}C,E}$**	**4.74 (0.6)$^{A\text{-}D}$**
p value	*0.52*	*0.06*	*0.50*	***<0.0001^{F}***	***<0.0001^{F}***
ADAS 13, points (SE)	0.37 (0.3)^{D,E}	**1.25 (0.4)^{D,E}**	0.53 (0.5)^{D,E}	**2.43 (0.3)$^{A\text{-}C,E}$**	**4.98 (0.7)$^{A\text{-}D}$**
p value	*0.27*	***0.0042^{F}***	*0.27*	***<0.0001^{F}***	***<0.0001^{F}***
Total EC thickness, mm (SE)	-0.0401 (0.022)$^{B\text{-}E}$	**-0.118 (0.023)^{A,D,E}**	**-0.118 (0.031)^{A,D,E}**	**-0.261 (0.018)$^{A\text{-}C}$**	**-0.295 (0.057)$^{A\text{-}C}$**
p value	*0.069*	***<0.0001^{F}***	***0.0003^{F}***	***<0.0001^{F}***	***<0.0001^{F}***
Total HP volume, mm^3 (SE)	**-59.4 (14.5)$^{B\text{-}E}$**	**-111.2 (18.2)^{A,D,E}**	**-145.9 (20.5)^{A,D,E}**	**-216.3 (11.9)$^{A\text{-}C}$**	**-230.8 (36.0)$^{A\text{-}C}$**
p value	***0.0001^{F}***	***<0.0001^{F}***	***<0.0001^{F}***	***<0.0001^{F}***	***<0.0001^{F}***

A – Significantly different from CN Aβ-
B – Significantly different from CN Aβ+
C – Significantly different from MCI Aβ-
D – Significantly different from MCI Aβ+
E – Significantly different from AD Aβ+

F – Statistically significant slope
Bold – Slope that is statistically different from zero
All significance at least $p<0.05$

Abbreviations: Aβ, amyloid-β status; AD, Alzheimer disease; ADAS 11; Alzheimer's Disease Assessment Scale-cognitive test, version 11 (higher score is worse performance); ADAS 13, Alzheimer's Disease Assessment Scale-cognitive test, version 13 (higher score is worse performance); CN, cognitively normal; EC, entorhinal cortex; HP, hippocampal; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination (0-30, with 30 as perfect score)

3.5 Discussion

Our primary finding is the decrease over time in the concentration of several different CSF markers of neuronal injury (Tau, P-tau, VILIP-1, SNAP-25, Ng) in individuals once they have developed symptomatic AD. In contrast, elevations in Tau, but not the other injury markers, were observed at earlier stages (amyloid-positive MCI and CN groups). These findings replicate similar longitudinal patterns (for Tau, P-tau and VILIP-1) reported in a cohort of autosomal-dominant AD[64], thus supporting a commonality in neuropathologic processes in sporadic and genetic forms of the disease. Knowledge of such within-person patterns of change has important implications for clinical trials in MCI and early stage AD in terms of the use of biomarker concentrations as pathologic endpoints in determining treatment efficacy for neuronal integrity.

While all the injury markers decreased over time in the AD+ group, the reduction in Ng was especially robust. Neurogranin is a calmodulin-binding postsynaptic neuronal protein[213,214] thought to be involved in activity-dependent synaptic plasticity and long-term potentiation[215]. Levels are reduced in AD brain[216,217] and elevated in AD CSF[73,106] , with high levels predictive of progression from mild cognitive impairment (MCI) to AD dementia[68,69,72,218]. Since elevations in CSF Ng are associated with brain atrophy[69,218] and reduced brain glucose uptake[218], it is considered a marker of synaptic dysfunction/loss.

Although less is known about SNAP-25 (a pre-synaptic t-SNARE molecule that plays a crucial role in calcium-dependent exocytosis of synaptic vesicles) in AD, like Ng, levels are reduced in brain[219] and elevated in CSF[80] compared to controls. Although both synaptic markers were decreasing longitudinally in the AD+ group, Ng was dropping at more than twice the rate as SNAP-25 (annual decreases of 6.9% vs 2.5%, respectively) and the other markers (1.8% Tau, 3.9% P-tau, and 3.4% VILIP). Interestingly, Aβ42 was also significantly decreasing annually by 5% in the early AD+ group but less so in the other groups. Although levels of Aβ42 are known to drop early in the disease and then plateau as amyloid continues to accumulate[204], 63% (10/16) of individuals in the current AD group were at very early symptomatic stages (CDR 0.5). Baseline levels of YKL-40, an astrocyte-derived protein with presumed neuroinflammatory properties[220], also increased with clinical severity as reported previously[221], but we observed a high level of within-group variability in longitudinal patterns. It is likely that YKL-40 reflects neuroinflammatory components not specifically due to AD. Interestingly, levels appeared to increase with age in the AD+ group (**Appendix Figure 1**) as has also been observed in cognitively normal middle-aged individuals[31]. Further studies regarding the role of YKL-40 in neurodegenerative diseases are warranted[222,223].

Despite the fact that there were strong positive correlations among levels of the various injury markers, consistent with previous reports[69,70], discordance in patterns of longitudinal change over time for tTau was observed in the amyloid-positive MCI group (robust increases in Tau but no statistical change in the other markers, including P-tau). CSF Tau levels are known increase in response to acute neuronal death as occurs in response to stroke, traumatic brain injury and

Creutzfeld-Jakob disease (CJD)[224], thus suggesting a robust phase of neuronal death and/or alterations in the normal metabolism of tau at the very early (MCI) symptomatic stage of AD, the time during which the first signs of cognitive impairment are evident. The reason(s) for a lack of within-person increase in these other injury markers remains unclear but may have something to do with the relatively short follow-up time in the current cohort (mean 4.0 ± 1.61 years) and/or the lack of information regarding how long a given individual had been in their designated clinical group at the time of baseline LP (i.e., where in the natural progression of the disease). Alternatively, such discordance may indicate that these markers reflect different processes associated with synaptic dysfunction and/or neuronal injury[70]. A full understanding of biomarker trajectories will require serial samples being collected from a larger number of individuals over a long period of time as they progress from one clinical stage to the next.

The biological reason(s) for reductions in CSF injury markers over time in early AD is unclear. In fact, very little is known about the normal metabolism of these markers that would lead to their appearance in the CSF in both normal and pathological settings. While it is conceivable that such reductions simply reflect a general dilution of CSF proteins that would come with increasing ventricular volume associated with overall brain atrophy, reductions were still observed after controlling for ventricular volume. It is possible that longitudinal reductions from an elevated baseline during early AD reflect a slowing of acute neurodegenerative processes with symptomatic disease progression and/or neuronal death leading to a smaller number of neurons that remain and contribute to the pool in CSF. Unlike structural MRI and amyloid (and tau) PET imaging measures that reflect cumulative change over the course of the disease, CSF measures reflect a snapshot in time, thus measuring different things. Indeed, hippocampal and EC atrophy continued over the course of the disease even in the face of decreasing levels of injury markers in

the CSF. It is therefore not unexpected that there exists some discordance when defining biomarker positivity (and notably for neuronal injury), as a function of imaging versus CSF[225]. This issue is important to consider when selecting biomarker modalities (CSF and/or imaging) for use in screening and/or outcome measures in clinical trials.

This study is not without limitations. The cohort with longitudinal CSF samples available for analysis was relatively small which, when divided into five groups, may limit statistical power to detect longitudinal changes, especially in the preclinical and early symptomatic AD groups. Although serial LP follow-up was longer than in previous longitudinal ADNI CSF studies[226,227], it was still relatively short (3-5 years). Also, despite the groups being dichotomized as amyloid-positive versus -negative in order to ascertain plaque status in the clinical groups, there was considerable overlap in clinical and biomarker patterns between individuals, especially in the MCI and AD groups. Finally, due to the small numbers of individuals in the clinical/biomarker groups, statistical models were not adjusted for multiple comparisons.

3.5.1 Conclusions

The present results underscore the importance of evaluation of true longitudinal, serial measures of CSF biomarkers from individuals as they progress through the normal course of the disease as opposed to the more traditional approach of inferring longitudinal change by comparing cross-sectional data from groups of individuals at different disease stages. Indeed, concentrations of each of the markers have been reported to be elevated in AD compared to MCI and cognitively normal controls[221]. While we also observed such elevations in baseline levels of these injury markers among the different clinical/amyloid groups, the within-person patterns of change over time were different. For clinical trial purposes, given the stage-specific differences in the direction of true longitudinal change in these biomarkers, a “positive” biomarker outcome would

be different depending on the characteristics of the trial cohort. For example, a slowing of the course of neuronal injury may be indicated by a slowing of the rate of increase in CSF Tau in individuals who are early in the disease process (MCI), but perhaps a stabilization or even a slowing or reversal of the downward trajectory later in the disease (mild AD), potentially reflected as a longitudinal increase or as no decrease in this marker. Such possibilities warrant consideration in clinical trial design.

Chapter 4: ACS and ADNI Case Studies

In the previous chapters, data was discussed that covered CSF and imaging biomarkers across two very different cohorts. Because biomarker investigation is not yet at the level of single-person analysis, the data presented thus far has involved the grouping of individuals within each cohort by similar risk factors – *APOE* genotype and age for the ACS in Chapter 2 and CSF amyloid positivity and cognitive status for the ADNI study in Chapter 3 – and outlining baseline and longitudinal characteristics within each risk factor grouping. Elucidating biomarker patterns in this way is only one step forward; for a biomarker or panel of biomarkers to be useful, they must be able to identify disease within an individual. The current chapter is dedicated to exploring biomarker profiles in specific individuals chosen from both the ACS and ADNI Cohorts.

4.1 Case Studies from the ACS Cohort

The ACS was a particularly interesting cohort to study longitudinally because of the relatively young age at baseline (45-74 years) of study participants. It also represented a significant challenge in that few participants showed cognitive decline during their participation in the study (14 of 169, 8%), even as of the writing of this document in October 2017, forcing reliance on biomarker behavior as the primary indicator of AD.

4.1.1 Methods

To best visualize longitudinal change, the case study cohort was restricted from the ACS cohort defined in Chapter 2 to only individuals with 3 or 4 serial LP's, then defined by amyloid status according to CSF Aβ42 levels, see **Table 4.1** for details. Ten individuals were Aβ- at baseline but converted to Aβ+ at a future LP, defined as having levels above and below 1,041 pg/ml CSF Aβ42, respectively, according to the cutoff defined in Chapter 2; this group is identified as the

converter group (Aβ42 Converter). Thirteen individuals had baseline levels of CSF Aβ42 below the cutoff at baseline and all follow-up LP's, hereafter identified as stable amyloid positive (Stable Aβ42+). Thirteen individuals had CSF Aβ42 levels >1,041 pg/ml at baseline and all follow up LP's, identified as stable amyloid negative (Stable Aβ42-).

Multiple graphing paradigms were considered for showing CSF Aβ42, Aβ40, Tau, P-tau$_{181}$, YKL-40, VILIP-1, and the Aβ42/Aβ40 and Tau/Aβ42 ratios on a single graph. It was determined after finalizing each paradigm that the Aβ42/Aβ40 ratio, VILIP-1, and YKL-40 were too variable to be of use on an individual basis in this cohort. The only CSF biomarkers graphed in **Figures 4.1-4.6**, therefore, were CSF Aβ42, Tau, P-tau$_{181}$, and the Tau/Aβ42 ratio. Each figure shows graphs for individuals within 5-year age bins by baseline LP: 45-49 years old, 50-54 years old, 55-59 years old, 60-64 years old, 65-69 years old, and 70-74 years old at baseline.

In determining which graphing paradigm to best illustrate the longitudinal changes of multiple biomarkers, a number of options were considered. Transforming each biomarker to a z-score, while putting all biomarkers on the same scale, gave a skewed picture of the case study data because the ACS dataset biomarkers are quite homogenous and therefore z-scores largely centered close to the mean for each of the 36 individuals in the case study cohort. Percent change from BL was also considered, but this only gave an indication of whether biomarkers were changing within an individual and in what direction – a critical piece of data was lost because percent change does not take in to account the original biomarker level, which is particularly important when considering biomarker cutoffs or positivity. The last considered paradigm is common in AD CSF biomarker research and involves splitting a cohort in to tertiles for analysis. However, because the ACS case study cohort was small, increased granularity was required to see differences within and between individuals, so quintiles were calculated for each CSF

biomarker based on the overall ACS cohort. For each of the 36 individuals in the case study cohort, the baseline level of each biomarker was graphed as a quintile (1-5), allowing a straightforward visualization of the baseline biomarker levels within each individual in the context of the ACS cohort as a whole.

Most individuals, though, remained within a given quintile for each followup LP which gave the impression of no longitudinal change when followup was also graphed as a quintile. As an example, the quintiles for $A\beta_{42}$ are: Q1, 310-768 pg/ml; Q2, 769-1226 pg/ml; Q3, 1227-1683 pg/ml; Q4, 1684-2141 pg/ml; and Q5 2142-2599 pg/ml. A given individual could have a baseline $A\beta_{42}$ level of 1224 pg/ml and a follow up of 770 pg/ml, a substantial drop in concentration, and still be graphed within the second quintile for both time points, resulting in the visual of a straight line between the baseline and first followup LP. The final paradigm considered was unorthodox, but for visualization purposes allowed the comparison of all CSF biomarkers by graphing baseline in quintiles, while each followup LP was graphed as absolute percent change. Again, as an example, if an individual had a baseline CSF $A\beta_{42}$ value of 1224 pg/ml, time 0 (T0) would be visualized at quintile 2. For a followup value of 770 pg/ml, the percent change from T0 to T1 is -63%. In the following figures, this translated to T0 = “2” and T1 = “1.26”. The absolute percent change is the same, whether discussing the original biomarker level or the quintile proxy, and graphing in this manner allowed the visualization of baseline status and the magnitude and direction of change for each of the 5 biomarkers on a single, within-person graph.

A second set of graphs was designed to show biomarker changes within the overall context of age because the non-core biomarkers VILIP-1, YKL-40, and the Aβ42/Aβ40 Ratio were difficult to interpret on a within-person level – potentially due to age-related influences on these CSF biomarker values. In this graphing paradigm all individuals in the stable Aβ42- (n=13), Aβ42

converters (n=10), and stable Aβ42+ (n=13) were graphed by group Aβ42 status. Instead of baseline biomarker being represented as a quintile at T0, baseline quintile is graphed at the age of each individual baseline LP; all follow up LP's are graphed as absolute percent change at the corresponding age at LP. Each Aβ42 status group is represented by two graphs, for a total of six graphs in **Figure 4.7**: the core biomarkers and Aβ40 are contained in one graph and the non-core biomarkers VILIP-1, YKL-40, and Aβ42/Aβ40 Ratio are on a second graph. Baseline quintile and absolute percent change for the core biomarkers and Aβ40 are identical to the data graphed in **Figures 4.1-4.6**.

4.1.2 Results

The case study cohort was split in to six groups based on baseline age matching – Group 1, 45-49 years; Group 2, 50-54 years; Group 3, 55-59 years; Group 4, 60-64 years; Group 5, 65-69 years; Group 6, 70-74 years. **Table 4.1** contains demographic information on each participant. Of the 36 individuals comprising the case study cohort: 23 were female (64%), 11 had at least one *APOE* ε4 allele (31%), 6 had one ε2 and one ε3 allele (17%), and 19 had two ε3 alleles (53%) (percentages add up to 101 due to rounding). After baseline LP, each of the 36 individuals had between 4 and 9 years of follow up LP data with LP's performed approximately every 3 years, and between 7 and 13 years of clinical follow up. Only 4 of 36 individuals were assigned a CDR >0 at any point during clinical follow up (11%), and these were exclusively in the stable Aβ42+ group. Statistical analyses were not performed on this cohort for two reasons: (1) individuals were selected from a volunteer population already considered to be different from the general population due to recruitment based on a family history of AD (as outlined in Chapter 2) and high levels of education and (2) only 36 individuals are represented in this cohort.

Table 4.1. Demographic Information on the 36 ACS Case Study Cohort Individuals.

	Stable Aβ42-	Aβ42 Converter			Stable Aβ42+
n	13	10			13
45-49 Years at BL LP	**A**	**B**	**D**	**E**	**C**
Avg. LP Interval (Yr)	4.0	3.0	2.9	3.2	2.9
LP Followup (Yr)	8	6	6	6	6
Clinical Followup (Yr)	12	12	9	9	10
Aβ42 Conversion	--	Year 3	Year 3	Year 7	--
Clinical Followup After Conversion (Yr)	--	9	6	2	--
Clinical Status	CN	CN	CN	CN	CN
PiB Status	Stable Negative	--	--	Stable Negative	Stable Negative
Sex	M	M	F	F	F
APOE ε Alleles	3/3	3/3	3/3	3/3	3/3

Table 4.1 Cont.

50-54 Years at BL LP	**F**	**I**	**K**	**G**	**J**	**H**
Avg. LP Interval (Yr)	2.9	3.1	3.2	3.0	3.1	3.2
LP Followup (Yr)	6	9	6	6	6	7
Clinical Followup (Yr)	9	12	12	12	9	12
Aβ42 Conversion	--	--	--	Year 3	Year 6	--
Clinical Followup After Conversion (Yr)	--	--	--	9	3	--
Clinical Status	CN	CN	CN	CN	CN	CN
PiB Status	Stable Negative	--	--	--	Year 6	--
Sex	F	M	M	F	M	M
APOE ε Alleles	3/4	3/3	2/3	4/4	3/4	2/3

Table 4.1 Cont.

55-59 Years at BL LP	**L**	**O**	**M**	**P**	**N**	**Q**	**R**
Avg. LP Interval (Yr)	3.1	2.9	3.4	3.1	3.0	3.3	3.1
LP Followup (Yr)	9	6	7	9	6	7	6
Clinical Followup (Yr)	12	13	10	13	11	9	9
Aβ42 Conversion	--	--	Year 7	Year 9	--	--	--
Clinical Followup After Conversion (Yr)	--	--	3	4	--	--	--
Clinical Status	CN	CN	CN	CN	CN	CN	CDR 0.5 at year 9
PiB Status	--	--	--	Year 9	Stable Negative	PiB+ at year 3	Stable Positive
Sex	F	F	F	M	M	F	F
APOE ε Alleles	3/3	2/3	3/3	3/3	3/3	3/3	4/4

Table 4.1 Cont.

60-64 Years at BL LP	**S**	**V**	**X**	**T**	**U**	**W**
Avg. LP Interval (Yr)	2.1	3.1	3.3	2.7	3.1	3.0
LP Followup (Yr)	6	6	7	5	6	9
Clinical Followup (Yr)	10	10	9		8	12
Aβ42 Conversion	--	--	--	Year 3	--	--
Clinical Followup After Conversion (Yr)	--	--	--		--	--
Clinical Status	CN	CN	CN		CN	CN
PiB Status	Stable Negative	Stable Negative	Stable Negative	--	Stable Positive	Stable Positive
Sex	F	M	F	F	F	F
APOE ε Alleles	3/3	3/3	3/3	3/4	3/4	3/3

Table 4.1 Cont.

65-69 Years at BL LP	**Y**	**BB**	**DD**	**Z**	**AA**	**CC**	**EE**
Avg. LP Interval (Yr)	2.1	2.5	2.6	3.0	3.0	3.1	3.1
LP Followup	4	5	5	6	6	6	6
Clinical Followup (Yr)	8	9		10	9	9	10
Aβ42 Conversion	--	--	--	Year 6	--	--	--
Clinical Followup After Conversion (Yr)	--	--	--	4	--	--	--
Clinical Status	CN	CN		CN	CN	CDR 0.5, Yr 4; CDR 1, Yr 9	CN
PiB Status	Stable Negative	--	Stable Negative	Stable Negative	Year 6	Stable Positive	Stable Negative
Sex	F	F	M	M	F	M	M
APOE ε Alleles	2/3	2/3	2/3	3/4	3/4	3/4	3/3

Table 4.1 Cont.

70-75 Years at BL LP	**FF**	**GG**	**HH**	**II**	**JJ**
Avg. LP Interval (Yr)	3.0	3.1	2.8	2.5	3.0
LP Followup (Yr)	6	6	6	5	6
Clinical Followup (Yr)	10	7	10	9	
Aβ42 Conversion	--	Year 3	--	--	--
Clinical Followup After Conversion (Yr)	--	4	--	--	--
Clinical Status	CN	CN	CN	CDR 0.5, Yr 6; CDR 1, Yr 9	
PiB Status	--	--	Stable Positive	Stable Positive	Stable Positive
Sex	F	F	F	F	F
APOE ε Alleles	3/3	3/3	3/4	3/3	3/4

The cohort was split in to 6 bins by baseline age: 45-49 years, 50-54 years, 55-59 years, 60-64 years, 65-69 years, and 70-74 years. Within each age group, individuals were further separated by amyloid status: stable Aβ42- (BL and Followup CSF Aβ42 values all above 1,041 pg/ml), Aβ42 Converter (declining from CSF Aβ42 >1,041 pg/ml at BL to <1,041 pg/ml during Followup), and stable Aβ42+ (BL and Followup CSF Aβ42 values all below 1,041 pg/ml).

