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VARIANCE OF THE AMYLOID BETA PEPTIDE AS A METRIC FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE

Christina Beckett
University of Kentucky, tinalbeckett@hotmail.com
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Christina Beckett, Student

Dr. M. Paul Murphy, Major Professor

Dr. Joe Springer, Director of Graduate Studies

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science
in the College of Medicine
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By

Christina Lisa Beckett

Lexington, Kentucky

Director: Dr. M. Paul Murphy, Molecular and Cellular Biochemistry

Lexington, Kentucky

Abstract of Thesis

VARIANCE OF THE AMYLOID BETA PEPTIDE AS A METRIC FOR THE DIAGNOSIS OF ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder associated with aging. AD is by far the best understood and most studied neurodegenerative disease. Substantial advances have been made over the last decade, however it is debatable how much closer we are to a clinically useful therapy. A long standing goal in the AD field has been to improve the accuracy of early detection, with the assumption that the ability to intervene earlier in the disease process will lead to a better clinical outcome. Major facets of this effort have been the continued development and improvement of AD biomarkers, with a strong focus on developing imaging modalities. AD is accompanied by two pathological hallmarks in the brain: extracellular neuritic plaques composed of the beta-amyloid peptide (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. Evidence of Aβ as the driving force behind the progression of AD (the amyloid cascade hypothesis) was first published by Hardy & Higgins in 1992, and this peptide has been the focus of therapeutic and diagnostic testing for decades. Significant technological advances in recent years now allow imaging of amyloid pathology *in vivo*. These methods evaluate Aβ burden in a living person, and could potentially serve as both a biomarker, and as a diagnostic tool to detect disease. Pittsburgh Compound B (PiB) is currently the best studied of these imaging agents, however, our current knowledge of the quantitative relationship between PiB binding and amyloid pathology in the brain is limited. A better understanding of how these variables relate to one another is essential for the continued development of reliable diagnostic biomarkers for AD. We analyzed increasingly insoluble pools of Aβ to quantify their relative contributions to the overall Aβ burden, and to determine if any of these measures could be used to predict disease status. We found that the amount of PiB binding in a cortical region of the brain could distinguish cases of mild cognitive impairment (MCI) when corrected to the amount of PiB binding in the cerebellum. As the Aβ peptide ages, the amino acid aspartate may spontaneously convert to an isoaspartate residue through a succinimide intermediary. The presence of iso-Asp Aβ has been used to indicate the presence of aged plaques in AD and Down syndrome cases. We sought to investigate the potential relationship between levels of 'aged' Aβ in the plasma as indicated by iso-Asp Aβ and disease state, as a potential biomarker for the presence of AD pathology. We found that AD cases had lower levels of all forms of Aβ in plasma when standardized to the group average, and that plasma levels of Aβ and iso-Asp Aβ were reversed between disease groups. A follow up study is required, however, these initial data are a promising step towards utilizing aged iso-Asp Aβ plasma levels as a potential biomarker to indicate disease state.

KEYWORDS: Alzheimer's Disease, Aβ, PiB, Isoaspartate, Biomarker

Christina Lisa Beckett
Student Signature

April 27th 2016
Date

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By

Christina Lisa Beckett

Dr. M. Paul Murphy
Director of Thesis

Dr. Joe Springer
Director of Graduate Studies

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Date

To my fellow disasters....

...you know who you are.

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CHAPTER 1: INTRODUCTION

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder associated with aging. Worldwide, the current number of individuals with AD is about 24 million, but could reach more than 80 million by the year 2040 (Ferri et al., 2005). AD is by far the best understood and most studied neurodegenerative disease. Although substantial advances have been made over the last decade, it is debatable how much closer we are to a clinically useful therapy. A long standing goal in the AD field has been to improve the accuracy of early detection, with the assumption that the ability to intervene earlier in the disease process will lead to a better clinical outcome. Major facets of this effort have been the continued development and improvement of AD biomarkers (Petersen et al., 2010; Trojanowski et al., 2010), with a strong focus on developing imaging modalities (Klunk, 2011).

First described by Dr. Alois Alzheimer over one hundred years ago, AD is characterized by progressive cognitive and behavioural decline and dementia (Cipriani, Dolciotti, Picchi, & Bonuccelli, 2011). AD is accompanied by two pathological hallmarks in the brain: extracellular neuritic plaques composed of the beta-amyloid peptide (Aβ) and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. Evidence of Aβ as the driving force behind the progression of AD (the amyloid cascade hypothesis) was first published by Hardy & Higgins (Hardy & Higgins, 1992), and this peptide has been the focus of therapeutic and diagnostic testing for decades.

Aβ is a product of proteolytic processing of the amyloid precursor protein (APP). APP is a single transmembrane domain protein which is expressed ubiquitously throughout the body. There are multiple pathways for the processing of APP – not all of which produce Aβ. Approximately ~90% of APP is processed through the non-amyloidogenic pathway, in which the

initial cleavage event is performed by α-secretase *within* the Aβ region, in the portion of APP located outside the membrane. This releases a secreted APP fragment (sAPP-α), and leaves behind a C-terminal fragment within the membrane (CTF-α). The second cleavage event is performed by γ-secretase within the membrane-bound region, which releases a p3 fragment and the APP intracellular domain (AICD) which may play a role in transcriptional regulation (O'Brien & Wong, 2011). The remaining ~10% of APP processing occurs through the amyloidogenic pathway. This processing event is similar to the non-amyloidogenic pathway except that the initial cleavage event is performed by β-secretase. Cleavage by β-secretase (the active component of which is BACE1) occurs outside the membrane and releases a secreted APP fragment (sAPP-β), leaving behind the membrane-bound fragment (CTF-β). The second cleavage event is also performed by γ-secretase, this time releasing the Aβ peptide and the AICD. Depending on the site at which γ-secretase performs the cleavage, there is variation in the length of Aβ peptide produced. The majority (~90%) of γ-secretase cleavage produces the more soluble, 40-amino acid long Aβ peptide (Aβ40). Approximately 5-10% of γ-secretase cleavage produces an Aβ peptide which is 42 amino acids in length (Aβ42). The remaining cleavage events produce Aβ of varying sizes, the next most common being the 38-amino acid long Aβ38 (~1% of Aβ produced). The Aβ42 peptide is more aggregate-prone, and is the major Aβ peptide species present in the neuritic plaques found in Alzheimer's disease brain (Ahmed et al., 2010).

Significant technological advances in recent years now allow imaging of amyloid pathology *in vivo*. These methods evaluate Aβ burden in a living person, and could potentially serve as both a biomarker, and as a diagnostic tool to detect incipient disease (Jagust et al., 2009). Pittsburgh Compound B (PiB, 2-[4′ -(Methylamino)phenyl]-6- hydroxybenzothiazole), a derivative of the amyloid dye Thioflavin T, (Wang et al., 2004) is currently the best studied of

these imaging agents. After labeling with ^{11}C for PET imaging, increased PiB retention can be quantified in brain regions known to accumulate Aβ deposits in AD patients (Klunk et al., 2004). The utility of PiB and other probes for determining how mild cognitive impairment (MCI) progresses to AD is being evaluated in a large, multicenter effort (Apostolova et al., 2010; Grimmer et al., 2009; Klunk et al., 2004). Despite progress in understanding the contribution of Aβ to neuronal dysfunction and neurodegeneration, the lack of a detailed analysis of the interrelationship between Aβ and the other common indices of AD pathology has hampered our understanding of the development and progression of the disease. For instance, our current knowledge of the quantitative relationship between PiB binding and amyloid pathology in the brain is limited (Bacskai et al., 2007; Ikonomovic et al., 2008; Klunk et al., 2005; Klunk et al., 2003; Leinonen et al., 2008). A better understanding of how these variables relate to one another is essential for the continued development of reliable diagnostic biomarkers for AD. We analyzed increasingly insoluble pools of Aβ to quantify their relative contributions to the overall Aβ burden, and to determine if any of these measures could be used to predict disease status.

