The Effect of Vitamin D Supplementation on Plasma Aβ in an Older Population: A Randomized Control Trial

by

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ABSTRACT

Vitamin D deficiency has been previously associated with a higher Alzheimer's disease (AD) risk, a condition marked by dependent living and severe cognitive impairment. AD is histologically defined by the presence of brain amyloid beta (Aβ) plaques and neurofibrillary tangles. Ways to enhance Aβ clearance have been examined in order to sustain cognition and delay AD onset. In vitro and in vivo studies suggest that vitamin D might enhance brain Aβ transportation to the periphery by up-regulating P-glycoprotein production. The purpose of this study was to examine the effect of vitamin D supplementation on plasma Aβ in an older population.

This study was a parallel-arm, double-blinded, randomized control trial. Participants consumed either a vitamin D supplement or placebo once a week for eight weeks (n=23). Only vitamin D insufficient (serum total 25-OH, D < 30 ng/mL) people were included in the study, and all participants were considered to be cognitively normal (MMSE scores > 27). Serum total 25-OH, D and plasma $A\beta_{1-40}$ measurements were recorded before and after the eight-week trial. The plasma $A\beta_{1-40}$ change was compared between the vitamin D group and control group.

The vitamin D group experienced a 45% greater change in plasma $A\beta_{1-40}$ than the control group. The effect size was 0.228 when controlling for baseline plasma $A\beta_{1-40}$ ($p=0.045$), 0.197 when controlling for baseline plasma $A\beta_{1-40}$ and baseline physical activity ($p=0.085$), and 0.179 when controlling for baseline plasma $A\beta_{1-40}$, baseline physical activity, and age ($p=0.116$). In conclusion, vitamin D supplementation might increase brain Aβ clearance in humans, but physical activity and age also appear to modulate Aβ metabolism.

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CHAPTER 1

INTRODUCTION

Problem

Approximately thirty-five million people have Alzheimer's disease (AD), a progressive neurodegenerative disorder characterized by substantial cognitive decline (Ferri et al., 2005). The global prevalence is projected to increase fourfold by 2050, causing economic and societal strain (Brookmeyer, Johnson, Ziegler-Graham, & Arrighi, 2007). To compound the problem, current AD treatments are only modest in efficacy and no cure exists (Wimo et al., 2014). Nonetheless, roughly one third of AD cases might be due to modifiable risk factors (Norton, Matthews, Barnes, Yaffe, & Brayne, 2014). Hence, there is a need to identify risk factors in order to create primary treatments.

The two histological consistencies of AD are neurofibrillary tangles and amyloid-beta peptide (Aβ) cerebral plaques (Karantzoulis & Galvin, 2011). Aβ is a normal byproduct of amyloid precursor protein (APP) metabolism, but will build up in cerebral interstitial space if excessively produced or inadequately cleared (Wijesuriya, Bullock, Faull, Hladky, & Barrand, 2010). Excessive Aβ accumulation atrophies the brain and disrupts cerebral activity (Mattsson et al., 2015). This manifests in severe memory loss and further executive function impairment (Karantzoulis & Galvin, 2011).

Aβ peptides are cleared across the blood-brain barrier (BBB) through two transport proteins: lipoprotein receptor-related protein 1 (LRP-1) and P-glycoprotein (P-gp). An inverse relationship between brain P-gp and Aβ has been observed in human post-mortem AD cases. P-gp is encoded by the multidrug resistance protein gene (MDR1) and requires the vitamin D receptor (VDR) as a transcription factor. VDRs are

abundantly expressed in the central nervous system and are especially saturated within the hippocampus (Saeki, Kurose, Tohkin, & Hasegawa, 2008). Being that VDR activation increases P-gp expression, those with insufficient vitamin D might not proficiently transport Aβ out of the brain. See Figure 1.

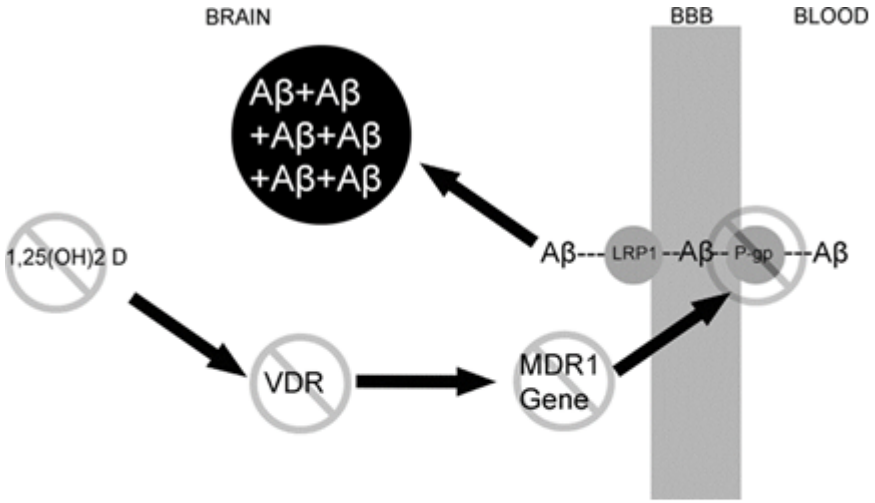

Figure 1. *Active transport clearance of Aβ across BBB. Aβ is transported to the periphery through LRP1 and P-gp (dashed line). Since calcitriol acts as a transcription factor for the MDR1 gene (black arrows), limited circulating vitamin D could lower P-gp production (interdictory circle), consequently leading to brain Aβ accumulation.*

Several experimental models confirm vitamin D enhances Aβ transportation out of the brain. In human brain microvessel cell lines, 100 nm of calcitriol decreased brain Aβ by 25-30% (Durk et al., 2012). In mice, calcitriol increased hippocampal P-gp expression and lowered brain Aβ levels by 30% (Durk et al., 2014). Hence, the Aβ modulating effects of vitamin D make for a potential lifestyle intervention

Although life style factors have been reported to influence AD risk, few trials have examined the effects of a lifestyle intervention on Aβ metabolism (Norton et al., 2014). Lifestyle interventions are valuable because they can be employed early in the

disease process. Lifestyle interventions could decrease AD risk by 4.8% and decrease Medicare costs by nearly $250 billion, while also being cost-effective complements to pharmaceuticals, as the projected drug development cost is $569.3 billion (Scott, O'Connor, Link, & Beaulieu, 2014; Sperling et al., 2011).

Epidemiological data justifies examining vitamin D as a potential therapeutic option. A significant association between vitamin D deficiency and dementia has been observed in multiple populations (Balion et al., 2012; Littlejohns et al., 2014). Vitamin D deficiency has also been associated with faster cognitive decline in several populations (Toffanello et al., 2014; Wilson et al., 2014). This is striking because nearly 1 billion people worldwide are suspected to have inadequate vitamin D levels (F Holick, 2011).

Proposed Research

The objective of this research was to examine the effect of vitamin D supplementation on plasma Aβ in healthy adults over the age of 50. The aim was to determine whether vitamin D supplementation increases plasma Aβ. No relationship between vitamin D supplementation and plasma Aβ was suspected.

Definition of terms

Apoptosis: Cell death

Calcitriol: The biologically active form of vitamin D.

Expression: The induction of protein production from genetic sequences.

Hippocampus: A brain structure involved in memory processing.

Modifiable risk factor: A risk factor for which an individual can amend.

Post-mortem: After death

Transcription factor: Proteins that help initiate RNA production from DNA sequences.

Abbreviations

AD: Alzheimer's disease

AMPAR: α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid receptor

α7 nAChR: α7 nicotinic acetylcholine receptor

APP: Amyloid precursor protein

Aβ: Amyloid beta

BBB: Blood-brain barrier

BDNF: Brain derived neurotrophic factor

BPSD: Behavioral and psychological symptoms

CSF: Cerebrospinal fluid

ER: Endoplasmic reticulum

HR: Hazard ratio

LTP: Long-term potentiation

MCI: Mild Cognitive Impairment

MMSE: Mini-mental state examination

MRI: Magnetic resonance imaging

NCI: Not Cognitively Impaired

NGF: Nerve growth factor

NMDAR: N-methyl-d-aspartate-receptor receptor

PET: Positron emission tomography

P-gp: P-glycoprotein

RCT: Randomized control trial

RDA: Recommended Dietary Allowance

RR: Relative risk

Tg: Transgenic

TrkA: Tyrosine kinase receptor A

TrkB: Tyrosine kinase receptor B

VDR: Vitamin D receptor

WT: Wild type

Delimitations and Limitations

There are several limitations to this study. First, Aβ can bind to various proteins in the blood, and other determinants of Aβ continue to be investigated (Costa, Ortiz, & Jorquera, 2012). Second, participants were not tested for ApoE4, the allele strongly associated with AD (Bennet et al., 2007). Third, participants' food intake and physical activity were collected using surveys, which are prone to substantial measurement error. Fourth, overall macronutrient and micronutrient data was not collected. Fifth, the sample size was small, categorizing this study as a pilot trial. Finally, results might not be generalizable to diabetic or demented individuals outside of the Phoenix metro area of Arizona who are taking statins.

CHAPTER 2

LITERATURE REVIEW

Alzheimer's Disease (AD)

Dementia and AD Overview

Dementia is a category of several types of reversible and irreversible neurodegenerative conditions, but AD represents nearly 60% of all dementia cases (American Psychiatric & Task Force on, 2000; Van Marum, 2008). Reversible types of dementia have identifiable causes (e.g., vitamin B12 deficiency, hypothyroidism, etc.), whereas the causes of irreversible dementia continue to be investigated. AD is an irreversible type, categorized alongside frontotemporal dementia and lewy body dementia (Tripathi & Vibha, 2009). AD is distinguishable from other irreversible forms based on two histological findings: plaques composed of amyloid beta peptides (Aβ) and intracellular neurofibrillary tangles (Selkoe, 2004). Experimental models suggest plaques and tangles disrupt the limbic and cortical system, which manifests in significant memory impairment and executive function deficiencies (Karantzoulis & Galvin, 2011). Even though there is a need for effective treatments, none have been developed partially because the etiological mechanisms are still unclear (Wimo et al., 2014).

Aβ Overview

Aβ gradually accumulates years before AD is detected. When patients express AD symptoms, there is already roughly 30-60% less neurons in the hippocampus (Price et al., 2001). Hence, disrupting Aβ accumulation early in the pathological progression might be a prime strategy.

Aβ production. Aβ peptides are a normal byproduct of amyloid precursor protein (APP) proteolyzation. Although its function is unclear, APP is rapidly produced in large quantities. APP is proteolyzed by two exclusive pathways in the trans-Golgi network; one produces toxic Aβ and the other produces non-toxic Aβ. The non-toxic Aβ pathway includes α- secretase and γ-secretase, whereas the toxic Aβ pathway includes BACE1 and γ-secretase (O'Brien & Wong, 2011).

Toxic Aβ is a derivative of two APP cleavages. First, BACE1 cleaves APP in the ectodomain region to produce sAPPβ. Second, γ-secretase cleaves the trasmembrane APP-C terminal fragment to generate $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$. The $A\beta_{1\text{-}40}$ form is produced at a higher rate than $A\beta_{1\text{-}42}$, but the more amyloidogeneic form is $A\beta_{1\text{-}42}$. Both isoforms, if not degraded, are transferred in vesicles to the extracellular space. Once in the extracellular space, Aβ must be cleared via non-enzymatic or enzymatic pathways. (V. W. Chow, Mattson, Wong, & Gleichmann, 2010).

Aβ aggregation occurs even at small concentrations in the neocortex and limbic cortex. The peptides amass gradually, starting as 4 kDa monomers, grouping into oligomers, and eventually forming the trademark fibrotic plaques. The core of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ neurotic plaques consists of internal hydrophobic C-terminals and external negatively charged N-terminals (Q. Wang et al., 2011). While the cause of Aβ accumulation is not fully understood, evidence points to overproduction and inadequate clearance (Mawuenyega et al., 2010; L.-B. Yang et al., 2003).

There are no useful therapies that sufficiently decrease brain Aβ. A therapy that lowers brain Aβ accumulation would be a breakthrough in AD care. Potential therapeutic targets include BACE1 or γ-secretase inhibition; however, targeting both these enzymes

in mice yielded discouraging results. One study that crossed BACE1$^{-/-}$ mice with transgenic (Tg) APP mice produced less Aβ and improved cognition, but consequently caused hypomyelination, decreased neurogenesis, vision problems, and seizures (Yan & Vassar, 2014). Likewise, another study that crossed γ-secretase$^{-/-}$ mice with Tg APP mice had several adverse outcomes because γ-secretase has nearly 50 substrates, all of which have diverse functions, such as gene expression, cell adhesion, ion regulation, and neurotrophin signaling (T. Yang, Arslanova, Gu, Augelli-Szafran, & Xia, 2008). Therefore, the consequences of BACE1 and γ-secretase inhibition justify the need to explore alternative strategies.

Aβ Clearance. Aβ will accumulate in brain interstitial space if not eliminated by enzymatic or non-enzymatic action. Four enzymes predominantly degrade Aβ: matrix metallopeptidase 9, glutamate carboxypeptidase II, neprilysin, and insulin-degrading enzyme. Three non-enzymatic pathways transport Aβ out of the brain. The first pathway transports Aβ from the brain to the cerebrospinal fluid (CSF), which then enters perivascular arterial spaces. The second pathway clears Aβ by microglial cell phagocytosis. The third pathway transports Aβ across the BBB via active transport receptors. BBB dysfunction is of particular interest to scientists studying Aβ clearance (Yoon & Jo, 2012).

The Aβ active transport pathway across the BBB includes two steps. First, Aβ enters the endothelial cell LRP1. Second, Aβ is excreted through P-gp, a luminal active transport protein encoded by the MDR1 gene (Silverberg et al., 2010). P-gp is substantially abundant in the BBB and also exports several substances from the neuroparenchyma to the blood (Miller et al., 2000). P-gp is necessary because the BBB

only allows for nonpolar molecules to passively diffuse (Wildsmith, Holley, Savage, Skerrett, & Landreth, 2013).

The relationship between Aβ and Pg-p has been extensively examined because of the potential for pharmaceutical utility. Lam et al. (2001) first hypothesized that Aβ is a substrate for P-gp in vitro. The theory was strengthened when an inverse relationship between Aβ deposits and P-gp was quantified in human post-mortem AD cases (Vogelgesang et al., 2002). Since then, several in vitro experiments reinforce the theory of Lam et al. (2001), suggesting that there is certainly an interaction between P-gp and Aβ (Bharate et al., 2015; Kuhnke et al., 2007; Park, Kook, Park, & Mook-Jung, 2014).