Average LP interval and LP followup indicate the frequency and duration of LP followup from LP baseline.

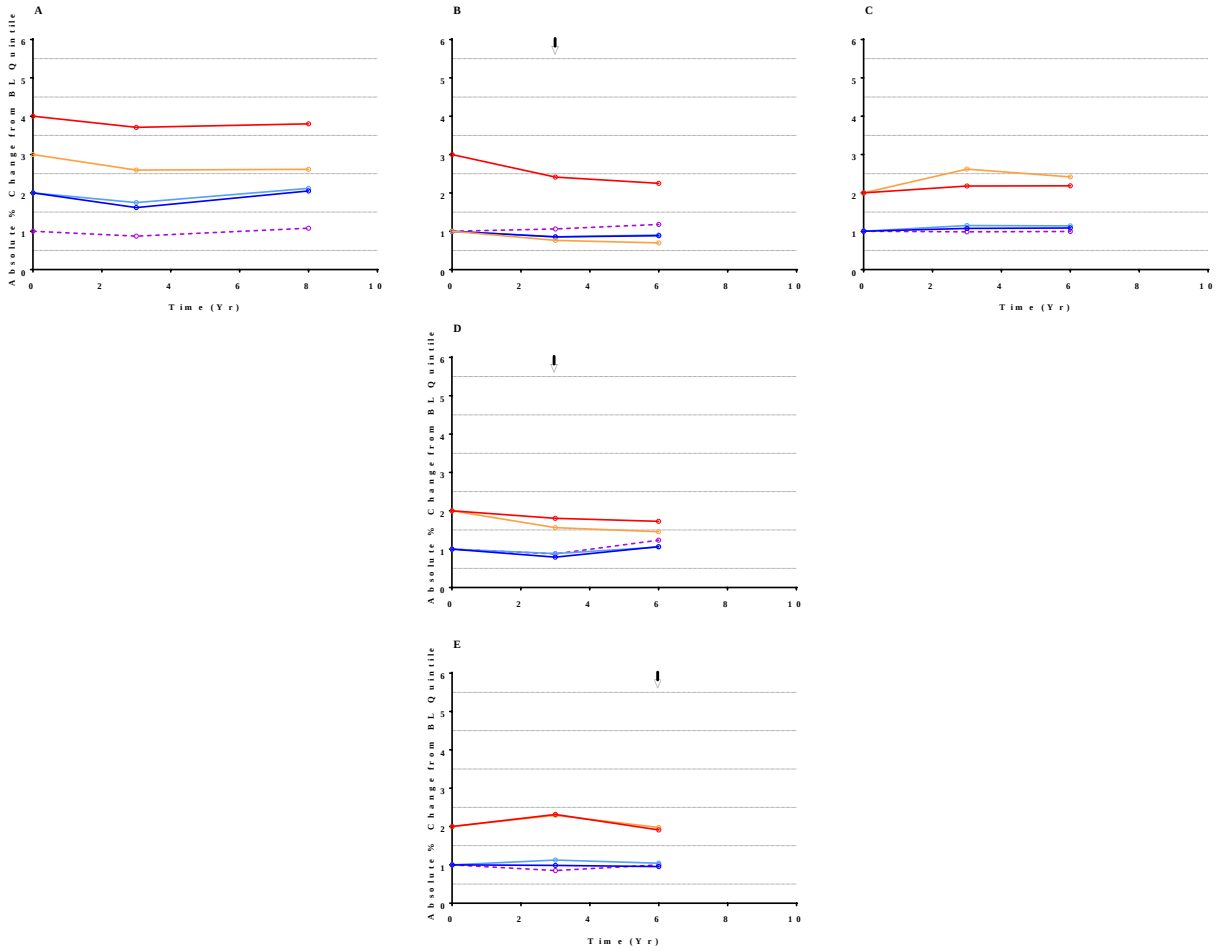
Clinical Followup in years accounts for years from LP baseline to the most recent clinical examination.

Aβ42 Conversion indicates number of years after baseline LP conversion occurred, while Clinical Followup After Conversion indicates the number of years after exhibiting Aβ42 positivity converters had clinical followup information available.

Clinical Status accounts for the latest clinical examination in the case of cognitively normal (CN) individuals while, for those who progress from CN to a CDR 0.5 or 1, the year of conversion is indicated.

The letters A-JJ identify each individual allowing comparison between **Table 4.1** and **Figures 4.1-4.6**.

Figure 4.1 Core CSF Biomarkers and CSF Aβ40 for Individuals from 45-49 Years Old at Baseline Lumbar Puncture

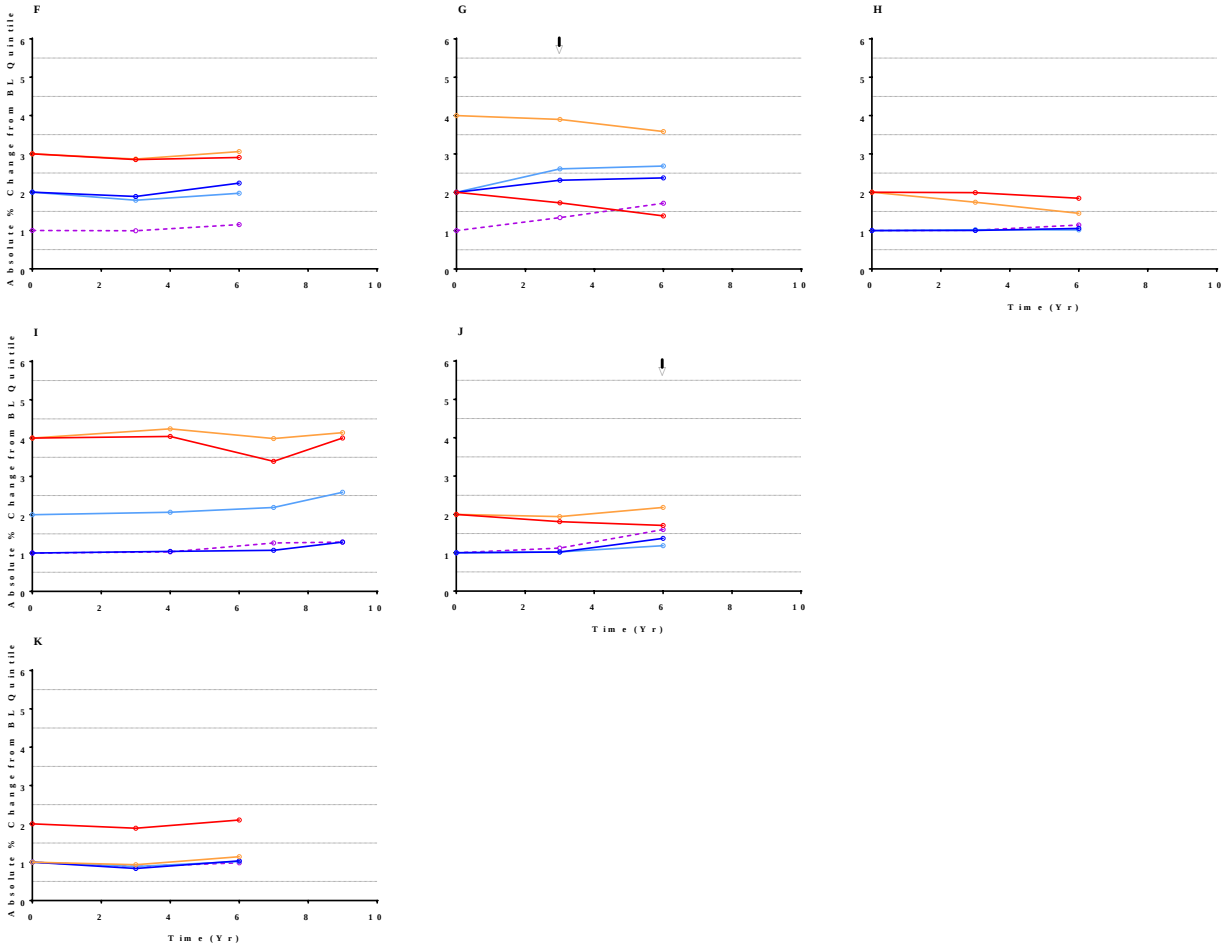


Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ_{42} Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate the time of Aβ42 transition from negative to positive during study follow up.

Figures 4.1 through **4.6** illustrate core and Aβ40 biomarkers on a within-person basis. The alphabetical labels in **Table 4.1** correspond to each individual graph in **Figures 4.1-4.6**. The core biomarkers (CSF Aβ42, Tau, and P-tau$_{181}$) and CSF Aβ40 are graphed on a single graph for each individual. Each figure encompasses a five-year age bin at baseline LP (**Figure 4.1**, 45-49 years old; **Figure 4.2**, 50-54 years old; **Figure 4.3**, 55-59 years old; **Figure 4.4**, 60-64 years old; **Figure 4.5**, 65-69 years old; and **Figure 4.6**, 70-74 years old), and within each figure the left column are stable Aβ42- individuals, the middle column are Aβ42 converters, and the right column are stable Aβ42+ individuals. There is a distinctly different biomarker profile for each Aβ42 classification, with stable Aβ42- individuals having lower Tau(s) at baseline, Quintile 1 or 2 with the exception of individual "O", and Tau/Aβ42 Ratios in the first quintile at BL exhibiting little change throughout follow up. Similarly, Aβ42 converters had low Tau(s) in Quintile 1 or 2 at baseline but were more likely to increase over time than stable Aβ42- individuals throughout follow up. In contrast, many of the stable Aβ42+ individuals exhibited levels of Tau(s) and Tau/Aβ42 in Quintile 1-3 at baseline, though over time levels of Tau(s) and, to a greater extent, Tau/Aβ42 increased more than in either the stable Aβ42- or Aβ42 converters. CSF Aβ40 in most individuals was in the same quintile as Aβ42 and followed similar longitudinal trajectories, but was less consistent than the core biomarkers.

Individuals in the center, Aβ42 converter columns of **Figures 4.1-4.6** were marked with a black arrow indicating the LP at which their CSF Aβ42 status changed from – to +. None of these individuals or the Aβ42- individuals exhibited a CDR >0.5 at any time from baseline LP through the most recent clinical assessment; for the stable Aβ42- group, clinical follow up lasted between 8 and 13 years from baseline LP and for the Aβ42 converter group, clinical follow up lasted

Figure 4.2 Core CSF Biomarkers and CSF Aβ40 for Individuals from 50-54 Years Old at Baseline Lumbar Puncture

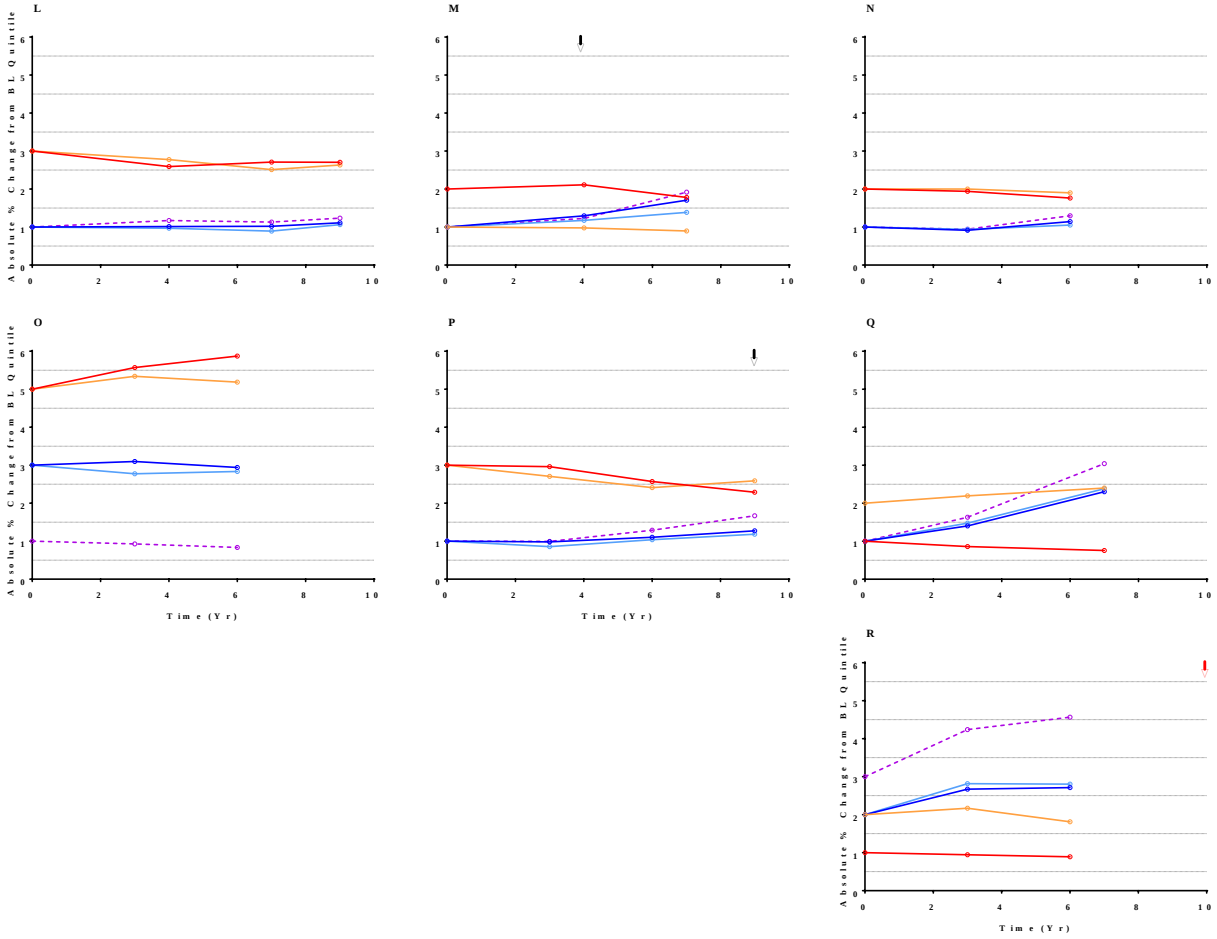


Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ$_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate time of Aβ42 transition from negative to positive.

between 7 and 13 years from baseline LP. In contrast, 4 of 13 individuals in the Aβ42+ group (31%) were assigned a CDR of 0.5 or 1 at some point during clinical follow up from baseline LP, which lasted between 8 and 12 years. Individual R (**Figure 4.3)** received a CDR 0.5 10 years after baseline LP; CSF Aβ42 was considered positive even at baseline and remained stable, while Tau and P-tau$_{181}$ were in the second quintile but increasing over time. The Tau/Aβ42 Ratio began in the third quintile and increased substantially throughout follow up. Individual CC (**Figure 4.5**) received a CDR 0.5 at 4 years after baseline LP, which further progressed to a CDR 1 9 years after baseline LP. The CSF Aβ42 level was in the second quintile, and decreasing slightly while the Tau(s) and Tau/Aβ42 Ratio were increasing over time. Individual HH (**Figure 4.6**) received a CDR 0.5 at 9 years after baseline LP, though this returned to a CDR 0 at 10 years. The Tau(s) and Tau/Aβ42 Ratio patterns show an increase followed by a decrease between years 4 and 6 after baseline LP while Aβ40 in individual HH is the only instance exhibiting a longitudinal increase in Aβ40 in the ACS Case Study cohort. Individual II (**Figure 4.6**) received a CDR 0.5 at 7 years after baseline LP and a CDR 1 at 9 years after baseline. While the Tau(s) were only in the first Quintile at baseline and increasing slightly over time, the Tau/Aβ42 Ratio was in Quintile 2 at baseline and exhibited large increases longitudinally.

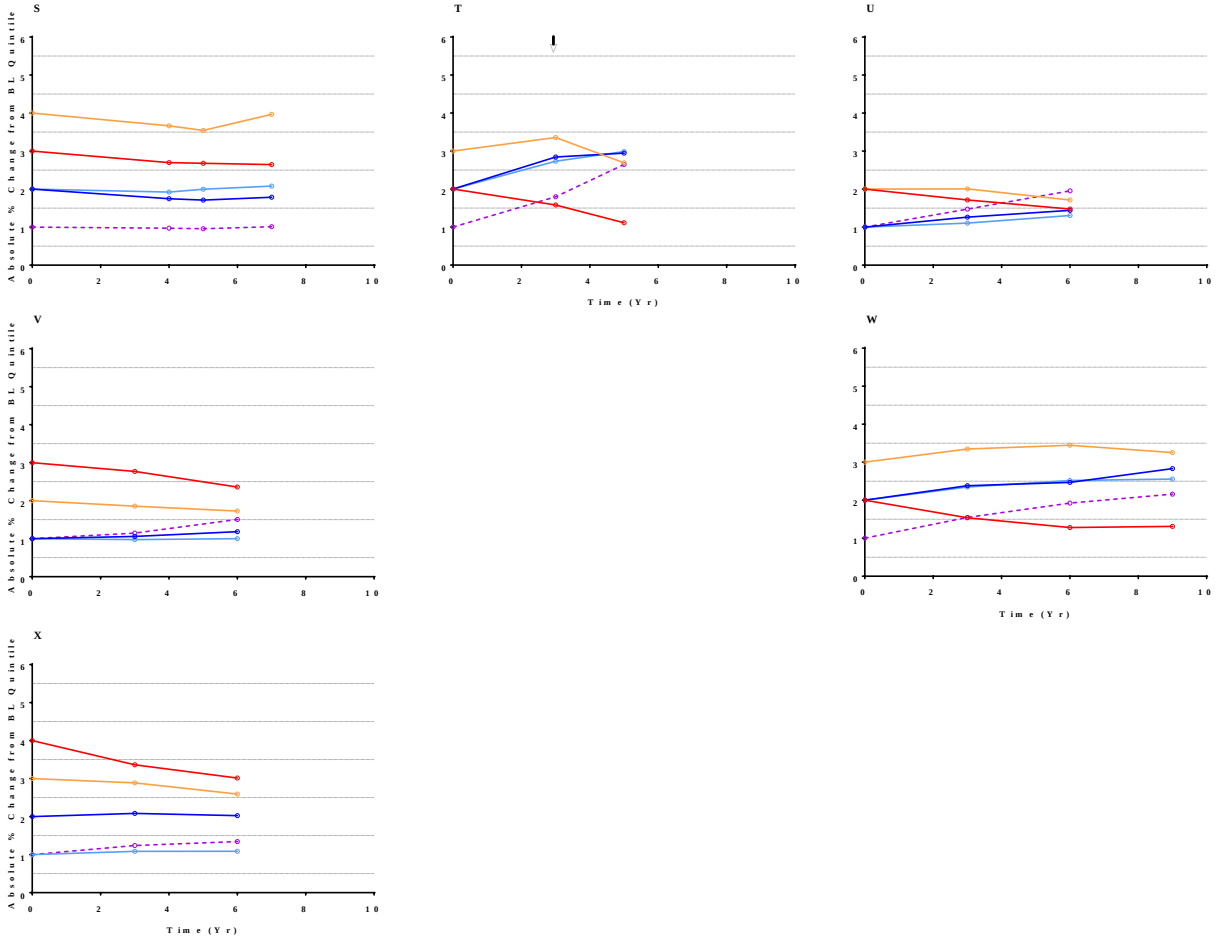
Graphing individuals by baseline age bin, while informative, does not give a visual sense of how longitudinal biomarker changes may perform across a large age range within Aβ42-, Aβ42 converter, and Aβ42+ groups. To better visualize this, **Figure 4.7** was created to show biomarker changes across the entire middle-age range of the ACS cohort (45-74 years of age). While difficult to pinpoint biomarker behavior on a single-individual basis, this did better represent biomarker changes across nearly a 30-year span during middle-age.