Alzheimer's disease is a progressive neurodegenerative disease, proceeding through several stages. Although the exact staging of the disease is still debated, there is almost certainly a preclinical or prodromal phase of the disease where some AD pathology is present in the absence of readily apparent clinical impairment (PCAD) (Price et al., 2009). After this stage, patients will pass through a state of mild cognitive impairment (MCI) (Petersen, 2004; Petersen et al., 2001) before progressing to early stage AD, defined by the presence of specific clinical features, including functional memory impairment. About twenty percent of otherwise cognitively normal elderly show PiB retention in one or more neocortical regions (Aizenstein et al., 2008), and this is associated with a risk of later cognitive decline, indicating the presence of a

possible stage of preclinical AD (Morris et al., 2009; Pike et al., 2007). Some of these cases can eventually be classified as amnestic MCI (Villemagne et al., 2008). About sixty percent of MCI patients show elevated PiB binding in some part of the neocortex (Kemppainen et al., 2007; Pike et al., 2007), and higher levels of PiB binding are associated with faster rates of cognitive decline, increased incidence of conversion to AD, and a greater degree of cerebral atrophy (Ewers et al., 2012; Koivunen et al., 2011).

The amino acid aspartate (present at positions 1, 7, and 23 in the human Aβ sequence) readily undergoes isomerization to produce isoaspartate (iso-Asp) under physiological conditions (Ahmed et al., 2010; Shimizu, Matsuoka, & Shirasawa, 2005). The reaction occurs when aspartate spontaneously converts to a succinimide intermediate; the succinimide intermediate is subsequently hydrolyzed to produce iso-Asp (Shimizu, Watanabe, Ogawara, Mori, & Shirasawa, 2000). The isomerization of aspartate is not a permanent modification. Isoaspartate can be converted back to aspartate by the highly conserved enzyme, protein L-isoaspartyl methyltransferase (PIMT), which is responsible for the recognition and repair of isoaspartate residues (Chondrogianni et al., 2014). Iso-Asp residues accumulate in aged proteins *in vitro* under physiologic temperature and pH (Reissner & Aswad, 2003), and its presence in Aβ peptides isolated from AD brains has been documented (Shimizu et al., 2005). The presence of predominantly (55.0%) iso-Asp at position 7 in Aβ (iso-Asp7 Aβ) from neuritic plaques was first reported by Roher (Roher et al., 1993), with the suggestion that these structural alterations in peptide composition could have an influence on Aβ deposition and/or clearance. The presence of aspartate at position 7 in Aβ was subsequently shown to be essential for classical complement pathway (CCP) activation; iso-Asp substitution at this position abolished the activation of the CCP (Velazquez, Cribbs, Poulos, & Tenner, 1997) indicating the significance of

modification to iso-Asp7 Aβ. The presence of iso-Asp7 Aβ has been used to indicate the presence of aged plaques in AD cases, and also in Down syndrome - a disorder which is also associated with the accumulation and deposition of the Aβ peptide within the brain, due to the presence of an extra copy of APP-containing chromosome 21 and resultant higher production of the Aβ peptide (Fonseca, Head, Velazquez, Cotman, & Tenner, 1999). Low levels of Aβ peptide are detectable in human plasma samples, however, although Aβ deposition in brain is a component of AD pathology, the presence of Aβ in plasma is not indicative of the presence or severity of Aβ plaques in the brain. We sought to investigate a potential relationship between levels of 'aged' Aβ in the plasma as indicated by iso-Asp7 Aβ and disease state, as a potential biomarker for the presence of AD pathology.

CHAPTER 2: MATERIALS AND METHODS

Human Subjects and Neuropathological Assessment

Samples were obtained from the tissue repository at the Alzheimer's Disease Center at the University of Kentucky Sanders-Brown Center on Aging (UK SBCoA). Details of the recruitment, inclusion criteria, and mental status test batteries have been described previously (Schmitt et al., 2000). Diagnoses followed the National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease (Hyman et al., 2012). Human tissue collection and handling followed PHS guidelines and the University of Kentucky IRB.

Cases used for set one of the PiB binding measures (Table 2.1) were as follows: control cases (n = 9, 5M / 4F; age, 84.3 ± 5.1 years) had no history of antemortem cognitive impairment (MMSE: 28.4 ± 1.5; last MMSE: 0.7 ± 0.4 years); AD cases (n = 10, 4M / 6F; age, 83.4 ± 5.7 years) showed substantial cognitive impairment (MMSE, 9.9 ± 6.0; last MMSE: 2.4 ± 2.3 years). Prodromal or preclinical AD (PCAD) cases (n = 10, 1M / 9F; age, 85.6 ± 3.7 years) were defined as those that met the NIA-Reagan neuropathology criteria for likely AD, but exhibited no clinical signs of dementia (MMSE, 29.4 ± 0.7; last MMSE: 0.8 ± 0.5 years) (Price et al., 2009). Amnestic MCI cases (n = 7, 3M / 4F; age, 89.0 ± 5.8 years) were defined as per Petersen et. al. (Petersen et al., 1999); MMSE scores (24.8 ± 3.1; last MMSE: 0.6 ± 0.3 years) were significantly lower ($p < 0.04$) in this group compared to the control group. Frontotemporal dementia (FTD) cases (n = 6, 3M / 3F; age, 61.0 ± 14.6 years) were included as an additional neurodegenerative disease and served as a specificity control (Cairns et al., 2007). FTD, which typically occurs at a younger age than AD, results in a significant cognitive impairment (MMSE, 7.8 ± 9.0; $p < 0.001$; last MMSE: 4.6 ± 3.1

TABLE 2.1: Case Set 1, PiB Binding

DISEASE STATE	n	AGE (yrs)	MMSE
CONTROL (CON)	9 (5M/4F)	84.3 ± 5.1	28.4 ± 1.5
PRECLINICAL AD (PCAD)	10 (1M/9F)	85.6 ± 3.7	29.4 ± 0.7
AMNESTIC MILD COGNITIVE IMPAIRMENT (MCI)	7 (3M/4F)	89.0 ± 5.8	24.8 ± 3.1
ALZHEIMER'S DISEASE (AD)	10 (4M/6F)	83.4 ± 5.7	9.9 ± 6.0
FRONTOTEMPORAL DEMENTIA (FTD)	6 (3M/3F)	61.0 ± 14.6	7.8 ± 9.0

Brain regions: superior and middle temporal gyri (SMTG), cerebellum (CB)

years) but does not typically show the same pattern of neuropathology as AD. Aβ deposition is not a feature of FTD (Cairns et al., 2007).

Cases for set two of the PiB binding experiments (Table 2.2) were as follows: control cases (N = 23; 87.0 ± 6.5 years) had no history of antemortem cognitive impairment and were age-matched to AD cases (N = 22; 85.8 ± 7.6 years). The average postmortem interval (PMI) was similar for both groups (Control: 3.0 ± 0.8; AD: 2.9 ± 0.7, hours).

Brain weights were determined and a gross neuropathological evaluation carried out at the time of autopsy. Tissue samples were dissected and frozen or formalin fixed. For histology, paraffin-embedded specimens were cut (8 μm) and stained with standard hematoxylin-eosin, modified Bielschowsky method, or Gallyas silver method. Braak staging (Braak & Braak, 1991) used both Gallyas and Bielschowsky-stained sections. Neurofibrillary tangles (NFTs), diffuse plaques (DPs; plaques without surrounding dystrophic, argyrophilic neurites), and neuritic plaques (NPs; plaques surrounded by dystrophic, argyrophilic neurites) were counted and averaged as described (Markesbery et al., 2006; Nelson et al., 2010; Nelson et al., 2007). There was no cause of death pattern in any disease group.