Studies have subsequently examined the relationship between P-gp inhibition and brain Aβ levels in mouse models. Cirrito et al. (2005) found that inhibiting P-gp expression decreased Aβ clearance. In this study, Tg APP/MDR1$^{-/-}$ mice had more Aβ than Tg APP mice that expressed MDR1. When Tg APP/MDR1$^{+/-}$ mice were given a P-gp inhibitor, the amount of Aβ accumulation predictably increased. Findings from Hartz et al. (2010) were similar. Rather than inhibiting P-gp expression, they found that prompting the expression of P-gp decreased brain Aβ. These results imply that P-gp is a viable therapeutic target to decrease brain Aβ.

Indeed, many studies show P-gp up-regulation increases Aβ clearance in mouse models and humans. Currently, known P-gp up-regulators include St. John's wort, caffeine, and rifampicin. Tg APP mice that were administered St. John's wort increased P-gp expression, subsequently increasing Aβ clearance (Brenn et al., 2014). Wild type (WT) mice that were given both caffeine and rifampicin cleared 20% more Aβ than controls, also a result from increased P-gp expression (Qosa, Abuznait, Hill, &

Kaddoumi, 2012). Just as rifampicin was beneficial for clearing Aβ in mice, so too was it beneficial in humans, as a three-month trial significantly improved cognition (Loeb et al., 2004). Therefore, therapies that increase P-gp expression might be a viable option to delay cognitive impairment.

Alzheimer's Disease and Brain Dysfunction

AD manifests in severe cognitive impairment because of several interlaced, degenerative mechanisms. In AD, synapses fail, cytosolic calcium increases, neurotrophin and neurotransmitter utility declines, and mitochondria stop functioning (Hirai et al., 2001; Hock, Heese, Hulette, Rosenberg, & Otten, 2000; Pierrot, Ghisdal, Caumont, & Octave, 2004; Purves, 2001; H. W. Querfurth & LaFerla, 2010).

Synapses. A synapse is a gap junction between a neuron and its target cell, the site where ions and organic molecules are exchanged. The axonal end of the first neuron is called the pre-synapse, and the dendritic end of the second neuron is called the post-synapse, with the small space in-between is called the synaptic cleft. The small synaptic cleft size (3 nm) allows for remarkably fast communication; therefore, transmission is sensitive to slight disturbances (Bear, Connors, & Paradiso, 2007, pp. 103-105). In AD, interneuron synapses are the types that degenerate (S. W. Scheff & Price, 2003).

The literature has consistently shown that synaptic failure is a distinct consequence of AD. Human brain and AD mouse models demonstrate that synaptic failure leads to severe cognitive impairment (DeKosky & Scheff, 1990). In fact, gradual degeneration of synapses has been observed many years before AD is clinically diagnosed. For example, Scheff et al. (2007) demonstrated that there is an inverse relationship between synaptic count and size in individuals with Mild Cognitive

Impairment (MCI) ($R=0.414$; $p<0.03$), a condition preceding the clinical definition of AD. Specifically, those with MCI had 5% larger synaptic volume than not cognitively impaired (NCI) individuals ($p<0.05$). An inverse relationship between synaptic count and size suggests that synapses grow larger to compensate for count loss. Not surprisingly, there was also a relationship between total hippocampal synapses and MMSE scores ($r=0.595$; $p<0.001$). Hence, neurodegenerative patterns mirror synaptic morphology.

Calcium Regulation. Calcium (Ca^{2+}) is ubiquitous in brain physiology. The functions of cytosolic Ca^{2+} include gene expression, neurotransmitter release, memory recall, cell survival, and action potential stimulation. Ca^{2+} homeostasis is crucially maintained by many mechanisms to ensure healthy brain physiology. For example, Ca^{2+} can enter the cytosol from the extracellular matrix, endoplasmic reticulum (ER), and mitochondria. From the extracellular space, Ca^{2+} enters the cytosol through activated N-methyl-d-aspartate receptors (NMDRs), α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid receptors (AMPARs), and voltage gated calcium channels. Once in the cytosol, Ca^{2+} can be stored within the endoplasmic reticulum (ER) and the mitochondria. To maintain homeostasis, the ER releases Ca^{2+} back into the cytosol through the membrane channels inositol trisphosphate receptor (IP_3R) and ryanodine receptor (RyR). Disruption to any of these Ca^{2+} regulatory mechanisms has deleterious effects (Woods & Padmanabhan, 2012).

Abnormal Ca^{2+} regulation has been linked to memory loss, mediated by neuronal death and Aβ production. Neuron survival and memory depends on a balance between cytosolic Ca^{2+} and mitochondrial Ca^{2+}. High cytosolic Ca^{2+} forces mitochondrial Ca^{2+} uptake, which in turn opens the permeability transition pore. This opening releases

cytochrome c and consequently starts the apoptosis cascade (Giacomello, Drago, Pizzo, & Pozzan, 2007). Additionally, high cytosolic Ca^{2+} induces memory loss independent of neuronal apoptosis. Normally, phosphorylation of AMPAR induces long-term potentiation (LTP), the mechanism by which memories are formed, but elevated cytosolic Ca^{2+} results in endocytosis of AMPARs (Supnet & Bezprozvanny, 2010). Furthermore, elevated cytosolic Ca^{2+} increases Aβ production. In vitro studies showed elevated cytosolic Ca^{2+} represses α- secretase activity, the first enzyme involved in producing the non-amyloidogenic form of Aβ, and increases production of $A\beta_{1-42}$ (Pierrot et al., 2004). Accordingly, stabilizing cytosolic Ca^{2+} would conceivably improve memory processing by protecting neurons and decreasing toxic Aβ levels.

Neurotransmitters. Neurotransmitters function as interneuronal chemical messengers. They are organized into vesicles and released into the synaptic cleft when the neuron is depolarized. The organic messengers thereafter bind to post-synapse voltage gated ion channels or G proteins (Bear et al., 2007, p. 141). Although there are several neurotransmitters, AD scientists are primarily concerned with acetylcholine and glutamate.

Acetylcholine and glutamate are involved in synaptic plasticity and APP metabolism. In AD, acetylcholine production is decreased because the basal forebrain structures degenerate, the site of the brain that produces acetylcholine (Mesulam, 2004). This is problematic because acetylcholine stimulates LTP by binding to the α-7 nicotinic acetylcholine receptor and muscarinic type 1 acetylcholine receptor, and binding of the muscarinic type 1 acetylcholine receptor also decreases Aβ production by promoting the non-amyloidogeneic APP processing pathway (H. Querfurth, 2010). In addition, synaptic

plasticity is further strengthened by the action of glutamate. Glutamate acts on NMDARs to stimulate cAMP response element binding protein (CREB) production, a necessary transcription factor for LTP (Snyder et al., 2005). However, evidence of decreased glutamate utility was found in human post-mortem AD brains, which specifically showed decreased hippocampal mRNA of NMDAR subunits (Hynd, Scott, & Dodd, 2004). Drug developers aim to alter the metabolism or binding affinity of both these neurotransmitters (Bond et al., 2012).

Neurotrophins. Neurotrophins support neuron growth, survival, and repair. AD affects mainly two neurotrophins: brain derived neurotrophin factor (BDNF) and nerve growth factor (NGF). BDNF binding prompts LTP and sustains the function of hippocampal, cortical, cholinergic, and dopaminergic neurons. NGF binding releases acetylcholine, activates muscarninic receptors, and limits α-secretase activity. Both neurotrophins bind to pan-neurotrophin receptors p75, but BDN binds to tyrosine kinase receptor B (TrkB) and NGF binds to tyrosine kinase A (TrkA) (Allen, Watson, & Dawbarn, 2011). As such, decreased expression of these neurotrophins or receptors would damage neurons and impair memory processing.

BDNF and NGF expression, as well as its receptors, are decreased in AD mouse models and post-mortem cases. In human post-mortem brain preparations, the levels of BDNF were much lower than healthy controls (Hock et al., 2000). In mice models, exogenous BDFN significantly improved synaptic integrity in the entorhinal cortex and dentate gyrus (Nagahara, 2009). Whereas BDNF levels are lower in AD, NGF levels did not differ between AD and NCI cases. However, there were 56% less TrkAs in AD brain models compared to NCI, reflecting poor NGF utility (Mufson et al., 2000). Increasing

the expression of neurotrophins and its receptors theoretically might sustain memory and delay neurodegeneration.

Mitochondria. The mitochondria are responsible for producing ATP. The brain uses nearly 25% of the body's total glucose supply for synaptic transmission, calcium homeostasis, and neurotrophin and neurotransmitter activity (Khatri & Man, 2013). Mitochondria additionally regulate reduction-oxidation potentials, reactive oxygen species (ROS) production, and caspase-mediated programmed cell death (Lin & Beal, 2006). Hence, damage to mitochondria would have injurious consequences.

The literature suggests that mitochondrial damage elicits cognitive impairment. Much of these observations have been noted using cell-line experiments. In one study, mitochondrial enzymes, including cytochrome oxidase, pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase, were found to be much lower in AD brains compared to NCI brains (Reddy & Beal, 2008). In another study, cytoplasmic mitochondrial DNA was found to be much higher in AD brains than NCI brains (Hirai et al., 2001). Predictably, Swerdlow et al. (2014) showed mitochondrial dysfunction was associated with decreased oxygen consumption and a lower NAD +/NADH ratio in cell models. This shift towards high NADH slows limits ATP production by slowing down glycolysis. Increased expression of COX2, the protein that converts arachidonic acid to prostaglandin H2 to mediate cell apoptosis was also observed. Altogether, mitochondrial damage might be one mechanism for which cognition is impaired.

The Effects of Aβ on Brain Physiology

Aβ disrupts memory by promoting brain atrophy and dysfunctional cerebral activity (Mattsson et al., 2015). Aβ impairs synaptic transmission, Ca^{2+} homeostasis,

neurotransmitter availability, neurotrophin availability, and mitochondrial function (Allen et al., 2011; Pagani & Eckert, 2011; Shipton et al., 2011; H. Y. Wang, 2000; Xie, 2004).

Aβ and Synapses. Many experimental models demonstrate that Aβ impairs synaptic transmission. One way for which Aβ can disrupt synaptic transmission is by influencing hyperphosphorylation of microtubule tau protein through glycogen synthase kinase 3 activation. Fittingly, inhibiting glycogen synthase kinase 3 reduced neurofibrillary tangles and prevented Aβ-induced memory impairment in mice (Shipton et al., 2011). Furthermore, Aβ oligomers change synaptic structure. Dendritic spines of hippocampal neurons grew extremely thin when exposed to Aβ in cell lines, an observation that resembled dendritic structure in mental retardation. Dendritic spin density even decreased by 50% only after 24 hours of Aβ exposure (Lacor et al., 2007). These results are consistent with previously reported dendritic spinal morphology in AD mouse models (Jacobsen et al., 2006).

Aβ and Ca^{2+} Homeostasis. The effect of Aβ on Ca^{2+} is apparent in the literature. Several in vitro experiments have shown that Aβ increases intracellular Ca^{2+} to an excitotoxic level. Aβ activates NMDARs and opens ER calcium channels in hippocampal neurons, causing a prolonged Ca^{2+} influx into the cytosol (Xie, 2004). Additionally, Aβ translocated into bilayer membrane models to form calcium channels, consequently increasing the amount of Ca^{2+} influx upon neuron depolarization (Arispe, Rojas, & Pollard, 1993). To compound the problem, Aβ prolongs neuron depolarization by blocking voltage-gated fast-inactivating potassium currents (Good, Smith, & Murphy, 1996). Reducing the amount of Aβ would, in theory, restore Ca^{2+} homeostasis.

Aβ and Neurotransmitters. High brain Aβ levels disrupt acetylcholine and glutamate utility. Aβ interacts with acetylcholine and glutamate receptors in vitro. Aβ limits acetylcholine uptake in the postsynapse and reuptake in the presynapse by binding to the α7 nicotinic acetylcholine receptor (α7 nAChR) (H. Y. Wang, 2000). LTP was significantly increased when an α7 nAchR partial agonist was administered to rat hippocampal cells (Kroker, Moreth, Kussmaul, Rast, & Rosenbrock, 2013). Findings from Snyder et al. (2015) demonstrated that Aβ also disrupts glutamate transmission by inducing the endocytosis of NMDARs at the surface of cortical neurons. NMDAR endocytosis disturbed CREB signaling, consequently decreasing LTP. When Aβ levels were decreased, NMDARs predictably increased at synaptic surface sites. These findings suggest Aβ reduction would sustain the ability of acetylcholine and glutamate to bind to its respective receptors**.**

Aβ and Neurotrophins. Several experimental models show that Aβ disrupts neurotrophin utility. Similar to its effects on neurotransmitter receptors, Aβ also binds to the receptors of NGF. This is problematic because NGF produces longer dendrites that improve synaptic plasticity, and high concentrations of Aβ were found to shorten dendrites by acting as an antagonist to p75NTRs (Arevalo, Roldan, Chacon, & Rodriguez-Tebar, 2009). Moreover, soluble Aβ decreases BDFN expression and disrupts BDNF signaling (Garzon & Fahnestock, 2007). Indeed, an inverse relationship between BDNF expression and Aβ count has been observed in mouse AD models (Peng et al., 2009). These findings might explains why a 23% decrease in mature BDNF was found in AD brains (Ferrer et al., 1999). Therefore, Aβ reduction load would conceivably sustain the activity of NGF and BDNF.

Aβ and Mitochondria. The mitochondria are sensitive to elevated Aβ concentrations. Even though the classical view is that extracellular Aβ deposits are the most injurious, many recent studies have also shown intracellular Aβ accumulation damages the mitochondria. For instance, monomeric and oligomer Aβ have been found to translocate within the mitochondrial membranes of AD brains (Pagani & Eckert, 2011). In mice, mitochondrial Aβ decreased cytochrome oxidase activity and stimulated reactive oxygen species (ROS) production. This fragemented DNA, suppressed dendritic growth, and decreased overall mitochondrial function (Manczak et al., 2010). Undeniably, subcellular Aβ induces mitochondrial damage, but Aβ deposits stimulate the amyloidogenic APP processing pathway, which might be the reason for excessive subcellular Aβ accumulation (Reddy et al., 2010). Nevertheless, Aβ unequivocally damages mitochondria even though the underlying mechanisms of Aβ secretion and formation still are not fully understood.