Figure 4.3 Core CSF Biomarkers and CSF Aβ40 for Individuals from 55-59 Years Old at Baseline Lumbar Puncture



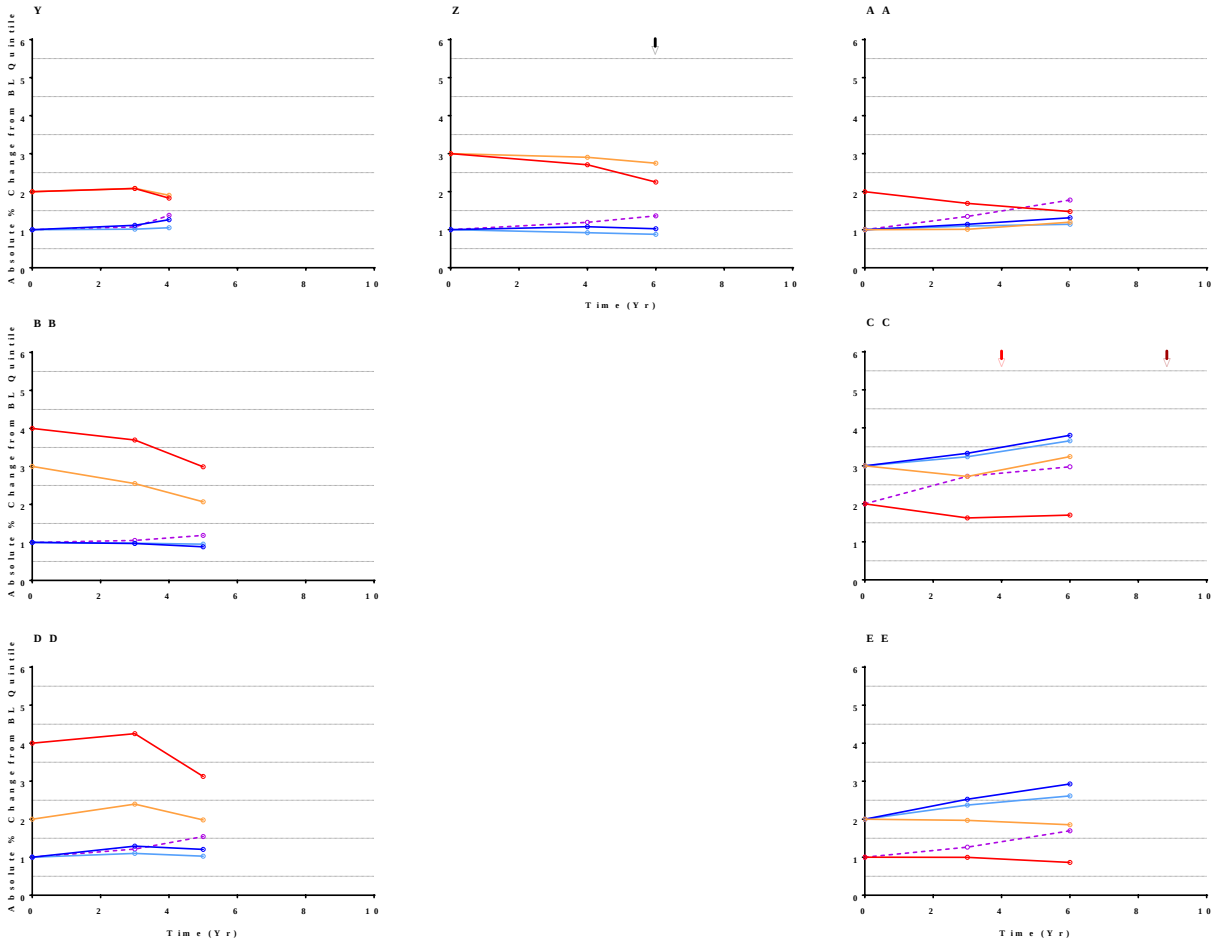
Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ$_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate time of Aβ42 transition from negative to positive, red arrows indicate a Clinical Dementia Rating Score of 0.5.

Figure 4.4 Core CSF Biomarkers and CSF Aβ40 for Individuals from 60-64 Years Old at Baseline Lumbar Puncture



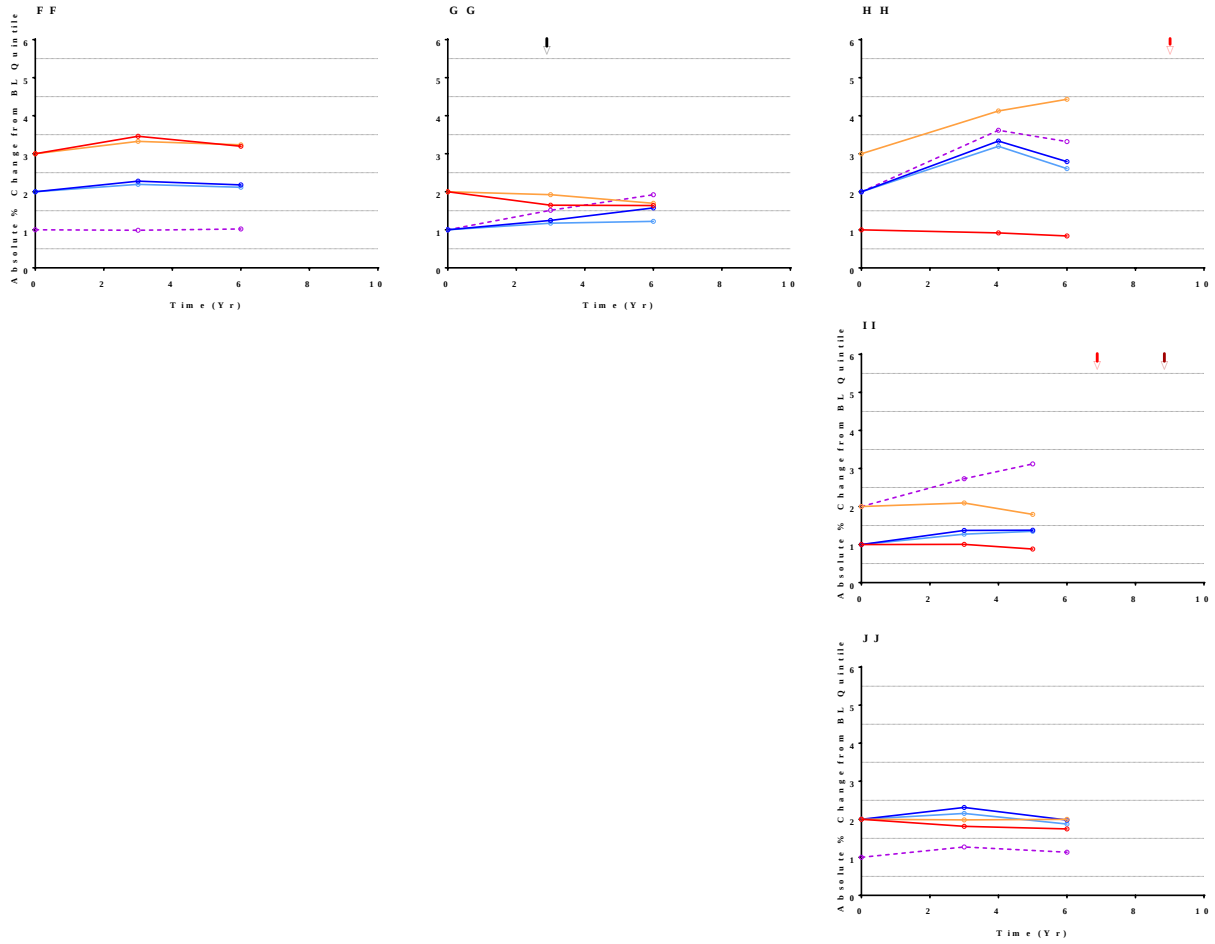
Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ$_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate time of Aβ42 transition from negative to positive.

Figure 4.5 Core CSF Biomarkers and CSF Aβ40 for Individuals from 65-69 Years Old at Baseline Lumbar Puncture



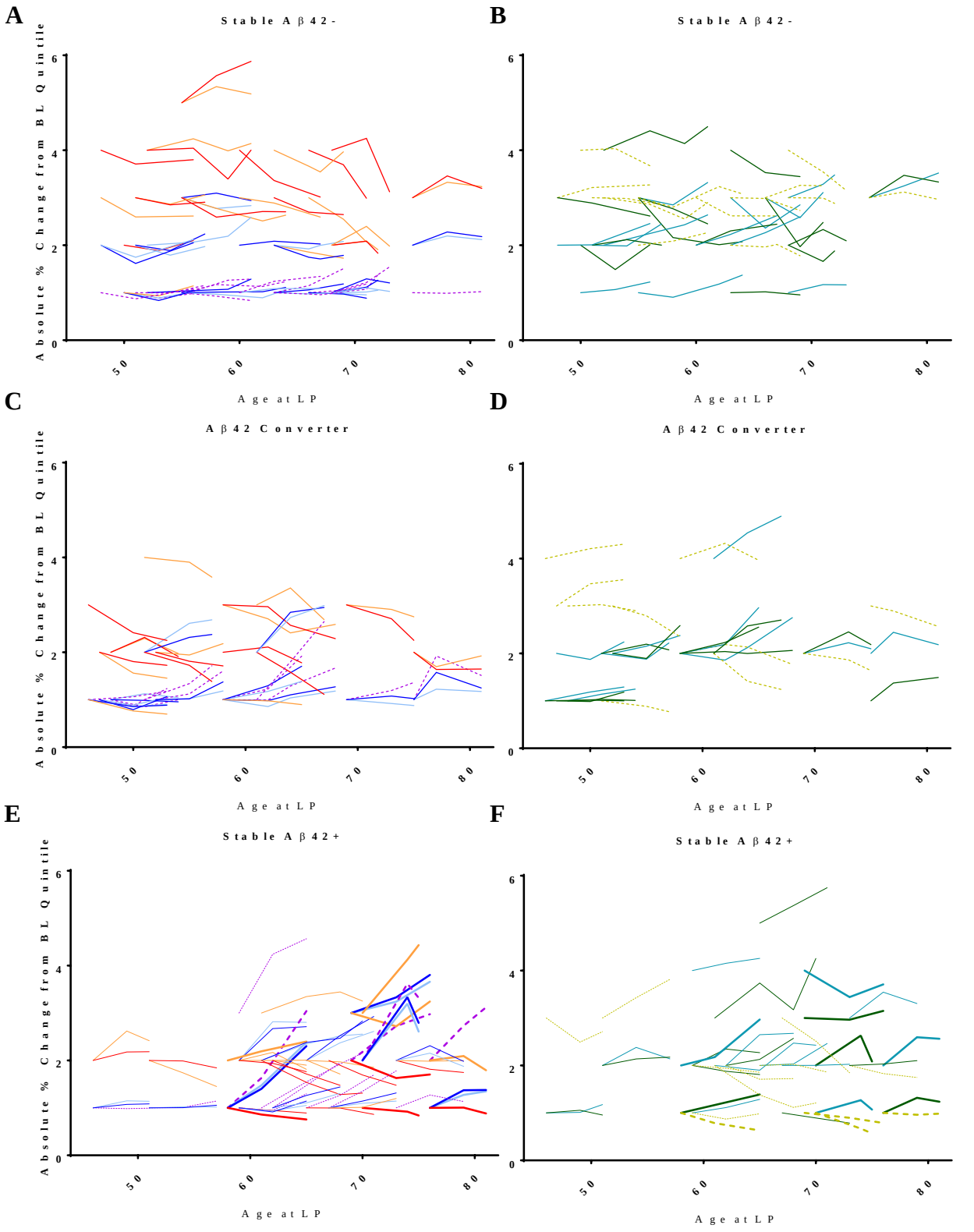
Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ$_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate time of Aβ42 transition from negative to positive, red arrows indicate a Clinical Dementia Rating (CDR) Score of 0.5, dark red indicate a CDR>1.

Figure 4.6 Core CSF Biomarkers and CSF Aβ40 for Individuals from 70-74 Years Old at Baseline Lumbar Puncture



Each graph represents a single individual from the ACS Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Orange, Aβ40; Dark Blue, Tau; Light Blue, P-tau$_{181}$; Purple Dash, Tau/Aβ_{42} Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. Left column, stable Aβ42-; middle column, Aβ42 converter; right column, stable Aβ42+. Black arrows indicate time of Aβ42 transition from negative to positive, red arrows indicate a Clinical Dementia Rating (CDR) Score of 0.5, dark red indicate a CDR>1.

Figure 4.7 Longitudinal Biomarker Change by Aβ42 classification, Core and Non-Core Biomarkers in the ACS Case Study Cohort



Longitudinal biomarker changes starting at baseline age. Left column, core biomarkers and Aβ40 (Red, Aβ42; Orange, Aβ40; Blue, Tau; Light blue, P-Tau_{181}; Purple dash, Tau/Aβ42 Ratio), right column, non-core biomarkers (Green, VILIP-1; Teal, YKL-40; Gold dash, Aβ42/Aβ40 Ratio).

4.1.3 Discussion

When visualizing the ACS case study cohort using the graphing paradigm shown, it becomes clear that there are indeed differences on a within-person level that indicate the very earliest stages of AD pathology. **Figures 4.1-4.6** show that levels of Aβ42, as expected, are comparatively higher (Quintile 2-5 at BL) in the Stable Aβ42- individuals than the Stable Aβ42+ individuals (Quintile 1-3 at BL), with Converters at intermediate levels (Quintile 2-3 at BL). Across the full age range of 45-74 years, Tau and $Ptau_{181}$ in Stable Aβ42- individuals remained low and unchanging (Quintile 1), except the oldest member of the cohort who had Tau(s) levels in Quintile 2 at BL which remained unchanged at followup for 9 years. In the Converter group, levels of Tau and $Ptau_{181}$, as well as the Tau/Aβ42 ratio, were in Quintile 1-2 at baseline and all but the two youngest showed increases over time. All individuals whose Tau/Aβ42 Ratio reached the third quintile, with the exception of individual Q, exhibited signs of cognitive decline.

Analysis on an individual level in the ACS cohort indicates that, at least for the core CSF biomarkers, there is a clear difference over time in individuals who exhibit eventual clinical decline compared to those who do not. The greatest strength and the greatest weakness of the ACS cohort, as a whole, is the preclinical nature of the cohort – middle-aged, cognitively normal individuals recruited to the study because of their family history of AD. On one hand, this relatively young cohort is unique because individuals with an AD CSF profile are in the preclinical stage until cognitive status begins to change which provides a perspective on very early CSF biomarker changes in AD; on the other hand, because this is such a young cohort very few individuals have exhibited cognitive decline even after 6 or more years of clinical follow up. Assessing longitudinal biomarker change in these individuals is interesting, but ultimately does not provide additional information on the relationship between CSF biomarkers and disease

status until an individual begins to show signs of dementia. However, this case study is an interesting and, ultimately, a net gain in the identification of AD on a within-person basis. The biggest barrier to clinical translation is that the quintile data would not be informative without longitudinal data – these case study analyses do not provide a single-timepoint identification with the available data.

Despite this, it remains clear that AD can be pinpointed even in individuals who remain cognitively normal for a number of years post-amyloid-biomarker positivity. Future work should focus on the biomarker trajectories of individuals who begin studies in the preclinical phase of disease and eventually progress to show cognitive decline. The combination of clinical follow up and CSF biomarkers alone will likely not be sufficient to account for environmental factors such as cognitive reserve, but investigation on a within-person basis on a large scale may elucidate trends in biomarker behavior that correspond to these environmental factors, thus rendering them easier to account for in studies of preclinical AD.

4.2 Case Studies from the ADNI Cohort

The ADNI cohort provided a unique perspective on within-person changes in CSF biomarkers due to the heterogeneous nature of the cohort. Individuals in the ADNI study may be cognitively normal, exhibit MCI, or have a diagnosis of AD at study entry. The longitudinal ADNI cohort in Chapter 3 also covered an extensive older age range, from 58 to 90 years old at baseline, compared with the ACS cohort.

4.2.1 Methods

The majority of the ADNI cohort had data from 3 or more LP's, therefore the case study cohort was defined by the number of Aβ42 converters. As with the original analyses in Chapter 3, Aβ42 status was defined by xMAP values. In the ADNI cohort, the cutoff value for Aβ42 has been

reported widely as 192 pg/ml, therefore individuals who transitioned from CSF Aβ42 >192 pg/ml to <192 pg/ml were classified as Aβ42 converters; 9 total individuals met these criteria. The availability of both xMAP and Elecsys data aided in selecting 9 stable Aβ42- and 9 stable Aβ42+ individuals. The Elecsys assay provided a much larger working range (1,472 pg/ml) compared with the xMAP assay (300 pg/ml) of Aβ42 values in the ADNI cohort. The 9 individuals with the highest Elecsys Aβ42 and lowest Elecsys Aβ42 values were chosen to represent stable Aβ42- and +, respectively, and compared with xMAP values to ensure that Aβ42 status was consistent between the two assays.

The graphing paradigm used for the ADNI cohort was identical to that used for the ACS cohort. Age matching was not possible because 9 of 27 individuals were either 77 or 78 years old at baseline, therefore the ADNI case studies are grouped by Aβ42 status in 3 groups. Additionally, the ADNI cohort did not have Aβ40 data available, so **Figures 4.7-4.9** represent only the core CSF biomarkers Aβ42, Tau, P-tau$_{181}$ and the Tau/Aβ42 ratio. **Figure 4.10** represents the core CSF biomarkers and the non-core biomarkers VILIP-1, SNAP-25, Ng, and YKL-40.

4.2.2 Results

The case study cohort visualized in 3 groups representing Aβ42 status as the age distribution was not even enough to bin by baseline age. **Table 4.2** contains demographic information on each participant. Of the 27 individuals comprising the case study cohort: 13 were female (48%), 5 had one *APOE* ε4 allele (19%%), 2 had two ε4 alleles (7%). After baseline LP, each of the 27 individuals had between 2 and 9 years of follow up LP data with LP's performed approximately every year, and between 2 and 9 years of clinical follow up. Of 27 individuals, 9 were diagnosed as having MCI at baseline (33%), and 2 were diagnosed with AD at baseline (7%).

Across the Aβ42 status groups, the Aβ42 converter group had 8 of 9 individuals diagnosed as cognitively normal at baseline, but 5 of those 8 exhibited cognitive decline which justified a diagnosis of MCI at some point during clinical followup (6-9 years). The individual diagnosed as having MCI at baseline showed no change over 8 years. The stable Aβ42- group had 3 MCI individuals. Of those, one showed no cognitive change over 8 years, one was diagnosed at a CDR 1 but reverted to CDR 0.5, and one reverted from CDR 0.5 to CDR 0. One CN individual was diagnosed at CDR 0.5 at followup but then reverted to CDR 0. The stable Aβ42+ group had five individuals diagnosed with MCI at baseline and 2 diagnosed with AD, of which 6 converted to CDR 1 or 2 during followup (2-6 years), and one MCI individual showed no change over 3 years. Two individuals were cognitively normal at baseline, one progressed to CDR 0.5 and CDR 2 during 8 years of followup and one converted to CDR 0.5 at year 3 but reverted to CDR 0 at year 4. Statistical analyses were not performed on this cohort for similar reasons as the ACS case study cohort, except that the ADNI study does not recruit based on family history of AD and has a much more varied cohort in terms of demographic variables such as education.

Table 4.2 Demographic Information on the 27 ADNI Case Study Cohort Individuals.

n	**9**								
Stable Aβ42-	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
Avg. LP Interval, Yr (SD)	2.4 (1.2)	1.6 (1.0)	1.0 (0.1)	1.5 (0.5)	1.5 (0.6)	1.7 (0.6)	1.6 (0.6)	1.4 (0.6)	1.6 (0.6)
LP Followup (Yr)	7	5	3	5	6	5	8	7	8
Clinical Followup (Yr)	7	8	7	6	7	8	8	7	8
Diagnosis at BL	CN	CN	CN	CN	CN	MCI	MCI	CN	MCI
Clinical Followup	No Change	No Change	CDR 0.5 Yr 6	No Change	No Change	No Change	CDR 1 Yr 2, Revert Yr 4	CDR 0.5 Yr 3, Revert Yr 7	Revert Yr 3
Age at BL (Yr)	70	79	76	62	90	74	83	72	86
Sex	M	M	F	F	F	F	F	F	M
APOE ε4 Alleles (#)	1	0	0	0	0	0	0	0	0

Table 4.2 Cont.

n	**9**								
Aβ42 Converters	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**
Avg. LP Interval, Yr (SD)	1.6 (0.8)	1.5 (0.5)	3.0 (3.1)	1.3 (0.5)	1.2 (0.5)	1.5 (0.7)	1.5 (0.7)	1.3 (0.5)	1.4 (0.8)
LP Followup (Yr)	8	9	6	5	5	3	6	8	7
Clinical Followup (Yr)	8	9	6	6	8	9	7	8	8
Aβ42 Conversion	Yr 5	Yr 1	Yr 6	Yr 3	Yr 4	Yr 3	Yr 6	Yr 3	Yr 3
Diagnosis at BL	CN	CN	CN	CN	MCI	CN	CN	CN	CN
Clinical Followup	No Change	CDR 0.5 Yr 6	No Change	CDR 0.5 Yr 3	No Change	CDR 0.5 Yr 9	CDR 0.5 Yr 7	No Change	CDR 0.5 Yr 8
Age at BL (Yr)	78	77	72	78	79	82	77	78	77
Sex	F	F	F	M	M	F	M	F	F
APOE ε4 Alleles (#)	0	0	0	0	0	0	0	0	o

Table 4.2 Cont.

n	**9**								
Stable Aβ42+	**S**	**T**	**U**	**V**	**W**	**X**	**Y**	**Z**	**AA**
Avg. LP Interval, Yr (SD)	1.2 (0.6)	1.3 (0.5)	1.0 (0.0)	1.3 (0.6)	1.0 (0.0)	1.0 (0.1)	1.3 (0.6)	1.0 (0.1)	1.0 (0.1)
LP Followup (Yr)	5	4	2	4	2	3	4	3	3
Clinical Followup (Yr)	6	4	2	4	2	3	8	5	6
Diagnosis at BL	MCI	MCI	AD	CN	AD	MCI	CN	MCI	MCI
Clinical Followup	CDR 1 Yr 3, CDR 2 Yr 4	CDR 2 Yr 4	CDR 1 Yr 1, CDR 2 Yr 2	CDR 0.5 Yr 3, Revert Yr 4	CDR 1 Yr 1	No Change	CDR 0.5 Yr 7, CDR 2 Yr 8	CDR 1 Yr 1, CDR 2 Yr 4	CDR 2 Yr 6
Age at BL (Yr)	70	71	69	76	68	73	77	77	78
Sex	M	M	M	M	F	M	M	M	M
APOE ε4 Alleles (#)	2	2	0	0	1	1	1	1	1

The cohort was split in to 3 bins by baseline amyloid status: stable Aβ42- (BL and Followup CSF Aβ42 values all above 192 pg/ml [xMAP] or 1,200 pg/ml [Elecsys]), Aβ42 Converter (declining from CSF Aβ42 >192 pg/ml at BL to <192 pg/ml during Followup), and stable Aβ42+ (BL and Followup CSF Aβ42 values all below 192 pg/ml [xMAP] or 1,200 pg/ml [Elecsys]).