TABLE 2.2: Case Set 2

DISEASE STATE	n	AGE (yrs)	MMSE
CONTROL (CON)	23 (7M/16F)	87.0 ± 6.5	28.6 ± 1.4
ALZHEIMER'S DISEASE (AD)	22 (8M/14F)	85.8 ± 7.6	12.1 ± 8.1

Brain regions: midfrontal gyri (BA9), superior and middle temporal gyri (SMTG), inferior parietal lobule (IP), hippocampal formation (HIPP), cerebellum (CB)

Aβ Extractions

Aβ was extracted from human brain samples under increasingly stringent conditions as described previously (Beckett et al., 2010). Briefly, frozen brain samples were weighed and homogenized via polytron in ice cold phosphate buffered saline (PBS, pH 7.4) including a complete protease inhibitor cocktail (PIC; Amresco, Solon, OH) at 200 mg/mL. An aliquot of raw homogenate was conserved for PiB binding measures; remaining raw homogenate was then centrifuged (20,800 x *g* for 30 mins @ 4°C), and the supernatant was collected. The pellets were re-extracted by sonication in 2% sodium dodecyl sulphate (SDS) with PIC (10 x 0.5 sec pulses @ 100 W; Fisher Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA), centrifuged (20,800 x *g* for 30 mins @ 14°C), and the supernatant collected. Finally, the remaining pellet was re-extracted in 70% (v/v) formic acid followed by centrifugation (20,800 x *g* for 1 h @ 4°C), and collection of the supernatant. The extracts were stored at -80°C until time of assay.

PiB Binding

^{3}H-PiB binding to brain homogenates was carried out similar to the filtration assay of Klunk et al. (Klunk et al., 2005), as per Rosen et al. (Rosen, Walker, & Levine, 2011). Briefly, unfractionated PBS homogenate was diluted into a 96-well polypropylene plate in triplicate. Two hundred µl of 1nM ^{3}H-PiB (custom synthesized by ViTrax Radiochemicals, Placentia, CA; a kind gift of Dr. Brian Ciliax, Emory University) was added to each of the first two wells, and 1 µM of an unlabeled competitor (BTA-1) was added to the third well to determine nonspecific binding (by subtraction). Femtomoles of ^{3}H-PIB bound were calculated per wet weight of tissue after correcting for counting efficiency.

Aβ ELISAs

Aβ40 and Aβ42 were measured using a well-characterized sandwich ELISA, details of which have already been published (Beckett et al., 2010; Das et al., 2003; McGowan et al., 2005; Murphy et al., 2007; Weidner et al., 2011). Briefly, 384-well plates (Immulon microtiter 4 HBX; Thermo Scientific, Rochester, NY) were coated with a capture antibody at a concentration of 0.5 µg/well, sealed, and incubated overnight @ 4°C. The following day, plates were washed once with PBS and blocked with 100 µL of blocking buffer (Synblock; AbD Serotec, Raleigh, NC) according to manufacturer's directions. Blocked plates were sealed and stored, dessicated, @ 4°C until use. Prior to use, plates were washed twice with PBS and standards and samples were loaded at least in triplicate. A standard curve was prepared using synthetic Aβ peptide (Aβ40 or Aβ42, as appropriate; rPeptide, Bogart, GA) diluted in antigen capture buffer (AC; 0.02 M sodium phosphate buffer (pH = 7), 0.4 M NaCl, 2 mM EDTA, 0.4% Block Ace (Serotec; Raleigh, NC), 0.2% BSA, 0.05% CHAPS, and 0.05% NaN_3). Samples were diluted in AC buffer prior to loading (PBS samples were diluted 1:4, SDS samples were diluted ranging from 1:20 to 1:100). Formic acid samples were neutralized 1:20 in TP buffer (1 M Tris base, 0.5 M Na_2HPO_4), followed by a further dilution in AC buffer for final dilution ranging between 1:100 and 1:400. The capture antibody used for the Aβ40 measures was Ab42.5 (specific for human Aβ1-16), and the capture antibody for the Aβ42 measures was 2.1.3 (c-terminal specific for Aβ42). Detection was performed with biotinylated 13.1.1 (1:1000, c-terminal specific for Aβ40) or biotinylated 4G8 (1:2000, specific for Aβ17-24; BioLegend, San Diego, CA), followed by NeutrAvidin-HRP (1:5000; Thermo Fisher, Waltham, MA). Finally, plates were developed with 3,3',5,5'-tetramethylbenzidine (TMB; Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD), stopped with 6% σ-phosphoric acid, and read at 450 nm.

Oligomeric Aβ was measured using a similar process, using the same antibody for both capture and detection (capture: 4G8 @ 0.1 µg/well, detection: biotinylated-4G8 @ 1:2000; BioLegend) followed by NeutrAvidin-HRP. The plates were developed with TMB (KPL), stopped with 6% σ-phosphoric acid, and read at 450 nm.

Iso-Asp7 Antibody Production and Screening

Monoclonal antibodies directed against iso-Asp7 Aβ were produced by abpro (Woburn, MA), and culture media from selected hybridomas were sent to our lab at UK for screening. Initial clones were screened for specificity via direct ELISA against iso-Asp7 Aβ1-16 and non-iso-Asp Aβ1-16. Immulon 4 HBX plates were coated with synthetic peptide (iso-Asp7 Aβ1-16 or non-iso-Asp Aβ1-16) @ 0.1 µg/well and incubated overnight @ 4°C. The following day, plates were washed once with PBS and blocked with Synblock (100 µL/well) for 1 hour @ room temperature. Next, the Synblock was removed and the plates were allowed to dry upside down for 1 hour at room temperature. The plates were then washed twice with PBS and the clonal supernatants were diluted into detection buffer (DB; 0.2 M sodium phosphate buffer (pH = 7), 2 mM EDTA, 0.4 M NaCl, 1% BSA, 0.002% thimerosal) to create a dilution series (no dilution, 1:10, 1:100, and 1:1000) and loaded at 100 µL/well. The plates were sealed and incubated at room temperature for 1 hour. The plates were then washed twice with PBST (PBS with 0.5% Tween-20) and twice with PBS. The plates were then incubated with goat-α-mouse-IgG-HRP (1:20,000 in DB; Pierce, Rockford, IL) for 1 hour at room temperature. Finally, the plates were washed four times with PBST and four times with PBS. They were then developed with TMB (KPL), stopped with 6% σ-phosphoric acid, and read at 450 nm. The ODs from the iso-Asp7 Aβ and the non-iso-Asp Aβ conditions were compared to each other. The clones which were reactive to iso-Asp7 Aβ and *not*

non-iso-Asp Aβ were selected for further subcloning. A total of five clones were selected for subcloning.

The supernatants from the subclones were screened following the procedure as described above. Two of the subclones (3A9.H7 and 7C2.E12) which showed high specificity for iso-Asp7 Aβ and non-reactivity to non-iso-Asp Aβ were chosen to be scaled up for antibody production and purification. Antibody 7C2.E12 was chosen for use in the screening of human samples for iso-Asp7 Aβ because it had a slightly better specificity profile than 3A9.H7.

Iso-Asp7 Aβ in Human Brain Samples

Serially extracted human brain samples from the inferior temporal region were screened for iso-Asp7 Aβ reactivity via sandwich ELISA. Antibody 2.1.3 (c-terminal specific for Aβ42) was used as a capture antibody (0.5 µg/well). PBS, SDS, and neutralized formic acid samples were loaded at appropriate dilutions in AC buffer (control samples: PBS @ 1:4, SDS @ 1:20, FA @ 1:100; AD samples: PBS @ 1:4, SDS @ 1:100, FA @ 1:400). Detection was performed using biotinylated antibodies 3A9.H7, 7C2.E12, or 6E10 (1:1000 in DB; 6E10 against human Aβ1-16; BioLegend), followed by NeutrAvidin-HRP @ 1:5000. The plates were then developed with TMB (KPL), stopped with 6% σ-phosphoric acid, and read at 450 nm.