Treatments

There are no treatments that reverse or prevent AD. Preliminary data suggests lifestyle factors might delay cognitive decline, but no large-scale intervention has yielded significant results (Norton et al., 2014). Those with AD are treated with pharmaceuticals only after diagnosis. The drugs used to treat AD include cholinesterase inhibitors, memantine, and several antipsychotics. However, findings show they are modestly effective at best, justifying the need to explore alternative strategies (Bond et al., 2012).

Cholinesterase Inhibitors. Cholinesterase inhibitors were created to offset cholinergic neuron degeneration. Currently, the American Academy of Neurology recommends the use of donepezil, rivastigmine, and galantamine to treat mild to

moderate AD (Doody et al., 2001). Recommendations have been reinforced by RCTs that show cognitive improvements (Kaduszkiewicz, Zimmermann, Beck-Bornholdt, & van den Bussche, 2005). Despite donepezil, rivastigmine, and galantamine differing in pharmacological properties, the underlying mechanisms are the same. These drugs stop the breakdown of acetylcholine in the synaptic cleft by blocking acetylcholinesterase. However, the most cholinesterase drugs can do is modify AD symptoms and cannot be used as a primary treatment (Di Santo, Prinelli, Adorni, Caltagirone, & Musicco, 2013).

Numerous RCTs have examined the effect of the three cholinesterase inhibitors on AD patients. A recent meta-analysis examined the efficacy of cholinesterase inhibitors on cognition scores in AD using data from 34 RCTs. Findings showed that the effect size of donepezil, rivastigmine, and galantamine was .38, .30, and 0.37 respectively ($p<.0001$) (Di Santo et al., 2013). However, cholinesterase inhibitors are not recommended to treat MCI because the evidence for efficacy is weak and usage is associated with substantial adverse health outcomes. Data from 5149 MCI cases revealed cholinesterase inhibitor usage was associated with a significantly higher relative risk (RR) of muscle spasms (RR: 7.52; 95% CI: 4.34-13.02), headaches (RR: 1.34; 95% CI: 1.05-1.71), gastrointestinal problems (RR: 2.10: 95% CI: 1.30-3.39), dizziness (RR: 1.62; 95% CI: 1.36-1.93), and insomnia (RR: 1.66; 95% CI: 1.36-2.02) (Russ & Morling, 2012). While cholinesterase inhibitors are mildly effective for those already with AD, they cannot be used as a preventive treatment.

Memantine. Memantine is used to reduce the severity of cognitive impairment. It reduces neuron toxicity by acting as an antagonist to NMDARs. This inhibits neuron apoptosis by normalizing intracellular Ca^{2+} levels (Lipton, 2005). Memantine also

decreases Aβ by inhibiting the amyloidogenic APP processing pathway (Alley et al., 2010). In 2003, the USA Food and Drug Administration approved memantine to treat moderately to severe AD (McShane, Areosa Sastre, & Minakaran, 2006). Memantine recommendations are polarized in the current literature, with some data suggesting they are not cost-effective and other data supporting usage (Tampi & van Dyck, 2007).

Like cholinesterase inhibitors, many studies have examined the effect of memantine on cognitive outcomes. What has been found is that the effects of memantine are larger in moderate to severe AD. Memantine usage after six months improved overall cognition and daily activity competence; however, in mild to moderate AD, memantine usage only improved cognition by 0.99 points on the 70-point Alzheimer's Disease Assessment Scale-cognitive subscale (95% CI: 0.21-1.78; p=0.01) (McShane et al., 2006). In regards to using memantine as a primary treatment, there are no reliable data that suggest memantine decreases cognitive decline in MCI. It is unlikely that there would be benefits because the effects of memantine in mild to moderate AD are minimal (Karakaya, Fußer, Schröder, & Pantel, 2013).

Studies have also evaluated combined dosages of memantine and cholinesterase inhibitors. There are limited data that suggests dual-therapy is better. The only evidence that it is more effective are drawn from retrospective analyses (Gareri et al., 2014), which is limited by small sample size, lack of control group, and selection bias. It is unlikely that combined usage would be successful for MCI because both therapies alone are modest.

Antipsychotic drugs. AD patients exhibit behavioral and psychological symptoms (BPSD), including delusions, anxiety, depression, aggression, agitation, and

apathy (Alexopoulos et al., 2007). BPSD is considered to be the most influential factor for increased caregiver burden (S. Zhang, Guo, Edwards, Yates, & Li, 2014). Therefore, to improve quality of life and reduce caregiver burden, several antipsychotic drugs are frequently prescribed to AD patients. To treat depression, serotonin reuptake inhibitors (SSRIs) are given, but its efficacy is questioned. Data show that SSRIs did not improve depression scores in AD patients in comparison to AD patients who received a placebo. In fact, caregivers of these AD patients who received the placebo paradoxically experienced higher quality of life and better mental health (Banerjee et al., 2011). To treat general BPSP, a variety of drug brands are prescribed, such as olanzapine, quetiapine, and risperidone. However, these are associated with faster cognitive decline than controls in AD patients. After 36 weeks, patients on antipsychotic drugs scored worse on the MMSE by 2.4 points than controls ($p=0.004$) (Vigen et al., 2011). Despite the rationale to administer antipsychotic drugs, some data suggest prescriptions should be reconsidered.

Healthcare clearly lacks worthwhile AD treatments. The effects of cholinesterase inhibitors are mild, with the greatest effect seen in mild to moderate AD. The effects of memantine are also mild, but more so in those with moderate to severe dementia (Karakaya et al., 2013). Even drugs that treat BPSD have promoted a faster cognitive decline and increased caregiver burden (Banerjee et al., 2011). Undeniably, there is a need to produce successful primary treatments.

Lifestyle Interventions. Previous researchers have hypothesized that diet and nutrient supplement interventions are protective against AD. Specifically, fatty acid supplementation and low-fat, low-glycemic index intervention studies have been

conducted. In a 39-subject RCT, AD patients who supplemented omega-3 fatty acid combined with lipoic acid experienced less MMSE score decline than did controls ($p<0.01$) (Shinto et al., 2014). Omega-3 fatty acid supplementation might delay cognitive decline epigenetically. Examination of 8,000 genes revealed that omega-3 supplementation up-regulated nine genes and down-regulated 10 genes, many of which suspected to be involved in inflammation and neuron survival (Vedin et al., 2012). In addition, low saturated fat and low simple sugar intake lowered CSF $A\beta_{1-42}$ in those with NCI, implying that foods high in saturated fat or have a high glycemic index increase Aβ production (Bayer-Carter et al., 2011). Nevertheless, a nutrition educational program in 11 AD centers did not slow down the rate of decline in AD patients when compared to AD patients who did not receive education (Salva et al., 2011). Even though there appears to be some relationship between diet and AD-related markers, more work is needed in order to incorporate successful diet interventions that are generalizable to the public.

Modifiable risk factors

Modifying risk factors might delay the onset of AD. Seven modifiable risk factors were identified in a 2010 US National Institutes of Health report: diabetes, midlife hypertension, midlife obesity, physical inactivity, depression, smoking, and low educational attainment, based on data from meta-analyses that investigated the association between these modifiable risk factors and AD (Norton et al., 2014). Determined based on pooled data from a variety of studies, the summary RR for AD of each modifiable risk factor was as followed: 1.46 for diabetes (95% CI: 1.20-1.88), 1.61 for midlife hypertension (95% CI: 1.16-2.24), 1.60 for midlife obesity (1.34-1.92), 1.82

for physical inactivity (95% CI: 1.19-2.78), 1.65 for depression (95% CI: 1.42-1.92), 1.59 for smoking (95% CI: 1.15-2.20), and 1.59 for low educational attainment (95% CI: 1.35-1.86) (Barnes & Yaffe, 2011; Cheng, Huang, Deng, & Wang, 2012; Gao et al., 2013; Hamer & Chida, 2009; Peters et al., 2008; TakkoucheéI, 2006). According to estimated population-attributable risks, modifiable risk factors make up 9.6 million of 33.9 million AD cases. AD would decrease by 8-15% if each risk factor decreased by 10-20% per decade (Norton et al., 2014). With such a large percentage of AD attributable to modifiable risk factors, further research exploring other potential risk factors should be considered.

Diagnostics

According to the National Institute of Neurological Disorders and Stroke (NINDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA), AD is diagnosed when criteria for dementia are met, symptoms worsen over months to years, and psychological batteries indicate amnestic and nonamnestic deficits. Diagnostic certainty is increased when combining CSF samples, structural magnetic resonance imaging (sMRI), functional MRI (fMRI), and positron emission tomography (PET). These tools not only reflect Aβ and tau pathology, but also show structural differences and real-time cerebral activity disruption. Still, confirmation of AD can only be made post-mortem by the identification of plaques and tangles (McKhann et al., 2011).

Physicians assess Aβ and tau pathology using CSF samples and PET scans. Concentrations of Aβ and tau are different in living AD patients compared to cognitively normal people. For instance, levels of CSF $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}40}$ are lower than cognitively normal controls, and hyperphosphorylated tau levels are much higher in living AD

patients (Mattsson et al., 2009). In combination, PET scans also differentiate between AD and NCI cases. An individual is classified as Aβ positive if a high amount of radiolabeled F 18-florbetapir-P binds to brain Aβ (Klunk et al., 2004). However, PET tracers are still being investigated to quantify tau (W. Zhang et al., 2011).

Physicians additionally assess structural and cerebral activity irregularities using several imaging tools, including PET, fMRI, and sMRI. Brain glucose utilization decreases in AD, measured by PET. Overall brain blood flow also decreases in AD, measured by functional MRI (fMRI). Findings from PET and fMRI can be supplemented with structural MRI (sMRI), a scan that quantifies the severity of brain atrophy (Wurtman, 2014). Altogether, CSF, sMRI, fMRI, and PET scans improve diagnostic certainty, but the expensive and invasive nature do not allow for large-scale screening.

Needs

Costs. Almost $226 billion will be spent on AD treatment in 2015, and 68% of such costs are coming from Medicare and Medicaid (Alzheimer's, 2015). The societal cost burden is similar to that of cancer or heart disease (Hurd, Martorell, Delavande, Mullen, & Langa, 2013). By 2050, it could cost nearly $1 trillion to cover the projected 13.5 million diagnosed AD cases (Alzheimer's, 2015). Delaying the onset of AD by 5 years would not only decrease Medicare costs by 25%, but total AD costs by 34% (Sperling et al., 2011).

Much of the costs for AD come from diagnosing and monitoring disease progression. Psychological examinations cost roughly $860; PET and MRI cost nearly $3,130; and a comprehensive panel consisting of CSF biomarkers, imaging, and psychological examination costs about $4,470 (Wimo et al., 2013). However,

psychological batteries might not accurately identify those in the early stages of AD. Even the ADAS-Cog, considered by the field to be the gold standard for AD diagnosis, has produced false-negatives in those with mild symptoms (Hobart et al., 2013). Hence, there is a need to develop more accurate and cost-effective diagnostic tools that can be used to detect early pathological changes and monitor treatments.

Peripheral biomarkers. No peripheral biomarkers are available to diagnose or track AD in living patients. Identifying peripheral biomarker would be a landmark in the field. The non-invasive nature of peripheral biomarkers would allow for early diagnosis in a cost-effective manner (Hampel et al., 2010).

Developing a blood-based biomarker is challenging. One reason is that the temporal association between Aβ plaques or neurofibrillary tangles and AD onset is unclear (Reddy, 2011). Another reason is that the cause of interstitial Aβ accumulation is debated. Both inadequate clearance and overproduction influence interstitial Aβ accumulation, but there is no clear consensus for which method is more influential. (Y. J. Wang, Zhou, & Zhou, 2006). Because of such challenges, no diagnostic cut-offs have been established.

Plasma Aβ as a Possible Biomarker. Plasma Aβ has been systematically examined because of its potential diagnostic and prognostic utility. Measuring plasma Aβ is justified because most interventions wish to alter Aβ metabolism and the assay is relatively inexpensive and commercially available. A recent meta-analysis conducted by Koyama et al. (2012) analyzed whether change in plasma Aβ levels over time predicted AD incidence. RR was determined comparing bottom and top quartiles of data from 13 prospective studies with 10,303 participants. No relationship between $A\beta_{1\text{-}42}$ and onset of

AD was observed (summary RR from nine studies: 1.01; 95% CI: 0.48-2.11; p=0.99), whereas those in the lowest quartile for plasma $A\beta_{1-40}$ were at a borderline significantly higher AD risk (summary RR from seven studies: 1.66; 95% CI: 0.98-2.83; p=0.06). Notably, those in the lowest $A\beta_{42:40}$ quartile were at a 1.60 times higher AD risk (95% CI: 1.04-2.46; p=0.03). Significant heterogeneity, however, exists amongst the examined studied and could be due to different assay methodology and blood collection protocols.

Plasma Aβ and AD risk was also assessed by Chouraki et al. (2014) using the Framingham Study cohort. In total, 2189 NCI participants were followed for 10 years after having plasma Aβ measured at baseline. This is one of the larger sample sizes used to assess plasma Aβ, and the single-center nature of the study eliminates the factors that influenced heterogeneity in prior meta-analyses (e.g., assay variability, blood collection differences, etc.). Higher $A\beta_{1-42}$ at baseline was associated with a lower AD risk (HR per one SD increase in plasma $A\beta_{1-42}$: 0.79; 95% CI: 0.69-0.90; p>0.001). Likewise, higher $A\beta_{42:40}$ was associated with a lower AD risk (HR per one SD increase in $A\beta_{42:40}$: 0.85; 95% CI: 0.73-0.98; p<0.012). However, no significant association for $A\beta_{1-40}$ and AD was observed. In addition, the investigators of this study updated the meta-analysis of Koyama et al. (2012) by adding data from the Framingham Study cohort and unpublished results. Those in the lowest plasma $A\beta_{42:40}$ quartile had a 1.53 greater AD risk than those in the highest quartile (95% CI: 1.09-2.51; p=0.014), while the association of plasma $A\beta_{1-42}$ and $A\beta_{1-40}$ with AD remained insignificant.