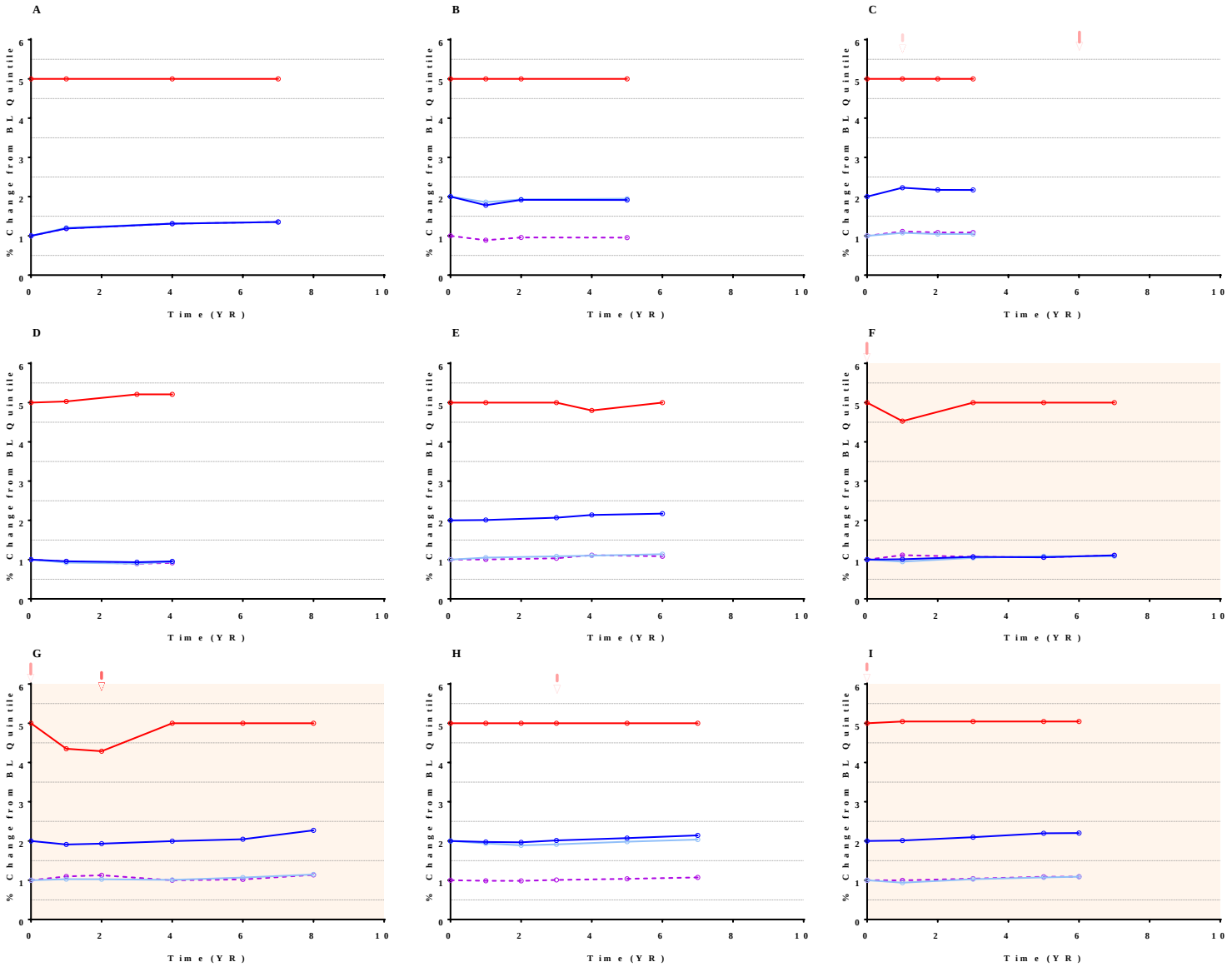
Average LP interval and LP followup indicate the frequency and duration of LP followup from LP baseline.

Clinical Followup in years accounts for years from LP baseline to the most recent clinical examination.

Aβ42 Conversion indicates number of years after baseline LP conversion occurred, while Diagnosis at BL and Clinical Followup indicate the cognitive status (CN, MCI, or AD) and change in cognitive status for each individual during followup.

The letters A-AA identify each individual allowing comparison between **Table 4.2** and **Figures 4.7-4.9**.

Figure 4.8 Core CSF Biomarkers for Stable Aβ42- ADNI Participants



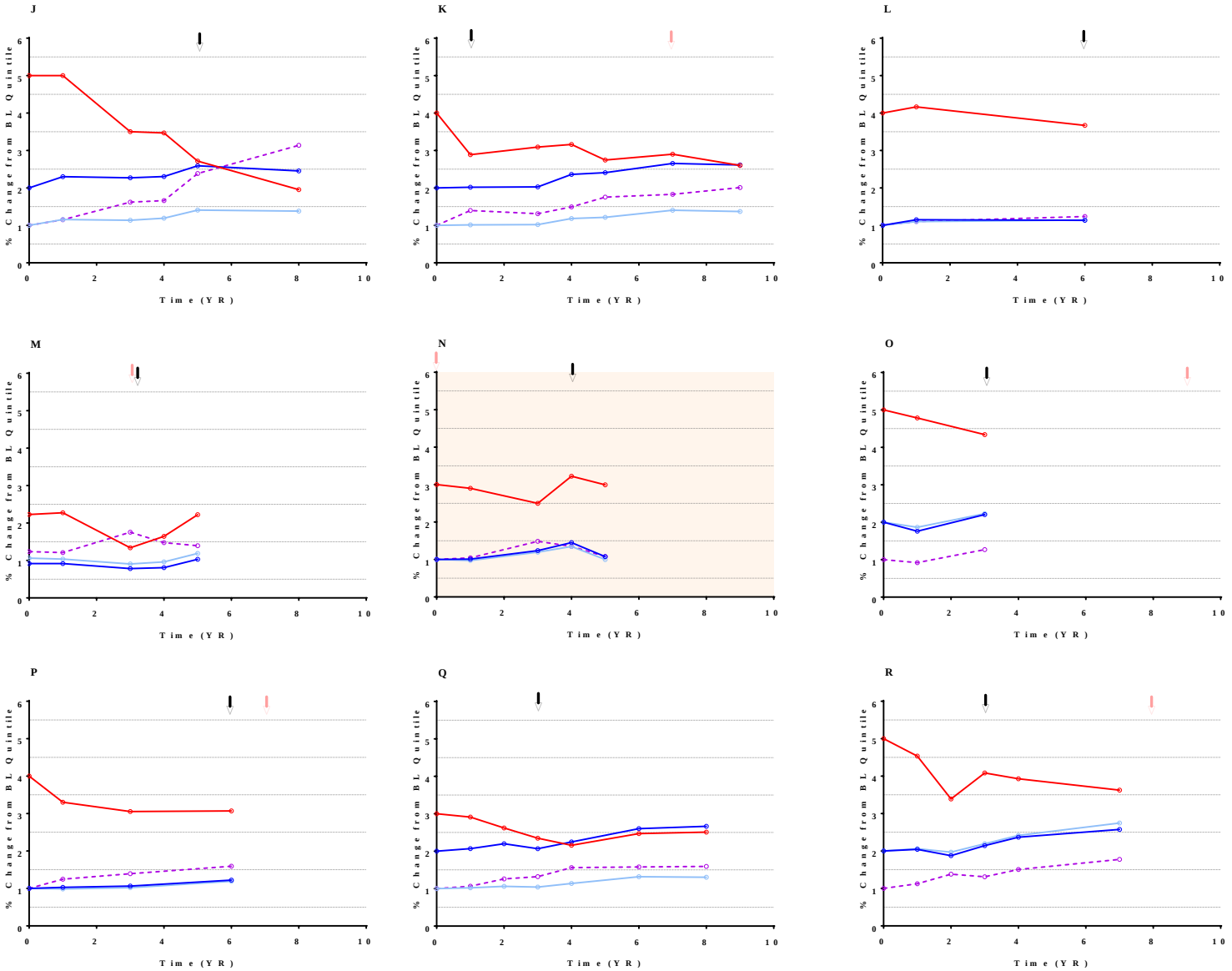
Each graph represents a single individual from the ADNI Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Dark Blue, Tau; Light Blue, P-tau_{181}; Purple Dash, Tau/$A\beta_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. A tan background indicates individuals diagnosed with MCI at baseline. Pink arrows indicate a Clinical Dementia Rating (CDR) Score of 0.5, red arrows indicate a CDR 1, dashed arrows indicate a CDR that reverted to a lower score at a later clinical followup.

Figures 4.7 through **4.9** illustrate ADNI core CSF biomarkers on a within-person basis. The alphabetical labels in **Table 4.2** correspond to each individual graph in **Figures 4.7-4.9**. The core biomarkers (CSF Aβ42, Tau, and P-tau$_{181}$) and the Tau/Aβ42 ratio are graphed on a single graph for each individual. Each figure represents 9 individuals with differing Aβ42 statuses: **Figure 4.7**, stable Aβ42-; **Figure 4.8**, Aβ42 Converters; **Figure 4.9**, stable Aβ42+. Biomarkers are more homogenous between Aβ42 classification groups than was seen in the ACS cohort. Stable Aβ42- individuals had lower Tau(s) at baseline, Quintile 1 or 2, and Tau/Aβ42 Ratios in the first quintile at BL, all exhibiting little change throughout follow up. Similarly, Aβ42 converters had low Tau(s) in Quintile 1 or 2 at baseline but were more likely to increase over time than stable Aβ42- individuals throughout follow up. Many of the stable Aβ42+ individuals exhibited levels of Tau(s) and Tau/Aβ42 in Quintile 1-2 at baseline and, unlike the ACS cohort, the levels of Tau, P-tau$_{181}$ and the Tau/Aβ42 ratio changed less over time.

The differential diagnoses of CN, MCI, and AD at baseline were coded by filling the corresponding graph background with tan (MCI) or pink (AD) to visually indicate individuals with evidence of cognitive decline at baseline. The stable Aβ42+ group had the largest number of impaired individuals at baseline. The stable Aβ42- group was next, but the majority of individuals that showed impairment or progression also reverted back to their baseline cognitive status over the course of the study. Interestingly, the Aβ42 converter group had the smallest number of both individuals with an MCI or AD diagnosis at BL, but did show a high rate of conversion from CN to a CDR 0.5.

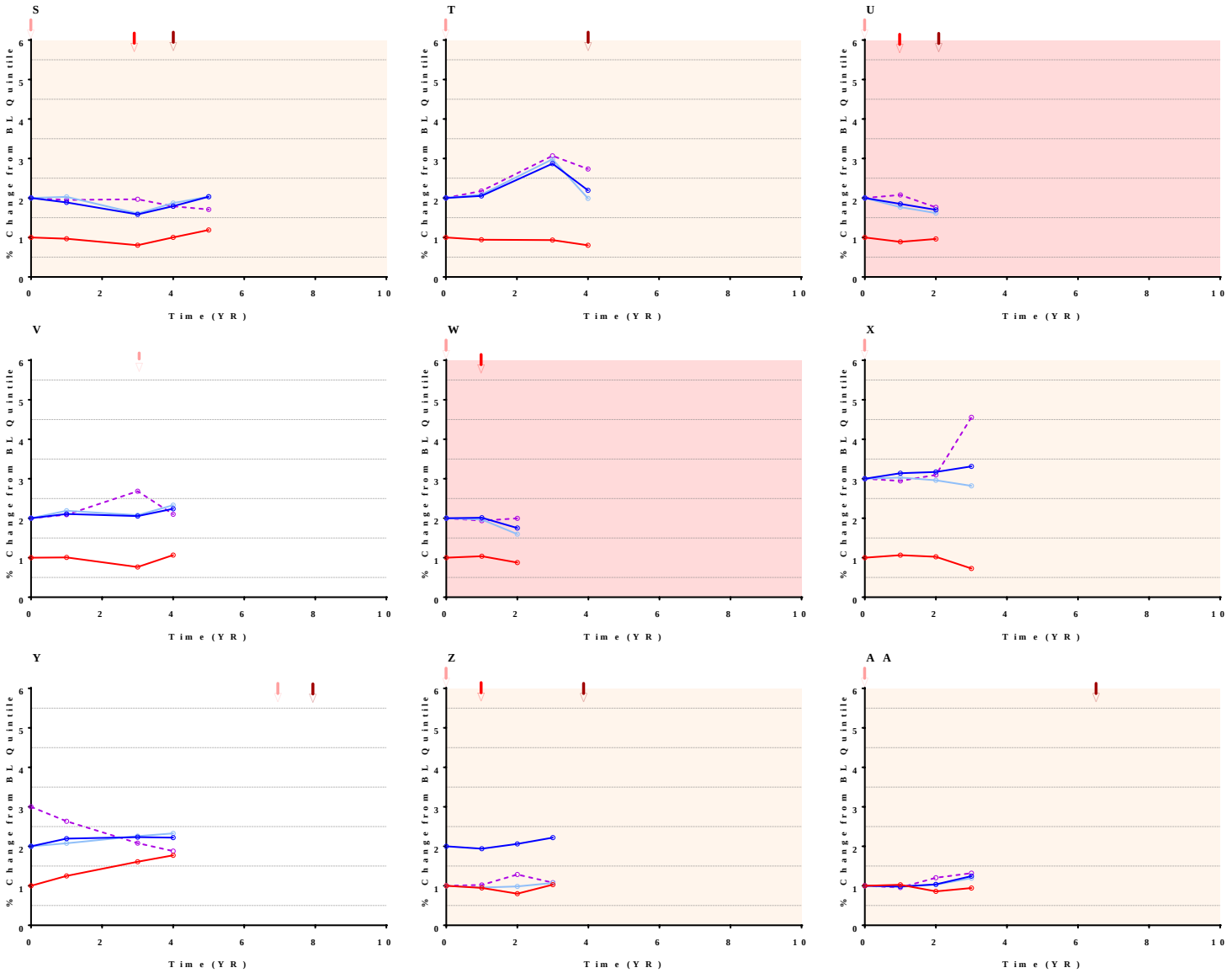
The Aβ42 converter group showed the most change in core biomarkers over time. Whereas the stable Aβ42- group remained stable throughout followup for all core biomarkers, the converter

Figure 4.9 Core CSF Biomarkers for Aβ42 Converter ADNI Participants



Each graph represents a single individual from the ADNI Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Dark Blue, Tau; Light Blue, P-tau_{181}; Purple Dash, Tau/$A\beta_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. A tan background indicates individuals diagnosed with MCI at baseline. Pink arrows indicate a Clinical Dementia Rating (CDR) Score of 0.5 clinical followup.

Figure 4.10 Core CSF Biomarkers for stable Aβ42+ ADNI Participants



Each graph represents a single individual from the ADNI Case Study cohort, with each color representing a different biomarker (Red, Aβ42; Dark Blue, Tau; Light Blue, P-tau_{181}; Purple Dash, Tau/$A\beta_{42}$ Ratio). Time (Yr) indicates time between lumbar punctures (LP) with T0 indicating baseline LP. Each LP, represented by an open circle, is presented as baseline quintile at T0 followed by the absolute percent change from the baseline biomarker level. A tan background indicates individuals diagnosed with MCI at baseline, a pink background indicates individuals diagnosed with AD at baseline. Pink arrows indicate a Clinical Dementia Rating (CDR) Score of 0.5 clinical followup, red arrows indicate a CDR 1 diagnosis, and dark red arrows indicate a CDR 2 diagnosis. Dashed pink arrows indicate a reversion from CDR 0.5 to CDR 0.

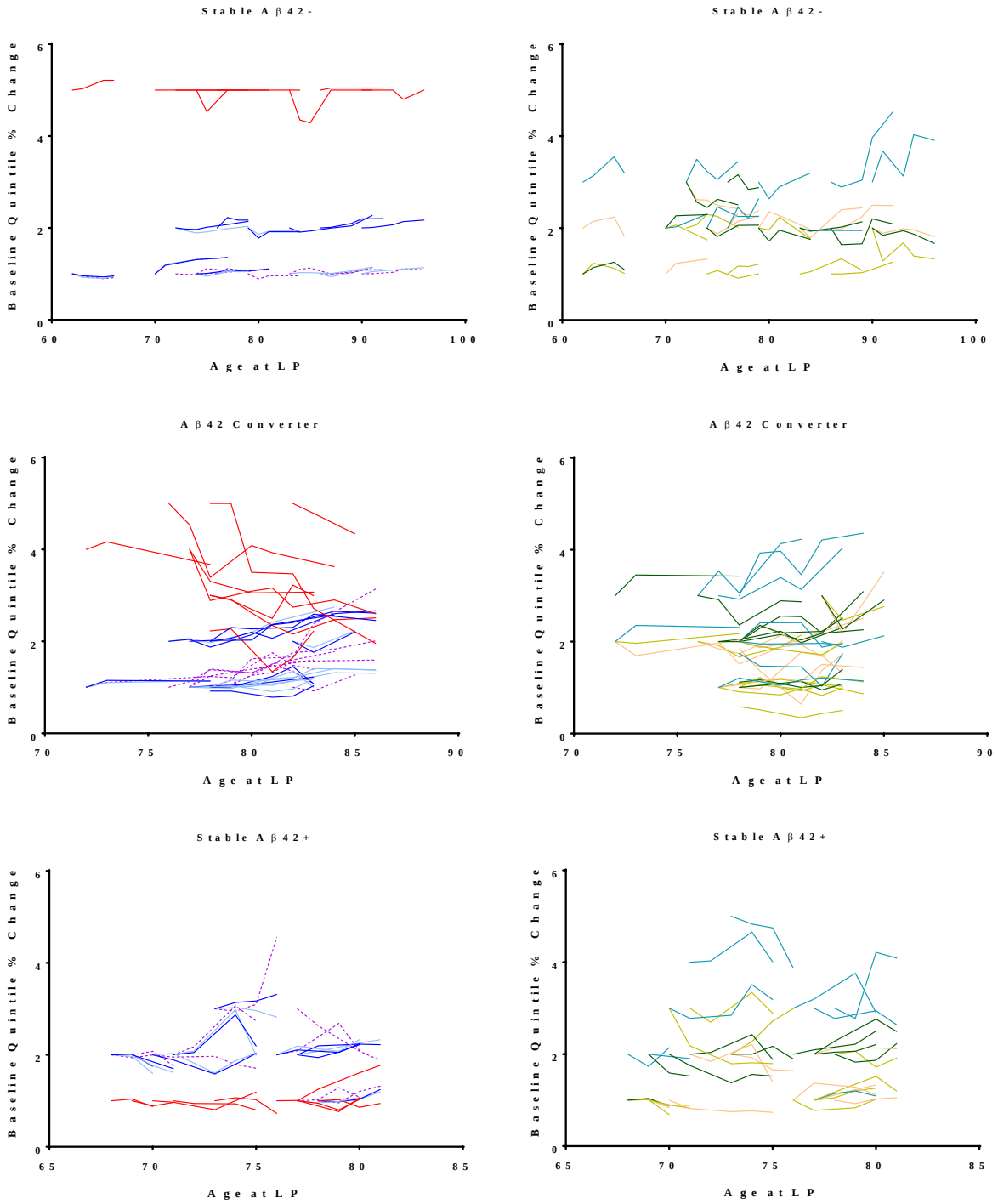
group showed concomitant longitudinal changes in Aβ42, Tau, P-tau$_{181}$ and the Tau/Aβ42 ratio. The stable Aβ42+ group did not show elevated levels of Tau, P-tau$_{181}$ or the Tau/Aβ42 ratio – in fact, in many individuals such as T, U, or W exhibited longitudinal decreases in these biomarkers.