Iso-Asp7 Aβ in Human Plasma Samples

Plasma from AD and control cases were received from the UK SBCoA Alzheimer's Disease Center (Table 2.3). Controls (N=10 4F/6M; 82.9 ± 2.6 years) had no history of ante-mortem cognitive impairment and were age-matched to AD cases (N=10, 5F/5M; 85.6 ± 1.7 years). The average postmortem interval (PMI) was similar for both groups (Control: 4.7 ± 1.0; AD: 6.9 ± 1.7, hours). Aβ40, Aβ42, iso-Asp7 Aβ40, and iso-Asp7 Aβ42 levels in plasma from AD

TABLE 2.3: Case Set for Plasma Iso-Asp7 Aβ Measures

DISEASE STATE	n	AGE (yrs)	MMSE
CONTROL (CON)	10 (6M/4F)	82.9 ± 2.6	27.7 ± 0.8
ALZHEIMER'S DISEASE	10 (5M/5F)	85.6 ± 1.7	15.9 ± 3.1

**Note: In this case set, the average MMSE score was calculated from only 9 of the 10 AD cases, as the MMSE test was not able to be completed for one of the participants due to behavioral/cognitive problems.*

cases and controls were measured via sandwich ELISA. Both Aβ40 and iso-Asp7 Aβ40 were captured with antibody 13.1.1 (0.5 μg/well, c-terminal specific for Aβ40); Aβ42 and iso-Asp7 Aβ42 were measured with capture antibody 2.1.3 (0.5 μg/well, c-terminal specific for Aβ42). Non-isomerized Aβ (both Aβ40 & Aβ42) was detected with biotinylated-6E10 (1:1000, specific for human Aβ1-16), and iso-Asp7 Aβ (40 & 42) was detected with biotinylated-7C2.E12 (1:1000, specific for iso-Asp7 Aβ1-16) followed by NeutrAvidin-HRP (1:5000). Plates were then developed with TMB (KPL), stopped with 6% σ-phosphoric acid, and read at 450 nm. A standard curve was prepared using synthetic non-isomerized Aβ (either Aβ40 or Aβ42, as appropriate) which was used to predict the values for the non-isomerized Aβ conditions. For the iso-Asp7 Aβ conditions, dilutions of synthetic iso-Asp7 Aβ1-16 were coated directly onto the plate, and were detected with biotinylated 7C2.E12.

CHAPTER 3: RESULTS

For case set one, quantitative measurements of Aβ by ELISA were performed from both SMTG and cerebellum (Figure 3.1). The total amount of Aβ found in the disease cases was increased in both the SMTG (SDS: $F[4,32] = 63.01$, $p<0.0001$; FA: $F[4,32] = 7.00$, $p<0.001$) and cerebellum (SDS: $F[4,31] = 11.49$, $p<0.001$; FA: $F[4,31] = 4.5$, $p<0.006$). There were no disease-related differences between the amounts of aqueous (PBS) soluble Aβ in either region (SMTG: $F[4,32] = 1.39$, n.s.; cerebellum: $F[4,31] = 2.07$, n.s.). The disease-related increase in the SMTG relative to the cerebellum was larger in the SDS fraction ($F[4,29] = 32.37$, $p<0.0001$) but not in the FA fraction ($F[4,29] = 0.97$, n.s.). The largest values were from the AD cases, with smaller increases observed in the PCAD and MCI cases. The PCAD and MCI groups were not significantly different from each other. There was ~8 times more SDS-soluble Aβ in the SMTG compared to the cerebellum in the AD group as compared to the controls ($t[9] = 6.77$, $p<0.01$), whereas there was no difference between the amounts of FA-soluble Aβ in the same cases ($t[9] = -0.63$, n.s.). Effects were similar for both Aβ40 and Aβ42 (Table 3.1). There was significantly more oligomeric Aβ in the SMTG ($F[4,32] = 5.00$, $p<0.003$), but not in the cerebellum ($F[4,31] = 1.15$, n.s.), in disease. When compared to the cerebellum, oligomeric Aβ was higher in the SMTG in the AD ($t[9] = 7.09$, $p<0.01$), PCAD ($t[9] = 3.32$, $p<0.04$), and MCI ($t[5] = 4.03$, $p<0.03$) cases. PiB binding was similarly elevated in the SMTG ($F[4,32] = 7.08$, $p<0.001$). PiB binding was minimal in the cerebellum, and did not show a strong disease-related increase ($F[4,31] = 2.2$, $p<0.1$) in this region. There were no significant differences between the FTD cases and controls.

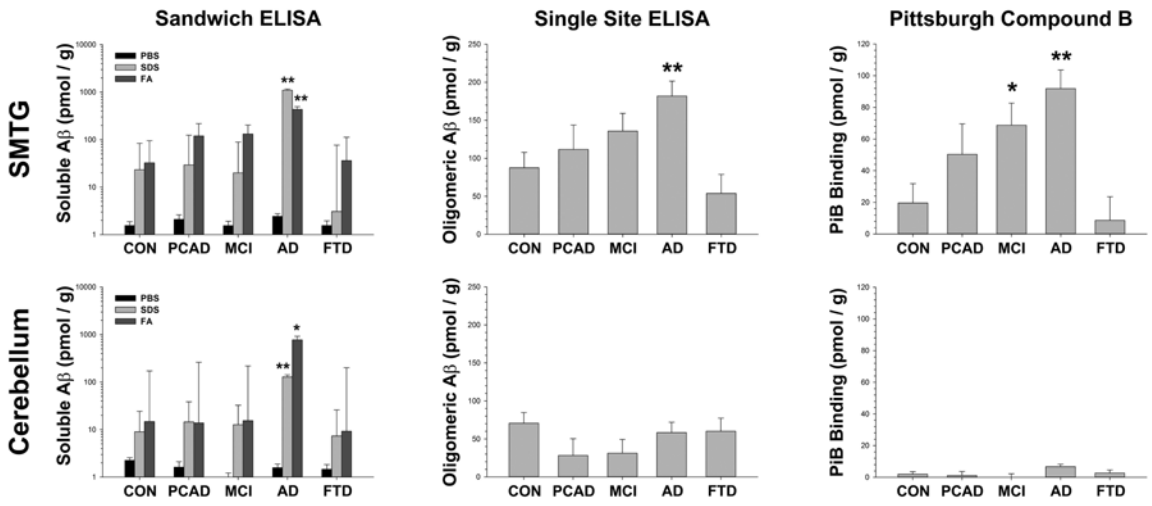

Figure 3.1 Soluble Aβ, Oligomeric Aβ, and Fibrillar Aβ.

Sandwich ELISA (soluble Aβ): The total amount of Aβ was increased in AD in both the SMTG and cerebellum in the SDS and FA fractions; PBS-soluble Aβ was not elevated in disease. Increases in PCAD and MCI cases were modest relative to controls, and were most apparent in the FA fraction isolated from the SMTG. FTD cases were essentially indistinguishable from control cases. Data shown are corrected for age and PMI. Similar results were obtained in separate evaluations of Aβ40 and Aβ42 (Table 3.1). Single-Site ELISA (oligomeric Aβ): Oligomeric Aβ was only significantly higher in the SMTG and not within the cerebellum. Values for PCAD and MCI cases were intermediate between control and AD cases; FTD cases were slightly lower than controls, although this was not a significant difference. PiB Binding (fibrillary Aβ): Fibrillar Aβ, as defined by PiB binding, was significantly higher in the SMTG than in the cerebellum. Values for PCAD and MCI cases were intermediate between control and AD cases; FTD cases were not significantly different from control cases and, similar to oligomeric Aβ, were slightly lower. Dunnett's test, * = $p < 0.05$, ** = $p < 0.01$. *Reprinted from (Beckett et al., 2012) with permission from IOS Press.*

Although we could detect clear differences in postmortem PiB binding between the SMTG and cerebellum, they were significant in the AD group ($t[9] = 6.61$, $p<0.01$), but only marginally significant for the PCAD ($t[9] = 2.60$, unadjusted $p<0.03$) and MCI ($t[5] = 2.84$, unadjusted $p<0.04$) groups. For amyloid imaging *in vivo*, values for cortical PiB retention are typically standardized to values obtained from the cerebellum in the same patient [16]. We therefore performed a similar comparison in our case series, by standardizing the SMTG value to the cerebellum from the same case (Figure 3.2). Since the oligomeric Aβ measures exhibited a pattern comparable to PiB binding, we did the same analysis in these cases. There were no differences between disease states when the amount of oligomeric Aβ in the SMTG was standardized in this manner ($F[4,31] = 0.86$, n.s.). The postmortem PiB binding ratio was significantly different in disease when the SMTG values were corrected to the cerebellum ($F[4,31] = 5.70$, $p<0.001$). When the data were analyzed in this way, the MCI group was notably higher than the other groups, an effect attributable to the very low levels of cerebellar PiB binding in this subgroup.