Studies have examined the association of plasma $A\beta_{1-40}$ and lifestyle modifications. One cross-sectional study assessed the relationship between physical activity levels and plasma $A\beta_{1-42}$ and $A\beta_{1-40}$. Findings suggest that higher levels of

physical activity might lower the $A\beta_{42}$:$_{40}$ ratio (Brown et al., 2013). Another study examined the effect of high-dosage B-vitamin supplementation on plasma $A\beta_{1\text{-}40.}$ Participants receiving B-vitamin supplements over two years had a 16% lower mean plasma $A\beta_{1\text{-}40}$ change than did controls, albeit results did not achieve traditional significance ($p=0.08$) (Flicker et al., 2008). The third study tested high-dosage curcumin supplementation on plasma $A\beta_{1\text{-}40}$. Those receiving curcumin significantly lowered plasma $A\beta_{1\text{-}40}$ after four weeks of treatment ($p<0.01$) (DiSilvestro, Joseph, Zhao, & Bomser, 2012). Such findings are inconsistent with the literature that suggests low Aβ increases AD risk (Chouraki et al., 2014).

Voids. The National Alzheimer's Project Act of 2011 reflects the urgency to treat AD. The field aims to provide effective treatments by 2025, according to the National Plan to Address Alzheimer's Disease (Naylor et al., 2012). Epidemiological evidence suggests low serum vitamin D increases AD risk (Littlejohns et al., 2014). Hence, examining the relationship between vitamin D status and plasma Aβ is merited.

Vitamin D and Alzheimer's Disease

Overview

Vitamin D is a fat-soluble derivative of cholesterol and a secosteroid. It is necessary for humans because its flexible chemical conformational binds to a variety of proteins (Gropper & Smith, 2008, p. 392). Vitamin D is widely recognized for its role in bone metabolism, but its non-skeletal benefits have become a recent focus in the literature (Fortmann et al., 2013).

Sources. The Recommend Dietary Allowance (RDA) for 51-70 year olds and those greater than 70 year olds is 600 IU and 800 IU respectively. The RDA was

established in order to reflect healthy bone metabolism (Del Valle, Yaktine, Taylor, & Ross, 2011). Dense amounts of vitamin D are found in animal products, including dairy products, fish, and liver. Other food products, such as milk, yogurt, cheese, margarine, and some orange juices, are even fortified with vitamin D in the US. The main form of vitamin D found in animal products is cholecalciferol, and the form found in plants is ergocalciferol. Both dietary ergocalciferol and cholecalciferol are absorbed into the intestinal cell with a micelle and released into the lymphatic system bound to chylomicrons, eventually entering the bloodstream. In addition to diet, vitamin D can be attained through sun exposure. Upon sun UVB exposure, skin-synthesized 7-dehydrocholeterol is converted to precholecalciferol, where UVB photons open the B ring and cause a volatile conformation. Precholecalciferol isomerizes to cholecalciferol after roughly three days to a more stable form, which in turn enters the bloodstream (Gropper & Smith, 2008, pp. 392-393).

Metabolism. Cholecalciferol is the predominant form of circulating vitamin D and must be hydroxylated twice in order to become biologically active. The first hydroxylation occurs in the liver, where cholecalciferol is converted to calcidiol (25-OH, D_3) by the enzyme 25-hydroxylase. Thereafter, calcidiol circulates in the blood stream tightly bound to vitamin D binding protein (DBP), with a half-life of 10 days to three weeks. Because of the long half-life, serum total 25-OH, D is used to assess vitamin D sufficiency. The second hydroxylation occurs in the kidney, where calcidiol is converted to its active form, 1, 25-OH, D_3 (calcitriol) by 1-hydroxylase (Gropper & Smith, 2008, pp. 394-395). Interestingly, although 1-hydroxylase is found primarily in renal tissue, the enzyme is also saturated in neurons and glial cells of the hippocampus, basal forebrain,

hypothalamus, prefrontal cortex, and cingulate gyrus (Eyles, Smith, Kinobe, Hewison, & McGrath, 2005). Upon reaching its target tissue, calcitriol is released from DBP and enters the cell through a vitamin D receptor (VDR). VDRs are not only abundantly expressed in the intestine, kidney, and skin, but also in the central nervous system (Haussler et al., 2008).

Function. Calcitriol is estimated to be a transcription factor for nearly 3.5% of all genes (Dong et al., 2012). It acts on nuclear VDR to induce a conformational change that starts the transcription cascade. The calcitriol-VDR-bound dimer forms a heterodimeric complex, consisting of retinoic acid or retinoid x receptor, which binds to the vitamin d response element (VDRE) on the promoter region of DNA. Additional comodulator proteins bind to the heterodimeric complex on VDRE, connecting more transcriptional cofactors (e.g., SRC family proteins and NCoA-62) with RNA polymerase II (MacDonald, Baudino, Tokumaru, Dowd, & Zhang, 2001). For this reason, limited circulating vitamin D would reduce transcription of several genes.

Low Vitamin D Prevalence

Over 1 billion people in the world are estimated to have insufficient vitamin D levels (serum total 25-OH, D < 30 ng/mL) (F Holick, 2011). Even those living in lower latitudes, where sun exposure is easily attainable, experience insufficiency. In a study conducted in a South Florida population, 38% and 40% of men and women had serum total 25-OH, D below 20 ng/mL (Tangpricha, 2007). Insufficiency is especially prevalent in older populations. According to the National Health and Nutrition Examination Survey (NHANES), 27% of men and 35% of women over 50 have insufficient vitamin D levels. In a review of 42 studies, the amount of vitamin D insufficient elderly individuals ranged

between 40-100% (Barnard & Colón-Emeric, 2010). This is not surprising because the elderly synthesize 75% less cholecalciferol than younger adults (Holick, Matsuoka, & Wortsman, 1989).

Molecular Plausibility for Vitamin D and AD

In addition to its potential for a lifestyle intervention, vitamin D metabolism could also be used as a way to identify pharmaceutical targets that might modulate AD pathology. For example, VDRE is a human multidrug resistance protein 1 (MDR1) promoter, the gene encoding the Aβ-clearing, active transporter P-glycoprotein. This has been shown using a variety of experimental models. In caco-2 cells, calcitriol induced MDR1 expression via VDR activation (Saeki et al., 2008). These results were consistent with studies that showed activated VDR increases MDR1 expression in rat kidney, rat liver, mouse liver, and mouse brain (E. C. Chow, Durk, Cummins, & Pang, 2011; Edwin CY Chow, Sun, Khan, Groothuis, & Pang, 2010). In Tg APP mice, calcitriol even increased hippomcampal P-gp by 250% ($p<0.05$) (Durk et al., 2012). Vitamin D metabolism shows that targeting VDR would yield more P-gp and, in theory, protect against Aβ accumulation.

Indeed, activating VDR using calcitriol increases Aβ clearance in experimental models. The Aβ clearance rate increased in human brain cells by 30% when exposed to calcitriol for three days ($p<0.05$). In Tg APP mice at the plaque-phase, calcitriol decreased soluble Aβ levels by 25-30%, with the highest reductions in the hippocampus and cortex ($p<0.05$) (Durk et al., 2014). These data suggests a noteworthy link between vitamin D and Aβ clearance that is mediated by VDR activation.

Serum Vitamin D and Cognition

There is an association between vitamin D status and cognition. Cross-sectional, prospective, and intervention studies all suggest optimal vitamin D sustains cognition.

Cross-sectional. A strong association between serum total 25-OH, D and cognitive impairment was found in French, Italian, US, and English populations. In a French 95-participant sample, those with serum total 25-OH, D between 4-16 ng/mL were more likely to be cognitively impaired than those with serum total 25-OH, D between 32-78 ng/mL (OR: 59.64; 95% CI: 4.62-533.28) (C Annweiler et al., 2012). In an Italian representative sample, those with serum total 25-OH, D below 10 mg/mL had a four-fold increased risk of cognitive impairment than those over 30 ng/mL (95% CI: 1.37-9.90) (Llewellyn, Lang, Langa, & Melzer, 2010). Specific to US community-dwelling residents, those with higher serum total 25-OH, D were more likely to have higher MMSE scores (β coefficient = 0.05; p= 0.05) (Buell et al., 2009). Risks for US citizens are similar to English citizens. English citizens with serum total 25-OH, D below 10 ng/mL had nearly a three-fold increased risk of cognitive impairment than those above 30 ng/mL (95% CI: 1.48-5.00) (Llewellyn, Langa, & Lang, 2009). These findings suggest that sufficient vitamin D status slows cognitive impairment.

Prospective. The relationship between serum 25-OH, D and cognitive decline has also been examined over time. Wilson et al. (2014) most recently analyzed this concept, enrolling 2,777 US community-dwelling subjects who were 70-79 years old and followed for an average of four years. Cognition was quantified using the 100-point Modified Mini-Mental State Examination. At follow-up, subjects with serum total 25-OH, D between 20 and 29 ng/mL experienced a four times greater cognitive decline than did

those with serum total 25-OH, D greater than or equal to 30 ng/mL ($p=0.05$). Findings from Llewellyn et al. (2010) were similar. Italian women with serum vitamin D less than 10 ng/mL were 60% more likely to have lower MMSE scores than those with serum vitamin D greater than 30 ng/mL (95% CI: 1.19-2.00; $p<0.05$). Also in alignment, findings from Slinin et al. (2012) demonstrated that US community-dwelling women with vitamin D less than 30 ng/mL were more likely to score worse on the MMSE after four years than those over 30 ng/mL (p for trend<0.053). Similarly, Toffanello et al. (2014) concluded that those with serum vitamin D less than 20 ng/mL were 36% more likely to score worse on the MMSE after four years than those over 30 ng/mL (95% CI: 1.14-1.62; $p<0.001$). Therefore, to imply causality, more randomized control trials are certainly justified.

Interventional. Only a few studies have examined the effect of vitamin D supplementation on cognition. One study evaluated the influence of vitamin D supplementation on a variety of cognitive domains. Forty-four subjects were assigned to a vitamin D or placebo group, and cognition was tested after 16 months of supplementation. The supplementation prescriptions varied in dosage according to the patient's physician recommendation (i.e., 800 IU or 100,000 IU month). Nevertheless, those receiving vitamin D supplements scored better on the MMSE, Cognitive Assessment Battery, and Front Assessment Battery after 16 months ($p<0.05$) (Cédric Annweiler, Bruno Fantino, et al., 2012). Another study showed that an 8-40 week supplementation period of cholecalciferol (9000 IUs) did not have a significant effect on cognition in 82 participants (Corless et al., 1987). However, much of the resources available today were not available then. Today, evidence-based vitamin D

supplementation trials have been established, cognitive exams are more robust, and vitamin D assays have improved (Hsieh, Schubert, Hoon, Mioshi, & Hodges, 2013; Kennel, Drake, & Hurley, 2010; Meunier, Montérémal, Faure, & Ducros, 2015). As such, caution needs to be taken when interpreting these interventional results.

Serum Vitamin D and AD Risk

Consistent with studies that suggest adequate vitamin D status sustains cognition, vitamin deficiency is also associated with a considerably higher AD risk. Recent evidence from a US population-based study conducted by Littlejohns et al. (2014) demonstrated that there is a significant association between vitamin D deficiency and AD. In total, 1547 dementia-free participants were analyzed after being followed for a mean of 5.6 years. Participants with serum total 25-OH, D less than 10 ng/mL had a 122% higher risk of developing AD than those greater than or equal to 20 ng/mL (95% CI: 1.02-4.83). In the same population, for each SD increase in serum total 25-OH, D, AD risk decreased by 20% (95 % CI: 0.65-0.99). Moreover, pooled data from six cross-sectional and case-control studies demonstrated that AD patients have significantly lower serum total 25-OH, D than NCI individuals (Balion et al., 2012). Altogether, these imply that vitamin D could be a pivotal factor in AD.

Vitamin D Attainment and AD Risk

How vitamin D is attained (i.e., sun exposure or dietary intake) and whether it is associated with AD has also been examined. One study assessed vitamin D intake with a 21-item food frequency question and a sun exposure question, then evaluated its association with AD after 7 years. Those in the lowest quintile versus the highest quintile of vitamin D intake had a 77% greater chance of developing AD (95% CI: 0.08-0.69). In

the same way, the lowest quintile of low sun exposure had a 45% greater chance of developing AD (95% CI: 0.24-0.85) (Cédric Annweiler, Yves Rolland, et al., 2012). Another study compared diet patterns with AD brain imaging markers. Higher intake of vitamin D was associated with increased brain glucose utilization, decreased brain atrophy, and lower Aβ accumulation (Berti et al., 2014). Both limited dietary vitamin D intake and sun exposure could progress AD.

Summary

Current State of AD and Vitamin D Literature

In AD, excessive brain Aβ accumulation manifests in severe cognitive dysfunction, which is partially mediated by brain atrophy and impaired cerebral activity (Mattsson et al., 2015). Even though Aβ is a natural byproduct of APP metabolism, it must be cleared or degraded (V. W. Chow et al., 2010). One way by which Aβ is cleared is through transportation across the BBB via P-gp. Indeed, evidence from in vitro and in vivo studies revealed that P-gp clears Aβ from the brain to the blood (Kuhnke et al., 2007; Lam et al., 2001). P-gp is coded by the MDR1 gene and requires VDR as a transcription factor. Several studies demonstrated that calcitriol increased P-gp expression and subsequently the rate of Aβ clearance (E. C. Chow et al., 2011; Durk et al., 2012). Such findings might explain why low serum total 25-OH, D was associated with greater cognitive impairment and AD risk.

Vitamin D and AD Voids

Little is known about the effect of vitamin D on AD-related markers. Recent evidence suggests that vitamin D improves cognition, but no study has examined the effect of vitamin D supplementation on outcome measurements that are not cognitive

outcomes (Cédric Annweiler, Bruno Fantino, et al., 2012). Furthermore, the field critically lacks robust peripheral biomarkers. Peripheral biomarkers would be a momentous breakthrough in AD care because they are non-invasive and relatively inexpensive (Hampel et al., 2010). One potential peripheral biomarker is plasma Aβ. Both curcumin and B-vitamin supplementation lowered plasma Aβ, and one cross-sectional analysis confirmed that physical activity was associated with lower plasma Aβ (DiSilvestro et al., 2012; Flicker et al., 2008). However, these findings contradict large-scale epidemiological data that suggest low plasma Aβ at baseline increases AD risk (Chouraki et al., 2014). The reason for the inconsistent findings could be because curcumin and B-vitamin supplementation decreased Aβ production, a pathway that does not directly influence Aβ transportation to the periphery. Another problem AD researchers have encountered is that it is unknown whether Aβ accumulation is a result of overproduction, inadequate clearance, or a combination of both (Mawuenyega et al., 2010; L.-B. Yang et al., 2003). Altogether, these voids need to be addressed in order to develop successful AD therapies.