As with the ACS case study cohort, it is difficult to visualize biomarker changes in relation to age. **Figure 4.10** shows biomarker levels by baseline quintile and followup percent change across the entire age range of the ADNI case study cohort. The age range for the stable Aβ42- group covered the full age range from approximately 60-90 years of age at baseline for included individuals. The Aβ42 converter group was more compact, approximately 70-90 years and the stable Aβ42+ group was approximately 65-85 years at baseline. The stable Aβ42- exhibits relatively stable levels of all core biomarkers; the Aβ42 converter group exhibits decreasing CSF Aβ42 levels and increasing Tau, P-Tau$_{181}$, and Tau/Aβ42 levels. The stable Aβ42+ group exhibits low Aβ42 levels and a mix of behavior for the Tau(s) biomarkers, including increasing over time, remaining relatively stable, and decreasing.

4.2.3 Discussion

The ADNI case study cohort is particularly interesting because many individuals included exhibited some form of cognitive impairment either at baseline or during clinical followup. This clinical data makes it a more conducive dataset for following changes through all stages of disease whereas the ACS cohort has greatest strength in the preclinical stage. A particular within-person characteristic that fits well with the longitudinal data presented in Chapter 3 – where significant decreases in markers of neuronal injury/death were seen in the AD group – are the relatively low and, in some cases, decreasing concentrations of the Tau(s) and Tau/Aβ42 ratio in the stable Aβ42+ group. Unfortunately, as the preclinical stage of AD lasts between

Figure 4.11 Longitudinal Biomarker Change by Aβ42 classification, Core and Non-Core Biomarkers in the ADNI Case Study Cohort



Longitudinal biomarker changes starting at baseline age. Left column, core biomarkers (Red, Aβ42; Blue, Tau; Light blue, P-Tau$_{181}$; Purple dash, Tau/Aβ42 Ratio), right column, non-core biomarkers (Green, VILIP-1; Teal, YKL-40; Gold, SNAP-25; Orange, Ng).

10-20 years, there is an important cross-section of disease that is under-represented in the ADNI case study cohort: Aβ42 converters that go on to show signs of cognitive decline. A particular confounding factor is the use of both xMAP and Elecsys Aβ42 data – Chapter 5 briefly explores the analyte differences between the two platforms, particularly with respect to when an individual would be considered a "converter", and the two assays do not agree in most of the 9 Aβ42 converter cases in the ADNI dataset. This detail complicates the identification of the Aβ42 conversion time point and, therefore, the downstream biomarkers of Tau(s) and the Tau/Aβ42 ratio. The ADNI longitudinal cohort dataset was defined and analyzed before the Elecsys data became available, thus necessitating the retention of xMAP Aβ42 data because the study had been defined with it, regardless of the fact that the data was not truly longitudinally assayed. On the other hand, the Elecsys is a new platform for measuring AD biomarkers and does not have the same wealth of legacy data. Both concerns have been recognized in research facilities around the world, so the most thorough option seemed to be using both xMAP and Elecsys Aβ42 data when looking at case studies.

Despite analytical differences, individuals J, K, O, Q, and R all exhibit strikingly similar core biomarker profiles to the ACS case study Aβ42 converter group. Individuals L and P appear to be in even earlier stages of preclinical disease, but further longitudinal followup is necessary to confirm these observations. Individuals M and N are notable due to unstable Aβ42 values – decreasing, then increasing – despite "N" having a baseline diagnosis of MCI. More individuals like these, with longer longitudinal followup, are necessary to get a clearer picture of disease progress in unstable Aβ42 cases.

Perhaps the most striking visualization in the ADNI case study cohort comes from the stable Aβ42- group who, across a period of nearly 40 collective years, exhibit remarkably stable and

consistent core biomarker profiles that indicate the absence of AD. The Aβ42 converter and stable Aβ42+ groups covered a much smaller age range (70-90 years old and 65-85 years old, respectively) and show much more change over time than the stable Aβ42- group. In general, the core biomarkers of neuronal death/injury are increasing in the converter group while the stable Aβ42+ group exhibits higher levels of these markers at baseline, but variable longitudinal behavior. The non-core biomarkers of neuronal injury and inflammation were inconclusive across all three groups, similar to the ACS cohort. There is potential that these non-core biomarkers of neuronal injury or inflammation may be more useful after better understanding the mechanistic or risk-factor-based causes that result in the longitudinal change seen in AD.

As with the ACS case study cohort, the ADNI case study cohort is too small to perform rigorous statistical analyses.

4.3 Conclusions

Visualizing multiple markers on a single graph, while not statistically rigorous, again points to the consistency of core biomarker changes that have been seen in both longitudinal and cross-sectional CSF biomarker studies – low Aβ42, high Tau(s) and high Tau/Aβ42 ratio are indicators of future cognitive decline. However, longitudinal studies such as the DIAN[27] and the ADNI longitudinal cohort tell a slightly different story in that markers of neuronal injury/death may begin to decline after a certain point in disease. The ACS and ADNI case study cohorts are of great interest because they provide a window in to the behavior of AD biomarkers as they relate to one another on an individual basis. Noting the distinct similarities between the Aβ42 converter groups, if nothing else, shows that despite obstacles with analytical aspects of these assays (Aβ42 assays in particular), biomarkers behave similarly enough to eventually combine data

from diverse and varied cohorts with CSF data to better understand and track disease progress on a within-person level as well as a group level.

A number of considerations also spring from the apparent lack of association - or weak association in the case of the ACS case study cohort - between non-core biomarkers of neuronal injury/death and inflammation. Though it is important to note that group-level changes in these biomarkers were seen in both the ACS and ADNI cohorts, associated with either disease risk and amyloid status or diagnosis and amyloid status. As CSF biomarkers were the only biomarker modality considered in this chapter, the addition of imaging biomarkers (e.g. longitudinal hippocampal volume) may pinpoint other aspects of disease that are more closely related to, for instance, the synaptic markers SNAP-25 or Ng. The current longitudinal datasets available are not large enough to allow such intricate analyses across all stages of disease. As stated earlier, preclinical longitudinal datasets have the drawback of tracking little cognitive change and datasets such as ADNI typically have very small AD groups due to the difficulty of retaining impaired individuals as part of a volunteer study. However, future work in assay development, continued longitudinal followup in these and similar studies, and more data from group-wise analyses should result in the sensitive and specific tracking of AD across all disease states.

Chapter 5: Assay Platform and Cohort Comparison

Portions of this chapter were published in the July 2015 issue of JAMA Neurology[31].

Some important pitfalls in AD biomarker investigation became apparent during the timeframe of the work covered in the current document. Analytical variability, particularly with the Aβ42 peptide, has become a focus of investigation as the field attempts to solve 3 major problems hindering the translation of CSF biomarkers from research use to widespread clinical use: (1) high inter- and intra-lab variability, (2), analytical challenges within and between assay platforms and (3) lack of a certified reference material. While not the main focus of the current work, a number of assay comparisons were performed to assess agreement between different assay platforms on samples treated in an identical manner from collection, through processing, storage, and finally, analysis.

5.1 Reasoning for Assay Comparison

Studies like those covered in Chapter 2 and Chapter 3 are of paramount importance because groups such as the Alzheimer's Association Quality Control (AAQC) Program, along with independent research labs, have discovered consistent undesirable characteristics when assessing CSF biomarkers – particularly Aβ42 – that may hinder diagnosis or prognosis in a transition to consistent clinical application.

In 2013, the AAQC program reported consistency between participating laboratories in AD diagnosis, using cutoffs established prior to the formation of the program, but noted this was in spite of high measurement variability[228]. Some of the variability is due to inter-lab differences in assay technique and sample handling, but another setback was in assay variability. An important

guidance issued in the AAQC program report was that assay kit manufacturers, in particular those of enzyme-linked immunosorbent assays (ELISASs) and Multi-Analyte Profiling (xMAP) should consider it "critically important" to improve product quality and minimize lot-to-lot variability[228] which, in an additional study, was considered a hindrance to applying biomarkers in a clinical setting[229]. Performing assay comparisons can help identify when an assay is not performing well, and can also serve as trials for new but promising assays.

A more recent study that stemmed from the longitudinal ACS cohort found that one of the most commonly used assays worldwide (INNOTEST ELISA) exhibited significant upward drift in CSF Aβ42 over more than a decade of production[230], and such assay drift has multiple implications for past and future data analysis. For example, individuals in the ACS cohort covered in Chapter 2 and Chapter 4 return for LP's once every 3 years, if assays are run shortly after each LP, the upward drift may obscure biologically important changes in CSF Aβ42 levels, particularly if protein levels are close to the cutoff for amyloid positivity. A second implication is a moving cutoff for amyloid positivity over time; Aβ42 cutoffs have risen in conjunction with both assay drift and an improved INNOTEST ELISA, which improved variability but raised the absolute average value of Aβ42 substantially[230]. In this case, performing a single-lot analysis on samples that had been collected and analyzed over a period of ten years on different lots was what led to the investigation on assay drift.

5.2 ACS Assay Comparisons

5.2.1 INNOTEST Versus EUROIMMUN ELISA in the ACS Cohort

As part of the design of the ACS longitudinal study (Chapter 2), the analysis of Aβ42, Aβ40, and Tau were performed simultaneously on the same set of samples. An experienced scientist performed "Improved" INNOTEST ELISA's for Aβ42, Aβ40, Tau and P-Tau_{181} and

immediately after, the author performed EUROIMMUN ELISA's for Aβ42, Aβ40, and Tau, as described in Chapter 2. All analyses were performed on the same set of samples, in parallel, to reduce variability in sample handling. Assay performance characteristics are reported in **Table 5.1**. Compared to the previous version of the INNOTEST assays, the improved assays each contain a set of ready-to-use calibrator series, run validation control samples (calibrator in buffer), and harmonized buffer reagents. The EUROIMMUN assays are considered second generation assays because they do not have matrix interference problems (i.e., they exhibit good dilutional linearity). The amount of CSF required for testing is lower in the EUROIMMUN assays, thereby reducing the issues with matrix interference[179,231]. So, in principle, the EUROIMMUN assay measures another fraction of the free, non-protein bound analyte in a sample, unlike many other Aβ42 ELISA's which show poor dilutional linearity. All ELISA kits passed in-house quality control measures including: 1) standard curve values having a CV <25% and no more than two standards either oversaturating or undersaturating using a Synergy 2 Multi-Mode Reader (Bio-Tek Instruments, Inc.), 2) no more than one provided kit control and one internal pooled CSF control failing due to reading outside the provided range or having a CV >25% with the exception of one assay in which both internal CSF controls failed due to high CV – in this case, both kit controls passed, and 3) individual samples failed if there was a CV >25%. The Singulex kits passed in-house quality control measures including: 1) standard curves were assessed according to software provided by Singulex, 2) no more than 2 of 3 internal pooled CSF controls falling outside the range determined by all previous runs using the same lot number of kits and 3) samples failed if there was a CV >25%.

Comparisons of CSF Aβ40, Aβ42, and total tau obtained with the 2 assays were positively correlated (Aβ40, n=412, Pearson r = 0.772 [95% CI, 0.730-0.808], P < .001; Aβ42, n=394,

Pearson r = 0.879 [95% CI, 0.855-0.900], P < .001; total tau, n = 410, Pearson r = 0.958 [95% CI, 0.949-0.965], P < .001). Although the absolute values for Aβ40 and Aβ42 differed between the assays (roughly 2- to 3-fold higher with EUROIMMUN compared with INNOTEST), absolute values for total tau were similar. Patterns of within-person biomarker changes over time were virtually identical between the 2 assays for Aβ42, total tau, and the total tau to Aβ42 ratio. However, baseline comparisons and longitudinal patterns for Aβ40 were slightly different between the assays and thus are difficult to interpret. Clinical observations were confirmed in both immunoassays for Aβ42 and total tau.

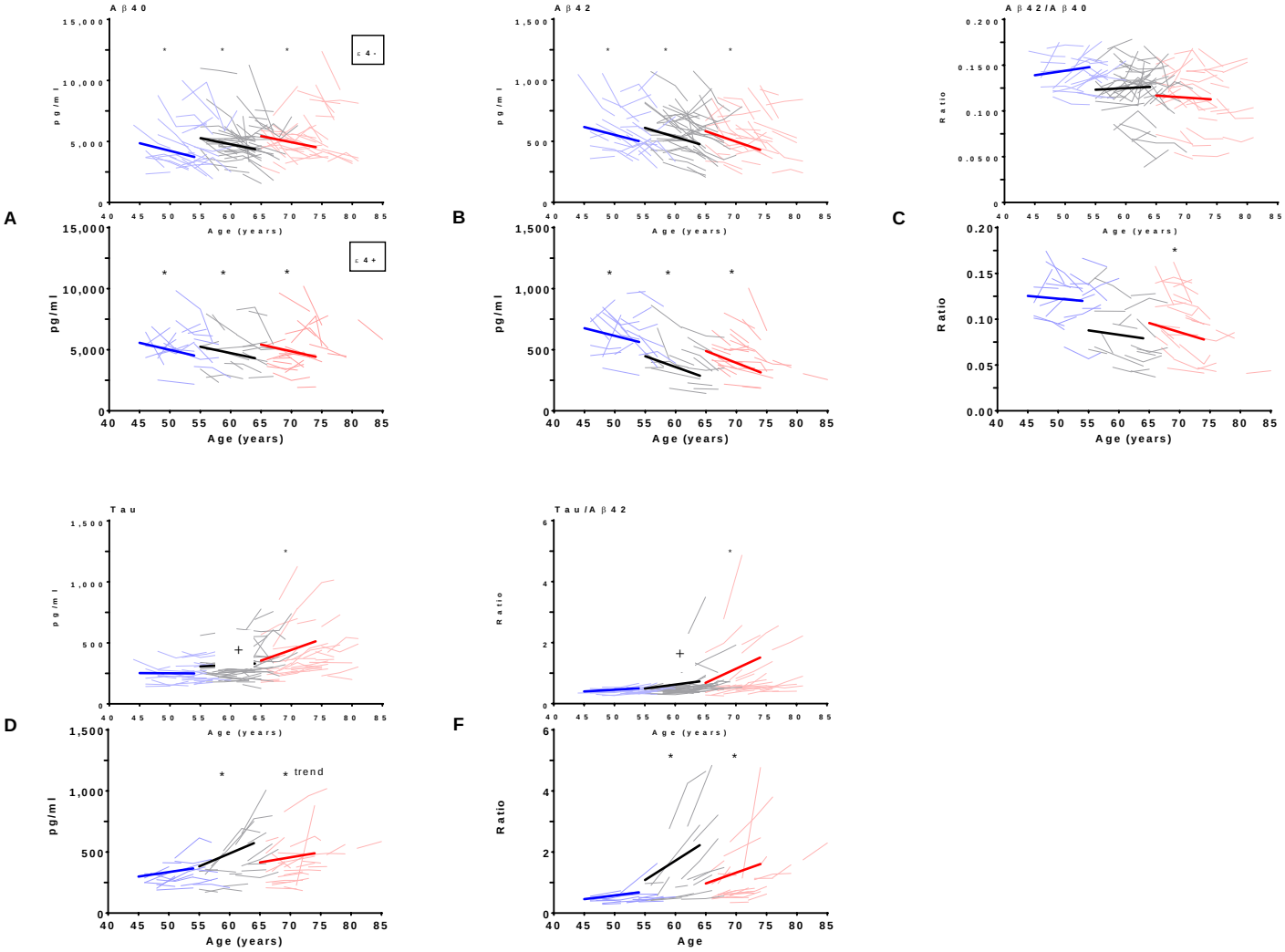
More recent work has highlighted the same disparity in absolute concentration for Aβ42, as well as good correlation between multiple assays, for the INNOTEST and EUROIMMUN assays[232]. Head-to-head comparisons such as these are of paramount importance for studying Aβ42 (and, to a lesser extent Tau(s)) and allow for a focus on assay performance as many of the pre-analytical and analytical variables are controlled for, e.g. limited or no changes in kit lots, uniformity in assay conditions, or uniformity in sample handling across a single study[233].

Table 5.1 Assay Performance Specifications as Provided by the Vendors

Vendor	Fujirebio (formerly Innogenetics)				EUROIMMUN			QUIDEL	SINGULEX
Analyte	**Aß1-40**	**Aß1-42**	**Total tau**	**Ptau181**	**Aß1-40**	**Aß1-42**	**Total tau**	**YKL-40**	**VILIP-1**
Technology	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	ELISA Colorimetric	Single Molecule Counting (SMC™)
Fluid Sample	CSF	CSF	CSF	CSF	CSF	CSF	CSF	CSF	CSF
Capture Antibody	2G3	21F12	AT120	HT7	2G3	21F12	ADx 201	Not Available	3A8.1
Capture Antibody Epitope	35-40	37-42	218-224	159-163	Aßx-40	Aßx-42	Proline-rich region	Not Available	Not Available
Detection Antibody	3D6	3D6	HT7/BT2	AT270	3D6	3D6	ADx 215	Not Available	Sheep 22
Detection Antibody Epitope	1-5	1-5	159-163/193-198	176-182	Aß1-x	Aß1-x	N-terminus	Not Available	Multiple
Calibrator Concentration Range, pg/mL ***(lot specific, for this study)***	7.8 - 1000 (n=8)	62.5 - 4000 (n=6)	50 - 2500 (n=6)	15.6 - 1000 (n=6)	54 - 711 (n=6)	83 - 1236 (n=6)	30 - 1050 (n=6)	20 - 300 (n=6)	3.9 - 3000 (n=10)
Limit of Detection (LOD), pg/mL, (range), number of runs	3.3 (0.7 - 4.2) 4 runs	65 (52-87) 7 runs	34 (25-47) 8 runs	13.4 (11.4 - 14.9) 4 runs	70 (58 - 85) 6 runs	7.5 (6.3 - 9.0) 6 runs	44.2 (41.1 - 46.8) 4 runs	5.4 Not available Not available	1.4 (0.077-6.9) 16 runs
Sample Specifications									
Sample Pre-dilution	1:100	No	No	No	1:21	No	No	1:03	No
Volume Sample Per Well (µL)	75	25	25	75	15	15	25	20	15
Total Well Volume	100	100	100	100	115	115	125	120	200
Number of Replicate Wells	2	2	2	2	2	2	2	2	2
Sample Incubation									
Time, hrs	14-18	1	14-18	14-18	3	3	3	1	2
Temperature	2-8°C	23-27°C	23-27°C	2-8°C	23-27°C	23-27°C	23-27°C	23-27°C	23-27°C
Reported Intra-Assay Variability[1], % CV, (range), number of runs	2.8 (0.3 - 7.8) 5 runs	4.6 (0.8 - 11.0) 3 runs	3.2 (0.0 - 13.2) 2 runs	1.7 (0.0 - 8.9) 4 runs	2.7 (2.4 - 3.1) 3 runs	3.9 (2.3 - 6.2) 3 runs	5.0 (3.9 - 6.5) 4 runs	6 (5.6 - 6.6) Not available	4.4 (3.1 - 7.0) 10 runs
Reported Inter-Assay Variability[1], %CV, (range), number of runs	4.4 (3.2 - 5.8) 4 runs	7.8 (1.4 - 18.0) 3 runs	11.5 (3.8 - 42.8) 2 runs	11.4 (6.6 - 19.2) 4 runs	8.3 (7.3 - 9.3) 5 runs	5.6 (4.4 - 7.6) 7 runs	7.4 (6.1 - 9.3) 5 runs	6.7 (6.0 - 7.0) Not available	6.2 (5.0 - 7.0) 1 run
Study Intra-Assay Variability[2], % CV, (range), number of runs	2.7 (0.4 - 12.7) 12 runs	4.4 (0.2 - 13.7) 12 runs	2.0 (0.0 - 11.4) 12 runs	1.0 (0.1 - 4.6) 12 runs	2.3 (0.1 - 6.0) 12 runs	3.0 (0.1 - 9.7) 12 runs	5.42 (0.8 - 22.9) 12 runs	1.9 (0.1 -6.4) 12 runs	4.5 (0.0 - 20.8) 16 runs
Study Inter-Assay Variability[2], %CV, (range), number of runs	3.8 (0.0 - 24.6) 12 runs	4.3 (0.0 - 20.6) 12 runs	2.1 (0.0 - 14.8) 12 runs	2.2 (0.0 - 17.5) 12 runs	14 (0.1 - 13.7) 12 runs	4.1 (0.0 - 16.7) 12 runs	4.1 (0.0 - 24.2) 12 runs	3.7 (0.0 - 21.6) 12 runs	4.3 (0.0 - 24.8) 12 runs

Samples were analyzed for Aβ1-40, Aβ1-42, Tau, and P-tau$_{181}$ using the "Improved" INNOTEST™ ELISA (Fujirebio Europe [formerly Innogenetics], Gent, Belgium). For comparison purposes, concentrations of Aβ1-40, Aβ1-42 and total tau were also measured using EUROIMMUN ELISAs (EUROIMMUN, Luebeck, Germany). For Aβ species, both assays utilize the same monoclonal antibodies, but they are obtained from a different process of culture, production, and purification. YKL-40 (also known as chitinase-3 like 1), an astrocyte-derived marker of gliosis/neuroinflammation, was measured with the MicroVue ELISA (Quidel, San Diego, CA). Visinin-like protein 1 (VILIP-1), a marker of neuronal injury, was measured using a two-site immunoassay implemented via a microparticle-based Erenna immunoassay system (Singulex, Alameda, CA). [1] performance characteristics as provided by the vendor. [2] actual performance characteristics in the current study.