TABLE 3.1: $A\beta_{40}$ and $A\beta_{42}$ Values (Uncorrected)

SMTG

	PBS		SDS		FA	
	$A\beta_{40}$	**$A\beta_{42}$**	**$A\beta_{40}$**	**$A\beta_{42}$**	**$A\beta_{40}$**	**$A\beta_{42}$**
CON	0.3 ± 0.2	1.3 ± 0.3	4.9 ± 3.1	17.6 ± 9.1	10.1 ± 1.6	24.0 ± 19.2
PCAD	0.3 ± 0.1	1.6 ± 0.2	2.6 ± 0.9	19.8 ± 6.3	13.0 ± 1.3	44.0 ± 20.6
MCI	0.4 ± 0.1	1.2 ± 0.5	3.1 ± 0.6	16.0 ± 6.1	27.3 ± 4.1	116 ± 82.4
AD	0.6 ± 0.3	2.0 ± 0.3	111 ± 52.5*	1032 ± 131*	310 ± 108.3*	98.7 ± 16.5
FTD	0.3 ± 0.1	1.3 ± 0.6	3.1 ± 0.7	0.0 ± 0.0	18.0 ± 1.5	18.3 ± 19.2

Cerebellum

	PBS		SDS		FA	
	$A\beta_{40}$	**$A\beta_{42}$**	**$A\beta_{40}$**	**$A\beta_{42}$**	**$A\beta_{40}$**	**$A\beta_{42}$**
CON	0.2 ± 0.0	2.1 ± 0.2	7.9 ± 1.6	0.7 ± 0.7	12.2 ± 1.3	2.5 ± 2.5
PCAD	0.2 ± 0.1	1.6 ± 0.3	11.4 ± 0.9	0.2 ± 0.2	13.4 ± 1.2	2.2 ± 1.5
MCI	0.0 ± 0.0	0.8 ± 0.3	11.1 ± 0.9	1.2 ± 1.0	14.8 ± 3.0	0.0 ± 0.0
AD	0.4 ± 0.2	1.2 ± 0.3	53.8 ± 28.8	62.0 ± 5.3*	544 ± 328.7	87.9 ± 37.6*
FTD	0.0 ± 0.0	1.4 ± 0.1	7.3 ± 1.4	0.0 ± 0.0	9.0 ± 0.9	0.2 ± 0.2

Values are given in pmol / g (see main text for a description of the methods)

** p < 0.05, Dunnett's test (relative to control group).*

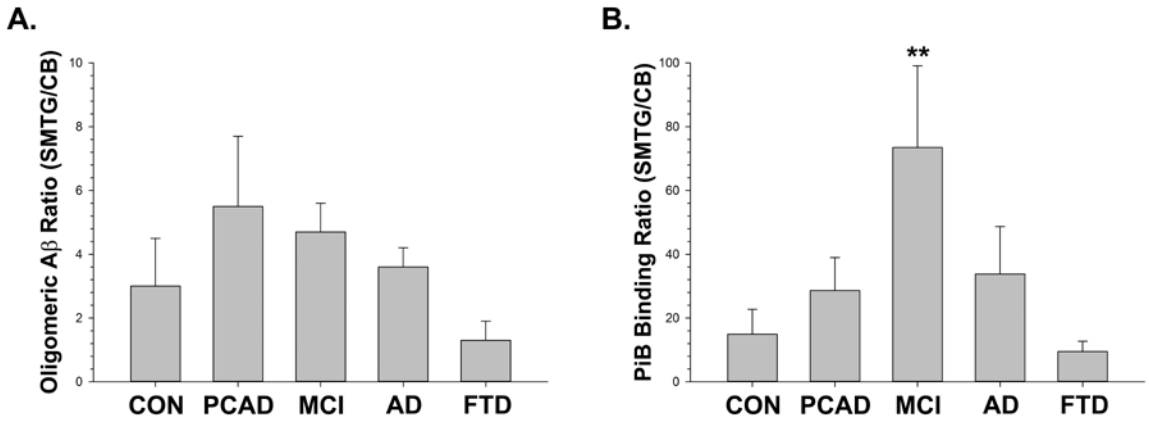

Figure 3.2 Correcting PiB Binding Values to the Cerebellum Distinguishes MCI Cases.

(A) There were no differences between disease states when the amount of oligomeric Aβ in the SMTG was standardized to the amount found in the cerebellum. (B) There was a significant difference detected for disease when PiB binding in the SMTG was corrected to the amount in the cerebellum. Interestingly, the most prominent effect of this standardization was to amplify the difference between the MCI cases as compared to the other groups. Dunnett's test, ** = $p < 0.01$. *Reprinted from (Beckett et al., 2012) with permission from IOS Press.*

Finally, for this case series we wanted to determine how PiB binding related to other aspects of Aβ pathology (Figure 3.3). Postmortem PiB binding was strongly correlated with the total plaque number ($R2 = 0.51$, $p<0.0001$; not shown). Whereas plaque counts are a reasonable means to determine disease status, it is likely that Aβ positive area is a stronger correlate of PiB binding (Ikonomovic et al., 2008). Oligomeric Aβ was only correlated with SDS soluble Aβ42 ($R2 = 0.16$, $p<0.01$; not shown). PiB binding was significantly correlated with both SDS- ($p<0.001$) and FA- ($p<0.05$) soluble Aβ, but not with PBS-soluble Aβ ($p<0.12$). These forms of the peptide are less soluble by definition (they require either a harsh detergent or acid to solubilize), and also show the most consistent increases with disease. In both cases, the strongest correlation was with Aβ42 (SDS: $R2 = 0.46$, $p<0.001$; FA: $R2 = 0.21$, $p<0.002$). PiB binding was also significantly correlated with the amount of oligomeric Aβ ($R2 = 0.22$, $p<0.01$). PiB binding was not significantly correlated with any of these forms of Aβ (including either Aβ40 or Aβ42) in the cerebellum (oligomeric Aβ, $p<0.3$; PBS-soluble Aβ, $p<0.4$; SDS-soluble Aβ, $p<0.15$; FA-soluble Aβ, $p<0.1$; data not shown). This was somewhat surprising, as at least two of these forms of Aβ (SDS- and FA-soluble) did show significant changes in the cerebellum with disease, and were higher in the AD cases (Table 3.1; Figure 3.1). We did not detect a relationship ($p<0.5$) between any aspect of cerebrovascular pathology and postmortem PiB binding in this case series.