Addressing Voids

More rigorous RCTs are needed to examine whether vitamin D modulates AD pathology. Since some studies have found AD pathology to begin years before symptoms manifest, RCTs that use non-psychological measures are needed (Morris, 2005). Assessing the effect of vitamin D supplementation on plasma Aβ in humans addresses several voids, including how vitamin D modulates AD pathology and what determinants for plasma Aβ exist. As a result of more research, the field might progress towards

establishing better and cost-effective treatment strategies, ultimately meeting the treatment demand for the growing disease prevalence.

CHAPTER 3

METHODS

Participants and Study Design

Participant Selection

The protocol was approved by the Arizona State University Institutional Review Board (Appendix A). Participants were eligible to participate if at least 50 years old and vitamin D insufficient (serum total 25-OH, D <30 ng/mL). Participants were excluded if diabetes, kidney disease, liver disease, or blood disorders were reported, or excluded if cognitive impairment was measured. Diabetes has been associated with aberrant plasma Aβ levels (Fishel et al., 2005). Kidney and liver disease impair vitamin D conversion to its active form (Gropper & Smith, 2008, p. 395). Blood disorders could alter plasma Aβ levels (Ghiso et al., 2004). The intention was to include healthy, older subjects and to eliminate known confounding variables of plasma Aβ.

Recruitment

Sites. Participants were recruited through retirement communities (Friendship Village and Beatitudes), Arizona State University (ASU), and email listservs of ASU, New Frontier, and the Arizona Academy of Nutrition and Dietetics. A poster was placed inside the fitness area of Friendship Village, and the investigator spoke at a monthly town hall meeting at Beatitudes.

Screening. The screening process involved two steps. First, the screening survey was administered to interested volunteers in order to assess diabetes, kidney disease, statin usage, and blood disorders (Appendix B). Volunteers could fill out the screening survey in-person or online at surveymonkey.com. Interested volunteers at Friendship

Village and Beatitudes were able to request a screening survey from an employee to return back to the same employee. The employee informed the investigator when screening surveys were ready for pick-up. Second, if the volunteer passed initial screening, a blood draw was scheduled at Friendship Village, Beatitudes, or the Arizona Biomedical Collaborative (ABC) lab of downtown Phoenix to assess serum total 25-OH, D levels.

Enrollment. A total of 152 people completed the screening questionnaire. At the start of recruiting, only people 65 years of age or older were eligible for participation; however, the age minimum was lowered to 50 years of age or older because recruiting was not robust. Out of 152 volunteers, 73 passed initial screening and were scheduled for the second screening, a fasting blood draw. After completing a fasting blood, 24 people qualified and were enrolled. One participant dropped before the trial began per a physician recommendation. A total of 23 started and completed the study. Four participants were removed from analysis due to missing data points. As such, 19 participants were included in analysis.

Design

This study was an eight-week, parallel-arm RCT. Twenty-four subjects were randomly assigned to either a vitamin D supplement group or placebo group. Participants were matched based on age, BMI, and baseline serum total 25-OH, D. Serum total 25-OH, D, plasma $A\beta_{1\text{-}40}$, vitamin D intake (IUs/d), and physical activity (METS/week) were measured at baseline and follow-up. The main outcome measurement was plasma $A\beta_{1\text{-}40}$ change.

Sample Size

A sample size of 601 per group was calculated using the Hedwig Harvard Sample Size Calculator. Significance was set at 0.05 (two-sided) and power was set at 0.8. The sample size was calculated using a previous curcumin supplementation trial and B-vitamin supplementation trial that used plasma Aβ as an outcome measurement (DiSilvestro et al., 2012; Flicker et al., 2008). These two studies aimed to decrease plasma Aβ levels by modulating Aβ production. Due to difficulty recruiting and the exploratory nature of this study, the sample size in this study was 24 (n=12 per group). Based on these two previous studies, the plasma $A\beta_{1\text{-}40}$ change was suspected to be 17.2 $\pm$ 11.1 pg/ml higher in the vitamin D group compared to the control group after the trial. See Appendix C.

Protocol and Procedures

Subjects participated in this trial for approximately eight weeks. Subjects met with the investigator three times. The first visit was step two in the screening process, where serum total 25-OH, D and baseline plasma Aβ were measured. The purpose of the second visit was to collect vitamin D intake and physical activity data, disperse supplements, and review instructions. The purpose of the third visit was to measure follow-up serum total 25-OH, D and plasma Aβ after the supplementation period concluded. See Appendix D.

Visit 1

Participants who passed the initial screening were scheduled for a fasting blood draw at Friendship Village, Beatitudes, or the ABC lab. Participants gave written consent (Appendix E), filled out a brief health-history questionnaire (Appendix F), and were

administered the Mini-Mental State Examination (MMSE) (Appendix G) before the blood draw. The MMSE is the most common tool used to assess incidence of cognitive impairment (Strauss, Sherman, & Spreen, 2006). The brief health-history questionnaire confirmed medicine and supplement usage and allowed participants to express additional health concerns. A trained phlebotomist or Registered Nurse performed the blood draw. Participants were informed of eligibility within 7-10 days (i.e., serum total 25-OH, D < 30 ng/mL). Blood samples and data were stored in the ABC lab.

Visit 2

Eligible participants were asked to schedule a second visit at Friendship Village, Beatitudes, or the ABC lab. Participants were randomized to either the vitamin D group or placebo group. Eight supplements were placed in a brown paper bag, identified by ID number, and given to participants. This was paper-clipped to a manila folder that included a copy of the consent form, supplement instructions, and a calendar. Participants also completed the Brief Vitamin D Questionnaire (BVDQ) (Appendix H) and Community Health Activities Model Program for Seniors questionnaire (CHAMPS) (Appendix I). Both questionnaires were self-administered. The BVDQ reflected intake over the last three months. The CHAMPS questionnaire reflected physical activity over the last four weeks. The investigator verbally explained how to complete both questionnaires. Participants were also reminded once during the eight-week trial to take the supplement once a week.

Visit 3

Participants were scheduled for a final fasting blood draw at Friendship Village, Beatitudes, or the ABC lab roughly one week after supplementation period ended. The

BVDQ and CHAMPS were completed once more. Participant were given a $25 Target gift card after the blood draw, thus concluding subject participation.

Measurements

Survey

Vitamin D intake and physical activity data were collected via self-administered surveys. Vitamin D intake (IUs/day) was quantified using the BVDQ. The BVDQ was validated against a three-day food record, the Block Health History and Habits Questionnaire 1998 (BHHHQ98), and serum total 25-OH, D. Participants in the validation study were postmenopausal women, with a mean age of 63.9 $\pm$ 7.8 years. The tool correlated mildly to the three-day food record ($r=0.43$; $p<0.001$) and BHHHQ98 ($R=0.51$; $p<0.001$), but did not correlate to serum total 25-OH-D ($p>0.05$) (Hacker-Thompson, Schloetter, & Sellmeyer, 2012). Physical activity was quantified using the CHAMPS questionnaire. A randomized control trial was used to determine whether CHAMPS could quantify changes in METs/week and frequency of activities as a result from an exercise trial. The intraclass correlation (ICC) for moderate and greater METs/week was 0.67 and 0.66 respectively after six months. METs/week and frequency were significantly correlated for moderate activities ($R=0.73$; $p<0.05$) and all activities ($R=0.55$; $p<0.05$). At the end of the trial, those in the exercise group increased METs/week by 687 ($p<0.0001$). Participants were older (mean=74 years; SD=6), and 64% of the sample were females (Stewart et al., 2001).

Blood-based

Serum vitamin D and plasma Aβ were analyzed. About 10 mL of blood was drawn into a serum separator tube (red top) for vitamin D analysis, and about 4 mL of

blood was drawn into a sodium heparin tube (green-top) for plasma Aβ analysis. All blood samples were centrifuged for 15 minutes at 3000 rpm within four to six hours after venipuncture. Immediately after centrifuging, one mL of serum was sent to Sonora Quest in a transport tube for total 25-OH, D analysis. Sonora Quest uses LC-MS/MS technology and examines both 25-OH, D_2 and 25-OH, D_3. Additionally, 0.495 mL of plasma was immediately aliquoted into pyrogen/endoxin-free tubes, treated with 0.005 mL of pefabloc (protease inhibitor), and frozen at -80°C. Plasma $A\beta_{1\text{-}40}$ was analyzed in the ABC lab using a commercially available assay according to the manufacture protocol (Aβ40 Human ELISA Kit; Invitrogen; Appendix J). Samples were run in duplicates and diluted two-fold. Dilutions were determined empirically. Samples were read on the Biotek H1 Synergy Monochromatic Plate Reader. A four parameter logistic curve was generated for the standard curve.

Compliance

Compliance was measured using self-reported data. Participants tracked supplement consumption using a calendar given to them. Supplements were to be taken once a week for eight weeks, and participants were to mark which day of the week the supplement was consumed. Calendars were returned at the final visit. Participants who lost the calendar were asked how many pills remained at the final blood draw.

Compliance was quantified by how many pills were consumed. A total of eight pills were given to each participant. For example, a score of "7" was recorded if the participant took seven of the eight pills.

Statistical Analyses

All data was analyzed using IBM SPSS Statistics v.22 (Chicago, IL). Shapiro-Wilk was used to examine normal distribution of data. Skewed data was log-transformed for analysis. Data was reported as mean $\pm$ SE. Baseline values and changes in values between the control group and vitamin D group were assessed.

Independent t-tests were used to evaluate mean baseline BMI, serum total 25-OH, D, plasma $A\beta_{1-40}$, METs/week, and IUs/day differences between the control group and vitamin D group. The Mann-Whitney test was used to assess mean age difference between the two groups, and to also determine whether supplement compliance differed between the two groups.

Univariate analyses of variance were used to examine mean serum total 25-OH, D and plasma $A\beta_{1-40}$ changes between the vitamin D group and control group. Four models were used to assess mean differences. The first model did not control for any variables. The second model controlled for the baseline measurement (i.e., either serum total 25-OH, D or plasma $A\beta_{1-40}$). The third model controlled for baseline measurement and METs/week. The fourth model controlled for baseline measurement, METs/week, and age. Covariates in models were determined using Pearson and Spearman correlations. A partial correlation was used to determine the relationship irrespective of group, using group as a covariate in the model.

CHAPTER 4

RESULTS

Baseline Values

Of the 19 participants, 10 (53%) were in the vitamin D group and 9 (47%) were in the control group. A total of 14 females and 5 males participated. Males made up 30% of the vitamin D group and 22% of the control group. There was no age difference between the two groups (p=1.00). Although not statistically significant, BMI was higher in the control group (2.2 $\pm$ 2.6; p=0.410), despite BMI being used to match cases during randomization. The reason for this is because four participants were removed from analysis after randomization due to missing data points. Descriptive statistics for all 23 participants are included in Appendix K. Serum total 25-OH, D, plasma $A\beta_{1\text{-}40}$, METs/week, and vitamin D IUs/day intake did not differ at baseline. However, plasma $A\beta_{1\text{-}40}$ and METs/week were higher in the control group, even though differences were not statistically significant. The control group had a higher mean plasma $A\beta_{1\text{-}40}$ by 25.0 pg/mL (p=0.079) and higher METs/week by 553.6 (p=0.059). See Table 1.

Table 1

Mean baseline values and mean follow-up values (n=19)[a]

Characteristics	Vitamin D group n=10	Control Group n=9	p value
Baseline values			
Male	3	2	
Female	7	7	
Age	62.5 ± 3.4	64.3 ± 4.3	1.000
BMI	24.9 ± 1.3	27.1 ± 2.3	0.410
Total 25-OH, D (ng/mL)	24.2 ± 1.1	23.2 ± 2.0	0.677
$A\beta_{1\text{-}40}$ (pg/mL)	106.6 ± 9.1	131.6 ± 9.9	0.079
PA (METs/week)[b]	1128.9 ± 219.5	575.3 ± 139.8	0.059
Vitamin D Intake (IUs/day)[b]	230.5 ± 77.0	231.9 ± 77.6	0.916
Follow-up values			
Total 25-OH, D (ng/mL)	50.7 ± 1.2	22.9 ± 2.1	
$A\beta_{1\text{-}40}$ (pg/mL)	121.1 ± 6.0	144.4 ± 8.4	
PA (METs/week)[b]	984.0 ± 253.3	555.6 ± 60.0	
Vitamin D Intake (IUs/day)[b]	323.9 ± 142.7	140.4 ± 38.9	

[a] All values are means ± SE; univariate analyses to examine group differences at baseline except age, which was not normally distributed and the Mann-Whitney test was used.
[b] One missing data point in vitamin D group.

Compliance

Compliance was determined to be sufficient, as all participants but two consumed all eight pills. The two participants who did not consume all pills were in the vitamin D group, but both did consume seven pills. There was no significant compliance difference between the vitamin D group and control group according to the Mann-Whitney test (p=0.167).

Changes in Outcome Measurements

The vitamin D group mean change in serum total 25-OH, D was 538% than that of the control group. The mean serum total 25-OH, D increased 26.6 $\pm$ 1.9 ng/mL in the vitamin D group, whereas its level decreased -0.3 $\pm$ 1.6 ng/mL in the control group. The vitamin D effect size was estimated using four different models. The effect size was 0.870 when not controlling for additional variables ($p<0.001$), 0.902 when controlling for baseline serum total 25-OH, D ($p<0.001$), 0.886 when controlling for baseline serum total 25-OH, D and baseline METs/week ($p<0.001$), and 0.903 when controlling for baseline serum total 25-OH, D, baseline METs/week, and age ($p<0.001$). See Table 2.

Following the intervention, the vitamin D group had a 45% greater change in plasma $A\beta_{1\text{-}40}$ than the control group. Mean plasma $A\beta_{1\text{-}40}$ increased 14.9 $\pm$ 12.0 pg/mL in the vitamin D group, compared to a 12.8 $\pm$ 12.8 pg/mL increase in the control group. Four models were used to assess effect size. The effect size was 0.001 when adjusting for no variables ($p=0.925$). However, the effect size was 0.228 and statistically significant when controlling for baseline plasma $A\beta_{1\text{-}40}$ ($p=0.045$). Although not significant, the effect size was 0.197 when controlling for baseline plasma $A\beta_{1\text{-}40}$ and baseline METS/week ($p=0.085$), and 0.179 when controlling for baseline plasma $A\beta_{1\text{-}40}$, baseline METS/week, and age ($p=0.116$). See Table 2.