Figure 5.1 Group Longitudinal Change Over Spaghetti Plots of EUROIMMUN CSF Biomarkers During Middle-age.



Estimated slopes and within-person patterns for A) Aβ40, B) Aβ42, C) the Aβ42/Aβ40 ratio, D) tau, and E) tau/Aβ42 are shown in three age bins for *APOE* ε4-negative (top graph of each panel, n=108participants) and ε4-positive (bottom graph of each panel, n=61 participants) groups. Annual slopes have been extrapolated to 9 years, and each slope begins at the mean baseline biomarker value of each age bin. Blue, EARLY middle-age (45-54 years at baseline); Black, MID middle-age (55-64 years at baseline); Red, LATE middle-age (65-74 years at baseline). * slopes significantly different from 0 ($p<0.05$). +, slopes significantly different between *APOE* ε4 groups within a given age group ($p<0.05$). Trend, $p=0.051$-0.06.

While no judgements were made as to the quality of either the INNOTEST or EUROIMMUN ELISAs, this comparison was informative in that consistent results were found with both assays. The exception was Aβ40: the slopes in the *APOE* ε4 non carrier group were declining in the early- and mid-middle age groups. However, the EUROIMMUN data shows a significant decline in Aβ40 slope across all 6 groups. The INNOTEST data shows no change and perhaps even a slight increase in Aβ40 over time in the three *APOE* ε4 carrier groups, though not significant. Because assay comparison has largely focused on the problematic Aβ42 peptide, it is difficult to speculate the reasons behind this difference. However, recent data from Vanderstichele and colleagues may offer a potential explanation: despite both INNOTEST and EUROIMMUN using the same capture antibodies (21F12 for Aβ42 and 2G3 for Aβ40), and the same detection antibody (CD6) across all four assays, INNOTEST displays a dose-dependent difference in Aβ42 levels that corresponds to Aβ40[232]. They speculate that this is due to Aβ40 interfering in the Aβ42 assay through CD6 binding, where higher levels of Aβ40 in the CSF result in a lower Aβ42 value. Because CD6 is a component of both the Aβ40 and Aβ42 assays there is some potential for interference in the Aβ40 assay, though Aβ40 is roughly ten times as abundant in CSF as Aβ42. It is additionally difficult to say how this interference is happening as the ELISA format goes through multiple washing steps before applying the detection antibody. Unless there is substantial binding of Aβ40 to the 21F12 antibody or oligomerization of Aβ40 on to Aβ42 that has bound to 21F12, there should be little to no interference as it is reported by Vanderstichele and colleagues. Interestingly, this interference is not seen with the EUROIMMUN assay. These questions are further support for the continued comparison of different assays and assay platforms as those used most frequently are not ready to transition in to clinical use at this time.

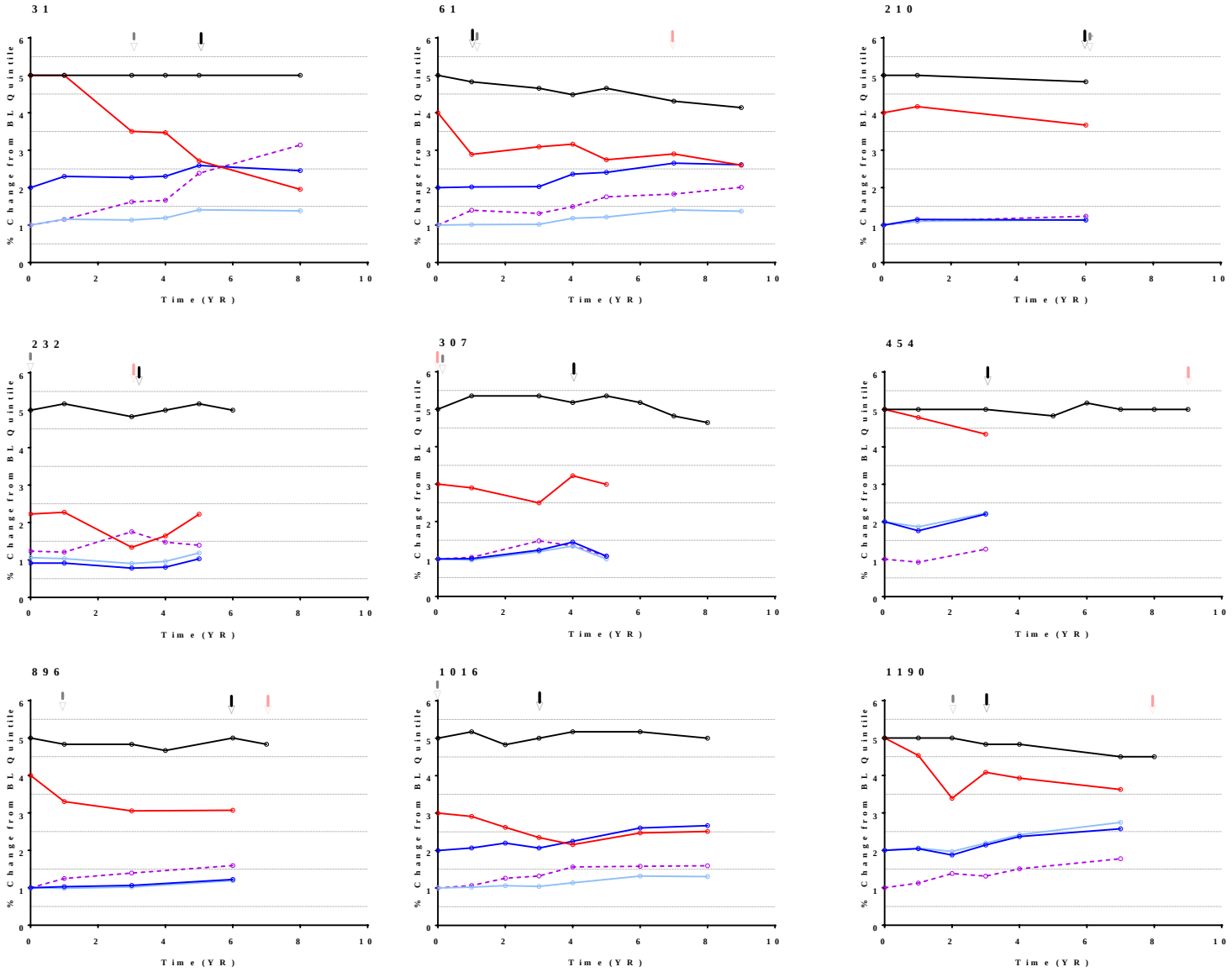
5.2.2 xMAP Versus Elecsys Comparison in the ADNI Cohort

Unlike the ACS cohort, assays for Aβ42, Tau, and P-Tau_{181} were not performed in-house in the ADNI cohort. Data for these analyses were downloaded from the ADNI, thus there is no way to compare specific performance characteristics of each assay. ADNI researchers analyze CSF samples on a rolling basis, though some longitudinal analyses have been completed. The ADNI study reported in Chapter 3 relied on two sets of xMAP data generated by re-analyzing longitudinal samples from an individual at the same time, rather than relying on data that was generated on the rolling basis. Unfortunately, not all samples were assayed in one longitudinal run and there is no way to determine which lot number of xMAP assay was used. Further, without access to the raw data, it is not possible to perform comparisons between the two sets of xMAP data to ensure continuity between assay lots. Fortunately, assay validation and assay comparisons are a major focus of numerous large studies, ADNI included, and data from the new Elecsys platform was released for Aβ42, Tau and P-Tau_{181} in early 2017. Correlations between xMAP and Elecsys data for Aβ42 and Tau were as expected – Tau was highly correlated (Pearson $r = 0.948$, $p<0.001$) and Aβ42 showed more variability but still correlated well between the two assay platforms (Pearson $r = 0.884$, $p<0.001$). Originally, P-tau_{181} was excluded from analysis due to clear variability between the two xMAP datasets. Typically, both Tau and P-tau_{181} show very close correlation, as with the INNOTEST and EUROIMMUN comparisons above, but in this case xMAP and Elecsys P-tau_{181} did not correlate as well as would be expected (Pearson $r = 0.743$, $p<0.001$).

Despite these close correlations, there were clear differences in diagnostic ability in individuals who converted from Aβ- at baseline LP to Aβ+ at a later LP. **Figure 5.2** illustrates all nine individuals in the ADNI longitudinal cohort who converted from Aβ42- to Aβ42 positive as

defined by the cutoff of 192 pg/ml for the xMAP assay. This paradigm was used to classify individuals as amyloid + or – for the analyses in Chapter 3. A poster presentation in July 2017 outlined potential cutoffs for amyloid positivity as defined by Elecsys values[234]. Three cutoffs were presented: 1,065 pg/ml when compared to xMAP Aβ42, 1,017 or 1,172 pg/ml when modeled by unweighted and weighted densities respectively, and 1,198 pg/ml when compared to amyloid imaging. The xMAP and Elecsys Aβ42 values showed good concordance, but better concordance was found when comparing to amyloid imaging. Mixture modeling identifies sub-populations in a given sample and weighting takes in to account the distribution of each sub-population in the overall population. The rich history of amyloid imaging and CSF biomarker analysis means that (1) concordance between amyloid imaging and CSF Aβ42 is high in most studies and (2) a general estimate for amyloid positivity in cognitively normal individuals can be established, therefore a cutoff of 1,200 pg/ml was chosen to indicate amyloid positivity using the Elecsys assay for the purposes of the current study.

Figure 5.2 ADNI Aβ42 Converter xMAP Versus Elecsys Positivity Comparison



Core CSF biomarkers, Tau/Aβ42 Ratio and MMSE score in the nine individuals in the ADNI longitudinal cohort that converted from Aβ42- to Aβ42+ at any LP after that baseline LP, as defined by xMAP Aβ42. Each graph represents a single individual, with T0 representing the baseline LP and representative baseline quintile for each biomarker. Follow up LP's or MMSE tests are marked by open circles. Each colored line represents a different biomarker (Red, Elecsys Aβ42; Blue, Elecsys Tau; Light blue, Elecsys P-Tau$_{181}$; Purple dash, Aβ42/Tau Ratio; Black, MMSE score). At the top of each graph, pink arrows indicate the time when an individuals' CDR score changed from 0 to 0.5; black arrows indicate the time point that an individual would be considered Aβ42+ according to xMAP data; grey dashed arrows indicate the time point an individual would be considered Aβ42+ according to Elecsys data; grey dashed arrows with a star indicate an Elecsys value very close to the Aβ42 cutoff of 1200 pg/ml.

Only one individual exhibited agreement between xMAP and Elecsys amyloid positivity – individual 61. Individual 210 was considered amyloid positive 6 years after baseline with the xMAP assay; with the Elecsys assay the CSF Aβ42 value was 1,271 pg/ml, very close to the 1200 pg/ml cutoff. Individual 454, though showing decline in Aβ42 over time, did not exhibit amyloid positivity when using the Elecsys cutoff, even though xMAP Aβ42 values converted to positivity 3 years after baseline LP and a CDR score of 0.5 was given 9 years after baseline LP. The remaining 6 individuals would be classified as Aβ42+ one to five years earlier using Elecsys data than they would using xMAP data. Visually, the earlier positive classifications using Elecsys data fit better with the hypothesis of a long period of amyloid accumulation before cognitive symptoms appear and also match data from the ACS longitudinal cohort showing that amyloid changes are detected earlier than tau and cognitive changes.

5.2.3 Conclusions

Reductions in CSF Aβ42 over the time-course of AD are now a well-established phenomenon, but recent discoveries regarding the variability in measurement over time for this important biomarker have added an element in longitudinal analysis that is difficult to control for, much less anticipate. The increase in assay comparison studies, alongside the emergence of new assay platforms such as the Elecsys assays for Aβ42, Tau and P-tau$_{181}$, is a barometer for the next important phase in AD research: the search for or development of reliable assays that can be used worldwide for the consistent measurement of Aβ42 from bodily fluids such as CSF.

The ADNI dataset provides an interesting opportunity to compare a widely used assay – xMAP – and a new assay platform – Elecsys – in the same set of samples. The biggest caveat to the ADNI observations above is that they cannot, at this time, be statistically validated due to the very small group of only nine individuals. However, these data do again showcase the necessity for

biomarker comparison across multiple assays and assay platforms in the effort to find an assay (or assays) that perform most sensitively and most specifically in identifying AD on a within-individual basis.

There is a delicate balance between adequate assay testing and the depletion of precious stores of CSF from cohorts such as the ACS and ADNI, which necessitates careful consideration of appropriate assay platforms and study design. The development and adoption of stable measurement techniques for Aβ42 and other AD biomarkers will likely contribute greatly to defining the within-person changes that will allow biomarker-based diagnosis and prognosis on an individual basis.

Chapter 6: Conclusions and Discussion

The development in CSF biomarkers of AD has progressed rapidly over the last decade. From the development of core biomarkers of disease through the identification and study of important non-core biomarkers, the field as a whole is learning important lessons about AD progression and about the difficulties involved in measuring biomarkers in a slow-developing disease where the organ of interest is not easily accessible. The current work is a step toward developing CSF biomarker profiles that may eventually provide, over the full course of the disease, diagnostic and prognostic applicability in conjunction with neuroimaging and psychometric analysis. The results reported in this document, alongside other work in the field, indicate that the diagnostic and prognostic paradigm for AD will likely require multi-factorial, longitudinal, assessment in order to pinpoint disease on a within-person basis. They also provide support for viewing AD as a continuum as outlined by Aisen et al[3].

The aim of the works presented here were primarily to define the behavior or CSF biomarkers in large, independent cohorts during the preclinical and early symptomatic stages of AD. In agreement with data from the DIAN cohort, changes in core CSF biomarkers of AD begin to differentiate individuals at higher risk of developing the disease than those at lower risk, as defined by APOE ε4 allele carriage in the ACS cohort, as early as 45 years of age. In many cases, these changes occurred earlier in ε4 carriers than non-carriers, though the rates of change did not differ between the two groups. In mid middle-age, core markers of neuronal injury and death, as well as non-core markers of neuronal injury and neuroinflammation in the ε4 carrier group increased at a faster rate than mid middle-age non-carriers, but this rate of change was not different between late or mid middle-aged carriers or late middle-aged non-carriers, indicating

that regardless of the age of disease onset, it progresses at roughly the same rate across individuals, when defined by biomarker status.

Further investigation in the ACS cohort – though of an n too small to perform statistical analyses – indicated that PiB positivity developed only after CSF Aβ42 levels were reduced. This perhaps was most plainly indicated by two individuals with stable low CSF Aβ42 who were PiB negative. Interestingly, CSF Tau, VILIP-1, and YKL-40 were increasing slightly or at mid-to-high concentrations compared to other individuals at similar ages, which suggests these individuals were in the beginning stages of amyloid deposition in the brain. Further follow-up with these individuals would be particularly interesting to confirm that indeed, this may be evidence of the very earliest stages of preclinical disease.

These visual associations with PiB imaging, alongside an association with cognitive changes in late middle-age are an indication that the utilization of multiple modalities may lead to a more accurate representation of the full continuum of disease. The ACS cohort provides an incredibly important glimpse of CSF changes during preclinical AD, from very early longitudinal decreases in CSF Aβ42, followed by longitudinal increases in CSF markers of neuronal injury or death alongside the development of PiB positivity in mid middle-age, followed lastly by evidence of cognitive changes in the oldest age group studied. A larger n, paired with continued follow-up of this group of study participants has the potential to allow analysis of biomarker trajectories throughout the continuum of early disease.

In contrast, the ADNI cohort provided the ability to study CSF biomarkers across the spectrum of cognitive impairment that physicians currently use to define AD in research and clinical settings. Only the core CSF biomarkers Aβ42, Tau, and P-tau showed longitudinal change in the

preclinical, cognitively normal amyloid negative and/or amyloid positive groups, mirroring changes seen in the ACS cohort. The non-core markers VILIP-1 and YKL-40 did not exhibit changes during this period, unlike the changes seen in the ACS cohort. However, the core biomarkers and non-core markers indicative of neuronal dysfunction (either neuronal injury, death, or synaptic injury) showed longitudinal decreases during the early symptomatic stages of disease with the exception of CSF Tau. Again based on data from the DIAN cohort, longitudinal decreases in CSF biomarkers of neuronal dysfunction were not unexpected[64]. However, the work published here supports these findings in a large, independent cohort of individuals with sporadic AD, providing further evidence that the pathologic disease processes of AD are indeed the same in both ADAD and sporadic AD, regardless of differing etiology.

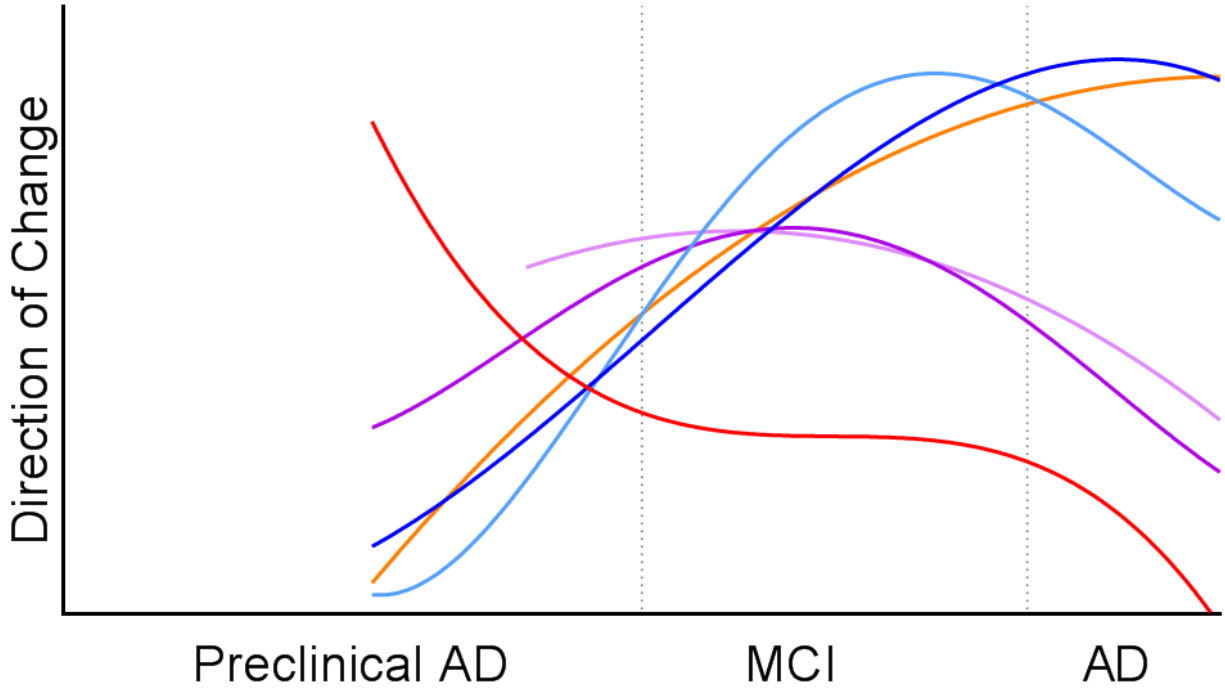
These data have important implications for clinical trials looking to enroll individuals during preclinical AD. For instance, a trial aiming to capture individuals across the spectrum of changes that occur during preclinical AD may enroll participants with only low CSF Aβ42, with low CSF Aβ42 and who are amyloid imaging positive, or who are CSF or amyloid imaging with evidence of high or increasing markers of neuronal dysfunction. Such separation may provide opportunities to test anti-amyloid therapeutics at varying states of disease prior to cognitive impairment to best pinpoint when during preclinical disease these agents are most efficacious. It may also provide a platform for testing alternative therapeutic agents such as anti-tau or secretase inhibiting drugs to determine if/when they are efficacious at preventing amyloid or tau accumulation or neuronal death. Further reasoning for determining in-depth biomarker trajectories over the full course of AD is apparent in the ADNI data. In many cases, reduction in a biomarker that is elevated during disease would be seen as an indicator of therapeutic efficacy in a clinical trial. However, if the core and non-core biomarkers are indeed decreasing

longitudinally during the early symptomatic stages of AD (individuals with a CDR score of 0.5 to 1), a reduction in, for instance, Tau or VILIP-1 might be indicative of the natural course of disease rather than therapeutic target engagement or treatment efficacy. This is particularly important in clinical trials enrolling individuals with mild cognitive impairments, where a reduction in markers of neuronal injury might well be considered an indicator of treatment downstream treatment efficacy, even with anti-amyloid therapeutics.