For the second case series, we determined which combination of Aβ measurements best discriminated AD cases from controls. To this end, we used a logistic regression model for the presence of disease. We then used a stepwise procedure to identify the subset of measurements that best predicted that a randomly selected subject was an AD case (by log odds ratio). A simple solution was obtained, as there was only one variable in the best subset: the total amount of SDS soluble Aβ, with an odds ratio of 1.06 ± 0.04 (95 % C.I.). A box plot of the

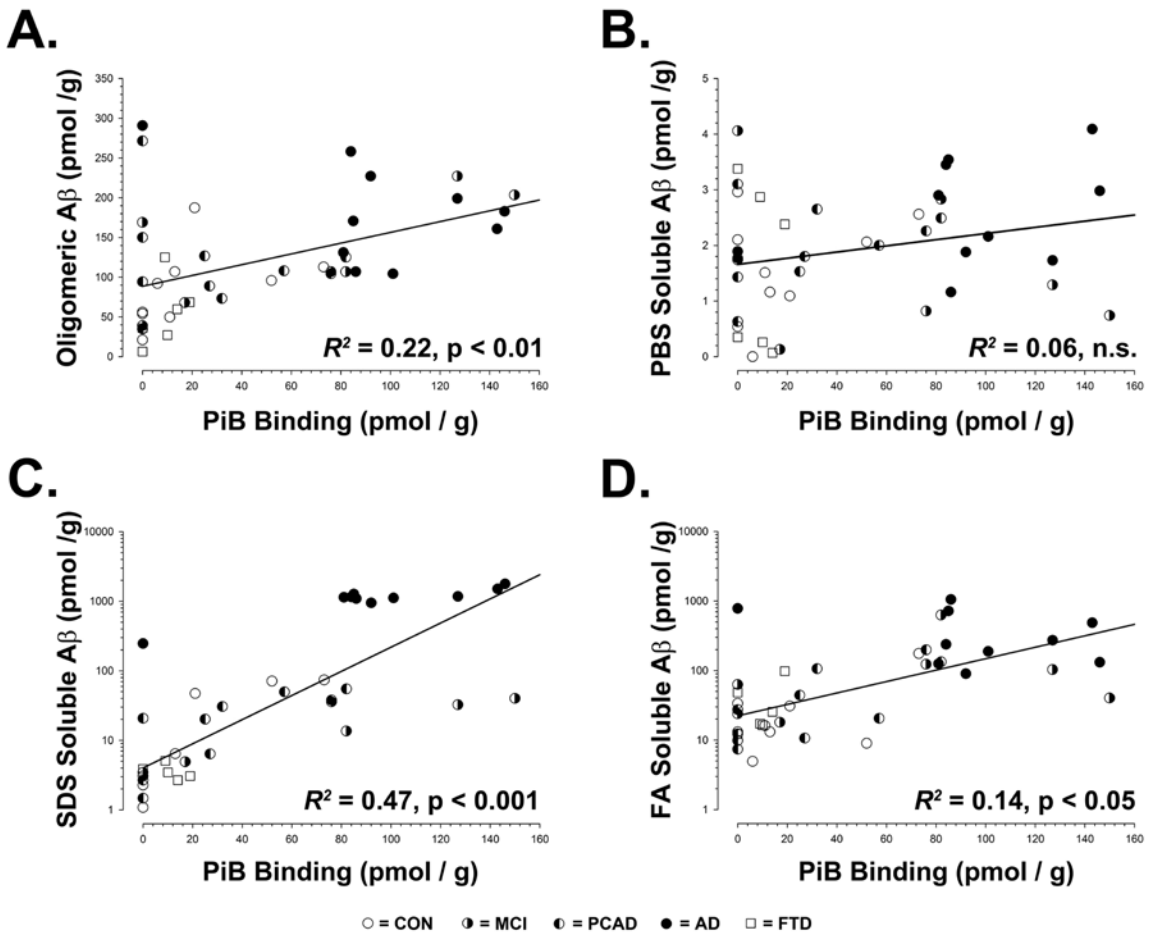

Figure 3.3 PiB Binding Correlates with Less Soluble Forms of Aβ in the SMTG.

(A) The amount of postmortem PiB binding was significantly correlated with oligomeric Aβ ($p<0.01$). (B) PiB binding was not correlated with Aβ extractable in PBS, the most soluble form of the peptide. However, PiB binding was significantly correlated with Aβ extractable in either SDS (C; $p<0.001$) or FA (D; $p<0.05$). *Reprinted from (Beckett et al., 2012) with permission from IOS Press.*

same data showed minimal overlap between AD cases and controls (Figure 3.4). While informative, the overall sample size was too small to conduct a training-validation approach to selecting the best predictor of disease status. Although values between 90 and 95 could be used as a cut-off point to declare an individual to be an AD case, the data set was not large enough to identify a more precise number. PiB binding offered no discernible advantage as a diagnostic tool in this context.

To test the hypothesis that iso-Asp7 Aβ might have predictive value in differentiating AD cases from controls, we evaluated a set of twenty cases from the UK ADC. For this study, we compared normal plasma Aβ40 and Aβ42 using our standard sandwich ELISA, alongside iso-Asp7 Aβ40 and iso-Asp7 Aβ42. In general, AD cases were lower on all forms of Aβ when standardized to the group average (Figure 3.5), although only iso-Asp7 Aβ40 was close to significant on this measure ($p<0.06$). It is possible given the uniform direction of the trend that the data might better segregate given a larger sample size. The Aβ values were highly variable in general (Figure 3.6). However, there was a potentially interesting crossover effect in the data. In this case, Aβ40 values in controls were lower than Aβ42, but reversed for iso-Asp7 (i.e., iso-Asp7 Aβ40 was higher than iso-Asp7 Aβ42). A somewhat similar effect was observed in the AD cases. We evaluated this change using a simple sign test. In this case, the change in direction in the Aβ40 values trended towards significance ($p<0.12$), but was significant for Aβ42 ($p<0.02$). However, although this is a promising outcome, there were not enough cases to determine if this could be used to distinguish AD cases from control cases. This will need to be determined in a larger, follow-up study.

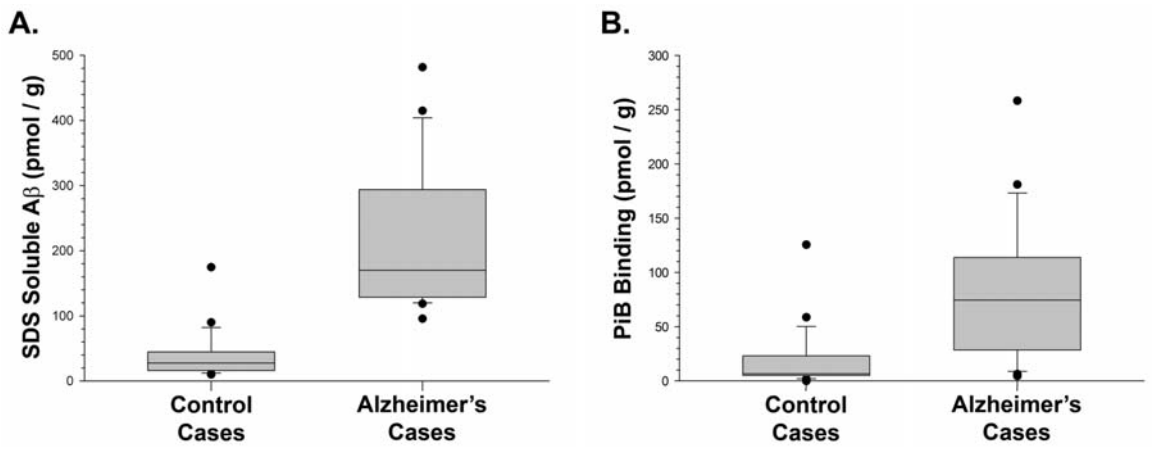

Figure 3.4 SDS Soluble Aβ Indicates Disease State.