Table 2

Mean serum vitamin D change and mean plasma $A\beta_{1-40}$ change (n=19)[a]

Characteristics	Vitamin D Group n=10	Control Group n=9	%Change[c]	η_p^2	p value
Total 25-OH, D Change (ng/mL)	26.5 ± 1.9	-0.3 + 1.6	+538%		
Unadjusted Model[b]				0.870	<0.001
Model 1				0.902	<0.001
Model 2				0.886	<0.001
Model 3				0.903	<0.001
$A\beta_{1-40}$ Change (pg/mL)	14.9 ± 12.0	12.8 ± 12.8	+45%		
Unadjusted Model[b]				0.001	0.925
Model 1				0.228	0.045
Model 2				0.197	0.085
Model 3				0.179	0.116

[a] All values are means ± SE; univariate analysis of variance used to examine mean differences.

[b] Unadjusted Model: Controlled for no additional variables. Model 1: Controlled for baseline value. Model 2: Controlled for baseline $A\beta_{1-40}$ and METS/week. Model 3: Controlled for baseline $A\beta_{1-40}$, METS/week, and age.

[c] Vitamin D group versus control group

Correlations amongst Baseline Values and Change Values

Table 3 reports the relationship amongst baseline age, BMI, serum total 25-OH, D, plasma $A\beta_{1-40}$, METS/week, vitamin D IUs/day, serum total 25-OH, D change, and plasma $A\beta_{1-40}$ change. Five statistically significant relationships were observed. First, baseline METs/week strongly correlated with baseline plasma $A\beta_{1-40}$ (Spearman R= -.83; p=0.002). Second, age mildly correlated with plasma $A\beta_{1-40}$ change (Spearman R= -.48; p= 0.039). Third, baseline physical activity was mildly associated with plasma $A\beta_{1-40}$ change (Spearman R= .60; p= 0.008). Fourth, age and baseline METS/week were mildly

related (Spearman R= -.61; p=0.008). Fifth, baseline plasma $A\beta_{1-40}$ was strongly related to plasma $A\beta_{1-40}$ change (Pearson R= -.75; p<0.001). Furthermore, the relationship between baseline plasma $A\beta_{1-40}$ and plasma $A\beta_{1-40}$ change remained significant independent to group difference (R= -.82; p<0.001), indicating that the reason for the relationship was not due to vitamin D supplementation. See Table 3. See Figure 2.

Table 3
Correlation matrix for baseline values and outcome measurements (n=19)

	1	2	3	4	5	6	7	8
1. Age[b]								
2 BMI[b]	-.26							
3. Total 25-OH, D[b]	-.19	.11						
4. $A\beta_{1-40}$[b]	.25	-.06	.03					
5. Physical Activity[ab]	-.61**	.12	-.06	-.83**				
6. Vitamin D Intake[ac]	.38	.2	-.22	-.07	.11			
7. Total 25-OH, D Change[c]	-.19	-.09	-.08	.46	.43	-.13		
8. $A\beta_{1-40}$ Change[c]	-.48*	.07	.09	-.75**	.60**	-.07	-.01	

[a] One missing data point in vitamin D group
[b] Spearman r
[c] Pearson r
*Significance at the 0.05 level.
**Significance at the 0.01 level.
Mild correlation: r= .30-.49; Moderate correlation: r= .50-.69; Strong correlation: r= .70-1.00

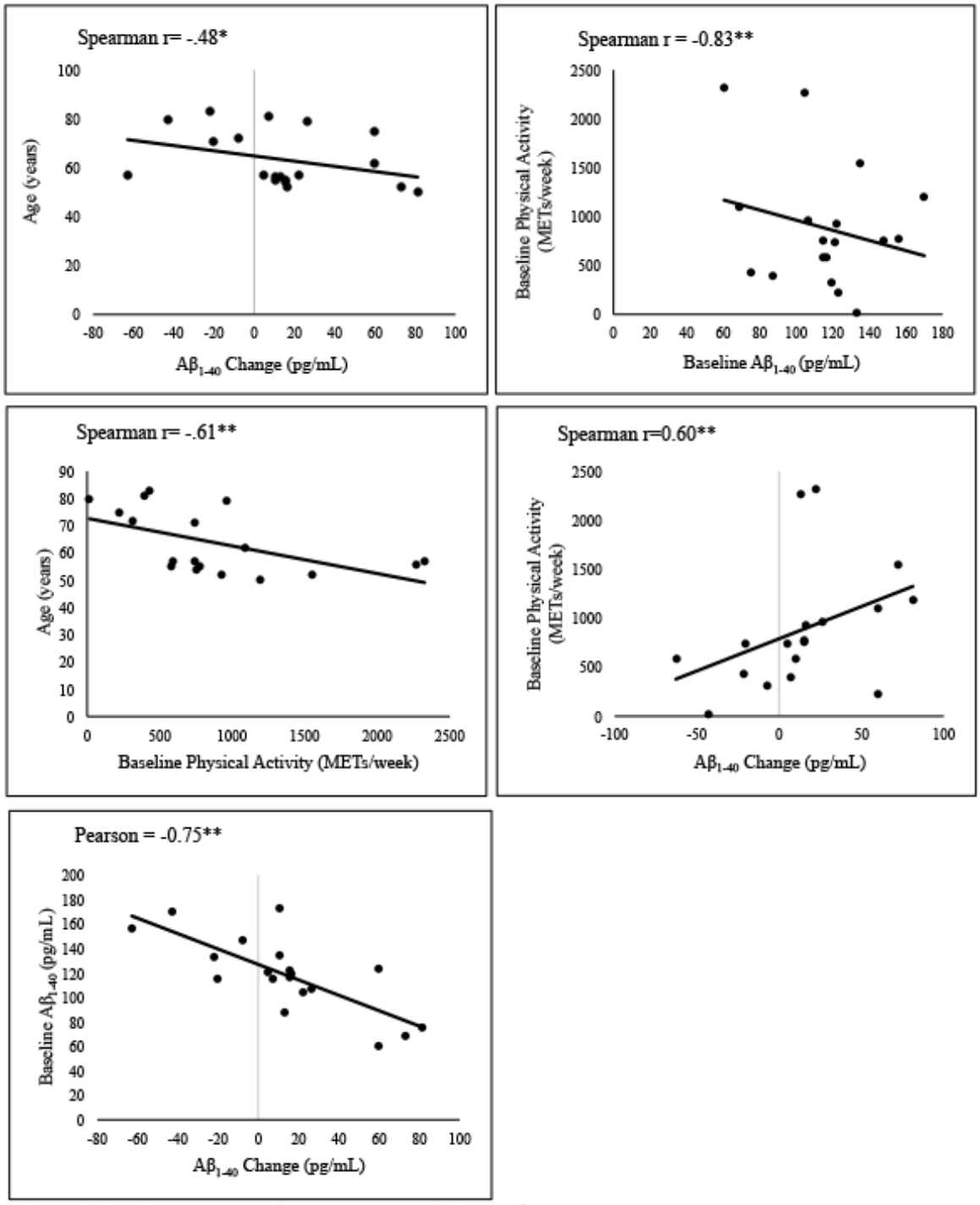

Figure 2. *Correlations amongst baseline values and plasma $A\beta_{1\text{-}40}$.*
n=18 for physical activity
**Significance at 0.05 level*
*** Significance at 0.01 level*

CHAPTER 5

DISCUSSION

The association between serum vitamin D and AD risk is established in the literature, but how vitamin D influences AD pathology has been unclear. Recent research suggests vitamin D up-regulates transporters that clear Aβ to the periphery, specifically P-gp. These studies were conducted using mouse models and human brain endothelial cell lines. Both experimental models showed vitamin D cleared more Aβ that was mediated by increased P-gp production (E. C. Chow et al., 2011; Durk et al., 2012; Durk et al., 2014).

This study was an attempt to expand upon these findings using a randomized, double-blinded RCT design, where humans consumed one vitamin D or placebo capsule once a week for eight weeks. Changes in plasma $A\beta_{1\text{-}40}$ and serum total 25-OH, D were compared between the vitamin D group and control group.

The vitamin D supplementation protocol needed to be successful in order to examine plasma $A\beta_{1\text{-}40}$ change differences between the groups. The vitamin D group almost doubled baseline serum 25-OH, D levels after finishing the eight supplements, whereas there was little change in serum 25-OH, D levels in the control group. Undeniably, the vitamin D protocol was robust.

The vitamin D group experienced a higher plasma $A\beta_{1\text{-}40}$ change than the control group, which was significant when controlling for baseline plasma $A\beta_{1\text{-}40}$ levels. However, significance was lost when further controlling for age and physical activity, suggesting that both factors influenced overall $A\beta_{1\text{-}40}$ change. This is not surprising since age has been previously correlated with plasma Aβ, and physical activity has been shown

to alter Aβ metabolism (Brown et al., 2013; Fukumoto et al., 2003). Therefore, vitamin D supplementation might have increased plasma $A\beta_{1\text{-}40}$, but its effect was smaller and considered non-significant when factoring in age and physical activity.

The observation that baseline $A\beta_{1\text{-}40}$ predicts overall $A\beta_{1\text{-}40}$ change is worth noting. The relationship was also observed independent to group difference, meaning that an increase in plasma $A\beta_{1\text{-}40}$ was greater in those started with lower levels regardless of vitamin D supplementation.

Peripheral Sink Hypothesis

The literature suggests that brain Aβ and peripheral free Aβ have homeostatic tendencies, essentially operating as a concentration gradient. It has been suggested that brain Aβ will only be cleared if the periphery Aβ concentration is lowered. Indeed, several experimental models have shown peripheral Aβ degradation reduces cerebral Aβ burden (Marques et al., 2009; Vasilevko, Xu, Previti, Van Nostrand, & Cribbs, 2007). On the contrary, recent evidence points against the periphery sink hypothesis. For example, selective peripheral BACE1 inhibition decreased peripheral Aβ, but did not alter cerebral Aβ in mice (Georgievska et al., 2015). Likewise, cerebral Aβ levels did not decrease following neprilysin-induced peripheral Aβ degradation in non-human primates (Henderson et al., 2014). However, BBB function was not evaluated in such studies. For Aβ to be cleared, a functional BBB with a sufficient amount of active transporters would theoretically have to be available (Wildsmith et al., 2013)

The fact that these study participants with low baseline $A\beta_{1\text{-}40}$ levels experienced a greater $A\beta_{1\text{-}40}$ change after vitamin D supplementation might be explained by a peripheral sink mechanism. Low baseline $A\beta_{1\text{-}40}$ levels in the periphery could suggest that

there are not enough P-gp transporters to transport Aβ, leading to an inability to achieve Aβ homeostasis. In theory, vitamin D would allow for Aβ homeostasis by producing more P-gp. The intent of this study was to increase $A\beta_{1\text{-}40}$ by increasing the amount of P-gp. Nevertheless, controls also experienced a greater $A\beta_{1\text{-}40}$ change when baseline $A\beta_{1\text{-}40}$ levels were low. Additionally, low plasma $A\beta_{1\text{-}40}$ might also indicate low brain $A\beta_{1\text{-}40}$ production. Therefore, it cannot be certain that a peripheral sink mechanism was observed.

Physical Activity Mechanisms

Epidemiological data suggest physical activity decreases AD risk (Scarmeas et al., 2009). The pathways for which physical activity decreases AD risk are related to the cardiovascular system, but recent evidence suggests it also modulates Aβ metabolism. Brown et al. (2013) examined the effect of physical activity on plasma $A\beta_{1\text{-}40}$ and plasma $A\beta_{1\text{-}42}$. Lower levels of physical activity were associated with a higher $A\beta_{42:40}$ ratio, suggesting that physical activity modulates Aβ metabolism. No association between plasma $A\beta_{1\text{-}40}$ and physical activity was found by Brown et al. (2013), whereas these findings did suggest an association. Nonetheless, this study is in agreement that Aβ metabolism might be influenced by physical activity levels. Being that the mean plasma $A\beta_{1\text{-}40}$ change between the vitamin D group and control group was not significant after controlling for physical activity, more research regarding the effect of physical activity on AD pathology is warranted.

Immunological Mechanisms

Macrophage activity has been found to be lower in AD brains, leading to an inability to degrade brain Aβ, but vitamin D has been shown to restore innate immunity

function in the brain. Experimental models have shown vitamin D to activate type II macrophages (Mizwickia et al., 2012). Hence, increasing circulating vitamin D has the potential to increase brain Aβ degradation via the restoration of innate immunity. Immune mechanisms were not evaluated in this study. Although evidence points towards vitamin D increasing Aβ transport out of the brain, future attempts to assess clearance must also consider its potential immunological effects.

Peripheral Etiology

The etiology of AD has been theorized to start in the periphery and manifest in the brain. Brain Aβ has been associated with AD, but the brain is not the lone source of Aβ production. For example, platelets are a major source of Aβ. Platelet-secreted can enter the brain from the periphery. Many prior attempts to alter Aβ pathology have focused specifically on disrupting brain Aβ metabolism, despite the fact that platelets produce nearly 90% of Aβ in the blood (Chen, Inestrosa, Ross, & Fernandez, 1995). New perspectives in AD treatment even involve targeting peripheral Aβ (Decourt et al., 2013).

The intent of this study was to measure brain Aβ clearance indirectly by assessing plasma $A\beta_{1\text{-}40}$ change. Being that platelets produce the majority of blood Aβ, the effects of vitamin D on peripheral Aβ metabolism need to be considered. Evidence suggests that vitamin D up-regulates neprilysin production, one of the enzymes that can degrade $A\beta_{1\text{-}40}$ even in the periphery (Grimm et al., 2014; Henderson et al., 2014). Therefore, perhaps vitamin D also influences peripheral-based Aβ degradation.

Validity and Reliability

Blood-based

For plasma $A\beta_{1-40}$, ELISA tests were run in duplicates and the mean intrassay coefficient of variation was 6.5%. Mean plasma $A\beta_{1-40}$ measurements were consistent with previously reported data that measured $A\beta_{1-40}$ using an ELISA technique (Brown et al., 2013). The amount of $A\beta_{1-40}$ change was also within the range of previously reported changes in intervention-based studies (DiSilvestro et al., 2012; Flicker et al., 2008). For serum total 25-OH, D, all participants in this study had levels less than 30 ng/mL. This cutoff was determined by examining when PTH levels plateaued with serum total 25-OH, D levels (Chapuy et al., 1997).

Questionnaire

The vitamin D IUs/d mean of this study population was similar to the range reported in the BVDQ validation study (Hacker-Thompson et al., 2012). The METs/week mean of this study population was within the ACSM recommendation of 500-1000 METs/week (Garber et al., 2011). Hence, both surveys did not produce abnormal measurements.