As mentioned in Chapter 1, the hypothetical models of biomarker change that have thus far been proposed were invariably represented as sigmoidal curves. Much work remains to allow cross-study comparisons of biomarkers in a way that would render modeling changes in the ACS and ADNI groups together a possibility. However, the pictures of core AD CSF biomarkers developed in the ACS, ADNI, DIAN and other studies are largely in agreement in terms of direction of change (increasing or decreasing) over the course of the disease. If the non-core CSF biomarkers are considered similarly, the ACS and ADNI data may also indicate how these biomarkers change, as a whole, over the course of disease. **Figure 6.1** outlines what the biomarker trajectories of CSF Aβ42, Tau, Ptau, VILIP-1, SNAP-25, Ng, and YKL-40 might look like as further data from longitudinal studies is added to our knowledge base. Current constraints do not allow for the ACS and ADNI data to be formally compared to one another, but for the purposes of visualizing biomarker change based on disease state, the ACS and ADNI data are considered on the same curve for each individual biomarker. The ACS data likely shows a more complete picture of preclinical AD as the CN Aβ- and Aβ+ groups were small. However, without enough longitudinal follow-up to show individuals progressing from cognitively normal and biomarker positive through clinically recognizable cognitive impairments, it is currently only possible to speculate which individuals might be in the very earliest stages of disease

(hypothesized to exhibit only CSF amyloid changes). Therefore the hypothetical curves do not extend through the full range of "preclinical AD" as labeled in **Figure 6.1**. Further, as SNAP-25 and Ng were only assessed in the ADNI cohort, the hypothetical curve (shown in lavender) begins later in the "preclinical AD" range than the other CSF biomarkers. The inflection points where markers of neuronal dysfunction hit a peak and then fall between the "MCI" and "AD" labels are hypothesized to occur in the ADNI group designated as MCI Aβ+, individuals who most likely are exhibiting impairment due to AD. Data from Chapter 3 indicates that these markers do not significantly change during MCI, potentially due to either a plateau once biomarker concentrations are at their maximum, followed by the decline seen in the AD group, or due to the slope of biomarker change being in effect washed out by individuals on one side of the inflection increasing longitudinal and individuals on the other side decreasing. Further research will be needed to develop these curves more fully.

Figure 6.1 Hypothetical Model of Combined ACS and ADNI CSF Biomarker Trajectories



Hypothetical trajectories of CSF biomarkers based on data from both the ACS and ADNI datasets combined. The x-axis indicates disease state as it progresses from Preclinical AD, through MCI, through AD; vertical dashed lines indicate, roughly, the transition from one disease state to the next. The y-axis indicates direction of longitudinal change, which can be interpreted as upward change corresponding to significant increases in biomarkers over time, downward change corresponding to significant decreases over time, and plateaus or inflection points corresponding to no significant change over time in the respective diagnostic groups. Each line indicates a single CSF biomarker trajectory: Red, Aβ42; Dark Blue, Tau; Light Blue, Ptau; Purple, VILIP-1; and Orange, YKL-40; Lavender represents both SNAP-25 and Ng as both biomarkers performed similarly in the ADNI cohort.

Biomarker research in AD is still only just beginning. The data generated by this project will be used moving forward for a number of projects: 1) analyzing VILIP-1 and YKL-40 across both the ACS and ADNI datasets to assess whether the two cohorts might be viably combined, thus increasing the n for at least two non-core biomarkers to 317 individuals; 2) assessing further promising non-core biomarkers in these populations such as NfL; 3) exploring further statistical analyses based on the addition of neuroimaging biomarkers or different classification schemes. The wealth of data offered by these studies allows for a multitude of avenues of investigation. In particular, the data presented lends itself well to investigation using groups defined by the proposed A/T/N classification from the NIA-AA. Grouping by APOE allele risk factor or by amyloid positivity only as defined by CSF Aβ42 may obscure longitudinal changes occurring in the later preclinical or MCI stages of AD. For instance, grouping individuals by CSF Aβ42 status only may not accurately capture differences in the rates of longitudinal change in biomarkers of neuronal dysfunction that could occur in an individual who is amyloid positive only, compared with an individual who is amyloid positive and tau positive. There is much left to learn from these studies.

By far, one of the biggest limitations of the current studies is the low n; despite both the ACS and ADNI being the largest longitudinal studies of AD reported to date, they are still quite small in terms of statistical power. However, these studies may be used to perform power calculations in further cohorts, allowing judicial allocation of precious CSF for testing at a time when enough samples have been collected. Another limitation was brought to the forefront in Chapter 5: assay variability. Particularly with CSF Aβ42, the reproducibility and comparability of Aβ42 when measured in labs around the world is not at a level that receives confidence from regulatory agencies as a validated diagnostic, prognostic, or theragnostic tool – though in Europe the

measurement of core CSF biomarkers is more widely approved and more common than in the United States. Additionally, non-core biomarkers may be especially valuable in tracking downstream indicators of therapeutic efficacy in clinical trials. However, further longitudinal validation is needed on each of the biomarkers reported here, as well as development of other promising biomarkers, to bring the level of confidence in such markers on par with the core biomarkers.

Large longitudinal studies such as the ACS and ADNI require rigorous planning, protocol adherence, abundant funding, and, most importantly, dedicated populations of volunteers willing to undergo a gamut of testing modalities on a regular basis. Despite being active for nearly two decades, the ACS is only now at a point to assess longitudinal CSF data from enough participants to allow rigorous statistical analysis. The ADNI study is just over a decade old though, as a study designed with the validation of AD biomarkers in mind, much more data has been collected and on a shorter time-scale than the ACS. Data gained through both cross-sectional and longitudinal data on these and other large, diverse cohorts is essential to the forward movement in understanding the full course of AD. Hopefully, these data will allow development of statistically validated trajectories of biomarkers throughout the course of disease, allowing clinical trials to accurately assess biomarker changes in relation to therapeutic agents and eventually allowing physicians to reliably diagnose individuals with AD at any stage and offer a prognosis or, someday, the appropriate treatment at the best possible junction.

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Appendix

A.1 Appendix Data and Figures for Chapter 3

A.1.1 Assay Details

VILIP-1

Mouse anti-human VILIP-1 and sheep anti-human VILIP-1 were used to develop a sandwich ELISA using an Erenna instrument (Singulex). Assay reagents include biotinylated mouse anti-human VILIP-1, clone 3A8.1 “capture” antibody, bound to streptavidin-coated magnetic micro particles and Invitrogen Alexa Fluor 647 dye labeling of sheep anti-human VILIP1. Additional materials purchased from Singulex include 10X Wash Buffer (02-0001-01), Elution Buffer (02-0297-xx) and elution step neutralization Buffer C (02-0298-00).

Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of recombinant human VILIP-1 ranging from 3.9 to 3000pg/mL in assay buffer (prepared daily for the assay and filtered before use contained per liter) containing 10 mm TRIS, 150 mm NaCl, pH = 8.1, supplemented with 0.1% each of Triton X (Sigma T-9284), and Sodium Azide, also with 1 gram BSA (Sigma A-7030), as well as 2 gram Equitech-Bio mouse IgG (SLM66) (2 mg/mL) and 2 mM CaCl2 (Sigma 21115), with each concentration assayed in triplicate. 15 µL standards or CSF sample were combined with 135ul assay buffer and 50µL antibody coated micro particles. The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex Wash Buffer. Fluorescent dye labeled detection antibody (20µL per

well) was added and incubated for one hour. After washing the magnetic micro-particles five times, 20µL per well of Singulex Elution Buffer was added for 10 minutes to separate detection antibody from the micro-particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

SNAP-25

Mouse anti-human SNAP-25 antibodies were used for development of a sandwich ELISA using an Erenna instrument (Singulex). Assay reagents included a preparation of the monoclonal capture antibody 6H07-2C12 for binding to Invitrogen (Carlsbad, CA) MyOne magnetic micro-particles and Invitrogen Alexa fluor dye labeling of monoclonal antibody 9E11, using Singulex labeling kits (capture antibody labeling kit 03-0077-xx and detection antibody labeling kit 03-0076-02). Additional materials purchased from Singulex include 10X Wash Buffer (02-0001-01), Elution Buffer (02-0297-xx) and elution step neutralization Buffer C (02-0298-00).

Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of recombinant human SNAP25 (CSI15602) from Cell Science, Inc (Seattle, WA) ranging from 0.078 to 90pg/mL in Thermo Scientific, Inc (Rockford IL) Blocker Casein in TBS plus 0.1% Tween-20 from Sigma-Aldrich, Inc (St Louis MO) and with each concentration assayed in triplicate. 100µL standards or CSF diluted 4-fold were combined with 100µL antibody coated micro particles diluted in Blocker Casein in TBS plus 1% Tween-20. The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro-particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex wash buffer. Fluorescent dye labeled detection antibody diluted in Blocker Casein in TBS plus 1% Tween-20 (20µL per

well) was added and incubated for one hour. After washing the magnetic micro-particles five times, 20µL per well of Singulex Elution Buffer was added for 10 minutes to separate detection antibody from the micro particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

Ng

Two epitope-specific rabbit anti-human NGRN antibodies were used for development of an ELISA using an Erenna instrument (Singulex). Assay reagents included a preparation of a C-terminal specific antibody (P-4793) for binding to Invitrogen (Carlsbad, CA) MyOne magnetic micro-particles and Invitrogen Alexa fluor dye labeling of N-terminal specific antibody (P-4794) using Singulex labeling kits (capture antibody labeling kit 03-0077-xx and detection antibody labeling kit 03-0076-02). Additional materials purchased from Singulex include 10X Wash Buffer (02-0001-01), and custom Elution Buffer (02-0002-01).

Prior to the assay all samples were centrifuged (11,000 g x 3 minutes) to remove particulates. All assay steps were performed at room temperature unless otherwise indicated. A calibration curve was prepared using dilutions of synthetic full-length NGRN purchased from AAPPTec, (Louisville KY), ranging from 1.75 to 3000pg/mL in standard diluent (TBS, 2 mg/ml rabbit IgG from Equitech-Bio [Kerrville, TX] plus 0.1% Tween-20), with each concentration assayed in triplicate. 50µL standards or CSF diluted 10-fold were combined with 100µL antibody coated micro-particles diluted in assay buffer (TBS, rabbit IgG plus 1% Tween-20). The assay plate was incubated for two hours on a plate shaker set to 525 revolutions per minute. Micro-particles were then magnetically separated and washed one time using an Agilent (Santa Clara, CA) Bravo Automated Liquid Handling Platform using Singulex Wash Buffer. Fluorescent dye labeled detection antibody diluted in assay buffer (20µL per well) was added and incubated for one hour.

After washing the magnetic micro-particles 5 times, 20µL per well of Singulex custom Elution Buffer (PN 02-0002-03) was added for 30 minutes to separate detection antibody from the micro-particles. Eluted antibodies were then transferred with the Bravo instrument to a clean 384 well plate for reading in the Erenna® immunoassay system.

YKL-40

YKL-40 was measured using the MicroVue YKL-40 ELISA assay (Quidel, San Diego, CA). Prior to the assay, all samples were lightly vortexed for 5 seconds. All assay steps were performed at room temperature unless otherwise noted. The complete standard curve of YKL-40 purified from osteosarcoma MG-63 cells is provided with the assay kit, and each standard and sample was assayed in duplicate. All CSF samples were diluted 1:2 in Standard A (0 ng/ml) on an ice cold pre-plate before transferring 20µl to the coated ELISA plate. After adding 100µl capture solution, the plate was incubated for 60 minutes followed by washing 4 times with 250µl wash buffer. Enzyme conjugate (100µl) was prepared prior to beginning the assay and added to the assay plate, followed by a 60 minute incubation. The substrate solution was prepared during this step to ensure dissolution of the substrate tablet. After another wash (four times with 250µl wash buffer), 100µl substrate was added to the assay plate followed by a 60 minute incubation. Finally, 100µl stop solution was added to the assay plate, and samples were read at an optical density of 405nm and analyzed with a linear regression curve-fit.

A.1.2 Assay Quality Control

All samples (each from the same freeze/thaw cycle) were run in triplicate for VILIP-1, SNAP-25 and Ng and in duplicate for YKL-40, all using a single assay lot number. Importantly, within-person longitudinal samples were run on the same assay plate to reduce inter- and intra-plate variability. Quality control (QC) for VILIP-1, SNAP-25 and Ng included analysis of three internal standard CSF pools run on each assay plate. For YKL-40, two internal standard CSF

pools were run on each plate. QC mean and tolerance limits for VILIP-1, SNAP-25 and Ng were established by computing the average of at least 15 values collected over at least four runs prior to running ADNI samples. Tolerance limits were defined at ± two standard deviations (2SD) and ± three standard deviations (3SD) from the mean. QC mean and tolerance limits for YKL-40 were determined by the kit manufacturer. For VILIP-1, SNAP-25 and Ng plates with two or more QC sample values greater than 2SD from the mean were reanalyzed. For YKL-40, plates with two or more internal pooled controls and/or kit-provided controls falling outside the 2SD limit were reanalyzed. In addition, any individual sample with a coefficient of variation (% CV) greater than 25% was reanalyzed. Samples that failed QC were refrozen and stored at -80°C for at least 48 hours before being reanalyzed. When samples were reanalyzed due to QC failure, all within-person longitudinal samples were reanalyzed as well, on the same freeze/thaw cycle. Due to the availability of only a single 500uL aliquot of ADNI CSF, workflows defined that VILIP-1, SNAP-25 and Ng were to be run on the first freeze/thaw cycle, while YKL-40 (and any required reruns for VILIP-1) was run on the second freeze/thaw cycle. Internal QC experiments previously revealed <10% loss of YKL-40 over four freeze/thaw cycles (unpublished observations). Any samples that required repeat SNAP-25 or Ng measurements were performed on the third freeze/thaw cycle samples. Due to protein loss from multiple freeze/thaw cycles, SNAP-25 reruns did not pass QC; therefore, any samples that failed QC from the first freeze/thaw were removed from the SNAP-25 dataset. In total, 21 of 587 (3.4%) samples failed initial QC for VILIP-1, 1 of 587 (0.2%) for SNAP-25, 126 of 587 (21.5%) for Ng, and 73 of 587 (12.4%) for YKL-40.

A.1.3 Statistical results adjusting for sex, *APOE* ε4 status, education and baseline age

Elecsys® tTau

When adjusting for sex, females had significantly higher levels of tTau at baseline (p=0.008), but such adjustment had no effect on longitudinal change. Adjusting for sex resulted in the loss of significant increase in the CN groups and a gain of significance of a longitudinal decrease in the AD+ group (p=0.02). Between-group comparisons were identical to the unadjusted model at baseline and longitudinally. Adjusting for education had no significant effects on baseline or longitudinal tTau or between-group comparisons, but the between group differences no longer reached statistical significance, likely due to inadequate statistical power. Adjusting for baseline age significantly affected the MCI+ group at baseline, lower in older individuals (p=0.04), but had no effect on longitudinal or between-group comparisons. The MCI+ group remained significantly higher at baseline than the CN- group (p=0.04). Adjusting for total ventricular volume significantly affected tTau in the MCI+ group at baseline (p=0.007), but did not influence longitudinal change or between-group comparisons. Adjusting for *APOE* ε4 status had no effect on baseline or longitudinal tTau patterns.

Elecsys® pTau

Adjusting for education and baseline age had no significant effect on baseline or longitudinal pTau values or between-group comparisons. However, the between-group differences seen in the unadjusted model no longer achieved statistical significance, nor did the longitudinal changes in the CN+ and AD+ groups, likely due to inadequate statistical power. Adjusting for sex showed that females had significantly elevated baseline pTau (p=0.01), but it did not change any longitudinal or between-group comparison findings compared to the unadjusted model. Adjusting for *APOE* ε4 status negated the significantly elevated baseline in the CN+ compared to the CN- group (p=0.07), but otherwise the results were the same as in the unadjusted model. Adjusting for total ventricular volume significantly affected pTau in the MCI+ group at baseline (p<0.001), but did not influence longitudinal change or between-group comparisons.

VILIP-1

Adjusting for education had no significant effect on baseline or longitudinal VILIP-1 or between-group differences, but the between-group differences observed in the unadjusted model no longer achieved statistical significance, likely due to inadequate statistical power. Adjusting for baseline age had a significant effect in the MCI+ group at baseline (higher levels with older age, $p<0.01$) but did not change the group differences between MCI+ and the MCI- and CN- groups. Adjusting for *APOE* ε4 status or sex had a significant effect on baseline VILIP-1 (ε4+ and female individuals were higher at baseline (both $p<0.03$)). The significant between-group comparisons at baseline were lost in the ε4 model. In the sex model, all aspects remained identical to the unadjusted model except that the MCI+ was now significantly higher than the CN+ group at baseline ($p=0.03$). Adjusting for total ventricular volume had a significant effect on baseline in the MCI+ group ($p<0.0001$), but not longitudinally. Between-group comparisons at baseline were lost in the AD+ compared with both CN- and MCI- groups as well as longitudinal comparisons in the AD+ compared with the CN-, CN+, and MCI- groups.

SNAP-25

Adjusting for *APOE* ε4 status had a significant effect on baseline SNAP-25 (higher in ε4+ individuals, $p<0.0001$) In addition, baseline differences between the MCI+ and the CN groups is lost, as is the significant longitudinal decrease in the AD+ group. Adjusting for baseline age and education had no significant effect on baseline or longitudinal SNAP-25 or between-group comparisons, but the between-group differences seen in the unadjusted model no longer reached significance, likely due to inadequate statistical power. Adjusting for sex had no effect on baseline or longitudinal SNAP-25 patterns. Adjusting for total ventricular volume significantly affected baseline SNAP-25 ($p=0.02$), but not longitudinal patterns. The baseline difference between the AD+ and CN+ groups was lost.

Ng

Adjusting for *APOE* ε4 status had no effect on baseline or longitudinal Ng; however, the MCI+ group at baseline was no longer higher than the MCI- and CN- groups (p=0.1 and p=0.057). Adjusting for sex had a significant effect on baseline Ng (higher in females, p=0.03), but did not have a significant effect on longitudinal Ng. Between group comparisons were identical to the unadjusted model except the MCI+ group now also showed significant decreases over time (p=0.04). Adjusting for baseline age and education had no significant effect on baseline or longitudinal Ng or between-group comparisons, but the between-group differences observed in the unadjusted model no longer reached significance, likely due to inadequate statistical power. Adjusting for total ventricular volume significantly affected baseline Ng (<0.0001), but not longitudinal patterns. The baseline difference between the AD+ and CN+ groups was lost as were longitudinal differences between the AD+ and CN-, CN+, and MCI- groups.

YKL-40

Adjusting for sex impacted many of the baseline and longitudinal patterns of YKL-40 among the groups. In general females showed higher levels of baseline YKL-40 compared to males (p=0.003). Baseline levels of YKL-40 were still significantly higher in the AD+ compared to the MCI- group (p=0.04), but was now also higher in the AD+ compared to the CN- group (p=0.03), as well as the MCI+ compared to the MCI- (p=0.005) and both CN groups (p=0.03 for CN+, p=0.001 for CN-). Longitudinally, YKL-40 levels no longer increased significantly in the MCI+ group, but instead now decreased in the AD+ group (p=0.003). Adjusting for baseline age and education had minimal effect on baseline or longitudinal patterns of YKL-40, but the between-group differences seen in the unadjusted model no longer reached significance, potentially due to high variability and inadequate statistical power. Adjusting for *APOE* ε4 status had no significant effect on baseline or longitudinal YKL-40, but the difference at baseline between the AD+ and

MCI- groups was no longer significant (p=0.08). Adjusting for total ventricular volume had a significant effect on longitudinal YKL-40 (p<0.001), but not on baseline levels, and the difference at baseline between the AD+ and MCI- groups was no longer significant (p=0.22).

Elecsys® Aβ42

Adjusting for *APOE* ε4 status affected baseline Aβ42 levels right at the statistical significance level (i.e., ε4+ had lower Aβ42, p=0.05), but did not influence longitudinal change or between-group comparisons. Adjusting for sex only impacted the longitudinal decline in the AD+ group (i.e., losing significance, now p=0.08). Adjusting for baseline age eliminated the statistical significance of decline in both the AD+ and CN- groups. In addition, most between-group comparisons at baseline lost significance, except that the AD+ group was still lower than the CN- group at the statistical significance level (p=0.05). Adjusting for total ventricular volume affected baseline levels and longitudinal change in the MCI- group (p=0.05 and p=0.01, respectively) but not between-group comparisons.