SDS soluble Aβ was identified as an exceptionally strong indicator of disease state. The area under the receiver operator curve was 0.978 ($p = 0.0016$), with a sensitivity of 100% and a specificity of 95.7%. The Hosmer-Lemeshow goodness of fit statistic ($p = 0.51$) indicated no evidence of a lack of fit. A boxplot of SDS soluble Aβ, segregated by disease state, shows almost no overlap between cases and controls (A); PiB binding, in contrast, shows considerable overlap (B). *Reprinted from (Niedowicz, Beckett, et al. 2012) with permission from John Wiley and Sons.*

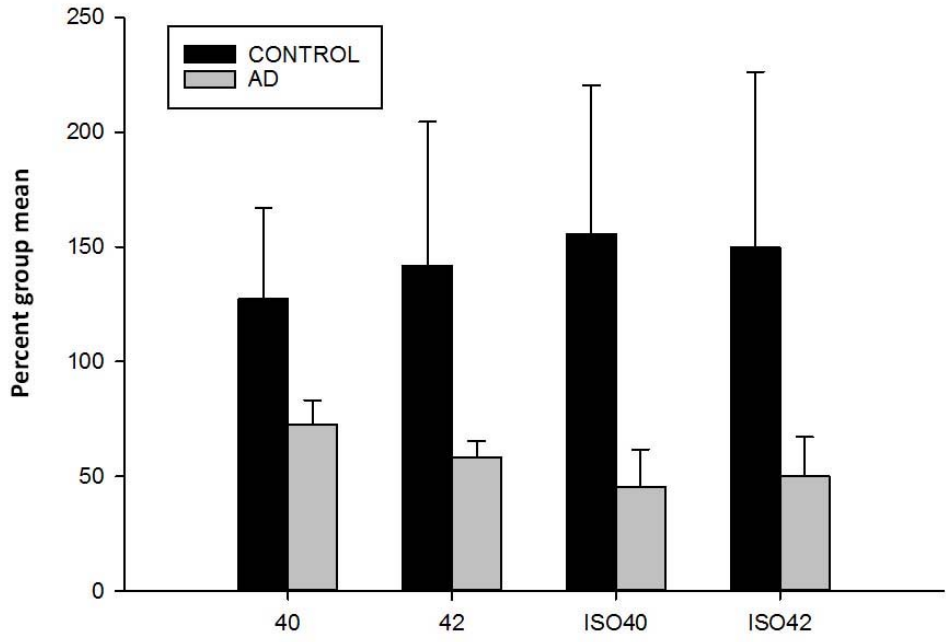

Figure 3.5 AD Cases Have Lower Standardized Levels of Plasma Aβ Than Control Cases.

Levels of plasma Aβ were measured from 20 human cases (Control: N=10, 6F/4M; 82.9 ± 2.6 years; AD: N=10, 5F/5M; 85.6 ± 1.7 years). When standardized to the group average, AD cases had lower levels plasma Aβ, regardless of form. However, only iso-Asp7 Aβ was close to significant ($p<0.06$).

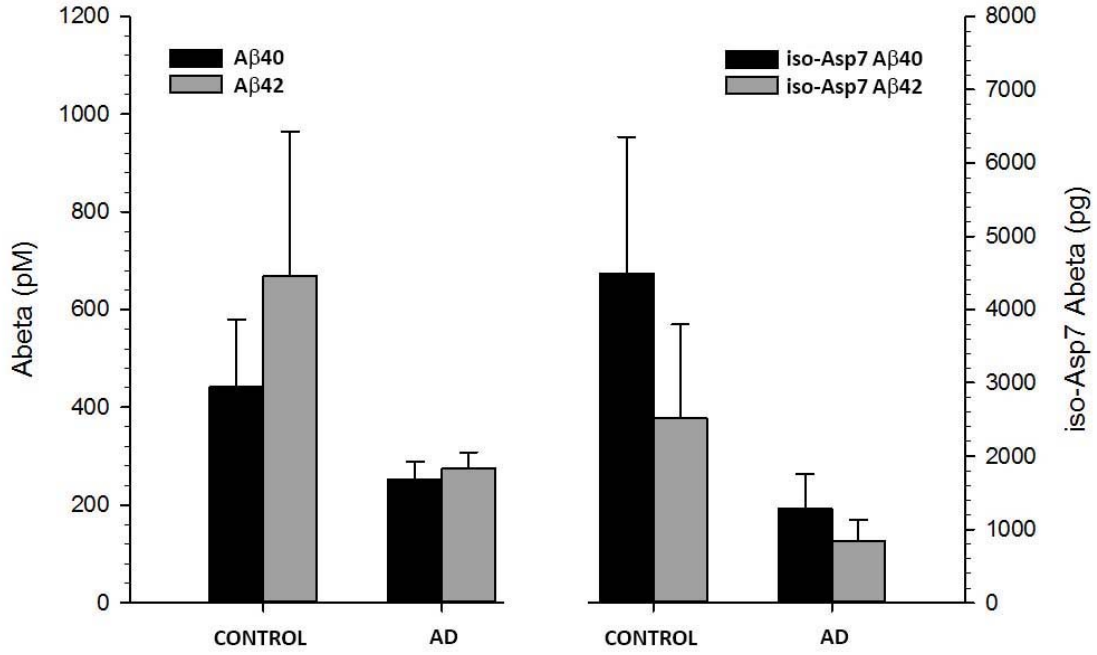

Figure 3.6 Levels of Aβ and Iso-Asp7 Aβ Were Reversed Between Groups.

Plasma levels of Aβ and iso-Asp7 Aβ were reversed between disease group and Aβ form. For example, Aβ40 was lower than Aβ42 in the control group; however iso-Asp7 Aβ40 was higher than iso-Asp7 Aβ42 in the control group. A similar effect was present in the AD cases. Using a simple sign test, we evaluated this change. The change in direction in the Aβ40 values trended towards significance ($p<0.12$). The direction change for the Aβ42 values was significant ($p<0.02$).

CHAPTER 4: DISCUSSION

We found significant quantities of higher order Aβ multimers (oligomeric Aβ) in the neocortex that increased with disease progression, and these were considerably less abundant in the cerebellum. Similarly, PiB binding, thought to mostly reflect Aβ in a fibrillar state, was increased in the SMTG but not in the cerebellum. However, in later stage AD, the amount of aggregated Aβ was similar between the SMTG and cerebellum. This implies that at least some higher order Aβ structures do not form solely in a concentration dependent manner, and that other processes must be involved. Differences between the amounts of oligomeric Aβ in the neocortex and the cerebellum are well known (Klein, 2002; Lambert et al., 2001), and the role of these species in AD neuropathology has been explored in some detail in recent years (Mc Donald et al., 2010; Walsh et al., 2002). Interestingly, we found correlations between PiB binding and oligomeric, SDS- and FA-soluble Aβ in the SMTG but not in the cerebellum. This further indicates that the Aβ deposited in the cerebellum is different from the Aβ deposited in the neocortex in some fundamental way, and that the diffuse amyloid deposits in the cerebellum are not strongly related to PiB binding.

There are both high (nM) and low (μM) affinity PiB binding sites on synthetic and biological Aβ fibrils (Klunk et al., 2005). The low affinity site is more abundant in synthetic fibrils, fibrillar Aβ from transgenic mice, and the Aβ found in the brains of aged non-human primates (Maeda et al., 2007; Rosen et al., 2011). A large proportion of the PiB binding under our assay conditions (1 nM ^{3}H-PiB) is to the high affinity site (Klunk et al., 2005). It is possible that these differences between the SMTG and cerebellum reflect an underlying difference in the disease process that could be useful in elucidating unknown, or at least unappreciated, aspects of AD. At a minimum, a comparison between the cerebellum and a neocortical region such as the SMTG

might be ideal for determining the molecular identity of the high density, high affinity PiB binding site in the AD brain. The identification and mapping of this site could represent an important step towards the development of new imaging agents.

Autopsy studies of patients subject to PiB neuroimaging are still relatively uncommon (Ikonomovic et al., 2008; Klunk et al., 2005; Svedberg et al., 2009), and there has been relatively little examination of earlier stage cases of disease of the type reported here. The longitudinal study of these individuals, and the integration of PiB neuroimaging data with established pathologies and other potential biomarkers, will be critical for developing a reliable clinical protocol for AD diagnosis and monitoring (Apostolova et al., 2010). It is intriguing that our data show that PiB may have some benefit in distinguishing MCI cases from not only normal elderly controls, but possibly from preclinical AD cases as well. We emphasize that this is a study in a relatively small number of cases, and demonstrating the true utility of PiB binding as an agent for identifying MCI will require a much larger cohort. These findings are particularly important given the recommendations from the National Institute of Aging (NIA) /Alzheimer's Association which focus on the use of imaging and CSF measures to define MCI (Albert et al., 2011). These recommendations emphasize the use of subjects from longitudinal studies and incorporation of CSF and/or imaging studies to define MCI as being caused by AD pathology. Similarly, both imaging and CSF play a larger role in the defining of AD in the NIA guidelines (Jack et al., 2011). Data from the present study contribute to both of these efforts by demonstrating the utility of imaging probes for both clinical imaging as well as *ex vivo* analysis in the laboratory setting to understand the pathological processes involved in AD. This has the potential to not only advance our understanding of the disease at the molecular level, but could also lead to better imaging reagents in the future.