Strengths

This study has many strengths. This was the first study to examine the effect of vitamin D supplementation on AD pathology in humans using non-psychological measurements. The only way to detect AD pathological modulations in younger people is with outcome measurements that are not cognitively based. Furthermore, only people with insufficient serum vitamin D, according to LC-MS/MS technology, were included. The LC-MS/MS measurement technology is considered to be the most accurate of all

vitamin D assays (Roth, Schmidt-Gayk, Weber, & Niederau, 2008). This ensured that the effect of vitamin D supplementation on vitamin D status could accurately be quantified. Overall, this study was a novel concept that expanded upon recent in vitro and in vivo studies quickly.

Limitations

There are several limitations to this study. First, although the vitamin D group experienced an increased plasma $A\beta_{1\text{-}40}$, so did the control group. Baseline plasma $A\beta_{1}$-40 also correlated with plasma $A\beta_{1\text{-}40}$ change independent to group difference. There are several possible reasons for this. One reason is that only free plasma $A\beta_{1\text{-}40}$ was measured. Another reason is that $A\beta_{1\text{-}40}$ is produced by peripheral organs, including the pancreas, liver, and platelets, and can also bind to plasma proteins (Kuo et al., 2000; Roher et al., 2009). Even the participant's state during the blood draw also might impact plasma Aβ measurements, (e.g., hydration status). Second, physical activity and vitamin D intake data were self-reported and ought to be interpreted with caution due to the possibility of measurement error. Third, additional dietary data was not collected, such as caffeine intake and overall macronutrient and micronutrient composition. Fourth, the ApoE4 allele was not controlled for, which is strongly associated with AD pathology. As a substitute, all participants on statins were excluded because ApoE4 is associated with high serum cholesterol (Bennet et al., 2007). Fifth, generalizability is limited because of a small sample size. Finally, participants were Arizona residents, non-diabetic, without dementia, not taking statins, without kidney disease, and without blood disorders. As such, results cannot be generalizable to individuals who do not have these characteristics.

CHAPTER 6

CONCLUSION

Findings from this research suggest that vitamin D supplementation might enhance brain Aβ transportation to the periphery. In this study, those receiving the vitamin D supplement experienced a greater plasma $A\beta_{1\text{-}40}$ change than those receiving a placebo. The effect was considered significant when controlling for baseline $A\beta_{1\text{-}40}$, but was considered to be non-significant when controlling for physical activity and age.

This study is considered a pilot study because it was the first of its kind, the sample size was small, there are additional plasma Aβ measurement tools, and supplementary measurements could be included to improve validity. This study sample size was 19, although a sample size of roughly 600 was calculated using previous studies that used plasma $A\beta_{1\text{-}40}$ as an outcome measurement. Additionally, the INNO-BIA plasma Aβ assay, which uses xMAP technology, has become increasingly popular in the literature because it measures $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42,}$ and its truncated forms simultaneously with high precision (Lachnoa et al., 2012). To ensure homogeneity and reproducibility, future studies should consider measuring plasma Aβ with this technology. Validity can also be improved by including supplementary outcome measurements to plasma Aβ. For example, including TNF-α, IL-6, neprilysin, and BACE1 blood measurements might provide further insight to how vitamin D modulates AD pathology. Evaluating TNF-α, IL-6, and neprilysin would offer more insight into possible immunological mechanisms, and BACE1 measurements would provide information about peripheral Aβ metabolism.

Using vitamin D as a mode to identify AD pharmaceutical targets is a justifiable strategy to develop successful treatments. Vitamin D can be used to monitor cellular

changes in order to help understand AD pathology. The ultimate conclusion as a result from more vitamin D research might not exclusively be a vitamin D supplementation recommendation, but also the identification of additional powerful agents that can modulate AD pathology.

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APPENDIX A

IRB APPROVAL

ASU Knowledge Enterprise Development

APPROVAL: MODIFICATION

Carol Johnston
SNHP - Nutrition
602/827-2265
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 2/27/2015 the ASU IRB reviewed the following protocol:

Type of Review:	Modification
Title:	The Effect of Vitamin D Supplementation on Plasma Aβ in an Elderly Population: A Randomized Control Trial
Investigator:	Carol Johnston
IRB ID:	STUDY00001924
Funding:	None
Grant Title:	None
Grant ID:	None
Documents Reviewed:	• Dietary vitamin D questionnaire, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • consent - modified, Category: Consent Form; • protocol , Category: IRB Protocol; • Friendship Village email, Category: Off-site authorizations (school permission, other IRB approvals, Tribal permission etc); • Physical activity questionnaire, Category: Screening forms; • Health history, Category: Screening forms; • research ad, Category: Recruitment Materials; • MMSE, Category: Screening forms;

The IRB approved the modification.

When consent is appropriate, you must use final, watermarked versions available under the “Documents” tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:

APPENDIX B

SCREENING SURVEY

1. Please provide your primary email address (if applicable):

_____

2. Please provide your phone number.

_____

2. What is your preferred way of communication?

Email OR Phone

3. Are you 50 years of age or older? Yes No

5. Are you consuming any medications? Yes No

6. If you answered yes to the above question, list medications currently using:

_____
_____
_____
_____
_____

7. Are you taking any nutritional supplements? Yes No

8. If you answered yes to the above question, list nutritional supplements currently using:

_____
_____
_____
_____
_____

9. Has your physician diagnosed you with any form of dementia?

Yes No

10. Has your physician diagnosed you, currently, with high cholesterol?

Yes No

11. Has your physician diagnosed you, currently, with diabetes?

Yes No

12. Has your physician diagnosed you with kidney disease?

Yes No

13. Has your physician diagnosed you with liver disease?

Yes No

14. Would you be willing to visit with ASU investigators at Friendship Village, Beatitudes, or the downtown Phoenix ASU lab three times within a 8 week span?

Yes No

15. Are you ok with providing a blood sample from the arm vein? (a trained phlebotomist will perform the blood draw)

Yes No

APPENDIX C

SAMPLE SIZE CALCULATOR

	Author	Year	Aβ40 Change	N per group	Calculated n per group	Age	Subject State	Test
1	DiSilvestro	2012	-2.0 ± 8.7 pg/ml	19	598	47 ± 5	Healthy, no incidence of dementia, CVD, smoking, cancer, gingivitis, use of phytochemical concentrates	Curcumin extract supplementation on plasma Aβ40 (parallel arm)
2	Flicker	2008	-19.8 ± 11.5 pg/ml	113	604	79.3 ± 0.2	Healthy, no incidence of dementia, depression, and outstanding medical problem	B vitamin supplementation on plasma Aβ40 (parallel arm
	AVERAGE		-17.2 ± 11.1 pg/ml	66	601	76.7		

All data reported as mean + SE

APPENDIX D

METHODS FLOW CHART

RECRUITMENT

- ASU, New Frontier, AZ Nutrition and Dietetics email listervs
- Poster boards and flyers placed at Friendship Village and downtown Phoenix ASU campus
- Presentation at Beatitudes
- Interested volunteers completed screning survey online or in-person

VISIT 1

- Consent
- Mini-mental state examination
- Health history survey
- Fasting blood draw (baseline vitamin D and plasma Aβ)

VISIT 2

- Disperse supplements
- Vit D and PA survey
- Instructions

SUBJECTS ENROLLED AND RANDOMIZED

N=12 per group
Double-blind
Placebo controlled
Parallel arm

8-WEEK SUPPLEMENT PERIOD

VISIT 3

- Fasting blood draw (follow-up serum vitamin D and plasma Aβ)
- Vitamin D and PA survey
- Target gift card

APPENDIX E

MEDICAL HISTORY QUESTIONNAIRE

HEALTH HISTORY QUESTIONNAIRE ID#_____

1. Gender: M F

2. To be filled in by investigator: Body weight _____ Height _____

3. Age: _____

4. Ethnicity: (please circle) Native American African-American Caucasian Hispanic Asian Other

5. Have you been treated by a physician for high cholesterol? Yes No
   If yes please explain:

_____

6. Have you lost or gained a significant amount of weight in the last 12 months? Yes No
   If yes, how much lost or gained? _____ How long ago? _____

7. Have you been diagnosed with diabetes?
   Yes No

8. Have you been diagnosed with kidney disease or liver disease?
   Yes No

9. Do you currently take any medications?
   Yes No

If yes, please list type and frequency:

Medication	Dosage	Frequency

10. Have you take vitamin D supplements in the past 2 years?
Yes No

11. Have you ever been told by a doctor that you are low in vitamin D?
Yes No

12. Do you currently take any other supplements (vitamins, minerals, herbs, etc.) ?
Yes No

If yes, list type and frequency:

Supplement	Dosage	Frequency

13. Have you been hospitalized within the last 2 years? Yes No

If yes, please explain:

_____
_____
_____
_____
_____

14. Have you been diagnosed with any form of autoimmune disease (e.g., Chron's, Celiac's, etc.)? Yes No

15. Do you get nervous or faint when blood is drawn from an arm vein?
Yes No

16. Will you have any concerns consuming a large gel capsule? (once weekly)
Yes No

17. Are there any other health-related issues you need to tell us before starting this trial?
Yes No

If yes, please explain:

_____
_____
_____
_____

APPENDIX F

CONSENT FORM

CONSENT FORM

Title of research study: Vitamin D supplementation for clearing amyloid proteins from the brain in senior citizens: a randomized controlled trial

Investigators: Carol Johnston, ASU Nutrition Professor; Brendan Miller, Nutrition Master's student

Why am I being invited to take part in a research study?

We invite you to take part in this research study because you are a healthy adult over the age of 50 with a vitamin D insufficiency and without diabetes, kidney disease, liver disease, or conditions that would affect platelet levels.

Why is this research being done?

The purpose of this research is to examine the effect of vitamin D on clearing harmful proteins out of the brain. There are a growing number of people who are developing Alzheimer's disease because of a buildup of amyloid proteins in the brain. Identifying factors that might contribute to progression of this disease is important because the number of Alzheimer's disease cases is expected to exponentially increase in the next decade.

How long will the research last?

We expect individuals to spend 8 weeks participating in the proposed activities.

How many people will be studied?

We expect about 40 people will participate in this research study.

What happens if I say yes, I want to be in this research?

You will be a participant in this study for approximately 8 weeks. Your participation includes three visits: one for initial screening and blood draw, the second for dispersion of supplement and instructions, and third for your final blood draw. You will only need to have your blood drawn twice: once at the beginning of the study and once at the end of the 8-week supplementation period. Approximately 2 tablespoons of blood will be collected at each blood draw. Your first visit will be a screening to evaluate vitamin D levels in the blood and memory. If you qualify and are fasting, your blood will be drawn at that time; or if you are not fasting, this visit will be rescheduled. At the second visit, you will be given either a vitamin D supplement or placebo to consume once a week for 8 weeks. In addition to instructions, you will be given a compliance calendar to note what day of the week the supplement was consumed and will be asked questions related to physical activity and diet. The day you consume the supplement will be the same day every week (e.g., once on Monday every week). The length of each visit will

approximately last 15-20 minutes. During the research process, you will interact with the research team, consisting of the investigator, a registered nurse, and a lab technician. The contact information of the investigators will be provided to you. The processing of the blood sample will be done at the Arizona Biomedical Collaborative laboratory (ABC) in downtown Phoenix. The research team is testing for the molecules of beta amyloid from your blood sample. There are two groups in this research trial. One group will be given a vitamin D supplement, whereas the other group will be given a placebo. You will not know which group you fall into, nor will the investigator. The research trial is expected to last from April 2015-May 2015.

What happens if I say yes, but I change my mind later?

You can leave the research at any time it will not be held against you. If you decide to leave the research, there will be no adverse consequences. If you decide to leave the research, contact the investigator so that the investigator can appropriately exclude you from the trial.

Is there any way being in this study could be bad for me?

While taking the vitamin D supplement is likely safe in the prescribed amounts (1 capsule, 1 time per week for 8 weeks), there are potential physical risks associated with consuming the vitamin D supplement in toxic amounts. If you accidentally consume too many capsules, calcium levels in your blood may rise. This calcium rise can leave you feeling weak, nauseated, with a headache, and at a risk for kidney stones. You may feel temporarily nauseous or faint during the blood draw. Blood draws are performed by a registered nurse or a trained phlebotomist who are experienced in handling these issues. Taking part in this research study may lead to added costs and time commitments. Driving to the ASU nutrition laboratory in Phoenix will entail gas costs, and you may need to pay the meter for curbside parking (costs should be < 50 cents).

Will being in this study help me any way?

We cannot promise any benefits to you or others from your taking part in this research. However, your participation will help the scientists advance knowledge regarding vitamin D and Alzheimer's disease.

What happens to the information collected for the research?

Efforts will be made to limit the use and disclosure of your personal information, including research study data, to people who have a need to review this information. We cannot promise complete secrecy. Organizations that may inspect and copy your information include the Institutional Review Board at Arizona State University and other representatives of this organization. All data and blood samples collected during this study will be identified only by a number assigned to you and stored in a secure setting in the ABC laboratory.

What else do I need to know?

If you agree to participate in the study, written consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury.

Who can I talk to?

If you have questions, concerns, or complaints, or think the research has hurt you, talk to the research team at Arizona State University. You may email or call the investigators [Carol.Johnston@asu.edu (602)827-2265 or Brendan.Miller@asu.edu (480)580-0099] to express any concerns.

This research has been reviewed and approved by the Bioscience Institutional Review Board at ASU. You may talk to them at (480) 965-6788 or research.integrity@asu.edu if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You cannot reach the research team.
- You want to talk to someone besides the research team.
- You have questions about your rights as a research participant.
  You want to get information or provide input about this research.

Your signature documents your permission to take part in this research.

Signature of participant	Date
Printed name of participant	Email or phone#
Signature of person obtaining consent	Date

APPENDIX G

MINI-MENTAL STATE EXAMINATION

ID#: _____

Right / Wrong? - 30 questions for 30 points

ORIENTATION – 10 points

Ask the following questions:

1. What is today's date?
2. What is the month?
3. What is the year?
4. What day of the week is it today?
5. What season is it?
6. What is the name of this clinic (place)?
7. What floor are we on?
8. What city are we in?
9. What county are we in?
10. What country are we in? **Orientation subtotal = /10**

IMMEDIATE RECALL – 3 points

Ask the subject if you may test his/her memory. Then say "ball", "flag", "tree" clearly and slowly, about 1 second for each. After you have said all 3 words, ask him/her to repeat them - the *first* repetition determines the score (0-3):

11. BALL
12. FLAG
13. TREE **Recall subtotal = /3**

ATTENTION – 5 points

NB PERFORM SERIAL 7S **OR** 'WORLD' BACKWARDS **BUT NOT BOTH**!