Adjusting for education had a significant effect on baseline Aβ42 in the CN+ group (p=0.006), with more education associated with a higher baseline Aβ42. Longitudinally, more education had a significant impact in the MCI- group which showed significant yearly increases in Aβ42. The significant longitudinal decline in the AD+ and CN- groups seen in the unadjusted model was lost. The significant difference at baseline between the MCI+ and MCI- groups was also lost, but the MCI+ group now had significantly lower Aβ42 than the CN+ group (p=0.03), possibly driven by the significant effect of education on the CN+ group.

MMSE

Adjusting for education had no effect on baseline or longitudinal MMSE, though all between-group comparisons at baseline lost significance. In this model, longitudinal MMSE in the AD+

and MCI+ groups still decreased at a significant rate (both $p<0.04$), but were not significantly different from each other.

Adjusting for baseline age had no effect on baseline MMSE, but did significantly affect longitudinal change in the AD+ group (slowed the decline) ($p=0.05$); however, the AD+ and MCI+ groups still decreased at a significant rate (both $p<0.03$). Adjusting for *APOE* ε4 status and sex had no effect on baseline or longitudinal MMSE or between-group comparisons, and the significant results were identical to those in the unadjusted model.

ADAS11 and ADAS13

Adjusting for education had no effect on baseline or longitudinal ADAS11; however, it did affect the between-group comparisons, with only the difference between the MCI+ and CN+ groups remaining significant ($p=0.05$). In this model, the AD+ group was still significantly increasing longitudinally ($p=0.0035$). Adjusting for baseline age had a significant effect on the slope in the AD+ and MCI+ groups (slowed the rate of increase) (both $p<.02$), but both groups retained the significant longitudinal increases observed in the unadjusted model (both $p<0.003$). The between group effects seen at baseline in the unadjusted model were absent, though the AD+ group still increased longitudinally at a faster rate than the MCI+ group ($p=0.05$). Adjusting for *APOE* ε4 status and sex had no effect on baseline or longitudinal ADAS11 and yielded results identical to the unadjusted model.

Adjusting for education had no effect on baseline or longitudinal ADAS13, but affected between-group comparisons similarly to ADAS11, with the MCI+ group being elevated compared to both CN groups (both $p<0.04$). In this model, the AD+ group was not significantly increasing longitudinally ($p=0.07$). Adjusting for baseline age showed similar results to those seen in ADAS11; however, the AD+ group was no longer increasing at a faster rate than the

MCI+ group (now p=0.13). Adjusting for *APOE* ε4 status did not affect baseline or longitudinal ADAS13, except that no significant difference was observed between the MCI+ and MCI- groups (p=0.09). Adjusting for sex significantly affected the longitudinal change in ADAS13, with females showing a faster increase (decline in performance)(p=0.03). Between-group comparisons at baseline remained identical to the unadjusted model. The longitudinal increase in the CN+ group was no longer significant (p=0.06), but between-group differences remained identical to the unadjusted model.

HP Volume

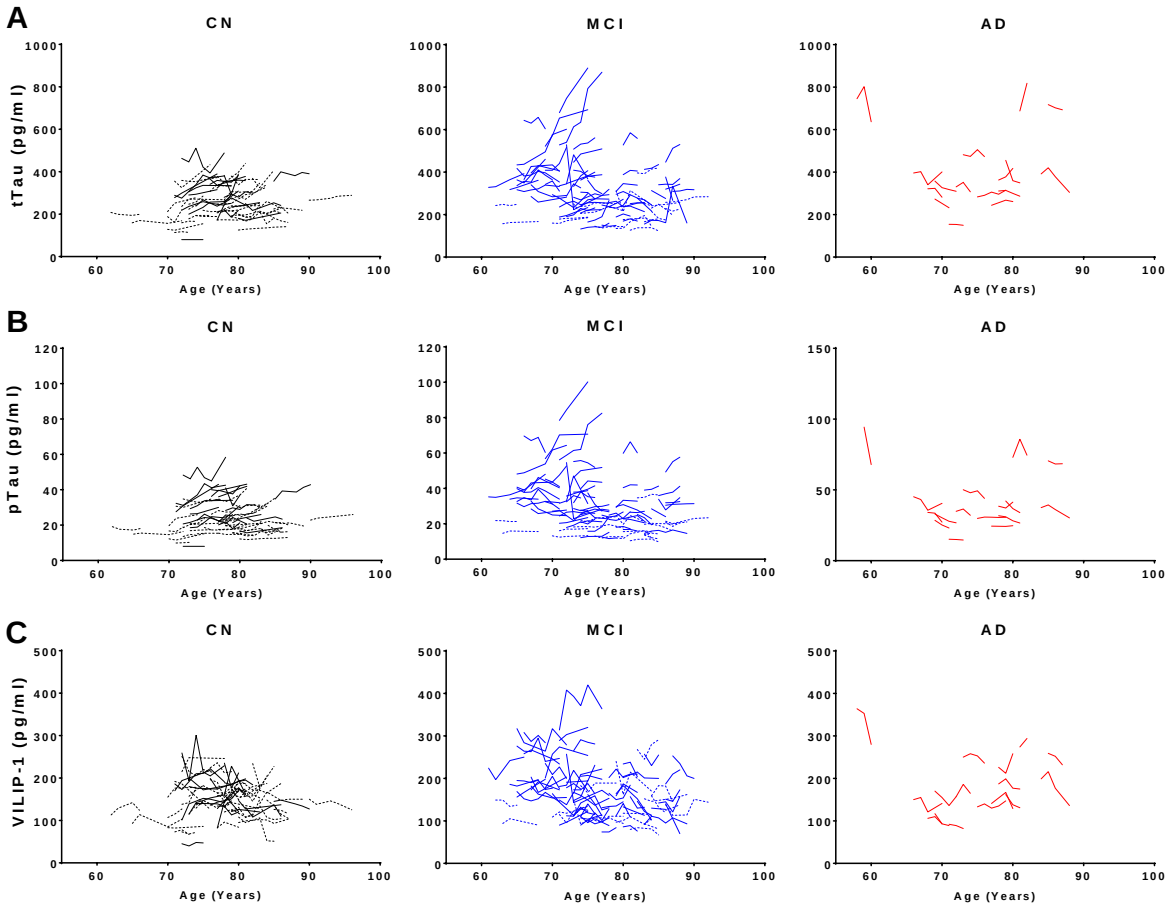
Adjusting for baseline age had no significant effect on baseline or longitudinal HP volume or between-group comparisons, but the slopes and between-group differences observed in the unadjusted model no longer reached significance, likely due to inadequate statistical power. The model adjusted for sex showed a significant effect on baseline HP volume, with females having a smaller volume (p=0.006). The significant differences at baseline between the AD+ and both MCI groups were lost (p=0.08 for MCI-, p=0.06 for MCI+), and longitudinally the AD+ group was no longer decreasing at a faster rate than the MCI- group (p=0.06). Adjusting for *APOE* ε4 status had no effect on baseline or longitudinal HP volume, but the longitudinal between-group differences were lost for the AD+ and MCI- groups, the CN+ and CN- groups, and the MCI+ and MCI- groups (all p=0.2).

EC Thickness

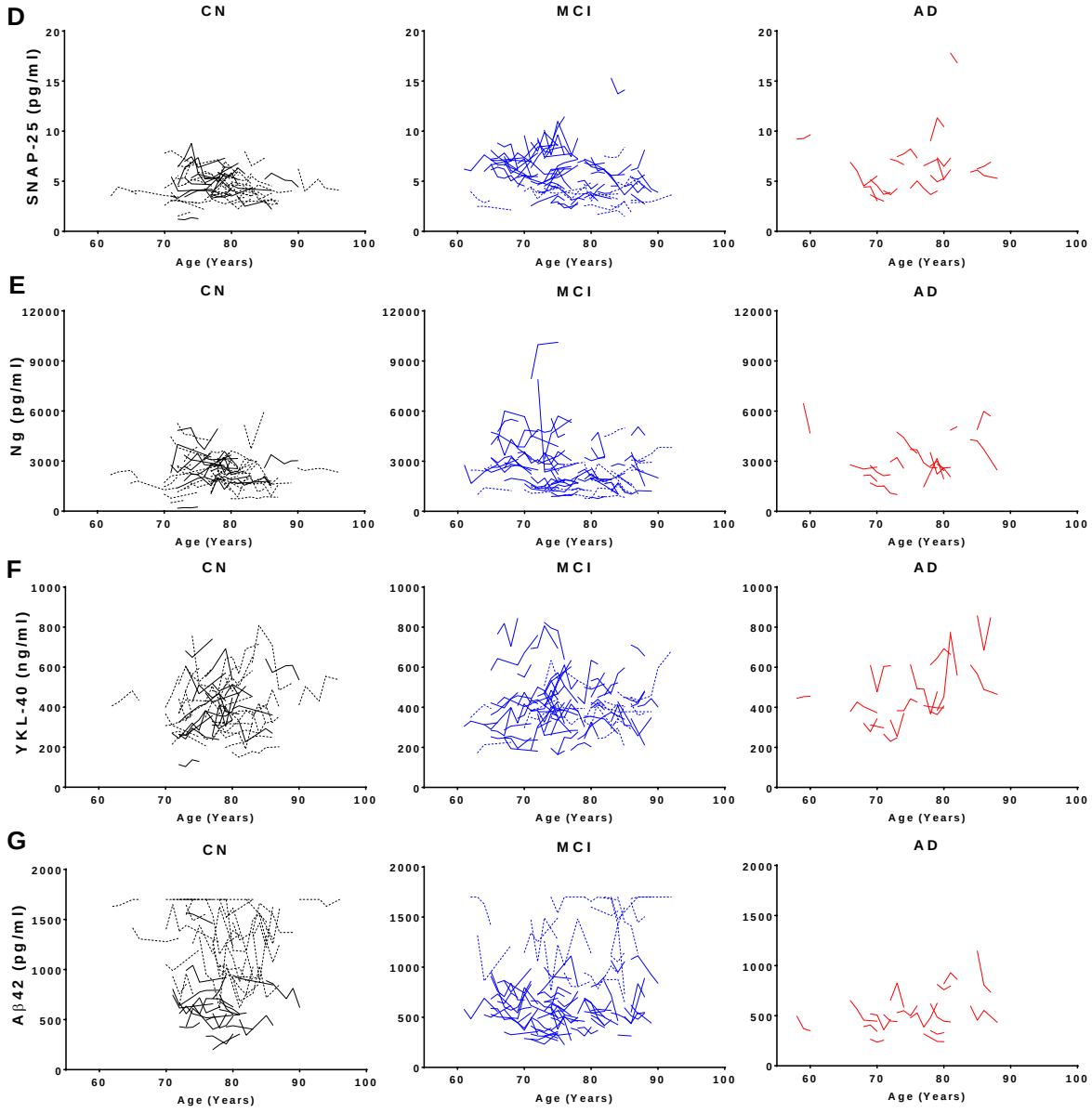
Adjusting for baseline age affected EC thickness the same way it did HP volume. Adjusting for sex had no effect on baseline or longitudinal EC thickness, and between-group comparisons remained identical to the unadjusted model. Adjusting for *APOE* ε4 status had a significant effect on longitudinal change, with ε4+ individuals thinning more rapidly (p=0.03), and the CN- group now also significantly declining (p=0.005). Longitudinally the AD+ group was thinning

significantly faster than the CN- group (p=0.0007), but the MCI- versus CN+ between-group difference was no longer significant when adjusting for *APOE* ε4 status, nor were the longitudinal between-group differences for the AD+ and MCI- groups or the MCI+ and MCI- groups.

Figure A.1 Spaghetti plots of longitudinal change in CSF biomarkers.

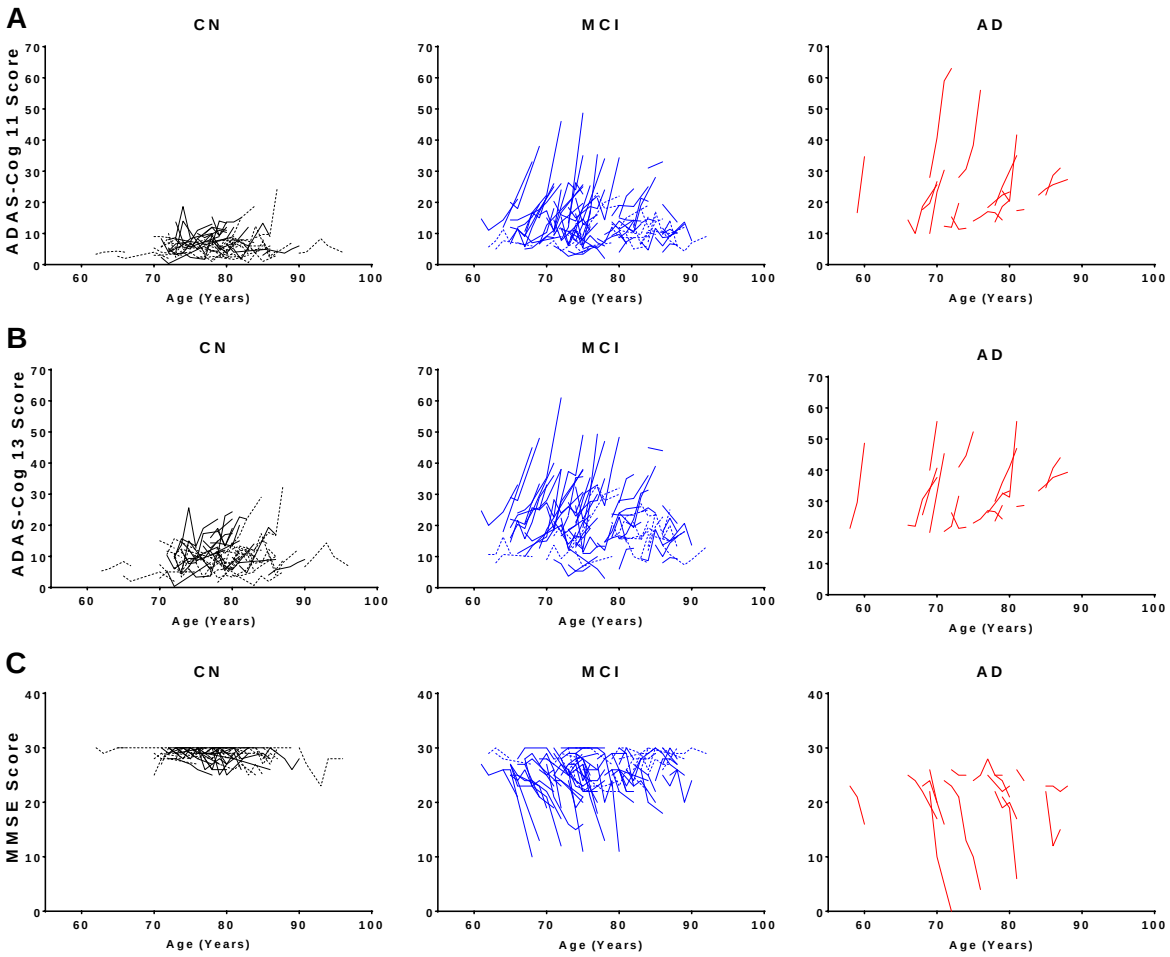


Supplemental Figure 1 (continued)



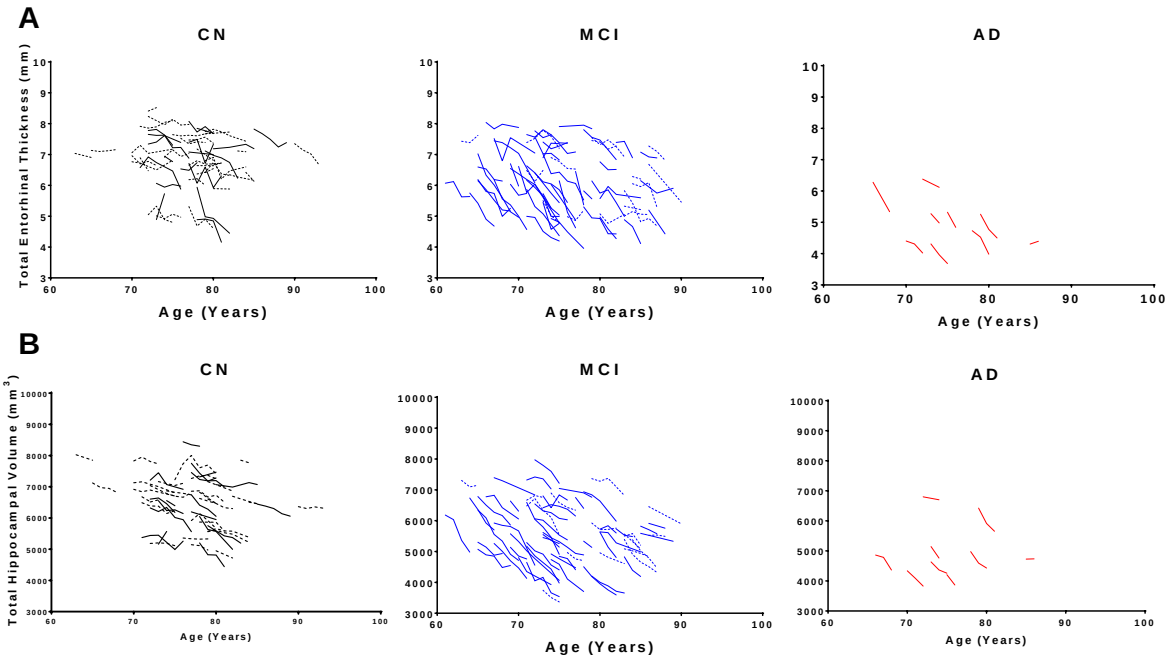
Within-person longitudinal changes for tTau (**A**), pTau (**B**), VILIP-1 (**C**), SNAP-25 (**D**), Ng (**E**), YKL-40 (**F**), and Aβ42 (**G**). Dashed lines indicate Aβ42-negative (Aβ-) individuals. Solid lines indicate Aβ42-positive (Aβ+) individuals. Each biomarker is shown according to diagnostic group: left column, cognitively normal at baseline (n=56); middle column, MCI at baseline (n=79); right column, AD at baseline (n=17).

Figure A.2 Spaghetti plots of longitudinal change in cognition



Within-person longitudinal changes in performance on ADAS-Cog 11 (**A**), ADAS-Cog 13 (**B**), and MMSE (**C**). Dashed lines indicate Aβ42-negative (Aβ-) individuals. Solid lines indicate Aβ42-positive (Aβ+) individuals. Each biomarker is shown according to diagnostic group: left column, cognitively normal at baseline (n=56); middle column, MCI at baseline (n=79); right column, AD at baseline (n=17). MMSE, 30 is the best possible score. ADAS-Cog 11/13, lower score is better performance, with 70 as the worst possible score.

Figure A.3 Spaghetti plots of longitudinal change in MRI measures.



Within-person longitudinal changes for total Entorhinal Cortical Thickness (**A**) and total Hippocampal Volume (**B**). Dashed lines indicate Aβ42-negative (Aβ-) individuals. Solid lines indicate Aβ42-positive (Aβ+) individuals. Each biomarker is shown according to diagnostic group: left column, cognitively normal at baseline (n=56); middle column, MCI at baseline (n=79); right column, AD at baseline (n=17).

Table A.1 Spearman R Correlation Matrix for CSF Biomarkers

	Aβ42	VILIP-1	SNAP-25	Ng	YKL-40	E-Aβ42	E-tTau
Aβ42							
VILIP-1	**-0.235**						
SNAP-25	**-0.413**	**0.72**					
Ng	**-0.22**	**0.849**	**0.619**				
YKL-40	-0.121[a]	**0.311**	**0.307**	**0.422**			
E-Aβ42	**0.868**	-0.131[a]	**-0.240**	-0.154[a]	0.007[a]		
E-tTau	**-0.416**	**0.798**	**0.688**	**0.853**	**0.389**	**-0.214**	
E-pTau	**-0.512**	**0.744**	**0.668**	**0.803**	**0.346**	**-0.324**	**0.975**

Bold, significant correlations (at least p<0.008)
[a]not significantly correlated

Abbreviations: Aβ42, AlzBio3 Aβ42; VILIP-1, visinin-like protein 1; SNAP-25, synaptosomal associated protein-25;Ng, neurogranin; E-Aβ42, Elecsys Aβ42; E-tTau, Elecsys tTau; E-pTau, Elecsys pTau181

A.2 Disclosures

A.2.1 ACS Data

This work was supported by grants P01AG026276, 5P30 NS048056, and 5P30 NS048056 from the National Institutes of Health, the Barnes-Jewish Hospital Foundation, the Fred Simmons and Olga Mohan fund, and a grant from Eli Lilly and Co.

A.2.2 ADNI Data

Data used in preparation of Chapters 3-6 this work were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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