In the second case set, we detected an approximate 11-fold elevation in extractable Aβ in the AD brain relative to controls, compared to more modest increases in the amount of PiB binding (~4 fold). We did not detect a robust elevation in oligomeric Aβ using the single site immunoassay method in this study. While these data do not rule out the possibility that oligomeric and fibrillar Aβ contribute to AD (Mc Donald et al., 2010; Walsh et al., 2002), they do indicate that any role these two species play in mediating AD pathogenesis occurs in the background of a tremendous amount of Aβ in other pools. It is possible that oligomeric and fibrillar species of Aβ contribute to AD via their synergy with other Aβ pools. It is also possible that the largest contribution of oligomeric Aβ to neuronal dysfunction and degeneration is of far greater importance earlier in the disease process (such as in preclinical AD (Price et al., 2009) or in cases of amnestic MCI (Petersen et al., 1999)). Alternatively, the location or local concentration of oligomeric species may be the key to their ability to promote disease onset and progression. Finally, the immunoassay approach that we used in this case series detects only relatively large forms of oligomeric Aβ; it is likely that smaller forms of oligomeric Aβ (e.g., dimers, trimers, etc.) escape detection by this method. It is possible that these smaller forms of oligomeric Aβ may be more important for the disease process (Walsh & Selkoe, 2004). Nevertheless, the lack of a clear increase in oligomeric Aβ in AD cases highlights that, in spite of recent advances, there are still limitations on our understanding of the disease process. It is noteworthy that PiB binding in the postmortem brain was unable to discriminate between cases and controls as well as SDS soluble Aβ. Postmortem PiB binding is evaluated under highly favorable experimental conditions, while binding *in vivo* occurs under less optimal conditions. Blood flow and sequestration on the time scale of the imaging session are significant *in vivo*

variables, whereas PiB has greater access to amyloid binding sites *ex vivo*. It is well known that there is considerable variability and heterogeneity in PiB retention in the human brain, with PiB retention increasing in a non-linear manner during the progression of disease and unable to uniformly discriminate AD from non-AD cases (Sojkova & Resnick, 2011). Postmortem PiB binding appears to involve only a small fraction of the total amount of Aβ in the brain, and it is likely that some portion of the unlabeled amyloid is significant to the disease process (Svedberg et al., 2009). It is also likely that differentially soluble pools of Aβ in the brain deposit at different rates (Murphy & LeVine, 2010), and it is unclear which of these pools PiB retention represents. The study of PiB binding *ex vivo* could shed light on these issues. Although these data do not diminish the potential clinical utility of PiB as an agent for detecting the deposition of Aβ in the brain of living patients, they nevertheless raise the possibility that *in vivo* imaging using PiB is largely detecting deposits of Aβ that are considered by neuropathologists as less important to the AD clinical phenotype than either NPs or NFTs. For instance, in this study and many others, the strongest relationship between MMSE and neuropathology is with neocortical NFTs (Nelson et al., 2010; Nelson et al., 2007), which are not related to the amount of PiB binding. This finding is in line with some of the earliest studies of PiB *in vivo*, which reported no relationship between PiB binding and MMSE scores (Klunk et al., 2004). However, recent work in defining the preclinical state of AD has raised the intriguing possibility that DPs are being overly discounted as a factor in pathology (Price et al., 2009). There are currently relatively few individuals that have come to autopsy following PiB neuroimaging. Further study of these individuals, and integration with other biomarker data (Apostolova et al., 2010), will be essential for developing a reliable clinical screening procedure for the detection of AD and monitoring its progression.

We were able to measure levels of Aβ40, Aβ42, iso-Asp7 Aβ40, and iso-Asp7 Aβ42 in plasma samples from AD and control cases. When standardized to the group average, AD cases had lower levels of all forms of Aβ. Although the trend was convincing, this did not reach significance. The difference in iso-Asp7 Aβ40 between AD and control cases was closest to reaching significance ($p<0.06$). Aβ deposition in diffuse and neuritic plaques are a hallmark of AD pathology. AD cases have significantly higher levels of Aβ in the brain compared to control cases (Figure 3.1). Iso-Asp7 Aβ is significantly deposited within Aβ plaques in AD brain (Roher et al., 1993). With this in mind, we expected to find higher levels of Aβ overall, and of iso-Asp7 Aβ in particular, in the plasma from AD cases compared to control cases. However, this was not the case. A possible explanation is that once Aβ plaques develop to the point of becoming neuritic, both the non-isomerized Aβ and iso-Asp7 Aβ peptides may be selectively sequestered within those plaques of the AD brain, and become less free to move about the periphery. If this is indeed the case, the significantly lower plaque burden among control cases could allow for more free-floating Aβ species throughout the periphery, resulting in the relatively higher levels of plasma Aβ seen in this study. If the trend we saw in plasma Aβ levels between AD and control cases were to hold true, we would expect the effect to reach significance with a larger case study. A follow-up study is required, however, these initial data are a promising step towards utilizing aged iso-Asp7 Aβ plasma levels as a potential biomarker to indicate disease state.

Appendix I: List of Abbreviations

AC	antigen capture buffer
AD	Alzheimer's disease
AICD	amyloid precursor protein intracellular domain
APP	amyloid precursor protein
Aβ	amyloid-beta
C	Celsius
CCP	classical complement pathway
CON	control
CTF-α	c-terminal fragment α
CTF-β	c-terminal fragment β
DB	detection buffer
DP	diffuse plaque
ELISA	enzyme-linked immunosorbent assay
F/M	female/male
FA	formic acid
FTD	frontotemporal dementia
Iso-Asp7 Aβ	isomerized aspartate 7 amyloid-beta
MCI	mild cognitive impairment
NFT	neurofibrillary tangle
NP	neuritic plaque
OD	optical density
PBS	phosphate buffered saline

PBST	phosphate buffered saline with tween-20
PCAD	pre-clinical Alzheimer's disease
PiB	Pittsburgh binding compound B
PIC	protease inhibitor cocktail
PMI	postmortem interval
sAPPα	secreted amyloid precursor protein alpha
sAPPβ	secreted amyloid precursor protein beta
SDS	sodium dodecyl sulfate
SMTG	superior and mid temporal gyrus
TMB	3,3',5,5'-tetramethylbenzidine
TP	tris phosphate buffer

Appendix II: Aβ Sequences

Aβ40	**DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV**
Aβ42	**DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA**
Iso-Asp7 Aβ40	**DAEFRH-isoD-SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA**
Iso-Asp7 Aβ42	**DAEFRH-isoD-SGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA**

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Vita

Name: Christina (Tina) Lisa Beckett

Birthplace: North York, Ontario, Canada

EDUCATION:

1994-2000 University of Toronto
Honours Bachelor of Science in Neuroscience and Biology
Toronto, ON, Canada

POSITIONS HELD:

2014-2016 Staff Scientist III, University of Kentucky, Lexington, KY

2008-2014 Research Analyst, Pr., University of Kentucky, Lexington, KY

2007-2008 Research Analyst, University of Kentucky, Lexington, KY

2005-2007 Laboratory Technician, Sr., University of Kentucky, Lexington, KY

2004-2005 Veterinary Technician, McGilvray Veterinary Hospital, Toronto, Canada

2002-2004 Manager, Folly, Toronto, Canada

2000-2002 Veterinary Assistant, Wychwood Animal Hospital, Toronto, Canada

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