A) Ask the subject to begin with 100 and count backwards by 7. Stop after 5 subtractions. Score the correct subtractions.

14. "93"
15. "86"
16. "79"
17. "72"
18. "65"

B) Ask the subject to spell the word "WORLD" backwards. The score is the number of letters in correct position. For example, "DLROW" is 5, "DLORW" is 3, "LROWD" is 0.

"D"
"L"
"R"
"O"
"W" **"DLROW" or Serial 7s subtotal = /5**

DELAYED VERBAL RECALL – 3 points

Ask the subject to recall the 3 words you previously asked him/her to remember.

19. BALL?
20. FLAG?
21. TREE? **Delayed verbal recall subtotal = /3**

NAMING –2 points

Show the subject a wrist watch and ask him/her what it is. Repeat for pencil.
22. WATCH
23. PENCIL

REPETITION – 1 point
Ask the subject to repeat the following :"No ifs, ands, or buts"
25. REPETITION

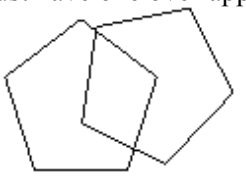
3-STAGE COMMAND - 3 points
Give the subject a plain piece of paper and say, "Take the paper in your hand, fold it in half, and put it on the floor."
25. TAKES
26. FOLDS
27. PUTS

READING – 1 point
Hold up the card reading, "Close your eyes", so the subject can see it clearly. Ask him/her to read it and do what it says. Score correctly only if the subject actually closes his/her eyes.
28. CLOSES EYES

WRITING 1 point
Give subject a piece of paper and ask him/her to write a sentence. It is to be written spontaneously. It must contain a subject and verb and be sensible. Correct grammar and punctuation are not necessary.
29. SENTENCE

Language subtotal = /8

COPYING – 1 point
Give subject a piece of paper and ask him/he to copy a design of two intersecting shapes. One point is awarded for correctly copying it. All angles on both figures must be present, and the figures must have one overlapping angle.

Example: 

30. PENTAGONS **Pentagon subtotal = /1**

TOTAL MMSE = /30
(MMSE maximum score =

APPENDIX H

CHAMPS PHYSICAL ACTIVITY QUESTIONNAIRE

CHAMPS Activities Questionnaire for Older Adults

Date:_____

Name or ID:_____

CHAMPS: Community Healthy Activities Model Program for Seniors
Institute for Health & Aging, University of California San Francisco
Stanford Center for Research in Disease Prevention, Stanford University

Contact: Anita L. Stewart, Ph.D., UCSF, anitast@itsa.ucsf.edu

This questionnaire is about activities that you may have done in the past 4 weeks. The questions on the following pages are similar to the example shown below.

INSTRUCTIONS

If you DID the activity in the past 4 weeks:

Step #1 **Check the YES box.**

Step #2 **Think about <u>how many</u> TIMES <u>a week</u> you usually did it, and write your response in the space provided.**

Step #3 **Circle how many TOTAL HOURS <u>in a typical week</u> you did the activity.**

Here is an example of how Mrs. Jones would answer question #1: Mrs. Jones usually visits her friends Maria and Olga <u>twice a week</u>. She usually spends <u>one</u> hour on Monday with Maria and <u>two</u> hours on Wednesday with Olga. Therefore, the total hours a week that she visits with friends is <u>3</u> hours a week.

In a typical week during the past 4 weeks, did you…							
X Visit with friends or family (other than those you live with)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
1. Visit with friends or family (other than those you live with)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
4. Go to the senior center? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
3. Do volunteer work? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
4. Attend church or take part in church activities? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
5. Attend other club or group meetings? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
6. Use a computer? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
7. Dance (such as square, folk, line, ballroom) (do <u>not</u> count aerobic dance here)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
8. Do woodworking, needlework, drawing, or other arts or crafts? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
9. Play golf, carrying or pulling your equipment (count <u>walking time</u> only)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
10. Play golf, riding a cart (count <u>walking time</u> only)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
11. Attend a concert, movie, lecture, or sport event? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
12. Play cards, bingo, or board games with other people? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
13. Shoot pool or billiards? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
14. Play singles tennis (do <u>not</u> count doubles)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
15. Play doubles tennis (do <u>not</u> count singles)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
16. Skate (ice, roller, in-line)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
17. Play a musical instrument? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
18. Read? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
19. Do heavy work around the house (such as washing windows, cleaning gutters)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
20. Do light work around the house (such as sweeping or vacuuming)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
21. Do heavy gardening (such as spading, raking)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
22. Do light gardening (such as watering plants)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
23. Work on your car, truck, lawn mower, or other machinery? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
****Please note: For the following questions about running and walking, include use of a treadmill.**							
24. Jog or run? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
25. Walk uphill or hike uphill (count only uphill part)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
26. Walk fast or briskly for exercise (do not count walking leisurely or uphill)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
27. Walk to do errands (such as to/from a store or to take children to school (count walk time only)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
28. Walk <u>leisurely</u> for exercise or pleasure? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
29. Ride a bicycle or stationary cycle? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
30. Do other aerobic machines such as rowing, or step machines (do <u>not</u> count treadmill or stationary cycle)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
31. Do water exercises (do <u>not</u> count other swimming)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
32. Swim moderately or fast? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
33. Swim gently? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
34. Do stretching or flexibility exercises (do <u>not</u> count yoga or Tai-chi)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
35. Do yoga or Tai-chi? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
36. Do aerobics or aerobic dancing? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
37. Do moderate to heavy strength training (such as hand-held weights of more than 5 lbs., weight machines, or push-ups)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
38. Do light strength training (such as hand-held weights of 5 lbs. or less or elastic bands)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
39. Do general conditioning exercises, such as light calisthenics or chair exercises (do not count strength training)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL hours a week did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

In a typical week during the past 4 weeks, did you …							
40. Play basketball, soccer, or racquetball (do <u>not</u> count time on sidelines)? ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours
41. Do other types of physical activity not previously mentioned (please specify)? _____ ☐ **NO** ☐ **YES** How many TIMES a week?_____ ➔	How many TOTAL <u>hours a week</u> did you usually do it? ➔	Less than 1 hour	1-2½ hours	3-4½ hours	5-6½ hours	7-8½ hours	9 or more hours

APPENDIX I

BRIEF VITAMIN D QUESTIONNAIRE

Food	day	**OR** week	**OR** month	Medium Serving Size	S	M	L
Milk, liquid all types				1 cup			
Milk, dry powder				1 Tbs			
Milk in coffee/tea				1 Tbs			
Cheese, cheddar				1 oz			
Cheese, parmesan				1 oz			
Cheese, swiss				1 oz			
Egg custard, pudding				1 cup			
Yogurt drink. Brand_____				11 oz			
Yogurt. Brand _____				4 oz			
Ice cream				1/2 cup			
Egg, whole or yolk (17g)				1 each			
Beef (lean cuts)				3 oz			
Hamburger/Cheeseburger				3 oz			
Hot dog (beef or pork)				1 dog			
Salami				3 slices			
Bologna				1 ounce			
Whitefish (cod, flounder)				3 oz			
Herring (Atlantic)				3 oz raw			
Herring (pickled)				3 oz			
Tuna (canned in oil)				3 oz			
Mackerel (Atlantic, Pacific, canned)				1 cup			
Oysters Eastern wild				6 medium raw			
Chum canned salmon				3 oz			
Salmon pink, canned				3 oz			
Sardines, canned in tomato sauce				1 sardine			
Sardines canned in oil				2 sardines			
Shrimp				3 oz raw			
Butter/Margarine				1 pat			
Cream				1 Tbs			
Cod Liver Oil				1 Tbs			
Mushrooms, raw				1/2 cup			
Mushroom, Shiitake, dried				1 each			
Orange juice, fortified with Vitamin D (MinMaid)				8 oz			
All kinds, fortified. Cereal_____				1 cup			
Rice Beverage Enriched				1 cup			
Soy milk enriched. Brand_____				1 cup			
Salmon unspecified filet				3 oz			

APPENDIX J

AMYLOID BETA 40 ELISA KIT, HUMAN PROTOCOL

Preparing Standard Reconstitution Buffer

Dissolve 2.31 grams of sodium bicarbonate in 500 mL of deionized water. Add 2 N sodium hydroxide until pH is 9.0. Filter solution through a 0.2 µm filter unit.

Note: This buffer is used to reconstitute the lyophilized standard. Do not use this buffer to dilute the standard or samples.

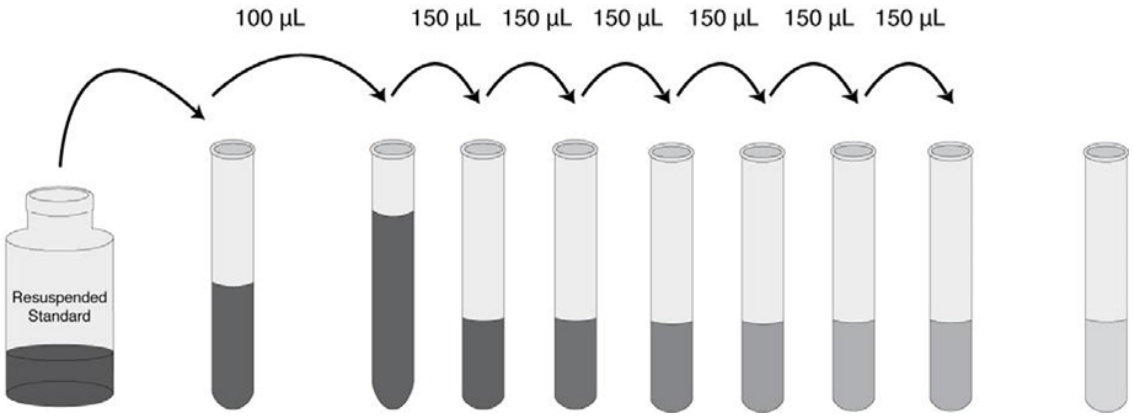
Dilution of Standard

Note: Polypropylene tubes may be used for standard dilutions.

The Hu Aβ40 Standard is calibrated against highly purified Hu Aβ where mass was corrected for peptide content by amino acid analysis.

1. Remove the *Hu Aβ40 Standard* vial from storage and let equilibrate to room temperature (RT).
2. Reconstitute standard to 100 ng/mL with *Standard Reconstitution Buffer* (55 mM sodium bicarbonate, pH 9.0). Refer to the standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Briefly vortex prior to preparing standard curve.
3. Generation of the standard curve using the Aβ peptide standard must be performed using the same composition of buffers used for the diluted experimental samples. For example, if brain extracts are diluted 1:10 with Standard Diluent Buffer, then the buffer used to dilute standards should be 90% Standard Diluent Buffer and 10% brain extraction buffer (including AEBSF at a final concentration of 1 mM).
4. Add 0.1 mL of the reconstituted standard to a tube containing 0.9 mL of the *Standard Diluent Buffer*. Label as 10,000 pg/mL Hu Aβ40. Mix.
5. Add 0.1 mL of the 10,000 pg/mL standard to a tube containing 1.9 mL *Standard Diluent Buffer*. Label as 500 pg/mL Hu Aβ40. Mix.
6. Add 0.15 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 250, 125, 62.5, 31.25, 15.63, 7.81, and 0 pg/mL Hu Aβ40.
7. Make serial dilutions of the standard as described in the following dilution diagram. Mix thoroughly between steps.

Note: Remaining reconstituted Hu A β40 standard may be stored in aliquots at -80°C for up to 4 months. Standard can be frozen and thawed one time only without loss of immunoreactivity.



Preparing Secondary Antibody

Note: Prepare within 15 minutes of usage. The *Anti-Rabbit IgG HRP (100X)* is in 50% glycerol, which is viscous. To ensure accurate dilution, allow *Anti-Rabbit IgG HRP (100X)* to reach room temperature. Gently mix. Pipette *Anti-Rabbit IgG HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100X concentrated solution with 1 mL of *HRP Diluent* for each 8-well strip used in the assay. Label as Anti-Rabbit IgG HRP Working Solution.
2. Return the unused *Anti-Rabbit IgG HRP (100X)* to the refrigerator.

# of 8-Well Strips	Volume of Anti-Rabbit IgG HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

Sample Preparation

Prepare one or more dilutions of each sample. These dilutions should be made in *Standard Diluent Buffer,* although the exact dilution must be determined empirically (e.g., 1:2 and 1:10 represent a reasonable range). This dilution must be performed because certain components in samples can interfere with the detection of the Aβ peptides or to bring the levels of Aβ within the range of this assay. AEBSF should be added to the diluted samples and the standards at a final concentration of 1 mM in order to prevent proteolysis of the Aβ peptides.

Refer to β-Amyloid Application (page 13) for procedure for homogenization of human or transgenic mouse brains.

Note: Analysis of plasma samples may require pretreatment to disrupt interaction

of Aβ with masking proteins.

Assay Procedure

Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 50 µL of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
3. Prepare samples and standards with appropriate diluents. Add 50 µL of Aβ peptide standards, controls, and samples to each well except for the chromogen blank (s).
4. Add 50 µL of *Hu Aβ40 Detection Antibody* solution to each well except for the chromogen blank(s).
5. Cover plate with *plate cover* and incubate for **3 hours at room temperature with shaking**.
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
7. Add 100 µL Anti-Rabbit IgG HRP Working Solution to each well except the chromogen blank(s). See **Preparation of Reagents**.
8. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
10. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
11. Incubate for **30 minutes at room temperature and in the dark**.
    ***Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceeds the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 15 to 20 minutes is suggested.
12. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
13. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution*.
14. Use a curve fitting software to generate the standard curve. A four

parameter algorithm provides the best standard curve fit.

15. Read the concentrations for unknown samples and controls from the standard curve. **Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.** (Samples producing signals greater than that of the highest standard should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

APPENDIX K

ORIGINAL BASELINE DESCRIPTIVE STATISTICS (N=23)

Table 4

Original baseline descriptive statistics (n=23)[a]

Characteristics	Vitamin D group n=12	Control Group n=11	p value
Baseline values			
Male	3	3	
Female	9	8	
Age	64.3 ± 3.0	64.5 ± 3.6	0.975
BMI	25.2 ± 1.1	26.7 ± 1.9	0.489

[a]All values are means + SE